Lecture Supplements

Plant Bacteriology

Bacterial Diagnosis-Part 1

Compiled by N. Hassanzadeh

Version 4.25

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Plant Pathology

Science and Art

Plant Pathology
Definition

- Plant Pathology is the study of plant diseases, their causes, and the interactions with the environment.
- The field/discipline consists of several sub-disciplines/courses including:

1. Phytomycology
2. Plant bacteriology (Prokaryotic Plant Pathogens)
3. Virology
4. Nematology
5. Epidemiology, and
6. Molecular biology of host-pathogen interactions.

- Plant pathology has its foundation in biology and agriculture and offers wide opportunities in both basic and applied areas of biology.

Molecular Plant Pathology

- Molecular plant pathology covers a wide range of activities, including:

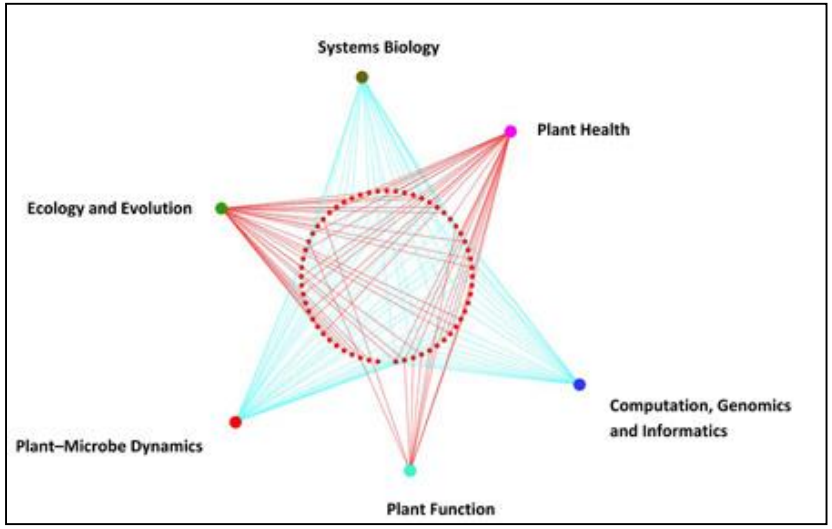
1. Biochemistry;
2. Genetics;
3. Physiology;
4. Tissue culture, and
5. Taxonomy.

- Pathologists have developed and adapted contemporary technologies which have significantly advanced the discipline.

Plant Pathology and Plant-Microbe Biology

Fundamental aspects

- Plant Pathology and Plant-Microbe Biology is a multi-faceted discipline that comprises various subjects including:
- Plant biology
- Plant health
- Plant function
- Genomics, evolution
- Ecology
- Plant medicine
- Biology of plant pathogens
- Plant-microbe and plant-insect interactions
- Plant associated bacteria and fungi
- Fate and action of pesticides
- Pesticide resistance management
- Epidemiology of pathogens
- Molecular genetics, and
- Disease diagnosis.



Oregon State University, Department of Botany and Plant Pathology, 2010; Cornell Departments of Plant Pathology and Plant-Microbe Biology,2014; Clemson University,2014;UNL Plant Pathology Dept.,2014.

Books on Plant Bacteriology

Reference Books in Plant Pathogenic Bacteria

Books on Plant Bacteriology (1905-2025)

In order of publication dates

1. Smith, Erwin F.1905. Bacteria in Relation to Plant Diseases. Volume 1. Methods of Work and General Literature of Bacteriology Exclusive of Plant Diseases. USA: Carnegie Institution of Washington, 285 pp.
2. Smith, Erwin F.1911. Bacteria in Relation to Plant Diseases. Volume 2. Vascular diseases, USA: Carnegie Institution of Washington. pp.?
3. Smith, Erwin F.1914. Bacteria in Relation to Plant Diseases. Volume 3. Vascular diseases, USA: Carnegie Institution of Washington. pp.?
4. Smith, Erwin F. 1920. Introduction to Bacterial Diseases of Plants. USA, Philadelphia: Saunders.
5. Bulloch, W, 1935. The History of Bacteriology. London, UK: Oxford University Press. pp.?
6. Elliott, C.1951. Manual of bacterial plant pathogens. 2nd ed. Waltham, Mass., USA: Chronica Botanica Company, 186 pages.
7. Dowson, W. J. 1949. Manual of Bacterial Plant Diseases. London; A. and C. Black, 183p. (2d ed. with title Plant Diseases Due to Bacteria. Cambridge, Eng.; Cambridge University Press, 1957. 231p.)
8. Ramamurthi, C.S.1959. Comparative studies on some gram positive phytopathogenic bacteria and their relationship to the Corynebacteria. Memoir /Cornell University Agricultural Experiment Station, 52 pages.
9. Stapp, C. 1961. Bacterial Plant Pathogens. Oxford University Press, Oxford. pp.?
10. Kado, C.I. 1971. Methods in Plant Bacteriology. Plant Pathology 228, University of California, Davis, 75 pp.
11. Mount, M.S. and G.H. Lacy.1982. Phytopathogenic Prokaryotes. Volumes I & II, Academic Press, Inc.

Books on Plant Bacteriology (1905-2025)

In order of publication dates

12. Rhodes-Roberts, M. E. and F.A. Skinner.1982. Bacteria and Plants (The Society for Applied Bacteriology Symposium Series, No. 10). Academic Press Inc. (London) Ltd., 264 pp.
13. Fahy, P.C. and G.J. Persley.1983. Plant Bacterial Diseases- A Diagnostic Guide. Academic Press, Australia, 393 pages.
14. Starr, M.P.1983. Phytopathogenic Bacteria. Selections from the Prokaryotes. New York, USA: Springer Publishing. 168 pages.
15. Bradbury, J. F. 1985. Guide to Plant Pathogenic Bacteria. CAB, International, Farnham House, Farnham Royal, UK, 332 pp.
16. Lelliott, R.A. and D.E. Stead.1987. Methods for the Diagnosis of Bacterial Diseases of Plants. Blackwell Scientific Publ., 216 pages.
17. Billing, Eve. 1987. Bacteria as Plant Pathogens. Chapman and Hall, 79 pages.
18. Schaad, N.W. 1988. Laboratory Guide for Identification of Plant Pathogenic Bacteria. Second ed., API Press, 158 pages.
19. Kleinhempel, H., K. Naumann and D. Spaar. 1989. Bakterielle Erkrankungen der Kulturpflanzen. (Germany Edition). Jena, Germany: Gustav Fischer Verlag. pp.?
20. Saettler, A.W., N.W. Schaad and D.A. Roth. 1989. Detection of Bacteria in Seed and Other Planting Material. Amer. Phytopathological Society, Spi edition, 122 pages.
21. Klement, Z., K. Rudolph, and D.C. Sands. 1990. Methods in Phytobacteriology. Akadémiai Kiad'o, Budapest, 563 pages.
22. Goto, M. 1992. Fundamentals of Bacterial Plant Pathology. AP Press, Inc. 342 pages. (Translated to Persian by M. Mohammadi, 1999).
23. Hassanzadeh, N. 1995. Principles and Methods of Plant Bacteriology. Scientific Publication of Islamic Azad University, Iran, 641 pages. (In Persian with English Summary).

Books on Plant Bacteriology (1905-2025)

In order of publication dates

24. Klement, Z. 1995. Plant Pathogenic Bacteria parts A/B. Akademiai Kiado, 1061 pages.
25. Goszczynaska,T., J.J. Serfontein and S. Serfontein. 2000. Introduction to Practical Phytobacteriology. ARC, South Africa, 83 pp.
26. Cullimore, D. Roy. 2000. Practical Atlas for Bacterial Identification, CRC Press.
27. Schaad, N.W., J. B Jones and W. Chun, eds. 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria. API Press, Third ed., 373 pages.
28. Jayaraman, J. and J.P. Verma. 2002. Fundamentals of Plant Bacteriology. Kalyani Publications, India. 129 pages.
29. Singleton, P. 1992. Introduction to Bacteria. 2nd edition. New York, USA: John Wiley & Sons. 221 pages.
30. Sigee, D.C. 1993. Bacterial Plant Pathology: Cells & Molecular Aspects. Cambridge University Press, 325 pp.
31. Zhu, Bian Zheng Ren Xin. 2000. Classification and identification of plant pathogenic bacteria (Chinese Edition). China Agriculture Press, pages??
32. Bradbury, J. F. and G. S. Saddler. 2002. A Guide to Plant Pathogenic Bacteria. Second Edition, CABI Publishing.
33. Mishra, S. R. 2003. Bacterial Plant Diseases. Discovery Publishing House, New Delhi, 218 p.
34. Griffith, C.S, T.B. Sutton and P.D. Peterson. 2003. Fire Blight: the Foundation of Phytobacteriology. Selected Papers of Thomsas J. Burrill, Joseph C. Arthur, and Merton B. Waite. St Paul, Minnesota, USA: APS Press.
35. Hassanzadeh, N. 2005. Identification and Classification of Phytopathogenic Bacteria. Scientific Publication of Science and Research Branch of Islamic Azad University, Iran, 520 pages. (In Persian with English Summary).

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In order of publication dates

36. Chakravarti. B.P. 2005. Methods of Bacterial Plant Pathology. Udaipur, Agrotech, 208 pages.
37. Srivastava, M., U. N. Ram and K. D. Rai. 2006. Introductory Phytobacteriology. 1st ed., Advance Publishing Concept, 312 pages.
37. Janse, J.D. 2006. Phytobacteriology: Principles and Practice. Cabi Publishing, 363 pp.
38. Saygili, H., F. Sahin, Y. Aysan. 2006. Fitobakteriyoloji. (Turkish Edition). Meta Basimevi, Turkey, 530 pp.
39. Civerolo, E.L., A. Collmer, R.E. Davis and A.G. Gillaspie. 2007. Plant Pathogenic Bacteria (Current Plant Science and Biotechnology in Agriculture). Springer, 1 edition, 1050 pages.
40. Tripathi, D.P. 2008. Introductory Plant Bacteriology. Kalyani Publishers /Lyall Bk Depot., 519 pages.
41. Jackson, R. 2009. Plant Pathogenic Bacteria: Genomics and Molecular Biology. Caister Academic Press, 356 pages.
42. Wang, Jin-sheng. 2009. Plant pathogenic bacteriology (Chinese Edition). Publisher: China's agriculture, pp.??
43. Kado, C. I. 2010. Plant Bacteriology. APS Press, 336 pages. (Translated to Persian by Hoseinzadeh *et al.*,2014).
44. Mondal, K.K. 2012. Plant Bacteriology. Kalyani Publishers /Lyall Bk Depot., pp. 190 pages.
45. Thind, B.S. 2012. Phytopathogenic Procaryotes and Plant Diseases. Scientific Publishers, 545+32 Colour Pages.
46. Fatmi, M'Barek, Ron R. Walcott and N.M. Schaad. 2017. Detection of Plant-Pathogenic Bacteria in Seed and Other Planting Material, Second Edition, APS Press, 360 pages.
47. Wezelmanm, T. 2017. Plant Bacteriology. Agri-Horti Press, 258 pages.

Books on Plant Bacteriology (1905-2025)

In order of publication dates

48. Lee, Young-geun. 2017. Understanding Plant Bacterial Pathogens (Korean Edition). With forest Publisher, pp.??
49. Borkar, S. 2017. Laboratory Techniques in Plant Bacteriology. CRC Press, 1st Edition, Kindle Edition, 342 Pages.
50. Burdman, S. and Ronald R. Walcott. 2018. Plant-Pathogenic *Acidovorax* Species. APS, 200 pages.
51. Thind, B.S. 2019. Phytopathogenic Bacteria and Plant Diseases. CRC Press. 398 Pages.
52. Trivedi, P.C. and T. Agarwal. 2021. Bacteriology: Structure, Reproduction, Plant Diseases and Management. Nova Science Pub Inc. 497 pages.
53. Borkar, S. G. and R. Anand Yumlembam. 2021. Bacterial Diseases of Crop Plants. CRC Press, 616 Pages.

Books on Agricultural Bacteriology

1. Ellis, D. 2008. Outlines of Bacteriology (Technical and Agricultural). Originally published in 1909. Cornell University Library, 286 pages.
2. Lohnis, F. 2009. Laboratory methods in agricultural bacteriology. Cornell University Library, 228 pages.
3. Percival, J. 2009. Agricultural Bacteriology: Theoretical and Practical. Originally published in 1920. Cornell University Library, 430 pages.
4. Russell, H.L.2009. Agricultural Bacteriology for Students in General Agriculture. Originally published in 1915. Cornell University Library, 324 pages.
5. Russell, H.L.2009. Agricultural Bacteriology for Students in General Agriculture. Originally published in 1915. Cornell University Library, 324 pages.
6. Greaves, J.E. 2012. Agricultural Bacteriology. Forgotten Books, 446 pages.

Plant Bacteriology Books
Published by Indian authors/publishers

1. Patel, P.N.1972. Plant Bacteriology: Bacterial Diseases of Plants in India. Volume 1. Indian Council of Agricultural Research, 522 pages.
2. Verma, J. P. 1998. The Bacteria. Malhotra Publ. House, New Delhi, 220 pp.
3. Dube, H.C. 1989. A Textbook of Fungi, Bacteria and Viruses. 4 editions, Vikas Publishing House, 240 pages.
4. Jayaraman, J. and J.P. Verma. 2002. Fundamentals of Plant Bacteriology. Kalyani Publishers, 129 pages.
5. Mishra, S. R. 2003. Bacterial Plant Diseases. Discovery Publishing House, New Delhi, 218 pages.
6. Mondal,K.K.2012. Plant Bacteriology. Kalyani Publishers /Lyall Bk Depot., 190 pages.
7. Srivastava, M., U. N. Ramand and K. D. Rai. 2006. Introductory Phytobacteriology. 1st ed., Advance Publishing Concept, 312 pages.
8. Tripathi, D.P. 2008. Introductory Plant Bacteriology. Kalyani Publishers /Lyall Bk Depot., 519 pages.
9. Singh, A. Kr.2010. Encyclopedia Of Plant Bacteriology. Anmol Publications,288 pp.
10. Narayanasamy,P.2010. Microbial Plant Pathogens-Detection and Disease Diagnosis: Bacterial and Phytoplasmal Pathogens. Vol.2, Springer, 279 pages.
11. Khan,I.M.2010. Encyclopedia of Bacterial Diseases in Plants. Anmol Publications, 312 pages.

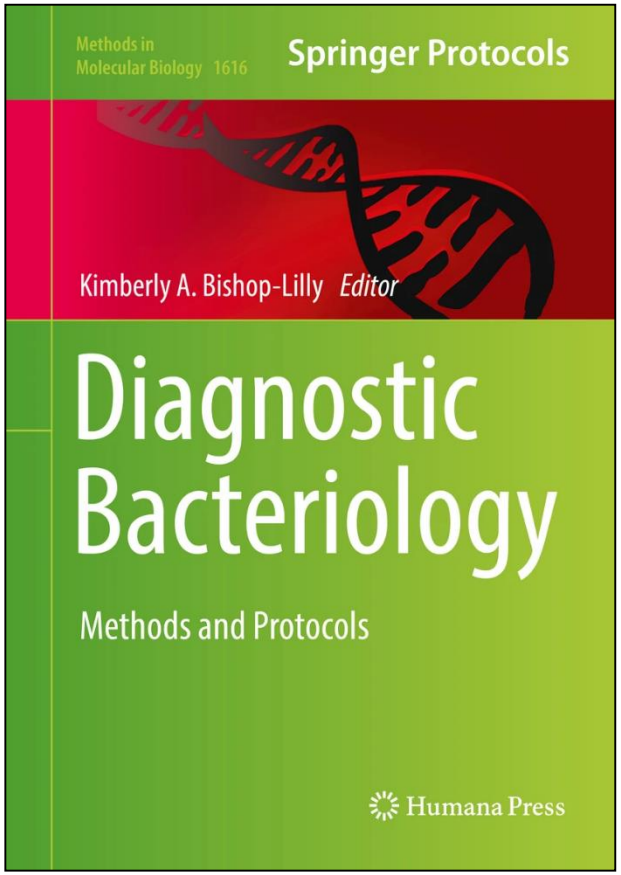
Plant Bacteriology Books

Published by Indian authors/publishers

12. Thind, B.S. 2012. Phytopathogenic Procaryotes and Plant Diseases. Scientific Publishers, 577 pages.
13. Borkar, S. G. 2017. Laboratory Techniques in Plant Bacteriology. CRC Press; 1 edition.342 pages.

Diagnostic Bacteriology
Methods and Protocols

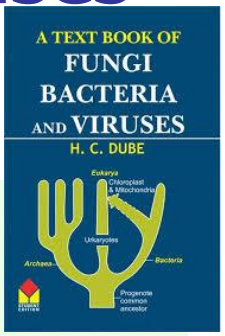
- **Diagnostic Bacteriology Methods and Protocols (Methods in Molecular Biology, 1616).**
- Kimberly A. Bishop-Lilly (Editor)
- Publisher: Humana
- 2017
- 276 pages



Chapters detail common bacterial pathogens, protocols that can be applied to diverse or even unknown pathogens, digital PCR, next generation sequencing, and bioinformatic analyses.

A Textbook of Fungi, Bacteria and Viruses

Book written by my respected PhD supervisor



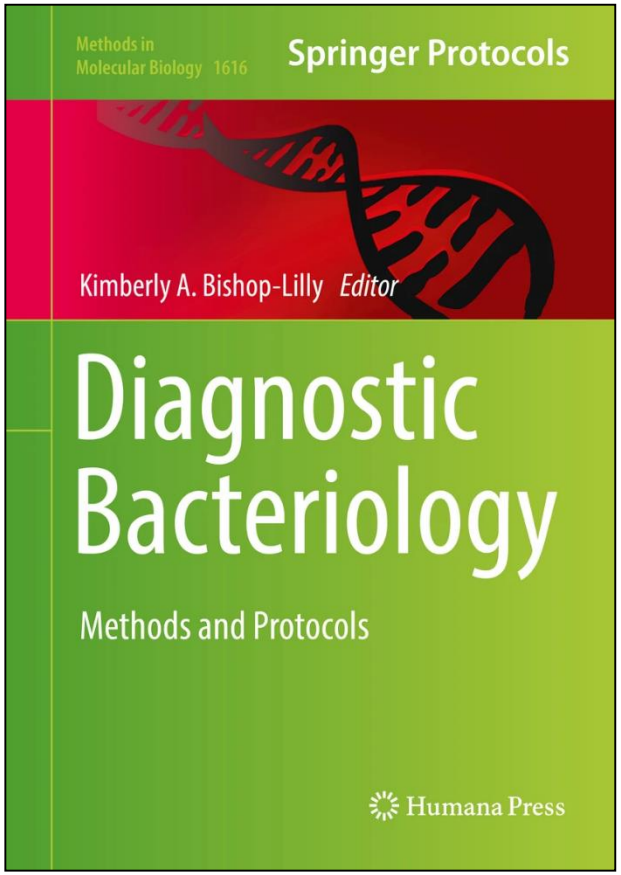
- Publisher: STUDENT EDITION
- 3rd Ed.,2015, 284 pages.
- Prof. H. C. Dube did his M.Sc. from Gorakhpur, D.Phil from Allahabad, under Prof. Bilgrami, and Postdoctoral at University of Madras with Padmabhushan Prof. T. S. Sadasivan. He also worked at Colorado State University (USA) with Prof. Ralph Baker and at Hannover (Germany) with Prof. S. Schonbeck.
- Dr. Dube was Professor and Head of the Department of Life Sciences at Bhavnagar University for 17 years before his retirement in November 2002.
- Earlier he was reader at Sardar Patel University, Vallabh Vidyanagar and lecturer at University of Udaipur.
- Prof. Dube has authored two books, published by Vikas, and edited four volumes published in India and USA., latest being the Annual Review of Plant Pathology 2002.
- Prof. Dube was elected FNASc in 1989 and made President of the Mycological Society of India in 2001.
- He has been on the Editorial Board of Indian Journal of Microbiology, Indian Phytopathology and Journal of Mycology and Plant Pathology.
- He was Director of UNESCO-sponsored project on water management, and Research Coordinator of Ocean Science and Technology Cell of the Department of Ocean Development.
- Govt, of India, to plan and promote research on Marine Ecology of the West Coast of India.
- Dr. Dube has produced 36 Ph.Ds on modern integrated areas in Biology and published over 200 research papers. Dr. Dube was honoured with the Outstanding Teacher Award in 2006.

Books related to Bacteria

Diagnostic Bacteriology

Methods and Protocols

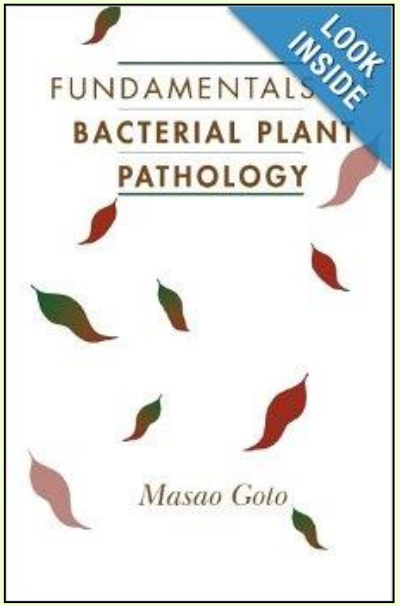
- **Diagnostic Bacteriology Methods and Protocols (Methods in Molecular Biology, 1616).**
- Kimberly A. Bishop-Lilly (Editor)
- Publisher: Humana
- 2017
- 276 pages



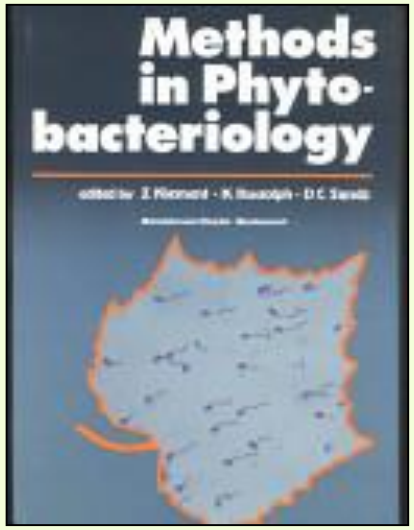
Chapters detail common bacterial pathogens, protocols that can be applied to diverse or even unknown pathogens, digital PCR, next generation sequencing, and bioinformatic analyses.

Three book front covers

Plant pathogenic bacteria books



Goto, M., 1992



Klement, Z., K. Rudolph, and D.C. Sands (editors),1990

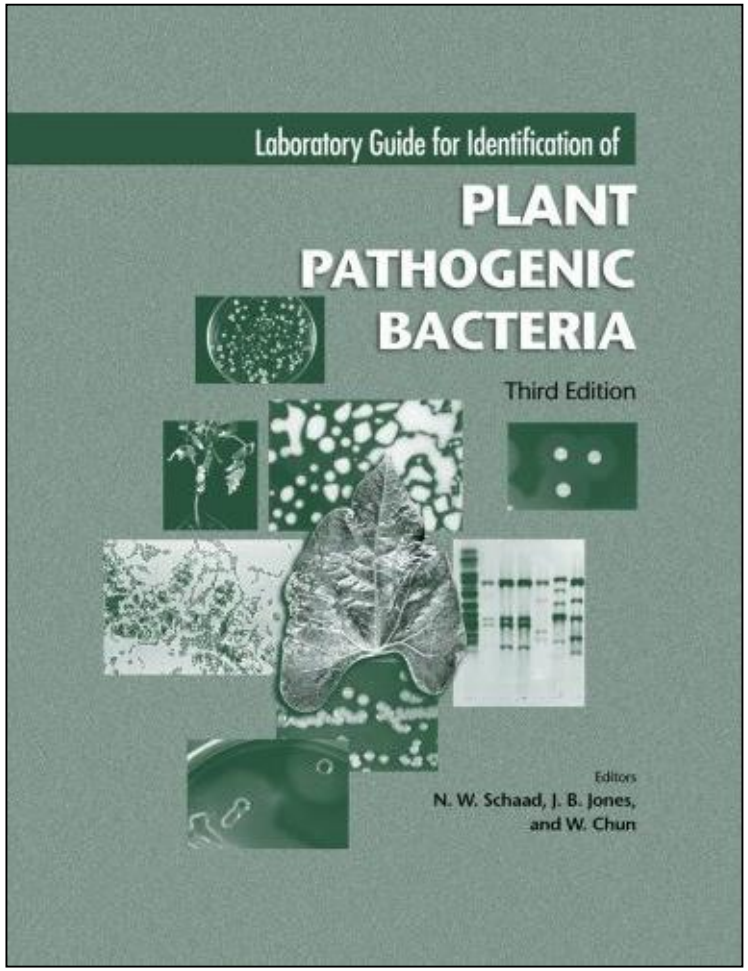
Bradbury, J. F. 1986

Frequently cited book

Laboratory Guide for the Identification of Plant Pathogenic Bacteria

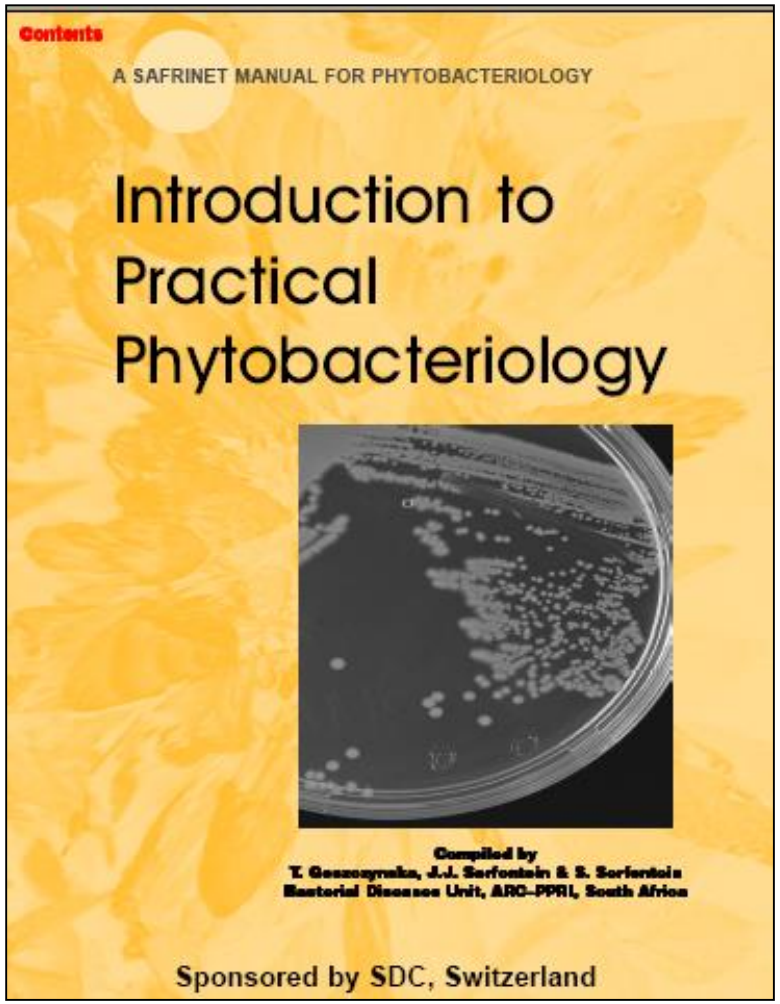
Product Details

- **Editors:** Schaad, N.W., J. B Jones and W. Chun.
  Publisher: API Press, 2001, 373 pages.
- To assist readers in correctly identifying bacteria, the Laboratory Guide for Identification of Plant Pathogenic Bacteria, Third Edition includes simplified methods, color photographs of biochemical results, full descriptive methods and a list of semi-selective agar media useful for isolating bacteria.
- This guidebook will be a useful resource for educators, students, researchers, extension personnel, diagnosticians or anyone involved in identifying plant bacterial diseases.



Frequently cited book

Introduction to Practical Phytobacteriology



Product Details

- **Editors:** Goszczynaska *et al.*, 2000
- 83 pages.
- This manual is a guide to a course in practical Phytobacteriology for technical assistants of SADC countries in the SAFRINET-LOOP of BioNET-INTERNATIONAL.

Frequently cited book

Phytobacteriology: Principles and Practice

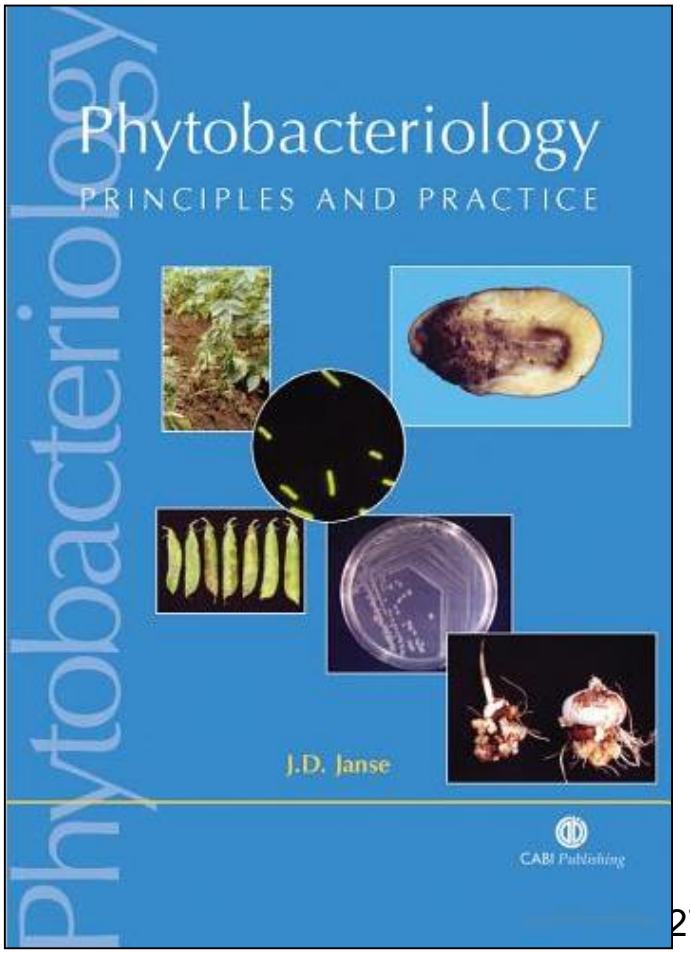
Product Details

- **Author: J. D. Janse**
- Publisher: Cabi Publishing, 2006.
- 360 pp.

Product Description

This book is a comprehensive manual of phytobacteriology and is heavily illustrated with over 200 color photographs and line illustrations.

- It starts by outlining the history and science of bacteriology and gives an overview of the diversity and versatility of complex bacteria.
- It goes on to explain characterization, identification, and naming of complex bacteria and how bacteria can cause disease and how plants react to this.
- It also discusses the economic importance of bacterial diseases and strategies for their control and reduction of crop losses.
- It concludes with 50 examples of well and lesser known plant pathogenic bacteria and the diseases that they cause.

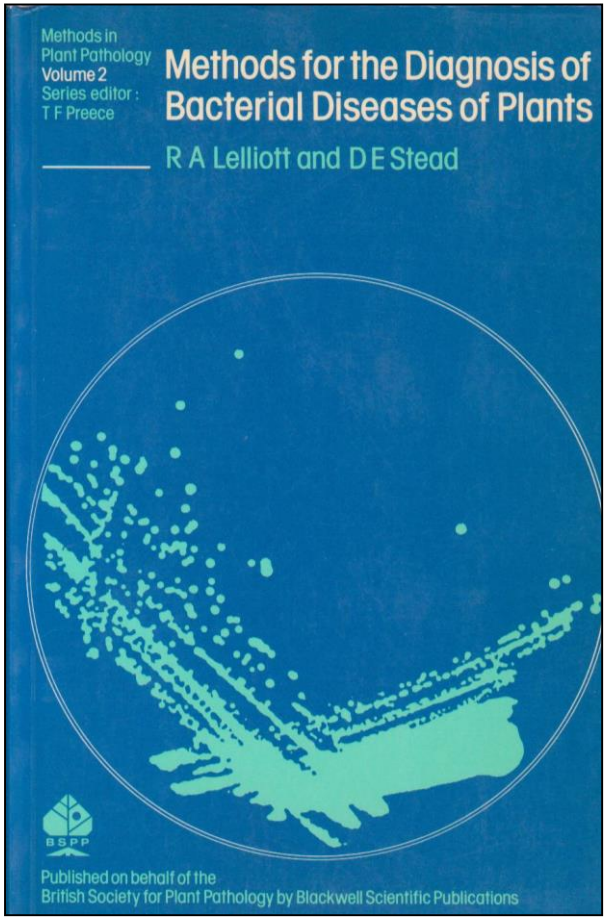


Frequently cited book

Methods for the Diagnosis of Bacterial Diseases of Plants

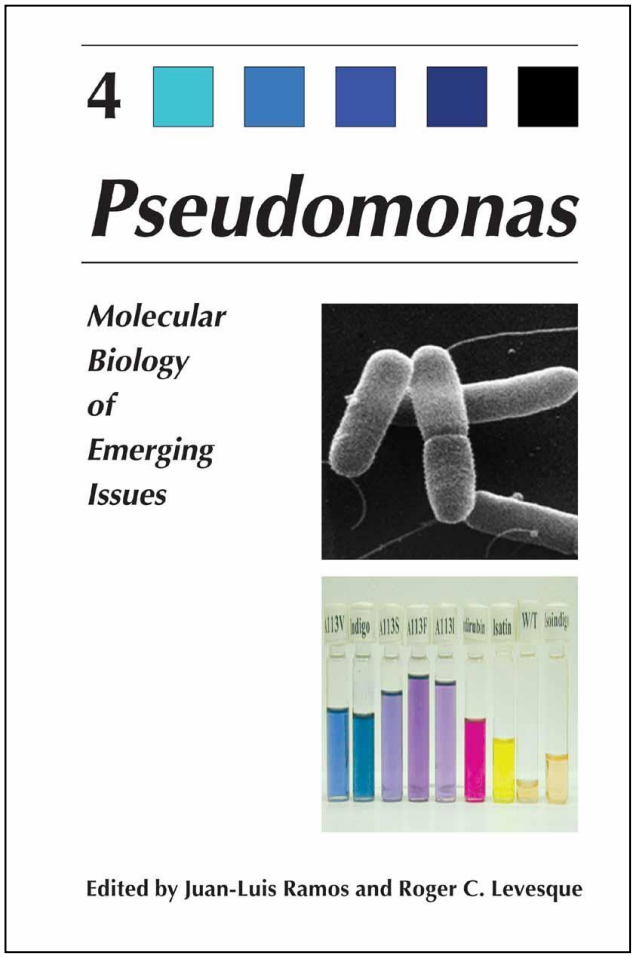
Product Details

- **Methods for the Diagnosis of Bacterial Diseases of Plants.** Authors: Ronald Alexander Lelliott and D.E. Stead
- Publisher: Blackwell Scientific Publications Inc., 1987.
- 216 pages.



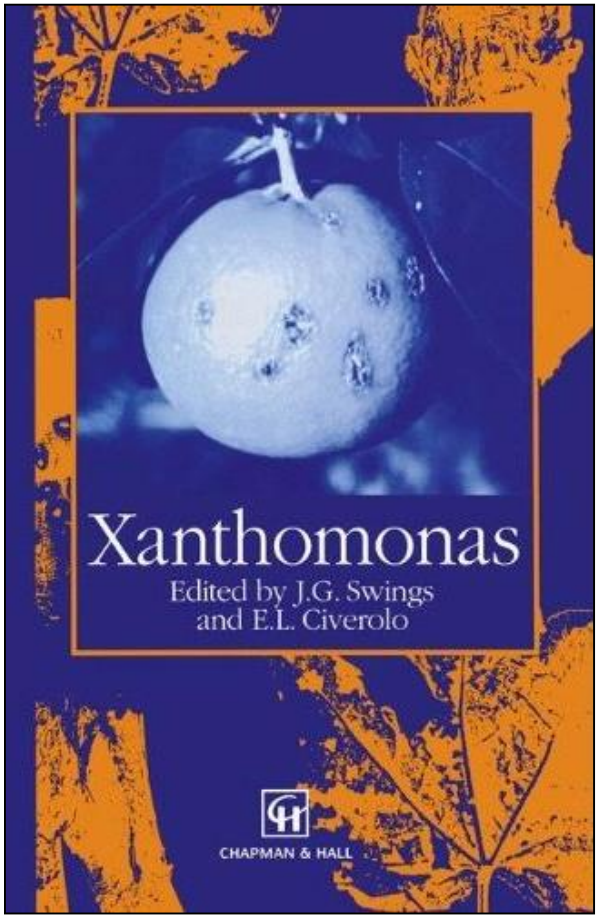
Pseudomonas

- Edited by Juan-Luis Ramos, CSIC, Granada, Spain,2006, Springer.
- Volume 1: Genomics, Life Style and Molecular Architecture.
- Volume 2: Virulence and Gene Regulation.
- Volume 3: Biosynthesis of Macromolecules and Molecular Metabolism.
- Volume 4: Molecular Biology of Emerging Issues.
- Volume 5: A Model System in Biology.



Xanthomonas

- Edited by Swings, J. and Civerolo E.L.
- 1993.
- Chapman & Hall,
- 399 pages.
- *Xanthomonas* is a bacterial plant pathogen which infects a wide range of crops worldwide.
- This book presents an overview of the host plants and the diseases caused by the pathogen on different crops.



Bacterial nomenclature

Bergey's Manuals

- The "bible" of bacterial identification is a book called Bergey's Manual of Determinative Bacteriology, based on morphology, differential staining, biochemical tests.
- He was the first doctor to isolate the bacterium *Actinomyces* from a human being, in 1907.
- Bergey is long dead, but the American Society for Microbiology continues to revise and publish the book.

- David Hendricks Bergey

Born	December 27, 1860, Pennsylvania
Died	September 5, 1937 (aged 76) Philadelphia, Pennsylvania
Alma mater	University of Pennsylvania
Fields	Bacteriology
Institutions	University of Pennsylvania

Alma mater is an allegorical Latin phrase for a university, school, or college that one formerly attended.

Bergey's Manual of Determinative Bacteriology
2nd-8th Editions (1923-1974)

- Bergey's Manual gets its name from Dr. David H. Bergey first chairman of the Editorial Board of the Manual published by the then Society of American Bacteriologists (now called the American Society for Microbiology).
- The Trust Funds provided through the generosity of Dr. Bergey before his death have been used in developing the present edition of the Manual and future funds are to be used in the same way under the management of a self-perpetuating Board of Editor-Trustee.

Bergey's Manual of Determinative Bacteriology
2nd-8th Editions (1923-1974)

- Classifies bacteria by cell wall composition, morphology, biochemical tests, differential staining, etc.
- The classification in Bergey's Manual is accepted by most microbiologists as the best consensus for prokaryotic taxonomy.
- Chester's Manual of Determinative Bacteriology,1901(long out of date).
- Bergey's Manual of Determinative Bacteriology (ed.1,1923);
- Bergey's Manual of Determinative Bacteriology (ed. 2,1925);
- Bergey's Manual of Determinative Bacteriology (ed. 3,1930);
- Bergey's Manual of Determinative Bacteriology (ed. 4,1936);
- Bergey's Manual of Determinative Bacteriology (ed. 5,1939);
- Bergey's Manual of Determinative Bacteriology (ed. 6,1948);
- Bergey's Manual of Determinative Bacteriology (ed. 7,1957);
- Bergey's Manual of Determinative Bacteriology (ed. 8,1974).

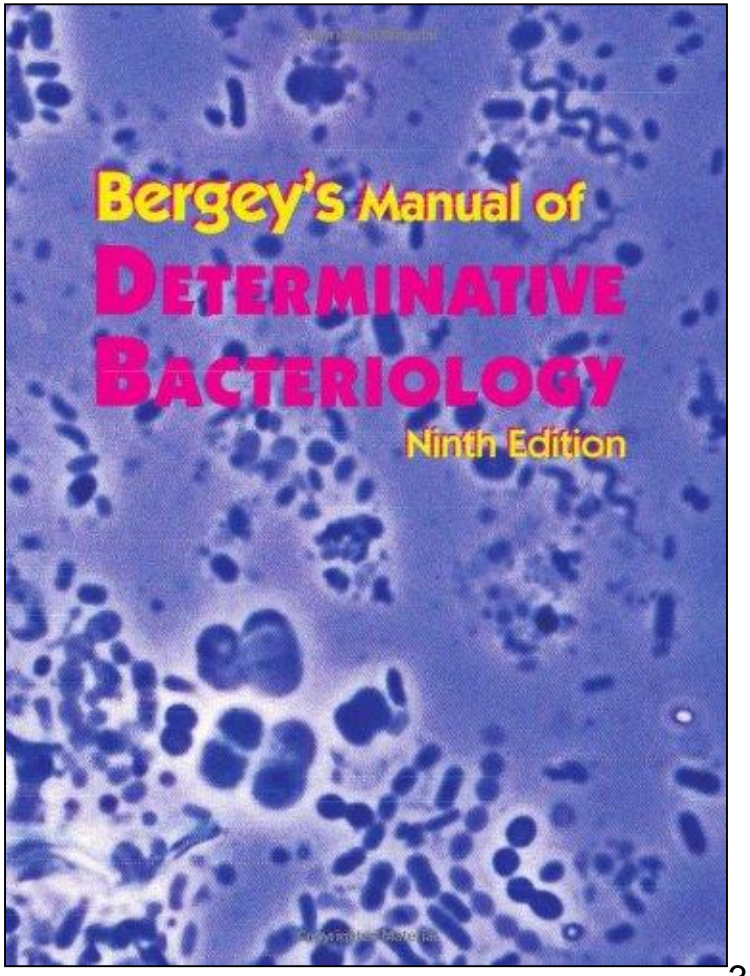
Systems of classification were based entirely on morphologic characters.

Bergey's Manual of Determinative Bacteriology

9th Edition(1994)

Product Details

- **Bergey's Manual of Determinative Bacteriology**
- by John G Holt
- Publisher: Lippincott Williams & Wilkins; 9 edition (January 15, 1994).
- 787 pages.



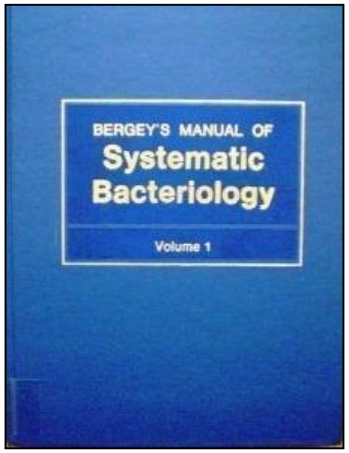
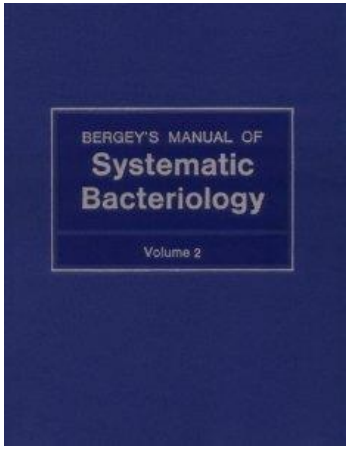
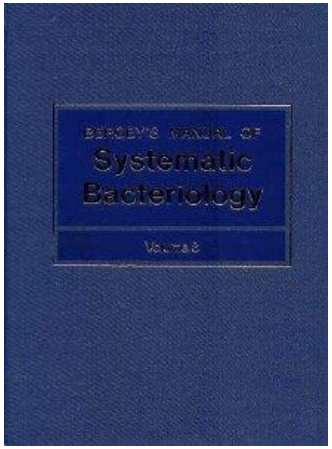
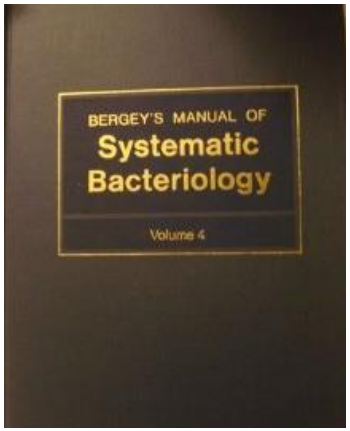
Bergey's Manual of Systematic Bacteriology (Vol.1-4)

1st edition(1984-1989)

- Classifies bacteria via evolutionary or genetic relationships.
- John G. Holt, Editor-in-Chief
- Williams & Wilkins, Baltimore, MD
- **Published in 4 volumes:**
- **Volume 1** (1984)
  - Gram-negative Bacteria of general, medical, or industrial importance
- **Volume 2** (1986)
  - Gram-positive Bacteria other than Actinomycetes
- **Volume 3** (1989)
  - Archaeobacteria, Cyanobacteria, and remaining Gram-negative Bacteria
- **Volume 4** (1989)
  - Actinomycetes

Bergey's Manual of Systematic Bacteriology(Vol.1-4)

1st edition(1984-1989)

			
Volume 1(1984) 964 pages	**Volume 2 (Aug.1986)** 672 pages	**Volume 3 (March 1989)** 744 pages	**Volume 4 (Dec.1989)** 440 pages

In these volumes many higher taxa are defined in terms of phenotype. Mix Phylogenetic/Phenetic-5 Kingdoms.

Bergey's Manual of Systematic Bacteriology(Vol.1-5)

2nd Edition(2001-2012)

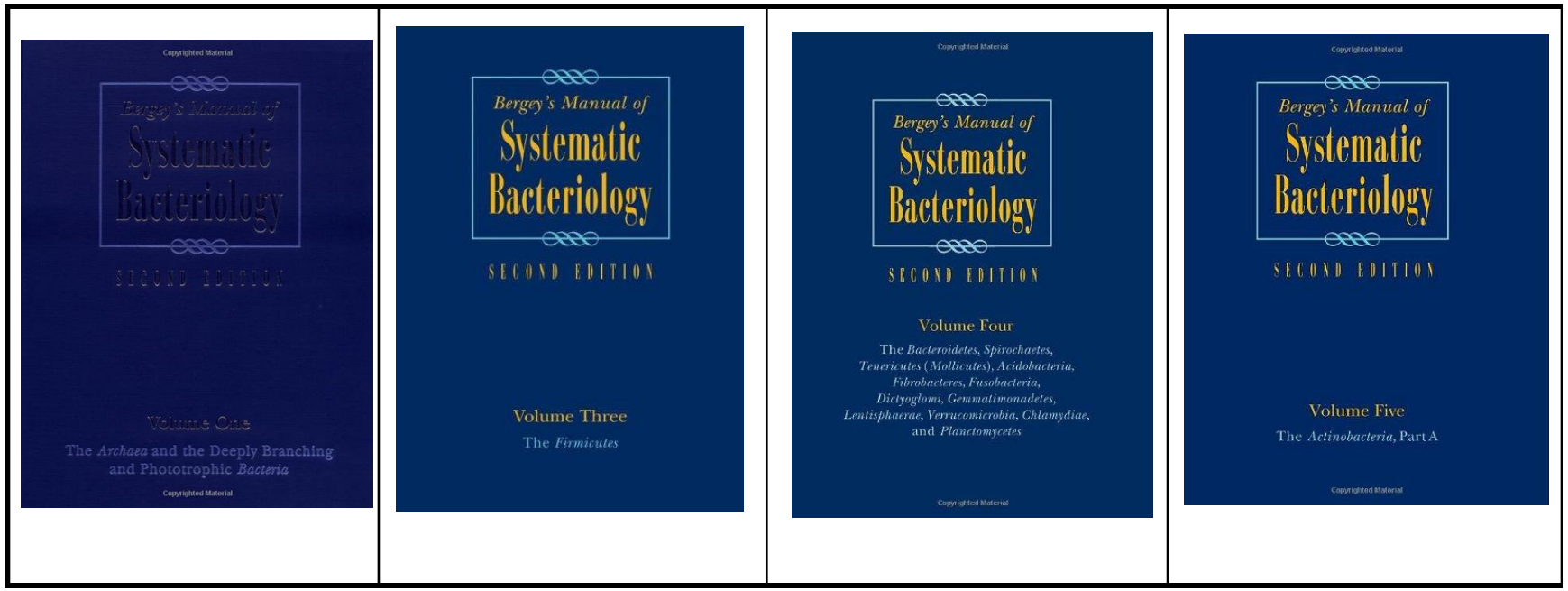
- The second edition is being published in 5 volumes.
- The current volumes differ drastically from previous volumes in that many higher taxa are not defined in terms of phenotype, but solely on 16S phylogeny.
- Based on Phylogenetic-3 Domains.
- Yet retains much of the layout of the first edition.

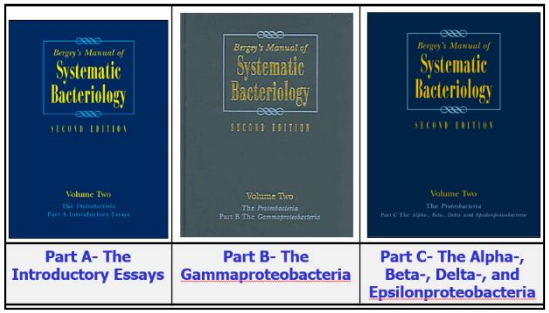
Bergey's Manual of Systematic Bacteriology(Vol.1-5)
2nd Edition(2001-2012)

- **The second edition is being published in 5 volumes.**
- **Volume 1** (2001)
  - The *Archaea* and the deeply branching and phototrophic *Bacteria*
  - **Editor-in-Chief:** George M. Garrity
  - **Editors:** David R. Boone and Richard W. Castenholz. 721 pp.
- **Volume 2** (2005) **divided into three books (Parts A, B & C)**
  The *Proteobacteria*
  - **Editor-in-Chief:** George M. Garrity
  - **Editors:** Don J. Brenner, Noel R. Krieg and James T. Staley. 2816 pp.
- **Volume 3** (2009)
  - The *Firmicutes*
  - **Editors:** Paul De Vos, George Garrity, Dorothy Jones, Noel R. Krieg, Wolfgang Ludwig, Fred A. Rainey, Karl-Heinz Schleifer and William B. Whitman. 1450 pp.
- **Volume 4** (2010)
  - The *Bacteroidetes*, *Planctomycetes*, *Chlamydiae*, *Spirochaetes*, *Fibrobacteres*, *Fusobacteria*, *Acidobacteria*, *Verrucomicrobia*, *Dictyoglomi* and *Gemmatimonadetes*
  - **Editors:** Noel R. Krieg, James T. Staley, Brian Hedlund, Bruce J. Paster, Naomi Ward, Wolfgang Ludwig and William B. Whitman. 974 pp.
- **Volume 5** (2012)
  - The *Actinobacteria*
  - **Editors:** Michael Goodfellow, Peter Kämpfer, Hans-Jürgen Busse, Martha Trujillo, Ken-ichiro Suzuki, Wolfgang Ludwig and William B. Whitman. 1750 pp.

Bergey's Manual of Systematic Bacteriology(Vol.1-5)

2nd Edition(2001-2012)





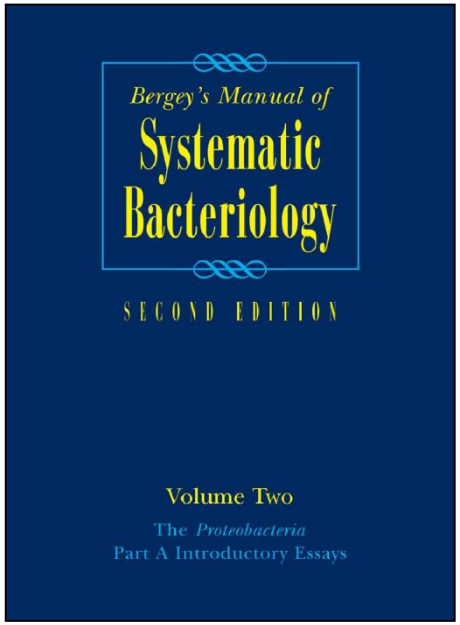
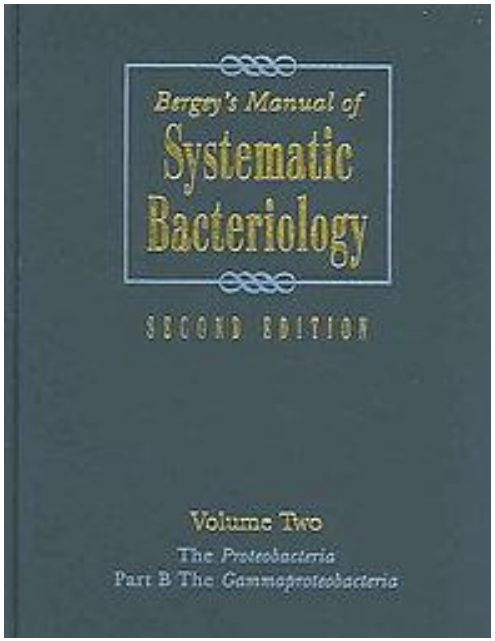
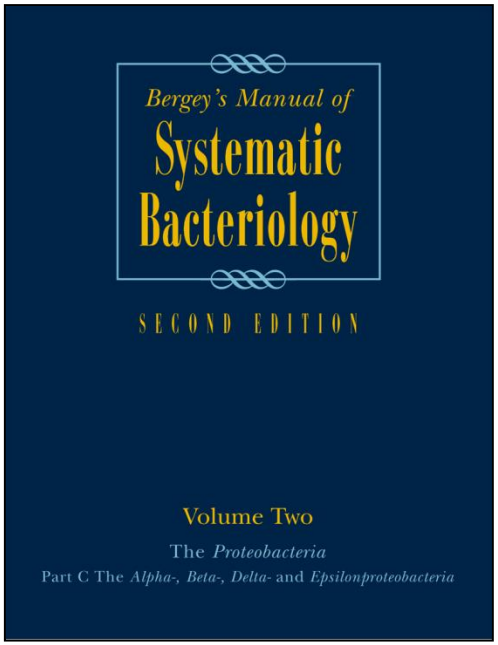
Bergey's Manual of Systematic Bacteriology 2nd Edition, Volume 2 (Parts A, B & C)

The Proteobacteria

- **Volume 2 "The Proteobacteria."** (2004). Don J. Brenner, Noel R. Krieg, James T. Staley (Volume Editors), and George M. Garrity (Editor-in-Chief) with contributions from 339 colleagues.
- Hardcover: 2816 pages
- The volume provides descriptions of more than 2000 species in 538 genera that are assigned to the phylum Proteobacteria.
- This volume is subdivided into three parts:

A. **Part A- The Introductory Essays** (332 pgs, 76 figures, 37 tables).
B. **Part B- The Gammaproteobacteria** (1136 pages, 222 figures, and 300 tables).
C. **Part C- The Alpha-, Beta-, Delta-, and Epsilonproteobacteria** (1256 pages, 512 figures, and 371 tables).

Bergey's Manual of Systematic Bacteriology
Volume 2 (Parts A, B & C)
The Proteobacteria

		
Part A- The Introductory Essays	**Part B- The Gammaproteobacteria**	**Part C- The Alpha-, Beta-, Delta-, and Epsilonproteobacteria**

The Prokaryotes:
A Handbook on Habitats, Isolation, and Identification of Bacteria
First Edition, 1981

- The first edition, published in 1981 in two volumes, set out to be an encyclopedic, synoptic account of the world of the prokaryotes- a collection of monographic descriptions of the genera of bacteria.
- The Archaea had not yet been formalized as a group.

M. P. Starr, H. Stolp, H. G. Trüper, A. Balows, and H. G. Schlegel (Eds.).1981. The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria. Berlin, Germany: Springer Verlag.

The Prokaryotes

A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications

Second Edition, 1992, in 4 volumes

Treatise on prokaryote taxonomy

- A more phylogenetically oriented, treatise on prokaryote taxonomy is the 2nd edition (1992) of The Prokaryotes; over 4,100 pages in 4 volumes.
- For the second edition in 1992, the editors made the decision to organize the chapters on the basis of the molecular phylogeny championed by Carl Woese, which increasingly provided a rational, evolutionary basis for the taxonomy of the prokaryotes.

Balows, A., H. G. Trüper, M. Dworkin, and K.-H. Schleifer (Eds.).1992. The Prokaryotes: A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications, 2nd ed. Springer-Verlag. New York, NY.

The Prokaryotes

A Handbook on the Biology of Bacteria

Third Edition, 2006, in 7 volumes

Product Details

7 Volume-Set- Part 1-4

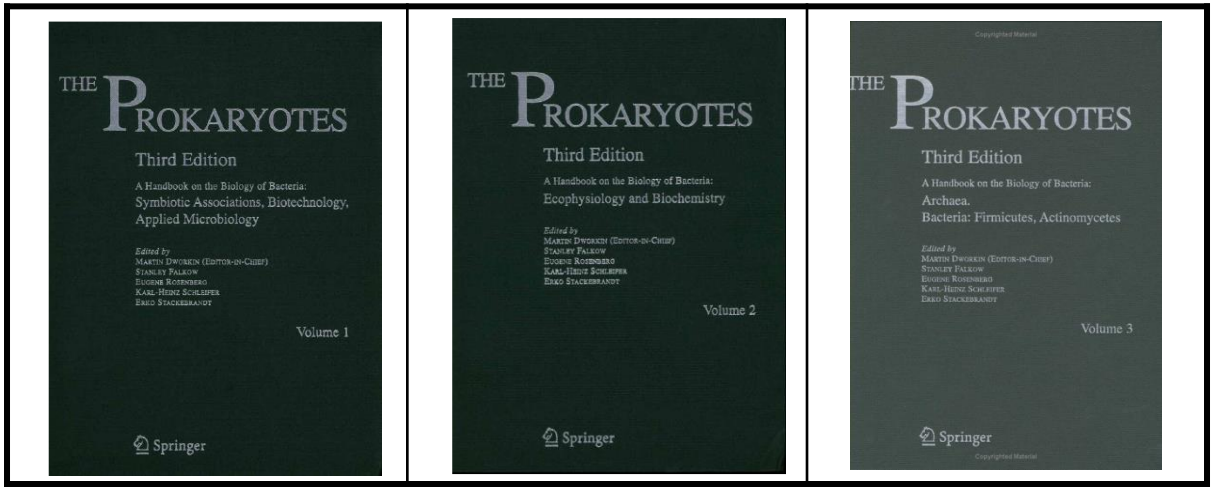
- Volume 1: Symbiotic Associations, Biotechnology, Applied Microbiology,959 pp.
- Volume 2: Ecophysiology and Biochemistry, 1107 pp.
- Volume 3: Archaea. Bacteria: Firmicutes, Actinomycetes, 1143 pp.
- Volume 4: Bacteria: Firmicutes, Cyanobacteria, 1140 pp.
- Volume 5: Proteobacteria: Alpha and Beta Subclasses, 919 pp.
- Volume 6: Proteobacteria: Gamma Subclass, 1193 pp.
- Volume 7: Proteobacteria: Delta and Epsilon Subclasses. Deeply Rooting Bacteria, 1073 pp.

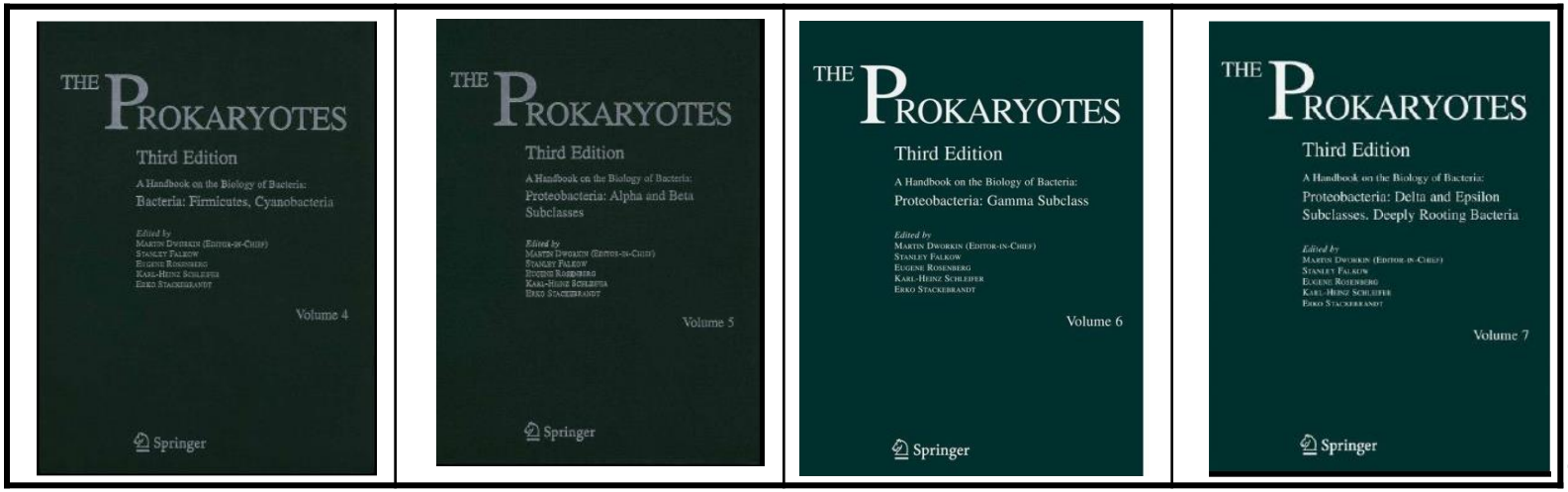
Dworkin, M., Stanley Falkow, Eugene Rosenberg, Karl-Heinz Schleifer, Erko Stackebrandt (Eds.).2006. The Prokaryotes: A Handbook on the Biology of Bacteria. 7 Volume-Set- Part 1-4. Springer Science+Business Media,Inc.

The Prokaryotes

A Handbook on the Biology of Bacteria

Third Edition, 2006

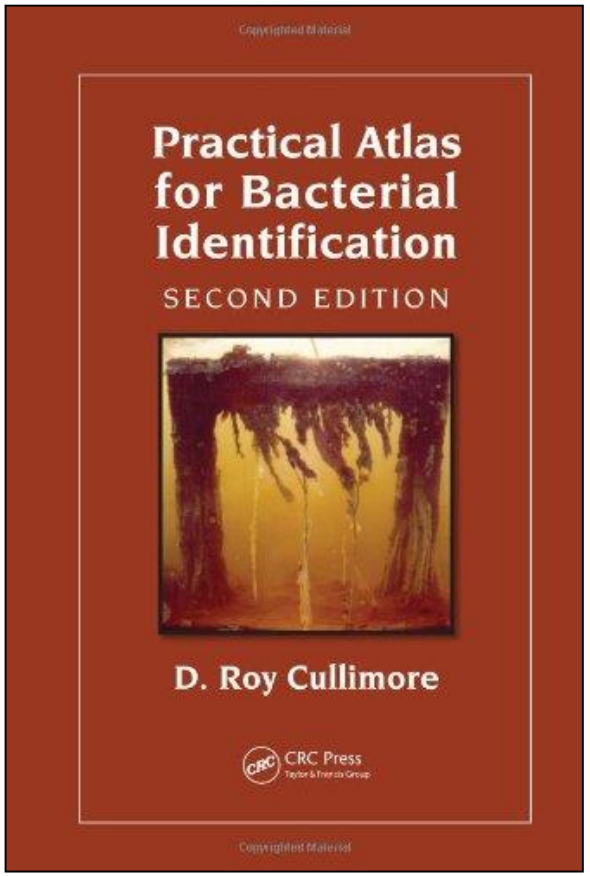




Practical Atlas for Bacterial Identification

Product Details

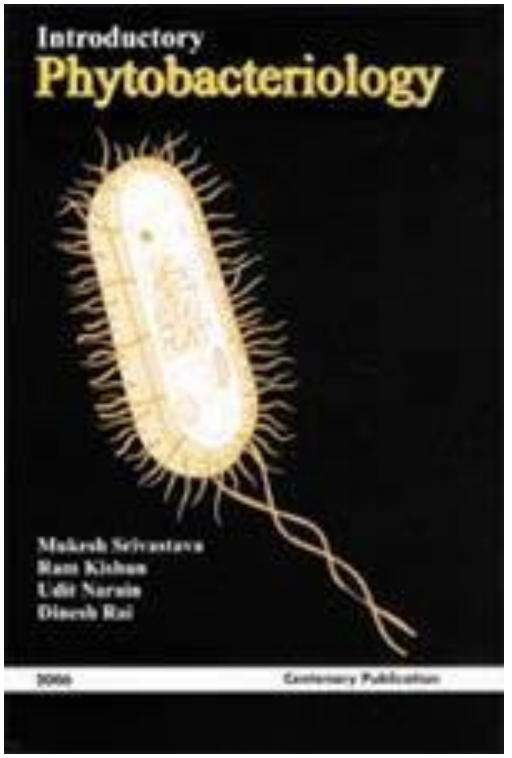
- **Practical Atlas for Bacterial Identification**
- D. Roy Cullimore
- Publisher: CRC Press; 2 edition (March 17, 2010).
- 327 pages.



Introductory Phytobacteriology

Product Details

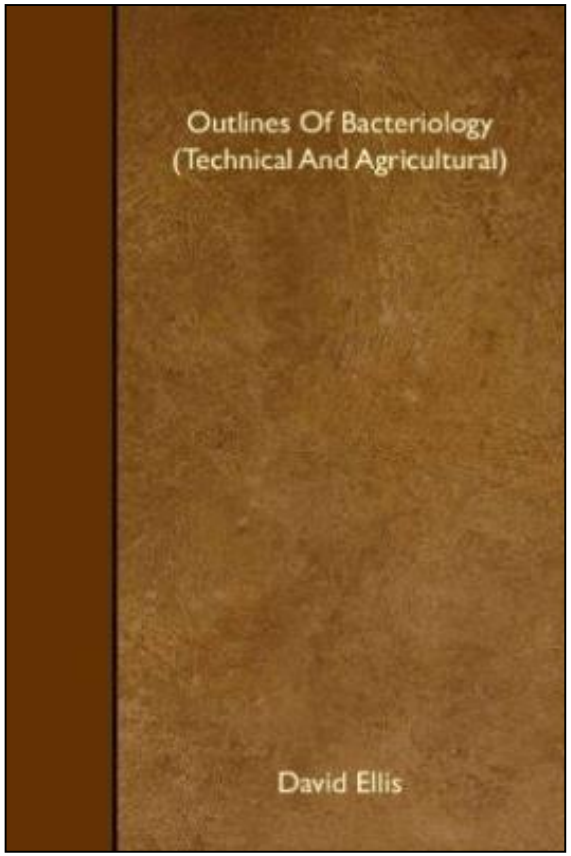
- **Introductory Phytobacteriology**
- Ram Kishun Udit Narain, Dinesh Rai and Mukesh Srivastava
- Pub. Date: Jan 2006, 1st ed.
- Publisher: Advance Publishing Concept
- 312 pp.



Outlines Of Bacteriology
Technical and Agricultural

Product Details

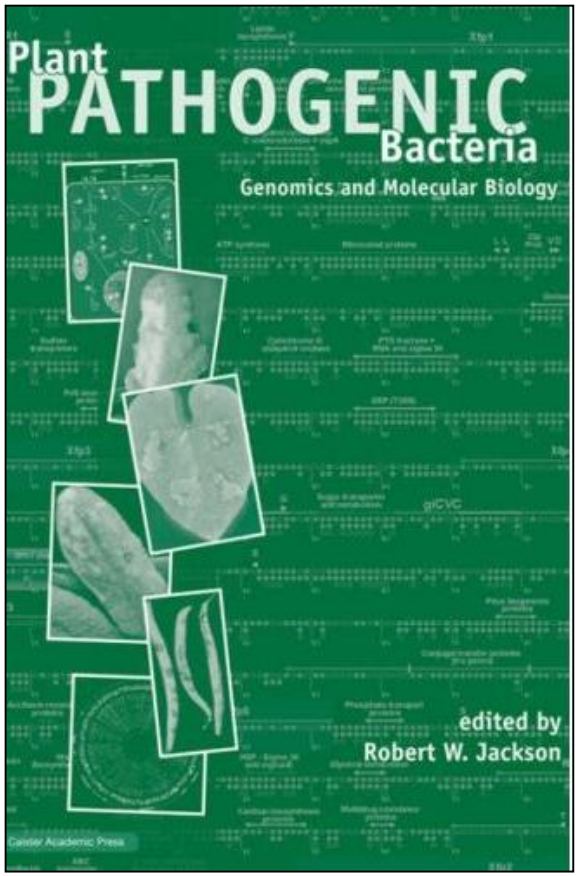
- **Outlines Of Bacteriology Technical And Agricultural**
- By D. Ellis
- Publisher: BiblioLife
- February 11, 2009
- Paperback: 276 pages



Plant Pathogenic Bacteria: Genomics and Molecular Biology

Product Details

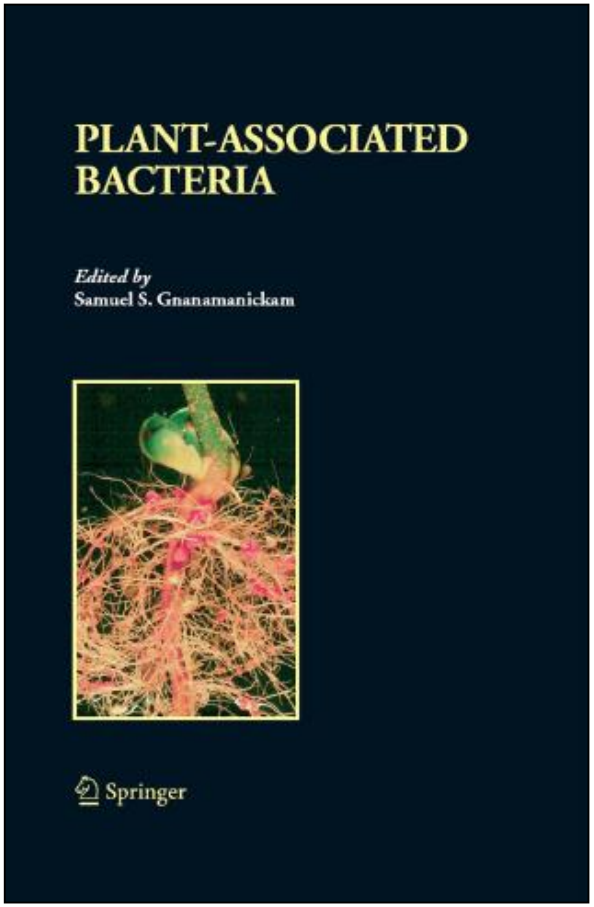
- **Plant Pathogenic Bacteria: Genomics and Molecular Biology**
- By Robert Jackson (Editor).
- Publisher: Caister Academic Press
- 2009
- 356 pages.



Plant-Associated Bacteria

Product Details

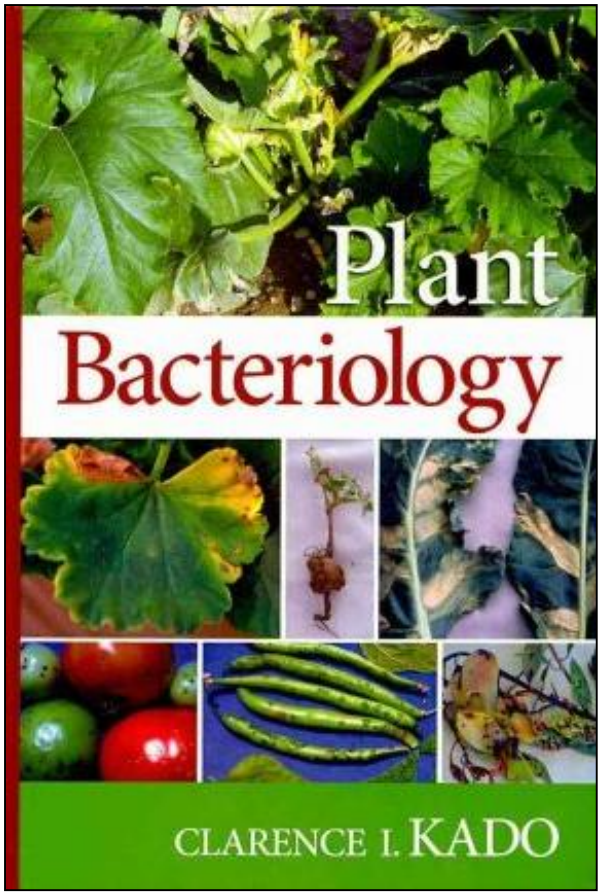
- **Plant-Associated Bacteria**
- Edited by Samuel S. Gnanamnickam
- University of Madras, Chennai, India
- 2006.
- Springer, The Netherlands.
- 712 pages.



Plant Bacteriology

Product Details

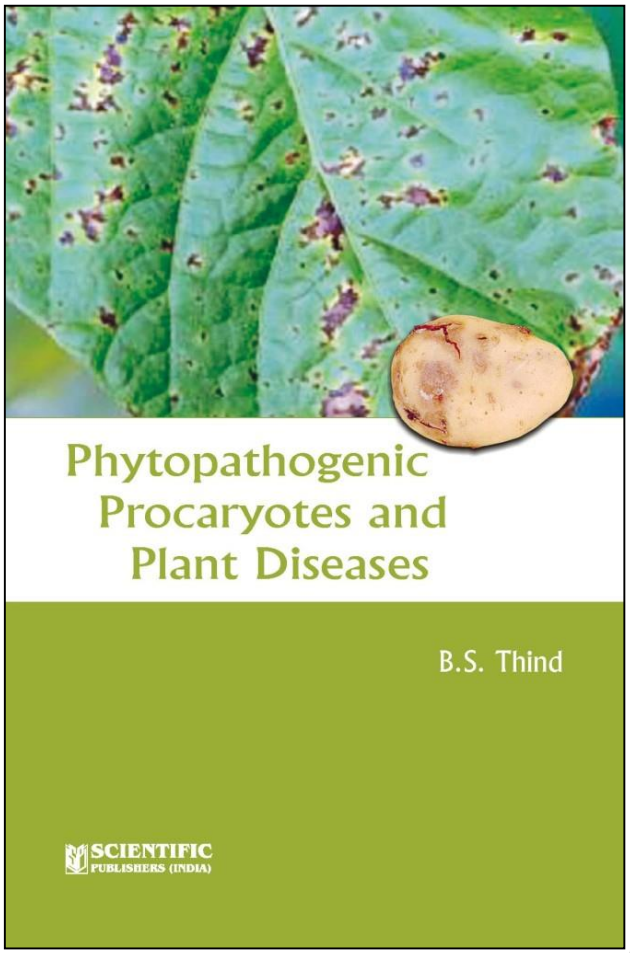
- **Plant Bacteriology**
- Clarence I. Kado
- American Phytopathological Society,
- 2010
- 336 pages



Phytopathogenic Procaryotes and Plant Diseases

Product Details

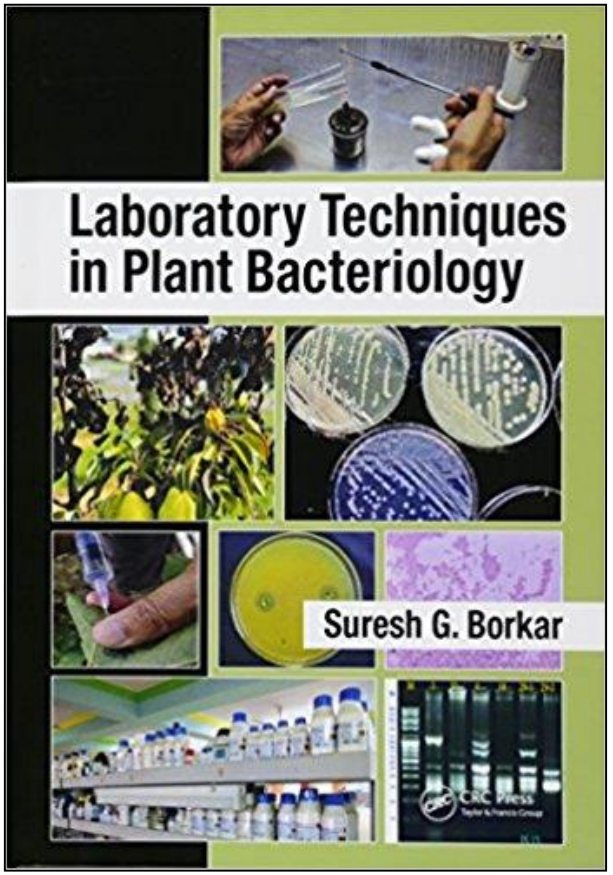
- **Phytopathogenic Procaryotes and Plant Diseases**
- B.S. Thind
- American Phytopathological Society,
- 2012
- 545 + 32 Colour Pages.



Laboratory Techniques in Plant Bacteriology

Product Details

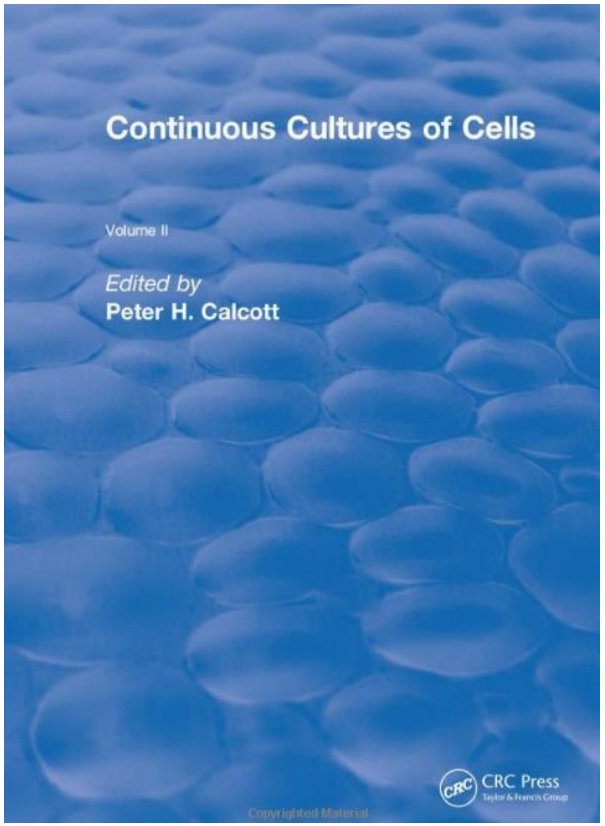
- **Laboratory Techniques in Plant Bacteriology**
- Suresh G. Borkar
- CRC Press
- 2017
- 342 Pages.



Continuous Cultures of Cells

Product Details

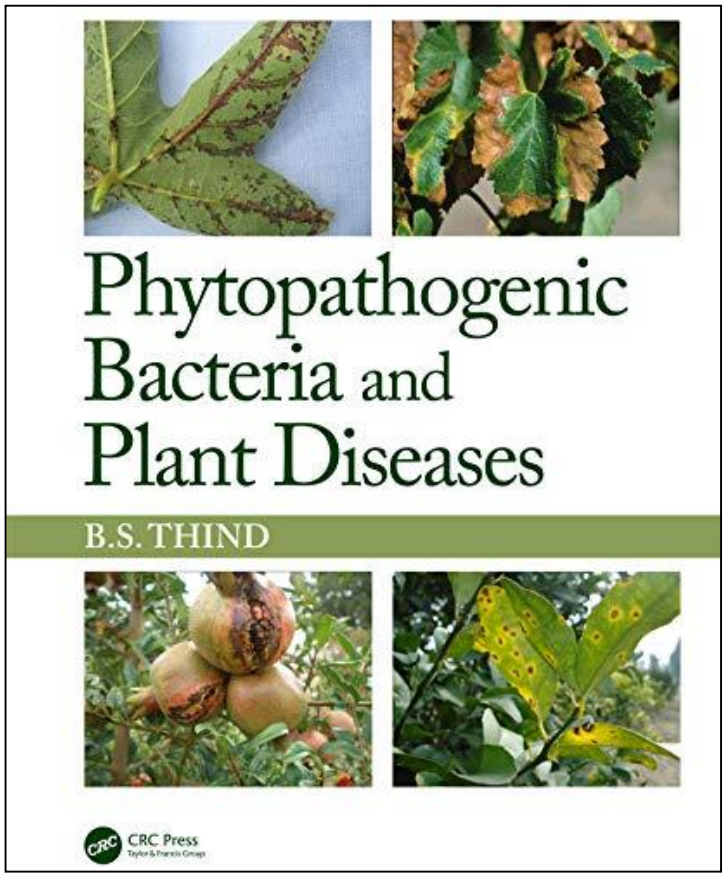
- **Continuous Cultures of Cells**
- Volume II (CRC Press Revivals)
- 1st Edition
- Peter H Calcott
- 2017
- 216 Pages.



Phytopathogenic Bacteria and Plant Diseases

Product Details

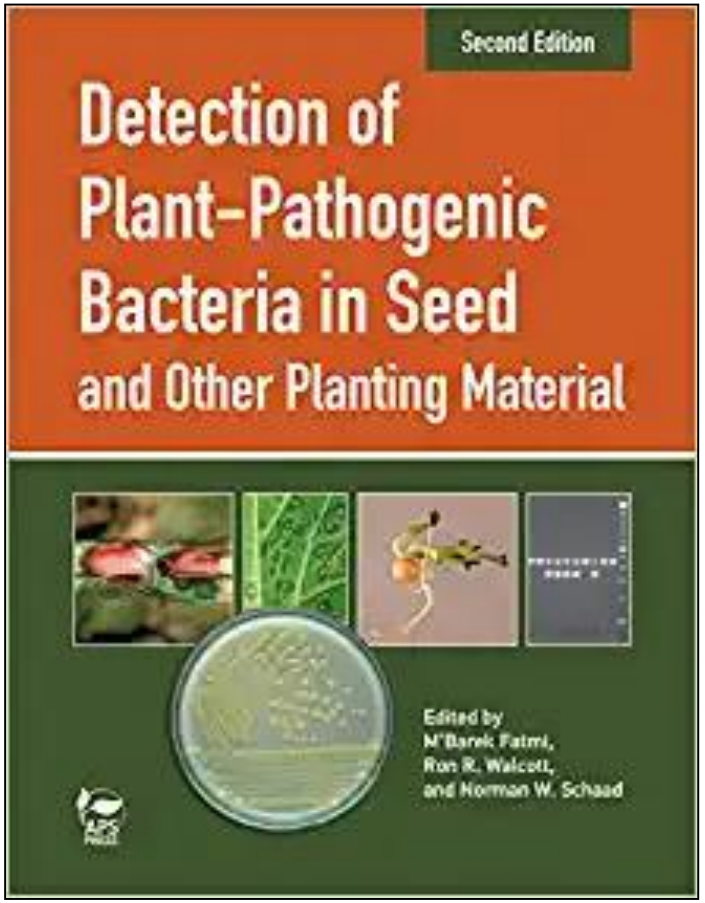
- **Phytopathogenic Bacteria and Plant Diseases**
- 1st Edition
- B.S. Thind
- 2017
- 398 Pages.



Detection of Plant-Pathogenic Bacteria in Seed and Other Planting Material

Product Details

- **Detection of Plant-Pathogenic Bacteria in Seed and Other Planting Material**
- Second Edition, APS
- Fatmi, M., R. R. Walcott and N.M. Schaad
- 2017
- 372 Pages.



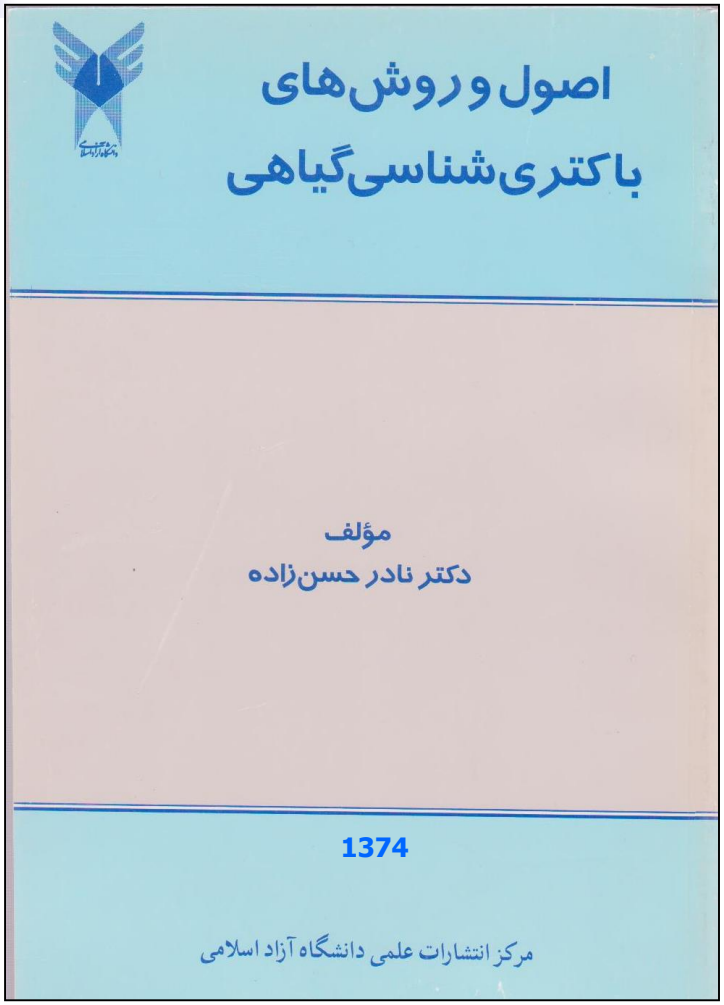
Advances in research on plant pathogenic bacteria

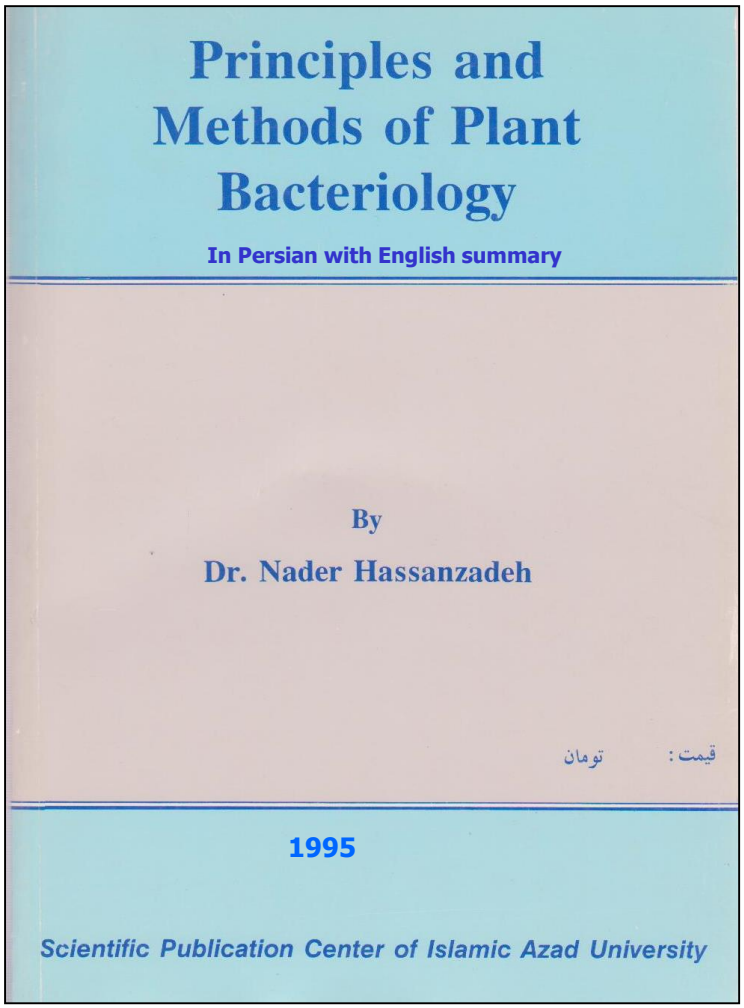
- Advances in research on plant pathogenic bacteria (International bioscience series).

Product Details

- Unknown Binding: 245 pages
- Publisher: Today & Tomorrow's Printers & Publishers
- 1988

Principles and Methods of Plant Bacteriology
N. Hassanzadeh
Scientific Publication of Islamic Azad University, 641 pp.

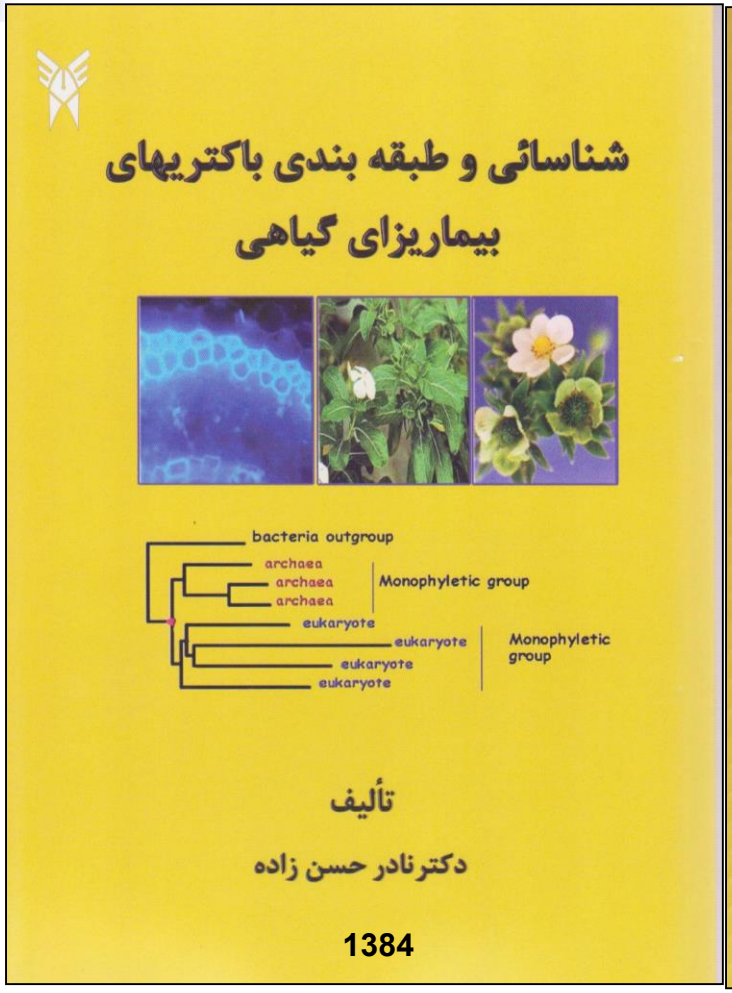


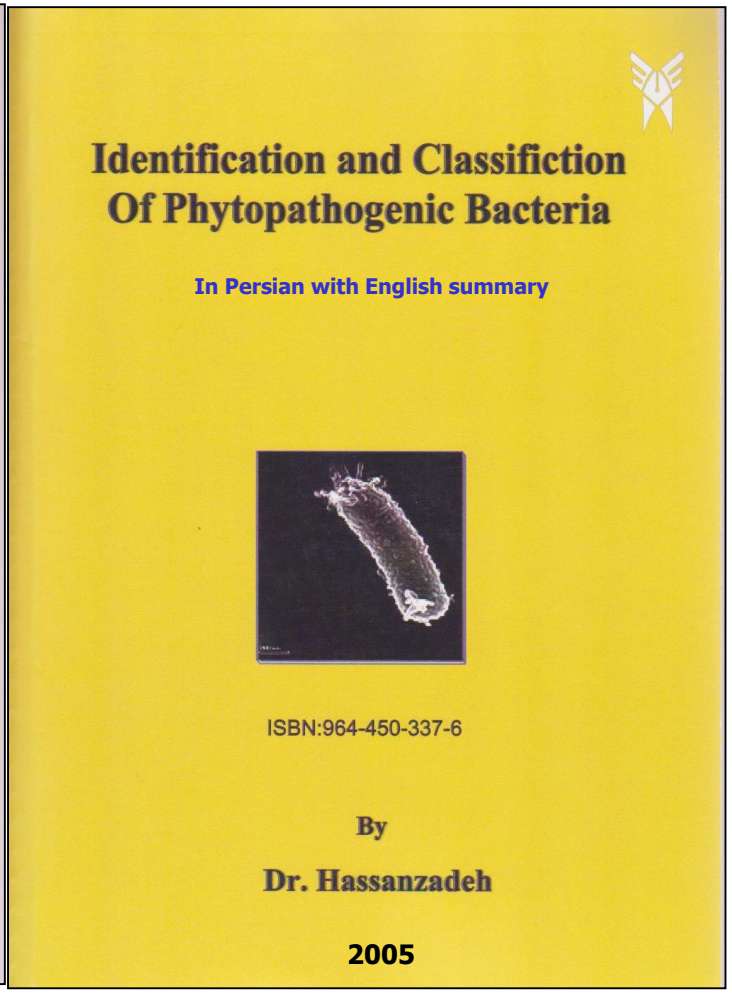


Identification and Classification of Phytopathogenic Bacteria

N. Hassanzadeh

Published by I. Azad University, Science and Research Branch, Tehran-Iran, 520 pp.





Proceedings/Reviews/Monographs/Book chapters/PowerPoints/PDF files

Plant Pathogenic Bacteria

Reviews/Monographs/Book chapters

- Abaca and Enset (ed. D.R. Jones).2000. Diseases of Banana. CAB International. Pp. 213-259.
- Alvarez, A.M. 2004. Integrated approaches for detection of plant pathogenic bacteria and diagnosis of bacterial diseases. Annu. Rev. Phytopathol. 42:339–66.
- Pérombelon, M.C.M. and J.M.,van der Wolf. 1998. Methods for the detection and quantification of *Erwinia carotovora* subsp.*atroseptica* on potatoes: A laboratory manual. Scottish Crop Research Institute Occasional Publication No. 10.
- Jones, J.B. 2006. Lecture 1- Phytobacteriology. 19 pages.
- Leben, C. 1974. Survival of plant pathogenic bacteria (Special circular 100), 21 pages, Ohio Agricultural Research and Development Center.
- Palacio-Bielsa, A., Cambra, M.A. and López, M.M. 2009. PCR detection and identification of plant pathogenic bacteria: updated review of protocols (1989-2007). Journal of Plant Pathology 91 (2), 249-297.
- Schaad, N.W. 2008. Emerging Plant Pathogenic Bacteria and Global Warming. In: Fatmi, M.B. editor. *Pseudomonas syringae* Pathovars and Related Pathogens. Dordrecht, The Netherlands: Springer Science + Business Media B.V. 369-379.
- Stead, D.E., Elphinstone, J.G., Weller, S.A., Smith, N.C. and Hennessy,J. 2000. Modern methods for characterising, identifying and detecting bacteria associated with plants. Acta Horticulturae 530: 45-59.

PowerPoints, PDF files, Lecture Notes

- Abedon, S.2011. Scope and history of microbiology. Chapter10 ID and classification of prokaryotes(Nester10) 2.51 MB. 33 slides.
- BacteriaFall2008.ppt. classes.plantpath.wsu.edu/plp429/lecture.
- Eric W. Brown. Chapter 2. Molecular differentiation of bacterial strains. Pp. 29-66.
- Cooper,J. 2006. Bacterial plant pathogens and symptomology.
- COST 873, StoneFruitNutHealth. Symptoms of bacterial diseases of nuts and stone fruits. CSL. pdf. www.atlasplantpathogenicbacteria.it.
- COST 873, StoneFruitNutHealth.2008 (Compiled by Elphinstone *et al.,* 2008). Short term training Mission-Plant Bacteriology Manual. Pdf file, 59 pp.
- COST 873, StoneFruitNutHealth.2010. *Pseudomonas* Pathogens of Stone Fruits and Nuts: Classical and Molecular Phytobacteriology. Pdf file, 71 pp.
- Cuppels.2007. Biology 418a.
- Diversity_1.pdf.2004. Microbial Diversity.
- Duffy, B, C. Pelludat, F. Pasquier, B. Frey, J.E. Frey. 2008. Diagnostic Microarrays. COST 873 Training Workshop, CSL, York UK.
- Fox, A.2008. Culture and identification of infectious agents.
- Fox, A.2008. Antibiotics affecting the cell envelope.
- Fox, A.2008. The Bacterial Cell.

PowerPoints, PDF files, Lecture Notes

- Ibrahim, N., A. Fataah and H. Reheem. Isolation of the bacteria *Clavibacter xyli* ssp *xyli* and production of polyclonal antibodies against it for detection of Ratoon stunting disease in Sugarcane crops. Sugarcane crops Ins., ARC, Giza, Egypt.
- Lab4-ss.pdf. Plant Disease diagnostics.
- Karlm.2004. Taxonomy.
- Keim Lab. Week3_ Exercise2_BC.ppt.
- Keller, B. Bre's Exercise 2.ppt.
- Mckay,T. 2011. Nonfermenting Gram Negative Rods. 20 slides,193 KB.
- Manceau,M.2008. Isolation, storage and management of plant pathogenic bacterial strains. COST 873,INRA, m_Manceau_StoringBacteria_CSL_1. pdf. Marshall, D.S. 2002. Plant diseases caused by bacteria.
- Mehan, V.K. and D. McDonald, 1995(eds). Techniques for diagnosis of *Pseudomonas solanacearum* and for resistance screening against groundnut bacterial wilt. ICRISAT, 68 pages.
- Miller, S. 2006. Diagnosing Plant Diseases Caused by Bacteria. IPDN Training Meeting, IITA, Cotonou, Benin. The Ohio state University.
- Nahson,J.B.2007. Plant disease diagnostic capacity and needs in crop production. Department of crop science and production, Tanzania.
- Nunney, L. Monitoring *Xylella fastidiosa* for changes in host range and virulence using genome-based methods. Dept of Biology, University of California, Riverside.

PowerPoints, PDF files, Lecture Notes

- Sridhar Rao P. N. Identification of bacteria. www.microrao.com. File Format: Shockwave Flash.
- Parkinson, N. Identifying Relatedness Between Bacterial Plant Pathogensm. Parkinson_MolID_CSL_1.pdf.
- Smith, J. Plant Pathogenic Bacteria. Characterization & Identification. eJulianIdentificationofbacteria. pdf.
- Stead, D. Classification and Nomenclature of Plant Pathogenic Bacteria - A Review(m_stead_CSL_1. pdf).
- Stead, D. Minimum requirements for diagnosis. (m_stead3_DiagnosticRequirements_CSL_1. pdf).
- Tavares *et al.*,2010. Genomic loci at the CUPID and Insignia intersections increase the reliability of phytopathogen-diagnostic markers. 31 slides,4.70 MB.
- Thwaites, R. DNA Fingerprinting of Bacteria. Plant Health Group, CSL (m_Thwaites_CSL_1. pdf).

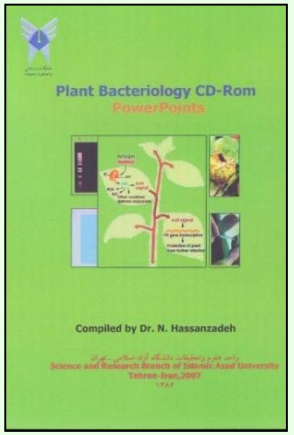
CDs in plant pathogenic bacteria

CD Presentations On Plant Pathogenic Bacteria

Plant Bacteriology CD-Rom

First version released in 2007

- **Plant Bacteriology CD-Rom(version I)**
- Hassanzadeh, N. 2007 (Compiled & Edited). Scientific Publication of Science and Research Branch of Islamic Azad University, 425 MB CD contains 4000 slides (In English).
- As a supplement to the Plant Bacteriology lecture the CD-Rom was presented in the form of eight PowerPoint Presentations:
  1. Bacterial disease symptoms
  2. Bacterial cell structure
  3. Bacterial diagnosis
  4. Bacterial classification
  5. Bacterial phylogeny
  6. Bacterial genetics
  7. Bacterial pathogenesis
  8. Bacterial disease management



- **Four appendices were also included:**
- Appendix 1. Names of Plant Pathogenic Bacteria (ISPP List) updated March 30,2004.
- Appendix 2. Bacterial Validation Lists updated March 02, 2007.
- Appendix 3. Video clips on bacterial motility, electrophoresis techniques, cloning, restriction enzymes, Northern Blot, PCR technique, signal transduction, horizontal gene transfer, DNA, etc.
- Appendix 4. Bacterial Phylogeny of Cavalier,2002.

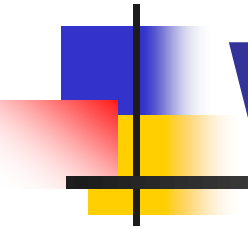
Plant Bacteriology CD-Rom
Second version released in 2009

- **Plant Bacteriology CD-Rom (version II).**
- Hassanzadeh, N. 2009 (Compiled & Edited). Scientific Publication of Science and Research Branch of Islamic Azad University,323 MB CD contains 5600 slides (In English).
- As a supplement to the plant bacteriology lecture, all old eight PowerPoint presentations were elaborated and included as follows:
  1. Bacterial disease symptoms
  2. Bacterial cell structure
  3. Bacterial diagnosis
  4. Bacterial classification
  5. Bacterial phylogeny
  6. Bacterial genetics
  7. Bacterial pathogenesis
  8. Bacterial disease management

- The current CD contains 5600 slides, showing considerable expansion in both quantity and quality of the slides.
- To quickly access the original files, all old files related to appendices and video clips were deliberately omitted.

Plant Diseases Caused by Bacteria:
An Image Database and Educational Resource CD
Released by APS in 2010

- **Plant Diseases Caused by Bacteria CD.**
- Milton N. Schroth, Eva I. Hecht-Poinar, and Anne M. Alvarez.2010 (Editors).
- The image database contains 1006 images of plants affected with bacteria diseases.
- The CD includes detailed pictures with a range of symptom types caused by bacterial diseases.
- In addition, this CD contains illustrated and detailed narratives on 34 major bacterial diseases and includes important information on various factors of the diseases: mode of transmission, host range, mechanisms of infection, survival, and management.
- APS PRESS, $259 Price.



Websites

Professional Websites on Plant Pathogenic Bacteria

Hasanzadeh's Website HomePage

www.phytobacteriology.com

Website HomePage (2009)

Placed single file: Plant Bacterial Diagnosis

Old Website HomePage (2010)

Placed files: Eight Microsoft PowerPoint files

Hasanzadeh's Website HomePage

www.phytobacteriology.com

Website HomePage (2011-12)

Placed files: Nine Microsoft PowerPoint files

Website HomePage (2013)

Placed files: The ten Microsoft PowerPoint files

Hasanzadeh's Website HomePage

www.phytobacteriology.com

Website HomePage (2014)

Placed files: Eleven Microsoft PowerPoint files

Website HomePage (2015)

Placed files: Twelve Microsoft PowerPoint files

Hasanzadeh's Website HomePage

www.phytobacteriology.com

Website HomePage (2016)

Placed files: Fourteen Microsoft PowerPoint files

Website HomePage (2017)

Placed files: Fiftheen Microsoft PowerPoint files

Hasanzadeh's Website HomePage

www.phytobacteriology.com

Website HomePage (2017)

Placed files: Fourteen Microsoft PowerPoint files

Website HomePage (2018)

Placed files: Fiftheen Microsoft PowerPoint files

Hasanzadeh's Website HomePage

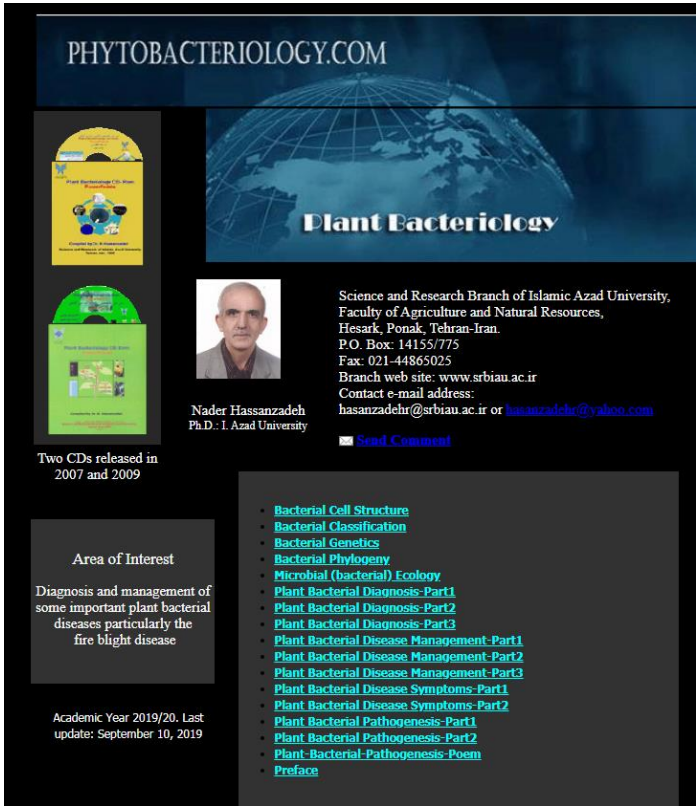
www.phytobacteriology.com

Website HomePage (2019)

Placed files: Fifteen Microsoft PowerPoint files

Website HomePage (2019)

Placed files: Sixteen Microsoft PowerPoint files



Hasanzadeh's Website HomePage

www.phytobacteriology.com

Website HomePage (2020)

Placed files: Sixteen Microsoft PowerPoint files

Website HomePage (2020)

Placed files: Sixteen Microsoft PowerPoint files

Hasanzadeh's Website HomePage

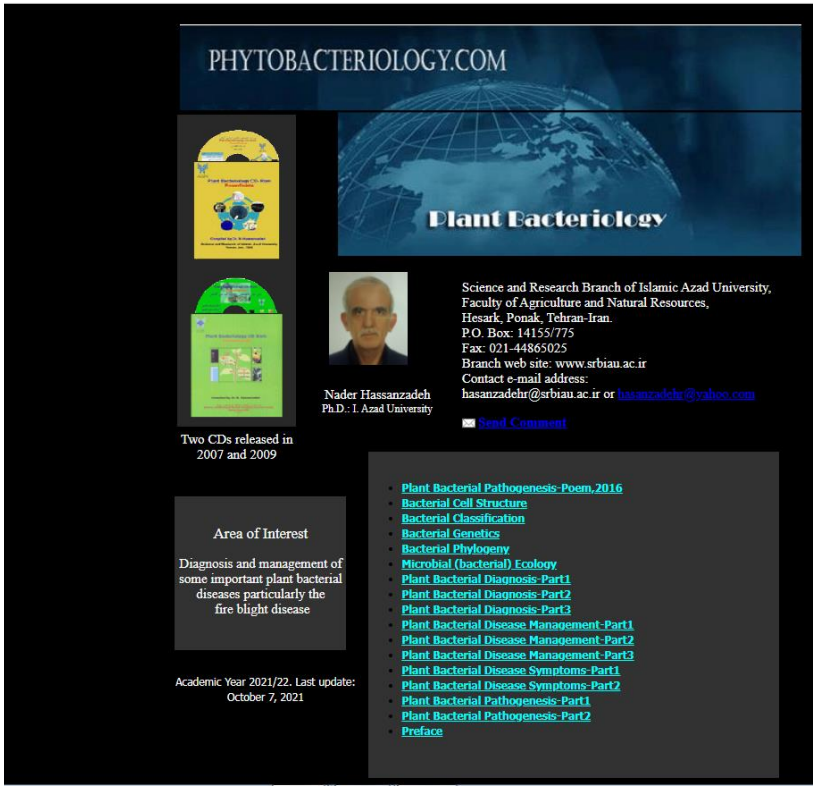
www.phytobacteriology.com

Website HomePage (2021)

Placed files: Sixteen Microsoft PowerPoint files

Website HomePage (2021)

Placed files: Sixteen Microsoft PowerPoint files

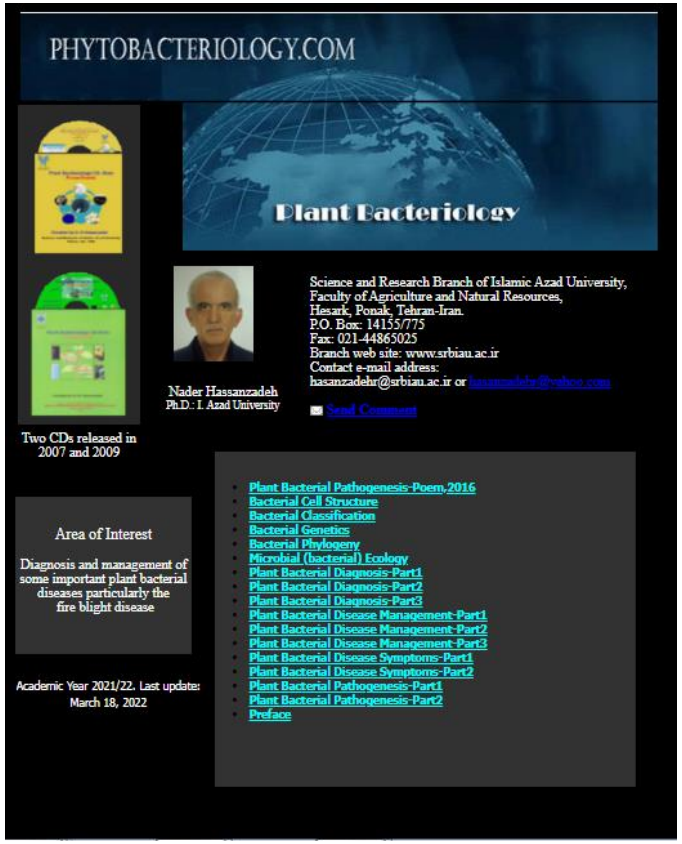


Hasanzadeh's Website HomePage

www.phytobacteriology.com

Website HomePage (2022)

Placed files: Seventeen Microsoft PowerPoint files



Website HomePage (2023)

Placed files: Seventeen Microsoft PowerPoint files

Ralstonia/ Bacterial wilt dedicated website

- Visit our *Ralstonia*/Bacterial wilt dedicated website:

  http://plantpath.ifas.ufl.edu/rsol/
- Pest and disease management guides
- Project description, accomplishments
- Real time pest alerts and first reports worldwide
- Protocols, book references and journal articles database
- Web resources
- Photo galleries
- Access to *Ralstonia*-mailing list.

Champoiseau *et al.*,2009

Plant Bacteriology Website

Hawaii University

Home page

Plant Bacteriology

University of Hawaii at Manoa

Welcome to the website of Dr. Anne Alvarez at the University of Hawaii at Manoa. Hopefully, this website will serve as a useful resource for those interested in plant bacteriology and related fields.

Please select from the following links to quickly enter the portion of the site you're interested in.

Happy surfing!

Here you will find the research and teaching tools (courses and student resources) at the University of Hawaii Department of Plant and Environmental Protection Sciences.

This site was created in the spirit of open, shared information. The creator of this site shall not be held liable for material contributed or posted by other authors.

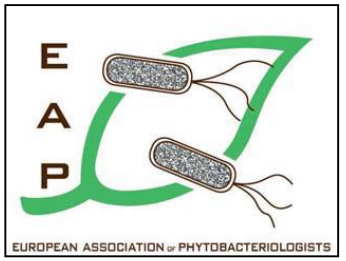
Dedicated to protecting agricultural and food industries from the deliberate or accidental introduction of plant and animals pests and diseases.

Bacterial Plant Pathogen Websites of Interest

Bacterial Plant Pathogen Websites of Interest		
Bacterial Nomenclature	Bacterial Nomenclature Up-to-date	http://www.dsmz.de/bactnom/bactname.htm
	List of Bacterial Names with Standing in Nomenclature	http://www.bacterio.cict.fr/
	Bergey's Manual of Systematic Bacteriology 2nd Edition – Taxonomic Outline of the Procaryotes	http://dx.doi.org/10.1007/bergeysoutline200210
Microbiology	Cells Alive	http://cellsalive.com/
	Microbe World	http://www.microbeworld.org/
	Bacteria Museum	http://www.bacteriamuseum.org
	The Microbiology Information Portal	http://www.microbes.info/
Plant Pathology	Plant Path Internet Guide Book	http://www.pk.uni-bonn.de/ppibg/ppigb.htm
	Plant Disease Control Picture Index	http://plant-disease.ippc.orst.edu/image_index.cfm

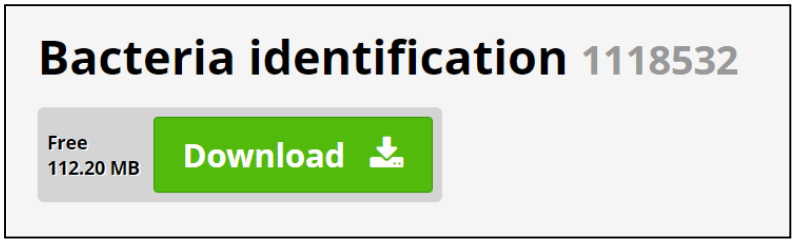
APS,2015

The European Association of Phytobacteriologists
Set up early 2007



- The Chair of the EAP is J.D. Janse, phytobacteriologist at the General Inspection Service (NAK), The Netherlands.
- The objectives of the EAP are:

1. Development, improvement and implementation of diagnosis, detection, identification and classification of bacterial plant pathogens.
2. Education and training in principles and practice of phytobacteriology.
3. Provision of advice and expertise to plant health regulatory bodies.
4. Promotion of technical and scientific collaboration.
5. Exchange of information through communication networking.



The Website ABIS

ABIS online
Automated Biometric Identification System

www.microrao.com/identify.htm

ABIS online

Automated Biometric Identification System

An Advanced Bacterial Identification Software

- **History**
  The project started in 2003 as a MS-DOS-based program for clinically significant Enterobacteriaceae identification, using biochemical characters, morphology, growth conditions, ecology and pathogenicity data.

ABIS online

Automated Biometric Identification System

An Advanced Bacterial Identification Software

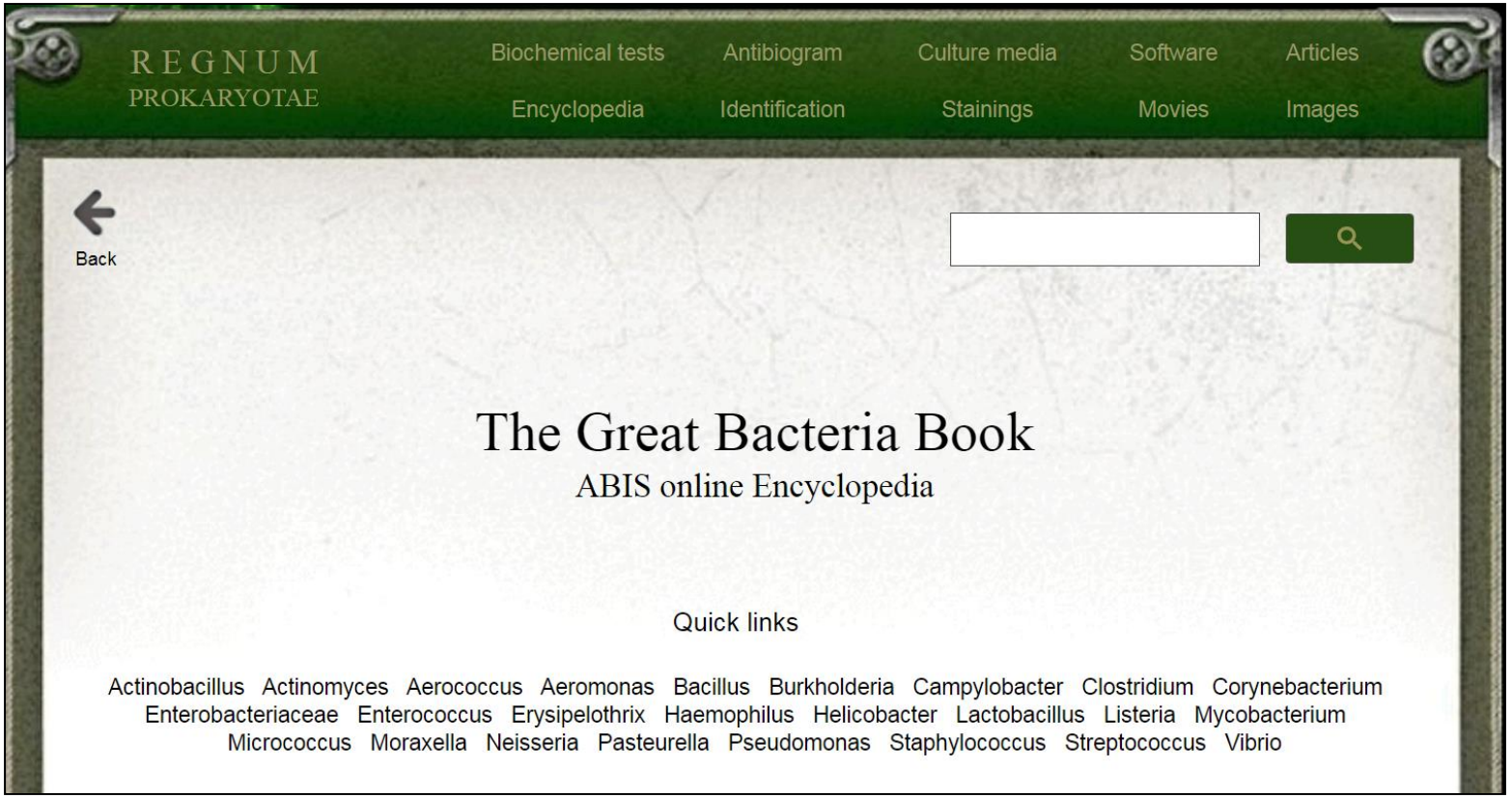
- ABIS online is a laboratory tool for bacterial identification, based on morpho-biochemical characters, cultural characteristics, growth conditions, ecology & pathogenicity data.
- Main identifiable taxa:
- Enterobacteriaceae, Pasteurellaceae, Campylobacteraceae, Bacillaceae, Lactobacillus, Staphylococcus, Streptococcus, Clostridium, Vibrio, Listeria, Erysipelothrix, Mycobacterium, Corynebacterium, Neisseria, Chromobacterium, Aeromonas, Pseudomonas and related species.
- The number of taxa included in database is constantly expanding, and at the time I wrote this article, the database had exceeded 1,900 species.

ABIS online Encyclopedia - ABIS Encyclopedia,2021

ABIS online

Automated Biometric Identification System

An Advanced Bacterial Identification Software



ABIS online Encyclopedia - ABIS Encyclopedia,2021

ABIS online

Automated Biometric Identification System

Library

The Great Bacteria Book

ABIS online Encyclopedia

Quick links

Actinobacillus Actinomyces Aerococcus Aeromonas Bacillus Burkholderia Campylobacter Clostridium Corynebacterium Enterobacteriaceae Enterococcus Erysipelothrix Haemophilus Helicobacter Lactobacillus Listeria Mycobacterium Micrococcus Moraxella Neisseria Pasteurella Pseudomonas Staphylococcus Streptococcus Vibrio

Alphabetical index

A
Abiotrophia
Abyssicoccus
Achromobacter
Acidovorax
Acinetobacter
Actinobacillus
Actinobaculum
Actinomyces
Actinotignum
Advenella
Aerococcus
Aeromonas
Aggregatibacter
Agrilactobacillus
Alcaligenes
Aliicoccus
Aliivibrio
Allobaculum
Alysiella
Amantichitinum
Ampullimonas
Amylolactobacillus
Anaerobacillus
Andreprevotia
Aneurinibacillus
Apilactobacillus
Aquaspirillum
Aquitalea
Arcanobacterium
Arcobacter
Arthrobacter
Arsenophonus
Auricoccus
Avibacterium
Azohydromonas

B
Bacillus
Bavariicoccus
Bergeriella
Bibersteinia
Blautia
Bombilactobacillus
Bordetella
Brackiella
Brenneria
Brevibacillus
Brevibacterium
Brevundimonas
Brochothrix
Budvicia
Bulleidia
Burkholderia
Buttiauxella

C
Caballeronia

G
Gallibacterium
Gemella
Geobacillus
Globicatella
Glutamicibacter
Grimontia
Gulbenkiania

H
Hafnia
Haematomicrobium
Haemophilus
Helicobacter
Holdemania
Holzapfelia
Hydromonas

I
Ignavigranum
Iodobacter

J
Jeongeupia
Jeotgalicoccus

K
Kerstersia
Kingella
Klebsiella
Kluyvera
Kocuria
Kyrpidia

L
Lacticaseibacillus
Lactiplantibacillus
Lactobacillus
Lactococcus
Lactovum
Lapidilactobacillus
Laribacter
Latilactobacillus
Lautropia
Leclercia
Leeia
Leminorella
Lentilactobacillus
Levilactobacillus
Ligilactobacillus
Limnobacter
Limosilactobacillus
Liquorilactobacillus
Listeria
Listonella
Loigolactobacillus
Lysinibacillus

Q
Quisquiliibacterium

R
Rahnella
Raoultella
Ralstonia
Rathayibacter
Riemerella
Rivicola
Robbsia
Rummeliibacillus

S
Saccharedens
Salimicrobium
Salinicoccus
Salinivibrio
Salmonella
Sanguibacter
Schleiferilactobacillus
Secundilactobacillus
Serratia
Shigella
Shimwellia
Silvimonas
Simonsiella
Sinomonas
Snodgrassella
Solibacillus
Solobacterium
Sphingomonas
Sporosarcina
Staphylococcus
Stenotrophomonas
Stenoxybacter
Streptococcus
Sulfurospirillum

T
Tatumella
Taylorella
Tetragenococcus
Thermothrix
Tolumonas
Trabulsiella
Trueperella
Turicella
Turicibacter

U
Uruburuella

V
Vagococcus
Varibaculum
Verticiella
Vibrio
Vitreoscilla
Virgibacillus
Viridibacillus
Vogesella
Volucribacter

W
Wolinella

X
Xenorhabdus

Y
Yersinia
Yokenella

Bulleidia
Burkholderia
Buttiauxella

C
Caballeronia
Caenimicrobium
Campylobacter
Candidimonas
Castellaniella
Catellicoccus
Cellulosilyticum
Cedecea
Chitinibacter
Chitinilyticum
Chitinimonas
Chitiniphilus
Chromobacterium
Citricoccus
Citrobacter
Clavibacter
Clostridium
Comamonas
Companilactobacillus
Conchiformibius
Corticicoccus
Corynebacterium
Cosenzaea
Crenobacter
Cupriavidus

D
Deefgea
Dellaglioa
Dermabacter
Derxia
Dickeya
Dolosicoccus

E
Edwardsiella
Eikenella
Enteric Groups
Enterobacter
Enterococcus
Enterovibrio
Eoetvoesia
Eremococcus
Erysipelothrix
Erwinia
Escherichia
Ewingella

F
Facklamia
Falsarthrobacter
Flaviflexus
Flavonifractor
Formivibrio
Formosimonas
Fructilactobacillus
Furfurilactobacillus

Liquorilactobacillus
Listeria
Listonella
Loigolactobacillus
Lysinibacillus

M
Macrococcus
Mannheimia
Melissococcus
Microbacterium
Micrococcus
Microvirgula
Mobiluncus
Moellerella
Moraxella (Branhamella)
Morganella
Morococcus
Mycoavidus
Mycobacterium

N
Neisseria
Neomicrococcus
Nosocomiicoccus

O
Obesumbacterium
Oceanimonas
Oligella

P
Paenalcaligenes
Paenarthrobacter
Paenibacillus
Paeniglutamicibacter
Paludibacterium
Pandoraea
Pantoea
Paralcaligenes
Paraburkholderia
Paracandidimonas
Parapusillimonas
Pasteurella
Paucilactobacillus
Pectobacterium
Pediococcus
Pelistega
Pelomonas
Phocoenobacter
Photobacterium
Photorhabdus
Pigmentiphaga
Pilibacter
Plesiomonas
Polynucleobacter
Pragia
Proilnoborus
Proteus
Providencia
Pseudarthrobacter
Pseudoglutamicibacter
Pseudogulbenkiania
Pseudomonas
Psychrobacillus
Pullulanibacillus
Pusillimonas

V
Vagococcus
Varibaculum
Verticiella
Vibrio
Vitreoscilla
Virgibacillus
Viridibacillus
Vogesella
Volucribacter

W
Wolinella

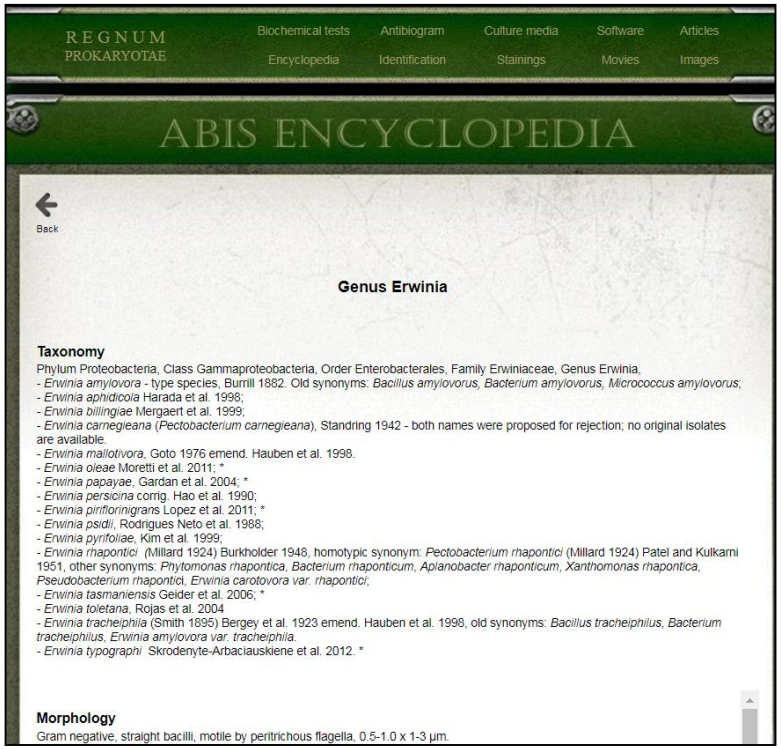
X
Xenorhabdus

Y
Yersinia
Yokenella

ABIS online

Automated Biometric Identification System

Genus: *Erwinia*



REGNUM PROKARYOTAE

Biochemical tests | Antibiogram | Culture media | Software | Articles
Encyclopedia | Identification | Stainings | Movies | Images

ABIS ENCYCLOPEDIA

Back

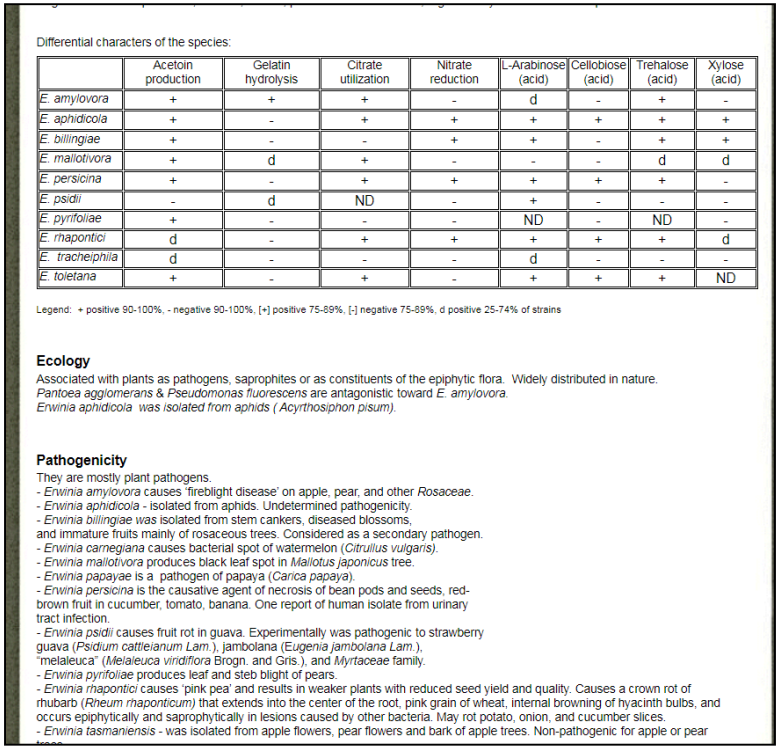
Genus Erwinia

Taxonomy

Phylum Proteobacteria, Class Gammaproteobacteria, Order Enterobacterales, Family Erwiniaceae, Genus Erwinia,
- *Erwinia amylovora* - type species, Burrill 1882. Old synonyms: *Bacillus amylovorus, Bacterium amylovorus, Micrococcus amylovorus*;
- *Erwinia aphidicola* Harada et al. 1998;
- *Erwinia billingiae* Mergaert et al. 1999;
- *Erwinia carnegieana* (*Pectobacterium carnegieana*), Standring 1942 - both names were proposed for rejection; no original isolates are available.
- *Erwinia mallotivora*, Goto 1976 emend. Hauben et al. 1998.
- *Erwinia oleae* Moretti et al. 2011; *
- *Erwinia papayae*, Gardan et al. 2004; *
- *Erwinia persicina* corrig. Hao et al. 1990;
- *Erwinia piriflorinigrans* Lopez et al. 2011; *
- *Erwinia psidii*, Rodrigues Neto et al. 1988;
- *Erwinia pyrifoliae*, Kim et al. 1999;
- *Erwinia rhapontici* (Millard 1924) Burkholder 1948, homotypic synonym: *Pectobacterium rhapontici* (Millard 1924) Patel and Kulkarni 1951, other synonyms: *Phytomonas rhapontica, Bacterium rhaponticum, Aplanobacter rhaponticum, Xanthomonas rhapontica, Pseudobacterium rhapontici, Erwinia carotovora var. rhapontici*;
- *Erwinia tasmaniensis* Geider et al. 2006; *
- *Erwinia toletana*, Rojas et al. 2004
- *Erwinia tracheiphila* (Smith 1895) Bergey et al. 1923 emend. Hauben et al. 1998, old synonyms: *Bacillus tracheiphilus, Bacterium tracheiphilus, Erwinia amylovora var. tracheiphila.*
- *Erwinia typographi* Skrodenyte-Arbaciauskiene et al. 2012. *

Morphology

Gram negative, straight bacilli, motile by peritrichous flagella, 0.5-1.0 x 1-3 µm.



Differential characters of the species:

	Acetoin production	Gelatin hydrolysis	Citrate utilization	Nitrate reduction	L-Arabinose (acid)	Cellobiose (acid)	Trehalose (acid)	Xylose (acid)
E. amylovora	+	+	+	-	d	-	+	-
E. aphidicola	+	-	+	+	+	+	+	+
E. billingiae	+	-	-	+	+	-	+	+
E. mallotivora	+	d	+	-	-	-	d	d
E. persicina	+	-	+	+	+	+	+	-
E. psidii	-	d	ND	-	+	-	-	-
E. pyrifoliae	+	-	-	-	ND	-	ND	-
E. rhapontici	d	-	+	+	+	+	+	d
E. tracheiphila	d	-	-	-	d	-	-	-
E. toletana	+	-	+	-	+	+	+	ND

Legend: + positive 90-100%, - negative 90-100%, [+] positive 75-89%, [-] negative 75-89%, d positive 25-74% of strains

Ecology

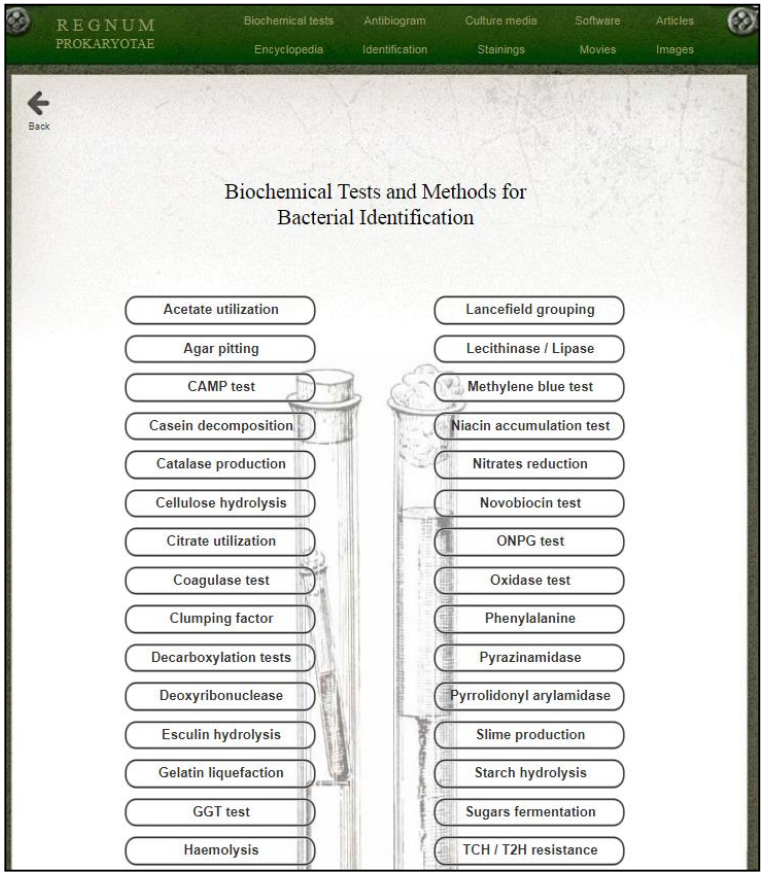
Associated with plants as pathogens, saprophites or as constituents of the epiphytic flora. Widely distributed in nature.
Pantoea agglomerans & *Pseudomonas fluorescens* are antagonistic toward *E. amylovora*.
Erwinia aphidicola was isolated from aphids (Acyrthosiphon pisum).

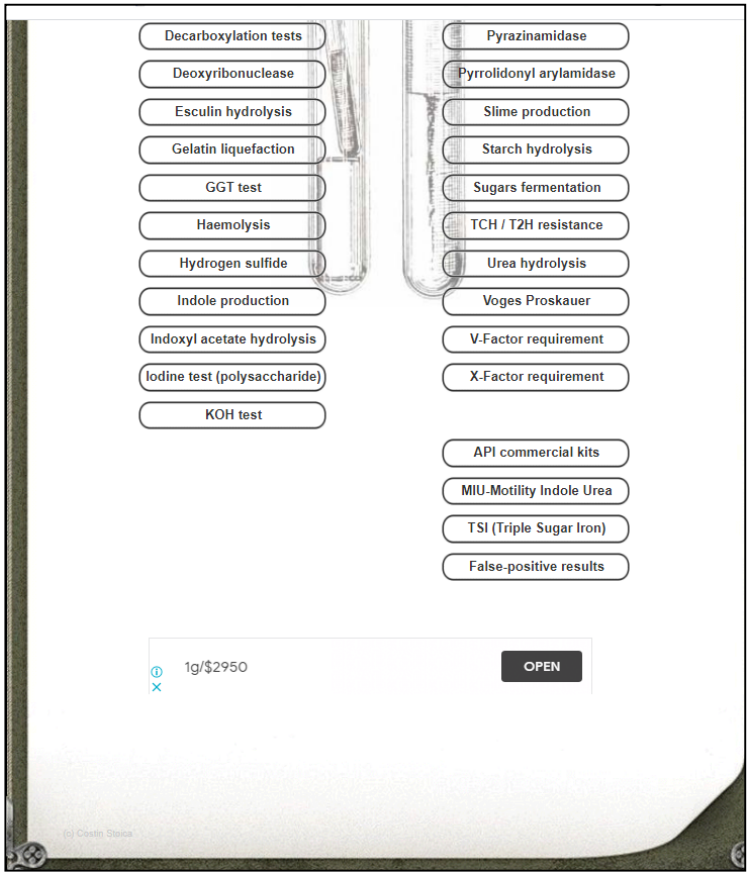
Pathogenicity

They are mostly plant pathogens.
- *Erwinia amylovora* causes 'fireblight disease' on apple, pear, and other *Rosaceae*.
- *Erwinia aphidicola* - isolated from aphids. Undetermined pathogenicity.
- *Erwinia billingiae was* isolated from stem cankers, diseased blossoms, and immature fruits mainly of rosaceous trees. Considered as a secondary pathogen.
- *Erwinia carnegiana* causes bacterial spot of watermelon (*Citrullus vulgaris).*
- *Erwinia mallotivora* produces black leaf spot in *Mallotus japonicus* tree.
- *Erwinia papayae* is a pathogen of papaya (*Carica papaya*).
- *Erwinia persicina* is the causative agent of necrosis of bean pods and seeds, red-brown fruit in cucumber, tomato, banana. One report of human isolate from urinary tract infection.
- *Erwinia psidii* causes fruit rot in guava. Experimentally was pathogenic to strawberry guava (*Psidium cattleianum Lam.*), jambolana (*Eugenia jambolana Lam.*), "melaleuca" (*Melaleuca viridiflora* Brogn. and Gris.), and *Myrtaceae* family.
- *Erwinia pyrifoliae* produces leaf and steb blight of pears.
- *Erwinia rhapontici* causes 'pink pea' and results in weaker plants with reduced seed yield and quality. Causes a crown rot of rhubarb (*Rheum rhaponticum)* that extends into the center of the root, pink grain of wheat, internal browning of hyacinth bulbs, and occurs epiphytically and saprophytically in lesions caused by other bacteria. May rot potato, onion, and cucumber slices.
- *Erwinia tasmaniensis* - was isolated from apple flowers, pear flowers and bark of apple trees. Non-pathogenic for apple or pear

ABIS online

List of biochemical tests used





ABIS online

Requests for adequate identification

A recommendation from a reader on Research Gate

- Putting your biochemical results into PIBWIN software your organism is identified as *Escherichia blatae*.
- But some more biochemical tests will be required for accurate identification.
- You can try on your own with this software (freely available in net) also you can put all your results into another online identification software named ABIS online.
- I think this will solve your purpose.

ABIS online

Automated Biometric Identification System

Comparative reference strains identification between ABIS online and apiweb™ software

#	Strain name and code	ABIS results id %[a]	apiweb™ results id %[b]
1	*Staphylococcus aureus* ATCC 25293	*Staphylococcus aureus* subsp. *aureus* 99%	*Staphylococcus aureus* 97.8%
2	*Helicobacter pylori* ATCC 43504	*Helicobacter pylori* 94%	*Helicobacter pylori* 99.9%
3	*Bacillus thuringiensis* NCIMB 9134	*Bacillus thuringiensis* 93%	*Bacillus cereus*, possibility of *B. thuringiensis* 98.9%
10	*Escherichia coli* ATCC 8739	*Escherichia coli* 98%	*Escherichia coli* 99.9%
14	*Pseudomonas aeruginosa* ATCC	*Pseudomonas aeruginosa* 92%	*Pseudomonas aeruginosa* 98.1%
16	*Lactobacillus paracasei* CCM 1837	*Lactobacillus paracasei* subsp. *paracasei* 94%	*Lactobacillus paracasei* subsp. *paracasei* 48.0 / 51.0%

Legend: [a]- ABIS %id represents the percentage of similarity with taxa from the database, all databases containing a matrix where probabilistic incidence values are allocated for every taxon and their corresponding morpho-biochemical characters; [b]- apiweb™ %id (API web) is a probabilistic calculation using bioMerieux own system procedure.

Costin and Ionut,2017

The history of phytobacteriology

The pioneers

The history of phytobacteriology

The pioneers

Scientists to Remember

Janse,2010

The history of phytobacteriology

The pioneers

Scientists to Remember

- E. F. Smith (U.S.)- Father of Phytobacteriology. Described 20 different bacterial diseases.
- A. de Bary (1831-1888), the Father of Plant Pathology and his students like A. Fischer believed that bacteria did not cause disease or they were unimportant.
- Fischer/Smith controversy
- Thomas Burrill (1878), showed that the fire blight of pear was caused by a bacterium which he identified as *Micrococcus amylovorus*.
- J.H. Wakker (1883) in Holland, who discovered the *Bacillus* which produces the "yellows" of the hyacinth.
- J. C. Arthur (1885-1887), confirmed Burrill's work during 1885-1887 using pure culture of the bacterium.
- L. Savastano (1886) (Italy) - Demonstrated that a bacterium incited olive knot.
- L. R. Jones (1910). Isolated *Erwinia carotovora* (*Pectobacterium*) from soft rotting vegetables.

B. Schreoder;Thind,2013

The history of phytobacteriology

The pioneers

Those who ignore history are condemned to repeat it (Prof. Don E. Ellis)

- There is an open question and controversies in the History of the Plant Pathology concerning who was the first to report a plant disease caused by a bacterium.

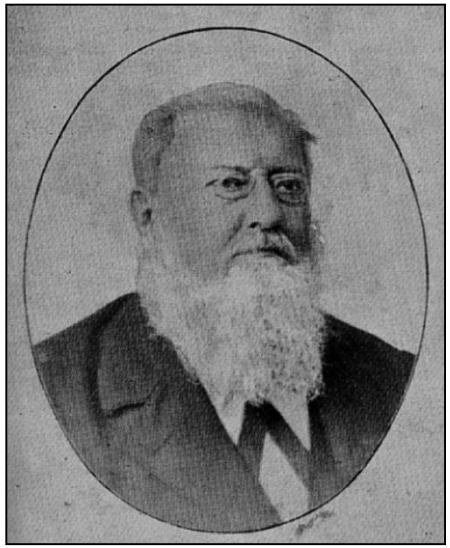
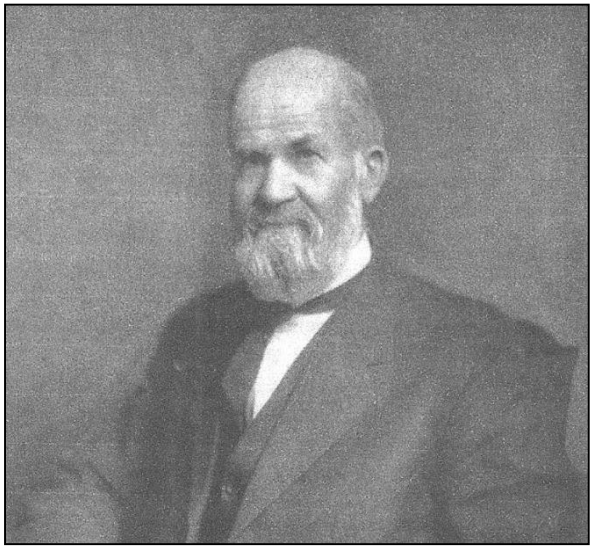
Kennedy, B.W., Widin, K.D. and Baker, I.S.F. (1979). Bacteria as the cause of disease in plants: A historical perspective. ASM News 45: 1-5.

Kennedy *et al.*,1979; Moura,2010

The history of phytobacteriology

The pioneers

Those who ignore history are condemned to repeat it (Prof. Don E. Ellis)

	
Frederic Murices Draenert, a German botanic, considered by the majority of the Brazilian plant pathologists the first to report a plant bacteria disease in the world. He reported(1869) the causal agent of sugarcane disease(gummosis) as caused the plant cells. At that time the bacteria were considered plants.	Thomas Jonathan Burrill, an American bacteriologist who was referred by the international phytopathological literature as the first to describe a plant bacteria disease in the world. His two publications was about the etiology of pear and apple blight (Burril, 1877 and 1878).

Moura,2010

The history of phytobacteriology

The pioneers

Those who ignore history are condemned to repeat it (Prof. Don E. Ellis)

- Other believe that bacteria can cause plant diseases was discovered almost simultaneously in four different countries, with the United States claiming first honors.

1. In 1878, Professor T. J. Burrill of the University of Illinois (USA) was the first to report a plant disease caused by a bacterium. His two publications on the etiology of pear and apple blight (Burril, 1877; 1878) advanced the theory that fire blight of apple and pear was due to the bacteria.
2. In 1879, the French scientist Prillieux published a paper on bacteria as the cause of rose-red disease of wheat;
3. In 1880 the Italian comes recognized bacteria as pathogenic to plants; and
4. In 1883, Walker in Holland reported the bacterial nature of yellows disease of hyacinth.

Moura,2010; Kenneth Horst,2013

Erwin Frink Smith (1854-1927)
The father of the Plant Bacteriology

- Erwin F. Smith the most famous plant bacteriologist in the History of Plant Pathology, strongly supported Burrill's papers and consequently, since than, almost all text books on Plant Pathology and Plant Bacteriology have given credit to Burrill's discovery and pointed him as the first to report a plant disease caused by a bacteria.

Phytobacteriology

Significant Contributions:

E. F. Smith 1893-1900
Studied crown gall disease: 'cancer' of the plants

1977: Mary Dell Chilton's group: crown gall bacterium physically transfers part of its plasmid

Smith, E.F. 1916. In memoriam of Thomas J. Burrill. Jour. Bact. 1: 269-271.

Bacteria Fall,2008;Moura,2010

Plant bacteriology
The Fischer-Smith controversy

Alfred Fischer and Erwin Smith engaged in a classic and bitter debate in the late nineteenth century regarding the existence of bacterial diseases of plants.

Given the times and what you know about bacterial diseases, why was this so controversial?

Smith, E.F. 1899a. Are there bacterial diseases of plants? Ibid. 5:271–278;
Smith, E.F. 1899b. Dr. Alfred Fischer in the role of pathologist. Ibid. 5:810-817.

Starr,1983

The Fischer-Smith controversy:
Are there bacterial diseases of plants?

- Alfred Fischer, a famous botanist, student of De Bary, was among the last scientists who did not accept that bacteria can cause diseases in plants.
- In 1881, he attacked in his book the work of E. Smith.
- Between 1899 and 1901, Smith demonstrated that bacteria may be plant pathogens, putting end to the controversy.
- Following the controversy, the area of phytobacteriology highly developed, and with the time it was found that bacteria can cause disease in all plant families, and sometimes the degree of the economic damage is bigger than with other parasites such as fungi and virus.

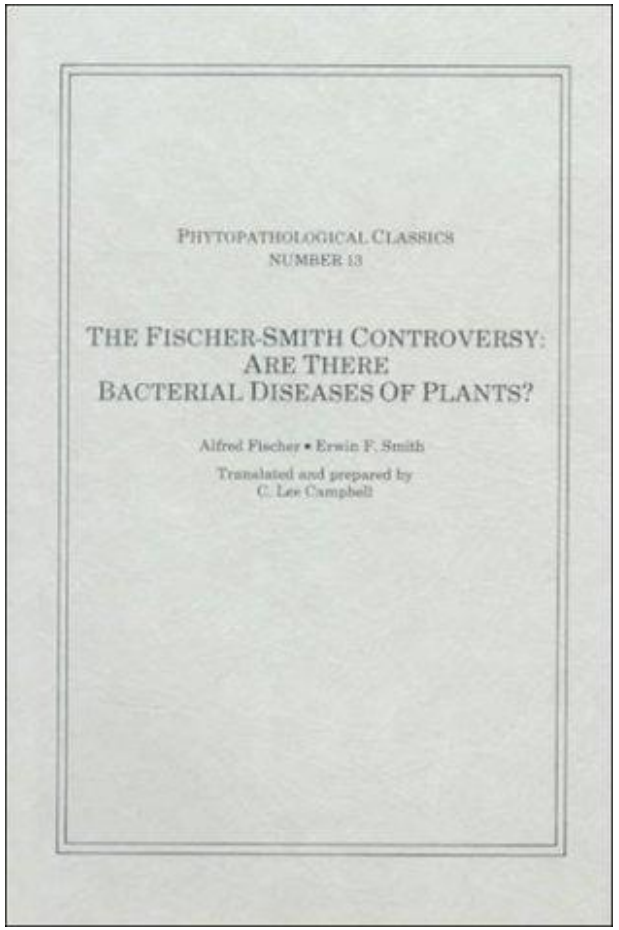
De La Fuente,2009

The Fischer-Smith Controversy:
Are There Bacterial Diseases of Plants?

Product Details

- By Alfred Fischer and Erwin F. Smith.
- Translated by C.L. Campbell.
- 65 pages
- **Publisher** : American Phytopathological Society (August,1998)
- **Language** :English

Articles describing the classic and bitter debate regarding the existence of bacterial diseases of plants that took place during the late nineteenth century.



De La Fuente,2009

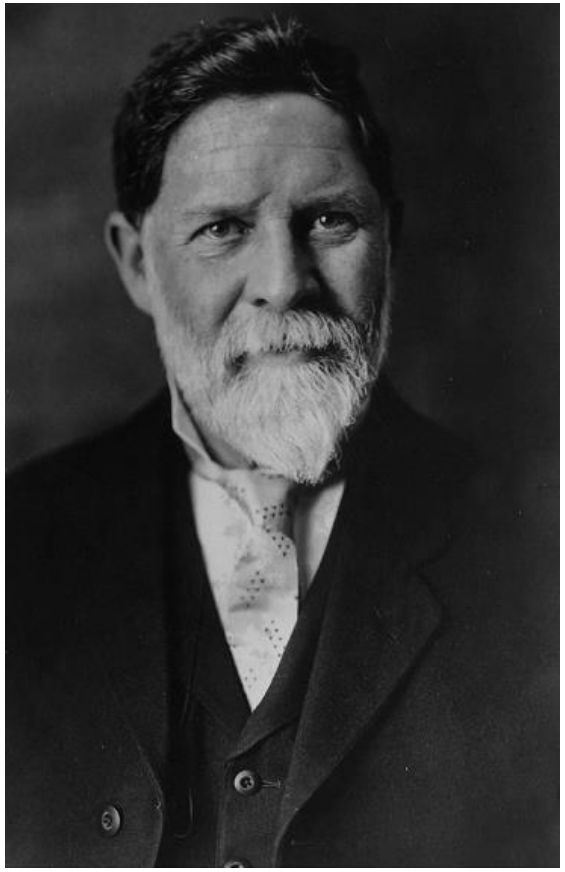
Plant bacteriology
The Fischer-Smith controversy

- Erwin F. Smith, of the U.S. Department of Agriculture won rancorous (hard) debate.
- He spent a lifetime in the process, starting with peach yellows, and going on to a study of crown gall and its relation to human cancer.
- In 1905 the first volume of his monumental work Bacteria in Relation to Plant Diseases was published.
- Genus *Erwinia* named for him.
- A. Fischer committed suicide in 1913.

Hughes,1996; Kenneth Horst,2013

Erwin Frink Smith (1854-1927)
The father of the Plant Bacteriology

- According to his biographies, E. F. Smith was a quite unique person.
- Besides sciences, he was interested in general literature and languages.
- He wrote considerable amount of poetry, some of which published in his book.
- He will remain in history as one of the main founders and key contributors to those important sciences for mankind (Jones, 1939).



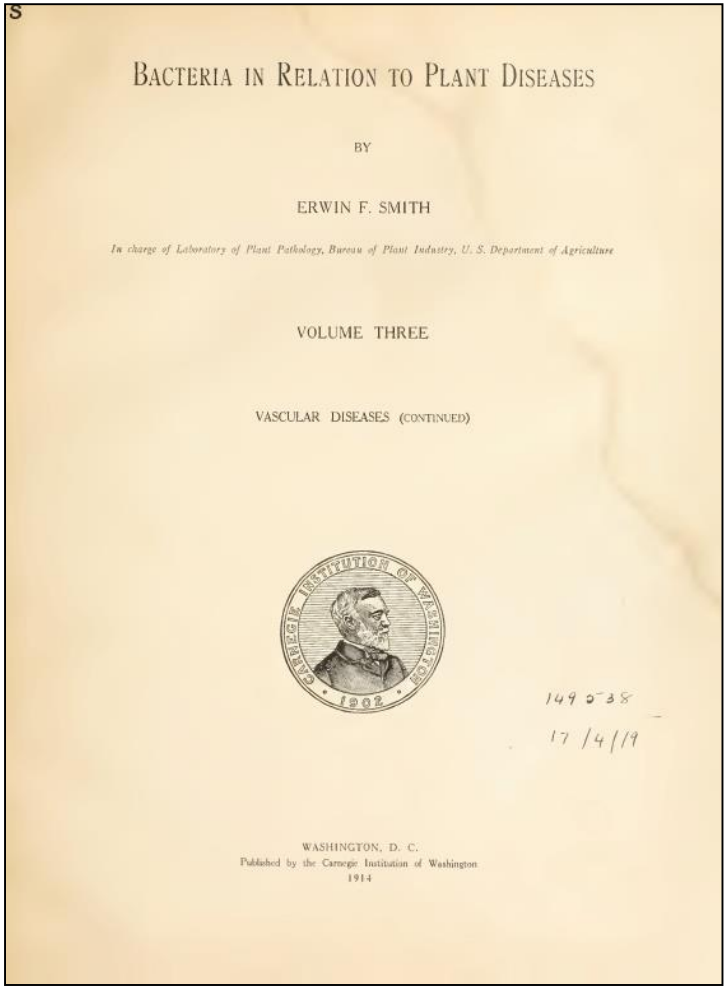
Moura,2010

Biographical Sketch of Erwin Frink Smith
The Founder of Phytobacteriology

- 1854 Born on January 21 in Gilbert Mills, Oswego County, New York.
- 1886 Received B.S. in Biology from University of Michigan.
- 1889 Received doctorate from University of Michigan, based on his work on the serious orchard disease, peach yellows.
- 1886-1892 Investigated peach yellows and peach rosette.
- 1894-1910 Researched *Fusarium* diseases of melons, cotton, cowpeas, tomatoes, potatoes, and cabbage.
- 1892 Started investigating bacteria as an important pathogen of plants (By 1880, T. J. Burrill).
- 1895 Studied the bacterial wilt of cucurbits. Published his first paper on bacteria as the cause of plant diseases in *Centralblatt fur Bakteriologie*.
- 1890's-1901 Replied to Fischer in German in *Centralblatt fur Bakteriologie* (1899,1901). Dr. Alfred Fischer of Germany had stated that there was no proof that bacteria caused plant diseases. Thus began the famous Fischer-Smith polemic.
- 1905-1911 Published 3-volume treatise, Bacteria in Relation to Plant Diseases.
- 1913 Received award from American Medical Association for his work on plant cancer.
- For years, he had researched crown galls- cancer of plants and their relationship to cancer in humans.
- 1920 Published An Introduction to Bacterial Diseases of Plants, dedicated to T. J. Burrill.
- 1926 Was honored in December at a dinner given by the American Phytopathological Society.
- He was lauded as "scientist, linguist, poet, friend, who for forty years has devoted his life's service to the broad field of pathology.
- 1927 Died on April 6 at the age of 73 in Washington, D.C, because of heart attack.

Bacteria in Relation to Plant Diseases, Volume 3

Original cover page



S

BACTERIA IN RELATION TO PLANT DISEASES

BY

ERWIN F. SMITH

In charge of Laboratory of Plant Pathology, Bureau of Plant Industry, U. S. Department of Agriculture

VOLUME THREE

VASCULAR DISEASES (CONTINUED)

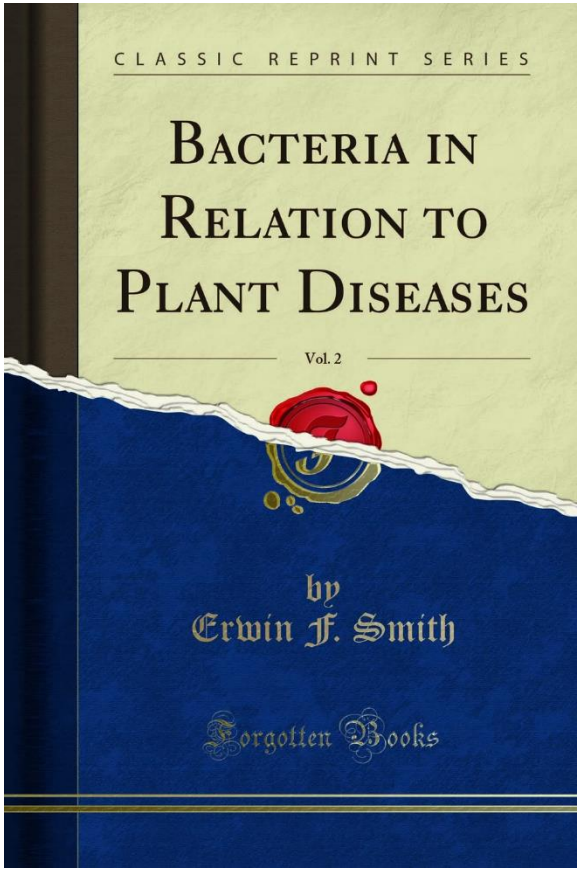
CARNEGIE INSTITUTION OF WASHINGTON 1902

149 5-38
17/4/19

WASHINGTON, D. C.
Published by the Carnegie Institution of Washington
1914

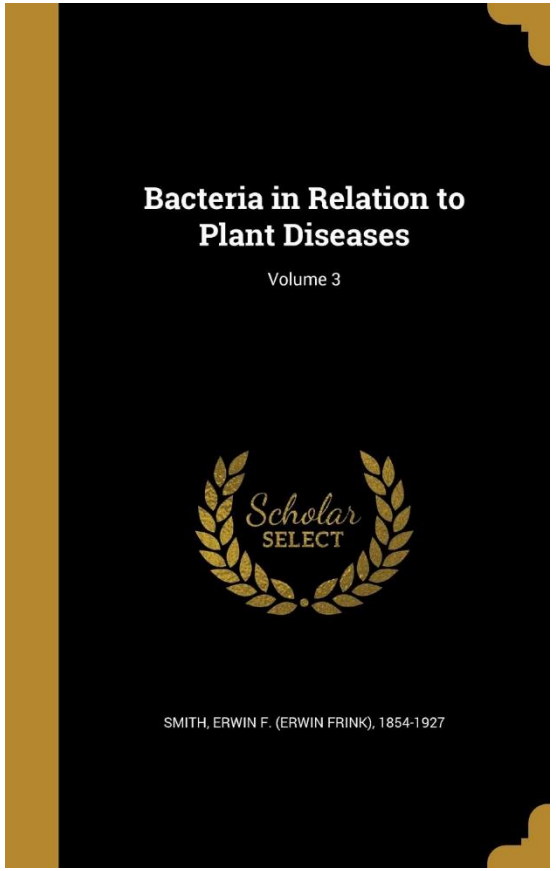
Bacteria in Relation to Plant Diseases, Volume 2

- **Bacteria in Relation to Plant Diseases**, Vol. 2 (Classic Reprint).
- by Erwin F. Smith (Author)
- **Publisher:** Forgotten Books 2020
- 424 pages.



Bacteria in Relation to Plant Diseases; Volume 3

- **Bacteria in Relation to Plant Diseases**, Vol. 3
- by Erwin F. Smith (Author)
- **Publisher:** Wentworth Press
- 2016
- 426 pages.



E. F. SMITH (1854-1938), USA, FOUNDER OF PHYTOBACTERIOLOGY

Fig. 26b
Symptoms of yellow disease in hyacinth; compare with drawings of E.F. Smith in **Fig. 26c**.

Fig. 26a
Cells of *Xanthomonas hyacinthi* as drawn by E.F. Smith from microscopic preparations. Source: as in **Fig. 26c**, page 345.

Fig. 26c

Symptoms of yellow disease caused by *Xanthomonas hyacinthi*. Plate 19 as it appeared in E.F. Smith, Bacteria in Relation to Plant Diseases, Volume 2, page 334, 1911.

Janse,2006

The Birth of Phytobacteriology:

Erwinia amylovora -The first plant pathogenic bacterium Named for the first phytobacteriologist, Erwin Smith

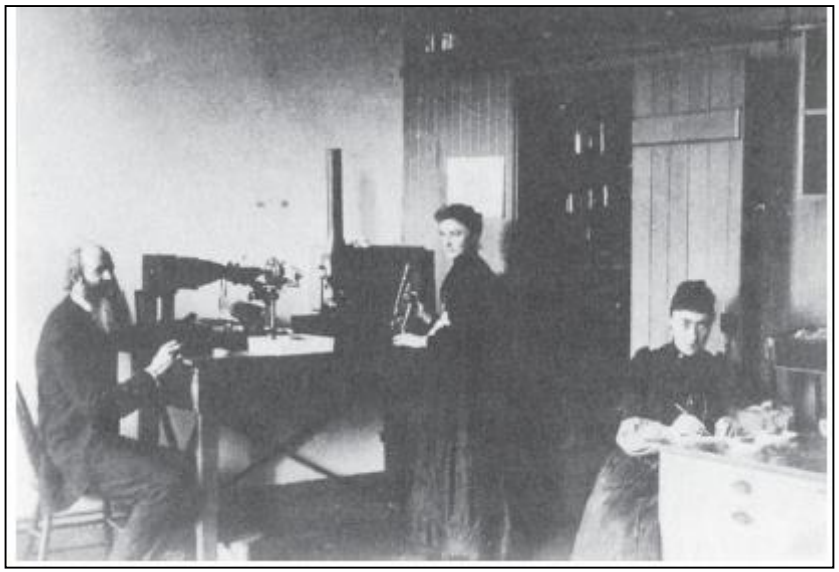
- Bacteria were shown in 1878 to be associated with fireblight of apples and pears in Illinois and New York, USA (Burrill,1878).
- 1878: 1st demonstration that bacteria can cause plant disease.
- In 1882 Burrill named his fire-blight organism *Micrococcus amylovorus*.
- Early discoveries about the nature of fire blight laid the foundation for phytobacteriology and had a major impact on general microbiology, since they occurred at the time when bacteria were being recognized as the cause of human and animal diseases.

Burill, T.J. 1883. New species of *Micrococcus* (bacteria) Am. Nat 17: 319.

Thomas J. Burrill

The first scientist to propose that bacteria can cause plant diseases

- In 1878, Thomas J. Burrill (1839-1916), who taught at the University of Illinois, was the first scientist to propose that bacteria can cause plant diseases.
- In his studies on fire blight, he found that …under our microscope, the field is seen to be alive with moving atoms known in a general way as bacteria.
- Burrill, however, did not use the pure culture of the bacterium in his work.
- Burrill's proposal was later proved correct when Koch's postulates were fulfilled by J. C. Arthur in his Ph.D. thesis work at Cornell University.

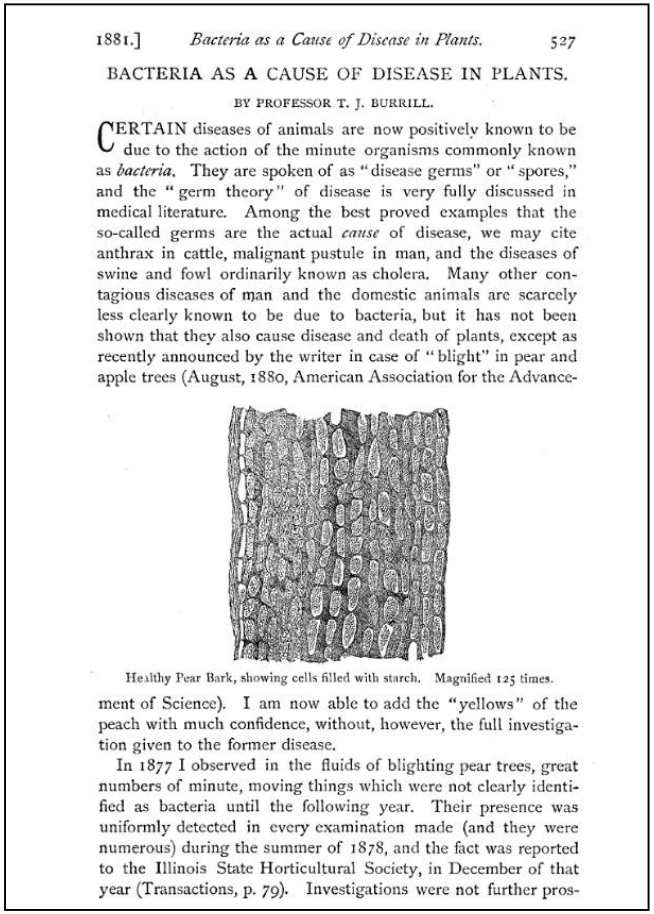


Thomas J. Burrill, one of world's first plant doctors in a laboratory with students,1882.

Thomas J. Burrill

The first scientist to propose that bacteria can cause plant diseases

- JOURNAL ARTICLE:
- **Bacteria as a Cause of Disease in Plants**
- T. J. Burrill.
- The American Naturalist Vol. 15, No. 7 (Jul., 1881), pp. 527-531.
- Published by: The University of Chicago Press for The American Society of Naturalists
- Page Count: 5
- Topics: Bacteria, Bark, Cell walls, Bacterial diseases, Starches, Inoculation, Plants, Blight, Viruses, Plant diseases.



1881.] *Bacteria as a Cause of Disease in Plants.* 527

BACTERIA AS A CAUSE OF DISEASE IN PLANTS.

BY PROFESSOR T. J. BURRILL.

CERTAIN diseases of animals are now positively known to be due to the action of the minute organisms commonly known as *bacteria*. They are spoken of as "disease germs" or "spores," and the "germ theory" of disease is very fully discussed in medical literature. Among the best proved examples that the so-called germs are the actual *cause* of disease, we may cite anthrax in cattle, malignant pustule in man, and the diseases of swine and fowl ordinarily known as cholera. Many other contagious diseases of man and the domestic animals are scarcely less clearly known to be due to bacteria, but it has not been shown that they also cause disease and death of plants, except as recently announced by the writer in case of "blight" in pear and apple trees (August, 1880, American Association for the Advance-

Healthy Pear Bark, showing cells filled with starch. Magnified 125 times.

ment of Science). I am now able to add the "yellows" of the peach with much confidence, without, however, the full investigation given to the former disease.

In 1877 I observed in the fluids of blighting pear trees, great numbers of minute, moving things which were not clearly identified as bacteria until the following year. Their presence was uniformly detected in every examination made (and they were numerous) during the summer of 1878, and the fact was reported to the Illinois State Horticultural Society, in December of that year (Transactions, p. 79). Investigations were not further pros-

The Pdf file of this article available http://about.jstor.org/terms

Phytobacteriology

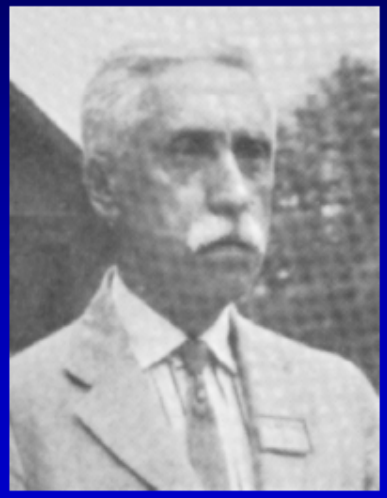
Significant Contributions

First to prove a microbe is the causative agent of a disease

Phytobacteriology

Significant Contributions:

Arthur 1885 (proved Koch's postulates for fireblight)



What was happening in 1885?

Chester Arthur President of the US;

Dedicates WA Monument

AT&T was incorporated

Rabies vaccine was tried by Pasteur

Statue of Liberty arrives in NY

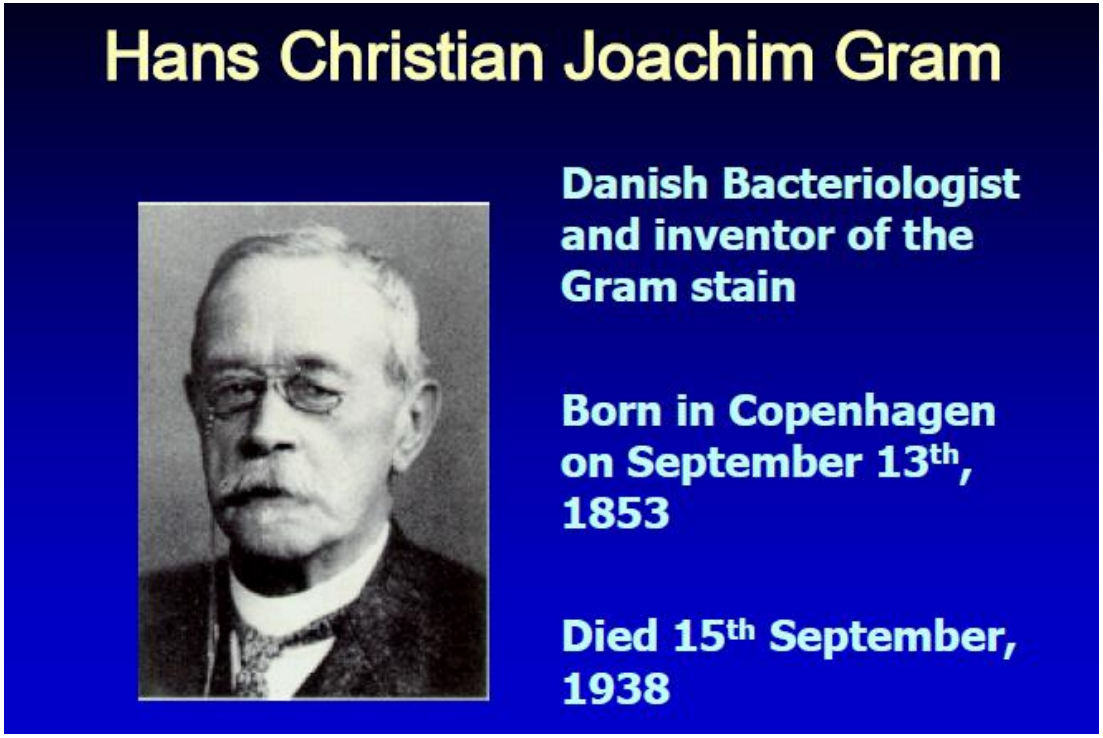
Adventures of Huckleberry Finn was published for the first time

Good Housekeeping was published for the first time

Arthur, J.C.1885. Proof that bacterial are the direct cause of the disease in trees known as pear blight. Botanic. Gaz. 10: 343–345.

Phytobacteriology
Significant Contributions
Inventor of the Gram stain

- The Gram staining method, named after the Danish bacteriologist Hans Christian Joachim Gram, who originally devised it in 1882 (published 1884).



Phytobacteriology

Significant Contributions

Introduction of pure culture techniques

- Julius Richard Petri (1852–1921) was a German microbiologist who is generally credited with inventing the device known as the Petri dish, which is named after him, while working as assistant to bacteriologist Robert Koch. He further developed the technique of agar culture to purify or clone bacterial colonies derived from single cells.

Wikepedia,2021

Systemic approach to diagnosing plant pathogenic bacteria

General approaches

Diagnosis of plant diseases

- **Systemic approach to diagnosing a plant problem**

Consists of five basic steps:

1. Determine if a problem exists (symptoms vs. signs);
2. Look for patterns (uniform vs. random damage patterns);
3. Determine the time of development;
4. Ask questions (history of problem, treatments, weather, etc.);
5. Synthesize the information.

Steps in Diagnosing Plant Diseases
Bacterial diseases

- Identify the host plant (common and botanical names).
- Visualize symptoms of common diseases and signs of the pathogen on specimen:
- Look for signs such as fungal fruiting structures, mycelia in or on tissue, sclerotia or bacterial ooze.
- Symptoms can be internal as well as external.
- The presence of general decline or wilt may indicate root problems.
- Use reference books to determine what diseases are recorded for the host plant.
- Refer to monographs, compendia, bulletins, host indices, and Fact Sheets that describe the common diseases and problems of the host plant.

Momol *et al.*,2009

Diagnosis of plant diseases

Correctly diagnose plant disease problems, follow a few basic steps:

CLOSE-UP VIEW

- The most obvious place to look first is up close. Use a hand lens if necessary.
- Look for symptoms on leaves, stems, roots, flowers and fruits.
- Cut open a branch or stem to look for vascular problems.

GENERAL VIEW

- Stand back and look at the overall picture.
- Consider the total environment: weather, soil, stage of development for plant and pathogens, cultural practices and condition of other plants in the area.

TIME

- Determine when the symptoms became apparent. Are plants of other species affected? Diseases are usually species-specific.

Diagnosis of plant diseases

KNOWLEDGE AND EXPERIENCE

- Gather all the information you can to help you make the diagnosis.
- When in doubt about a diagnosis, turn to agents, state specialists or the pest and plant disease clinic for assistance or a second opinion.

NONLIVING OR ABIOTIC AGENTS

- Nonliving or abiotic agents can indirectly result in plant problems.
- Additionally, several factors in the plant's environment can produce disease-like symptoms: weather extremes, high winds, high or low temperatures, nutrient deficiencies, physical damage and poor cultural conditions.
- Plant disease can result from a combination of abiotic agents and biotic agents.

Diagnosis of plant diseases

RANDOM OR UNIFORM PATTERNS

- Random distribution of symptoms on injured plants is usually caused by a biotic factor, such as infectious disease pathogens or an insect/animal.
- Uniform patterns are generally associated with abiotic or noninfectious agents like:

1. pesticides,
2. fertilizers,
3. environmental or site stress, and
4. mechanical damage.

Diagnosis of plant diseases

Sample size and sampling pattern

- **Sampling for detection:**
- Presence or absence of a pathogen or disease in a given field requires a different sampling pattern and sample size than sampling to determine the disease incidence or severity in the field.
- The number of samples collected should be representative of the impacted area.
- Among the systematic sampling designs, entire-field X- and W-shaped patterns are equivalent to each other and superior to diagonal or partial-field sampling patterns.

Diagnosis of plant diseases

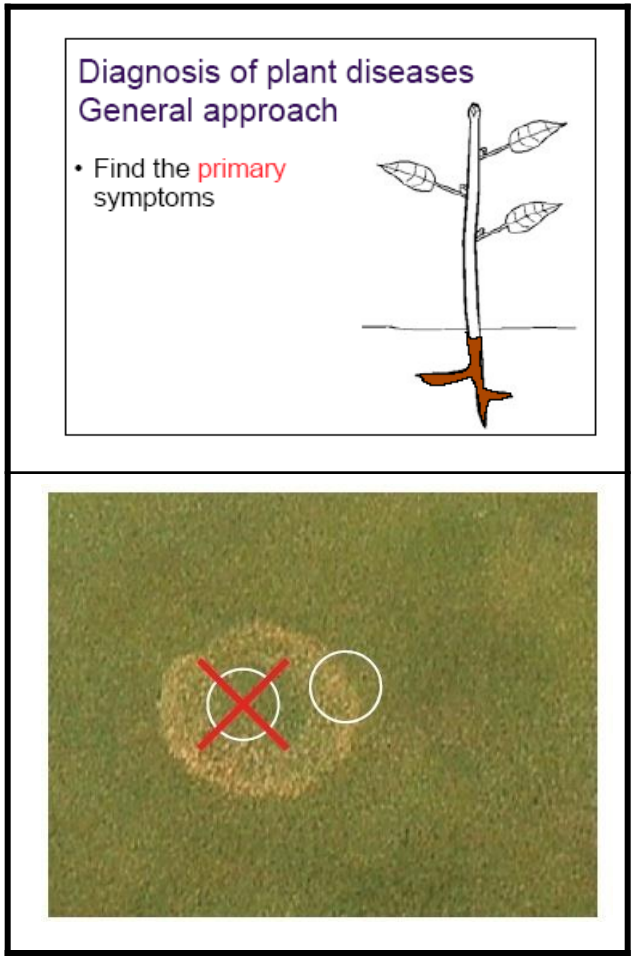
COLLECTING SPECIMENS

- Plant specimens that are to be diagnosed should be taken from the area where symptoms are showing on living tissue.
- Dead plants are often invaded by secondary pathogens which may hide the original problem.
- Collect several representative samples showing various stages of disease development.
- A generous sampling will assist in diagnosis.
- If possible, collect the entire plant, including roots.
- Place the soil and roots in a plastic bag, seal, and accurately label samples.
- Rapid delivery may be critical.

Diagnosis of plant diseases

Sample collection

- A fresh sample is required.
- Find the primary symptoms.
- Wrap the specimens in dry paper.
- Do not moisten them or seal them in plastic wrap or plastic bags.
- Never mix different specimens in a single bag.
- Label properly.



Diagnosis of plant diseases

Sample collection

- Collect several plant specimens showing various stages of disease development.
- Select plants that are still alive.
- Collect the entire plant whenever possible.
- Plants should be dug (not pulled) to keep the roots intact.
- First, wrap roots of plant in plastic bag so that they do not dry out.
- Second, wrap entire sample in plastic bags.

Lab 4-ss.pdf

Sample Quality
Packing and Shipping

Examples of good packaging

Amanda Hodges

Diagnosis of plant diseases
Sample collection

- Collect in the field.
- Always collect disease and healthy parts of plant as samples for isolation.

4 March 2008 COST 873, York

Manceau,2008

Plant Sample Submission

Incidence

A percentage of the crop affected

Severity

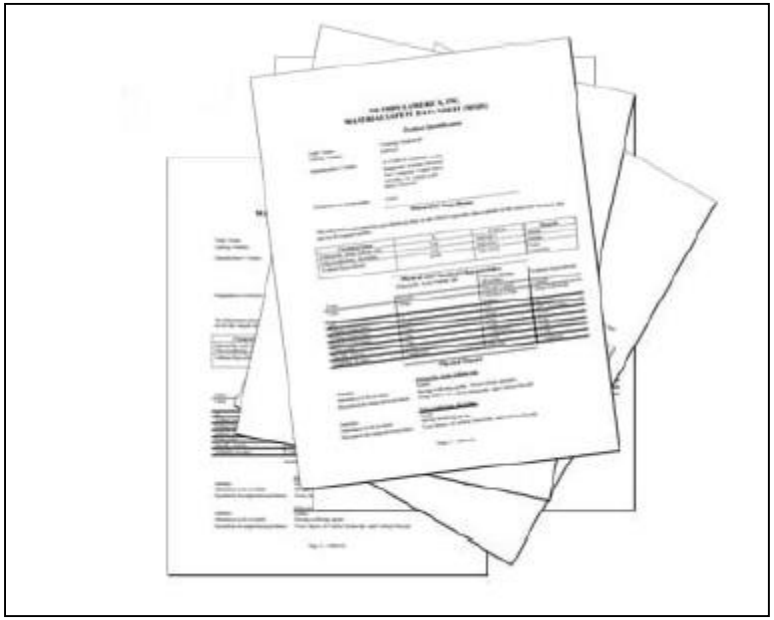
A measure of impact on plants or crops

Amanda Hodges

Diagnosis of plant diseases

Record problem history

- Complete the diagnostic form as thoroughly as possible.
- This will result in better diagnosis.



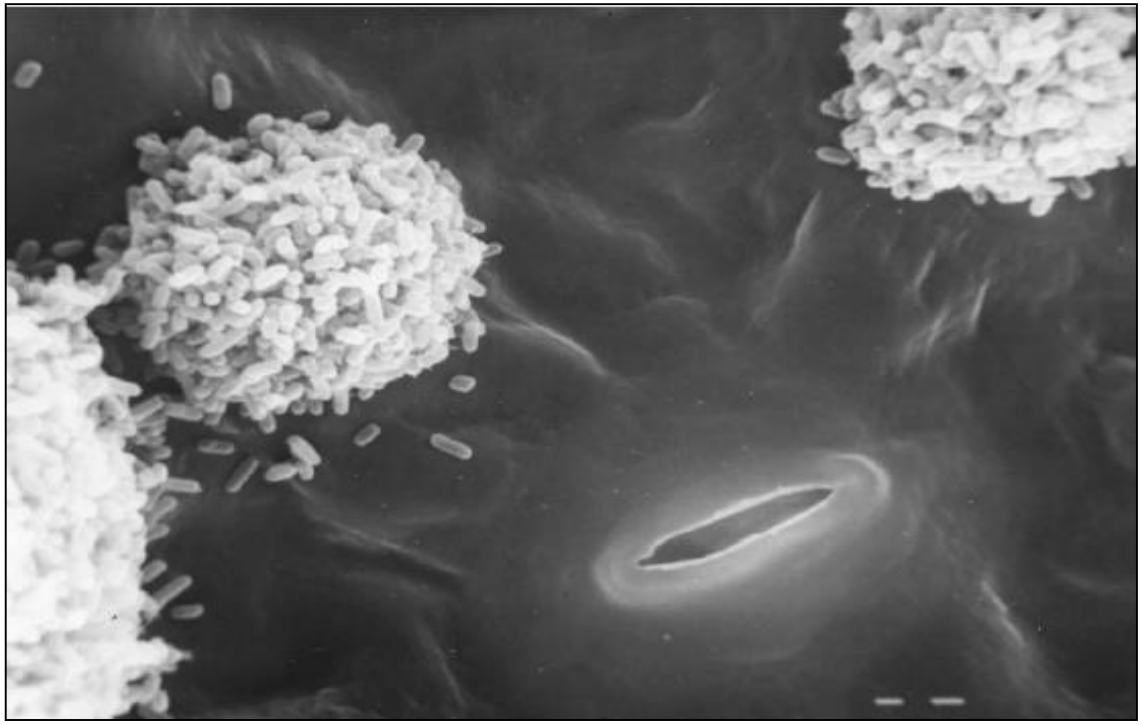
Lab 4-ss.pdf

Diagnosis of plant diseases

Symptoms assessment

- **Observe symptoms for diagnostic symptoms and signs:**
- Bacterial oozing/streaming,
- Odor,
- Water-soaking,
- Leaf spots,
- Excrescences(outgrows) and galls,
- Tumors,
- Wilting (vascular diseases),
- Necrosis and cankers,
- Rotting.
- Consult references (Publications, Web references & Personal knowledge).

Symptoms assessment
Exudation



Pseudomonas syringae* pv. *morsprunorum
exuding from stomata of infected cherry leaves.

Photograph courtesy of Roos and Hattingh, 1983

Agrios,2005

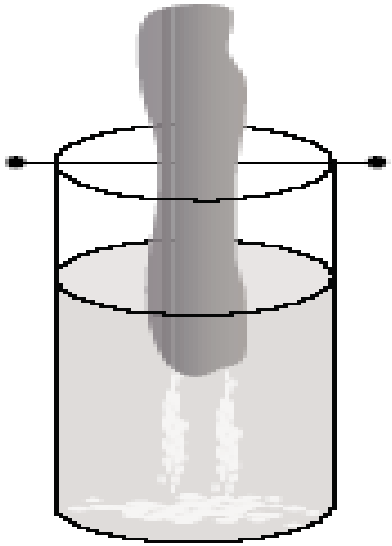
Bacterial exudation test

- In the case of vascular wilt, bacterial exudation can be observed by the naked eye by placing a piece of the disease tissue in water.
- The formation of a milky stream coming out from the plant tissue indicates that the problem is caused by a bacterial infection.
- For leaf spots, the bacterial stream can be also observed by light microscopy examination of the diseased tissue.
- The lesions with no fungal sporulation were submitted to the pus exudation test in a water-drop (Mafia *et al.*,2007) and observed under light microscope (200x).

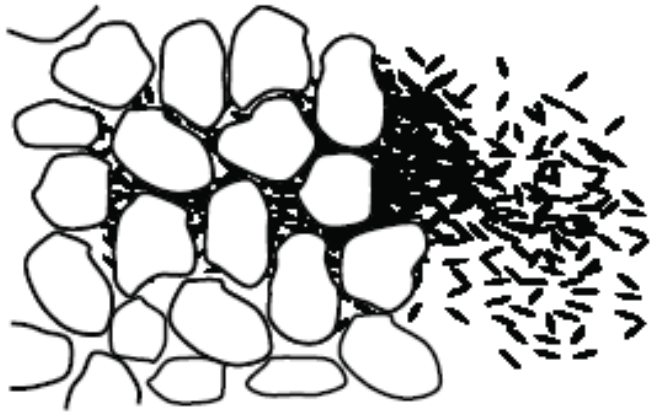
Rui Pereira Leite Jr.,2001

Bacterial oozing

Testing for bacterial streaming



Milky exudates from tomato stem infected by *R. solanacearum*



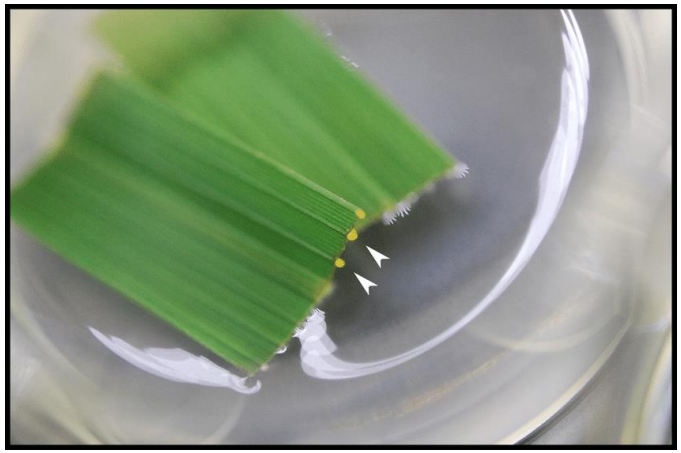
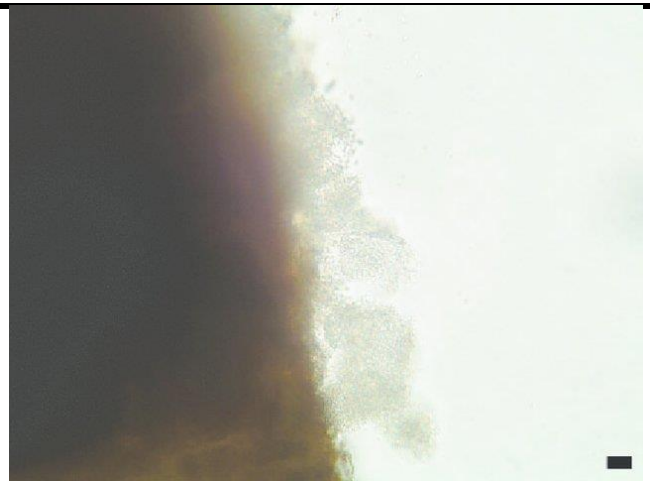
Schematic drawing illustrating bacterial steaming from diseased tissue.

Goszczynaska *et al.*,2000

Symptoms assessment

Bacterial oozing

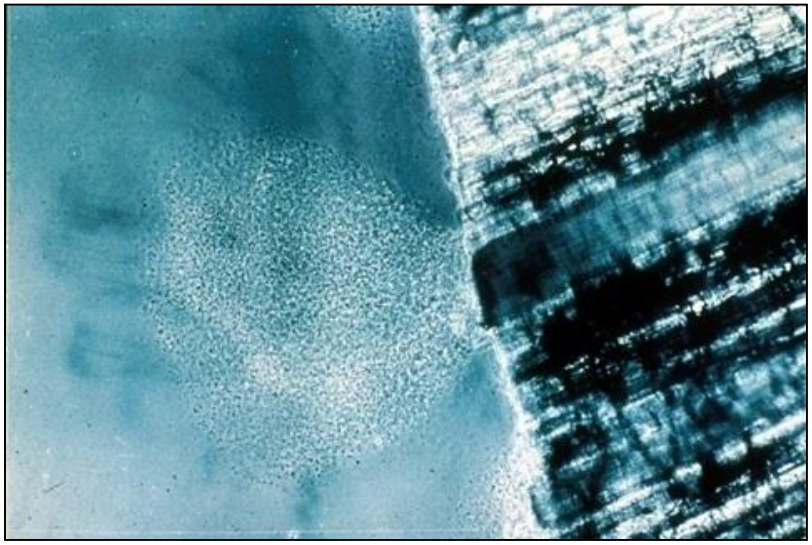
- In heavily infected plants, bacterial populations in leaves or lesions may reach 10^8 or 10^9 CFU/gram of plant tissue, and actually visibly ooze from leaves or stems.

	
Bacterial oozing (white arrowheads) was observed from rice leaf xylem vessels three days post infection(Th omas *et al.*,2016).	Bacterial oozing from cut lesion (bar represents 10 μm). *Burkholderia andropogonis* blight of golden cane palms (Grice *et al.*,2007).

Bacterial oozing

Testing for bacterial streaming

- A simple way to determine if a disease is caused by a bacterium is to cut a typical lesion or discolored area near its boundary with healthy tissue and suspend it in a droplet of water on a microscope slide.
- If a mass of moving small rods or 'dots' is seen at 200-1000x magnification flowing from the cut tissue under a microscope, you are observing bacterial streaming which is an indicator of a bacterial disease.
- However, not all bacterial infections show streaming, or it may not be visualized without special microscope attachments.



White masses or streams of *Pseudomonas syringae* cells oozing from the edge of a wheat ample observed under a dark fields microscope.

Isolation procedures

Isolation of common bacterial pathogens on common culture media

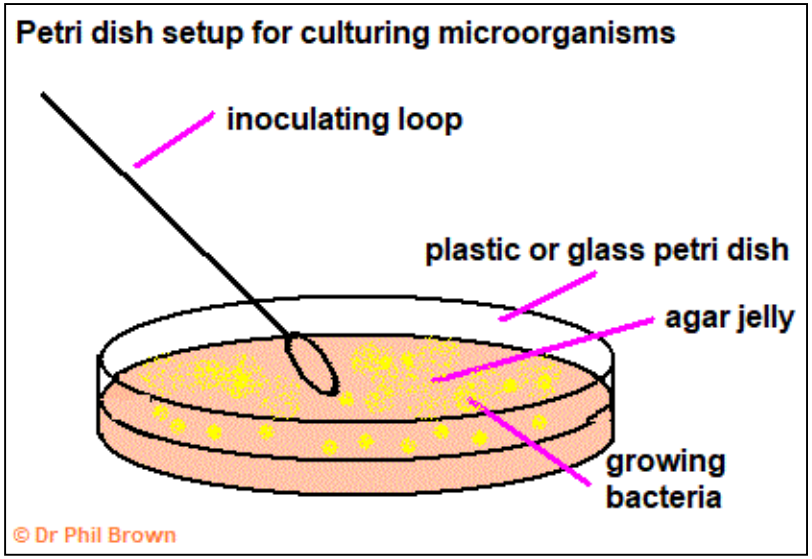
- Isolation is another process open to abuse and creation of more work than is necessary.
- As a visual infection develops, the symptomatic tissue may contain the pathogen but often in a decline phase of growth or in competition with secondary invaders.
- The leading edge of the symptomatic tissue, that zone that is either side or usually just in front of the actual edge is where you are most likely to find viable pathogen and usually in the absence of competitors.
- Your skill at selecting the correct leading edge is defined usually by the purity of the isolation you make.
- Same applies to spots, cankers, dieback, rots.

Isolation procedures

How to grow bacteria in the laboratory

Equipment and materials

- The experiments are conducted in petri dishes - shallow round plastic/glass containers over which a tight fitting lid can be fitted.
- The bacteria are grown ('cultured') in a culture medium.



Preparation of culture media

Equipment and materials

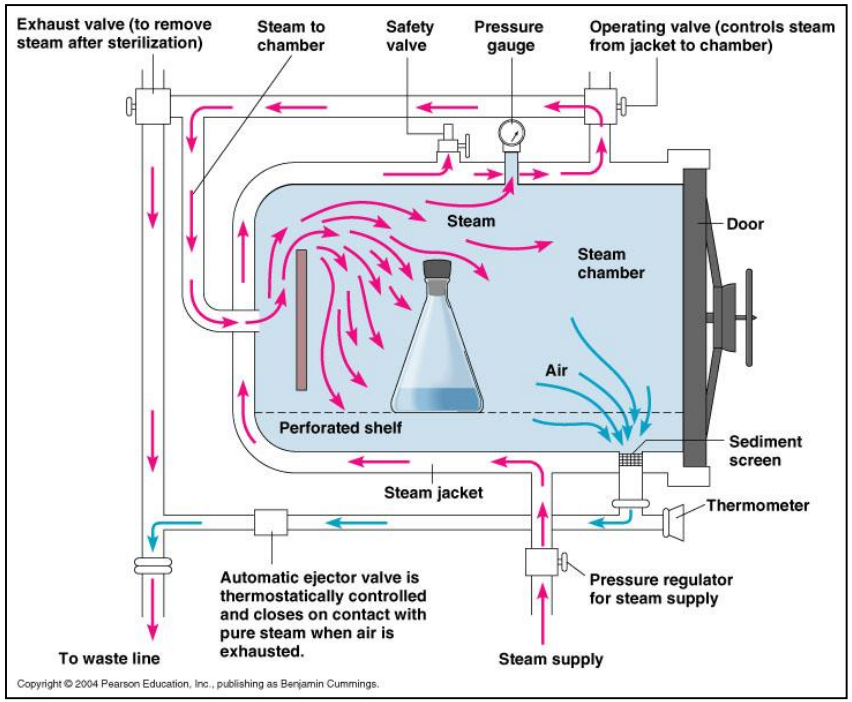
Sterilization

- Most media (including agar) can be sterilized by treatment with steam under pressure (Moist Heat) in an autoclave, the usual treatment being 15-20 minutes at a pressure of two atmospheres.
- This raises the steam temperature to 121°C.
- Note:
- Foil or cloth wrap solid materials;
- Must use on liquids;
- Never overfill an autoclave since this will upset the pressure/volume relationship and the correct temperature will not be attained;
- Screw caps on bottles must be left slightly open during sterilization and screwed down on removal from the sterilizer.

Pacarynuk,2006

Sterilization

Autoclave: 120-130°C at 15-20 ps
(pounds per square or lbs./in^2)



A pound (symbol: lb) is a unit of mass.
1 Ib=0.45 Kg.

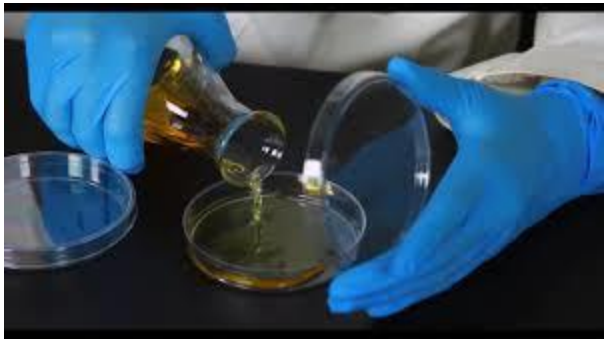
TABLE 7.3 **The Relationship Between the Pressure and Temperature of Steam at Sea Level***

Pressure (psi in excess of atmospheric pressure)	Temperature (°C)
0 psi	100
5 psi	110
10 psi	116
15 psi	121
20 psi	126
30 psi	135

*At higher altitudes the atmospheric pressure is less, which must be taken into account in operation of an autoclave. For example, in order to reach sterilizing temperatures (121°C) in Denver, Colorado, whose altitude is 5280 feet (1600 meters), the pressure shown on the autoclave gauge would need to be higher than the 15 psi shown in the table.

Sterilization

Sterile nutrient agar(NA)prepared plates ready to pour plates



Erlenmeyer flask/Autoclaving PYREX® Glassware

Making agar plates
LB-agar or NA-agar

- Pour molten agar into 'petri plates' under sterile conditions and allow it to set.

Isolation procedures

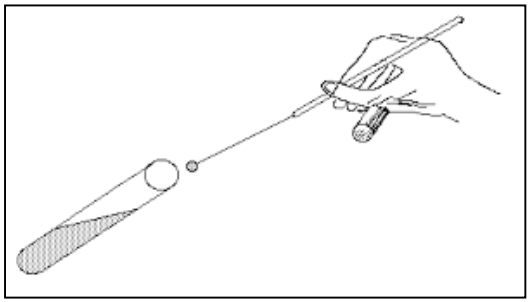
Isolation of common bacterial pathogens on common culture media

- Wash tissue containing lesions in sterile water or surface sterilize in 10% Clorox for 3 min., followed by sterile water rinse.
- Chop tissue in a drop of sterile water; incubate several minutes, then streak plates.
- Use several media for unknowns:
- YDC, NBY,NA, NGA, King's B.
- Water agar if *Streptomyces* is expected.
- Bacteria vary in robustness of growth on these media.

Miller,2006

Isolation procedures

Isolation of common bacterial pathogens



Sutula,2008;..

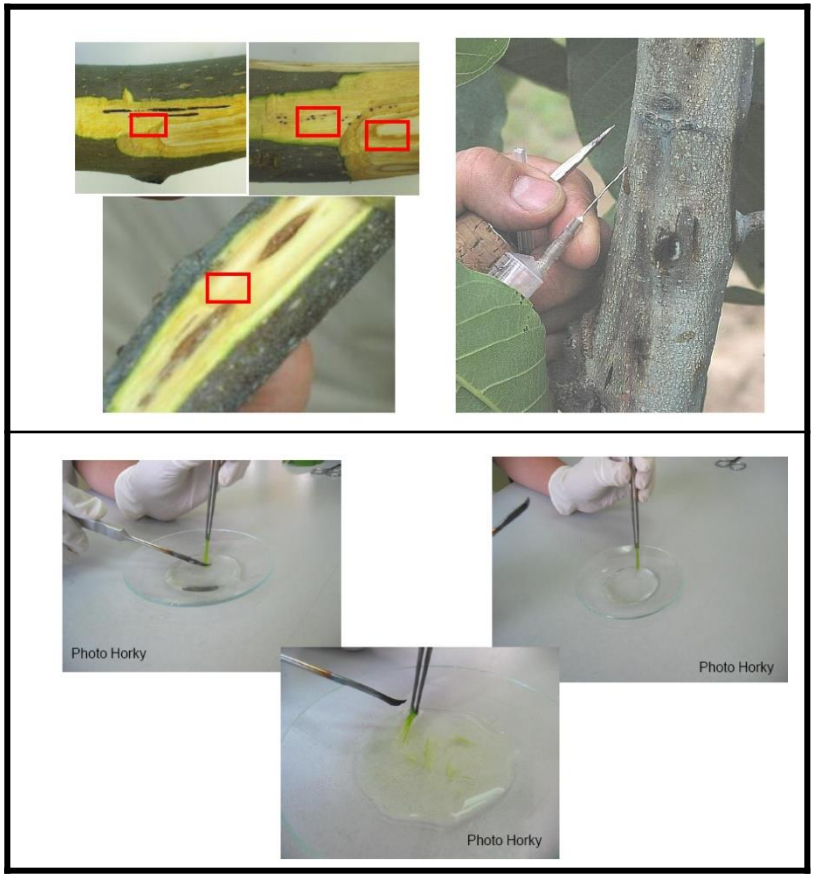
Isolation procedures

Steps of bacterial isolation

Preparing sample suspension

- Crushing and /or washing in buffer.
- Prepare test sample suspension by grinding the samples in a sterile pestle and mortar with a small volume of D.H_2O or buffer saline.
- Homogenised suspensions of the dissected/ macerated tissues was grown on certain common culture media.

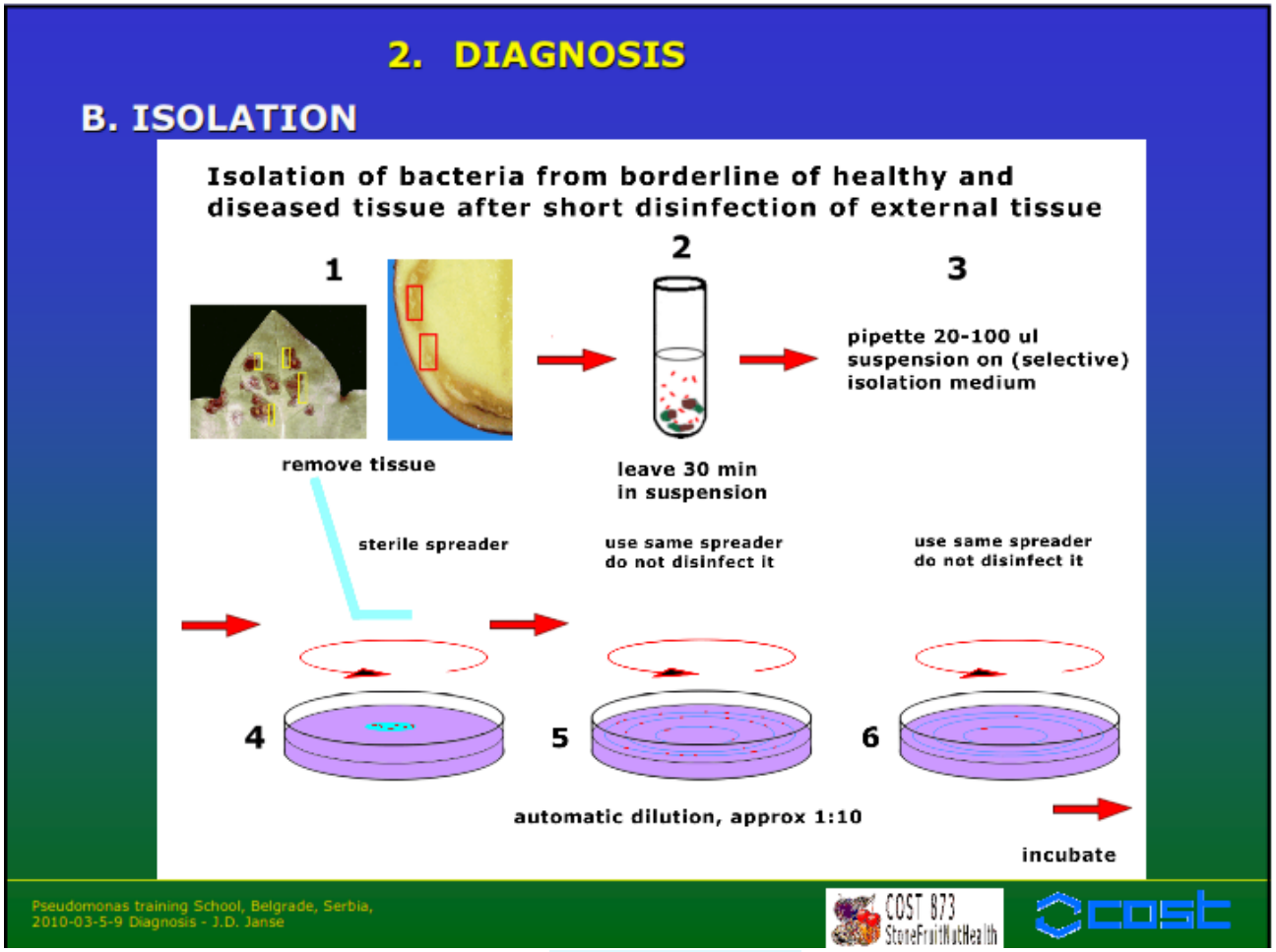
Physiological saline is a 0.9% sodium chloride solution.



Manceau,M.2008;..

Isolation procedures

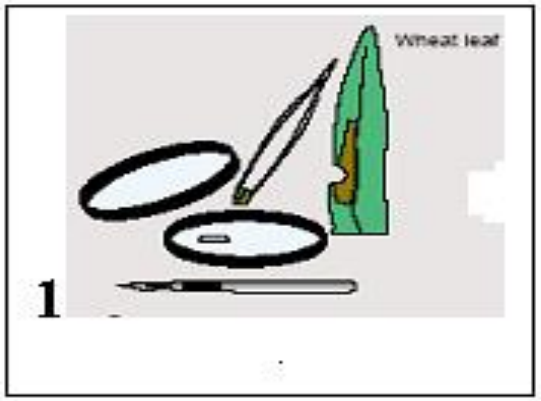
Steps of bacterial isolation on common culture media

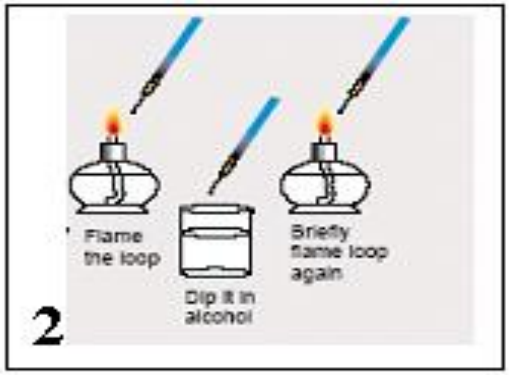


Janse,2010

Isolation procedures

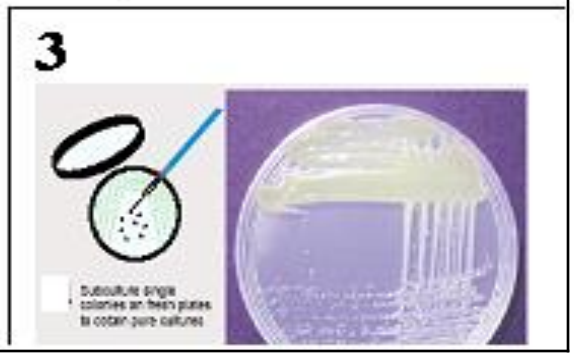
Steps of bacterial isolation on common culture media





laminar flow cabinet

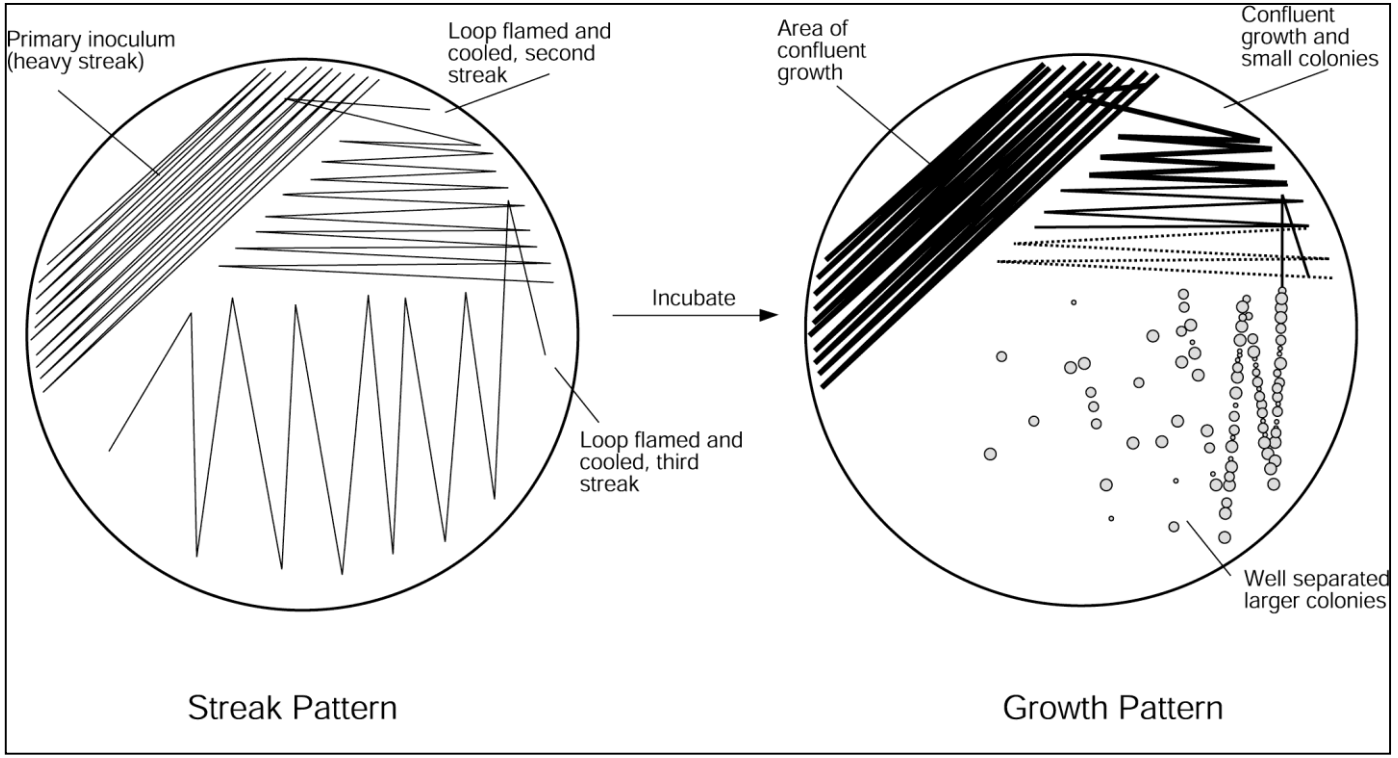
Sterile or distilled water



Isolation procedures

The streak-plate procedure

Isolation streak pattern



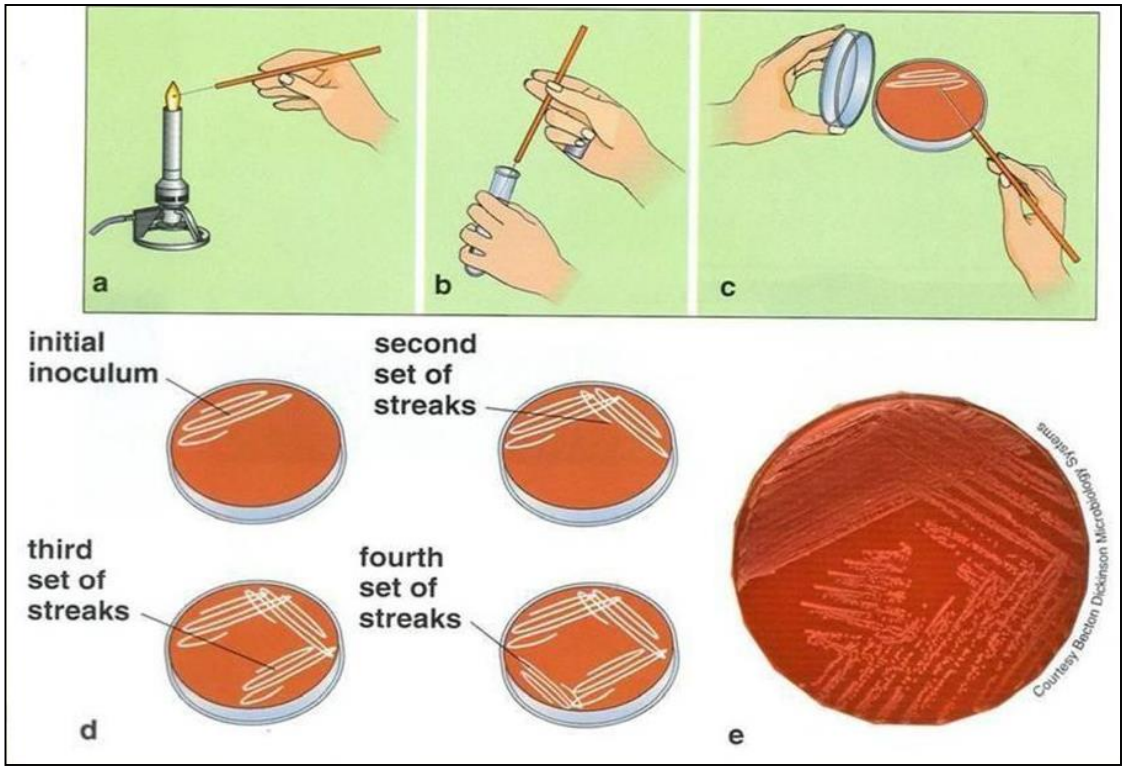
Streak inoculum onto one portion of the plate. Sterilize the loop
Streak through the first inoculum and spread into second section
Repeat several times. Incubate it for 24 h., a single colony will be observed.

Week3_ Exercise2_BC.ppt;..

Isolation procedures

Streak plate method

Isolation streak pattern



Streak inoculum onto one portion of the plate. Sterilize the loop
Streak through the first inoculum and spread into second section
Repeat several times. Incubate it for 24 h., a single colony will be observed.

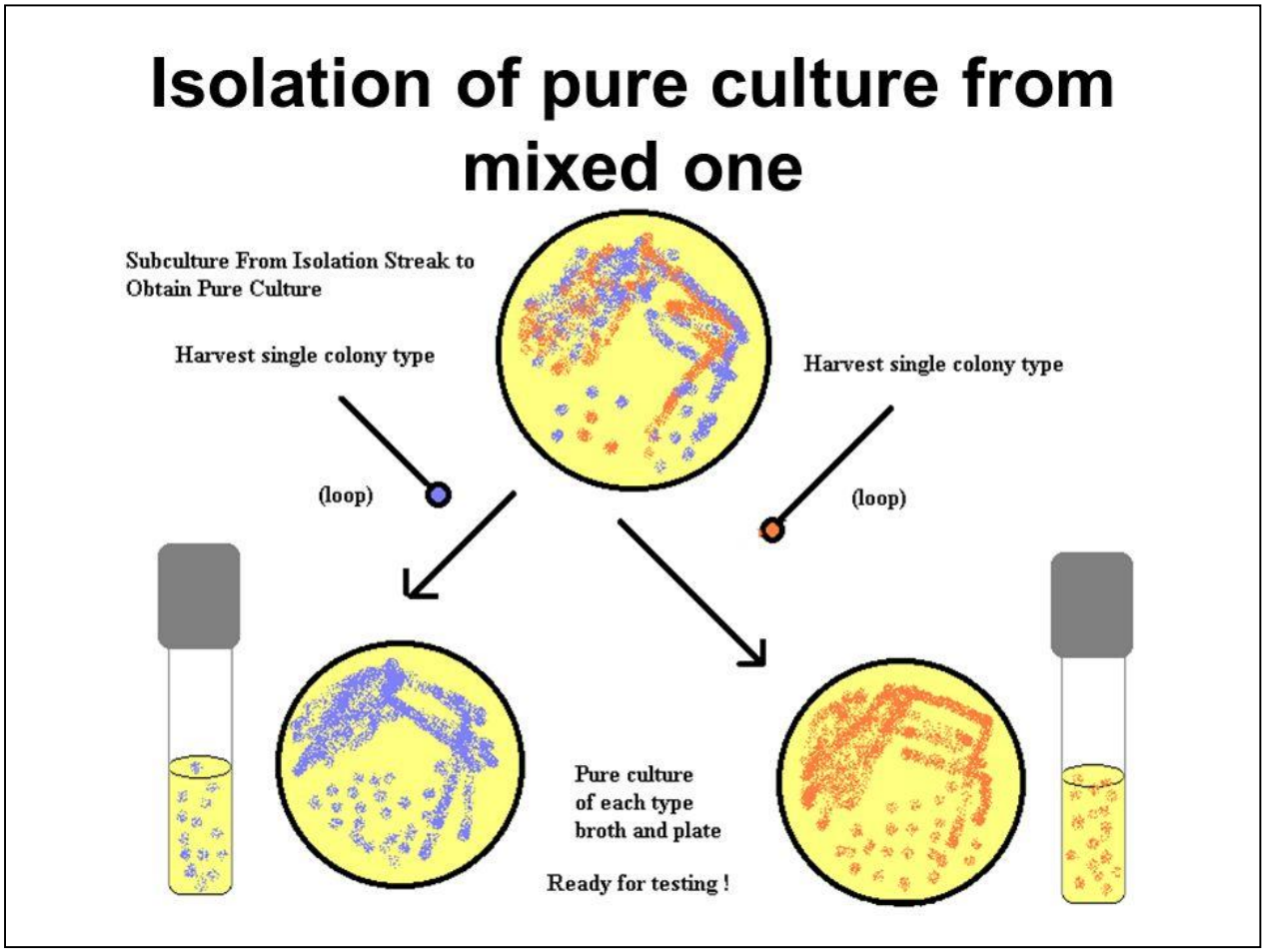
Pure culture

A pure culture is the basis for all further work in identification and diagnosis

- First, the mixture must be diluted until the various individual microorganisms become separated far enough apart on an agar surface that after incubation they form visible colonies isolated from the colonies of other microorganisms. This plate is called an isolation plate.
- Then, an isolated colony can be aseptically "picked off" the isolation plate and transferred to new sterile medium.
- After incubation, all organisms in the new culture will be descendants of the same organism, that is, a pure culture.

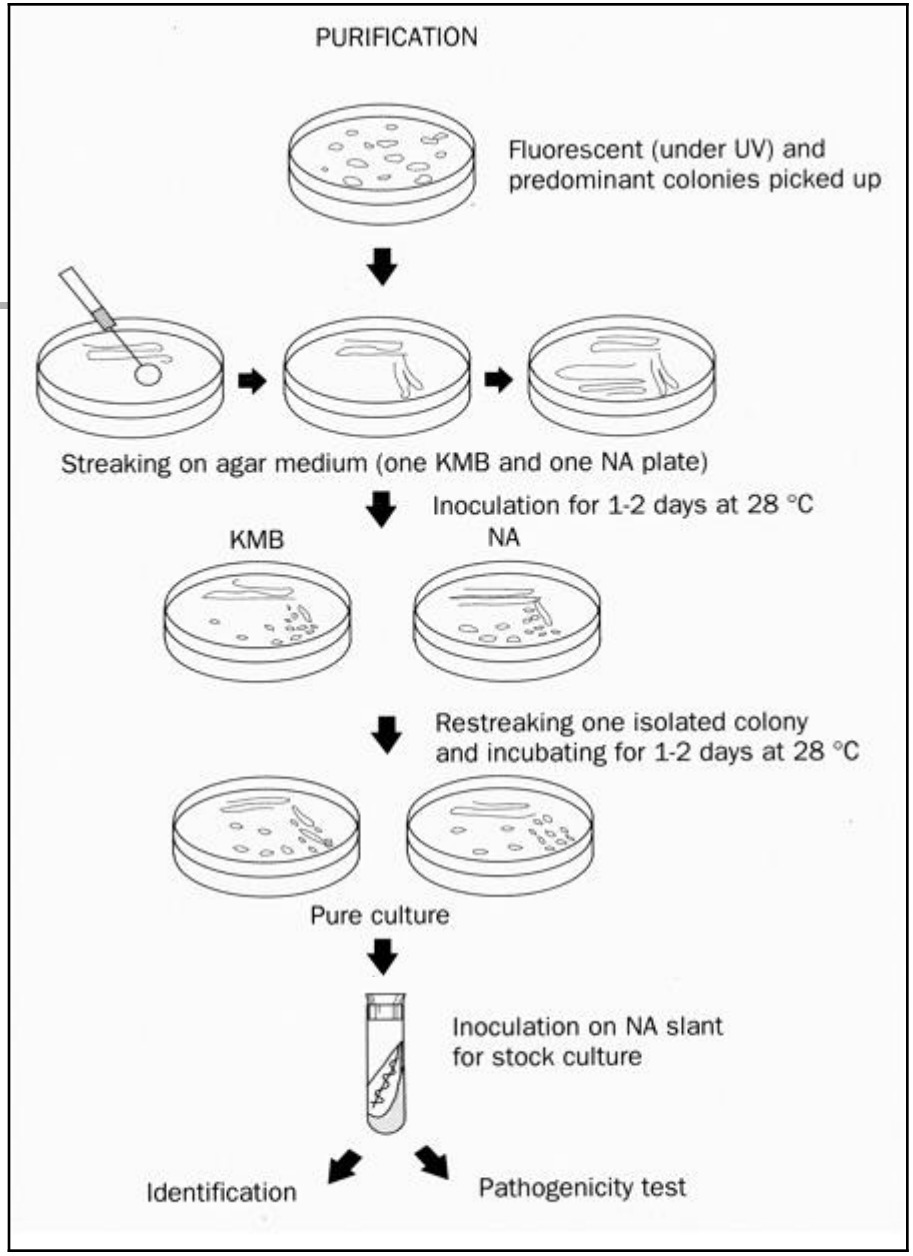
Pure culture

Isolation of a pure culture may be enhanced by providing a mixed inoculum with a medium favouring the growth of one organism to the exclusion of others



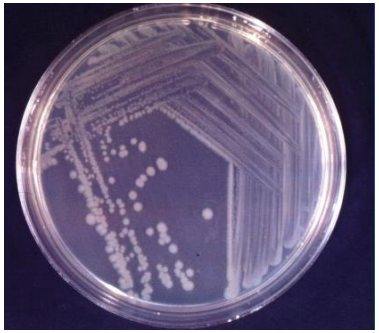
Leslie Robinson

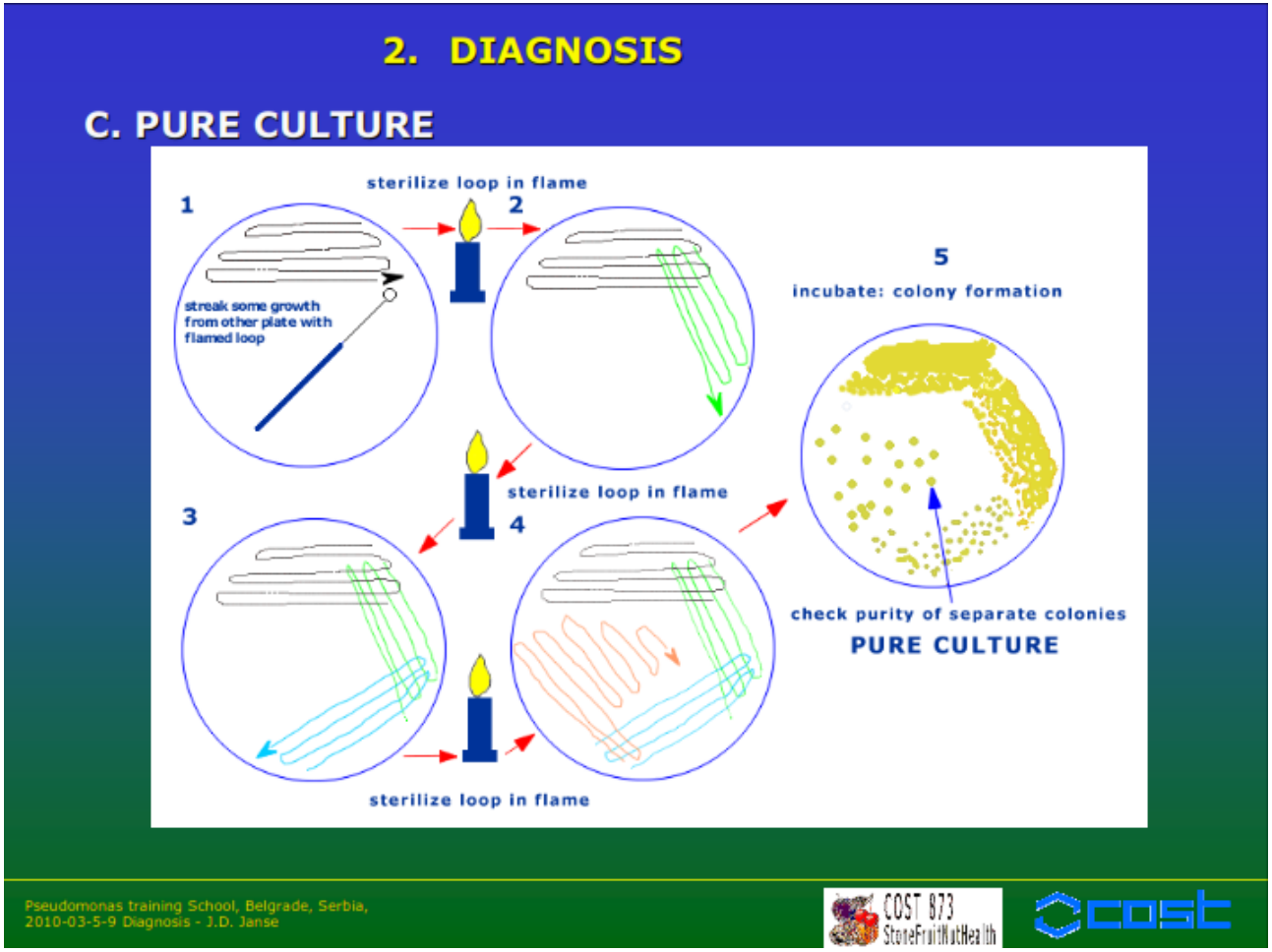
Purification procedures



Mew and Misra,1994

Culture media
Pure culture

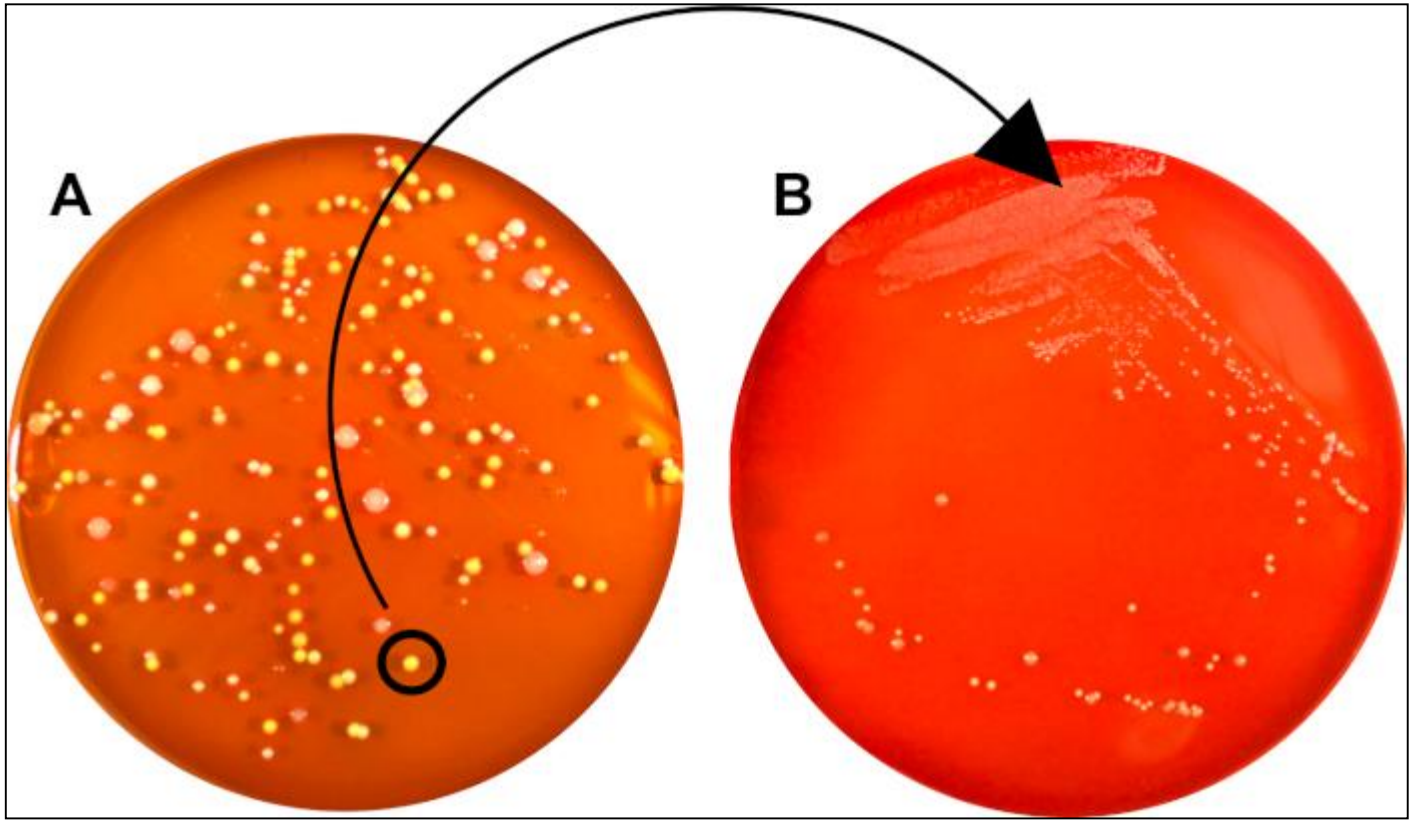




Janse,2010

Bacterial colony purification

Pure cultures and streak plating: Isolation of single bacterial colonies from a mixed sample

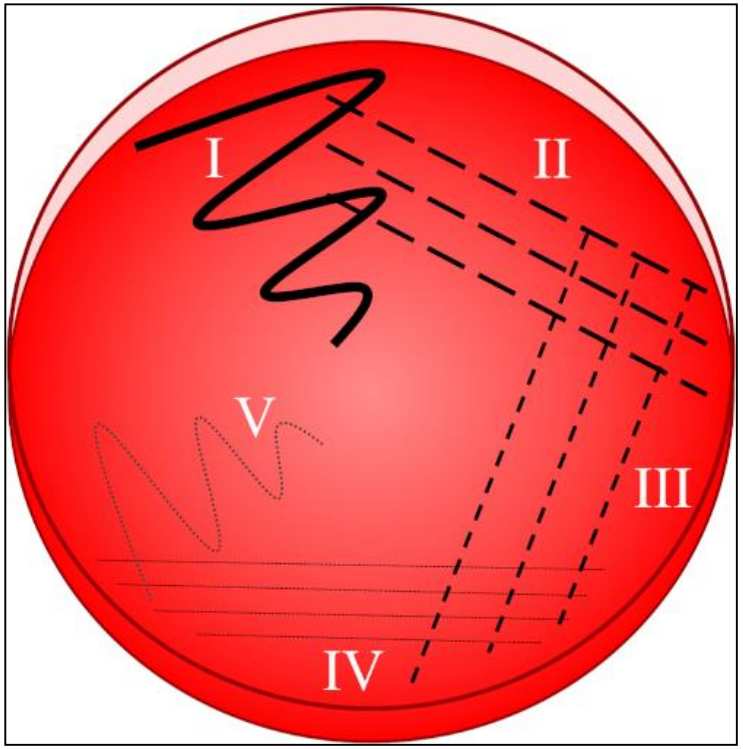


Andersson and Lood,2019

Bacterial colony purification

Pure cultures and streak plating: Isolation of single bacterial colonies from a mixed sample

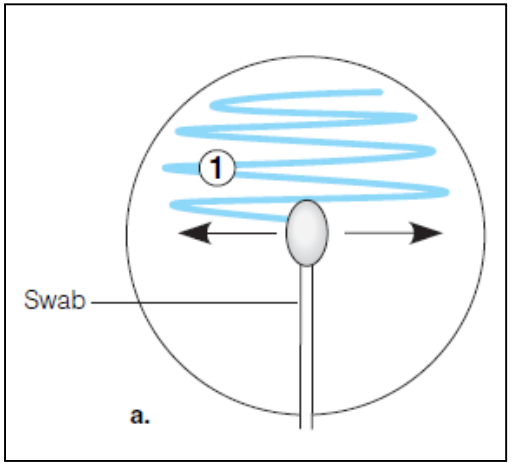
- **I)** The inoculum is initially dispersed using a zig-zag motion, creating an area with a relatively dense bacterial population.
- **II-IV)** Streaks are drawn from the preceding area, using a sterile inoculation loop each time, until the fourth quadrant is reached.
- **V)** A final zig-zag motion directed towards the middle of the plate forms a region where the inoculum has been markedly diluted, allowing colonies to appear separate from one another.



Andersson and Lood,2019

Spread plate technique
Swab culture

- Spread or 'lawn' plates should result in a heavy, often confluent growth of culture spread evenly over the surface of the growth medium.
- This means they can be used to test the sensitivity of bacteria to many antimicrobial substances, for example, mouthwashes, garlic, disinfectants and antibiotics.
- A Pathology lab technician using a cotton swab on a Petri dish with a bacteria culture.



Isolation procedures

Isolation of endophytic bacteria from fruits

- The Black grapes and Avacado samples were rinsed with autoclaved distilled water, disinfected with Hydrogen peroxide for 2 minutes.
- Then were rinsed for 5 minutes with 70% ethanol followed by 3% hypochlorite + tween 20 (0.1%) and finally rinsed with autoclaved distilled water.

Prasad and Dagar,2014

Isolation procedures

Processing of samples and isolation of endophytic bacteria from fruits

- Two methods were employed in the present study in order to isolate endophytic bacteria from the fruits.

1. First method involved cutting samples were into 2 halves; and each half was impregnated on nutrient agar plates and incubated at room temperature for 24 h.
2. The second method involved macerating the surface sterilized samples using a sterile pestle and mortar.

- 1 g of the macerated sample was serially diluted and the dilutions were plated on nutrient agar media and incubated at room temperature for 24h.
- After overnight incubation, isolated colonies were selected and used further.

Prasad and Dagar,2014

Isolation procedures

Isolation of endophytic bacteria from sugarcane(SST) and from xylem fluids of grapevine

- Samples were collected from stem tissue of sugarcane(SST) and from xylem fluids of grapevine(GXF).
- Sugarcane stem tissue of approximately 5 inches was surface sterilized with 20% sodium hypochlorite for 10 minutes followed by two 10-minute washes of sterile distilled water.
- 100µL of each GXF sample was plated onto potato dextrose bacterial agar medium (PDA).
- From the GXF bacteria recovery medium (PDA), 30 distinct bacterial colonies were identified, isolated and re-plated to produce pure colonies.

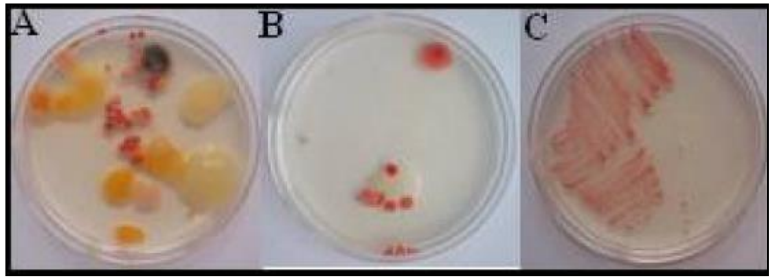
Hudson *et al.*,2010

Isolation procedures

Isolation of of endophytic bacteria from sugarcane(SST) and from xylem fluids of grapevine

- To isolate SST endophytes, internal tissue was dissect under sterile conditions and place into 100 ml of PD broth media and incubated at 30°C for 48 hours with shaking at 250 rpm.
- To facilitate isolation of pure colonies from SST, serial dilutions were conducted from 10^{-1} to 10^{-10}.
- One 100µL of the samples ranging from10^{-5}-10^{-10} was plated onto five different agar media: Luria broth, nutrient agar, potato dextrose, colony isolation, and R2A (Fig. 2).

Sample of GXF endophytes recovered on PDA strain purification.
GXF isolates recovered from grape plants in row 1, plant 19 (R1/19) and R3/13, respectively, A and B; purified endophyte isolate GXF4 isolated from grape plant R1/19,C.



Hudson *et al.*,2010

Isolation procedures

Isolation of of endophytic bacteria from agronomic crops and prairie plants

- For corn and sorghum, endophytic populations were collected from the pith tissue of stalks.
- Individual plants were severed aseptically 3 cm above the soil level, and the stalks were stripped of leaves, put into plastic bags, and kept on ice until further processing.
- In the laboratory, the stalks were wiped with 70% ethanol for 1 to 2 min. and flame sterilized, and each stalk was dissected into a segment containing the third, fourth, and fifth nodes.
- A crosscut through the stalk 2 cm above the third node was made, and a sterile no. 8 cork borer was inserted to a depth of at least 2 cm.
- The outer stalk was removed, exposing a cylinder of tissue inside the cork borer.

Zinniel *et al.*,2002

Isolation procedures

Isolation of of endophytic bacteria from agronomic crops and prairie plants

- Soybean, wheat, and prairie plants(plants that live in it must be adapted to floods, drought, heat and cold) were collected.
- Leaves and stems were surface sterilized for 10s with 2% sodium hypochlorite containing 0.1% Tween 20.
- To remove the disinfectant, sections were rinsed five times each in two washes of non sterile deionized distilled water and a wash of sterile water; the sections were dried with sterile paper towels.
- All plant samples were placed into polyethylene bags or dissected into ca. 1-cm pieces and macerated with either a sterile mortar and pestle or a sterile Polytron.
- Tissue extracts were then serially diluted in 12.5 mM potassium phosphate buffer (pH 7.1) (phosphate buffer) and plated in triplicate to recover any bacterial endophytes present in the plant tissue.

Zinniel *et al.*,2002

Isolation procedures

Isolation of endophytic bacteria from roots

- On a glass plate, cut transversally the plant roots with a scalpel in order to obtain small pieces of approximately 0.5 to 1 cm.
- Disinfection of plant roots by and ethanol, sodium hypochlorite solutions and sterilized distilled water.
- To verify the efficiency of the disinfection, place some pieces of disinfected roots(prior grounding or macerating the treated roots) in a Petri dish containing solid King B medium, in triplicate, and incubate 48 h at 28° to 30°C.
- If the disinfection was efficient no growth of microorganisms should appear on this plate.
- Then after, macerate the treated roots, prepare sample suspensions and culture in some solid media such as King B medium, in triplicate.

Ambrosini and Passaglia,2017

Culture media

Common nutrient media

Undefined media

- Nutrient media:
- Undefined media (also known as basal or complex media) is an undefined media that contains:

1. A carbon source such as glucose for bacterial growth.
2. Water
3. Various salts need for bacterial growth.
4. A source of amino acids and nitrogen (e.g. beef, yeast extract).

Tap water is not used in media preparation because it may contain undesirable compounds such as chlorine, copper, lead, and detergents.

Culture media

Common(non-selective) to semi-selective and selective media

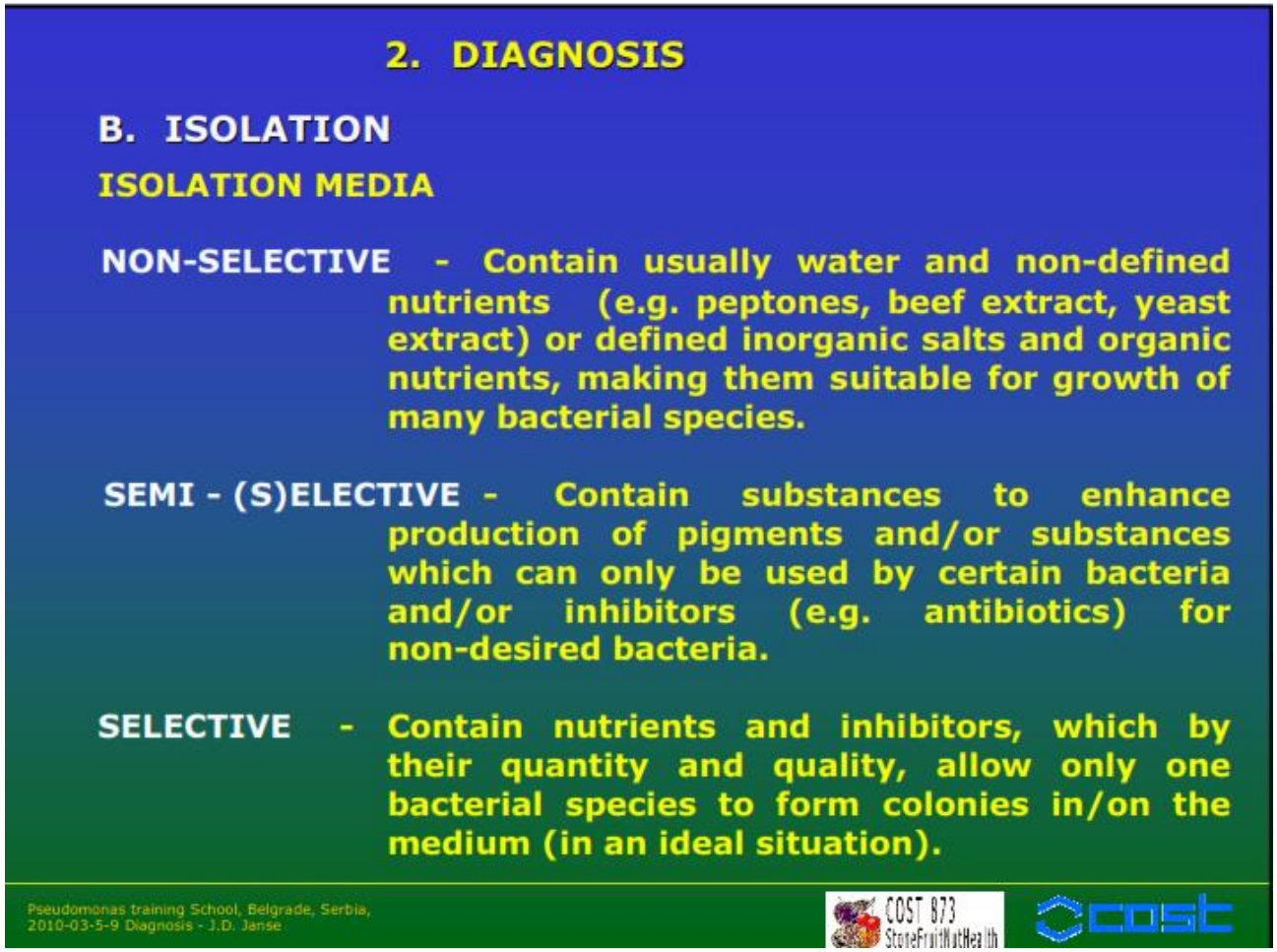
Diagnostic media

1. Standard/General media: Nutrient agar, peptone-glucose.
2. Differential media(appearance differs): Some sort of indicator, typically a dye, is added, that allows for the differentiation of particular chemical reactions occurring during growth. e.g. peptone-glucose plus TZC, EMB(Eosin methylene-blue lactose sucrose agar), KB, Levan (NA+sucrose).
3. Semi-selective: With low antibiotics, inhibitors.
4. Selective(growth or no growth): With high amounts of antibiotics, inhibitors and dyes.
5. Storage: e.g. Yeast-dextrose-calcium carbonate(YDC).
6. Enrichment: Contains vitamins, nitrogen sources.
7. Minimal media: Those that contain the minimum nutrients possible for colony growth, generally without the presence of amino acids.

Both selectivity and plating efficiency
are important for selective and semi-selective media.

Culture media

Non-selective to semi-selective and selective media



Janse,2010

Culture media

Selective media

Eosin methylene blue

- The inhibitory substance is added to a solid media to inhibit commensal or contaminating bacteria such as:

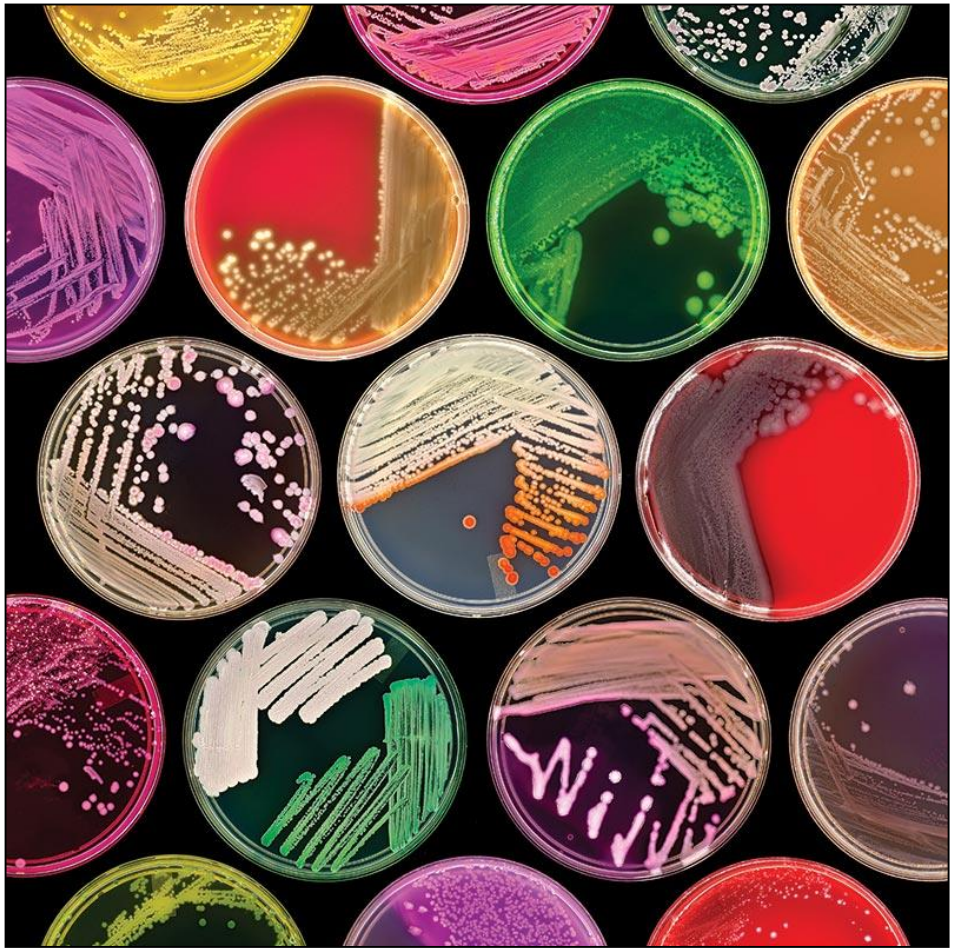
1. Antibiotics
2. Dyes
3. Chemicals
4. Alteration of pH.

- Selective for gram negative bacteria.
- The dye methylene blue in the medium inhibit the growth of gram positive bacteria.

Culture media

Common, differential to semi-selective and selective media

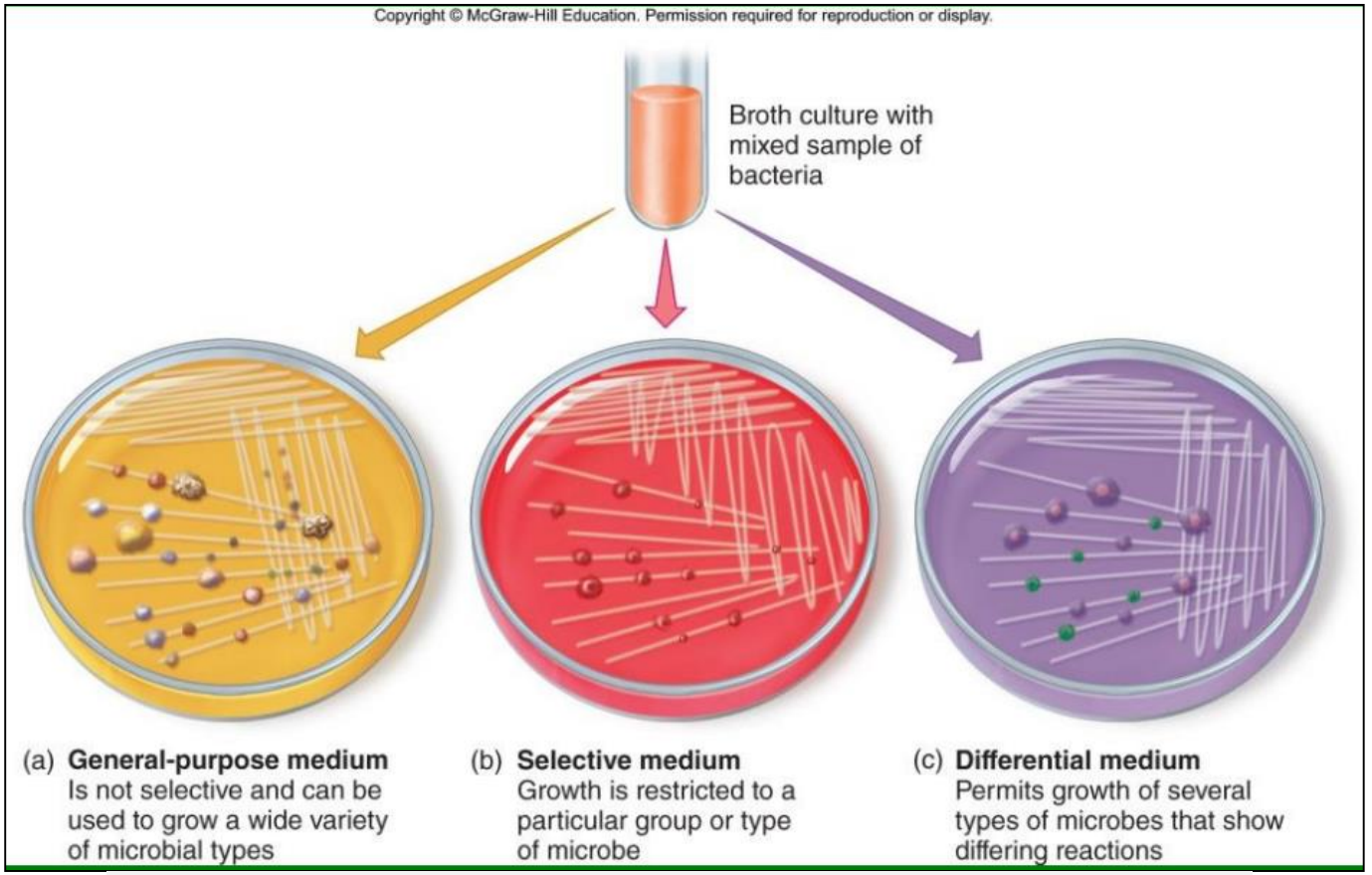
The dye methylene blue in the medium inhibit the growth of gram positive bacteria.



ATCC

Culture media

Common, non-selective and differential media



Selective medium promote the growth of one organism and retards the growth of the other organism.

Common media
Nutrient media

- **Nutrients(proteins/peptides/amino-acids):**
- Meat infusions: contain water-soluble fractions of protein (amino-acids and small peptides) along with other water-soluble products such as vitamins, trace metals, minerals and carbohydrates (glycogen).
- Such infusions or extracts may have been regarded as`peptones' but their amino-nitrogen content was usually too low to sustain the growth of large numbers of bacteria.
- Gelatin Peptone: containing amino acids, short chained peptides derived from enzymatic digestion of high quality gelatin.

Thermo Fisher Scientific Inc.,2021;..

Common media
Nutrient media

- **Nutrients (proteins/peptides/amino-acids):**

1. Meat Peptone: Derived from enzymatic hydrolysis of meat. Source of amino acids and short chained peptides. Their amino-nitrogen content was usually too low to sustain the growth of large numbers of bacteria.
2. Gelatin Peptone: containing amino acids, short chained peptides derived from enzymatic digestion of high quality gelatin.
3. Yeast extract: Derived from an aqueous extract of brewer's yeast. It is a rich source of vitamin content provides excellent growth conditions for more microbes.

Thermo Fisher Scientific Inc.,2021

Nutrient media

Nutrient media

- **Energy:**
- The most common substance added to culture media as a source of energy to increase the rate of growth of organisms is glucose.
- Other carbohydrates may be used as required.
- Carbohydrates added to media at 5-10 grammes per litre are usually present as biochemical substrates to detect the production of specific enzymes in the identification of organisms.
- It is usual to add pH indicators to such formulations.

Thermo Fisher Scientific Inc.,2021

Common media
Nutrient media

- **Essential Metals and Minerals(salts):**
- The inorganic essential components of culture media are many and can be divided on a semi-quantitative basis:

1. Typical macro-components (gm/litre): Na, K, Cl, P, S, Ca, Mg, Fe.
2. Typical micro-components (mgm-microgm/litre): Zn, Mn, Br, B, Cu, Co, Mo, V, Sr, etc.

Thermo Fisher Scientific Inc.,2021

Common media
Nutrient media

- **Buffering Agents:**
- It is important that the pH of a culture medium is poised around the optimum necessary for growth of the desired micro-organisms.
- The use of buffer compounds at specific pK values is especially necessary when fermentable carbohydrates are added as energy sources.
- Phosphates, acetates, citrates, zwitterion compounds and specific amino-acids are examples of buffering agents that may be added to culture media.

Thermo Fisher Scientific Inc.,2021

Common media

Complex medium for the growth of common and some fastidious bacteria

Component	Amount	Function of component
Beef extract	1.5 g	Source of vitamins and other growth factors
Yeast extract	3.0 g	Source of vitamins and other growth factors
Peptone	6.0 g	Source of amino acids, N, S, and P
Glucose	1.0 g	C and energy source
Agar	15.0 g	Inert solidifying agent
D. H_2O	1000 ml	
NaCl	5g	pH=7

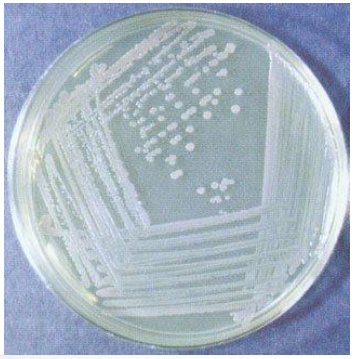
Growth factors are proteins that promote cell growth.

Todar,2000;..

Common media

Nutrient agar (NA)

Difco Nutrient Agar



- Typical composition of NA medium (g/litre):
- The carbohydrate source(e.g. glucose) is added at 10 g/L (1% w/v) to a basal medium prepared. pH=7.

Sodium chloride	5.0 g
Peptone	5.0 g
Yeast extract	2.0 g
Beef extract	1.0 g
Agar No.3	15.0 g
Deionized water	1000 ml

Common media

Trypticase Soy Agar (TSA)

- Used for cultivation of a variety of microorganisms.
- Composition (g/L):
- Bacto tryptone 15.0 g
- Bacto soytone 5.0 g
- NaCl 5.0 g
- Agar 15.0 g
- Dissolve in distilled water to a final volume of 1 L, autoclave for 15 min at 121°C, and pour into sterile Petri dishes.

Common media

Sucrose peptone agar (SPA)

- SPA medium is a nonselective medium useful for general purpose bacterial isolations.
- It is particularly useful when looking for levan-producing pseudomonads. On SPA, *R. solanacearum* colonies are white and fluidal with characteristic whorls.
- *Xanthomonas campestris* is characteristically mucoid on this medium.
- Composition (g/L):
- Sucrose 20 g
- peptone 5 g
- $MgSO_4$. $7H_2O$ 0.25 g
- K_2HPO_4 0.5 g
- Agar 15.0 g
- Adjust pH to 7.2-7.4 with 40% NaOH.

Common media

Potato semi synthetic agar/broth PSA/PSB or WPSB

- Wakimoto's potato semi synthetic broth:
- $Na_2HPO_4.12H_2O$ 2.0 g
- $Ca\ (NO_3)_2.4H_2O$ 0.5 g
- Peptone 5.0 g,
- Sucrose 20 g
- 15 g agar added to 1 liter of boiled 300 g potato, pH 7.0.

Common media

Peptone sucrose agar (PSA)

- Bactopeptone 10 g,
- Sucrose 10 g,
- Sodium glutamate 1 g,
- Distilled water 1 L
- Peptone sucrose broth (PSB): The same composition without agar.

Common media

Yeast Extract Glucose Agar(YPGA)

- Used for cultivation of a variety of bacteria like *R. solanacearum*, *Xylophilus ampelina* (ex. *X. ampelinus*), *Xanthomons* spp. and *C. m. michiganensis*.
- Composition (g/L):
- Yeast extract 5.0
- Bacto peptone 5.0
- D(+) glucose 10.0
- Agar 15.0
- pH=7-7.2
- Dissolve in distilled water to a final volume of 1 L, autoclave for 15 min at 121^{o}C, and pour into sterile Petri dishes.

Differential medium

King's medium B Base

Useful for detection of fluorescent Pseudomonads

- **Ingredients Gram/Liter**
- Proteose peptone 20
- Dipotassium hydrogen phosphate(K_2HPO_4) 1.5
- Magnesium sulphate. Heptahydrate ($MgSO_4.7H_2O$) 1.5
- Agar 20
- Final pH (at 25°C) 7.2±0.2
- **Directions**
- Suspend 42.23 grams of dehydrated medium in 1000 ml distilled water containing 15 ml of glycerol. Heat to boiling to dissolve the medium completely. Mix well. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Aseptically pour into sterile Petri plates.

Glycerol also called glycerin is a type of carbohydrate called a sugar alcohol.

Differential medium

Pseudomonas agar F(PAF/PSF)

For fluorescent Pseudomonads

- Equivalent to KB medium.
- Palleroni and Doudoroff (1972) medium contains:
- Tryptone 10 g
- Proteose peptone 10 g
- $MgSO_4$ 1.5 g
- Dipotassium phosphate 1.5 g
- Agar 15 g
- Glycerol 10 g
- $D.H_2O$ 1 L
- Autoclave for 15 min at 121°C, and pour into sterile Petri dishes.

Differential media

Fluorescent pseudomonads

Fluoresce on KB medium

- After incubation the plates were exposed to UV light at 365 nm for few seconds.

1. *Pseudomonas* (pyroverdins or fluorescent pigments) fluoresce both under short (254 nm) and long wave length (366 nm) ultraviolet light.
2. Nonfluorescent bacteria fluoresce just in short wavelength (254 nm) ultraviolet light.

Fluorescence of some strains(8,29,7,17) under UV lamp.

Sajben *et al.*,2004;..

Differential medium

Casamino-sucrose-gelatin medium

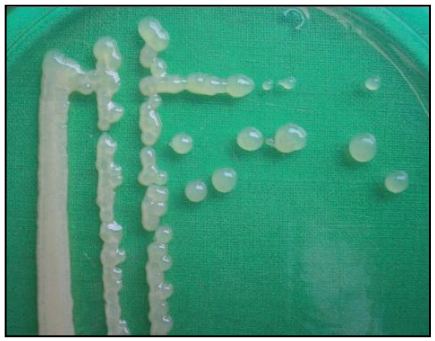
Alternative culture medium for pseudomonads that do not produce fluorescent on KB medium

- Vitamin free casamino acids 1 g
- $MgSO_4.7H_2O$ 0.1 g
- Dipotassium phosphate 0.1 g
- Agar 2 g
- Sucrose 1 g
- Gelatin 3 g
- $D.H_2O$ 100 ml
- Autoclave for 15 min at 121°C, and pour into sterile Petri dishes.

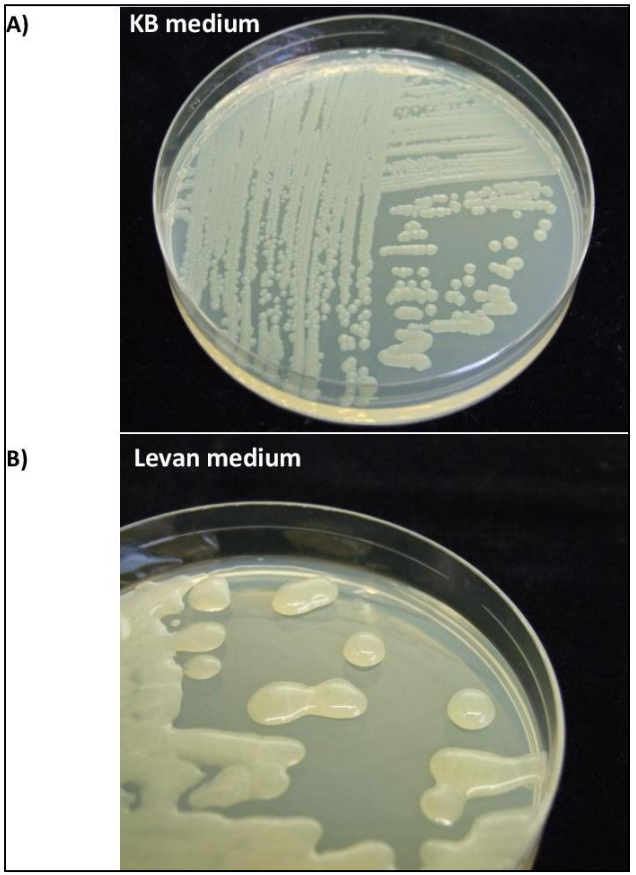
Lelliott and Stead, 1987

Differential media

Levan vs. KB



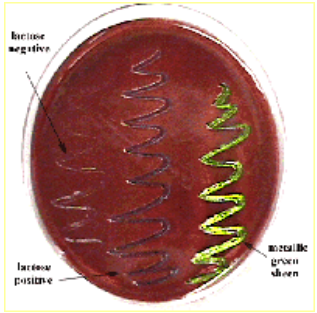
- Streak culture onto nutrient agar to which 5% (w/v) sucrose (NAS medium) has been added.
- White mucoid, dome-shaped colonies after 3 to 5 days incubation indicate a positive reaction.
- Polymer of fructose produced by levansucrase converting sucrose to glucose and fructose by which the fructose is transferred to the reducing end of an oligosaccharide chain resulting in levan.



Bereswill *et al.*,1998; Clares, 2017

Differential media

EMB Agar



- **Eosin Methylene-blue Lactose Sucrose Agar**
- Contains methylene blue: toxic to Gram+bacteria, allowing only the growth of Gram-bacteria such as *E. coli*, *Enterobacter cloacae*, etc.
- Typical Composition (g/litre): Peptone 10.0; di-potassium hydrogen phosphate 2.0; lactose 5.0; sucrose 5.0; eosin Y 0.4; methylene blue 0.07 and agar-agar 13.5.
- Appearance of Colonies:

1. In *Escherichia coli*, colonies are greenish, metallic sheen in reflected light, blue-black centre in transmitted light.
2. In case of *Enterobacter cloacae*, +/- Metallic sheen.

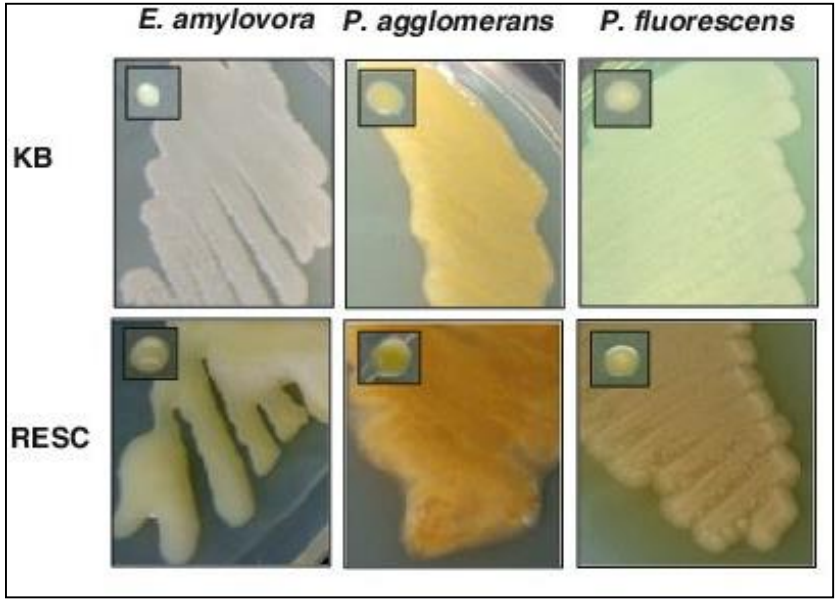
Methylene-blue a bright greenish blue organic dye;
Eosin (Y and D): A class of rose-colored dyes, soluble in water or alcohol, used as antiseptic.

Culture media

Two non-selective differential media

KB and RESC

- Growth and colonial morphology of *E. amylovora*, *P. agglomerans* and *P. fluorescens* (strains CFBP1430, EPS411, EPS347, respectively) on differential King's B (KB) medium and non-selective differential RESC medium containing 1.5 mM CuSO4, after 48 h at 26°C.



RESC (Recovery Erwinia amylovora- Stressed Cells) medium was used to improve the recovery of *E. amylovora* from plants under unfavorable conditions i.e. unfavorable weather conditions, copper-treated samples, nutrient starvation, etc.. its colonies were easily distinguished by a light yellow color and a high mucus production.

Ordax *et al.*,2011

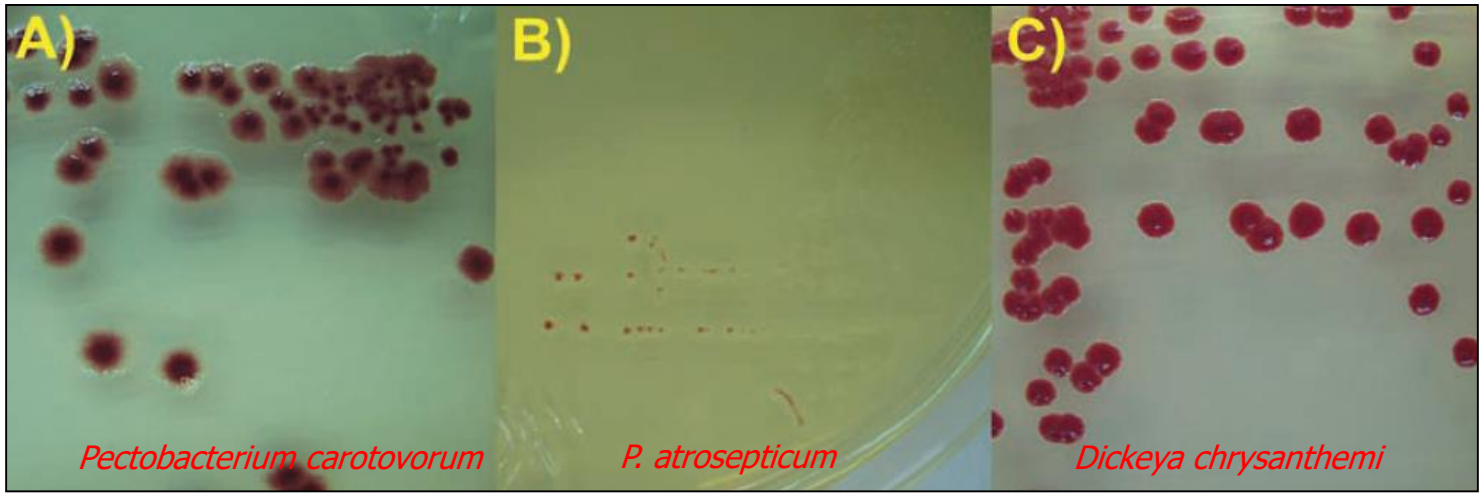
Differential medium

Modified tetrazolium chloride agar (TTC/TZC)

- Kelman TZC agar (Kelman,1954):
- Dextrose(glucose) 10 g
- Peptone 10 g
- Casein hydrolysate (casimic acid) 1 g
- Agar 15 g
- D. H_2O 1L
- Autoclave, and add 0.05% aqueous solution (filter-sterilized or autoclave separately) of 2,2,3-triphenyl-tetrazolium chloride (TTC) before pouring into petri dishes.

Differential medium

Modified triphenyltetrazolium chloride agar (TTC/TZC)



A. *P. carotovorum* subsp. *carotovorum* (1.5 mm colonies with red-pinky center in 24 h).
B. *P. atrosepticum* (0.5 mm colonies with red center after 48 h).
C. *Dickeya chrysanthemi* (2 mm colonies with completely red center within 24 h).

- Living cells reduce the tetrazole to a water-insoluble red colored formazan.

Ivanović *et al.*,2006;..

Differential medium

***Xanthomonas* spp. are easily differentiated from other yellow pigmented bacteria. Their colonies are mucoid, convex, and shiny on YPGA and Wilbrink-N media**

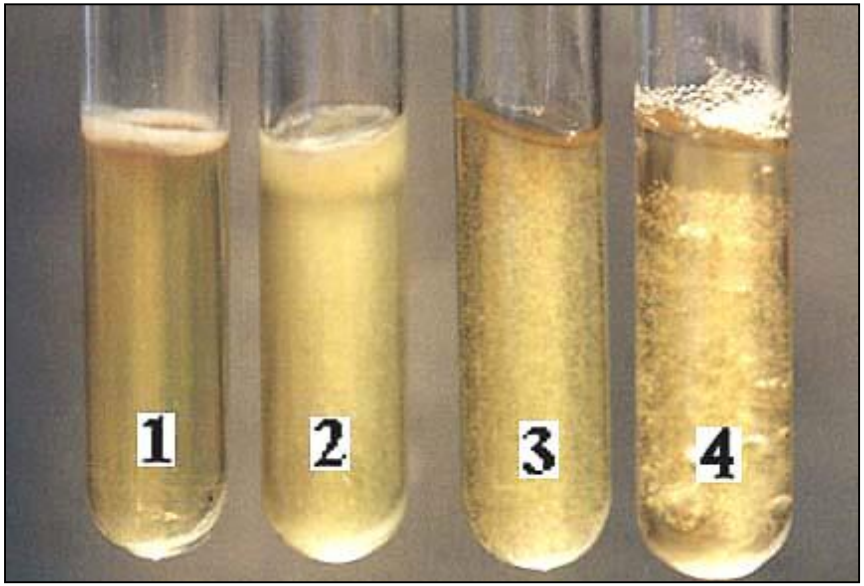
Xanthomonas fragariae

- **Wilbrink-N media (Koike, 1965;with nitrate):**
- Sucrose 10 g
- Proteose peptone 5 g
- K_2HPO_4 0.5 g
- $MgSO_4.7H_2O$ 0.25 g
- $NaNO_3$ 0.25 g
- Purified agar (L28 Oxoid) 15 g
- distilled water 1 L
- Adjust pH to 7.0-7.2.
- Prepare this medium with cycloheximide if fungal presence is suspected.
- After autoclaving, add 0.25 g cycloheximide per L (prepare a stock solution of 2.5 g per 33.33 mL of absolute ethanol and keep at -20°C).

Dorothy Noble

Enrichment media

- Supplemented with highly nutritious materials, such as serum, or yeast extracts, for the cultivation of fastidious organisms.
- It is used to encourage the growth of particular bacteria in mixed.



Enrichment medium
Luria-Bertani (LB)

- A rich medium used in molecular biology.
- **Enrichment Recipe for 1 Liter of LB:**
- Tryptone 10.0 g
- Yeast extract 5.0 g
- NaCl 10.0 g
- Agar 15.0 g
- pH 7.0
- Glucose can be added (2.0 g/L) for growth of organisms that cannot utilize other sugars.

CV026 was cultured in Luria-Bertani (LB) agar (1% w/v peptone, 0.5% w/v distilled water).yeast extract, 0.5% w/v NaCl, agar 1.5% per 100 mL.

Culture media
Mineral salt medium(MSM)

- The Mineral Salt Medium was used for isolation of bacteria from soil.
- MSM contained (g/l):
- $(NH_4)_2SO_4$ 2.0
- KH_2PO_4 1.5
- Na_2HPO_4 1.5
- $MgSO_4.7H_2O$ 0.2
- $CaCl_2.2H_2O$ 0.01
- $FeSO_4.7H_2O$ 0.001
- The pH of the medium was adjusted to 7.0 ± 0.1 with 2M NaOH.

Mahiudddin *et al.*,2014

Culture media
Soil Extract Agar

- Soil Extract Agar is used for isolation of soil microorganisms.
- Ingredients g/l:

Glucose	1
Dipotassium phosphate	0.5
Soil extract	17.75
Agar	15
Final pH (at 25°C)	6.8±0.2

- Directions
- Suspend 34.25 grams of soil in 1000 ml distilled water. Heat to boiling to dissolve the medium completely and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour into sterile Petri plates.

Glucose serves as readily metabolizable carbon source whereas dipotassium phosphate buffers the medium.

HiMedia Laboratories,2011

Culture media
Anaerobic media

- Anaerobic bacteria need special media for growth because they need low oxygen content, reduced oxidation-reduction potential and extra nutrients.
- Media for anaerobes may have to be supplemented with nutrients like hemin and vitamin K.
- Boiling the medium serves to expel any dissolved oxygen.
- Addition of 1% glucose, 0.1% thioglycollate, 0.1% ascorbic acid, 0.05% cysteine or red hot iron filings can render a medium reduced.
- Robertson cooked meat that is commonly used to grow *Clostridium* spp. medium contain a 2.5 cm column of bullock heart meat and 15 ml of nutrient broth.
- Before use the medium must be boiled in water bath to expel any dissolved oxygen and then sealed with sterile liquid paraffin.
- Methylene blue or resazurin is an oxidation-reduction potential indicator that is incorporated in the thioglycollate medium.
- Under reduced condition, methylene blue is colourless.

Semi-selective medium

Pseudomonas viridiflava

- Gitaitis and co-workers (1997) have published a diagnostic medium which was effective for the semi-selective isolation and enumeration of *Pseudomonas viridiflava*, the causal agent of bacterial streak and rot of onion.
- The medium contained:

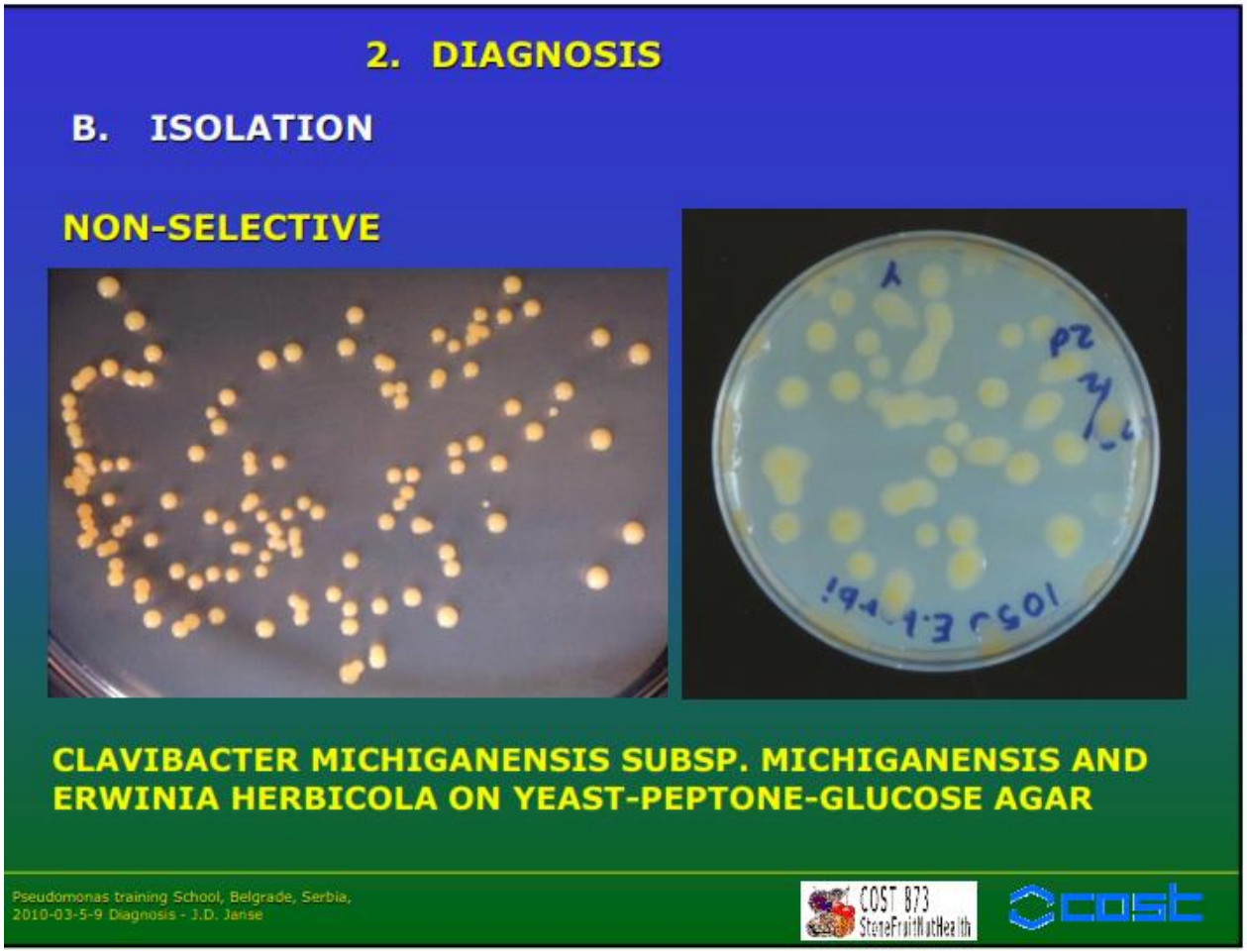
1. Tartrate as a carbon source, and
2. The antibiotics, bacitracin, vancomycin, cycloheximide, novobiocin and penicillin G.

- Selectivity was enhanced by incubation at 5°C rather than higher temperatures.

Semi-selective medium

Yeast-peptone-glucose agar (YPGA)

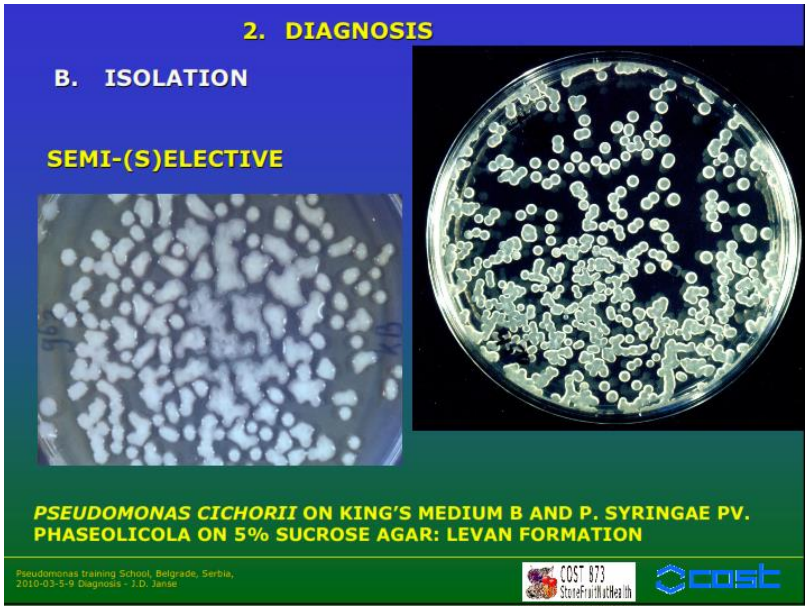
Clavibacter michiganensis and *Pantoea herbicola*

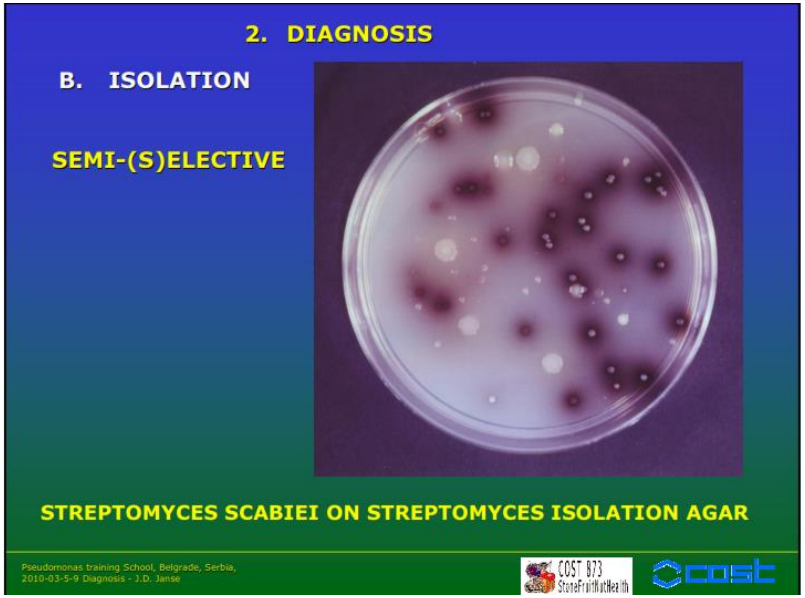


Janse,2010

Semi-selective medium

Pseudomonas cichorii, P. phaseolicola and *Streptomyces scabiei*





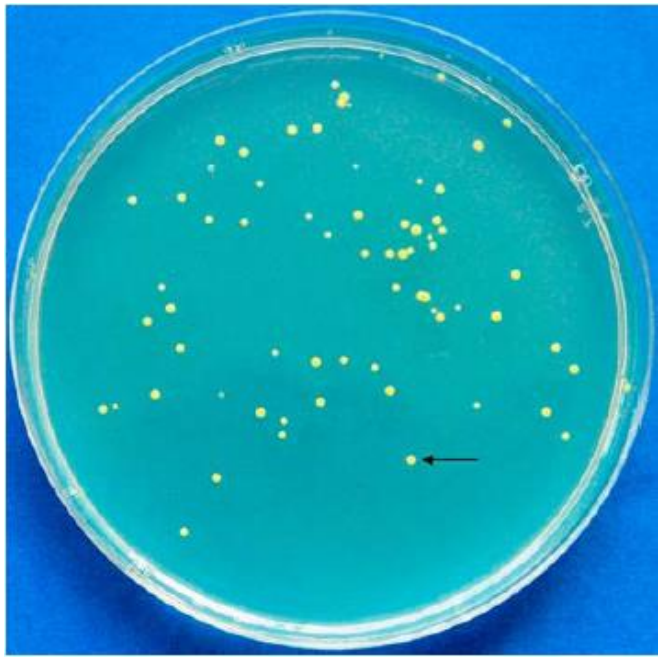
Janse,2010

Semi-selective medium

YTSA-CC

X. c. pv. *musacearum*

- A semi-selective medium YTSA-CC was have developed containing yeast extract (1%), tryptone (1%), sucrose(1%), agar (1.5%), cephalexin (50 mg l^{-1}) and cycloheximide (150 mg l^{-1}), pH 7.0.
- The pathogen *X. c.* pv. *musacearum* was easily identified as yellowish, mucoid and circular colonies on YTSA-CC medium.
- This simple semi-selective medium was effective for isolation of *X. c.* pv. *musacearum* from infected banana tissues and soil, and it should be a valuable tool in ecological and epidemiological studies.



Tripathi *et al.*,2007

The plating efficiency

Comparison of YTSA[a] and YTSA-CC[b] semi-selective medium in recovering colonies of *Xanthomonas campestris* pv. *musacearum* plated at different dilutions

- Efficiency of the semi-selective medium was quantified in comparison with YTSA according to the following formula:
- Efficiency % = [(colonies on YTSA-CC)/colonies on YTSA)] X 100.

Dilution of *Xcm* culture	Mean (±SE) No. of colonies		Mean (±SE) efficiency of YTSA-CC[c]	Mean (±SE) size of colonies (mm)	
	YTSA[a]	YTSA-CC[b]		YTSA	YTSA-CC
10^{-2}	Uncountable	Uncountable	–	–	–
10^{-4}	863.0 ± 26.6	843.7 ± 26.5	99.9 ± 5.1	1.3 ± 0.2	1.4 ± 0.3
10^{-6}	88.7 ± 5.8	87.0 ± 1.1	98.8 ± 4.9	2.7 ± 0.3	2.4 ± 0.3
10^{-8}	6.5 ± 2.2	6.1 ± 2.0	95.4 ± 2.4	3.3 ± 0.3	3.2 ± 0.2

[a]YTSA contains 1% yeast extract, 1% tryptone, 1% sucrose and 1.5% agar.
[b] YTSA-CC medium is YTSA containing also 50 mg l^{-1} cephalexin and 150 mg l^{-1} cycloheximide.
[c] Efficiency % = [(colonies on YTSA-CC)/(colonies on YTSA)] x 100.

Selective medium
CVP(Crystal violet pectate) medium

- **CVP per 500 ml water:**
- 1 N NaOH 4.5 ml 10%
- $CaCl_2.2H_2O$ 3.0 ml
- $NaNO_3$ 1.0 g
- Agar 1.5 g
- Sodium polypectate 10 g
- 10% SDS 0.5 ml
- 0.075% crystal violet 1.0 ml
- Pour 300 ml of boiling water into a preheated blender jar, then add the first 4 ingredients.
- See the next slide

Cuppels and Kelman,1974

Selective medium

CVP(Crystal violet pectate) medium Continued

- Blend at high speed for 15 seconds while adding the polypectate.
- Blend for another 15 seconds while adding 200 ml of hot water.
- Pour the medium into a 1L bottle and add the SDS and crystal violet.
- Autoclave for 25 min.
- The medium will be very soft and translucent with a brown-violet tint.
- Great care must be used to not disturb the medium when spreading or streaking bacteria onto CVP plates.
- The cavities formed by pecteolytic bacteria.

Cuppels and Kelman,1974

Selective medium

Modified crystal violet pectate medium(CVPD)

- Sodium polypectate obtained from other sources does not give satisfactory results. This might be due either to poor gelling quality, or failure of some bacteria to break down the polypectate.
- In CVPD medium, sodium polypectate was replaced with tryptone in both basal and upper layer(0.6 and 0.3 % tryptone, respectively) to improve growth and cavity formation.
- Also incorporation of 2,3,5-triphenyltetrazolium chloride and L-asparagine in the basal medium and bromthymol blue in the upper layer resulted in pink to red colonies of the soft rot *Erwinia* species in addition to deep cavity formation.
- While colonies of contaminating bacteria appeared white, red, green or yellowish without cavity formation.

Bdliya *et al.*,2004

Selective medium

Modified crystal violet pectate medium(CVPD)

Polymyxin B is an antibiotic primarily used for resistant Gram-negative infections. It is derived from the bacterium *Bacillus polymyxa*

- The basal medium contained per 250 ml of distilled water, 2 g peptone, 0.6 g yeast extract, 1 g NaCl, 3 g agar, 1.5 g tryptone, 0.5 g L-asparagine and 50 mg sodium dodecyl sulphate (SDS).
- After autoclaving, 0.5 ml of filter sterilized solution of 1% 2, 3, 5-triphenyl tetrazolium chloride and 0.8 mg of polymyxin B sulphate(mixture of Polymyxin B1 and B2) were added to 250 ml and shaken properly.
- About 10 ml was poured per plate and allowed to set at room temperature for 30-60 min before pouring the upper layer.
- The upper layer was basically CVPB medium (Perombelon and Burnett, 1991) modified by replacing 1 g $NaNO_3$, 0.5 g tryptone and novobiocin with 1 g KNO_3, 1.5 g tryptone and polymyxin B sulphate, respectively, per 500 ml and the addition of 0.5 ml of 0.5 % bromthymol blue solution.
- After autoclaving the medium, it was allowed to cool to about 40 °C and then filter sterilized solution of 0.6 mg polymyxin B sulphate was added to 500 ml instead of the novobiocin as in the CVPB medium.
- The medium was then shaken properly and about 15 ml poured per plate of the basal medium.

Bdliya *et al.*,2004

Isolation and pigment development
Semi-selective media
C. michiganensis subsp. *michiganensis*

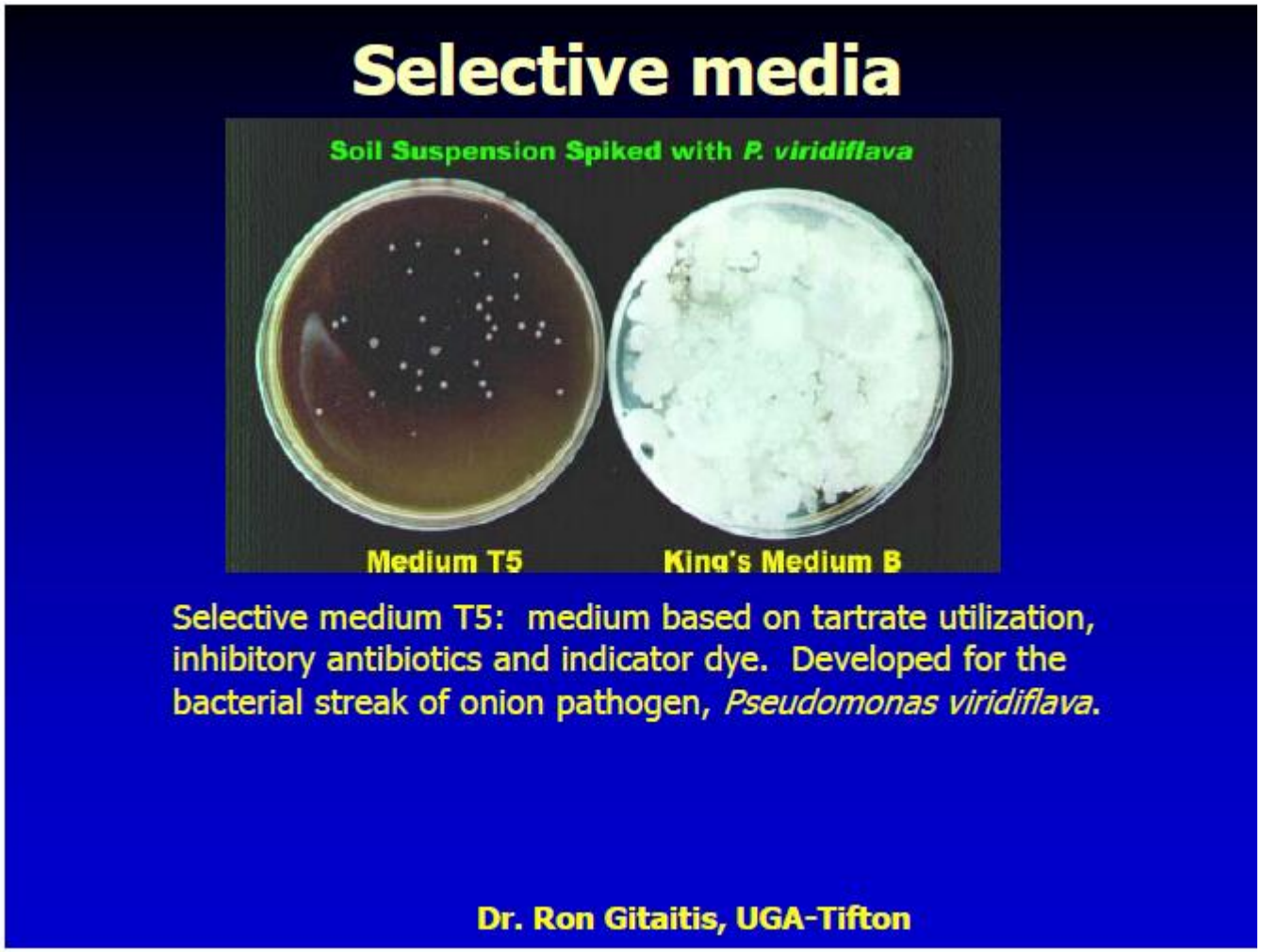
- Six representative semi-selective media for the detection of *C. michiganensis* subsp. *michiganensis*, namely CMM1T, SCM, SCMF, BCT, and SMSMM:
- The composition of each medium per 1 L were as follows:
- CMM1T (10 g sucrose, 3.32 g Tris base, 11.44 g Tris-HCl, 0.25 g $MgSO_4.7H_2O$, 5 g LiCl, 2 g yeast extract, 1 g NH_4Cl, 4 g casamino acids, 15 g agar, 10 mg polymyxin B sulfate, 28 mg nalidixic acid, 100 mg nystatin).
- SCM (10 g sucrose, 0.1 g yeast extract, 1.5 g H_3BO_3, 0.25 g MgSO4.$7H_2O$, 2 g K_2HPO_4, 0.5 g KH_2PO_4, 18 g agar, 30 mg nalidixic acid, 50 mg nicotinic acid, 100 mg nystatin, 10 mg potassium tellurite),
- SCMF (10 g sucrose, 2 g yeast extract, 1.5 g H_3BO3, 0.25 g $MgSO_4.7H_2O$, 2 g K_2HPO_4, 0.5 g KH_2PO_4, 18 g agar, 20 mg nalidixic acid, 50 mg nicotinic acid, 100 mg nystatin, 10 mg potassium tellurite, 80 mg trimethoprim),
- BCT (2.5 g mannitol, 2 g yeast extract, 1 g K_2HPO_4, 0.1 g KH_2PO_4, 0.05 g NaCl, 0.1 g $MgSO_4$. $7H_2O$, 0.015 g MnSO4. H_2O, 0.015 g $FeSO_4$. $7H_2O$, 0.6 g H_3BO_3, 15 g agar, 20 mg nalidixic acid, 100 mg trimethoprim, 20 mg polymyxin B, 4.2 mg epoxiconazole, 12.5 mg fenpropimorph), and
- SMCMM (20 g glycerol, 5 g peptone, 3 g yeast extract, 2 g K_2HPO_4, 0.5 g KH_2PO_4, 0.25 g $MgSO_4$. 7H2O, 5 g LiCl, 15 g agar, 20 mg nalidixic acid, 40 mg cycloheximide, 80 mg $K2Cr_3O_7$, 2 mg NaN_3, 2.1 mg 2,4,5,6-tetrachloroisophthalonitrile).
- All tested semi-selective media contained nystatin or cycloheximide. Both antibiotics inhibit the growth of fungi. Moreover, it is considered that other antimicrobial agents influence selectivity for *C. michiganensis* subsp. *michiganensis* in bacteria.
- R2A (per 1 L: 0.5 g glucose, 0.5 g soluble starch, 0.5 g yeast extract, 0.5 g proteose peptone no. 3, 0.5 g casamino acid, 0.3 g K_2HPO_4, 0.05 g MgSO4. 7H2O, 0.3 g sodium pyruvate, 15 g agar) medium containing nystatin at 100 μg mL^{-1} was used to detect total cultural bacteria in seed samples(SOMEYA *et al.*,2020).

Note: Conc. lactic acid(1ml/L) reduces the pH of the medium to about 4.8, which is low enough to inhibit the growth of most bacteria. If lactic acid is added prior to autoclaving or to very hot medium, it can prevent solidification of the agar.

Selective medium

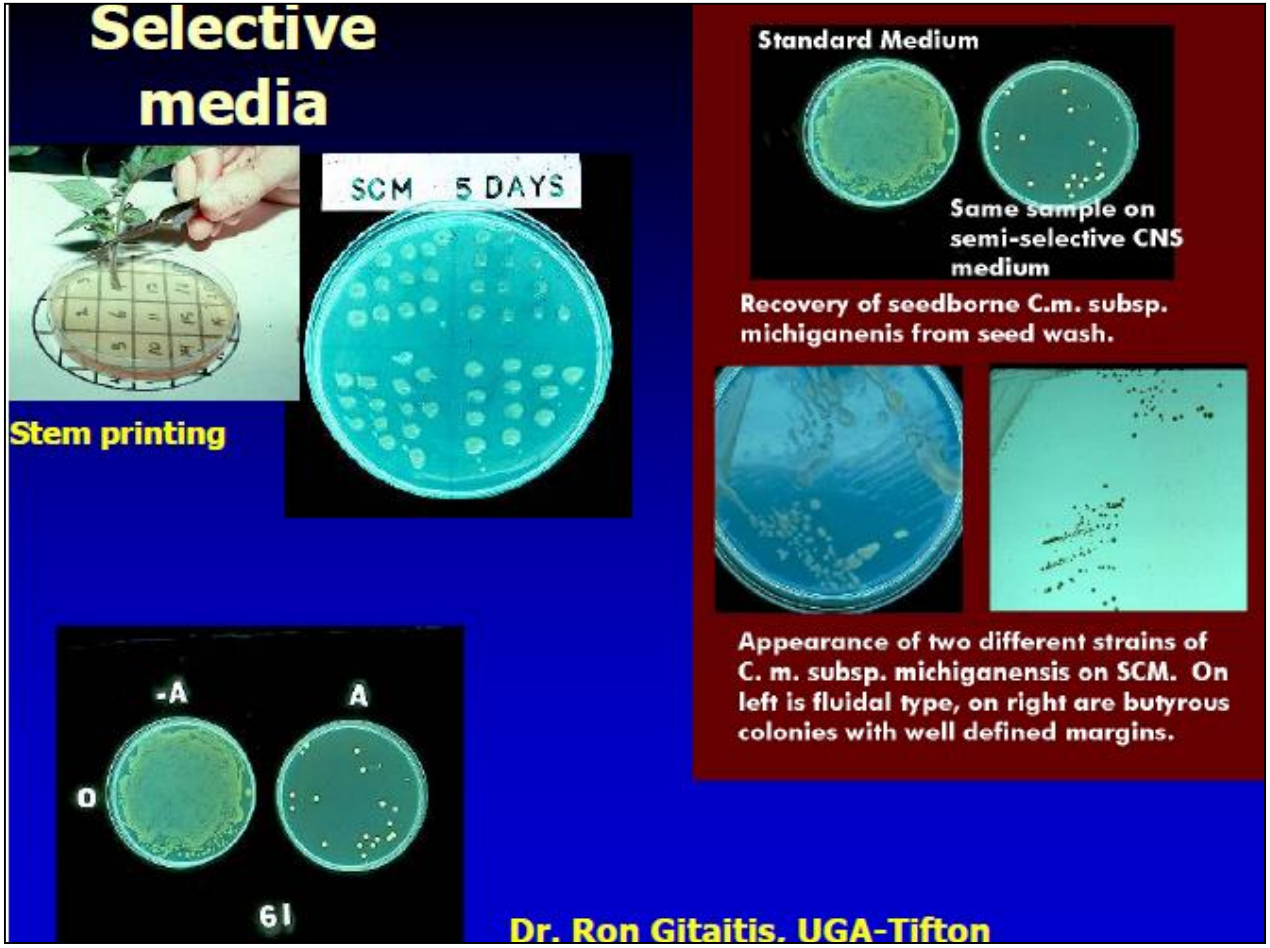
Medium T5

Pseudomonas viridiflava



Selective media

SCM and CNS for coryneforms



Selective media

- Tween A medium
- YSSM-XP medium
- Stock Salt Solution (SSS)
- NSCAA
- KBC medium
- SCM medium
- MT medium
- NASA medium
- YMA+C medium
- SX agar
- DIM (Modified D1 medium)
- NASA medium

Selective media

Comparison of the compositions of reported selective media

Target bacteria	*Burkholderia glumae*	*Acidovorax avenae*	*Pectobacterium carotovorum*		
Selective medium	CCNT medium Kawaradani *et al.*, 2000	AAC medium Shirakawa *et al.*, 2000	D medium Kado and Heskett 1970	CVP medium Cuplles and Kelman 1974	PEC-YA medium Starr *et al.*, 1977
natural materials	peptone yeast extract	yeast extract	hydrolysate casein		yeast extract
carbon source	inositol	adipic acid	arabinose glycine sucrose	citrate pectate	pectate
basal salts*		NH_4SO_4 KH_2PO_4 Na_2HPO_4 $MgSO_4$ $Na2MoO_4$	LiCl NaCl $MgSO_4$	$CaCl_2$	
antimicrobial	ceramide chloramphenicol novobiocin TPN**	ampicillin cycloheximide novobiocin phenethicillin	SDS****	SDS	
colony indicator		BTB***	acid fuchsin BTB	crystal violet	BTB

Selective media

Comparison of the compositions of reported selective media

Target bacteria	***Xanthomonoas campestris***			***Ralstonia solanacearum***	
selective medium	SM medium	CCA medium	YTSA-CC medium	FSM medium	SM-1 medium
	Chun and Alvarez, 1983	Mwangi *et al.*, 2007	Tripathi *et al.*, 2007	Nesmith and Jenkins, 1979	Granada and Sequeira, 1983
natural materials	potato starch	beef extract peptone yeast extract	tryptone yeast extract	casein hydrolysate peptone yeast extract	casein hydrolysate peptone
carbon source	glucose methionine	cellobiose glucose	sucrose	citrate glucose	glucose
basal salts[a]	NH_4Cl KH_2PO_4 Na_2HPO_4 $MgSO_4$ and others	NH_4Cl K_2HPO_4 $MgSO_4$		$MgSO_4$ $FeC_6H_5O_7$ $MnSO_4$ $ZnSO_4$ and others	
antimicrobial	cycloheximide	cephalexin cycloheximide fluorouracil	cephalexin cycloheximide	benomyl chloroneb cycloheximide dichloran and others	chlorothalonil cycloheximide polymyxin thimerosal and others
colony indicator	TZC*****			TZC	crystal violet TZC

Selective media

Comparison of the compositions of reported selective media

Foot-note

* Including sources of nitrogen, sulfur, phosphorus, and minerals

** Tetrachloroisophthalonitrile

*** Bromothymol blue

**** Sodium dodecyl sulfate

***** Tetrazolium chloride(TTC/TZC)

Selective media

- Selective media have a fairly high detection level (10^3-10^5 cells ml^{-1}).
- Use known controls.
- Use general media at the same time.

Goszczynaska *et al.*,2000

13. Tween A medium (modified)

For isolation of *Xanthomonas* spp.

	Per 1000 ml distilled H_2O
Peptone	10.0 g
KBr	10.0 g
$CaCl_2$	0.25 g
Agar	15.0 g
• Autoclave.	
• Cool to 50 °C and add:	
Tween 80 (autoclaved)	10.0 ml
Cephalexin (10 mg/ml water)	3.5 ml
5-Fluorouracil (6 mg/ml water)	2.0 ml
Cyclohexamide (100 mg/ml 75 % ethanol)	0.5 ml

14. YSSM-XP medium

For isolation of *X. axonopodis* pv. *phaseoli* from seed and plants.

Stock salt solution (SSS)

	Per 1000 ml distilled H_2O
$NH_4H_2PO_4$	2.5 g
K_2HPO_4	2.5 g
$MgSO_4 \times 7\ H_2O$	1.0 g
NaCl	25.0 g

Medium

SSS	200 ml
Yeast extract	5 g
H_2O	800 ml
• Adjust pH to 7.4.	
Soluble potato starch	10.0 g
Agar	15.0 g
• Autoclave, cool to 50 °C and add:	
Bravo 500F (15 mg/ml water)	1 ml
Cephalexin (10 mg/ml water)	2 ml
Brilliant cresyl blue (1 mg/ml water)	1 ml
Kasugamycin (10 mg/ml water)	2 ml
Methanol	20 ml

15. NSCAA (nutrient starch cycloheximide antibiotic agar)

For isolation of *X. campestris* pv. *campestris* from seed.

	Per 1000 ml distilled H_2O
Nutrient agar (Difco)	23.0 g
Soluble starch	15.0 g

Selective media

- Autoclave, cool to 50 °C and add:

Cycloheximide (100 mg/ml 75 % ethanol)	2 ml
Vancomycin (10 mg/ml water)	50 µl

16. KBC medium

For selective isolation of *P. syringae* pv. *syringae* from bean seed and plants.

I. Difco proteose peptone No. 3	20.0 g
Glycerol	15.0 ml
K_2HPO_4 (anhydrous)	1.5 g
$MgSO_4 \times 7\ H_2O$	1.5 g
Agar	15.0 g
Distilled water	900 ml
II. Boric acid	1.5 g
Water	100 ml

- Autoclave I and II separately, cool to 50 °C, mix and add:

Cephalexin (10 mg/ml water)	8 ml
Cyclohexamide (100 mg/ml 75 % ethanol)	2 ml

17. SCM medium

For selective isolation of *C. michiganense* subsp. *michiganense*.

	Per 1000 ml distilled H_2O
Sucrose	10.0 g
Yeast extract	0.1 g
K_2HPO_4	2.0 g
KH_2PO_4	0.5 g
$MgSO_4 \times 7\ H_2O$	0.5 g
Boric acid	1.5 g
Agar	15.0 g

- Autoclave, cool and add:

Cyclohexamide (100 mg/ml 75 % ethanol)	2 ml
Naladixic acid (salt) 10 mg/ml 2 % 0.1N NaOH)	1 ml
Difco Chapman tellurite (1 % solution)	1 ml
Nicotinic acid (free acid) (10 mg/ml aq.)	10 ml

18. MT medium (milk–tween)

Semi-selective medium for isolation and differentiation of *Pseudomonas syringae* and *P. savastanoi* pathovars, and *Xanthomonas* spp.

I. Proteose peptone No. 3 (Difco)	10.00 g
$CaCl_2 \times 2\ H_2O$	0.33 g
Tyrosine	0.50 g
Agar	15.00 g
Distilled water	500 ml

Goszczynaska *et al.*,2000

Selective media

II. Skimmed milk	10.0 g
Distilled water	500 ml
III. Tween 80	10 ml
• Autoclave I, II and III separately and mix when still hot.	
• Cool to 50 °C and add:	
Cephalexin (10 mg/ml water)	8 ml
Cycloheximide (100 mg/ml 75 % ethanol)	2 ml
Vancomycin (10 mg/ml water)	1 ml

19. NASA medium

For isolation of *Agrobacterium* spp.

Add to 1000 ml of autoclaved nutrient agar (5):

Cellenite (0.5 g/100 ml water)	10 ml
Cycloheximide (actidione) (0.5 g/100 ml water)	10 ml

20. YMA+C medium

For isolaton of *Agrobacterium* spp.

	Per 1000 ml distilled H_2O
Mannitol	10.0 g
K_2HPO_4	0.5 g
$MgSO_4 \times 7H_2O$	0.2 g
NaCl	0.1 g
Yeast extract	0.4 g
Congo Red (1 g/400 ml w/v)	10.0 ml
• Adjust pH to 7.0.	
Agar	12.0 g

21. SX agar

For isolation of *Xanthomonas campestris* pv. *campestris*

	Per 1000 ml distilled H_2O
Potato starch (soluble)	10.0 g
Beef extract (Difco)	1.0 g
NH_4Cl	5.0 g
K_2HPO_4	2.0 g
Methyl violet 2B (1 % in 20 % ethanol)	0.4 ml
Methyl green (1 % in water)	2.0 ml
Agar	15.0 g
• Autoclave, cool to 50 °C and add:	
Cycloheximide (100 mg/ml in ethanol)	2.0 ml
• Mix and pour	

Goszczynaska *et al.*,2000

SMART Media

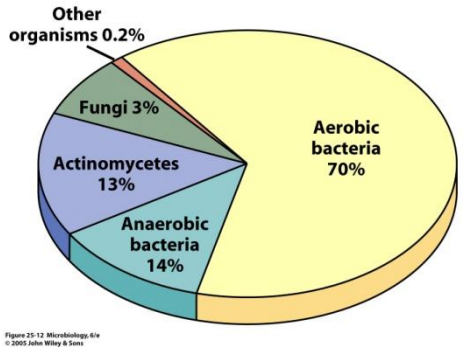
Selective Medium-Design Algorithm Restricted by Two Constraints

New Detection Systems of Bacteria Using Highly Selective Media

- Culture techniques have been indispensable to microbiological research since the 1870s, when they were first established by Louis Pasteur, Robert Koch, and other scientists.
- Among the media used for culturing, some possess a degree of selectivity that enables simple, efficient multiplication of a specific microorganism from samples with a large quantity of saprophytes; these are called selective media.
- Selective media can reliably isolate pathogenic and commercially useful microorganisms.

Kawanishi *et al.*,2011

Soil microorganisms
Including soil bacteria



- Enormous numbers of microorganisms exist in soil, plant tissues, seawater, and other environments.
- For example, the number of species in 1 gram of soil has been variously estimated as approximately
- 10,000 species (Torsvik *et al.*,1990);
- 10,000,000 species (Wolinsky and Dunbar,2005); and
- 2,000 species (Schloss and Handelsman,2006; Hong *et al.*,2006).

Kawanishi *et al.*,2011

Selective media

Old detection systems for isolation of target bacteria

- Selective media must have two functions:

1. Enabling the proliferation of the target microorganism, and
2. Suppressing unintended microorganisms on the medium.

- Moreover, selective media are efficient means for growing fastidious microorganisms.
- Due to their usefulness, many selective media have been developed for various microorganisms.

Kawanishi *et al.*,2011

List and compositions of selective media

Old selective media

- The compositions of ten reported selective media are summarized in Table S1, and categorized as:

1. Natural materials,
2. Carbon sources,
3. Basal salts,
4. Antimicrobials, and
5. Colony indicators (Table S1).

- Most reported selective media contain natural materials (e.g. potato extract, peptone and yeast extract).

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Old selective media

Table S1

Table S1. Comparison of the compositions of reported selective media.

target bacteria	*Burkholderia glumae*	*Acidovorax avenae*	*Pectobacterium carotovorum*		
selective medium	CCNT medium Kawaradani *et al.*,2000	AAC medium Shirakawa *et al.*,2000	D medium Kado and Heskett,1970	CVP medium Cuplles and Kelman,1974	PEC-YA medium Starr et al.,1977
natural materials	peptone yeast extract	yeast extract	casein hydrolysate		yeast extract
carbon source	inositol	adipic acid	arabinose glycine sucrose	citrate pectate	pectate
basal salts*		NH4SO4 KH2PO4 Na2HPO4 MgSO4 Na2MoO4	LiCl NaCl MgSO4	CaCl2	
antimicrobial	ceramide chloramphenicol novobiocin TPN**	ampicillin cycloheximide novobiocin phenethicillin	SDS****	SDS	
colony indicator		BTB***	acid fuchsin BTB	crystal violet	BTB

Old selective media

Table S1(Continued)

target bacteria	*Xanthomonoas campestris*			*Ralstonia solanacearum*	
selective medium	SM medium Chun and Alvarez,1983	CCA medium Mwangi *et al.*,2007	YTSA-CC medium Tripathi *et al.*,2007	FSM medium Nesmith and Jenkins,1979	SM-1 medium Granada and Sequeira,1983
natural materials	potato starch	beef extract peptone yeast extract	tryptone yeast extract	casein hydrolysate peptone yeast extract	casein hydrolysate peptone
carbon source	glucose methionine	cellobiose glucose	sucrose	citrate glucose	glucose
basal salts[a]	NH4Cl KH2PO4 Na2HPO4 MgSO4 and others	NH4Cl K2HPO4 MgSO4		MgSO4 FeC6H5O7 MnSO4 ZnSO4 and others	
antimicrobial	cycloheximide	cephalexin cycloheximide fluorouracil	cephalexin cycloheximide	benomyl chloroneb cycloheximide dichloran and others	chlorothalonil cycloheximide polymyxin thimerosal and others
colony indicator	TZC*****			TZC	crystal violet TZC

* including sources of nitrogen, sulfur, phosphorus, and minerals;** tetrachloroisophthalonitrile; *** bromothymol blue
**** sodium dodecyl sulfate; ***** tetrazolium chloride

SMART Media

New Detection Systems of Bacteria Using Highly Selective Media

- We named our strategy SMART for highly Selective Medium-design Algorithm Restricted by Two constraints.

1. A carbon source, and
2. An antimicrobial(antibiotic).

- Note prior to this, there were no design theories for developing selective media, and each ingredient in selective media has been determined using trial-and-error methods.

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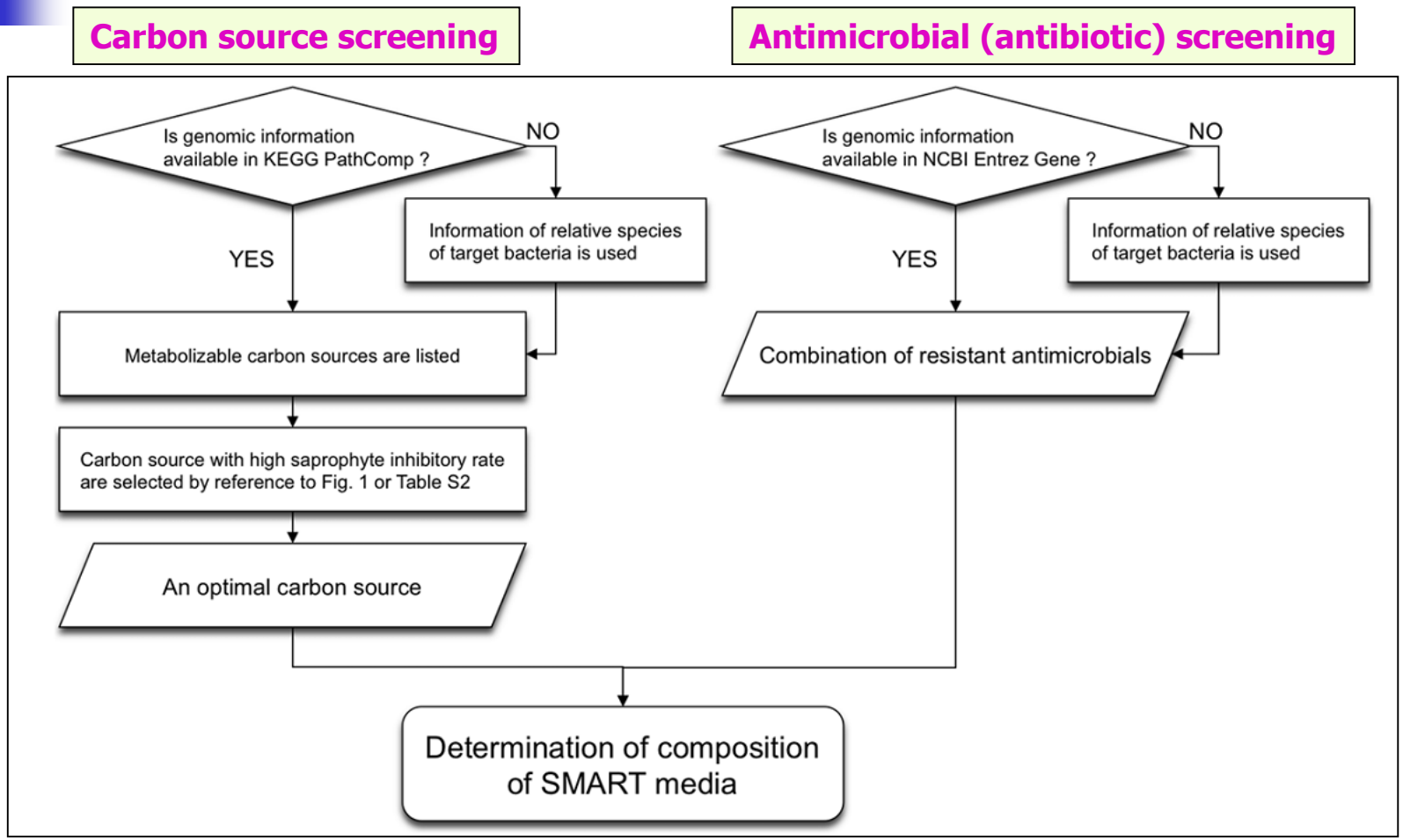
Flowchart of the SMART method

An optimal carbon source and a combination of antimicrobials to which the target bacterium is resistant should be chosen for designing a selective medium using SMART



KEGG (Kyoto Encyclopedia of Genes and Genomes)
NCBI(National Center for Biotechnology Information

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KEGG PathComp

http://www.genome.jp/kegg/

Quick detection of signaling pathways

- **KEGG (Kyoto Encyclopedia of Genes and Genomes):**
- It is a collection of online databases dealing with:

1. Genomes,
2. enzymatic pathways, and
3. biological chemicals.

KEGG PathComp

http://www.genome.jp/kegg/

Quick detection of signaling pathways

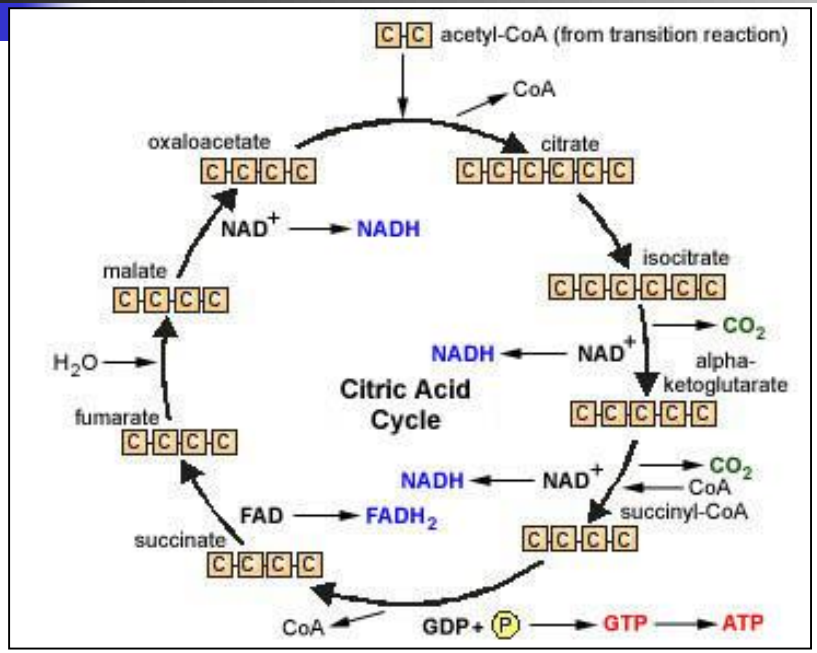
- **KEGG PathComp: Generate Possible Reaction Paths:**
- PathComp in KEGG is a computational tool that proposes possible reaction pathways between an initial and final compound using information about the presence or absence of known enzymatic reactions.
- With few exceptions, pathogenic bacteria encode a complete gene set for the

1. pentose phosphate pathway,
2. citrate cycle, and
3. glycolysis pathway.

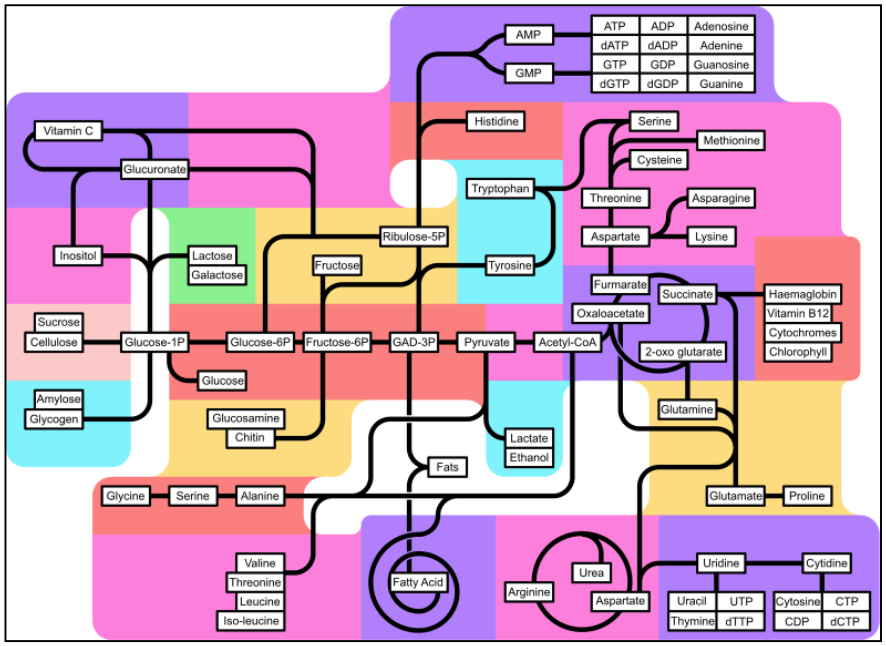
SMART Media

Carbon source

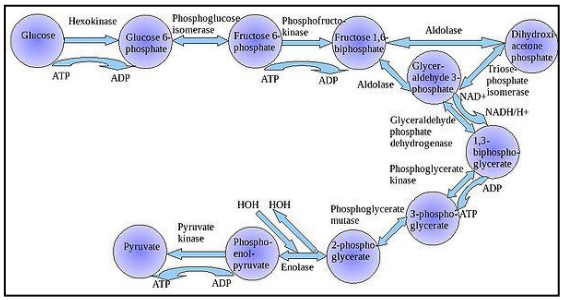
The three most important metabolic pathways



Citrate cycle (Krebs circle, aerobic pathway)



Pentose (aerobic and anaerobic pathway)



Glycolysis (Krebs aerobic and anaerobic pathway pathway)

SMART Media

Pentose phosphate pathway

Selection of carbon sources for *B. glumae* (*Bgl*)

- The metabolizable carbon sources were selected for Bgl based on genomic information available for a wide range of microorganisms in Kyoto Encyclopedia of Genes and Genomes or KEGG
- For *Burkholderia glumae* (*Bgl*) metabolic pathway links to alpha-D-glucose-6-phosphate, the starting material of the pentose phosphate pathway.
- *B. glumae* (*Bgl*) use L-glutamate, glucose, and 20 other substrates both the computed and experimental methods (Next fig. and Table).

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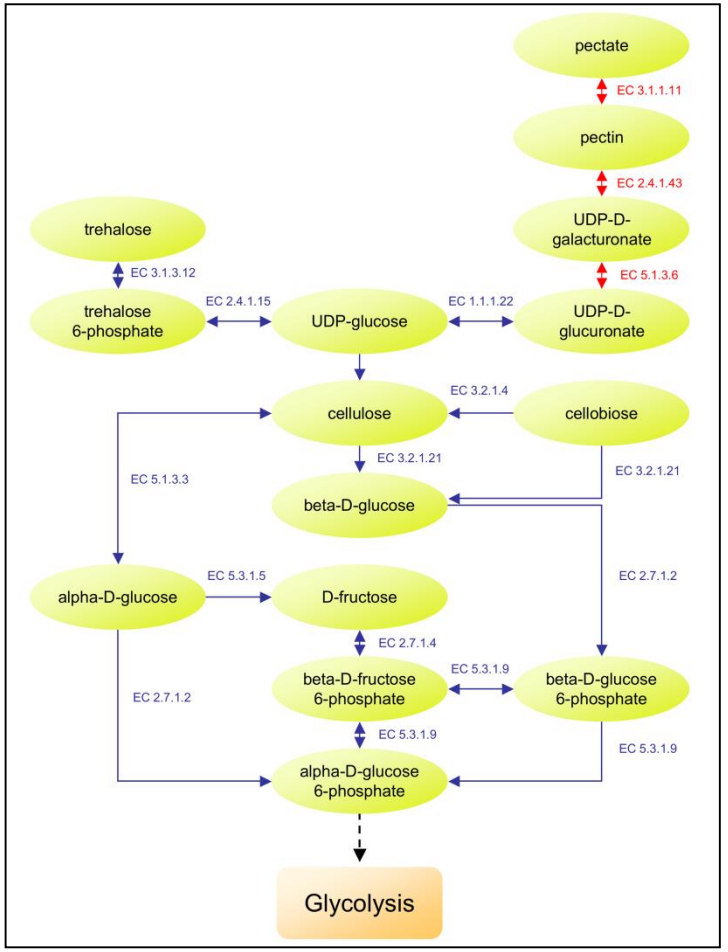
SMART Media

Pentose phosphate pathway

Selection of carbon sources for *B. glumae*

- Reaction enzymes that *B. glumae* encodes (blue arrows) and does not encode (red arrows).
- *B. glumae* has a pathway from cellobiose, trehalose, and D-fructose, but not from pectate to alpha-D-glucose-6-phosphate.
- Therefore, an a priori methodology predicts that:

1. Cellobiose, trehalose, and D-fructose are metabolizable carbon sources for *B. glumae*, while pectate is not.



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Pentose phosphate pathway

Selection of carbon sources for *B. glumae*

***Burkholderia glumae* (*Bgl*) use L-glutamate, glucose, and 20 other substrates both the computed and experimental methods**

Carbon sources	KEGG entry ID	Experimental data by colony formtaion	Prediction by genomic information*
L-glutamate	C00025	+	+
glucose	C00031	+	+
glycine	C00037	-	+
L-lysine	C00047	+	+
L-aspartate	C00049	+	+
L-arginine	C00062	+	+
L-glutamine	C00064	+	+
L-serine	C00065	+	+
L-methionine	C00073	+	+
L-tryptophan	C00078	+	+
L-phenylalanine	C00079	+	+
L-tyrosine	C00082	+	+
sucrose	C00089	+	+
D-fructose	C00095	+	+
L-leucine	C00123	+	+
L-histidine	C00135	-	+
myo-inositol	C00137	-	-
L-proline	C00148	+	+
L-valine	C00183	+	+
cellobiose	C00185	+	+
L-threonine	C00188	+	+
L-sorbose	C00247	+	+
D-mannitol	C00392	-	-
L-isoleucine	C00407	+	+
pectate	C00470	-	-
ribitol	C00474	-	-
D-sorbitol	C00794	+	+
trehalose	C01083	+	+

*Metabolizable carbon sources were predicted using KEGG PathComp.

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SMART Media

An appropriate carbon source

Growth promotion of target bacteria and growth inhibition of soil saprophytes

- To choose an optimal carbon source from these candidates, their inhibitory effect on the growth of soil saprophytes was calculated.
- The inhibitory ability of carbon sources against the soil saprophytes was shown in next slides.
- The carbon sources used in SMART plays both roles of:

1. energy source of the target bacterium, and
2. growth inhibitor of saprophytes.

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Recommended carbon source list

Growth inhibition of soil saprophytes (rice, turnip, and tomato soils)among carbon sources

*Of the 20 candidate carbon sources, D-sorbitol-supplemented basal medium resulted in the fewest unintended colonies (i.e., it had the highest rate of saprophyte growth inhibition.

Therefore, D-sorbitol was chosen as the optimal carbon source for selective *B. gladioli* medium.

Growth inhibitory rate [(1- the number of colony forming units (cfu) on each carbon source-added medium/the number on a sucrose-added medium) x100%].

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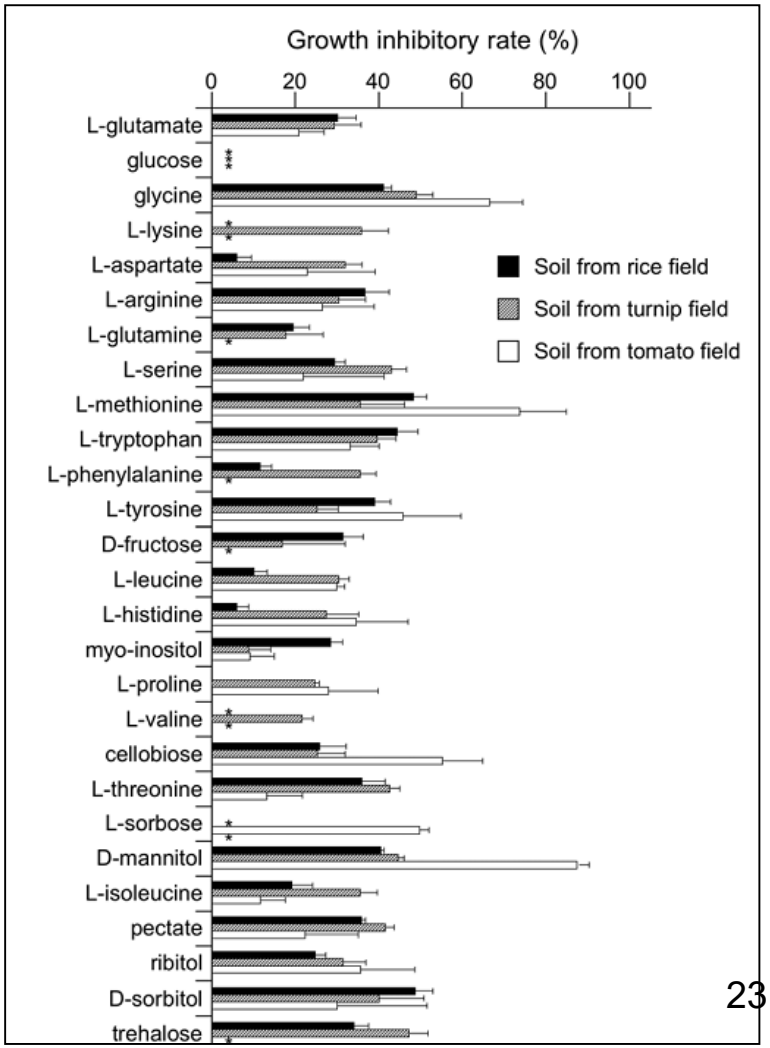
Table S2. Recommended carbon source list.

Carbon sources	Growth inhibition ranking* against soil saprophytes of:		
	rice	turnip	tomato
glycine	4	1	3
L-methionine	1	10	2
D-mannitol	5	3	1
D-sorbitol	2	7	10
trehalose	10	2	21
L-serine	13	4	16
L-tryptophan	3	8	9
L-tyrosine	6	18	6
cellobiose	14	20	4
ribitol	16	14	7
L-glutamate	12	17	17
L-arginine	7	15	13
L-leucine	20	16	11
L-histidine	22	19	8
L-threonine	8	5	18
pectate	9	6	15
L-aspartate	21	13	14
D-fructose	11	24	21
myo-inositol	15	25	20
L-proline	23	21	12
L-sorbose	23	26	5
L-isoleucine	18	12	19
glucose	23	26	21
L-lysine	23	9	21
L-glutamine	17	23	21
L-phenylalanine	19	11	21
sucrose	23	26	21
L-valine	23	22	21

SMART Media

Growth inhibition of soil saprophytes among carbon sources

- Each bar indicates the growth inhibitory rate [(1- the number of colony forming units (cfu) on each carbon source-added medium/the number on a sucrose-added medium) x100%].
- Asterisks indicate that the saprophyte inhibitory rate was less than that of sucrose.



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SMART Media

2. Antimicrobials

Determination of resistant antimicrobials in SMART using NCBI Entrez Gene Database

- In this study, we established the procedure for SMART, comparing genome-based predictions with experimental data on:

1. *Burkholderia glumae* (Bgl), and
2. Four other phytopathogenic bacteria.

- Antimicrobials for selective media for *Aav*, *Pca*, *Rso*, and *Xca* were selected using the NCBI Entrez Gene Database.

The SMART method is applicable to different Bacteria such as *Acidovorax avenae* subsp. *avenae* (Aav), *Pectobacterium carotovorum* subsp. *carotovorum* (Pca), *Ralstonia solanacearum* (Rso), and *Xanthomonas campestris* pv. *campestris* (Xca).

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SMART Media

Determination of resistant antimicrobials(antibiotics) in SMART

- In the SMART method, there is no need to test all possible combinations of carbon sources and antimicrobials manually to develop a synthetic selective medium.
- Genomic information can be used to select both the metabolizable carbon source and the appropriate antimicrobials.
- Because genomic information is accumulating rapidly with recent progress in DNA sequencing technology, such information is increasingly available to researchers.

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SMART Media

Determination of resistant antimicrobials in SMART

Antimicrobial resistance of *Burkholderia glumae* predicted by the NCBI database compared to experimental data

Antimicorbial Name	Reported Resistance Gene Name	COG*	Predicted Resistance**	Experimental Data***
ampicillin	beta-lactamase	COG2367V	R	R
	multidrug efflux pump *acrB*	-		
cephalosporine	beta-lactamase	COG2367V	R	R
cetrimonium	quaternary ammonium compound resistance protein	COG2076P	R	R
chloramphenicol	chloramphenicol acetyltransferase	-	R	R
	multidrug efflux pump *mdtC*	COG0841V		
gentamicin	aminoglycoside phosphotransferase aac3	-	S	S
	aminoglycoside adenyltransferase aadB	-		
neomycin	aminoglycoside phosphotransferase aac6	-	S	S
penicillin	beta-lactamase	COG2367V	R	S
polymyxin	polymixin resistance glycosyltransferase	-	S	R
streptomycin	streptomycin phosphotransferase strA	-	S	S
	streptomycin phosphotransferase strB	-		
trimethoprim	dihydrofolate reductase type I	-	S	S
	dihydrofolate reductase type X	-		
gramicidin	hydantoin racemase	-	S	S

*COG stands for clusters of orthologous groups of proteins (http://www.ncbi.nlm.nih.gov/COG/).
**R and S indicate resistant and susceptible, respectively.
***The concentration of antimicrobials added to the medium was 10 ppm.

SMART Media

New SMART media for some important plant pathogenic bacteria

Species	Strain	Growth on SMART-Bgl	Growth on SMART-Aav	Growth on SMART-Pca	Growth on SMART-Rso	Growth on SMART-Xca
Burkholderia glumae	MAFF 301441	+	-	-	-	-
Acidovorax avenae	MAFF 301502	-	+	-	-	-
Agrobacterium rhizogenes	MAFF 301724	-	-	-	-	-
	MAFF 301725	-	-	-	-	-
Agrobacterium tumefaciens	MAFF 301001	-	-	-	-	-
Burkholderia andropogonis	Am	-	-	-	-	-
Pectobacterium carotovorum	MAFF 301394	-	-	+	-	-
Pseudomonas cichorii	u1	-	-	-	-	-
	u2	-	-	-	-	-
Pseudomonas syringae	MAFF 301499	-	-	-	-	-
	MAFF 301430	-	-	-	-	-
Ralstonia solanacearum	chiba_tomato8945A1	-	-	-	+	-
	kouchi_tomato3-2	-	-	-	+	-
Xanthomonas campestris	MAFF 106641	-	-	-	-	+
	MAFF 106644	-	-	-	-	+
	MAFF 211374	-	-	-	-	+
	stock1-1	-	-	-	-	+
	NL 7756	-	-	-	-	+

MAFF; Ministry of Agriculture, Fisheries and Food of Japan.
doi:10.1371/journal.pone.0016512.t003

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SMART Media

New SMART media for some important plant pathogenic bacteria

SMART-Bgl medium plate

For *Burkholderia glumae*

D-sorbitol	1 g
Na2HPO4	3 g
KH2PO4	3 g
NH4Cl	1 g
MgSO4	0.25 g
FeSO4	5 mg
Crystal violet	3 mg
Agar	15 g
DW	1 L
Cycloheximide	50 mg
Ampicillin	10 mg
Cetrimonium	10 mg
Chloramphenicol	10 mg

SMART-Aac medium plate

For *Acidovorax avenae*

L-methionine	1 g
Na2HPO4	3 g
KH2PO4	3 g
NH4Cl	1 g
MgSO4	0.25 g
FeSO4	5 mg
Crystal violet	3 mg
Agar	15 g
DW	1 L
Cycloheximide	50 mg
Ampicillin	10 mg
Cetrimonium	10 mg
Polymixin	10 mg

SMART-Pca medium plate

For *Pectobacterium carotovorum*

Trehalose	1 g
Na2HPO4	3 g
KH2PO4	3 g
NH4Cl	1 g
MgSO4	0.25 g
FeSO4	5 mg
Crystal violet	3 mg
Agar	15 g
DW	1 L
Cycloheximide	50 mg
Cetrimonium	10 mg
Tyrothricin	10 mg

SMART-Rso medium plate

For *Ralstonia solanacearum*

D-Mannitol	1 g
Na2HPO4	3 g
KH2PO4	3 g
NH4Cl	1 g
MgSO4	0.25 g
FeSO4	5 mg
Crystal violet	3 mg
Agar	15 g
DW	1 L
Cycloheximide	50 mg
Chloramphenicol	10 mg
Polymixin	10 mg

SMART-Xca medium plate

For *Xanthomonas campestris*

Glycine	1 g
Na2HPO4	3 g
KH2PO4	3 g
NH4Cl	1 g
MgSO4	0.25 g
FeSO4	5 mg
Crystal violet	3 mg
Agar	15 g
DW	1 L
Cycloheximide	50 mg
Cepharexin	10 mg
Penicillin	10 mg

Each selective medium was comprised of one optimal carbon source and a few antibacterials.

Kawanishi *et al.*,2011

SMART Media

Selective Medium-Design Algorithm Restricted by Two Constraints

New SMART media for some important plant pathogenic bacteria

SMART-Bgl LSM		SMART-Aac LSM		SMART-Pca LSM		SMART-Rso LSM		SMART-Xca LSM	
For *Burkholderia glumae*		For *Acidovorax avenae*		For *Pectobacterium carotovorum*		For *Ralstonia solanacearum*		For *Xanthomonas campestris*	
D-sorbitol	1 g	L-methionine	1 g	Trehalose	1 g	Mannitol	1 g	Glycine	1 g
Na2HPO4	3 g	Na2HPO4	3 g	Na2HPO4	3 g	Na2HPO4	3 g	Na2HPO4	3 g
KH2PO4	3 g	KH2PO4	3 g	KH2PO4	3 g	KH2PO4	3 g	KH2PO4	3 g
NH4Cl	1 g	NH4Cl	1 g	NH4Cl	1 g	NH4Cl	1 g	NH4Cl	1 g
MgSO4	0.25 g	MgSO4	0.25 g	MgSO4	0.25 g	MgSO4	0.25 g	MgSO4	0.25 g
FeSO4	5 mg	FeSO4	5 mg	FeSO4	5 mg	FeSO4	5 mg	FeSO4	5 mg
Crystal violet	3 mg	Crystal violet	3 mg	Crystal violet	3 mg	Crystal violet	3 mg	Crystal violet	3 mg
DW	1 L	DW	1 L	DW	1 L	DW	1 L	DW	1 L
Cycloheximide	5 mg	Cycloheximide	5 mg	Cycloheximide	5 mg	Cycloheximide	5 mg	Cycloheximide	5 mg
Ampicillin	1 mg	Ampicillin	1 mg	Cetrimide	1 mg	Chloramphenicol	1 mg	Cepahrexin	1 mg
Cetrimonium	1 mg	Cetrimide	1 mg	Tryothricin	1 mg	Polymixin	1 mg	Penicillin	1 mg
Chloramphenicol	1 mg	Polymixin	1 mg						

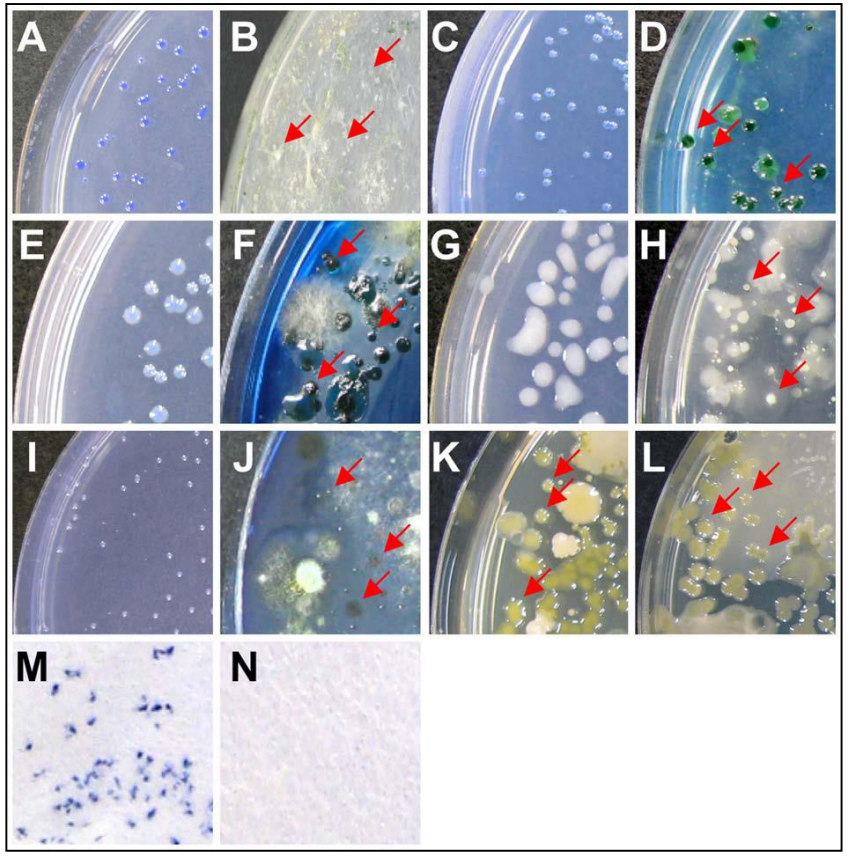
Kawanishi *et al.*,2011

SMART Media

Comparison of colonies formed on selective media produced by SMART and previous methodologies

- A suspension of pathogen-inoculated soil was plated on both a SMART medium and an existing selective medium.

1. *Burkholderia glumae* (*Bgl*) formed colonies on:
   A. SMART-Glu medium and
   B. CCNT medium.
2. *Acidovorax avenae* subsp. *avenae* (*Aaa*) formed colonies on
   C. SMART-Aav medium and
   D. AAC medium.



Kawanishi *et al.*,2011

SMART Media

Comparison of colonies formed on selective media produced by SMART and previous methodologies

3. *Pectobacterium carotovorum* (*Pc*) formed colonies on:

E. SMART-Pca medium and

F. CVP medium.

4. *Ralstonia solanacearum* (*Rso*) formed colonies on:

G. SMART-Rso medium and

H. SM-1 medium.

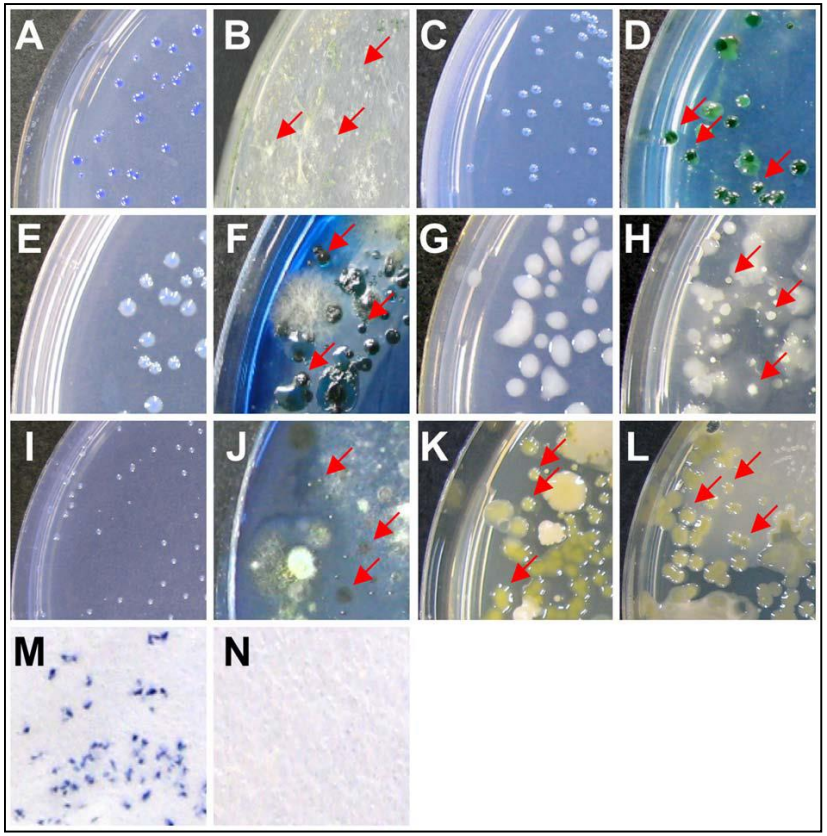
5. *Xanthomonas campestris* (*Xc*) formed colonies on:

I. SMART-Xca medium and

J. SM medium,

K. CCA medium,

L. YTSA-CC medium.



Kawanishi *et al.*,2011

Colony morphology

Pigmentation

Cultural conditions

- Probably the most visual characteristic is pigmentation (color).
- However, formation of pigment depends on environmental factors such as:

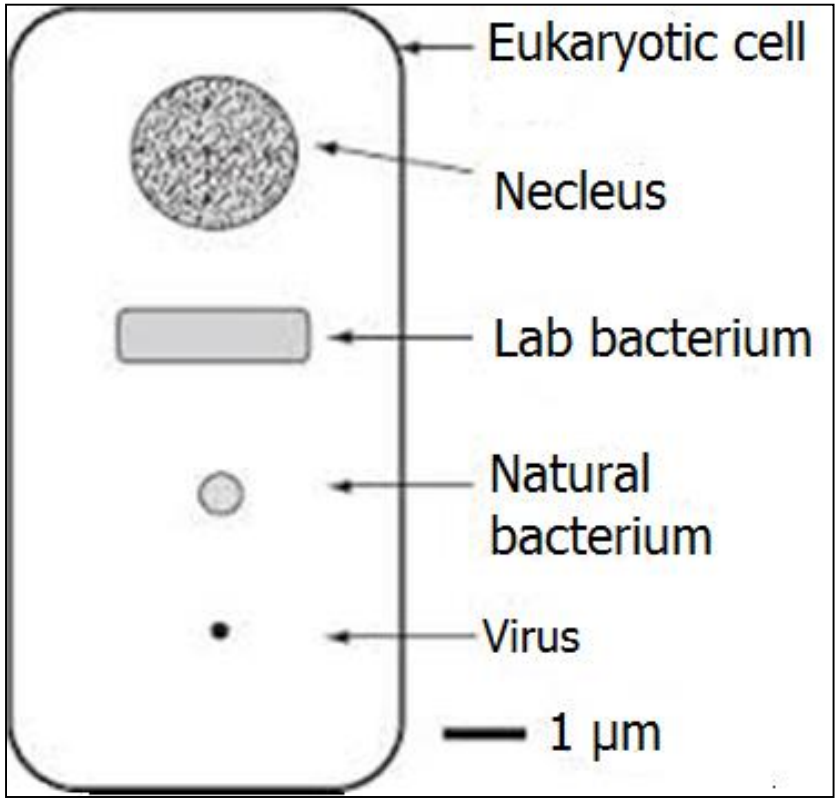
1. temperature,
2. nutrients,
3. pH, and
4. moisture.

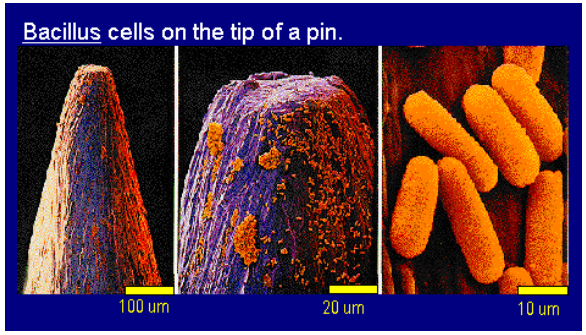
- For example, *Serratia marcescens* produces a deep red pigment at 25°C, but does not produce pigment at 37°C.

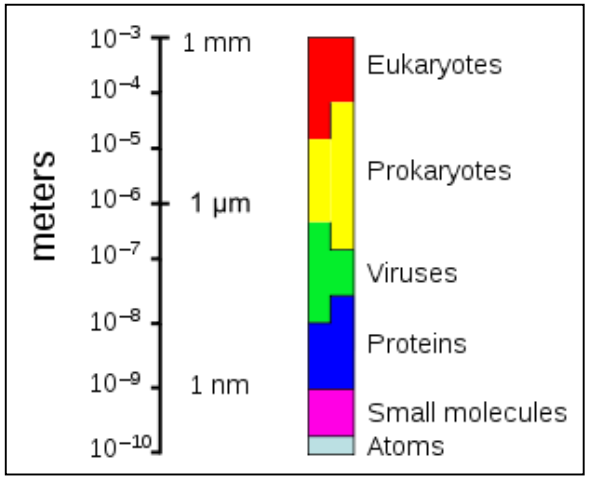
Kaiser,2009

Cell morphology

Bacterial cell size





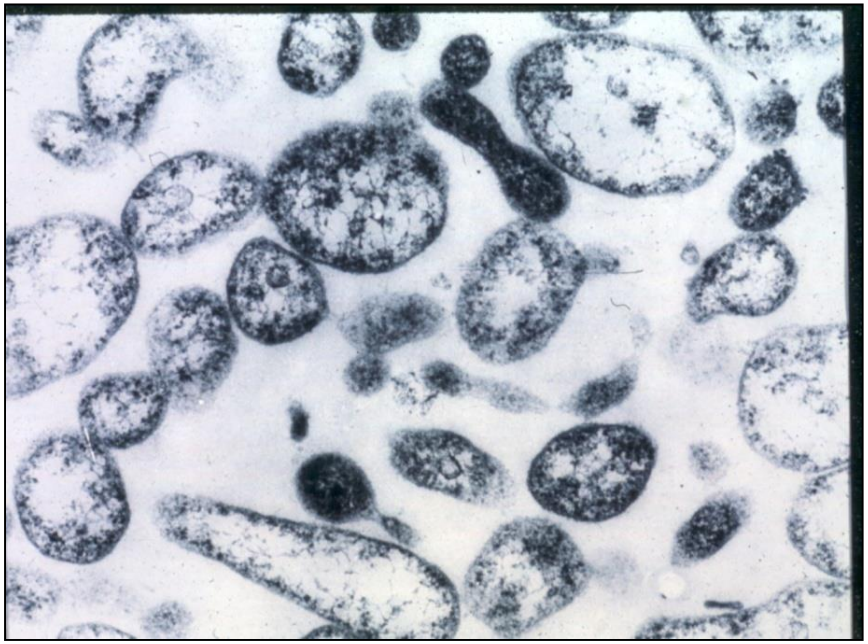


Wikipedia;...

Cell shape in living plant cells

Electron micrograph of bacterial cells

- A substantial portion of bacteroid development is triggered and directed by chemicals from the host plant, which therefore control bacterial morphology and/or cell division.
- If bacterial shape is important enough that the host plant devotes resources to modifying it, then shape may be a significant element in the overall process.

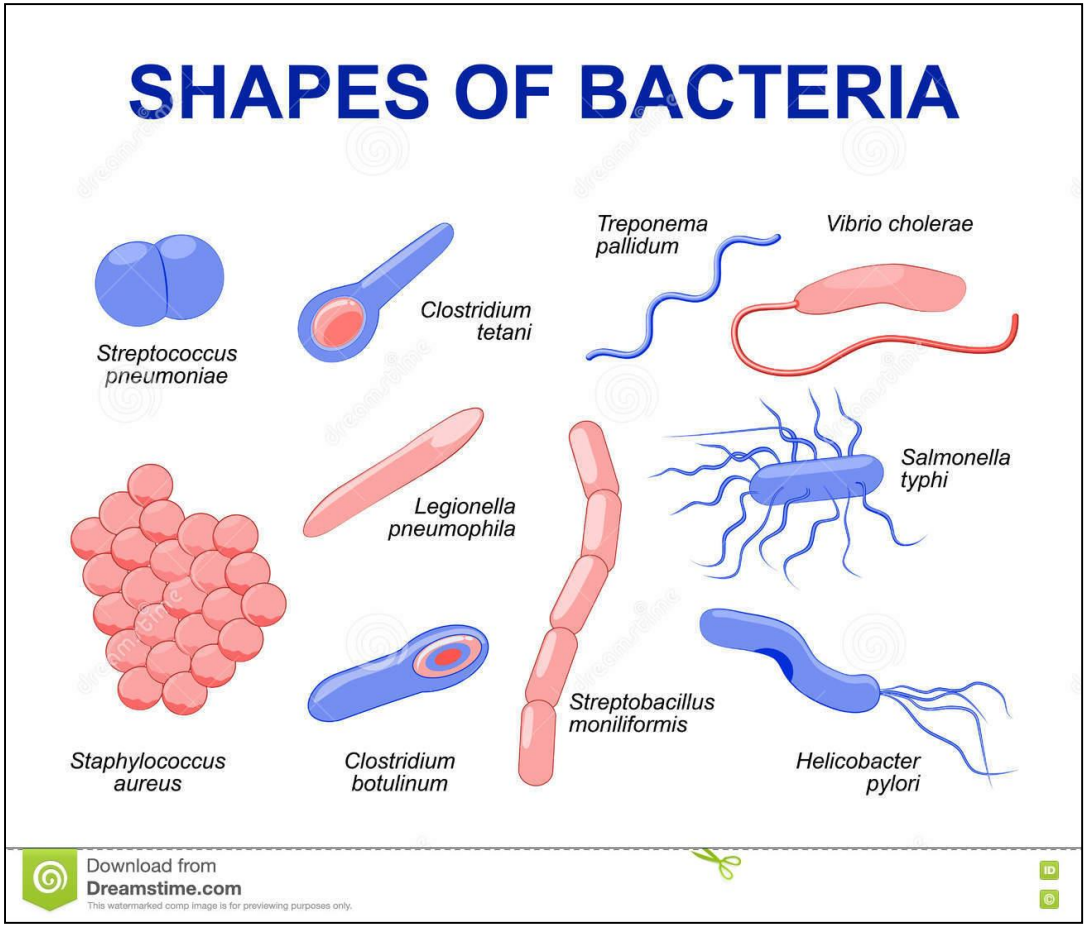


scanning electron microscope (SEM) reaching about 100,000x magnification and the **transmission electron microscope** (TEM) capable of 1,000,000x magnification.

Young,2006;..

Bacterial morphological shapes

Variety of prokaryotic shapes



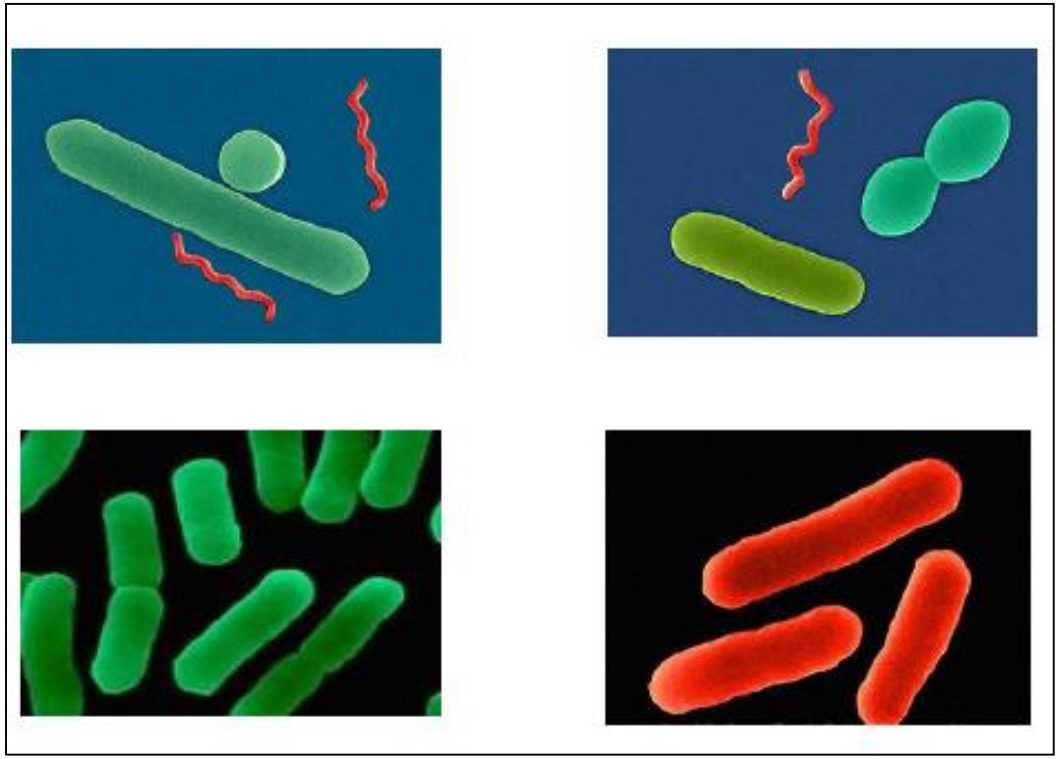
Dreamstime

Cell morphology

On culture media

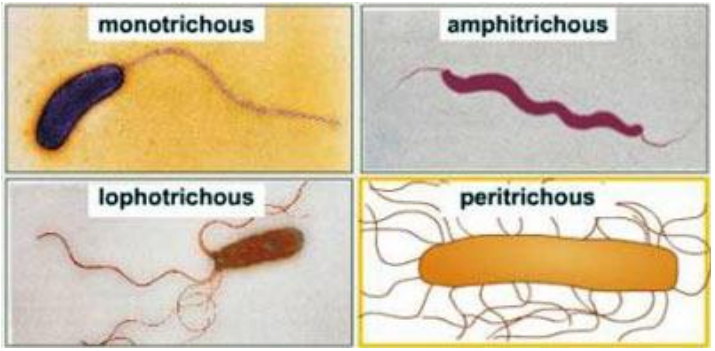
Bacterial cell shapes

- The majority of plant-associated bacteria are rods.



Hiremath,2009

Morphology
Rod-shaped bacteria



- Electron micrographs of some of the most important genera of plant-pathogenic bacteria:

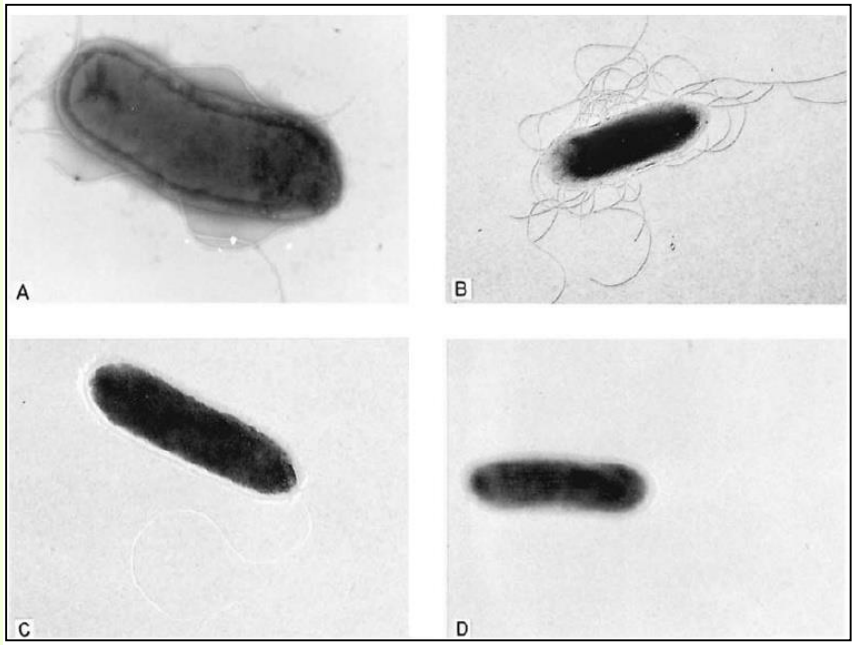
A. *Agrobacterium*,

B. *Erwinia*,

C. *Pseudomonas*, and

D. *Xanthomonas*.

- [Photographs courtesy of (A) R. E. Wheeler and S. M. Alcorn and (B–D) R. N. Goodman and P. Y. Huang.] Magnified 1600x.



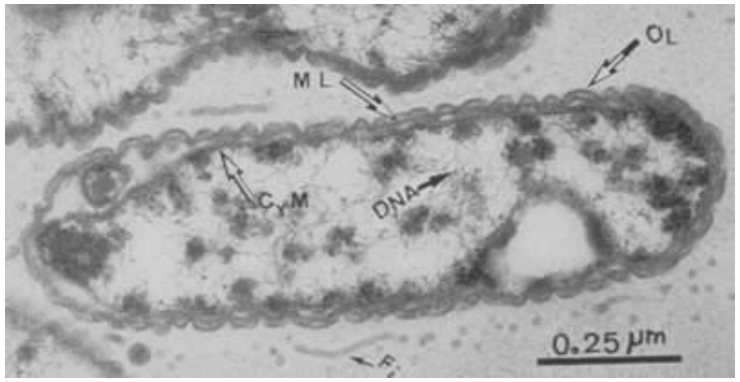
Agrios,2005;..

undulate

Cell shape

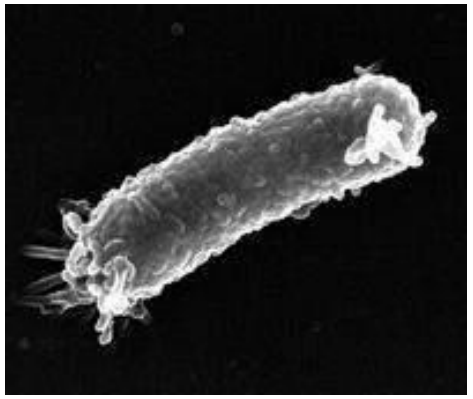
The ripple/undulating/wavy cell wall

Xylella fastidiosa



TEM micrograph

Note wavy outer wall layer.



Scanning electron micrograph

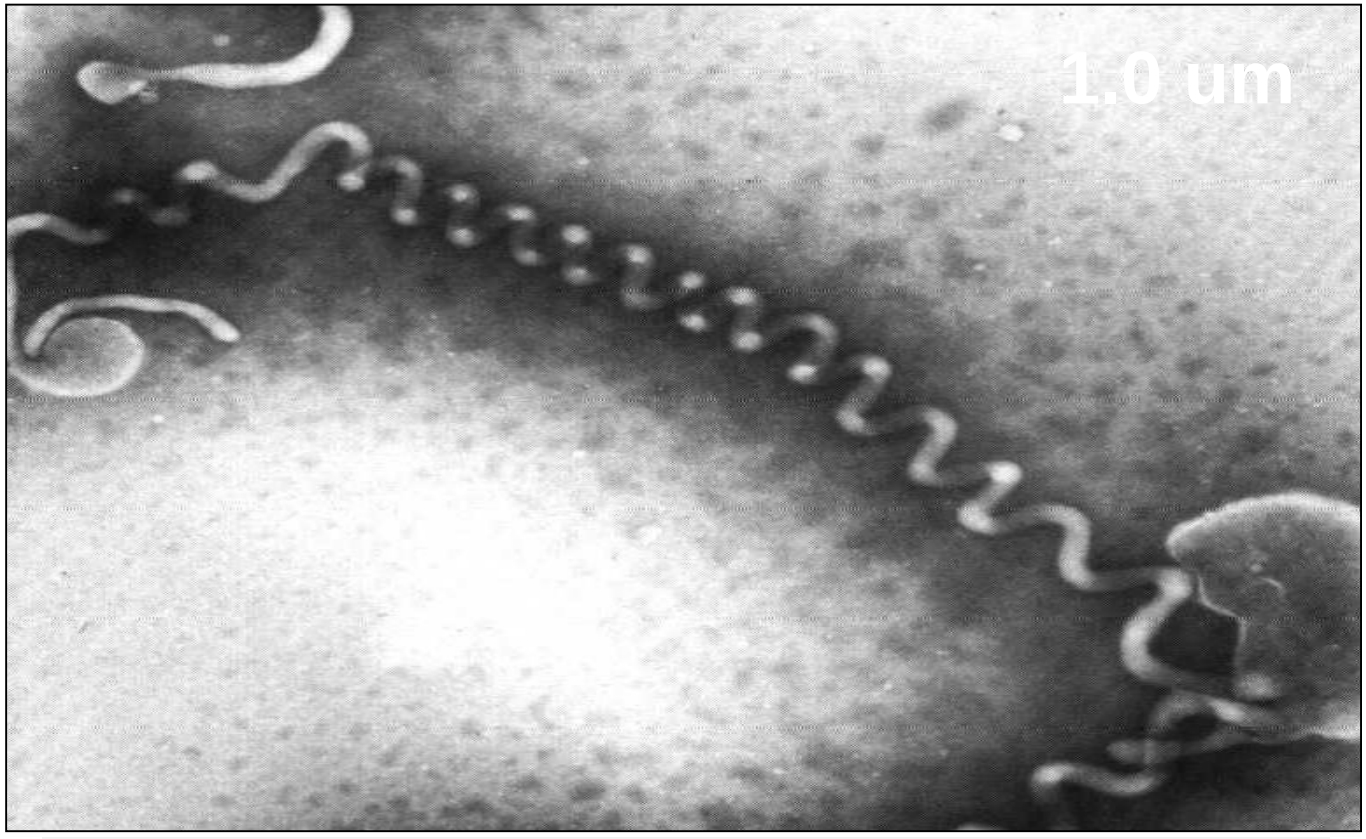
Xylella fastidiosa cells are small (0.25-0.5x1.0-4.0 µm), Gram negative, have no flagella. The outer layer of the cell wall is usually undulating or rippled.

The wall consists of:

1. an outer,
2. an inner layers (each comprised of 3-layered unit membrane structure), and
3. a middle peptidoglycan layer.

Cell shape

Filament detail of a 2 day old culture of *Spiroplasma citri*



Spiroplasmas form a helical cell shape and swim without flagella in viscous media. Cells of *Spiroplasma kunkelii* are just 0.15-0.2 μm in diameter while being 2.0-15 μm in length.

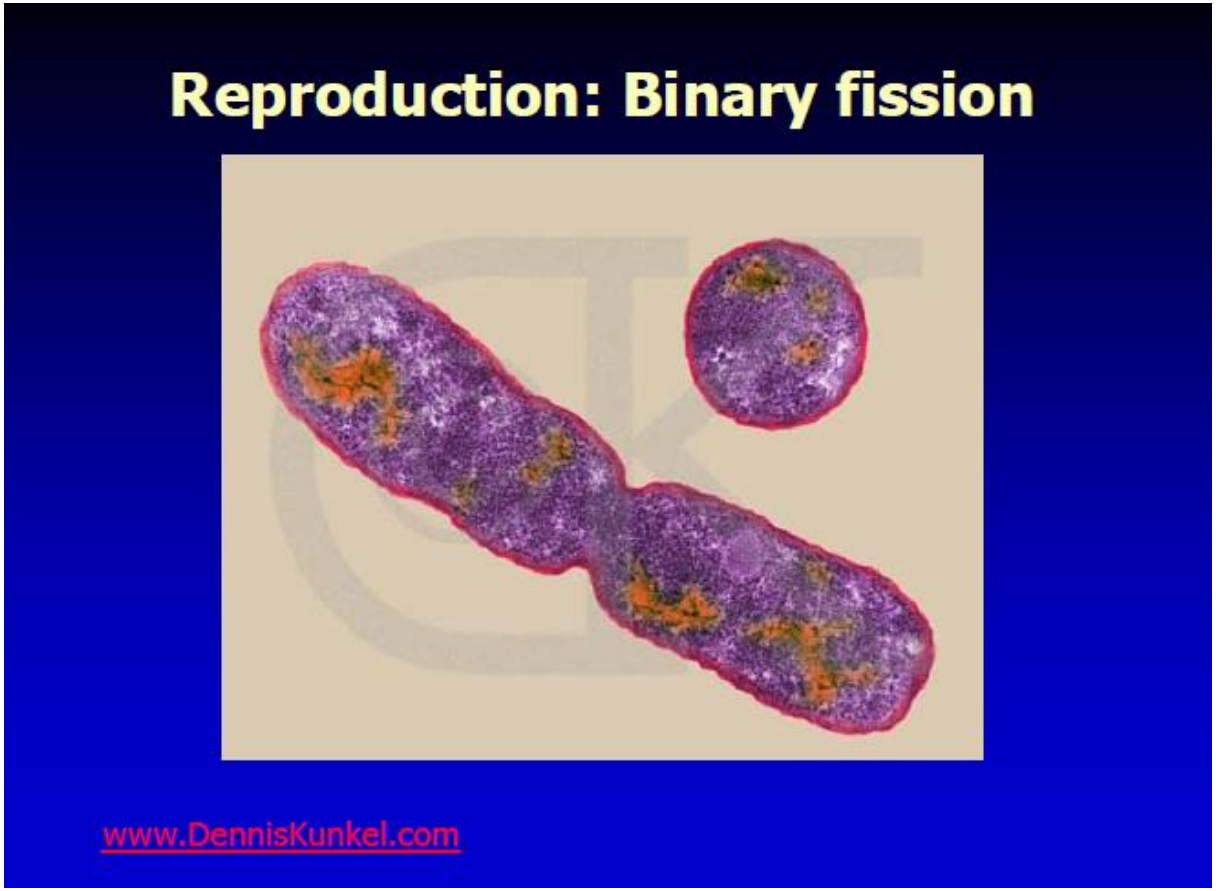
Cell division

Plant pathogenic bacteria act in groups, power in numbers

- Growth occurring in the absence of cell division usually results in an increase in the size and weight of the cell.
- The new cell formed from mother cell eventually attain the same size as the original cell (Jenkins, E.M.).

Bacterial growth

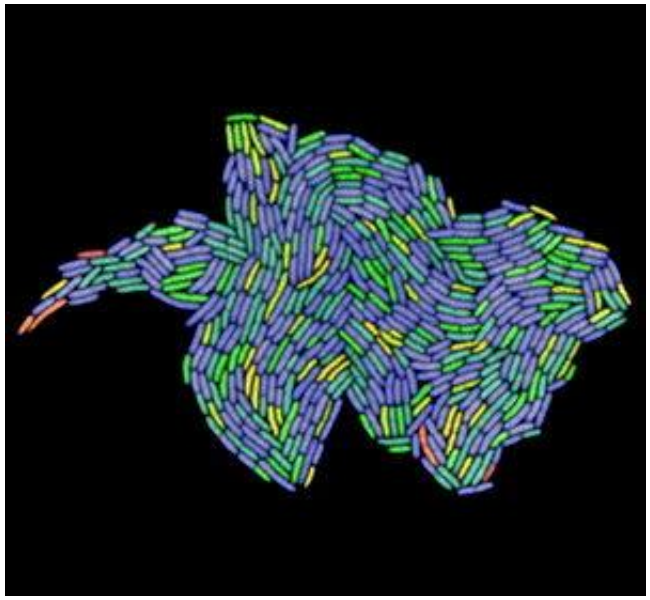
Cell division: binary fission



Bacteria Fall,2008

Cell division
Growing colony

- Under optimal conditions, bacteria can grow and divide extremely rapidly, and bacterial populations can double as quickly as every 9.8 minutes.
- In some of the faster multiplication can produce over 47 million descendants in 12 hours.
- Normally pin-point size colonies contain 1,000 or more cells each(Zaho *et al.*,2007).



A growing colony of *Escherichia coli* cells.

Wikipedia,2008;…

Enumeration methods

Methods used to determine bacterial concentrations

- Several methods can be used to determine bacterial concentrations.
- These include:

1. Direct counts,
2. Plate counts,
3. Turbidimetric measurements,
4. McFarland standards.

Direct microscopic method

Using a counting chamber(hemocytometer)

Total cell count

- The most widely used type of chamber is called a hemocytometer(hemo=blood), since it was originally designed for performing blood cell counts.
- Counted number is used to determine the total number of bacteria per ml.
- The concentration per ml of the original suspension is determined by multiplying to adjust for dilution factors.

Direct count, however does not allow an assessment of cell viability, nor can one distinguish cell types.

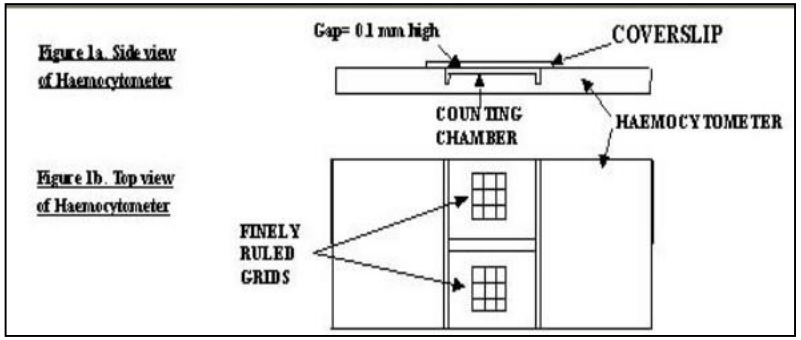
Direct microscopic method

Cytometers

Neubauer chamber and Petroff Hausser chamber

- Both Neubauer chamber, and Petroff-Hauser cytometers has the dimensions (3mm x 3mm)
- The counting region consists of two square shaped ruled areas.
- The price of counting chamber BLAUBRAND® Neubauer improved, with and without double ruling:

Cat.-No.	Product name	Price per	From**	Price***		
717805	Counting chamber BLAUBRAND® Neubauer improved, w/o dips double ruling	1 piece(s)	1	49,05 €	BUY →	INQUIRY
717820	Counting chamber BLAUBRAND® Neubauer improved, with dips double ruling	1 piece(s)	1	69,30 €	BUY →	INQUIRY



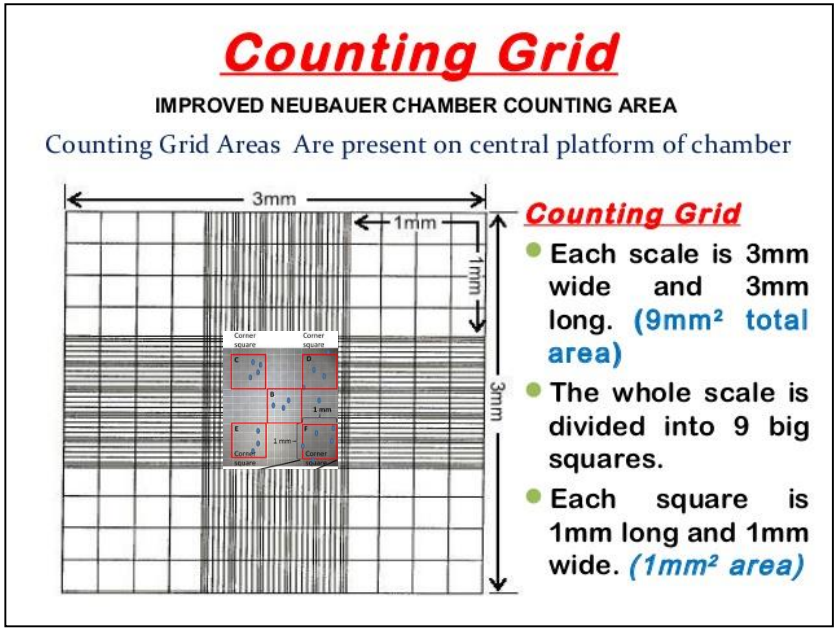
Choudhary,2018;..

Direct microscopic method

Cytometers

Neubauer chamber and Petroff Hausser chamber

- The Neubauer chamber is a thick crystal slide with the size of a glass slide (30 x 70 mm and 4 mm thickness.
- Neubauer chamber, and
- Petroff-Hauser chamber have the same dimensions i.e. 3mm x 3mm.
- Use microscope and coverslip for counting.

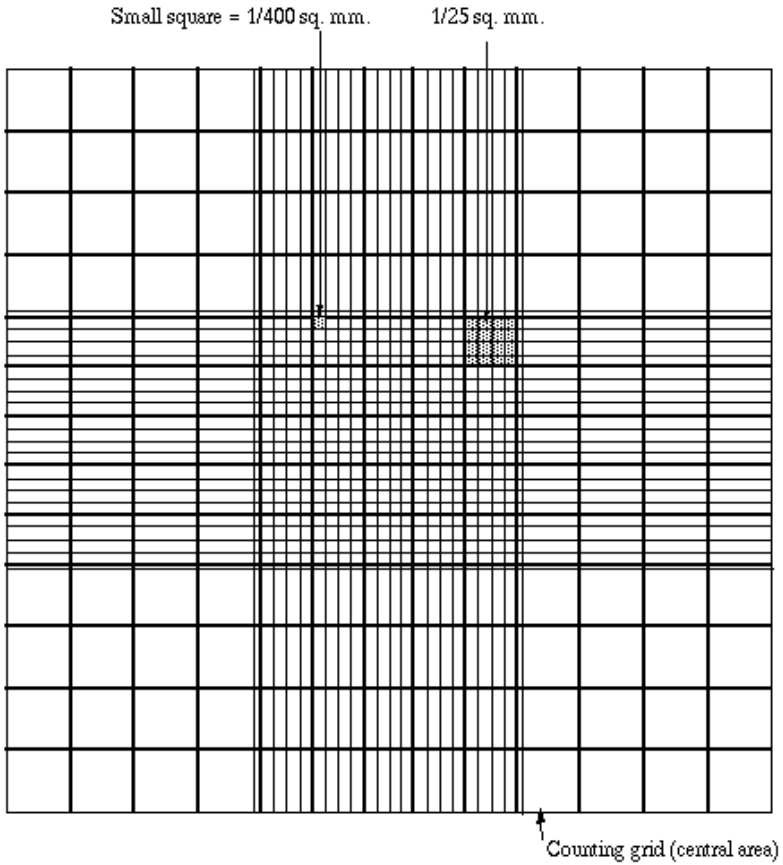


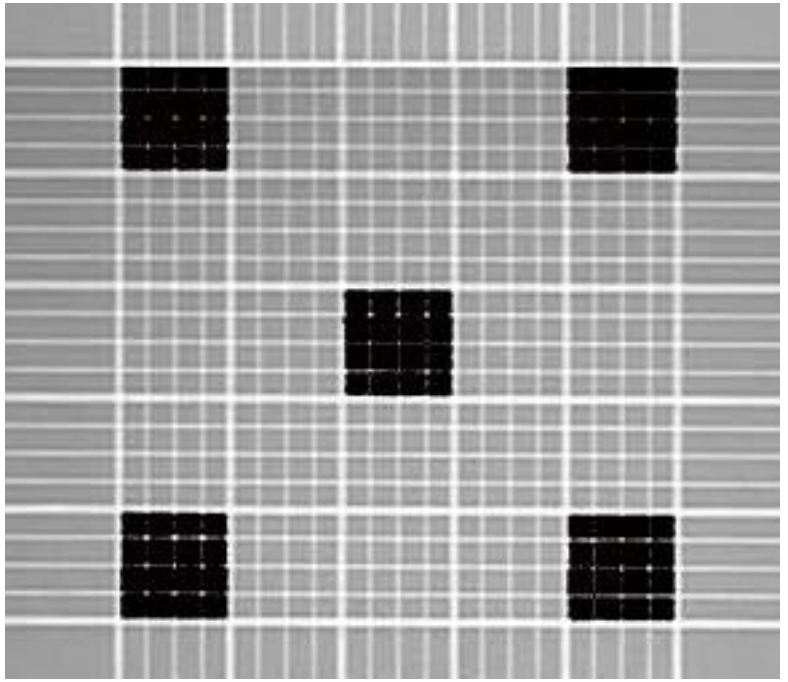
Choudhary,2018

Direct microscopic method

Using a counting chamber

Neubauer hemocytometer



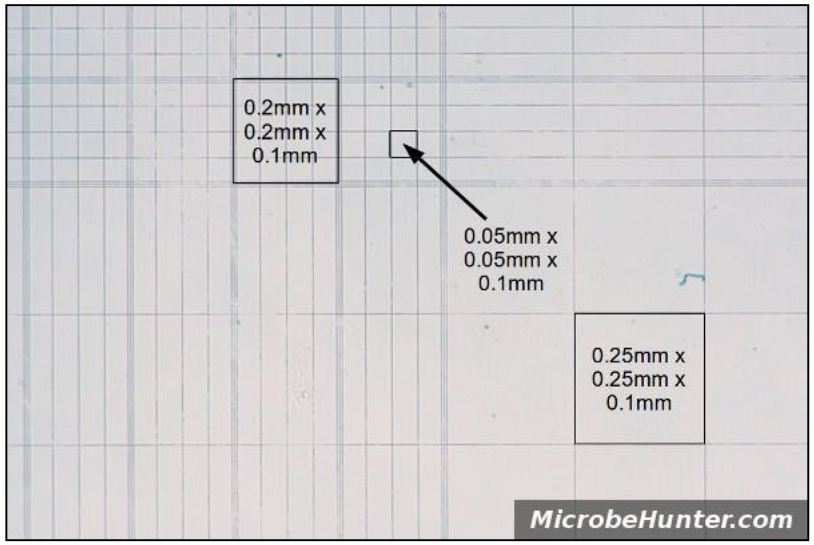


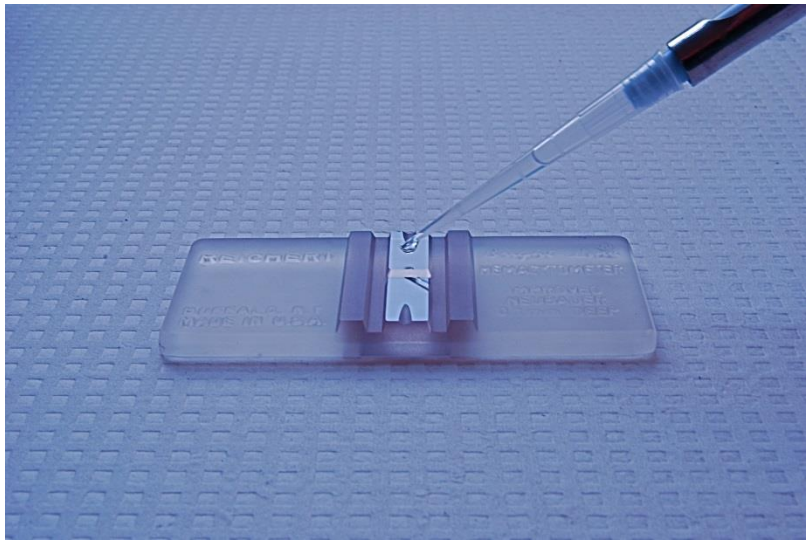
Virtual Microbiology; Caprette,2012

Direct microscopic method

Using a counting chamber

Neubauer hemocytometer



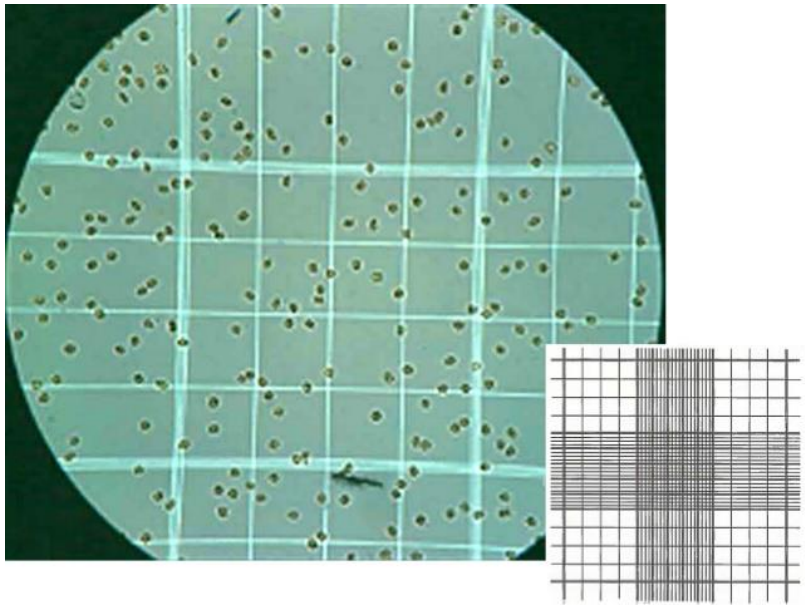


Direct microscopic method

Using a counting chamber

Neubauer hemocytometer

- The center of the slide contains a precisely machined grid, with 16 small squares, each square (1/20 or 0.05 mm length x 1/20 or 0.05 mm width and 1/10 or 0.1 mm depth).



Note: The depth of the standard Neubauer system has to be 0.1 mm. Petroff-Hausser counter using Improved Neubauer rulings is used for bacteria & sperm counts & is offered with varying chamber depths.

Direct microscopic method

Using a counting chamber

Neubauer hemocytometer

- Suppose that you conduct a count as described above, and count 187 particles(bacterial cells) in the five small squares described.
- Each square has an area of 1/25 mm-squared(contains 16 small squares), that is, 0.04 mm-squared and depth of 0.1 mm.
- The total volume in each square is (0.04)x(0.1) = 0.004 mm-cubed.
- You have five squares with combined volume of 5x(0.004) = 0.02 mm-cubed.
- Thus you counted 187 particles in a volume of 0.02 mm-cubed, giving you 187/(0.02) = 9350 particles per mm-cubed.
- There are 1000 cubic millimeters in one cubic centimeter (same as a milliliter), so your particle count is 9,350,000 per ml.

Caprette,2012

Direct microscopic method
Formula for the counting chamber

- If an average of 2.31 cells if found in a 10^{-1} dilution, the formula would appear as shown here with a result of 4.62 x 10^8 cells per ml of culture.
- N_c is the average number of cells counted per square and D is the dilution of the samples placed on the slide.

$$\frac{N_c \times 10^3}{\frac{1}{20} \times \frac{1}{20} \times \frac{1}{50} \times \frac{1}{D}}$$ or $$\frac{2.31 \times 10^3}{0.05 \times 0.05 \times 0.02 \times 0.1} =$$

$$\frac{2.31 \times 10^3}{\frac{1}{20} \times \frac{1}{20} \times \frac{1}{50} \times \frac{1}{10}} = 4.62 \times 10^8$$

Plate count

Viable count

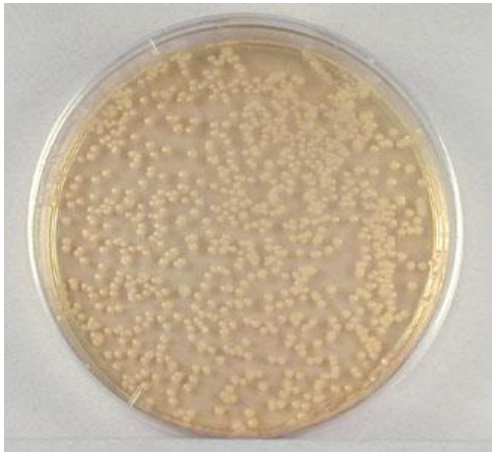
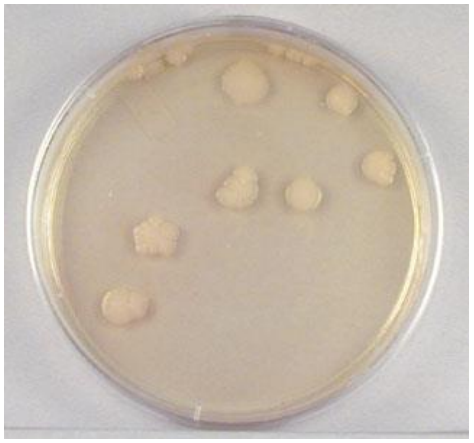
- Standard plate count (viable count) is valid if the number of colonies growing on the agar is between 30 and 300.

1. Any plate which has more than 300 colonies is designated as "too many to count" (TMTC).
2. Plates with fewer than 30 colonies do not have enough individuals to be statistically acceptable.

- When a suspension contains bacteria in higher concentrations, serial dilutions are prepared and plated.
- It is rare to find samples from nature with higher concentrations.

Standard plate count

Determining the number of Colony-Forming Units (CFUs)

1/1,00,000 Dilution of Bacterium (10^{-5})	1/1,000,000 Dilution of Bacterium (10^{-6})	1/10,000,000 Dilution of Bacterium (10^{-7})
		
This plate has over 300 colonies and cannot be used for counting.	This plate has between 30 and 300 colonies and is a suitable plate for counting.	This plate has less than 30 colonies and cannot be used for counting.

Kaiser,2002

Plate count dilution procedure

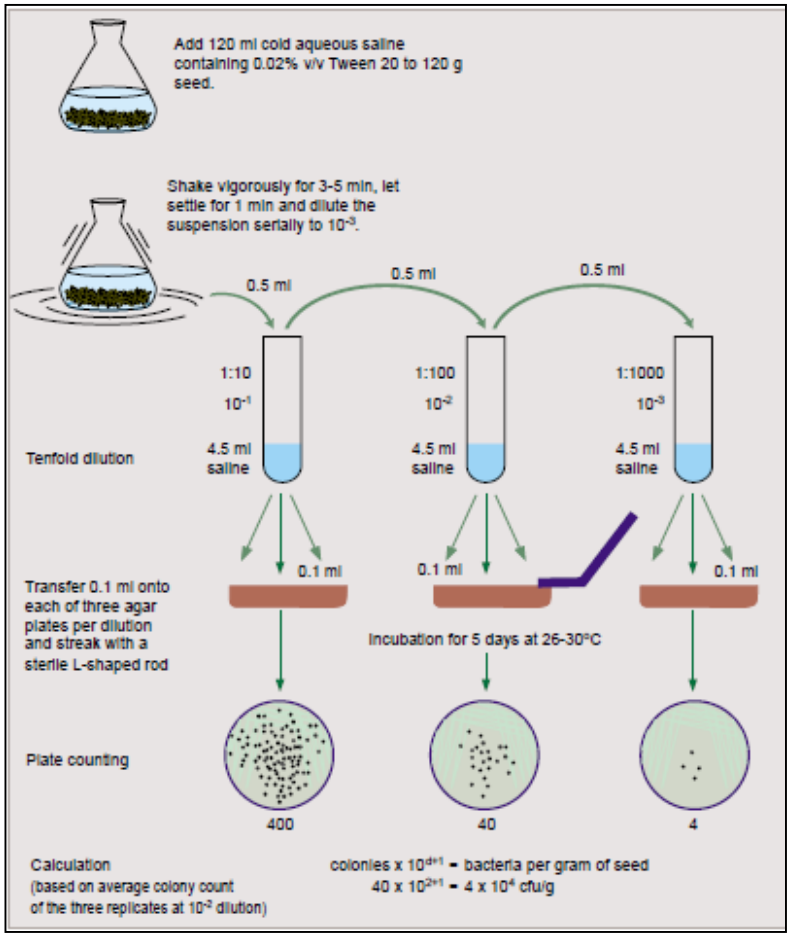
Counting of bacteria by serial dilution method

- The best estimate is obtained by counting colonies on plates where the number of colonies ranges between 50 and 300.
- Hence, when *x* grams of seed are washed in *x* ml of saline solution (w:v = 1:1) and 0.1 ml is plated onto agar medium,

  cfu/ gram = n*d* x 10 $^{(d+1)}$,
- where n represents the number of colonies counted on the medium at a dilution fold *d*.

Colonies x 10^{d+1}= bacteria per gram of seed
e.g. if 40 colonies are on a plate of 1:100 dilution, then the count is $40x10^{2+1}= 4x10^4$. d=dilution. Dilutions can be made up to 10 to -6 level.

colonies x 10^{d+1}= bacteria per gram of seed
$40 \times 10^{2+1} = 4 \times 10^4$ cfu/g



Duveiller *et al.*,1997

Pathogenicity and virulence

Pathogenicity traits

Colony-forming unit (CFU or cfu) and cells/ml

- The CFU/ml can be calculated using the formula:

$$\text{cfu/ml} = \frac{\text{no. of colonies x dilution factor}}{\text{volume of culture plate}}$$

- For example, suppose the plate of the 10^6 dilution yielded a count of 130 colonies.
- Then, the number of bacteria in 1 ml of the original sample can be calculated as follows:
- Bacteria/ml = (130) x (10^6) = 1.3 × 10^8 or 130,000,000.
- The usual estimate is that OD_{600}=1 (1 cm path length) represents on the order of 10^9 CFU/ml (see McFarland section).

Standard plate count
Calculation formula

- To determine the number of CFUs per ml in the original sample, the following formula is used:

B = N/D

B = number of bacteria
N = number of colonies counted on a plate
D = dilution factor (either 1, 10 or 100)

$$\text{Viable cell count (CFU/g soil)} = \frac{\text{Number of colonies}}{\text{Volume of inoculum}} \times \text{Dilution factor}$$

McFarland Scale

Different ratio of H_2SO_4 and $BaCl_2$

- The McFarland Scale is a scale numbered from 1 to 10 which represents specific concentrations of bacteria/ml.
- It is designed to be used for estimating concentrations of Gram negative bacteria.
- The advantage of these standards are that no incubation time or equipment is needed to estimate bacterial numbers.

Joseph McFarland, M. D., JAMA, XLIX, 14,1176-1178 (1907)

McFarland Scale

0.5 McFarland standard

- Mixing the two compounds forms a barium sulfate precipitate, which causes turbidity in the solution.
- Standardized inoculum has a concentration of 1-2 × 10^8 cfu/ml (about 0.5 McFarland standard).
- A 0.5 McFarland standard is prepared by mixing 0.05 ml of 1% barium chloride dihydrate ($BaCl_2.2H_2O$), with 9.95 ml of 1% Concentrated sulfuric acid (H_2SO_4).
- The standard can be compared visually to a suspension of bacteria in sterile saline or nutrient broth.

If the bacterial suspension is too turbid, it can be diluted with more diluent. If the suspension is not turbid enough, more bacteria can be added. Store the McFarland standard at room temperature (25°C) when not in use.

Wikimedia,2009

MacFarland Nephelometer Standards

McFarland Standard No.	0.5	1	2	3	4
1% Barium chloride (ml)	0.05ml	0.1	0.2	0.3	0.4
1% Conc. Sulfuric acid (ml)	9.95ml	9.9	9.8	9.7	9.6
Approx. cell density ($1X10^8$ CFU/mL)	1.3	3.0	6.0	9.0	12.0
% Transmittance*	74.3	55.6	35.6	26.4	21.5
Absorbance*	0.132	0.257	0.451	0.582	0.669

*at wavelength of 600 nm

A McFarland standard of 0.5 should have an OD_{600} between about 0.08 and 0.1. The absorbance (or optical density) is directly proportional to the cell concentration. The greater the absorbance, the greater the number of bacteria.

چگالی صحیح کدورت استاندارد برای نیم مک فارلند با استفاده از اندازه گیری جذب در اسپکترو فوتومتر با طول مسیر نوری cm ۱، مشخص می شود. میزان جذب (OD) نیم مک فارلند در طول موج nm ۶۲۰ تا ۶۳۰ بین 0.08 تا 0.13 می باشد.

Wikimedia,2009; Aryal,2020;..

McFarland Scale and Standards

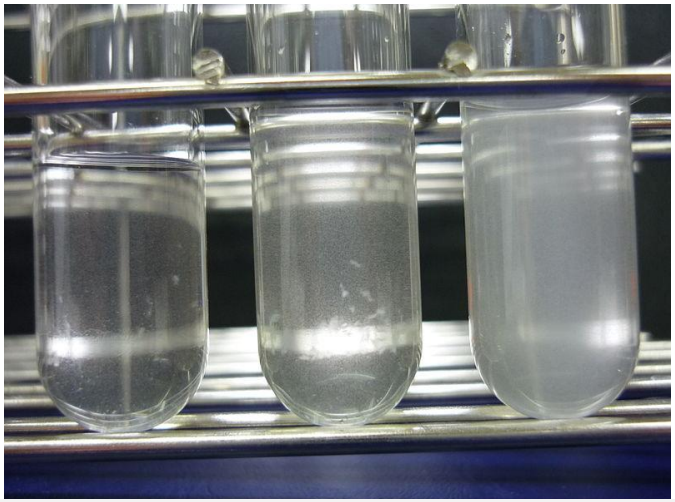
Different ratio of H_2SO_4 and $BaCl_2$

McFarland Scale (McFarland Standard No.)	No. Bacteria ($x10^6$/ml)
1	300
2	600
3	900
4	1200
5	1500
6	1800
7	2100
8	2400
9	2700
10	3000

Estimate the concentration of a broth culture using McFarland standards

Mcfarland standards BBS(Barium sulfate suspension) turbidity

- Compare the turbidity of the test bacterial suspension with that of:
- 0.5,
- 1, and
- 2 McFarland standards.



McFarland standards. No. 0.5, 1 and 2.

Wikimedia,2011

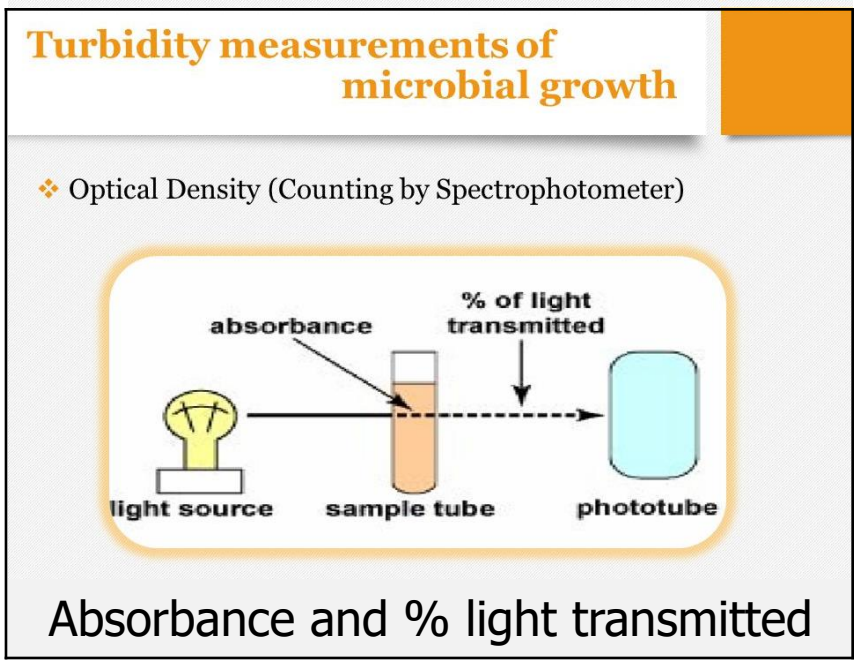
Turbidity
The Spectrophotometer

- It consists of:

1. A light source,
2. A filter which allows only a single wavelength of light to pass through,
3. The sample tube or cuvette, which is standardized for wall thickness containing the bacterial suspension, and
4. A photocell that compares the amount of light coming through the tube with the total light entering the tube.

Turbidity
The Spectrophotometer

- The ability of the culture to block the light can be expressed as either percent of light transmitted through the tube or the amount of light absorbed in the tube.



Absorbance and % light transmitted

Kaiser,2009

Turbidity

The Spectrophotometer

- The amount of light passing through the sample is indicated on a meter and can be read either as:

1. percent transmittance, or
2. optical density (O.D.).

- The percent of light transmitted is inversely proportional to the bacterial concentration. The greater the percent transmittance, the lower the number of bacteria.
- The absorbance (or optical density) is directly proportional to the cell concentration. The greater the absorbance, the greater the number of bacteria.
- Optical density is a measure of absorbance and is related to transmittance by the following equation:

O.D. = 2 - log of % transmittance

The greater the percent transmittance< the lower the number of bacteria<less absorbance or OD.

The greater the absorbance or OD> the greater the number of bacteria <less percent transmittance).

Turbidity

The Spectrophotometer

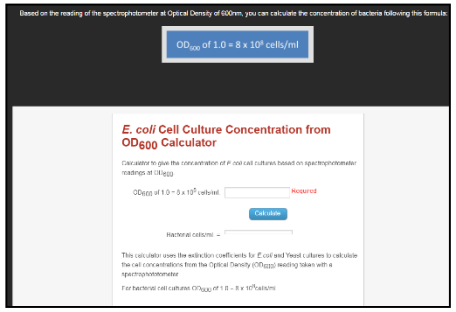
Bacterial cell number (OD_{600})

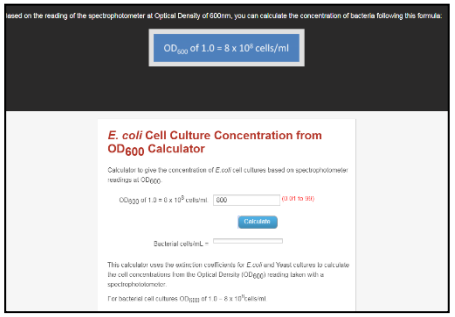
- Based on the reading of the spectrophotometer at Optical Density of 600nm, you can calculate the concentration of bacteria following this formula:

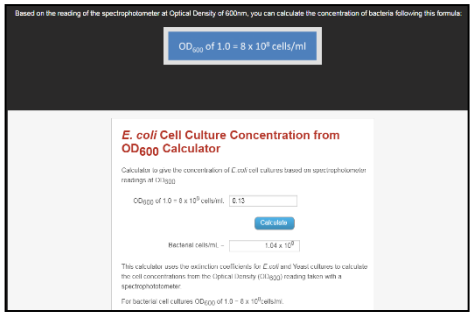
OD_{600} of 1.0 = 8 x 10^8 cells/ml

- To calculate different Ods, add required value in upper column ranges 0.01 to 99.
- e.g.

1. For bacterial cell cultures OD_{600} of 1.0 = 8 x 10^8cells/ml.
2. For bacterial cell cultures OD_{600} of 0.13 = 1.04 x 10^5cells/ml.



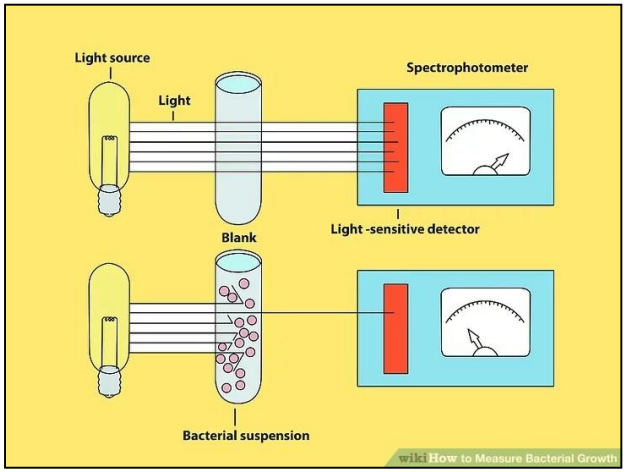




Free online tabtools

A photoelectric spectrophotometer

- By connecting the photoelectric tube to a device that measures electric current (a galvanometer), a means of directly measuring the intensity of the light is achieved.
- The galvanometer has two scales:
- One indicates the % transmittance, and the other, a logarithmic scale with unequal divisions graduated from 0.0 to 2.0, indicates the absorbance. E.g.



Absorbance 0.1 at 540 nm=10^8 cfu/ml.

Pacarynuk,2006; M. Eraqi

Logarithmic scale vs. linear scale

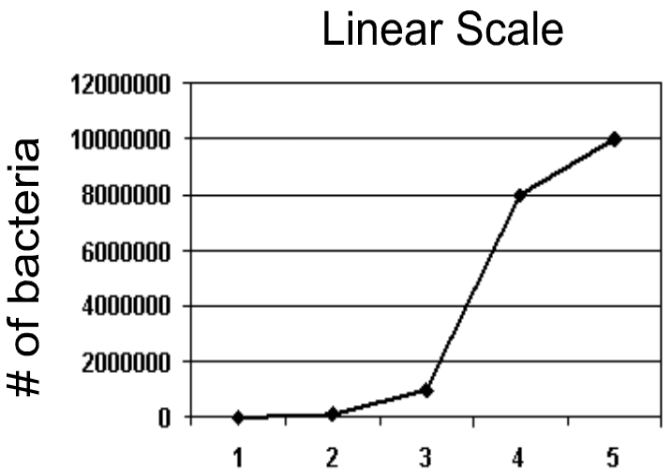
What is a log scale?

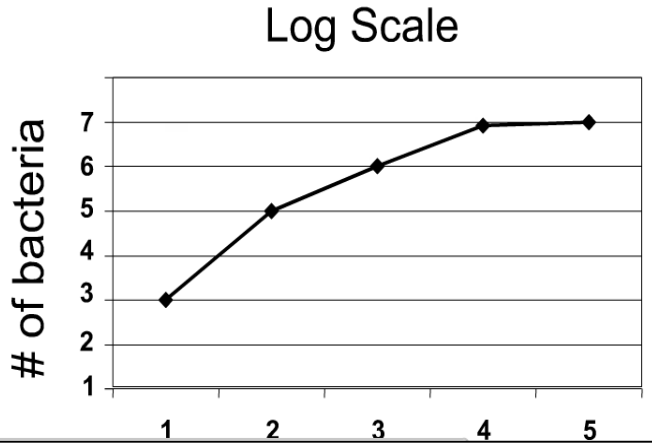
Day	# bacterial cells
1	1000
2	100,000
3	1,000,000
4	8,000,000
5	10,000,000

$1000 = 10x10x10 = 10^3$

So, 1000 -> 3

Day	# bacterial cells
1	3
2	5
3	6
4	6.9
5	7





Logarithmic scale vs. linear scale
Examples of log reduction

1. Example of one log reduction in bacterial population:
- 10,000,000(10^6) bacteria reduced to 1,000,000(10^5) bacteria
- 1 log reduction= 90% reduction

2. Example of two log reduction in bacterial population:
- 10,000,000(10^6) bacteria reduced to 100,000 bacteria(10^4)
- 2 log reduction= 99% reduction

3. Example of two log reduction in bacterial population:
- 10,000,000(10^6) bacteria reduced to 10,000 bacteria(10^3)
- 3 log reduction= 99.9% reduction

Preparation of spectrophotometer

Calculate optical density from transmittance or vice versa

A. The instrument should be plugged in and turned on using the power/zero control knob at least 20 minutes before measurements are taken.

B. Set the wavelength at 686 nm by rotating the wavelength control knob.

C. "Zero" the machine by turning the zero control knob until the meter reads 0% transmittance. Make sure that the lid on the cuvette holder is closed.

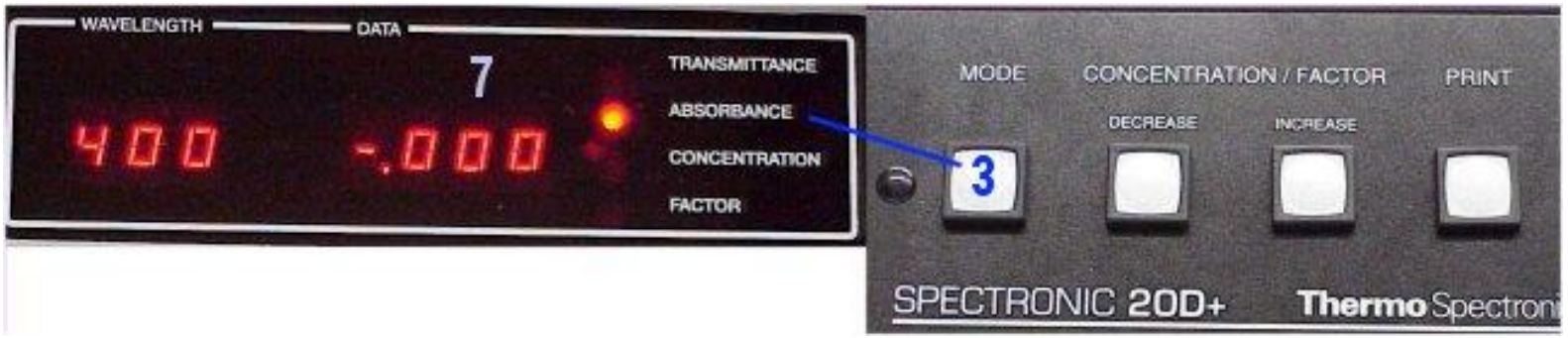
D. Your standard is uninoculated medium. Place a clean cuvette containing uninoculated medium into the cuvette holder making sure that the cuvette is properly aligned in the holder. Turn the light control knob to adjust the meter to 100% transmittance.

E. Remove the cuvette and check to see that the machine reads 0%. If not, repeat steps c and d. When the machine is stable, you are ready to take measurements.

The micro-cuvette must contain 1 ml for the spectrophotometer to read the fluid.

Spectrophotometry

Data collection

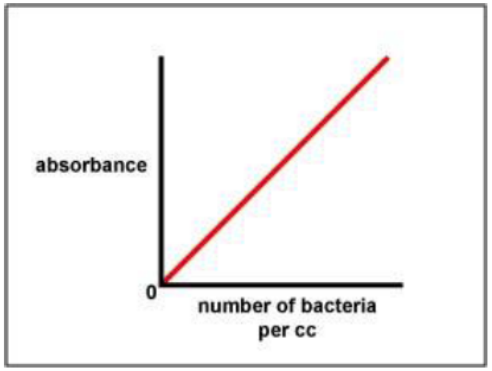
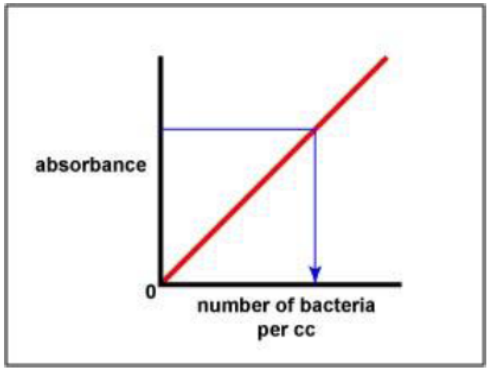


NO.	Dilutions	(X) absorbance	# of bacteria (Y)
1	Original tube		
2	1/2		
3	1/4		
4	1/8		
5	1/16		

A standard curve

Plotting the number of bacteria per cc versus absorbance

- A standard curve comparing absorbance to the number of bacteria can be made by plotting absorbance versus the number of bacteria per cc (left curve).
- Once the absorbance is determined, the standard curve can be used to determine the corresponding number of bacteria per cc (right curve).

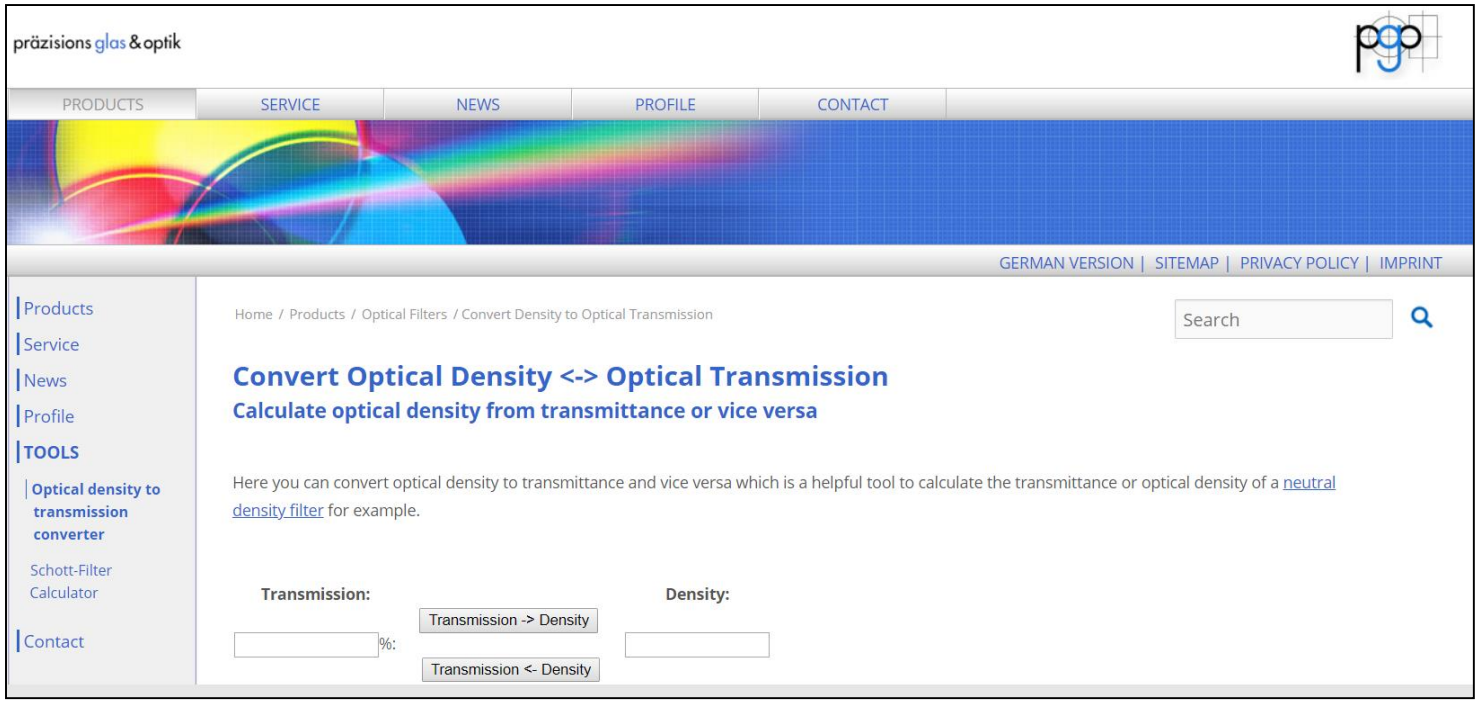
A Standard Curve Plotting the Number of Bacteria per cc versus Absorbance	Using a Standard Curve to Determine the Number of Bacteria per cc in a Sample by Measuring the Sample's Absorbance
	

Kaiser,2009

Spectrophotometer

Convert Optical Density <-> Optical Transmission

Calculate optical density from transmittance or vice versa



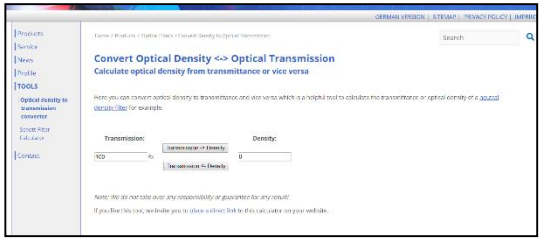
Converter App | Convert transmittance to optical density. https://www.pgo-online.com/intl/optical-density-transmission-converter.html

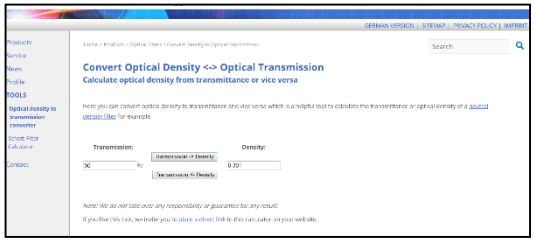
Spectrophotometer

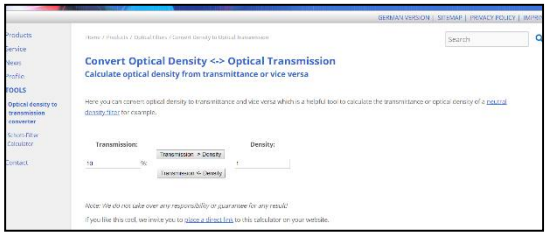
Convert Optical Density <-> Optical Transmission

Calculate optical density from transmittance or vice versa

- transmittance 100% = OD 0
- transmittance 50% = OD 0.301
- transmittance 10% = OD 1
- transmittance 1% = OD 2
- transmittance 0.08% = OD 3.097







✓ The greater the percent transmittance, the lower the number of bacteria(less absorbance or OD).
✓ The greater the absorbance or OD, the greater the number of bacteria (less percent transmittance).

Converter App | Convert transmittance to optical density. https://www.pgo-online.com/intl/optical-density-transmission-converter.html

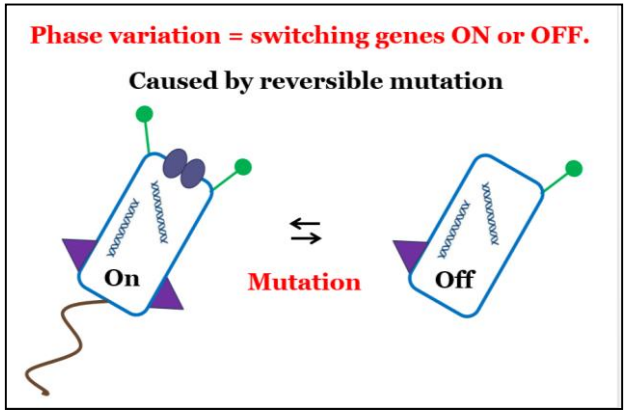
Phenotypic variation or phase variation

Colony phase variation

Influence the production of diverse traits based on virulence determinants

- Phase variation, a type of phenotypic variation, refers to reversible, ON-and-OFF production of a molecule, structure, or complex characteristic.

1. Andrewe reported "diphasic salmonellas";
2. Later, phase variation has been discovered in many bacteria, particularly pathogens.

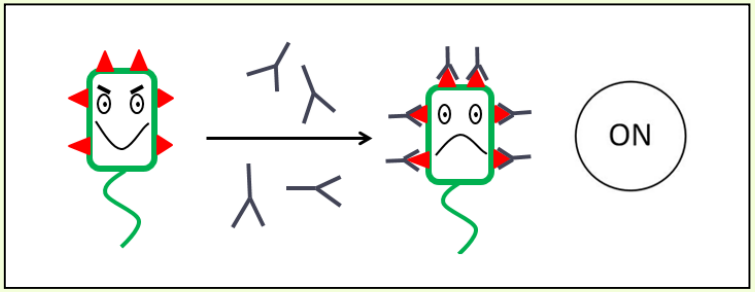


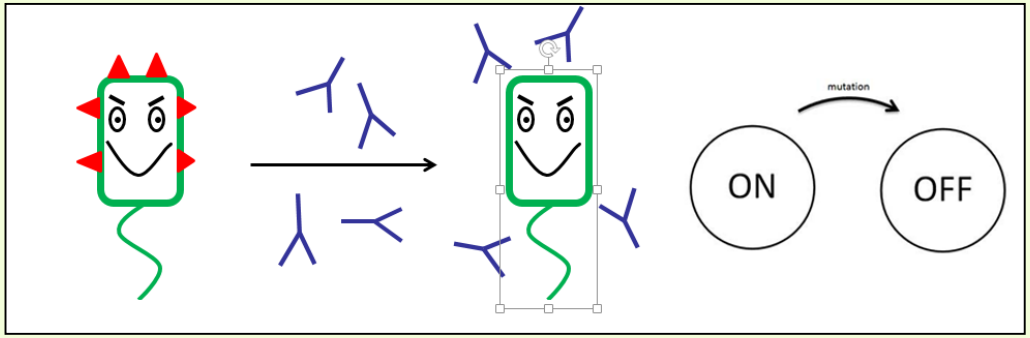
Li and Zhang,2021;..

Phase variation

How can phase variation benefit bacteria?

- Switching some genes off can help the bacteria to escape the immune system.





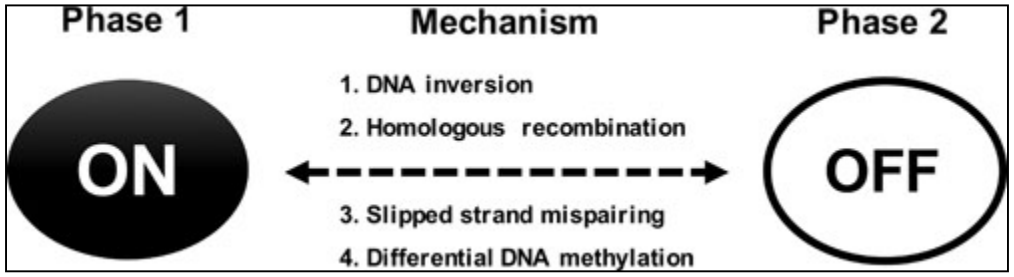
Phenotypic variation or phase variation

Colony phase variation

Influence the production of diverse traits based on virulence determinants

- The vast majority of the well-described phase variations are caused by one or combinations of the following four major mechanisms:

1. DNA inversion,
2. homologous recombination,
3. slipped-strand mispairing, and
4. differential DNA methylation.



Li and Zhang,2021

Phenotypic variation or phase variation

Colony phase variation

Influence the production of diverse traits based on virulence determinants

- Phase variation, as a regulatory system, can influence the production of diverse traits such as the production of:

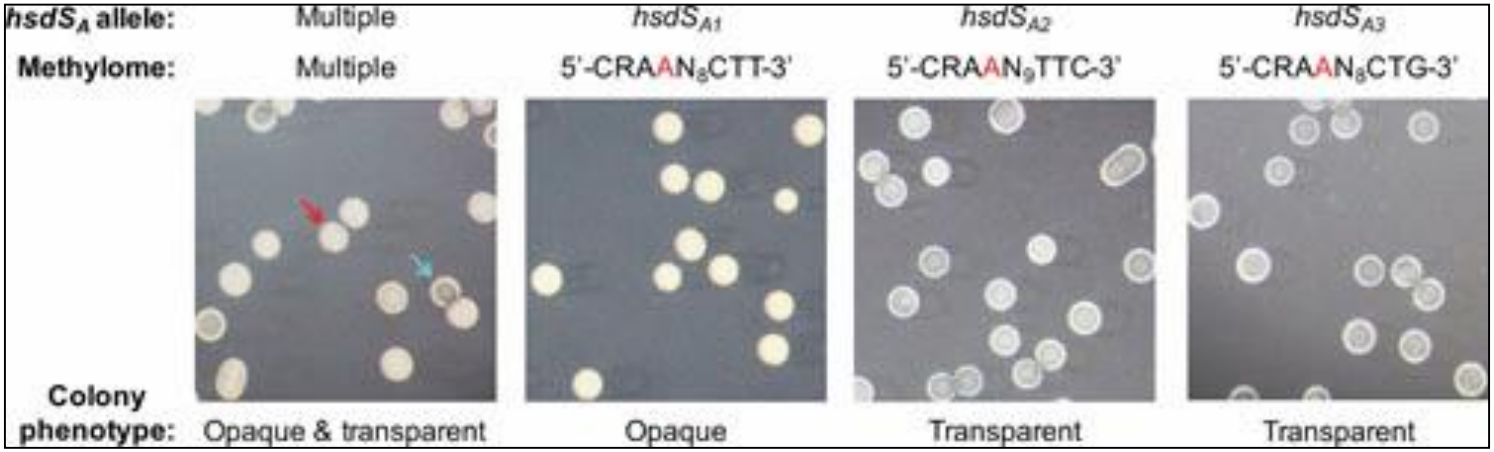
1. fimbriae/pili,
2. Flagella,
3. Capsule,
4. modification of lipopolysaccharides and lipooligosaccharides, and
5. Colony morphology.

Van den Broek *et al.*,2003; Li and Zhang,2021

Colony phase variation

Mutation in *hsdS* gene cause colony phase variation

- Colony phenotypes associated with hsdSA-locked derivatives.
- The representative opaque and transparent colonies in the wild-type strain are indicated by blue and red arrowheads, respectively.



The hsdS gene encodes the sequence recognition subunit of the type I RM DNA methyltransferase.

Li and Zhang,2021

Colony phase variation

Mutation in *gacS* and *mutS* genes cause colony phase variation

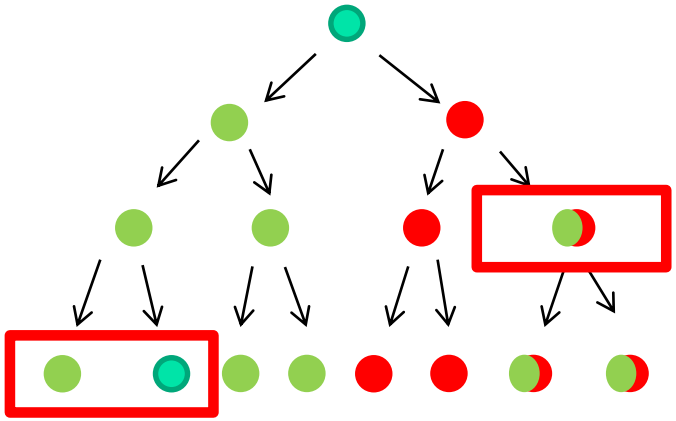
- Mutations in the *mutS* gene increased the frequency of switching from phase I (opaque colonies) to phase II (translucent colonies) up to 1000-fold.
- Phase variation is based on structural changes at the DNA level and results in subpopulations of bacteria, as is often demonstrated by the presence of distinct morphological phases **between colonies** or **within a colony**.

Mutator gene (mutS):DNA repair gene. *gacS* :sensor gene.

Van den Broek *et al.*,2003

Phase variation

What does phase variation look like?

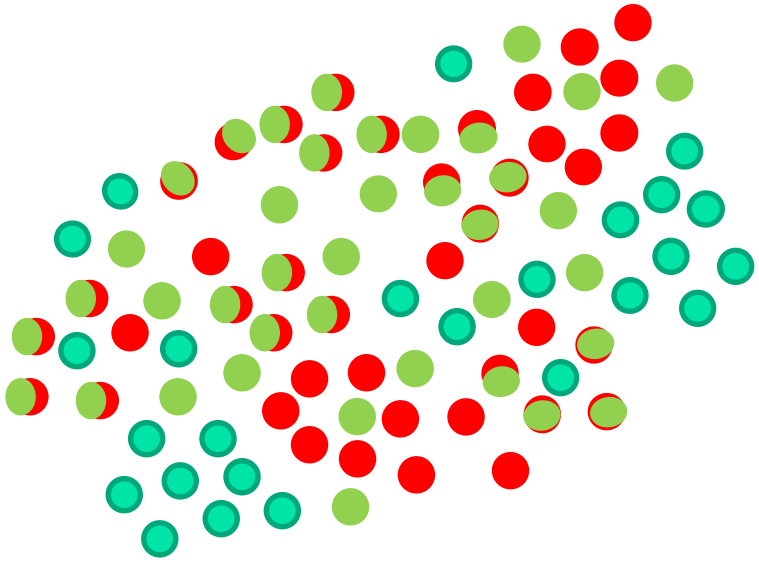


* Many bacteria have more than one phase variable gene.
* Both genes can mutate at the same time.

* Mutation isn't this quick in real bacteria.

Phase variation

What is the effect of phase variation on populations of cells?



All of the different cells have the same DNA but have different antigens expressed.

Colony phase variation

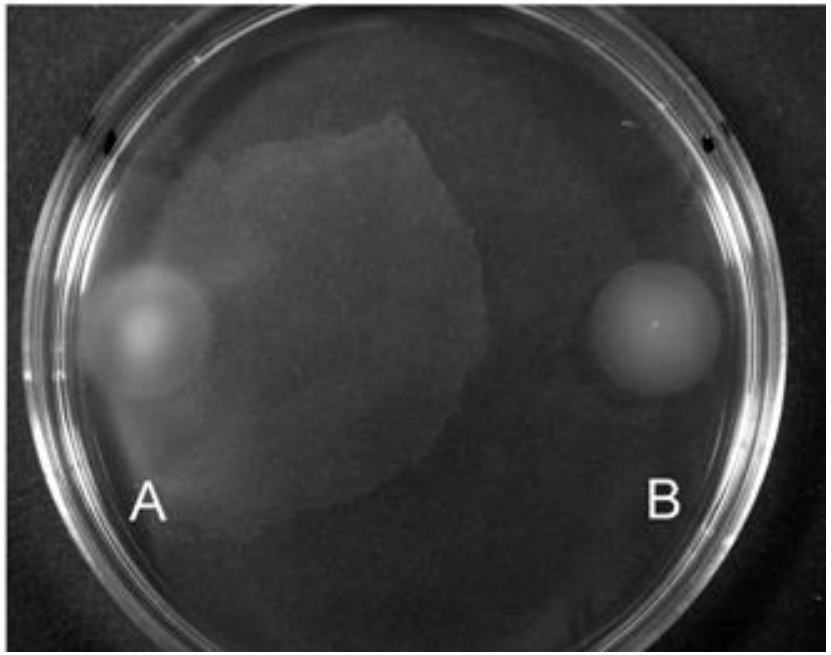
From phase I morphology (opaque colonies) to phase II morphology(translucent colonies)

- Motility of PCL1171 phase I and phase II cells.
- Cells of PCL1171

A. phase I, and

B. phase II

- were inoculated on 1/20 King's medium B agar and were grown overnight at 28°C.
- Motility is absent in colony with phase II morphology(B).



Van den Broek *et al.*,2003

Phenotypic variation or phase variation
Colony phase variation
Loss of virulence associated with *gacS* or *gacA* mutations

- In *Pseudomonas*, the occurrence of phenotypic variants is clearly correlated to stress conditions.
- Culture conditions, medium composition and the scale of the cultures strongly influence the percentage of *gac* mutants in *Pseudomonas fluorescens* CHAO.

- *gacS* and *gacA* are global regulatory genes.
- *gac* genes regulate alginate(EPS) production in *Pseudomonas* species and probably among other gram-negative bacteria.
- There is a link between the *gacS-gacA* regulon in these bacteria and the production of alginate and pathogenicity.
- Mutations in the global regulatory genes *gacS* and *gacA* render *Pseudomonas syringae* pv. *syringae* strain B728a completely nonpathogenic in foliar infiltration assays on bean plants.

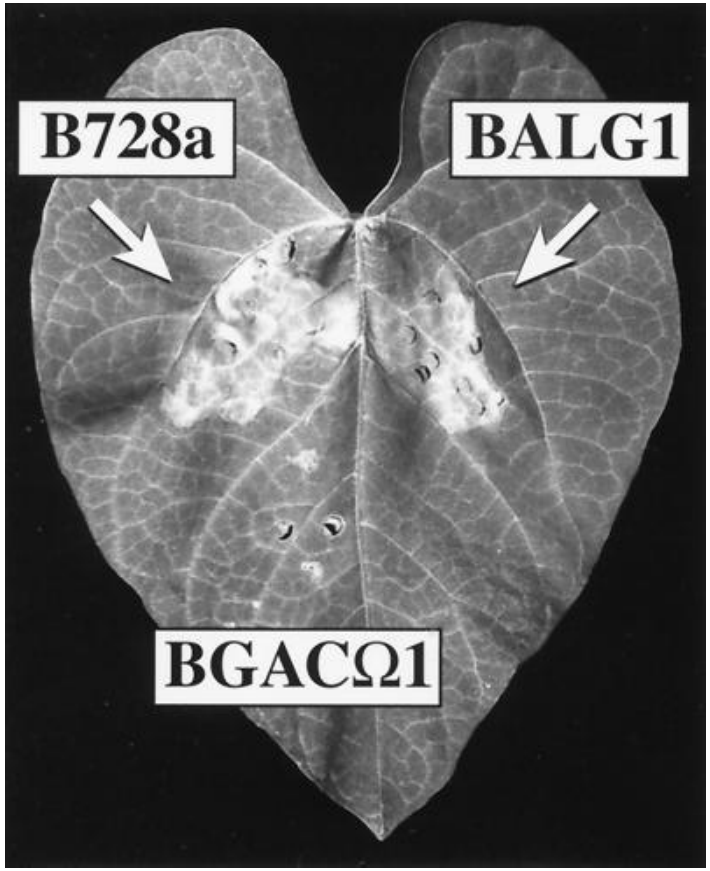
Willis *et al.*,2001;Van den Broek *et al.*,2005

Phenotypic variation or phase variation

Colony phase variation

Loss of virulence associated with *gacS* or *gacA* mutations

- Lesion formation by *Pseudomonas syringae* pv. *syringae* strain B728a and mutant derivatives on bean leaves.
- When the *gacA* mutant BGACΩ1 was inoculated along with the other wild strains in the same experiment, no disease symptoms were observed for mutant strain



Willis *et al.*,2001

Phenotypic variation or phase variation
Colony phase variation
Pseudomonas avellanae

- It was reported that *P. avellanae*, isolated directly from diseased hazelnut specimens in central Italy, showed, in some cases, a colony phase variation with the presence of transparent colony variants (i.e., water drop-like colonies) on nutrient agar medium supplemented with 5-7% sucrose (NSA).
- These variants appeared encapsulated, were possibly not pathogenic and did not induce a hypersensitivity reaction in tobacco leaves.
- Reversal towards typical *P. avellanae* colonies (i.e., creamy-white colour) was also observed following the re-streaking of transparent variants onto NSA plates.

Grenga *et al.*,2012

Colony phase variation
Root-tip colonizing *Pseudomonas* strains

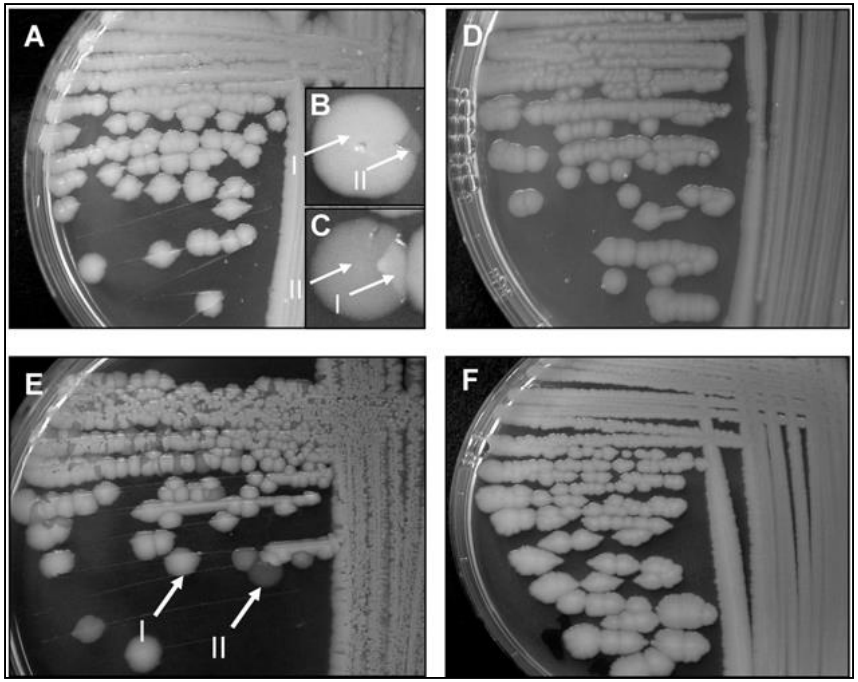
- Of 214 *Pseudomonas* strains isolated from maize rhizosphere, 46 turned out to be antagonistic, of which 43 displayed clear colony phase variation.
- The latter strains formed both opaque and translucent colonies, designated as phase I and phase II, respectively.
- It appeared that important biocontrol traits, such as motility and the production of antifungal metabolites, proteases, lipases, chitinases, and biosurfactants, are correlated with phase I morphology and are absent in bacteria with phase II morphology.
- Phase variation can negatively influence competitive root-tip colonization and, therefore, biocontrol.

Van den Broek *et al.*,2003

Colony phase variation

Pseudomonas sp. strain PCL1171 and its mutants

A. Wild-type PCL1171, in which colonies with a phase I morphology are dominant;
B. enlargement of a single colony of this strain, in which phase II appears as a sector;
C. enlargement of a single colony of this strain, in which a phase I sector appears;
D. stable phase II colony morphology of PCL1572 (Tn*5luxAB*::*gacS*);
E. colony morphology of PCL1555 (Tn*5luxAB*::*mutS*), in which the frequency of colony phase variation is increased; and
F. colony morphology of PCL1556 (Tn*5luxAB*::*mutS*) complemented by pMCS5-*mutS*, which decreases the frequency of colony phase variation of the mutant to wild-type levels.



The arrows indicate phase I (I) and phase II (II) colonies, respectively.

Van den Broek *et al.*,2003

Preservation of bacterial cultures
Loss of pathogen virulence in culture

- If the culturing of the pathogen is prolonged sufficiently, the pathogen may lose virulence completely.
- Such partial or complete loss of virulence in pathogens is sometimes called attenuation, and it has been shown to occur in bacteria, fungi, and viruses.
- Pathogens that have experienced partial or complete loss of virulence in culture or in other hosts are often capable of regaining part or all of their virulence if they are returned to their hosts (reinoculation of the proper host) under proper conditions.
- Sometimes, however, the loss of virulence may be irreversible.

Preservation of bacterial cultures
Loss of pathogen virulence in culture

- Loss of virulence in culture, or in other hosts, seems to be the result of:

1. In culture: Selection of individuals of less virulent or avirulent pathogen strains that happen to be capable of growing and multiplying in culture;
2. In the other host: less virulent or avirulent pathogen strains grow much more rapidly than virulent ones.

Preservation of bacterial cultures

Subculturing working cultures to stock cultures

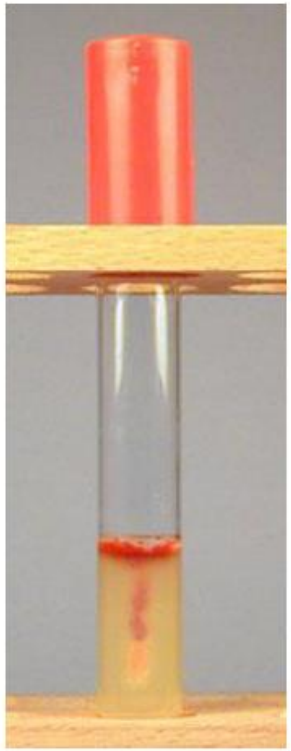
Culture collection methods

- Long-term storage of microbial samples prior to analysis may be necessary; thus, great care must be taken to **preserve the integrity and security of the samples.**
- Some plant pathogens may be stored more successfully than others.
- Storage of viable pathogen cultures is a matter very different from preservation of desiccated leaf tissue, seeds, or fruits.
- Documentation of environmental conditions during storage is required.

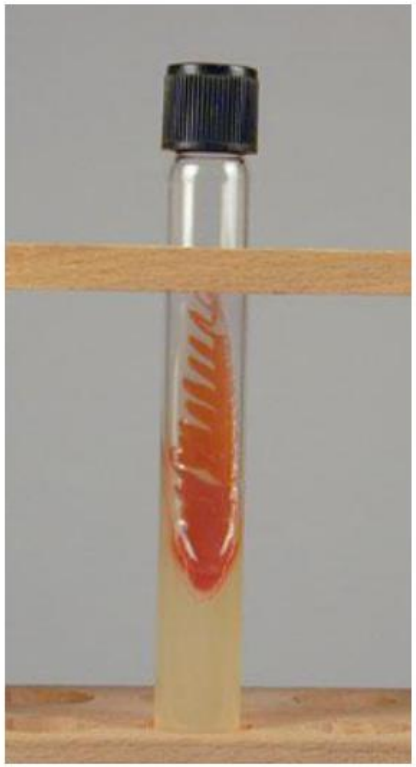
Culture collections

Bacterial growth in a stab tube/on a slant tube

Bacterial Growth in a Stab Tube



Slant Culture



Kaiser,2009

Methods of preservation other than by lyophilization

Bacterium	Preferred method of preservation	Growth medium	Subculturing interval (years)
Agrobacterium spp.	Soil/oil	NA	1–2
Coryneforms	Soil/oil	YDCA	1
Erwinia spp. (other than soft rot group)*	Oil/soil	NA	2
Erwinia spp. (soft rot group)†	Oil slopes at −20°C/ distilled water	NA	1
Pseudomonas spp. (green fluorescent types)	Soil/oil	NA	1
Pseudomonas spp. (non-fluorescent types)	Oil	YDCA	0.5
Xanthomonas spp.††	Oil/soil	YDCA	1

NA = Nutrient agar
YDCA = Yeastrel dextrose chalk agar
**Erwinia tracheiphila* rapidly loses virulence during storage.
†*Erwinia chrysanthemi* is less viable than *E. carotovora*.
††*Xanthomonas ampelina* and *Xanthomonas populi* do not remain viable for more than a few months.

Lelliott and Stead,1987

Preservation of bacterial isolates
In screw-capped bottles

- Most plant pathogenic bacteria can be stored on agar slants (preferably yeast-glucose-chalk agar, or Wilbrink's medium) in screw-capped bottles for some time (months).
- However, bacteria easily perish or change due to mutation and loss of pathogenicity.
- Screw caps containing a rubber stopper, stored in the refrigerator at 4°C.

Bacterial cultures preserved on agar slants.

Janse,2006

Preservation of bacterial isolates

In screw-capped bottles or eppendorf tubes

- Fluorescent pseudomonads responsible for the yellowing of oyster mushroom such as *Pseudomonas tolaasii* and *Pseudomonas 'reactans'* were maintained at 4°C on 2% glycerine nutrient agar slants (Cantore and Iacobellis,2014).

- Bacterial strains were isolated from blotched mushroom samples were preserved by mixing overnight LB culture with 50% glycerol (1:1, vol/vol) and storage at -80°C (Munsch *et al.*,2000).

Preservation of bacterial isolates
In sterile water

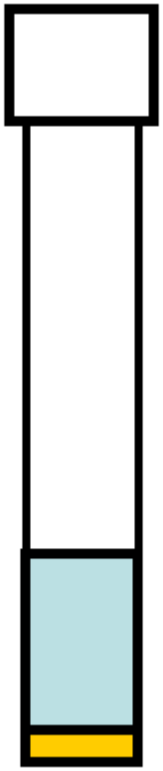
- Some bacteria like *Pectobacterium carotovorum* and *Ralstonia solanacearum* can be very well kept without transfer in sterile tap water(unless it contains too much chlorine) at room temperature from 1 month to several years depending on the species.
- However, this way of storage does not prevent change by mutation or loss of pathogenicity.

Janse,2006

Preservation of bacterial isolates

Under water layer

- Store at +4°C
- Until one year
- Easy to use



Falcon tube

Manceau,2008

Preservation of bacterial isolates

Under sterile oil layer

Double sterile liquid paraffin

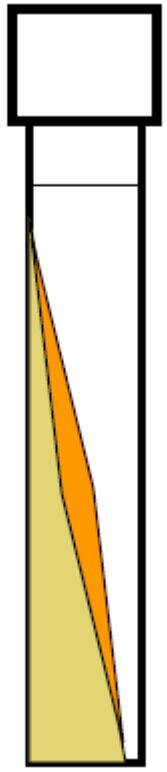
- Mineral oil prevents dehydration and slows down the metabolic activity and growth through reduced oxygen tension.
- Mature healthy cultures are covered by 10 mm of sterile mineral oil (liquid paraffin or medicinal paraffin specific gravity 0.830-0.890 sterilized by autoclaving twice at 121°C for 15 min).
- At CABI *Bioscience* the universal bottles are stored with their caps loose in racks in a temperature controlled(15-18°C) room.

Preservation of bacterial isolates

Under sterile oil layer

Double sterile liquid paraffin

- On agar slant under oil layer.
- Store at room temperature;
- 2-3 years;
- Easy to use.



Manceau,2008

Preservation of bacterial isolates

Frozen at -20°C or preferably at -80°C in glycerol/beads

- For prolonged periods storage is also possible in 15% or 30% glycerol solutions or commercial equivalent cryoprotectants at 15-20° or better 70°-80°C in screw-capped bottles.
- Frozen at -20° or preferably at -80°C on sterile beads covered with a thin film of sterile cryoprotectant fluids such as glycerol or dimethylsulfoxide(DMSO).
- There is little or no risk of mutation or loss of pathogenicity.
- All Agrobacteria and Rhizobia isolates can be kept at -20°C in YM broth with 25% (v/v) glycerol.

Janse,2006; Bashan *et al.*,1993

Preservation of bacterial isolates

Glass beads

Long-term storage of a wide range of bacteria, yeasts and fungi

- For use in spreading bacterial cultures.
- Frozen storage of fastidious and non fastidious organisms.
- The porous nature of the glass beads allows adherence of the bacteria onto the bead surface.
- Beads require no flame or sterilization procedures and can be autoclaved for repeated use.
- 1-oz container, 150 beads per bag.

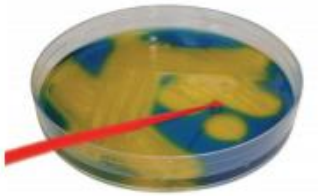
Preservation of bacterial isolates

Glass beads

Long-term storage of a wide range of bacteria, yeasts and fungi

Simple to set up

1: Make a suspension of the organism in the vial liquid



2: Shake to coat the beads

3: Remove vial liquid using fine tip pastette

4: Place in freezer - done!

Easy to recover

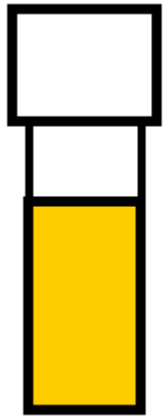
1: Aseptically remove one bead using a sterile needle

2: Roll bead directly onto medium or place in broth and incubate

Preservation of bacterial isolates

Frozen at -20°C or preferably at -80°C in glycerol/beads

- Freezed
- Store at -20°C, or
- Store at -80°C
- Bacterial suspension in nutrient broth (2/3) and sterile glycerol (1/3) or dimethylsulfoxide (DMSO).
- Possibility to use glass beds.
- Several years
- Better reliability at -80°C than -20°C.



Bacterial isolates can be also stored at 20°C in a 40% King's B and 60% glycerol liquid medium.

Manceau,2008; Thiel,1999;..

Preservation of bacterial isolates
Frozen at -20°C in glycerol

- Grow cells to late log or early stationary phase under optimal conditions on the medium of choice.
  - If broth culture is used, centrifuge to harvest the cells. Pour off the supernatant.
- Resuspend the cells in a smaller volume of the same growth medium, and add an equal amount of 20% (vol/vol) glycerol.
  - If cells were grown on agar slants, wash off the growth with the appropriate broth and add an equal amount of 20% glycerol.
  - Alternatively, 10% glycerol can be added directly to the cells to wash them off the agar surface.
- Aliquot into small plastic vials and freeze. The lower the temperature, the longer the cells will survive.
- Liquid nitrogen storage is the most ideal.

ATCC,2016

Preservation of bacterial isolates

Frozen at -20°C or preferably at -80°C in glycerol/beads

- **To recover a strain from freezer:**

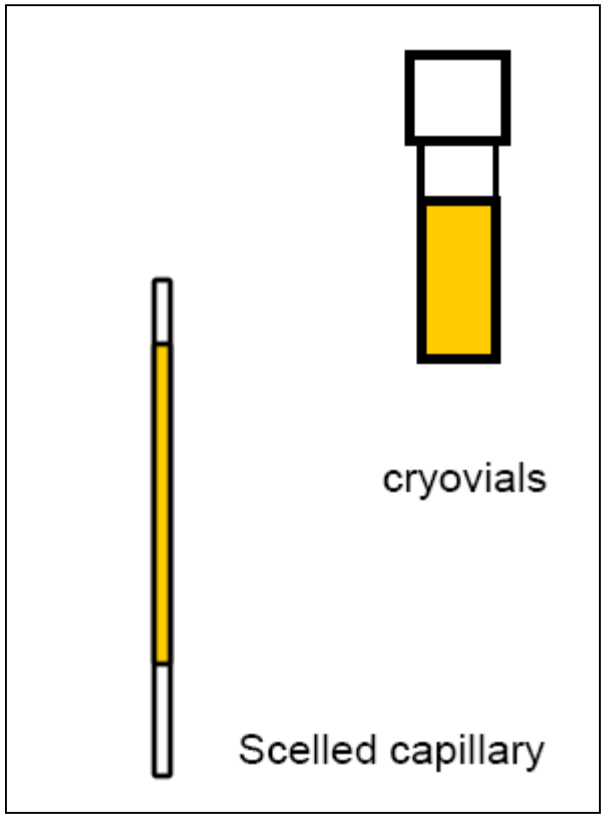
1. Label the bottom of a petri plate containing an appropriate medium with the name of the strain and the date.
2. Carry the plate and a beaker holding several sterile toothpicks to the freezer.
3. Remove the cap.
4. Quickly scrape a small amount of the frozen cells onto the toothpicks.
5. Transfer the material to the agar surface of the plate.
6. Quickly replace the cap and put the tube back in the freezer before it thaws.
7. Take the plate back to the lab and use a sterile inoculating loop to streak from the melted material to make a typical plate.
8. Colonies should appear in 1-2 days.

- If no colonies appear, thaw the entire tube and transfer the contents to a flask of broth.

Thiel,1999

Preservation of bacterial isolates
Deep-freezing

- Deep-freezed
- Store at -196°C in liquid nitrogen.
- Bacterial suspension in nutrient broth (2/3) and sterile glycerol (1/3).
- Possibility to use glass beds.
- Several years.
- Better reliability at than at -20°C or -80°C because no risk of electric failure.



Manceau,2008

Preservation of bacterial isolates
Freeze-Dry (lyophilization)

- Freeze-drying, also called lyophilization or cryodesiccation, is the process of:

1. removing water from a product after it's frozen, and then
2. placed under a vacuum, allowing ice to change from a solid to a vapor, without going through a liquid phase.

- This process can take as little as three hours, and as long as 24 hours, not including culture growth time.

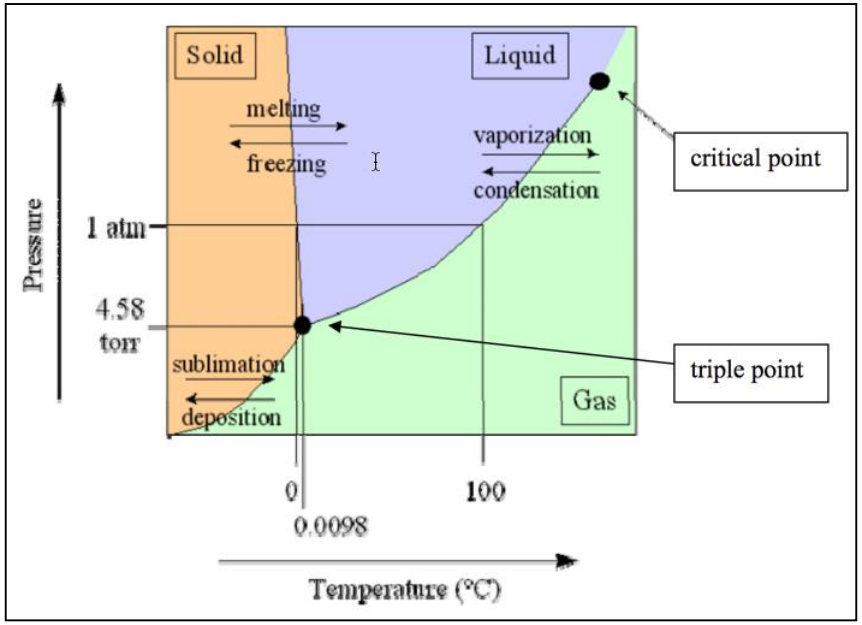
Phillips,2018

Preservation of bacterial isolates

Freeze-Dry (lyophilization)

- Lyophilization is a technique of dehydration by refrigeration and vacuum.
- A balance between the pressure and temperature helps in drying aqueous preparation while respecting:

1. the integrity of living cells (e.g. bacteria), as well as,
2. complex and delicate structures (e.g. enzymes, proteins).



Manceau,2008; John Barley

Preservation of bacterial isolates

Freeze-Dry or lyophilization

Products You'll Need

- Freeze-dryer
- Nutrient or other appropriate agar plates (and incubator to grow culture)
- Glass rod
- Lyophilization buffer
- Crimp-top(compress) vials with rubber stoppers (and a crimper to apply the caps)
- Freezer.

Phillips,2018;..

Preservation of bacterial isolates

Freeze-Dry (Lyophilization)

The step-by-step process of lyophilization

- First, grow an overnight culture, or lawn, of the microorganism on LB or other appropriate nutrient agar plate.
- Next, prepare sterile crimp-cap vials by autoclaving ahead of time, with the caps (rubber stoppers) placed loosely on top. Place paper labels printed with the culture's identification inside the tubes prior to autoclaving. Alternatively, use tubes with caps designed for sterility.
- Add 4 mL lyophilization buffer to the plate. If necessary, the cells can be suspended using a sterile glass rod.
- Quickly transfer the culture suspension to the sterilized vials. Add approximately 1.5 mL per vial. Seal with the rubber cap.
- Freeze the culture suspension inside the vials by placing the vials in a -20 degrees Celcius freezer.
- Store the lyophilized culture collection at room temperature.

Phillips,2018

Preservation of bacterial isolates
The step-by-step process of lyophilization
Continued

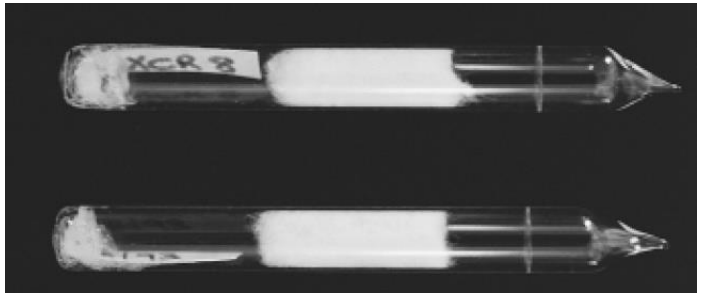
- Once the cultures are frozen, prepare the freeze-dryer by turning it on and allowing time for the appropriate temperature and vacuum conditions to stabilize. Do this according to the manufacturer's instructions for the particular brand of freeze-dryer you're using.
- Carefully, and aseptically, place the vial caps loosely on top of the vials, so that moisture can escape during the freeze-drying process, and place the vials into a freeze-dryer chamber. Apply the vacuum to the chamber according to the manufacturer's instructions.
- Allow the culture time to completely lyophilize (dry out). This may take from a few hours to overnight depending on the volume of each sample and how many samples you have.
- Remove the samples from the freeze-dryer chamber according to the manufacturer's instructions and immediately seal the vials with the rubber caps, then the crimp tops.
- Store the lyophilized culture collection at room temperature.

Phillips,2018

Preservation of bacterial isolates
Lyophilization for indefinite storage

- The best way of preservation is by freeze-drying (lyophilization) using a commercial lyophilization apparatus, where viability and pathogenicity/virulence are best preserved for many years.
- Usually bacteria are freeze-dried in ampoules or small screw-capped bottles.

Some lyophilization ampoules and bottles.

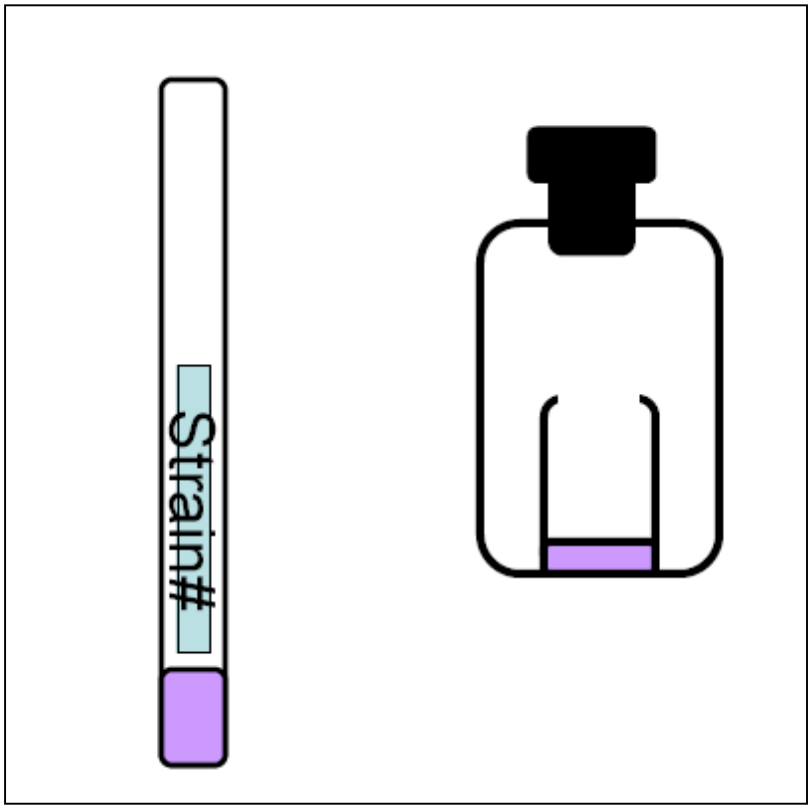


Ampoules with freeze-dried bacterial cultures.

Goszczynaska *et al.*,2000 & Janse,2006

Preservation of bacterial isolates
Lyophilization for indefinite storage

- Lyophilized:
- In vials
- In sealed glass tube.



Manceau,2008

Viability of lyophilized cultures of plant-pathogenic bacteria

	Half-life (years)	Shelf-life (years)
Coryneforms	3.0	>50
Agrobacterium	0.8	16
Erwinia spp.	0.9	18
E. chrysanthemi	0.8	16
E. tracheiphila	0.8	16
Pseudomonas spp.	0.9	18
P. solanacearum	0.8	16
P. rubrisubalbicans	0.8	16
Xanthomonas campestris	2.0	40
X. albilineans	0.8	16
X. populi *X. ampelina* *X. axonopodis*	Not determined but low	

Shelf-life: the length of time for which an item remains usable.
Half-life: is the time required for exactly half of the entities to decay on average.

Lelliott and Stead,1987

Initiating lyophilized cultures
Revival of freeze-dried bacteria

Revival of freeze-dried bacteria

The revival of the strains must be done by qualified staff, in an appropriate laboratory with equipment providing aseptic conditions.

Material needed

- A diamond tool
- Pasteur pipettes
- A Pasteur pipette with round end or a loop

Goszczynaska *et al.*,2000

Media

- 5 ml of peptone nitrated water or other nutrient broth
- Nutrient agar plates, adapted to the strains

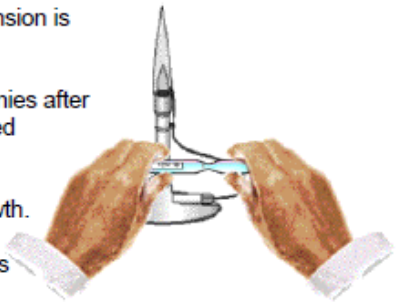
Reviving freeze-dried bacteria :

-**Make stripes** all arround the narrow part of the vial with the diamond tool. **Sterilize** the narrow part of the vial. **Break** the vial in aseptic conditions.

- **Resuspend** the lyophilisat with 300µl of the nutrient broth by multiple pipeting. Ensure that the lyophilisat is well resuspended.

- **Two drops** are laid down on the agar plate, the residual bacterial suspension is added to the broth.

- One drop is used to **spread** the inoculum in order to obtain isolated colonies after incubation. The other is kept untouched in order to preserve a concentrated inoculum.



- Agar plates and tubes are **incubated** at the optimal temperature for growth.

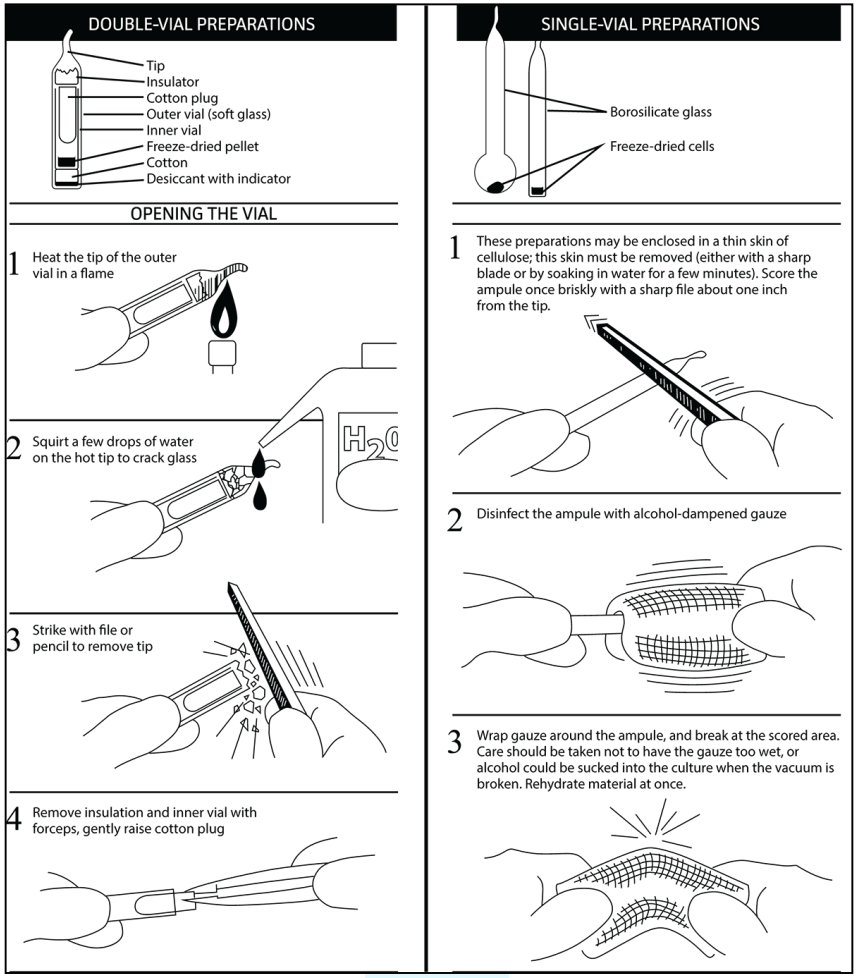
- If no growth occurs on the plates after recommended incubation time, it is possible to spread the nutrient broth on new nutrient agar plates.

Initiating lyophilized cultures
Revival of freeze-dried bacteria

- Using a Pasteur pipette, aseptically add 0.5 mL of the recommended growth medium to the freeze-dried material. Mix well.
- Transfer the entire suspension to a test tube containing 5 to 6 mL of the recommended medium. Additionally, transfer several drops of the suspension to an agar slant.
- Incubate cultures under the appropriate temperature and atmospheric conditions as recommended for the product.
- Additional test tubes can be inoculated by transferring 0.5 mL of the primary culture to additional secondary cultures.
- Examine cultures after the recommended incubation period. The incubation period will vary between strains. Most freeze-dried cultures will grow within a few days.

Initiating lyophilized cultures

Revival of freeze-dried bacteria



ATCC

Details of bacterial culture collection methods.

Short-term storage

Periodic subculture

This method is traditionally used to maintain frequently used isolates. Bacteria can be stored on sealed agar plates or slants at room temperature or in a refrigerator (Fig. 21). The humidity of the storage area is important because slow dehydration leads to a loss of viability. Several agar media can be used: glucose yeast extract $CaCO_3$ agar, tryptone glucose extract agar, nutrient agar etc. (see chapter 'Media and Diagnostic Tests').

Periodic subculturing is not recommended for long-term preservation. The likelihood of contamination, loss in pathogenicity, mislabelling and risk of culture loss are great. Some bacteria like *Pseudomonas* spp. generally survive for long periods without subculturing.

Storage under mineral oil

Sterile paraffin oil must cover the slants completely. Cultures are stored at room temperature or at 4 °C.

Long-term storage

Storage in distilled water

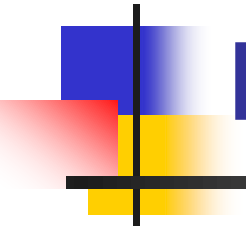
A dense suspension of culture is prepared in sterile distilled water and stored at room temperature. This method is suitable for *Ralstonia solanacearum, A. tumefaciens* and *X. albilineans.* Some isolates stored in this way can survive for up to 20 years.

Deep-freezing

Most bacteria can survive in the frozen state for a long time. The metabolic rate is greatly reduced and the lower the temperature the less is the loss of viability of most micro-organisms.

Before deep-freezing, prepare a dense suspension of bacteria, from a 3–4-day-old growth on agar, in milk-glycerol (10 % skimmed milk, 15 % glycerol in distilled water) or in NGY (0.8 g nutrient broth, 15 ml glycerol, 0.2 g yeast extract, 0.5 g glucose in 100 ml distilled water). Place 0.5 ml of the suspension in a small, labelled tube or bottle, seal it

Goszczynaska *et al.*,2000



Diagnostic Tests

Phenotypic and genotypic traits analyses

Diagnostic Tests

From bioassays to real-time PCR

- Diagnostic tests are still evolving (Schaad *et al.*, 2001), so that few are standardized and validated by multiple users, including governments.
- With some practice, most bacterial diseases can be easily diagnosed.
- However, the variation that can occur with different strains may require more sophisticated testing.
- Variations may be determined:

1. Genetically (genotypic), or
2. By the environment (phenotypic).

- In the latter case all cells of a culture vary.

Diagnostic Tests
From classical methods to modern ones

- **The methods used for diagnosis includes:**
- Range from classical methods such as nutritional tests,
- Use of (semi-)selective media,
- PCR,
- Fatty acid analysis,
- Serology and
- Pathogenicity tests,
- **As well as (more) modern ones such as:**
- Free flow capillary electrophoresis,
- real-time PCR,
- rep-PCR,
- fAFLP,
- Sequencing of open reading frames (ORFs) and housekeeping genes such as *gyrB* and *rpoD* (also known as sigma 70).
- In general both classical and more modern tests appear to be useful and necessary and often complement each other.

Janse,2009

Comparison of microbial identification methods

Phenotypic, molecular and chemotaxonomic method

	Phenotypic methods	Molecular methods		Chemotaxonomic methods
	Traditional methods	RT-PCR	Sequencing	MALDI-TOF MS
Sensitive and specific	1	4	3	2
Rapid	1	3	2	4
Easy to perform, not labor intensive	1	3	2	4
Data easy to interpret	4	2	1	3
Cost effective	4	3	1	2
Automatization	1	3	2	4
Total	12/24	18/24	11/24	10/24

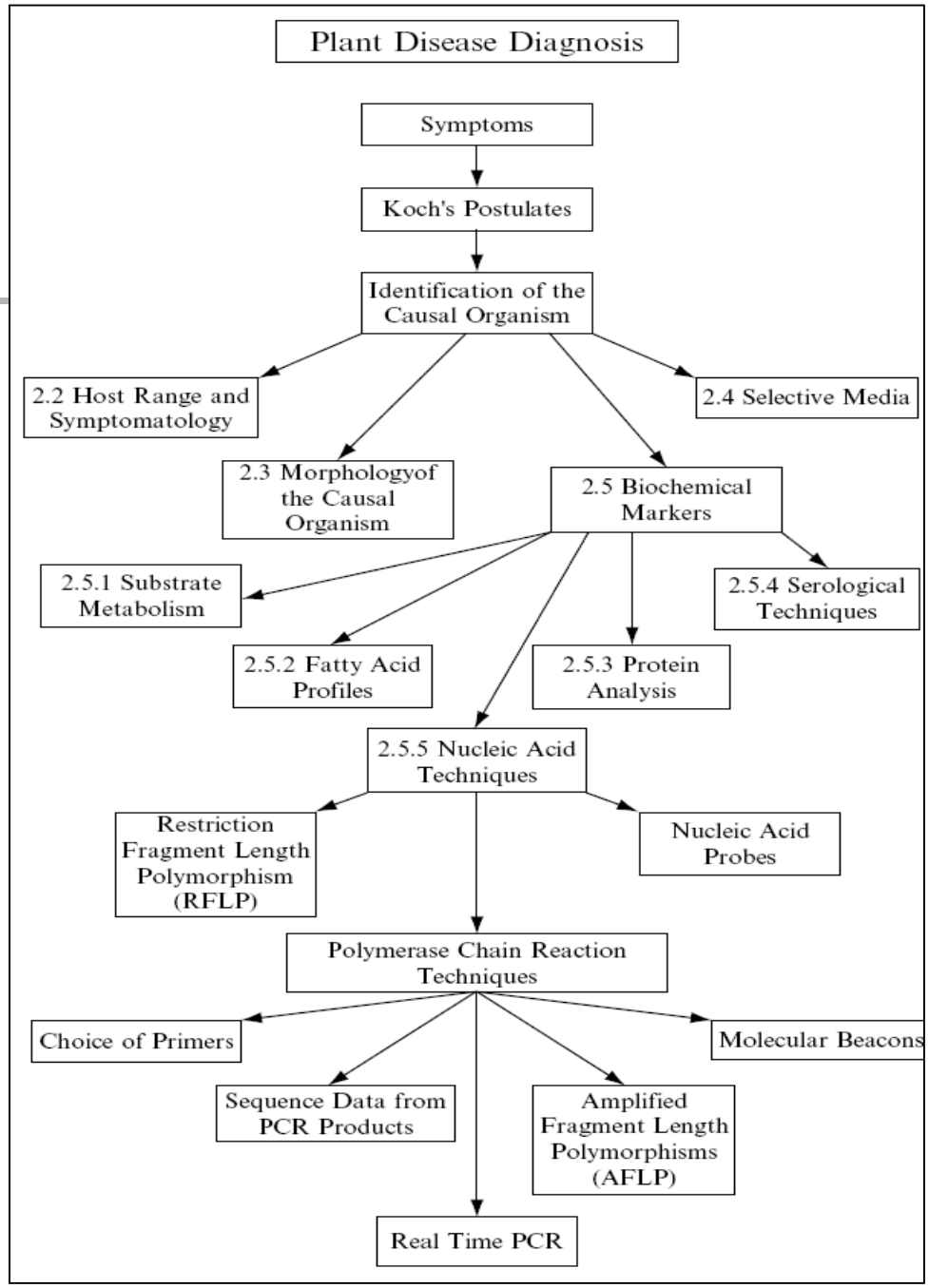
RT-PCR= Repetitive PCR fingerprinting; MALDI-TOF MS=Mass spectrometry for protein and lipid analyses

See similar slide comparing phenotypic (Biolog) and genotypic techniques

Elek,2010

Detection and Diagnosis

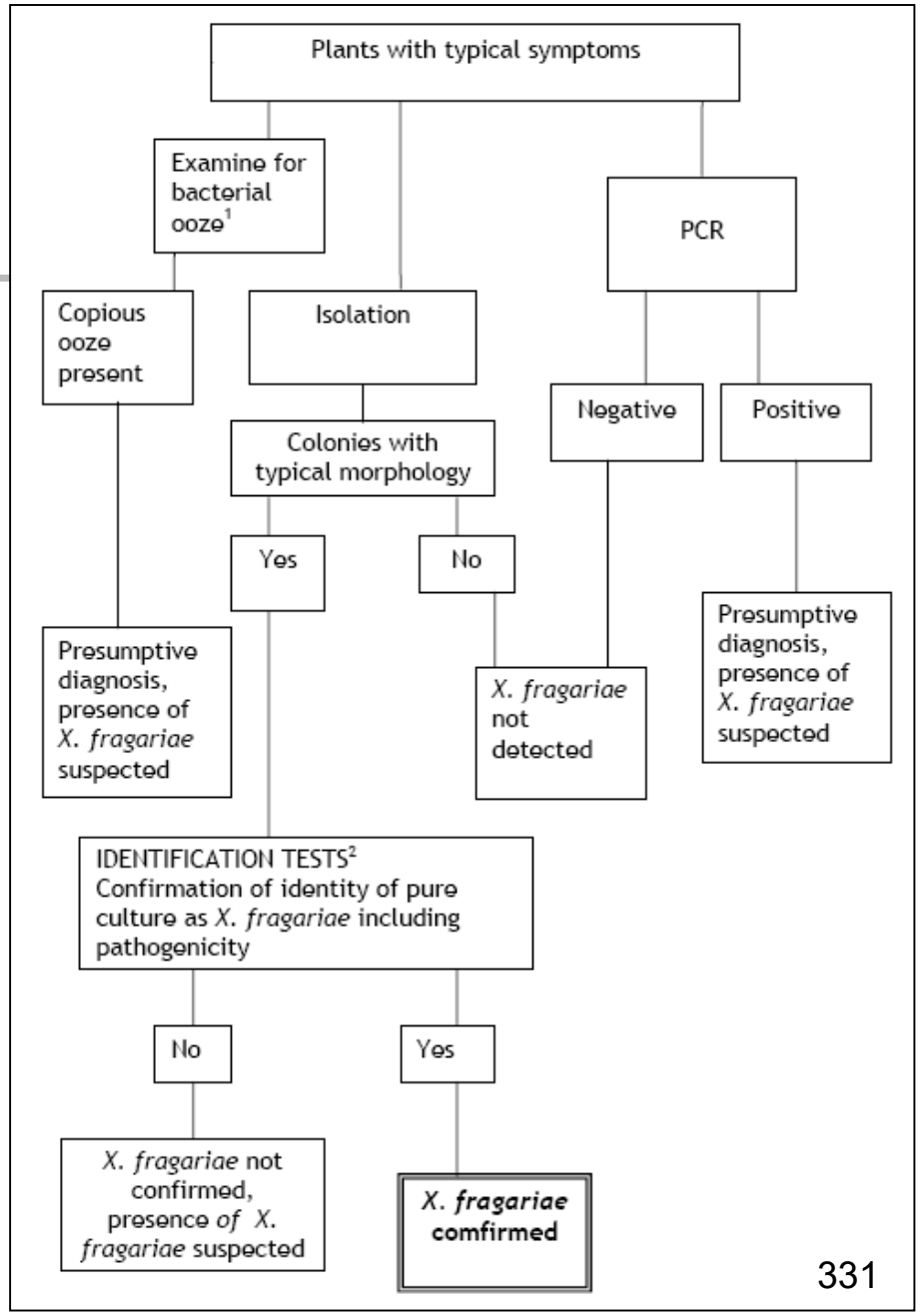
- Characters differentiating culturable plant pathogenic bacteria.
- Diagram to show the range of techniques available for the detection and diagnosis of plant diseases and plant pathogens.



Strange,2003

Detection and Diagnosis

- Diagnostic flow chart for plants with symptoms.
- If isolation of the pathogen is unsuccessful or if the tissue does not have symptoms (Symptomless tissue), then PCR must be used.



Fingerprinting methods

PCR- and non-PCR-based fingerprinting techniques

1. **Fingerprinting methods based on multitest kits:**

- The Biolog performs 95 discrete tests simultaneously and gives a characteristic reaction pattern called a "fingerprint".

2. **Fingerprinting methods based on chemotaxonomic category:**

- Protein patterns (determined by electrophoretic techniques).
- Fatty acid patterns (determined by gas chromatography).
- Chemical composition of cell walls, etc.

3. **DNA fingerprinting (non-PCR-based fingerprinting techniques):**

- Microarrays, oligonucleotide probes,...

4. **DNA fingerprinting (PCR-based fingerprinting techniques):**

- Repetitive PCR fingerprinting (REP-PCR) very good for species, subspecies and often pathovar.;
- Combined REP/BOX/ERIC often as good as AFLP.
- rDNA PCR of the 16S-23S intergenic spacer region, ITS ('ribotyping');
- Randomly amplified polymorphic DNA PCR(RAPD-PCR).
- Need to compare with reference strains.

Minimal standards

Bacterial identification for publication: when is enough enough?

- All systems used to identify bacteria, whether phenotypic or genotypic, have limitations, because no single test methodology will provide results that are 100% accurate.
- Reliance on a single identification system, phenotypic or genotypic, to identify an organism provides more opportunity for misidentifying bacterial species.
- Ideally, identification of any taxon is based upon a polyphasic approach that includes a combination of:

1. Phenotypic testing methods
2. Genotypic testing methods

Janda and Abbott,2002

Minimal standards

Limitations of biochemical tests

Traditional tube methods or kits

- Because of the limited number of biochemical and phenotypic tests available, characterization of proposed species was inadequate and imprecise.
- This problem often led to confusion and resulted in the discovery and rediscovery of the same bacterial species by different investigators who gave the same taxa its new names based upon slightly different morphological, cultural, and phenotypic criteria.

Tan wan Leng,2005

Minimal standards

Limitations of biochemical tests

Traditional tube methods or kits

- Biochemical properties do not accurately reflect the entire extent of the genomic complexity of a given species.
- Phenotypic properties can be unstable at times and expression be dependent an upon changes in environmental conditions.
- The drawbacks with some commercial systems is their constant number of the tests.
- One of the consequences of all of these limitations is that some commercial systems have great difficulty identifying certain groups of bacteria.

Janda and Abbott,2002

Minimal standards

Limitations of molecular biology and molecular techniques

- **Limitations of DNA hybridization:**
- The downside of DNA hybridization is that it is an expensive, technically complex, and labor-intensive procedure.
- **Limitations of 16S rDNA gene sequencing:**
- Although this technique relies on sequencing of the DNA that encodes the 16S rRNA subunit, like phenotypic tests, it surveys only a small portion of the microbial genome.

Janda and Abbott,2002

Proposed guidelines for identification of bacterial species for publication purposes

Minimum standard for description of new species, case reports and publication studies

- According to Christensen and colleagues,2001, recognition of new species should not be based upon a single strain but rather upon a minimum of 5 to 10 strains from geographically and epidemiologically unrelated areas.
- We believe perhaps expanded guidelines should be applied to case reports or a limited series of case reports involving unusual (rare) bacterial species or infrequent biotypes (genotypes) of established (traditional) pathogens.
- Proposals to recognize new species would require confirmation of the bacterial species or unusual phenotype or genotype by two independent laboratories (Table 1).

Janda and Abbott,2002

Proposed guidelines for identification of bacterial species for publication purposes

An alternative proposal that should be both technically and financially feasible and would help to reduce the number of publications with misidentifications

Type of study	Circumstances	Minimum requirements
Description of new species	Description of an organism and proposal of a new bacterial species	Identification based upon at least five strains (i) that have been demonstrated to be different by at least one molecular technique (e.g., pulsed-field gel electrophoresis, PFGE) and (ii) that are not related temporally, geographically, or epidemiologically.
Case report (single)	Isolation of a species identified as unusual or rare (e.g., unusual biotype of a common species or a common species associated with a new disease, disease syndrome, or anatomic site of isolation)	(i) Species or biotype identification confirmed by two independent laboratories (preferably, one of the two serves in a reference capacity); (ii) identification methods must differ (e.g., a commercial system and a traditional [tube] method) and must generate results indicating very good to excellent identification likelihood; and (iii) relevant phenotype(s) or genotype(s) used for identification and method(s) of detection (e.g., AP120E, septyl code, and identification probability [results showing excellent, very good, or good likelihood], and/or percent similarity or divergence from a published 16S rDNA gene sequence already in a database) must be reported.
Case report (series)	Isolation of a single agent or multiple agents, some or all of which are species that are uncommon or at least rarely identified in the clinical laboratory.	(i) For a single species, identification of at least two isolates confirmed by two independent methods; (ii) for multiple species, identification of at least one isolate of each species confirmed by two or more methods; and (iii) laboratory data indicating that isolates of the same species do not represent the same strain must be provided.
Population studies	Isolation of multiple strains belonging to multiple taxa (e.g., in epidemiological investigations or validation studies of identification systems).	Identification of each strain by at least a single universally available (i.e., commercial) method.

Janda and Abbott,2002

Minimal standards

Minimal requirements for a description of a new species

- Sneath in 1977, suggested the minimum number for description of a new species is about 10 strains.
- If a prokaryote strain is considered as a new species, authors should at first explain why they consider this as a new species.
- They should carry out a thorough comparison between the new and the known species (and genera) of the bacterial group with which the new organism shows most similarities.
- If, as is usually the case, the new organism can be allotted to one of the major groups of prokaryotes, the description of a new species (and/or genus) should contain at least the information presented in the following Table.

The Prokaryotes (chapter 1.4),2006

Minimum data required for the description of a new species

	Required data	Desired/required data, if applicable
Cell morphology	Cell shape[a] Cell size (diameter, length) Motility Visible internal or external structures[a] Formation of typical cellular aggregate[a] Occurrence of cell differentiation[a] Ultrastructure (general)[b]	Color Flagellation type[a] Spores[a], appendages[a], capsules[a], sheaths[a] Life cycle[a], heterocysts[a], hormogonia[a] Ultrastructure of flagella, envelope, cell wall[b]
Colonial morphology	Appearance of cell suspensions Appearance of colonies	Color of suspension (absorption spectra) Color of colonies Motility of colonies Formation of fruiting bodies[a] Formation of mycelia[a]
Staining behavior **Cell constituent**	Gram stain DNA base ratio Reserve materials	Acid-fast stain, spore stain, flagellum stain Nucleic acid homology; rRNA sequences Cellular pigments Cell wall and membrane constituents Typical enzymes
Physiology	Temperature range and optimum pH range and optimum Modes of energy metabolism (phototrophy, chemotrophy, lithotrophy, organotrophy) Relation to oxygen List of electron acceptors List of carbon sources List of nitrogen sources List of sulfur sources	Salinity or osmolarity requirements Vitamin requirements, Typical metabolic products formed (acids, osmolytes, pigments, antibiotics, toxins, antigens) Tolerances and susceptibilities
Ecology	Natural habitat(s)	Pathogenicity, host range Antigen formation Serology Phage susceptibility Symbiosis

[a]To be demonstrated by light microscopy.
[b]To be demonstrated by electron microscopy.

Hormogonia are motile filaments of cells formed by some cyanobacteria. Many multicellular cyanobacteria produce specialized nitrogen-fixing heterocysts.

Minimal standards

Revised minimal standards for description of new species of the class *Mollicutes*

- **The mandatory requirements are:**

1. Deposition of the type strain into two recognized culture collections, preferably located in different countries;
2. Deposition of the 16S rRNA gene sequence into a public database, and a phylogenetic analysis of the relationships among the 16S rRNA gene sequences of the novel species and its neighbours;
3. Deposition of antiserum against the type strain into a recognized collection;
4. Demonstration, by using the combination of 16S rRNA gene sequence analyses, serological analyses and supplementary phenotypic data, that the type strain differs significantly from all previously named species; and
5. Assignment to an order, a family and a genus in the class, with an appropriate specific epithet.

Brown *et al.*,2006

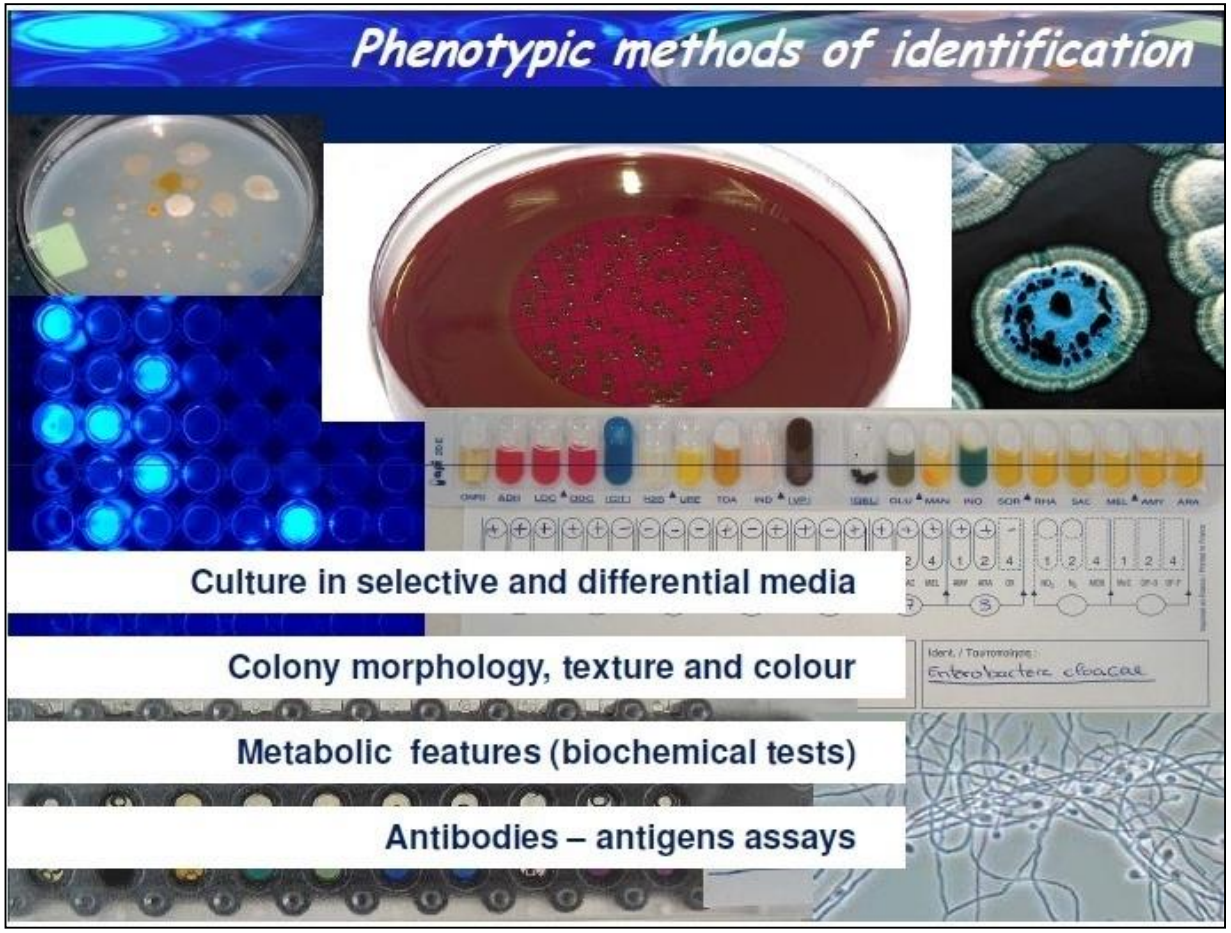
1. Phenotype-based methods:
Classical or conventional methods

The old methods still have great value

Speciation or sub-typing strains based upon metabolic or housekeeping enzymes

1. **Morphological traits(Cultural characteristics)**
2. **Biochemical tests/kits**
3. **Total protein analysis**
4. **Fatty acids analysis**
5. **Phage typing**
6. **Serological methods**

Phenotype-based methods

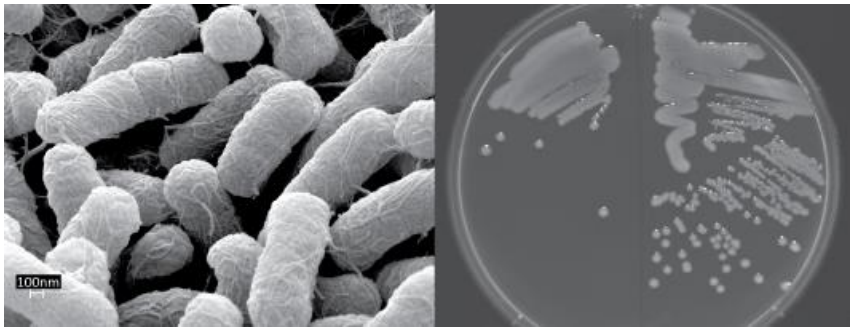


Tavares *et al.*,2010

Cultural characteristics
Bacterial observation

- Bacterial genera may be differentiated in two ways:

1. By the cellular morphology which is observed microscopically;
2. By the colony morphology which is observed on a plate culture.



Cultural characteristics

1. Cellular morphology

- These include:

1. Cell size,
2. Cell shape,
3. Motility,

- Arrangement and absence or presence of:

1. Flagella,
2. Storage material (granules of glycogen(a stored form of glucose), polyphosphate or poly-ß-hydroxybutyrate, PHB),
3. Gas vacuoles (in photosynthetic, aquatic bacteria),
4. Capsules/loose slime, and
5. Spores.

Cultural characteristics

2. Colony morphology

- The characteristics of a colony(shape, size, pigmentation, etc.) are termed the colony morphology.
- Although bacterial and fungi colonies have many characteristics and some can be rare, there are a few basic elements that you can identify for all colonies.

Cultural characteristics

2. Colony morphology

1. Form: circular, filamentous, etc.
2. Margins: round, undulate (wavey), rhizoid.
3. Topography (Surface): raised, flat, rugose (wrinkled), glistening, rough, dull (opposite of glistening), etc.
4. Opacity(color): transparent (clear), opaque, translucent,….
5. Pigmentation (chromogenesis): white, dull, buff, red, purple, colorless, beige, yellow, orange-yellow, tan, salmon, pink, blue, green, golden-brown, brown, agar or whitish,...
6. Size: measured in mm: <0.100 = small
   > 2.0 mm = large

- Three additional elements of morphology should be examined only in a supervised laboratory setting:

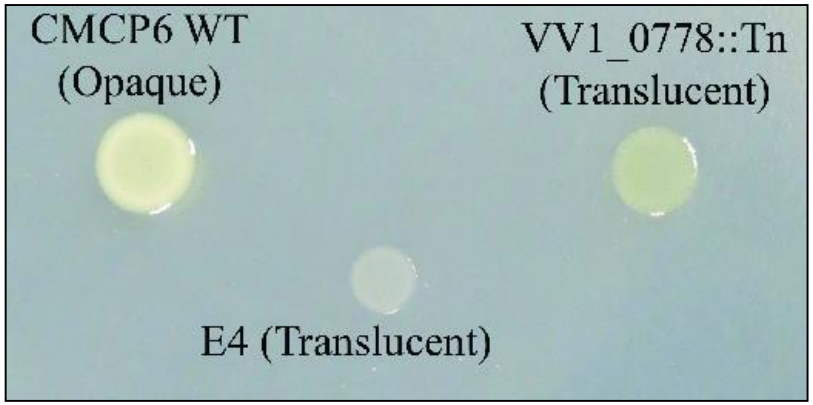
1. Texture(consistency): dry, moist, mucoid, brittle, viscous, butyrous (buttery).
2. Emulsifiability,
3. Odor.

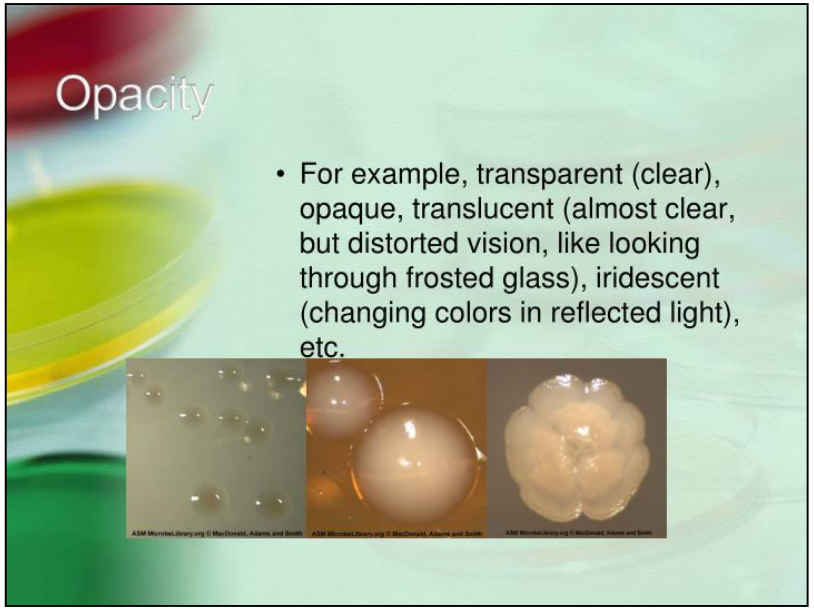
Colony morphology
Opacity(color)

1. *Proteus mirabilis* (clear diffusible beige on beige background);
2. *Enterococcus faecalis* (blue colour);
3. *klebsiella pneumonia* (mucoid, metallic blue);
4. *P. aeruginosa* (transparent, yellow serrated edges, diffuse);
5. *E. coli* (Small, pink-red);
6. *Staphylococcus aureus* (golden-yellow or whitish).

Samra *et al.*,1998;..

Colony morphology
Opacity(color)

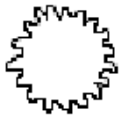




Opacity(color): transparent (clear), opaque, translucent (almost clear, but distorted vision, like looking through frosted glass), iridescent (changing colors in reflected light), milky white, whitish grey, opalescent (colourless or white like an opal, synonym for iridescence).

Colm Banks;..

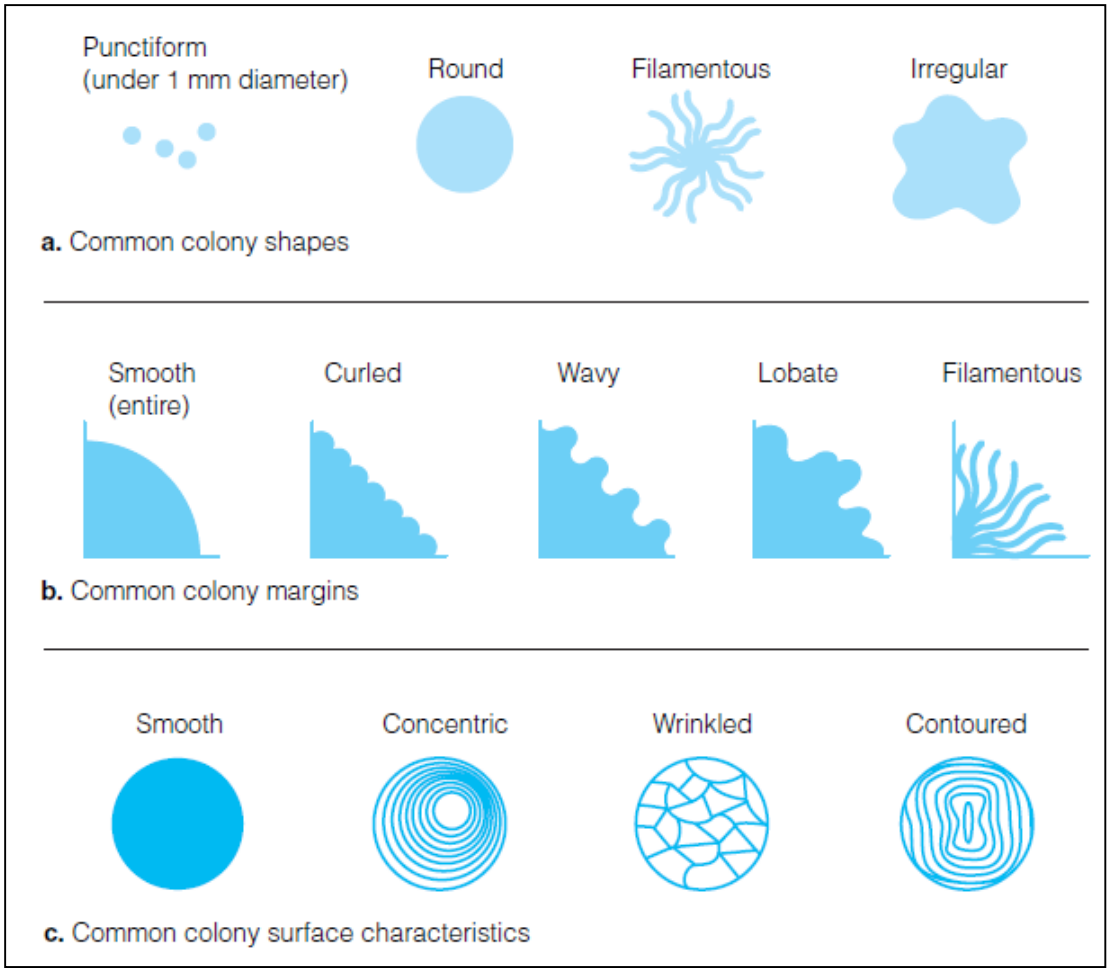
Colony morphology:
Shapes of bacterial colonies.

MARGIN	COLOUR	ELEVATION	TEXTURE	SHAPE
 Curled	 Orange	 Raised	Slimy, moist	 Round
 Entire (smooth)	 Red or pink	 Umbonate	Matte, brittle rough	 Punctiform (pinpoint or dot)
 Filamentous	 Black	 Flat	Shiny, viscous	 Rhizoid (root-like)
 Undulate (wavy)	 Brown	 Convex	Dry, mucoid	 Filamentous
 Lobate	 Opaque or white	 Pulvinate (Cushion-shaped)	Translucent clear, transparent	 Irregular
 **Erose (serrated)** uneven	**Milky**	**Growth into culture medium**	**Iridescent (changes colour in reflected light)**	 **Spindle**

Source: MicroDok

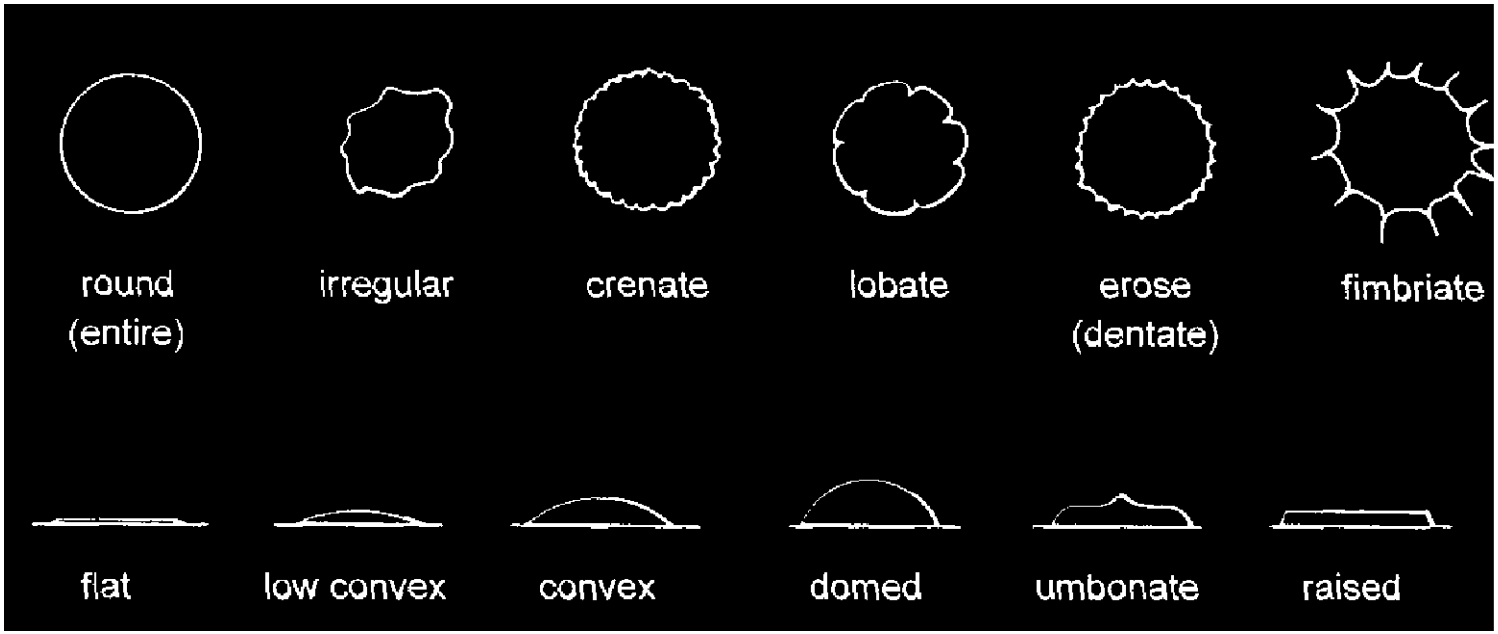
Colony morphology

Shapes of bacterial colonies



Colony morphology

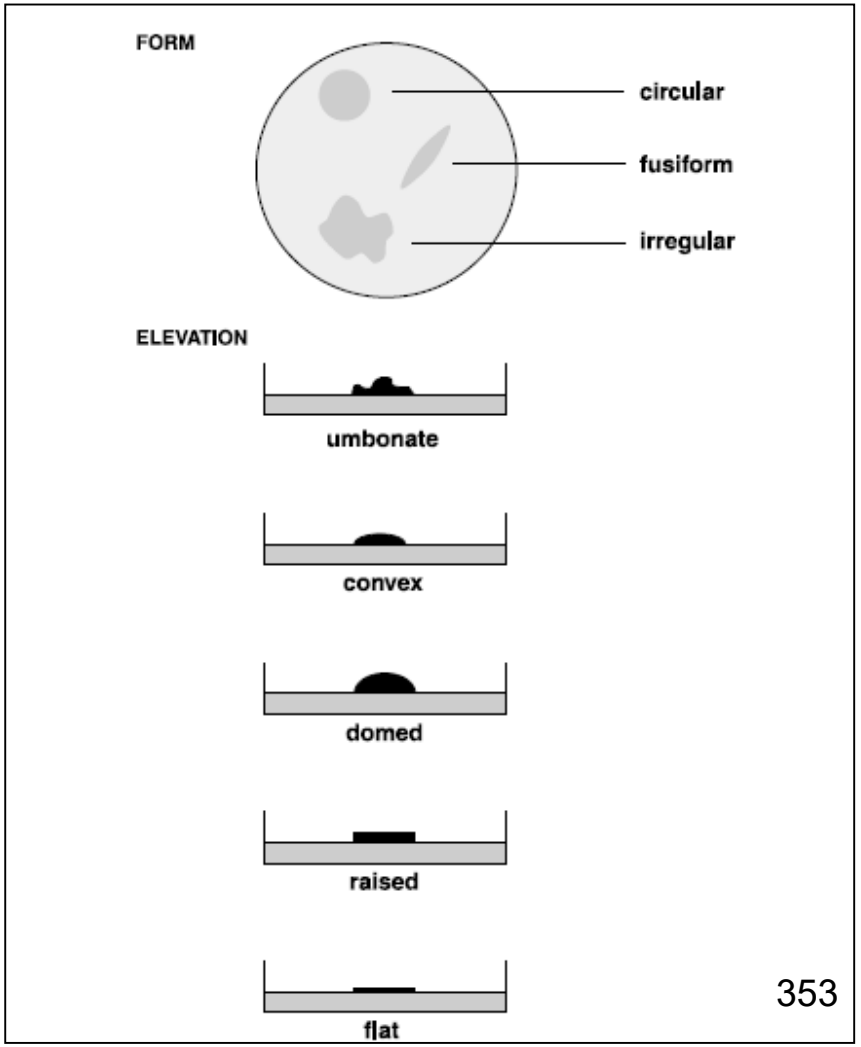
Shapes of bacterial colonies



Singleton,2004

Colony morphology

Elevation of the bacterial colony: This describes the side view of a colony



Goszczynaska *et al.*,2000

Smelling bacteria

Odor bacteria

Colonies with unpleasant odor

- Bacteria make a lot of smells, mostly ones that we'd rather not think about.
- Some bacteria produce hundreds of volatile compounds, often with an unpleasant odor.
- Think of the smell of rotten potatoes, for example.
- In humans, the formation of body odors is caused by factors such as diet, gender, health, and medication, but the major contribution comes from bacterial activity.
- Body odor is the perceived unpleasant smell our bodies can give off when bacteria that live on the skin break down sweat into acids.

Smelling bacteria

Odor bacteria

Colonies with unpleasant odor

- An electronic-nose device was designed to rapidly identify phytopathogenic bacteria based on their profile of volatile metabolites.
- This technique could be utilized for detection and identification of plant pathogenic bacteria in plant diagnostic clinics and quarantine laboratories.

Dog's nose: possess up to 300 million olfactory(smell sense) receptors in their noses, compared to about six million in us. And the part of a dog's brain that is devoted to analyzing smells is about 40 times greater than ours.

Colorful microorganisms

Pigment producing bacteria

Chromogenic bacteria

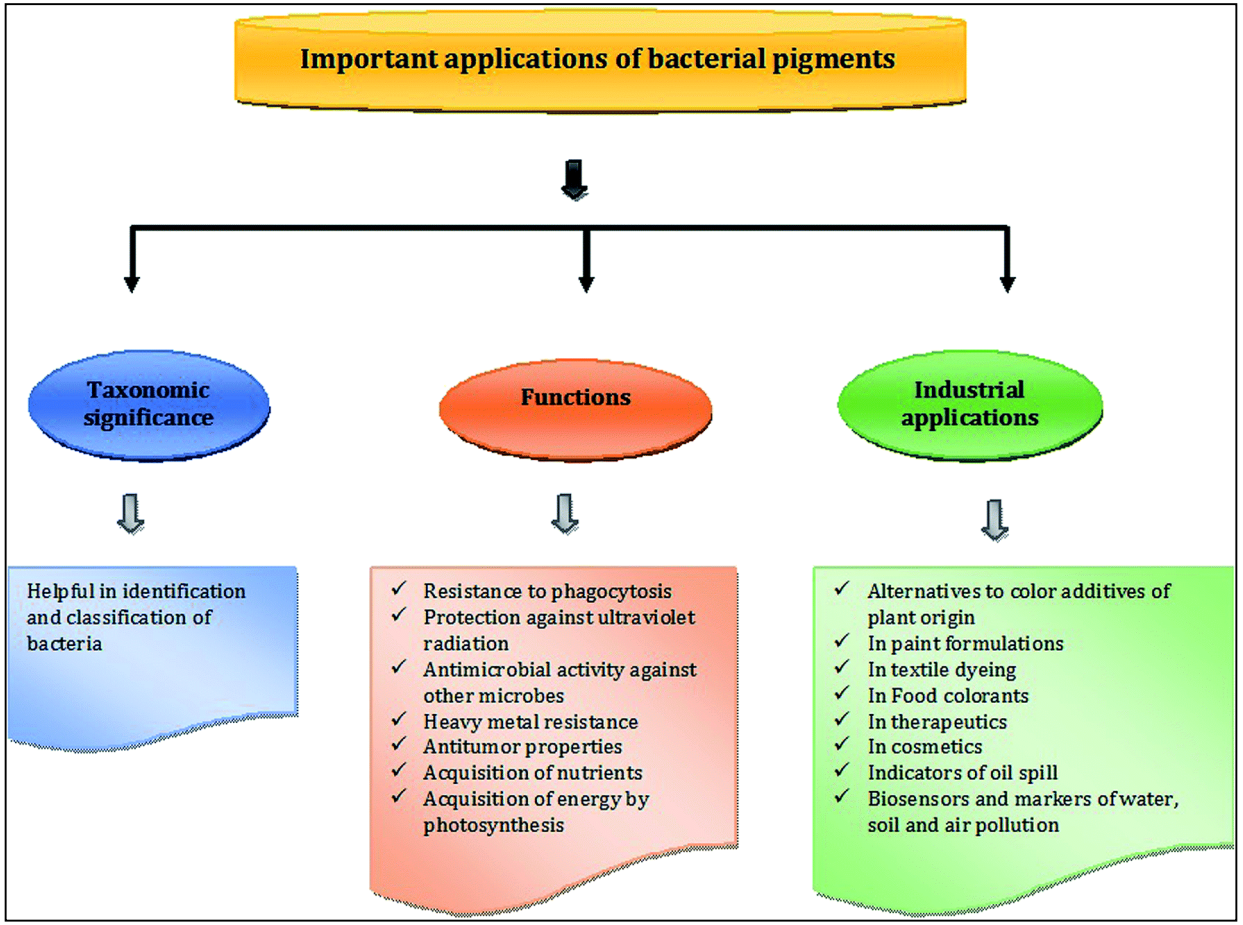
- Microbial cells accumulate pigments under certain culture conditions, which have very important industrial applications.
- Microorganisms can serve as sources of carotenoids, the most widespread group of naturally occurring pigments.
- More than 750 structurally different yellow, orange, and red colored molecules are found in both eukaryotes and prokaryotes with an estimated market of $ 919 million by 2015.

Kirti *et al.*,2014

Colorful microorganisms

Chromogenic bacteria

Microbial pigments



Venil *et al.*,2014

Colorful microorganisms

Chromogenic bacteria

Microbial pigments

Pigment	Microorganism
Indigoidine (blue-green)	*Streptomyces aureofaciens* CCM 323, *Corynebacterium insidium*
Carotenoid	*Gemmatimonas aurantiaca* T-27[7]
Melanin (black-brown)	*Kluyveromyces marxianus, Streptomyces chibanensis, Cryptococcus neoformans, Aspergillus* sp., *Wangiella dermatitidis, Sporothrix schenckii,* and *Burkholderia cepacia*
Prodigiosin (red)	*Serratia marcescens, Rugamonas rubra, Streptoverticillium rsubrireticuli, Serratia rubidaea, Vibrio psychroerythrus, Alteromonas rubra,*and *Vibrio gaogenes*
Zeaxanthin	*Staphylococcus aureus, Vibrio psychroerythrus, Streptomyces* sp., and *Hahella chejuensis*
Canthaxanthin (orange)	*Monascus roseus, Bradyrhizobium* sp.
Xanthomonadin (yellow)	*Xanthomonas oryzae*
Astaxanthin (red)	*Phaffia rhodozyma, Haematococcus pulvialis*
Violacein (purple)	*Janithobacterium lividum*
Anthraquinone (red)	*Pacilmyces farinosus*
Halorhodopsin and rhodopsin	*Halobacterium halobium*
Rosy pink	*Lamprocystis roseopersicina*
Violet/purple	*Thiocystis violacea, Thiodictyon elegans*
Rosy peach	*Thiocapsa roseopersicina*
Orange brown	*Allochromatium vinosum*
Pink/Purple violet	*Allochromatium warmingii*

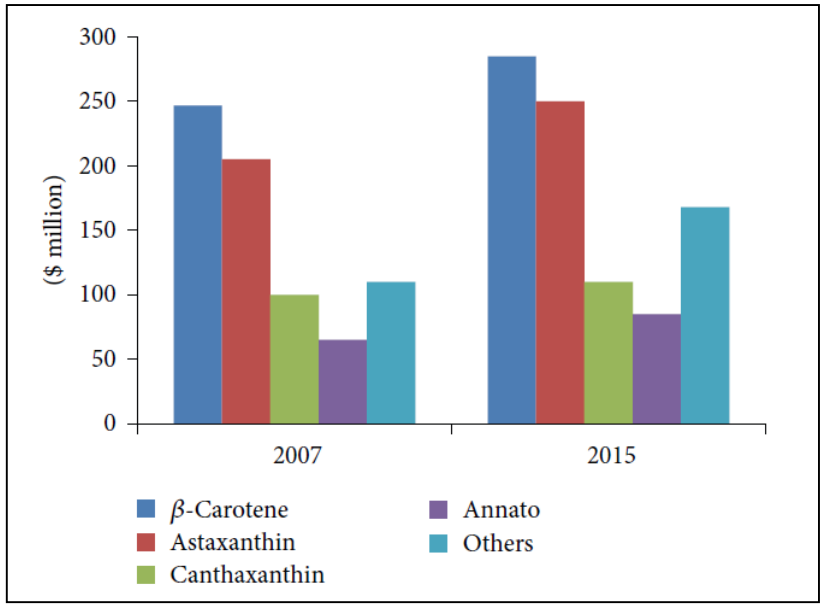
Kirti *et al.*,2014

Colorful microorganisms

Chromogenic bacteria

Microbial pigments in food industry

- Canthaxanthin(A bacterium *Bradyrhizobium* sp.);
- Astaxanthin(A microalga *Haematococcus pluvialis*);
- β-Carotene(A fungus *Blakeslea trispora*).

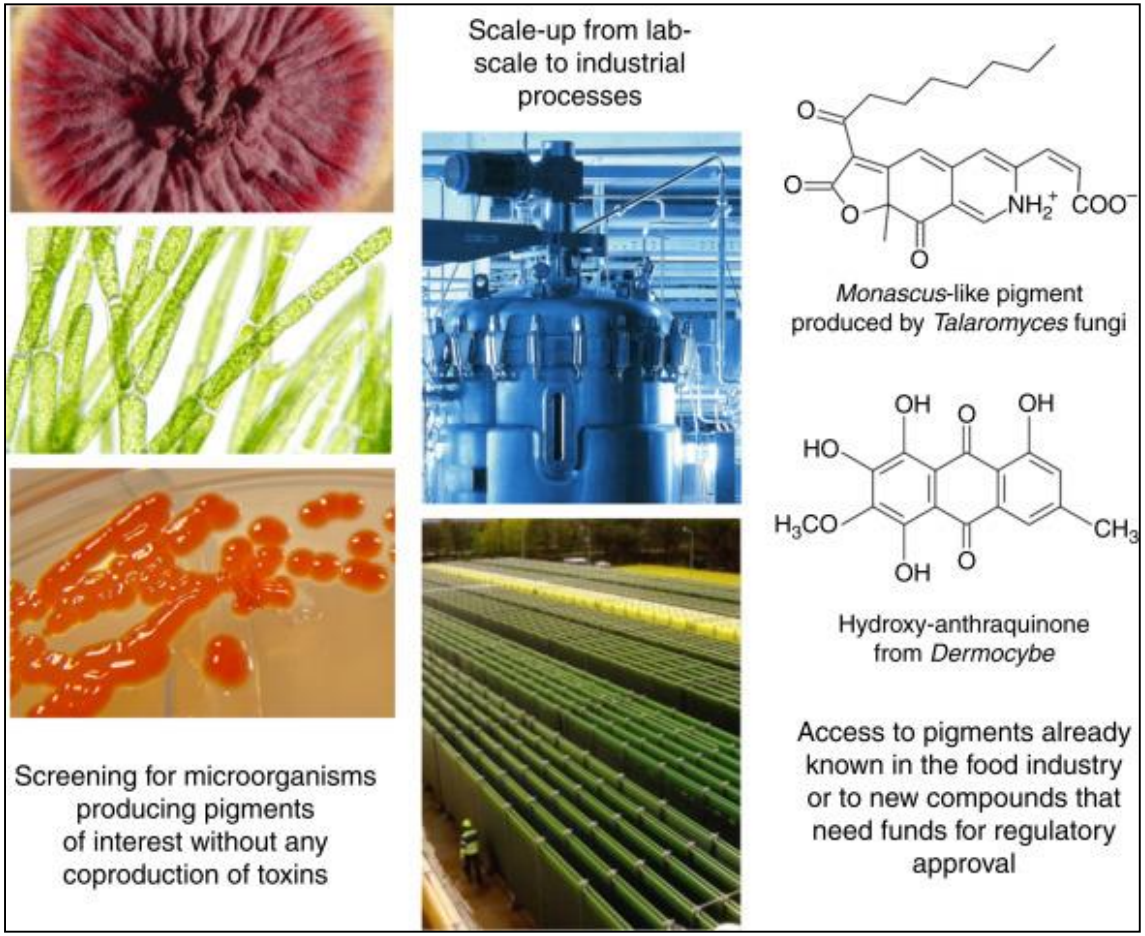


Global carotenoid product market in 2007 and 2015 ($ million): Analyst-Ulrich Marz.

Kirti *et al.*,2014

Colorful microorganisms

Microbial pigments from bacteria, yeasts, fungi, and microalgae for the food and feed industries



Laurent Dufossé,2018

Colorful microorganisms

Chromogenic bacteria

Bacterial pigments in food industry

Bacteria	Pigment	Application in food
Pseudomonas aeruginosa	Pyocyanin	Colorant in beverages, cakes, confectionaries, pudding, decoration of food items
Flavobacterium	Zeaxanthin	As an additive in poultry feed to increase yellow color of animal's skin and eggyolk. Colorant in cosmetic and food industry
Agrobacterium auranticum	Astaxanthin	Food colorant
Streptomyces echinoruber	Rubrolone	Food colorant
Streptomyces coelicolor	Actinorhodin	Edible natural pigment and food colorant

Kirti *et al.*,2014

Bacterial pigmentation

Carotenoids

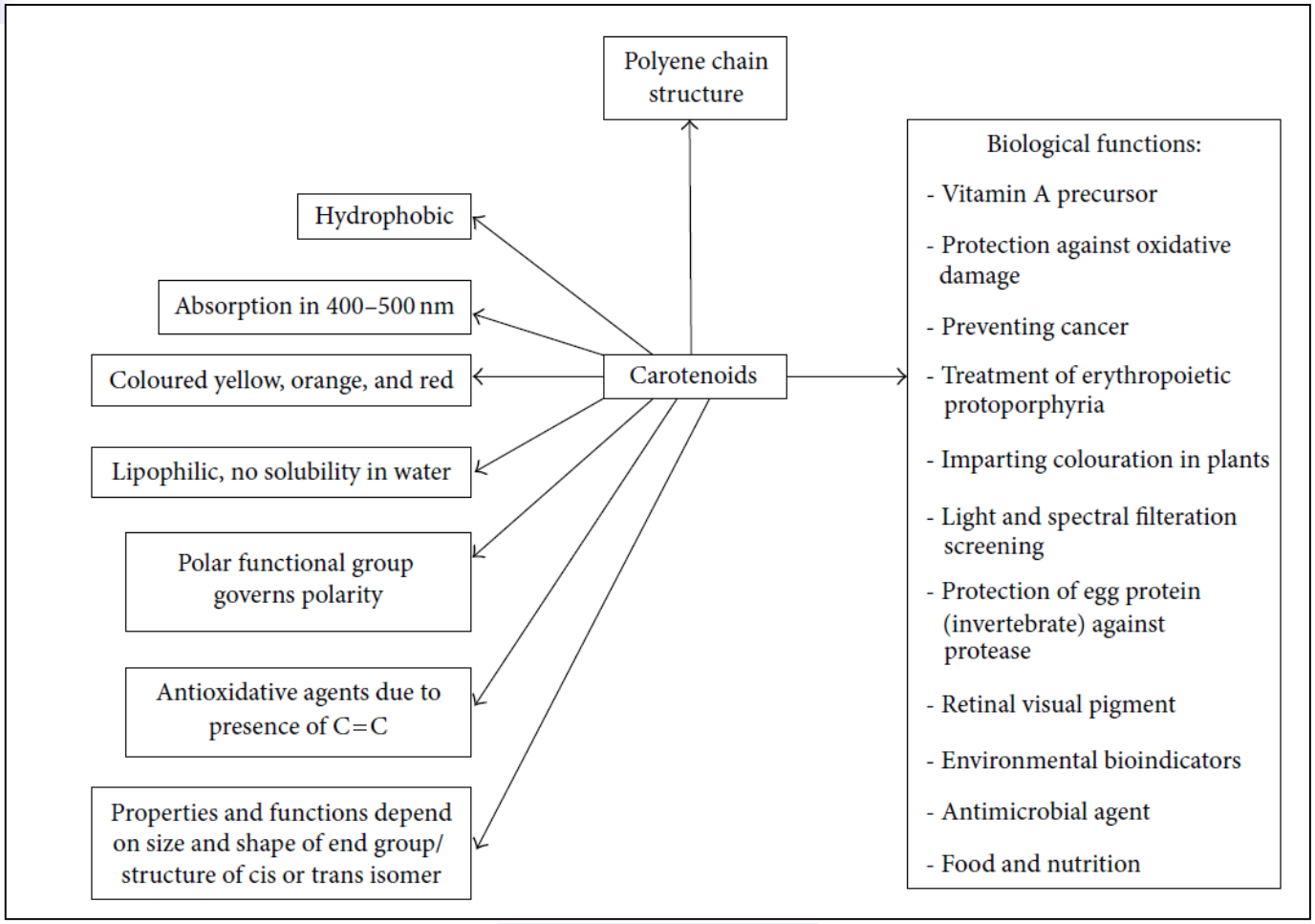
Important physical, chemical, and biological properties of carotenoids

- Carotenoids are among the most diverse natural products;
- They are synthesized by many organisms, including animals, plants, and microorganisms including bacteria, and absorb light in the 400- to 550-nm range, which gives them their yellow-orange color.
- Carotenoids are lipid soluble classes of molecules associated with the lipidic fractions.
- Carotenoids protect cells against photooxidative damage and oxygen radicals.

Bacterial pigmentation

Carotenoids

Important physical, chemical, and biological properties of carotenoids



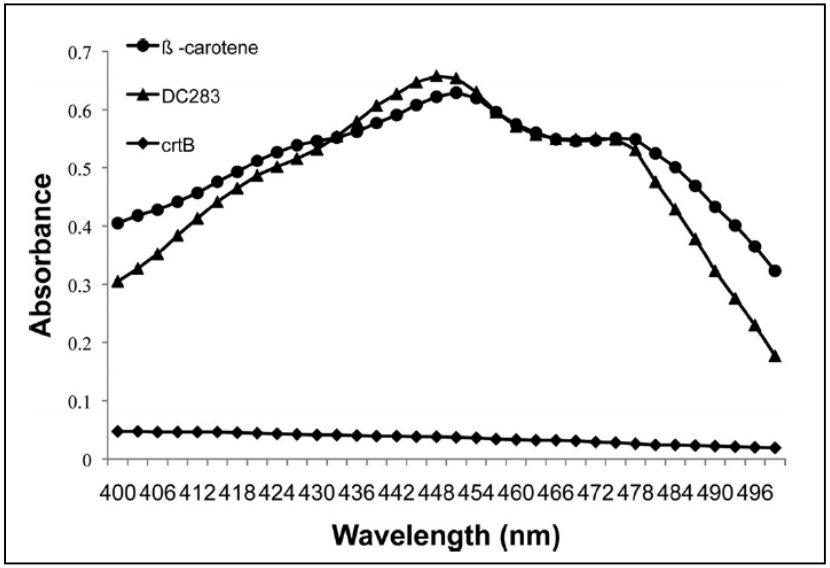
Kirti *et al.*,2014

Bacterial pigmentation

Carotenoids

Spectral analysis of the *P. stewartii* carotenoid pigment

- The methanole xtractable pigment from wild-type *P. stewartii* (DC283) has an absorption spectrum with a peak at 451 nm and a shoulder at 475 nm and with the maximum absorbance (max) at 450 nm, which was similar to that of the β-carotene standard.
- The *crtB*::*Mar2xT7* (*crtB*) mutant did not have detectable pigment in the methanol extract.



Mohammadi *et al.*,2012

Bacterial pigmentation

As potential virulence agents

ROS reactive oxygen species

Pigment	Chemistry	Color	Bacterium	Virulence functions
Pyocyanin	Phenazine derived Zwitterion	Blue green	*Pseudomonas* sp.	Cytotoxicity, neutrophil apoptosis, ciliary dysmotility, proinflammatory
Melanin	Polyacetylene or polypyrrole polymers	Dark-brown, black	*Streptomyces* spp.	Antioxidants Antiphagocytic Block antimicrobials
Violacein	Rearranged pyrrolidone scaffold	Purple	*Chromobacterium violaceum*	Antioxidant, detoxify ROS
Prodigiosin	Linear tripyrrole	Red	*Serratia marcescens*	Immunosuppressant

A zwitterion(dipolar ion) is a neutral molecule with both a positive and a negative electrical charge that can easily penetrate biological membranes.

Kirti *et al.*,2014

Bacterial pigmentation

As potential virulence agents

ROS reactive oxygen species

Pigment	Chemistry	Color	Human pathogens	Virulence functions
Staphyloxanthin	Carotenoid	Golden	*Staphylococcus aureus*	Antioxidant, detoxify ROS [5, 6]
Pyocyanin	Phenazine-derived zwitterion	Blue green	*Pseudomonas* spp.	Cytotoxicity [37, 38, 40] Neutrophil apoptosis [51] Ciliary dysmotility [44] Proinflammatory [50]
Melanin	Polyacetylene or polypyrrine polymers	Dark brown, black	*Cryptococcus neoformans*, *Aspergillus* spp., *Wangiella dermatitidis*, *Sporothrix schenckii*, *Burkholderia cepacia*	Antioxidant [12, 31, 32, 33] Antiphagocytic [17] Block antimicrobials [19, 20]
Porphyrin	Heteromacrocycle	Black	*Porphyromonas gingivalis*	Antioxidant, detoxify ROS [75]
Granadaene	Ornithine rhamnopolyene	Orange red	*Streptococcus agalactiae*	Antioxidant, detoxify ROS [65]
Violacein	Rearranged pyrrolidone scaffold	Purple	*Chromobacterium violaceum*	Antioxidant, detoxify ROS [70]
Prodigiosin	Linear tripyrrole	Red	*Serratia marcescens*	Immunosuppressant [80]
Hemozoin	β-hematin aggregates	Brown black	*Plasmodium* spp.	Detoxification [54] Macrophage suppression [57] Pro-inflammatory [59]

Liu and Nizet,2009

Bacterial pigment protects against UV radiation

Microbiocidal activity of UV

- The ultraviolet portion of the light spectrum includes all radiations with wavelengths from 100 nm to 400 nm.
- The microbicidal activity of ultraviolet (UV) light depends on:

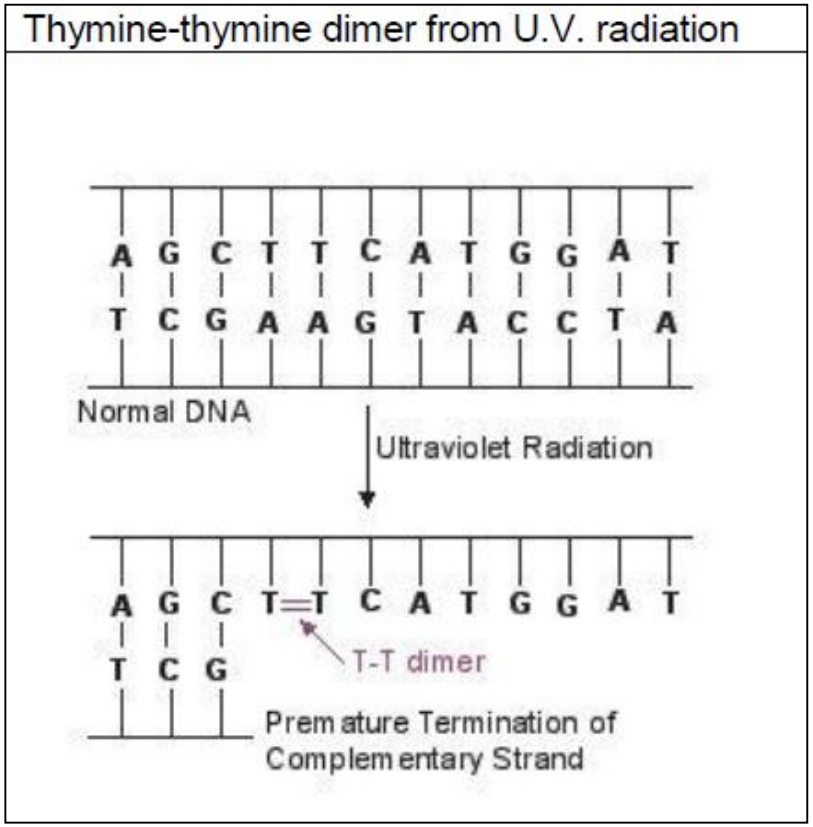
1. The length of exposure: The longer the exposure the greater the cidal activity.
2. Wavelength of UV: The most cidal wavelengths of UV light lie in the 260 nm-270 nm range where it is absorbed by nucleic acid.

Kaiser,2009

Bacterial pigment protects against UV radiation

Microbiocidal activity of UV

- UV light is absorbed by microbial DNA and causes adjacent thymine bases on the same DNA strand to covalently bond together, forming what are called thymine-thymine dimers.
- As the DNA replicates, nucleotides do not complementary base pair with the thymine dimers and this terminates the replication of that DNA strand.
- Only microorganisms on the surface of a material that are exposed directly to the radiation are susceptible to destruction.



Kaiser,2009

Colorful bacteria

Chromogenic bacteria

Bacterial pigmentation

- Characteristic pigments may form:

1. within the colony, or
2. can be excreted into the growth medium, and
3. may require special lighting (e.g. UV) for detection(fluorescent bacteria).
4. Occasionally, bacterial pigments can be detected in seed.

Cultural characteristics

Chromogenic bacteria

Bacterial pigmentation

- Pigments can be divided into two basic types:

1. Water-insoluble, and
2. Water-soluble.

- If the pigment is water-insoluble, as in the case of most chromogenic bacteria, it does not diffuse out of the organism. As a result, the colonies are pigmented but the agar remains the normal color.
- If the pigment is water-soluble, as in the case of *Pseudomonas aeruginosa* it will diffuse out of the organism into the surrounding medium.
- Both the colonies and the agar will appear pigmented.

Kaiser,2009

Cultural characteristics

Bacterial pigmentation

Water Insoluble and Water-Soluble Pigment in Chromogenic Bacteria

Water-insoluble pigment	Water-soluble pigment
	

Kaiser,2009

Colony morphology

Pigmentation

Cultural conditions

- Probably the most visual characteristic is pigmentation (color).
- However, formation of pigment depends on environmental factors such as:

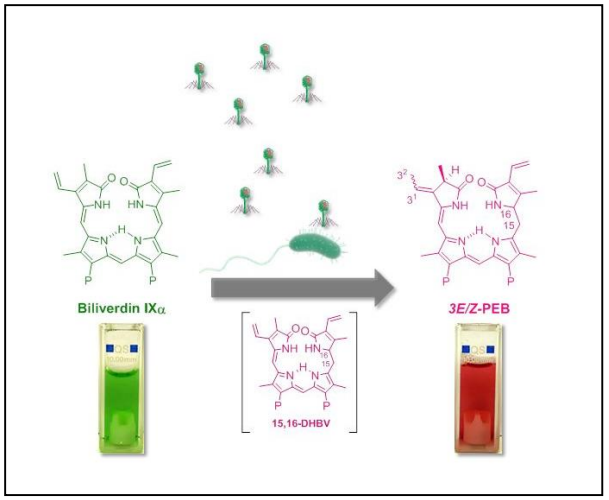
1. temperature,
2. nutrients,
3. pH, and
4. moisture.

- For example, *Serratia marcescens* produces a deep red pigment at 25°C, but does not produce pigment at 37°C.

Kaiser,2009

Viruses cause bacteria to produce pink pigments

- The viruses carry genetic information which can be used to produce the pink-coloured pigments.
- We were able to show that the viruses most likely affect those microbes for which we do not yet know what purpose the pigment serves.



Viruses from the ocean carry the genetic information for the turnover of the green pigment biliverdin to the pink pigment phycoerythrobilin. During infection this information is transferred to the bacteria which enable them to produce the pink pigment.

Colony morphology

Fluorescent and non-fluorescent pigmentation

- *Clavibacter michiganensis* (carotenoid);
- *Pantoea* (carotenoid);
- *Xanthomonas* (xanthomonadins);
- *Pseudomonas* (pyocyanine, pyorrubin(red), pyomelanin (brown) fluoresce under short wave length (254 nm) UV radiation.

1. Fluorescent *Pseudomonas* (pyroverdins or fluorescent pigments) fluoresce both under short (254 nm) and long wave length (366 nm) ultraviolet light.
2. Nonfluorescent bacteria fluoresce just in short wavelength (254 nm) ultraviolet light.

- *Dickeya chrysanthemi* (indigoidine or blue);
- *Clavibacter insidiosum* (indigoidine);
- *Burkholderia* (melanins).

Melanins are polymers of phenolic compounds. Found in both Pro- and Eukaryota. Protect from environmental stress and in pathogenic microorganisms it correlates with an increased virulence (Plonka & Grabacka,2006).

Colony morphology

Fluorescent and non-fluorescent pigmentation

Some pigments of plant pathogenic bacteria

Bacterium	Pigment(s)	Colour	Function
Burkholderia glumae	fervenulin,toxoflavin	fluorescent	chlorosis, inhibition of leaf
Clavibacter michiganensis	carotenoids	yellow, non-water soluble	?
Dickeya (Ex. *Erwinia*) *chrysanthemi*	indigoidine	blue, water-soluble	?
E. rhapontici	Proferrorosamine A (pFR A)	pink	iron-chelating
B. rubrifaciens	rubrifacine	pink	inhibition of electron transport
Fluorescent *Pseudomonas* spp.	pyoverdins	green or blue fluorescent, diffusible	binding and transport through cell wall or membrane of Fe, so-called siderophores
Streptomyces scabiei	melanin	brown, water soluble	Factor of virulence
Ralstonia solanacearum	melanin	brown, water soluble	Factor of virulence
Xanthomonas spp.	xanthomonadins	yellow, water insoluble	Protection from visible light damage

Janse,2006

Colony morphology

Fluorescent pigmentation

Characteristic of pigments in *P. aeruginosa*

- The characteristic blue-green appearance of colonised/infected pus or of an organism culture is due to the mixture of:

1. pyocyanin (blue), and
2. pyoverdin (fluorescein, yellow).

- Production of blue-green pigment is indicative of *Pseudomonas aeruginosa*.
- *P. aeruginosa* can lose its fluorescence under UV if the cultures are left at room temperature for a short time.
- Fluorescence reappears when plates are re-incubated.

Fluorescent pseudomonads
Fluoresce on KB medium

- After incubation the plates were exposed to UV light at 365 nm for few seconds.

1. *Pseudomonas* (pyroverdins or fluorescent pigments) fluoresce both under short (254 nm) and long wave length (366 nm) ultraviolet light.
2. Nonfluorescent bacteria fluoresce just in short wavelength (254 nm) ultraviolet light.

Fluorescence of some strains(8,29,7,17) under UV lamp.

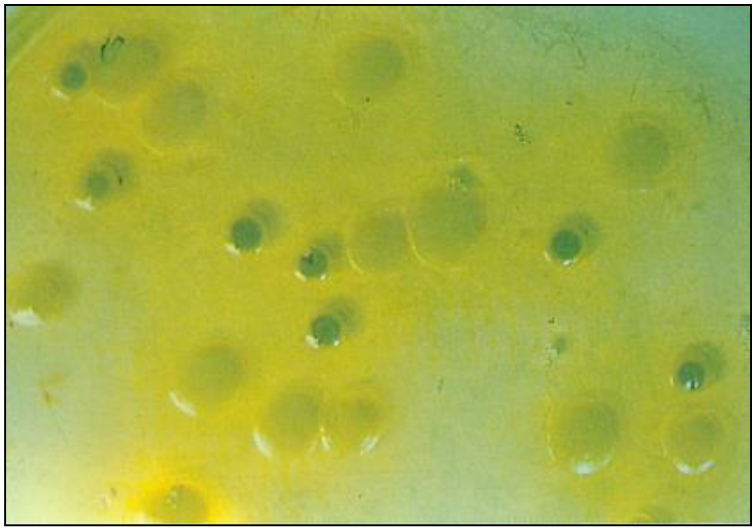
Sajben *et al.*,2004;..

Pseudomonas fuscovaginae

Bacterial sheath brown rot of rice

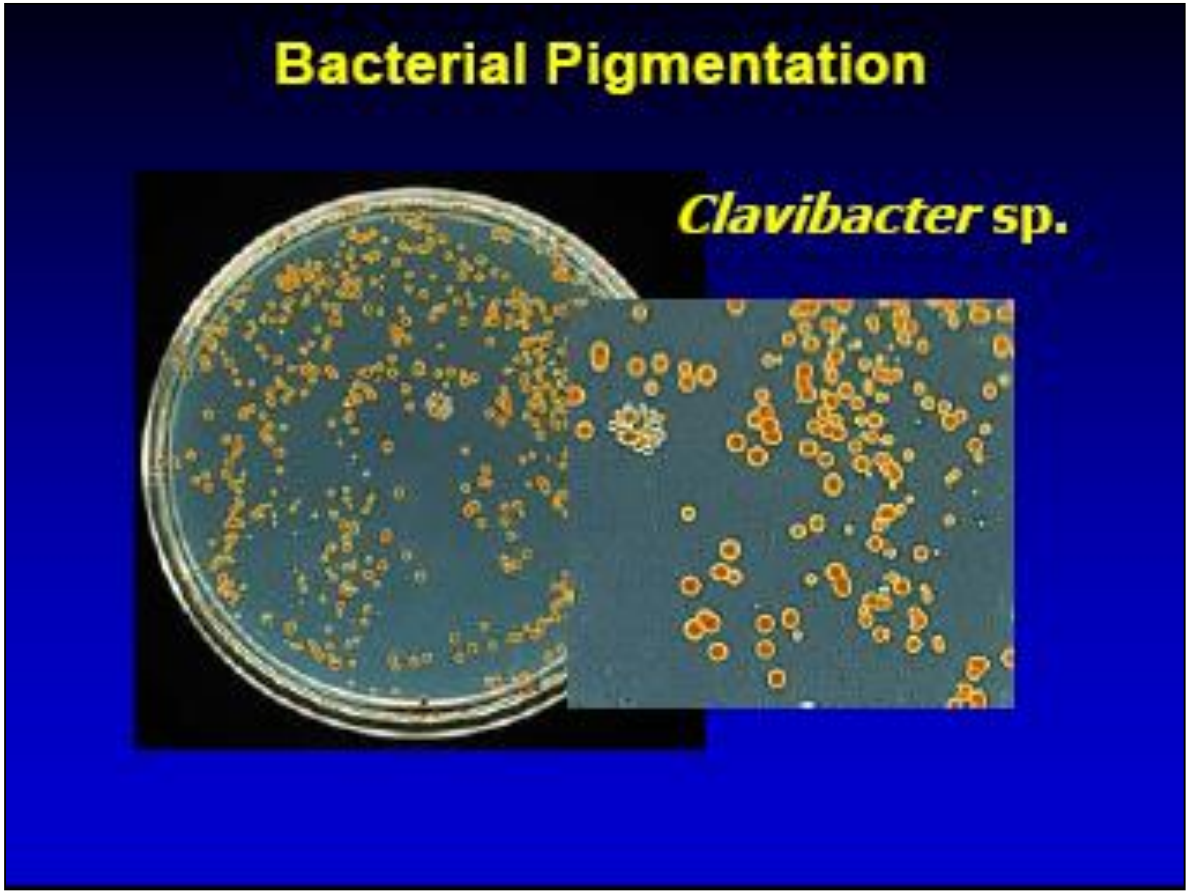
- *Pseudomonas fuscovaginae* on Miyajima's medium showing beige or cream-colored colonies.
- Note some colonies with green pigment at the center.

Selective medium for P. fuscovaginae (Miyajima 1989)	
Penicillin G	750,000 units
Novobiocin	45 mg
Cycloheximide	75 mg
75% ethanol	3 ml
Distilled water	50 ml
King's medium B	940 ml



Mew and Misra,1994

Colony morphology
Non-fluorescent pigmentation

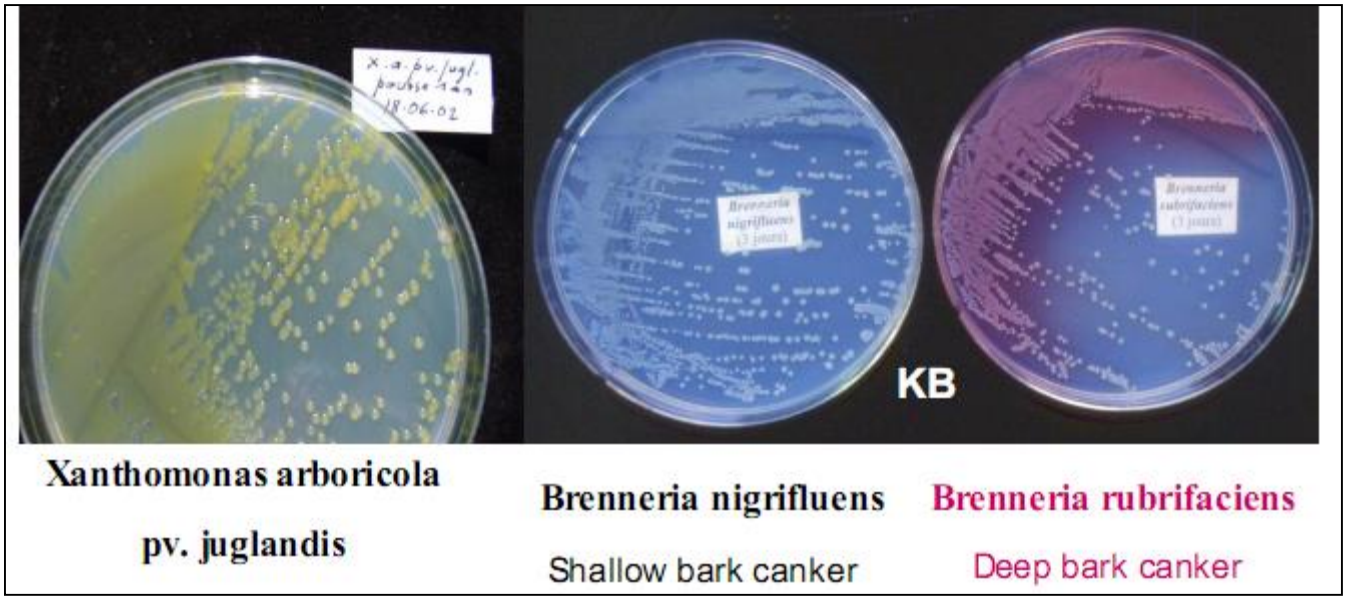


Colony morphology

Non-fluorescent pigmentation

Bacterial pigmentation

- *Xanthomonas juglandis* produces xanthomonadins;
- *Brenneria rubrifaciens* produces rubrifacine.



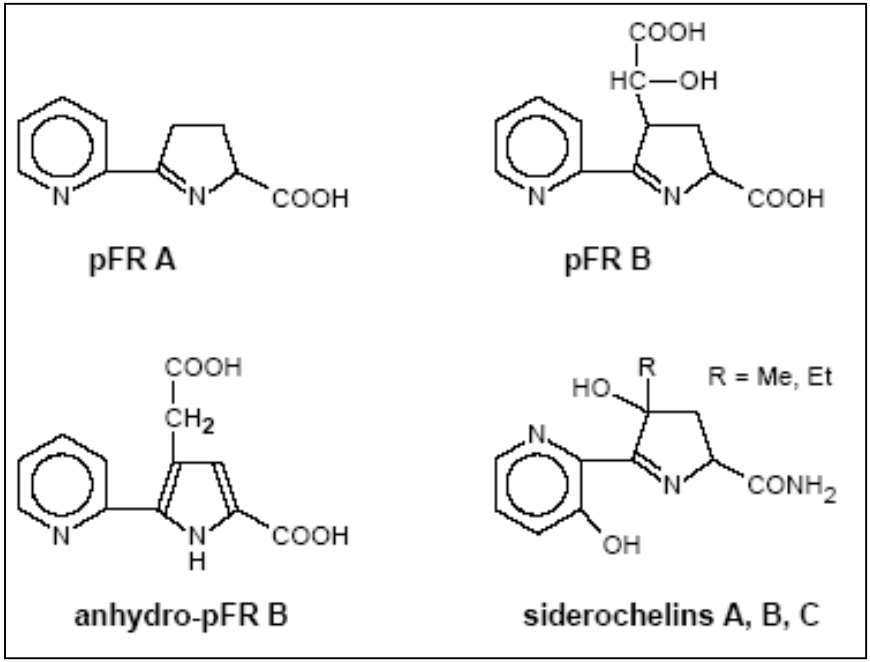
Meyer *et al.*,2007

Proferrorosamines

Structures and siderochelins

Erwinia rhapontici and *E. persicina*

- Proferrorosamines belong to the family of the rare microbial iron(II) chelators.
- *Erwinia rhapontici* produces Proferrorosamine A (pFR A).
- *E. persicina* was shown to produces proferrorosamines (pFR A and pFR B).
- pFR A clearly inhibited germination and seedling development in both wheat and cress.
- Therefore, proferrorosamines were considered as microbial virulence factors.



Feistner *et al.*,1997

Red pigment (prodigiosin)

Non-fluorescent pigmentation

Serratia marcescens and *S. rubidaea*

- Bacteria were grown on peptone/glycerol agar (peptone, 5 g; glycerol, 10 ml; agar, 20 g; distilled water, 1L).
- Cells were harvested by centrifugation at 13,000 rpm for 5 min, and then intracellular prodigiosin(red pigment) was extracted from the cells by suspending them in an acidified ethanol solution (4% [vol/vol] 1 M HCl in ethanol).
- Prodigiosin exhibits a characteristic maximum absorption spectrum at 534 nm in acidified ethanol.
- Most strains including strain NIMA as a control strain were shown maximum absorbance at 535 nm, shoulder at 510 nm in ethanol/HCl.

Grimont *et al.*,1976; Kwon *et al.*,2010

Red pigment(prodigiosin) assay

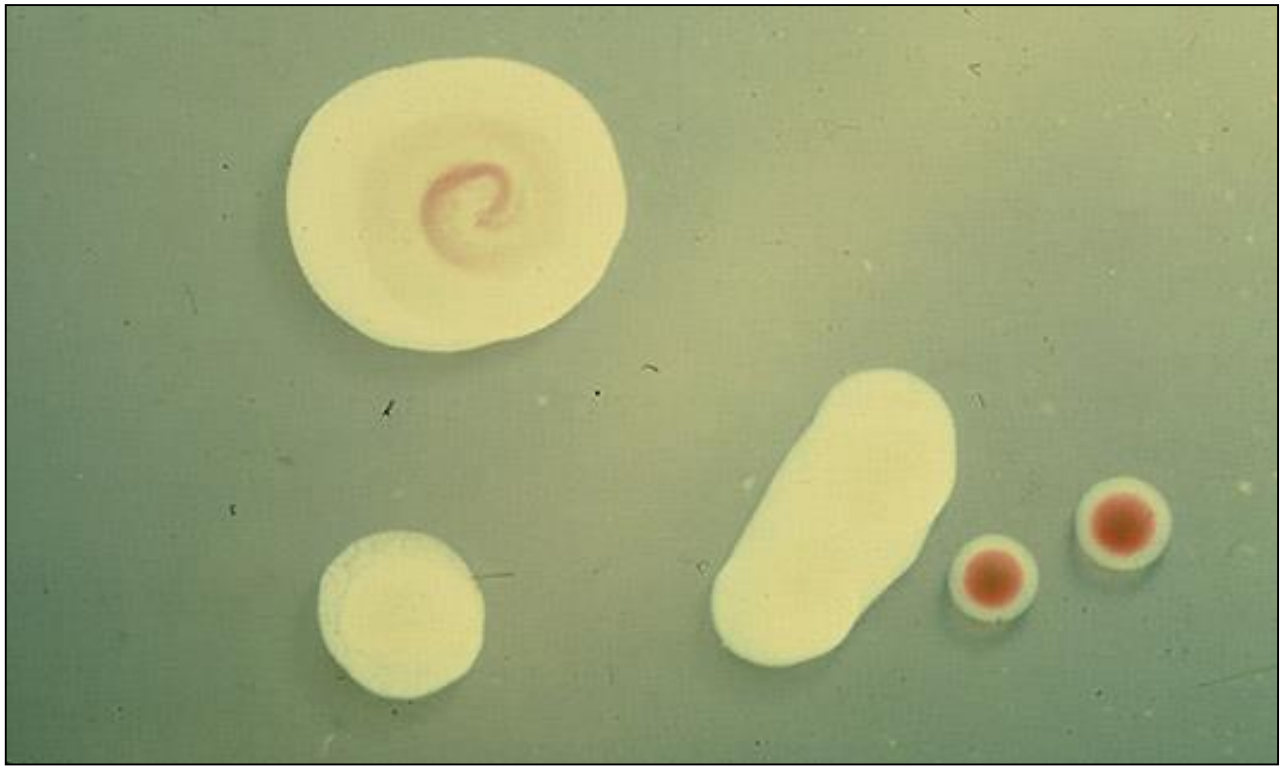
Serratia marcescens and *S. rubidaea*

Modified method

- Prodigiosin in representative culture samples were estimated after centrifugation at 4,000 rpm for 10 minutes.
- The pellet was suspended in acidic methanol (4.0 ml of 1 M HCl and 96 ml of methanol) and left at 4[degrees]C overnight for extraction.
- The samples were then centrifuged at 4,000 rpm for 15 minutes, and the supernatant was assayed spectrophotometrically at 535 nm.

Venil *et al.*,2009

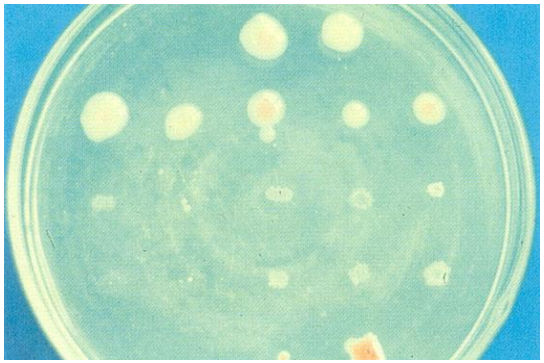
Colony morphology
Interpreting plates



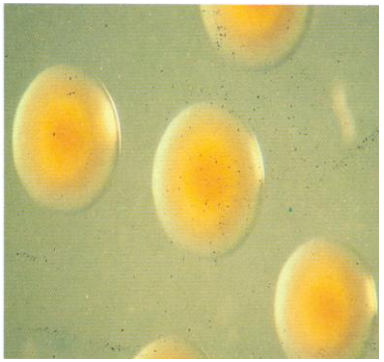
Different colony morphologies on TTC medium.

Colony morphology

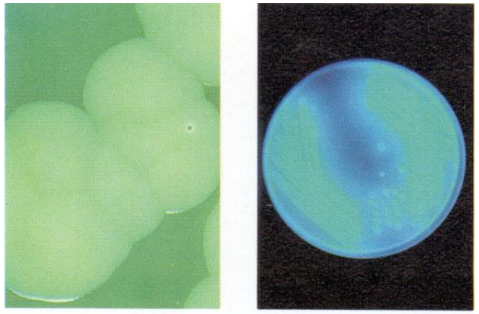
Colony shapes/color and texture



Agrobacterium

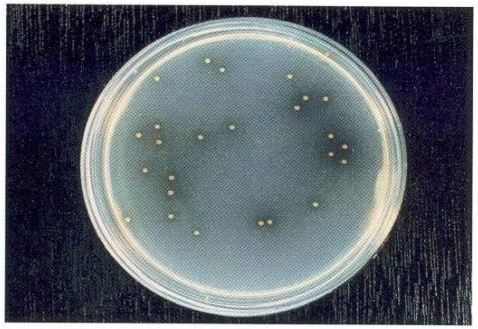


Pantoea

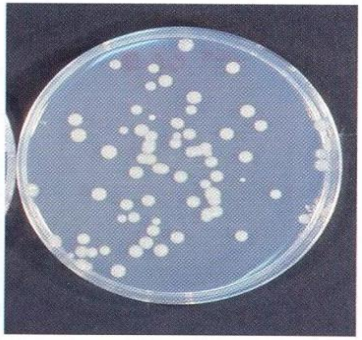


Pseudomonas

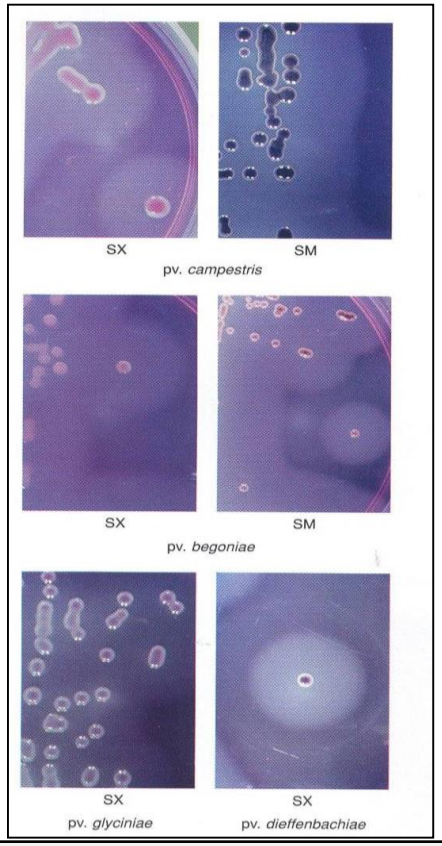
Colony morphology



Colonies of *Xanthomonas* on ET medium



Colonies of *Xanthomonas* on CTA medium



Colonies of *Xanthomonas* on SX and SM agars

Colony morphology

Culture	Reaction	Description	Culture	Reaction	Description
Escherichia coli		This microbe forms medium sized colonies with a regular margin and convex elevation.	***Klebsiella pneumoniae***		This microbe forms slightly gummy/wet looking colonies that are circular, convex with an entire margin.
Serratia marcescens		Colonies of this microbe are red in appearance, circular and have an entire margin.	***Pseudomonas fluorescens***		Colonies are circular, convex with an entire margin.

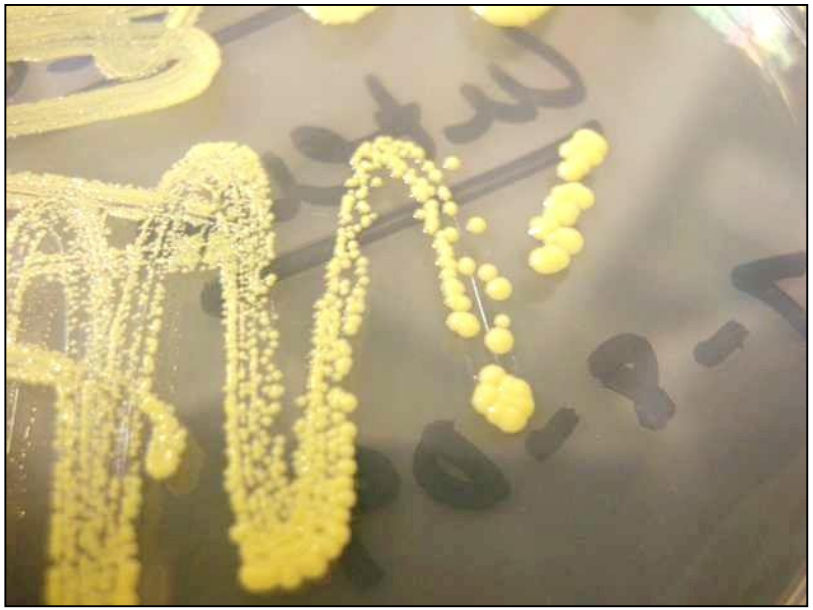
Colony morphology

Culture	Reaction	Description	Culture	Reaction	Description
Chromobacterium Violaceum Gram-negative, facultative anaerobe		Colonies are circular, convex with an entire margin and appear purple on most medium.	***Micrococcus luteus***		Colonies are punctiform (pin-point), convex with an entire margin and appear yellow on most medium.
Enterococcus faecalis		Colonies are punctiform, convex with an entire margin. The smallness of the colonies is due to the inefficient metabolism of these microbes. They can take 3-4 days to reach appreciable size.	***Lactobacillus plantarum***		Colonies are punctiform, convex with an entire margin. The smallness of the colonies is due to the inefficient metabolism of these microbes. They can take 3-4 days to reach appreciable size.

Colony morphology

This is your lab friend *Micrococcus luteus*

- Gram-positive, facultative anaerobe, cocci.
- Found in many places throughout the environment human skin, animals, water, dust, and soil.

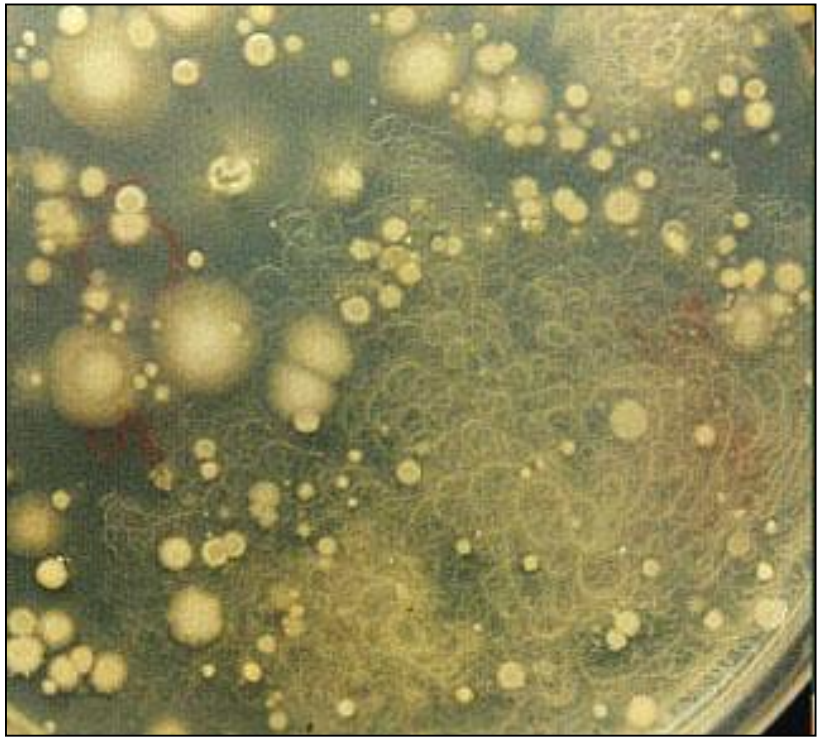


Colony morphology

Rapidly spreading bacteria

Bacillus mycoides

- Because of the heat treatment of the inoculum and the aerobic incubation of the NA plates, virtually all colonies on this plate would be those of various species of *Bacillus*.
- The spreading, filamentous growth is that of *Bacillus mycoides*, one of the few bacterial species which are identifiable by colony appearance.
- The "filaments" of *B. mycoides* always curve counterclockwise upon the surface they are growing.



Bacteriology 102 Website,2011

Cell morphology
Staining

- Bacteria are almost transparent and hence, unstained bacteria are not readily visible without special techniques such as:
- phase contrast microscopy, or
- dark-field microscopy.

Pacarynuk,2006

Types of staining

Simple/differential stains or negative/positive stains

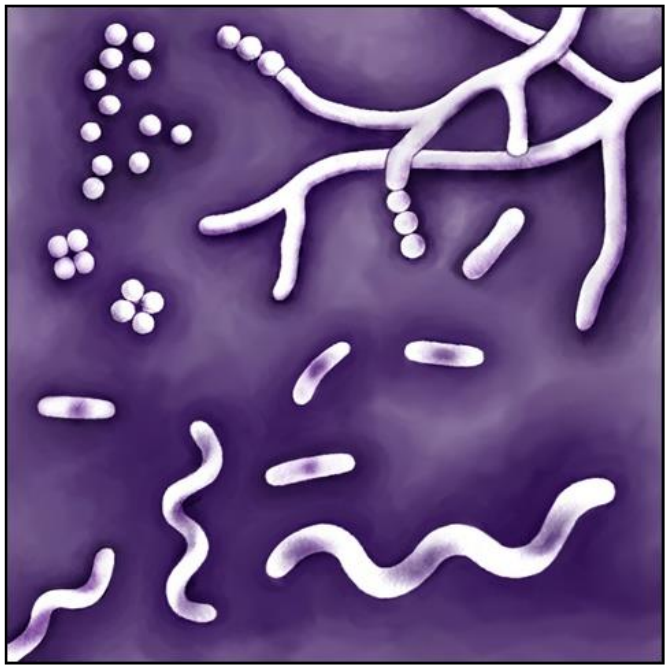
1. Simple stains use a single dye to observe cellular morphology and arrangement i.e. determine the morphology of an organism):
   - Methylene Blue is a simple stain.
2. Differential stains use multiple dyes to differentiate between two different cells, or two different parts of the same cell i.e. differentiate the organisms from one another:
   - Gram stain differentiates between cell types.
   - Capsule stain differentiates between a cell and its capsule.

Bre's Exercise 2.ppt; Week3_ Exercise2_BC.ppt

Types of staining

Negative stains

- A drawing of bacteria with a negative stain.
- The most practical use for a negative stain is to determine cell size and morphology (shape) because there is no need to heat-fix the slide.
- Heat-fixing causes the cells to shrink.

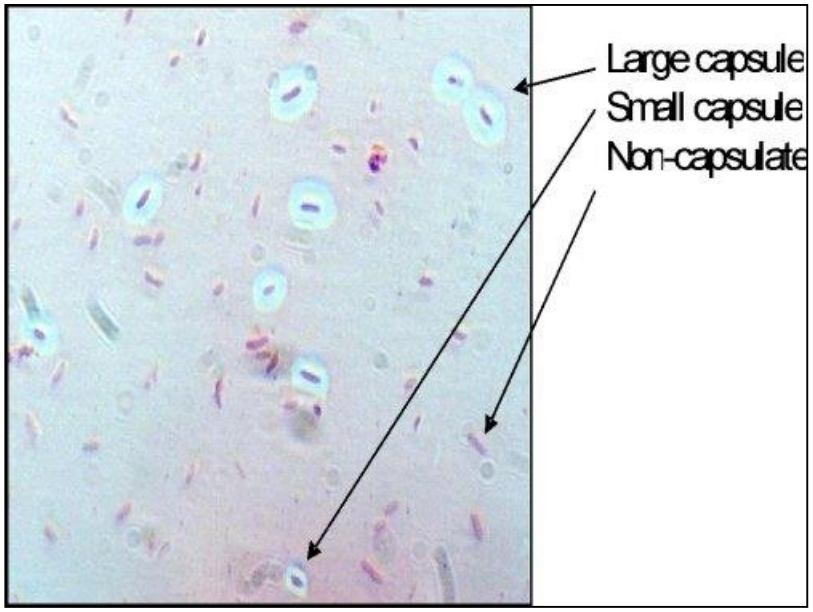


Cell shape
Light microscopic image of bacterial cells.

Capsule staining

Note colorless capsules surrounding bacterial cells

- The molecules in the capsule repel the stain and only the background and the cytoplasm are stained (A negative, differential stain).
- Capsule:
  - Protective layer around a bacterium
  - Made of glycoprotein or polypeptides
  - Impervious to stains
  - Many pathogens have a capsule.



Bacteria with capsules form smooth (S) colonies;
Those without a capsule form rough (R) colonies.

Capsule stain

Staining method of Maneval

- Procedure given by Maneval gives very satisfactory results.
- Place a drop of 1% congo red on a clean glass slide.
- Mix a loopful of bacterial suspension and spread gently over a slide. Allow it to air dry at room temperature.
- Do not heat fix the smear.
- Flood the smear with Maneval's stain and allow it to react for 1 minute.
- Wash with distilled water, air dry and examine under oil immersion objective.
- Capsules will be seen colourless against red background and bacterial cell will be stained red or reddish brown in colour.

Preparing of Maneval's stain:

Phenol(5%)	30 ml
Acetic acid (20%)	60 ml
$FeCl_3$ (30%)	4 ml
Acidic fuschin (1%)	2 ml

Types of staining

Cationic stains

Positive stains

- Cationic stains: These stains are positively charged, so they bind to the negative cell wall (generally, bacterial membranes are negatively charged).
- Any procedure that results in the staining of whole cells or cell parts is referred to as positive staining.
- Most positive stains used involve basic dyes where basic means that they owe their colored properties to a cation (positively charged molecule).
- e.g. Gram stain (See the file diagnosis II).

Pacarynuk,2006

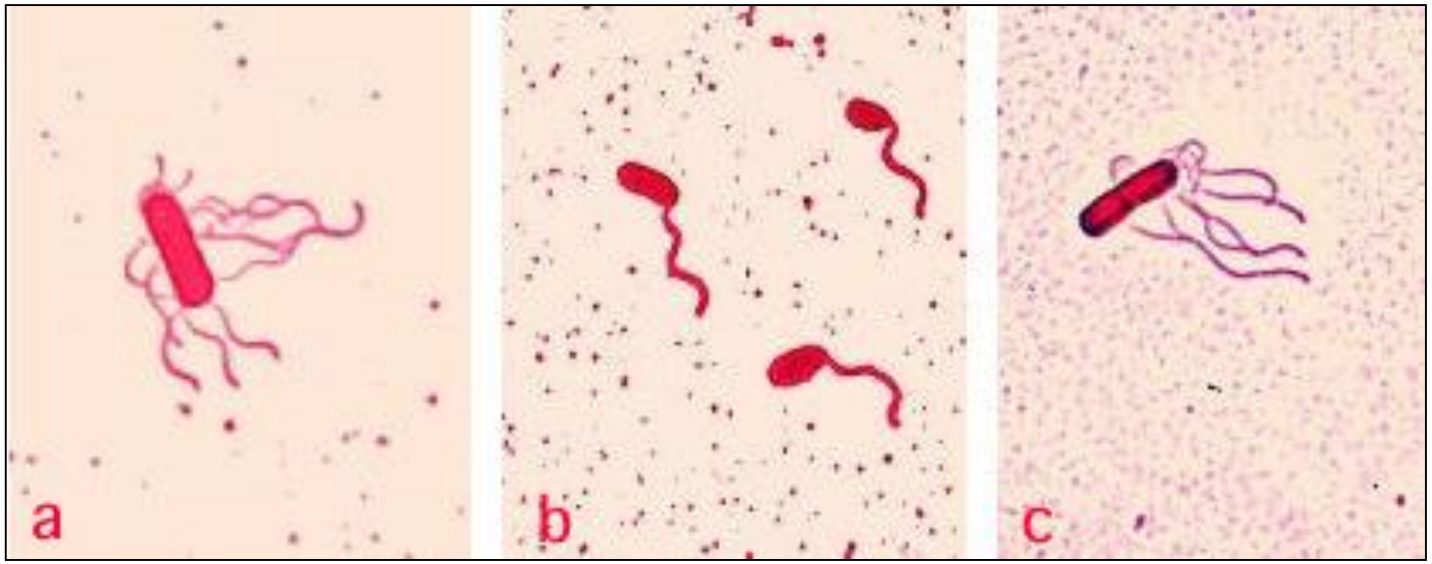
Flagella staining
Procedures

- Flagella staining is the most difficult histochemical staining and usually requires some amount of practice.
- Also note that no heat-fixation is used when staining bacteria flagella!

1. Prepare a thoroughly cleaned and well dried microscope slide;
2. With forceps and a scalpel, cut out the colony in which you detected motile bacteria;
3. Gently touch the culture side of the agar piece to a clean microscope slide;
4. Allow the imprint to air-dry. **Do not heat-fix!**
5. Flood the dried bacteria film with pararosaniline (basic fuchsin) stain until a golden film with precipitate forms. This may take up to 15 min.
6. Rinse very gently with water.
7. Air dry preparation, do not blot dry!
8. Cover dry preparation with immersion oil. Observe first with low power (20× objective) to locate a good view field.
9. Add a drop of immersion oil and swing the 100× oil immersion objective lens into the oil.

Flagella staining

Light microscopy

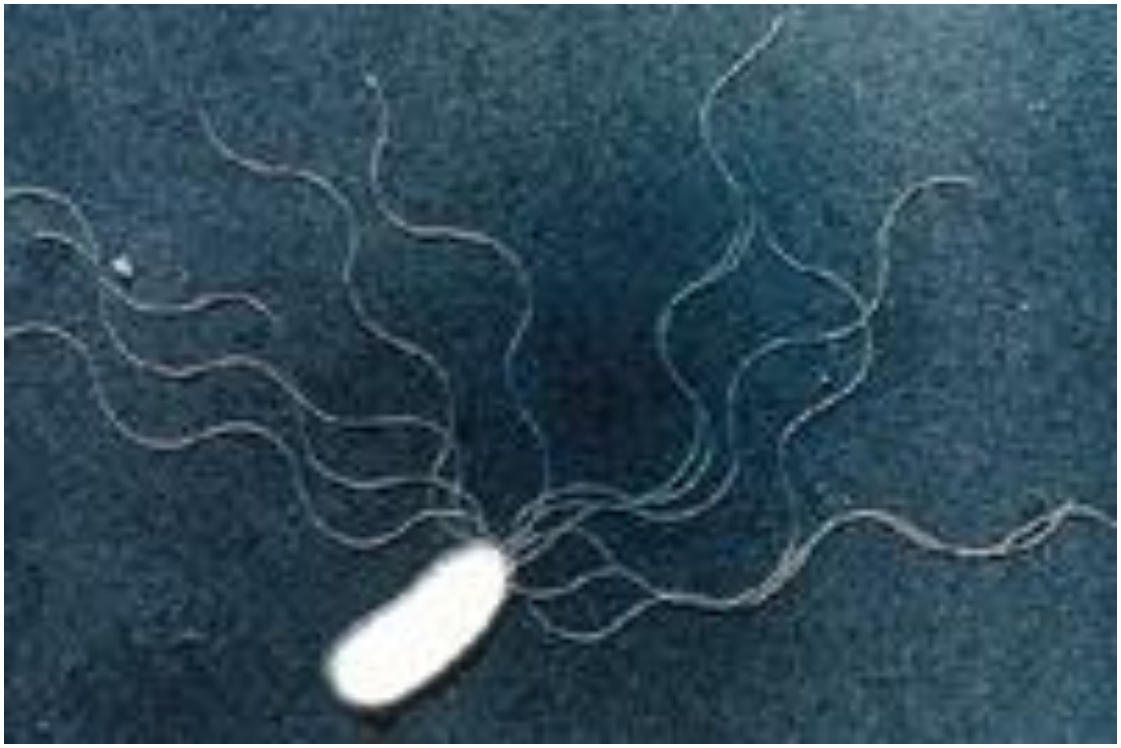


Flagella staining
Electron Microscope

- In most cases, bacteria were adsorbed to TEM grids by floating the grids Formvar side down on an undiluted drop of culture for at least 5 min.
- The bacteria on grids were stained by submerging the grids for 20 s in 0.5% (wt/vol) uranyl acetate and then were rinsed three times (10 s each) by submersion in aliquots of Milli-Q water.
- The grids were examined with a Hitachi model H-500 TEM by using an accelerating voltage of 80 to 100 kV.

Grossart *et al.*,2000

Flagella staining
Electron Microscope



Polar flagellation of *Pseudomonas marginalis* shadowed with carbon-platinum.

Poly-β-hydroxybutyric acid (PHB) staining

- PHB granules are common inclusion bodies in bacteria.
- These granules are storage depots for carbon and energy.
- Monomers of β-hydroxybutyric acid are connected by ester linkages forming long polymers which aggregate into granules.
- These granules have an affinity for fat-soluble dyes such as sudan black.

1. PHB granules easily can be stained with sudan black, and
2. Cytoplasm with safranin.

Pacarynuk,2006

PHB staining
Procedures

- Bacterial cell was screened using two different stains:

1. Sudan Black B, and
2. Nile Blue A to detect the accumulation of PHA granules in the bacteria.

Zargoun *et al.*,2015

PHB staining

1. Sudan Black B Stain

Staining of the colonies on slide:

- Bacterial smears were made on sterile glass slides, heat fixed and stained with an ethanolic solution of Sudan Black B to visualize the PHB granules.
- The prepared smears of the cultures were stained for 10 minutes with Sudan Black solution.
- The slides were then washed with running tap water and air dried.
- It was then counter stained with 0.5% Safranin for 5 minutes and washed with running water.
- Blue coloration is an indicator for the presence of lipophilic compounds.

Fathima and Krishnaswamy, 2017

PHB staining

Sudan Black B staining of colonies and cells

1. Staining of the colonies on ager media:

- The bacteria colony on the agar plate was stained with Sudan Black B to detect the presence of PHB granules. The dye solution was prepared by dissolving 0.3 g of Sudan powder in 100 ml alcohol.
- The prepared solution was spread over the plate and then left for 30 minutes. The plate was washed with ethanol (96% v/v) to remove the excess stain. Counterstain for 1 min with safranin (Juan *et al.*,1998).

2. Staining of the bacterial cells on slide:

- For more confirmation, the same of colony of bacteria was smeared onto clean slide, then stained with 0.3 g of Sudan black B in 70% (v/v) (ethanol) for 10 minutes. Subsequently, the smear was immersed with xylene to decolorize the cells, after that, 5% (w/v) Safranin water solution was used for 10 seconds as counter stain. Finally, the slide was washed with distilled water and dried before observation under an optical microscope:

1. Cytoplasm is pink,
2. Lipids are dark grey or black.

Zargoun *et al.*,2015

PHB staining
2. Nile Blue A Stain

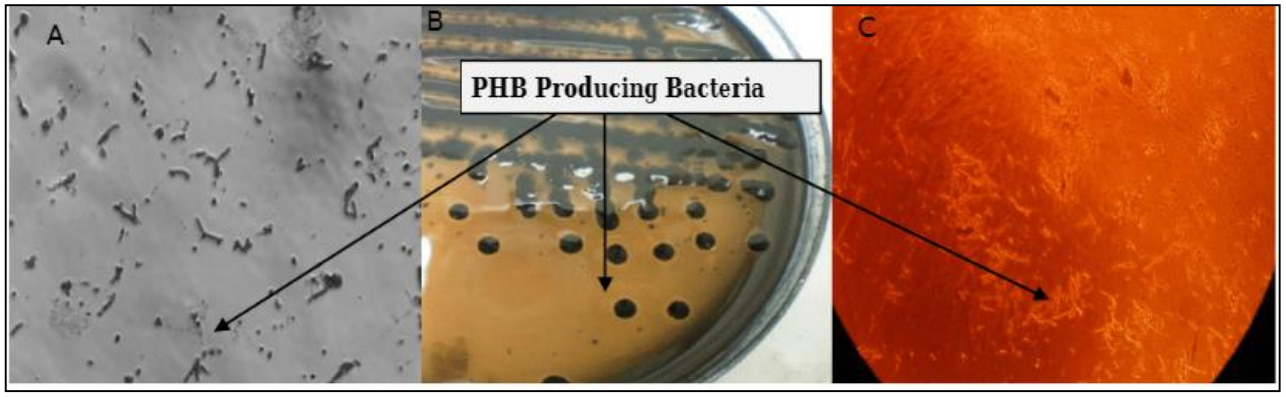

Coplin staining jar

- Nile Blue A as a sensitive dye for detecting PHB granules.
- An aqueous solution of 1% (w/v) of Nile Blue A was prepared after dissolving at 50°C, and then filtered before use.
- Bacterial smear was fixed on the slide by heating, before adding the Nile Blue A.
- The slide was inserted into a coplin staining jar filled with aqueous solution at 55°C for 10 minutes.
- Afterwards, the slide was washed with tap water to remove excess stain and then with 8 % (w/v) acetic acid for 1 minute.
- The smear was washed again with tap water and dried with bibulous paper.
- Finally, the smear was covered with a cover slip and then examined under a Nikon fluorescence microscope (Ostle and Holt,1982).

Zargoun *et al.*,2015

PHB staining
Sudan Black B Stain

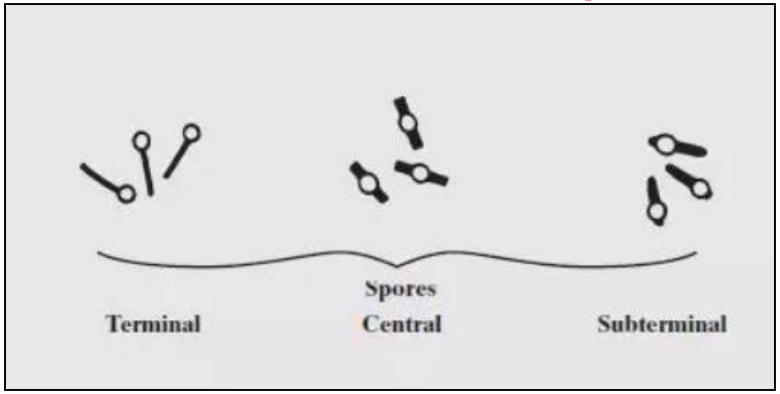
- The Figure A and B showed screening images of the PHB producing bacteria stained with Sudan Black B on the slide and agar plate, respectively.
- Figure C showed the PHB producing bacteria exhibited a yellow-orange color under a fluorescent microscope after stained with Nile blue A. The orange fluorescence of the PHB granules was investigated under UV-light.
- All the images were positive PHB producing bacterium.



Zargoun *et al.*,2015

Endospore staining

- Endospores may be formed in a central, terminal, or sub-terminal position in the cell and their shape varies from ellipsoidal to spherical.
- The location of the endospore in the cell is usually characteristic of the species.
- For example, the location and shape of the *Bacillus subtilis* endospore is different from the location and shape of the *Clostridium* endospore.



Pacarynuk,2006; Tankeshwar,2015

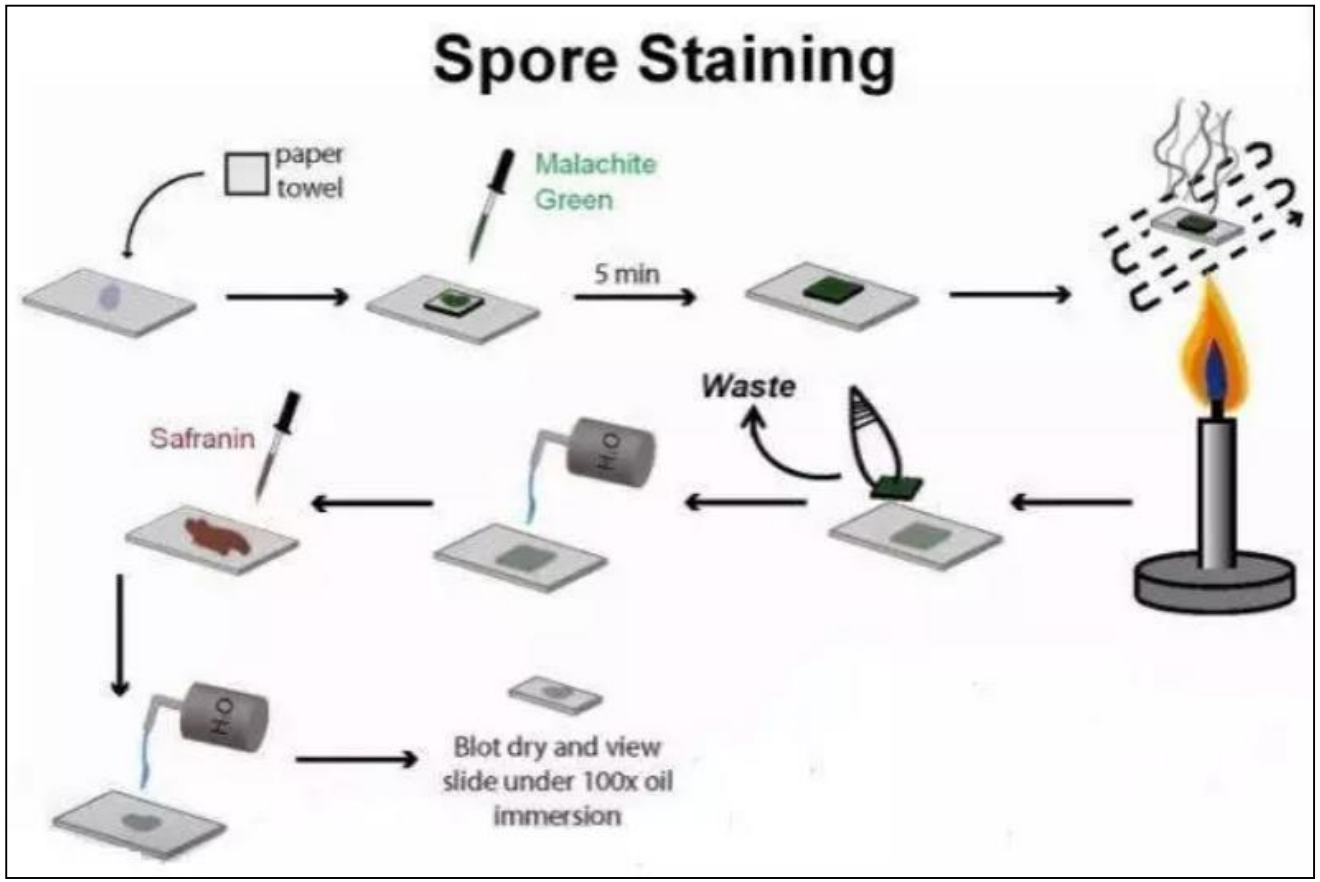
Endospore staining
Procedures

- Prepare smear and heat fix. Cover the dried fixed film with a small piece of paper towel.
- Saturate this with 5% malachite green (Dissolve 5 g of malachite green oxalate in 100 ml of distilled water).
- Pass the Bunsen burner flame under the slide until the stain steams; continue for 5 minutes.
- Add additional stain as needed (Do not allow stain to boil or completely evaporate).
- Allow the slide to cool, then rinse with water.
- Tap over a paper towel to remove excess water
- Counterstain with safranin for 30-60 seconds.
- Rinse slide with water.
- Allow to air dry, and view.

1. Endospores will stain green,
2. The rest of the cell pink.

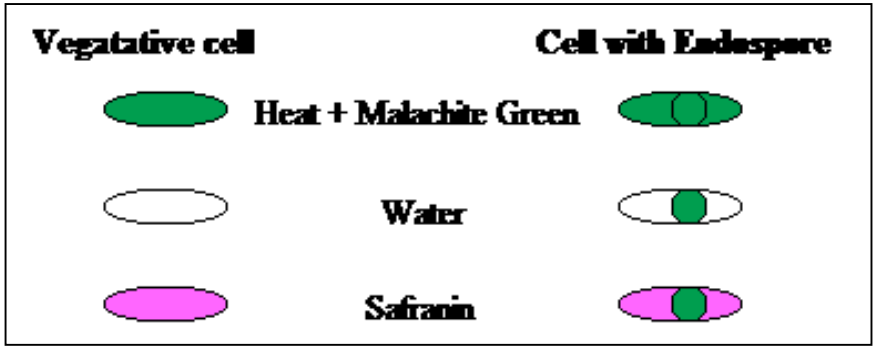
Pacarynuk,2006

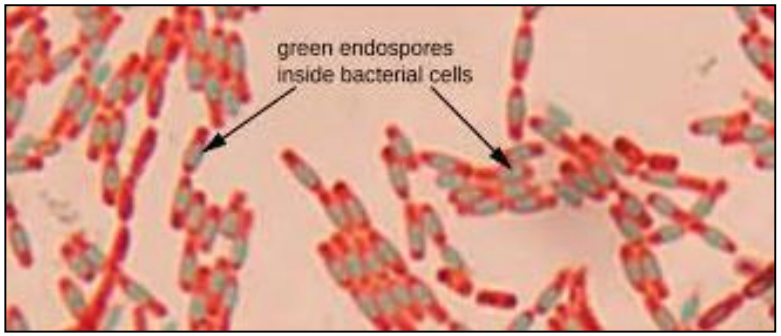
Endospore staining
Procedures



Tankeshwar,2015

Endospore staining
Procedures





Endospores will stain green, the rest of the cell pink.

Micrometry

Bacterial cell size measurement

Haemocytometer

- When studying bacteria or other microorganisms, it is often essential to evaluate the size of the organism.
- By tradition, the longest dimension (length) is generally stressed, although width is sometimes useful for identification or other study.

Pacarynuk,2006

Bacterial cell size measurement

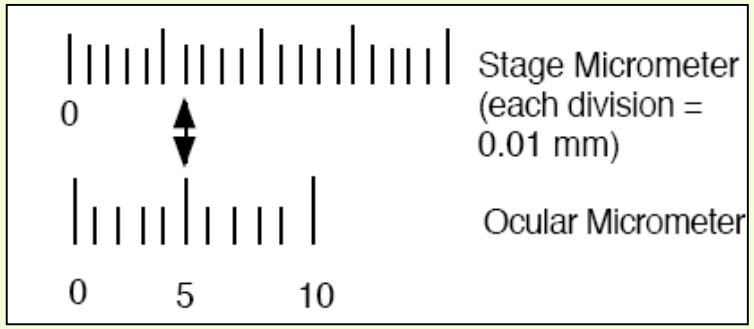
Materials

- Compound light microscope
- Various prepared slides of bacteria
- Stage micrometer
- Ocular micrometer
- Immersion oil.

Pacarynuk,2006

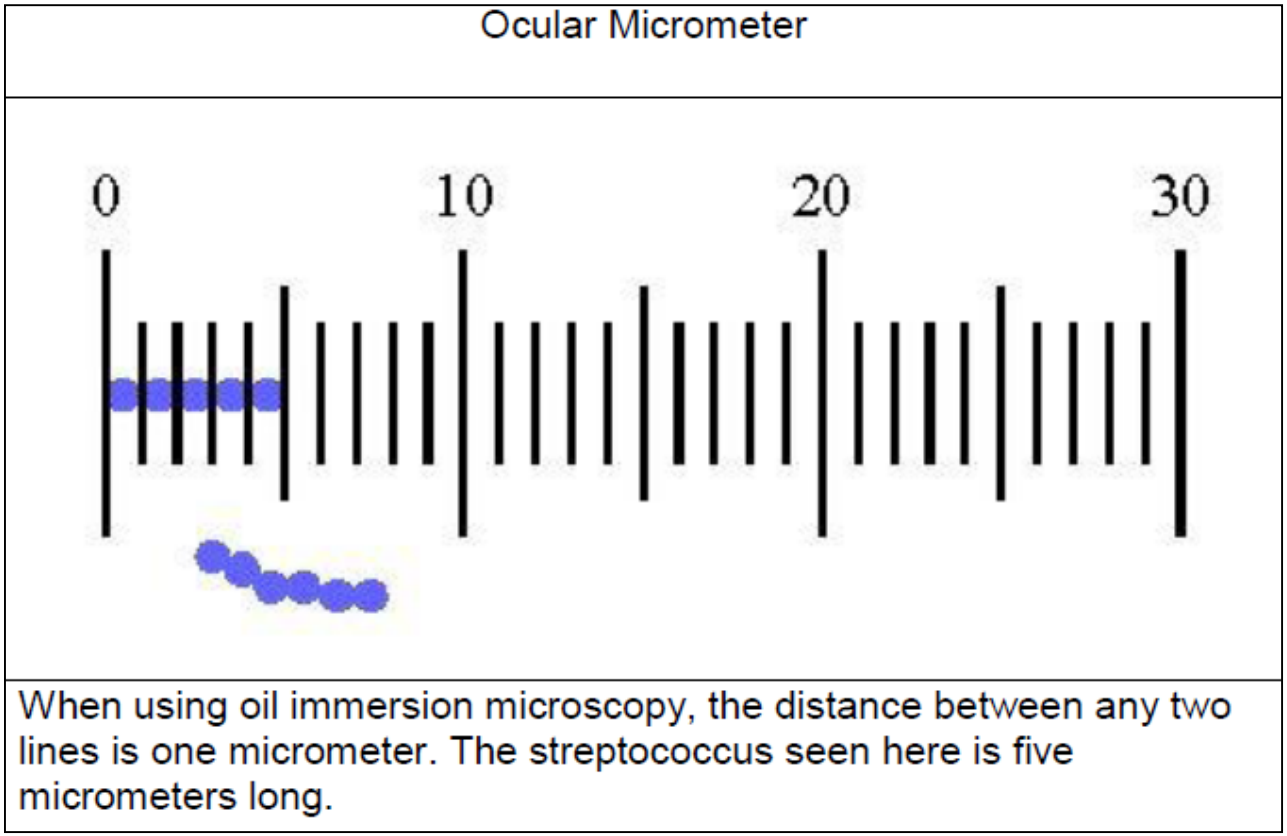
Ocular Micrometer/ Stage micrometer

- The distance between the each lines of stage micrometer is 0.01 mm (10 µm).
- The distance between any two lines in ocular micrometer is arbitrary and need to be calibrated.
- To calibrate the ocular micrometer scale, count the number of its lines required to reach between any two consecutive lines on the stage micrometer scale.
- Then place your count into the following equation.



$$\text{DISTANCE BETWEEN CALIBRATION (for ocular micrometer)} = \frac{.01 \text{ mm (dist. between lines of stage micrometer)}}{\text{NUMBER OF LINES ON OCULAR MICROMETER}}$$

Ocular Micrometer



Kaiser,2009

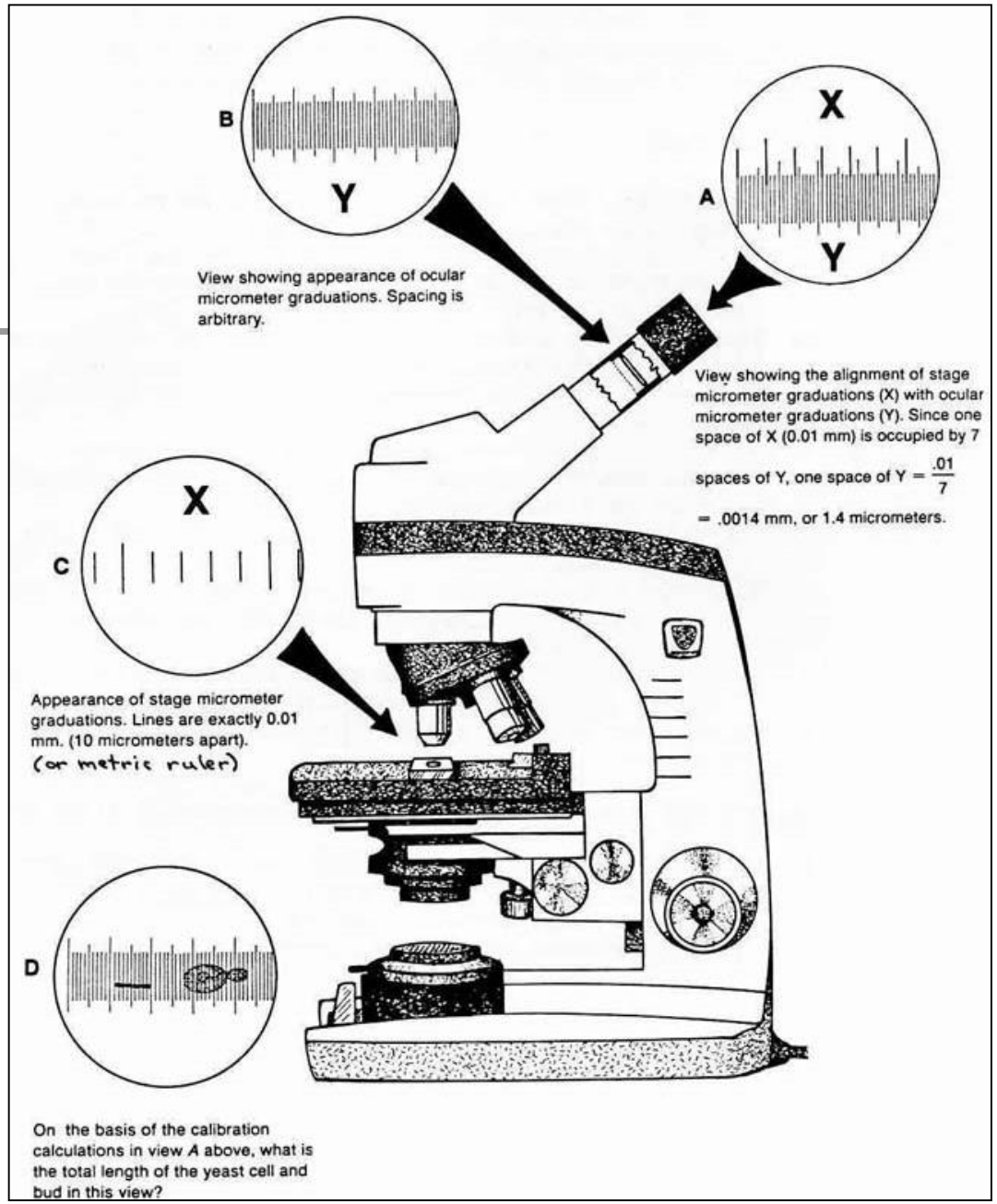
Calibration of an ocular micrometer
Procedure

1. Place a micrometer slide on the stage and focus the scale using the 40x objective.
2. Turn the eyepiece until the graduations on the ocular scale are parallel with those on the micrometer slide scale and superimpose the micrometer scale.
3. Move the micrometer slide so that the first graduation on each scale coincides.
4. Look for another graduation on the ocular scale that exactly coincides with a graduation on the micrometer scale.
5. Count the number of graduations on the ocular scale and the number of graduations on the micrometer slide scale between and including the graduations that coincide.
6. Use the compound microscope to observe the prepared slides of bacteria.
7. Calibrate the ocular divisions for the 40x and the 100x objective lenses.

- **Do not attempt to calibrate the 100X oil immersion lens**.
- Once an ocular micrometer has been calibrated, objects may be measured in ocular divisions and this number converted to µm using the conversion factor determined.

Pacarynuk,2006

Calibration of ocular micrometer



Physiological tests

Bacterial physiology and growth nutritional requirements

1. **Water:** 70 to 80% of a bacterial cell is water.
2. **Carbon and energy source.**
3. **Nitrogen:** Inorganic source e.g. ammonia and organic source e.g., amino acids (e.g., glutamate, glutamine, proline).
4. **Inorganic ions**(anions and cations)
5. **Oxygen**
6. **Other growth factors:** Many heterotrophs (particularly pathogens) require additional organic growth factors, e. g., vitamins, amino acids, purine or pyrimidine bases, heme, etc.

Physiological tests
Bacterial physical requirements

- The main physiological traits include:

1. Oxygen requirements,
2. Temperature tolerance,
3. NaCl tolerance,
4. pH range.

- Bacteria require higher water activity for growth than fungi.

Physiological traits

Oxygen requirements

ATP

- Many species of bacteria are facultative aerobes, i.e. they can grow under aerobic or anaerobic conditions, the latter ability being dependent upon the presence of some substance that can be utilized as an electron acceptor by the species concerned.

1. Some bacteria are obligate aerobes, unable to use anything but oxygen as a final electron acceptor.
2. Others are obligate anaerobes that cannot use oxygen as an electron acceptor. They use something other than oxygen as a terminal electron acceptor.
3. A few bacteria are somewhat intermediate, growing best in low oxygen tensions. These are called microaerophilic bacteria.

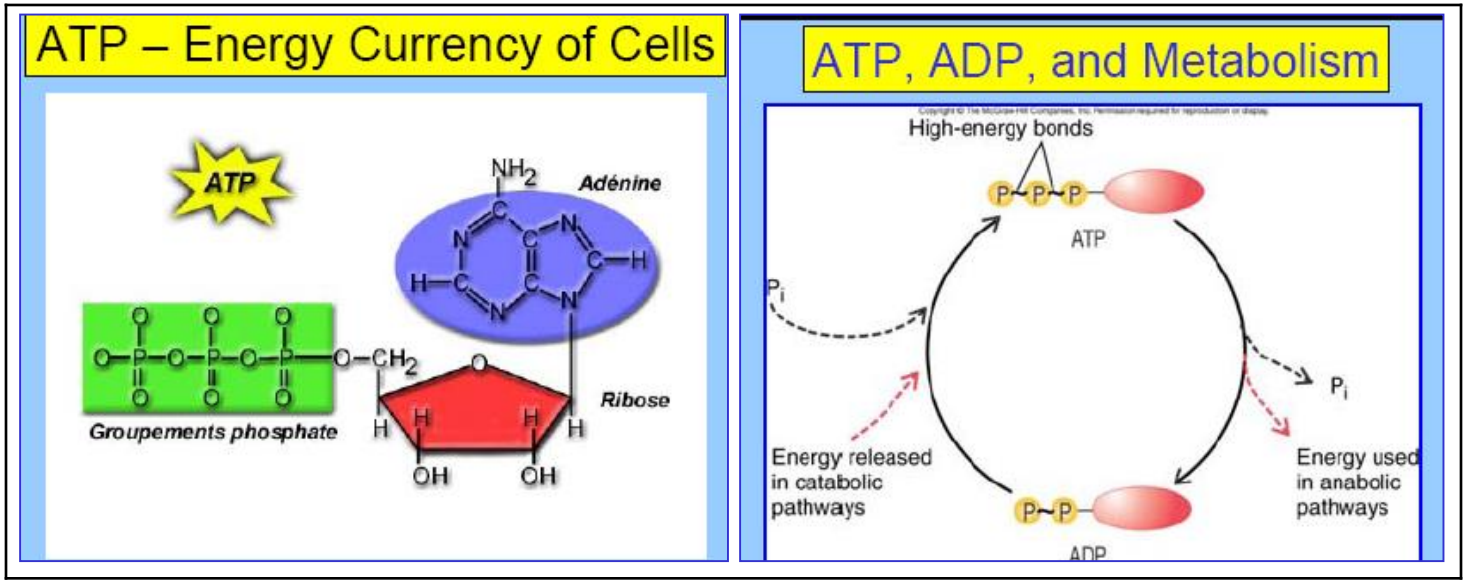
Pacarynuk,2006

ATP
All cells require energy

- Energy is conserved in the cell in the form of ATP= Adenosine Triphosphate.
- Three ways energy can be obtained:

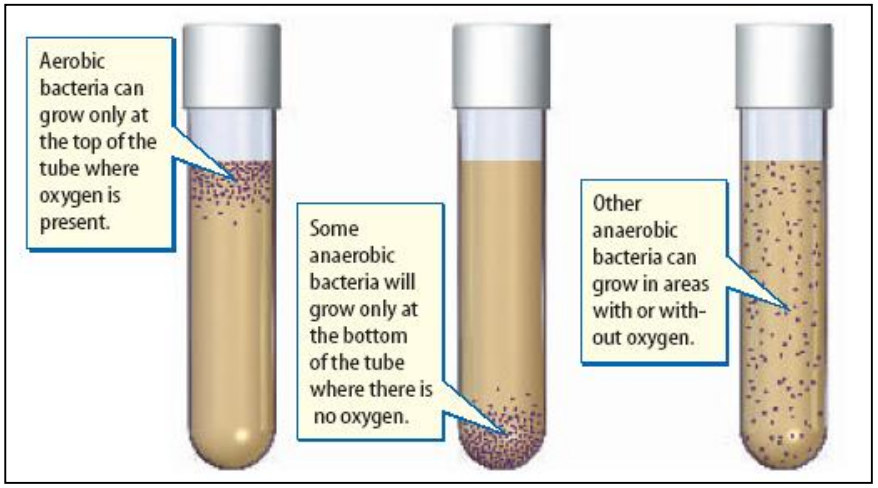
1. From organic chemicals
2. From inorganic chemicals
3. From light

- How is energy obtained? By oxidizing the compound(removing e- from).



Physiological traits
Oxygen requirements

- Bacterial oxygen range from strict aerobes to strict anaerobes.
- Most plant pathogens are aerobic,
- Some are facultative anaerobes (i.e. they can grow with or without oxygen), and
- A rare few are anaerobes.



Physiological traits

Oxygen Requirements

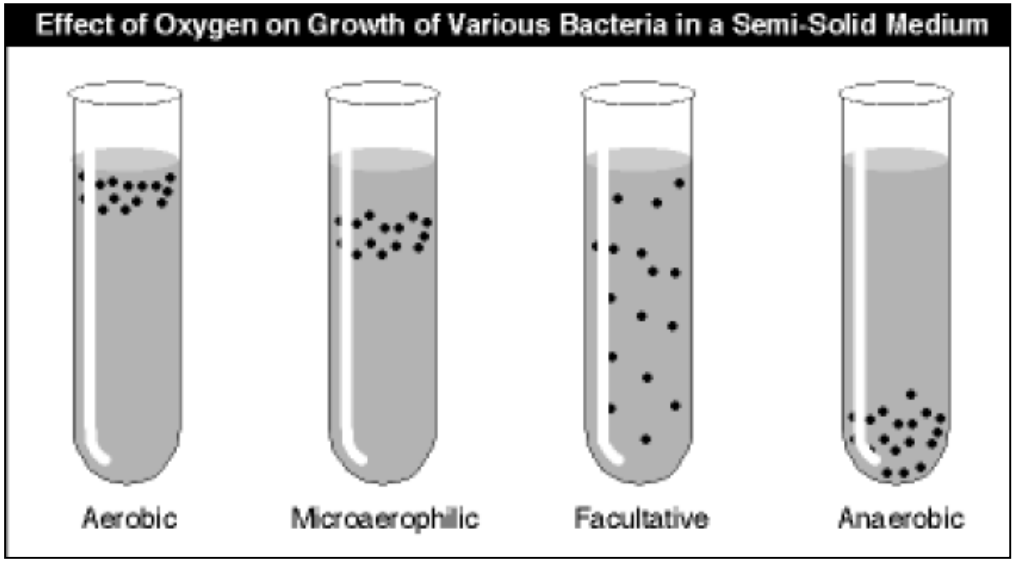
Fluid Thioglycollate Medium

- The media contains glucose, cystine, and sodium thioglycollate to lower the oxidation-reduction potential.
- The oxygen tension is high at the surface of the media (allowing the media to grow) and decreases toward the bottom of the media (for anaerobic growth). Resazurin (a dye) causes the media to turn pink in the presence of oxygen.
- Procedure:
- Boil and cool media with the screw cap loose
- Inoculate media with the organism using a wire loop. DO NOT SHAKE THE MEDIA.
- Incubate at optimum temperature for 24 hours.
- Interpretation:

1. Aerobe- Growth at the top of the media.
2. Facultative- Growth throughout the media.
3. Anaerobe- Growth at the bottom of the media.

Physiological traits
Oxygen requirements

1. Strict aerobes: Growth only at the top of the medium. e.g. *Burkholderia*.
2. Microaerophiles: Growth appear slightly underneath the surface.
3. Facultative anaerobes: Growth appear throughout the medium with growth heaviest near the top.
4. Strict anaerobes: Growth only at the very bottom of the medium.



Anaerobic chamber/jar
Obligate anaerobes

- In order to sample material containing anaerobes, specimens must be obtained and immediately placed into an environment containing an oxygen-free gas and an indicator that changes color when oxidized to indicate when oxygen has contaminated the sample.
- Organisms may then be cultured in sealed jars containing gas mixtures of N_2 and CO_2 or even by cultivation in an anaerobic chamber.
- Note that plant pathologists are reluctant to work with anaerobes.

Pacarynuk,2006;Allen *et al.*,2009

Anaerobic chamber/jar

Obligate anaerobes

- Production of vacuum:
- Incubate the cultures in a vacuum desiccator.
- Displacement of oxygen with other gases
- Displacement of oxygen with hydrogen, nitrogen, helium or CO_2.
- E.g. Candle jar.

Anaerobic chamber/jar

Obligate anaerobes

Gaspak

- Commercially available disposable envelope.
- Contains chemicals which generate H_2 and CO_2 on addition of water.
- Cold catalyst – in the envelope
- Indicator is used – reduced methylene blue.
  - Colourless – anaerobically,
  - Blue colour – on exposure to oxygen.

Physiological traits
Temperature requirements

- The terms used to describe microorganisms according to their temperature requirements are as follows:

1. Thermophiles require temperatures of 45°C-65°C.
2. Extreme thermophiles (which are usually archaebacteria) will grow at temperatures above 65°C.
3. Mesophiles grow best at temperatures of 20°C-45°C.
4. Psychrophiles require low temperatures - below 15°C.

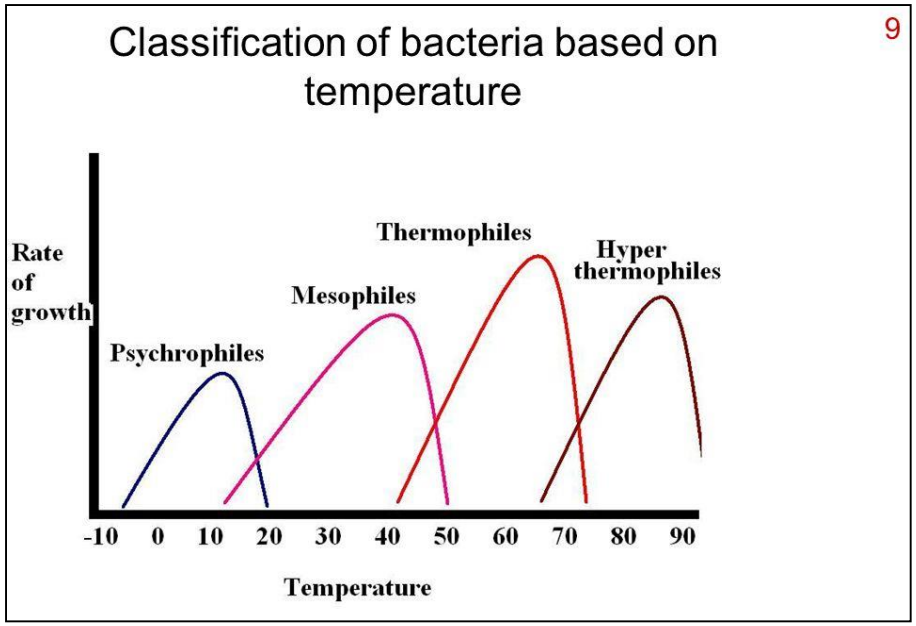
- Soil organisms and plant pathogens are normally incubated at 20-30°C.

Pacarynuk,2006

Physiological traits

Temperature requirements

All microorganisms



Group	Minimum	Optimum	Maximum	Comments
Psychrophile	Below 0	10-15	Below 20	Grow best at relatively low T
Psychrotroph	0	15-30	Above 25	Able to grow at low T but prefer moderate T
Mesophile	10-15	30-40	Below 45	Most bacteria esp. those living in association with warm-blooded animals
Thermophile	45	50-85	Above 100 (boiling)	Among all thermophiles is wide variation in optimum and maximum T

Todar,2000

Physiological traits
Temperature requirements
Plant pathogenic bacteria

- On laboratory media, plant pathogens usually grow more slowly than non-pathogenic bacteria isolated from plants, with optimal temperatures of 20-30°C.
- This makes isolation sometimes very challenging.
- Allow culture to grow several days to rule out contamination.

1. A few grow at 37°C or higher, the temperature at which human pathogens, e.g. *Burkholderia cepacia*, are able to grow.
2. Some can grow slowly at 10-12°C.

- Thermal death point (for plant pathogenic bacteria usually 50-55°C, when kept for 10 minutes at this temperature in liquid medium).

Physiological traits
Temperature requirements
Plant pathogenic bacteria

- No plant pathogenic pseudomonads can grow at 41°C and only *P. savastanoi* grows at 37°C.
- *Xanthomonas fragariae* fails to growth at 33°C.
- *Agrobacterium* spp. above 32°C inhibit pathogenicity.
- Optimal growth temperature for *Erwinia amylovora* is 27-33°C.

Physiological traits

Test temperature effects on soft rot *Pectobacterium* spp.

- Inoculate the tissue slices of whole vegetables in a refrigerator:
- At room temperature,
- At 26 degrees C,
- At 45 degrees C if incubators are available.
- The soft rot bacteria e.g. *Pectobacterium carotovorum* can grow and are active

1. over a range of temperatures from 3-35°C but,
2. are killed by extended exposure at about 50°C.

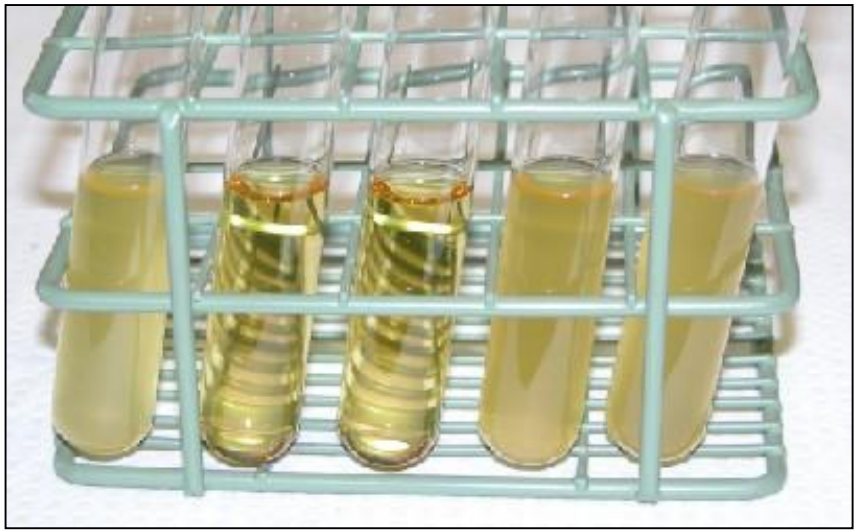
Carroll and Evans,2001

Physiological traits
Salt tolerance

- Bacteria don't tolerate well a very low ionic strength medium like water.
- Halophilic bacteria can tolerate and even require high salt, e.g., in 30% NaCl.
- Isolates were inoculated to nutrient broth with 0%, 1%, 2%, 3%, 4% and 5% NaCl concentration to evaluate their salt tolerance (Hayward,1964).
- Inoculated salt free (0%) nutrient broth was used as positive control, and
- Uninoculated broth of each salt concentration was used as negative control, and
- The presence or absence of growth was recorded.

Physiological traits
NaCl tolerance

- Touch a well-isolated colony with a sterile loop and inoculate a tube of broth/solid media containing different levels of NaCl, e.g. 2, 5 and 7%, etc.
- Incubate at 25-30^{o}C and check for growth.
- Visible turbidity is evidence of growth.



Physiological traits
NaCl tolerance

- *Rathayibacter caricis* shows weak growth with 5% (w/v) NaCl.
- *Ralstonia solanacearum* is inhibited in culture by low concentrations (2%) of sodium chloride (NaCl).
- The maximum NaCl tolerance value of *Xanthomonas fragariae* is 0.5-1.0%.

Physiological traits

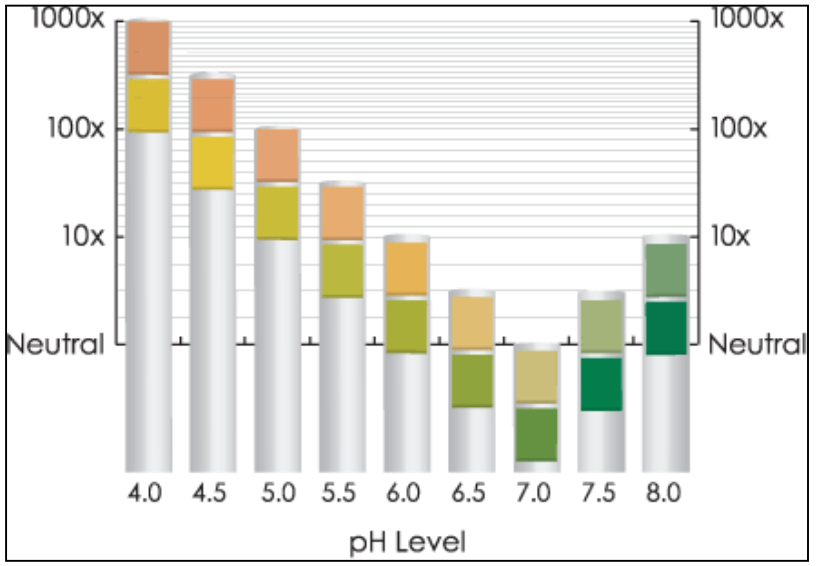
pH tolerance

Bacteria have narrower range of pH tolerance than yeasts and molds

- In general, the optimum pH value for bacterial growth is 7.0.
- But the pH tolerance may vary in different genera.
- Most bacteria prefer neutral pH (6.5-7.5).
- Molds and yeast grow in wider pH range, but prefer pH between 5 and 6.
- The pH value of the medium that tolerated by *Erwinia amylovora*, and *Xanthomonas vesicatoria* was found lower about (pH 5).

Physiological traits
pH tolerance

- *Pseudomonas* spp. and *Burkholderia* could not grow at pH value above 9.
- When the pH value was below 6, the growth of the pathogens was inhibited.
- Few bacteria are acidophiles, and survive the low pH (2-4) E.g.
- *Acetobacter aceti*, a bacterium that produces acetic acid (vinegar) from the oxidation of ethanol.
- It has an acidified cytoplasm.



Production of ammonia will raise pH.

pH strip

Heat test for spores

Alternative method for endospore staining

- In order to test whether a sample of inoculum contains endospores, the sample is exposed to high heat (80°C) for 10 minutes.
- This treatment would kill all cells but endospores would survive and could grow when incubated at an appropriate temperature.

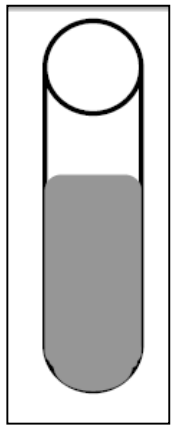
1. If endospores are present, the resultant inoculum in NB medium will give rise to a turbid culture (indicating bacterial growth);
2. If there are no endospores present, no growth is observed.

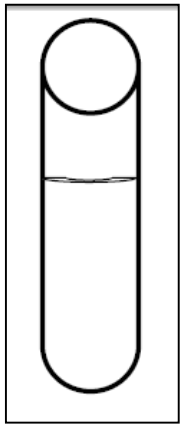
Christopher and Bruno,2003;..

Heat test for spores

Alternative method for endospore staining

- A turbid culture will form following incubation if the original inoculum contained endospores.
- Control tube: uninoculated and Negative result: No growth occurs following extreme heat exposure if the original inoculum did not contain endospores.



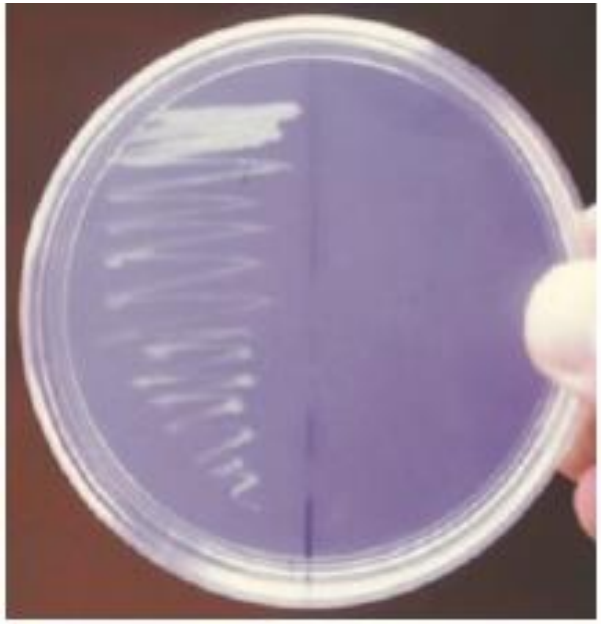


Christopher and Bruno,2003

Heat test for spores

Alternative method for endospore staining

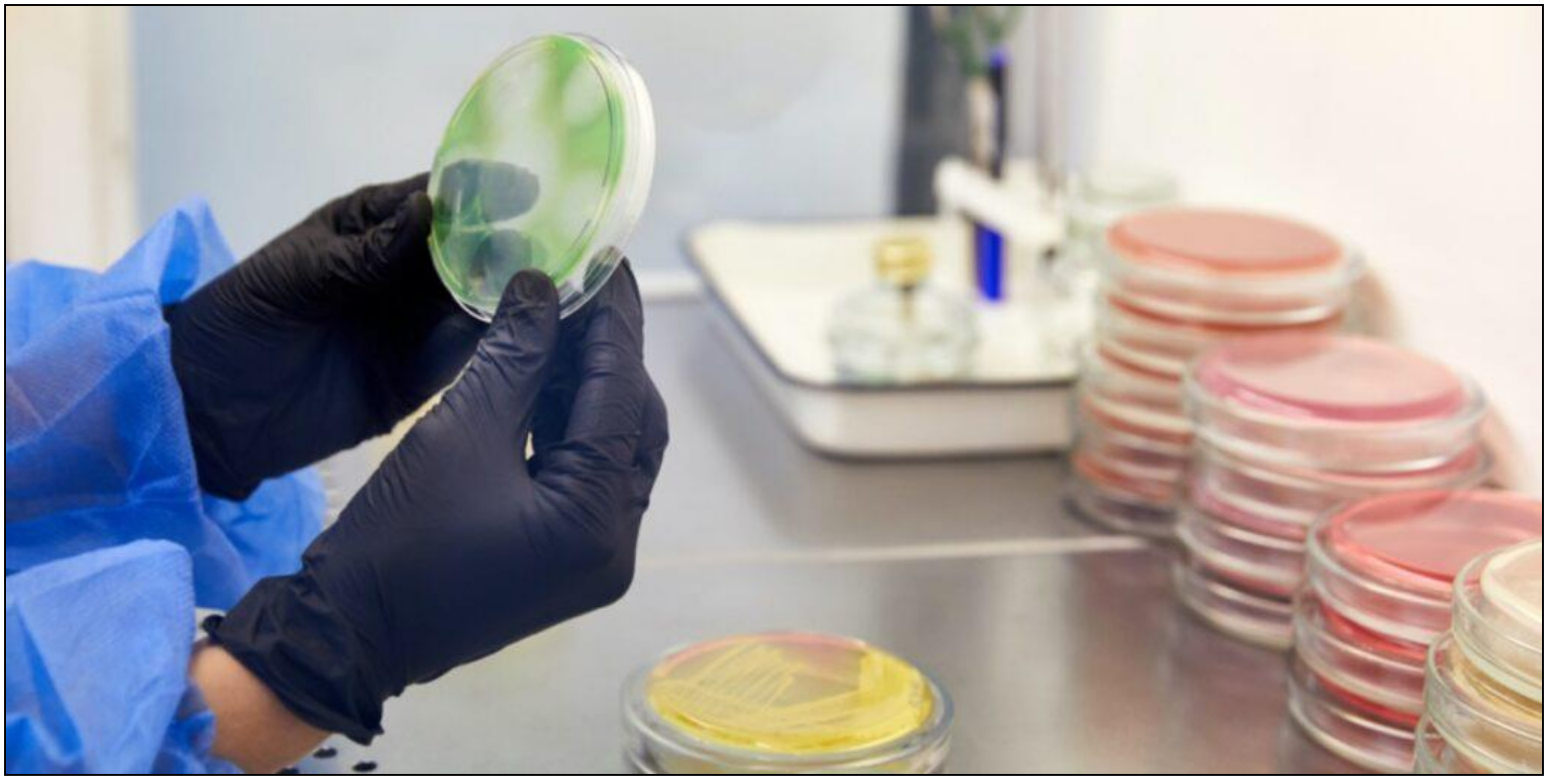
- In this test, bacterial suspensions were exposed to 80°C for 10 minutes.
- One loopful was streaked on NA medium.
- The Growth on the agar plate is limited to the area streaked with bacterial inoculum containing endospore.



See also endospore staining method

Biochemical Tests

Enzymes by functions



Bitesize Bio,2021

Identification of bacteria

Some key morphological methods

- Microscopic appearance:
- Gram staining(otherwise alternative macroscopic methods)
- Capsule staining
- Cell size and cell shape
- Motility (otherwise alternative macroscopic methods)
- Spore staining(otherwise alternative macroscopic methods)
- Macroscopic appearance:
- Colony morphology
- Physiological tests
- Biochemical tests

Identification of Bacteria

depend on:

1 – **Microscopic appearance** :-under the compound light microscope which include

a . Reaction with gram stain .
b .Morphology and arrangement .
c . Capsulated or not .
d . Motile or not .
e . Spore forming or not .

2 – **Macroscopic appearance** :include

a . Culture appearance .
b . Biochemical tests .
c . Gene tests .

Biochemical Tests
Enzymes by functions

- Many bacteria produce enzymes called hydrolases.
- Hydrolases catalyze the splitting of organic molecules into smaller molecules in the presence of water.
- Triglycerides (lipids) are hydrolyzed by the enzymes called lipases into glycerol and free fatty acid molecules.
- The hydrolysis of urea is catalyzed by specific enzyme urease to yield 2 moles of ammonia.
- Casein (milk proteins) is broken down by protease (caseinase) into peptones and amino acids.
- Use of starch is accomplished by an enzyme called alpha-amylase.
- Deaminases (e.g. indole)- Enzymes that remove amino groups from individual amino acids.
- Nitrate reductase: Reduction of nitrate (NO_3) to nitrite (NO_2).

Biochemical Tests

Enzymes by functions

- Oxidase test
- Arginine dihydrolase test
- Gelatin hydrolysis test
- Casein hydrolysis
- Fat hydrolysis
- Starch hydrolysis test
- Lecithinase test

Oxidase (Kovacs test)

Used to determine the presence of oxidase enzyme

A respiratory system similar to that of mitochondria

1. This enzyme forms part of the bacterial electron transport chain.
2. Bacteria that test positive for oxidase test are aerobic.
3. They can make use of oxygen in respiration. i.e. respiratory system is present in aerobic bacteria similar to that of mitochondria, which are capable to use oxygen as the final hydrogen acceptor.
4. On the other hand, bacteria that test negative for oxidase test can be aerobic, anaerobic, or facultative.
5. Respiratory system is present in aerobic bacteria which are capable to use oxygen as the final hydrogen acceptor.
6. The enzyme cytochrome c oxidase is the last enzyme in the respiratory electron transport chain of cells located in the membrane.

Oxidase(Kovacs test)

Used to determine the presence of oxidase enzyme

A respiratory system similar to that of mitochondria

OXIDASE TEST

- **Oxidase:** an enzyme of the bacterial electron transport chain.
- When present, the cytochrome c oxidase oxidizes the reagent (tetramethyl-p-phenylenediamine) to (indophenols) purple color end product.
- When the enzyme is absent, the reagent remains reduced and is colorless.
- **Principle:**

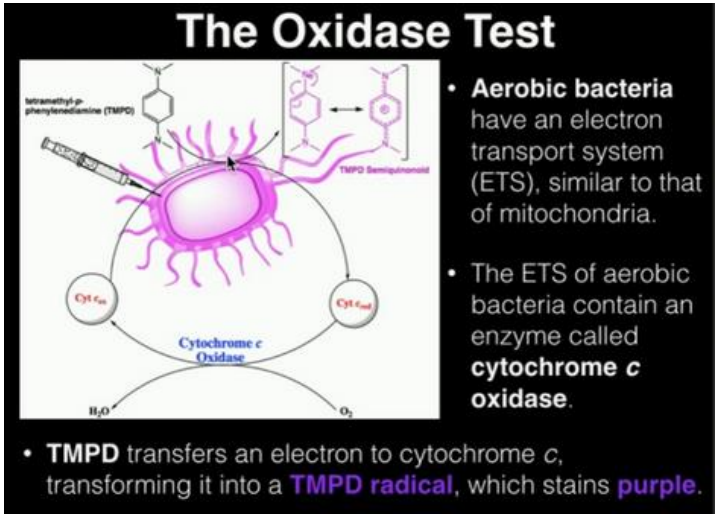
2 oxidized cytochrome C + p-aminodimethylalanine oxalate ⟶ **2 reduced cytochrome C + Wurster's blue (purple color)**

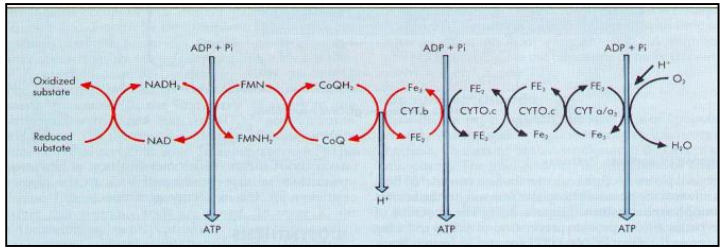
Oxidase (Kovacs test)

To identify bacteria containing the respiratory enzyme Cytochrome C Oxidase

A respiratory system similar to that of mitochondria

- Aerobic organisms obtain their energy by respiration, which is responsible for the oxidation of various substrates through the cytochrome oxidase systems (ETC).
- The ETS of aerobic bacteria contain an enzyme calles cytochrome C oxidase.
- This enzyme plays a key role in electron transport chain during aerobic respiration, similar to that of mitochonderia.





Oxidase (Kovacs test)

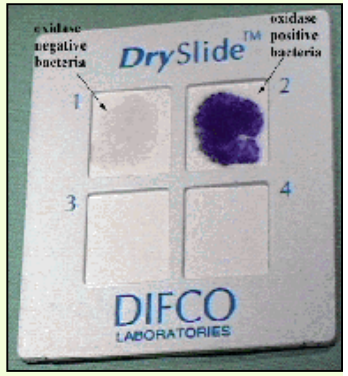
Used to determine the presence of oxidase enzyme

Three different methods

- Obligate aerobes have this enzyme.
- There are several ways to perform oxidase test:

1. Filter paper method
2. Using a swab, or
3. Adding the reactive directly on plate.

- For all methods a blue color appears within 1 minute if reaction is positive.



$$4\,Fe^{2+}\text{-cytochrome } c_{red} + 8H^{+}_{in} + O_2 \rightarrow 4\,Fe^{3+}\text{-cytochrome } c_{ox} + 2H_2O + 4H^{+}_{out}$$

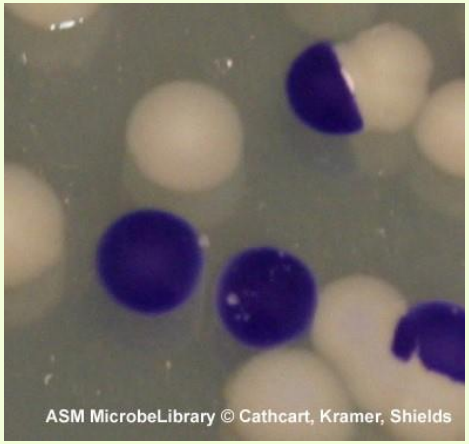
Oxidase (Kovacs test)

Used to determine the presence of oxidase enzyme

Direct and swab methods

1. **Direct method:**

- Make an isolation streak of the organism on the TSA (tryptic soy agar plate) plate
- Incubate at optimum temperature for 24-48 hours
- Add several drops of oxidase test reagent directly to organism.
- Observe for color changes.



http://www.microbelibrary.org

Oxidase (Kovacs test)

Used to determine the presence of oxidase enzyme

Oxidase test reagent and result interpretation

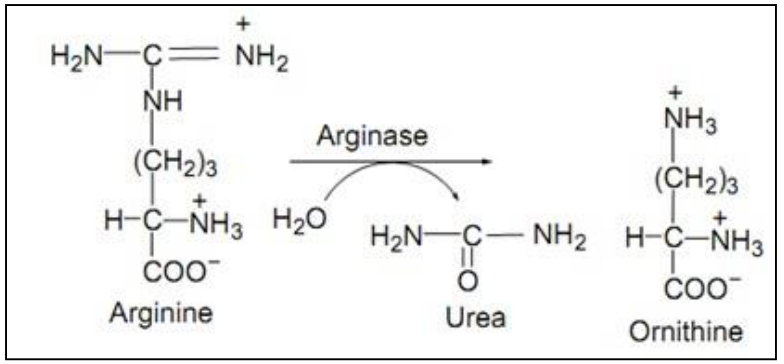
- Oxidase test reagent: Soak a piece of filter paper in 1% Kovacs reagent (N,N,N',N' tetra-methyl-p-phenylenediamine dihydrochoride) fresh solution.
- Store refrigerated in a dark bottle no longer than 1 week
- Results:

1. Microorganisms are oxidase positive when the color changes to dark purple within 5 to 10 seconds.
2. Microorganisms are delayed oxidase positive when the color changes to purple within 60 to 90 seconds.
3. Microorganisms are oxidase negative if the color does not change or it takes longer than 2 minutes.

Arginine dihydrolase test

Microbe can use the amino acid arginine as a source of carbon and energy for growth

- Arginine is first converted to citrulline via dihydrolase reaction, in which NH_2 group is removed from agrinine.
- Citrulline in next converted to ornithine.
- Ornithine then undergoes decarboxylation(carboxyl group was removed and carbon dioxide (CO_2) was releases.
- This elevates the pH and the pH indicator bromo cresol purple gives purple color in the medium (alkaline condition).



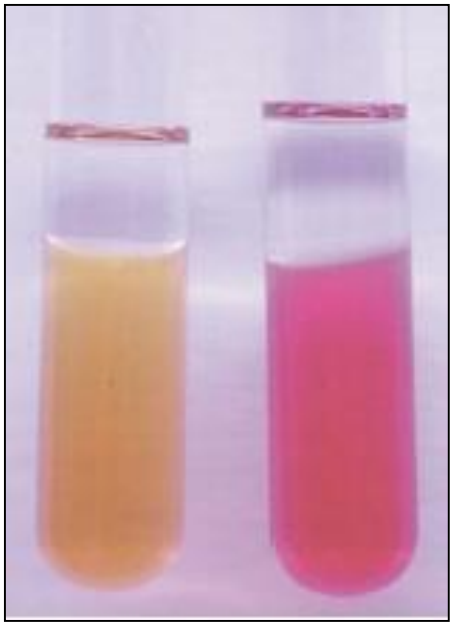
Pumpkin seeds have one of the highest concentrations of arginine. Other seeds with a high amount of arginine include watermelon, sesame, and sunflower seeds.

Arginine dihydrolase test

Microbe can use the amino acid arginine as a source of carbon and energy for growth

- In the arginine dihydrolase test, a positive reaction is characterized by the appearance of:

1. A reddish purple color after stab inoculation (right).
2. Controls are pink (left).



Ingredients gr/Litre
L-Arginine, monohydrochloride 5/L, Yeast extract 3, Glucose 1, Bromocresol purple 0.015, agar 3 g. Final pH (at 25°C) 6.8±0.2.

Directions
Distribute in 13 x 100mm tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Allow the tubes to cool in an upright position. Arginine reaction is strictly anaerobic; therefore the broth tubes after inoculation must be overlayed with mineral oil.

Indole test

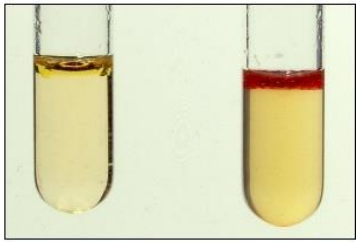
Tryptophan hydrolysis test

- Indole, a bacterial product of tryptophan degradation.
- Medium composed of tryptone, 10 g; $CaCl_2$, 0.03M; NaCl, 5 g in 1 liter of distilled water.
- Each isolate was inoculated into the tryptone broth and incubated at 28^{o} C.
- After 2 and 5 days of incubation, 0.5 ml of Kovak's Reagent (P-dimethyl amino benzaldehyde 5 g, Butanol 75 ml, and concentrated HCl 25 ml) was added into each tube and shook gently.
- A dark red color in the surface layer was taken as positive for indole production (Aneja, 1996).

L-Tryptophan is an essential amino acid producing indole, skatole (methyl indole) and indole acetate. Dissolve while warming water. It should be sterilized through a sterile filter, not autoclaved, and is stored at 4°C.

Indole test

Tryptophan hydrolysis test

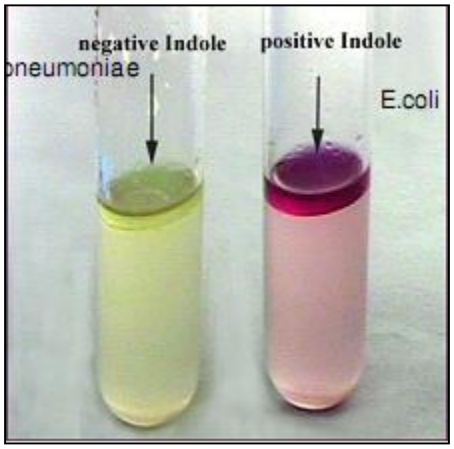


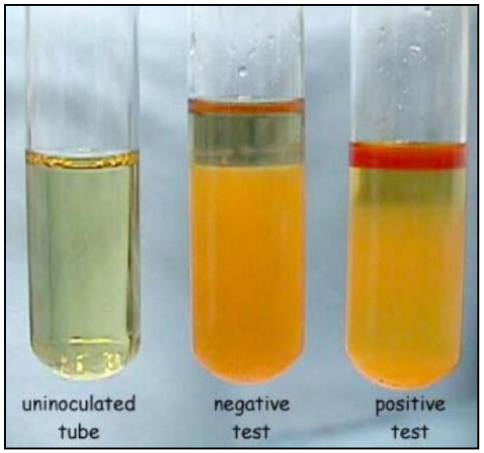
VetBact

- If the bacterium produces the tryptophanase enzyme to break down tryptophan into molecules of indole, pyruvic acid, and ammonia, the Kovac's reagent will turn red, indicating the organism is indole-positive.

tryptophanase
tryptophan ----------> indole + pyruvic acid + ammonia

- If the Kovac's reagent remains yellow, no indole was produced and the organism is indole-negative.





Sridhar Rao;Wetts,2001;..

MR-VP test

Methyl red/Voges Proskauer test

- MR-VP medium is two tests in one:

1. the Methyl Red Test, and
2. the Voges-Proskauer test.

- MR-VP medium was composed of peptone, 5 g; glucose 5 g and K_2HPO_4, 5 g in 1 liter of distilled water.
- pH was adjusted 6.9.
- Dispense 3 ml of the medium into each tube, sterilized at 121°C.

MR-VP test

Methyl red/Voges Proskauer test

- **Methyl Red Test:**
- MR positive bacteria ferment the glucose in the media to produce a mixture of fermentation acids – lactic, acetic, and formic acids.
- The acids produced by *E. coli* are strong enough to overcome the buffering capacity of the phosphate buffer in the media, thus lowering the pH.
- A red color indicates a positive MR reaction, indicating a pH of 4.4 or lower.

Cain *et al*., 2012

MR-VP test
Methyl red/Voges Proskauer test

- **Voges-Proskauer Test:**
- Some bacteria ferment glucose, but only produce one acid end-product, usually acetic acid.
- The acetic acid produced initially lowers the pH of the media, but is quickly converted to acetylmethylcarbinol, which leads to a pH of approximately 6.2.
- The chemicals in VP reagent will react with acetylmethylcarbinol, and a positive reaction will show a dark red band at the top of the broth in the tube which will diffuse over time into the rest of the media.
- NOTE: An organism will NEVER be positive for both the MR and VP tests!!

Cain *et al.*, 2012

MR-VP test

Methyl red/Voges Proskauer test

- **Methyl Red Test:**
- Some bacteria produce large amounts of various acids (lactic, acetic, succinic, formic) plus H_2 and CO_2. The large amounts of acids lower the pH to lower than 5.0.
- Methyl red test determines whether acidic fermentation products result from growth.
- **Voges-Proskauer Test:**
- Some bacteria produce acetoin. Acetoin is a precursor to 2,3 butanediol.
- This test determines whether the microbe produces 2,3 butanediol as a fermentation product from glucose.

MR-VP test

Methyl red/Voges Proskauer test

- Inoculate two MR-VP broths with the organism.
- Incubate at optimal temperature for 3-5 days
- Add 3-4 drops of Methyl Red reagent to one tube
- Interpretation for methyl red test:
- Positive- Red color develops
- Negative- Yellow color develops
- Pipette 1 ml of culture from the other MR-VP tube into a small screw cap test tube.
- To the extracted 1 ml of culture, add 18 drops of Barritt's Solution A (alphanapthol) and 18 drops of Barritt's Solution B (KOH).
- Agitate vigorously for 1-2 minutes. Let stand for 1-2 hours (Min. 25 minutes).
- Interpretation for VP test:
- Positive- Wine red (burgundy) color develops
- Negative- Brown color develops. May diffuse over time into the rest of the medium.

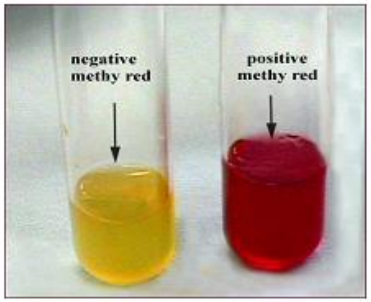
MR-VP test

Methyl red/Voges Proskauer test

Methyl red:

Glucose oxidation

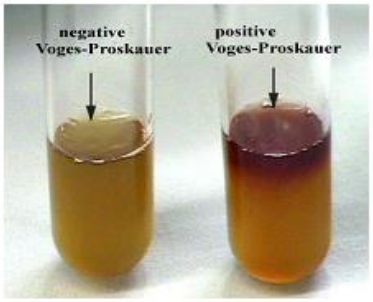
- It tests the ability of organism to produce and maintain strong, mixed acid from buffered-dextrose peptone broth
- *E. coli* is one of the bacteria that produces acids, causing the pH to drop below 4.4.
- When the pH indicator methyl red is added to this acidic broth it will be cherry red (a positive MR test)



Vogues Proskauer

Production of neutral end products

- *Klebsiella* and *Enterobacter* produce more neutral products from glucose (acetoin)
- pH rises above 6.2.
- The reagents: alpha-napthol and potassium hydroxide.
- If acetoin is present reagents turn a pink-burgundy color (a positive VP test).
- color may take 20 to 30 minutes to develop.



VP intrepretation test:

Positive: Pinkish red color at the surface of the medium.

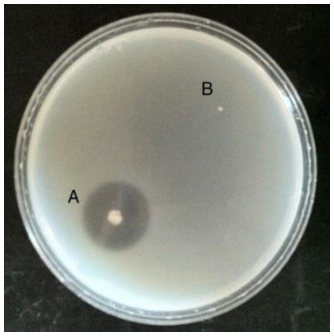
Negative : Yellow color at the surface of the medium

Wetts,2011; R.Kavitha

Gelatin hydrolysis test

Gelatinase

The plate method



- In this method, a heavy inoculum of an 18-24 hour old test bacteria is stab-inoculated onto culture plates pre-filled with Nutrient Gelatin (23 g/L Nutrient Agar+0.4% gelatin).
- Inoculated Nutrient Gelatin plates are incubated at 35°C for 24 hours.
- Gelatin hydrolysis is indicated by clear zones around gelatinase-positive colonies (A).
- In some cases, plates are flooded with saturated ammonium sulphate or mercuric chloride ($HgCl_2$) on agar plates.
- $HgCl_2$-HCl solution (D.H_2O, 80ml, Conc. HCl, 16ml, $HgCl_2$,12 g) making the clear zones easier to see.

ASM MicrobeLibrary;..

Gelatin hydrolysis test

Gelatinase

Tube test method

- The medium contain peptone 5.0 g, beef extract 3.0 g, gelatin 120.0 g/liter. Final pH: 6.8 ± 0.2 at 25°C.
- Partial or total liquefaction of gelatin in the test tubes indicates a positive result.
- Complete solidification of gelatin at 4°C represents a negative result.

Casein hydrolysis

Milk agar medium

Caseinase

- Caseins are the major family of proteins in milk.
- These proteins are commonly found in mammalian milk, comprising c. 80% of the proteins in cow's milk and between 20% and 60% of the proteins in human milk
- Casein is a large protein responsible for the white color of milk.

Márta *et al.*,2011;..

Casein hydrolysis

Milk agar medium

Caseinase

- Skim milk powder 28.0 g, tryptone 5.0 g, yeast extract 2.50 g, dextrose (glucose) 1.0 g, agar 15.0 g/L. Final pH (at 25°C)
- Clear zone around the growth of the isolates was recorded as positive for casein hydrolysis.

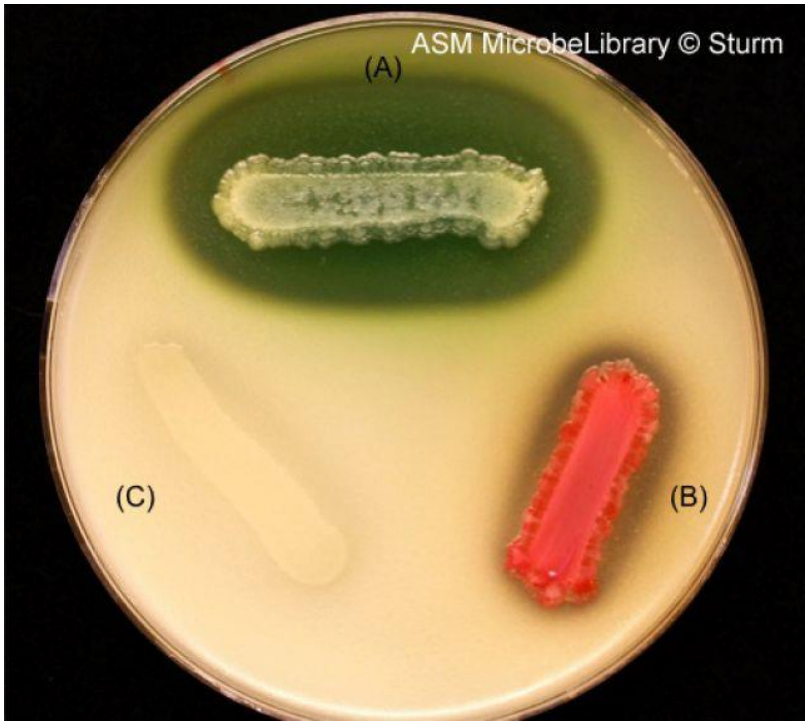
microbiologyinfo.com

Casein hydrolysis test

Alternative medium

Caseinase

- Hold the plate up to the light to see the zones.
- Positive- A zone of clearing occurs along the streak line
- Negative- No zone of clearing.
- There is no reagent or indicator in the agar.



To see clear zones, flood the plates with $HgCl_2$-HCl solution (D.H_2O, 80ml, Conc. HCl, 16ml, $HgCl_2$,12 g).

Two different casease positive organisms: (A and B) and a casease negative organism (C). Note: compare results with the Litmus milk test.

Litmus milk test

Litmus milk is a complex medium that can produce a variety of results

- Used to differentiate organisms in skim milk agar according to metabolic properties:

1. Lactose fermentation
2. Reduction of litmus
3. Clot formation
4. Peptonization (digestion).

- The addition of litmus, other than explaining the pH type, acts as an oxidation-reduction indicator.

1. In un-inoculated milk, litmus will be a purple/blue (pH 6.8).
2. In acidic solution (pH 4.5) litmus will be pink, and
3. In alkaline solution (pH 8.3) litmus will be blue.

Litmus milk test

Litmus Milk Tube

1. Lactose fermentation:

- If the organism can ferment lactose, an acidic condition occurs and the media will be pink.

Lactose → Glucose + Galactose
Glucose → Pyruvic Acid → either Lactic acid, Butyric acid, or $CO_2 + H_2$

- If the organism cannot ferment lactose, it may act on nitrogenous substances in the milk to release ammonia and the media will be blue.

Litmus milk test
Litmus milk tube

2. Reduction of litmus:

- Litmus is used as an E_h indicator.
- An organism capable of reducing litmus will cause the media to turn white.

3. Clot formation:

- Proteolytic enzymes (rennin, pepsin, or chymotrypsin) cause the hydrolysis of milk proteins, which result in the coagulation of milk.

4. Peptonization (digestion):

- Hydrolysis of casein by caseinase causes the casenogen precipitate (clot) to be converted to a clear liquid.

Oxidation can occur either by addition of oxygen or removal of hydrogen ion. Litmus acts as the acceptor of the hydrogen ion. Litmus is purple in the oxidised state, but when it accepts hydrogen, it becomes reduced and turns white or milk-coloured.

Litmus milk test

Procedure

1. The ingredients of litmus milk broth medium or its ready-made powder required for 100 ml of the broth is weighed and dissolved in 100 ml of distilled water in a 250 ml conical flask by shaking and swirling.
2. Its pH is determined using a pH paper or pH meter and adjusted to 6.8 using 0.1N HCI if it is more or using 0.1N NaOH if it is less. The flask is heated, if required, to dissolve the ingredients completely.
3. The broth is distributed into five test tubes (approximately 10 ml each), cotton-plugged, covered with craft paper and tied with thread or rubber band.
4. The broth tubes are sterilised at 121°C for 15 minutes in an autoclave.
5. The broth tubes are allowed to cool to room temperature.
6. The test bacteria is inoculated aseptically, preferably in a laminar flow chamber, into the broth with the help of an inoculating loop sterilised over bunsen flame. The loop is sterilised after each inoculation.
7. The inoculated broth tubes are incubated at 37°C for 24 to 48 hours (Max. 1 week) in an incubator.

Litmus milk test

Litmus milk is a complex medium that can produce a variety of results

- Interpretation 1:

1. Lactose fermentation: Colour of the broth changes to pink.
2. Gas formation: Broth becomes solid curd with fissures in it.
3. Litmus reduction: Colour of the broth changes to white with purple ring at top.
4. Curd formation: Broth becomes white solid curd with purple ring at top.
5. Proteolysis: Broth becomes whey-like brownish translucent with deep purple ring at top:
6. Alkaline reaction: Broth colour remains unchanged or changes to deep blue.

Litmus milk test

Litmus milk is a complex medium that can produce a variety of results

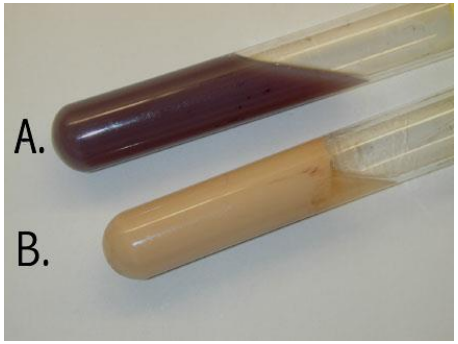
- Interpretation 2:
- Your result will be one or more of the following:

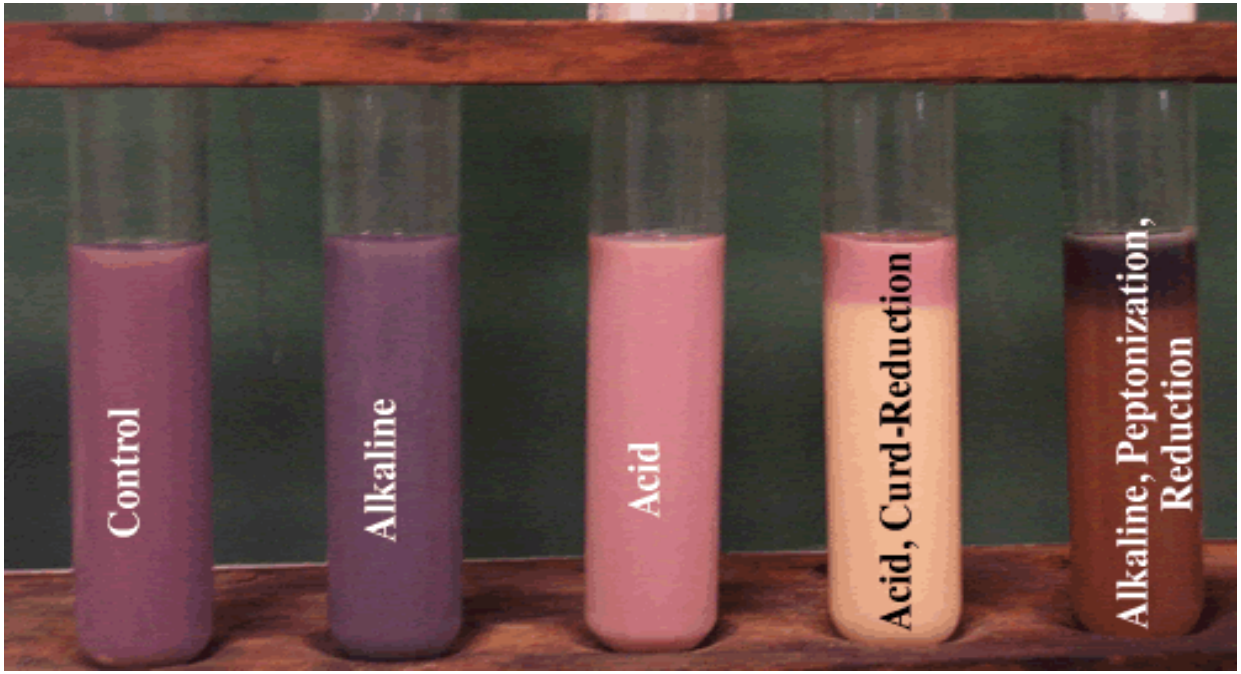
1. Pink: Acid reaction, lactose fermented (verify with carbohydrate fermentation test if possible);
2. Purple/blue: No fermentation of lactose;
3. Blue: Alkaline reaction, no fermentation of lactose; organism attacks nitrogenous substances;
4. White: Reduction of litmus;
5. Clearing of the media: peptonization (casien digestion);
6. Clot/Curd: Milk protein coagulation.

Litmus milk test

Litmus milk tube

A. Represents a tube that produced a curd and reduced litmus.
B. Represents an uninoculated control.





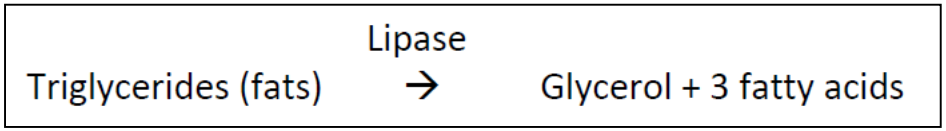
Fat hydrolysis test

Lipase

Lipolytic activity

- **Media with Tween 80**
- Cultures were grown on nutrient medium (heart infusion agar or nutrient agar) and minimal medium (Difco Bacto agar) supplemented with 1% Tween 80 (a stearic acid) and 0.01% $CaCl_2$.
- An opaque zone with crystals (Tween 80) or a clear zone (triglyceride tributyrin) around the colonies shows esterase (lipolytic) activity.

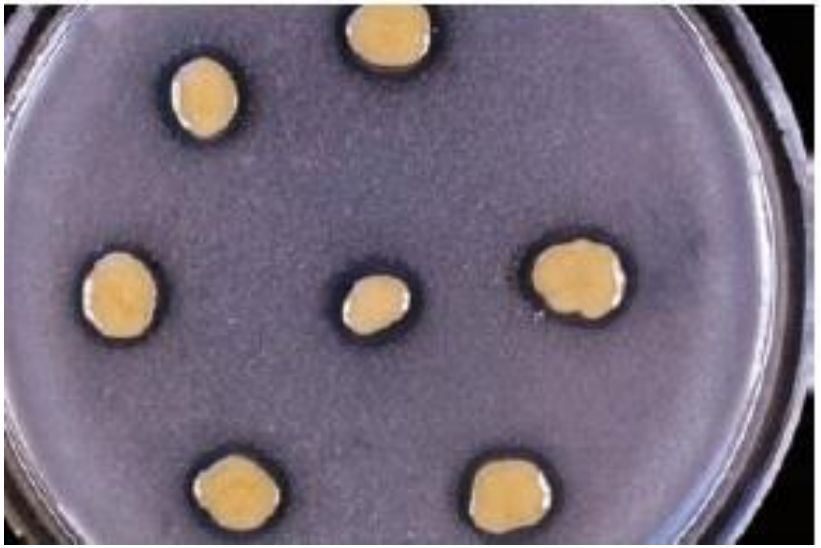
Note: crystals of the calcium salt of the fatty acid liberated by lipolysis.



Sierra,1957;Janse,2006

Fat hydrolysis
Lipolytic activity

- Hydrolysis of fat, demonstrated on a medium containing tributyrin, seen as clear zones around colonies of *Xanthomonas axonopodis* pv. *begoniae* (7 days after inoculation).



Janse,2006

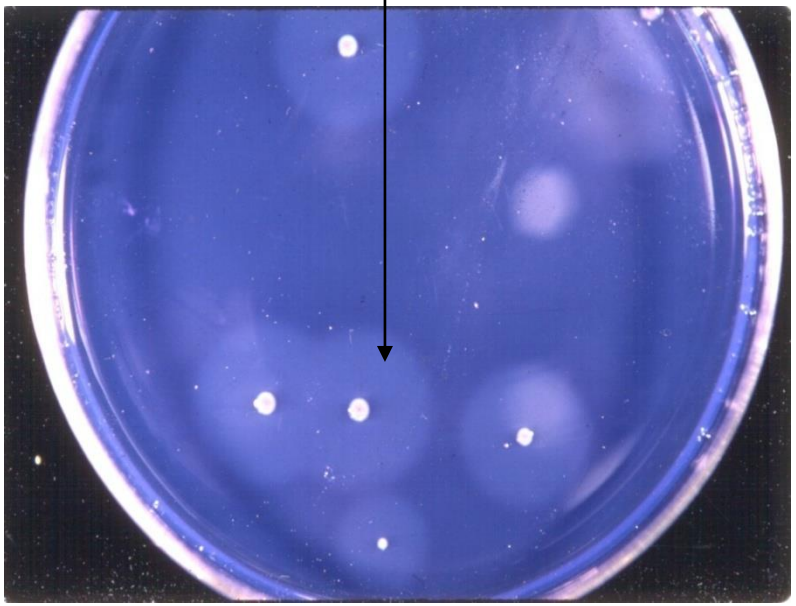
Starch hydrolysis test
Amylase

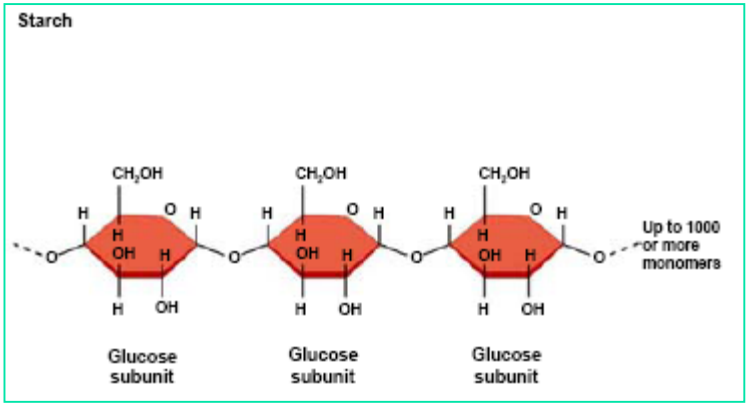
- In this demonstration, the action of bacterial species is compared on starch agar (NA+0.2% starch).
- The plates were incubated for 24 hours at 37°C.
- Iodine (Lugol's iodine,1g iodine and 2g potassium iodide in 100 ml D.H_2O), which changes color from a yellow-brown to blue-black in the presence of starch, was applied to the agar surface and allowed to stand for 10 minutes.
- If the species produces and releases amylase, starch hydrolysis in the agar should occur.
- A clear zone around the growth which is a positive reaction.

Starch hydrolysis test

Amylase

Zones of starch hydrolysis





Amylase

Starch → Maltose + Glucose + Dextrin

Lecithinase test

Lecithinase activity

- Egg yolk emulsion was prepared from a fresh hen egg, which was washed and sterilized in 70% ethanol for 5 min.
- The egg was flamed, broken aseptically, and the yolk separated into a sterile measuring cylinder and diluted to 1.5% (v/v) with sterile water.
- 100 ml of this egg yolk emulsion were added to 900 ml of molten Nutrient agar (NA) medium, cooled to 55°C and poured into Petri plates.

Lecithinase test

Lecithinase, Lipase and proteolytic activity

- Lecithin is a normal component of the egg yolk.
- An egg yolk suspension is incorporated to medium to detect the production of lecithinase and lipase and proteolytic activity.
- Examine for growth after 48 hours of incubation.

1. Lecithinase degrades Lecithin into phosphorylcholine and an insoluble diglyceride, which results in the formation of a precipitate in the medium.
2. Lipase breaks down free fats present in the egg yolks, causing an iridescent, "oil on water" sheen to form on the surface of the colonies.
3. Proteolysis is indicated by clear zones in the medium surrounding growth.

Lecithinase test

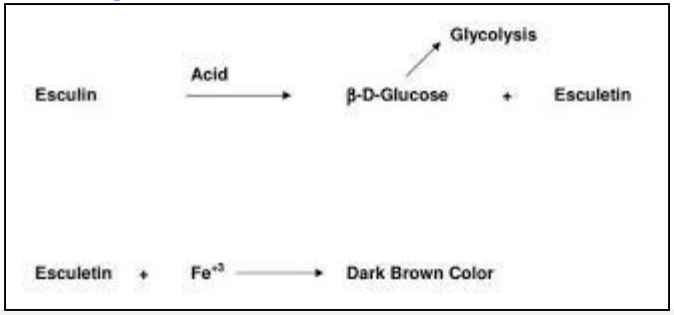
Egg yolk lecithinase hydrolysis test

Aryal,2019

Aesculin hydrolysis

Semi-selective esculin-trehalose (ET) medium

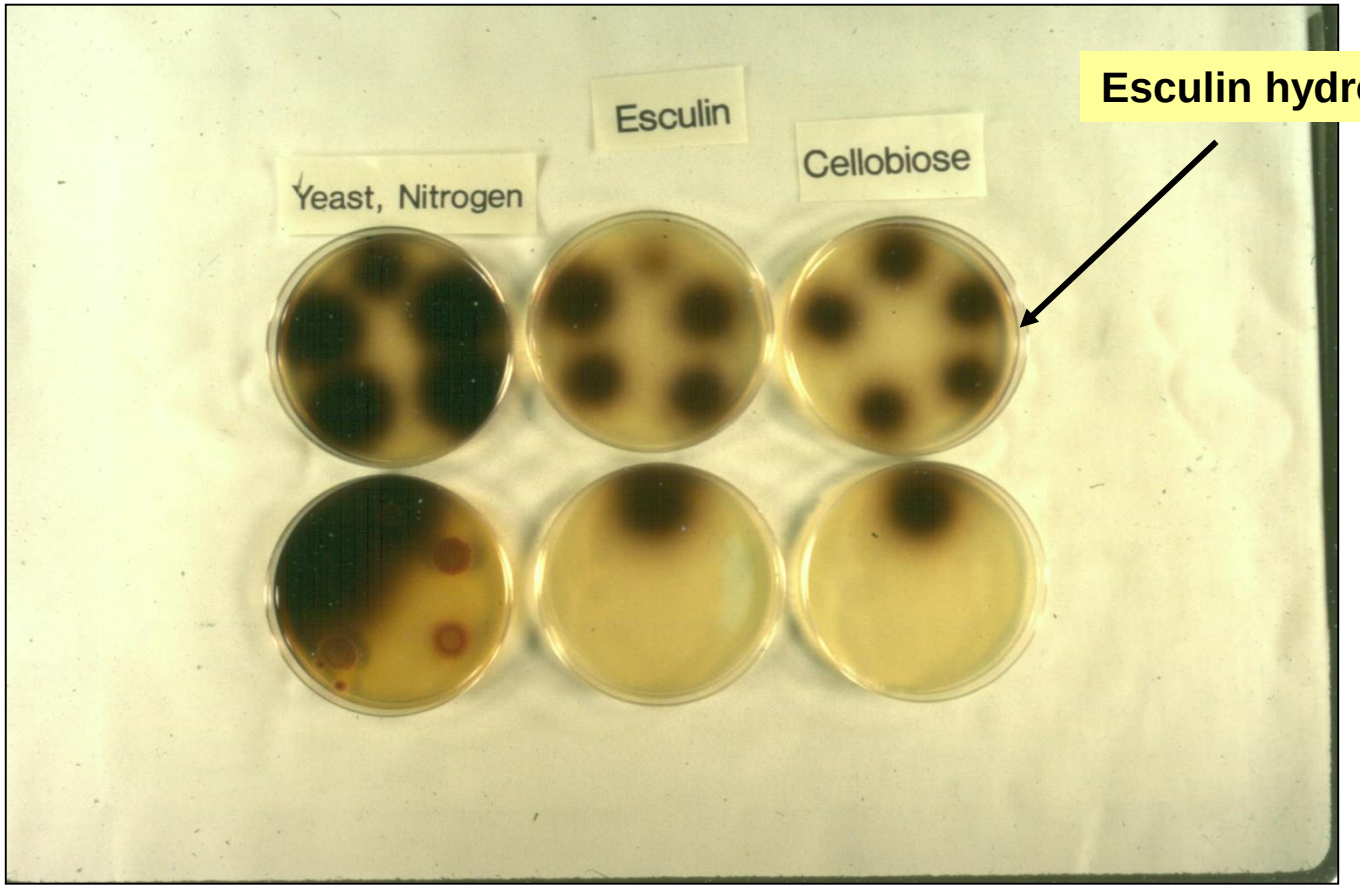
- Esculin, a water soluble glycoside, is hydrolyzed by certain bacteria to yield glucose and esculetin.
- Esculetin reacts with the ferric ions to produce a black colored complex that surrounds the colonies.
- Microorganisms that are not capable of hydrolyzing esculin will not produce a blackening in the medium.



Glycosides can be defined as the compounds in which one or more sugars are combined with nonsugar molecules through glycosidic linkage.

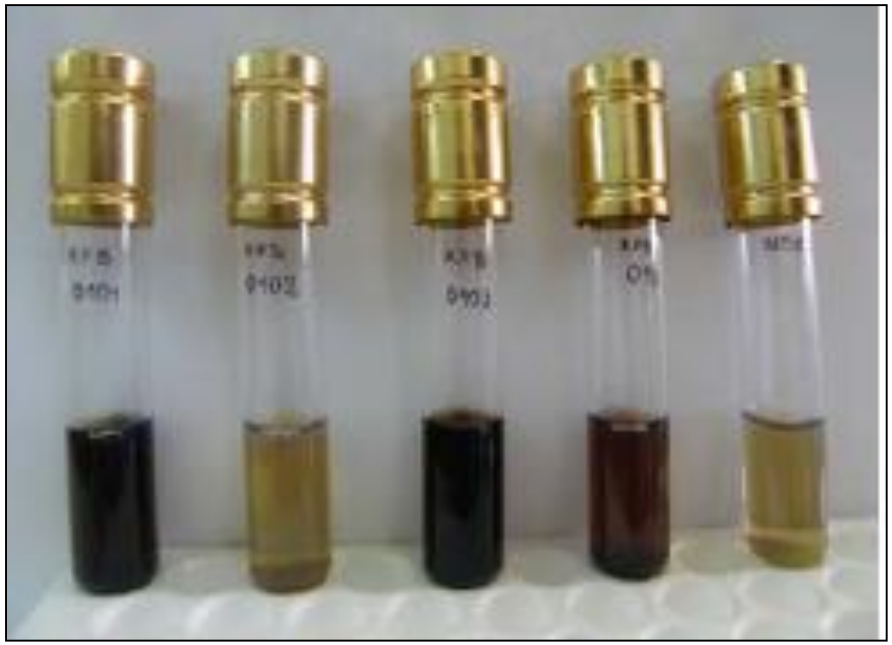
Aesculin hydrolysis

Semi-selective esculin-trehalose (ET) medium



Aesculin hydrolysis

- Dark brown discoloration of medium= positive reaction.



Obradovic,2010

Nitrate reduction test

Nitratase

Exceptional cases

- If there is no color change at this step, nitrite is absent. If the nitrate is unreduced and still in its original form, this would be a negative nitrate reduction result.
- However, it is possible that the nitrate was reduced to nitrite but has been further reduced to ammonia or nitrogen gas. This would be recorded as a positive nitrate reduction result.

$$NO_3 \text{ ----> } NO_2 \text{ ----> } NH_3 \text{ or } N_2$$

- To differentiate between these possibilities, a speck of zinc dust is added to the tubes.
- **RECORD the Following as RESULTS:**

1. reagents = RED = (+) for nitrate reduction
2. reagents + zinc dust = RED = (-) for nitrate reduction
3. reagents + zinc dust = no change in color = (+) for nitrate reduction and conversion to ammonia or nitrogen gas.

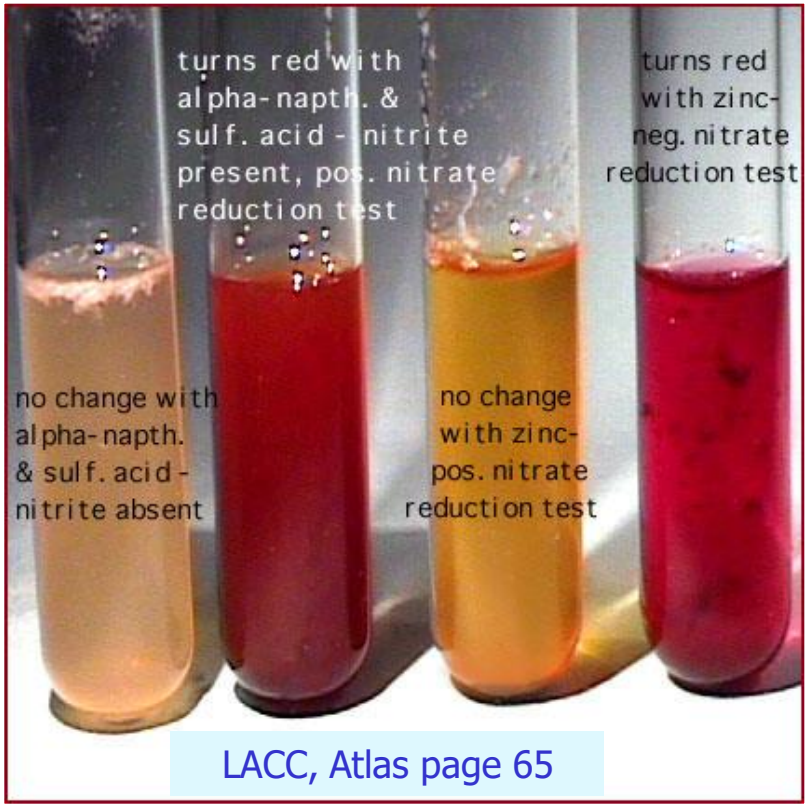
Mehan and McDonald,1995; Watts,2011; LACC, Atlas page 65

Nitrate reduction test

Nitrate reduction assay (in Broth)

Nitratase

- After incubation,
- the Nitrate Reagent A: 0.8 g of sulphanilic acid was dissolved in 100 ml of 5N acetic acid) and Nitrate Reagent B: 0.5 g of α-napthylamine was dissolved in 100 ml of 5N acetic acid are added.
- Use 1/2 dropper-full of each in the order given.
- These two compounds react with nitrite and turn red in color, indicating a positive nitrate reduction test.



LACC, Atlas page 65

Test medium: KNO_3, 1 g; Peptone, 5 g; Yeast extract, 3 g and Agar, 3 g in 1 liter distilled water.

Nitrate reduction test

The agar plate nitrate reduction assay

Sandwich overlay technique

- The agar plate nitrate reduction assay uses a similar principle of the culture broth (tube) nitrate reduction test but is performed directly on colonies on petri plates.
- The bacteria were surface plated on pre-poured M17 agar plates, allowed to dry (adsorb), and then overlaid with 10-12 mL nitrate agar (HiMedia) to entrap the plated colonies in a sandwich overlay technique.
- The overlaid plates were allowed to incubate overnight at 37°C, and then plates with a range of 25-250 colonies were selected to be overlaid with reagent agar layers tempered at 46-48°C before use.
- A plain agar layer containing addition of nitrate reagent A (2 mL reagent A is added in 50 mL 0.5% soft agar) was mixed and ~6-8 mL is overlaid onto the colony-sandwiched plate. After 5-10 min, another 6-8 mL plain agar layer containing nitrate reagent B (2 mL reagent B is added in 50 mL 0.5% soft agar) was overlaid on top of the nitrate reagent A layer and then tilted to facilitate the soft agar in running to the other side of the plate.
- The chemicals in the two separate overlays diffuse to the lower levels, reacting with nitrite in the order of addition as in the liquid nitrite test, and zones of red color observed around colonies indicated the presence of nitrite and nitrate-reducing bacteria.

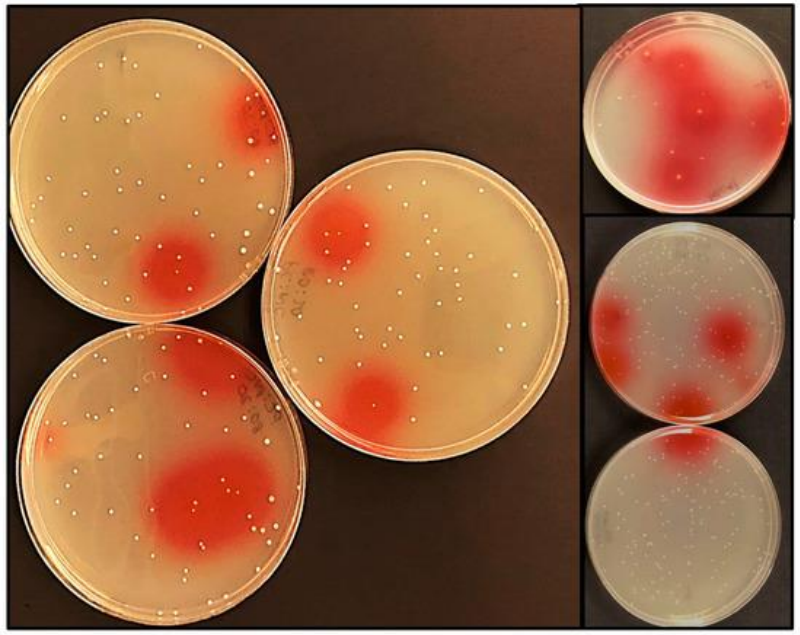
Bhusal and Muriana,2021

Nitrate reduction test

The agar plate nitrate reduction assay

Sandwich overlay technique

- Nitrate reducing bacterial colonies showing red color zones after sandwich overlays with soft agar containing nitrate reagents A and B.
- Colonies with surrounding red color were isolated using the inverted agar method and streak plated on new plates and confirmed for nitrate reduction in nitrate broth (the plates above are shown from the bottom side of the petri plate).

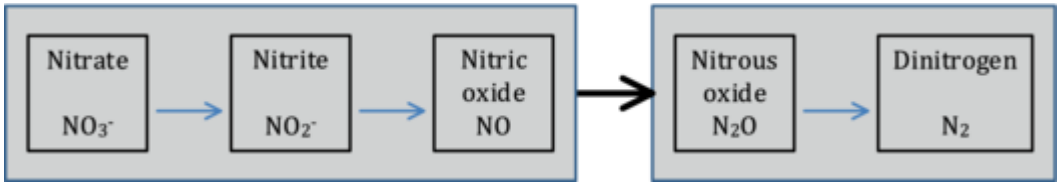


Bhusal and Muriana,2021

Nitrate Reduction
Denitrification test

- In denitrification, dissimilatory nitrate reduction occurs in the following steps:

Nitrate (NO3)	→ Nitratase	Nitrite (NO2)	→ (further reduction)	N2 + NH3



Denitrification

$NO_3^- \Rightarrow NO_2^- \Rightarrow NO \Rightarrow N_2O \Rightarrow N_2$

Primary nitrate reducers: *Thiobaccillus, Pseudomonas,Alcaligenes,Rhodo-pseudomonas*. This process takes place under anoxic conditions

Nitrate Reduction
Denitrification test

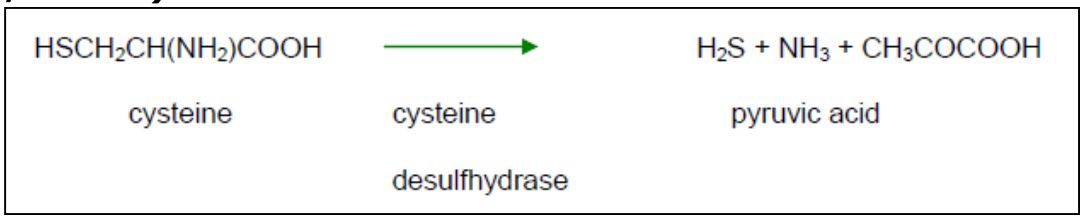
- The ability of the isolates to reduce nitrate to nitrite was evaluated in a test medium that contains KNO_3, 1 g; Peptone, 5 g; Yeast extract, 3 g and Agar, 3 g in 1 liter distilled water.
- Each tube was inoculated by stabbing and sealed with 3 ml-sterilized molten agar to avoid false positives and incubated at 28^{o}C.
- Observation was made after 3, 5 and 7 days of inoculation.
- Bubble formation beneath the upper agar layer was taken as positive result for nitrate reduction (Dickey and Kelman,1988).

Hydrogen sulfide production
Cysteine desulfhydrase
Dye method

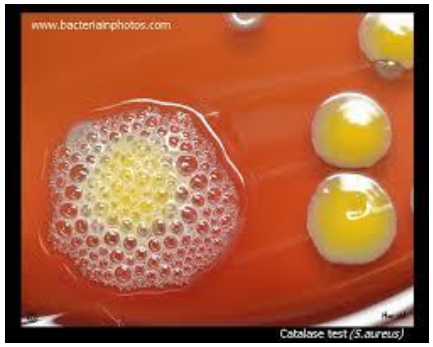
- H_2S production from cysteine, a sulphur rich compound gave good and more consistent result.
- The medium for H_2S production test contained (in gL^{-1}): $NH_4H_2PO_4$ 0.5; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NCl, 5.0; yeast extract and 5; cysteine hydrochride, 0.1.
- The medium was dispensing 5 mL quantities into tubes and then autoclaved and allowed to set.
- A lead acetate strip was prepared by immersing cut filter paper strips in 5% lead acetate, air dried and autoclaved.
- The lead acetate strip was suspended over the medium after inoculation and held by a screw cap and allowed to stand for 14 days.
- Black lead paper strips indicate H_2S production from cysteine.

Hydrogen sulfide production
Alternative medium

- The isolates were evaluated for H_2S production using Sulphide Indole Motility (SIM) agar medium (Peptone, 30 g; Beef extract, 3 g; Ferrous ammonium sulfate, 0.2 g; Sodium thiosulphate, 0.025 g and Agar,3 g in 1 liter distilled water).
- The isolates were inoculated by stabbing and incubated at 28^{o}C for 48-72 hours.
- The presence of black coloration along the line of stab inoculation was recorded as positive for H_2S production (Aneja,1996).



Catalase test



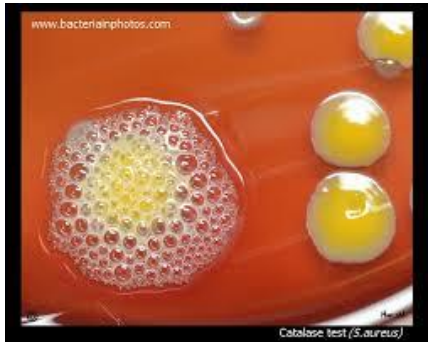
- Used to test for the presence of enzyme catalase.
- Hydrogen peroxide (H_2O_2) is formed as an end product of the aerobic breakdown of sugars. When H_2O_2 accumulates, it becomes toxic to the organism. Catalase decomposes H_2O_2 and enables the organism to survive. Only obligate anaerobes lack this enzyme.

$$2\,H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

- Procedure:
- Streak nutrient agar slant with the organism
- Incubate at optimum temperature for 24-48 hours
- Place a few drops of 3% H_2O_2 on the slant culture
- Interpretation:
- Positive- Bubbling (O_2 gas is liberated from the H_2O_2)
- Negative- No bubbling.

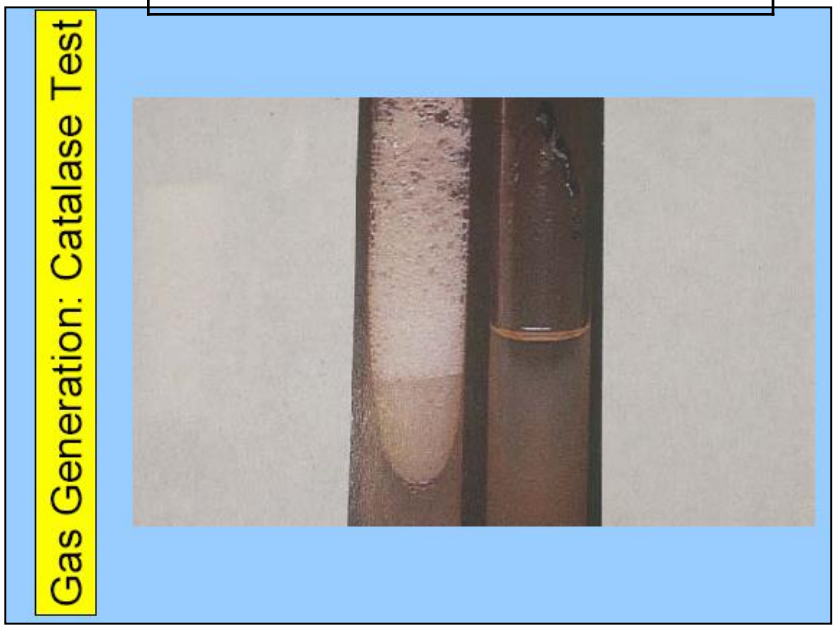
Abedon,2011

Catalase test



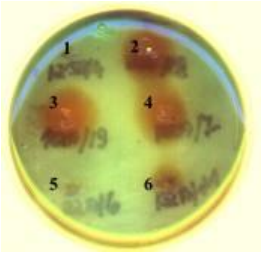
- The catalase enzyme decomposes hydrogen peroxide (H_2O_2) to water and O_2.
- Hydrogen peroxide is very toxic for bacteria.
- Bacteria are smeared in a drop of hydrogen peroxide and checked for formation of oxygen gas bubbles.
- Most bacteria are catalase positive.
- Catalase positive bacteria are obligatory aerobic organisms.

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2$$



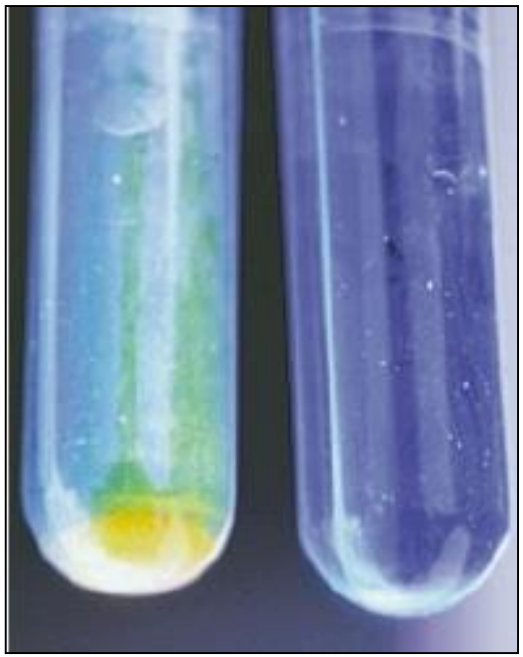
Note: Hydrogen peroxide (H_2O_2) was used for the activation of lactoperoxidase system on preservation of milk.

Abedon,2011



Reducing compounds from sucrose

- Bacteria were grown in sucrose broth containing peptone 10 g, beef extract 5 g and sucrose 40 g/L (Fahy and Persley,1983).
- After 48 h, an equal volume of Benedict's reagent is added and tubes held in a boiling water bath for 10 min.
- A yellow-orange to brown colour change (with or without precipitate) indicates production of 2- ketogluconate (left), compared to the control (right).

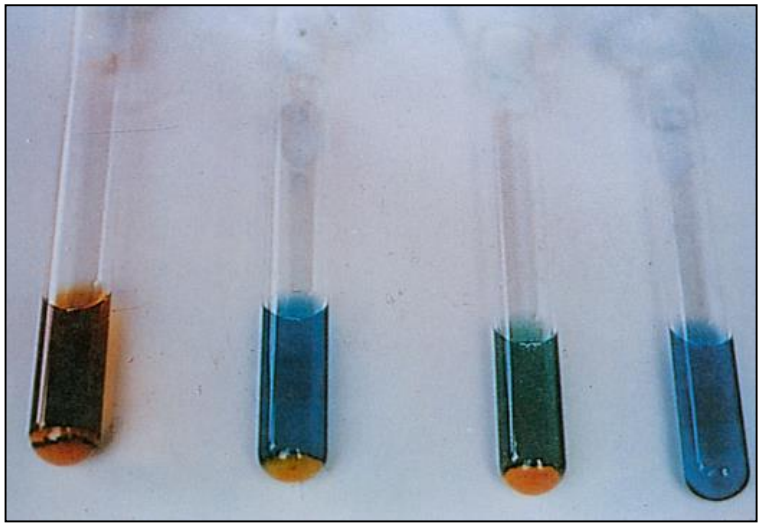


Benedict's reagent is prepared as follows:

Add 173 g sod. citrate and 100 g $Na_2CO_3.H_2O$ in 800 ml D. H_2O. Heat the solution and filter it. Bring volume to 850 ml and while stirring add 17.3 g $CuSO_4.5\ H_2O$. Increase volume to 1 L and store in brown bottle (Schadd,1998).

2-Ketogluconate production

- Bacteria were grown in broth containing peptone 10 g, beef extract 5 g and sodium or potassium gluconate 40 g/L (Fahy and Persley,1983).
- After 48 h, an equal volume of Benedict's reagent is added and tubes held in a boiling water bath for 10 min.
- The yellow precipitates in the three tubes on the left show a positive reaction.
- The clear blue solution with no precipitate in the tube on the right indicates a negative reaction.



Mew and Misra,1994

2-Ketolactose test

- Lactose agar medium contains: lactose 10 g, yeast extract 1 g, agar 12 g/L (Lelliott and Stead,1987).
- Sterilize by autoclaving and pour plates.
- Inoculate a spot heavily and incubate for 2 days.
- Flood with Benedict's reagent.
- Presence or absence of a yellow zone of Cu_2O indicating 3-ketolactose production, in the agar around the colony within 1 hour.
- Among different biovars of *Agrobacterium* only Biovar I (*A. tumefaceins*) produce this compound.

Benedict's reagent is prepared as follows:
Add 173 g sod. citrate and 100 g $Na_2CO_3.H_2O$ in 800 ml D. H_2O. Heat the solution and filter it. Bring volume to 850 ml and while stirring add 17.3 g $CuSO_4.5\ H_2O$. Increase volume to 1 L and store in brown bottle(Schadd,1998).

α-Methyl-d-glucoside utilization

- The α-Methyl-d-glucoside medium is made up of 5 parts:

A- KH_2PO_4 2.0 g

- K_2HPO_4 7.0 g
- NH_4Cl 1.0 g
- Dist. Water 500 ml

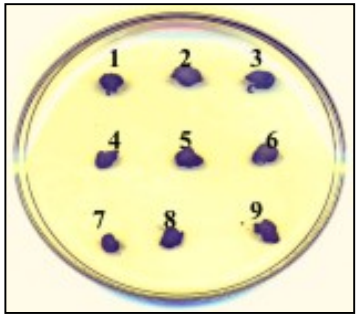
B- Agar 15.0 g

- Casamino acids (Difco) 1.0 g
- Dist. water 500 ml

C- $MgSO_4$ X 7 H_2O 1.0 ml (10% solution)

D- α-Methyl-d-glucoside 50 ml (20% solution)

E- 2,3,5-Triphenyl-tetrazolium chloride 2.0 ml (1% solution)



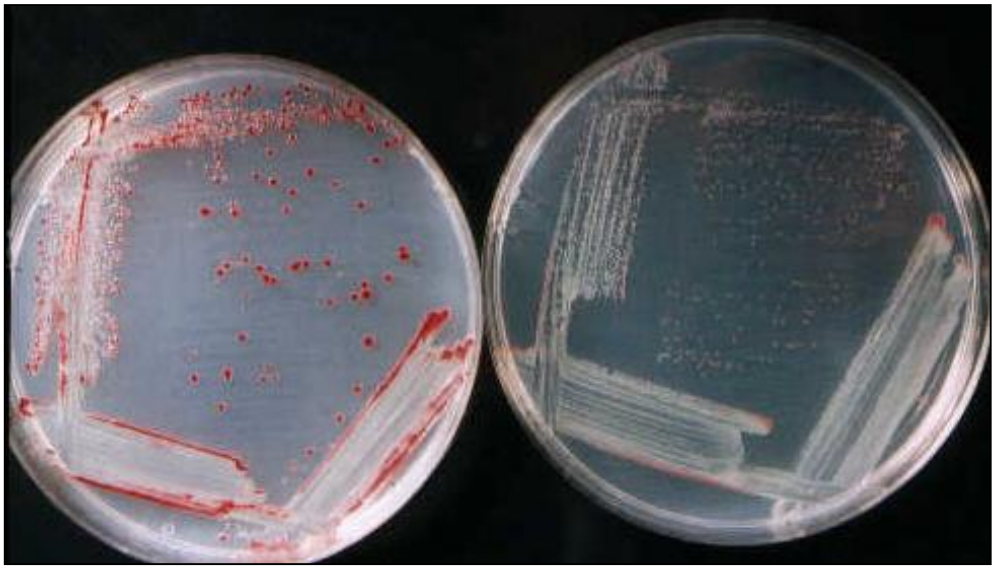
- Each solution of the 5 parts is autoclaved separately.
- Mix A and B, cool down to 50°C, add solutions C, D and E, and pour into Petri plates.

Mamdoh Ewis Esmael Ahmed,2001

α-Methyl-d-glucoside utilization

Only strains of *Erwinia carotovora* subsp. *atroseptica* (Eca) show a positive reaction, but not those of *Erwinia carotovora* subsp. *carotovora* (Ecc) and *Erwinia chrysanthemi* (Ech)

- Differentiation between *Erwinia carotovora* subsp. *atroseptica* (Left) and *Erwinia carotovora* subsp. *carotovora* (right) using the ∝-methyl-d-glucoside medium after incubation at 27°C for 24 h.
- A positive reaction was indicated by a pink colour of the colony.

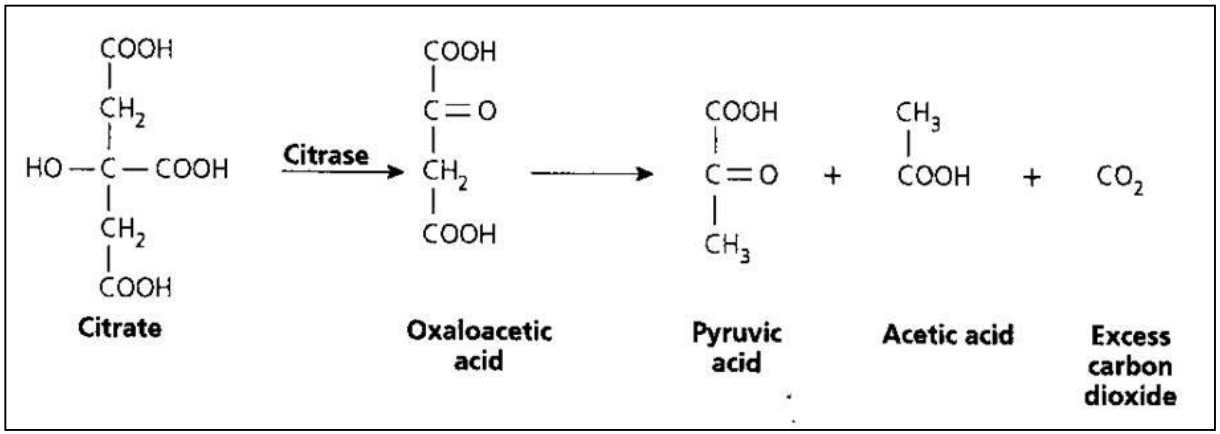


Mamdoh Ewis Esmael Ahmed,2001

Citrate utilization

Simmons citrate agar

- Used to determine if an organism is capable of using citrate as the sole source of carbon with production of the enzyme citratase (citrase).
- An organism that uses citrate breaks down the ammonium salts to ammonia, which creates an alkaline pH.



Biochemic2; AP Biology-Benskin

Citrate utilization

Simmons citrate agar

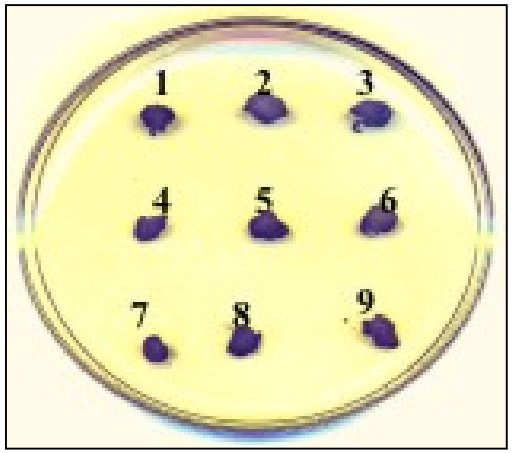
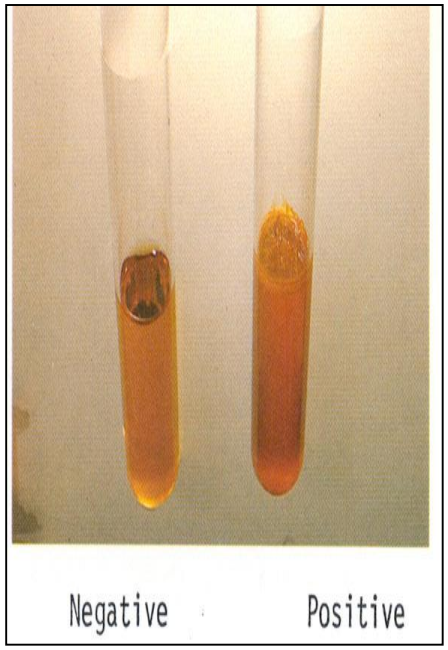
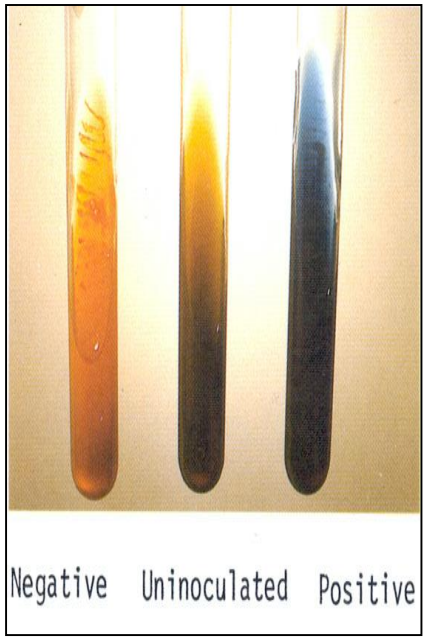
- Citrate utilization of the isolates was tested using Simmons citrate agar slants ($NH_4H_2PO_4$, 1 g; K_2HPO_4, 1 g; NaCl, 5 g; $MgSO_4$. $7H_2O$, 0.2 g; sodium citrate, 2 g; Agar,15 g; Bromothymol blue, 0.08 g in 1 liter distilled water (pH 6.9).
- A loopful from each isolate was streaked on the slant and incubated at 28^o C for 48-72 hours.
- Positive: Alkaline pH causes media to change from green to metallic blue.
- Negative: No color change.

Malate utilization

Malate is a salt or ester of malic acid

- Malate utilization of the isolates was tested using a broth medium containing malic acid, 2 g; $NH_4H_2PO_4$, 1 g; K_2HPO_4, 1 g; $MgSO_4.7H_2O$, 0.2 g; Yeast extract, 1 g; Bromothymol blue, 12.5 ml of 0.2% solution in 1 liter distilled water (pH 6.9).
- Forty-eight hours old bacterial isolates were inoculated and incubated at 28^o C for 48-72 hours.
- A color change from green to blue was taken as positive for malate utilization and uninoculated tubes used as negative control (Aneja,1996).

The more tree biochemical tests

		
Production of acid from methyl-D-glucoside	**Ferric ammonium citrate test**	**Citrate utilization test**

Marshall,2002; Firefox document

Amino acid decarboxylation

Glutamic acid, lysine and ornithine decarboxylase test

- Decarboxylase broth tests for the production of the enzyme decarboxylase, which removes the carboxyl group from an amino acids.

1. If the inoculated medium is yellow, or if there is no color change, the organism is decarboxylase-negative for that amino acid.
2. If the medium turns purple, the organism is decarboxylase-positive for that amino acid.

Amino acid decarboxylation

Glutamic acid, lysine and ornithine decarboxylase test

- Decarboxylase broth containing (g/L):

1. Peptone 5.00
2. Amino acid (L-Lysine or Glutamic acid or ornithine) 5.00
3. Yeast Extract 3.00
4. Dextrose(glucose) 1.00
5. Bromocresol Purple 0.02.

- pH was adjusted to 6.6-6.8. Dispense quantities of 5 ml into screw-capped tubes. Sterilize in autoclave at 121°C for 15 minutes. The color is violet.
- The tubes are inoculated with the microorganism samples, overlaid with a layer of sterile mineral oil and incubated.
- +ve result: presence of purple-red color.
- -ve result: A yellow color.

Amino acid decarboxylation

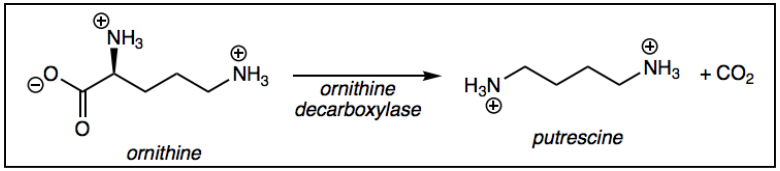
Ornithine Decarboxylase Test

Ornithine decarboxylase broth

Ornithine decarboxylase test

It is a test used to detect ability to decarboxylase ornithine

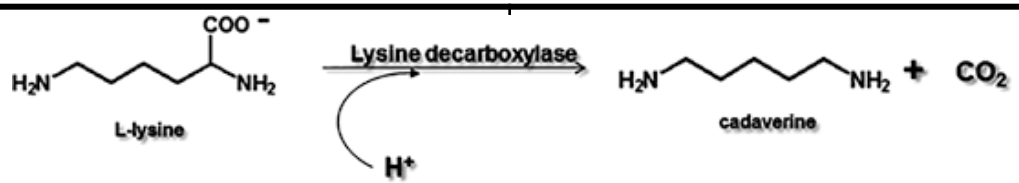
- Many species of bacteria can degrade ornithine, arginine or lysine into alkaline amine and CO_2.
- Moeller decarboxylase broth contains any of the three amino acids, glucose, beef extract and bromocresol purple (pH indicator)
- The broth is inoculated with test suspension and overlaid with sterile liquid paraffin to seal it from environmental oxygen and incubated at 37°C overnight.
- Initially, glucose is fermented and pH falls. The colour changes to yellow. Later, as amino acid is decarboxylated, pH rises and the colour reverts back to purple.
- A control tube must also be placed simultaneously.
- Lysine : E coli (positive), Proteus (negative)



Sridhar Rao;..

Amino acid decarboxylation

Lysine decarboxylase test

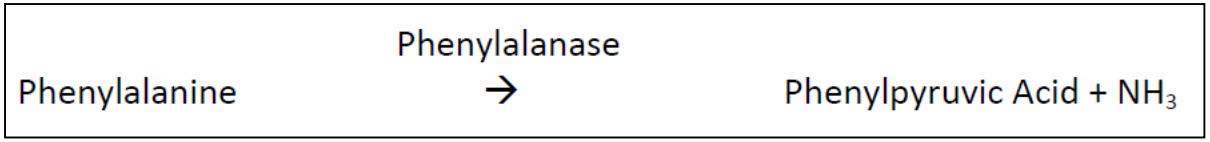


	
Serratia marcescens, displays a positive reaction, as the medium has turned purple.	*Proteus vulgaris* (has too much sterile mineral oil in the overlay), displays a negative result.

Phenylalanine Deamination

Phenylalanine Agar

- Used to determine the ability of an organism to deaminate the amino acid phenylalanine resulting in the production of phenylpyruvic acid and ammonia.
- This reaction is catalyzed by the enzyme phenylalanase.



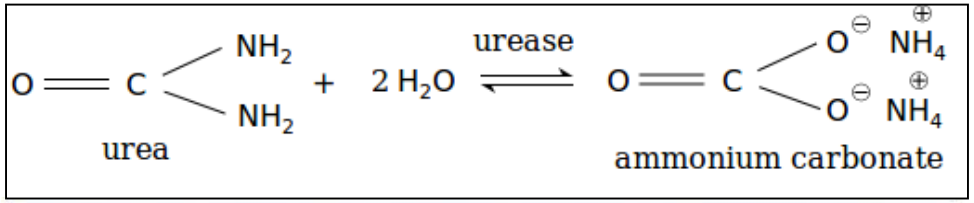
- Procedure:
- Streak phenylalanine agar slant with the organism.
- Incubate at optimum temperature for 24-48 hours
- Place 5-10 drops of 10% Ferric Chloride on the slant culture. Use a loop to mix the organism into the solution.
- Interpretation:
- Positive- A deep green color appears within 1-5 minutes
- Negative- An amber color develops.

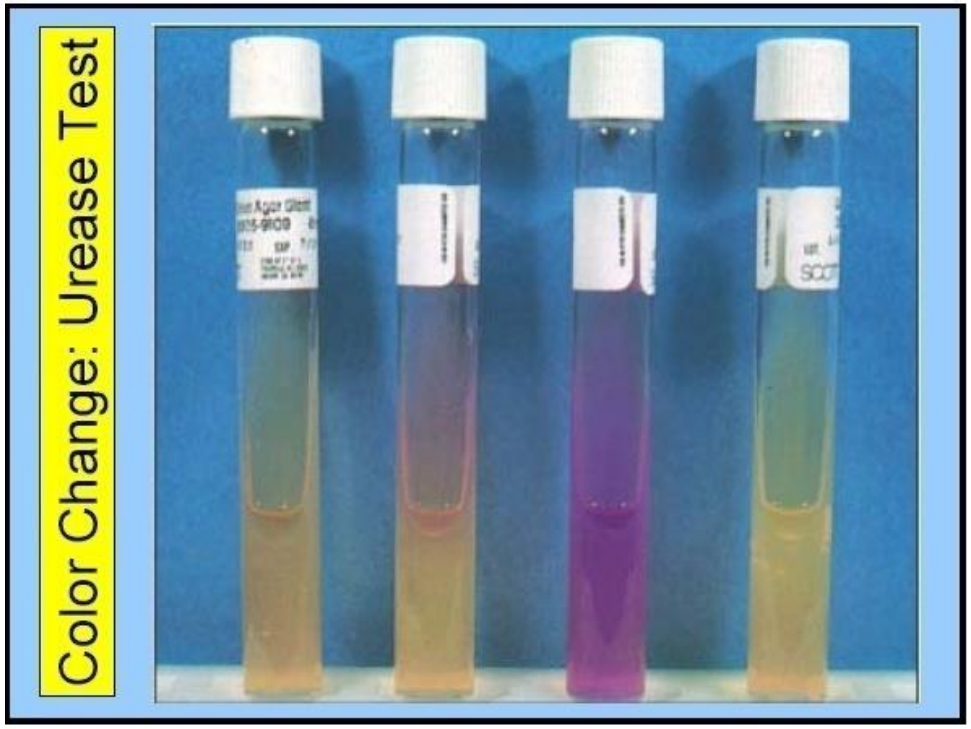
Urease test

- Used to determine the ability of an organism to split urea to form ammonia (an alkaline end product) by the action of the enzyme urease.
- Media also contains the pH indicator phenol red, which turns an intense pink at alkaline pH.
- Procedure:
- Inoculate urea broth with the organism
- Incubate at optimum temperature for 24-48 hours
- Interpretation:
- Positive: Intense pink/red color
- Negative: No color change
- Note- Continue incubation of negative tubes for a total of 7 days to check for slow urease producers.

Urease test

Note: A blood urea nitrogen (BUN) test **measures the amount of nitrogen in your blood** that comes from the waste product urea.
Urea is made when protein is broken down in your body.
Urea is made in the liver and passed out of your body in the urine.
A BUN test is done to see how well your kidneys are working.

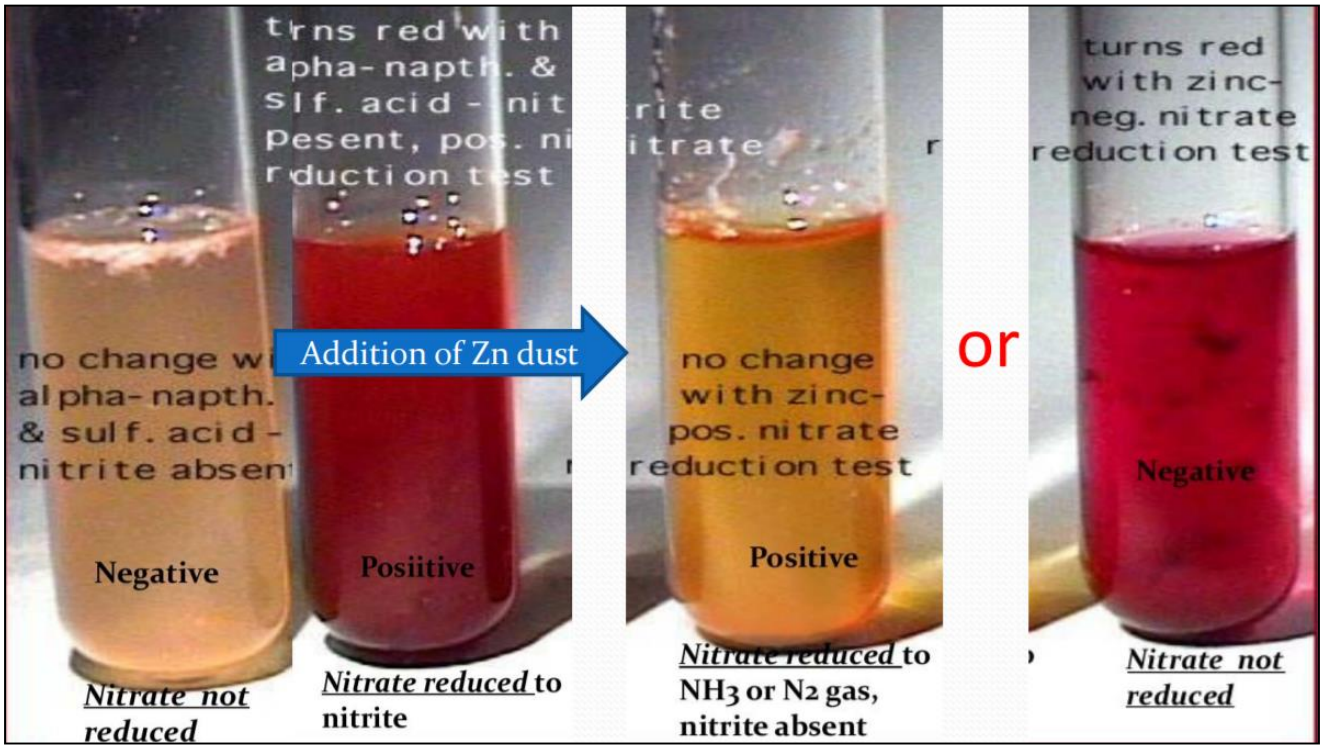




Abedon,2011

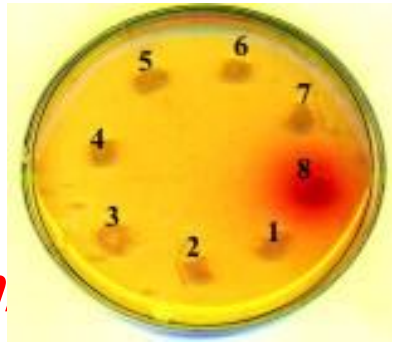
Urease test

Addition of zinc powder

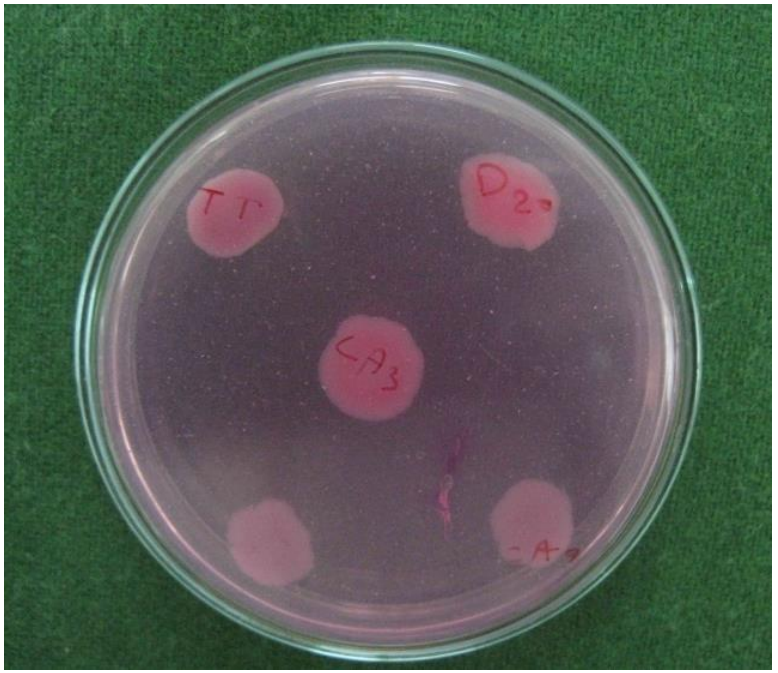


Phosphatase test

For identifying *Dickeya chrysanthem*



- Prepare NA and when partially cooled add a filter-sterilized solution of phenolphathalin diphosphate sodium salt at final conc. of 0.05%.
- Spot inoculate the bacteria for 48 h.
- Then place a drop of concentrate ammonia in the lid of the Perti dish and invert the medium over it.
- Colonies with positive Phosphatase activity turn a bright pink colour immediately.



Schaad *et al.*,2001

DNAse test
Deoxyribonuclease

VetBact

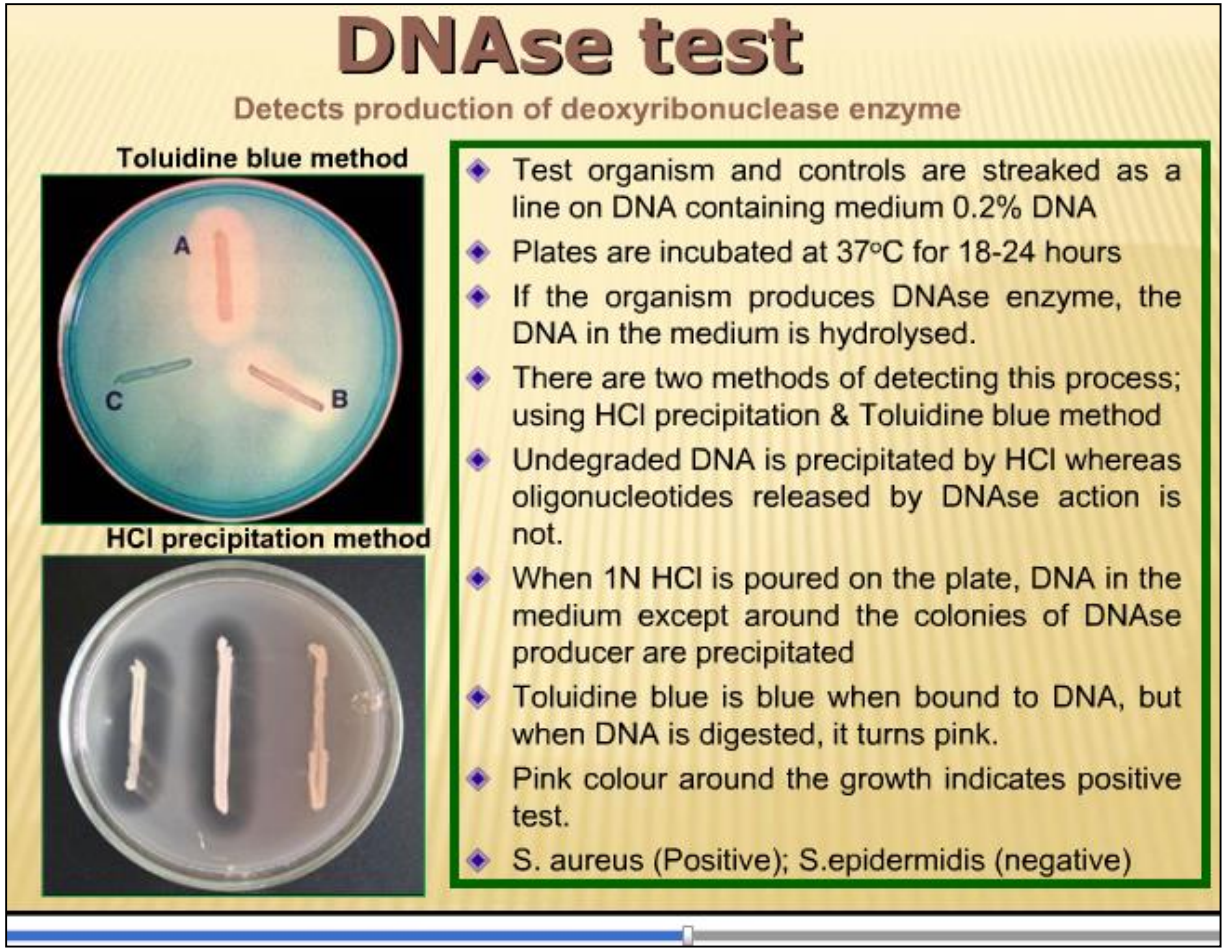
- Bacteria were grown on DNAase medium (BBL).
- After 3 days the plates were flooded with 1 M-HCl and inspected for areas of clearing.
- The test was repeated for negative strains after 5 days incubation.

Grimont *et al.*,1976

DNAse test

Deoxyribonuclease

Alternative method



Coagulase test
Most useful for clinical bacteria

- Bacteria that produce coagulase use it as a defense mechanism by clotting the areas of plasma around them, thereby enabling themselves to resist phagocytosis by the host's immune system.
- The sample in question is usually inoculated onto 0.5 ml of rabbit plasma and incubated at 37 degrees celsius for one to four hours.
- A positive test is denoted by a clot formation in the test tube after the allotted time.

El-Safey,2011

Cellulose hydrolysis

Cellulase production

CMC

- Since crystalline cellulose is degraded at very slow rates, most assays were adapted to use more easily degradable soluble cellulose derivatives like carboxymethylcellulose (CMC).
- The detection of the cellulolytic activity in these cases is achieved by staining or precipitation of undigested CMC in plate regions which were not exposed to cellulolytic activity, while areas exposed to cellulase give clear halos surrounding the source of the enzyme.

Johnsen and Krause,2014

Cellulose hydrolysis

Cellulase production

CMC

- Carboxymethylcellulose (CMC), which measures endo-β-1,4- glucanase activity, is one of the most popular artificial substrates for measuring cellulase activity because of its high solubility in water.

Tokuda *et al.*,2005

Cellulose hydrolysis
Cellulase production

- Cellulase production was determined in M9 medium (Miller,1974) amended with yeast extract (1.2 g L^{-1}) and cellulose (10 g L^{-1}).
- After 8 d of incubation at 28°C, isolates surrounded by clear halos were considered positive for cellulase production.
- *D. chyrsanthemi* as a cellulolytic bacterium is able to hydrolyze CMS, while other Erwinia's are not.

M9 minimal medium (5X concentrate): To 800mL of distilled/deionized water add: 64g Na_2HPO_4;15g KH_2PO_4; 2.5g NaCl; 5.0g NH_4Cl. Make to 1 litre with dH_2O. Sterilize by autoclaving or filter sterilization if autoclave is not available.

Cellulose medium

Na-taurocholate	5 g
$NH_4H_2PO_4$	1 g
$MgSO_4.7H_2O$	0.2 g
KCl	0.2 g
Bromothymolblue	0.05 g
$CaCO_3$	3 g
Carboxymethylcellulose (Sigma)	80 g

Cattelan *et al.*,1999; Janse,2010

Enzyme assays
Extracellular cellulase activity
Congo red clearing zone assay

- Nutrient agar plates were supplemented with 1% carboxy methyl cellulose (CMCA plates).
- Strains were streaked and petri plates were incubated at 37ºC for 48 hours.
- Petri plates were flooded with 0.1% Congo red reagent and left for 20 minutes.
- Then the plates were washed with 1M NaCl.
- Clearance zones called halo zones are seen against the red color of Congo red for the positive test.
- The NaCl solution elutes the dye in the clearing zone where the cellulose has been degraded into simple sugars by the enzymatic activity.

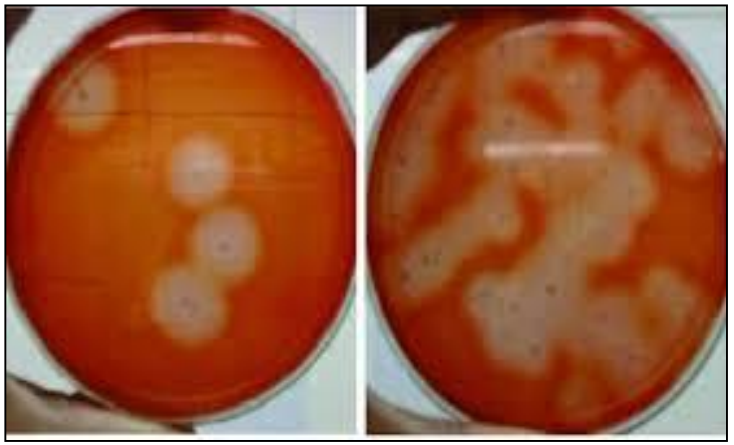
Ponnambalam *et al.*,2011; Upadhyaya *et al.*,2012

Enzyme assays

Extracellular cellulase activity

Congo red clearing zone assay

- Clearance zones (halo zones) are seen against the red color of Congo red for the positive test.



Upadhyaya *et al.*,2012;..

Enzyme assays

Extracellular cellulase activity

Gel diffusion assay

- In gel diffusion assay two layers of solidified medium are created.

1. The lower layer contains only CMC and the agar, and
2. The upper layer contains CMC, agar and 0.1 percent congo red dye.

- The isolated organisms are surface inoculated into the upper layer.
- The dye will diffuse from the upper layer to the lower layer if there is cellulolytic activity.

Ponnambalam *et al.*,2011

Determination of extracellular cellulase activity

Xanthomonas malvacearum

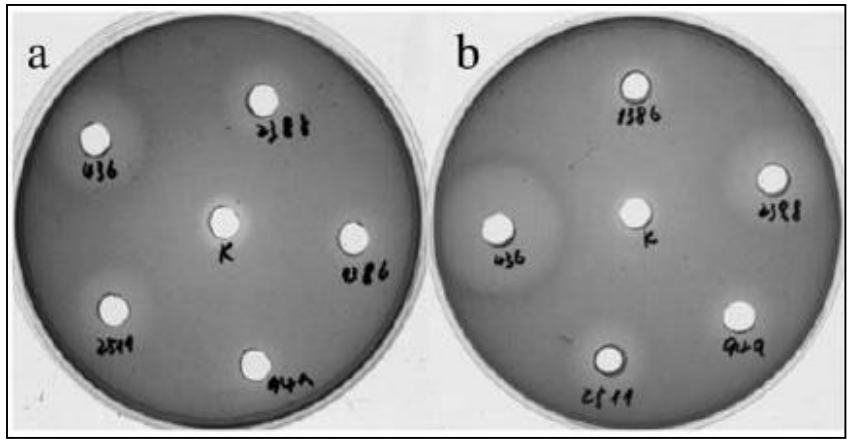
- Bacteria were pre-cultured on basal medium with 0.75% glucose, 0.5% CMC, and 0.1% yeast extract for 1 day, then transferred to fresh medium (basal medium with 0.5% CMC and 0.1% or 1% yeast extract) and cultivated for 3 days.
- The bacterial suspension was centrifuged and extracellular cellulase activity in the supernatant was determined by digesting a soluble chromogenic substrate, carboxyl methylcellulose-remazol brilliant blue R (CMC-RBB).
- The bacterial supernatant was incubated on CMC-RBB containing-agar, where the cellulase activity was expressed as a clear zone around a well.

Huang *et al.*,2008

Determination of extracellular cellulase activity

Xanthomonas malvacearum

- Determination of cellulase activity on agar medium containing chromogenic substrate with supernatants from strains:
- GSPB 1386 (race 18),
- GSPB 2388 (race 20) of *X. malvacearum*,
- GSPB 2511 (*X. axonopodis manihotis*,),
- GSPB 436 (*E. carotovara* subsp. *carotovora*, 436),
- ATCC 49294 (Xam).



Control (k), cultured in medium containing: 0.1% yeast extract (a), or 1% yeast extract (b).

Huang *et al.*,2008

Pectin hydrolysis

Pectic enzymes

- Pectic enzymes are important for degradation by bacteria of middle lamellae and cell walls of plants.
- Media with sodium polypectate such as CVP have been developed (single or double layer) that allow detection of pectinolysis in the form of pits in the medium.
- Pectic enzymes have different pH optima.
- Pseudomonads usually produce low pH pectin lyases (PL), whereas erwinias usually produce high pH pectin methylgalacturonases (PMG).

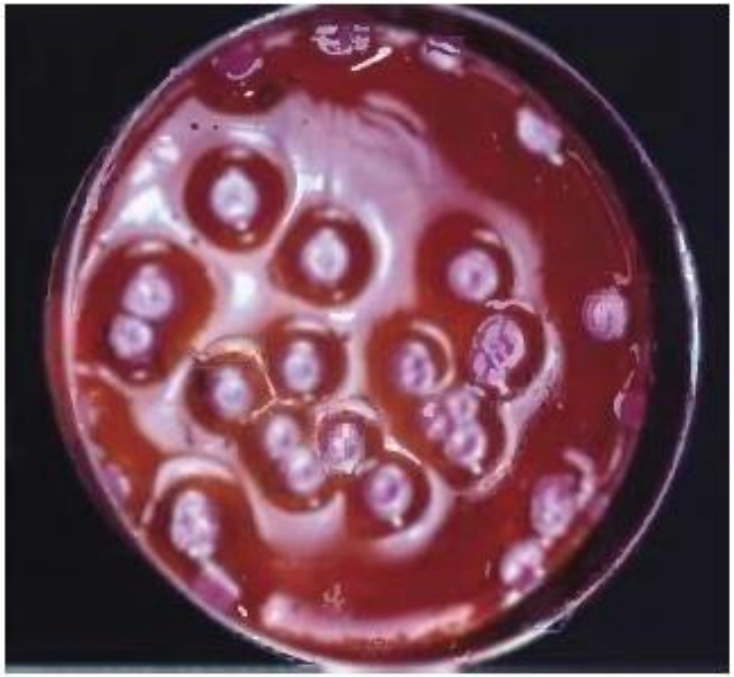
Janse,2006

On modified crystal violet sodium polypectate (CVPD) double layer medium, with 2,3,5-triphenyltetrazolium chloride and *L*-asparagine in the basal medium and bromthymol blue in the upper, Pcc isolates formed red to pink colonies with pits (Bdliya *et al.*,2004; Hibar *et al.*,2007).

Pectin degradation

Pectinases or pecteolytic enzymes

- Demonstration of pectin hydrolysis by pectinolytic enzymes of *Pectobacterium carotovorum* on the double-layer medium of Perombelon after 48h of growth.
- The top layer is pure pectin and by hydrolysis the bacteria form pits in the surface.



Janse,2006

Pectin degradation

Pectinases or pecteolytic enzymes

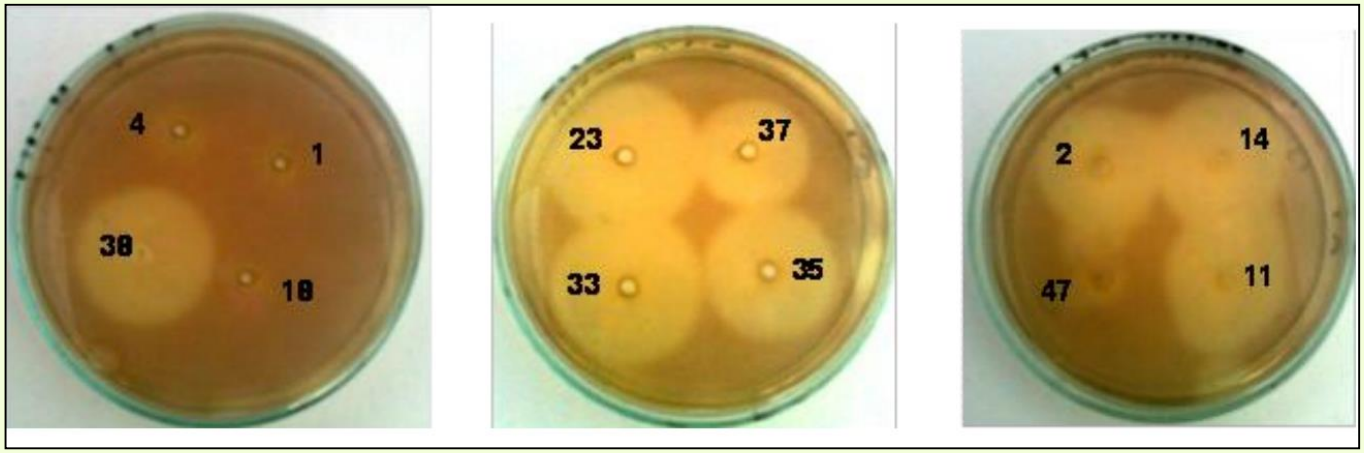
- Petri plates containing autoclaved modified MS medium containing 0.3% KH_2PO_4, 0.6% Na_2HPO_4, 0.2% NH_4Cl, 0.5% NaCl, 0.01% $MgSO_4.7H_2O$ and 1.5% agar (Gerhardt *et al.*, 1994) supplemented with 0.2% pectin were prepared.
- After solidification of the medium, four wells of 5 mm in diameter were cut in the agar with the help of cork borer.
- Each well was filled with 25 µl of cell supernatant.
- After incubating for 24 h at 37°C, plates were observed for pectinase activity by flooding them with iodine solution containing 0.25% iodine, 0.5% potassium iodide and 31 ml of 20% ethanol (Cappuccino and Sherman,2007).
- The enzyme activity was observed by measuring the diameter of clear zone around the well in millimeter.

Tariq an Latif,2012

Pectin degradation

Pectinases or pecteolytic enzymes

- Zone formation by different bacterial cultures showing pectin depolymerization on MS medium supplemented with 0.2% pectin and flooded with iodine solution.
- The following indexation was used, based on the diameter of the halo: -, no halo; +,10-20 mm; ++, 21-30 mm; +++, 30 mm and above.



Tariq an Latif,2012;..

Pectinase production

Pectinolytic activity on citrus pectin agar

Alternative method

- Pectinase production was determined in the same M9 medium (described in cellulose production) except the cellulose was replaced with pectin such as citrus pectin (4.8 g L^{-1}).
- After 2 d of incubation at 28°C, the plates were flooded with 2 M HCl (T. Denny, 1997, personal communication), and isolates surrounded by clear halos were considered positive for pectinase production.

Cattelan *et al.*,1999;..

Chitinase production
Chitin hydrolysis

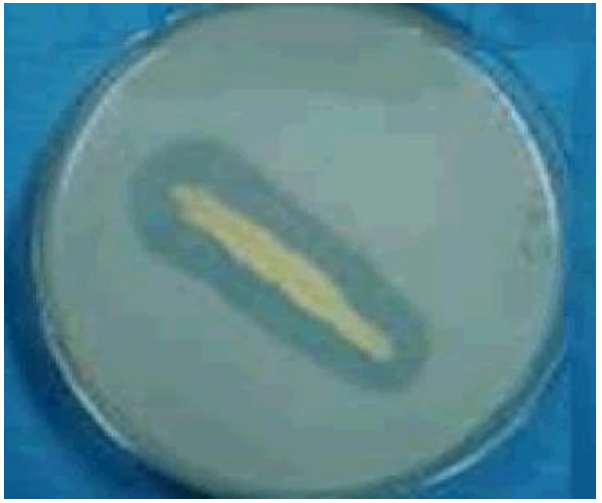
- Colloidal chitin was prepared by dissolving 2 g chitin in 40 ml cold concentrated HCl, allowing the solution to stand for 3 h and then filtering it through glass wool.
- The filtrate was diluted 1:10 in cold distilled water, and the precipitate was washed three times with distilled water, suspended in distilled water, neutralized with 0.1 M-NaOH, and again washed three times with distilled water.
- The chitin was then suspended in 40 ml distilled water, sterilized by heating at 80°C for 30 min on three successive days, then added to molten nutrient agar to a final concentration of 4% (w/v).
- After inoculation and incubation, plates were examined for up to 5 days for clearing around the growth.

Grimont *et al.*,1976; Roberts & Selitrennikoff,1988

Chitinase production

Agar plate assay for chitinase degradation of chitin and bacterial cell walls

- A based medium containing 1% colloidal chitin was used as sole carbon source.
- Bacterial colony was evenly spread on this medium.
- After incubation at 30°C for 3 days, bacterial colony with clear halo around indicate positive reaction.



Energy and carbon source types

- Prokaryotes can be grouped into four categories according to how they obtain energy and carbon:

1. Species that use light energy are phototrophs.
2. Species that obtain energy from chemicals in their environment are chemotrophs.
3. Organisms that need only CO_2 as a carbon source are autotrophs.
4. Organisms that require at least one organic nutrient as a carbon source are heterotrophs.

The main types of energy-capturing metabolism

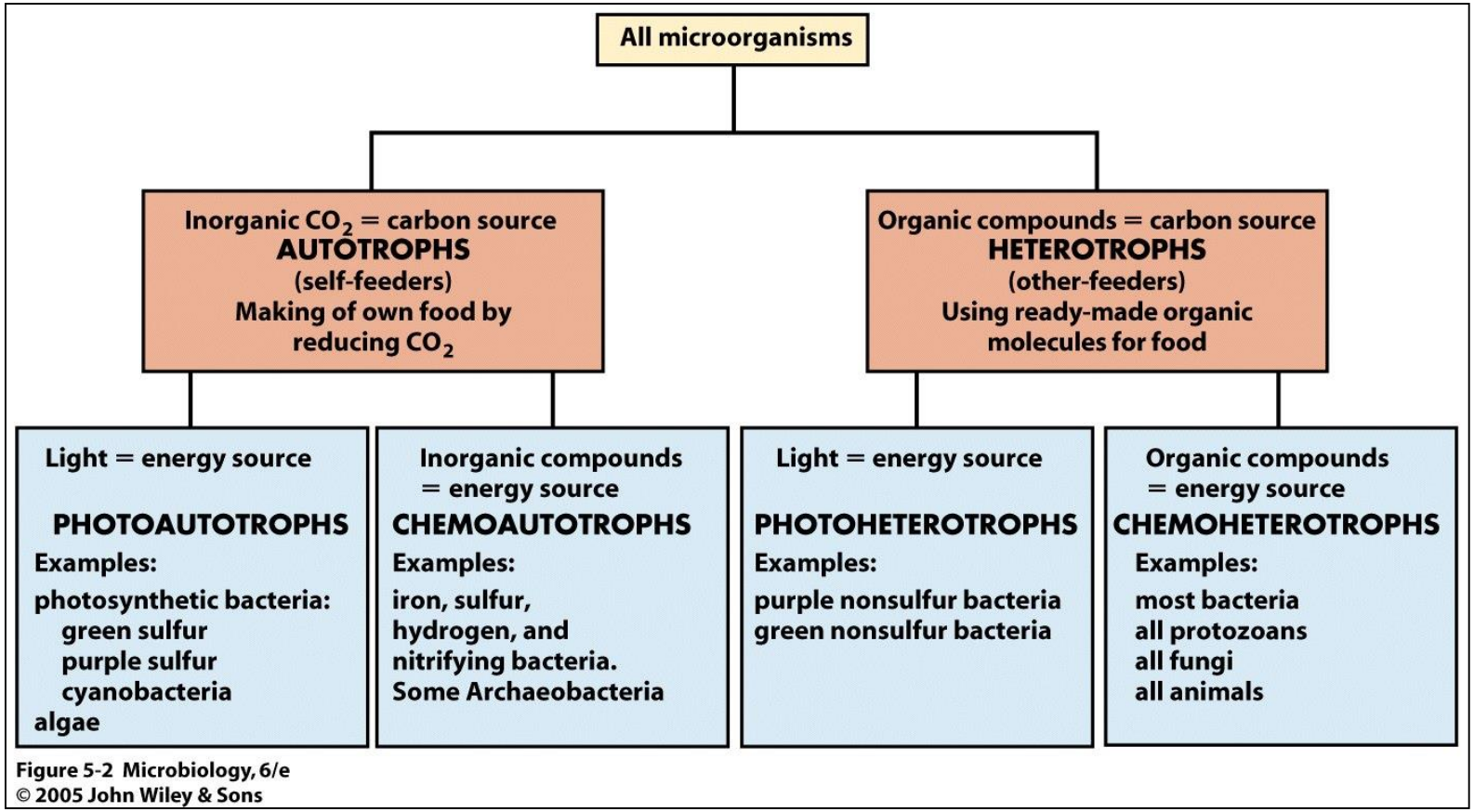
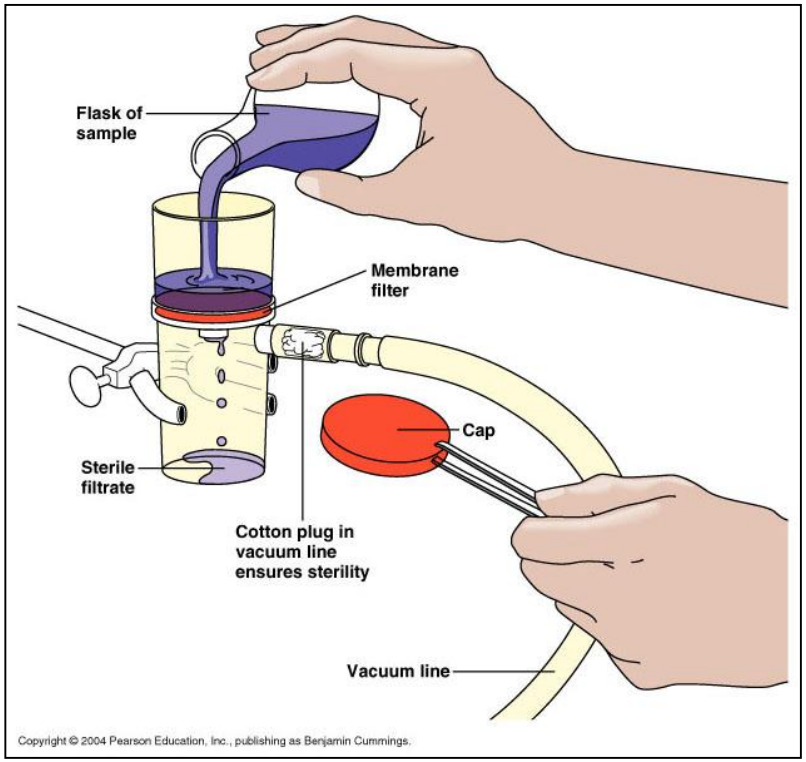


Figure 5-2 Microbiology, 6/e

C/N sources for utilization

Millipore filtration for C/N utilization tests

- Filtration was used for heat sensitive liquids such as serum, urea or C or N source- containing media.
- 0.45 µm or 0.22 µm pores in membrane.
- Decomposition and utilization of a C or N source can be determined by checking if growth or no growth takes place after spot-inoculation on a solidified minimal medium, containing the C or N source.



0.2>0.45>0.22
0.2 divided by 0.22 ≈1
0.45 divided by 0.22 ≈2
0.2 divided by 0.45 ≈0.4

C/N sources for utilization

Acid formation from carbon sources

- Tests have been developed to determine if bacteria are able to decompose certain C sources (e.g. sugars, alcohols, organic acids, glycosides) and/or N sources (e.g. amino acids).
- This may be done by inoculation of tubes containing a sole C or N source in minimal medium, including a pH indicator.
- If the bacterium is able to decompose the C or N source to acid or alkali products, the pH indicator (and therefore the colour of the medium) will change.

Acid formation from D-trehalose, maltose, α-methylglucoside and glucose (O-tube):

Check for yellow color, score green as negative (-), faint green as doubtful (d), a little yellow in the top layer as weak positive (+/-)and a strong yellow color as positive (+).

Glycoside=Sugar + a small organic compound

C/N sources for utilization

Akaline formation from carbon sources

- The same principle as for acid formation from carbon sources, but now bacteria form alkaline products following decomposition of the carbon compound.
- The indicator dye will change from green to blue when the pH is raised.

Alkali formation from NA-malonate:

Check for a blue color, score green or yellowish as negative (-), dark green as doubtful (d), a little blue in the top layer as weak positive (+/-) and a strong blue colour as positive (+).

Janse,2010

C/N utilization tests

Incompatibility of sugars with amino acids in the autoclave

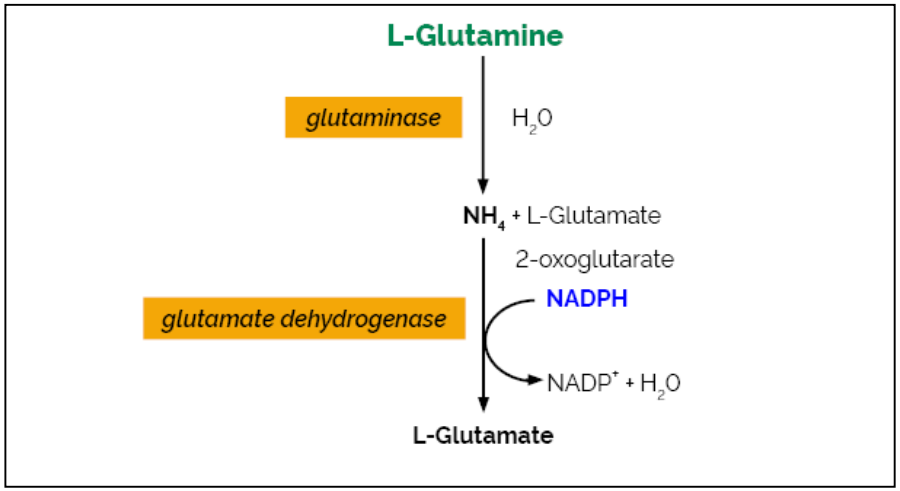
- The base for almost all media is amino acid-rich powders such as yeast extract and tryptone. There isn't any apparent incompatibility with sugars and amino acids, and there isn't – at room temperature.
- In the autoclave, however, sugars and amino acids fall victim to the Maillard reaction.
- In Maillard reaction, the amino group of an amino acid reacts with a carbonyl group of a sugar, fusing the two molecules together.
- This is a common reaction in our kitchens, making bread crusts brown, and changing the flavors of foods as we cook them.
- Therefore, sugars should be autoclaved separately from amino-acid (and protein) containing solutions, if at all.

Plank,2017

C/N utilization tests

L-glutamine is a highly thermolabile and should not be autoclaved as it is temperature-sensitive

- L-glutamine is a highly thermolabile and non-volatile biomolecule that is chemically modified into different compounds at high temperatures.
- When autoclaved at 121°C and 15 psi for 20 min, L-glutamine was structurally modified into 5-oxo proline and 3-amino glutarimide (α-amino glutarimide), respectively.

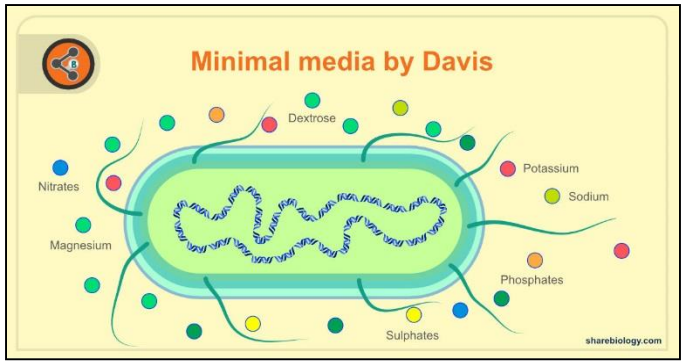


Sandal *et al.*,2011

C/N utilization tests

Minimal medium

- For experiments when a chemically defined medium is required.
- Carbon source can vary.
- Ammonium sulfate (1.0 g/L)
- Dipotassium phosphate (7.0 g/L)
- Monopotassium phosphate (3.0 g/L)
- Magnesium sulfate (0.1 g/L)
- Carbon source (1-10 g/L)
- Agar (15.0 g/L)
- pH 7



C/N utilization tests

Minimal medium

Ingredients	For 100mL	For 500mL	For 1000ml
Dextrose	0.1 gm	0.5 gm	1 gm
Ammonium sulphate	0.1 gm	0.5 gm	1 gm
Dipotassium phosphate	0.7 gm	3.5 gm	7 gm
Monopotassium phosphate	0.2 gm	1 gm	2 gm
Sodium citrate	0.05 gm	0.25 gm	0.5 gm
Magnesium sulphate	0.01 gm	0.05 gm	0.1 gm
Agar	1.5 gm	7.5 gm	15 gm
Distilled water	Up to 100 ml	Up to 500 ml	Up to 1000 ml

C/N utilization tests
Dye's medium C

- The was prepared with the composition of:
- $NH_4H_2PO_4$, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4.7H_2O$, 0.2 g; NaCl, 5 g; Yeast extract, 10 g; carbon source, 5 g; bromocresol purple (1.5% in alcohol solution), 0.7 ml and agar 12 g in 1 liter-distilled water (Dye,1962 cited in Bradbury,1984).
- The medium was poured to test tubes and autoclaved at 121^{o}C for 15 minutes.
- The isolates were stab inoculated and incubated at 28 ^{o}C.
- After 2, 4, 7, 14 and 21 days of incubation, a change in color from blue to yellow was recorded as positive for utilization of the carbohydrate.
- Uninoculated tubes were taken as negative control.

Bobosha,2003

C/N utilization tests

Ayers, Rupp and Johnson Medium

Without carbon sources

- $NH_4H_2PO_4$ 1.0 g
- KCl 0.2 g
- $MgSO_4$ 0.2 g
- Bromothymol blue 75 ml (solution 0.2%)
- Carbon source 10 g
- Distilled water to 1 L.
- Adjust pH to 7.
- Distribute base medium into tubes and sterilize by autoclaving at 120°C for 15 min.
- To solidify the medium add 12.0 g of Oxoid Agar.

Ayers, S. H., Rupp, P. & Johnson, W. T. 1919. A Study of Alkali-forming Bacteria in Milk. Bulletin no. 782. Washington, DC: US Department of Agriculture.

Antibiotic susceptibility test
Antibiogram

- Testing sensitivity to an antibiotic is important when this antibiotic is used to control a particular bacterium in the field (e.g. streptomycin against *Erwinia amylovora*).
- These tests can be carried out either:

1. Diffusion disc method (Antibiogram), or
2. Broth tube dilution method.

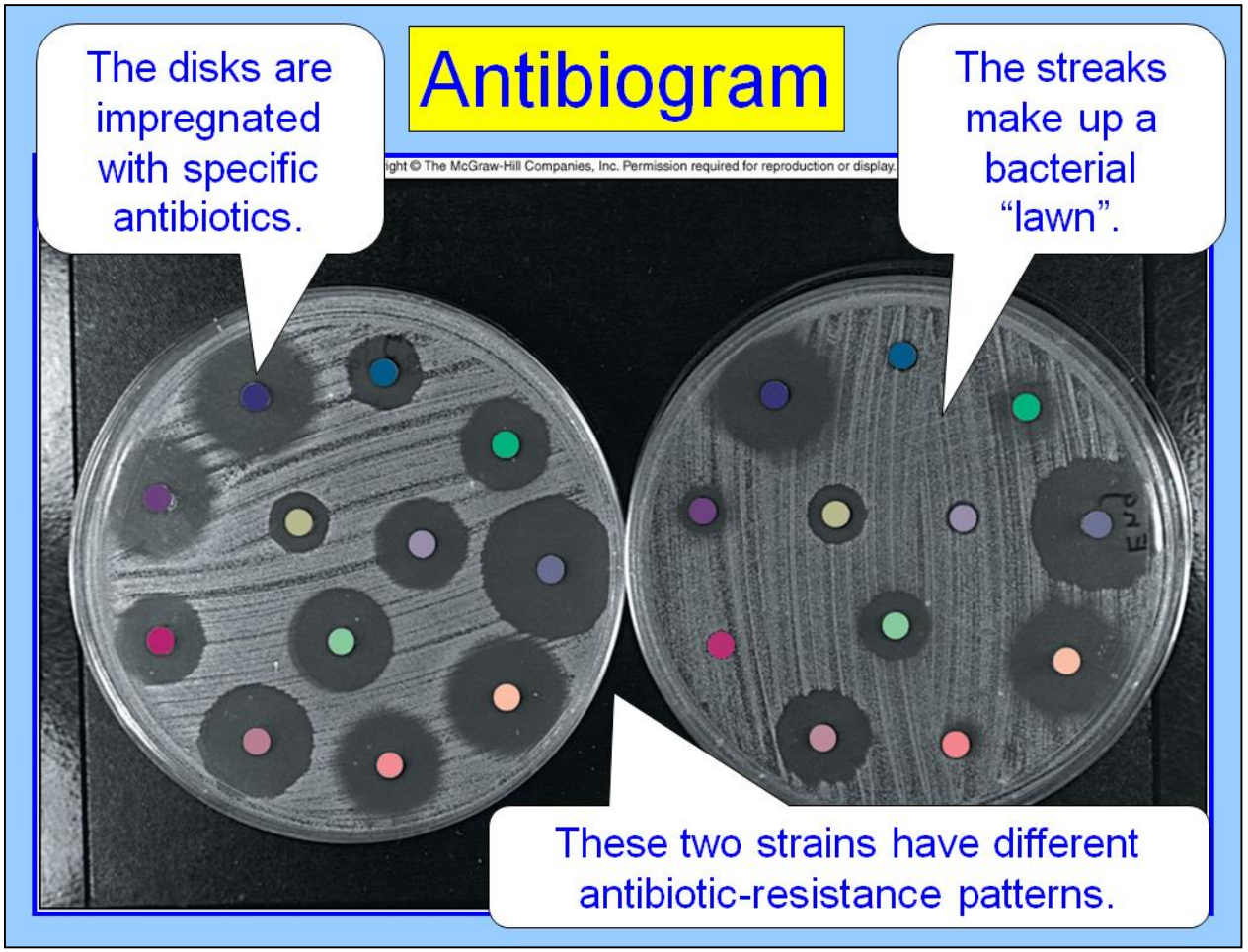
Diffusion disc method

Antimicrobial discs

- Position disks such that the minimum center - center distance is 24 mm and no closer than 10 to 15 mm from the edge of the Petri dish.
- A maximum of six disks may be placed in a 9-cm Petri dish and 12 disks on a 150 mm plate.
- Reduce the number of disks applied per plate if overlapping zones of inhibition are encountered.

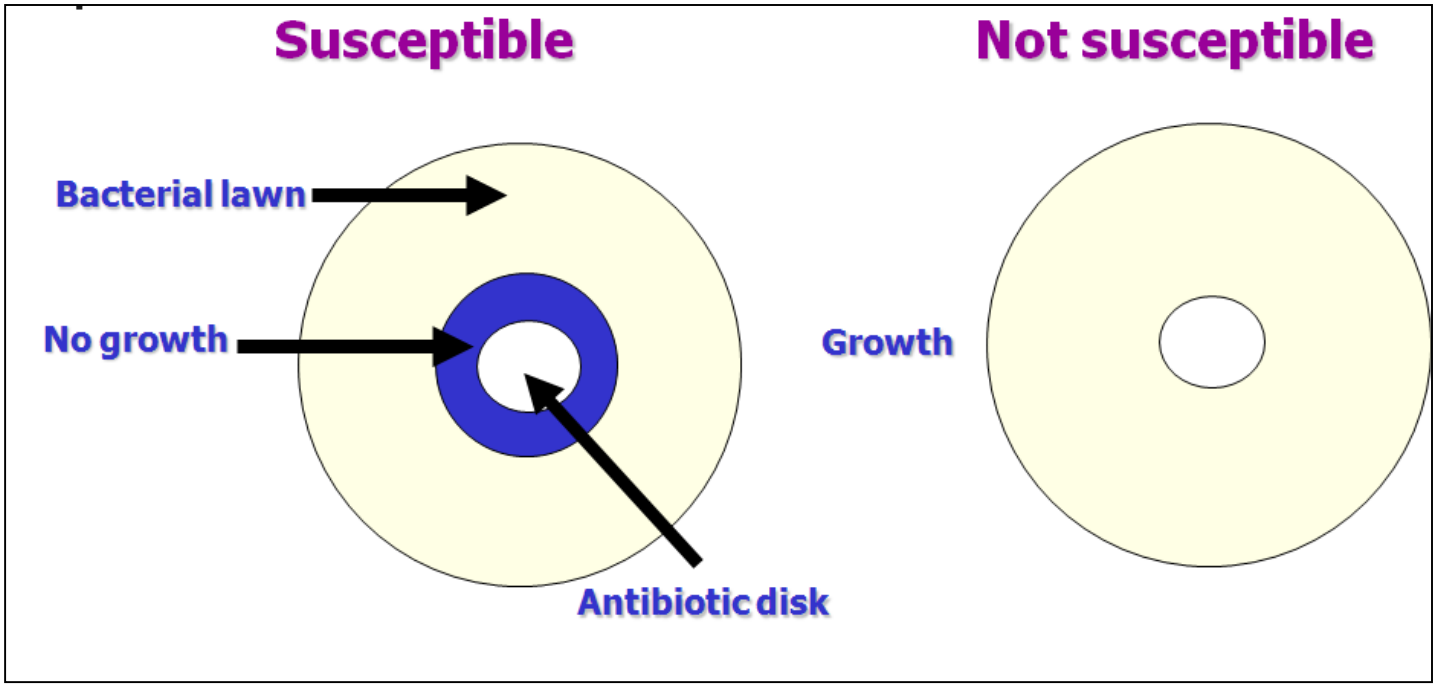
Diffusion disc method

Antimicrobial susceptibility testing



Abedon,2011

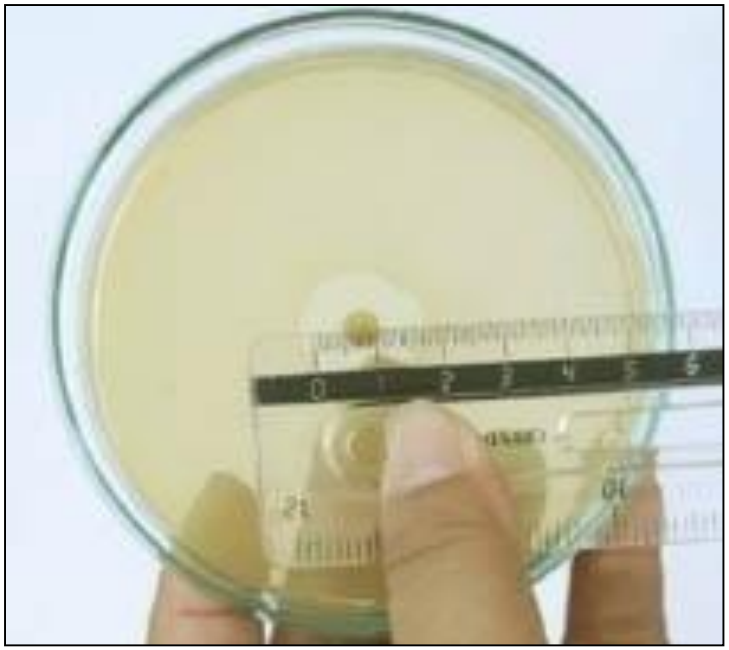
Antibiotic susceptibility testing



Fox,2008

Diffusion disc method
Reading

- Read and record the diameter of the zones of inhibition using a ruler graduated to 0.5 mm.
- Round up the zone measurement to the nearest millimeter.
- Determine the MIC's for all antibiotics:
- Negative = NO GROWTH
- Positive = GROWTH



MIC definition: MIC was defined as the lowest concentration that exhibits no growth by visual reading, and the strains were considered susceptible for each drug, if their MICs were below or equal to the critical concentration.

Broth tube dilution method

Broth tube dilution method for determining the MICs

- Specific amounts of the antibiotic are prepared in decreasing concentrations.
- This dilution series of the antibiotic is then inoculated with a culture of the organism to be tested.
- The turbidity of the inoculum is based on comparison to a McFarland standard for turbidity.
- The susceptibility of the organism is determined after suitable incubation by macroscopic observation for the presence or absence of growth in the varying concentrations of the antimicrobial agent.
- This bacteriostatic end-point value is known as the Minimal Inhibitory Concentration (MIC).

Commercial kits
Phenetic identification

Miniaturized and automated identification techniques

Automation is the need of the hour.

Need to integrate medicine and innovative technology in our health system

RAPID – ACCURATE – COST EFFECTIVE

Rapid multitest panels for identification of bacteria

Commercial kits

- A major innovation in laboratory sciences occurred in the late 1960s, when manual miniaturized identification systems(packaged microbial identification systems)were first introduced into the clinical microbiology laboratory.
- These early systems included:

1. The nine-test Enterotube, and
2. Two-tube R-B systems for identifying members of *Enterobacteriaceae*.

Janda and Abbott,2002

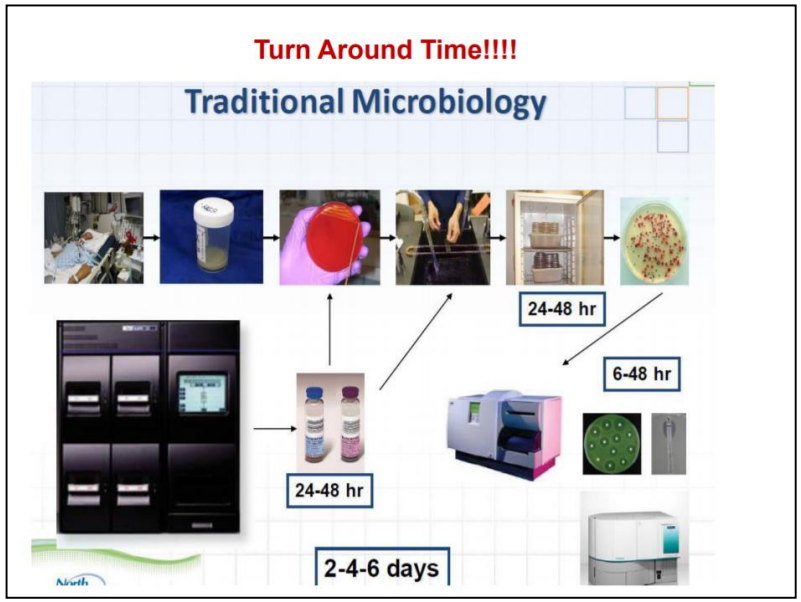
Rapid multitest panels for identification of bacteria

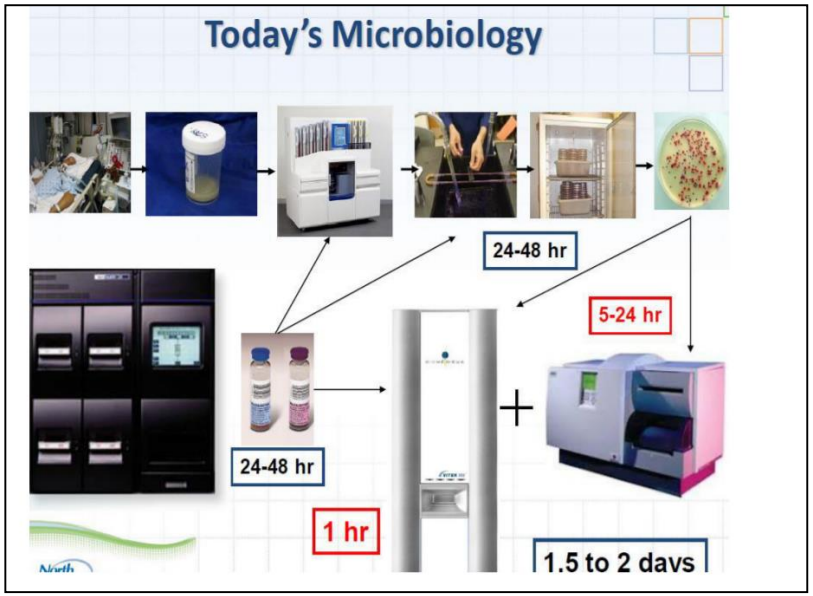
Commercial kits

- Today there are numerous standardized and miniaturized versions of those biochemical procedures.
- Today there are numerous standardized and miniaturized versions of those biochemical procedures.
- These automated diagnostic systems identify bacteria as little as 2 to 4 h.

Janda and Abbott,2002

Traditional vs. Today's Microbiology
Commercial kits





Commercial kits developed on the basis of biochemical tests/Acid production or utilization of C/N sources.

Example: Differentiated characteristics of *Pectobacterium/Dickeya* spp.

Test	Ecc	Eca	Ecb	Eco	Ecw	Ech	Ect
Growth at 37°C	+	-	+	+	-	+	+
Reducing sugars from sucrose	-	+	+	+	-	-	-
Phosphatase activity	-[b]	-	-	-	-	+	V
Sensitivity to erythromycin	-	-	-	ND	-	+	-
Indole production	-	-	-	-	-	+	-
Acid produced from :							
sorbitol	-	-	-	+	ND	-	-
melibiose	+	+	-	+	-	+	-
citrate	+	+	-	+	+	+	ND
raffinose	+	+	-	ND	-	+	-
arabitol	-	-	-	+	ND	-	ND
lactose	+	+	+	+	+	+	-
Utilization of keto-methyl glucoside	-	+	+	+	-	-	-

+, 80% or more strains positive; V, between 21-79% of strains positive; -, 80% or more strains negative; ND, not determined.

[a] Ecc = *E. carotovora* subsp. *carotovora*
Eca = *E. carotovora* subsp. *atroseptica*
Ecb = *E. carotovora* subsp. *betavasculorum*
Eco = *E. carotovora* subsp. *odorifera*
Ecw = *E. carotovora* subsp. *wasabiae*
Ech = *E. chrysanthemi*
Ect = *E. cacticida*

[b] Some strains of *E. carotovora* subsp. *carotovora* may be weakly positive

Schaad *et al.*,2001

Concepts of technology

Commercial kits

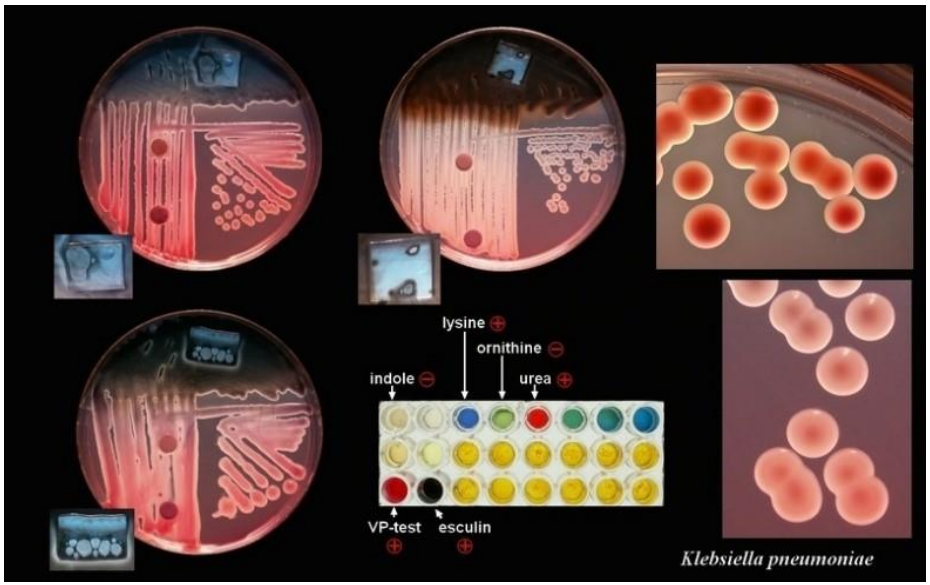
Mechanism of actions

- All commercial identification systems are based on one of five different technologies or a combination thereof.
- These include:
- pH-based reactions that require from 15 to 24 h of incubation,
- Enzyme-based reactions that require 2 to 4 h,
- Utilization of carbon sources,
- Visual detection of bacterial growth,
- Detection of volatile or nonvolatile fatty acids via gas chromatography.

O'Hara,2005

Manual of some diagnostics systems
Commercial kits

- API 20E
- API 20NE
- RapiD 20E
- Crystal E/NF
- Enterotube II
- EPS
- GN2 Microplate
- ID 32E
- ID Tri-Panel
- Micro ID
- RapiD NF Plus
- RapID onE
- RapID SS/u
- UIUID/UID-3
- Uni-N/F-Tek
- Oxi/Ferm II
- R/B Enteric Differential System



O'Hara,2005;..

Automated identification systems
Commercial kits

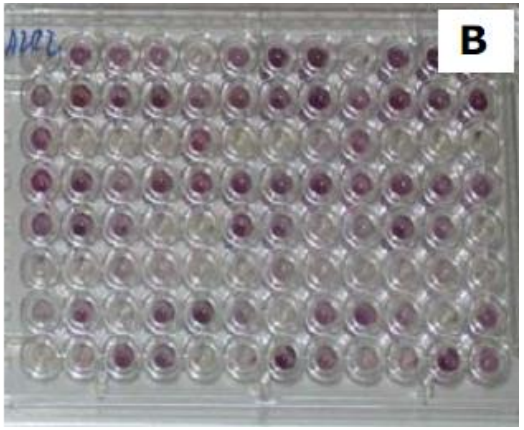
- **BD Phoenix 100**
- **NID**
- **bioMérieux Vitek**
- **GNI+**
- **ID-GNB**
- **2GN**
- **Dade Behring Microscan**
- **Neg ID type 3**
- **Rapid Neg ID type 3**
- **Sherlock Microbial Identification System**
- **Trek Diagnostic Systems**
- **Sensititre® GPID and GNID**

O'Hara,2005

Miniaturized and automated identification techniques

API and Biolog kits

API (Analytical Profile Index) system: Results are recorded as either a substrate colour change or as growth.



Biolog test: Tetrazolium dye turn to purple as the carbon sources are oxidized by bacteria.

Olamendi,2005

Comparison of commercial test kits for Gram negative organisms

- Various methods have different numbers and types of organisms within their database.
- Figure below, compares several popular kit-based methods.
- The Biolog GN2 MicroPlate has a much larger number of tests, which provides greater fingerprint discrimination and a larger database.

Manufacturer	Number of aerobic species in database*	Number of tests used for identification
Biolog, Inc MicroLog	501	95
BioMérieux®Vitek GNI+	104	29
BioMérieux API 20E® & NFT	~180	20
BBL®Crystal™	~105	28

* Need to update

Fenollar, 2000;..

BACTID system

Bactid: Bacteriological Identification System For Resource-Poor Plant Pathology Laboratories

- BACTICD analysis was used to eliminate non-pathogens and further identify the bacterial isolates.
- The basic motivation was to develop a scheme that would providees sufficient in formation for diagnostic purposes without necessarily resorting to complete identification.
- Originally conceived by the senior author and J.Kolkowsky at the International Mycological Institute, and based on Bradbury's key (Bradbury, 1970), the flow chart for the scheme is given in next Fig.

See Bradbury,1988 (appendix 2, part 2)

BACTID system

Bactid: Bacteriological Identification System For Resource-Poor Plant Pathology Laboratories

- BACTID describes a kit for bacterial identification based on modifying traditional methods for identification of all plant pathogenic bacteria into a cost-effective kit format.
- The key issue in the identification of plant pathogenic bacteria is the need to distinguish a potential pathogen from contaminating non-pathogenic bacteria.
- Colony type and colour and cell morphology are rarely sufficient for this purpose.
- Hence the need for many different specialised, selective or semi-selective media and a host of reagents for confirmatory tests.

BACTID system

A computer program

Scheme for preliminary identification

- Preparing these media conventionally in Petri dishes is time-consuming and the media so prepared have a limited shelf-life.
- The BACTID kit uses Eppendorf tubes instead of Petri dishes; the tubes can be prepared in bulk and kept for several months so the media are instantly available for use.
- Much smaller quantities of media ingredients and reagents are necessary for these small tubes.
- The BACTID kits were validated by using them for identifying plant pathogenic bacteria in working plant pathology laboratories (Plant Clinics) attached to agricultural research stations.

BACTID system

A computer program

Scheme for preliminary identification

- The Bactid system/kit has been developed to provide an accessible approach to bacteriological identification in plant pathology laboratories which are not equipped for, or used to, routine bacteriology work.
- The system comprises:

1. The Bactid scheme,
2. The Bactid kit, and
3. The Bactid program.

- The accompanying Bactid software provides full details of media and reagents and instructions on how to do the tests.

Tests for bacterial identification included in the BACTID kit

Bact ID

Nitrate reduction test (NIT) (P. fusco is neg.)

Tetrazolium chloride tolerance (TTC) test (P. fusco is pos.)

Oxidation/Fermentation of carbohydrates test (P. fusco is + for oxd'n and – for fermentation)

EH GRAHAM CENTRE for Agricultural Innovation

Bactid-Identification system for *R. solanacearum*

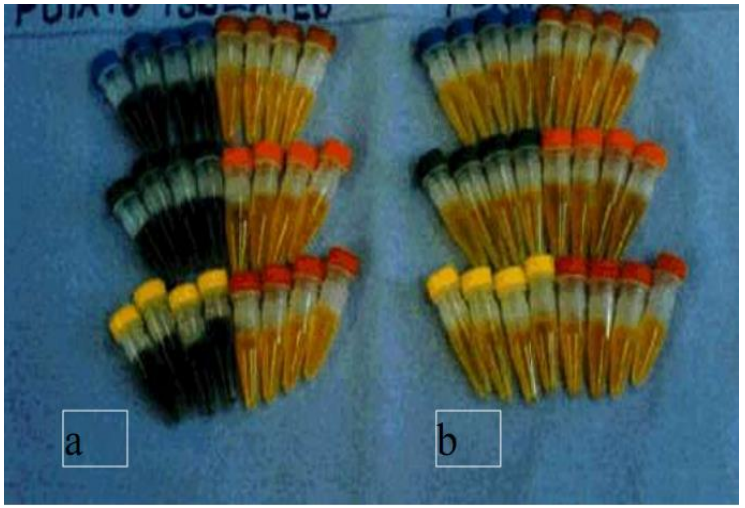
BACTID biovar kit

- BACTID biovar kit used to determine the biovars of isolates of *R. solanacearum* from tomato and potato:

a) biovar 2 (potato isolate);

b) biovar 3 (tomato isolate).

- A yellow colour indicates the metabolism of one of the differential substrates of the biovar test.
- Biovar 2 only metabolises disaccharides (right hand side of each set), biovar 3 metabolises disaccharides and sugar alcohols (left hand side of each set).



The system characterize at subspecies level.

Black and Seal,1999

BACTID analysis

Phenotypic characterization of *E. chrysanthemi* pv. *dieffenbachiae*, *X. campestris* pv. *dieffenbachiae*, *P. syringae* and a non-fluorescent *Pseudomonas*

Isolate no.	KOH	TTC	Oxidase	KMB	Starch	Gelatin	Nitrate	O/F	Leavan	Potato rot
E. chrysanthemi pv. *dieffenbachiae*										
1	+	-	(+)	-	(+)	+	+	-/-	n	n
10	+	-	-	-	(+)	+	+	+/+	n	n
X. campestris pv. *dieffenbachiae*										
2	+	-	-		-	+	-	+/-	n	n
4	+	-	-	-	-	+	-	+/-	n	n
5	+	-	-	-	-	+	-	-/-	n	n
6	+	-	-	-	-	+	-	+/-	n	n
7	+	-	-	-	-	+	-	+/-	n	n
Pseudomonas										
3	+	-	-	+	-	+	-	+/-	+	-
8	+	+	-	-	-	+	-	+/-	n	n
9	+	-	-	+	-	+	-	+/-	+	-

KEY: +: strong positive reaction; (+): weak positive reaction; -: negative reaction; n: not done.
TTC: Triphenyl tetrazolium chloride. KMB: Kings Medium B. O/F: Oxidation/Fermentation. KOH: Potassium hydroxide

Brown and Asemota,2009

Analytical Profile Index (API)

API 20E strip as gold standard

- These kits were rapidly followed by the landmark API 20E strip test which was a micromethod employing 20 different biochemical tests.
- The 20E strip generated a septyl (7-digit) code in 18 to 21 h based upon biochemical reactions plus a screening oxidase test.
- The API 20E strip test was so advanced for its time that it is still commonly used in clinical microbiology labs throughout the world and is considered by many to be the "gold standard" commercial system.

Janda and Abbott,2002

Biochemical Identification Kits

Analytical Profile Index (API)

API kits

- API tests provided useful alternatives to conventional procedures and identify usually as genus and species.
- Gram Negative Identification
- api 20E
- api 20E
- api NE
- api NH
- api Rapid 20E
- Gram Positive Identification
- api STAPH
- RAPIDEC® STAPH
- api 20 STREP
- api CORYNE
- Anaerobe Identification
- api 20A
- Rapid ID 32A
- Yeast Identification
- api 20C AUX or api 32C system
- The API 20C requires less preparation of reagents.

Analytical Profile Index (API)
API 20E, API 20NE and API RapiD 20E

- **API 20E**
- The bacterial database has expanded from 87 taxa in 1977 to 102 taxa in 2003. For identification of the Enterobacteriaceae.
- **API 20NE**
- The bacterial database contains 32 genera and 64 species of nonfastidious gram-negative rods not belonging to the Enterobacteriaceae.
- **API RapiD 20E**
- The API RapiD 20E system is designed to identify Enterobacteriaceae in 4 h.
- The database contains 26 genera and 65 species.
- **API 50 CH**
- Performance of carbohydrate metabolism tests.
- **API 20C AUX**
- Identification of yeasts.

API kits

The main and supplementary tests مشخصات تیوب ها

- A plastic strip with 20 miniaturized chambers containing dehydrated substrates and a plastic incubation chamber with a loose fitting lid.
- Each chamber is divided into two parts, the tube portion and the cupule(small cup) portion.
- Each chamber has a small hole at the top through which the bacterial suspension can be inoculated with a pipette.
- The 20 cupules strip contains 8 conventional substrates and 12 assimilation tests.
- Any color change in microtubes demonstrates enzymatic activity or fermentation of carbohydrates.
- Apart these, oxidase test is considered as an important initial differentiation for the flow chart.

API kits

The main and supplementary tests

- The oxidase test is considered an important part of the 21 tests in this strip.
- Oxidase positive organisms would be presumptively identified as non-fermenters; oxidase negative organisms would be presumptively identified as fermenters.
- The oxidase test may be performed in either the negative ONPG or H_2S cupules, and the nitrate test in the glucose tube.
- Read the explanation of the oxidase test in the Atlas.
- Be sure to include the oxidase (a positive is 4, negative is 0).
- The seven-digit profile number that is compiled from the test reactions is entered into the APILAB software.
- Identifications are also available by using the Analytical Profile Index.

API kits

Materials for API-20E strip and Enterotube II kits

- Culture media
- API-20E strip or Enterotube II
- Clean test tube
- Sterile saline or water
- Mineral oil
- 10% $FeCl_3$
- Kovac's reagent (Indole)
- 40% KOH
- 6% Alpha-naphthol
- 0.8% Sulfanilic acid
- 0.5% N, N-dimethyl alpha-naphtylamine

The API 20E System

The main and supplementary tests

- **The test consists of the following:**
  ONPG: Galactosidase
  ADH: Arginine Dihydolase
  LDC: Lysine Decarboxylase
  ODC: Ornithine Decarboxylase
  CIT: Citrate utilization
  H_2S: Hydrogen sulfide production
  URE: Urease
  TDA: Tryptophan Deaminase
  IND: Indole Production
  VP: Acetoin production (Voges Proskauer)
  GEL: Gelatinase
  GLU: fermentaion / oxidation (Glucose)
  MAN: fermentaion / oxidation (Mannitol)
  INO: fermentaion / oxidation (Inositol)
  SOR: fermentaion / oxidation (Sorbitol)
  RHA: fermentaion / oxidation (Rhamnose)
  SAC: fermentaion / oxidation (Saccharose)
  MEL: fermentaion / oxidation (Melibiose)
  AMY: fermentation / oxidation (Amygdalin)
  ARA: fermentation / oxidation (Arabinose)
- **Supplemental Tests also may be done with the API test:**
  OXI: Oxidase
  nitrates to nitrites (NO_2):
  N2 GAS:
  Motility(MOT):
  Growth on Mac Conkey agar medium(MAC):
  Oxidation of glucose (OF-O):
  Fermentation of glucose (OF-F):

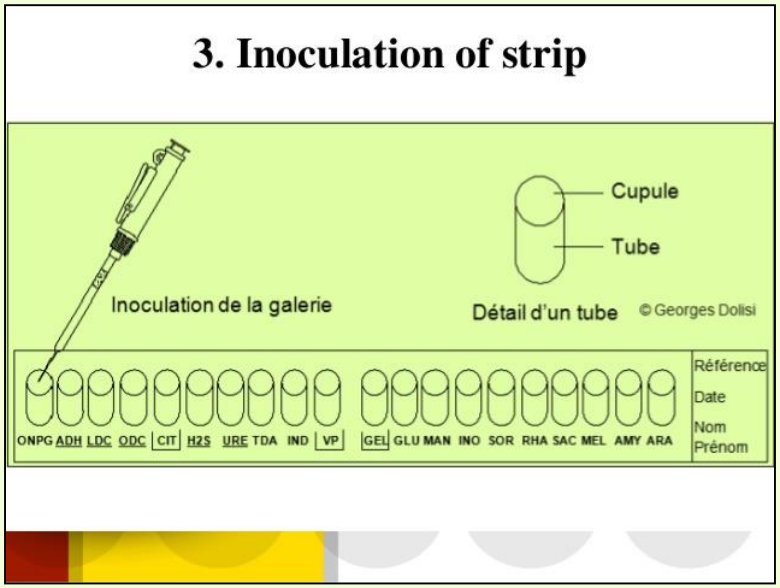
Inoculation procedure
API-20E strip

- For all strains, a cell suspension having a 0.5 McFarland optical density (MF) standard was made with 0.85% NaCl.
- Appropriate amounts of this material were added to the wells of an API strips (bioMerieux).
- Some tubes are overlaid with mineral oil such that anaerobic reactions can be carried out (ADH, LDC, ODC, H2S, URE).
- Fermentation is shown by an acid reaction (yellow color of indicator).
- Hydrogen sulfide production (H_2S) and gelatin liquefaction or hydrolysis (GEL) result in a black color throughout the tube.

Inoculation procedure

API-20E strip

- The strips were incubated for 48 h at 30°C, and the results were recorded by visual inspection and scored with an APILAB PLUS software package as proposed by the manufacturer.
- The seven-digit profile number is converted to an identification by using the APILAB software, version 3.3.3.

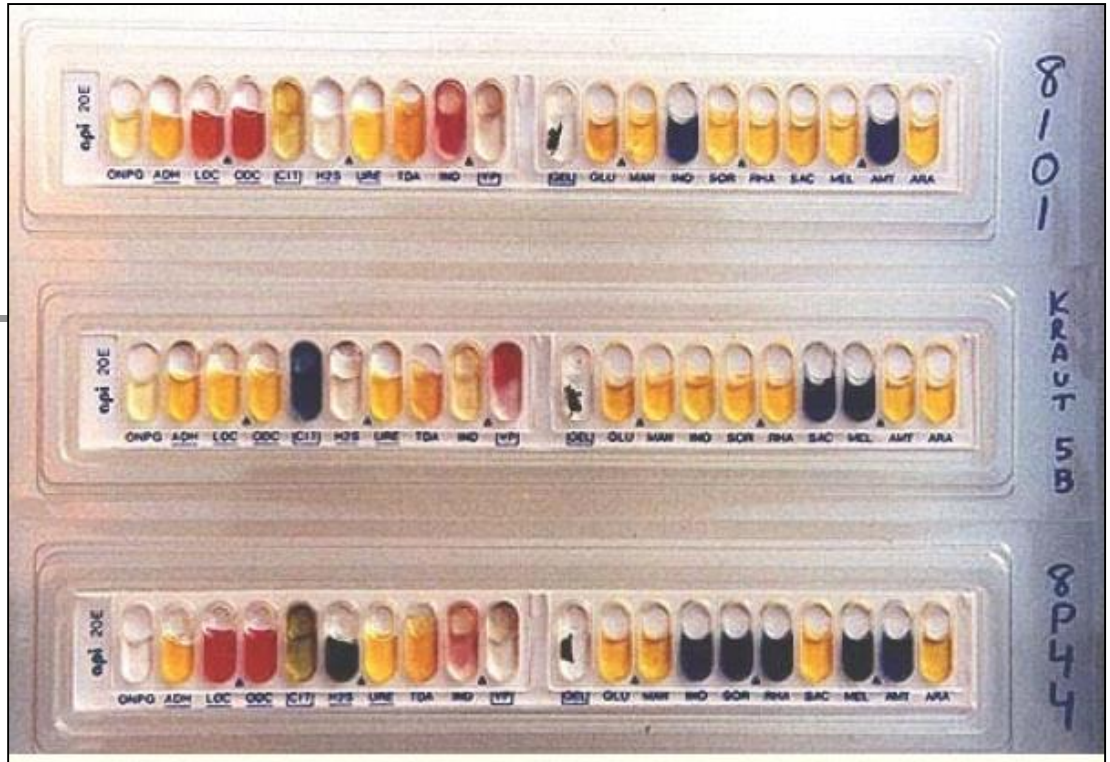


Noman Farrukh

API 20E

®API-20E The Enteric Identification (designed System for members of the Family *Enterobacteriaceae* and associated organisms).

ONPG: Galactosidase
ADH: Arginine Dihydolase
LDC: Lysine Decarboxylase
ODC: Ornithine Decarboxylase
CIT: Citrate utilization



culture no.	ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	identification
8101	+	–	+	+	–	–	–	–	+	–	–	+	+	–	+	+	+	+	–	+	*Escherichia coli*
5B	+	–	–	–	+	–	–	–	–	+	–	+	+	+	+	+	–	–	+	+	*Enterobacter agglomerans*
8P44	–	–	+	+	–	+	–	–	+	–	–	+	+	–	–	–	+	–	–	+	*Edwardsiella hoshinae*

The oxidase reaction constitutes the 21st test and has a value of 4 if it is positive.

Interpretation of results
API-20E strip

- **Main tests:**

1. A very pale yellow should also be considered positive.
2. An orange color after 36-48 hours incubation must be considered negative.
3. Reading made in the cupule (aerobic).
4. Fermentation begins in the lower portion of the tube; oxidation begins in the cupule.
5. A slightly pink color after 10 minutes should be considered positive.

Tests	Reactions or Enzymes	Results	
		NEGATIVE	POSITIVE
ONPG	ß-galactosidase	colorless	yellow (1)
ADH	arginine dihydrolase	yellow	red / orange (2)
LDC	lysine decarboxylase	yellow	red / orange (2)
ODC	ornithine decarboxylase	yellow	red / orange (2)
CIT	citrate utilization	pale green / yellow	blue-green / blue (3)
H_2S	H_2S production	colorless / greyish	black deposit / thin line
URE	urease	yellow	red / orange (2)
TDA	tryptophane deaminase	TDA / immediate	
		yellow	reddish brown
IND	indole production	KOVAC'S /immediate	
		colorless pale green / yellow	pink
VP	acetoin production (Vogues Proskauer)	VP 1 + VP 2 / 10 min.	
		colorless	pink / red (5)
GEL	gelatinase	no diffusion	diffusion of black pigment
GLU	fermentation or oxidation (4)	blue / blue-green	yellow / greyish yellow
MAN	fermentation or oxidation (4)	blue / blue-green	yellow
INO	fermentation or oxidation (4)	blue / blue-green	yellow
SOR	fermentation or oxidation (4)	blue / blue-green	yellow
RHA	fermentation or oxidation (4)	blue / blue-green	yellow
SAC	fermentation or oxidation (4)	blue / blue-green	yellow
MEL	fermentation or oxidation (4)	blue / blue-green	yellow
AMY	fermentation or oxidation (4)	blue / blue-green	yellow
ARA	fermentation or oxidation (4)	blue / blue-green	yellow
OX	cytochrome oxidase	oxidase test -- See test package insert	

Identification
Based on seven-digit profile numbers

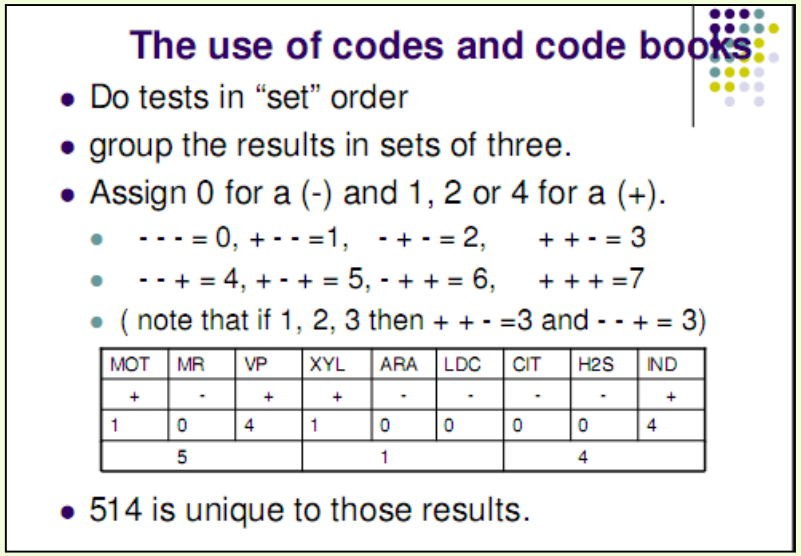
- Identification is obtained with the numerical profile.
- **Determination of the numerical profile:**
- On the result sheet, the tests are separated into:

1. groups of 3, and
2. a value 1, 2 or 4 is indicated for each.

- By adding together the values corresponding to positive reactions within each group, a 7-digit profile number is obtained for the 20 tests of the API 20 E strip.
- The oxidase reaction constitutes the 21st test and has a value of 4 if it is positive.

Identification
Based on seven-digit profile numbers

- Each compartment has a corresponding points.
- Every 3 compartment (or test) is given a number.
- **The points for positive results of every 3 set is 1, 2, and 4 (in order). Zero for negative results.**
- No point is given to e.g. ADH because the result is negative.
- Based on profile number identification of the test bacterium is made.



API 20E

Based on seven-digit profile numbers

Brenneria nigrifluens and *Brenneria rubrifaciens*

- Isolates that were Gram negative, oxidase-negative and with oxidative and fermentative metabolism were submitted to the API 20E procedure.
- *Brenneria nigrifluens*, the causal agent of shallow bark canker of walnut including the reference strains generated a 7-digit code=0005773 in the API 20E system, identical for the type strain of *B. nigrifluens*.
- *Brenneria rubrifaciens*, the causal agent of deep bark canker of walnut generates different 7-digit codes (0004022 or 0004122).

Moretti *et al.*,2007

Identification

Based on 27 tests and nine-digit profile numbers

- In some cases, the 7-digit profile is not discriminatory enough and the following supplementary tests need to be carried out.

Identification

Based on 27 tests and nine-digit profile numbers

- If in case all 27 tests were used, then THERE SHOULD BE 9 NUMBERS [27 tests/compartments divided into 3 groups each is 9].
- A test result for a specific bacteria such as *Escherichia coli* will be:

  + - + + - - - - + - - + + - + + - + - + - + - + + + +

  or

  5 1 4 4 5 5 2 5 7
- The nine-digit profile number is then converted to identify *E. coli*.

Identification

API 50 CH strip

apiweb identification software (Ref. 40 011), ATB instrument or mini API (consult bioMérieux)

- CONTENT OF THE KIT (Kit for 10 tests):

1. 10 ampules of API 50 CHB/E Medium,
2. 1 package insert provided in the kit or downloadable from www.biomerieux.com/techlib

Identification

API 50 CH strip

apiweb identification software (Ref. 40 011), ATB instrument or mini API (consult bioMérieux)

- API 50 CHB/E Medium is intended for the identification of *Bacillus* and related genera, as well as Gram-negative rods belonging to the Enterobacteriaceae and Vibrionaceae families.
- It is a ready-to-use medium which allows the fermentation of the 49 carbohydrates on the API 50 CH strip to be studied.
- Reading the strips:
- A positive test corresponds to acidification revealed by the phenol red indicator contained in the medium changing to YELLOW.
- For the esculin test (tube no. 25), a change in color from red to BLACK is observed.

Item	C5	C6	C13	R2	R3	R9	A3	A4	A6	F7	F8
Control	–	–	–	–	–	–	–	–	–	–	–
Glycerol	w	+	w	+	+	+	+	+	+	+	–
Erythritol	w	w	w	w	w	w	w	w	w	w	w
D-Arabinose	+	+	w	+	w	+	–	w	+	+	w
L-Arabinose	+	+	+	+	+	+	w	+	+	+	+
D-Ribose	+	+	+	+	+	+	+	+	+	+	+
D-Xylose	+	+	w	+	w	+	w	w	+	+	+
L-Xylose	w	w	w	w	w	w	–	w	–	w	w
D-Adonitol	w	w	w	w	–	–	–	–	–	–	–
Methyl-D-xylopyraniside	w	w	w	w	–	–	–	–	–	w	w
D-Galactose	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+	+	+
L-Sorbose	+	+	+	+	w	+	w	+	+	+	–
L-Rhamnose	+	+	+	+	w	+	w	+	+	+	+
Dulcitol	+	+	+	w	w	w	w	w	w	w	+
Inositol	+	+	+	w	w	w	w	w	w	–	–
D-Mannitol	+	+	+	+	+	+	+	+	+	+	+
D-Sorbitol	+	+	+	+	+	+	+	+	+	+	+
Methyl-D-mannopyranoside	+	+	+	+	+	+	w	w	+	+	+
Methyl-D-glucopyranoside	+	+	+	+	+	w	+	+	+	+	+
N-acetylglucosamine	+	+	+	+	+	+	+	+	+	+	+
Amygdaline	+	+	+	+	+	+	+	+	+	+	+
Arbutine	+	+	+	+	+	+	+	+	+	+	+
Esculine	–	–	–	–	–	–	–	–	–	–	–
Salicine	+	+	+	+	+	+	+	+	+	+	+
D-Celiobiose	+	+	+	+	+	+	+	+	+	+	+
D-Maltose	+	+	+	+	+	+	+	+	+	+	+
D-Lactose	+	+	+	+	+	+	+	+	+	+	+
D-Melibiose	+	+	+	+	+	+	w	+	+	+	+
D-Saccharose	+	+	+	+	+	+	+	+	+	+	+
D-Trehalose	+	+	+	+	+	+	+	+	+	+	+
Inuline	+	+	+	+	+	w	w	+	+	w	–
D-Melezitose	+	+	+	+	+	+	+	+	+	+	+
D-Raffinose	+	+	+	+	+	+	+	+	+	+	+
Amidon	+	+	+	+	+	+	+	+	+	+	w
Glycogen	+	+	w	w	w	w	–	w	w	+	w
Xylitol	+	w	w	w	w	w	w	w	w	+	w
Gentiobiose	+	+	+	+	+	+	+	+	+	–	–
D-Turanose	+	+	+	+	+	+	w	+	+	+	w
D-Lyxose	+	+	+	w	w	w	w	w	w	w	w
D-Tagatose	w	+	+	+	+	+	+	+	+	w	w
D-Fucose	w	w	w	w	w	w	w	w	w	w	w

Strain's carbohydrates fermentation patterns on API 50 CH panel

Identification

API 50 CH strip

apiweb identification software (Ref. 40 011), ATB instrument or mini API (consult bioMérieux)

Identification

API 50 CH strip

apiweb identification software (Ref. 40 011), ATB instrument or mini API (consult bioMérieux)

- The media and strips are systematically controlled at various stages of their manufacture. For those users who wish to perform their own quality control tests with the strip, the following strain may be used:
- Example: Test For identification of *Bacillus* spp.
- *Paenibacillus polymyxa* ATCC®43865 was used as control strain.

For *Bacillus* : ***Paenibacillus polymyxa*** **ATCC® 43865**

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
24	-	+	-	-	+	+	+	-	-	V	+	+	+	+	-	V	-	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-
48	-	+	-	-	+	+	+	-	-	+	+	+	+	+	-	V	-	-	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	V	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-

Results obtained after incubation at 30°C.

Identification of *Bacillus* spp. by API 50 CH strip

TABLEAU D'IDENTIFICATION / IDENTIFICATION TABLE / PROZENTTABELLE / TABLA DE IDENTIFICACION / TABELLA DI IDENTIFICAZIONE / QUADRO DE IDENTIFICAÇÃO / ΠΙΝΑΚΑΣ ΤΑΥΤΟΠΟΙΗΣΗΣ / IDENTIFIERINGSTABELL / IDENTIFIKATIONSTABEL / TABELA IDENTYFIKACYJNA

Bacillus

% de réactions positives dans les conditions d'incubation précisées à droite du tableau / % of positive reactions in the incubation conditions specified on the right-hand side of the table / % der positiven Reaktionen unter den in der Tabelle angegebenen Inkubationsbedingungen / % de las reacciones positivas en las condiciones de incubación indicadas a continuación / % di reazioni positive nelle condizioni di incubazione sotto indicate / % das reacções positivas nas condições de incubação indicadas aqui abaixo / % θετικών αντιδράσεων στις συνθήκες επώασης που ορίζονται στη δεξιά πλευρά του πίνακα /% positiva reaktioner under de inkubationsförhållanden som anges i tabellens högra kant / % positive reaktioner under inkubationsbetingelser som specificeret i tabellens højre side / % pozytywnych reakcji po inkubacji w warunkach określonych po prawej stronie tabeli

API 50 CHB V4.0	0	1 GLY	2 ERY	3 DARA	4 LARA	5 RIB	6 DXYL	7 LXYL	8 ADO	9 MDX	10 GAL	11 GLU	12 FRU	13 MNE	14 SBE	15 RHA	16 DUL	17 INO	18 MAN	19 SOR	20 MDM	21 MDG	22 NAG	23 AMY	24 ARB	25 ESC	26 SAL	27 CEL	28 MAL	29 LAC
Aneurinibacillus aneurinilyticus	0	50	0	0	9	33	9	0	0	0	33	33	33	9	0	0	0	0	9	0	9	0	9	9	9	9	9	9	9	33
Bacillus anthracis	0	49	1	0	1	99	1	0	0	0	0	100	79	2	0	0	0	1	0	1	0	0	99	0	79	97	33	71	99	1
Bacillus cereus 1	0	74	0	1	1	99	1	0	0	1	8	100	99	26	0	1	0	1	1	1	1	3	97	30	99	99	88	88	100	3
Bacillus cereus 2	0	11	1	0	0	77	1	0	0	0	3	100	99	1	0	0	0	0	0	0	0	0	99	0	43	68	24	2	100	10
Bacillus circulans	0	48	0	18	96	80	98	1	1	68	94	100	97	97	1	45	1	20	89	20	32	64	84	99	96	100	99	99	99	92
Bacillus coagulans	0	71	0	4	47	66	52	0	1	1	98	100	100	98	1	42	1	9	28	38	4	66	100	71	76	83	76	76	100	66
Bacillus firmus	0	41	0	0	4	20	7	0	0	0	1	88	50	11	0	0	0	1	66	2	0	2	63	1	4	55	1	11	92	1
Bacillus lentus	0	40	0	1	50	55	10	0	0	10	55	95	95	95	0	40	17	1	60	17	1	35	82	65	82	98	75	75	98	82
Bacillus licheniformis	0	90	1	1	99	97	87	1	1	1	75	100	100	99	8	32	1	69	99	92	1	99	62	99	99	100	99	99	100	44
Bacillus megaterium	0	80	1	1	87	86	76	1	1	1	82	100	99	28	1	8	1	55	97	39	3	30	87	73	80	97	84	83	99	76
Bacillus mycoides	0	20	0	0	1	98	1	0	0	0	28	100	99	4	0	0	0	1	4	1	0	1	99	12	80	77	80	31	98	20
Bacillus pumilus	0	72	1	1	88	97	65	0	0	0	49	99	100	99	1	14	1	11	99	2	37	27	64	62	98	100	99	99	35	14
Bacillus smithii	0	89	0	0	36	97	97	0	0	0	36	100	100	75	0	45	0	2	100	2	0	89	0	0	2	24	10	24	100	0
B. subtilis / B. amyloliquefaciens	0	77	0	0	84	91	56	0	0	0	12	95	98	87	1	1	1	65	95	86	0	83	29	70	80	100	86	97	98	23
Bacillus non reactive *	0	1	1	0	1	1	2	0	1	1	2	6	10	2	1	1	0	2	9	1	1	1	24	1	1	19	1	1	5	2
Brevibacillus agri	0	21	0	0	0	0	0	0	0	0	0	42	42	0	0	0	0	21	94	0	0	0	0	0	0	5	0	0	0	0
Brevibacillus laterosporus	0	72	0	0	1	61	20	0	0	0	0	98	98	66	0	1	0	1	61	5	0	1	98	38	88	94	79	66	88	0
Brevibacillus non reactive **	0	13	0	0	1	6	2	0	0	1	0	23	19	1	0	1	1	3	22	1	0	1	6	1	2	37	2	5	6	1
Geobacillus stearothermophilus	0	45	0	0	4	17	1	0	0	0	25	100	98	98	1	0	1	1	10	4	0	50	4	1	4	30	25	17	100	10
Geobacillus thermoglucosidasius	0	36	0	0	47	68	73	0	0	0	31	100	95	95	0	63	0	4	63	26	0	63	73	31	31	89	42	63	100	0
Paenibacillus alvei	0	100	0	0	0	100	0	0	96	0	28	87	3	28	0	0	0	40	0	0	0	50	96	71	87	100	87	50	100	0
Paenibacillus amylolyticus	0	26	0	0	84	84	100	0	0	93	100	100	100	100	0	53	0	1	99	0	46	100	93	100	100	100	100	100	100	100
Paenibacillus glucanolyticus	0	61	0	38	100	100	99	11	0	72	88	88	100	88	0	29	11	14	94	11	20	94	100	100	100	100	100	100	100	100
Paenibacillus lautus	0	73	0	46	100	93	100	0	26	66	100	100	100	99	0	0	6	6	99	6	6	98	100	100	100	100	100	100	100	100
Paenibacillus macerans	0	77	0	51	99	82	99	1	1	82	100	100	100	99	0	58	0	11	93	22	22	77	40	97	99	100	97	99	100	100
Paenibacillus polymyxa	0	83	0	2	93	100	97	0	0	83	97	100	99	97	0	2	0	1	99	0	6	71	46	100	100	100	100	99	100	97
Paenibacillus thiaminolyticus	0	94	0	0	0	87	0	0	22	0	100	100	94	94	0	0	0	58	0	0	35	70	70	87	98	98	98	87	100	94
Paenibacillus validus	0	93	0	0	6	100	93	0	0	6	100	100	100	43	0	0	85	100	100	6	0	100	0	0	0	100	0	25	100	6
Virgibacillus pantothenticus	0	55	0	44	0	88	0	0	0	0	98	100	100	98	0	79	0	20	0	50	1	100	100	88	100	100	100	38	100	20

API 50 CHB V4.0	30 MEL	31 SAC	32 TRE	33 INU	34 MLZ	35 RAF	36 AMD	37 GLYG	38 XLT	39 GEN	40 TUR	41 LYX	42 TAG	43 DFUC	44 LFUC	45 DARL	46 LARL	47 GNT	48 2KG	49 5KG	API 20 E ONPG	ADH	LDC	ODC	CIT	H2S	URE	TDA	IND	VP	GEL	NIT	TEMP	INCUB
Aneurinibacillus aneurinilyticus	9	33	9	0	0	9	0	0	0	9	0	0	9	0	0	0	0	0	0	0	9	9	0	0	66	0	9	0	0	9	9	90	29°	48 h
Bacillus anthracis	1	99	99	1	1	1	51	84	0	1	1	0	1	0	1	0	1	14	0	2	1	1	1	1	31	1	1	1	0	26	93	78	29°	48 h
Bacillus cereus 1	1	55	99	1	1	2	83	77	1	3	2	1	0	0	1	0	1	47	0	1	4	71	1	1	28	1	2	1	1	43	93	74	29°	48 h
Bacillus cereus 2	0	74	97	0	0	0	65	63	0	0	1	0	0	0	1	0	0	20	0	0	1	52	1	1	54	1	2	1	1	30	99	61	29°	48 h
Bacillus circulans	92	99	99	52	50	96	99	92	26	98	85	1	1	1	21	21	10	61	11	8	87	1	1	1	4	1	1	1	1	28	15	13	29°	48 h
Bacillus coagulans	100	95	98	1	1	61	95	23	0	61	61	0	0	0	1	57	0	61	4	0	73	19	1	1	1	1	1	1	1	57	1	17	29°	48 h
Bacillus firmus	1	77	58	1	0	1	48	3	0	1	0	0	1	0	0	0	0	7	0	0	18	4	1	2	13	1	2	1	2	39	48	70	29°	48 h
Bacillus lentus	55	82	82	25	30	75	75	55	0	50	40	0	10	0	0	4	0	1	0	0	98	0	1	0	25	0	30	0	0	25	40	60	29°	48 h
Bacillus licheniformis	26	99	99	50	1	44	99	87	1	60	75	1	91	1	1	1	1	39	0	0	99	91	1	1	48	1	15	1	1	83	86	68	29°	48 h
Bacillus megaterium	90	98	99	60	49	89	94	95	11	81	73	0	1	0	1	11	0	4	0	1	92	1	1	1	11	1	1	1	1	40	95	15	29°	48 h
Bacillus mycoides	1	57	98	1	1	1	99	99	0	0	4	1	0	0	0	0	0	12	0	0	27	48	1	1	34	1	1	1	1	55	89	68	29°	48 h
Bacillus pumilus	15	99	99	1	0	15	1	1	1	67	31	3	90	1	1	0	0	1	1	0	99	1	1	1	40	1	1	1	1	86	95	2	29°	48 h
Bacillus smithii	10	54	100	0	0	0	0	0	0	0	54	0	0	0	0	24	0	10	0	0	1	1	1	1	3	1	1	1	1	3	85	3	55°	16/24h
B. subtilis / B. amyloliquefaciens	48	90	88	58	0	62	78	79	1	52	50	0	0	1	1	1	0	7	0	0	73	1	1	1	44	1	2	1	1	90	99	57	29°	48 h
Bacillus non reactive *	1	6	5	2	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	12	1	1	1	23	1	32	1	1	17	49	18	29°	48 h
Brevibacillus agri	0	0	21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	94	0	0	0	0	94	100	0	29°	48 h
Brevibacillus laterosporus	0	5	98	0	0	0	5	11	0	12	0	0	1	0	0	0	0	1	0	0	1	1	0	0	11	0	1	0	5	66	66	42	29°	48 h
Brevibacillus non reactive **	0	5	8	1	0	2	1	1	1	1	1	0	1	1	0	1	0	0	0	1	19	7	1	1	15	1	9	1	1	40	41	39	29°	48 h
Geobacillus stearothermophilus	55	95	75	0	75	55	95	82	0	4	50	1	4	0	0	0	0	0	0	1	10	1	1	1	1	1	1	1	1	10	57	11	55°	6/24h
Geobacillus thermoglucosidasius	10	73	100	0	1	10	52	0	0	31	73	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	27	99	66	55°	16/24h
Paenibacillus alvei	50	40	28	0	0	40	50	28	0	40	28	0	0	0	0	0	0	12	0	0	87	0	0	0	0	0	12	0	100	96	3	1	29°	48 h
Paenibacillus amylolyticus	100	100	100	6	53	100	100	100	0	100	100	0	0	0	6	0	0	0	0	0	99	1	1	1	1	1	8	1	1	30	2	69	29°	48 h
Paenibacillus glucanolyticus	100	100	100	55	75	100	99	99	5	100	100	0	0	0	27	0	0	27	0	1	91	1	1	1	1	1	5	1	1	1	12	35	29°	48 h
Paenibacillus lautus	100	100	98	33	53	100	99	93	6	100	97	0	6	0	40	6	0	53	0	0	99	7	1	1	1	1	7	1	1	64	1	8	29°	48 h
Paenibacillus macerans	100	100	100	88	62	99	99	99	5	97	93	1	0	0	37	51	0	74	1	3	93	1	1	1	1	1	1	1	1	76	38	12	29°	48 h
Paenibacillus polymyxa	100	100	100	69	22	99	99	93	0	97	99	0	0	0	2	0	0	35	0	1	97	2	1	1	2	6	1	1	1	56	70	37	29°	48 h
Paenibacillus thiaminolyticus	70	94	87	0	77	70	87	70	0	87	94	0	0	0	47	0	0	58	0	0	71	7	1	1	1	71	92	1	92	1	71	42	29°	48 h
Paenibacillus validus	25	100	100	50	0	43	68	68	6	6	100	0	25	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	98	0	62	29°	48 h
Virgibacillus pantothenticus	0	94	100	0	0	0	98	5	0	27	66	0	98	0	61	0	0	33	0	0	22	5	1	1	29	12	1	1	1	1	70	25	29°	48 h

* *Bacillus* non réactif / *Bacillus* non reactive / *Bacillus* nicht reaktiv / *Bacillus* no reactivo / *Bacillus* non reattivo / *Bacillus* não reactivo / *Bacillus* μη ενεργό / icke reaktiv *Bacillus* / *Bacillus* ikke reaktiv / *Bacillus* nie dający reakcji = *Bacillus sphaericus / fusiformis / badius*

** *Brevibacillus* non réactif / *Brevibacillus* non reactive / *Brevibacillus* nicht reaktiv / *Brevibacillus* no reactivo / *Brevibacillus* non reattivo / *Brevibacillus* não reactivo / *Brevibacillus* μη ενεργό / icke reaktiv *Brevibacillus* / *Brevibacillus* ikke reaktiv / *Brevibacillus* nie dający reakcji = *Brevibacillus choshinensis / centrosporus / borstelensis / brevis*

API 20E
Erwinia amylovora

- Typical readings of *Erwinia amylovora* in API 20E tests after 48 h.

Test	Reaction (48 h)
ONPG	Variable
ADH	– (or weak +)
LDC	–
ODC	–
CIT	–
SH_2	–
URE	–
TDA	–
IND	–
VP	+ (or variable)
GEL	Variable
GLU	+
MAN	Variable
INO	Variable
SOR	Variable
RHA	–
SAC	+
MEL	– (or weak +)
AMY	–
ARA	+ (some –)

Bulletin OEPP/EPPO Bulletin **34**,155–157

API 50CH
Erwinia amylovora

- Prepare a suspension of OD=1.0 in PBS.
- Add 1 ml of the suspension to 20 ml of Ayers medium.
- Follow the manufacturer's instructions for inoculation of the strip.
- Incubate at 25-26ºC in aerobiosis and read after 24 and 48.
- Utilization of the different carbohydrates is observed by a yellow colour in the well.

Test[1]	*Reaction*
L-arabinose	+
Ribose	+
D-xilose	Variable
Galactose	Variable (mostly +)
D-glucose	+
D-fructose	+
D-manose	Variable
Mannitol	+
Sorbitol	+
N-acetylglucosamine	+
Melibiose	Variable
Sucrose	+
Trehalose	+
ß-gentiobiose	+

[1]= The remaining sugars are not utilised by *E. amylovora* but some strains can utilise glycerol and D-fucose (Donat et al., unpublished results).

Enterotube II

It consists of a tube with a flat side and contains 12 compartments

5-digitbiocode number

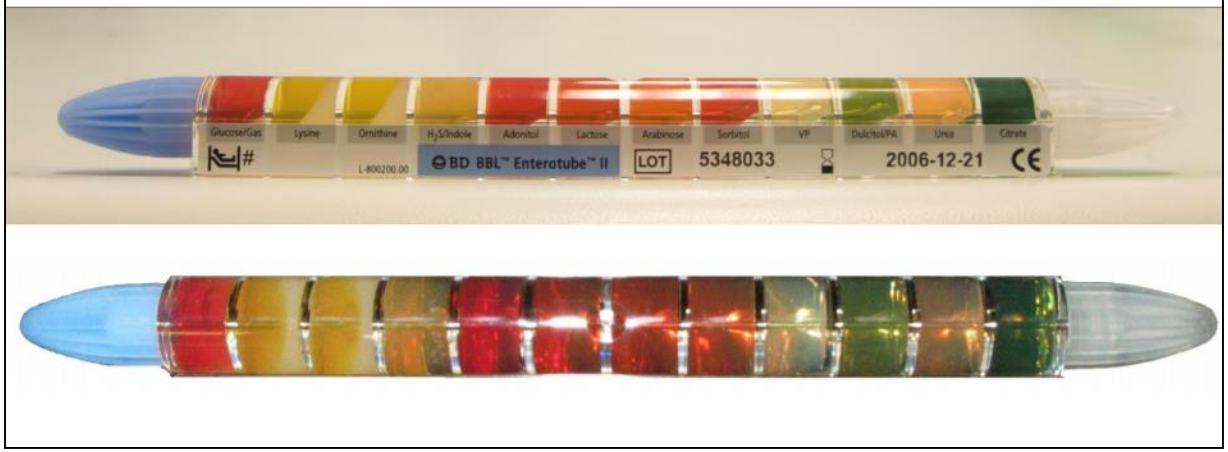
- Materials are the same as API-20E strips.
- The Enterotube II System™ is a plastic shell that contains 12 mini-media "ports".
- Subsequent performance of 15 biochemical tests (glucose, gas production, lysine decarboxylase, ornithine decarboxylase, H_2S, indole, adonitol, lactose, arabinose, sorbitol, Voges-Proskauer, dulcitol, phenylalanine deaminase, urea and citrate) from a single bacterial colony together with the Interpretation Guide (order separately) allow identification of *Enterobacteriaceae*.

Enterotube

5-digitbiocode number

12 individual compartments

Filled with different media



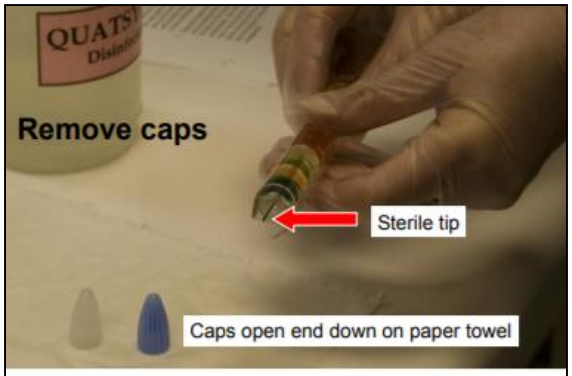
Ex. 21: Rapid Identification Methods-Enterotube™ II

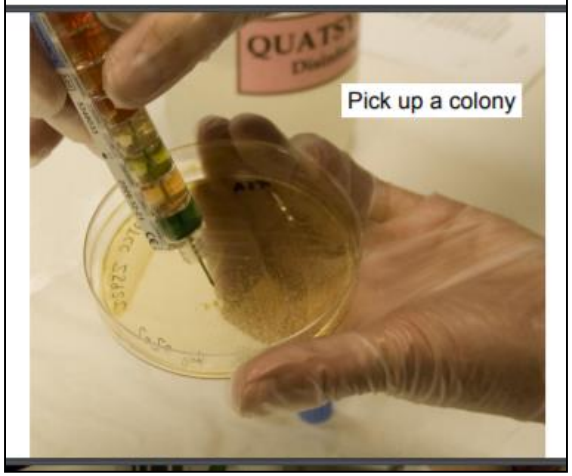
Enterotube II
Inoculation

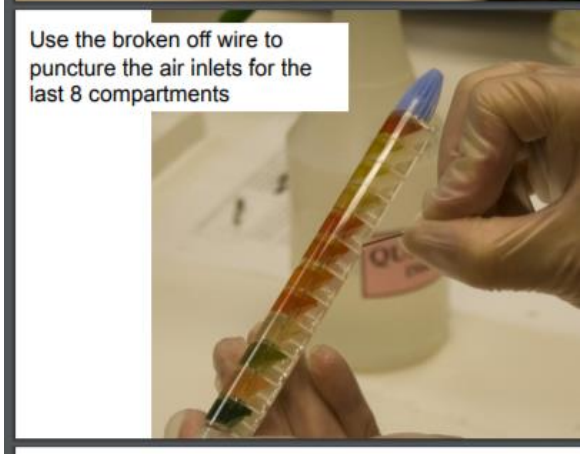
- Unscrew the two caps from the ends of the Enterotube making certain that you do not touch the needle end of the wire.
- From a bacterial culture, touch one colony with the needle end of the wire and inoculate the system by pulling and twisting the wire by its handle out of the system, then running it back through the system.
- After you have run the wire back through the system, pull it out to the notch in the wire and snap the wire off.
- Screw the caps back on the system.

Enterotube

5-digitbiocode number







From a bacterial culture, touch one colony with the needle end of the wire and inoculate the system by pulling and twisting the wire by its handle out of the system, then running it back through the system.

Enterotube II

Interpretation of the results

Positive results(scores) with different colors

Results for media in the Enterotube II System

Medium	Positive Results (Score)	Negative Results (Score)
Glucose	Yellow color (2)	Red color
Gas Production (in with Glucose)	wax moved (1)	wax not moved
Lysine decarboxylase	Purple color (4)	Yellow color
Ornithine decarboxylase	Purple color (2)	Yellow color
H_2S	Black color (1)	Beige color
Indole	Rose color (4)	Colorless
Adonitol	Yellow color (2)	Red color
Lactose	Yellow color (1)	Red color
Arabinose	Yellow color (4)	Red color
Sorbitol	Yellow color (2)	Red color
Voges-Proskauer	Red color	Colorless
Dulcitol	Yellow (1)	Green
Phenylalanine Deaminase	Black-smoky-gray color (4)	Green
Urease	Red/purple color (2)	Beige color
Citrate	Blue color (1)	Green color

Enterotube II

Interpretation of the results

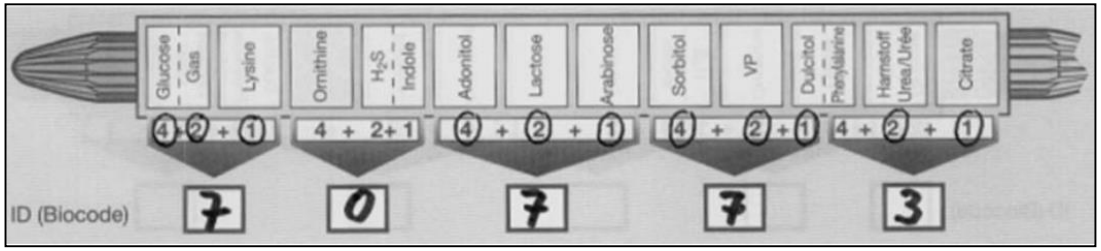
In the table below, record the score for each compartment

Glucose (2) Gas (1)	Lysine decarboxylase (4) Ornithine decarboxylase (2) H_2S (1)	Indole (4) Adonitol (2) Lactose (1)	Arabinose (4) Sorbitol ((2) Dulcitol (1)	Phenylalanine deaminase (4) Urease (2) Citrate (1)
SCORE:	SCORE:	SCORE:	SCORE:	SCORE:

In the box below, write your identification number:

With which bacteria in the table above do your results match?:

~~Use hardcopy Enterotube profile book for identification.~~
Bacterium are attempted to identify with the help of computer generated coding system available on report sheet from the instructor.

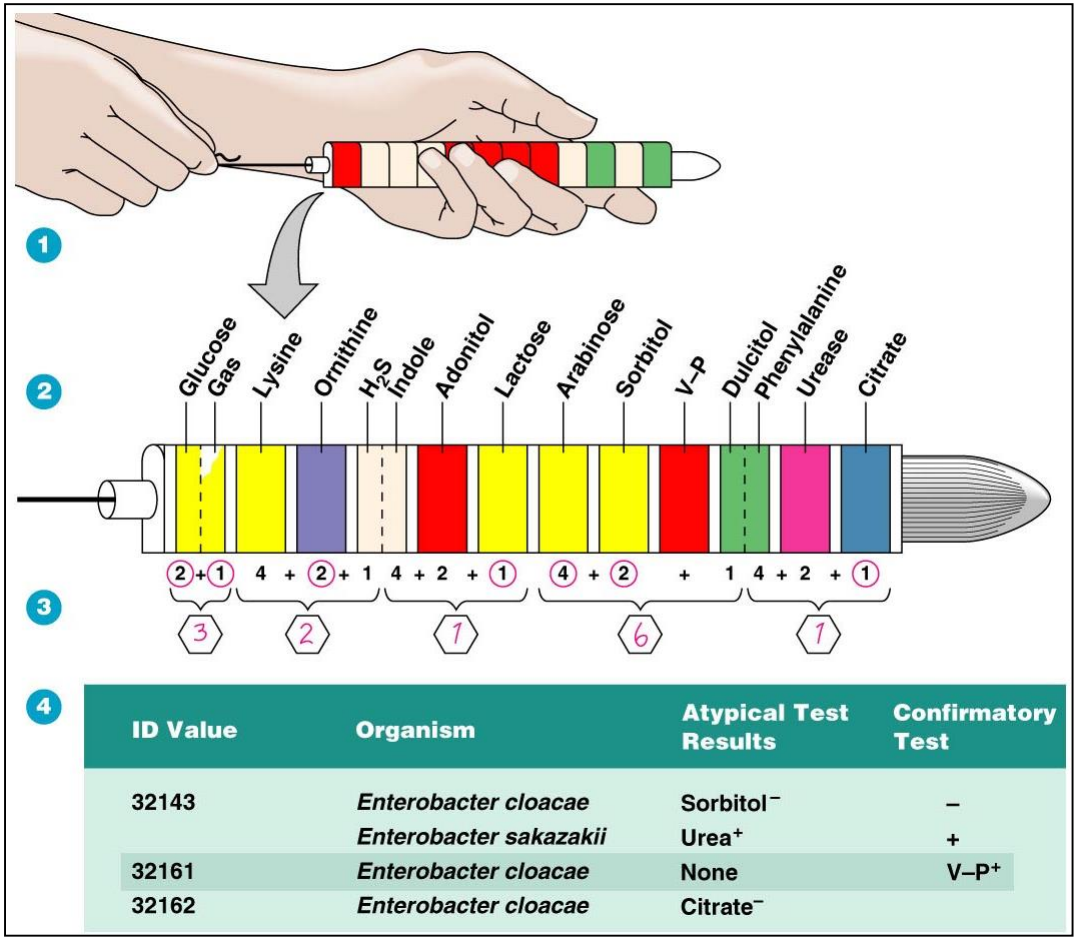


Enterotube

5-digitbiocode number

Three with dual test analyses:

1. Gucose+gas
2. H_2S+indole tests
3. Dulcitol+phenyalanine



ID Value	Organism	Atypical Test Results	Confirmatory Test
32143	*Enterobacter cloacae*	Sorbitol$^-$	–
	Enterobacter sakazakii	Urea$^+$	+
32161	*Enterobacter cloacae*	None	V–P$^+$
32162	*Enterobacter cloacae*	Citrate$^-$	

Enterotube

Enterotube II & Oxyferm II

Prices

- This tube has a series of media which are simaltaneously innoculated to ascertain the Enterobacteriaceae species.

ENTEROTUBE II	PK/25	$ 332.24
OXY/FERM II	PK/25	$ 335.81
ENTEROTUBE II INTERTPRETATION GUIDE	EA	$ 43.20
OXY FERM II CODE BOOK	EA	$ 43.20

The BD BBL™ Crystal™ identification system

Crystal E/NF(old version)

- The BBL Crystal Enteric/Nonfermenter (E/NF) ID kit is for the identification of:

1. Enterobacteriaceae, and
2. more commonly isolated glucose-fermenting, and
3. nonfermenting gram-negative bacilli.

- The plastic panels include 30 tests for the fermentation, oxidation, degradation, or hydrolysis of various substrates.
- The current software version is 4.0 and contains 38 genera and 104 species.

O'Hara,2005

The BD BBL™ Crystal™ identification system

Crystal E/NF(new version)

- The BD BBL™ Crystal™ identification system is a miniaturized identification system that requires only one step for inoculation.
- Once inoculated, the panels provide a safe and easy-to-handle closed system.
- Reagent addition and oil overlay are completely eliminated by the panel design.
- The system has been proven to accurately identify nearly 500 organisms.

BD,2021

Biotype 100

Biotype 100 gallery

- The BIOTYPE gallery, in the form of Biotype 100 from BioMérieux, a standardized method for establishing nutrient profiles.
- The Biotype 100 strip contains 100 tubes for the study of the metabolism of carbon sources using assimilation tests.
- Tubes are inoculated with an assimilation medium adapted to the nutritional requirements of the organism to be examined:
- **Liquid Biotype Medium 1:** Containing 16 growth factors for most Gram-negative bacteria, or
- **Liquid Biotype Medium 2:** Containing 31 growth factors for more fastidious bacteria (Gram-positive bacteria).
- After incubation, reactions are read visually, either as a cloudy appearance or as the development of a colour.

Biotype 100
Procedure

- A suspension with a turbidity of 3 on McFarland's scale is prepared from a pure culture of the bacterium under investigation.
- 2 ml of this suspension is transferred into Biotype Medium.
- After homogenization, the tubes are filled with suspension and incubated under aerobic conditions, at 28° C for Gram-negative bacteria, or at 26°C for Gram-positive bacteria.
- Strips are read:

1. After 2, 4 and 6 days for Gram-negative bacteria,
2. After 5, 10, 15 and 20 days for Gram-positive.

Poliakoff *et al.*,2005

ID Tri-Panel

- The ID Tri-Panel was introduced in 1984.
- The Panel will accommodate the testing of three isolates at one time or can be used as part of a combination MIC-ID configuration.
- It contains 30 colorimetric-based substrates.
- A profile number is generated, and the answer is obtained from either:
- An Electro-Code computer program, or
- The data management system.
- A total of 91% (93 of 102 isolates) of the *Pseudomonas-Xanthomonas* group and the *Acinetobacter* group were correctly identified to species level.

Kinds of VITEK Test Cards

Environmental microbes of plants, animals and clinical pathogens

- **GNI+ Card** (Gram-Negative Plus) V1316
  Identifies enterics, Vibros, *P. aeruginosa* and other "non-fermenters" in as little as two hours; 104 bacterial species.e.g. *Burkhoderia* spp.
- **GPI Card** (Gram-Positive) V1305
  Identifies *Streptococci*, *Staphylococci*, *Enterococci* sp. and other Gram-positive organisms such as *Listeria* and *Corynebacteria*; 49 Gram-positive organisms.
- **YBC Card** (Yeasts) V1303
  Identifies most clinically relevant yeasts (36 species).
- **ANI Card** (Anaerobes) V1309
  Identifies more than 80 anaerobes (direct reading).
- **BAC Card** (Bacillus Identification) V1304
  Automated identification of Gram-positive microorganisms of the family Bacillaceae.
- **BIO Card** (Bioburden) V1103
  Automated enumeration of microbial populations in a liquid sample.
- **NFC Card** (Non-Fermenter Identification) V1310
  Automated identification of oxidase-positive and some oxidase-negative Gram-negative non-fermenting bacilli.

Bioburden is normally defined as the number of bacteria living on a surface that has not been sterilized.

VITEK®2 Compact
BioMerieux

- The VITEK® 2 Compact is a fully automated bacterial identification and antibiotic susceptibility testing system that uses fluorescent technology to provide broad profiles for the reliable identification of the most relevant organisms.
- The average time to result on:

1. Gram-positive and Gram-negative card identifications is 2-8 hours,
2. On gram-positive spore forming bacilli are read at 14 hours, and
3. Yeast-like organisms are read at 18 hours.

Vitek test card

Procedure

- Prepare unknown bacterial suspension by adding one to three colonies in the 1.8 ml 0.45% sterile saline tube.
- Adjust the suspension to a 1.0 McFarland standard by using the Vitek colorimeter.
- After labeling the cards with a marker, place the card in the filling stand with a transfer tube that is in the bacterial suspension.
- Inoculate the cards via the filling module for a period of 20 min.
- A portion of each bacterial suspension was streaked on a agar plate to check for inoculum purity.
- Seal the cards via the sealer module and incubate at 30°C in an incubator for a period of time ranging from 4 to 12 hours.
- The instrument periodically scans each card and compared the color changes or gas production of each tiny well with the database of known cultures.
- Each VITEK unit can automatically scan 120 cards or more simultaneously.

Vitek test card
General procedure

- Provide rapid, accurate species-level identification of >350 clinically relevant bacteria and yeast within self-contained, disposable cards, for use with VITEK® 2 instruments.
- The cards, filled with the suspensions, were placed in a specific tray, which was placed in the Vitek combined reader-incubator.

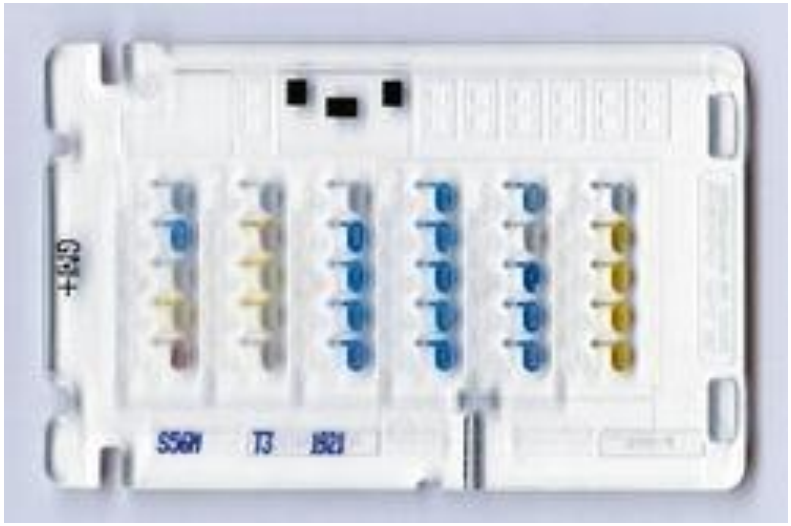
Vitek identification system

- In addition to the kit, the following supplies or equipment is needed.
  (1) Sterile wooden applicator sticks,
  (2) Sterile tubes containing 1.8 ml of 0.45 to 0.5% saline,
  (3) Colorimeter (Vitek colorimete),
  (4) Filling stand
  (5) Vitek System Senior, which includes the following:
    (a) Filler-sealer module,
    (b) Reader incubator unit,
    (c) Printer,
    (d) Computer (with a minimum of an R4.01, 1989 updated data base),
    (e) Data terminal,
  (6) Fine-tip markers,
  (7) Incubator (30°C),
  (8) Agar plates.

VITEK Test Card

Used in clinical microbiology laboratory

- The most successful and sophisticated miniaturized automated identification system is the VITEK system.
- This AutoMicrobic system is the size of a playing card and is made up of 30 or 45 microwells in each of which there is a different reagent.



Vitek card (developed on the NASA space shuttle to identify bacteria in space).

The Biolog systems

- The Biolog system presents an extended array of biochemical tests (C/N utilization).
- A positive result is seen as a purple colour change.
- The plate can be read by eye or by a plate reader.

The Biolog Systems
Bacterial/Yeast/Fungi Identification

- The GN2 MicroPlate was designed in 1989 by Biolog, Inc. (Hayward, Calif.).
- The Biolog systems can rapidly identify over 2,900 species of:
- Aerobic and anaerobic bacteria,
- Yeasts,
- Filamentous fungi.

The Biolog Systems

The perceived advantages and limitations of Biolog GN

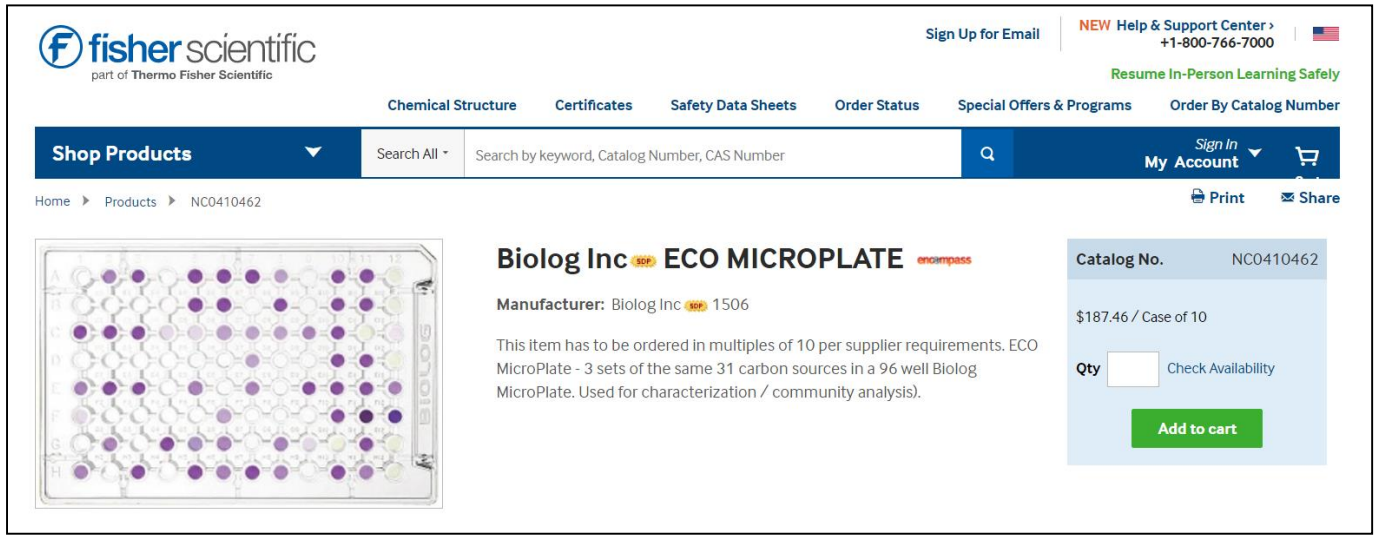
- Advantages:
- Is not requiring of expensive equipment;
- Quick system is quick, reproducible and easy to perform;
- Data can be shared between laboratories;
- Can provide a reasonable identification to the genus and species level.
- Limitations:
- Requires investment [access] to the library;
- Has limitation in resolving below species level (pathovar separation).
- Library stronger on human microbials than plant pathogenic bacteria.

Julian Smith

The Biolog Systems

Price: $187.46/case of 10

- This item has to be ordered in multiples of 10 per supplier requirements.
- ECO MicroPlate - 3 sets of the same 31 carbon sources in a 96 well Biolog MicroPlate.



Note: ECO MicroPlate were used for characterization/community analysis.

BIOLOG

Identification Databases – 2,900 Species/Taxa

Identifiaction patterns for pvs. of *Pseudomonas syringae* and *Xanthomonas campestris*

Pseudomonas syringae pv aceris Pseudomonas syringae pv antirrhini Pseudomonas syringae pv apii Pseudomonas syringae pv aptata Pseudomonas syringae pv atrofaciens Pseudomonas syringae pv coronafaciens Pseudomonas syringae pv cunninghamiae Pseudomonas syringae pv delphinii Pseudomonas syringae pv eriobotryae Pseudomonas syringae pv glycinea Pseudomonas syringae pv helianthi Pseudomonas syringae pv lachrymans Pseudomonas syringae pv mori Pseudomonas syringae pv myricae Pseudomonas syringae pv oryzae Pseudomonas syringae pv papulans Pseudomonas syringae pv persicae Pseudomonas syringae pv phaseolicola Pseudomonas syringae pv pisi Pseudomonas syringae pv porri Pseudomonas syringae pv primulae Pseudomonas syringae pv sesami Pseudomonas syringae pv syringae Pseudomonas syringae pv tabaci A Pseudomonas syringae pv tabaci B Pseudomonas syringae pv tagetis Pseudomonas syringae pv tomato Pseudomonas syringae pv zinaniae	Xanthomonas albilineans Xanthomonas campestris pv begoniae A Xanthomonas campestris pv begoniae B Xanthomonas campestris pv campestris Xanthomonas campestris pv carotae Xanthomonas campestris pv corylina Xanthomonas campestris pv dieffenbachiae Xanthomonas campestris pv hyacinthi Xanthomonas campestris pv juglandis Xanthomonas campestris pv malvacearum Xanthomonas campestris pv nigromaculans Xanthomonas campestris pv pelargonii Xanthomonas campestris pv phaseoli Xanthomonas campestris pv poinsettiicola Xanthomonas campestris pv raphani Xanthomonas campestris pv sesami Xanthomonas campestris pv tardicrescens Xanthomonas campestris pv translucens Xanthomonas campestris pv vesicatoria Xanthomonas oryzae pv oryzicola

GN2 MicroPlate™

Gram Negative Identification Test Panel

Carbon Sources in GP2 MicroPlate

A1 Water	A2 α-Cyclodextrin	A3 Dextrin	A4 Glycogen	A5 Tween 40	A6 Tween 80	A7 N-Acetyl-D-Galactosamine	A8 N-Acetyl-D-Glucosamine	A9 Adonitol	A10 L-Arabinose	A11 D-Arabitol	A12 D-Cellobiose
B1 i-Erythritol	B2 D-Fructose	B3 L-Fucose	B4 D-Galactose	B5 Gentiobiose	B6 α-D-Glucose	B7 m-Inositol	B8 α-D-Lactose	B9 Lactulose	B10 Maltose	B11 D-Mannitol	B12 D-Mannose
C1 D-Melibiose	C2 β-Methyl-D-Glucoside	C3 D-Psicose	C4 D-Raffinose	C5 L-Rhamnose	C6 D-Sorbitol	C7 Sucrose	C8 D-Trehalose	C9 Turanose	C10 Xylitol	C11 Pyruvic Acid Methyl Ester	C12 Succinic Acid Mono-Methyl-Ester
D1 Acetic Acid	D2 Cis-Aconitic Acid	D3 Citric Acid	D4 Formic Acid	D5 D-Galactonic Acid Lactone	D6 D-Galacturonic Acid	D7 D-Gluconic Acid	D8 D-Glucosaminic Acid	D9 D-Glucuronic Acid	D10 α-Hydroxybutyric Acid	D11 β-Hydroxybutyric Acid	D12 γ-Hydroxybutyric Acid
E1 p-Hydroxy Phenylacetic Acid	E2 Itaconic Acid	E3 α-Keto Butyric Acid	E4 α-Keto Glutaric Acid	E5 α-Keto Valeric Acid	E6 D,L-Lactic Acid	E7 Malonic Acid	E8 Propionic Acid	E9 Quinic Acid	E10 D-Saccharic Acid	E11 Sebacic Acid	E12 Succinic Acid
F1 Bromosuccinic Acid	F2 Succinamic Acid	F3 Glucuronamide	F4 L-Alaninamide	F5 D-Alanine	F6 L-Alanine	F7 L-Alanyl-glycine	F8 L-Asparagine	F9 L-Aspartic Acid	F10 L-Glutamic Acid	F11 Glycyl-L-Aspartic Acid	F12 Glycyl-L-Glutamic Acid
G1 L-Histidine	G2 Hydroxy-L-Proline	G3 L-Leucine	G4 L-Ornithine	G5 L-Phenylalanine	G6 L-Proline	G7 L-Pyroglutamic Acid	G8 D-Serine	G9 L-Serine	G10 L-Threonine	G11 D,L-Carnitine	G12 γ-Amino Butyric Acid
H1 Urocanic Acid	H2 Inosine	H3 Uridine	H4 Thymidine	H5 Phenyethyl-amine	H6 Putrescine	H7 2-Aminoethanol	H8 2,3-Butanediol	H9 Glycerol	H10 D,L-α-Glycerol Phosphate	H11 α-D-Glucose-1-Phosphate	H12 D-Glucose-6-Phosphate

GN2 MicroPlate™

Gram Positive Identification Test Panel

Carbon Sources in GP2 MicroPlate

A1 Water	A2 α-Cyclodextrin	A3 β-Cyclodextrin	A4 Dextrin	A5 Glycogen	A6 Inulin	A7 Mannan	A8 Tween 40	A9 Tween 80	A10 N-Acetyl-D-Glucosamine	A11 N-Acetyl-β-D-Mannosamine	A12 Amygdalin
B1 L-Arabinose	B2 D-Arabitol	B3 Arbutin	B4 D-Cellobiose	B5 D-Fructose	B6 L-Fucose	B7 D-Galactose	B8 D-Galacturonic Acid	B9 Gentiobiose	B10 D-Gluconic Acid	B11 α-D-Glucose	B12 m-Inositol
C1 α-D-Lactose	C2 Lactulose	C3 Maltose	C4 Maltotriose	C5 D-Mannitol	C6 D-Mannose	C7 D-Melezitose	C8 D-Melibiose	C9 α-Methyl-D-Galactoside	C10 β-Methyl-D-Galactoside	C11 3-Methyl Glucose	C12 α-Methyl-D-Glucoside
D1 β-Methyl-D-Glucoside	D2 α-Methyl-D-Mannoside	D3 Palatinose	D4 D-Psicose	D5 D-Raffinose	D6 L-Rhamnose	D7 D-Ribose	D8 Salicin	D9 Sedoheptulosan	D10 D-Sorbitol	D11 Stachyose	D12 Sucrose
E1 D-Tagatose	E2 D-Trehalose	E3 Turanose	E4 Xylitol	E5 D-Xylose	E6 Acetic Acid	E7 α-Hydroxybutyric Acid	E8 β-Hydroxybutyric Acid	E9 γ-Hydroxybutyric Acid	E10 p-Hydroxy-Phenylacetic Acid	E11 α-Ketoglutaric Acid	E12 α-Ketovaleric Acid
F1 Lactamide	F2 D-Lactic Acid Methyl Ester	F3 L-Lactic Acid	F4 D-Malic Acid	F5 L-Malic Acid	F6 Pyruvatic Acid Methyl Ester	F7 Succinic Acid Mono-methyl Ester	F8 Propionic Acid	F9 Pyruvic Acid	F10 Succinamic Acid	F11 Succinic Acid	F12 N-Acetyl-L-Glutamic Acid
G1 L-Alaninamide	G2 D-Alanine	G3 L-Alanine	G4 L-Alanyl-Glycine	G5 L-Asparagine	G6 L-Glutamic Acid	G7 Glycyl-L-Glutamic Acid	G8 L-Pyroglutamic Acid	G9 L-Serine	G10 Putrescine	G11 2,3-Butanediol	G12 Glycerol
H1 Adenosine	H2 2'-Deoxy Adenosine	H3 Inosine	H4 Thymidine	H5 Uridine	H6 Adenosine-5'-Monophosphate	H7 Thymidine-5'-Monophosphate	H8 Uridine-5'-Monophosphate	H9 D-Fructose-6-Phosphate	H10 α-D-Glucose-1-Phosphate	H11 D-Glucose-6-Phosphate	H12 D-L-α-Glycerol Phosphate

The Biolog Systems

Carbon sources in FF MicroPlate designed for filamentous fungi and yeast

BiOLOG — Yeast Identification Test Panels

YT MicroPlate™

A1 water	A2 acetic acid	A3 formic acid	A4 propionic acid	A5 succinic acid	A6 methyl succinate	A7 L-aspartic acid	A8 L-glutamic acid	A9 L-proline	A10 D-gluconic acid	A11 dextrin	A12 inulin
B1 cellobiose	B2 gentiobiose	B3 maltose	B4 maltotriose	B5 D-melezitose	B6 D-melibiose	B7 palatinose	B8 D-raffinose	B9 stachyose	B10 sucrose	B11 D-trehalose	B12 turanose
C1 N-acetyl-D-glucosamine	C2 α-D-glucose	C3 D-galactose	C4 D-psicose	C5 L-sorbose	C6 salicin	C7 D-mannitol	C8 D-sorbitol	C9 D-arabitol	C10 xylitol	C11 glycerol	C12 tween 80
D1 water	D2 fumaric acid	D3 L-malic acid	D4 methyl succinate	D5 bromo succinic acid	D6 L-glutamic acid	D7 γ-amino butyric acid	D8 α-keto-glutaric acid	D9 2-keto-D-gluconic acid	D10 D-gluconic acid	D11 dextrin	D12 inulin
E1 cellobiose	E2 gentiobiose	E3 maltose	E4 maltotriose	E5 D-melezitose	E6 D-melibiose	E7 palatinose	E8 D-raffinose	E9 stachyose	E10 sucrose	E11 D-trehalose	E12 turanose
F1 N-acetyl-D-glucosamine	F2 D-glucosamine	F3 α-D-glucose	F4 D-galactose	F5 D-psicose	F6 L-rhamnose	F7 L-sorbose	F8 α-methyl D-glucoside	F9 β-methyl D-glucoside	F10 amygdalin	F11 arbutin	F12 salicin
G1 maltitol	G2 D-mannitol	G3 D-sorbitol	G4 adonitol	G5 D-arabitol	G6 xylitol	G7 i-erythritol	G8 glycerol	G9 tween 80	G10 L-arabinose	G11 D-arabinose	G12 D-ribose
H1 D-xylose	H2 methyl succinate + D-xylose	H3 N-acetyl-L-glutamic acid + D-xylose	H4 quinic acid + D-xylose	H5 D-glucuronic acid + D-xylose	H6 dextrin + D-xylose	H7 α-D-lactose + D-xylose	H8 D-melibiose + D-xylose	H9 D-galactose + D-xylose	H10 m-inositol + D-xylose	H11 1,2-propanediol + D-xylose	H12 acetoin + D-xylose

The layout of tests in the YT MicroPlate is shown above.

Oxidation Tests (shaded) | Assimilation Tests (unshaded)

BIOLOG — Fungi Identification Test Panel

FF MicroPlate™

A1 Water	A2 Tween 80	A3 N-Acetyl-D-Galactosamine	A4 N-Acetyl-D-Glucosamine	A5 N-Acetyl-D-Mannosamine	A6 Adonitol	A7 Amygdalin	A8 D-Arabinose	A9 L-Arabinose	A10 D-Arabitol	A11 Arbutin	A12 D-Cellobiose
B1 α-Cyclodextrin	B2 β-Cyclodextrin	B3 Dextrin	B4 i-Erythritol	B5 D-Fructose	B6 L-Fucose	B7 D-Galactose	B8 D-Galacturonic Acid	B9 Gentiobiose	B10 D-Gluconic Acid	B11 D-Glucosamine	B12 α-D-Glucose
C1 Glucose-1-Phosphate	C2 Glucuronamide	C3 D-Glucuronic Acid	C4 Glycerol	C5 Glycogen	C6 m-Inositol	C7 2-Keto-D-Gluconic Acid	C8 α-D-Lactose	C9 Lactulose	C10 Maltitol	C11 Maltose	C12 Maltotriose
D1 D-Mannitol	D2 D-Mannose	D3 D-Melezitose	D4 D-Melibiose	D5 α-Methyl-D-Galactoside	D6 β-Methyl-D-Galactoside	D7 α-Methyl-D-Glucoside	D8 β-Methyl-D-Glucoside	D9 Palatinose	D10 D-Psicose	D11 D-Raffinose	D12 L-Rhamnose
E1 D-Ribose	E2 Salicin	E3 Sedoheptulosan	E4 D-Sorbitol	E5 L-Sorbose	E6 Stachyose	E7 Sucrose	E8 D-Tagatose	E9 D-Trehalose	E10 Turanose	E11 Xylitol	E12 D-Xylose
F1 γ-Amino-butyric Acid	F2 Bromosuccinic Acid	F3 Fumaric Acid	F4 β-Hydroxy-butyric Acid	F5 γ-Hydroxy-butyric Acid	F6 p-Hydroxyphenyl-acetic Acid	F7 α-Keto-glutaric Acid	F8 D-Lactic Acid Methyl Ester	F9 L-Lactic Acid	F10 D-Malic Acid	F11 L-Malic Acid	F12 Quinic Acid
G1 D-Saccharic Acid	G2 Sebacic Acid	G3 Succinamic Acid	G4 Succinic Acid	G5 Succinic Acid Mono-Methyl Ester	G6 N-Acetyl-L-Glutamic Acid	G7 Alaninamide	G8 L-Alanine	G9 L-Alanyl-Glycine	G10 L-Asparagine	G11 L-Aspartic Acid	G12 L-Glutamic Acid
H1 Glycyl-L-Glutamic Acid	H2 L-Ornithine	H3 L-Phenylalanine	H4 L-Proline	H5 L-Pyroglutamic Acid	H6 L-Serine	H7 L-Threonine	H8 2-Amino Ethanol	H9 Putrescine	H10 Adenosine	H11 Uridine	H12 Adenosine-5'-Monophosphate

FIGURE 1. Carbon Sources in FF MicroPlate

The Biolog Systems

Biolog GN2 Microplate

- The GN2 MicroPlate was designed in 1989 by Biolog, Inc. (Hayward, Calif.) for use with any one of three Microbial Identification/Characterization systems:

1. the OmniLog ID, a fully automated system;
2. the MicroLog MicroStation, a semiautomated system; and
3. the MicroLog 1 and 2, which are manual-read systems.

The Biolog Systems

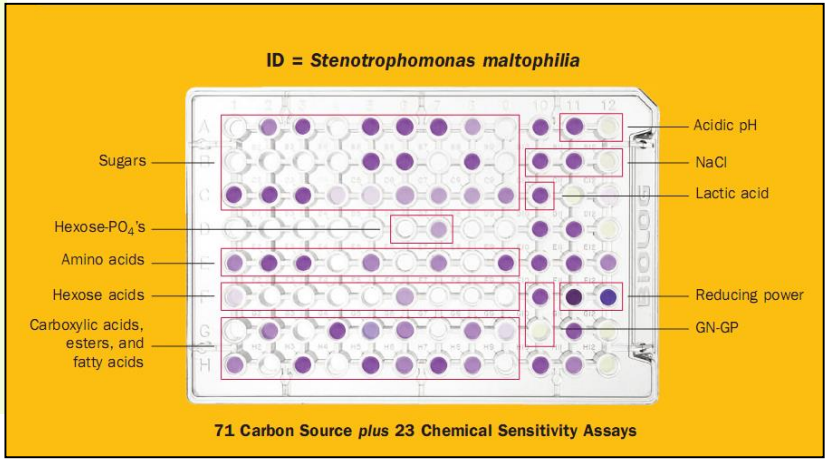
Biolog GN Microplate

- The GN2 MicroPlate was designed for use with any one of three Microbial Identification/Characterization systems:
- **Fully automated system:**
- GEN III OmniLog® ID System: For the ultimate in broad identification capability.
- **Semi-automated system:**
- GEN III MicroStation System: Allows rapid interpretation of results.
- **Manually Read systems:**
- Gen III MicroLog Mnual Systems(MLM): GEN III MicroLog M System without computer (MLM).

GEN III OmniLog® ID System

OmniLog: the fully automated system

- Biolog Omnilog GEN III Plus Automated Microbial Identification System.
- The Biolog GEN III Microplate provides a standardized micro method using 94 biochemical tests (71 carbon source utilization assays and 23 chemical sensitivity assays) to profile and identify a broad range of Gram-negative and Gram-positive bacteria.
- The test panel provides "Phenotypic Fingerprint" of the microorganism which can be used to identify it at the species level.



The semi-automated MicroStation

GEN III MicroStation System

- GN Microlog microplate were inoculated with 150 µl of bacterial suspension per reaction well and incubated at 28°C.
- Microplates were read at 590 nm at 4 and 24 h with a computer-controlled MicroPlate reader.
- Each metabolic profile was compared automatically with the GN Microlog database.
- Strains were identified with the Microlog software.

Absorbance was measured with a Dynatech MR-700 microtiter plate reader using Microlog 2N®software (Biolog).

The manual Biolog Systems
Gen III MicroLog Mnual Systems(MLM)
MicroLog 1 and MicroLog 2

- **GEN III MicroLog M System without computer (MLM).**
- Biolog offers two manual MicroLog Systems for laboratories with low testing volumes or limited budgets, MicroLog 2 and MicroLog 1.
- Manual systems deliver the same state-of-the-art reference-level capability as the fully automated MicroStation.
- This is accomplished by replacing the automated reading capability with visual reading and manual reaction entry.
- Visual reading of gram negative and gram positive MicroPlates is easy because of our unique one color reaction chromophore.

The Biolog Systems
Identification procedure

- Each of the 96 wells of the microtiter-style plate contains tetrazolium dye.
- Tetrazolium dye in each of the wells turn darker shades of purple as the carbon sources are oxidized by the microorganism.
- The microplates were incubated for 4 and 24 hours.
- The test yields a characteristic pattern of positive (purple) and negative wells(colorless) which provide a metabolic signature of the inoculated organism.
- Biolog's MicroLog software was used to identify the bacterium from its metabolic pattern(signature).
- The calculations for the identification of bacteria as to genus, species and other taxonomic units are based on similarity indices (Schaad *et al.*,2001).
- e.g. the similarity values in most Xcc strains exceed 0.5.

Biolog MicroLog MicroStation Identification System

Four basic steps

STEP 1

Isolate a pure culture on Biolog media and gram stain the organism to determine testing protocol.

STEP 2

Prepare inoculum at specified cell density.

STEP 3

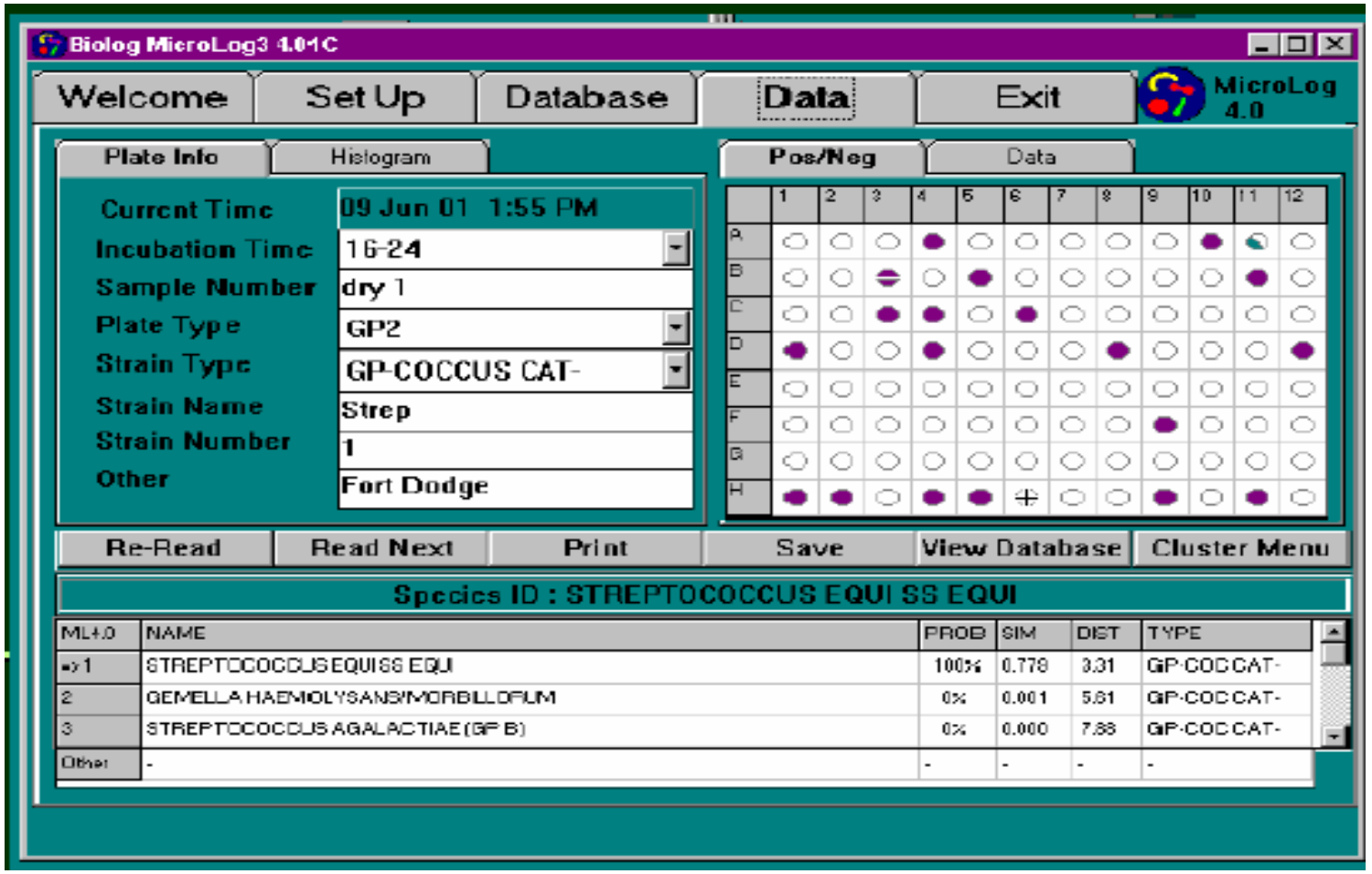
Inoculate and incubate MicroPlate

STEP 4

Read MicroPlate and determine ID

OmniLog: the fully automated system

Microphenotypic arrays – redox reactions

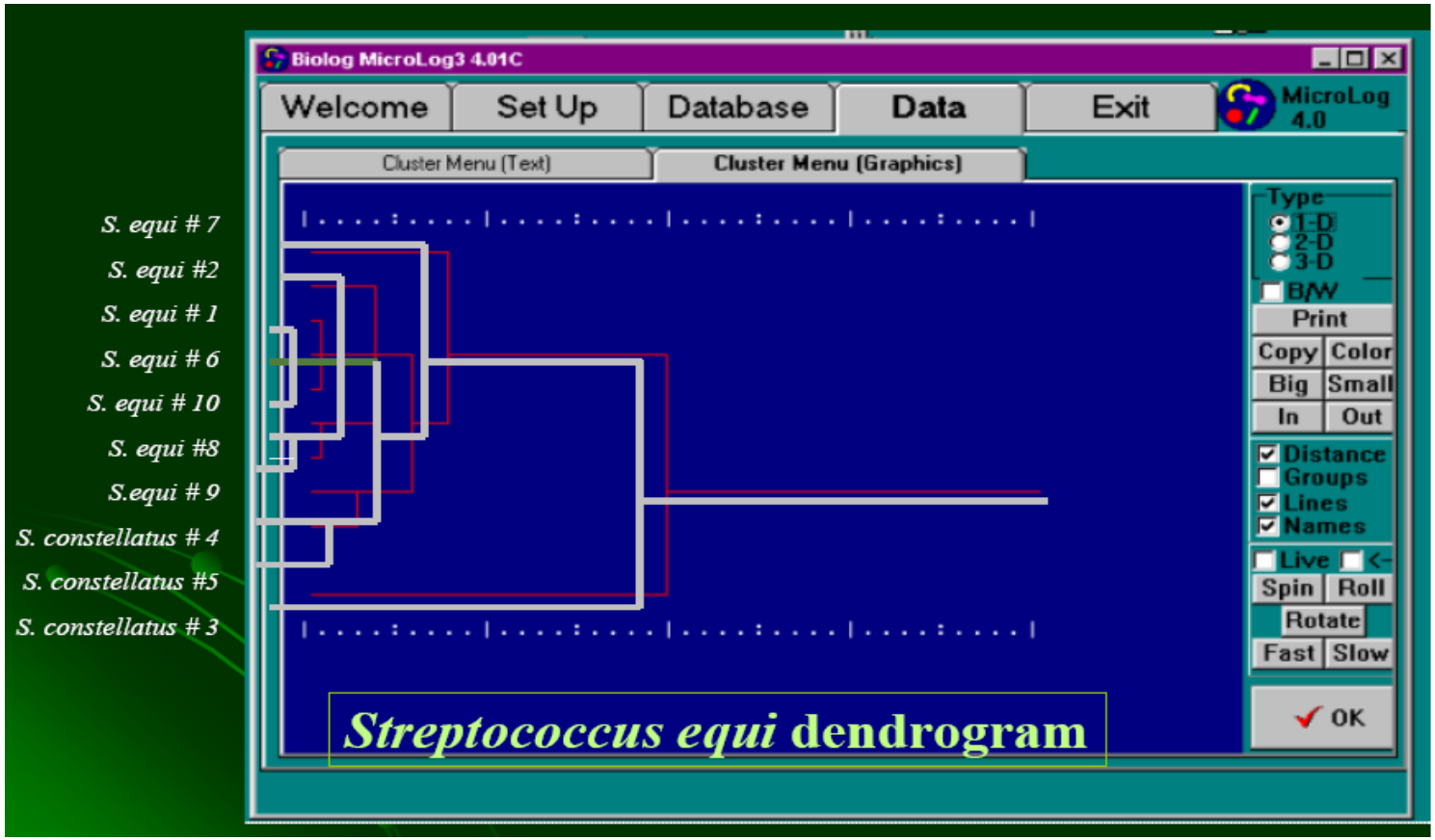


Biolog metabolic fingerprinting
Phenogram generation

- The metabolic fingerprints of each bacterial strain were entered into a computer and compared with the Biolog database.
- Furthermore, the fingerprints of the strains were changed into binary codes and analysed by the MIX program (mixed algorithm Version 3.572c), included in the PHYLIP package.
- A phenogram was generated using the DRAWGRAM program (Felsenstein,1993).

Massamo *et al.*,2003

Automated system
Dendrogram



Comparison of phenotypic and genotypic techniques

- The GN2 MicroPlate has demonstrated accuracy comparable to molecular methods and without the expense.
- Figure below, provides representative results from an independent study with non-routine isolates compared to a molecular method:

% Correct to the Species Level	Biolog	Molecular Method
Fermenters	**80%**	**72%**
Nonfermenters	**88%**	**100%**
Overall	**85%**	**89%**

Biolog GN2 microplates
B. nigrifluens

- **Procedure:**
- Nutritional requirements and the use of specific carbon sources for growth were tested with GN2 Microplates™ (Biolog Inc., Hayward, CA).
- The optical density of the suspension was adjusted as recommended by the manufacturer.
- Microplates™were inoculated with 150 µl of suspension per well, incubated at 28°C overnight and read visually.

van der Merwe *et al.*,2010

Biolog GN2 microplates

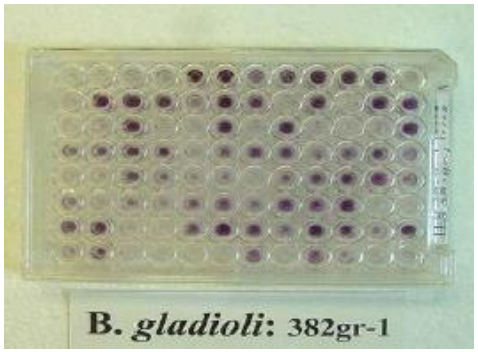
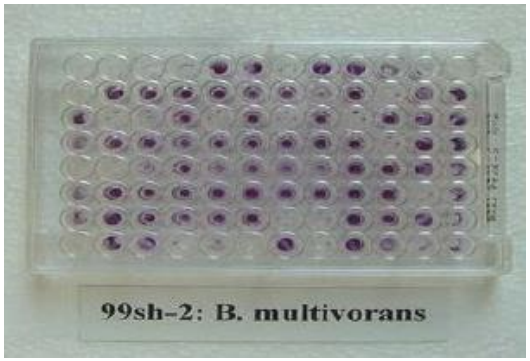
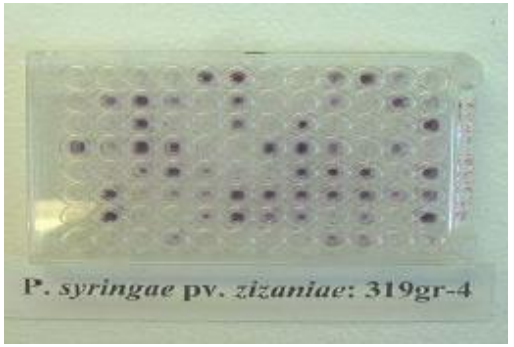
B. nigrifluens

- **Procedure:**
- Bacterial isolates were tested twice for their metabolism of 95 organic substrates using Biolog GN2 microplates.
- They were grown on Tryptic soy for 24 h at 27±1°C.
- Bacterial cells, washed twice with 25 ml of 0.85% NaCl to remove extracellular polysaccharides, were suspended in the GN/GP inoculating fluid and the absorbance at 595 nm adjusted to between 0.3 and 0.5.
- Metabolism of substrates by bacterial isolates was visually evaluated 24 h after incubation of the microplates at 27±1°C.
- Data, expressed in 3 categories (positive, negative and doubtful), were analyzed using the Biolog Microlog™ 4.1 software for bacterial identification and the GN database, version 6.01.

Moretti *et al.*,2007

Biolog GN2 Profile

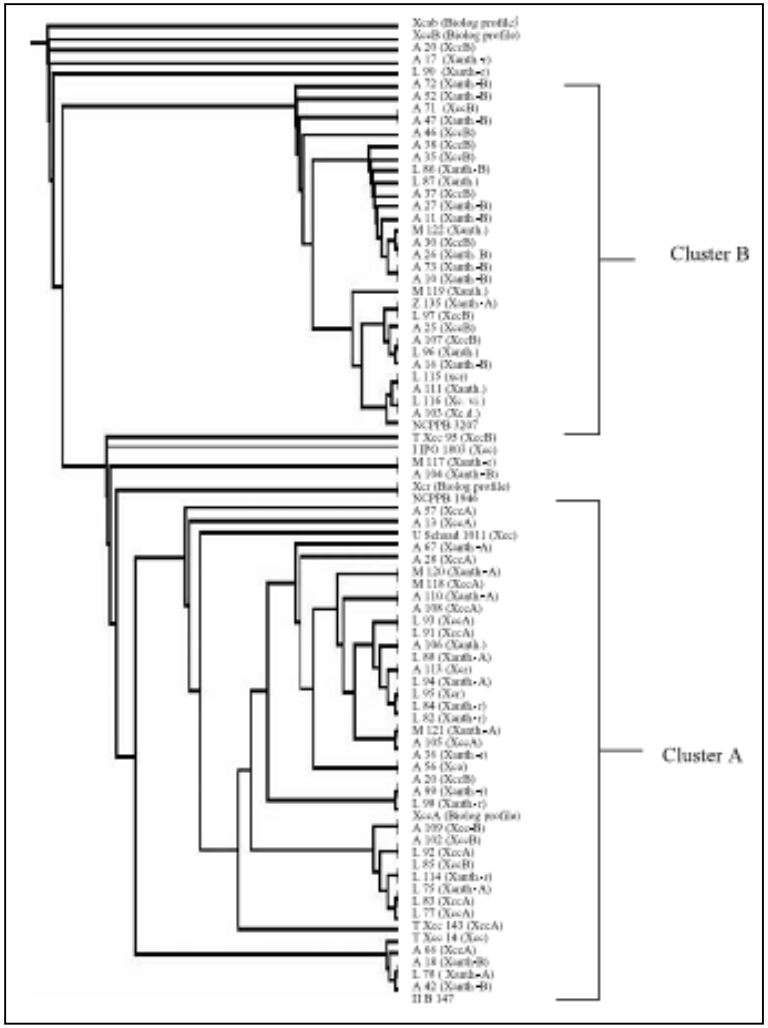
Burkholderia spp. and *P. syringae* pv. *zizaniae*

		
Biolog GN2 profile of *B. gladioli*	**Biolog GN2 profile of *B. multivorans***	**Biolog GN2 profile of *P. syringae* pv. *zizaniae***

Yuan,2004

Identification of *X. campestris* using Biolog automated aystem

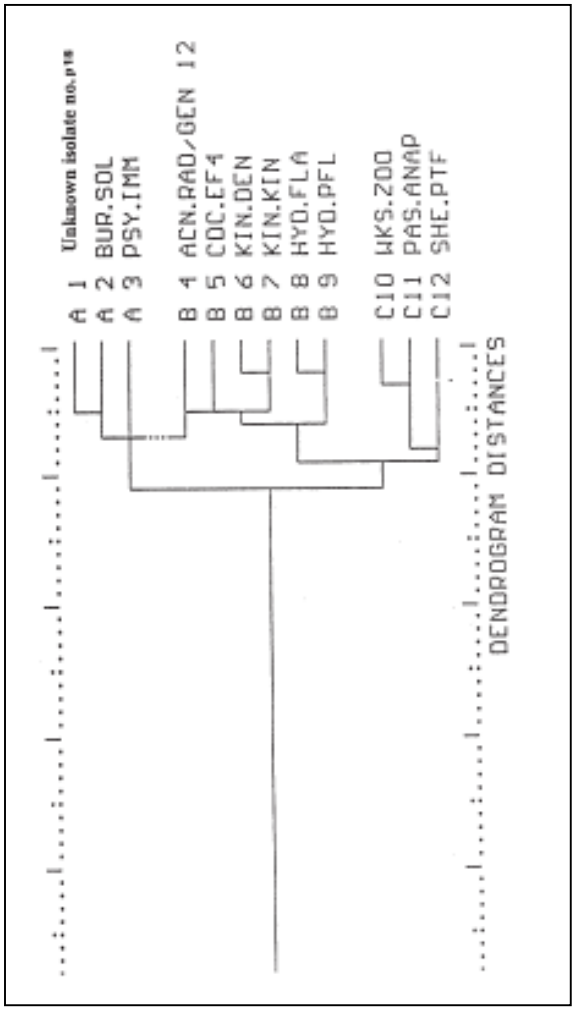
- A phenogram showing the relationships among the strains of *X. campestris* based on differential utilization of the 95 carbon substrates available in the Biolog GN MicroPlate.
- Xcc strains which belonged to the two Biolog types XccA and XccB were generally grouped in clusters A and B, respectively.



Massomo *et al.*,2003

Identification of *Ralstonia solanacearum* using Biolog automated system

- A simplified dendrogram was obtained using Biolog GN.
- It is showing the Biolog clusters of isolate number 18F of *Ralstonia solanacearum*.



Tawfik *et al.*,2008

Serological Methods
Serotaxonomy

Serotyping

Serological diagnosis
Definition

1. Measuring antibody in serum or secretions;
2. Identifying antigens in blood, tissue or secretions using immunochemical techniques.

In general, serological methods are robust, cheap and appropriate for a first screening and for massive analyses.

L Ross Whybin;..

Serological diagnosis

Uses

1. Immunity/past infection
2. Screening blood /tissue donors
3. Recent/Congenital infection
4. Epidemiological studies.

In general, serological methods are robust, cheap and appropriate for a first screening and for massive analyses.

L Ross Whybin;..

Serological diagnosis
Serological methods vs. molecular tests

- In general, the accuracy of the results obtained in each laboratory for the ELISA tests was lower than the accuracy values obtained using the molecular tests.
- The majority of the deviations were recorded for the samples containing the lowest bacterial concentration ($5x10^4$ CFU/ml), with several replicates testing negative, i.e. producing negative deviations.

EPPO,2017

Serological techniques

Sensitivity of different serological techniques used for antibody detection (ug/mL)

Method	Sensitivity (µg/mL)
Gel-diffusion	30
Ring precipitation	18
Bacterial Agglutination	0.05
Complement Fixation	0.05
Complement Fixation	0.01
Haemagglutination Inhibition	0.005
Immunofluorescence	0.005
ELISA	0.0005
Bacterial Neutralisation	0.00005

Serological techniques

Used for detection and identification of bacteria

Lateral Flow dipstick tests

- Serological techniques utilized for bacterial pathogens are mainly:

1. Indirect immunofluorescence staining (IF),
2. Several types of enzyme-linked immunosorbent assay (ELISA), and
3. The recent so-called lateral flow devices(principle used in pregnancy tests).

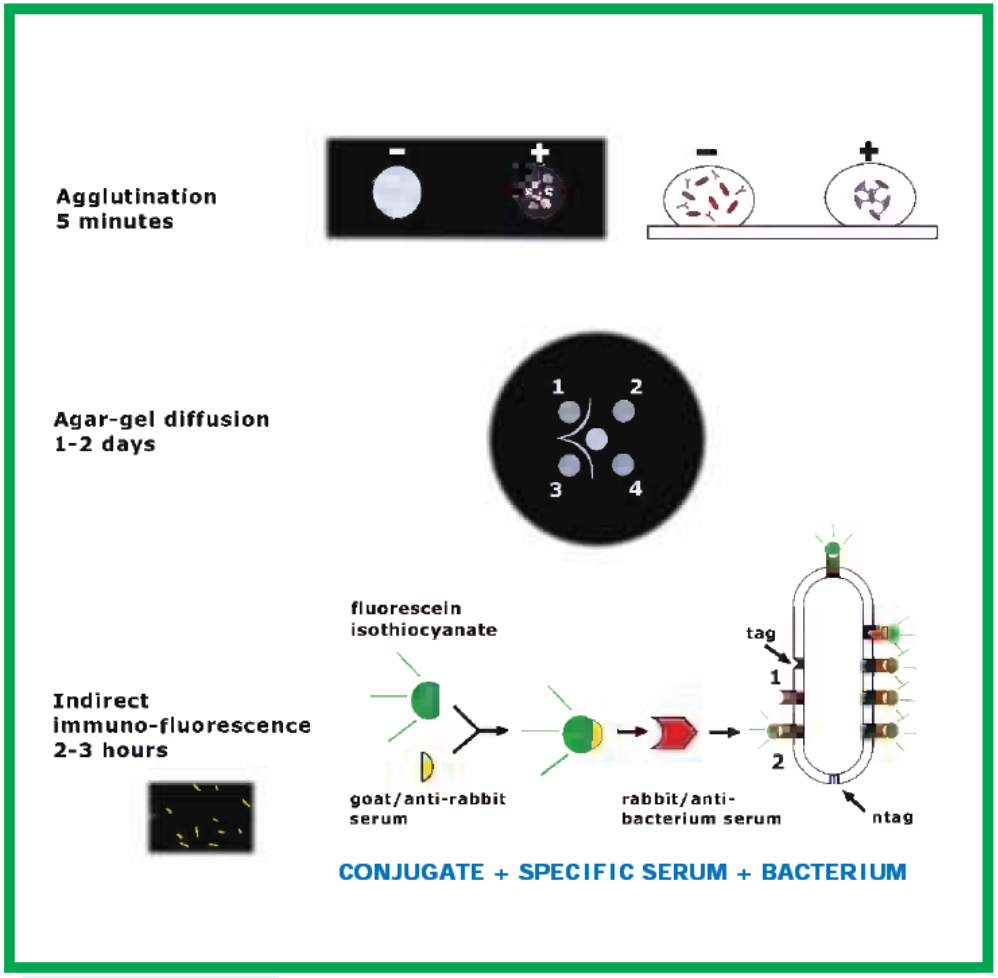
- Their accuracy is correlated with the quality of the antibodies utilized and when possible, the use of monoclonal antibodies is advisable.

López *et al.*,2010;..

Serological techniques

Used in detection and identification of bacteria

- tag= target antigen.
- ntag= non-target antigen.



Janse,2006

Serological techniques
Advantages and disadvantages

- Advantages of these techniques are:

1. Less time-consuming;
2. Simple and robust;
3. Have a fairly high detection level (e.g. the IF test has a detection level of 10^3-10^4 cells ml^{-1});
4. Have possibilities for screening many samples and automation.

- A disadvantage of ELISA is its higher detection level (10^5-10^6 cells ml^{-1}).
- Both IF and ELISA have the disadvantage of showing disturbing cross-reactions of non-target bacteria with antisera used.

Janse,2006

Definition

Antibodies vs. antigens

- **Antigen:**
- A foreign macromolecule capable of inducing antibody formation.
- Most effective antigens are proteins, though some polysaccharides are antigenic.
- **Antibodies:**
- Defence proteins in the blood serum called immunoglobulins or antibody moelcules.
- e.g. Immunoglobulin G (IgG) which are formed by animals against compounds (antigens) introduced in their body.

Definition

Antibodies

Polyclonal and monoclonal antibodies

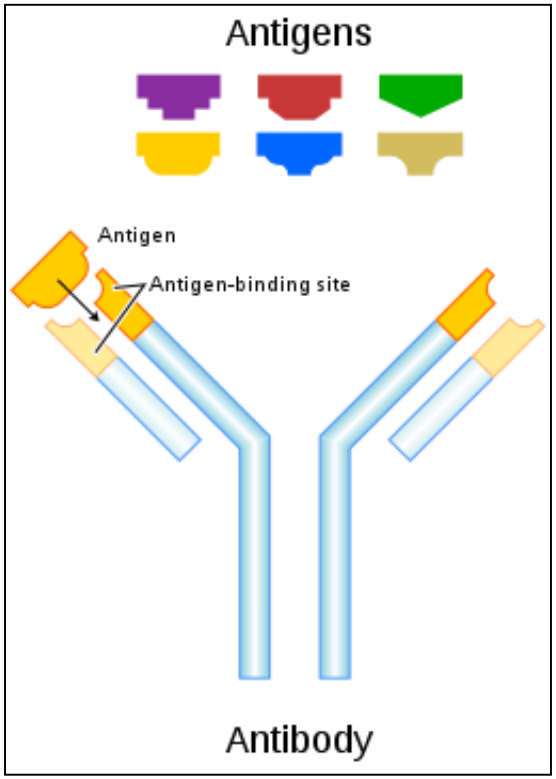
- The antiserum taken from such a rabbit (New Zealand albino rabbits weighing 2-4 kg) is called a polyclonal antibody/serum (Pabs).
- In this technique, different cell types of the rabbit produce normally many types of antibodies.
- In the monoclonal antibodies (MAbs) technique which is injected into a mouse, only one type of antibodies against one antigen (or epitope) of the bacterium is produced.

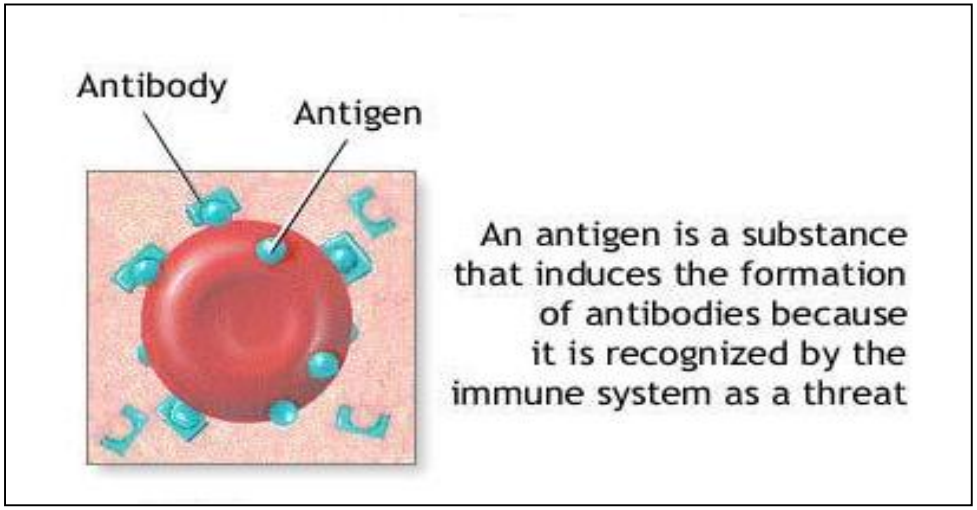
The antibodies
Specificity

- The basic premise behind all of these tests is that antibodies are highly selective in terms of the proteins (or other cell structures) to which they bind, to the point that they are able to distinguish the proteins coming:

1. From one bacterial species, or
2. Even one strain among many strain.

Structures
Antibodies vs. antigens





Each antibody binds to a specific antigen; an interaction similar to a lock and key.

Wikipedia,2018;..

The antigens
Epitopes

- The actual portions or fragments of an antigen that react with free antibody molecules, are called epitopes or antigenic determinants.
- The size of an epitope is generally thought to be equivalent to 5-15 amino acids or 3-4 sugar residues.
- Some antigens, such as polysaccharides, usually have many epitopes, but all of the same specificity.
- As a result, most "shapes" along the polysaccharide are the same (Fig. 1).
- Other antigens such as proteins usually have many epitopes of different specificities.
- This is because proteins are usually hundreds of amino acids long and are composed of 20 different amino acids (Fig. 2).

Kaiser,2009

The antigens
Epitopes

Fig. 1:
Epitopes of a polysaccharide antigen.

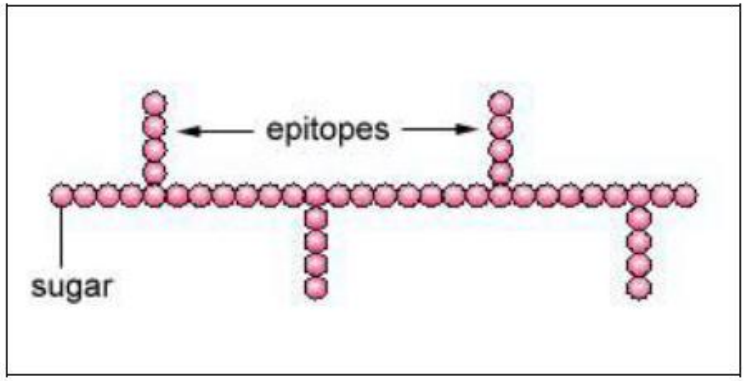
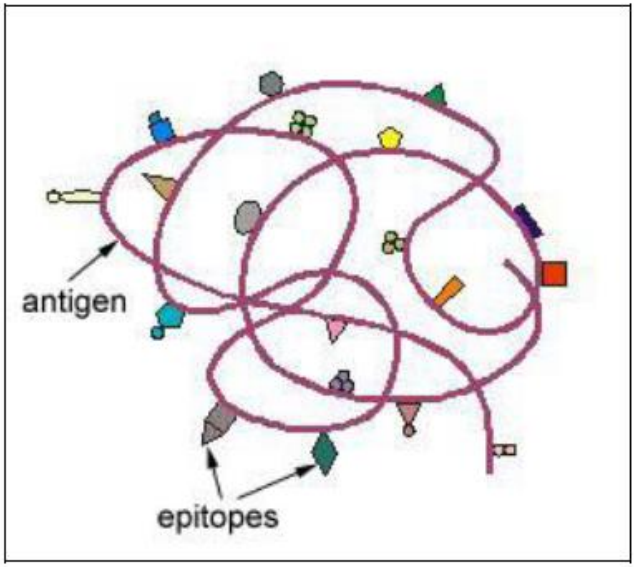


Fig. 2:
Epitopes of a protein antigen.



Kaiser,2009

The antibodies
Specificity

- Antibodies are proteins made by the immune system to destroy foreign invaders.
- There are five major types of antibodies:

1. Immunoglobulin A (IgA),
2. Immunoglobulin G (IgG),
3. Immunoglobulin M (IgM),
4. Immunoglobulin E (IgE), and
5. Immunglobulin D (IgD).

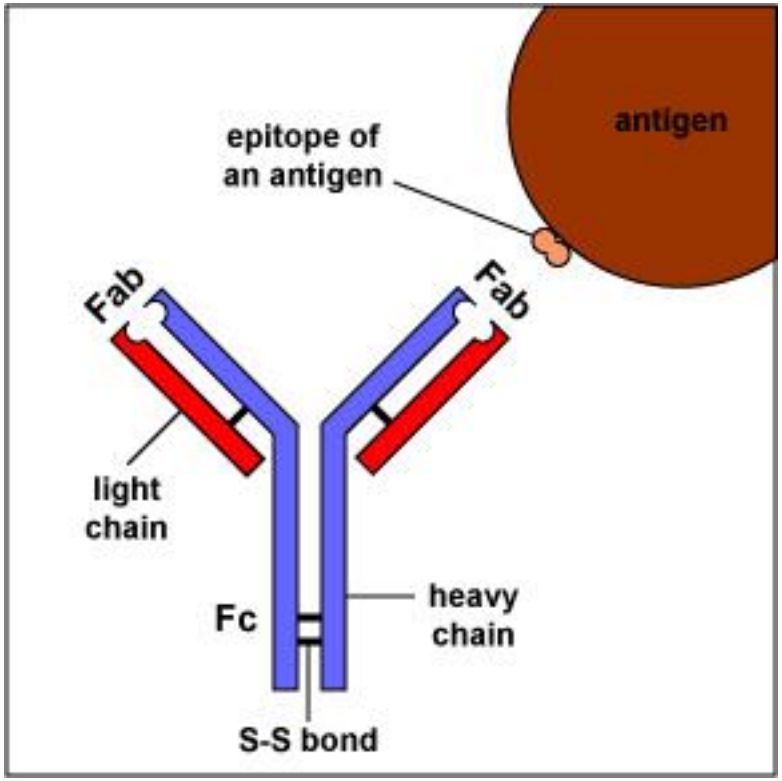
- An IgG antibody it is the smallest, yet most abundant, human antibody helps to battle bacterial and viral infections.

WebMD, 2005-2011

The antibody molecules

Antibody structure (IgG)

- The simplest antibodies, such as IgG, IgD, and IgE, are "Y"-shaped macromolecules called monomers.
- A monomer is composed of four glycoprotein chains:
1. two identical heavy chains, and
2. two identical light chains.
- Each IgG has two antigen binding sites.
- The four glycoprotein chains are connected to one another by disulfide (S-S) bonds and noncovalent bonds.
- The Fab portions provide specificity for binding an epitope on an antigen.
- The Fc portion directs the biological activity of the antibody.



Antibody structure (IgG)

Kaiser,2009; LibreTexts libraries,2018

Purification of antiserum

Purified antibodies

- The antiserum is further purified to obtain the gamma-globulin fractions.
- The gamma-globulin molecules with antibody activity are referred to as immunoglobulins (Ig), and the most abundant is IgG.
- Purified gamma-globulin is be used in ELISA assays.
- These purified antibodies against the virus/bacteria are used to prepare conjugate-IgG antibodies linked to an enzyme (usually peroxidase or alkaline phosphatase).
- These enzymes produce a colored product when incubated with a specific substrate.

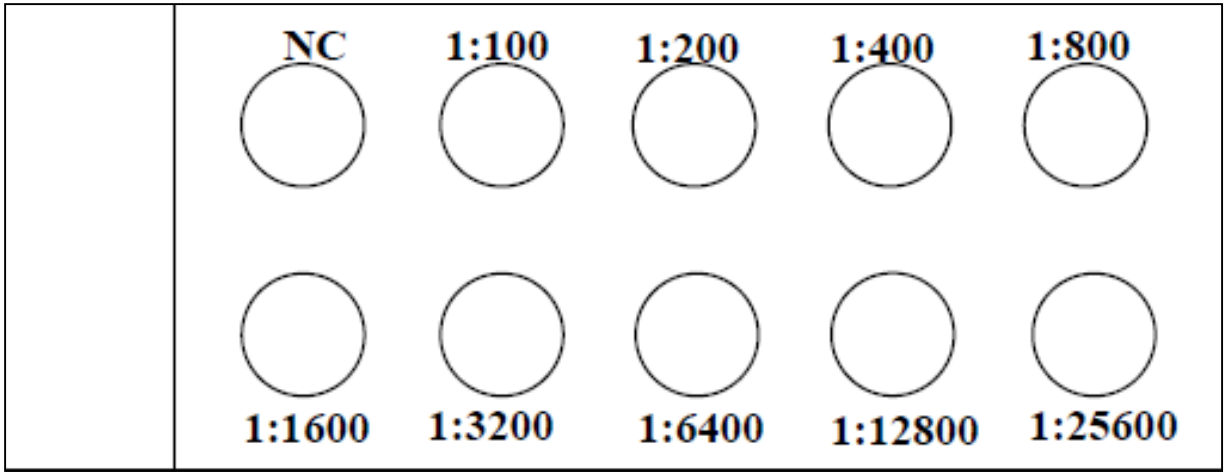
Storage of antibodies

- It is recommended to store crude antisera mixed 1:1 with 87% glycerol and antibodies (ca. 1 mg ml^{-1} in phosphate buffered saline containing 43% glycerol), both in small aliquots (0.5 ml) in cryotubes at -20°C or -80°C.
- Thaw at room temperature and mix before use but avoid repeated freezing and thawing.
- Alternatively, add 0.05% sodium azide as a preservative, when storage can be at 4°C.

Pérombelon & van der Wolf,2002

Titration of antiserum

Scheme of dilution of antiserum



Janse,2010

Monoclonal antibodies (MA)
Specificity

- **Flagella:**
- Antisera against flagellar proteins can be prepared and used for refined identification of bacterial strains.
- Bacterial extracellular polysaccharides (EPS):
- EPS may also be used to raise antisera with a high level of specificity.
- **Lipopolysaccharide (LPS):**
- Antiserum raised against LPS may be so specific that cultivar-specific strains of *Pseudomonas syringae* may be detected which are not differentiated by antiserum raised to intact bacteria.

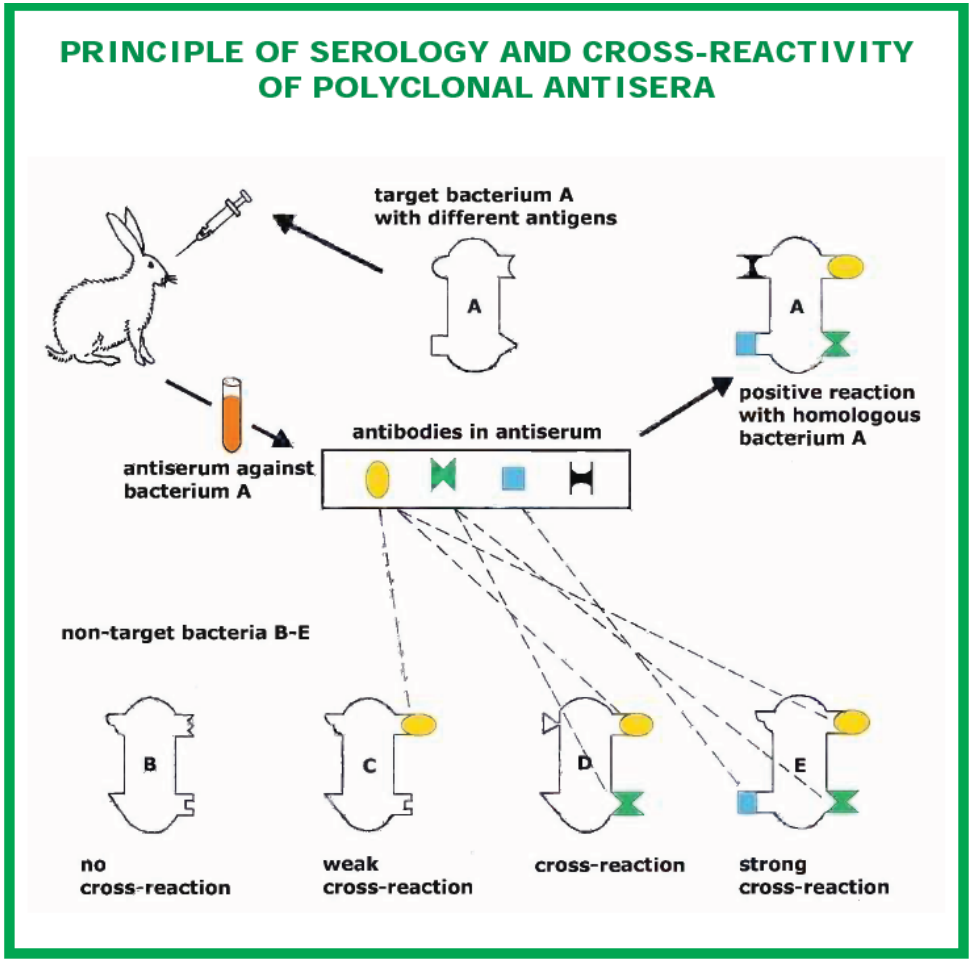
Monoclonal antibodies (MA)

Specificity and reactivity

- Monoclonal antibodies can be produced with a very constant quality and of a high specificity.
- Reactivity of monoclonal sera is usually lower than that of polyclonal sera, because only one epitope of the bacterium will show the binding reaction.
- To enhance reactivity of monoclonal sera mixtures of monoclonals can be made, if available (De Boer, 1987).

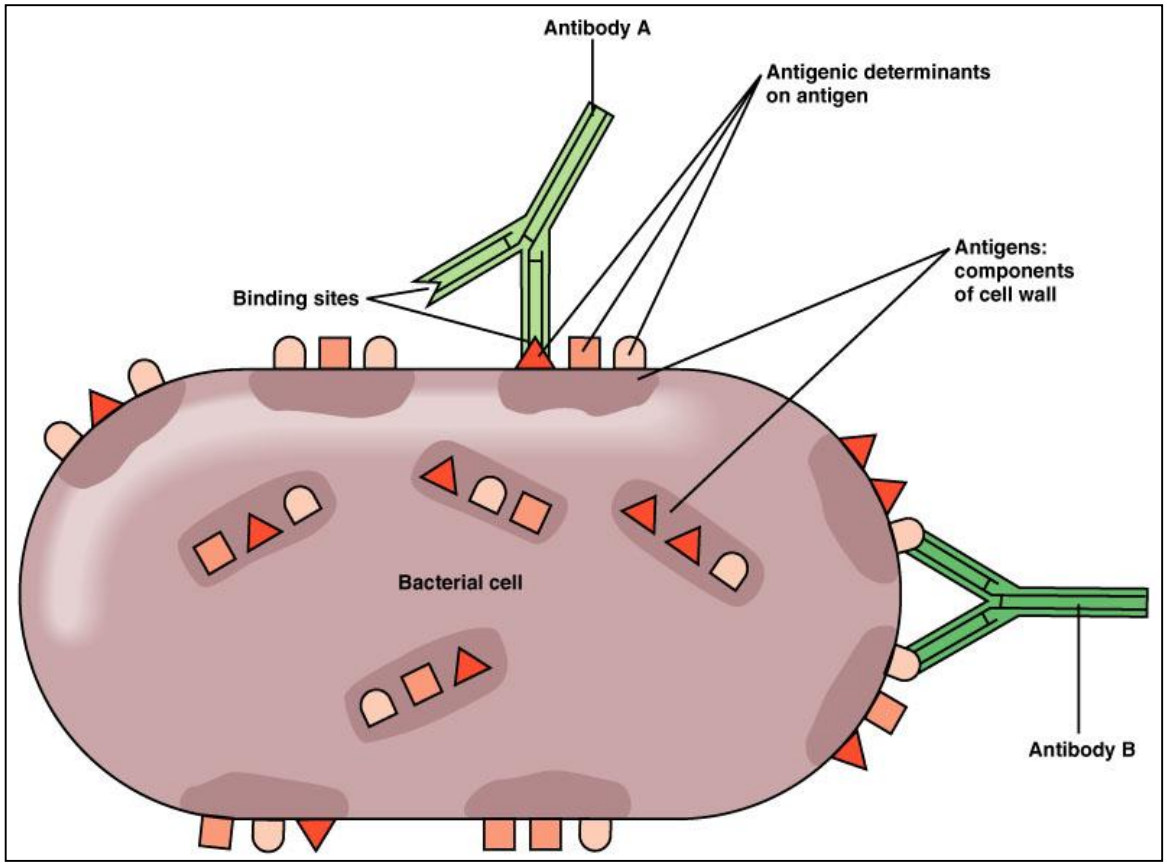
Janse,2006

Principle of serology
Cross-reactivity of polyclonal antisera



Janse,2006

Antigen-antibody reactions



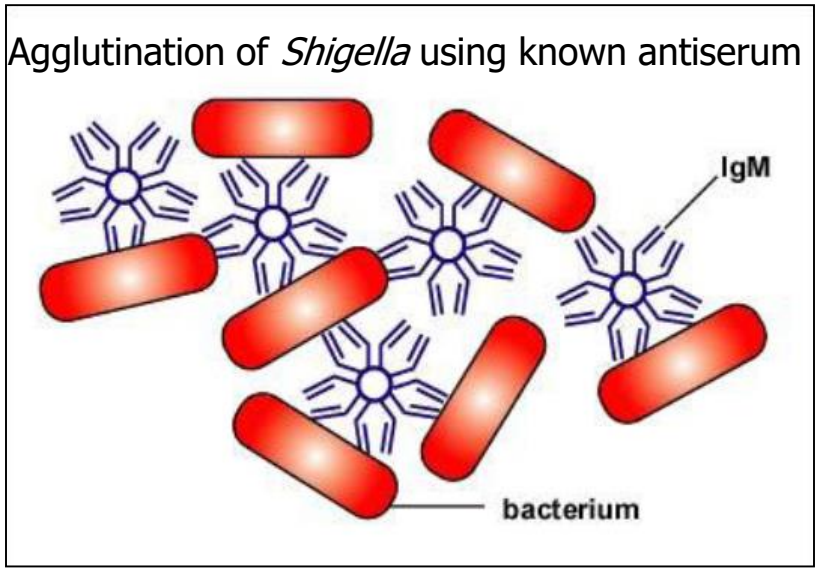
Karlm,2004

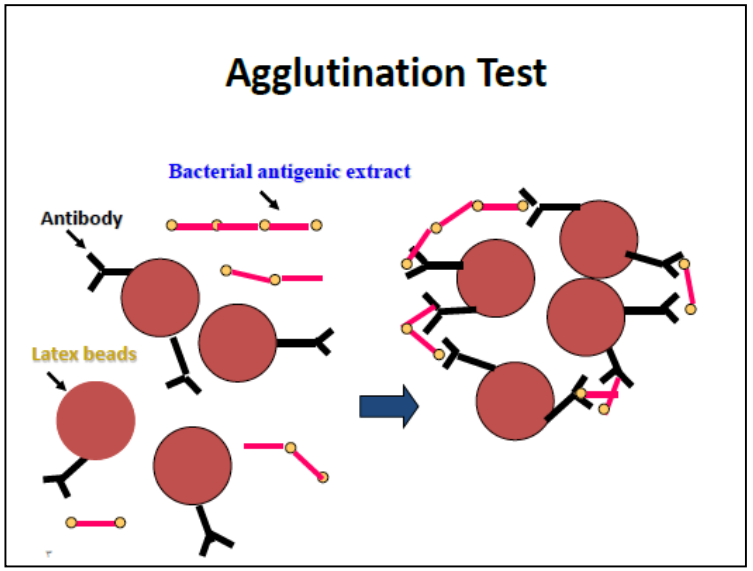
1. Agglutination test

- Agglutination test on a microscopic slide.
- If antibodies (in antiserum) and a suspension of bacteria are mixed in a certain concentration they will clump together and an agglutination reaction takes place.
- In the so-called latex agglutination test antibodies are coated to sensitized latex beads.
- In this way the antibodies are enlarged so to say, and the reaction is more readily visible, making the test more sensitive than slide agglutination.
- Unfortunately the agglutination test is often liable to disturbing cross-reactions.
- New is the development of so-called lateral flow kits (e.g. by CSL, York, UK) where an agglutination test can be performed with a kit in the field on symptomatic material.

Janse,2006

Agglutination Test

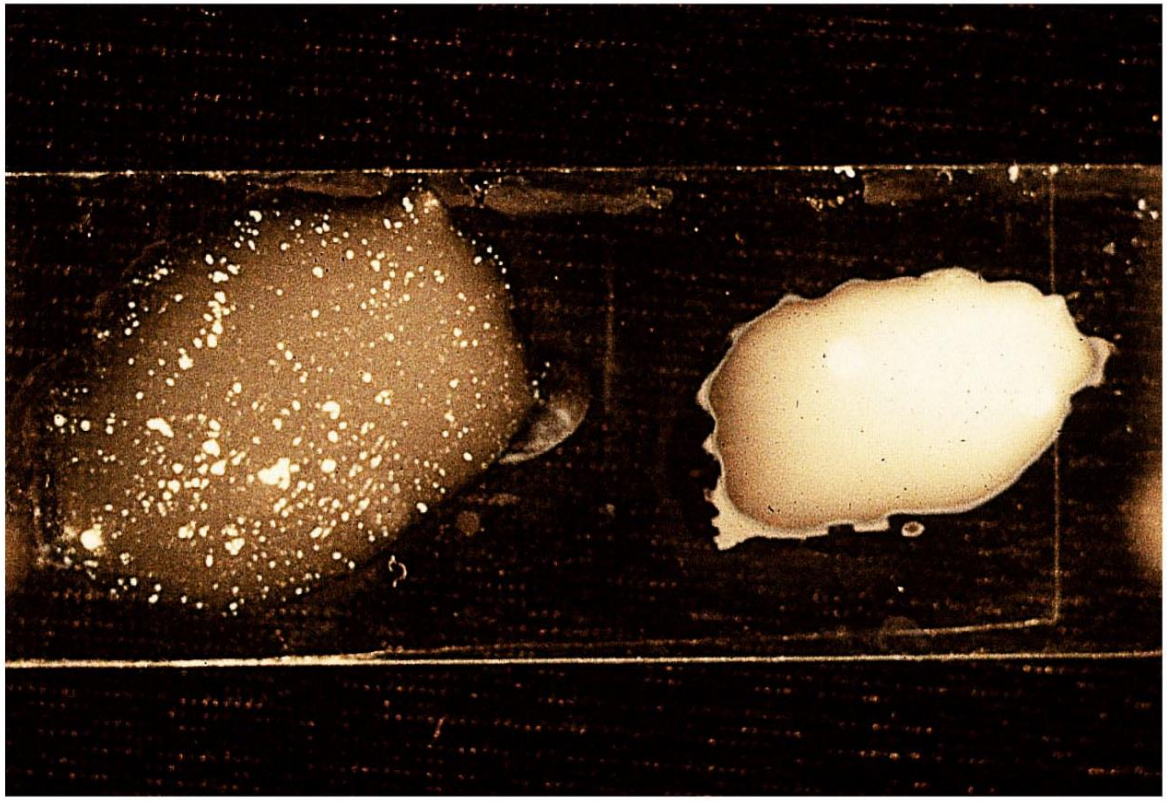




Kaiser,2009; Fox,2008

Agglutination test

Combine known antiserum+unknown bacterium Slide agglutination



(a) Positive test (b) Negative test

2. Precipitation test

- In this test only certain soluble antigenic proteins or polysaccharides of the bacteria react with the antibodies.
- Bacteria, often killed and disrupted by phenol, are placed in wells in the agar plate.
- Soluble antigens and antibodies diffuse in the agar.
- Where they meet in a certain concentration they bind, form flakes and precipitate, visible as a white line.
- How the lines of two different bacteria meet each other in the agar can say something about their serological relationship.
- The precipitation test is very insensitive.

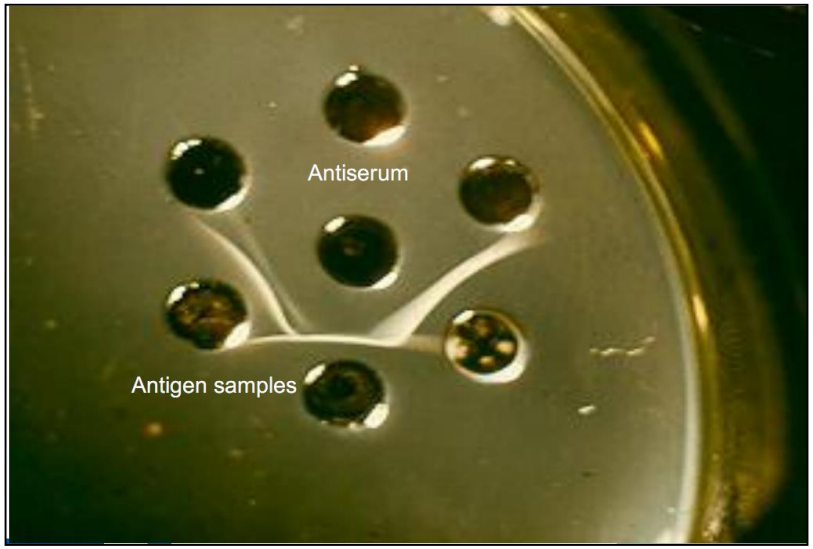
Janse,2006

Precipitation test

Ouchterlony double diffusion technique

Immunodiffusion test

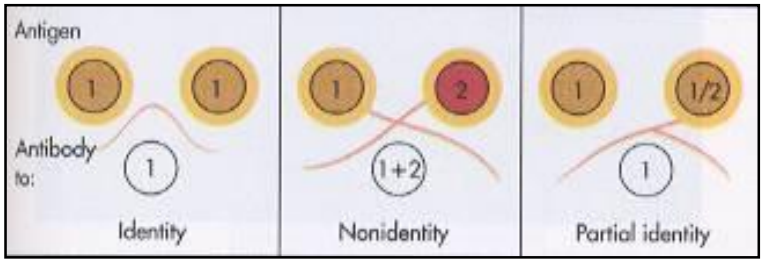
- Double diffusion plate with six wells surrounding the center antiserum (AS) well demonstrates the white precipitin bands formed in the plate by a positive reaction.



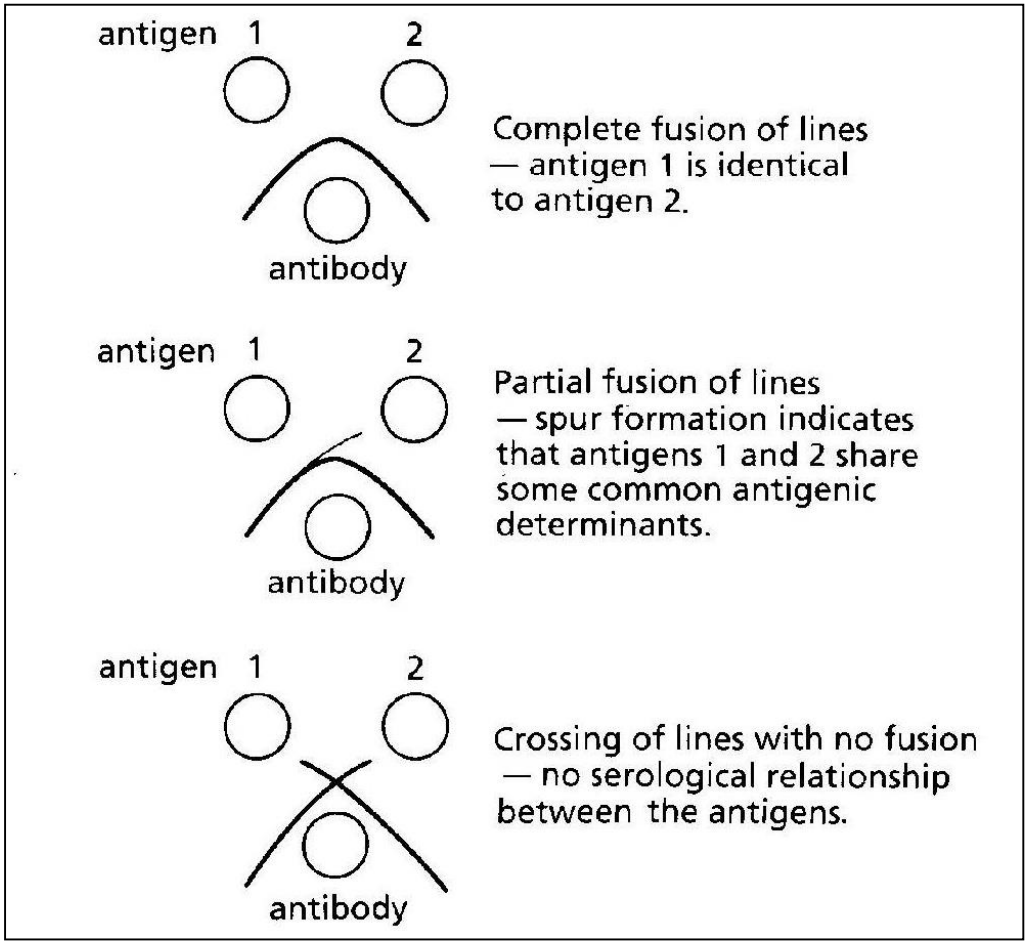
Trigiano *et al.*,2006; Gáborjányi,2010

Precipitation test

Basic precipitation patterns in double immunodiffusion



http://faculty.evansville.edu



Lelliott and Stead,1987;..

3. Immunofluorescence (IF) test
Direct IF method

- Immunofluorescence (IF) microscopy is a very sensitive and robust serological test (detection level of c. 10^3-10^4 cells ml^1(per ml) of plant extract) because the primary reaction of antigen and antibody is made visible.
- Binding reactions can be observed at very high titers.
- **Direct IF antiserum:**
- In so-called direct IF antiserum against a certain plant pathogen is already labeled with FITC.
- In the IF test antibodies are marked with a chemical dye that fluoresces in blue light, mostly fluorescein isothiocyanate (FITC).
- For IF a light microscope fitted for epi-fluorescent light is necessary with the suitable excitation and barrier filters for FITC.

Titer= highest dilution of the antiserum where a clear reaction is still visible) of antiserum.

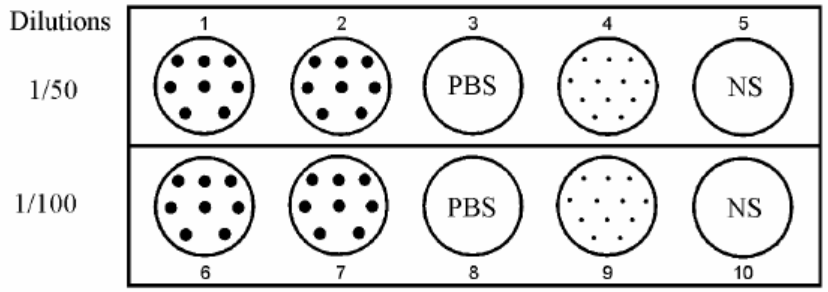
3. Immunofluorescence (IF) test
Indirect IF method

- The bacteria are first treated with a pathogen-specific rabbit or mouse antiserum (against the target bacterium).
- After incubation and washing, a second, labelled anti-rabbit or anti-mouse serum, prepared in another animal (e.g. goat), is applied.
- This anti-rabbit or antimouse serum is called the conjugate.
- Only the antibodies bound to the bacteria will fluoresce, while the others are removed by washing.
- Indirect IF is slightly more sensitive and less specific than direct IF.

Janse,2006

Indirect immunofluorescence antibody staining (IFAS) Procedures

- At least two dilutions of the antiserum should be used for IFAS.
- One should be equal to half the titre of the antiserum and the other close to the antiserum titre (Mazzucchi *et al.*, 1983).
- The first antiserum dilution will be used in wells 1, 2, 6 and 7;
- The second in wells 4 and 9.
- Wells 3 and 8 will be treated with PBS and wells 5 and 10 with preimmune serum.



Slide for IFAS

EPPO Standard in 1998

Immunofluorescence (IF)test

Bacterial cells+antibody attached to a fluorescein+ immunofluorescence microscopy

- Bacterial cells are fixed onto a microscope slide using heat or ethanol and labeled with a specific antibody.

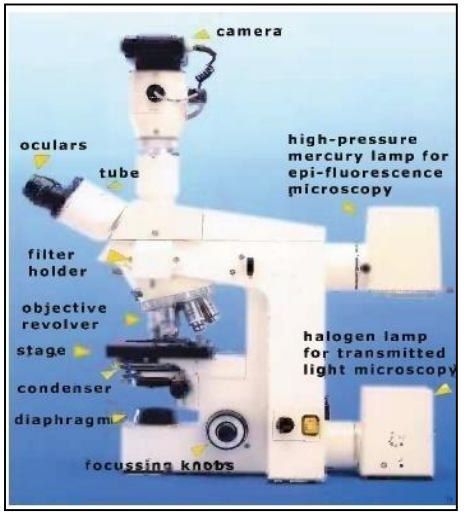
1. Direct IF method:

- The bacterium reacts to an antibody attached to a fluorochrome (usually fluorescein or rhodamine).
- The fluorochrome is observed under epi- UV light using a UV microscope and immersion oil; fluorescing cells appear green on a dark background.

2. Indirect IF method:

- The bacterium reacts against a first antibody, which in turn reacts with a second fluorochrome-labeled standard antibody.

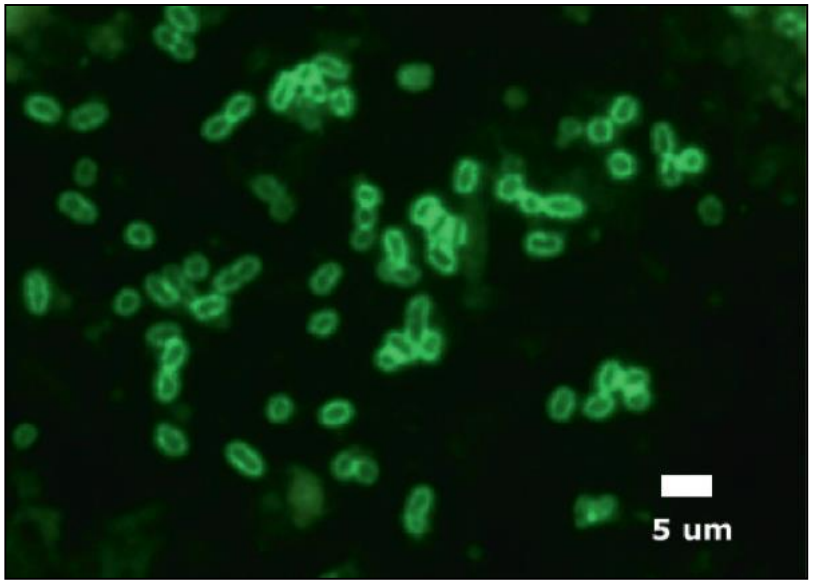
Figure 1.16. *Xanthomonas translucens* pv. *undulosa* cells observed under immunofluorescence microscopy.



Immunofluorescence (IF)test

Bacterial cells+antibody attached to a fluorescein+ immunofluorescence microscopy

- Typical cell morphology of *Ralstonia solanacearum* cells in naturally infected potato tissue extract at a dilution of 1:3,200 of a polyclonal antiserum and using X100 planapochromatic oil immersion objective.



Burns,2009

4. Enzyme Immunoassay(EIA)

- Enzyme Immunossay (EIA) is a simple, effective and powerful tool for identification and analysis of biological materials.
- A few EIAs routinely used in plant pathology laboratories are:

1. Enzyme Linked Immunosorbent Assay (ELISA),
2. Tissue Blot Immunoassay (TBIA) and
3. Dot Blot Immunoassay (DBIA).

Burns,2009

4.1. ELISA
The Enzyme Linked Immunosorbent Assay

- The ELISA test is a commonly used method to detect and identity but not to quantify, bacterial pathogens.
- The typical sensitivity of ELISA for bacteria is about 10^{6-5} cfu/ml.
- At the lower concentration, the target pathogens might be more difficult to detect.
- The standard ELISA does not differentiate between viable and non viable-cells.

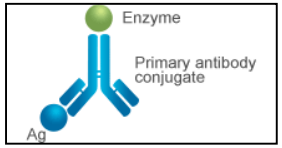
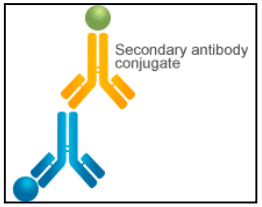
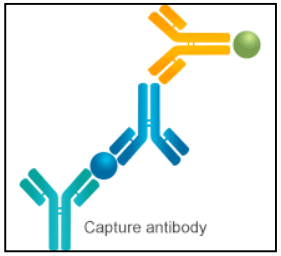
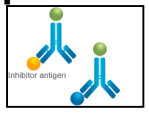
For more details
see the diagnostic file Part-3/Xylella section.

ELISA

Four Typical ELISA Formats

1. Direct ELISA
2. Indirect ELISA
3. Sandwich ELISA
4. Competition or Inhibition ELISA

Advantages and disadvantages of each ELISA type

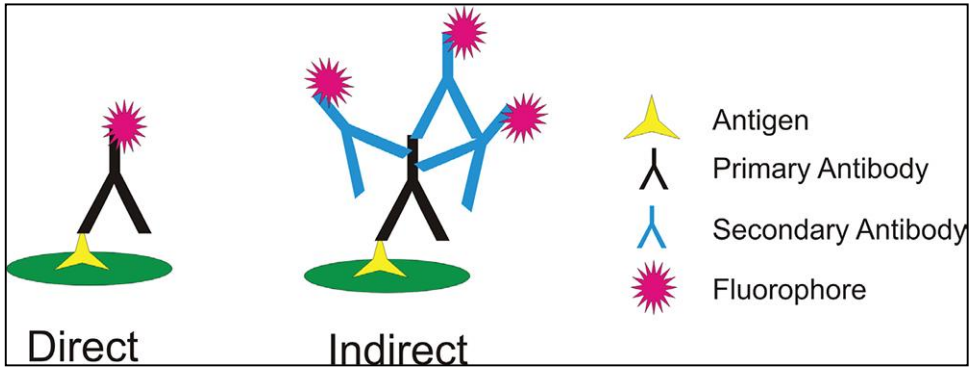
Direct ELISA 	1. Simple protocol, time-saving, and reagents-saving. 2. Only primary antibody is used, meaning that secondary antibodies are not needed. 3. It is the antigen that is immobilized to the plate. 4. No cross-reactivity from secondary antibody.	1. High background. 2. No signal amplification, since only a primary antibody is used and a secondary antibody is not needed. 3. Low flexibility, since the primary antibody must be labeled.
Indirect ELISA 	1. Both a primary antibody and a secondary antibody are used. 2. It is the antigen that is immobilized to the plate. 3. Signal amplification, since one or more secondary antibodies can be used to bind to the primary antibody. 4. High flexibility, since the same secondary antibody can be used for various primary antibodies.	1. Complex protocol compared with direct ELISA. 2. Cross-reactivity from secondary antibody.
Sandwich ELISA 	1. Unlike In direct and indirect ELISA, in sandwich ELISA, it is the antibody that is immobilized to the plate, and this antibody is called capture antibody. 2. High flexibility. 3. High sensitivity. 4. High specificity, since different antibodies bind to the same antigen for detection.	1. The antigen of interest must be large enough so that two different antibodies can bind to it at different epitopes. 2. It's sometimes difficult to find two different antibodies that recognize different epitopes on the antigen of interest and cooperate well in a sandwich format.
Competitive ELISA 	1. High flexibility. 2. High sensitivity. 3. Best for the detection of small antigens, even when they are present in low concentrations.	1. Relatively complex protocol. 2. Needs the use of inhibitor antigen.

ELISA

There are two main types of ELISA

Direct and Indirect ELISA

- **Direct ELISA:**
- uses a primary antibody;
- requires higher antigen amounts;
- quicker, cheaper but lower sensitivity process.
- **Indirect ELISA:**
- requires the addition of a secondary antibody;
- requires smaller amounts of an antigen;
- more sensitive.

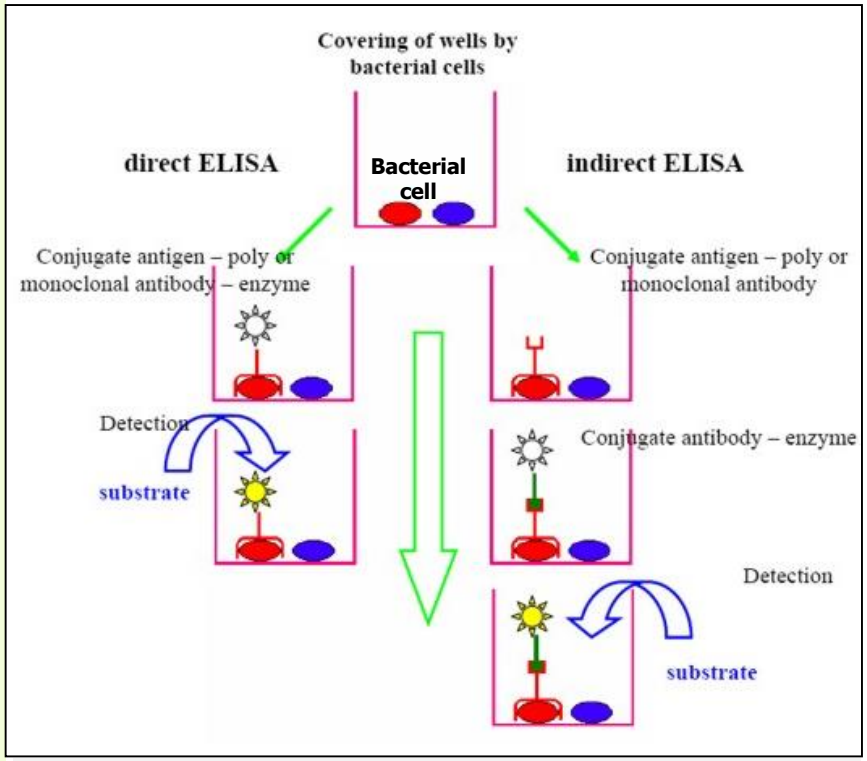


ELISA

There are two main types of ELISA

Direct and Indirect ELISA

- **Direct ELISA:**
- One-step ELISA.
- The specific antibody conjugated with enzyme i.e. alkaline phosphatase.
- **Indirect ELISA:**
- Two-step ELISA.
- The primary antibody is incubated with the antigen followed by the incubation with the secondary antibody conjugated with enzyme i.e. alkaline phosphatase.



The substrate like p-nitrophenylphosphate is added to the well to obtain the yellow-coloured product.

DAS-ELISA

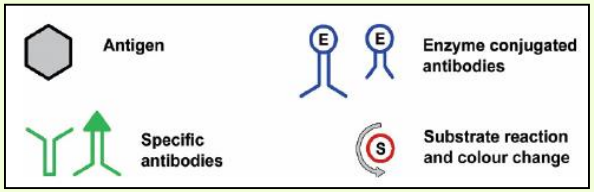
A more common ELISA test

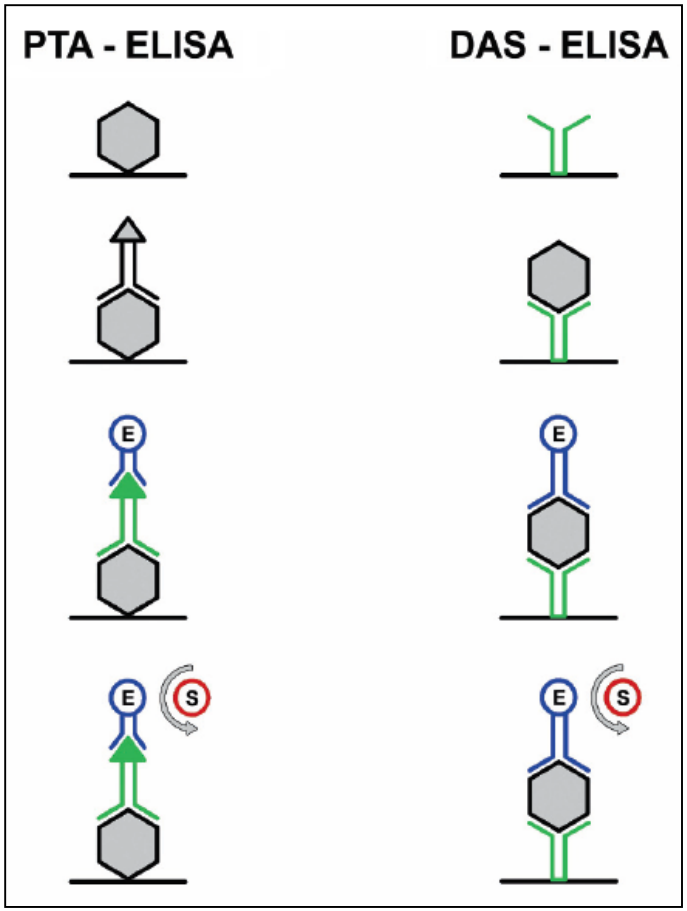
- A more commonly used format is the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA).
- In DAS-ELISA, firstly, the specific antibody is used to coat the microtitre plate, which then traps the target antigen from the test sample.
- An enzyme-labelled specific antibody conjugate is subsequently used for the detection of bacteria in sample.

Burns,2009

ELISA
PTA-ELISA and DAS-ELISA

- Schematic illustration of indirect PTA-ELISA (plate-trapped antigen) and DAS-ELISA (double antibody sandwich) formats.





Burns,2009

ELISA

Double antibody sandwich (DAS)-ELISA test

The procedures

1. Non-specific antibody i.e. gamma globulin was added to bound to the wells of a microtitre plate.
2. Sample containing antigen of interest was added.
3. Wells washed well with buffer.
4. Specific antibody conjugated with an enzyme (enzyme-linked antiserum) i.e. alkaline phosphatase was added ('sandwiched').
5. Wells containing excess antiserum were washed with phosphate buffered saline.
6. The substrate (e.g. p-nitrophenylphosphate) of the enzyme is added to the well to obtain the coloured product.

- The substrate is hydrolysed by the enzyme (usually alkaline phosphatase) bound to antiserum and releases p-nitrophenol, changing the colour of the reaction mixture (from colourless to yellow).
- Develop the color for 30 minutes and immediately read plate with plate reader at 405-410 nm.

ELISA test
Ralstonia solanacearum

- Plate layout for the *Ralstonia solanacearum* ELISA.
- Wells in rows 1-11 are coated with anti-*Ralstonia solanacearum* antibodies.
- Wells in row 12 are left uncoated.
- Different samples are pipetted into demarcated blocks of wells:

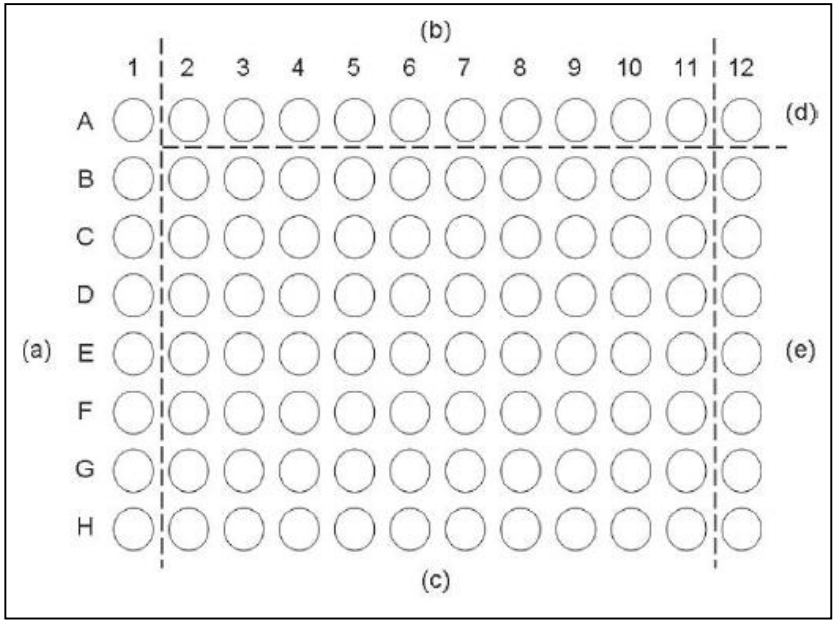
a) Wells containing buffer only to serve as blanks;

b) the dilution series of positive control samples;

c) Tuber sap samples;

d) A single dilution of the positive control sample;

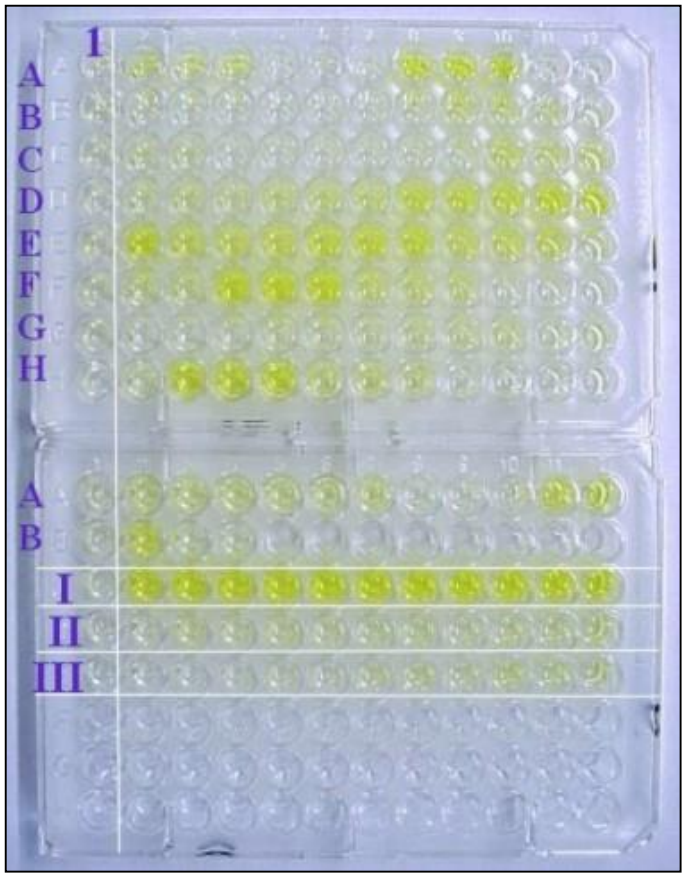
e) Duplicates of the tuber sap samples pipetted into the wells of row 11.



Burns,2009

ELISA plate

- ELISA plates typically have 96 shallow wells.
- Row I: positive control (*Pectobacterium carotovorum* subsp. *carotovorum*),
- Row II and III: negative controls (buffer& non-specific bacterium).



ELISA test

ELISA readers

- Normally the intensity of the colour is determined spectrophotometrically to give a measure of the amount of antigen, using a plate reader.
- i.e. intensity of the colour developed is proportional to the amount of antigen.

ELISA readers

4.2. Tissue and dot blot Immunoassays
TBIA and DBIA

- Dot-immunobinding assay (Lazarovits 1990), also known as DIA or DIBA, is a variation of ELISA modified in two ways.

1. First, the antigen is trapped on a nylon or nitrocellulose membrane, not on the microtitration plate; this can be done with a blot-dot microfiltration apparatus.
2. Second, the enzymatic reaction peroxidase with perhydrol in the presence of the chromogenic substrate (e.g., 4-chloro-1-naphtol) produces a precipitate that appears as colored dots on the membrane.

Duveiller, E., L. Fucikovsky and K. Rudolph, eds. 1997

Tissue and dot blot Immunoassays
TBIA and DBIA

- Antigens or antibodies are detected using a novel membrane based immunoassay.
- Known antigens or antibodies which will form complexes with antigens/antibodies to be assayed are spot filtered with pressure through a membrane.
- The membrane, either by itself or attached to a base material as a test strip, is incubated with a test fluid.
- Consequently, the resulting antibody-antigen complex is incubated directly or after an intermediate anti-antibody incubation with enzyme conjugated immunoglobulin and exposed to substrate which produces a colored insoluble product if the test target is present.

Tissue and dot blot Immunoassays
TBIA and DBIA

- Similar to coating procedure in ELISA, proteins can be immobilized on nitrocellulose membranes.
- Membranes 0.45 µm are suitable for TBIA or DBIA.
- Tissue blot is a process of transfer of protein antigens from a freshly cut tissue surface to nitrocellulose membrane.
- It is achieved simply by bringing a freshly cut tissue surface in direct contact with a dry nitrocellulose membrane.
- The tissue imprint is made by application of a slight pressure of the cut tissue surface while it is in contact with the nitrocellulose membrane.
- Do not squeeze juice out from tissues. This will smear the imprint.

Burns,2009

Tissue and dot blot Immunoassays
TBIA and DBIA

- In the Dot Blot procedure, the samples are applied to nitrocellulose membrane while it is attached and clamped into a manifold.
- Add 0.25-100 µL tissue extract containing pathogen protein to each well.
- Membranes are removed.
- From here on both membranes from tissue blot or dot blot are processed similarly as microtiter plates in ELISA.
- Either direct or indirect procedure can be used.

Burns,2009

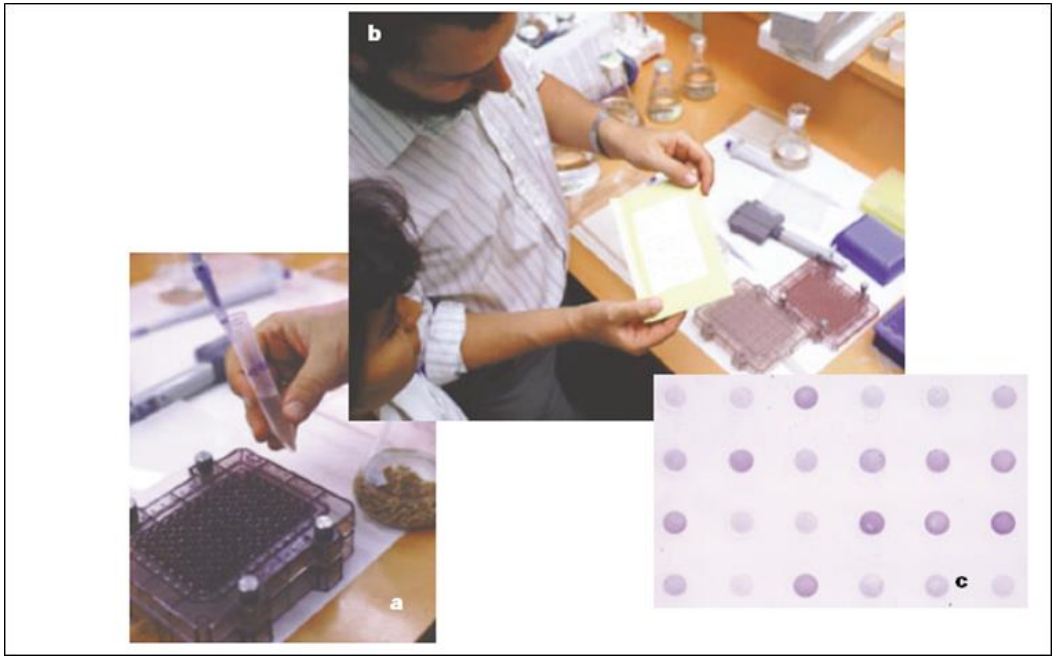
TBIA and DBIA
Procedures

1. 0.2- or 0.45- μm nitrocellulose membrane.
2. Razor blades.
3. Gloves or forceps.
4. Small (about 10 × 12 cm) glass tray.
5. Phosphate suffered saline solution (PBS): pH 7.5.
6. PBS-Tween: 0.05% Tween-20 in PBS.
7. Blocking solution: 1% nonfat dry milk, 0.5% BSA in PBS Tween.
8. Alkaline phosphatase-labeled antibody conjugate.
9. Substrate buffer: Solution A: 35.5 mL distilled water, 2.0 mL 2.5 M Tris–HCl, pH 9.5, 2.0 mL 11% sodium chloride, 0.2 mL 1 M magnesium chloride; Solution B: 14 mg nitro tetrazolium in 300 μL methanol; Solution C: 7 mg 5-bromo-4-chloro-3-indolyl *p*-toluidine salt in 50 μL dimethylsulfoxide.
   - Prior to use, add B to A, mix well.
   - Add C drop wise with shaking, mix completely.
   - Stop solution. 0.01 M Tris-HCl , 1 mM EDTA (pH 7.5).
10. Shaker.

Burns,2009

Dot-immunobinding assays
DIA or DIBA or Dot-Iba

a. A seed wash water sample is pipetted into the Bio-dot apparatus.
b. The membrane removed from the apparatus is exposed to chromogenic substrate 4- chloro-1-naphtol and perhydrol.
c. Results of the assay: the test is positive if a gray precipitate is clearly observed.



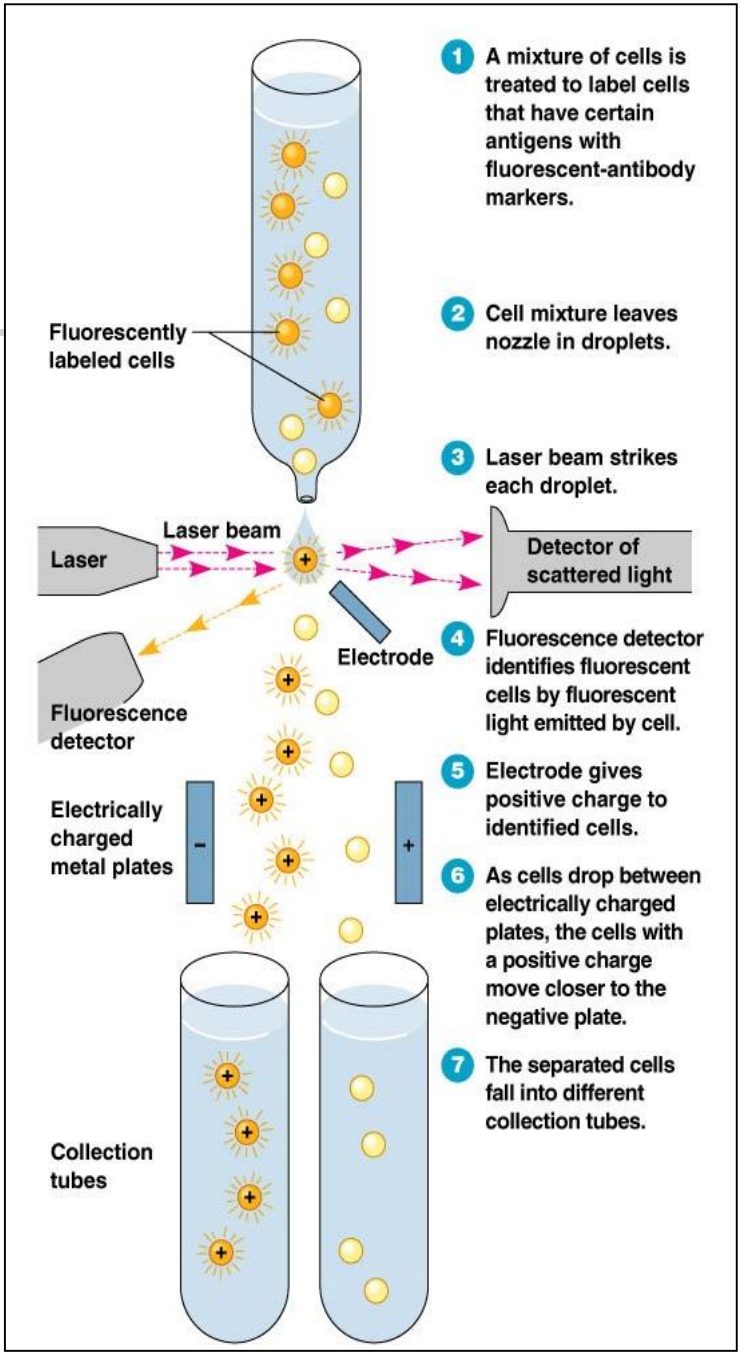
Duveiller, E., L. Fucikovsky and K. Rudolph, eds. 1997

Flow Cytometry

- Differences in electrical conductivity between species.
- Fluorescence of some species.
- Cells selectively stained with antibody plus fluorescent dye.

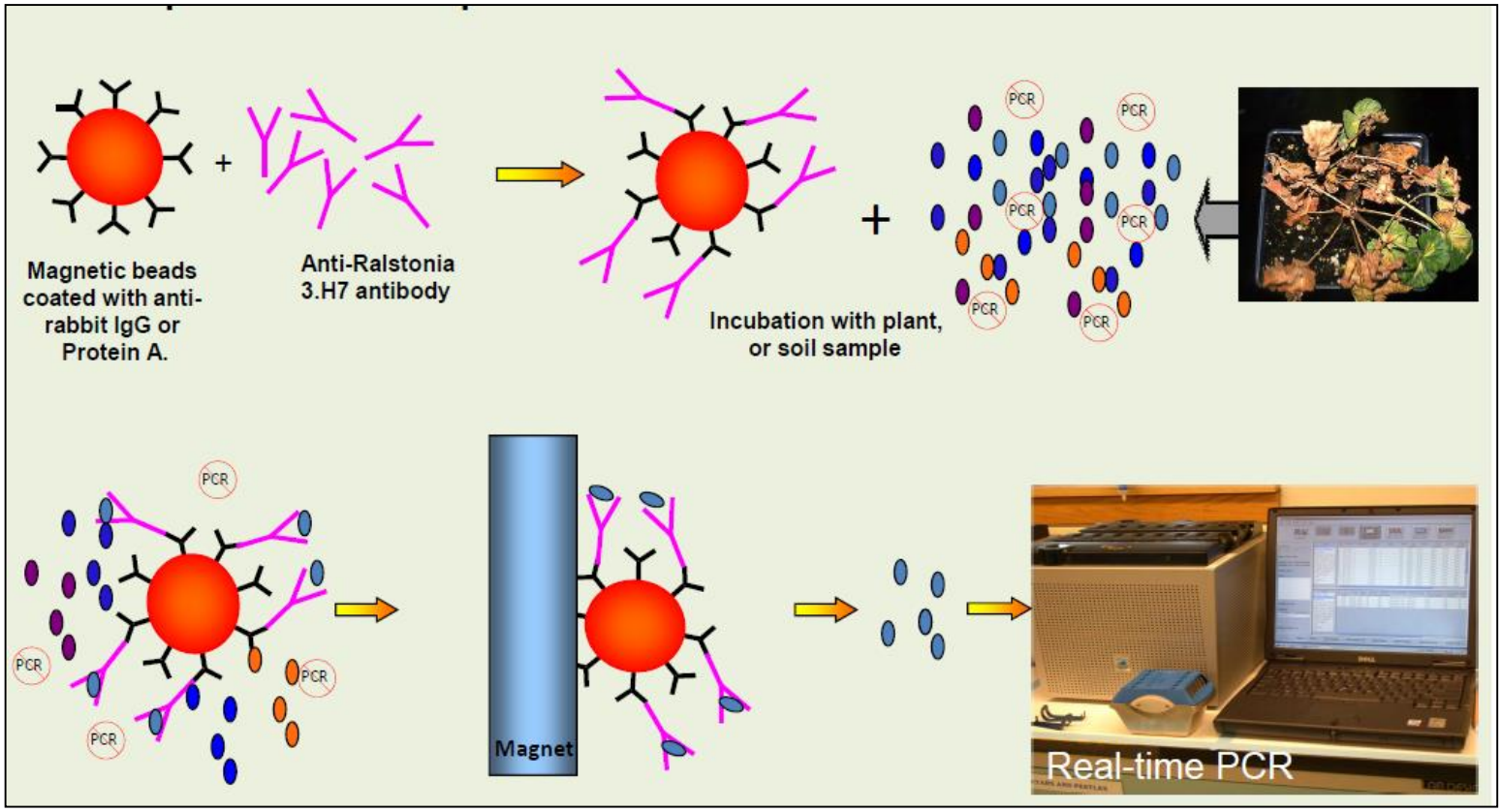
Flow cytometry, a technique adapted to the analysis of viability, metabolic state, and antigenic markers of bacteria. In particular, flow cytometry can be readily applied to the enumeration of viable bacteria in a sample.

Karlm,2004



ImmunoMagnetic Separation (IMS)

Use magnetic beads coated with Ab 3.H7 to simply and easily purify plant or soil samples from other bacteria rather than *R. solanaceraum* and PCR inhibitors



Champoiseau,2009

Chemotaxonomic methods

- Classify organisms based on differences and similarities in chemical markers:

1. Cell wall constituents,
2. Whole cell proteins
3. Lipids.

- Chemotaxonomic fingerprints:

1. SDS-PAGE for whole cell protein analysis
2. Capillary isoelectric focusing (CIEF) for separation of proteins and peptides
3. MALDI-TOF MS for proteins, lipids and DNAs analyses
4. FAME GC for lipid analysis

Protein analysis
1. SDS-PAGE of whole cell proteins

A chemotaxonomic method

Very good at the species and subspecies level

1D/2D SDS-PAGE method

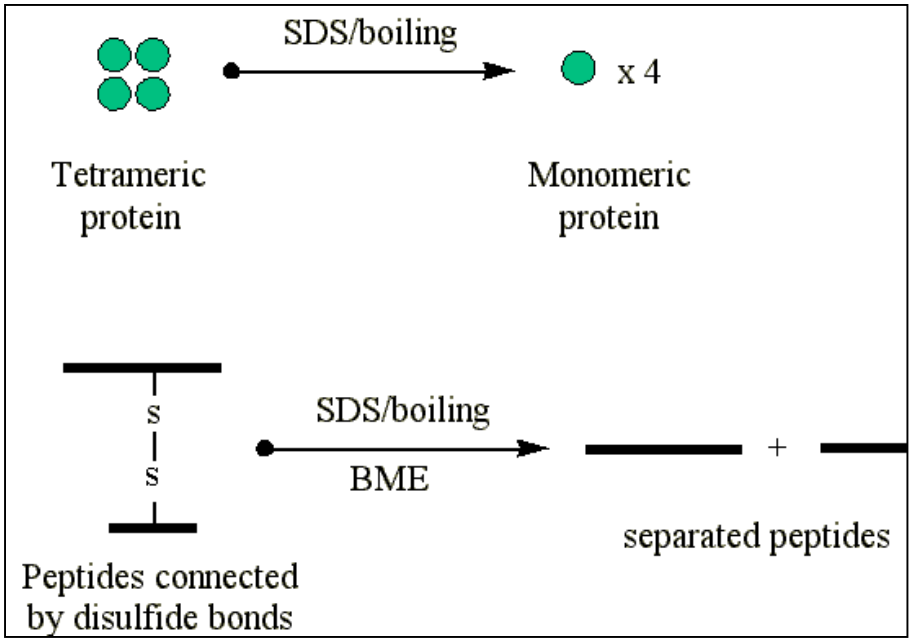
Detection of mono- and multimeric forms of protein and impurities

- Profiling of whole-cell proteins is a relatively simple and fairly inexpensive, although somewhat laborious method of identifying the pathogen.
- Standardization is required to minimize variation in the protein profiles.
- SDS-PAGE has proven to be extremely reliable for comparing and grouping large numbers of closely related strains at the species and subspecies level.
- Numerous studies have revealed a correlation between high similarity in whole-cell protein content and DNA-DNA hybridization (Costas,1992).

Note that another name for many SDS is sodium lauryl sulfate ,a detergent you will find in shampoos.

Protein extraction
SDS or β-mercaptoethanol

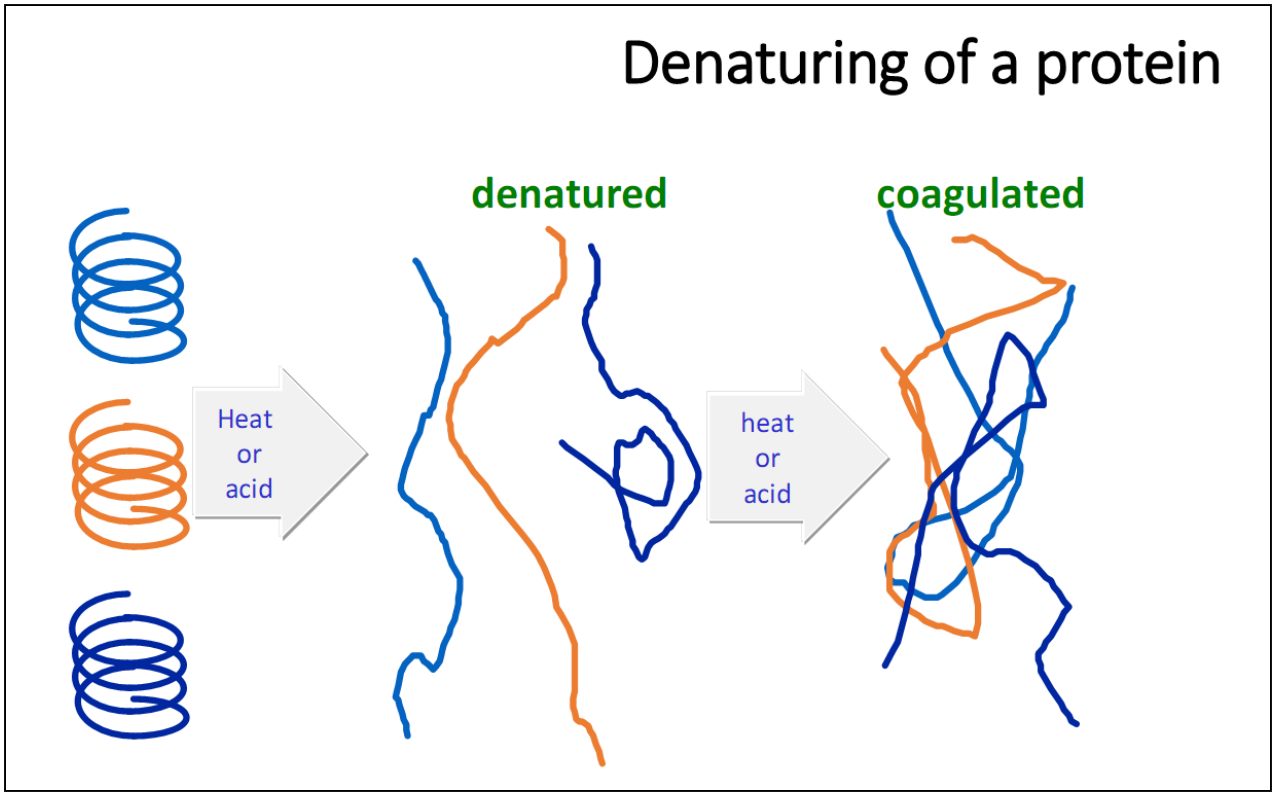
1. Protein gels are usually performed under denaturing conditions in the presence of the detergent sodium dodecyl sulfate (SDS).
2. PAGE may also be run in the presence of reducing agents, such as β-mercaptoethanol (BME).



Blaber,1998

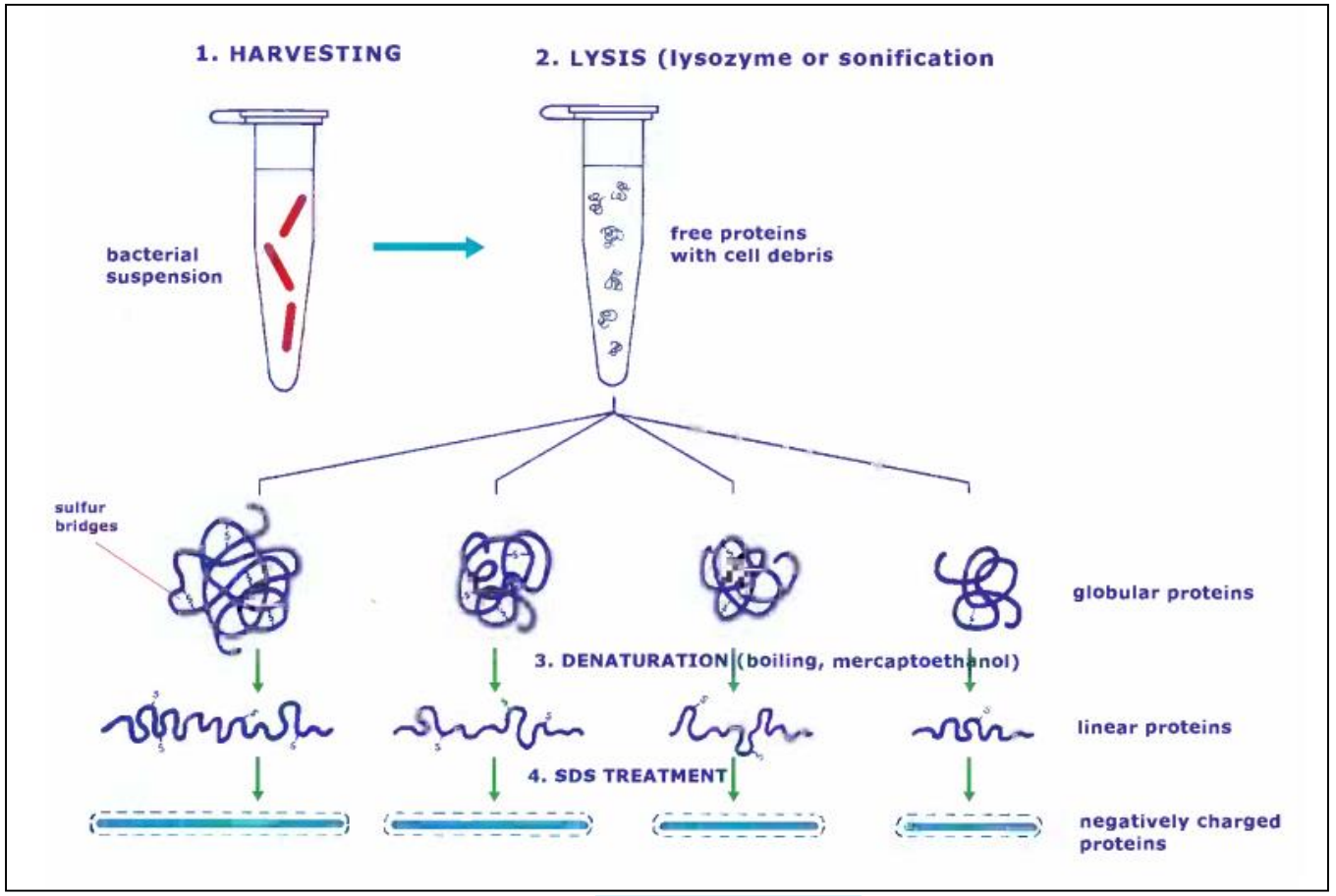
Denaturing of a protein

By heat or acid



Protein extraction

Principle of whole cell protein electrophoresis



Janse,2006

Gel electrophoresis for proteins
Whole-cell protein electrophoresis

- Gel electrophoresis of proteins almost exclusively utilizes polyacrylamide.
- The acrylamide solution usually contains two components:

1. Acrylamide, and
2. bis acrylamide.

- The bis acrylamide is essentially a cross-linking component of the acrylamide polymer.
- A typical value for the acrylamide: bis ratio is 19:1.
- The total acrylamide concentration in the gel affects the migration of proteins through the matrix (as with the concentration of agarose).
- The proteins migrate towards the anode.
- Protein PAGE gels are usually polymerized between two glass plates and run in the vertical direction.

Preparation of polyacrylamide gel
Stock solutions

- Acrylamide solution:
  30% (w/v) acrylamide,
  0.8% (w/v) bisacrylamide
- 10 X TBE buffer:
  0.9 M Tris
  0.9 M boric acid
  20 mM EDTA (pH 8.0)
- 87% Glycerine
- 10% (w/v) TEMED (TetraMethylEthyleneDiamine)
- 10% (w/v) APS (Ammonium persulphate)

APS and TEMED in are used to polymerize the Acrylamide gel.
These two are free radical polymerization reagents may lead to modification of the protein and peptide.

Formulation for preparing acrylamide gels

Solutions	Percentage of gel		
	3%	5%	10%
Acrylamide solution	0.4 ml	0.7 ml	1.44 ml
TBE-buffer	0.4 ml	0.4 ml	0.4 ml
Dm water	3.2 ml	2.9 ml	1.75 ml
TEMED	25 µl	25 µl	25 µl
Glycerine	-	-	0.4 ml
APS	25 µl	25 µl	25 µl

zampbioworld.org.,2011

Gel electrophoresis for proteins

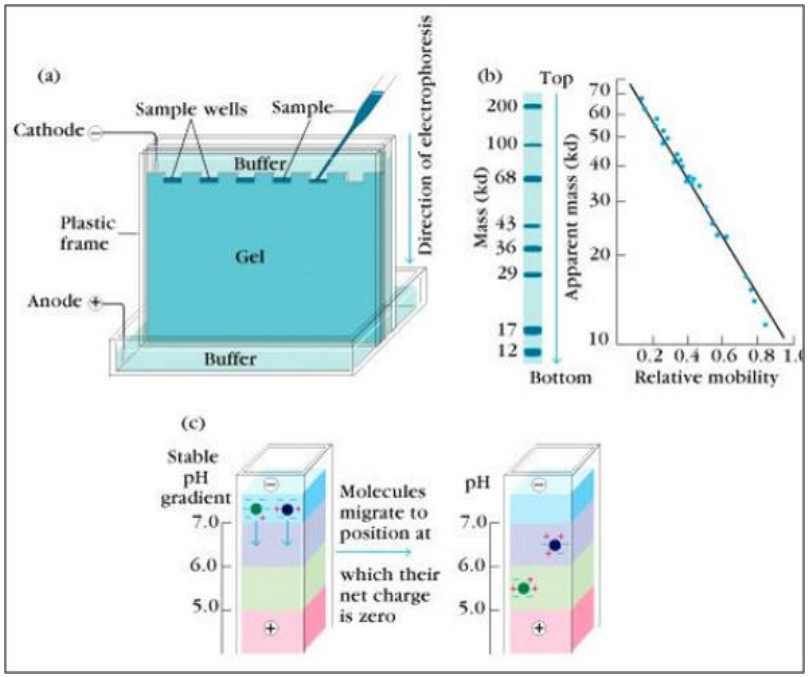
Whole-cell protein electrophoresis

Acrylamide (%)	Range of separation of polypeptides (in kilodaltons)
8	200-25
10	15-100
12.5	70-10
15	60-6
20	4-40

Gel electrophoresis for proteins

Whole-cell protein electrophoresis

- Denaturing protein gel, aka SDS-PAGE gel (Poly-acrylamide Gel Electrophoresis with Sodium Dodecyl Sulfate).
- Isoelectric focusing: Separates proteins on the basis of their charge (isoelectric points).

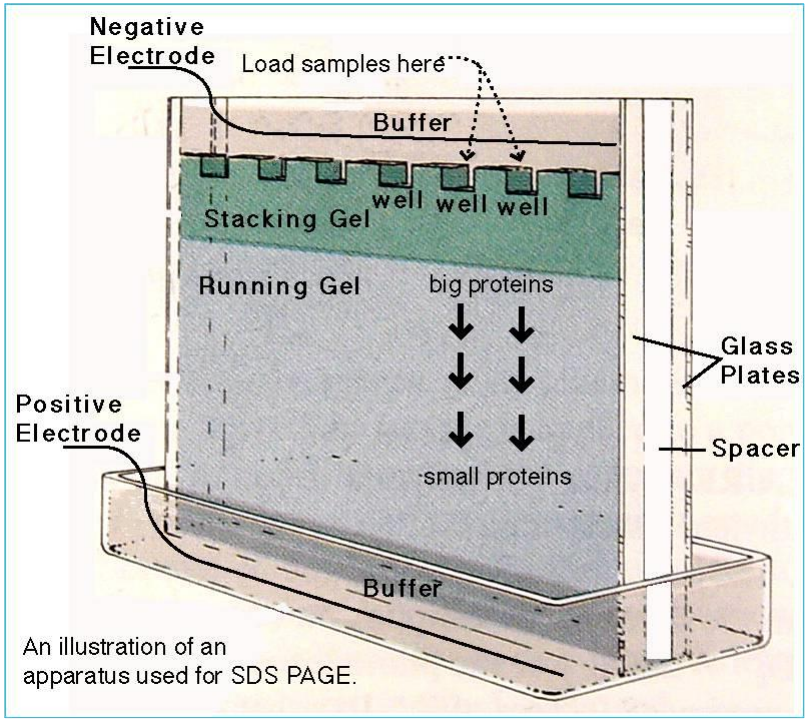


The isoelectric point (pH value) is different for each amino acid:

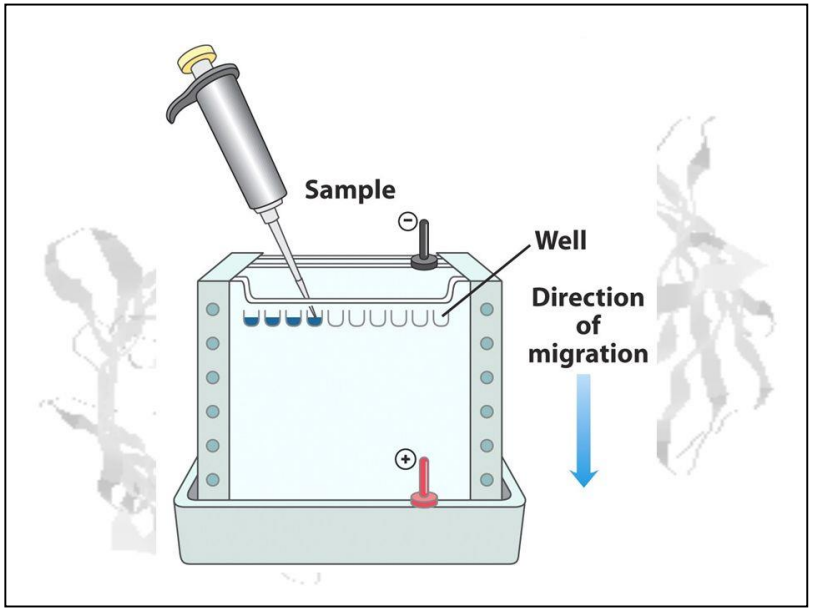
1. Amino acids with hydrocarbon R groups attain their isoelectric point between pH 5.0 and 7.0. ex. Leucine pH = 6.0
2. Basic amino acids need high pH values to reach their isoelectric points. ex. Arginine pH = 10.8

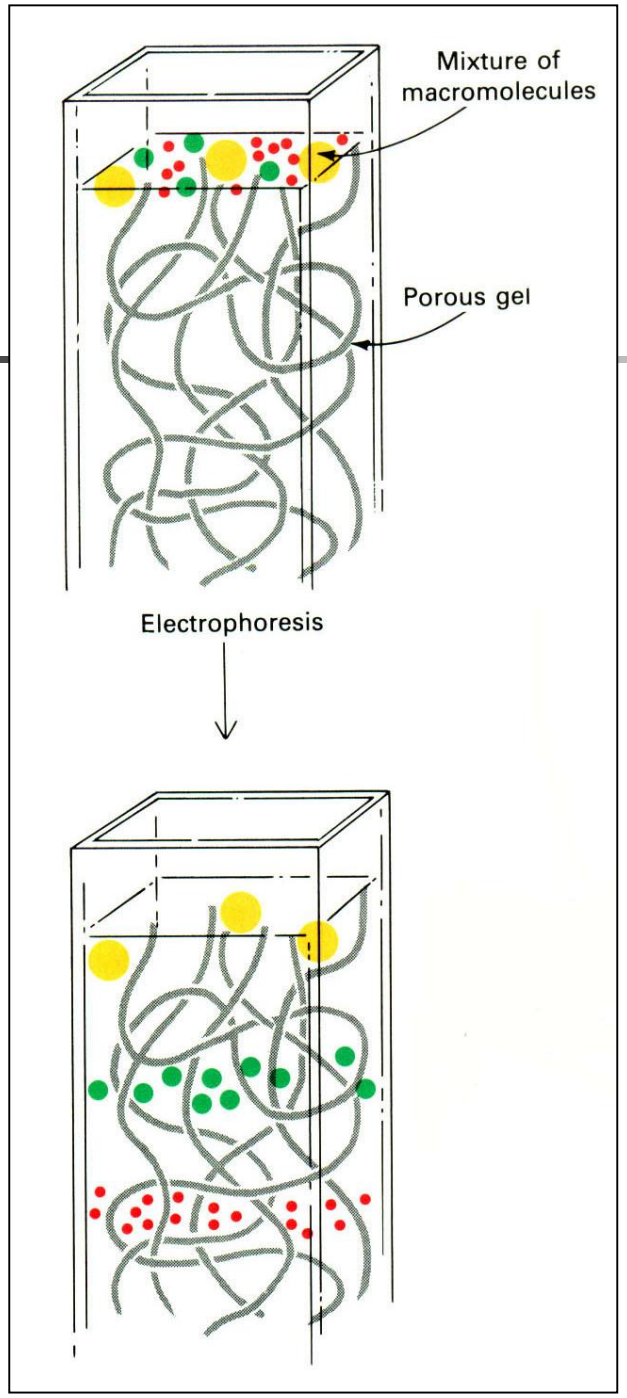
Gel electrophoresis for proteins

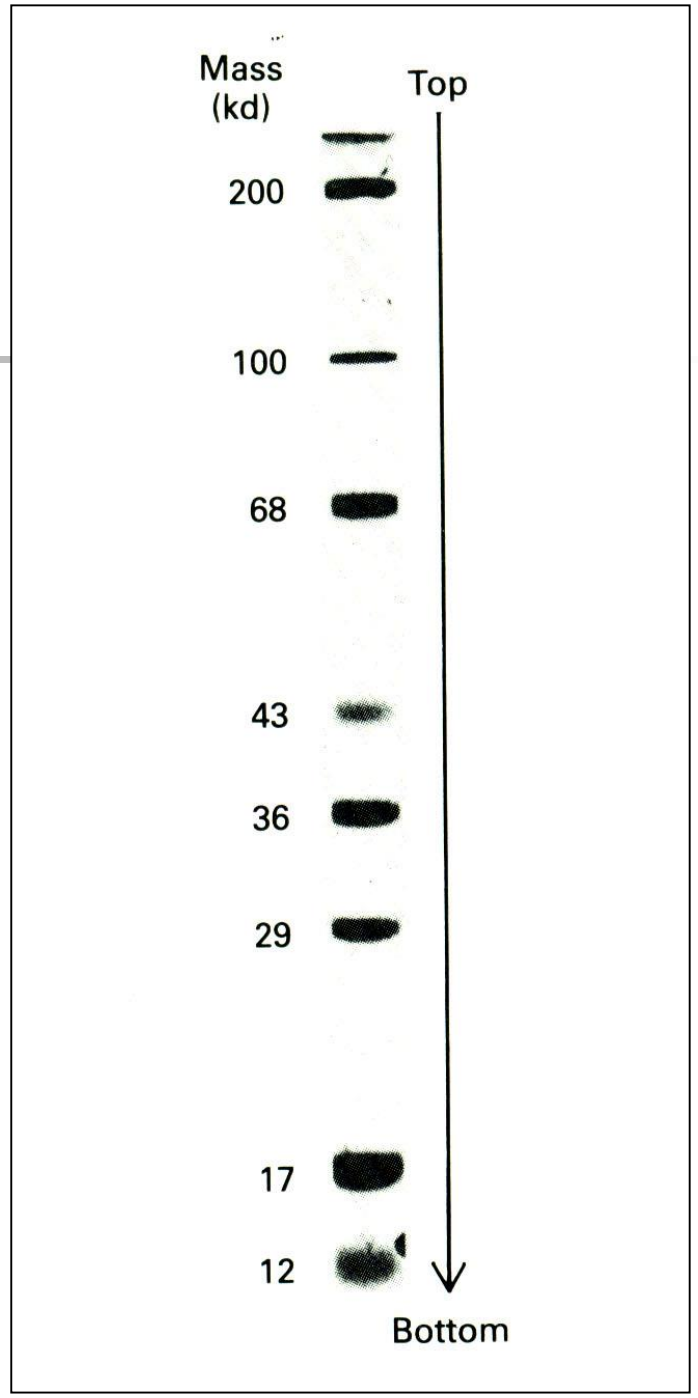
Whole-cell protein electrophoresis

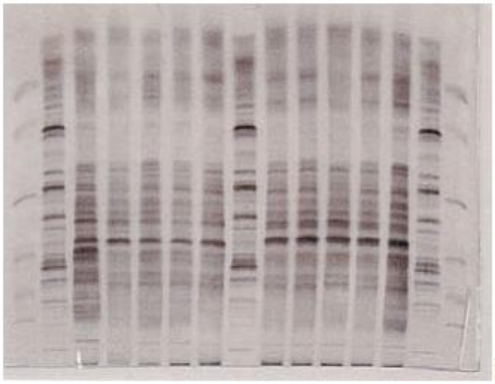


An illustration of an apparatus used for SDS PAGE.

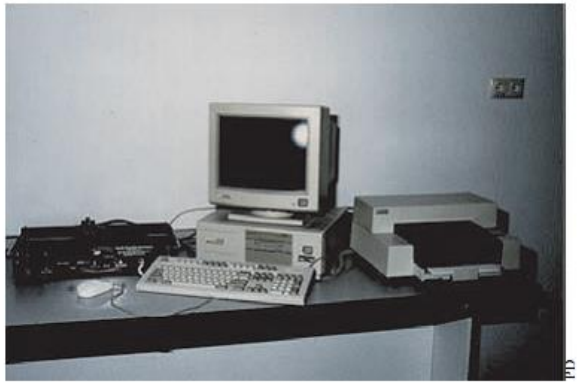



Mixture of macromolecules
Porous gel
Electrophoresis


Mass (kd)
Top
200
100
68
43
36
29
17
12
Bottom

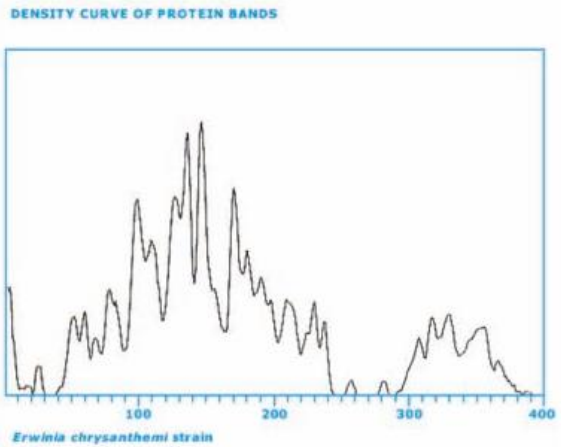


PAGE gel with protein profiles of several bacteria after electrophoresis and staining

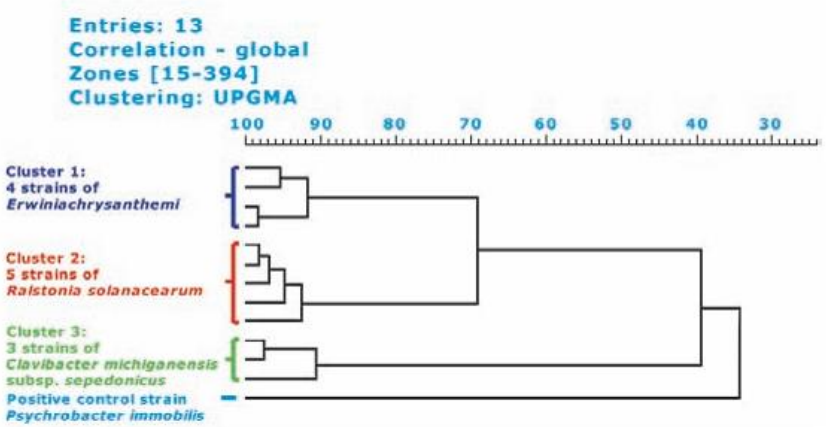


Computer with GelCompar software and gel scanner: digitizing protein profiles and identification and/or taxonomic analysis

D. SCANNING, IDENTIFICATION AND CLUSTERING WITH SOFTWARE



Peak pattern of one digitized protein profile



Cluster diagram of protein profiles of different bacteria after cluster analysis

Janse,2006

Gel electrophoresis for proteins

Recommended media, buffers and reagents

- NaPBS-EDTA pH 7.3: 0.2 m $Na_2HPO_4 \cdot 12H_2O$ 40.5 mL; 0.2 m $NaH_2PO_4 \cdot 12H_2O$ 9.5 mL; NaCl 8.0 g; EDTA 18.6 g; distilled water 950 mL. Dissolve ingredients and check pH. Aliquot and sterilize by autoclaving at 121°C for 15 min.
- Glucose Yeast Extract Calcium Carbonate Agar (GYCA): D(+) glucose 10.0 g; Difco Yeast extract 5.0 g; Calcium carbonate 30.0 g; Difco Bacto agar 15.0 g; distilled water 1 L. Prepare 500 mL of medium in 1-L Erlenmeyer flasks. Dissolve ingredients and sterilize by autoclaving at 121°C for 15 min.
- Sample buffer: Tris-HCl 0.98 g; 2-mercaptoethanol 5.0 mL; glycerol 10.0 mL; ultra-pure water 85.0 ml.

Whole cell protein analysis

Clavibacter michiganensis

- The cultures are suspended in 10 mL NaPBS-EDTA, collected in centrifuge tubes, made up to 30 mL with NaPBS-EDTA, and centrifuged (20 min, 10.000 *g*).
- The supernatant is gently decanted and the pellet resuspended in cold NaPBS-EDTA.
- This is repeated until excess EPS has been removed. After the final centrifugation and decanting of the supernatant, the centrifuge tubes are inverted on tissue paper to drain off the remaining liquid.
- 75 mg of pellet is transferred into a sterile 2-mL microvial and resuspended with 1.215 mL of sample buffer.
- 135 µL of freshly prepared lysozyme stock solution (4% w/v in 50 mm Tris-HCl pH 7.0) is added, and the vial is vortexed, and incubated for 2 h at 37°C.
- 150 µL of 20% w/v SDS solution is added and the vial is vortexed again. The samples are heated for 10 min at 95°C, removed from the heating block, put onto ice and centrifuged (5 min, 10.000 *g*). 50-µL aliquots of the supernatant are transferred into sterile microvials.
- One is used for electrophoresis and the remainder are stored at -18°C (< 2 months) or at -80°C (> 2 months).

EPPO,2005

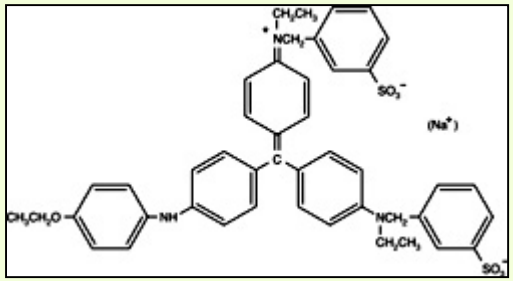
Whole cell protein analysis

Staining procedures

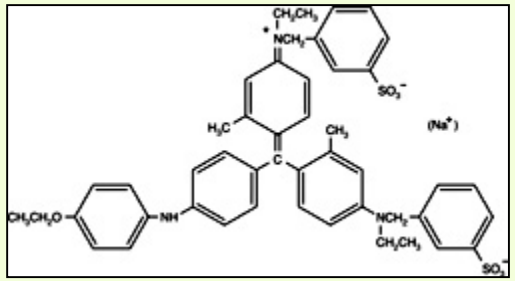
- Staining Gels with:

1. Coomassie Blue R250, or
2. Coomassie Blue G250.

- The two Coomassie blue dyes, R250 and G250, differ by only two methyl groups.
- **Stock solutions**:
- Staining solution: 50% Methanol, 7.5% Acetic Acid, 0.1% Brilliant Blue R250.
- Destaining solution: 30% Methanol, 7.5% Acetic Acid.



Coomassie Blue R-250



Coomassie Blue G-250

Whole cell protein analysis

Staining procedures

Standard protocol-Coomassie Blue R-250

- Gel may be prefixed in 50% MeOH(methanol), 10% HoAC (Acetic Acid), 40% H_2O for 30 minutes to overnight.
- Stain gel in the above solution, with 0.25 Coomassie Blue R-250, for 2-4 hours, until the gel is a uniform blue color.
- Staining is complete when the gel is no longer visible in the dye solution.
- Prior to complete staining, the gel will appear as a lighter area against the dark staining solution.
- Destain for 4-24 hours in 5% MeOH, 7.5% HoAC, 87.5% H_2O.
- Bands will begin to appear in 1-2 hours.
- Destain until background is clear.
- This method will detect as little as 0.1µg/band.
- Store gels in 7% HoAC.

Whole cell protein analysis

Staining procedures

Rapid protocol-Coomassie Blue G-250

- To make the Coomassie Blue G250 staining reagent, dissolve 0.2g dye in 100 ml H_2O (this will require warming to approximately 50°C).
- Cool and add 100 ml 2N H_2S0_4.
- Incubate at room temperature 3 hours to overnight, then filter.
- To filtered solution, CAREFULLY add 22.2 ml 10N KOH, then add 28.7g TCA (trichloroacetic acid).
- Allow to stand > 3 hours, then filter again if necessary to obtain an amber-brown solution without blue precipitate.
- To stain, immerse gel in above solution.
- Bands will begin to appear within 15 minutes.
- Intensity and sensitivity will continue to improve for several hours.
- Staining solution is stable for 2-3 weeks at 25°C.

Whole cell protein analysis
1- Dimensional SDS-PAGE

- Protein profiles are determined in a denaturing discontinuous electrophoresis system which involves a 12% polyacrylamide separation gel and a 5% polyacrylamide concentration gel.
- The electrophoresis system should be connected to a cooling thermostat set at 6°C.
- Volumes of 25 µL of sample mixed with tracking dye are applied to each well and a low molecular weight standard is used in the range from 10 kDa to 100 kDa.
- Electrophoresis is performed at constant power (6 W per gel) or at constant current (20 mA per gel), and should continue until the tracking dye has migrated a distance of approximately 10 cm from the top of the separation gel.
- The protein profile is visualized by staining with Coomassie Brilliant Blue.
- The stained gels should be dried by mounting between two sheets of glycerol-immersed cellophane and clamping in a frame.
- Any application of heat or vacuum may irreversibly damage the gel.
- The profiles should preferably be compared by scanning densitometry, with computer-assisted data processing, e.g. the commercially available GelCompar system.

One Dimensional SDS-PAGE
Xanthomonads

- Vauterin *et al.*,1991 have applied SDS-PAGE of whole-cell proteins to 307 *Xanthomonas* strains and delineated 19 protein electrophoretic clusters.
- The most aberrant protein patterns were these of the *Stenotrophomonas maltophilia* belonged to the Xanthomonadaceae.
- The most important and unexpected result of this study was the demonstration of the heterogeneity of many pathovars.
- e.g., *X. campestris* pv. *vesicatoria* and *X. campestris* pv.*dieffenbachiae*.
- These protein electrophoretic clusters have been used to select strains for subsequent DNA-DNA hybridizations.

Drawback of one Dimensional SDS-PAGE

- For its application in polyphasic taxonomy, protein electrophoresis has an important drawback:
- In contrast to, e.g. whole cell fatty acid analysis, it does not supply descriptive information, because in general, the identity of none of the protein bands is revealed.

Whole cell protein analysis

2-D Separation is based on size and charge

- A high throughput approach to identify proteins.
- Two-dimensional (2-D/2DE) gel spreads the proteins from a single sample out in two dimensions:

1. 1st dimension: Isoelectric focusing electrophoresis
2. 2nd dimension: SDS-PAGE

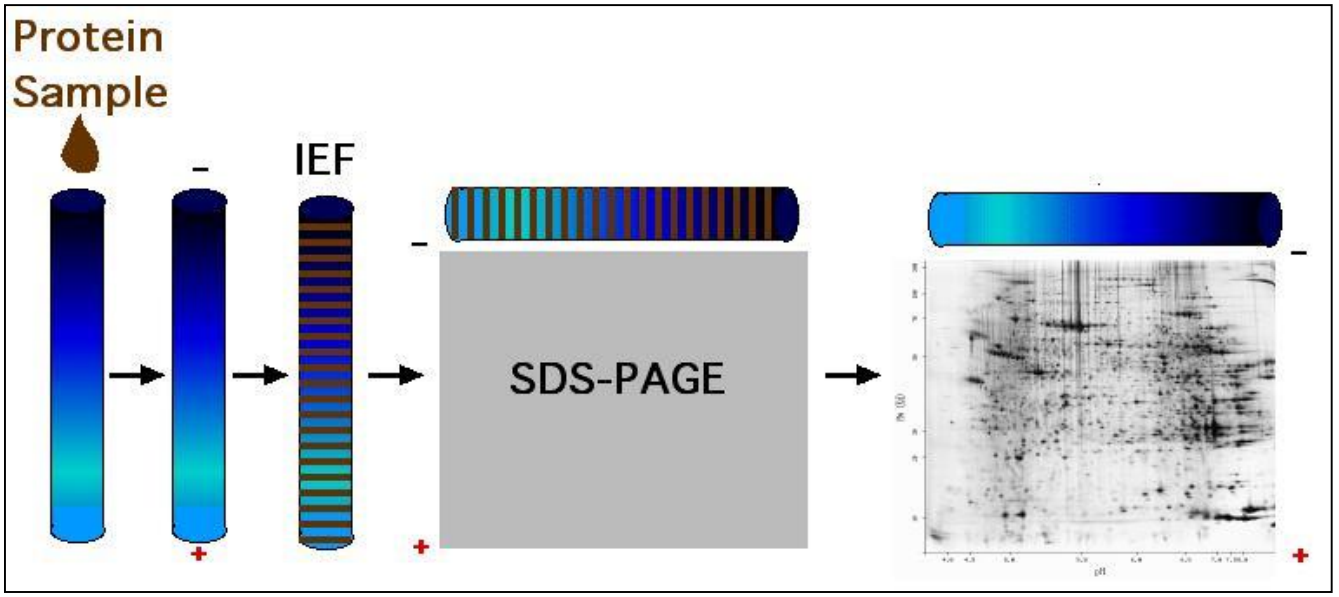
- Proteins are separated according to charge or isoelectric point (pH at which they have neutral net charge) in the first dimension, and
- Proteins are separated according to their molecular weight in the second dimension.

The IEF method is accomplished by electrophoresis of proteins or peptides through a stable pH gradient until they reach the pH equal to their isoelectric points (pI), at which point the net charge and mobility are zero.

Whole cell protein analysis

2-D.Separation is based on size and charge

- First step is to separate based on charge or isoelectric point, called isoelectric focusing.
- Then separate based on size (SDS-PAGE).



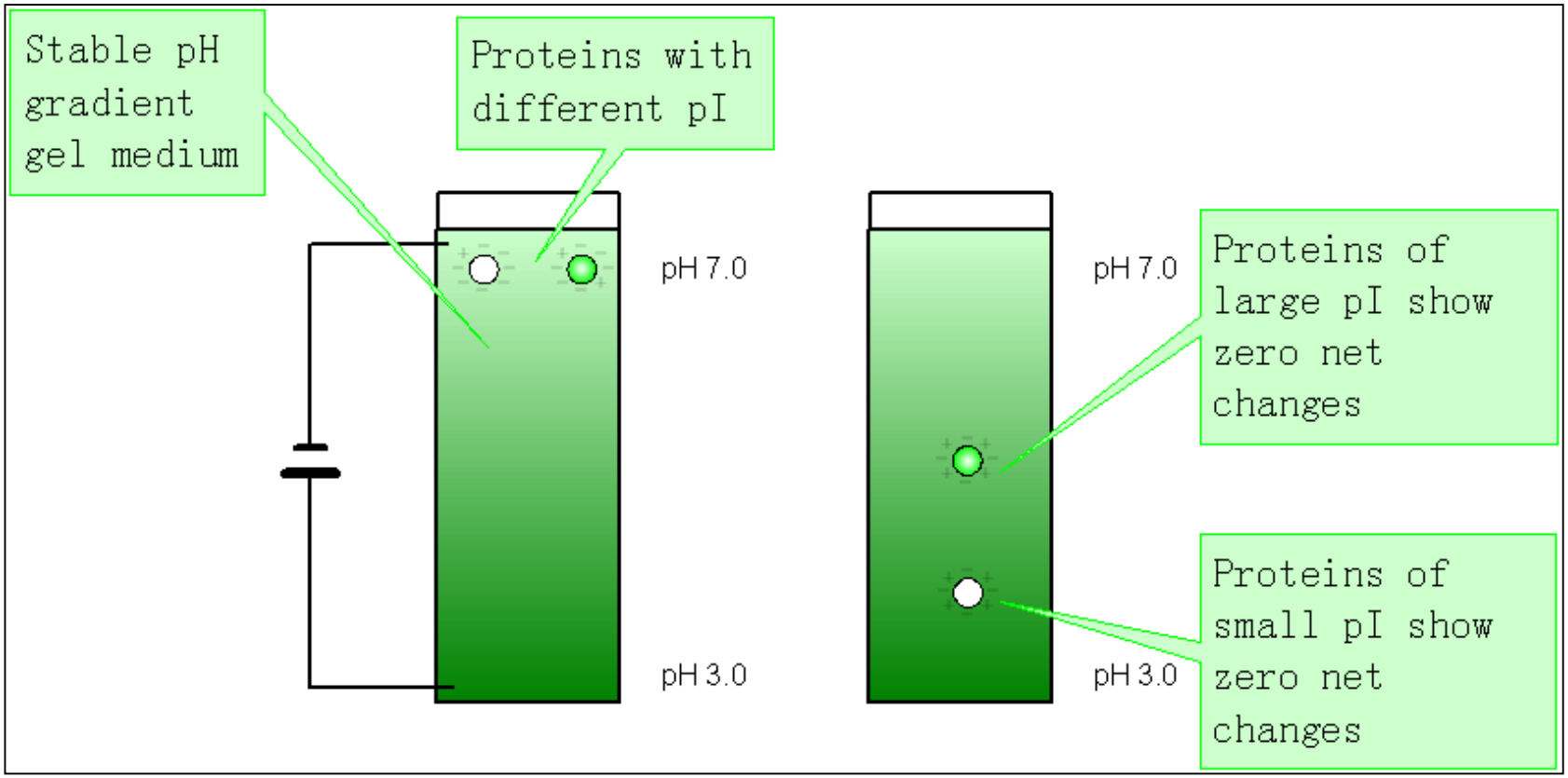
Isoelectric focusing

Principle of IFE

- Protein can be charged depending upon the pI or pH of the solution, and move toward the electrode with opposite.
- Depend upon the electric properties of proteins, the charged proteins, either positively or negatively, will migrate in the electric field.
- The proteins having net zero charges stop moving in the electric field.

Isoelectric focusing

Principle of IFE



Whole cell protein analysis

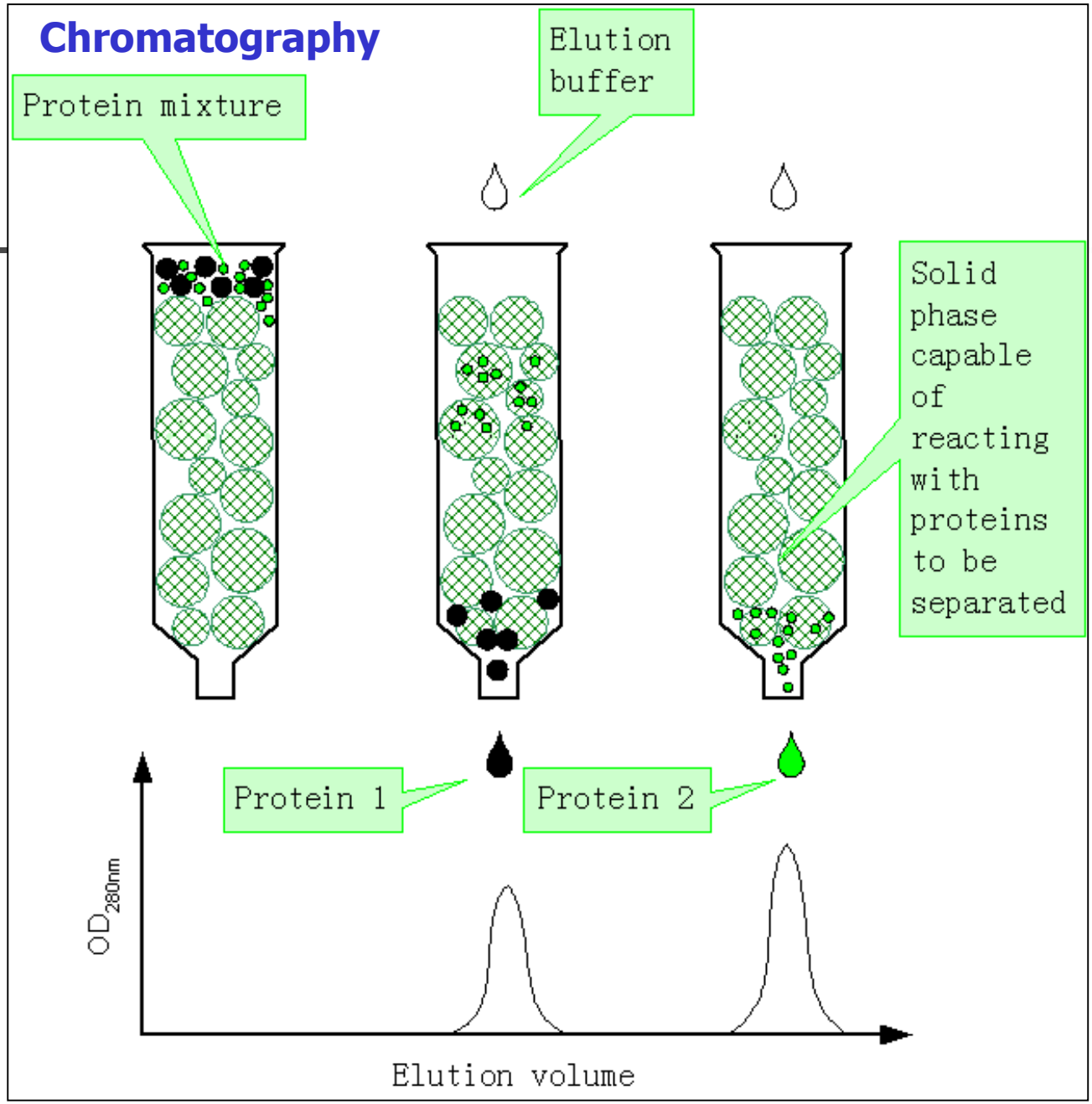
2-D.Separation is based on size and charge

- The of 2-DE separation technique has the potential to resolve thousands of protein spots with:
- Specific isoelectric points (pI), and
- MW on gels.
- Analysis of 2-DE gels simultaneously provides several pieces of information about the hundreds of proteins that are visualized including the MW, pI, quantity, as well as PTMs.
- Separated protein spots are visualized on the gel by staining with Coomassie Blue (CBB-R, Colloidal), SYPRO (ruby, red, orange) or silver stain.

Kav *et al.*,2006

Chromatography

- When a protein solution (called as mobile phase) passes through a stationary phase, proteins can interact with the stationary phase due to the differences in size, charge, and affinity, making the different proteins flow through the stationary phase at different speeds.


Chromatography
Protein mixture
Elution buffer
Solid phase capable of reacting with proteins to be separated
Protein 1
Protein 2
OD_{280nm}
Elution volume

Protein analysis
2. Capillary isoelectric focusing (CIEF)

A chemotaxonomic method

Very good at the species and pathovar level

Capillary Isoelectric Focusing (cIEF)method

Determination of the isoelectric point (pI) of a protein and assessment of isoform distribution and impurity profiling

- Capillary electrophoresis (CE) provides fast, highly efficient, and automated separation of proteins and peptides.
- A number of CE modes are available for separation of proteins and polypeptides.
- These modes include:

1. Capillary zone electrophoresis (CZE),
2. Capillary sieving electrophoresis (CSE),
3. Capillary isoelectric focusing (CIEF), and
4. Capillary electrochromatography (CEC)

Quigley and Dovichi,2004

The capillary isoelectric focusing with UV/Vis detection

- The capillary isoelectric focusing with UV/Vis detection were successfully used for the on-line rapid separation and detection of the bacteria.
- The possibilities to distinguish pathovars of some plant pathogenic bacteria (including *Pseudomonas*), according their isoelectric point by isoelectric focusing techniques are introduced here.

Horký *et al.*,2009

The capillary isoelectric focusing with UV/Vis detection

- These methods need further modification to detect or eventually identify and specify plant pathogenic bacteria for their successful use in phytosanitary diagnostic microbiology, but are helpful for determination of phytopathogenic *Pseudomonas* when there are controversial results after commonly used techniques.

Horký *et al.*,2009

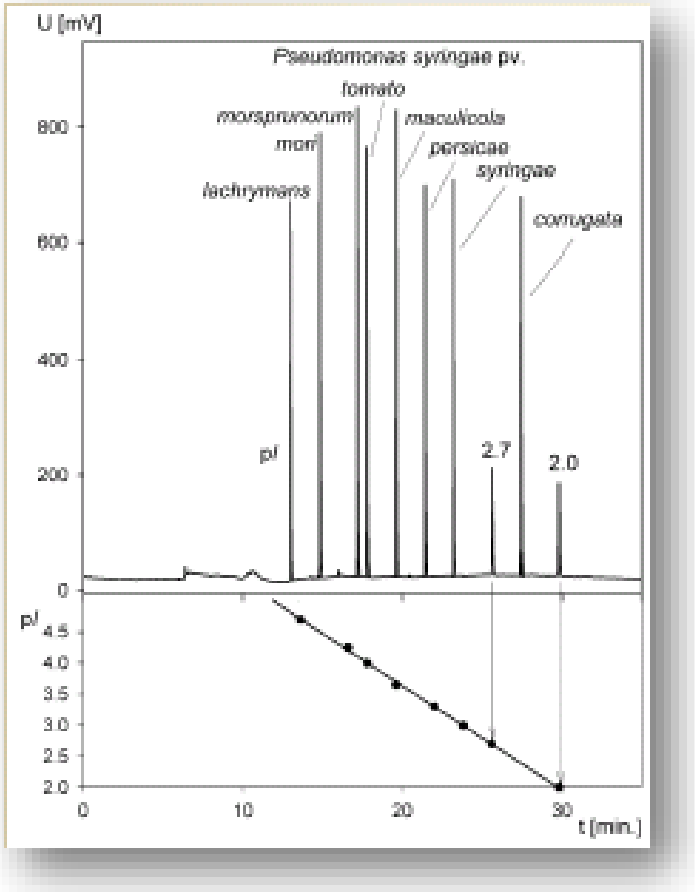
Capillary isoelectric focusing (CIEF) Method

- CIEF separates proteins or peptides based on isoelectric point, pI.
- In CIEF, the sample is normally mixed with ampholytes and the capillary is filled.
- Next, one end of the capillary is placed in a reservoir containing high pH solution, the other is placed in a low-pH solution, and an electric field is applied.
- A pH gradient is quickly formed within the capillary, and proteins or peptides migrate and are focused at the region where the pH is equal to their pI.
- UV-Vis absorbance detection is used as the most widely used detection system in CE.
- CIEF can be performed in one step or two steps.

Quigley and Dovichi,2004

Capillary isoelectric focusing (CIEF)
Discrimination of phytopathogenic *Pseudomonas* pathovars

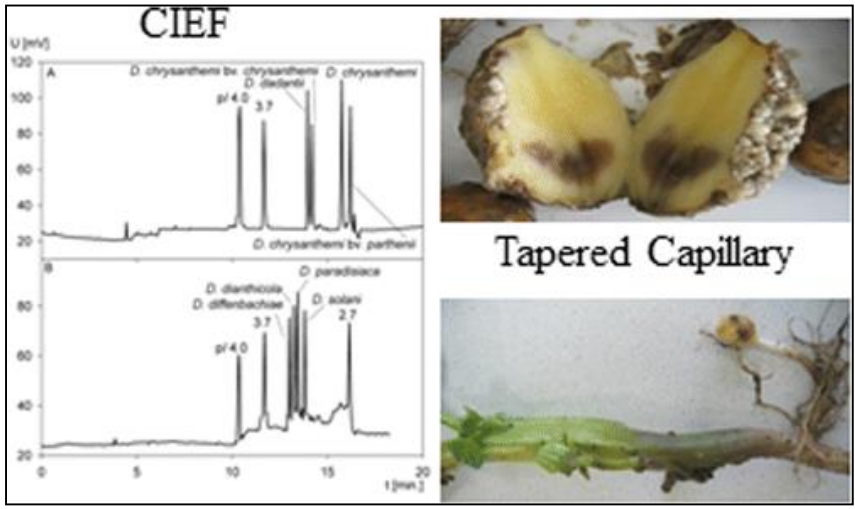
- Commonly used chemical and biochemical diagnostic techniques such as BIOLOG™ and fatty acid analysis are not sufficiently discriminating all pathovars in the genus *Pseudomonas* (*P. syringae* pathovars).
- CIEF was based on the movement of molecules in an electric field.
- It is fast and offers many advantages in terms of labor saving per test and reproducibility.



Quigley and Dovichi,2004

Capillary isoelectric focusing (CIEF)
Discrimination of different species of the genus *Dickeya*

- Eight strains representing different species of the genus *Dickeya* were selected on the basis of close proximity of their isoelectric points:
- *D. chrysanthemi*,
- *D.chrysanthemi* bv. *parthenii*,
- *D.chrysanthemi* bv. *chrysanthemi*,
- *D. dadantii*,
- *D. paradisiaca*,
- *D. solani*,
- *D. diffenbachiae*, and
- *D. dianthicola*.



Horka *et al.*,2013

Capillary isoelectric focusing (CIEF)

Capillary Isoelectric Focusing (pH gradient 2-5)

Plant pathogenic bacteria

Strain,isolate	Percentage of probability by GC identification	CIEF - pI
Pseudomonas syringae* pv. *syringae		
CCM 2870	*Pseudomonas syringae* pv. *syringae* 71,7 %	3,1
CCM 2114	*Pseudomonas syringae* 94,5 %	3,1
CCM 2868	*Pseudomonas syringae* pv. *syringae* 73,0 %, 73,7 %	3,1
PPBOL 1048	*Pseudomonas syringae* pv. *syringae* 84,0 %	4,8
PPBOL 1049	No result	3,1
PPBOL 1050	*Pseudomonas syringae* pv. *syringae* 84,0 %	3,1
PPBOL 1054	Not identified	3,1
PPBOL 1055	Not identified	3,1
B 32/08	*Pseudomonas syringae* pv. *syringae* 92,0 %	3,1
Pseudomonas syringae* pv. *lachrymans		
CFBP 1644	*Pseudomonas syringae* 94,2 %	4,8
CFBP 6465	*Pseudomonas syringae* pv. *syringae* 75,0 %	4,8
CFBP 6462	*Pseudomonas syringae* pv. *syringae* 93,9 %	4,8
Pseudomonas syringae* pv. *maculicola		
LMG 5071	*Pseudomonas syringae* pv. *maculicola* 63,9 %	3,7
Pseudomonas syringae* pv. *mori		
IVIA 10003.1a	*Pseudomonas viridiflava* 80,3 %, *Pseudomonas syringae* pv. *syringae* 74,6 %	4,5
CFBP 1642	*Pseudomonas viridiflava* 94,1 %, *Pseudomonas syringae* pv. *syringae* 88,7 %	4,5
B 141/08	*Pseudomonas viridiflava* 92,8 %, *Pseudomonas syringae* pv. *syringae* 92,7 %	4,5
B 104/08	*Pseudomonas syringae* 95,5 %, *Pseudomonas syringae* pv. *morsprunorum* 95,5 %	4,5
B 46/04	*Pseudomonas syringae* pv. *syringae* 92,9 %	4,5
Pseudomonas syringae* pv. *tomato		
IVIA 1733.3	*Pseudomonas syringae pv. tomato* 74,7 %	4,0
CFBP 1326	*Pseudomonas syringae* pv. *syringae* 92,9 %, *Pseudomonas viridiflava* 84,3 %	4,0

Continue:		
CCM 7018	*Pseudomonas viridiflava* 91,2 %, *Pseudomonas syringae* 91,1 %	4,0
CCM 7019	*Pseudomonas viridiflava* 93,6 %, *Pseudomonas syringae* 92,5 %	4,0
CFBP 2212	*Pseudomonas syringae pv. tomato* 86,1 %	4,0
CFBP 5422	*Pseudomonas syringae* 93,2 %, *Pseudomonas syringae* pv. *syringae* 89,1 %	4,0
CFBP 2546	*Pseudomonas syringae* 90,1 %, *Pseudomonas syringae pv. tomato* 66,2 %	4,0
B 106/08	*Pseudomonas syringae* 92,9 %, *Pseudomonas syringae* pv. *syringae* 92,9 %	4,0
Pseudomonas syringae* pv. *persicae		
LMG 5184	*Pseudomonas viridiflava* 93,1 %, *Pseudomonas syringae* pv. *syringae* 90,3 %	3,4
LMG 5078	*Pseudomonas syringae* 35,9 %	3,4
LMG 5566	*Pseudomonas syringae* 34,2 %	3,4
LMG 5568	*Pseudomonas syringae* 41,0 %	3,4
LMG 5569	*Pseudomonas syringae* 37,0 %	3,4
Pseudomonas corrugata		
IVIA 614.5.3	*Pseudomonas syringae* 86,3 %	2,4
CFBP 5324	*Pseudomonas putida* 82,0 %	2,4
CFBP 4901	*Pseudomonas putida* 78,0 %, *Pseudomonas corrugata* 40,3 %	2,4
CFBP 5465	*Pseudomonas corrugata* 66,4 %	2,4
CFBP 6663	*Pseudomonas corrugata* 63,1 %, 61,8 %	2,4
Pseudomonas syringae* pv. *morsprunorum		
CCM 2859	*Pseudomonas syringae* pv. *mori* 94,6 %	4,1
CCM 2534	*Pseudomonas syringae pv. morsprunorum* 96,3 %, 96,9 %	4,1
CCM 2860	*Pseudomonas syringae, Pseudomonas syringae* pv. *mori* 95,7 %	4,1

Quigley and Dovichi,2004

Protein analysis

3. MALDI mass spectrometry (MALDI-TOF MS)

A chemotaxonomic method

Good for species or strain identification

Mass spectrometry

- The general principle of Mass spectrometry (Mass Spec or MS) is to produce, separate, and detect gas-phase ions.
- Mass spectrometry uses high energy electrons to break a molecule into fragments.
- Separation and analysis of the fragments provides information about:

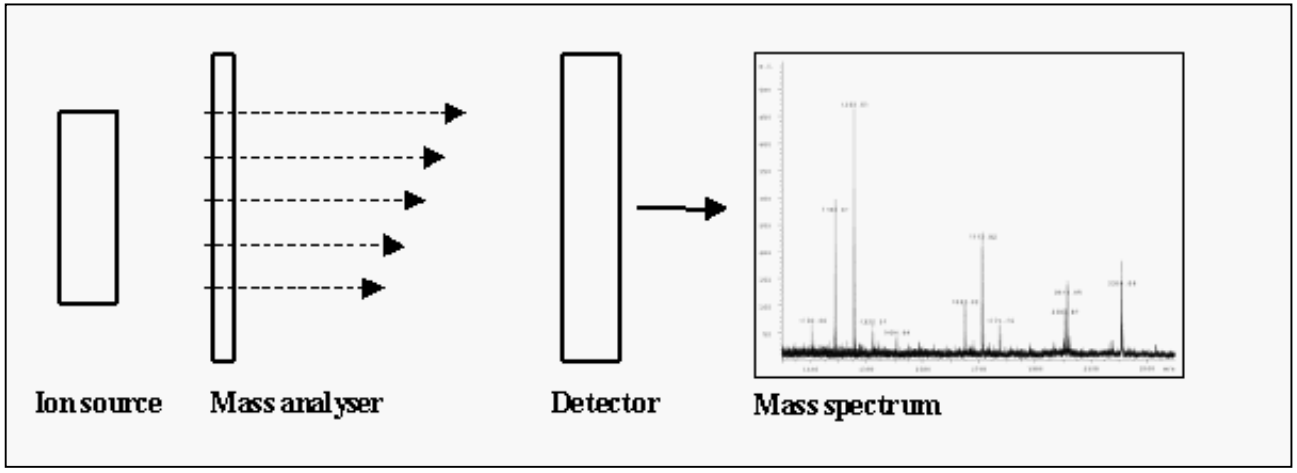
1. Molecular weight
2. Structure

- Two known "soft" ionization techniques are:

1. Electrospray ionization mass spectrometry (ESI-MS), and
2. Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-MS).

Mass spectrometry

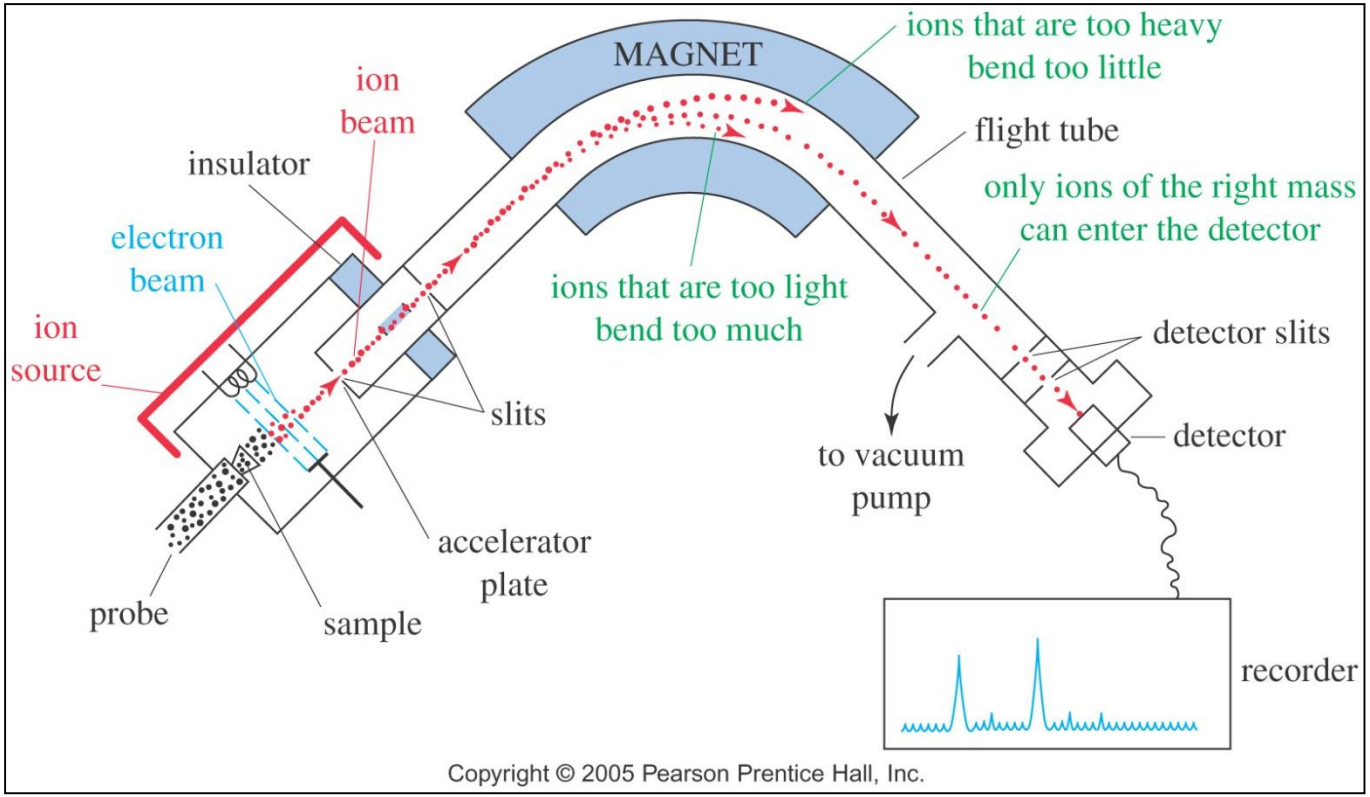
- Mass spectrometry is a chemical analysis technique that is used to measure the mass of unknown molecules (proteins, lipids and oligonucleotides) by ionizing, separating and detecting ions according to their mass-to-charge ratios(m/z).
- The data are recorded as mass spectra.



Elek,2010

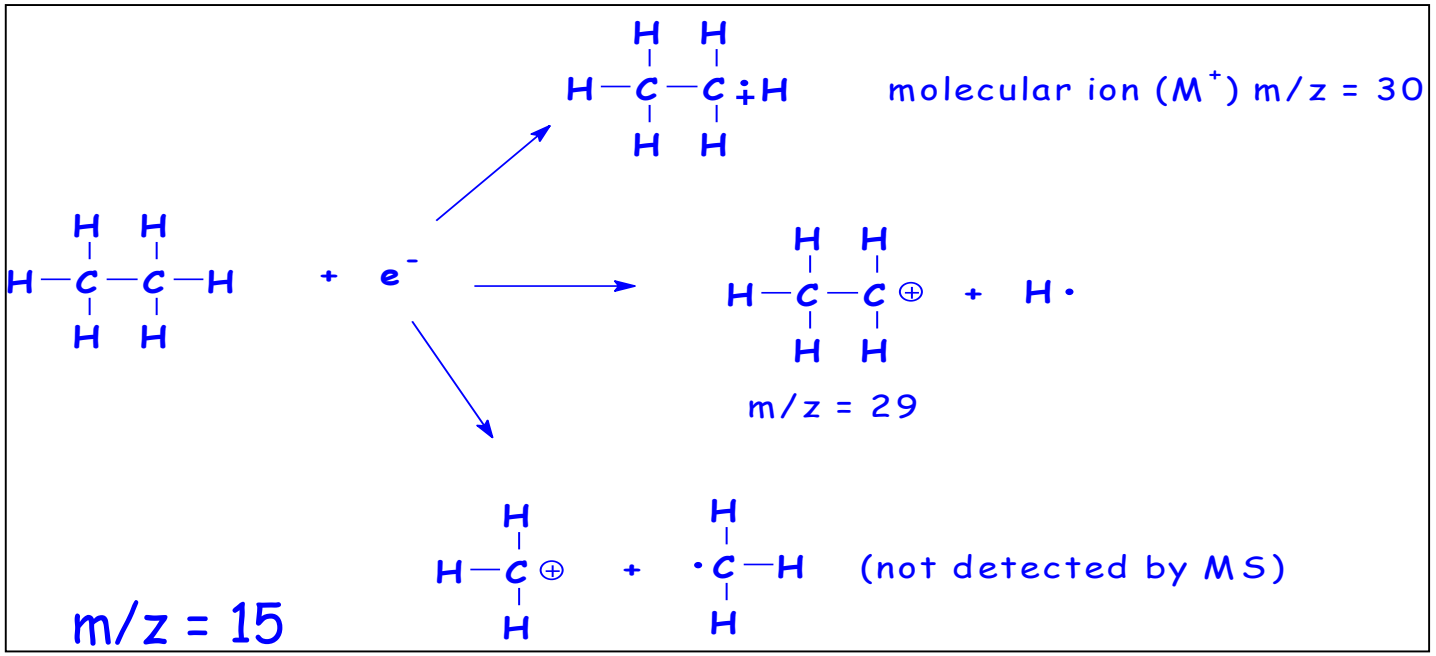
Mass spectrometry

- The cations that are formed are separated by magnetic deflection(amount of deviation).



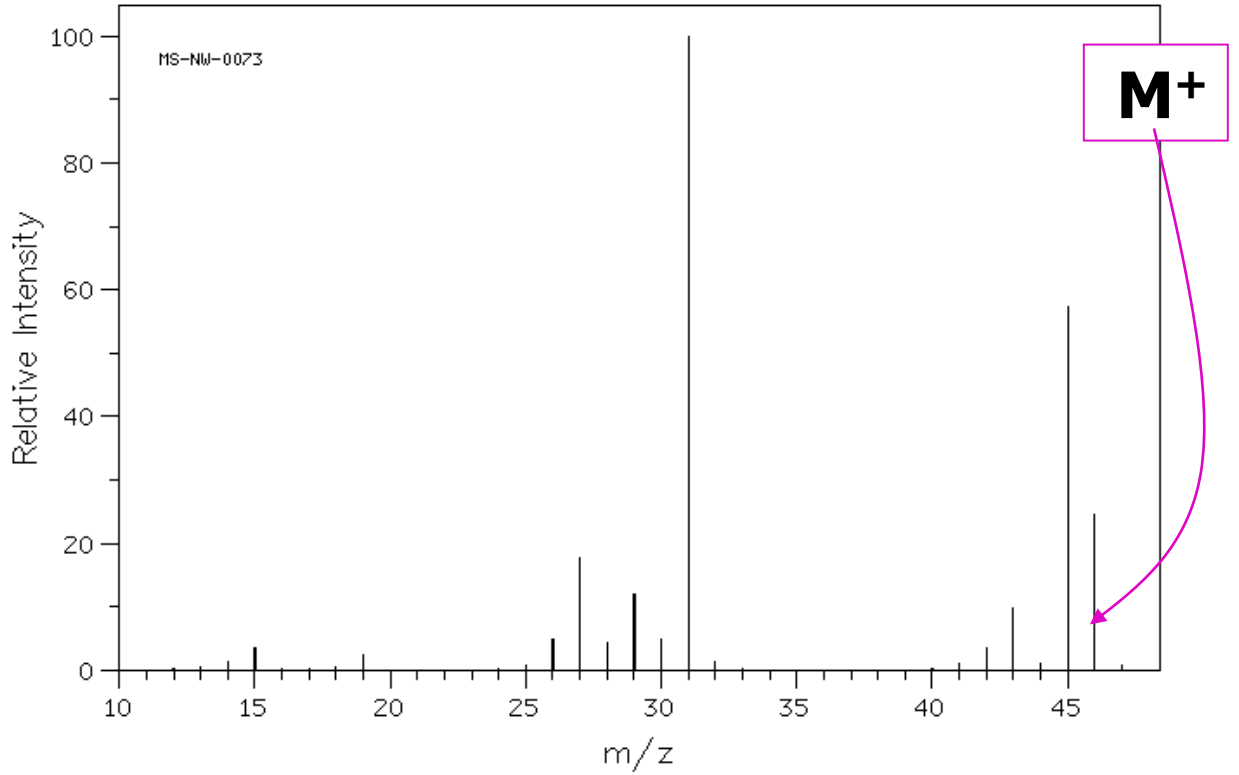
Mass spectrometry

- The impact of the stream of high energy electrons can also break the molecule or the radical cation (+) into fragments.



Mass spectrometry

- Mass spectrum of ethanol (MW=46).



SDBSWeb : http://riodb01.ibase.aist.go.jp/sdbs/ (National Institute of Advanced Industrial Science and Technology, 11/1/09)

Mass spectrometry

1. Electrospray ionization mass spectrometry (ESI-MS)

- The MS principle consists of ionizing chemical compounds to generate charged molecules and to measure their mass-to-charge ratio (m/z).
- Such molecular "signatures" can be used for rapid bacterial identification (ID) from isolated colonies.
- The use of mass spectrometry for bacterial ID is especially suitable and cost-efficient for laboratories with high volumes of samples.

Mass spectrometry
2. MALDI-TOF mass spectrometry method

- MALDI-TOF Mass spectrometry is a simple, rapid and reproducible method that has proven to be powerful for the identification of bacteria within minutes.
- The method presented requires only a simple time-of-flight (TOF) mass spectrometer.
- It can be used for bacterial identification in a variety of applications without (extensively) analyzing mass spectra.

Mass spectrometry

MALDI-TOF mass spectrometry method

Proteins, lipids and DNAs analyses

- Mass spectrometry is an important emerging method for the characterization of larger molecules such as proteins, lipids and oligonucleotides(DNAs).
- MALDI (Matrix-assisted laser desorption/ionization) is a primary method for ionization of whole proteins.
- Whole protein mass analysis is primarily conducted using time-of-flight (TOF) MS.
- The procedure can be easily applied by a microbiologist with essential knowledge in mass spectrometry.
- But it is high instrument cost in contrast to low analysis costs.

Mass spectrometry

MALDI mass spectrometry (MALDI-TOF MS)

Protein analysis

- MALDI-TOF technology (Matrix Assisted Laser Desorption Ionization Time-of-Flight) examines the patterns of proteins detected directly from intact bacteria.
- The sample to be analyzed is mixed with another compound, called a matrix.
- The mixture is applied to a metal plate target slide and irradiated ionized with a laser.
- The matrix absorbs the laser light and vaporizes, along with the sample, and in the process gaining gains an electrical charge (ionization).

Jurinke *et al.*,2004

Mass spectrometry

MALDI mass spectrometry (MALDI-TOF MS)

Protein analysis

- Electrical fields then guide the ions into the time of flight mass spectrometer, ions are separated according to their mass to charge (m/z) ratio, and ultimately the quantity of each ion is measured.
- Detection is achieved at the end of the flight tube.
- Ion masses (mass-to-charge ratios, m/z) are typically calculated by measuring their TOF, which is longer for larger molecules than for smaller ones (provided their initial energies are identical).

Jurinke *et al.*,2004

Mass spectrometry

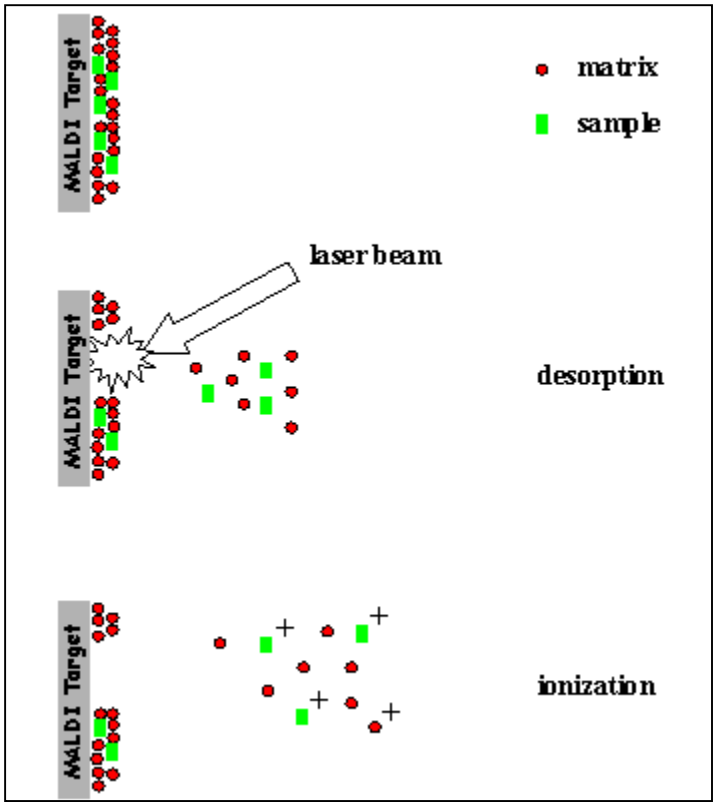
MALDI mass spectrometry (MALDI-TOF MS)

- **Matrix:**

1. Absorbs laser light;
2. Minimize sample/sample interactions;
3. Transports the sample in the expanding plume;
4. Donates protons to the analyte.

Ion source = MALDI

Acronym for matrix assisted laser desorption ionization



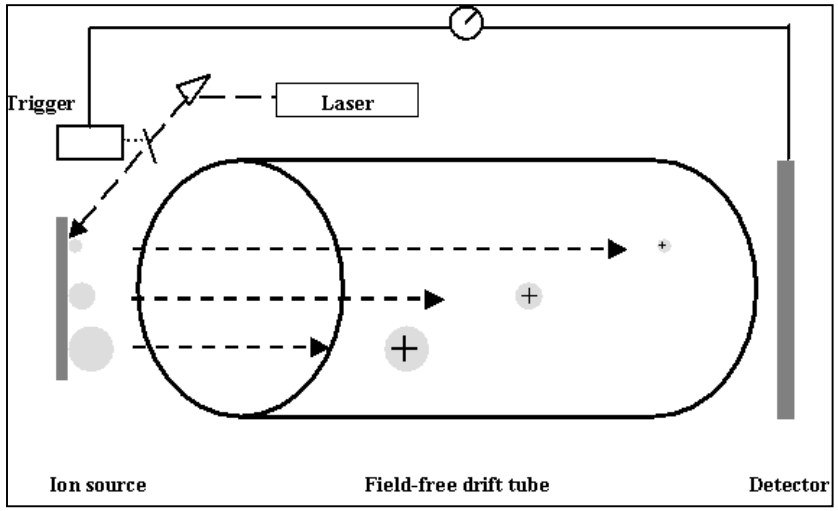
Elek,2010

Mass spectrometry

MALDI mass spectrometry (MALDI-TOF MS)

Mass analyzer = Time Of Flight

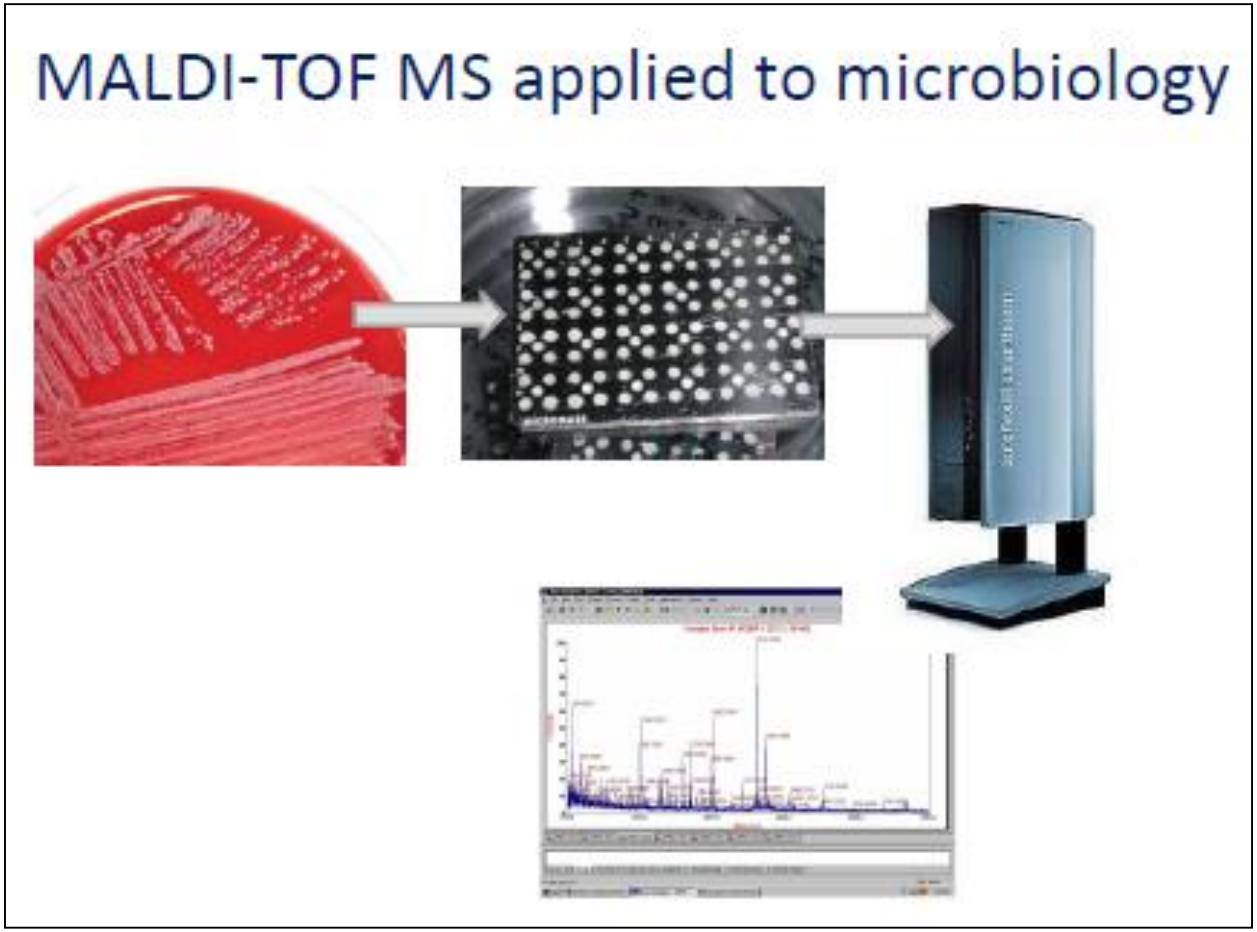
- Based on the principle that time is related to mass (fixed$_{Ekin}$=1/2 m.v²);
- Measured under high vacuum conditions;
- The higher the mass, the lower its velocity and the longer it takes before the ion strikes the detector.



Elek,2010

Mass spectrometry

MALDI-TOF mass spectrometry



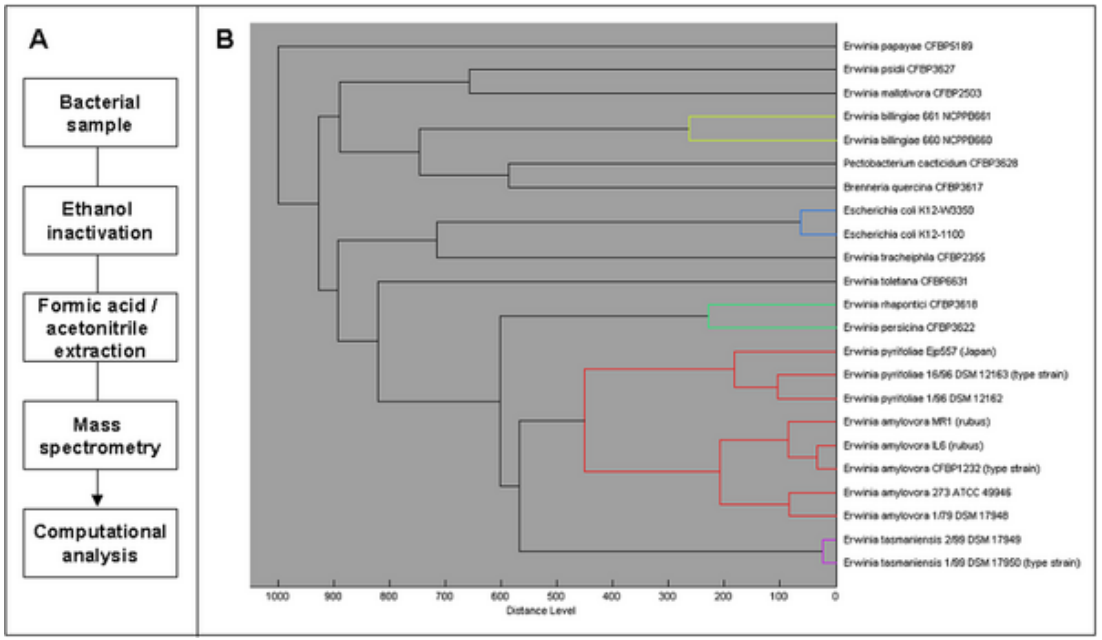
Elek,2010

Mass spectrometry

MALDI-TOF mass spectrometry

A general scheme of the procedure

- Bacterial colonies are subjected to chemical treatment.
- Samples can be analyzed within a few minutes by MALDI mass spectrometry and mass spectra are transferred to analysis and identification software.



Sauer *et al.*,2008

Mass spectrometry

MALDI-TOF mass spectrometry

A general scheme of the procedure

1. **Inactivation of bacteria:**

- The bacterial colonies were suspended in 300 µl water and inactivated by the addition of 800 µl ethanol at room temperature.

2. **Protein extraction:**

- The solution was centrifuged at 25,000×g for 2 minutes and the supernatant was discarded.
- Again, centrifugation was performed for 2 minutes at 25,000×g and residual supernatant was discarded.
- Five to 20 µl of 70% formic acid were added to the "pellet" (1 to 5 mg, or less bacterial material), and mixed to re-suspend the bacteria.
- Then 5-20 µl acetonitrile were added, accordingly, and the sample was mixed carefully.
- The solution was centrifuged at 25,000×g for 2 minutes.
- The supernatant (~5-20 µl) was transferred to a new tube immediately.

Sauer *et al.*,2008

Mass spectrometry
MALDI-TOF mass spectrometry
A general scheme of the procedure

3. MALDI preparation:

- This step was performed at room temperature and at 20–80% air humidity.
- One microliter of the supernatant was placed onto a stainless steel target plate and led dry in air.
- Then, 1 µl of matrix (3 mg/ml solution of alpha-cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluor acetic acid) was overlaid onto the dried sample and led dry in air.
- This simple preparation method provided homogenous samples to enable automated measurements and sufficiently reproducible mass spectra.
- To increase data reliability, we applied each bacterial sample six times onto the target plate.

Sauer *et al.*,2008

Mass spectrometry
MALDI-TOF mass spectrometry
A general scheme of the procedure

4. Mass spectrometry detection:

- Mass spectra were acquired using an Ultraflex I MALDI-TOF mass spectrometer.
- We performed measurements in linear positive ion detection mode, using a Nd:YAG laser at maximum frequency of 66 Hz.
- Pulsed ion extraction (PIE) was set to zero.
- Acceleration voltage (IS1) was set to 20 kV.
- The mass range of spectra was from 2,000 to 20,000 m/z.
- The final resolution in the mass range of 7,000-10,000 m/z was optimized to be higher than 600 and absolute signal intensities were about 10^3.
- At least 10 intensities were about 10^7 bacterial cells were required for high quality mass spectra.

Sauer *et al.*,2008

Mass spectrometry
MALDI-TOF mass spectrometry
A general scheme of the procedure

5. **Data analysis:**

- Mass spectra were analyzed with Flex Analysis software 2.4 (Bruker Daltonics).
- Further bacterial data analysis was performed by software developed and tested by us that we termed BioTyper.
- Spectra were pre-processed using default parameters for reference spectra libraries that we call main spectra libraries (MSPs).
- A maximum of 100 peaks with a signal-to-noise (S/N) ratio of 3 were selected in the range of 3,000-15,000 Da.
- Afterwards the main spectra were generated as a reference using all spectra given for a single microorganism.
- In general, 75 peaks were picked automatically, which occurred in at least 25% of the spectra and with a mass deviation of 200 ppm.
- For the evaluation of mass spectra reproducibility, we loaded the spectra into the ClinProTools 2.1 software.

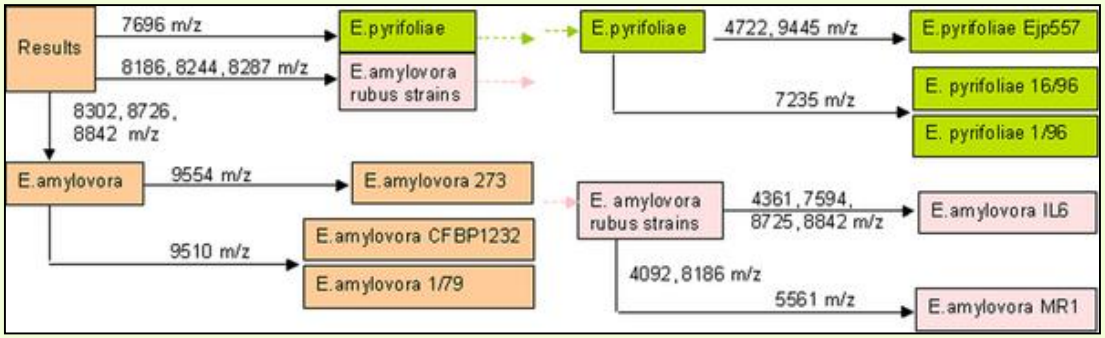
Sauer *et al.*,2008

MALDI-TOF mass spectrometry

Weighted pattern matching

Erwinina spp.

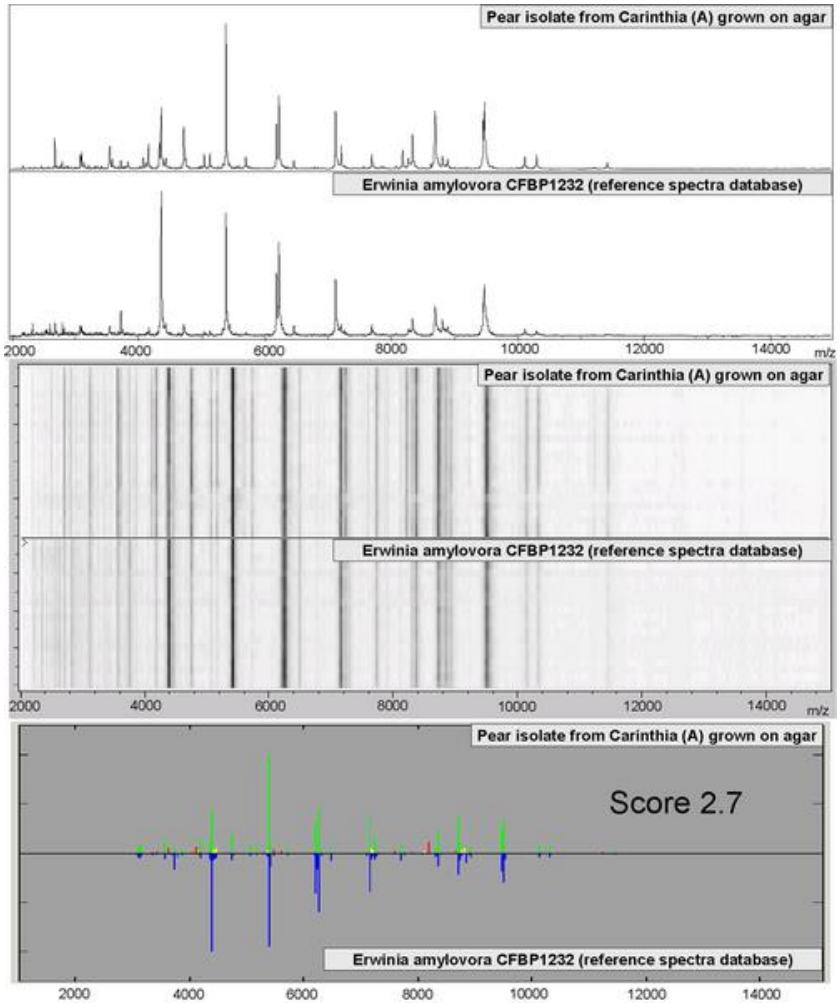
- An initial pattern matching analysis revealed two potential candidate strains, *E. amylovora* and *E. pyrifoliae*.
- The mass signals at 7696 m/z and the mass peaks at 8186, 8244, 8287 m/z are specific for *E. pyrifoliae* and *E. amylovora* rubus strains, respectively, which distinguish them from *E. amylovora*.
- The mass peak at 9554 m/z distinguished the North American type strain *E. amylovora* 273 from the other *E. amylovora* strains of American and European origin, which instead have an additional peak at 9510 m/z.



Sauer *et al.*,2008

A typical mass spectrum of a bacterial sample taken from necrotic wood compared with a matching spectrum from the reference library *Erwinina amylovora*

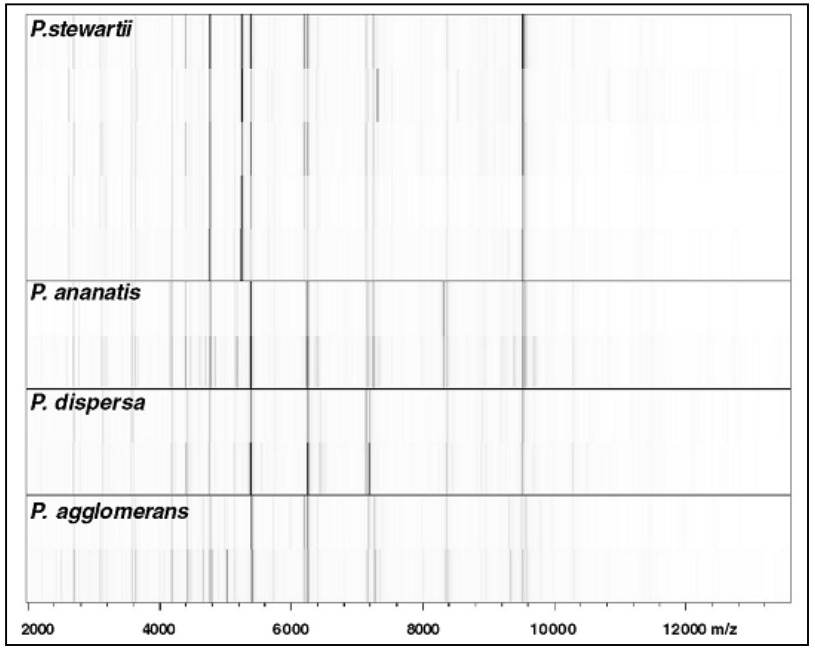
- Top: Original mass spectra,
- Middle: Respective pseudo gel-view showing a bar-code of masses and their intensities,
- Bottom: Identification by comparison of experimental and reference mass spectra using a pattern matching algorithm.
- In this example, a highly reliable identification score of 2.7 was obtained from the identification of *E. amylovora* (CFBP1232).



Sauer *et al.*,2008

Bar code representation of protein profiles
Pantoea spp.

- Protein spectra of various *Pantoea* isolates were visualized in a pseudo-gel representation with peak heights converted to color intensity.
- Lines are as follows (top to bottom):
- *P. stewartii* strains DC283, DC116, SW2, DC145, and DC405;
- *P. ananatis* strains DC129 and DC532;
- *P. dispersa* strains NCPPB 2285 and DSM 30073; and
- *P. agglomerans* strains DSM 3493.



Wensing *et al.*,2010

Comparison of microbial identification methods

Phenotypic, Molecular and chemotaxonomic method

	Phenotypic methods	Molecular methods		Chemotaxonomic methods
	Traditional methods	RT-PCR	Sequencing	MALDI-TOF MS
Sensitive and specific	1	4	3	2
Rapid	1	3	2	4
Easy to perform, not labor intensive	1	3	2	4
Data easy to interpret	4	2	1	3
Cost effective	4	3	1	2
Automatization	1	3	2	4
Total	12/24	18/24	11/24	10/24

Elek,2010

Whole-cell fatty acids analysis

GC analysis of FAMEs (fatty acid methyl esters)

A chemotaxonomic method

Very good for genera and species
Less good for pathovars, subspecies

Fatty acids analysis

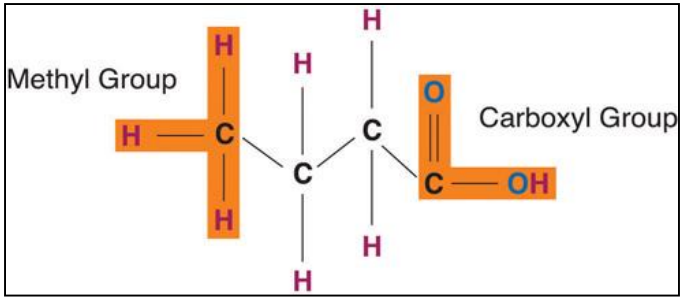
Fatty acid structure

- Fatty acids (FA) consist of carbon, hydrogen, and oxygen, arranged as a linear carbon chain skeleton of variable length, generally:

1. with an even number of atoms,
2. with a carboxyl group at one end.

- Fatty acids contain:

1. long reduced-carbon chains at one end, and
2. a hydrophilic carboxylic acid group at the other end.



www.tuscany-diet.net;..

Fatty acids analysis
Fatty acid chain length

- Depending only on chain length they can be functionally divided into:

1. **short chain FA** (SCFA): up to 6 carbon atoms;
2. **medium chain FA** (MCFA): from 8 to 12 carbon atoms;
3. **long chain FA** (LCFA): from 14 to 18 carbon atoms;
4. **very long chain FA** (VLCFA): from 20 carbon atoms onwards.

Fatty acids analysis

Type and fatty acid chain length in bacteria

- As many as 50 different fatty acids may be present in a cell.
- Most bacteria contain between 5 and 30 of the fatty acids which are generally 8-20 carbon atoms in length.

Fatty acids analysis

Fatty acid classification

Saturated and unsaturated fatty acids

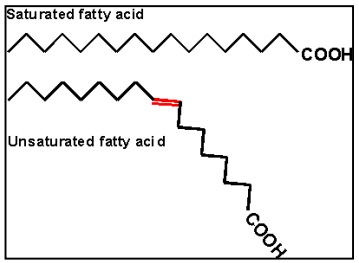
- Depending on their degree of saturation/unsaturation in the carbon chain, they can be divided into three classes:

1. saturated fatty acids (SFA), if no double bond is present;
2. monounsaturated fatty acids (MUFA), if only one double bond is present;
3. polyunsaturated fatty acids (PUFA), if two or more double bonds are present.

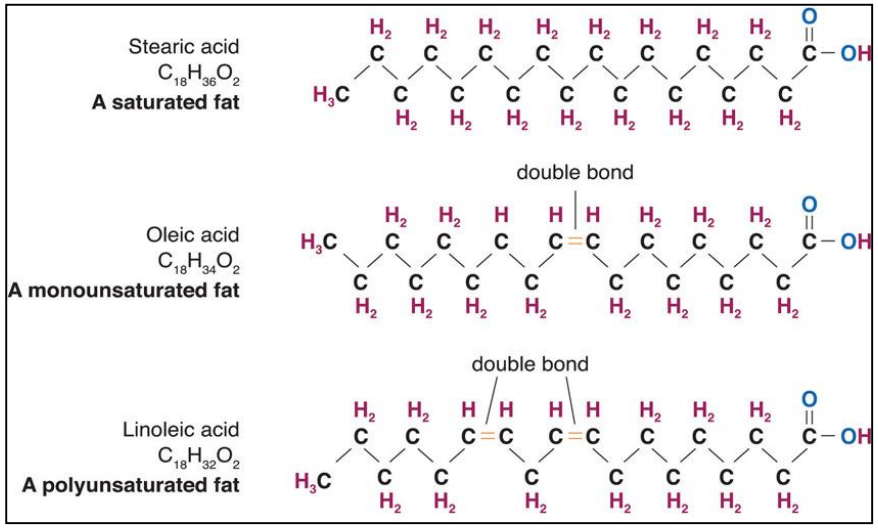
Fatty acids analysis

Fatty acid classification

Saturated and unsaturated fatty acids



- **Saturated fatty acids:**
- Among straight-chain fatty acids, the simplest are referred to as saturated fatty acids.
- **Unsaturated fatty acids ("missing" hydrogen):**
- When double bonds are present, fatty acids are said unsaturated.

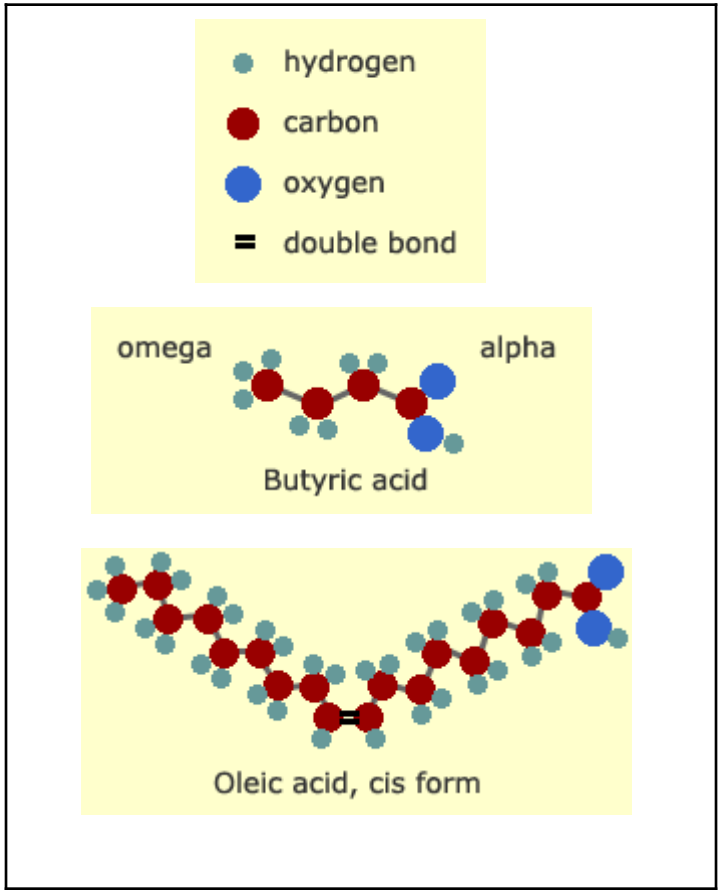


Fatty acids analysis

Fatty acid classification

Saturated and unsaturated fatty acids

- In the diagrams, the red circles are carbon atoms, forming the spine at the center of the molecules.
- The blue circles are oxygen, and the green are hydrogen.
- All saturated fats e.g. butyric acid are essentially straight, and the molecules can be packed tightly together.
- In unsaturated fats e.g. Oleic acid some of these hydrogen atoms are missing.



Wyn Snow,2004

Fatty acids
Nomenclature

- The first letter C represents carbon.
- The number after C and before the colon indicates the number of carbons.
- The letter after the colon shows the number of double bonds.
- The letter n (or w) and the last number indicate the position of the double bonds.

Examples of Fatty Acid Nomenclature:

Palmitic	C 16 : 0
Linolenic	C 18 : 2 n 6
Eicosapentaenoic	C 20 : 5 n 3

Structure of fatty acids

Saturated fatty acids

Systematic names

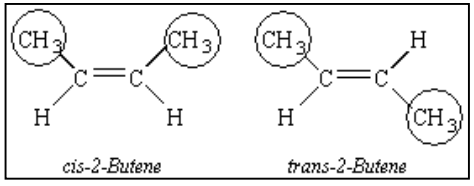
Systematic name	Trivial name	Shorthand designation	Molecular wt.
butanoic	butyric	4:0	88.1
pentanoic	valeric	5:0	
hexanoic	caproic	6:0	116.1
octanoic	caprylic	8:0	144.2
nonanoic	pelargonic	9:0	158.2
decanoic	capric	10:0	172.3
dodecanoic	lauric	12:0	200.3
tetradecanoic	myristic	14:0	228.4
hexadecanoic	palmitic	16:0	256.4
heptadecanoic	margaric (daturic)	17:0	270.4
octadecanoic	stearic	18:0	284.4
eicosanoic	arachidic	20:0	312.5
docosanoic	behenic	22:0	340.5

Structure of fatty acids

Mono-Unsaturated fatty acids

Systematic names

Systematic name	Trivial name	Shorthand designation	Molecular wt.
cis-9-tetradecenoic	myristoleic	14:1(n-5)	226.4
cis-9-hexadecenoic	palmitoleic	16:1(n-7)	254.4
cis-9-octadecenoic	oleic	18:1(n-9)	282.4
tr-9-octadecenoic	elaidic	tr18:1(n-9)	282.4
cis-11-octadecenoic	vaccenic (asclepic)	18:1(n-7)	282.4
cis-11-eicosenoic	gondoic	20:1(n-9)	310.5
cis-13-docosenoic	erucic	22:1(n-9)	338.6



The isomer with similar substituents on the same side of the double bond is called cis, a Latin stem meaning "on this side." The isomer in which similar substituents are across from each other, is called trans, a Latin stem meaning "across."

Structure of fatty acids

Odd- and even-chain fatty acids

- Most fatty acids are even chain, e.g. stearic (C16) and oleic (C18).
- But some fatty acids are odd chain. e.g. pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0).
- Odd-chain fatty acids are those fatty acids that contain an odd number of carbon atoms.

Structure of fatty acids

Profiles of major classes of fatty acids in bacteria

Saturated and unsaturated, iso- and anteiso fatty acids

- Normally, the fatty acyl chain is saturated and the branch is a methyl-group.
- However, unsaturated branched-chain fatty acids are found in marine animals, and branches other than methyl may be present in microbial lipids.
- Iso or anteiso a methoxylated C15-C16 fatty acids were reported from phospholipids of bacteria.
- Multimethyl branched acids are found mainly in bacteria.

Structure of fatty acids

Profiles of major classes of fatty acids in bacteria

Saturated and unsaturated, iso- and anteiso fatty acids

- There are several major classes of fatty acids in bacteria:

1. Primarily straight chain saturated(16:0),
2. Straight chain mono-unsaturated(16:1 w7cis),
3. Cyclopropanes (17:0 cyclopropane);
4. Iso-branched (15:0 iso); anteiso-branched(17:0 anteiso); hydroxy(12:0 3OH) and,
5. Mixed (13:0 iso 3OH or 17:0 cyclopropane)

Iso-fatty acids are often found in bacteria but not commonly in other microorganisms. Some bacteria have also rare acids e.g. the presence of 13:0 iso3OH is virtually exclusive to *Xanthomonas*.

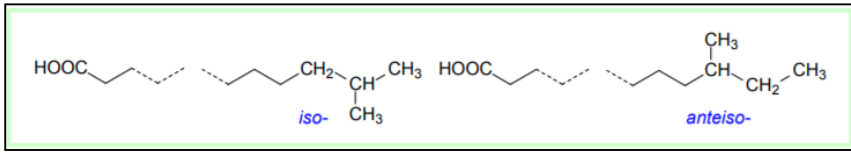
Fox,2008;..

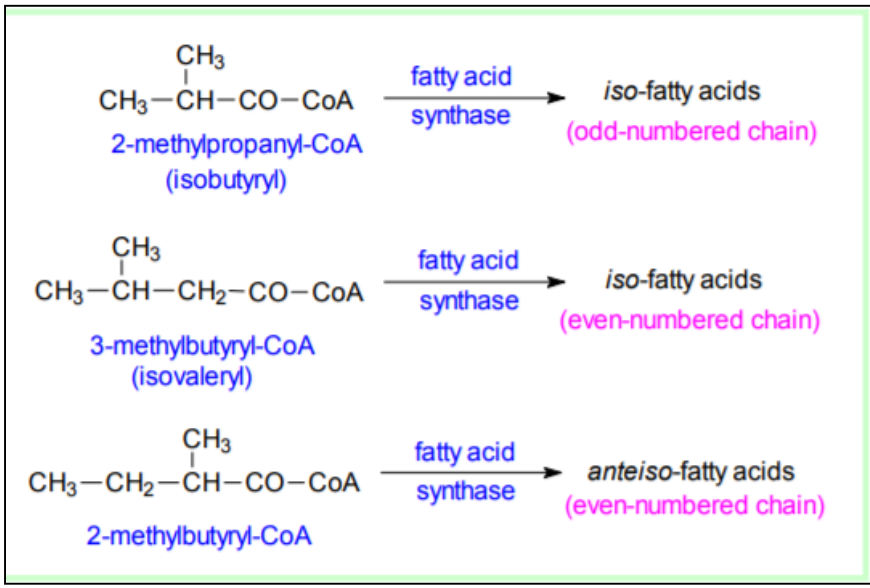
Structure of fatty acids

Profiles of major classes of fatty acids in bacteria

Saturated and unsaturated, iso- and anteiso fatty acids

- iso-methyl branched fatty acids have the branch point on the penultimate carbon (one from the end), while
- anteiso-methyl-branched fatty acids have the branch point on the ante-penultimate carbon atom (two from the end) as illustrated.
- odd-numbered iso-methyl acids and even-numbered anteiso-methyl acids.





Christie,2012

Structure of fatty acids

Profiles of major classes of fatty acids in bacteria

Major differences of fatty acids in Gram-negative and Gram-positive bacteria

- Gram-negative bacteria:

1. Have unique series of hydroxy acids
2. Few branched acids
3. Some cyclopropanes

- Gram positives bacteria:

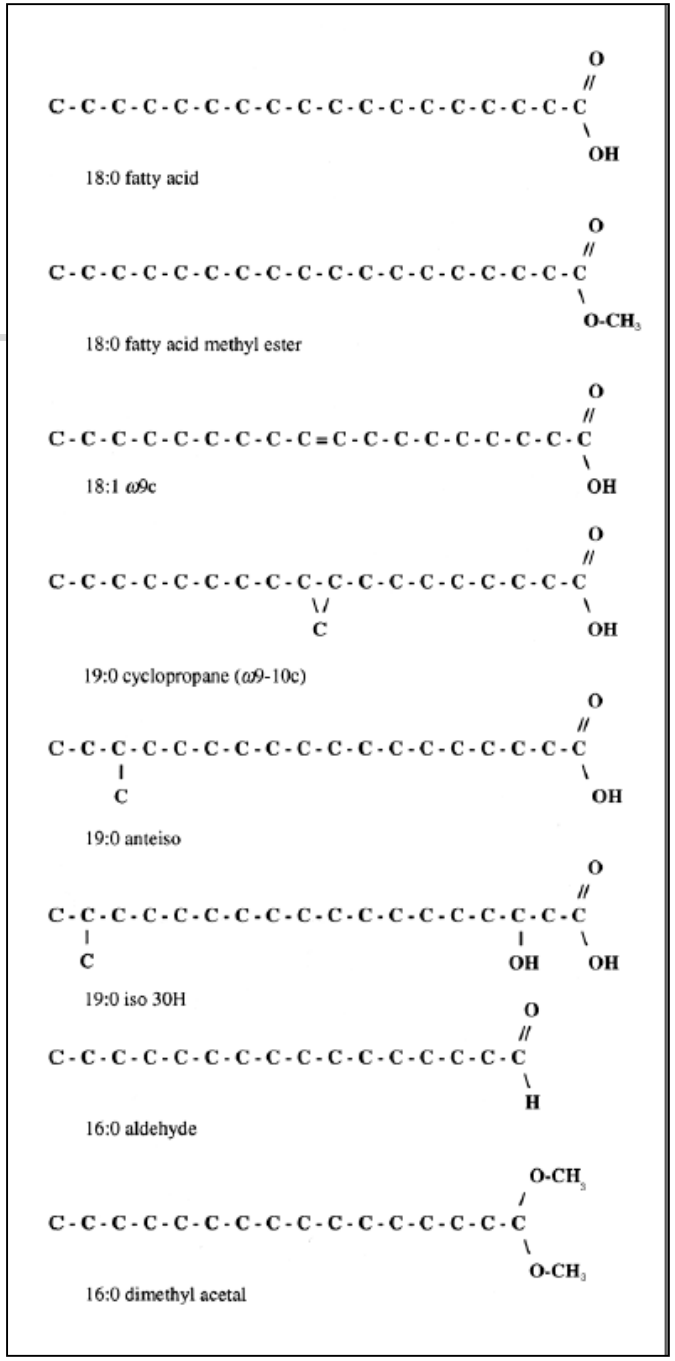
1. Lack hydroxy acids
2. Many branched acids
3. Very few hydroxy and cyclopropane acids.

Profiles of major classes of fatty acids in bacteria

In Gram-negative and Gram-positive bacteria

- More than 300 fatty acids and related compounds have been found in bacteria.
- The peaks are automatically named and quantitated by the system.
- Branched chain acids predominate in some Gram positive bacteria, while short chain hydroxy acids often characterize the lipopolysaccharides of the Gram negative bacteria.
- The structures of a few of these compounds are shown here.

Fatty acid profiles were prepared according to standard protocols using the Microbial Identification System based on software available from MIDI. MIDI Sherlock® Identification Systems uses fatty acids 9-20 carbons in length.



Analysis of bacterial fatty acids

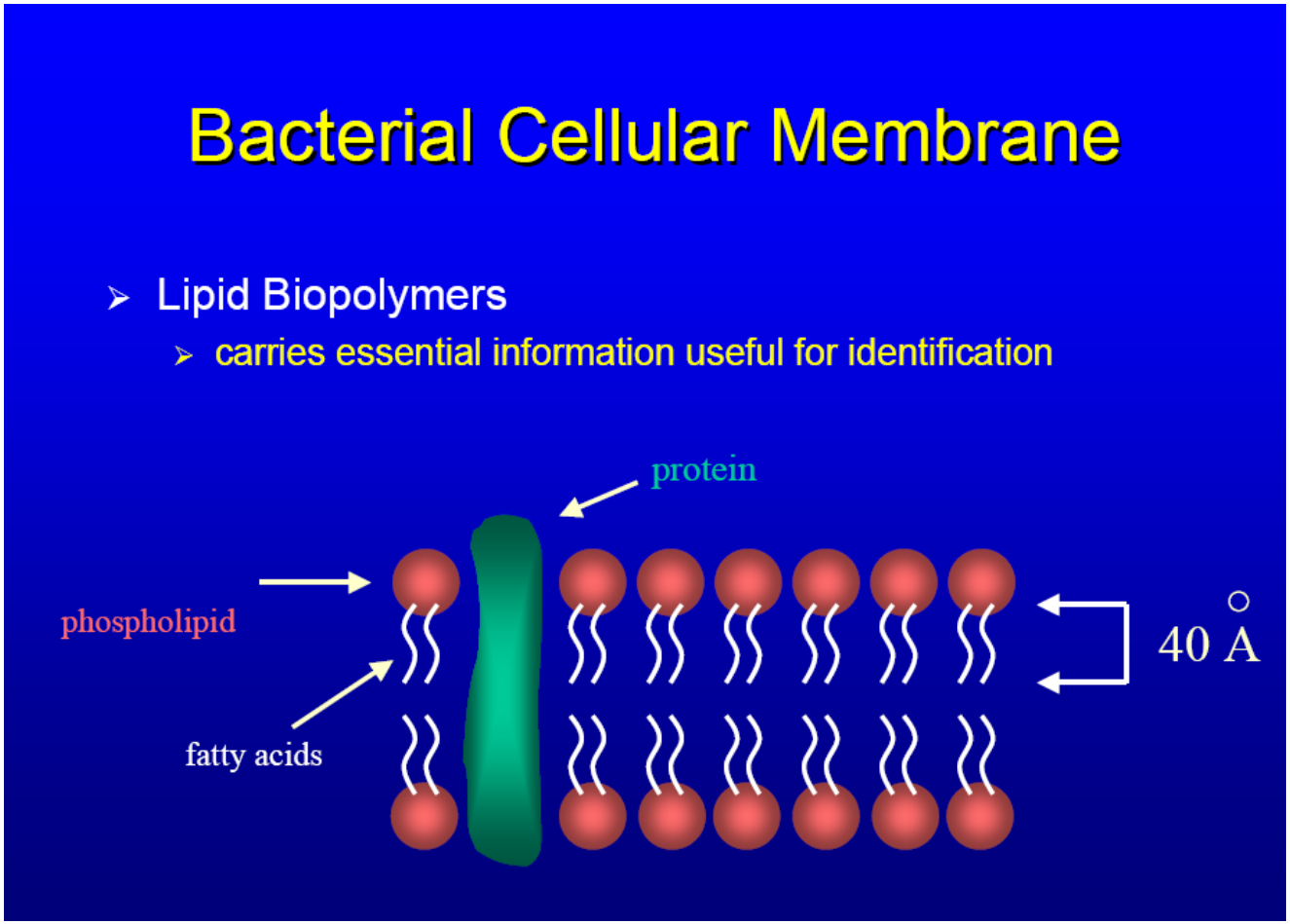
Whole cell fatty acids analysis in bacteria

- Sasser and his colleagues have developed a sophisticated chromatographic method with computer analysis to identify many bacterial plant pathogens.
- Analysis of derivatives of fatty acids obtained from the plasma membrane and outer membrane of bacteria can be used to identify organisms.

Analysis of bacterial fatty acids

Based on fatty acid composition of whole cells

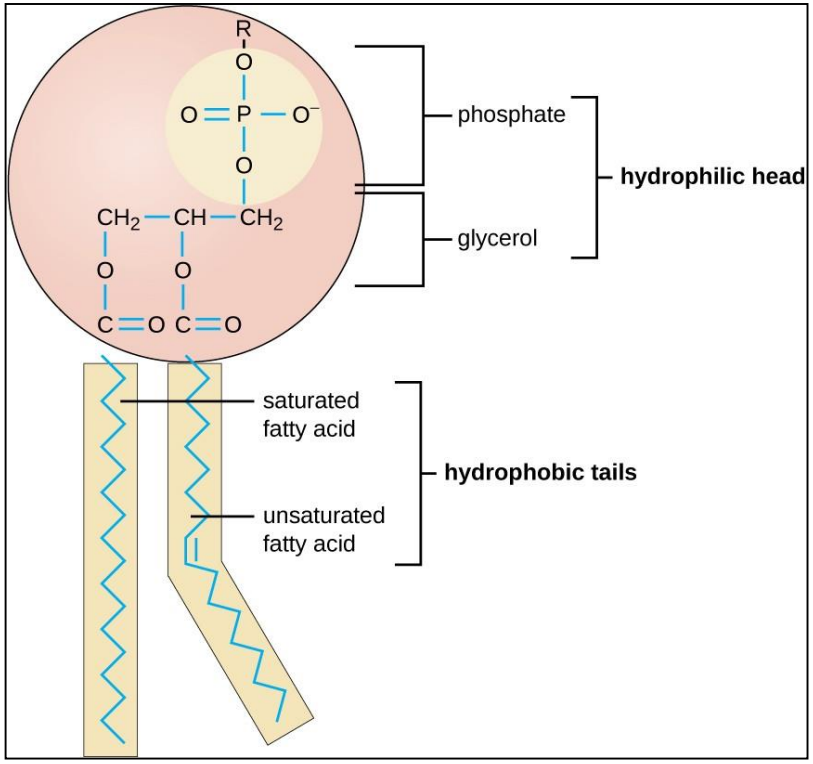
Lipoproteins, lipopolysaccharides, phosphlipids

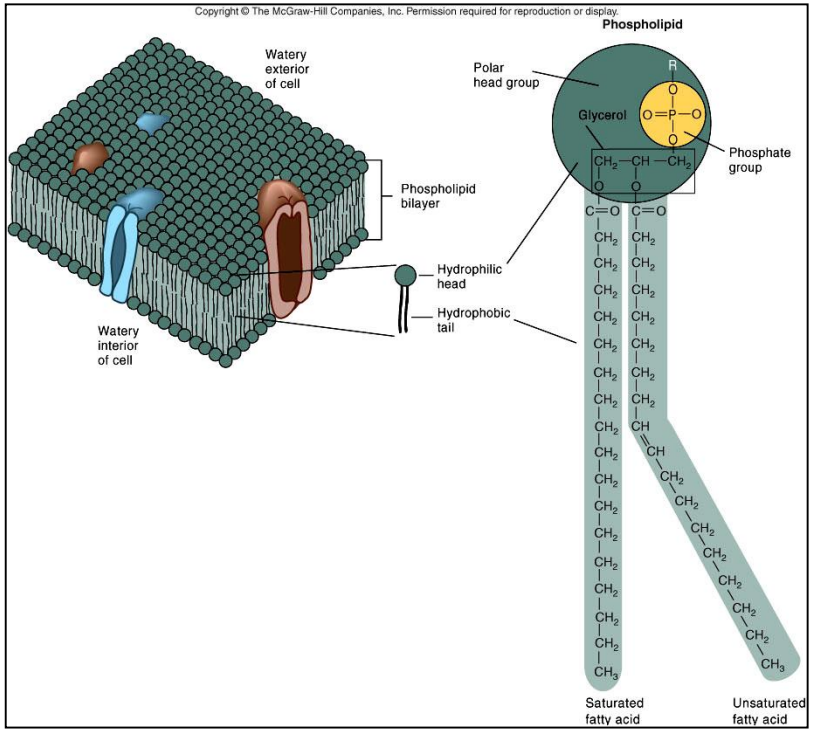


Analysis of bacterial fatty acids

Based on fatty acid composition of whole cells

Lipoproteins, lipopolysaccharides, phosphlipids





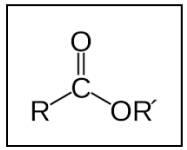
Bacteria cell membrane:
A typical unit membrane of phospholipid bilayer.

Seelke,2010; Lipids Microbiology

Analysis of bacterial fatty acids

The fatty acid methyl esters (FAME)

FAME GC



Ester bond

- Advantages:
- The type and percentage of individual fatty acids present in bacteria:

1. Not only varies from genus to genus, and species to species, but
2. In some cases it also unique at biovar or pathovar level.

- It can provide a reasonable identification to the genus and species level.
- System is quick, reproducible and easy to perform;
- Data can be shared between laboratories.

Esters have the general formula RCOOR′, where R may be a hydrogen atom, an alkyl group (e.g. methyl), or an aryl group group(e.g. phenyl), and R′ may be an alkyl group or an aryl group but not a hydrogen atom.

Julian Smith;…

Fatty acids analysis

The fatty acid methyl esters (FAME)

FAME GC

- Limitation:
- This system allows for identification of many, but not all bacteria.
- Requires investment in GC equipment and MIDI library.
- Has limitation in resolving below species level (pathovar).
- Library stronger on human microbials than plant pathogenic bacteria.

Julian Smith;...

Fatty acid extraction process

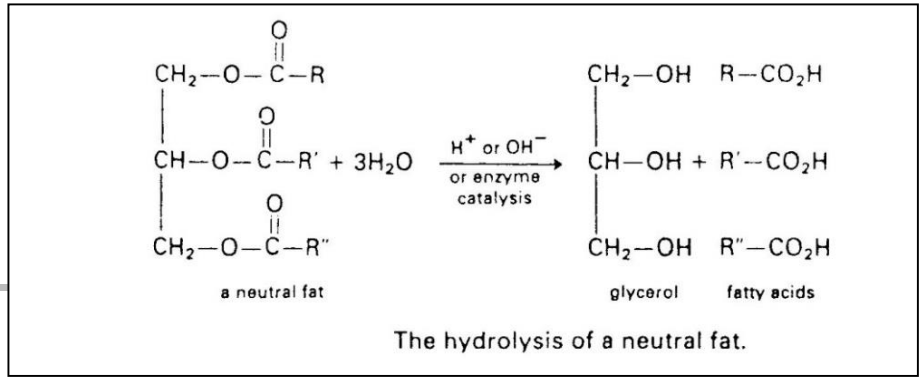
Sample processing

- **Harvesting, saponification & methylation:** Bacterial cells are harvested from agar or broth agar plates e.g. trypticase soy broth(TSB) grown for 24 h at 28°C. HCl is most widely used as an acid catalyst(acidification). The latter was suspended in sodium hydroxide and methanol and in tubes which are sealed and heated (saponification followed by methylation).

- **Extraction:** The fatty acid methyl esters (FAME) are extracted in an ether-hexane mixture and the esters are subsequently separated by gas chromatography.

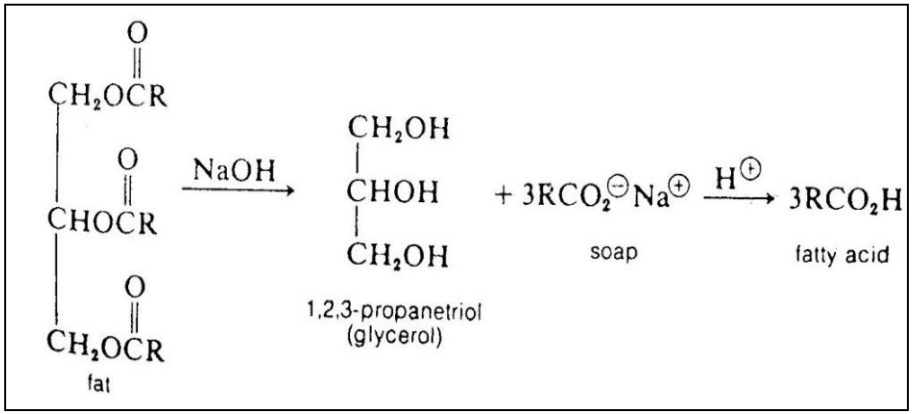
- **Fatty acids profiles/chromatograms:** Areas of the resulting peaks on the chromatograms are calculated and compared with profiles of known reference strains by computer programs.

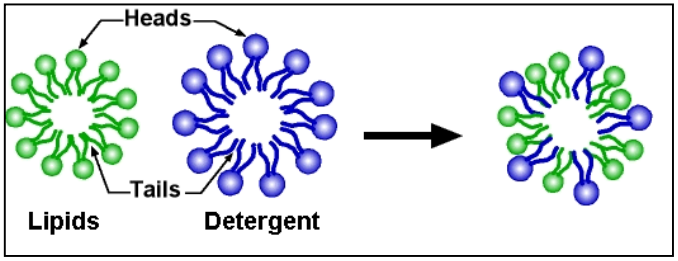
Fatty acid extraction process
Sample processing
Products of each process



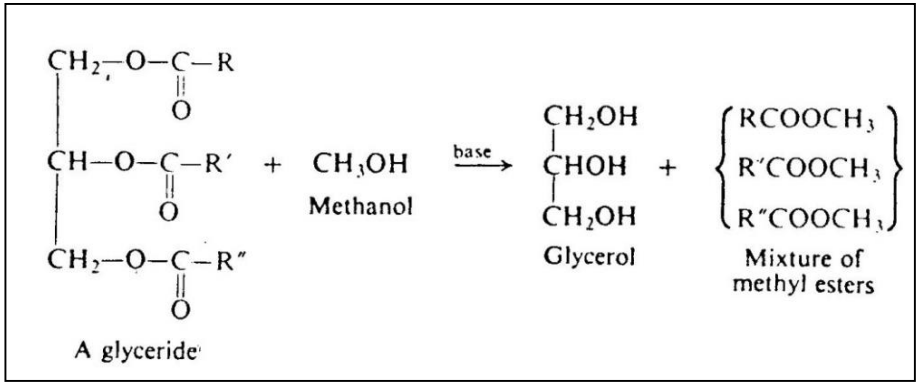
The hydrolysis of a neutral fat.

Conversion of fatty acids to their fatty acid methyl ester (FAMs) through: acidification, saponification and methylation procedures.



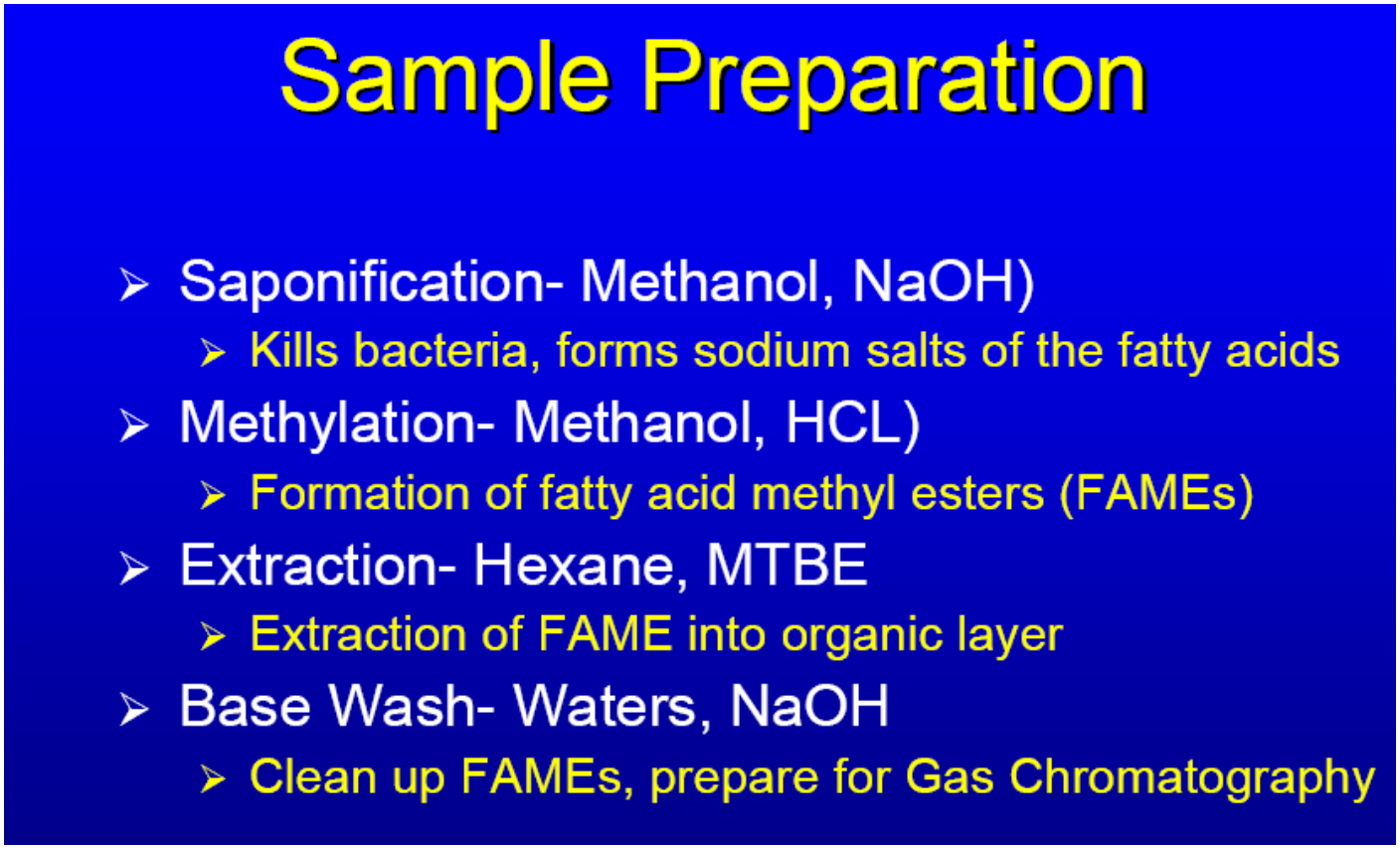


Lipid solubilization



MIDI system(The Microbial Identification System)

The five steps to prepare GC ready extracts

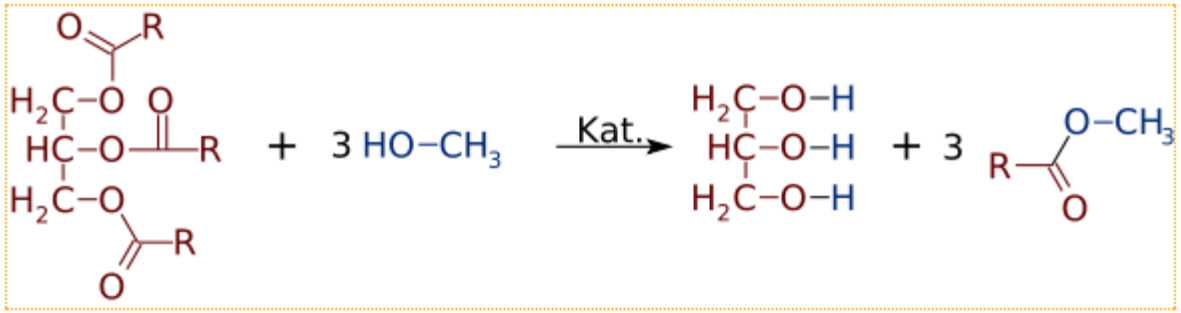


Sample processing

Conversion of fatty acids to their fatty acid methyl ester (FAMs)

- A **f**atty **a**cid **m**ethyl **e**ster (FAME) can be created by an alkali catalyzed reaction between fats or fatty acids and methanol through:

1. Saponification
2. Methylation
3. Extraction
4. Sample cleanup(base wash).



Fatty acid extraction process
Materials and equipment

- Analysis of fatty acids contains four steps:

1. saponification (liberation of fatty acids from fats and oils),
2. Esterification of fatty acids (to form fatty acid methyl esters),
3. Extraction of fatty acid methyl esters with solvents, and
4. Purification of the extract (washing step).

Tóth *et al.*,2013

Fatty acid extraction process
Materials and equipment

- TSA (Trypticase Soy Agar)plates (see next slides)
- 10 mL glass test tubes with Teflon-lined screw caps
- Reagent 1, 2, 3 and 4 (see next slide)
- vortex mixer
- overhead mixer
- degreased glass tubes
- water bath
- slush ice
- degreased Pasteur pipettes
- 1 mL glass tubes for sample storage
- microlitre syringe
- gas chromatograph (GC)
- BAME (Bacterial Methyl Ester) standard solution

Tóth *et al.*,2013

Fatty acid extraction process
Reagents 1, 2, 3 and 4

Reagent 1	
Sodium hydroxide (certified)	45.0 g
Methanol (reagent grade)	150.0 mL
Deionized distilled water	150.0 mL
Reagent 2	
6N HCl(49.8ml of conc. HCl in 50.2 mL of dH_2O)	325.0 mL
Methanol (reagent grade)	275.0 mL
Reagent 3	
Hexane (HPLC grade)	200.0 mL
Methyl-tert butyl ether (HPLC grade)	200.0 mL
Reagent 4	
Sodium hydroxide (certified)	10.8 g
Deionized distilled water	900.0 mL

Tóth *et al.*,2013

Fatty acid extraction process
Materials and equipment

1. Add 1 mL Reagent 1 to 2-3 loopfuls of bacteria in glass tubes, close with Teflon-lined screw caps, vortex and incubate in 100°C water bath for 5 minutes (saponification).
2. Vortex again and put the tubes back to the 100°C water bath, then cool them down suddenly in slush ice.
3. Add 2 mL Reagent 2, vortex and incubate in 80°C water bath for 10 minutes, then cool them suddenly in slush ice.
4. After this methylation process, add 1.25 mL Reagent 3 and mix with overhead mixer for 10 minutes.
5. Discard the lower phase using Pasteur pipette and add 3 mL Reagent 4 to the upper "solvent" phase, mix for 5 minutes. 1 µL of this liquid can be directly injected into a gas chromatograph.
6. Conditions of GC analysis: splitless injection, capillary column (SPB-1, 30x32 mm id.), heating condition: 150-250°C, heating rate: 4°C/min, carrier gas: Helium, detector: ,, 280°C.
7. Storage of these fatty acid samples is possible in 1 mL test tubes at -20°C for a few weeks.
8. Evaluate the obtained data with the help of BAME (Bacterial Methyl Ester) standard and compare the results of different bacteria.

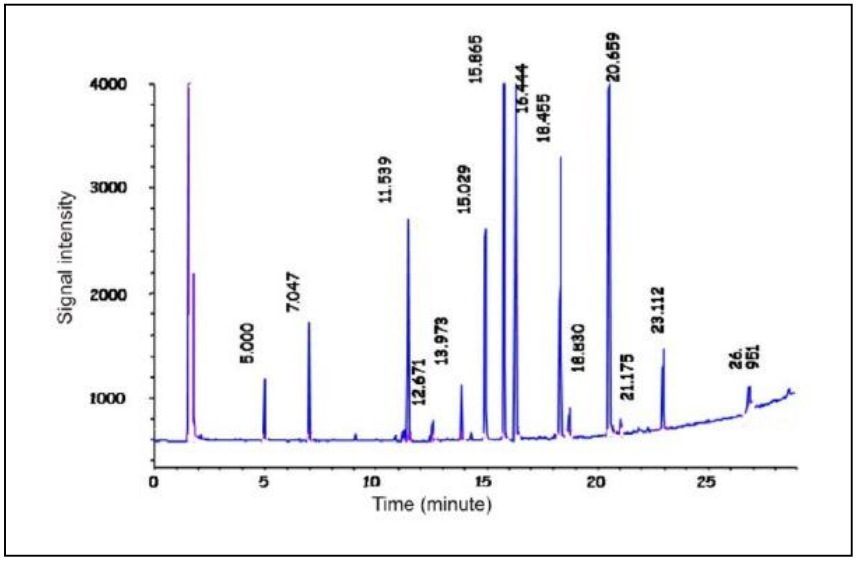
Splitless injection is the most sensitive method because all the analyte mass in a 1 µL injection goes on column, and is not split.

Tóth *et al.*,2013

Fatty acids analysis

Fatty acid methyl ester profile of a bacterial strain

- The profile was prepared with gas chromatograph(GC).
- Peaks were identified on the basis of their retention time using BAME (bacterial methyl ester) standard.



Tóth *et al.*,2013

Fatty acids analysis in details

1. Culturing and Harvesting

Effects of growth medium and temperature on fatty acid analysis

- Fatty acid analysis requires:

1. Rigid standardization as to the medium on which the organism is grown, and
2. At what temperature it is grown.

Fatty acids analysis in details

Culturing and Harvesting

Effects of growth medium and temperature on fatty acid analysis

- Bacterial cells are harvested from standard ager or broth media.
- Trypticase Soy Broth Agar (TSBA) consists of 30 g Trypticase Soy Broth and 15 g of agar (BBL) is often recommended for fatty analysis (Sasser,2001).
- King's medium B agar(KB) is used for cellular fatty acids of *Erwinia* species in the Amylovora group (*E. amylovora*, *E. nigrifluens*, *E. quercina*, *E. rubrifaciens*, *E. salicis* and *E. tracheiphila*). The bacteria were grown on KB for 1,3, and 6 days at 28°C(Wells *et al.*,2008).
- Diogo *et al.*,1999 used buffered charcoal-yeast extract agar for cells harvesting.

Fastidious bacteria were incubated longer.

Fatty acids analysis in details

Culturing and Harvesting

Effects of growth medium and temperature on fatty acid analysis

- Because of slow growth rate of many of the strains of *Xanthomonas* and *Stenotrophomonas maltophilia*, three different media were used:
- Yeast Sucrose Peptone medium (Dye,1962) for *X. albilineans*.
- Wilbrink's medium (Ricaud and Ryan,1989) for *X. fragariae*.
- All other strains were grown on Difco Trypticase Soy Agar amended with 5% sucrose (TSA) for 48 h at 28 (±) 1C (Norman *et al.*,1997).

Fatty acids analysis in details

Culturing and Harvesting

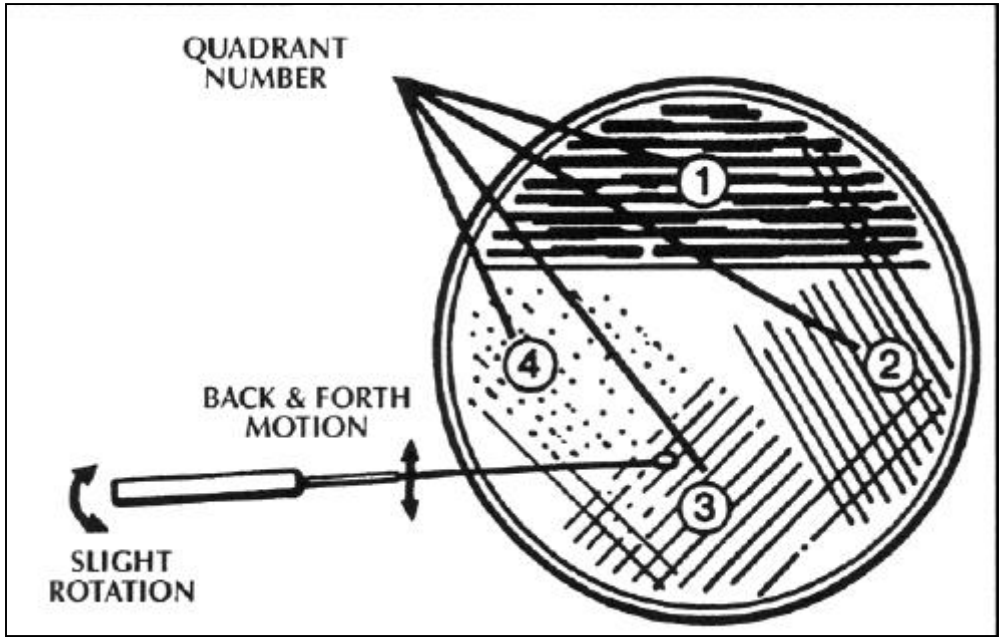
Effects of growth medium and temperature on fatty acid analysis

- Casano *et al*.,2008 were used 4 different agar media (King's medium B (KB), nutrient broth agar (NA), trypticase soy agar (TSA) and glucose yeast-extract carbonate agar (GYCA)), for total cellular fatty acids analysis of three strains of *Erwinia amylovora* by GLC.
- Saturated and odd-carbon and cyclic fatty acids were most affected by growth medium.
- On NA and TSA average percentage of saturated odd-carbon and cyclic acids were 7-10% and 4-5%, respectively.
- On KB and GYCA the averages were 1-2% and 2-3%, respectively.
- Ratios of even-to-odd saturated straight chain acids were above 30 for NA and TSA, and below 6.5 for KB and GYCA.

Fatty acids analysis in details

Culturing and Harvesting

Effects of growth medium and temperature on fatty acid analysis



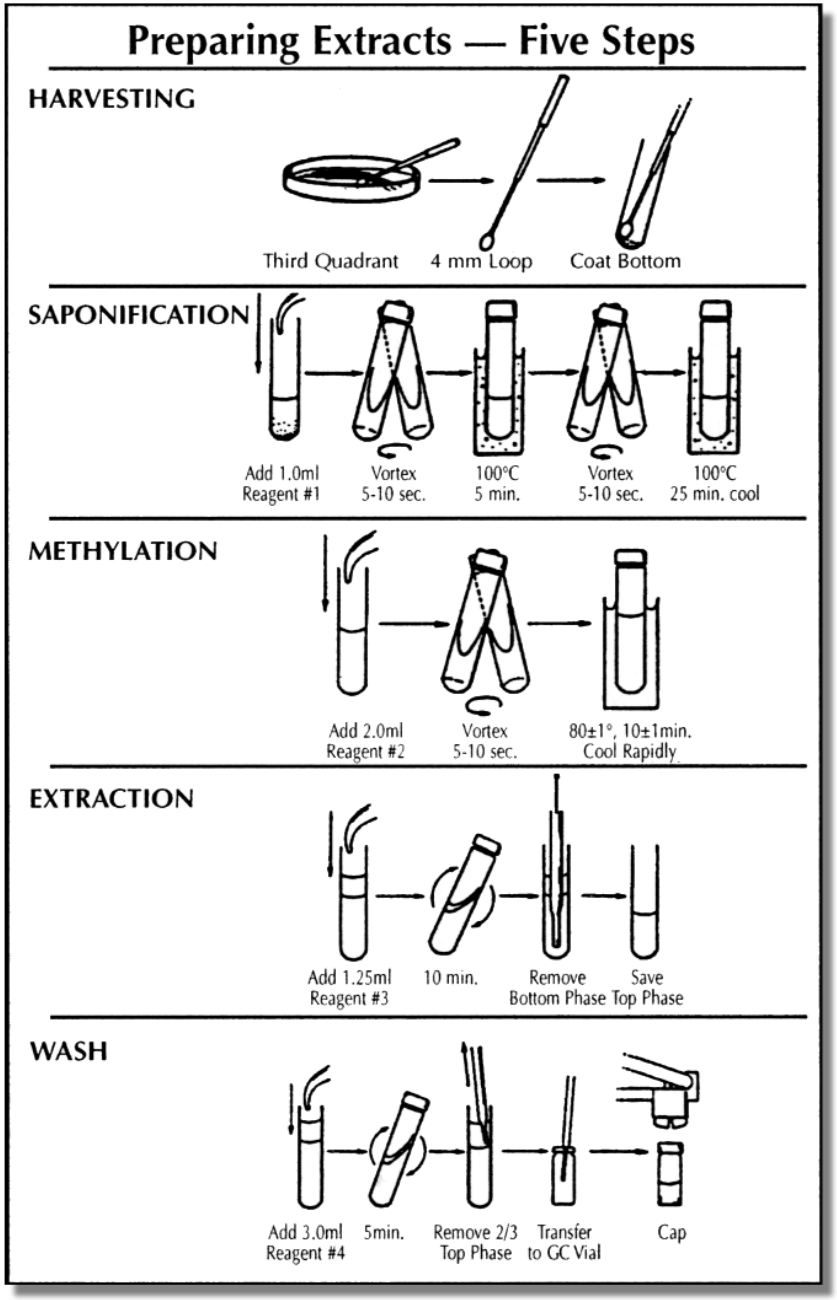
Quadrant streak

Sasser,2001

Fatty acids analysis in details

Preparing extracts in five steps

1. Culture cells. 24 hr on Trypticase Soy Broth Agar (TSBA).
2. Harvest cells.
3. Saponify lipids.
4. Methylate Fatty Acids (FAMEs).
5. Extract and purify.
6. GC analysis.
7. Comparison to library.



Sasser,2001

Reagents

Four reagents are required to cleave the fatty acids from lipids

- **Reagent 1, Saponification**:
- 45 g NaOH, 150 ml methanol, and 150 ml distilled water.
- Dispensing through use of an autopipet assures reproducibility and allows for large numbers of assays in a day.
- **Reagent 2, Methylation**:
- 325 ml certified 6.0 N HCl and 275 ml methyl alcohol(methanol).
- This drops the pH of the solution below 1.5 and causes methylation (for the increased volatility in a partially polar column) of the fatty acid.
- The fatty acid methyl ester(FAME) is poorly soluble in the aqueous phase at this point.

Reagents

Four reagents are required to cleave the fatty acids from lipids

- **Reagent 3, Extraction**:
- 200 ml hexane and 200 ml methyl tert (tertiary) butyl ether.
- This will extract the fatty acid methyl esters into the organic phase(top phase) for use with the gas chromatograph.
- **Reagent 4, Sample Cleanup(base wash):**
- An additional step based on solid-phase extraction was necessary to produce clean samples.
- This procedure reduces contamination of the injection port liner, the column, and the detector(see next slide).
- 10.8 g sodium hydroxide dissolved in 900 ml distilled water.
- The organic phase (top phase) is then washed with sodium hydroxide, removed, transferred to GC vial, capped.
- This solution is ready for injection into gas chromatograph.

Reagents

Why need for sample cleanup (base wash)?

- The base wash removes residual acid and reagent which would be damaging to the column.
- Tailing of hydroxy acid peaks is evident after light use of fused silica capillary column.
- The tailing is assumed to be a result of residual acid and reagent being injected onto the column.
- Degradation of hydroxy acid peaks after several injections on a fused silica capillary column need to proceed with base wash before injection.
- A simple base wash of the extracts before injection prevented the tailing of the hydroxy acid peaks even after extended use.
- The fused silica capillary column may be used extensively for bacterial FAME analysis without hydroxy acid peak tailing.
- More than 10,000 analyses can be performed on a column prior to needing any maintenance.

Miller,1982;…

MIDI system

Sample processing

The five steps to prepare GC ready extracts

- **Harvesting** - A 4 mm loop is used to harvest about 40 mg of bacterial cells from the third quadrant (second or first quadrant if slow growing) of the quadrant streaked plate. The cells are placed in a clean 13x100 culture tube.
- **Saponification** - 1.0 ml of Reagent 1 is added to each tube containing cells. The tubes are securely sealed with teflon lined caps, vortexed briefly and heated in a boiling water bath for ca. 5 minutes, at which time the tubes are vigorously vortexed for 5-10 seconds and returned to the water bath to complete the 30 minute heating.
- **Methylation** - The cooled tubes are uncapped, 2 ml of Reagent 2 is added. The tubes are capped and briefly vortexed. After vortexing, the tubes are heated for 10 ± 1 minutes at 80° ± 1°C. (This step is critical in time and temperature).
- **Extraction** - Addition of 1.25 ml of Reagent 3 to the cooled tubes is followed by recapping and gentle tumbling on a clinical rotator for about 10 minutes. The tubes are uncapped and the aqueous (lower) phase is pipetted out and discarded.
- **Base Wash** - About 3 ml of Reagent 4 is added to the organic phase remaining in the tubes, the tubes are recapped, and tumbled for 5 minutes.
- Following uncapping, about 2/3 of the organic phase is pipetted into a GC vial which is capped and ready for analysis.

Fatty acids analysis

Protocol for *P. tolaasii*-like strain and *P. tolaasii*

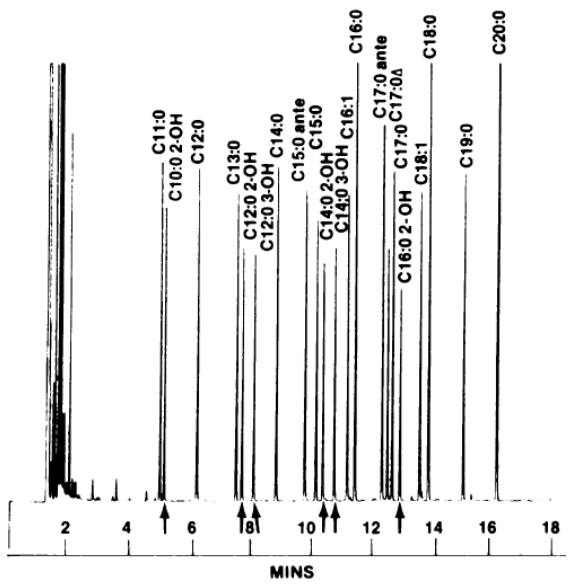
- Cultures of strain *P. tolaasi*-like PS 3aT and *P. tolaasi* LMG 2342T grown on trypticase soy agar for 24 h at 25°C were collected from four plates, washed three times with sodium phosphate buffer (10 mM, pH 7.0) and freeze dried.
- Whole-cell fatty acids were analysed as fatty acid methyl esters, after transmethylation had been done as described by Nikkilä *et al.*,1996.
- GC analyses were carried out using a Hewlett Packard (HP) series 6890 gas chromatograph, equipped with a HP-5MS (30 m x 0.25 mm x 0.25 µm) column, and a mass-selective detector (model HP 5973).
- Data analyses were realized with the HP chemstation software.
- The fatty acid methyl esters were identified by their mass (Wiley mass spectra library database) and by their retention times.
- Lactic acid, dihydrosterulic acid, 9,10- methylenehexadecanoic acid and hydroxy fatty acid methyl ester standards were obtained from Larodan Fine Chemicals.

Munsch *et al.*,2002

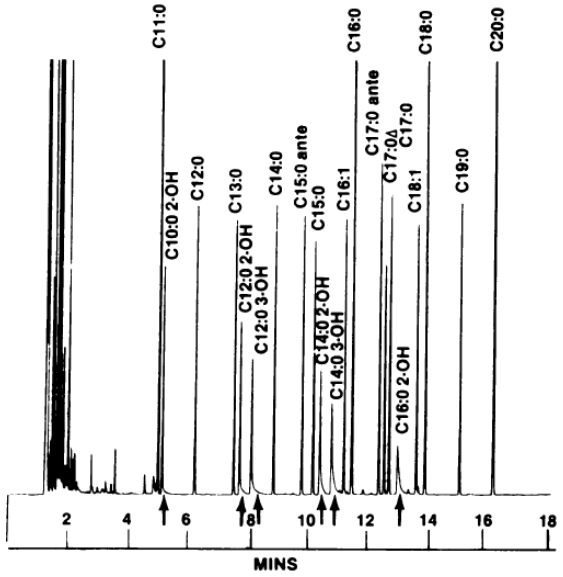
Fatty acids analysis

MIDI system print out

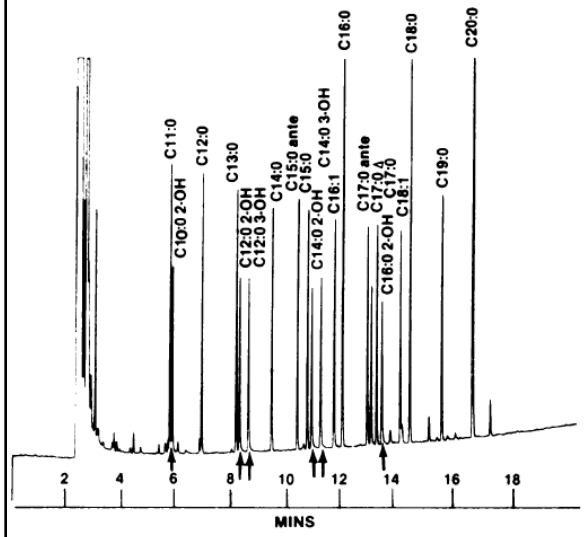
GC traces



New column
Arrows indicate hydroxy acid FAME peaks



Column with 10 injections without base wash
Hydroxy acid FAME peaks (indicated by arrows) are tailed



Column with 500 injections with base wash
Hydroxy FAME peaks show only very slight tailing

Miller,1982;…

The Column

Ultra 2 capillary column

Fused silica capillary column

- A bacterial FAME standard (Supelco, Bellefonte, Pa.) containing representative compounds of the functional groups common in bacteria was injected onto a fused silica capillary column (25 m by 0.2 mm) coated with SE-54 (Hewlett- Packard, Avondale, Pa.).

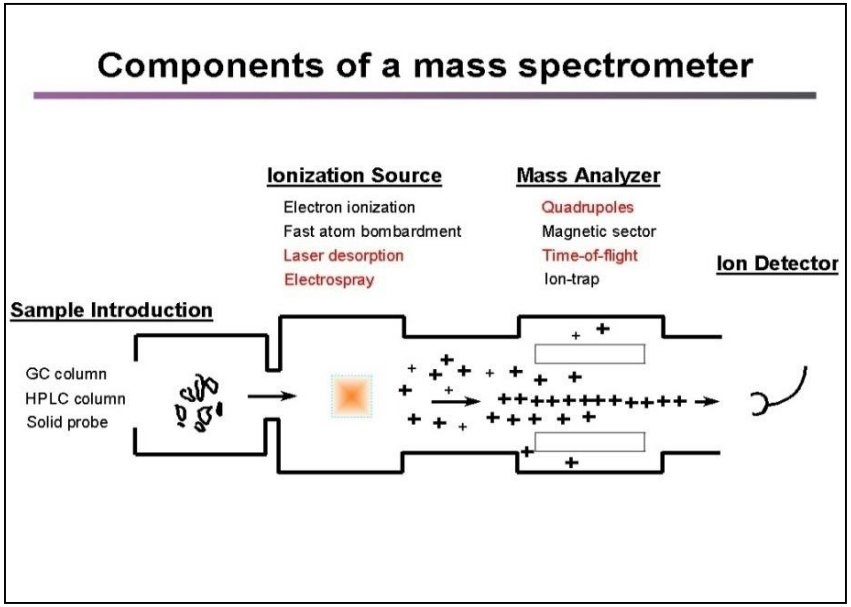
Miller,1982;…

The Column

Ultra 2 capillary column

Fused silica capillary column

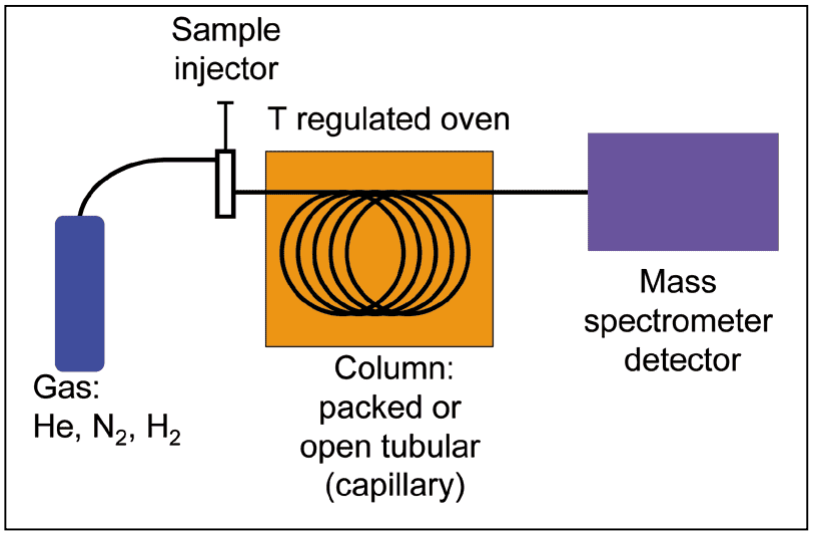
- A 25 m x 0.2 mm phenyl methyl silicone fused silica capillary column was used for routine analysis of bacterial extracts.
- 1 or 2-µl injection of bacterial FAME extracts was injected into GC-MS.



Sasser,2006

Gas chromatography
1. With mass spectrometer detector

- The GC-MS is composed of two major building blocks:
1. The gas chromatography, and
2. The mass spectrometer.
- The molecules take different amounts of time (called the retention time) to come out of (elute from) the gas chromatograph, and this allows the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately.
- The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their mass to charge ratio.



See also protein analysis section for mass spectrometry.

Wikipedia,2011

Gas chromatography
2. With flame ionization detector

- The organic layers were removed and injected into a gas chromatographic system equipped with:

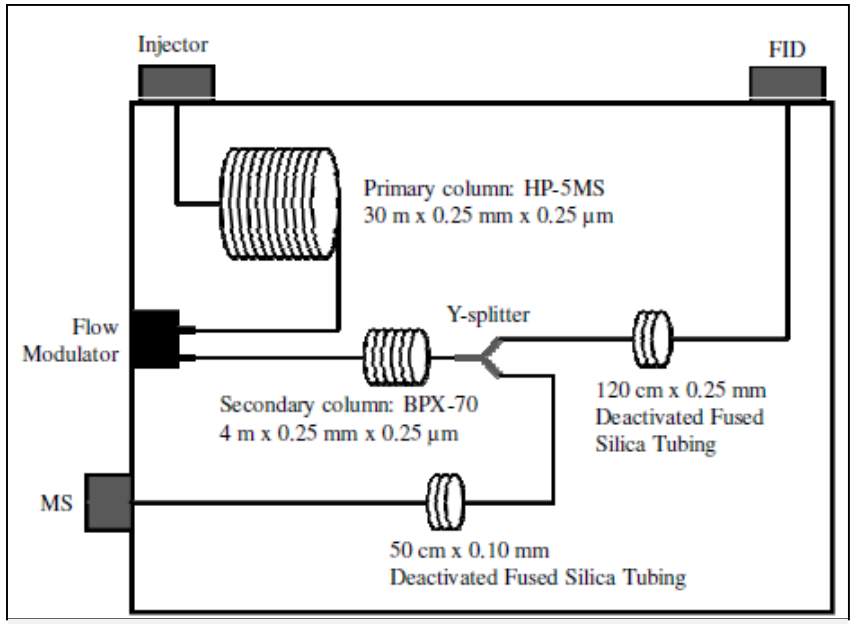
1. A 5% phenylmethyl silicone capillary column (Hewlett Packard Co., Ultra 2, 25 m X 0.2 mm X 0.33 pm),
2. A split/splitless capillary inlet system,
3. A flame ionization detector(FID),
4. A model 6890 injector with automatic sampler, and
5. A model Vectra XU 5/90 C computer with HP 3365 Series I1 ChemStation (version 3.34).

Sasser,2006

Gas chromatography
3. With FID and MS detectors

- The flame ionization detector(FID) allows for a large dynamic range and provides good sensitivity.
- Analysis of bacterial fatty acids by flow modulated comprehensive two dimensional gas Chromatography with parallel:

1. Flame ionization detector(FID), and
2. Mass spectrometry(MS).



The parallel FID/MS set-up is useful since the MS allows identification and confirmation, while the FID allows comparison of the relative fatty acid composition with existing databases.

Gu *et al.*,2010

Hardware factors

- **Gas chromatograph**:
- The temperature program ramps from 170°C to 270°C at 5°C per minute.
- Following the analysis, a ballistic increase to 300°C allows cleaning of the column during a hold of 2 minutes.
- Hydrogen is the carrier gas, nitrogen is the "makeup" gas, and air is used to support the flame.

Sasser,2001

Hardware factors
Computer and library

- The electronic signal from the GC detector is passed to the computer where the integration of peaks is performed.
- The electronic data is stored on the hard disk and the fatty acid methyl ester composition of the sample is compared to a stored database using the Sherlock pattern recognition software (Sherlock (MIDI) software).
- Fatty acid profiles were compared with libraries available commercially (MIDI) or self-generated usingMIDI library generation software.

Sasser,2001;Stead *et al.*,1998

MIDI system
Sherlock Microbial Identification System

- MIDI manufactures three microbial identification solutions:

1. The Sherlock® Microbial Identification System(MIS);
2. Sherlock® Mycobacteria Identification System;
3. Sherlock® DNA Sequencing Software.
4. MIDI Sherlock Microbial Identification System is used worldwide in clinical and environmental laboratories to identify over 1,500 aerobic and anaerobic bacterial species.

MIDI Sherlock® Microbial Identification System

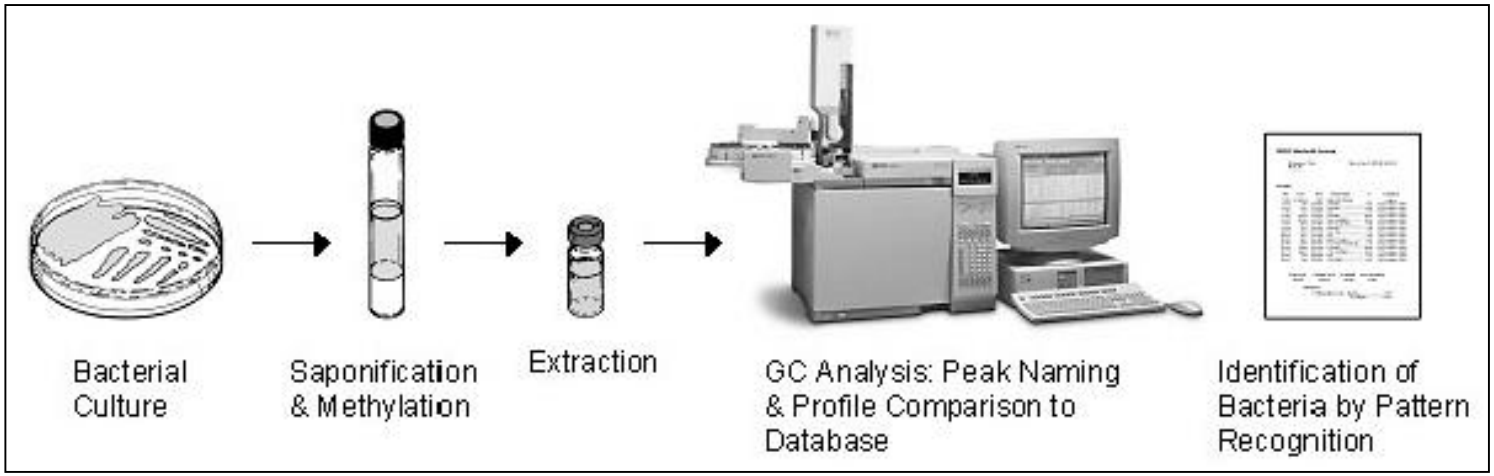
Gas chromatographic analysis of fatty acid methyl esters (GC-FAME)

MIDI System with Agilent 6850 GC

MIDI system

Sherlock Microbial Identification System Workflow

- There are also several companies hat will provide a fatty acid profile.
- Sample preparation for fatty acid analysis takes several hours, and each GC run generally takes around 20 to 30 min.



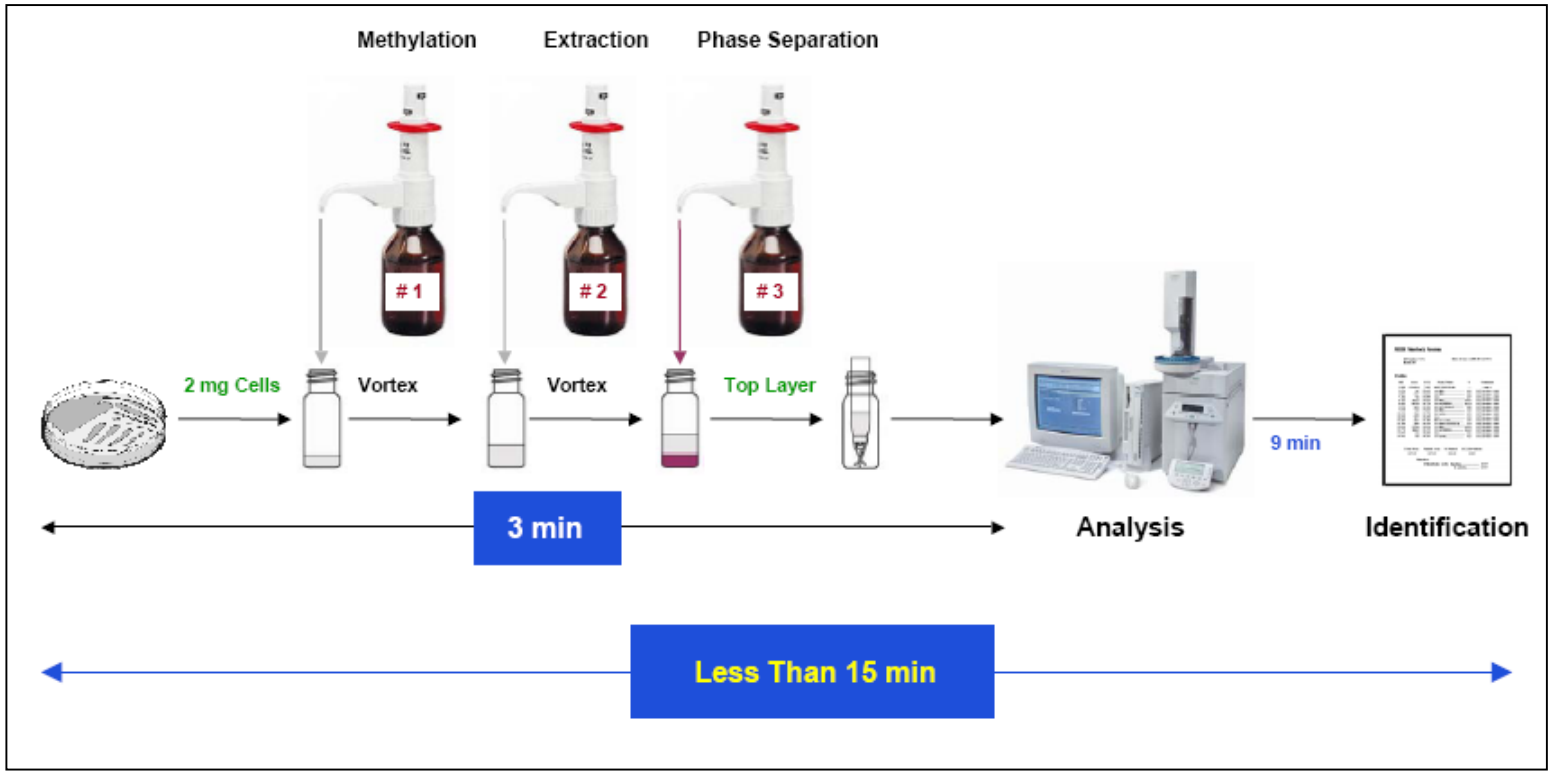
The system can analyze over 200 samples per day.

Inexpensive Consumables- under $5.00 USD per test.

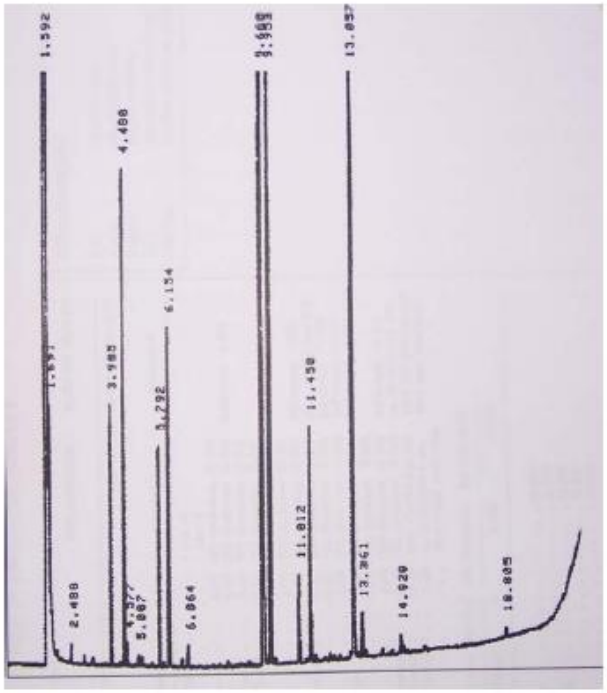
Sasser,2001; Fox,2006

MIDI system

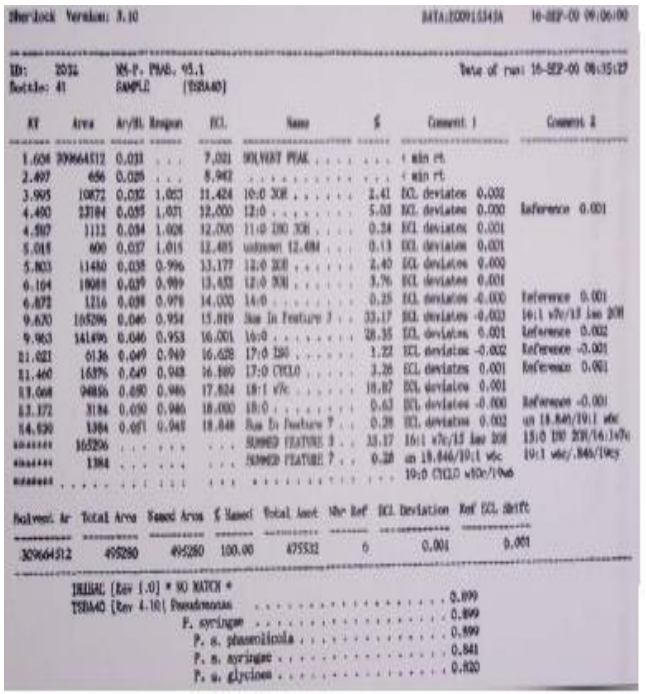
Sherlock Microbial Identification System Workflow



The Microbial Identification System
MIDI system print outs



GC trace



Library analysis

Julian Smith

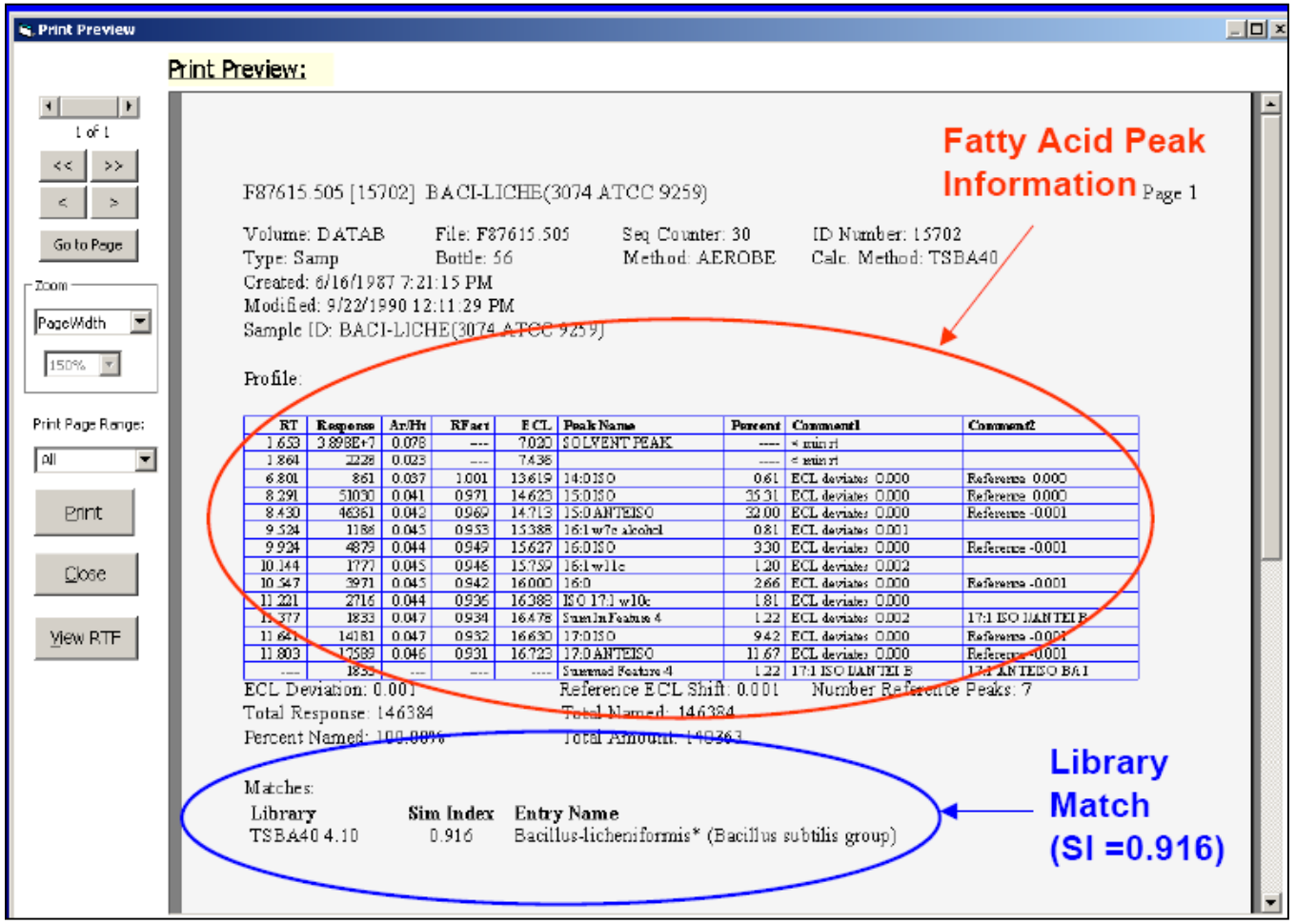
The Microbial Identification System
MIDI Sherlock standard libraries

- A library of profiles from known strains are part of the software which enable a comparison with unknown bacteria.
- The Sherlock MIS Software can only be used with the Agilent technologies 5890, 6890 or 6850 gas chromatographs.
- The Sherlock System's unique configuration is designed for optimal analysis of Fatty Acid Methyl Esters by gas chromatography.

Package	Library	Library Description
AEROBE	TSBA6	Aerobes, 28°C, 24hr, on Trypti-case Soy Broth Agar
	RTSBA6	
	CLIN6	Clinical Aerobes, 35°C, 24hr, on Blood Agar, Chocolate, etc.
	RCLIN6	
	BTR3	Bioterrorism Clinical Aerobes, 35°C, 24hr, on Blood Agar, Chocolate, etc.
	RBTR3	
	M17H10	Mycobacteria, 35°C, 5-10% CO_2, on Middlebrook 7H10 Agar with OADC enrichment
ANAEROBE	BHIBLA	Anaerobes, 35°C, 48hr, on BHIBLA plates in Gas Packs
	MOORE6	VPI Broth-grown Anaerobe Library, 35°C, in PYG Broth
YEAST	YST28	Yeasts, 28°C, 24hr, SAB Dextrose Agar
	YSTCLN	Yeasts, 28°C, 24hr, SAB Dextrose Agar
	FUNGI	Fungi, 28°C, 2-5 days, in SAB Dextrose Broth, 150 RPM shake culture
	ACTIN1	Actinomycetes, 28°C, 3-10 days, in Trypticase Soy Broth, 150 RPM shake culture

Composition Report

Sherlock sample profile



Sherlock Similarity Index (SI)

Identification of bacteria by computer-assisted fatty acid profiling

- The results for each isolate were computed as a similarity index.
- SI is a "statistical" measure of closeness of an unknown sample to each library entry
- SI is between 0.000 and 1.000 (perfect match).
- Similarity indices from 0.5 to 0.9 were considered reliable for identifying individual species.

Type: Samp Bottle: 20 Method: TSBA60
Created: 2/8/06 4:07:34 PM
Created By: rredding

Sample ID: UN-Company X-5E(1728-010692B)

Profile:

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.679	3.453E+8	0.027	—	7.012	SOLVENT PEAK	—	< min rt	
1.785	—	—	—	7.225		—	< min rt	
6.758	771	0.034	1.004	13.619	ISO	1.17	ECL deviates 0.000	Reference 0.009
8.233	12955	0.041	0.973	14.823	15:0 ISO	19.11	ECL deviates 0.000	Reference 0.009
8.370	28869	0.040	0.971	14.713	15:0 ANTEISO	42.48	ECL deviates 0.000	Reference 0.008
9.451	663	0.039	0.954	15.387	16:1 w7c alcohol	0.96	ECL deviates 0.000	
9.850	2118	0.041	0.949	15.627	16:0 ISO	3.05	ECL deviates 0.000	Reference 0.008
10.067	1936	0.040	0.946	15.758	16:1 w11c	2.78	ECL deviates 0.001	
10.465	1913	0.040	0.941	15.999	16:0	2.73	ECL deviates -0.001	Reference 0.007
11.140	2839	0.046	0.934	16.390	ISO 17:1 w10c	4.02	ECL deviates 0.002	
11.294	1631	0.042	0.933	16.479	Sum In Feature 4	2.31	ECL deviates 0.003	17:1 ISO I/ANTEI B
11.555	5794	0.046	0.930	16.631	17:0 ISO	8.17	ECL deviates 0.001	Reference 0.008
11.714	9398	0.043	0.929	16.723	17:0 ANTEISO	13.23	ECL deviates 0.000	Reference 0.007
—	1631	—	—	—	Summed Feature 4	2.31	17:1 ISO I/ANTEI B	17:1 ANTEISO B/i I

ECL Deviation: 0.001 Reference ECL Shift: 0.008 Number Reference Peaks: 7
Total Response: 68887 Total Named: 68887
Percent Named: 100.00% Total Amount: 65965

Matches:

Library	Sim Index	Entry Name
TSBA60 6.00	0.785	Bacillus-subtilis

MIDI system vs. splitless method
Different requirements

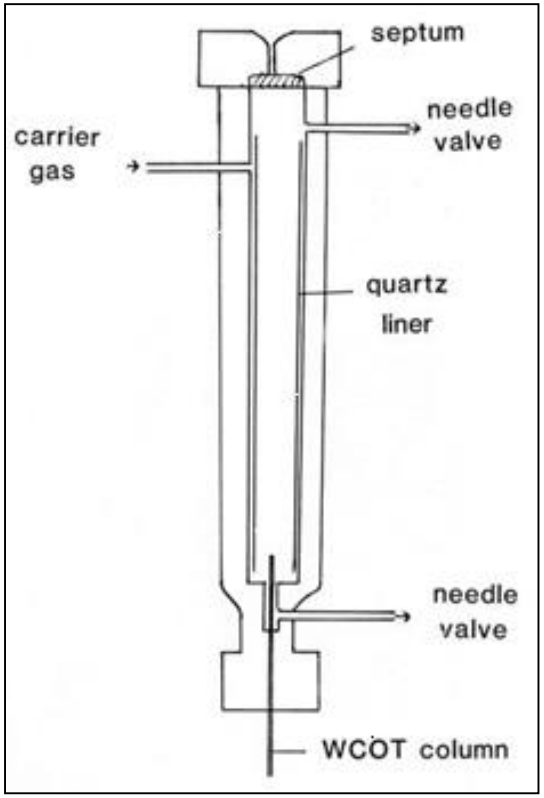
- **MIDI introduced methods (RTSB50, RCLN50, RBTR50) require:**

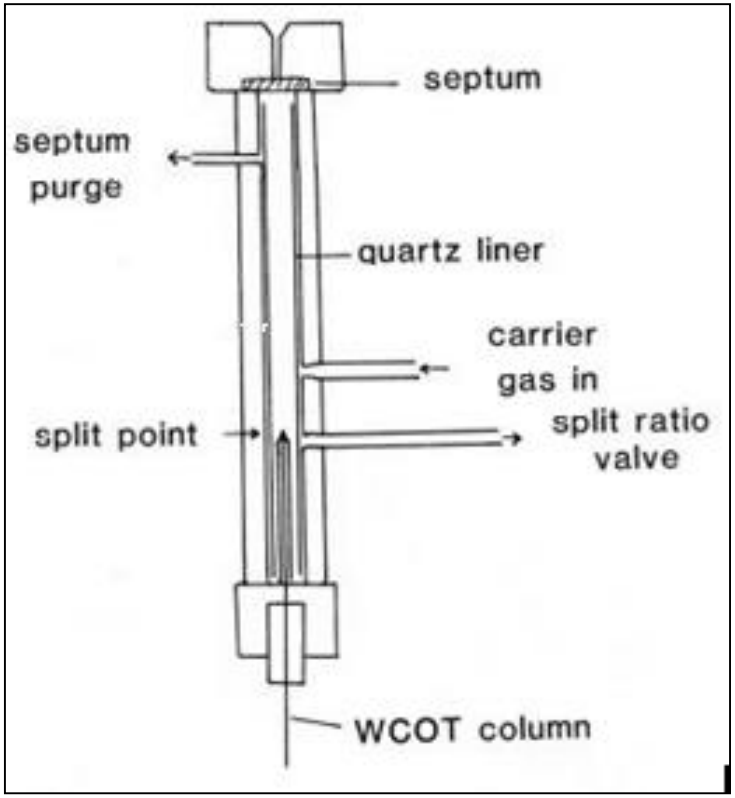
1. 40 mg of biomass (wet weight);
2. Had chromatographic run times of 5.83 min;
3. With final sample volume (1.25 ml), and
4. The chromatographic split ratio (40:1) i.e. 40 parts are injected and 1 part goes on column.

- **Splitless method (FAST6)requires:**
- A cold trap is used at the head of the chromatography column.

1. Sample size was reduced from 20 mg to 1-2 mg;
2. Total chromatographic run time was 5.93 min;
3. Sensitivity was increased four-fold by reducing the split ratio from 40:1 to 10:1. i.e. 10 parts are injected and 1 part goes on column.
4. Final volume of the sample was reduced from 1.25 ml to 300 µl.

Split vs. splitless injections

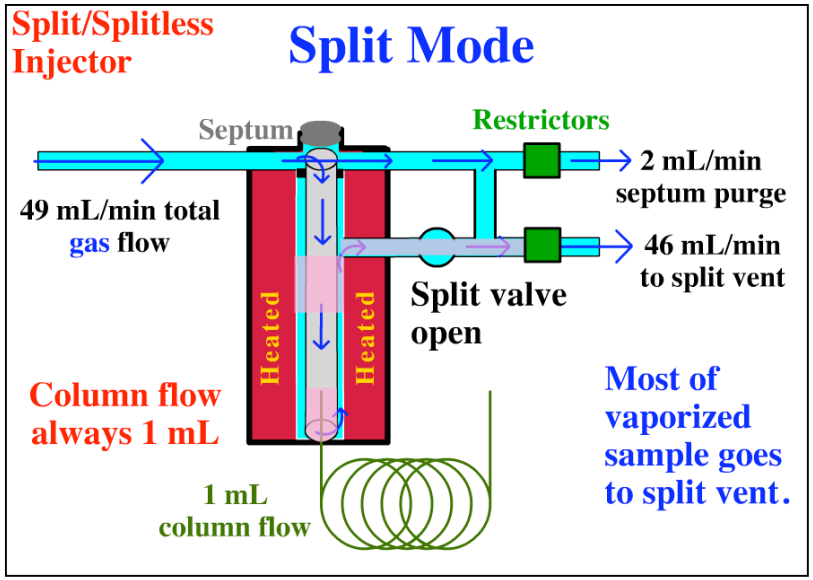


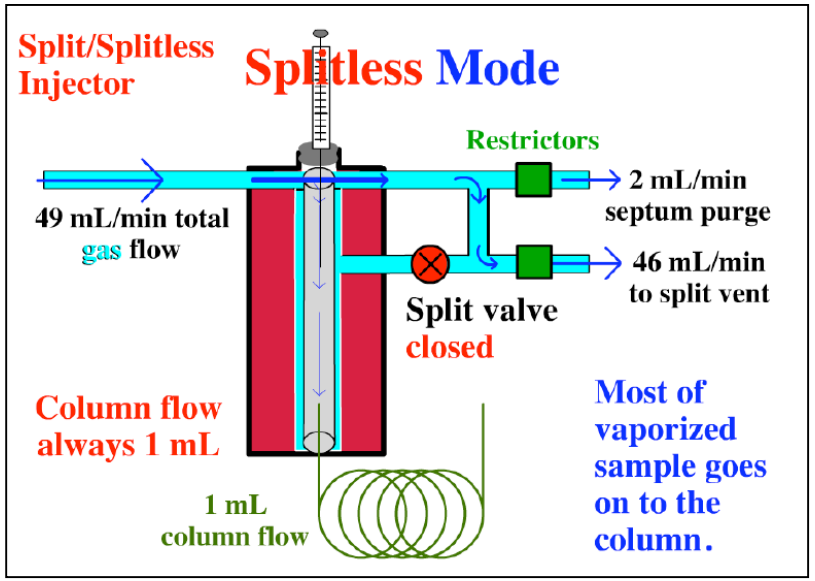


In splitless injection almost all the analyte mass in a 1 µL injection goes on column, and is not split.

Christie,2011

Split vs. splitless injections





Splitless injection is the most sensitive method because all the analyte mass in a 1 µL injection goes on column, and is not split.

Chasteen,2000

MIDI system vs. splitless method

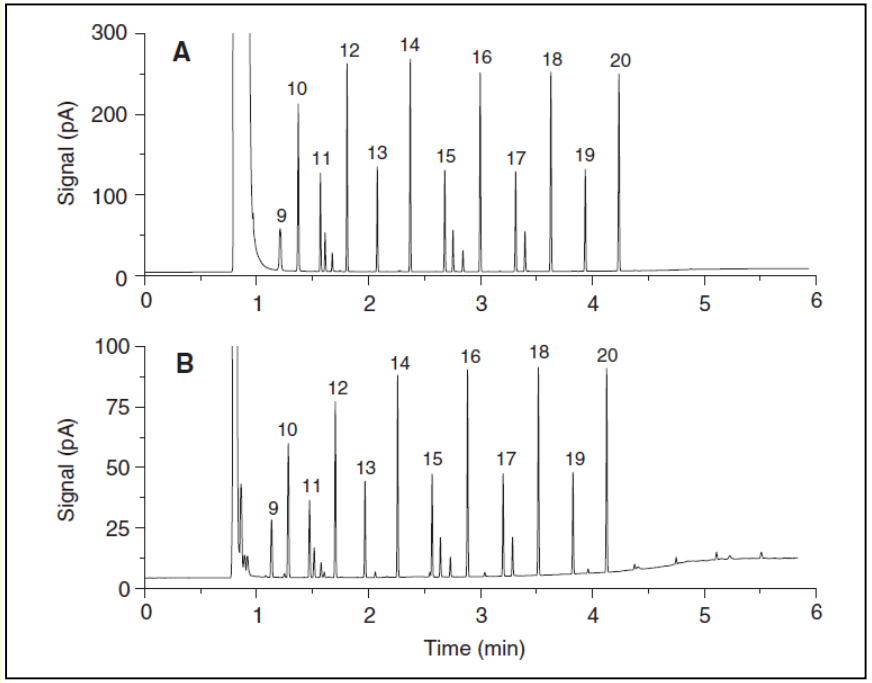
Gas chromatographic conditions for RTSB50 and FAST6 methods

Item	RTSB50 method	FAST6 method
Column	Ultra 2, 25 m long × 0.2 mm ID × 0.33 µm film thickness	Ultra 2, 25 m long × 0.2 mm ID × 0.33 µm film thickness
Carrier gas	Hydrogen, 1.3 ml/min, constant flow	Hydrogen, 1.3 ml/min, constant flow
Oven program (°C)	170 to 288 at 28/min 288 to 310 at 40/min Hold 1.25 min	170 hold 0.1 min 170 to 288 at 28/min 288 to 310 at 40/min Hold 1.25 min
Split ratio	40:1	10:1
Injection volume	2 µl	2 µl
Valve 5 (AirSharp)	None	Initially on Off 0.1 min On 5.8 min
Run time	5.83 min	5.93 min

Buyer, 2006

MIDI system vs. splitless method
Requirements

- Chromatograms of the MIDI calibration mix run by the
  A. FAST6 method and
  B. RTSB50 method.
- In (A), MIS Calibration Mix 1 is diluted 1:10.
- While in (B) MIS Rapid Calibration Mix is used without dilution.
- Straight-chain fatty acids are numbered, while hydroxy fatty acids present in the calibration mix are not labeled.



Buyer,2006

Fatty acids analysis

Accuracy of identification of plant-associated bacteria at different levels based on fatty acid profiling

- Accuracy of identification was generally 100% at genus and species levels.
- In those cases where it was less than 100%, it is likely that some strains had been wrongly classified on accession or were from taxa with as yet unresolved taxonomic problems such as in the *Pantoea-Erwinia* complex.
- At subspecies and biovar levels, accuracy of identification varied from 84 to 100% and 87 to 100%, respectively.
- At pathovar level, accuracy of identification varied widely, but was generally higher for *Xanthomonas campestris* pathovars than for *Pseudomonas syringae* pathovars.

Stead *et al.*,1998

Taxon	Rank	% strains listed as 1st choice	No. strains tested
Agrobacterium	Genus	100	20
Agrobacterium biovar 1	Biovar	87	15
Burkholderia	Genus	100	50
B. cepacia	Species	100	14
Ralstonia	Genus	99	155
R. solanacearum	Species	98	150
Acidovorax	Genus	100	48
A. avenae subsp. *avenae*	Subspecies	98	35
Comamonas	Genus	100	10
C. testosteroni	Species	100	5
Erwinia	Genus	99	307
E. amylovora	Species	97	76
E. herbicola	Species	82	17
E. carotovora subsp. *carotovora*	Subspecies	98	57
E. chrysanthemi	Species	100	21
E. chrysanthemi bv. 3	Biovar	100	7
Pantoea	Genus	100	43
P. agglomerans	Species	100	6
Pseudomonas	Genus	100	185
P. syringae	Species	100	175
P. syringae pv. *syringae*	Pathovar	12	16
P. syringae pv. *berberidis*	Pathovar	100	8
P. aeruginosa	Species	100	11
Stenatrophomonas maltophilia	Species	100	12
Xanthomonas	Genus	100	48
X. campestris pv. *campestris*	Pathovar	97	33
X. hyacinthi	Species	100	15
Bacillus	Genus	100	21
B. pumilus	Species	100	6
Clavibacter michiganensis	Species	100	41
C. michiganensis subsp. *michiganensis*	Subspecies	84	31
Curtobacterium	Genus	95	48
C. flaccumfaciens	Species	95	38
C. flaccumfaciens pv. *flaccumfaciens*	Pathovar	90	10
Rhodococcus fascians	Species	100	24

Some examples for fatty acid analyses
Some Gram negative/positive bacteria

Fatty Acid	Genus									
	1	2	3	4	5	6	7	8	9	10
14:0	+	+	+	(+)	(+)	+	(+)	+	+	+
15:0	(+)	+	(+)	(+)	-	+	-	(+)	-	-
14:0 ISO	+	+	+	(+)	-	-	+	-	-	-
15:0 ISO	+	+	+	+	(+)	-	+	-	-	-
15:1 ANTEISO A	-	-	-	-	+	-	-	-	-	-
15:0 ANTEISO	+	+	+	+	+	-	+	-	-	-
17:0 ISO	+	+	+	(+)	(+)	-	+	-	-	-
17:0 ANTEISO	+	+	+	+	+	-	+	-	-	-
17:1 ω9c	-	(+)	+	-	-	-	-	-	-	-
16:1 ω7c	+	(+)	(+)	(+)	-	-	+	-	+	+
18:1 ω9c	+	(+)	-	-	-	-	+	-	+	-
Hydroxy acids	(+)	-	-	-	-	-	-	(+)	-	(+)
10 methyl 18:0	-	-	-	-	-	+	-	-	-	-

-: normally absent in strains from most species of the genus; +: present in most strains within species of the genus; (+): present in most strains within some species of the genus. 1, *Bacillus*; 2, *Arthrobacter*; 3, *Micrococcus*; 4, *Curtobacterium*; 5, *Clavibacter*; 6, *Rhodococcus*; 7, *Staphylococcus*; 8, *Streptococcus*; 9, *Enterococcus*; 10, *Lactobacillus*.

Stead *et al.*,1998

Key fatty acids in genera of Gram-negative bacteria

Mostly plant pathogenic bacteria

Fatty Acid	Genus																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
10:0 3OH	+	+	+	-	-	-	(+)	-	-	-	-	-	-	+	-	+	-	-	-
12:0 2OH	-	-	(+)	-	-	-	(+)	-	-	-	-	-	(+)	+	-	+	-	-	-
12:0 3OH	-	-	+	+	-	-	(+)	-	-	-	-	-	+	-	-	+	-	-	-
14:0 3OH	-	-	(+)	-	+	+	+	-	-	(+)	(+)	+	(+)	-	+	-	+	+	+
14:0 2OH	-	-	(+)	-	(+)	(+)	(+)	(+)	+	-	+	-	-	-	-	-	-	-	-
16:0 2OH	-	-	-	-	-	-	-	(+)	-	-	+	+	-	-	-	-	-	+	-
16:0 3OH	-	(+)	(+)	-	-	-	-	+	+	+	+	+	-	-	(+)	-	+	(+)	+
16:1 2OH	-	(+)	-	-	-	-	-	(+)	-	(+)	-	-	-	-	-	-	-	(+)	-
18:1 2OH	-	-	-	-	-	-	-	-	-	-	-	+	-	-	(+)	-	-	+	+
11:0 ISO 3OH	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13:0 ISO 3OH	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15:0 ISO 3OH	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
17:0 ISO 3OH	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
12:0	+	+	+	-	+	(+)	+	-	-	-	-	-	+	+	-	+	-	-	-
14:0	+	+	(+)	+	+	+	+	(+)	+	+	+	(+)	(+)	-	-	(+)	-	+	-
15:0	(+)	+	(+)	+	(+)	(+)	(+)	(+)	-	+	-	-	(+)	-	-	-	-	-	-
16:0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17:0 CYCLO	-	(+)	(+)	(+)	+	(+)	+	-	-	-	-	-	-	+	-	-	-	+	-
19:0 CYCLO	-	-	(+)	-	(+)	(+)	+	-	-	-	+	-	-	-	(+)	-	+	+	+
15:0 ISO	-	-	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
15:0 ANTEISO	-	-	-	+	-	-	-	(+)	(+)	+	-	-	-	-	-	-	-	-	-
18:1 ω7c	+	+	+	(+)	+	+	+	-	-	-	+	+	(+)	+	+	+	+	+	+
18:1 ω9c	-	-	-	(+)	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
20:3 ω6,9,12c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(+)	-	-	-	-

1, *Acidovorax*; 2, *Comamonas*; 3, *Pseudomonas*; 4, *Xanthomonas;* 5, *Enterobacter*; 6, *Erwinia*; 7, *Serratia*; 8, *Flavobacterium*; 9, *Sphingobacterium*; 10, *Cytophaga*; 11, *Acetobacter*; 12, *Gluconobacter*; 13, *Acinetobacter*; 14, *Janthinobacterium*; 15,*Methylobacterium*; 16, *Flavimonas*; 17, *Agrobacterium*; 18, *Burkholderia*; 19, *Phyllobacterium*.

Stead *et al.*,1998

Some examples for fatty acid analyses
Gram negative plant pathogenic bacteria

Table 1 **Prediction of Gram-negative genera of plant pathogenic bacteria and other plant associated bacteria based on hydroxy fatty acids**

	8:0 3OH	10:0 3OH	12:0 2OH	12:03OH	14:0 2OH	14:0 3OH	16:0 2OH	16:0 3OH	16:1 2OH	18:1 2OH	11:0 ISO 3OH	13:0 ISO 3OH	15:0 ISO 3OH	17:0 ISO 3OH
Acetobacter					+	+	+	+						
Acidovorax		+												
Acinetobacter			+	+		(+)								
Agrobacterium						+		+		(+)			(+)	
Burkholderia		(+)				+	+	+	+	+				
Cytophaga								(+)	(+)				+	+
Enterobacter					(+)	+								
Erwinia					(+)	+								
Flavobacterium								+					+	+
Gluconobacter							+	+		+				
Herbaspirillum		+	+	+	+				+					
Hydrogenophaga	(+)	(+)												
Pseudomonas		+	+	+		(+)		(+)						
Ralstonia						+	+		+	+				
Serratia		(+)	+		(+)	+								
Sphingobacterium					+			+					+	+
Sphingomonas					+									
Stenatrophomonas				+							+	+		
Xanthomonas		(+)		+							+	+		
Xylophilus	(+)													

+ = thought to occur in all strains
(+) = occurs in some taxa/strains

Schaad *et al.*,2001

Characterization of Proteobacteria genera by presence/absence of specific hydroxy acids

Key acids from some genera

- Most Gram-negative bacteria have unique series of hydroxy acids.

FATTY ACID	10:0 3OH	12::0 2OH	12:0 3OH	14:0 3OH	16:0 2OH	16:0 3OH	16:1 2OH	18:1 2OH	11:0 iso3OH	13:0 iso3OH
Alphaprot										
Agrobacterium				+		+		(+)		
Betaprot										
Acidovorax	+									
Burkholderia				+	+	+	+	+		
Ralstonia				+	+		+	+		
Gammaprot										
'Erwinia'				+						
Pseudomonas	+	+	+	(+)						
Xanthomonas			+						+	+

Stead,2008

Characterization of Proteobacteria genera by presence/absence of specific hydroxy acids

Key acids from four genera

- Most Gram-negative bacteria have unique series of hydroxy acids.

Acid	*Acidovorax*	*Ralstonia*	*Pseudomonas*	*Burkholderia*
10:0 3OH	+		+	+
12:0 2OH			+	
12:0 3OH			+	
14:0 3OH		+	+	+
16:0 2OH		+		+
16:0 3OH			+	+
16:1 2OH		+		
18:1 2OH		+		+

Julian Smith

Characterization of Proteobacteria genera by presence/absence of specific hydroxy acids

Key acids from five genera

- Since many Gram-negative bacteria have unique series of hydroxy acids, the fatty acid profiles based on hydroxy acids are very useful in identification of these bacteria.

Pathogens	10:0 3OH	12:0 2OH	16:0 2OH	16:0 3OH
Acidovorax	+	-	-	-
Burkholderia	(+)	-	+	+
Ralstonia	-	-	+	-
Herbaspirillum	+	+	-	-
Xylophilus	-	-	-	-

David Stead;…

Fatty acids analysis

Cellular-fatty-acid composition of *P. tolaasii* -like strain(PS3a^T) and *P. tolaasii* strain(LMG2342)

- *$C_{10:0\ 3\text{-}OH}$, 3-Hydroxydecanoic acid;
- $C_{12:0}$, dodecanoic acid;
- $C_{12:0\ 2\text{-}OH}$, 2-hydroxydodecanoic acid;
- $C_{14:0,}$ tetradecanoic acid;
- $C_{15:0}$, pentadecanoic acid;
- $C_{16:1}$, hexadecenoic acid;
- $C_{16:0}$, hexadecanoic acid;
- $C_{17:0\ cyc}$, methylene hexadecanoic acid;
- $C_{17:0}$, heptadecanoic acid;
- $C_{18:1}$, octadecenoic acid;
- $C_{18:0}$, octadecanoic acid;
- $C_{19:0\ cyc}$, methylene octadecanoic acid.
- †Significantly different for 2/3 replicates.
- ‡ Significantly different for 3/3 replicates.
- §□, Trace amount <0.1%).
- The ±SD values for the results are shown in parentheses.

Fatty acid*	PS 3a^T	LMG 2342^T
$C_{10:0\ 3\text{-}OH}$	0·8 (0·06)	0·7 (0·3)
$C_{12:0}$	1·6 (0·09)	1·2 (0·04)
$C_{12:0\ 2\text{-}OH}$	4·4 (0·16)	4·3 (0·03)
$C_{14:0}$	0·5 (0·04)	0·3 (0·03)
$C_{15:0}$	0·2 (0·1)	0·2 (0·1)
$C_{16:1}$	25·0 (2·2)	24·5 (5·3)
$C_{16:0}$	20·9 (0·8)	21·7 (1·7)
$C_{17:0\ cyc}$	10·8 (0·4)†	14·9 (4·7)†
$C_{17:0}$	0·4 (0·2)	0·5 (0·3)
$C_{18:1}$	31·9 (2·1)‡	26·7 (0·4)‡
$C_{18:0}$	2·3 (0·3)	1·7 (0·6)
$C_{19:0\ cyc}$	T§	2·3 (1·2)
Unknown	1·5 (0·4)	0·9 (0·2)

Munsch *et al.*,2002

GC whole cell fatty acid profiles representative of the range of bacteria antagonistic towards *Pseudomonas tolaasii* Pt 28

Pseudomonas tolaasii Pt 28 and *Pseudomonas 'reactans'* NCPPB 3149 T2

		Antagonist								
		AN18	AN70	AN101	AN117	AN138	AN195	AN207	AN208	
Fatty acid	Pt 28	Fatty acid peak area (%)								NCPPB 3149 T2
Unknown 10·928	—	—	0·94	—	—	—	—	—	—	—
C10:0	0·06	—	—	0·90	0·09	—	—	0·30	—	0·11
C10:0 30H	3·63	—	—	5·36	3·01	2·57	3·30	2·89	3·20	2·99
Unknown 1·798	—	—	—	—	—	—	—	2·45	—	—
C11:0 ISO 30H	0·20	—	—	—	—	—	—	—	—	—
C12:0	2·20	—	3·71	1·71	3·58	2·08	3·49	4·89	2·20	3·28
Unknown 12·486	—	—	—	0·28	0·10	—	—	0·36	—	0·09
C12:0 20H	5·60	—	0·29	4·92	4·02	4·33	4·18	—	4·67	4·12
C12:1 30H	—	—	—	1·24	—	—	—	0·95	4·19	—
C12:0 30H	4·62	—	—	4·09	4·19	3·89	4·32	3·85	—	3·83
C13:0 ISO 20H	0·12	—	—	0·30	—	—	—	0·25	—	0.17
C14:0	0·36	0·76	7·80	0·43	0·58	0·36	0·54	0·78	0·36	0·29
C14:0 ISO	—	1·41	—	—	—	—	—	—	—	—
C14:0 ANTE ISO	0·11	—	—	—	—	—	—	—	—	—
C14:1 ISOE	—	—	—	0·48	—	—	—	—	—	—
Unknown 14·502	—	—	1·00	—	—	—	—	—	—	—
C15:0	0·44	—	0·48	—	0·13	—	—	1·05	—	0·10
C15:0 ISO	0·11	29·61	—	0·62	—	—	—	—	—	—
C15:0 ANTEISO	0·09	30·58	—	0·47	—	—	—	—	—	—
Unknown 15·549	0·09	—	—	—	—	—	—	—	—	—
Unknown 15·665	0·11	—	—	—	—	—	—	—	—	—
C16:CIS 9	28·19	—	26·8	23·90	42·63	26·49	15·55	25·66	24·14	24·08
C16:1 TRANS 9/15; 20H	—	—	—	—	—	—	—	5·55	—	—

The fatty acid profile of *Pseudomonas 'reactans'* NCPPB 3149 T2 gave best fits with *Ps. tolaasii* (0.628) and *P. chloroaphis* (0.500). *Pseudomanas tolaasii* Pt 28 most closely fitted *Ps. tolaasii* (0.834). Fatty acid profiling of bacterial antagonists ranged C_9-C_{20}chain length.

GC whole cell fatty acid profiles representative of the range of bacteria antagonistic towards *Pseudomonas tolaasii* Pt 28

Pseudomonas tolaasii Pt 28 and *Pseudomonas 'reactans'* NCPPB 3149 T2

Continued

C16:0 ISO	—	1·51	—	—	—	—	—	—	—	—
C16:0	28·72	3·21	30·35	26·97	27·70	29·77	31·36	17·48	32·86	30·12
C16:1 A	—	1·32	—	—	—	—	—	—	—	—
C17:0 ISO	0·24	4·82	—	—	0·10	—	—	0·22	—	—
C17:1 ISO E	—	—	3·31	—	—	—	—	—	—	—
C17:1 ISO I/ANTEI B	—	0·85	—	—	—	—	—	—	—	—
C17:0 CYCLO	7·76	—	6·75	5·92	0·12	10·10	20·14	—	11·44	12·46
C17:1 B	0·36	—	—	—	—	—	—		0·93	—
C17:1 C	—	—	—	—	—	—	—	0·51	—	—
C17:0	0·52	—	0·41	—	0·10	0·18	—	0·58	—	—
C17:0 ANTEISO	—	5·53	—	—	—	—	—	—	—	—
C18:2 CIS 9, 12/18:Oa	0·27	—	—	—	—	—	—	—	—	—
C18:1 CIS 9	0·20	—	—	—	—	—	—	—	—	—
C18:1 TRANS9/ t6/c11	14·27	—	—	19·10	13·43	17·82	13·76	30·32	15·45	16·61
C18:0	0·71	8·55	—	1·23	0·23	1·54	0·74	0·97	1·13	0·72
C19:0 CYCLO 11–12	0·41	—	—	—	—	0·87	2·62	—	0·36	0·83
C19:0 ISO	—	0·67	—	—	—	—	—	—	—	—
C19:0 ANTEISO 0·83	—	—	—	—	—	—	—	—	—	—
C20:0	—	7·32	—	—	—	—	—	—	—	—
C18:1 CIS 11/t/9/t6	—	—	11·97	—	—	—	—	—	—	—
C14:0 30H/16:1 ISO I	—	—	—	—	—	—	—	9·06	—	—

Fermor and Lynch,1988

Fatty acids analysis
B. gladioli pv. *agaricicola*

- The major fatty acid components of *B. gladioli* pv. *agaricicola* strain OM1 were:
- 16:0 (18.0%),
- 18:1w7c (14.4%),
- Sum in feature 3 (13.4%, comprising 16:1 w7c/15 iso 2OH),
- Sum in feature 2 (12.5%, comprising 14:0 3OH/16:1 iso I),
- 17:0 cyclo (11.7%),
- 16:0 3OH (6.3%),
- 19:0 cyclo w8c (5.53%),
- 10:0 3OH (4.67%),
- 18:1 2OH (1.93%),
- 16:1 2OH (1.79%), and
- 16:0 2OH (0.81%).

Lee *et al.*,2010

Fatty acids analysis

More reliable, fast, accurate methods to identify bacterial strains

Xanthomonas spp.

- Norman and co-workers,1997 differentiated three species of *Xanthomonas*, *X. albilineans*, *X. fragariae* and some pathovars of X. *campestris* as well as *Stenotrophomonas maltophilia* using fatty acid analyses with 100 per cent accuracy.
- Other pathovars of *X. campestris* were more diverse and therefore FAME analysis was not suitable for them.

Some bacteria have rare acids e.g. the presence of 13:0 iso3OH is virtually exclusive to *Xanthomonas*.

Fatty acids analysis

FAME profile of *Xanthomonas* spp.

Compound	% in:																			
	X. albilineans	*X. arboricola*	*X. axonopodis*	*X. bromi*	*X. campestris*	*X. cassavae*	*X. codiaei*	*X. cucurbitae*	*X. fragariae*	*X. hortorum*	*X. hyacinthi*	*X. melonis*	*X. oryzae*	*X. pisi*	*X. populi*	*X. sacchari*	*X. theicola*	*X. translucens*	*X. vasicola*	*X. vesicatoria*
Saturated fatty acids																				
10:0	0.7 ± 0.4[a]	0.8 ± 0.2	0.8 ± 0.4	0.3 ± 0.3	0.6 ± 0.3	0.1 ± 0.1	0.7 ± 0.0	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.3	1.6 ± 0.2	0.7 ± 0.1	1.5 ± 0.8	0.7 ± 0.0	2.0 ± 0.7	0.8 ± 0.1	1.2 ± 0.2	0.6 ± 0.2	0.9 ± 0.2	0.7 ± 0.1
14:0	0.1 ± 0.1	1.4 ± 0.4	1.1 ± 1.2	1.3 ± 0.1	0.8 ± 0.4	0.2 ± 0.2	1.0 ± 0.0	2.0 ± 0.3	2.6 ± 1.2	1.4 ± 0.3	0.7 ± 0.1	0.6 ± 0.1	0.4 ± 0.4	1.1 ± 0.0	5.1 ± 0.9	1.0 ± 0.1	1.5 ± 0.1	1.8 ± 1.1	1.8 ± 0.2	0.8 ± 0.3
15:0	0.3 ± 0.3	1.3 ± 0.7	1.3 ± 0.9	2.0 ± 0.3	1.2 ± 0.6	0.1 ± 0.2	2.3 ± 0.0	1.9 ± 0.7	1.5 ± 0.5	0.7 ± 0.7		1.5 ± 0.3	0.1 ± 0.2	1.2 ± 0.0	4.6 ± 0.6	0.4 ± 0.0	1.3 ± 0.2	0.4 ± 0.5	1.6 ± 0.6	0.8 ± 0.3
16:0	13.4 ± 2.4	4.0 ± 1.1	4.1 ± 2.0	4.9 ± 0.4	3.6 ± 1.0	1.4 ± 0.4	6.3 ± 0.0	6.7 ± 1.8	3.7 ± 0.9	2.0 ± 1.1	3.8 ± 0.5	4.3 ± 0.3	18.5 ± 3.8	3.4 ± 0.0	7.4 ± 1.8	4.2 ± 0.3	8.8 ± 0.6	4.3 ± 1.6	15.8 ± 1.2	3.3 ± 0.3
17:0	0.1 ± 0.1		0.0 ± 0.1				0.4 ± 0.0					0.2 ± 0.2	0.0 ± 0.1		0.2 ± 0.3	0.1 ± 0.1	0.5 ± 0.0			
18:0	0.1 ± 0.2		0.0 ± 0.1												0.0 ± 0.1	0.0 ± 0.1	0.5 ± 0.1			
20:0	0.2 ± 0.2				0.0 ± 0.1															
Unsaturated fatty acids																				
Summed group 1[b]		0.0 ± 0.1	0.0 ± 0.1							0.3 ± 0.4			0.0 ± 0.1	0.3 ± 0.0		0.3 ± 0.0		0.1 ± 0.2		
15:1 ω8c															2.9 ± 0.7					
15:1 ω6c		0.3 ± 0.3	0.4 ± 0.5		0.6 ± 0.4	0.1 ± 0.2	0.5 ± 0.0	0.1 ± 0.2		1.3 ± 0.6		0.4 ± 0.2		1.0 ± 0.0		0.5 ± 0.0	0.4 ± 0.0	0.3 ± 0.3		0.1 ± 0.2
16:1 ω9c		0.9 ± 0.9	0.7 ± 0.8	0.7 ± 0.8	0.9 ± 0.7	0.5 ± 0.5	1.5 ± 0.0	0.1 ± 0.3		0.1 ± 0.3		1.6 ± 0.1	2.3 ± 0.8	1.0 ± 0.0	16.9 ± 1.9	1.1 ± 0.1	0.2 ± 0.3	1.0 ± 1.2	0.7 ± 1.5	0.8 ± 0.5
16:1 ω7c	13.2 ± 2.7	15.0 ± 1.5	16.2 ± 4.2	8.9 ± 0.5	12.7 ± 2.0	10.3 ± 1.9	15.1 ± 0.1	16.0 ± 1.0	11.7 ± 1.4	18.6 ± 1.9	12.1 ± 0.7	10.4 ± 0.4	24.9 ± 3.3	13.8 ± 0.0		15.0 ± 0.4	12.9 ± 0.7	21.1 ± 3.1	25.0 ± 1.9	15.6 ± 0.9
16:1 ω5c	0.4 ± 0.3							0.0 ± 0.1	0.7 ± 0.5				0.0 ± 0.1		0.5 ± 0.4			0.1 ± 0.1		
17:1 ω8c	0.5 ± 0.3	1.1 ± 0.5	1.1 ± 0.9	0.5 ± 0.5	1.4 ± 0.5	0.6 ± 0.3	2.5 ± 0.0	0.6 ± 0.2	0.6 ± 0.9	0.5 ± 0.5		2.2 ± 0.4	0.1 ± 0.2	1.5 ± 0.0	4.0 ± 0.8	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.3	1.1 ± 0.3	0.8 ± 0.3
18:1 ω9c	0.2 ± 0.6	0.2 ± 0.2	0.3 ± 0.4		0.2 ± 0.3	0.4 ± 0.3	0.8 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.1	0.1 ± 0.1	0.8 ± 0.1	1.7 ± 0.8	0.5 ± 0.0	6.5 ± 1.9	0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.4	0.7 ± 0.3	0.1 ± 0.2
Summed group 7[b]	1.9 ± 0.5	0.0 ± 0.1	0.2 ± 0.4		0.1 ± 0.2	0.2 ± 0.3	0.5 ± 0.0	0.1 ± 0.1	0.0 ± 0.1		0.4 ± 0.2		1.3 ± 3.1	0.4 ± 0.0		0.3 ± 0.0	0.5 ± 0.1	0.0 ± 0.1	0.2 ± 0.3	
Hydroxy fatty acids																				
10:0 3OH		0.1 ± 0.2	0.2 ± 0.2		0.0 ± 0.1		0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.2	0.0 ± 0.1	0.4 ± 0.1		0.4 ± 0.3	0.3 ± 0.0	0.5 ± 0.4	0.2 ± 0.0	0.4 ± 0.1		0.1 ± 0.2	
11:0 3OH		0.1 ± 0.2	0.1 ± 0.2		0.1 ± 0.2				0.2 ± 0.2	0.0 ± 0.1		0.3 ± 0.3		0.4 ± 0.0	0.9 ± 0.5		0.4 ± 0.1		0.0 ± 0.1	
12:0 3OH	1.1 ± 0.5	3.1 ± 0.5	3.0 ± 1.1	2.0 ± 0.5	2.6 ± 0.5	1.6 ± 0.3	2.3 ± 0.0	2.5 ± 0.3	3.6 ± 0.8	2.5 ± 0.5	4.1 ± 0.4	2.5 ± 0.2	3.5 ± 2.1	3.4 ± 0.0	4.6 ± 1.5	2.2 ± 0.1	3.0 ± 0.4	1.7 ± 0.4	3.0 ± 0.4	3.0 ± 0.4
13:0 2OH		0.5 ± 0.2	0.2 ± 0.4		0.3 ± 0.3	0.1 ± 0.2		0.9 ± 0.2	0.5 ± 0.3	0.3 ± 0.4	1.3 ± 1.8	0.2 ± 0.3		0.4 ± 0.0		0.3 ± 0.1	0.6 ± 0.1	0.1 ± 0.2		0.2 ± 0.3
Branched-chain fatty acids																				
10:0 iso											0.4 ± 0.1					0.4 ± 0.0	0.4 ± 0.0			
11:0 iso	5.5 ± 2.0	4.9 ± 0.7	5.1 ± 1.5	6.1 ± 0.9	4.5 ± 0.7	3.5 ± 0.3	4.1 ± 0.1	4.1 ± 0.3	5.7 ± 0.8	4.7 ± 0.8	6.2 ± 0.9	5.2 ± 0.2	4.6 ± 1.2	4.1 ± 0.0	4.6 ± 1.3	3.9 ± 0.3	4.7 ± 0.5	5.9 ± 1.0	3.1 ± 0.4	4.2 ± 0.5
11:0 anteiso			0.0 ± 0.1					0.0 ± 0.1	0.1 ± 0.1		1.5 ± 0.1					0.2 ± 0.0	0.6 ± 0.1	0.0 ± 0.1		
12:0 iso											0.1 ± 0.2					0.5 ± 0.0	0.3 ± 0.0			
13:0 iso	0.0 ± 0.1	0.3 ± 0.3	0.2 ± 0.3		0.0 ± 0.2	0.1 ± 0.2		0.2 ± 0.2	1.5 ± 0.7	0.1 ± 0.2	0.4 ± 0.2			0.5 ± 0.0	1.3 ± 0.8	0.5 ± 0.0	0.4 ± 0.0	0.9 ± 0.7		
13:0 anteiso			0.0 ± 0.1						0.1 ± 0.2		0.3 ± 0.1							0.0 ± 0.1		
14:0 iso		0.8 ± 0.2	0.5 ± 0.9		0.7 ± 0.5		0.6 ± 0.0	1.2 ± 0.2	0.2 ± 0.2	0.7 ± 0.5	3.7 ± 1.0	0.2 ± 0.2		0.7 ± 0.0	0.0 ± 0.1	14.0 ± 0.7	9.8 ± 1.7	2.6 ± 1.8		0.9 ± 0.4
15:0 iso	2.4 ± 0.8	31.1 ± 3.4	28.2 ± 6.7	21.3 ± 1.8	26.5 ± 3.4	31.8 ± 2.6	26.0 ± 0.3	31.5 ± 1.8	36.8 ± 4.9	33.2 ± 3.5	11.1 ± 1.4	28.5 ± 2.9	6.3 ± 3.9	31.8 ± 0.0	23.4 ± 3.6	17.3 ± 0.7	12.9 ± 0.3	29.4 ± 4.0	15.5 ± 1.7	24.6 ± 0.5
15:0 anteiso	0.1 ± 0.1	13.7 ± 2.0	9.3 ± 3.5	2.6 ± 0.2	13.9 ± 2.2	9.5 ± 2.9	7.4 ± 0.0	10.2 ± 1.2	10.5 ± 1.4	15.7 ± 2.0	26.8 ± 1.5	6.6 ± 2.0	0.7 ± 0.8	9.4 ± 0.0	1.6 ± 1.8	5.6 ± 0.1	15.9 ± 0.6	5.0 ± 1.5	3.0 ± 0.6	15.5 ± 1.7
16:0 iso	4.7 ± 1.3	2.0 ± 0.7	2.6 ± 1.5	1.7 ± 0.3	3.2 ± 1.3	1.1 ± 0.5	3.6 ± 0.2	3.2 ± 0.8	0.9 ± 1.1	1.7 ± 0.6	6.6 ± 1.3	1.9 ± 0.5	0.2 ± 0.3	1.0 ± 0.0	0.0 ± 0.1	18.8 ± 0.6	9.9 ± 2.0	3.6 ± 2.8	1.3 ± 0.3	4.1 ± 0.8
17:0 iso	19.3 ± 3.3	4.5 ± 1.1	6.7 ± 2.8	10.9 ± 1.6	6.8 ± 1.4	9.5 ± 1.2	7.4 ± 0.3	3.9 ± 0.4	3.7 ± 2.0	2.7 ± 1.4	2.1 ± 0.3	10.2 ± 1.3	14.6 ± 2.9	5.3 ± 0.0	2.5 ± 1.0	2.7 ± 0.2	2.4 ± 0.1	4.8 ± 2.3	7.3 ± 1.2	6.8 ± 1.1
17:0 anteiso	2.9 ± 0.5	0.3 ± 0.3	0.4 ± 0.5	0.1 ± 0.3	0.8 ± 0.5	1.0 ± 0.4	0.5 ± 0.0	0.2 ± 0.2	0.2 ± 0.2	0.1 ± 0.3	1.1 ± 0.2	0.2 ± 0.3	0.3 ± 0.4	0.5 ± 0.0		0.2 ± 0.0	1.2 ± 0.1	0.0 ± 0.1	1.1 ± 0.3	1.1 ± 0.1
17:0 10-methyl	0.2 ± 0.2										0.4 ± 0.3									
18:0 iso	0.1 ± 0.1															0.5 ± 0.1	0.6 ± 0.1			
19:0 iso	1.6 ± 0.6																			

Vauterin *et al.*,1996

Fatty acids analysis

FAME profile of *Xanthomonas* spp.

Compound	% in:																			
	X. albilineans	*X. arboricola*	*X. axonopodis*	*X. bromi*	*X. campestris*	*X. cassavae*	*X. codiaei*	*X. cucurbitae*	*X. fragariae*	*X. hortorum*	*X. hyacinthi*	*X. melonis*	*X. oryzae*	*X. pisi*	*X. populi*	*X. sacchari*	*X. theicola*	*X. translucens*	*X. vasicola*	*X. vesicatoria*
Branched-chain unsaturated fatty acids																				
15:1 iso F		0.3 ± 0.3	0.1 ± 0.4	0.1 ± 0.2	0.4 ± 0.4	0.4 ± 0.2	0.5 ± 0.0	0.7 ± 0.3	0.3 ± 0.3	0.0 ± 0.1		0.8 ± 0.1		0.6 ± 0.0		0.5 ± 0.0	0.7 ± 0.1	1.0 ± 0.9	0.0 ± 0.1	0.1 ± 0.2
15:1 iso G			0.0 ± 0.1												1.6 ± 0.8					
16:1 iso H																0.4 ± 0.0				
iso 17:1 ω9c	23.3 ± 3.6	3.8 ± 0.8	6.5 ± 3.1	25.7 ± 1.1	8.5 ± 2.1	17.5 ± 2.0	7.5 ± 0.2	6.1 ± 1.0	3.5 ± 2.2	3.9 ± 1.4	1.7 ± 0.1	10.4 ± 1.2	9.1 ± 3.0	6.2 ± 0.0		2.2 ± 0.1	2.4 ± 0.1	7.4 ± 2.0	11.2 ± 1.6	8.3 ± 1.4
Summed group 5[b]	1.0 ± 0.6		0.0 ± 0.1			0.1 ± 0.2			0.1 ± 0.2											
Branched-chain hydroxy fatty acids																				
11:0 iso 3OH	3.1 ± 1.5	3.0 ± 0.5	2.6 ± 1.0	3.8 ± 0.8	2.8 ± 0.4	3.0 ± 0.2	2.6 ± 0.1	2.2 ± 0.1	4.4 ± 0.7	2.8 ± 0.6	2.4 ± 0.5	3.2 ± 0.2	2.7 ± 0.7	2.7 ± 0.0	2.8 ± 0.9	0.9 ± 0.1	1.8 ± 0.3	3.4 ± 0.8	2.0 ± 0.3	2.3 ± 0.3
12:0 iso 3OH	0.1 ± 0.2	0.1 ± 0.2	0.1 ± 0.3		0.2 ± 0.3		0.1 ± 0.1	0.5 ± 0.2	0.1 ± 0.1		3.1 ± 0.6	0.1 ± 0.2				2.8 ± 0.2	2.0 ± 0.0	0.6 ± 0.5		0.1 ± 0.1
13:0 iso 3OH	2.4 ± 0.7	4.4 ± 0.7	5.8 ± 2.0	5.5 ± 0.5	4.7 ± 0.7	5.2 ± 0.5	3.8 ± 0.1	2.6 ± 0.4	4.3 ± 1.2	4.1 ± 0.7	2.4 ± 0.4	5.2 ± 0.4	4.6 ± 1.6	4.7 ± 0.0	4.3 ± 1.4	1.0 ± 0.1	0.9 ± 0.1	2.7 ± 0.6	2.8 ± 0.3	3.9 ± 0.4
17:0 iso 3OH	0.5 ± 0.2		0.0 ± 0.1		0.1 ± 0.2	0.1 ± 0.2	0.2 ± 0.2		0.2 ± 0.2				0.1 ± 0.2	0.3 ± 0.0				0.0 ± 0.1	0.0 ± 0.1	0.1 ± 0.2
Others																				
16:1 ω7t alcohol																0.3 ± 0.1				
16:0 alcohol	0.3 ± 0.5										1.6 ± 0.3							0.0 ± 0.1		
17:0 cyclo			0.0 ± 0.1								1.0 ± 0.3									
Unknown 11.798[c]		1.9 ± 0.3	1.7 ± 0.7	1.6 ± 0.3	1.7 ± 0.3	1.7 ± 0.2	1.7 ± 0.0	1.2 ± 0.1	0.7 ± 0.6	1.5 ± 0.3		1.7 ± 0.1	1.7 ± 0.5	2.2 ± 0.0	1.4 ± 0.8				1.6 ± 0.3	1.7 ± 0.2
Unknown 14.258[c]											2.1 ± 0.4									
Summed group 2[b]			0.0 ± 0.1			0.0 ± 0.1				0.2 ± 0.4				0.3 ± 0.0		0.2 ± 0.0				

[a] Average ± standard deviation.

[b] Group containing two or more fatty acids that could not be separated by gas-liquid chromatography when the MIDI system was used. Summed group 1 is composed of 14:1 ω5t and/or 14:1 ω5c; summed group 2 is composed of 13:0 3OH and/or 15:1 iso I and/or 15:1 iso H; summed group 5 is composed of 17:1 iso I and/or 17:1 anteiso B; and summed group 7 is composed of 18:1 ω7c and/or 18:1 ω9t and/or 18:1 ω12t.

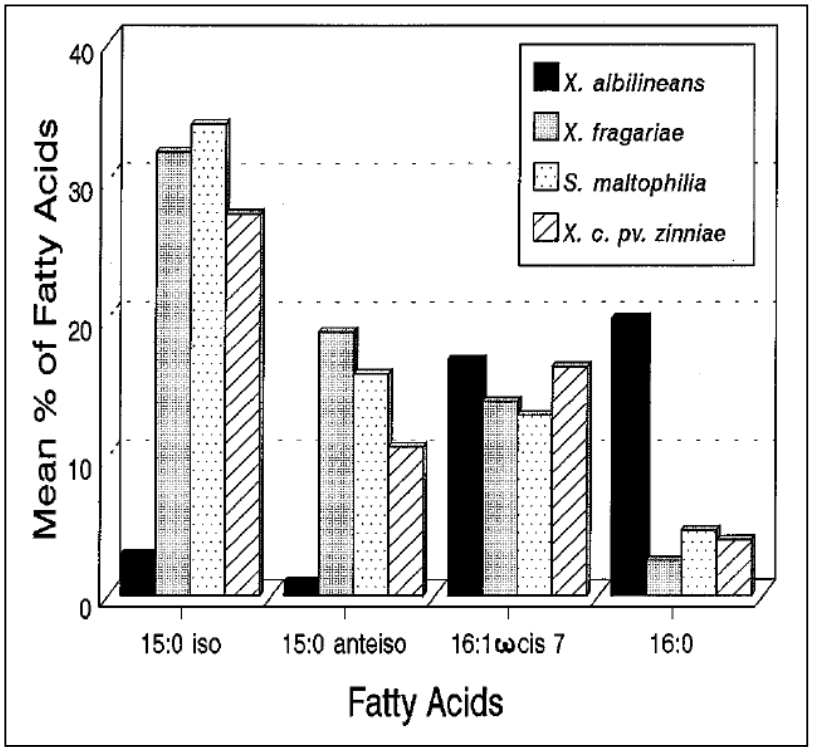
[c] Fatty acid that could not be identified by the Microbial Identification System and therefore is represented by its equivalent chain length.

Vauterin *et al.*,1996

Fatty acids analysis

FAME profile of *Xanthomonas* spp.

- Percentages of four major fatty acids in the FAME profiles of *X. albilineans, X. fragariae, S. maltophilia* and *X. c.* pv. *zinniae*.
- The four fatty acids comprise 35-60% of the total profile, and can be used to differentiate species of *Xanthomonas*.



Norman *et al.*,1997

Fatty acids analysis

Xanthomonas fragariae

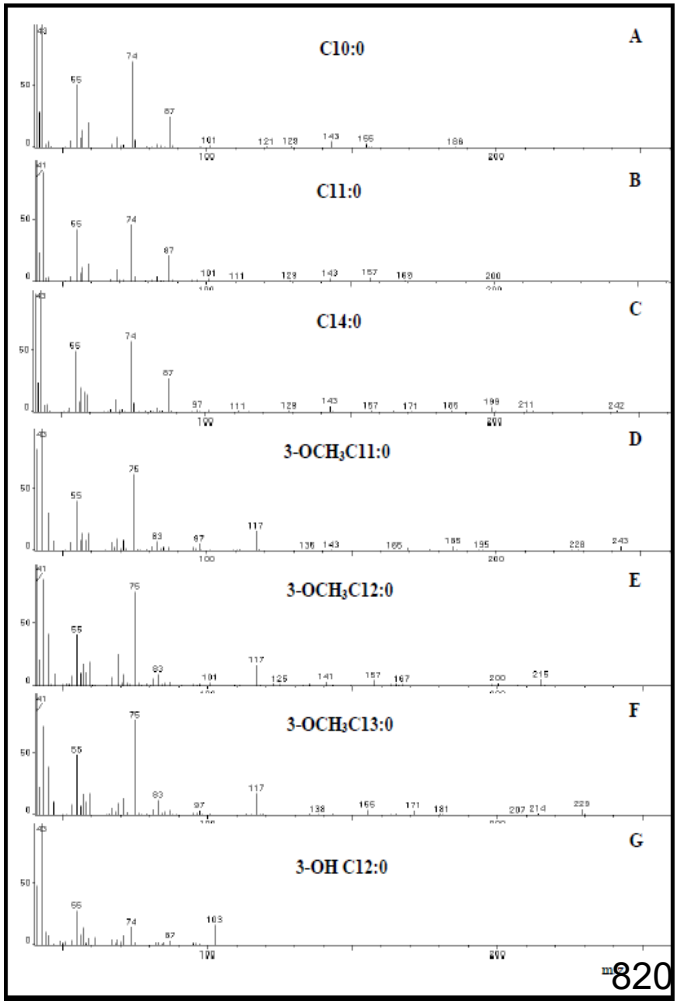
- Range and percentage of principal fatty acids used to discriminate *Xanthomonas fragariae* from other species.
- From the MIDI database library TSBA 6.

Index	Feature Name	Percent
4	10:0	1.09
6	11:0 iso	3.81
15	11:0 iso 3OH	1.87
26	12:0 3OH	3.18
27	14:0 iso	1.14
30	14:0	2.77
31	13:0 iso 3OH	1.60
37	15:0 iso	25.28
38	15:0 anteiso	10.58
48	16:0 iso	2.62
51	16:1 w9c	1.06
52	16:1 w5c	0.63
53	16:0	7.58
61	17:0 iso	3.90
103	Summed Feature 3	27.68
109	Summed Feature 9	4.00

Mass spectrometry

Matrix-assisted laser desorption/ionization(MALDI) mass spectra have been widely used to gain knowledge about lipid A heterogeneity in bacteria *X. axonopodis* pv. *citri*

- Mass spectra of the methylated fatty acids obtained wild type LPS from lipid A released from Xac.



See protein analysis for Mass spectrometry and m/z determination method.

Casabuono *et al.*,2004

Fatty acids analysis

Acidovorax

Fatty acid	1	2	3	4	5	6	7	8	9
9:0 3-OH		0·2±0·1							
10:0			0·2±0·1	0·2±0·2				0·2±0·2	
10:0 3-OH	2·1±0·2	1·9±0·3	3·1±0·5	3·7±0·2	3·2±0·4	2·6±0·6	2·3±0·1	3·0±0·1	2·7±0·1
11:0		0·3±0·1			0·1±0·1				
12:0	2·9±0·1	2·3±0·2	2·3±0·1	2·3±0·1	2·4±0·2	3·1±0·5	3·1±0·2	2·5±0	2·6±0·2
12:1 3-OH				0·6±0·1	0·2±0·3				
13:0		0·4±0·1			0·1±0·1				
14:0	3·6±0·1	1·6±0·2	2·2±0·2	1·7±0·1	1·6±0·2	3·5±0·4	3·0±0·1	3·0±0	0·7±0·1
15:1ω6*c*	0·4±0·3	2·8±1·0			1·6±0·9				0·2±0·3
15:0	3·8±0·9	12·5±2·6	0·2±0·1	0·5±0·1	5·5±2·2	0·1±0·2	0·1±0·1	0·4±0·1	1·8±1·6
16:1ω7*c*	46·3±0·5	37·9±1·6	42·9±1·0	40·6±0·3	41·9±0·3	40·9±0·3	43·3±0·6	43·1±0·4	36·3±3·9
16:0	29·4±1·0	20·4±1·7	32·2±1·2	34·9±0·4	32·9±2·7	25·9±0·7	26·0±0·1	29·3±0·5	31·9±1·2
17:1ω8*c*	0·1±0·2	1·0±0·2							0·1±0·1
17:1ω6		1·4±0·3			0·1±0·1				
17:0 cyclo	0·2±0·3		0·3±0·3	0·5±0·2		0·2±0·3	0·1±0·2		6·2±3·2
17:0	0·9±0·2	5·0±0·8	0·1±0·1	0·3±0·2	1·4±0·5			0·3±0	0·7±0·8
18:1ω7*c*	10·3±0·5	11·8±1·9	15·9±0·8	14·4±0·6	9·1±1·6	23·8±0·8	21·9±0·5	17·8±0·6	15·9±1·8
18:0		0·1±0·1	0·2±0·1	0·2±0·2			0·2±0·2	0·2±0	0·2±0·1

Standard deviations divided by mean values were <0.56 for all fatty acid methyl esters present in all strains.

Gardan *et al.*,2003

Fatty acids analysis

Enterobacter complex (*E. cloacae*, *E. asburiae* and *Enterobacter* sp.)

Mulberry wilt disease (MWD)

- Fatty acid similarity index analyses matched the four MWD isolates to *E. cancerogenus*.
- Comparisons of the fatty acid profiles of the four MWD strains with those of known bacteria in the MIDI database were matched to *E. cancerogenus* with similarity index of 0.59 to 0.84.
- MIDI instructions suggest that:

1. A Euclidean distance ≥10 is indicative of separate species, while
2. values of ≤6 suggest that two strains are in the same subspecies or biotype (Sherlock 2002).
3. Bacteria with Euclidean distances ≤2.5 are recommended for assignment to the same species.

- The four MWD isolates were in the same cluster with a Euclidean distance of ≤10, along with *E. cancerogenus* and *E. asburiae* (Euclidean distances of 3.7 and 6.7, respectively).

Wang *et al.*,2009

Fatty acids analysis

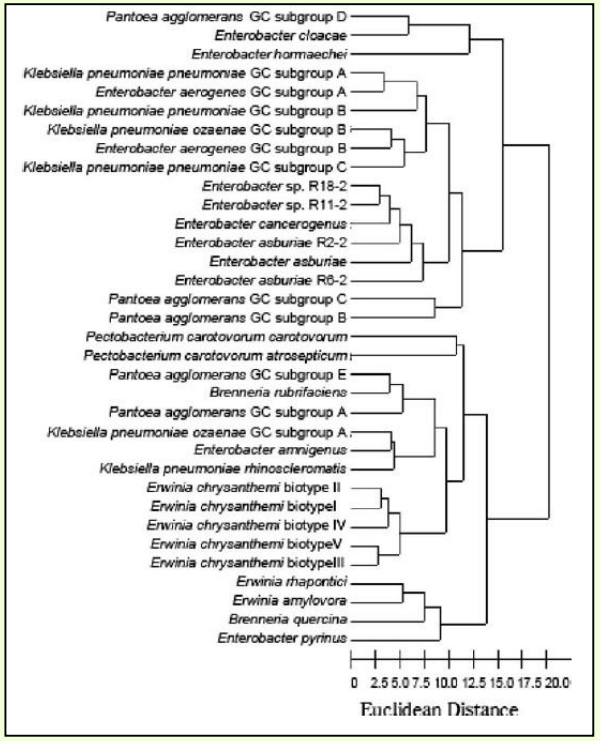
Enterobacter complex (*E. cloacae*, *E. asburiae* and *Enterobacter* sp.)

Mulberry wilt disease (MWD)

- Total cellular fatty acid composition similarity index (SI) identified as *Enterobacter cancerogenus*.

Strains	FAME SI[a]
R2-2	0.59
R6-2	0.64
R11-2	0.84
R18-2	0.73
LMG2683T	0.47[d]
LMG2693T	0.53

- Dendrogram cluster analysis of unweighted pair matchings based on fatty acid compositions.



Wang *et al.*,2009

Some examples for fatty acid analyses
Gram positive plant pathogenic bacteria

	15:0 ISO	15:0 ANTEISO	15:1 ANTEISO A	17:0 ANTEISO	10 Methyl 18:0	18:1 ù9cis
Bacillus	+	+				
Clavibacter	+	+	+	+		
Curtobacterium	+	+		+		(+)
Rathayibacter	+	+	(+)	+		
Rhodococcus					+	

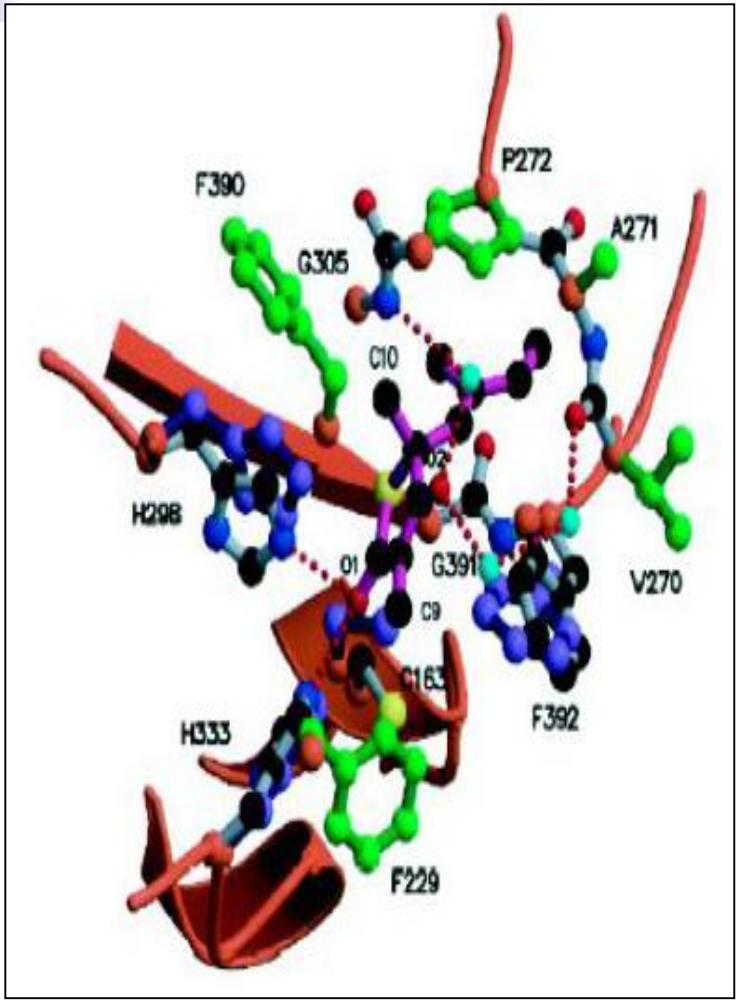
*Many Gram positive plant associated bacteria such as *Bacillus* spp., *Arthrobacter* spp. and *Curtobacterium* spp. have very similar fatty acids in their profiles.
+ = thought to occur in all strains
(+) = occurs in some taxa/strains

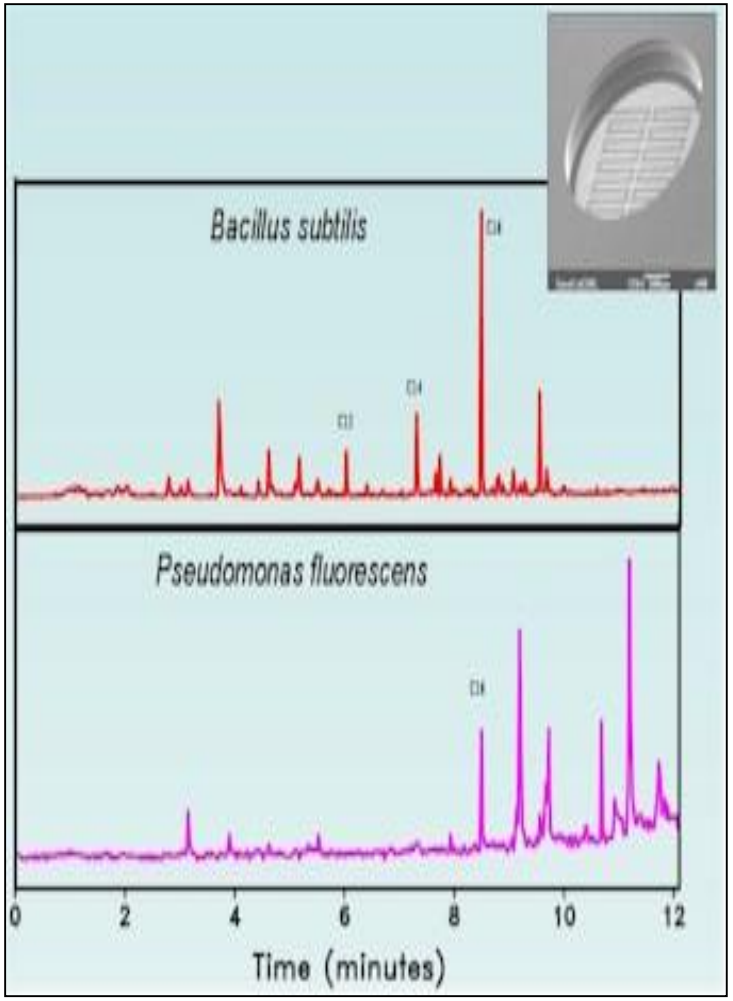
Prediction of Gram positive genera of plant pathogenic bacteria based on fatty acids.

Schaad *et al.*,2001

Fatty acid chromatograph

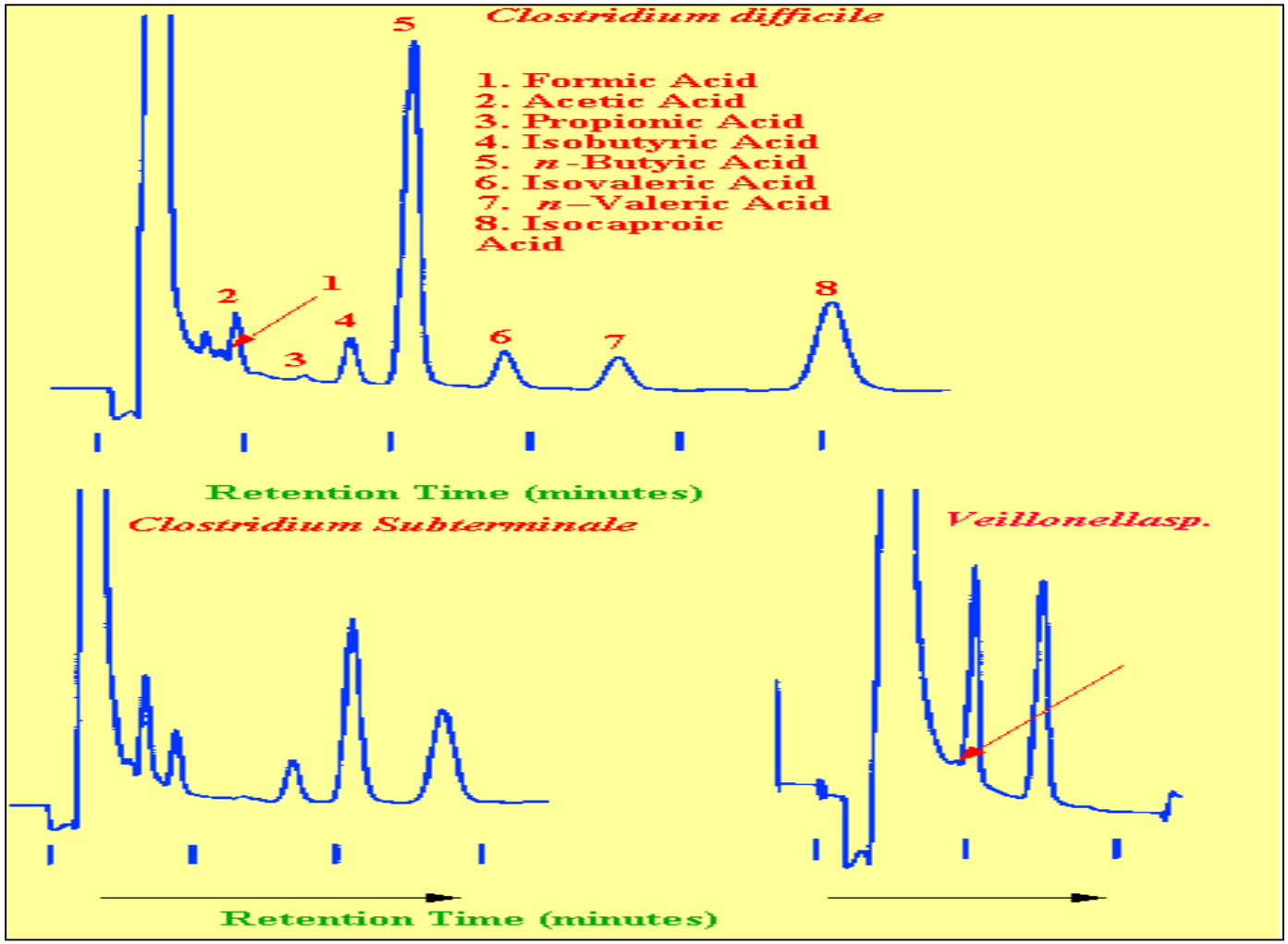
Bacillus subtilis/P. fluorescens





Fatty acid chromatograph

Clostridium spp.



Electronic Nose(EN or e-nose)

Sensor systems

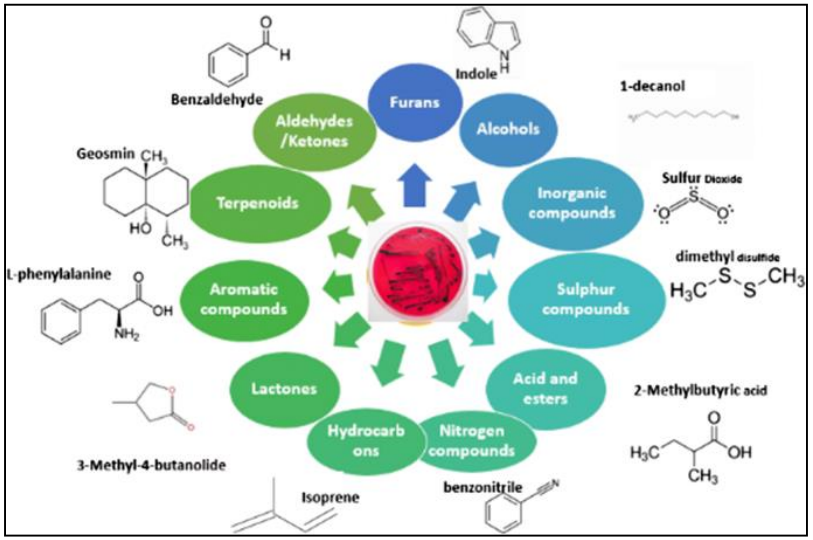
Modern method for detection

Discrimination of Plant Pathogenic Bacteria Using an Electronic Nose

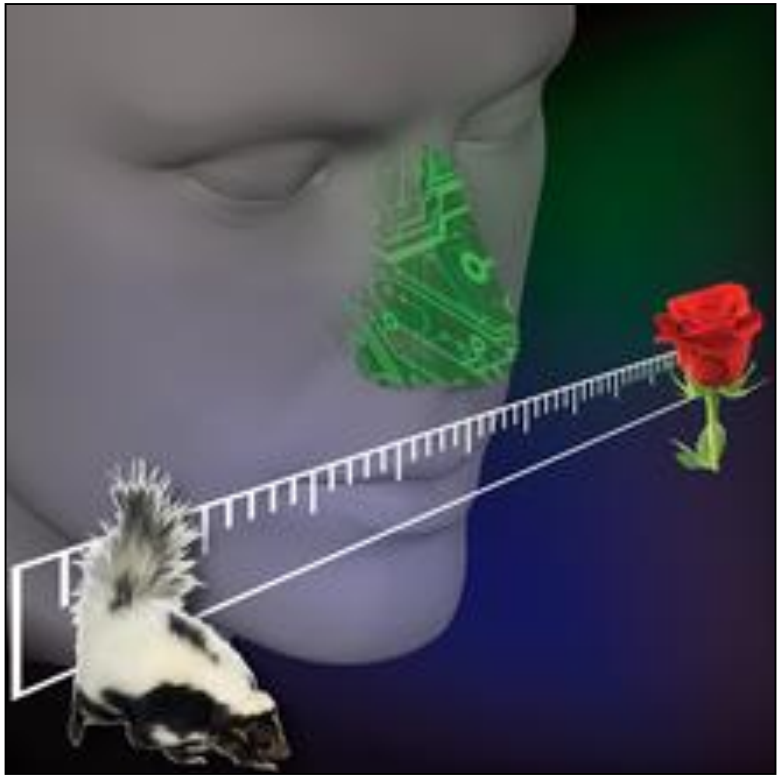
Dog's nose: possess up to 300 million olfactory(smell sense) receptors in their noses, compared to about six million in us. And the part of a dog's brain that is devoted to analyzing smells is about 40 times greater than ours.

Electronic Nose

An electronic nose (e-nose) is a device intended to detect odors or flavors



Application of electronic nose (E-nose) for food safety, with emphasis on classification and detection of foodborne pathogens.



Wikipedia,2017; Bonah *et al.*,2019

Electronic Nose

Possible and future applications in the fields of health and security

- The detection of dangerous and harmful bacteria.
- Detection and identification of plant pathogenic bacteria.
- The detection of lung cancer or other medical conditions by detecting the VOC's (volatile organic compounds) that indicate the medical condition.
- The quality control of food products as it could be conveniently placed in food packaging to clearly indicate when food has started to rot or used in the field to detect bacterial or insect contamination.

Electronic nose (E-nose) devices have been used for the evaluation of volatiles from fruit and vegetables to assist in fruit grading.

Wikipedia,2017;..

Electronic Nose

Possible and future applications in the fields of health and security

- Detection and identification of plant pathogenic bacteria:
- Sensor technology based on conducting polymers, quartz-resonator sensors.
- The use of the EN for the identification of plant pathogens was reported previously (Wilson& Lester,1997).
- A rapid, sensitive, specific, nondestructive and easy-to-use technique such as the EN could be utilized for detection and identification of plant pathogenic bacteria in plant diagnostic clinics and quarantine laboratories.

Momol *et al.*,2004

Electronic Nose (EN)

Useful for accurate identification of pure cultures detection

- EN with 12 polymer sensors discriminated between plant pathogenic bacteria.
- The system operates through an electronic nose containing a set of sensors selected for their sensitivity to marker volatile organic compounds (VOCs) and the resulting data are captured, displayed and recorded by computer.
- The electronic nose appears promising and this technology would be applicable to the detection of statutory organisms by plant health and seed inspectors (de Lacy Costello *et al.*,2006) but in addition it could be very useful for accurate identification of pure cultures.

López *et al.*,2008

Electronic nose
Diagnosis of *E. amylovora*

- Considering that pathogen-induced plant responses include changes in volatiles, in our experiments we tested the electronic nose (e-nose) as an alternative approach for the early diagnosis of fire blight on entire and still asymptomatic plants.
- The advantages the e-nose include:
- Fast execution, limited sample pre-processing and easy use even in field and nursery conditions.
- The VOCs profile of infected plants was determined by means of a bag-enclosure system combined with adsorption on solid materials and followed by GC-MS analysis.
- The results obtained by the GC-MS analysis were successively used to optimize the e-nose equipment.

Spinelli *et al.*,2009

Electronic Nose

Sensor types used in the lab-made e-nose and the targeted volatile compounds

- The lab-made e-nose used in this study consisted of 8 types of metal oxide semiconductor(MOS gas sensors) (from Figaro Inc., Osaka, Japan), as listed in the Table, namely TGS 813, TGS 822, TGS 823, TGS 826, TGS 2600,TGS 2603, TGS 2612, and TGS 2620. The e-nose was also equipped with an SHT31-D sensor (Sensirion Inc., Tokyo, Japan) for air temperature and humidity monitoring in the inside of the sensor chamber.

Type of Sensor	Targeted Volatile Compounds
TGS813	Methane, ethanol, propane, isobutane, hydrogen, and carbon monoxide
TGS822	Ethanol, acetone, benzene, n-hexane, isobutane, carbon monoxide, and methane
TGS823	Combustible gas, i.e., ethanol
TGS826	Ammonia
TGS2600	Methane, carbon monoxide, isobutane, ethanol, and hydrogen
TGS2603	Hydrogen, H_2S, ethanol, methyl mercaptan, and trimethylamine
TGS2612	Ethanol, methane, isobutane, and propane
TGS2620	Methane, carbon monoxide, isobutane, hydrogen, and ethanol

Astantri *et al.*,2020

Electronic Nose

Comprised of a sampling system, a data acquisition unit (DAQ), and a signal processing framework

- In addition, the e-nose device comprised of:

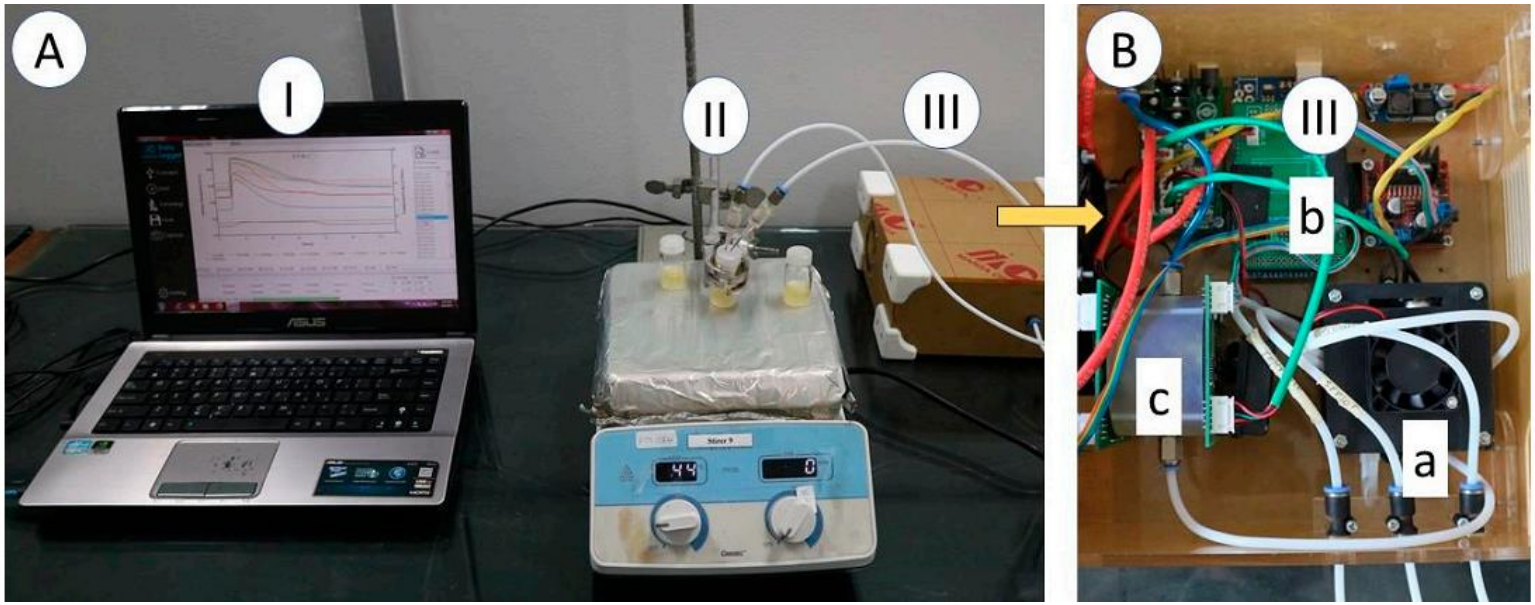
1. a sampling system,
2. a data acquisition unit (DAQ), and
3. a signal processing framework. See next Figure.

- The DAQ for sensor output signal acquisition was built using a microcontroller board, based on the ATmega2560 (or otherwise known as Arduino Mega 2560).

Astantri *et al.*,2020

Electronic Nose

Sensor types used in the lab-made e-nose and the targeted volatile compounds

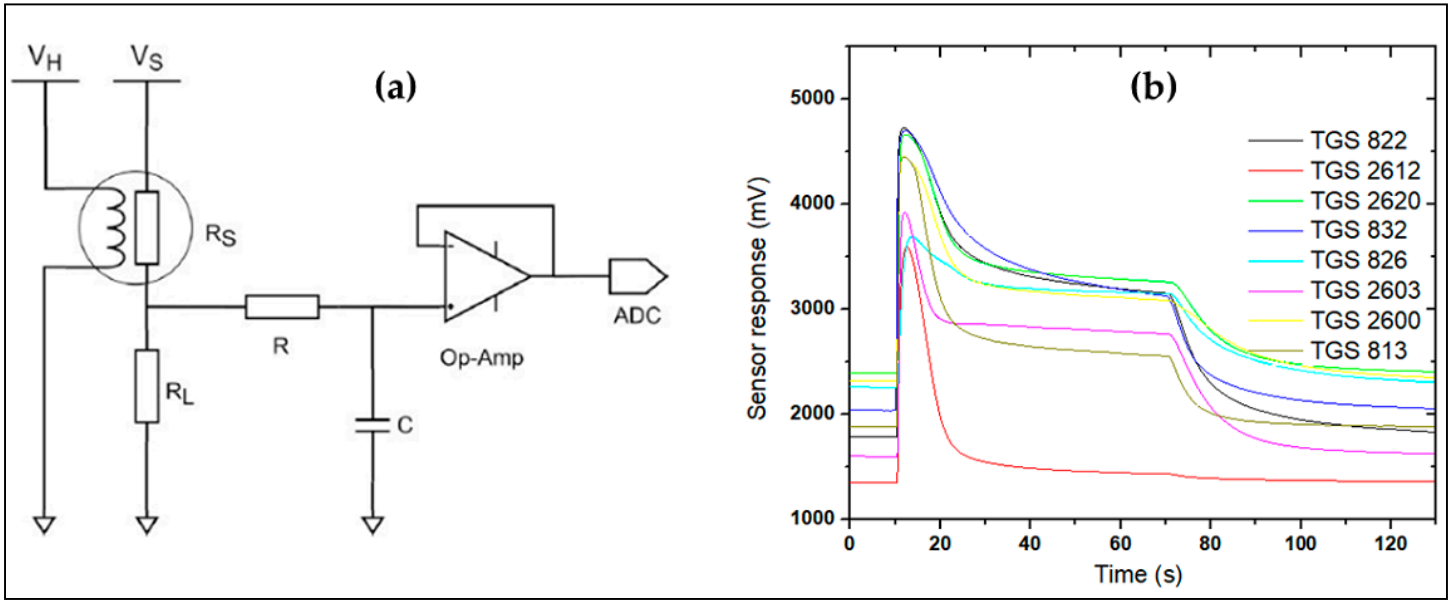


(A) E-nose measurement set-up. I: a personal computer with a software of data acquisition unit (DAQ) and chemometric models, II: sample on the hot plate, and III: the main part of e-nose; (B) III: main part of electronic nose device. a: sampling system, b: DAQ and controller, c: sensor chamber.

Astantri *et al.*,2020

Electronic Nose

Comprised of a sampling system, a data acquisition unit (DAQ), and a signal processing framework



(a) Electrical schematic of recording a signal from a sensor in this e-nose. VH and VS are voltage sources for heater and sensor, respectively; RS and RL are sensor resistance and load resistance, respectively; R and C for the low-pass filter. (b) Typical of gas sensor response of e-nose during the delay, sampling and purging processes.

Astantri *et al.*,2020

Electronic Nose

This study demonstrates the perspective of using a lab-made e-nose with MOS gas sensors coupled with chemometric models to correctly classify the existence of *L. monocytogenes* and *B. cereus* on TSB media

MVOCs	Functional Groups	Active Sensors
	Listeria monocytogenes	
2-undecanone	methyl vinyl ketone	TGS 822
2-nonanone/1-undecene	acrylic alkanes	TGS 813, TGS 2612, TGS 2620, TGS 2600
dimethyl trisulfide	2,3,4-trithiapentane	TGS 813, TGS 2612, TGS 2620, TGS 2600
aldehydes	aldehyde	TGS 823, TGS 2600, TGS 2603
ketones	ketones	TGS 822
3-methyl-butanal	butanal	TGS 823, TGS 2600, TGS 2603
acetone	propanone	TGS 813, TGS 2612, TGS 2620, TGS 2600
2-methyl-butane	isopentane	TGS 813, TGS 2612, TGS 2620, TGS 2600
3-hydroxy-2-butanone	methyl acetoin	TGS 813, TGS 2612, TGS 2620, TGS 2600
	Bacillus cereus	
2-undecanone	metal ketones	TGS 822
dimethylsulfide	2,3,4-trithiapentane	TGS 813, TGS 2612, TGS 2620, TGS 2600
4-hydroxy-2-butanone	methyl acetone	TGS 822
ethyl acetate	ester	TGS 813, TGS 2612, TGS 2620, TGS 2600

Astantri *et al.*,2020

Method

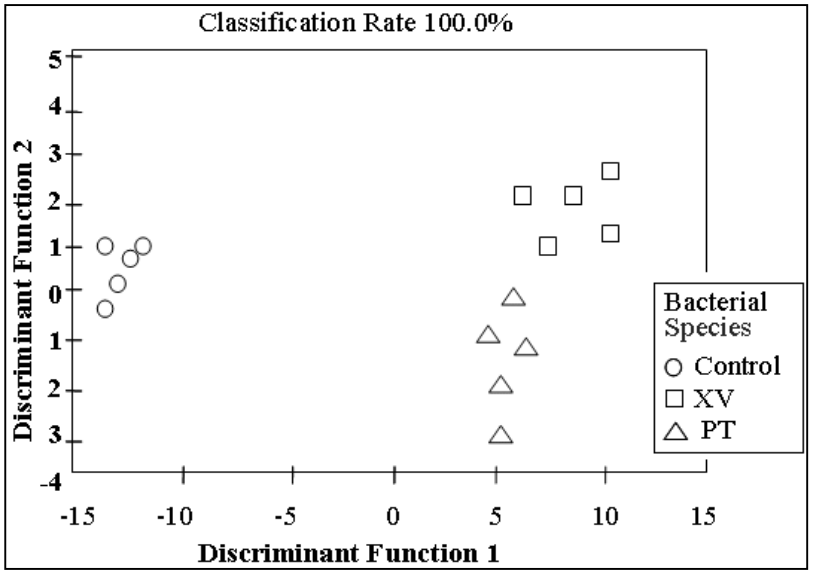
- All bacteria were grown on Trypticase soy agar (trypticase soy broth [BBL] containing Bacto Agar [Difco]) poured in 60-mm glass Petri plates at 28°C for 24 ± 2 h.
- Before incubation, plates were sealed with parafilm until processing.
- Each species or strain was grown in a separate plate.
- For all experiments, the same number (four or five replications) of bacteria-free control media samples were prepared and subjected to the same conditions as bacterial cultures.
- An EN (e-Nose 4000, EEV Inc., Elmsford, NY) was used for odor measurements of the bacterial species.
- Before each experiment, the electronic nose was calibrated with a polypropylene glycol solution (75% v/v water).
- Four or five samples (replicates) of each bacterial species and the control sample (uninoculated media) were read once separately with the EN.

Method

- Experiments were conducted by using two species, two strains of two species, five species, and seven species of plant pathogenic bacteria during the same run and bacteria-free control media.
- For each bacterial species and each combination, the same experiment was repeated twice.
- Analysis of electronic nose sensor data of bacterial species and the control was performed with Statistical.
- The identity of all strains was confirmed by fatty acid methyl esters profile data analyses.

The correct classification rate for two bacterial species at a time

- Discriminant function analysis of two bacterial species based on electronic nose readings.
- Abbreviations used:
- Control= bacteria free,
- XV = *Xanthomonas campetris* pv. *vesicatoria*
- PT = *Pseudomonas syringae* pv. *tomato*.

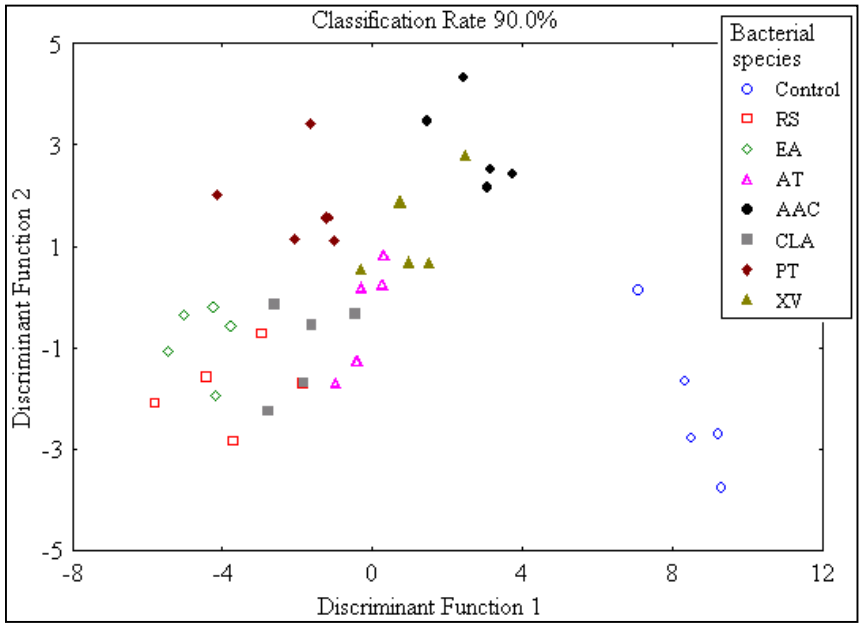


The correct classification rate for two bacterial species at a time (i.e. *Xanthomonas campestris* pv. *vesicatoria* versus *Pseudomonas syringae* pv. *tomato*) and control samples was 100.0%.

Momol *et al.*,2004

Discriminant function analysis of seven bacterial species based on electronic nose readings

- **Abbreviations used:**
- Control = bacteria free,
- RS= *Ralstonia solanacearum*
- EA= *Erwinia amylovora*
- AT= *Agrobacterium tumefaciens*
- AAC= *Acidovorax avenae* subsp. *citrulli*
- CLA= *Clavibacter michiganensis*
- PT= *Pseudomonas syringae* pv. *tomato*
- XV= *Xanthomonas campestris* pv.*vesicatoria*.



Momol *et al.*,2004

Result

- In this study, the EN with 12 polymer sensors discriminated between plant pathogenic bacteria belonging to up to seven species from seven different genera.
- Fatty acid analysis proved to be successful in performing overall correct classification (90.0%) of the seven bacterial species used in these experiments.
- Correct classification rate was higher if five or two species were tested in the same run.
- The EN technology is novel and in its infancy for application in plant pathology.
- This study is reported to encourage plant pathologists in industry and public sectors to explore EN technologies for plant pathology applications.

Electronic nose
Diagnosis of *E. amylovora*

- Considering that pathogen-induced plant responses include changes in volatiles, in our experiments we tested the electronic nose (e-nose) as an alternative approach for the early diagnosis of fire blight on entire and still asymptomatic plants.
- The advantages the e-nose include:
- Fast execution, limited sample pre-processing and easy use even in field and nursery conditions.
- The VOCs profile of infected plants was determined by means of a bag-enclosure system combined with adsorption on solid materials and followed by GC-MS analysis.
- The results obtained by the GC-MS analysis were successively used to optimize the e-nose equipment.

Spinelli *et al.*,2009

Electronic Nose

Diagnosis of *R. solanacearum* and kiwifruit pathogens

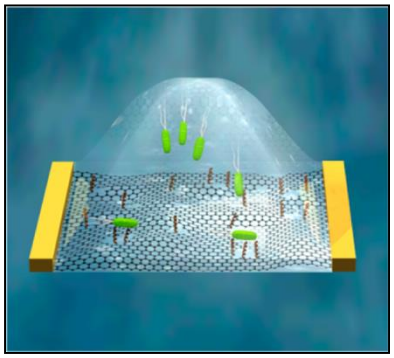
- Sensor systems for the easy detection of *R. solanacearum* potato tubers have been developed recently (Stinton *et al.*,2006).
- Preliminary results on the use of the electronic nose for the diagnosis of some important postharvest diseases of kiwifruit are reported.

López *et al.*,2008; Spinelli *et al.*,2009

Electronic Detection of Bacteria Using Holey Reduced Graphene Oxide

Carbon nanomaterials: Biosensing

A broad-spectrum bacterial probe



Electronic detection of bacteria
Carbon nanomaterials
Biosensing

- Carbon nanomaterials have been widely explored for diverse biosensing applications including bacterial detection.
- However, covalent functionalization of these materials can lead to the destruction of attractive electronic properties.
- To this end, we utilized a new graphene derivative, holey reduced graphene oxide (hRGO), functionalized with Magainin I to produce a broad-spectrum bacterial probe.

Magainin I, was covalently functionalized to hRGO, yielding a gram-negative specific biosensor that operates by taking advantage of the electrostatic interaction between positively charged Magainin I and anionic lipopolysaccharides.

Chen *et al.*,2014

Electronic detection of bacteria
Carbon nanomaterials
Biosensing

- Single-walled nanotubes (SWNTs) and graphene, because of their nanometer-scale sizes and unique electronic properties, are considered to be ideal materials for biosensing applications.
- SWNT field-effect-transistor (FET) biosensors have been functionalized with:

1. Antibodies, or
2. aptamers for the fast detection of multiple bacterial species.

Chen *et al.*,2014

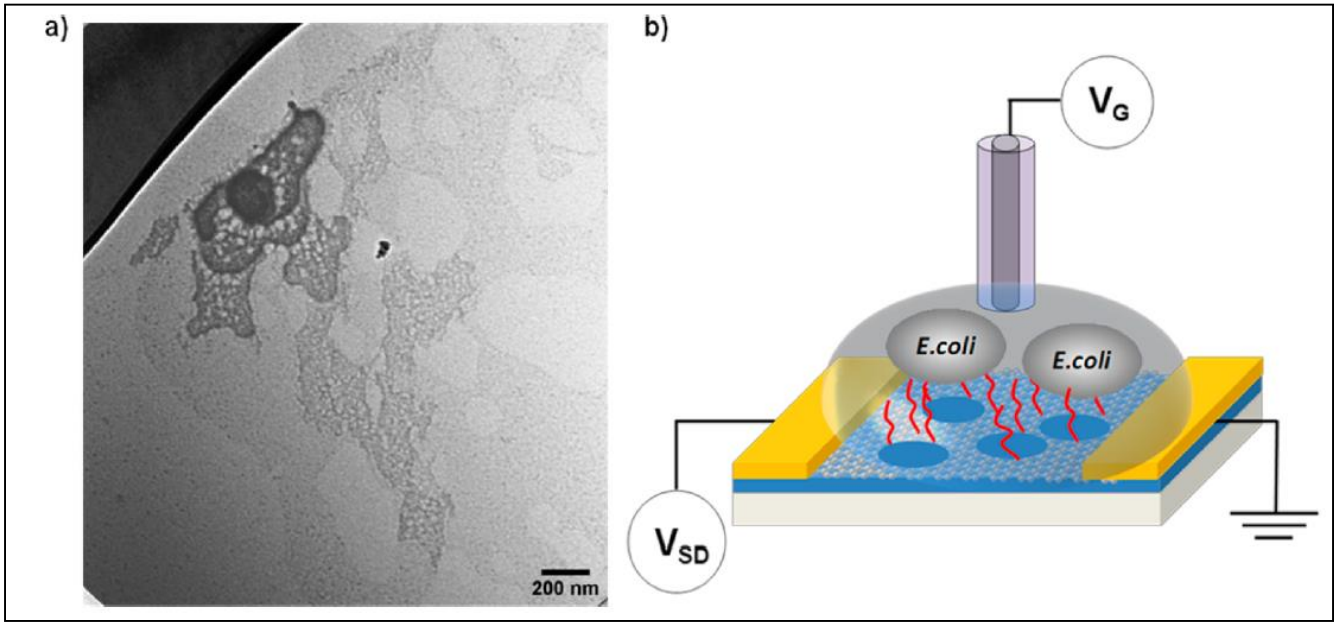
Electronic detection of bacteria

Carbon nanomaterials

Biosensing

a) TEM image of hRGO(holey reduced graphene oxide);

b) Schematic illustration of an AMP-functionalized hRGO FET for the selective detection of gram-negative bacteria cells.



Chen *et al.*,2014

Electronic detection of bacteria

Carbon nanomaterials

Biosensing

a) Optical micrograph of a single functionalized device after exposure to 10^7 cfu/mL *E. coli*.

b) Same device under a red fluorescent protein filter, showing PI-stained cell fluorescing. Scale bar for parts a and b is 50 μm.

c) SEM image of the functionalized device surface after incubation with *E. coli* O157:H7. The scale bar 2 μm.

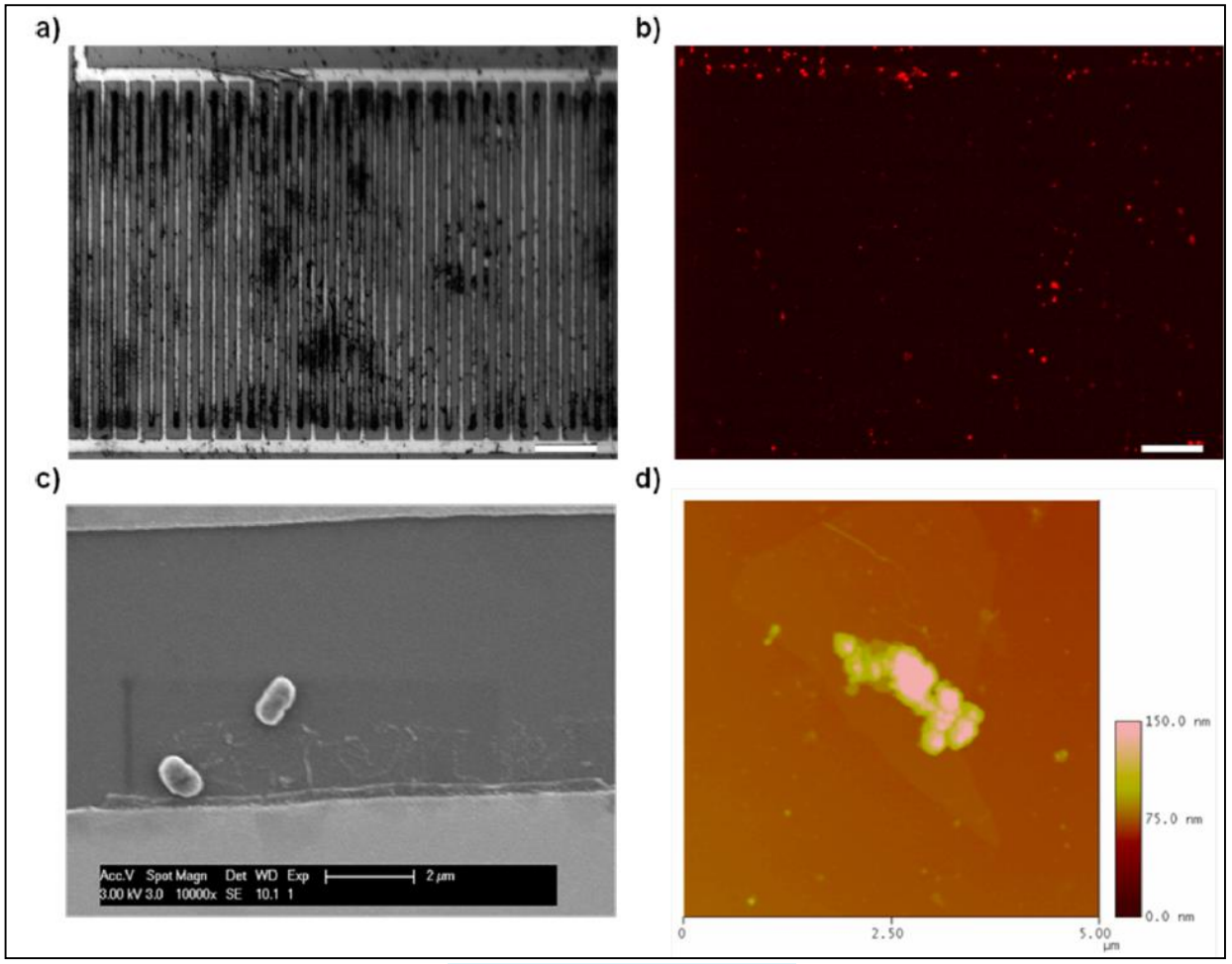
d) AFM image over an area of 5 μm^2 depicting the attachment of bacteria to the surface of hRGO (See figures of the next slide).

Chen *et al.*,2014

Electronic detection of bacteria

Carbon nanomaterials

Biosensing



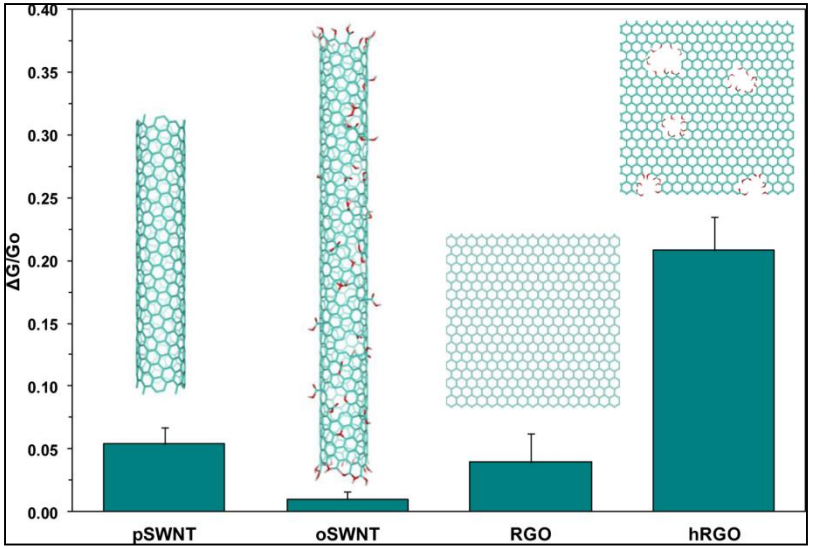
Chen *et al.*,2014

Electronic detection of bacteria

Carbon nanomaterials

Biosensing

- Comparison of the mean normalized responses (V_g=−0.5 V) of four Magainin I functionalized carbon nanomaterials to 10^7 cfu/mL *E. coli*.
- Averaged from four devices; the error bars represent 1 standard deviation.

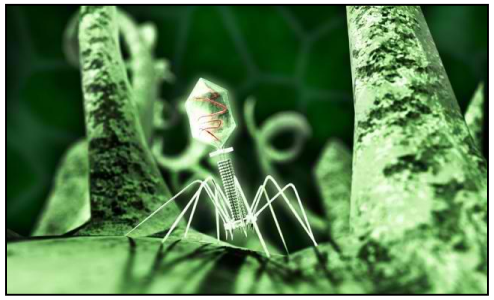


Chen *et al.*,2014

Phage typing

Bacterial viruses or bacteriophages

Identification of Bacteria by phage specificity tests



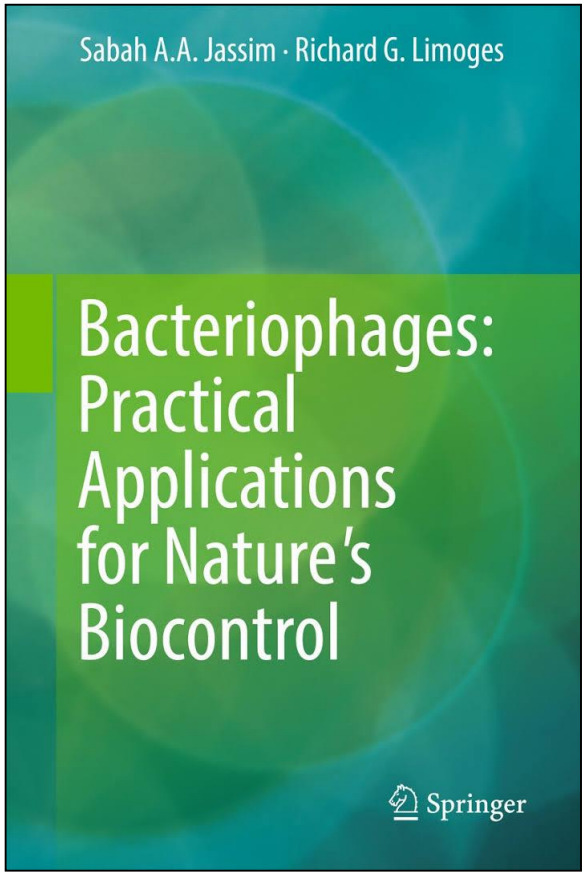
www.phage.org. abedon.1@osu.edu

Bacteriophages

- **Bacteriophages**
- Edited by Dr. Ipek Kurtboke
- Publisher: InTech
- 2012
- 256 pages

Bacteriophages: Practical Applications for Nature's Biocontrol

- **Bacteriophages: Practical Applications for Nature's Biocontrol**
- Richard G. Limoges and Sabah A.A. Jassim
- Publisher: Springer Cham
- 2017
- 242 pages.



Bacteriophages
Definition

- A bacteriophage, also known informally as a phage, is a virus that infects and replicates within bacteria and archaea.
- The term was derived from "bacteria" and the Greek phagein, "to devour/eat".
- Therefore, a bacteriophage is a kind of virus that can infect and replicate itself inside bacterial cells.

Management

Bacteriophages of *Pseudomonas tolaasii*

Terminology

- What are phage lysates?
- A plate lysate is simply a concentrated liquid sample of phage.
- It is obtained by infecting a plate of bacteria with the phage of interest, letting the phage lyse the cells, then adding buffer directly to the plate surface to collect the phages.
- What does a titer of a lysate express?
- Phage titer is expressed in PFU (Plaque Forming Units)/mL.

1. Lysates with a final concentration greater than 10^9 PFU/ml are "High Titer" lysates.
2. Current data suggest that the higher the titer, the more stable the lysate.
3. Full plate titers have higher degree of accuracy.

Bacteriophages

Distinct biological entities of viruses

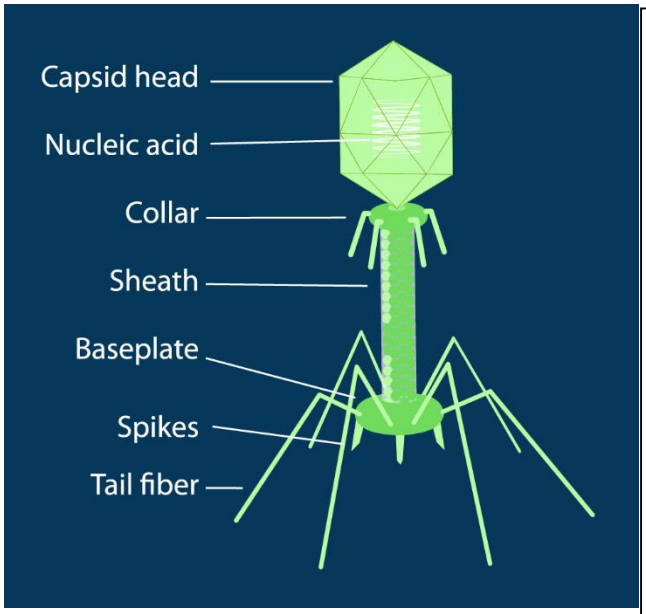
1. Prokaryotes as host;
2. Subcellular structure without metabolic machinery;
3. Double stranded DNA, single stranded DNA, RNA;
4. Virulent phage vs. temperate phage.

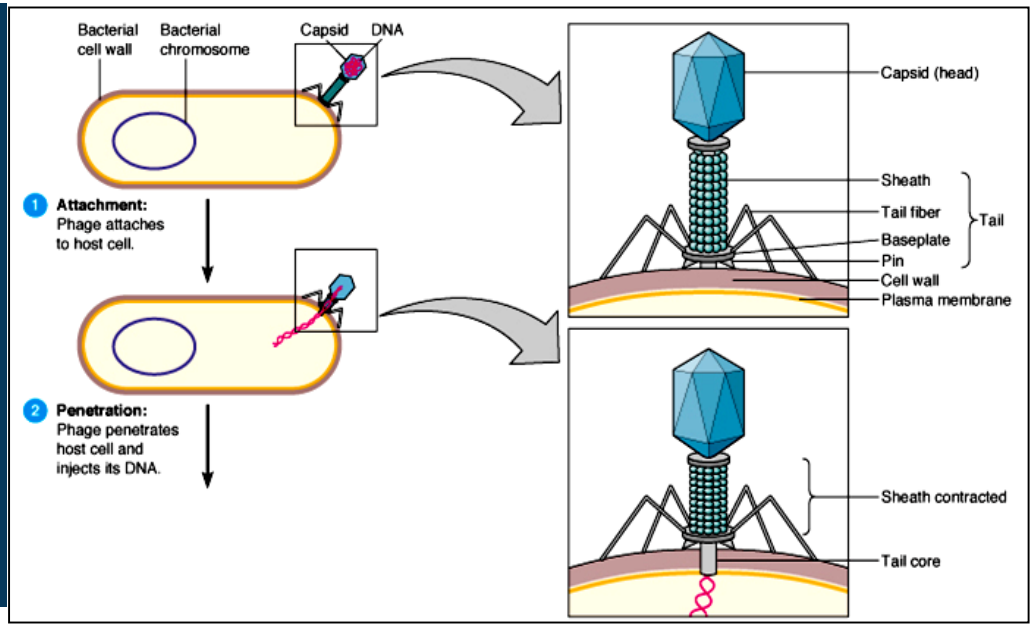
1. Viruses lack all metabolic machinery and do not produce ATP because they do not perform energy-requiring processes.
2. Bacteriophages can not independently reproduce. They must rely on host cell for reproductive machinery and components.

BC Yang;Todar,2012;..

Bacteriophage structure

Tail spike protein: Responsible for initial absorption of the phage to the host bacterium





The adsorption and infection of bacteriophage P2 is mediated by tail fibres and tail spikes. The tail consists of a hollow core through which the DNA is injected into the host cell.

Types of bacteriophages

- Like other types of viruses, bacteriophages vary a lot in their

1. Shape, and
2. genetic material.

- Phage genomes can consist of either:

1. DNA, or
2. RNA

- And can contain:

1. as few as four genes, or
2. as many as several.

Types of bacteriophages
DNA and RNA bacteriophages

- Bacteriophage have either DNA or RNA as their genetic material,

1. in either circular or linear configuration,
2. as a single- or a double-stranded molecule.

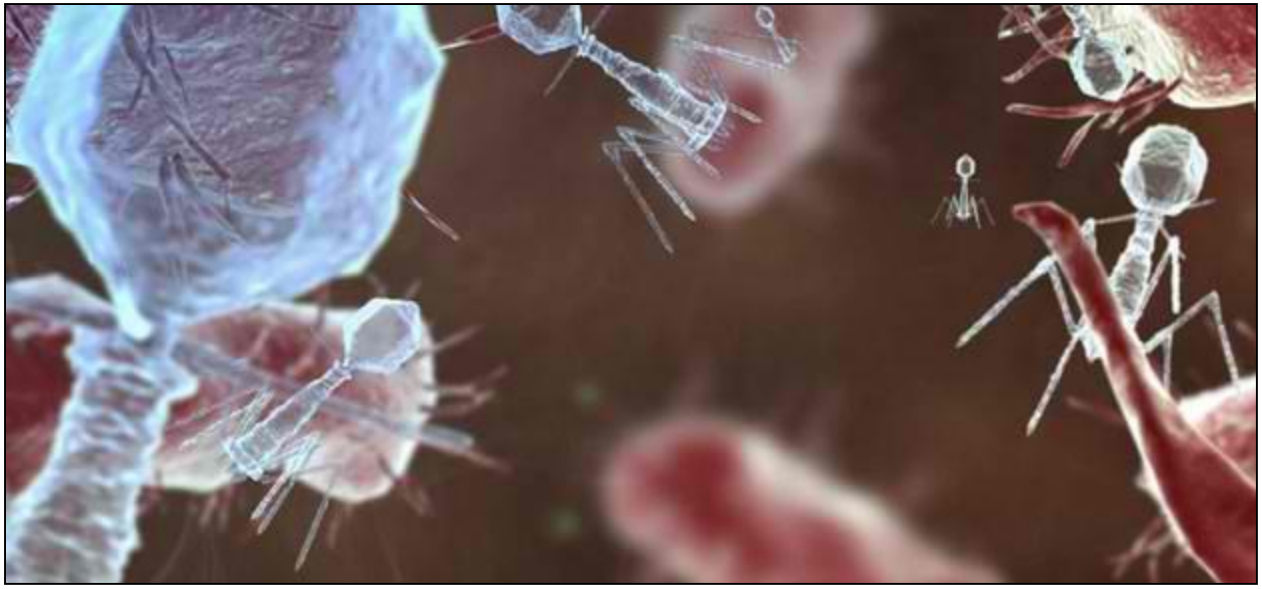
- Double-stranded DNA phages require multiple lysis proteins, including at least one enzyme that degrades the cell wall (peptidoglycan,PG).
- In contrast, the lytic ssDNA and ssRNA phages have a single lysis protein that achieves cell lysis without enzymatically degrading the peptidoglycan (PG).

Chamakura and Young,2018;..

Classification of Bacteriophages

Phage families and genera

- At present, over 5500 bacteriophages have been studied by electron microscopy and can be divided into 14 virus families.



nano.aau.dk/nano3/microbiology,2009; Everything about Bacteriophage,2013

Bacteriophages
Phage families and genera

1. Tailed phages:

- Comprise 96% of phages and are the largest virus group known. e.g. T-even lytic or virulent phages such as T2, T4 and T6 that infect *E. coli*.

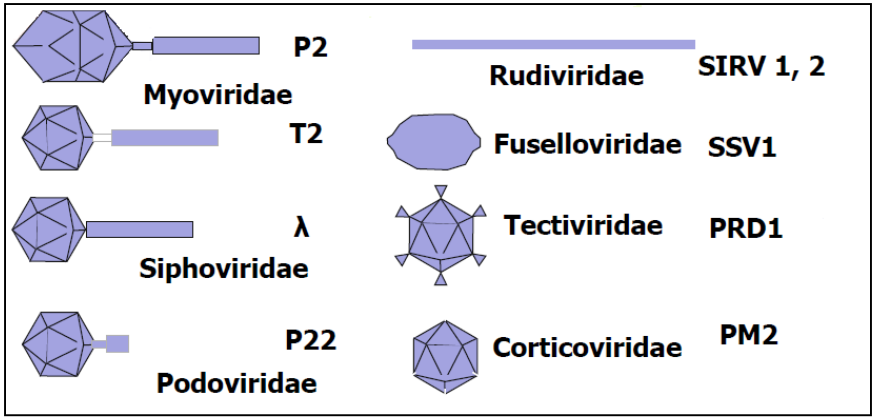
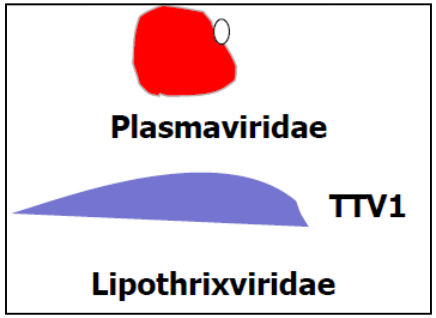
2. Cubic, filamentous, and pleomorphic phages:

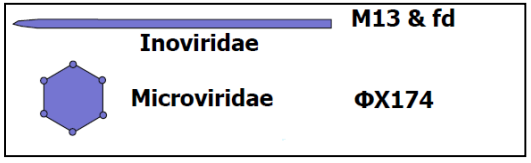
- This group includes 10 small phage families that correspond to approximately 4% of phages.
- These phages differ greatly in:

1. nucleic acid nature;
2. particle structure;
3. sometimes have a single member;
4. Host ranges are mostly narrow.

Ackermann,2004

Bacteriophage families

Double stranded DNA, Non-enveloped	Double stranded DNA, Enveloped
	

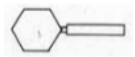
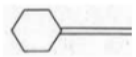
Single-stranded DNA	Single stranded RNA	Double stranded RNA
		

nano.aau.dk/nano3/microbiology,2009

Classification of Bacteriophages

Overview of phage families

One order and 10 families

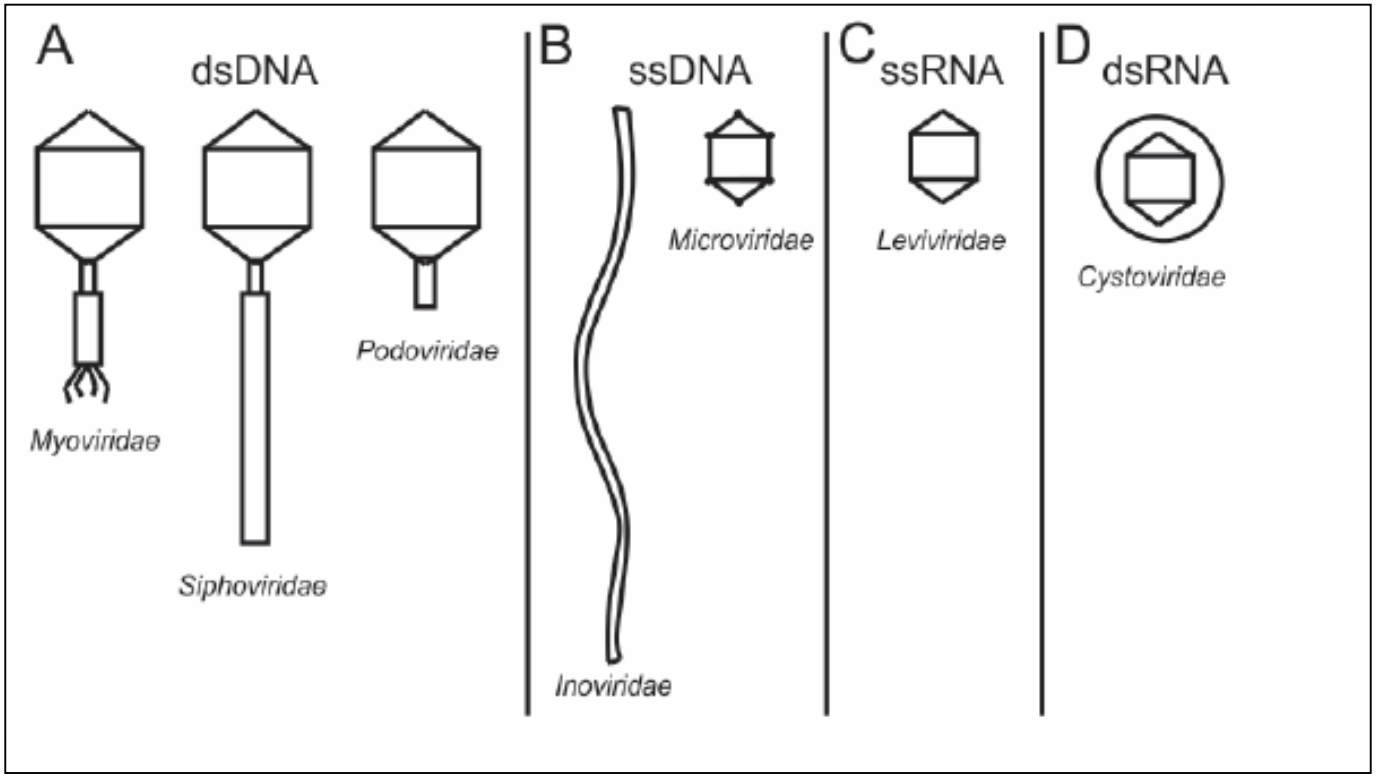
Shape	Order or family	Nucleic acid, particulars, size	Member	Number[a]
	Caudovirales	dsDNA (L), no envelope		
	*Myoviridae*	Tail contractile	T4	1312
	*Siphoviridae*	Tail long, noncontractile	λ	3262
	*Podoviridae*	Tail short	T7	771
	*Microviridae*	ssDNA (C), 27 nm, 12 knoblike capsomers	ϕX174	38
	*Corticoviridae*	dsDNA (C), complex capsid, lipids, 63 nm	PM2	3?
	*Tectiviridae*	dsDNA (L), inner lipid vesicle, pseudo-tail, 60 nm	PRD1	19
	*Leviviridae*	ssRNA (L), 23 nm, like poliovirus	MS2	38
	*Cystoviridae*	dsRNA (L), segmented, lipidic envelope, 70–80 nm	ϕ6	3
	*Inoviridae*	ssDNA (C), filaments or rods, 85–1950 x 7 nm	fd	66
	*Plasmaviridae*	dsDNA (C), lipidic envelope, no capsid, 80 nm	MVL2	5

[a] From reference 1. C, circular; L, linear.

Ackermann,2011

Classification of Bacteriophages

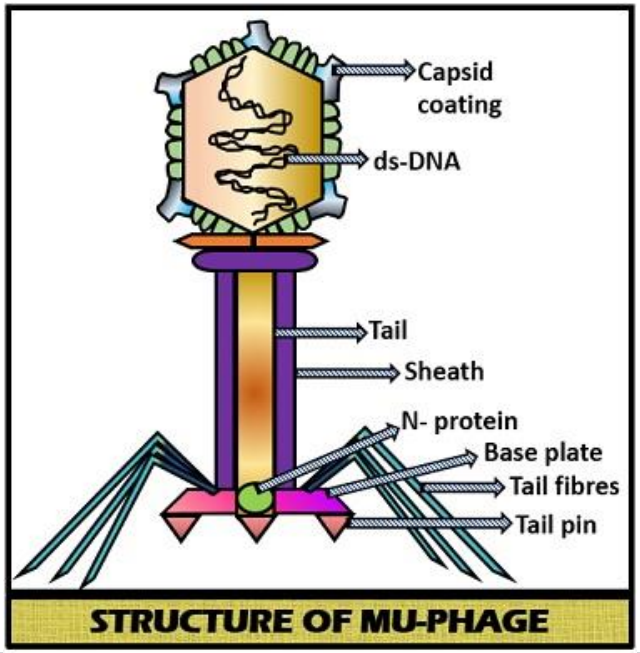
Families of bacteriophages grouped according to their most common nucleic acid composition



Dias *et al.*,2013

Types of bacteriophages

DNA and RNA bacteriophages



Family	Morphology	Nucleic acid	Examples
Myoviridae	Nonenveloped, contractile tail	Linear dsDNA	T4,Mu,P1,P2
Siphoviridae	Nonenveloped, noncontractile tail (long)	Linear dsDNA	λ, T5, HK97, N15
Corticoviridae	Nonenveloped, isometric	Circular dsDNA	PM2
Inoviridae	Nonenveloped, filamentous	Circular dsDNA	M13

Types of bacteriophages
DNA and RNA bacteriophages
ssRNA> dsRNA

- More than 1900 phage genomes are currently deposited in NCBI.
- Only 6 dsRNA bacteriophages, and
- 12 ssRNA bacteriophages genome sequences are reported.
- The 6 dsRNA bacteriophages were isolated from legume samples or lakes with *Pseudomonas syringae* as the host.

Yang *et al.*,2016

Types of bacteriophages

DNA and RNA bacteriophages

ssDNA>dsDNA

- DNA bacteriophages are currently classified by the International Committee for the Taxonomy of Viruses (ICTV) into eight separate families with a total of 494 species,

1. 55 single-stranded DNA (ssDNA), and
2. 439 double-stranded DNA (dsDNA) bacteriophage species.

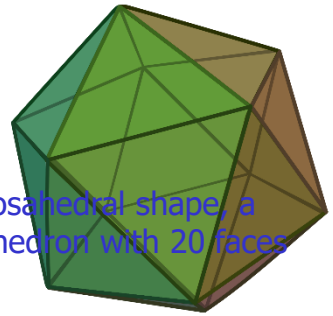
Double-stranded DNA phages require multiple lysis proteins, including at least one enzyme that degrades the cell wall (peptidoglycan). In contrast, the lytic ssDNA and ssRNA phages have a single lysis protein that achieves cell lysis without enzymatically degrading the peptidoglycan (PG).

Yang *et al.*,2016; Chamakura and Young,2018

Types of bacteriophages

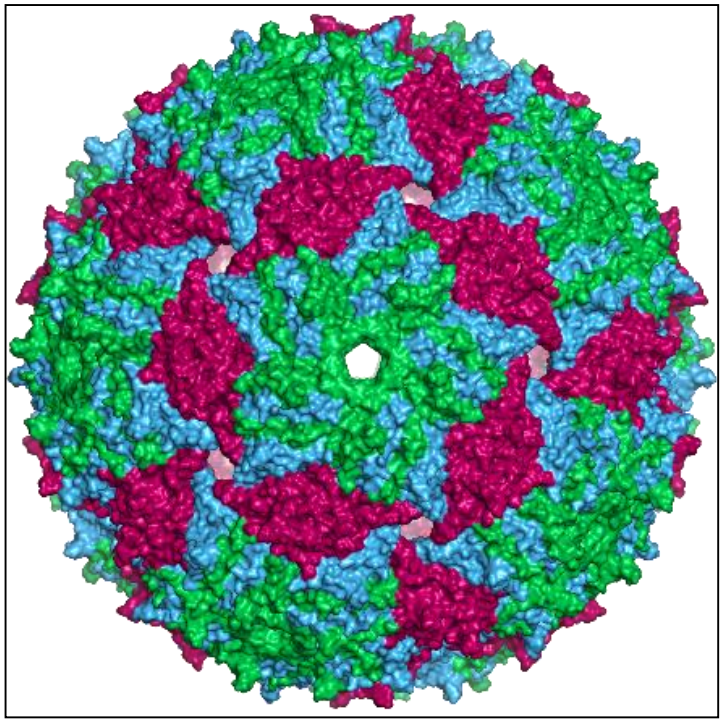
RNA bacteriophages

MS2



Icosahedral shape, a polyhedron with 20 faces

- RNA phage such as MS2 have the smallest genome, of only a few kilobases.
- Bacteriophage MS2 is an icosahedral virus with 180 copies of a coat protein forming a shell around a single-stranded RNA molecule.
- Infects the bacterium *Escherichia coli* and other members of the Enterobacteriaceae.



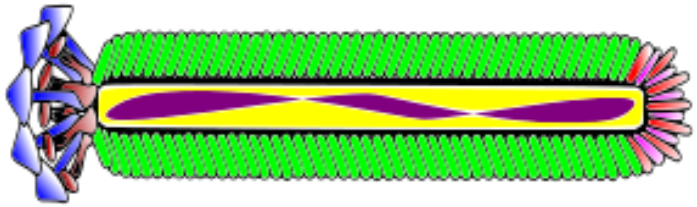
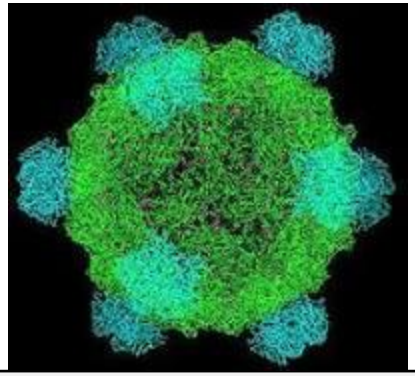
MS2 capsid surface

Wikipedia,2019

Types of bacteriophages

DNA bacteriophages

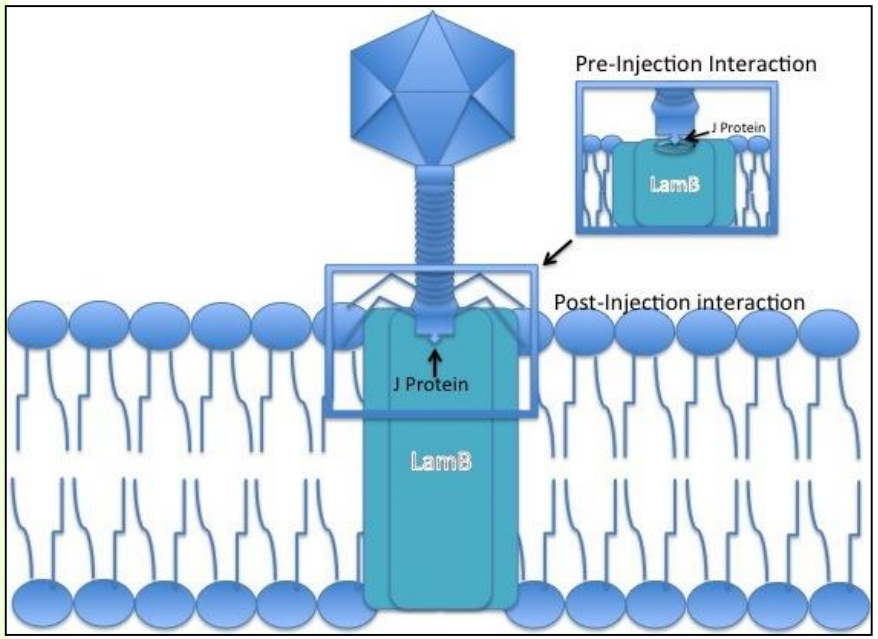
M13 and Phix174

	
M13 is a filamentous bacteriophage composed of circular single-stranded DNA (ssDNA). Infects enterobacteria, especially *E. coli*.	The phi X 174 bacteriophage is a single-stranded DNA virus that infects *Escherichia coli*.

Types of bacteriophages

Enterobacteria phage λ (lambda phage, coliphage λ, officially *Escherichia virus Lambda*)

- Bacteriophage Lambda binds to an *E. coli* cell by means of its J protein in the tail tip.
- The J protein interacts with the maltose outer membrane porin of *E. coli*.
- The linear phage genome is injected through the outer membrane.



Wikipedia,2019

Bacterial viruses or bacteriophages

Virulent (lytic) phages

T-even phages vs. T-odd phages

- Every bacterial species is parasitized by various specific bacteriophages.
- Those which attack *Escherichia coli* are called Coliphages and are designated T type.
- These were numbered T1, T2, T3.....T7 by Max Delbruck (1938).

1. T-even phages are T2, T4, and T6, and
2. T-odd phages are T3, T5,...

The T4 phage of the *Myoviridae* family infects *E. coli* bacteria and is one of the largest phages.

Bacteriophages

Morphology of the T series of phages

Name	Plaque size	Head (nm)	Tail (nm)	Latent period (min)	Burst size
T1	medium	50	150 x 15	13	180
T2	small	65 x 80	120 x 20	21	120
T3	large	45	invisible	13	300
T4	small	65 x 80	120 x 20	23.5	300
T5	small	100	tiny	40	300
T6	small	65 x 80	120 x 20	25.5	200-300
T7	large	45	invisible	13	300

Structure of bacteriophages

T-phages

Structure of T4 bacteriophage

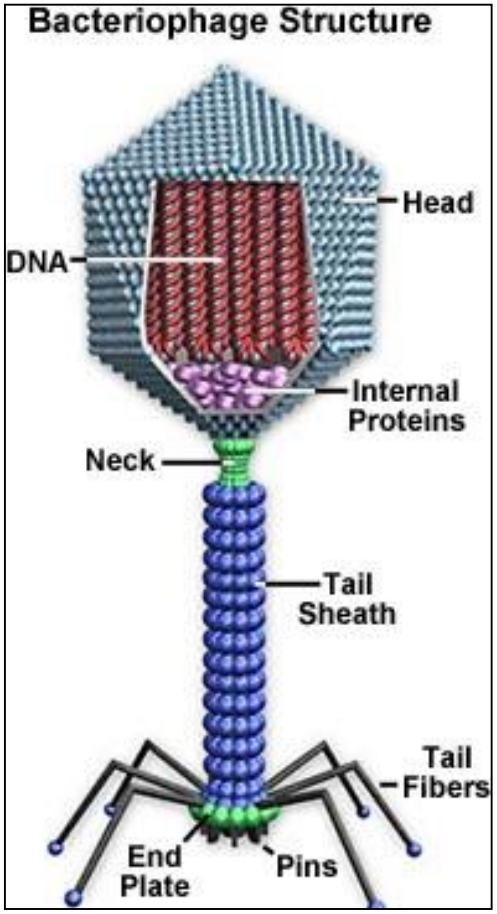
Structure of bacteriophages

T-phages

Structure of T4 bacteriophage

- Bacteriophages are in many different sizes and shapes.

1. Size: The phage T4 is among the largest phages; it is approximately 200 nm long and 80-100 nm wide.
2. Head or Capsid: All phages contain a head structure inside the head is found the nucleic acid.
3. Tail: The tail is a hollow tube through which the nucleic acid passes during infection.



Mayer,2010;..

Two types of bacteriophages infections

Lytic and lysogenic infections/Virulent phage vs. temperate phage

- Bacterial cells can undergo one of two types of infections by viruses:

1. lytic (virulent) infections. e.g. T-even phages such as T2, T4 and T6 that kill the host cells.
2. lysogenic (temperate) infections. e.g. phage lambda (phage λ). Temperate phages establish a persistent infection of the cell without killing the host cells.

BC Yang;Todar,2012

Bacteriophages

Temperate phages not suitable for the phagetherapy

- Under certain conditions, some species were able to change the mode of infection, especially if the number of host cells was falling down.
- The lysis-lysogeny decision of bacteriophage lambda (lambda) is a paradigm for developmental genetic networks.
- Therefore, temperate phages such as lambda phage are not suitable for the phagetherapy.

Orlova,2012;..

Bacterial viruses or bacteriophages

Differences between lytic and lysogenic cycles

- **In the Lytic Cycle:**

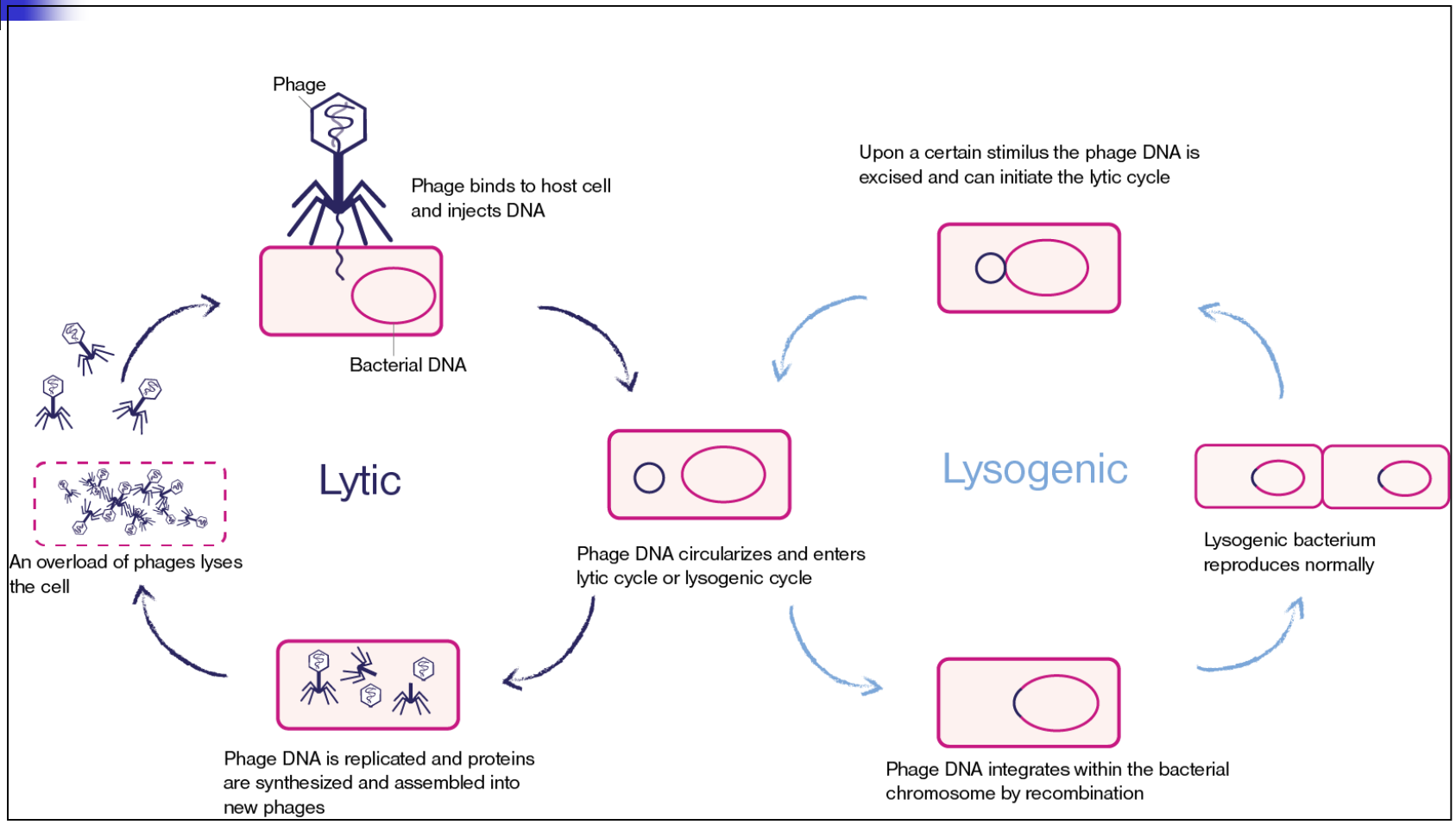
1. Viral DNA destroys cell DNA, takes over cell functions and destroys the cell.
2. The Virus replicates and produces progeny phages.
3. There are symptoms of viral infection.
4. Virulent viral infection takes place.

- **In the Lysogenic Cycle:**

1. Viral DNA merges with cell DNA and does not destroy the cell.
2. The Virus does not produce progeny.
3. There are no symptoms of viral infection.
4. Temperate viral replication takes place.

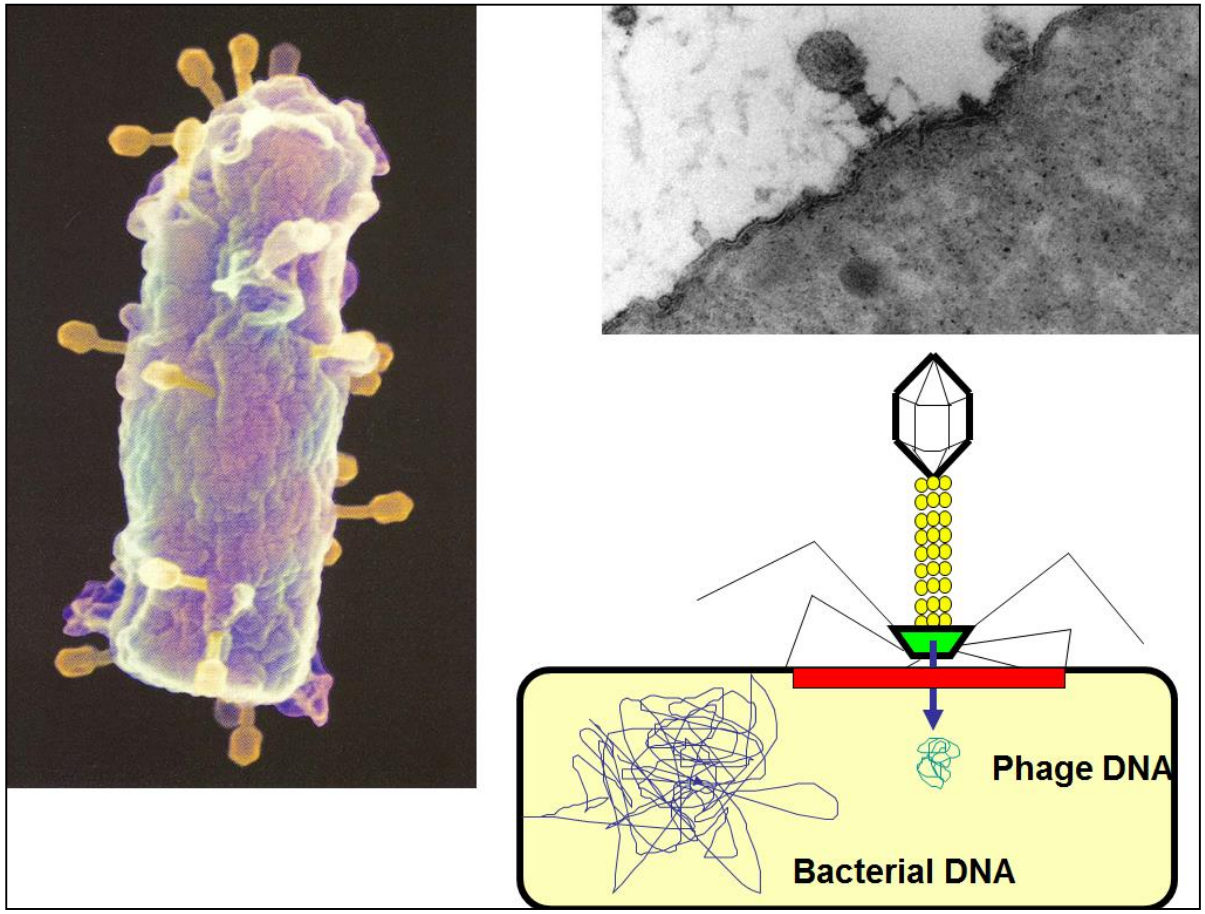
Bacteriophages

Differences between lytic and lysogenic cycles



Lytic (virulent) phage infection

Kills (lysis) the infected cell



Kelly Doran

Bacteriophage

Escherichia coli phage T4: Coliphage T4

The Lytic Life Cycle of Coliphage T4.

Fig. 2: Adsorption
Tail fibers of Coliphage T4 adsorb to receptors on the cell wall of *E. coli* B.

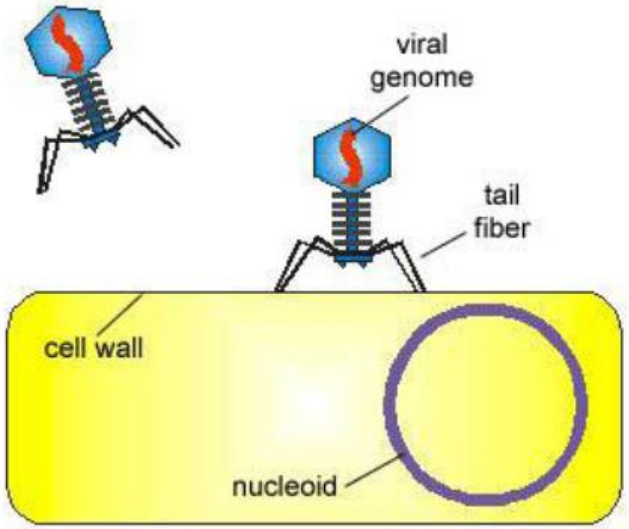
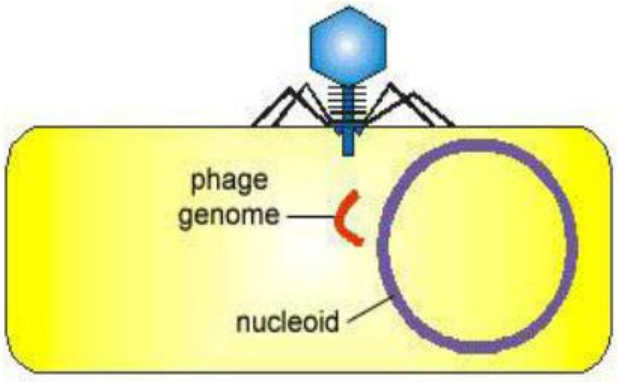


Fig. 3: Penetration
The bacteriophage injects its DNA genome into the cytoplasm of the bacterium.

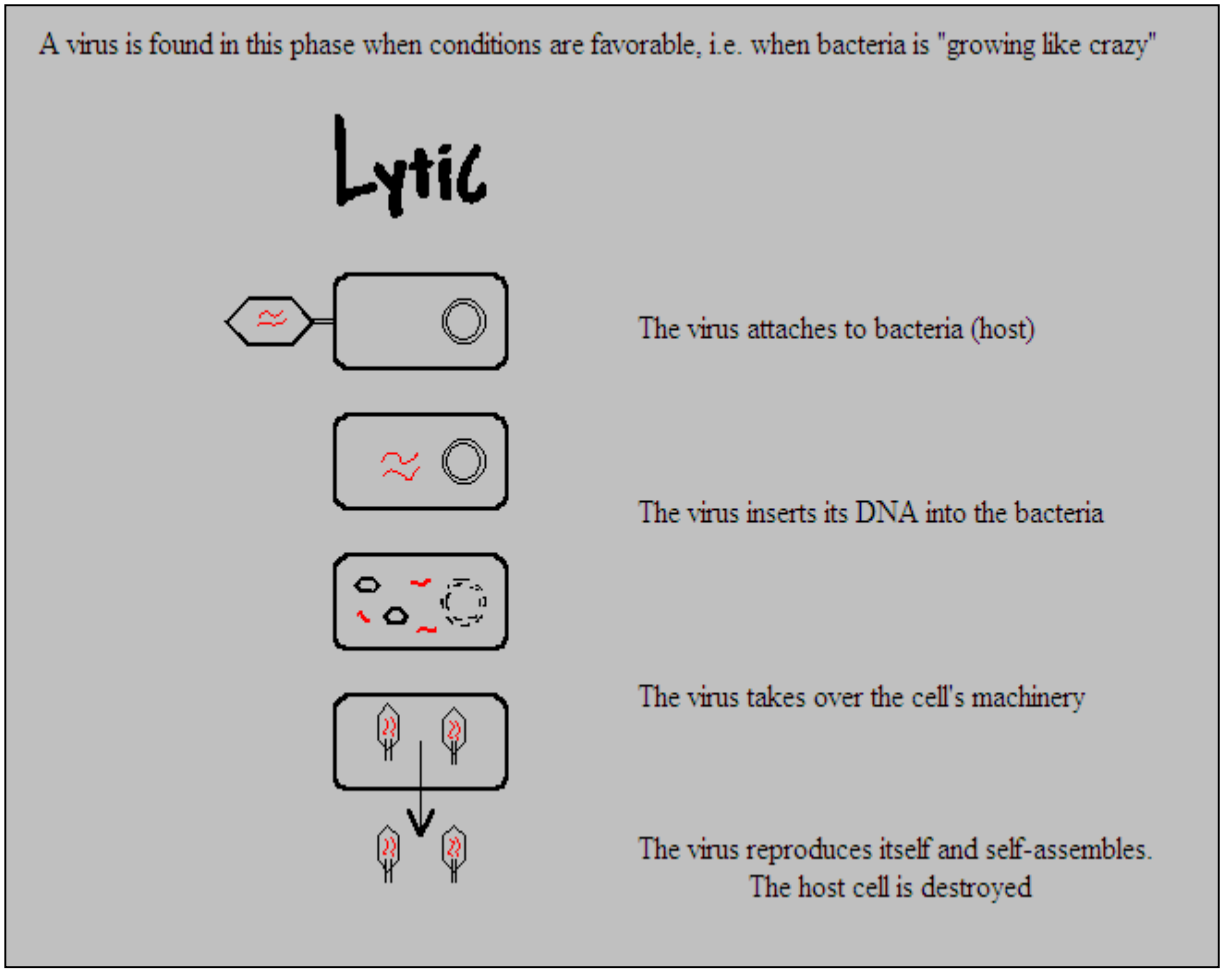


The phages that infect the bacterium *Escherichia coli* are called coliphages.

Kaiser,2009

Lytic (virulent) phage infection

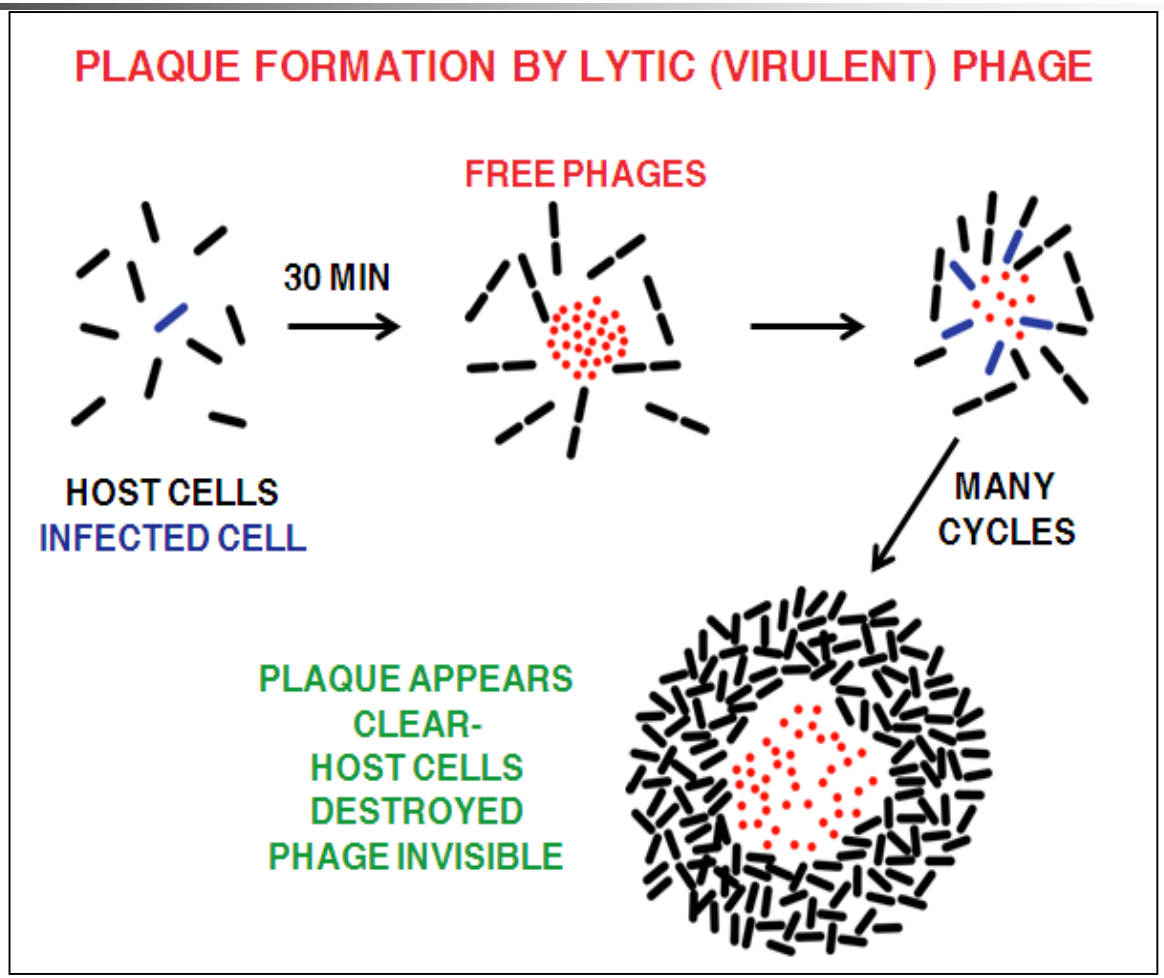
Lysis and plaque formation by lytic phage



2002-2003 Verizon Academic All-District IV Team

Lytic (virulent) phage infection

Lysis and plaque formation by lytic phage

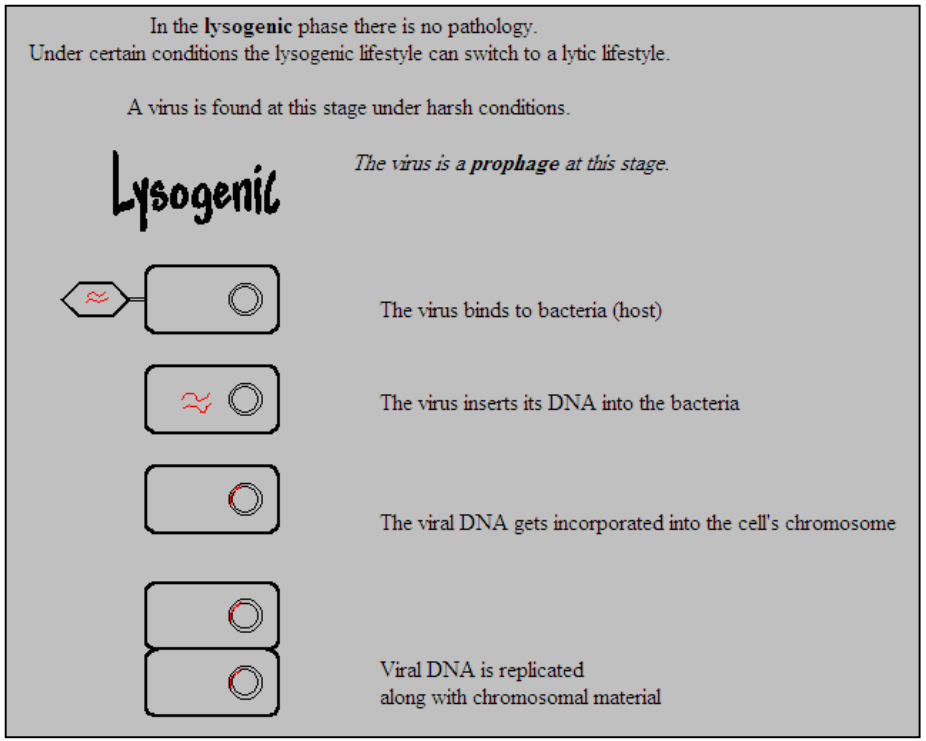


web.biosci.utexas.edu

Lysogenic (avirulent) phage infection Without killing(lysis) of the infected cell

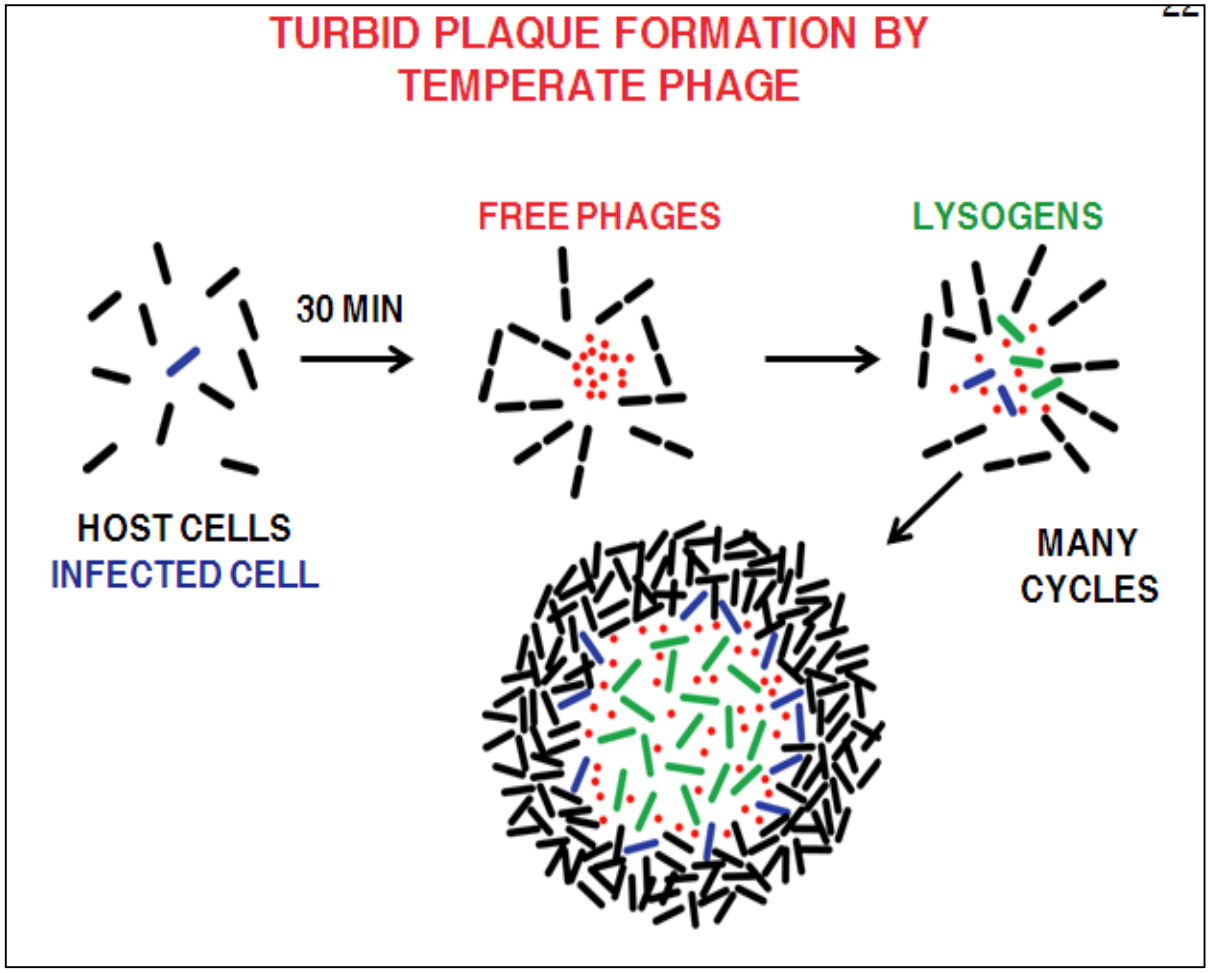
Also known as prophage

- Prophage:
- The latent form of a bacteriophage in which the viral genes are incorporated into the bacterial chromosomes without causing disruption (lysis) of the bacterial cell.
- The host is termed a lysogen when a prophage is present.



Turbid plaque formation by temperate (avirulent) phage

Without bacterial cell lysis



web.biosci.utexas.edu

Bacteriophages

Temperate phages, integrated phages or prophages

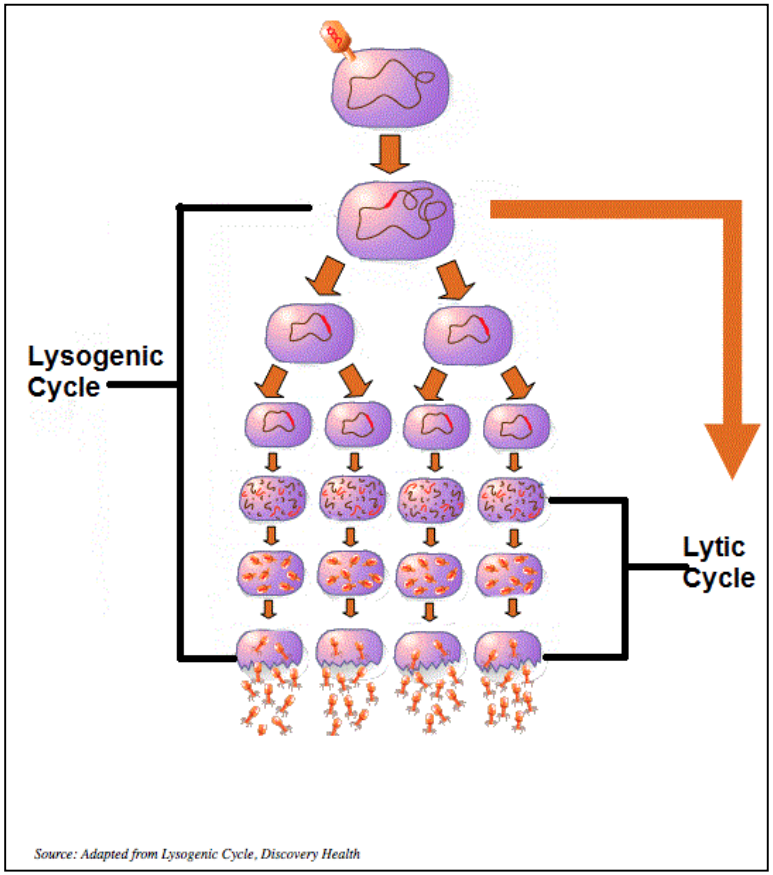
- A temperate phage such as lambda phage can integrate its genome into its host bacterium's chromosome, becoming a lysogen known as a prophage.
- The genetic material of the bacteriophage, called a prophage, can be transmitted to daughter cells at each subsequent cell division.
- Temperate bacteriophages (or phages) have the ability to enter a prophage dormant state upon infection, in which they stably replicate with the bacterial genome.

Paepe *et al.*,2014;..

Temperate phages, integrated phages or prophages
Using both the lytic and the lysogenic cycle
Horizontal gene transfer

- Temperate phages (such as lambda phage) can reproduce using both:

1. the lytic, and
2. the lysogenic cycle.



Oppenheim *et al.*,2005; Paepe *et al.*,2014

Temperate phages, integrated phages or prophages
Using both the lytic and the lysogenic cycle
Horizontal gene transfer

- Temperate phages (such as lambda phage) can reproduce using both the lytic and the lysogenic cycle.
- After infection of the host bacterium, a decision between lytic or lysogenic development is made that is dependent upon:

1. Environmental signals, and
2. The number of infecting phages per cell.

- Genomic comparisons suggest a remarkable level of horizontal gene transfer among temperate phages, favoring a high evolution rate.

Oppenheim *et al.*,2005; Paepe *et al.*,2014

Defective prophage(probacteriophage)

Using both the lytic and the lysogenic cycle

Transduction

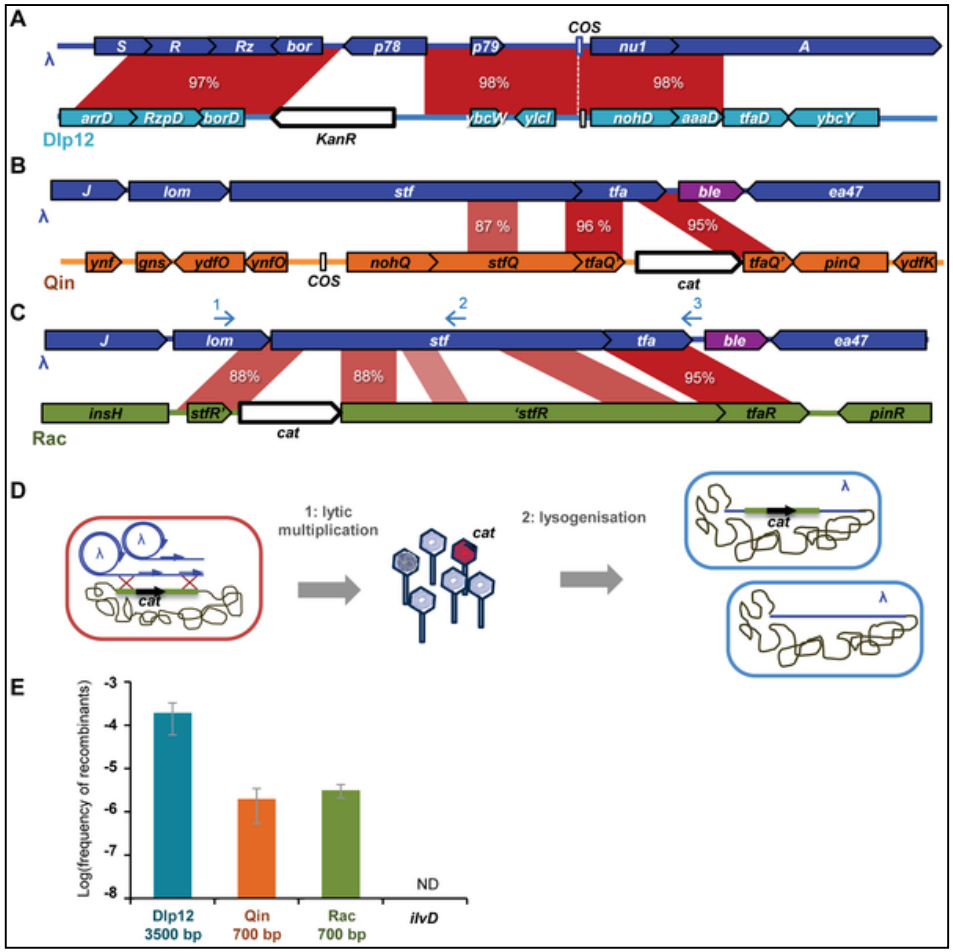
- A temperate bacteriophage mutant that cannot fully infect but that can replicate in the bacterial genome as a defective probacteriophage.
- A temperate bacteriophage mutant the genome of which does not contain all of the normal components and cannot become a fully infectious virus, but it can replicate indefinitely in the bacterial genome as a defective probacteriophage; many defective bacteriophages are mediators of transduction.

Paepe *et al.*,2014;..

Bacterial viruses or bacteriophages
Temperate phages or prophages

Recombinants between λ and defective prophages are formed during lytic cycle.

The mechanisms of genomic exchanges between an invasive infectious phage and defective prophages residing in the host (bacterial) chromosome.



Paepe *et al.*,2014

Bacterial viruses or bacteriophages

Temperate phages, integrated phages or prophages

Their contribution in increasing bacterial virulence

- Integrated phages (prophages) are major contributors to the diversity of bacterial gene repertoires.
- A majority of bacterial genomes contain multiple active or defective prophages, and numerous bacterial phenotypes are modified by these prophages, such as increased virulence.
- These mobile genetic elements are subject to high levels of genetic exchanges, through which new genes are constantly imported into bacterial genomes.

Paepe *et al.*,2014

Bacteriophages

Phage typing

- Phage can be very specific in what bacteria they infect and the pattern of infection by many phage may be employed in phage typing to distinguish bacterial species and strains.

Bacteriophages
Association with plant hosts

- Phage have been found in association with:
- Buds, leaves, root nodules (leguminous plants), roots, rotting fruit, seeds, stems and straw; crown gall tumors…
- healthy or diseased alfalfa, barley, beans, broccoli, Brussels sprouts, buckwheat, clover, cotton, cucumber, lucerne, mulberry, oats peas, peach trees, radish, rutabaga, ryegrass, rye, timothy, tobacco, tomatoes, [and] wheat".

Gill and Abedon,2003

Phage therapy

The application of bacteriophages in agricultural practices and food product industries

- The application of bacteriophages in agricultural practices and food product industries has been legally approved in several countries(Fernández *et al*.,2018).
- Bacteriophages have been found to be effective for the control of several pathogenic phytobacterial strains, such as *Erwinia*/ *Pectobacterium* spp., causing bacterial soft rot and fire blight on apple and pear (Park *et al*.,2018), *Xanthomonas* sp., pathogenic strains of bacterial spot of tomato, peach, geranium, citrus, walnut blight, leaf blight of onion, and citrus canker (Dong et al.,2018), *Ralstonia solanacearum*, provoking bacterial wilt of tomato (Wang *et al*.,2019), and *Streptomyces scabies*, producing potato scab (Abdelrhim *et al*.,2021).

Yun *et al*.,2022

Host range of most phages
Rhizosphere and phyllosphere phages

- The host range of most phages is relatively narrow- typically limited to only a single bacterial genus, species, or, often, even to only a limited number of strains within a given species.
- The phages may be found in:

1. Rhizosphere (The phage rhizosphere),
2. Aerial parts of plants (The phage phyllosphere).

- Phages, however, are usually too specific or not specific enough.

Gill and Abedon,2003

Bacteriophages
Association with plant hosts

- Bacteriophages were evaluated based on:

1. Plaque morphology,
2. Chloroform sensitivity,
3. Host range,
4. Genome size,
5. DNA restriction profile, and
6. Virion morphology (the virus particle as it exists outside an infected host cell).

Phage isolation

- The tissue samples were placed in plastic freezer bags or 125 ml flasks and after the addition of 50 ml deionized (DI) or sterilized tap water were shaken for 20 min.
- 2 ml were collected and centrifuged at 10,000 g for 10 min to remove debris.
- The supernatants were either treated with chloroform or filter-sterilized and then were checked for the presence of bacteriophages by spotting 20 µl onto freshly prepared lawns of the indicator bacteria.
- If lysis was observed after 24 h incubation at 28°C, the phage was purified by three successive single plaque isolations and then propagated and stored.

Isolation of phages
Isolation from nature

- All samples must be liquid.
- Soil and other solid material are homogenized and suspended in an appropriate medium.
- Solids and bacteria are removed, usually by filtration preceded or not by centrifugation.
- Very rich samples can be assayed directly on indicator bacteria.
- In most cases, phages must be enriched by incubating the sample in a liquid medium inoculated with indicator bacteria.
- The culture is then filtered and titrated and phages are purified by repeated cloning of single plaques.
- Large samples must be concentrated before enrichment.
- This is done by centrifugation, filter adsorption and elution, flocculation, or precipitation by polyethylene glycol 6000.
- Adsorption-elution techniques may involve strongly acidic or alkaline conditions that inactivate phages.

Ackermann,2004

Isolation of phages

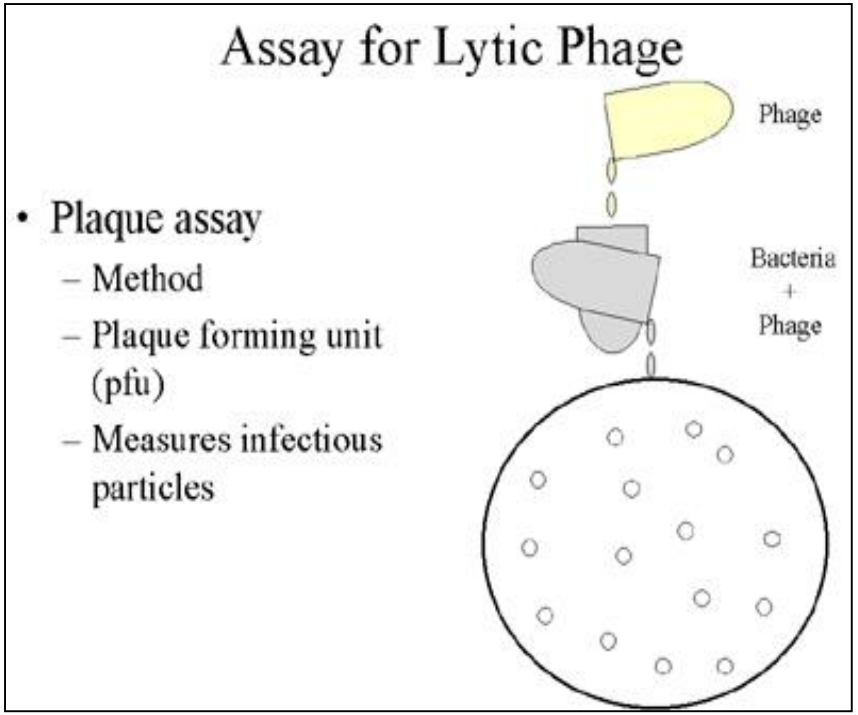
Isolation from lysogenic bacteria

- Many bacteria produce phages spontaneously.
- These phages may be detected by testing culture filtrates on indicator bacteria.
- It is generally preferable to induce phage production by mitomycin C, ultraviolet (UV) light, or other agents.
- A suspension of growing bacteria is exposed to the agent (e.g., 1g/ml of mitomycin C for 10 min or UV light for 1 min), incubated again, and then filtered.
- After mitomycin C induction, the bacteria should be separated from the agent by centrifugation and transferred into a fresh medium.
- Bacteriocins (see Section II.C), which are a source of error, are easily identified because they cannot be propagated and do not produce plaques when diluted.

Ackermann,2004

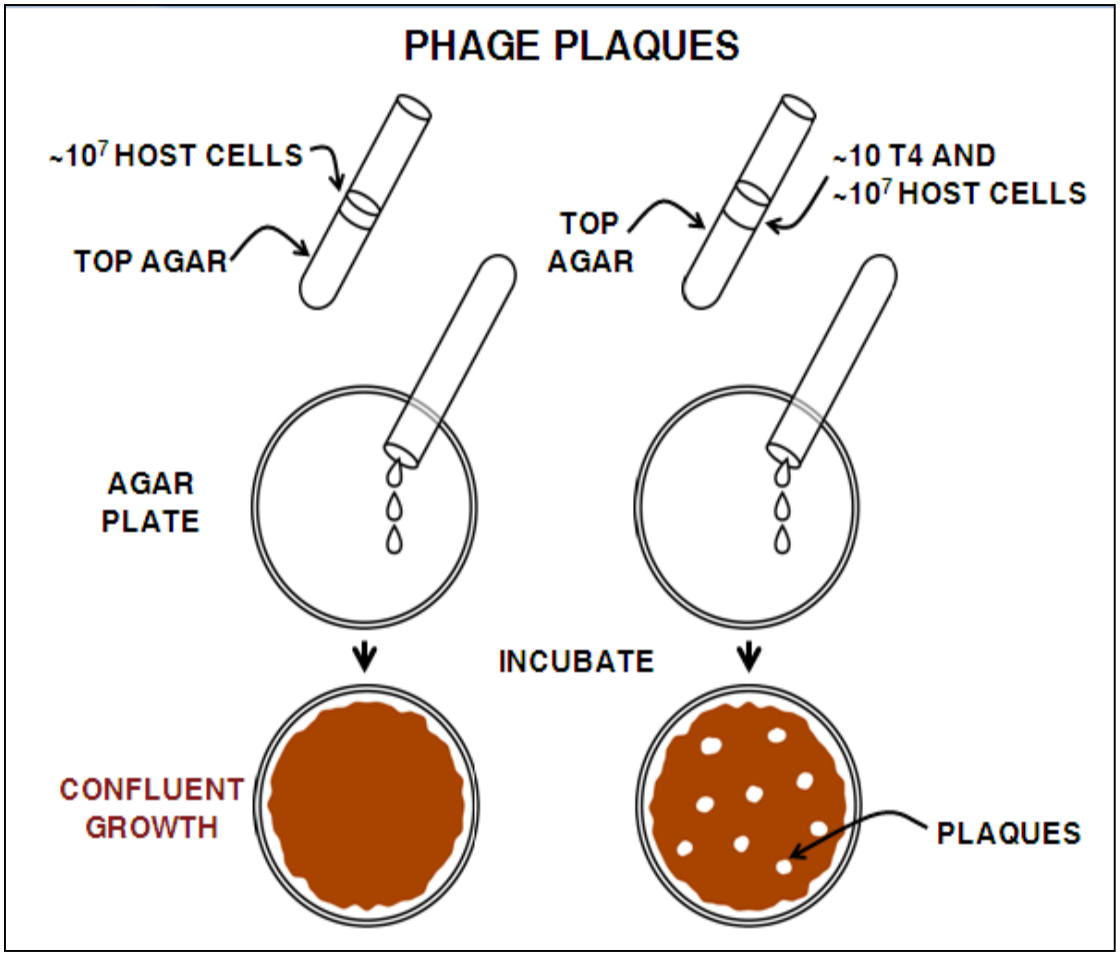
Evaluation of bacterial sensitivity
Plaque assay

- Lytic phage are enumerated by a plaque assay.
- A plaque is a clear area which results from the lysis of bacteria.
- Each plaque arises from a single infectious phage.
- The infectious particle that gives rise to a plaque is called a pfu (plaque forming unit).



Mayer,2010

Evaluation of bacterial sensitivity
Plaque assay

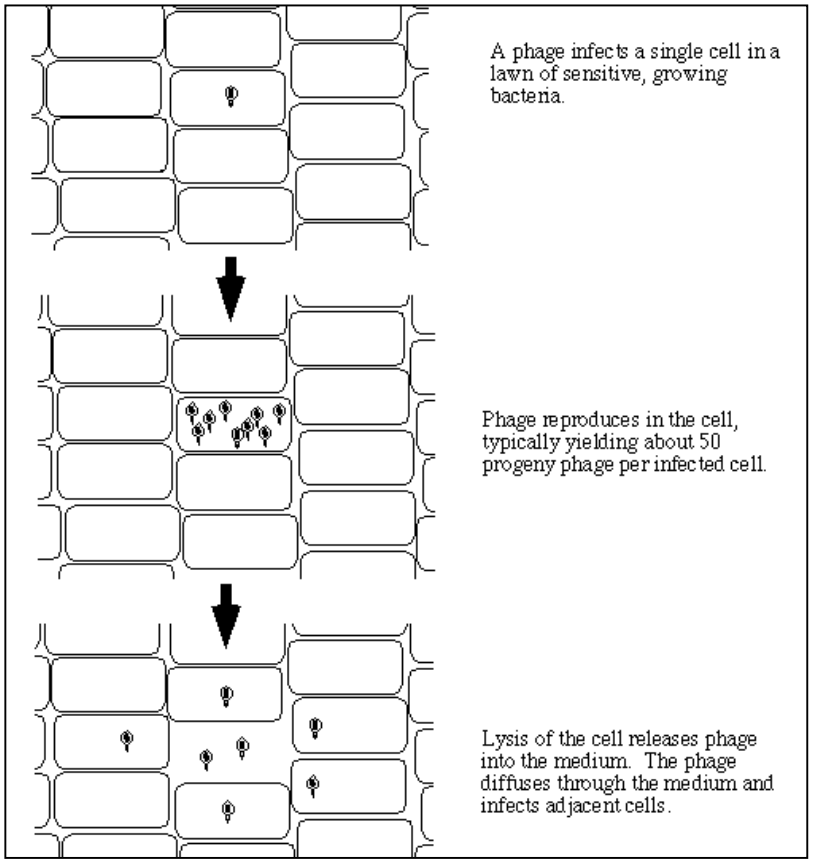


web.biosci.utexas.edu

Plaque assay

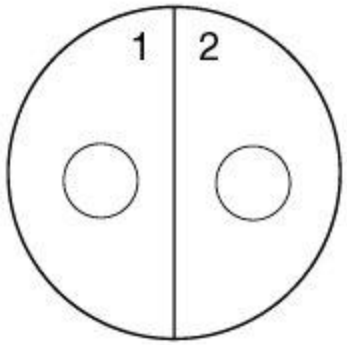
Plaques are clear zones formed in a lawn of cells due to lysis by phage

- At a low multiplicity of infection (MOI) a cell is infected with a single phage and lysed, releasing progeny phage which can diffuse to neighboring cells and infect them, lysing these cells then infecting the neighboring cells and lysing them, etc, ultimately resulting in a circular area of cell lysis in a turbid lawn of cells.



maloy@sciences.sdsu.edu

Phage typing
Spot test

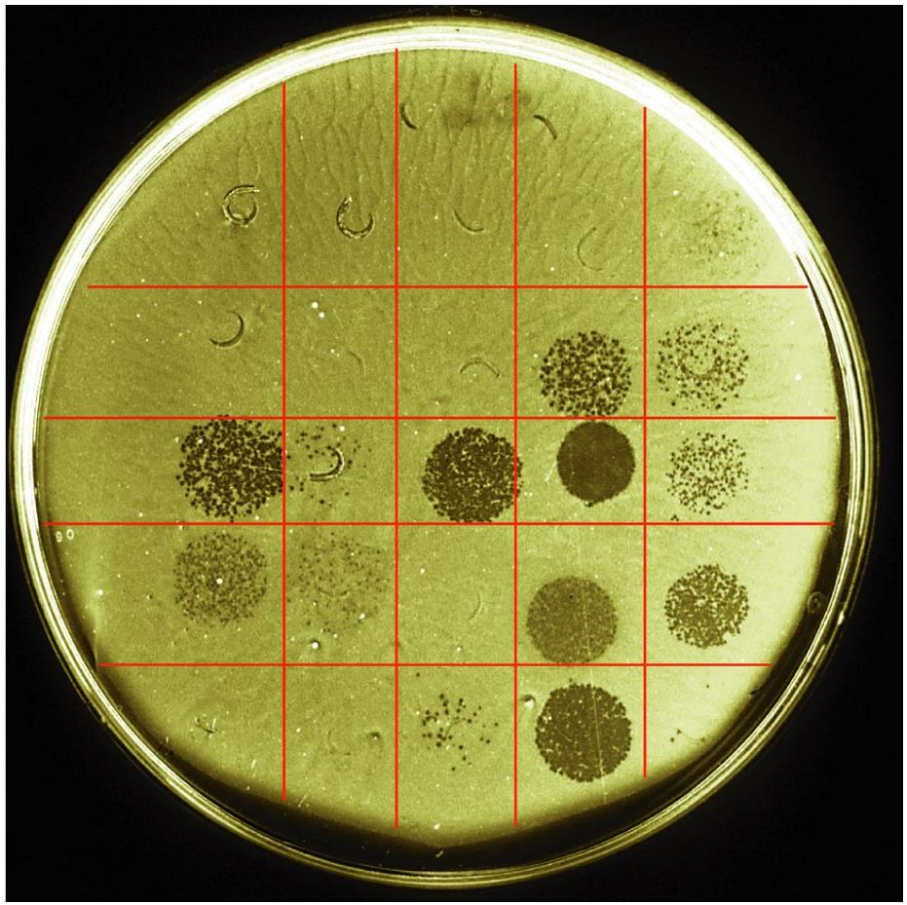


- Quickly pour the bacteria-bacteriophage mixtures onto separate plates of Trypticase Soy agar and swirl to distribute the contents over the entire agar surface.
- Using a wax marker, draw a line on the bottom of plates dividing them in half or many more sectors.
- Number the sectors 1, 2, 3,…
- Draw a circle about the size of a dime in the center of each of the sectors.
- Using a sterile Pasteur pipette and rubber bulb, add 1 drop of Coliphage T4 or any other phages to each sector in the area outlined by the circle.
- Incubate the 2 TSA plates right side up at 37C.
- Observe plaques formation.

Kaiser,2009

Phage typing

Spot test



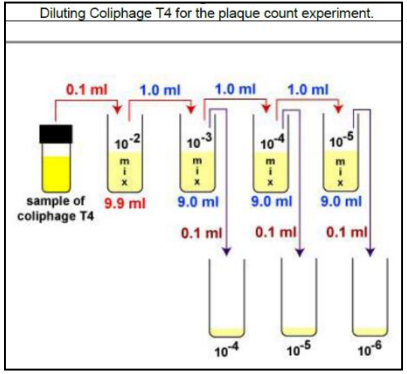
Karlm,2004

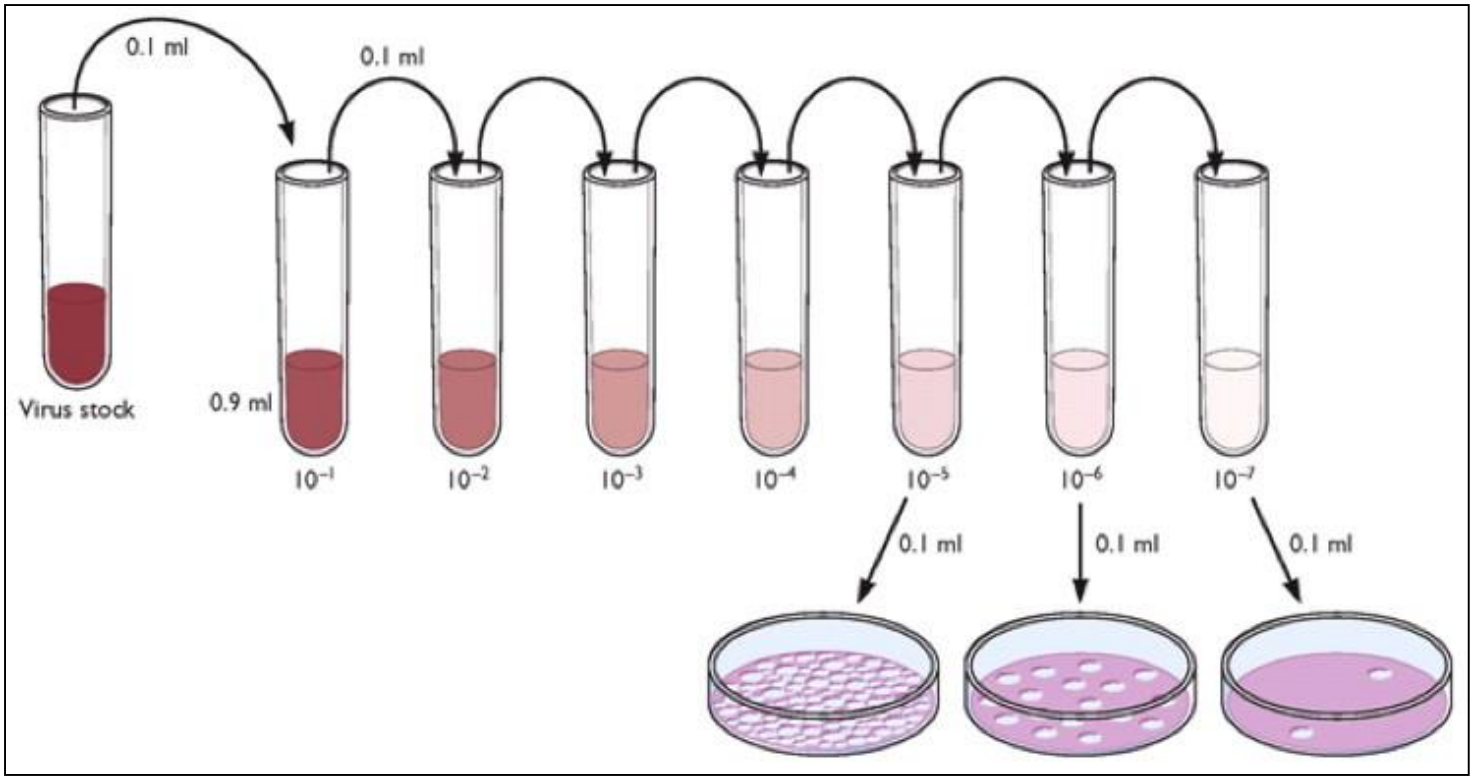
Plaque count
Determination of titer

- Phage concentrations were determined by dilution-plating-plaque count assay on NYA plates without bottom agar as previously described.
- One hundred microliter aliquots of dilutions of phage suspensions were mixed with 100 µL of concentrated bacterial suspension in empty Petri dishes and then 12 ml warm (48°C) NYA medium was poured in each dish.
- The dishes were gently swirled to evenly distribute the bacteria and the phages within the medium.
- After the medium solidified, the plates were transferred to 28°C incubators and the plaques were counted on the appropriate dilutions after 24 or 48 hours.
- The phage concentration was calculated from the plaque number and specific dilution and was expressed as PFU/mL.

Plaque count

Determination of titer

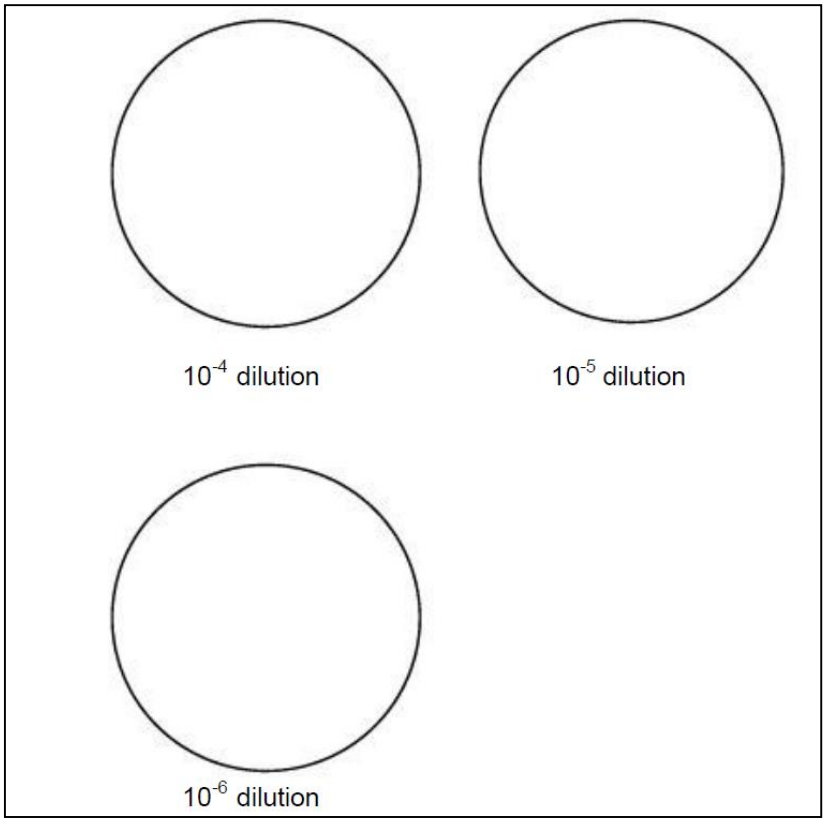




Virology blog,2009; Kaiser,2009

Plaque count
Determination of titer

- Observe the 3 plates for plaque formation and make a drawing.



Kaiser,2009

Phage propagation

- Phages were recovered from storage, purified by single plaque isolations and then mass streaked on the freshly prepared lawn of the propagating host.
- The next day phages were eluted from the plate, sterilized and enumerated, as described above.
- The eluate was used for infecting 500 mL actively growing culture of the propagating strain (10^8 cfu/mL) grown in NB liquid medium in 1 liter flasks, at 0.1 multiplicity of infection (MOI), (i.e., the phage concentration at the beginning of the incubation was 10^7 PFU/mL).
- After addition of the phage and 5-min incubation on the bench top, the culture was shaken at 150 rpm at 28ºC for 18 h.
- The resulting culture was sterilized; phages were enumerated and stored at 4°C in the dark until use.
- This method yielded phage titers of approximately 10^{10} PFU/mL.

Phage purification and storage

- Phages were purified by three subsequent single plaque isolations.
- Single plaque isolations were carried out by transferring phages from isolated plaques to a fresh lawn of the host bacterium using sterile toothpicks and then quadrant streaking them with sterile plastic transfer loops.
- Following purification the phages were propagated by mass streaking on fresh lawns of the host.
- After a 24-h incubation at 28ºC, the phages were eluted(removed) by pouring 5 ml sterilized tap water into the 100 mm×15 mm Petri dishes and gently shaking the plates (~20 rpm) for 30 min.

Phage purification and storage

- The eluate was centrifuged (10,000 g, 10 min), treated with chloroform or filter-sterilized, depending on the phage, then quantified as described below, and stored in 2-mL plastic vials at 4°C in complete darkness.
- The concentrations of these suspensions were approximately 10^9 plaque forming units (PFU) per ml.

Phage storage

- No single technique is suitable for all phages.
- Many phages can be kept as lystes at 4°C or in lyophile, but others are quickly inactivated under these conditions.
- Lystes should be kept without additives such as thymol or chloroform.
- The best procedures seems to be preservation at 70°C in 50% glycerol.
- Phages may also be preserved in liquid nitrogen, by drying on filter paper, and, in the case of endospore-forming bacteria, by trapping phage genomes in spores.
- Ideally, any phage should be preserved by several techniques.

Ackermann,2004

Phage identification

- Phase identification relies greatly on the observation that most phages are specific for their host genus; however, enterobacteria, in which polyvalent phages are common, are considered in this context as a single "genus."
- Phages are first examined in the electron microscope.
- This usually provides the family diagnosis and often indicates relationships on the species level.
- If no phages are known for a given host genus or only phages of different morphology, the new isolate may be considered as a new phage.

Ackermann,2004

Phage identification

- If the same host genus has phages of identical morphology, they must be compared to the isolate by:

1. DNA–DNA hybridization, and/or
2. serology.

- Further identification may be achieved by:

1. Determining restriction endonuclease cleavage patterns, or
2. Constitutive proteins.

Ackermann,2004

Bacteriophages

Main properties and frequency of phage families[a]

Talked, cubic, filamentous and pleomorphic shaped

Shape	Nucleic acid	Family	Genera	Particulars	Example	No. of Members[b]
Tailed	DNA, ds, L	Myoviridae	6, see text	Tail contractile	T4	1143
		Siphoviridae	6, see text	Tail long, noncontractile	λ	3011
		Podoviridae	3, see text	Tail short	T7	698
Cubic	DNA, ss, C	Microviridae	*Microvirus* *Bdellomicrovirus* *Chlamydiomicrovirus* *Spiromicrovirus*	Conspicuous capsomers	ϕX174	40
	DNA, ds, C, S	Corticoviridae	*Corticovirus*	Complex capsid, lipids	PM2	3?
	DNA, ds, L	Tectiviridae	*Tectivirus*	Double capsid, pseudo-tail, lipids	PRD1	18
	RNA, ss, L	Leviviridae	*Levivirus* *Allolevirirus*		MS2	39
	RNA, ds, L, M	Cystoviridae	*Cystovirus*	Envelope, lipids	ϕ6	1
Filamentous	DNA, ss, C	Inoviridae	*Inovirus*	Long filaments	fd	57
			Plectrovirus	Short rods	L51	
	DNA, ds, L	Lipothrixviridae	*Lipothrixvirus*	Envelope, lipids	TTV1	6?
	DNA, ds, L	Rudiviridae	*Rudivirus*	Stiff rods, no envelope, no lipids	SIRV1	2
Pleomorphic	DNA, ds, C, S	Plasmaviridae	*Plasmavirus*	Envelope, no capsid, lipids	MVL2	6?
	DNA, ds, C, S	Fuselloviridae	*Fusellovirus*	Lemon-shaped, envelope, lipids	SSV1	8?

[a]Modified from Ackermann (1987) with permission of Blackwell Scientific Publications Ltd. C, circular; L, linear; M, multipartite; S, supercoiled; ss, single-stranded; ds, double-stranded.
[b]Exluding phage-like bacteriocins and known defective phages. Computed October 31, 2000.

Phi (Φ) = most often pronounced fi is simply an irrational number like pi (p= 3.14).

Ackermann,2004

Bacteriophages
Phage naming

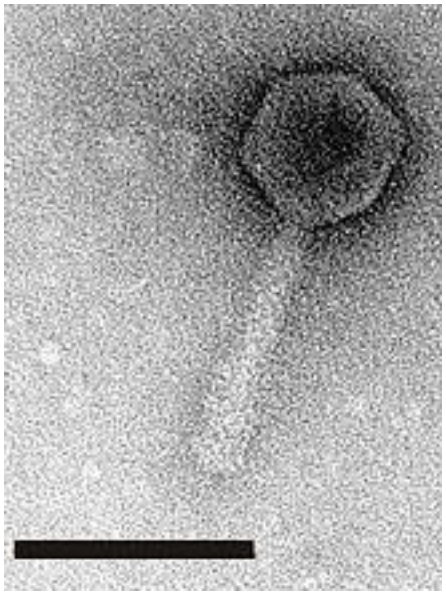
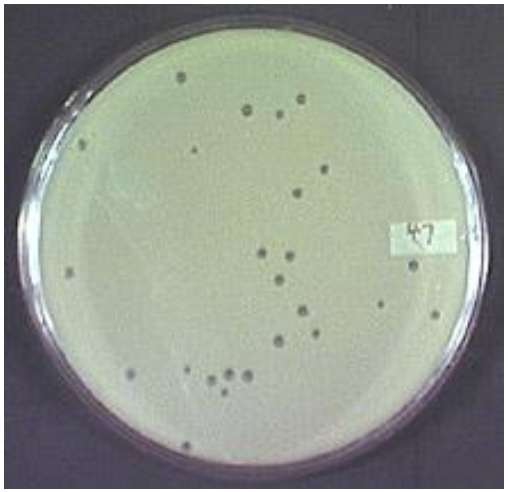
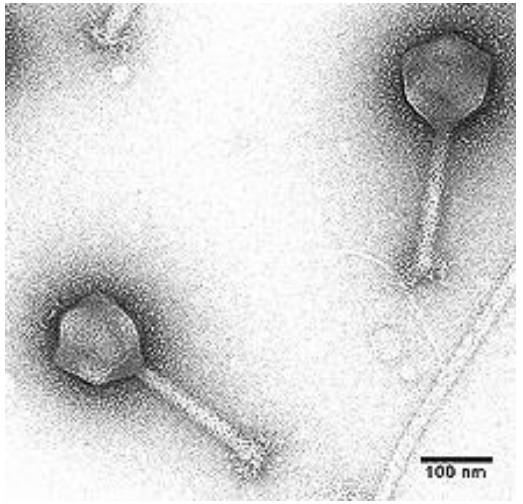
- Bacterial viruses are specific to one or a limited number of bacteria; thus, they are named after:

1. the bacteria group,
2. strain, or
3. species that they infect.

- E.g.
- *Erwinia amylovora* phage isolate PEa 31-2;
- Phages CP1 and CP2 of *Xanthomonas citri*, the causal agent of citrus canker disease.

Everything about Bacteriophage,2013;..

Phages of Enterobacteriaceae

		
LG1 plaques on a wide range of *E. coli* and a few Enterobacteriaceae	**Phage plaques on a *Erwinia* lawn**	***Erwinia amylovora* phage isolate PEa 31-2**

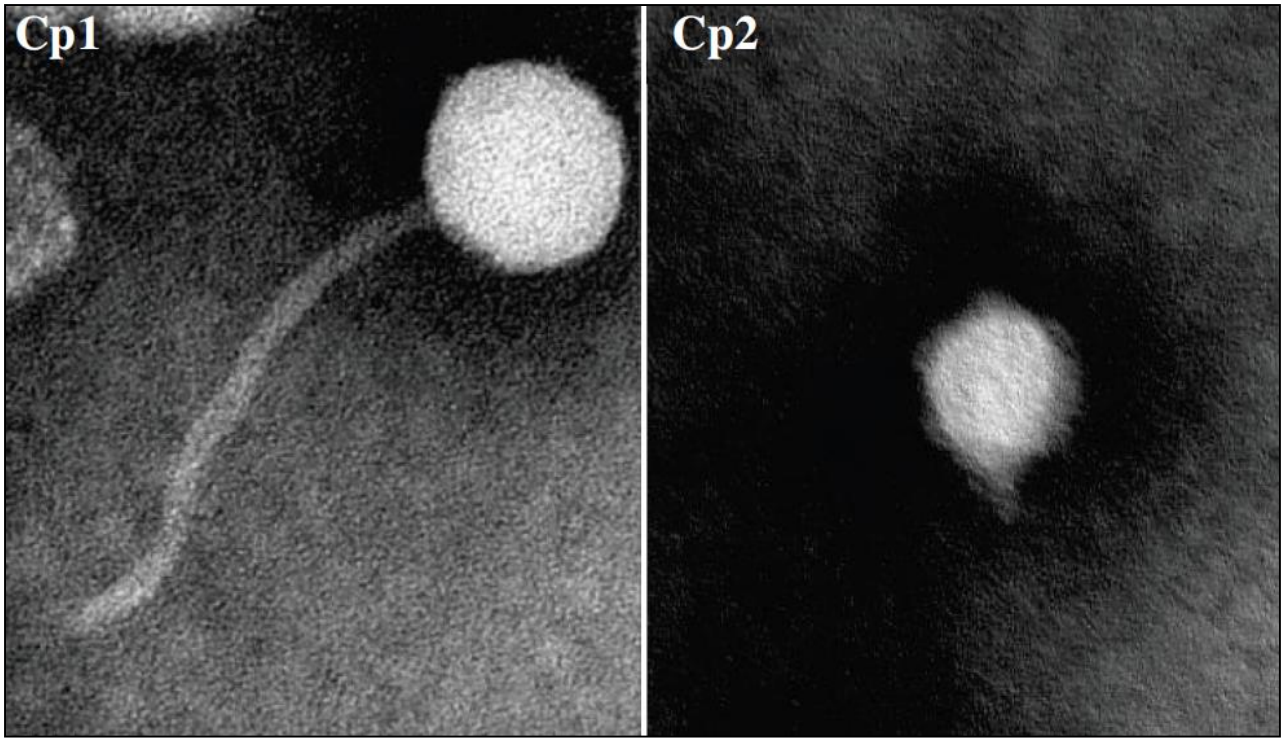
Gill and Abedon,2003

Phage typing of *Xanthomonas* strains

- Twelve phages were used in the phage typing study:
- ɑ-MME, Φ5536, ΦXacm4-11, ΦXv3-21, ΦXaacA1, ΦXaacF1, ΦXaacF8, Φcc19-1, Φcc13-2, CP1, CP2 and CP3.
- The bacterium-phage interactions were scored as:
- Sensitive=2
- Moderately sensitive=1
- Resistant=0
- Similarity matrix was calculated from the phage typing scores using the Pearson correlation, and a dendrogram of relatedness was prepared in which the clustering was achieved by UPGMA (unweighted pair group method using arithmetic averages) with Bionumerics software package version 3.0.

Balogh,2006

Phage typing of *Xanthomonas* strains
Morphology of Cp1 and Cp2 particles



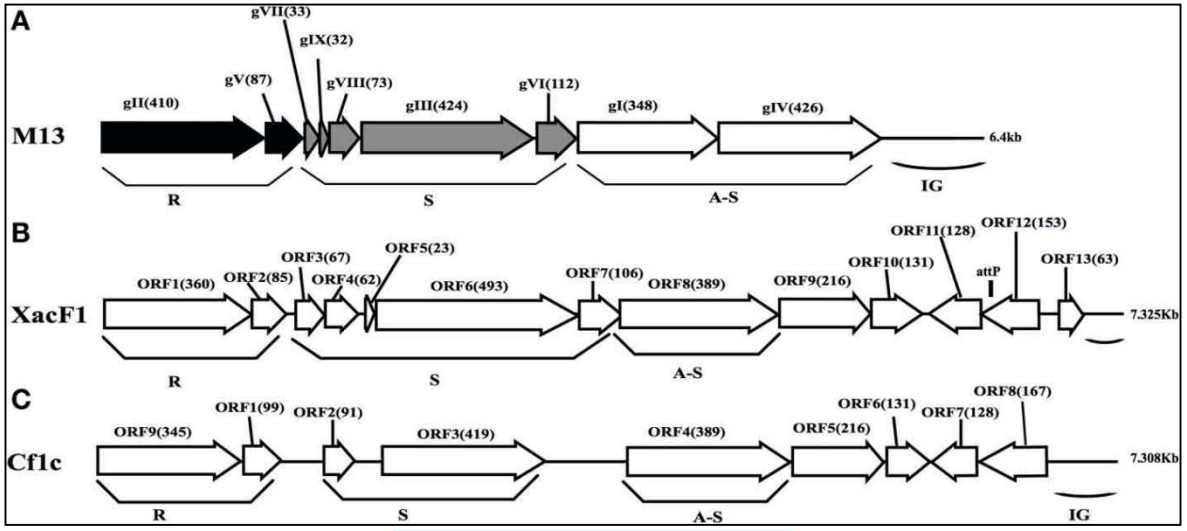
Ahmad *et al.*,2015

Phage typing of *Xanthomonas* strains

Genomic organization of phage XacF1

Linear genomic maps of *E. coli* phage M13 (A), XacF1 (B), and Cf1c (C) are compared

- Arrows oriented in the direction of transcription represent ORFs or genes. The functional modules for replication(R), structure (S), and assembly-secretion(A-S) are indicated according to the M13 model. Map for Cf1c was drawn according to the genomic sequence. ORF sizes (in amino acids) are in parentheses. IG (intergenic region), and *att*P are also shown.

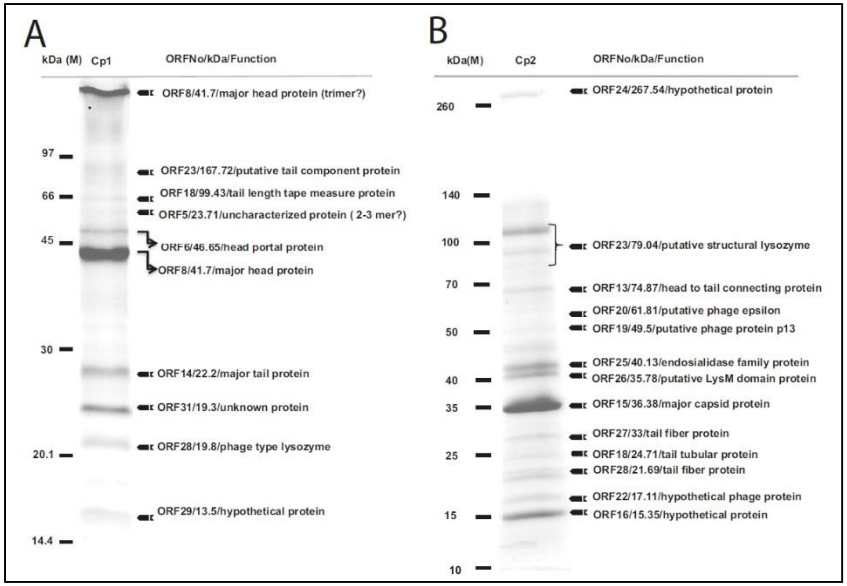


Ahmad *et al.*,2014

Phage typing of *Xanthomonas* strains

Proteomic analysis of Cp1 (A) and Cp2 (B) particles

- Proteins of purified phage particles were separated in 10% (wt/vol) polyacrylamide gel by SDS-PAGE and stained with Coomassie brilliant blue.
- The protein bands were recovered, digested in gel, and subjected to LC-MS/MS analysis.
- On the right are the descriptions of the genes, their deduced molecular sizes based on the ORF sequences, and their possible functions.
- Positions of size markers are shown on the left.
- For some protein bands, possible oligomerization of the phage protein was observed.



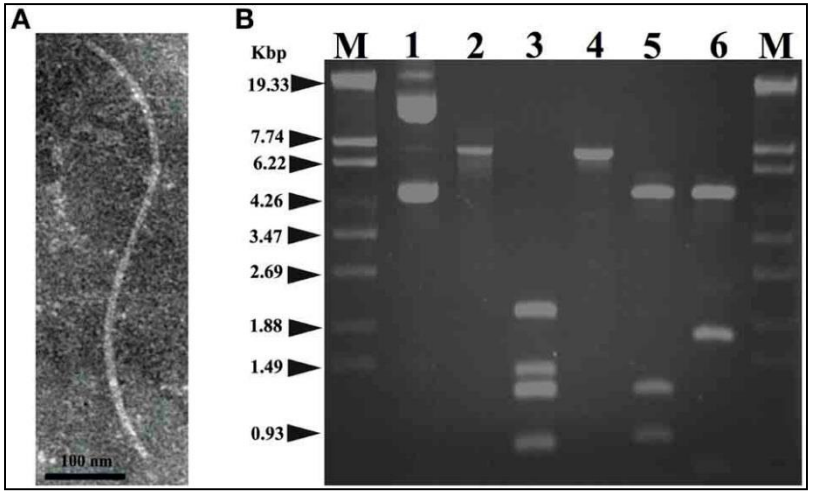
Ahmad *et al.*,2014

Phage typing of *Xanthomonas* strains
Morphology and genomic DNA of XacF1

A. Morphology of the filamentous XacF1 phage. The purified particles of XacF1 were negatively stained with phosphotungstate and examined by transmission electron microscopy. A filamentous structure was observed (approximately 600nm in length).

B. Restriction patterns of the replicative form of the XacF1 genomic DNA.

- Lanes:1,undigested XacF1DNA(RF);2, digested with S1 nuclease;3, *Hin*cII; 4, *Eco*RI; 5, *Eco*RV;6, *Cla*I; M, λ *Sty*I marker.



Ahmad *et al.*,2014

Phage typing of *Xanthomonas* strains

Chloroform sensitivity, plaque morphology and host range of bacteriophages originating in Florida, Argentina and Japan.

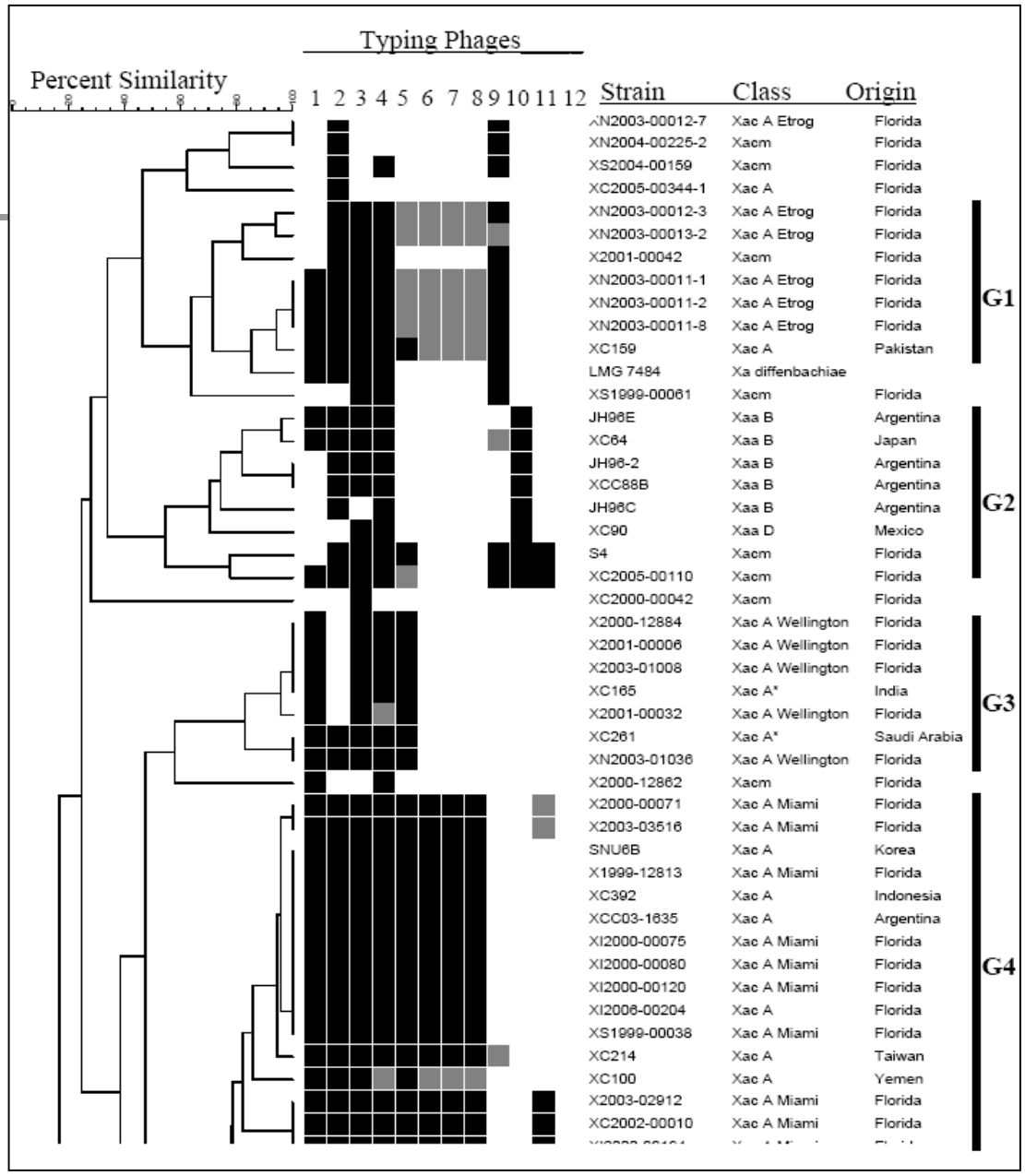
	Chloroform sensitivity	Plaque type[b], diameter (mm)	Host range[d]				
			BV38	XC90	Xac41	Xac65	Xac15
ΦXaacF1	R[a]	clear, 2-3	-[c]	-	++	++	-
ΦXaacF2	R	clear, 2-3	-	-	++	++	-
ΦXaacF3	R	clear, 2-3	-	-	++	++	-
ΦXaacF4	R	clear, 2-3	-	-	++	++	-
ΦXaacF5	R	clear, 2-3	-	-	++	++	-
ΦXaacF6	R	clear, 2-3	-	-	++	++	-
ΦXaacF7	S	turbid, 1	++	-	-	++	++
ΦXaacF8	S	turbid, 1	++	-	-	++	++
ΦXaacF9	S	turbid, 1	++	-	-	++	++
ΦXaacF10	R	clear, 2-3	-	-	++	++	-
ΦXaacF11	S	turbid, 1	++	-	-	++	++
ΦXaacF12	R	clear, 2-3	-	-	++	++	-
ΦXaacF13	S	turbid, 1	++	-	-	++	++
ΦXaacF14	R	clear, 2-3	-	-	++	++	-
ΦXaacF15	R	clear, 2-3	-	-	++	++	-
ΦXaacF16	R	clear, 2-3	-	-	++	++	-
ΦXaacF17	R	clear, 2-3	-	-	++	++	-
ΦXaacF18	R	clear, 2-3	-	-	++	++	-
ΦXaacF19	R	clear, 2-3	-	-	++	++	-
ΦXaacF20	R	clear, 2-3	-	-	++	++	-
ΦXaacF21	R	clear, 2-3	-	-	++	++	-
ΦXaacF22	R	clear, 2-3	-	-	++	++	-
ΦXaacF23	R	clear, 2-3	-	-	+	++	-
ΦXaacF24	R	clear, 2-3	-	-	++	++	-
ΦXaacF25	S	turbid, 1	++	-	-	++	++
ΦXaacF26	R	clear, 2-3	-	-	++	++	-
ΦXaacF27	R	clear, 2-3	-	-	++	++	-
ΦXaacF28	R	clear, 2-3	-	-	++	++	-
ΦXaacF29	R	clear, 2-3	-	-	++	++	-
ΦXaacF30	R	clear, 2-3	-	-	++	++	-
ΦXaacF31	R	clear, 2-3	-	-	++	++	-
ΦXaacF32	R	clear, 2-3	-	-	++	++	-
ΦXaacF33	R	clear, 2-3	-	-	++	++	-
ΦXaacF34	R	clear, 2-3	-	-	++	++	-
ΦXaacF35	R	clear, 2-3	-	-	++	++	-
ΦXaacF36	R	clear, 2-3	-	-	++	++	-
ΦXaacF37	R	clear, 2-3	-	-	++	++	-
ΦXaacA1	R	clear, 3-4	++	-	++	++	-
ΦXaacA2	R	clear, 3-4	++	-	++	++	-
ΦXaacA3	R	clear, 3-4	++	-	++	++	-
ΦXaacA4	R	clear, 3-4	++	-	++	++	-
ΦXaacA5	R	clear, 3-4	++	-	++	++	-
ΦXaacA6	R	clear, 3-4	++	-	++	++	-
ΦXaacA7	R	clear, 3-4	++	-	++	++	-
ΦXaacA8	R	clear, 3-4	++	-	++	++	-
ΦXaacA9	R	clear, 3-4	++	-	++	++	-

[a] S: sensitive, R: resistant.
[b] Plaque morphology was evaluated on the propagating host (Table 3-2) after 36 h.
[c] ++: sensitive, -: resistant.
[d] Strains Xacm36, Xacm45, Xacm47 and 306 were resistant to all phages. Strain BV42 had identical profile to Xac65.

Balogh,2006

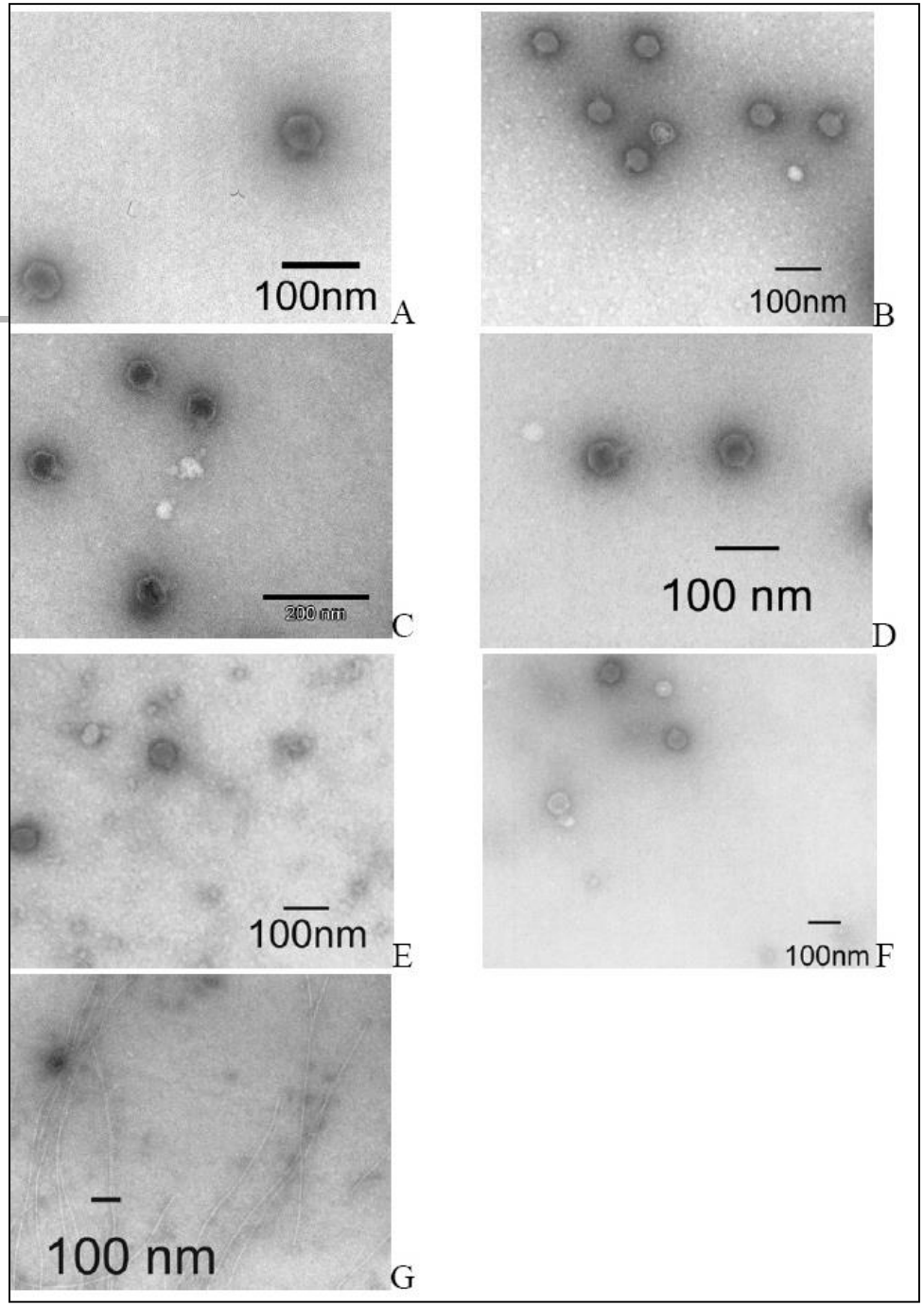
Dendogram and phage sensitivity matrix showing relationship amongst *Xanthomonas* strains causing citrus canker and citrus bacterial spot based on similarity of sensitivity profile against a battery of 12 phages.

Balogh,2006



- **Transmission electron micrographs of representative phages.**

A. **CP2,**
B. **ΦXaacA1,**
C. **ΦXaacF1,**
D. **ΦXaacF2,**
E. **ΦXaacF3,**
F. **ΦXaacF5,**
G. **ΦXaacF8.**



Balogh,2006

Phage sensitivity test
Xanthomonas axonopodis pv. *citri*

- Bacteriophages Cp1, Cp2, Cp3 were deposited separately in 10 µl drops on the surface of different strains of *Xac* seeded soft agar overlays.
- Plaque formation was observed at the routine test dilution (RTD).
- These strains were insensitive to bacteriophage Cp3.

	Xac-A	*Xac*-A*	*Xac*-B[1]	*Xac*-c[1]
Phage sensitivity				
CP1	v	-	-	-
CP2	v	-	-	-
CP3	v	-	+	-

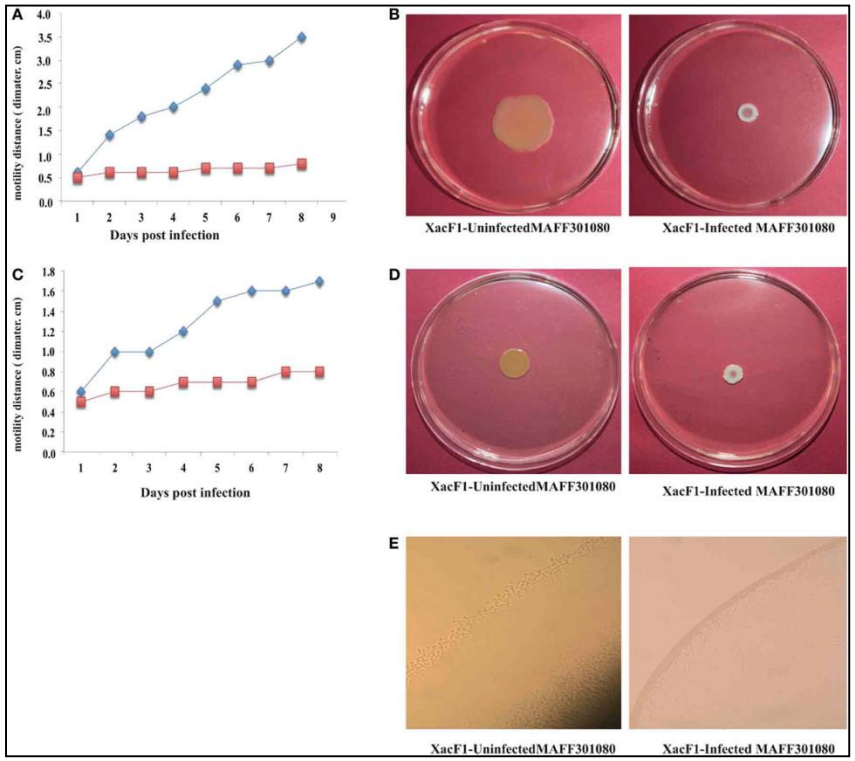
Typical Asiatic (*Xcc*-A) and atypical Asiatic (*Xcc*-A*) form of *X. citri* pv. *citri* (ex. Xac); *X. citri* pv. *aurantifolii* B; *X. citri* pv. *aurantifolii* C.

Phage sensitivity test

Motility assay

Impact of XacF1 on motility reduction of *Xac* cells

- Two microliters of bacterial solution [10^8 CFU)/mL] were inoculated in the swimming assay[0.3%(w/v)agar](A,B), swarming assay [0.7%(w/v) agar](C,D), and twitching motility assay (minimal agar medium) (E).
- The movement of bacterial cells was photographed 5 and 8 days post inoculation (dpi)on the swimming and swarming plates, respectively.
- Twitching motility of bacteria was observed under a microscope 5 dpi on the twitching plates.



Ahmad *et al.*,2014

Phage sensitivity test

Virulence assay

Impact of XacF1 on virulence reduction of *Xac* cells

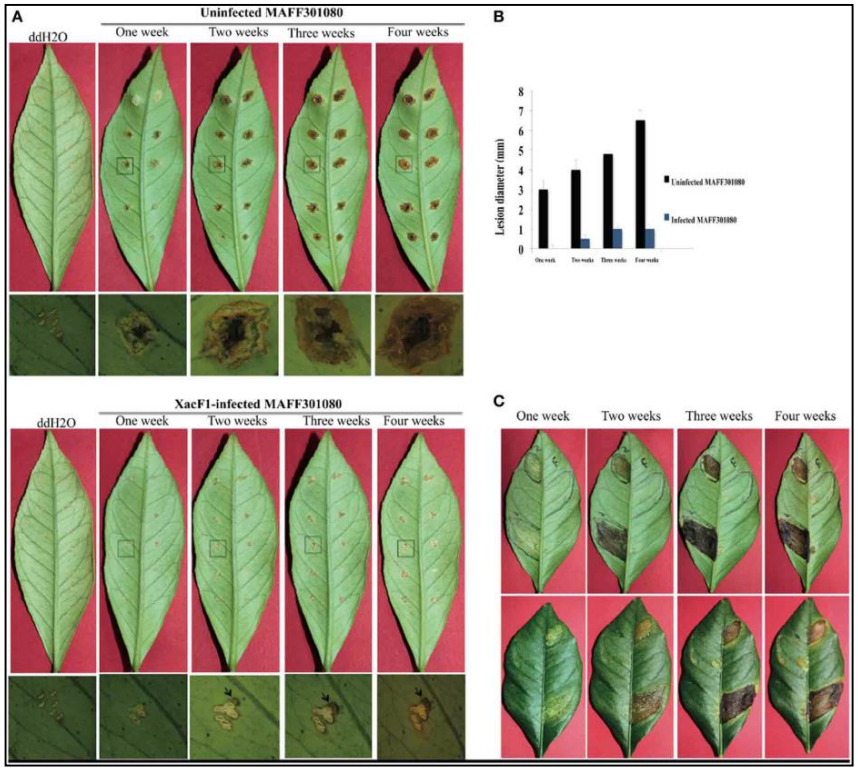
- Lesions on detached lemon leaves inoculated with cells of *Xac* MAFF301080.

A. Canker symptoms that had developed on leaves 1,2, 3, and 4 weeks post-infection by the needle-pricking method.

B. Comparison of the size of lesions formed on lemon leaves.

C. Lesions formed on lemon leaves by infiltration of bacterial cells, expanding with time.

- No lesions formed on either surface of the leaves infected with XacF1-infected cells.



Ahmad *et al.*,2014

Phage sensitivity test
X. arboricola pv. *junglandis*

- Sensitivity response one of three different *Xanthomonas junglandis* strains to six bacteriophage types isolated from the walnut canopy.

	Bacterial strain		
Phage type	**134[2] Lincoln**	**143 Lincoln**	**6494 Auckland**
$Bp60C_1$	+++	+	+
$Bp60C_2$	+++	+	+++
$Bp60C_3$	+++	+	+++
Bp_{10}	+++	+	-
Bp_{20}	+++	-	-
Bp_{22}	+++	+	-

+++Reactive (total lysis); -Not reactive; +Reactive (lysogenic).
[2]=strain used for initial isolation.

Mcneil *et al.*,2001

Host range phages of *P. carotovorum* subsp.*carotovorum*

Bacterial isolates	Group 1			Group 2	
	ΦEcc1	ΦEcc3	ΦEcc5	ΦEcc13	ΦEcc14
Erwinia carotovora subsp. *carotovora*					
Ecc1[a]	(+)	(+)	(+)	+	+
Ecc26	–	–	–	–	–
Ecc48	(+)	(+)	(+)	+	+
Ecc71	–	–	–	–	–
Ecc83	–	–	–	–	–
Escherichia coli DH5α	–	–	–	–	–
Erwinia chrysanthemi EchS80	–	–	–	–	–
Erwinia amylovora	–	–	–	–	–
Pantoea agglomerans	–	–	–	–	–

Note: (+), weak plaque formation; +, plaque formation; –, no plaque formation.
[a]Bacterial isolate on which the phages were initially isolated and propagated.

Ravensdale *et al.*,2007

2. Genotype-based methods:
Molecular Diagnosis of Plant Pathogenic Bacteria

Molecular methods Genotyping – DNA "Gold Standard"

1. **Nucleotide composition**
2. **DNA-DNA hybridization**
3. **Single locus sequence typing=SLSA**
4. **Unique 16S to 23S rRNA gene recognition sequences**
5. **Total genomic sequences**
6. **Multilocus sequence typing = MLST/MLSA (see also part 2)**

Note: The terms MLST and MLSA are very often considered interchangeable. This is however not correct as each analysis method has its distinctive features and uses.

Genotype identification of bacteria
Molecular methods

- **Molecular methods are no magic bullets, but welcome additional tools to study organisms, especially at a low taxonomic level.**

Janse,2006

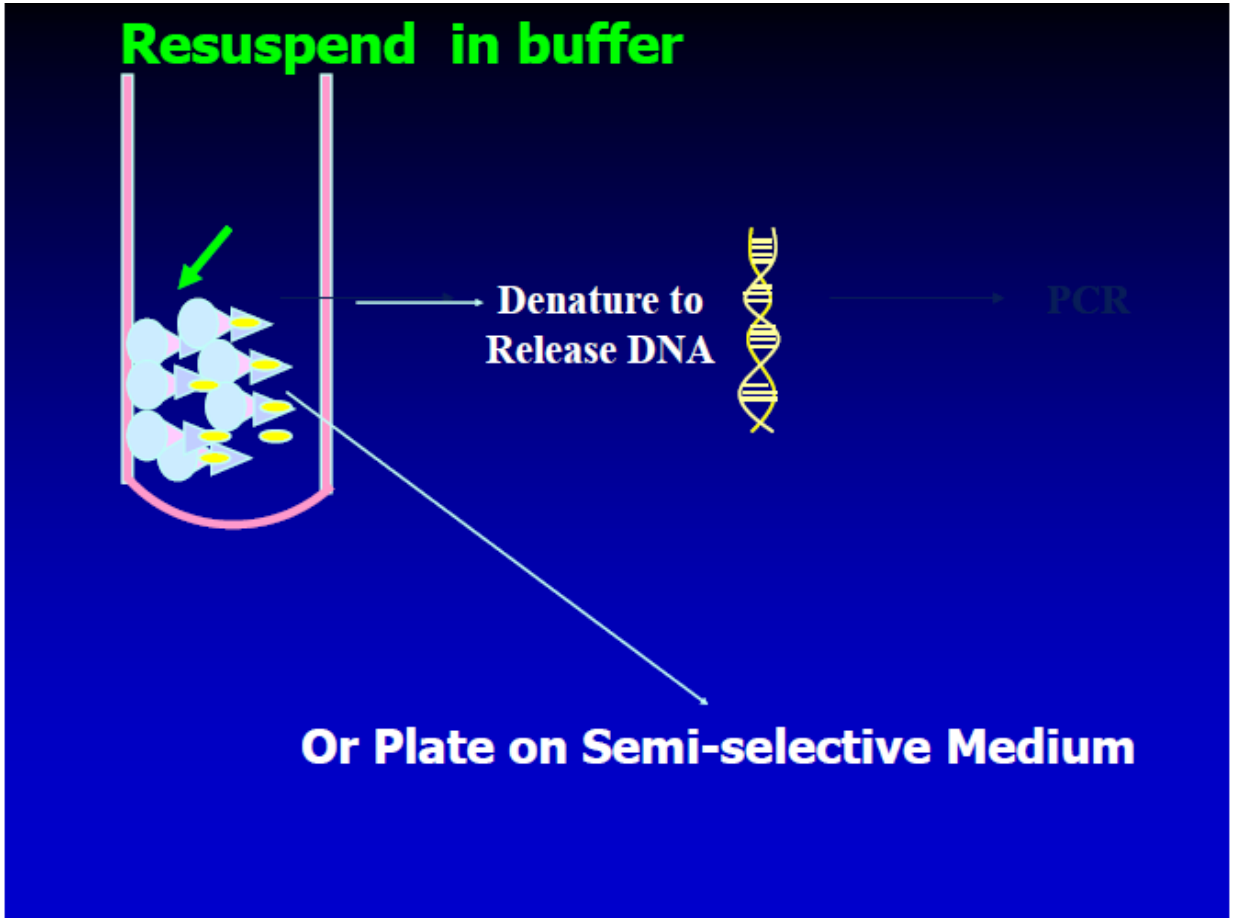
Molecular diagnosis

Molecular diagnosis

- Reduce reliance on culture
- Faster
- More sensitive
- More definitive
- More discriminating
- Techniques adaptable to all pathogens
- Technically demanding
- Relatively expensive
- Can be too sensitive
- Provides no information if results are negative

http://faculty.evansville.edu

Molecular techniques for bacterial identification



Bacteria Fall,2008

DNA approaches to identification
Advantages & Limitations

- Advantages
- Commercial services available for sequencing;
- Data can be shared between laboratories;
- By a combination of approaches identification to the genus, species and pathovar level can be achieved;
- Limitations
- Requires investment in PCR and gel equipment;
- Cost of molecular consumables is high;
- Technically demanding; PCR is notorious for random problems.

DNA approaches to identification
Advantages & Limitations

- Limitations
- DNA-based methods, though sensitive and specific, are not very practical since they only accommodate a small sample volume, usually 1-5 microliters, which may not contain enough bacterial DNA for detection.
- E.g. Detection of low *Ralstonia solanacearum* (*Rs*) in field soil and irrigation water was not possible when aliquots from large water samples were directly added to PCR.

Peckham et al

Possible pitfalls of molecular biological methods

- Specificity, sensitivity and reproducibility unknown or only tested to a limited extent (not validated);
- Negative influence of conditions and biochemicals (experimental error);
- Impossibility to discriminate between viable and non-viable cells and free nucleic acid in a sample;
- False negatives and false positives difficult to verify, Koch's postulates cannot be fulfilled;
- Changing probes/primers/enzymes/methods/chemicals may yield different (conflicting) patterns or no patterns at all;
- Only small part of structural elements of an organism used (sampling error);
- Answers from automated identification systems are as good as standard libraries and present-day taxonomy;
- Points of reference usually determine choice of patterns.

Janse,2006

Possible perspectives of molecular biological methods

- In current identification methods, which are based mainly on biochemical and phenotypic characteristics, rapid and accurate identification is not always possible.
- As a consequence, isolates may be misidentified or, when results do not fit those expected, may be labeled as atypical.

Toth *et al.*,2001

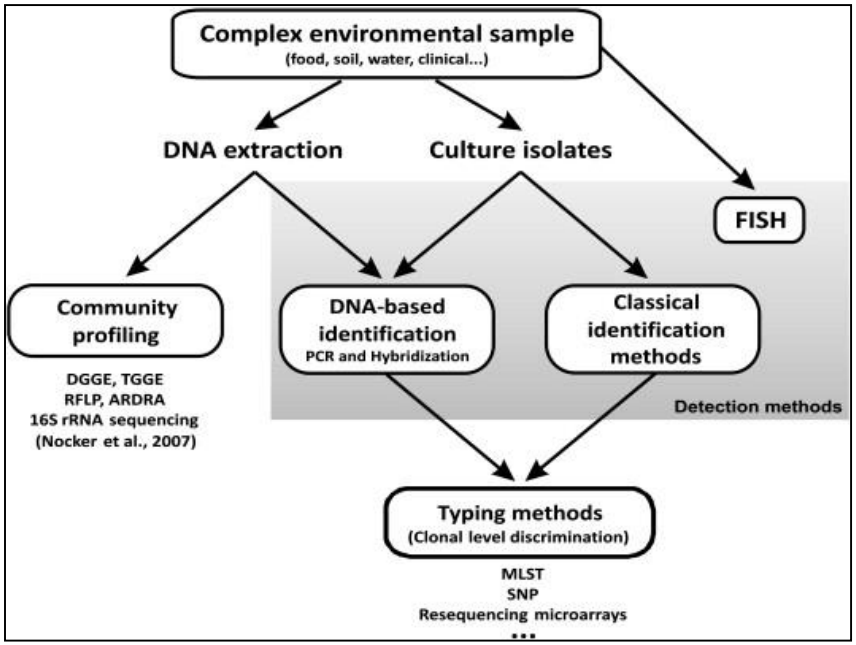
Possible perspectives of molecular biological methods

- Rapid, sensitive and cost effective,
- Integration into certification/inspection schemes,
- Commercially available, standardized test kits,
- Non-culturable organisms such as phytoplasmas can be analyzed,
- Genetically modified organisms can be traced in the environment more easily,
- Less sensitive to mutation or variation,
- Discrimination at low taxonomic level, often strain level.

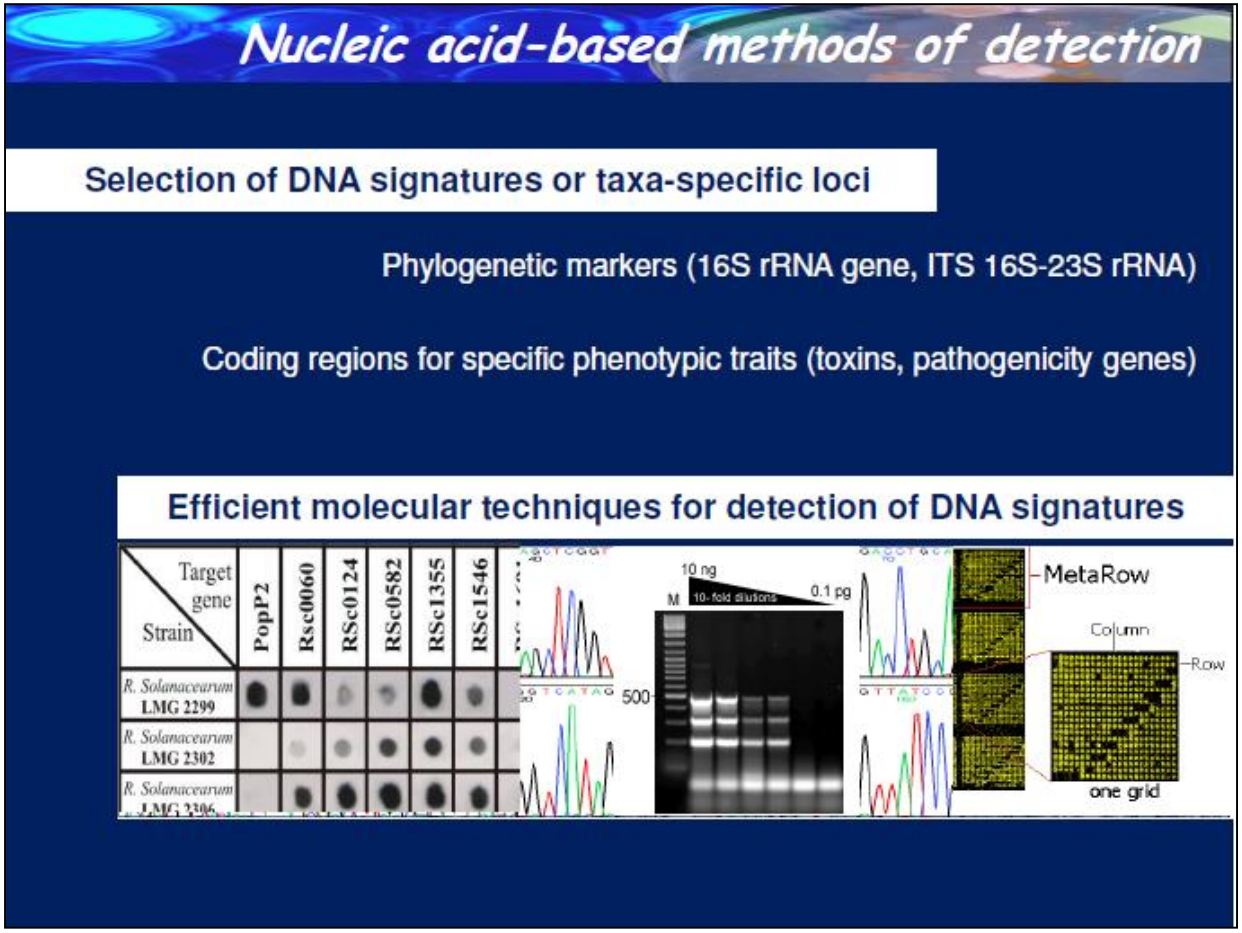
Janse,2006

Genotype identification
Genotype-based methods

- A perspective of the methodological alternatives for the detection of bacteria in complex environmental samples.
- Different levels of taxonomic and ecological discrimination are considered (species, strains, biovars or communities).



Genotype-based methods



Tavares *et al.*,2010

Genotype-based methods

Two kinds of genes

- Housekeeping genes
- e.g. Ribosomal RNA genes (16S, 5S, and 23s rRNAs)
  - Needed for cell metabolism
    - Required for nearly all cell types
- Regulated genes
- e.g. Transcription factors, Kinases and Phosphatases
  - Controls cell phenotype and function

Identification of bacteria

Single locus sequence vs. multiple loci typing

SLST vs. MLST

- We will first identify the isolates by 16S rDNA sequencing.
- If the 16S sequence is different, you can be sure that the isolates are different strains.
- Further characterization is necessary for those isolates that have identical 16S sequences and can be done using SLST or MLST.
- Multilocus sequence typing (MLST) has become a useful tool for studying the genetic diversity of important public health pathogens.
- This technology is designed to deliver the most informative data and have the ability to obtain an unambiguous result from all strains within a species.

Identification of bacteria

Single locus sequence vs. multiple loci

Benefits of single locus sequence analysis in diagnosis of pathogens

- The level of discrimination from a single locus is sufficient to facilitate rapid and cost effective identification to species and subspecies level.
- It has been demonstrated that the 578 *rpoD* locus and 530 bp *gyrB* locus provide identification to sub-clade and sequevar level of pathogens within:

1. *Xanthomonas* (e.g. *X. arboricola* sequevars), and
2. The *P. syringae* complex.

Parkinson and Elphinstone,2010

Identification of bacteria

Single locus sequence vs. multiple loci typing

MLST vs. Multiplex PCR

- Multiplex PCR: a novel method of simultaneous amplification of multiple DNA fragments (usually all target genes). The multiplex PCR assay is useful for low-cost screening of large numbers of isolates with rapid analytical capacity and could be utilized in most laboratories.
- Multilocus sequence typing (MLST): a reliable method for simultaneous amplification of multiple DNA fragments (usually housekeeping genes). However, the method is costly, time-consuming and difficult to use for screening large numbers of isolates.

PubMLST

Genotype identification of bacteria
MLST vs MLSA

- Both methods depend on the sequencing of multiple (usually four to eight) housekeeping genes, i.e., genes conferring a basic metabolic function.
- **Multilocus Sequence Typing (MLST):**
- MLST is usually applied to strains that belong to a well-defined species. Here a sequence of multiple (usually 5-7) housekeeping genes(HKGs) is analysed.
- **Multilocus Sequence Analysis (MLSA):**
- MLSA is more often used when species boundaries are not well known and MLSA data are used to improve species descriptions.

PAMBD.org;..

Genotype identification of bacteria
MLST vs MLSA

- **MLST** is based on the analysis of the combination of alleles at each locus, defining a sequence type (ST), while
- **MLSA** relies on the comparison of partial DNA sequences of each gene or of concatenated sequences among strains.
- **MLST** usually allows strains to be distinguished below the species level, while
- **MLSA** provides a framework for species definition and allows the identification of species by electronic taxonomy.

Jacques *et al.*,2012

Genotype identification of bacteria
MLST vs MLSA

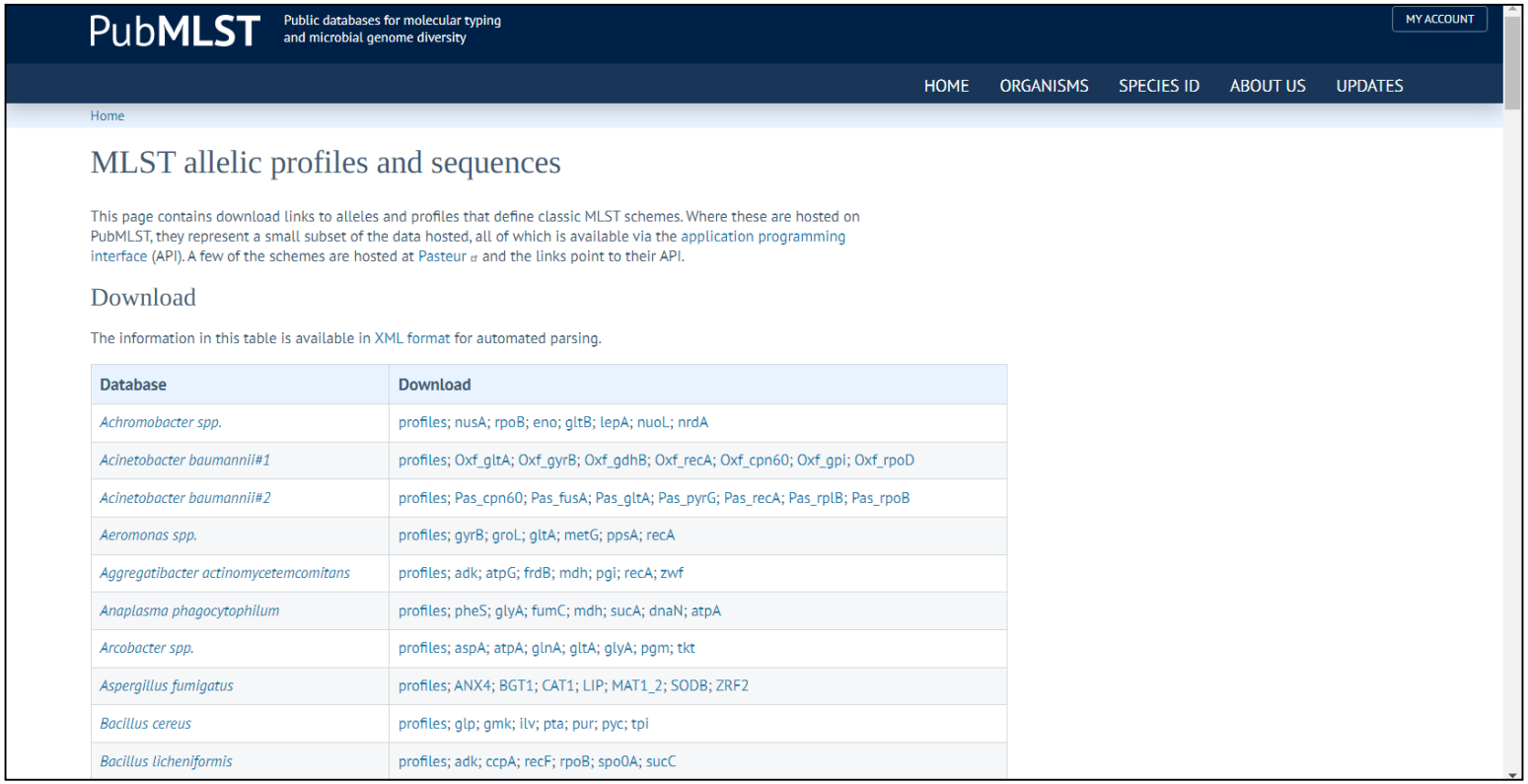
- MLSA and MLST studies have been conducted on diverse Gram-negative and Gram-positive bacteria; however, no full MLSA study on any Gram-positive plant-pathogenic bacteria has been published so far.

Jacques *et al.*,2012

Genotype identification of bacteria

MLST allelic profiles and sequences

Concatenated(series) housekeeping genes(HKGs) for each genus



PubMLST Public databases for molecular typing and microbial genome diversity

MY ACCOUNT

HOME ORGANISMS SPECIES ID ABOUT US UPDATES

Home

MLST allelic profiles and sequences

This page contains download links to alleles and profiles that define classic MLST schemes. Where these are hosted on PubMLST, they represent a small subset of the data hosted, all of which is available via the application programming interface (API). A few of the schemes are hosted at Pasteur and the links point to their API.

Download

The information in this table is available in XML format for automated parsing.

Database	Download
Achromobacter spp.	profiles; nusA; rpoB; eno; gltB; lepA; nuoL; nrdA
Acinetobacter baumannii#1	profiles; Oxf_gltA; Oxf_gyrB; Oxf_gdhB; Oxf_recA; Oxf_cpn60; Oxf_gpi; Oxf_rpoD
Acinetobacter baumannii#2	profiles; Pas_cpn60; Pas_fusA; Pas_gltA; Pas_pyrG; Pas_recA; Pas_rplB; Pas_rpoB
Aeromonas spp.	profiles; gyrB; groL; gltA; metG; ppsA; recA
Aggregatibacter actinomycetemcomitans	profiles; adk; atpG; frdB; mdh; pgi; recA; zwf
Anaplasma phagocytophilum	profiles; pheS; glyA; fumC; mdh; sucA; dnaN; atpA
Arcobacter spp.	profiles; aspA; atpA; glnA; gltA; glyA; pgm; tkt
Aspergillus fumigatus	profiles; ANX4; BGT1; CAT1; LIP; MAT1_2; SODB; ZRF2
Bacillus cereus	profiles; glp; gmk; ilv; pta; pur; pyc; tpi
Bacillus licheniformis	profiles; adk; ccpA; recF; rpoB; spo0A; sucC

PubMLST

Identification of bacteria

Multilocus Sequence Typing (MLST) Scheme

- Multilocus sequence typing (MLST) is an unambiguous procedure for characterizing isolates of bacterial species using the sequences of internal fragments of (usually) seven house-keeping genes.
- Approximately 450-500 bp internal fragments of each gene are used, as these can be accurately sequenced on both strands using an automated DNA sequencer.

See also *Xylella fastidiosa* section in Plant Bacterial Diagnosis-Part3

Identification of bacteria

Multilocus Sequence Typing (MLST) Scheme

- Briefly, in MLST each allele of a given gene is assigned a number, so different strains of bacteria can be characterized by a series of numbers, representing one allele for each locus analyzed.
- The combination of the allele numbers at each locus determine the so called sequence type (ST) for each analyzed strain.
- For each house-keeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST).

Identification of bacteria

Multilocus Sequence Typing (MLST) Scheme

- In MLST the number of nucleotide differences between alleles is ignored and sequences are given different allele numbers whether they differ at a single nucleotide site or at many sites.
- The rationale is that a single genetic event resulting in a new allele can occur by a point mutation (altering only a single nucleotide site), or by a recombinational replacement (that will often change multiple sites) - weighting according to the number of nucleotide differences between alleles would erroneously consider the allele to be more different than by treating the nucleotide changes as a single genetic event.

PubMLST

Identification of bacteria

Multilocus Sequence Typing (MLST) Scheme

- Most bacterial species have sufficient variation within house-keeping genes to provide many alleles per locus, allowing billions of distinct allelic profiles to be distinguished using seven house-keeping loci.
- For example, an average of 30 alleles per locus allows about 20 billion genotypes to be resolved.
- MLST is based on the well established principles of multilocus enzyme electrophoresis, but differs in that it assigns alleles at multiple house-keeping loci directly by DNA sequencing, rather than indirectly via the electrophoretic mobility of their gene products.

PubMLST

Identification of bacteria

Multilocus Sequence Typing (MLST) Scheme

- Multilocus sequence typing (MLST) is a molecular typing technique whereby a number of well chosen housekeeping genes (loci) are sequenced, usually in part.
- In a typical MLST approach, recombination is expected to occur with a much higher frequency than point mutations. Therefore, one does not look at the total sequence similarity between strains. Instead, each sequence for a given locus is screened for identity with already known sequences for that locus.
- If the sequence is different, it is considered to be a new allele and is assigned a unique (arbitrary) allele number.

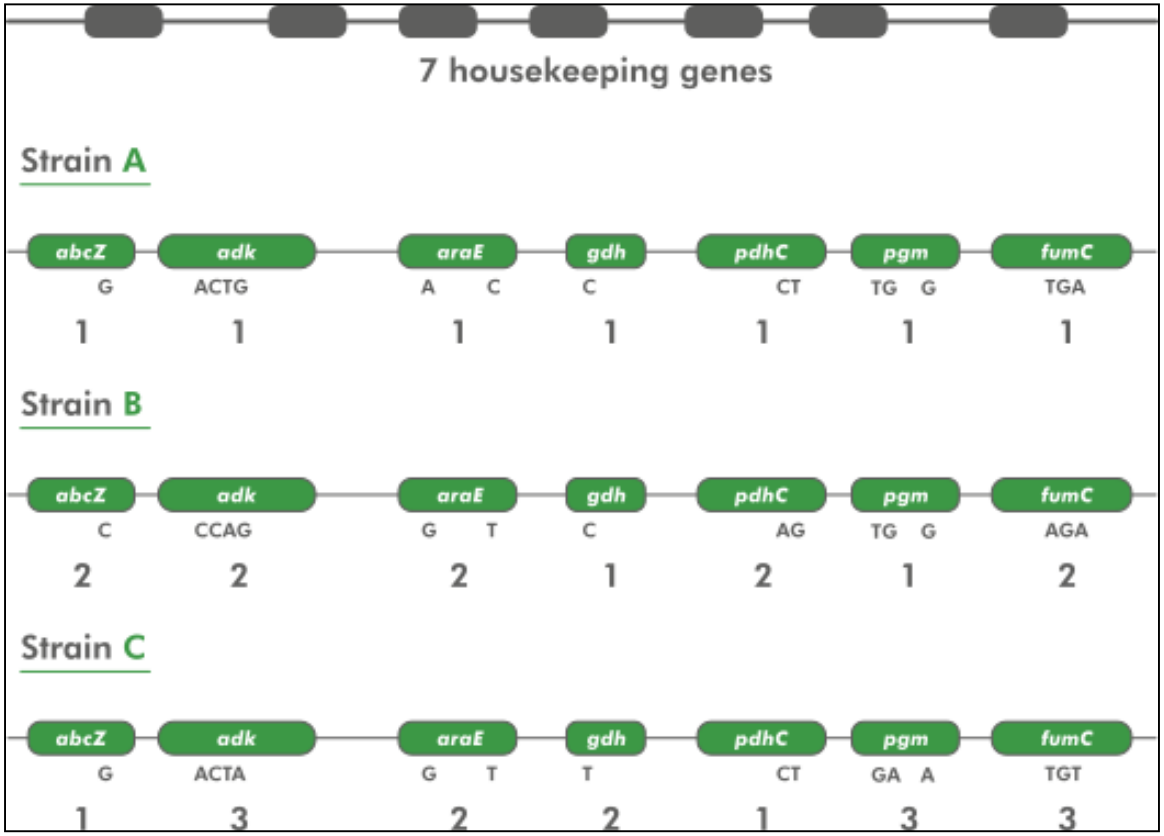
Identification of bacteria

Multilocus Sequence Typing (MLST) Scheme

- In case seven housekeeping genes are studied, each strain is thus characterized by a profile of seven allele numbers.
- The allelic profiles can be considered as a character set of 7 categorical characters.
- MLST has been used successfully to study population genetics and reconstruct micro-evolution of epidemic bacteria and other micro-organisms.

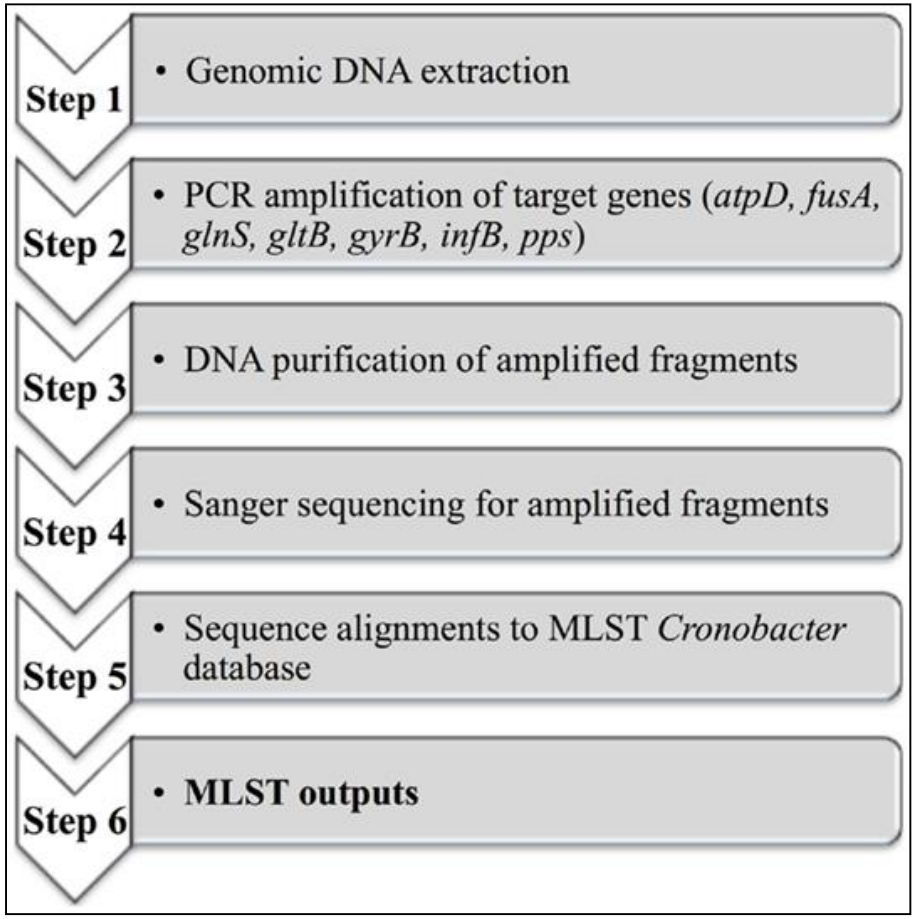
Identification of bacteria

Multilocus Sequence Typing (MLST) Scheme



Identification of bacteria

Multilocus Sequence Typing (MLST) Scheme



Yand and Fanning,2015

Identification of bacteria

Multilocus Sequence Typing (MLST) Scheme

Analysis of MLST sequences in BIONUMERICS

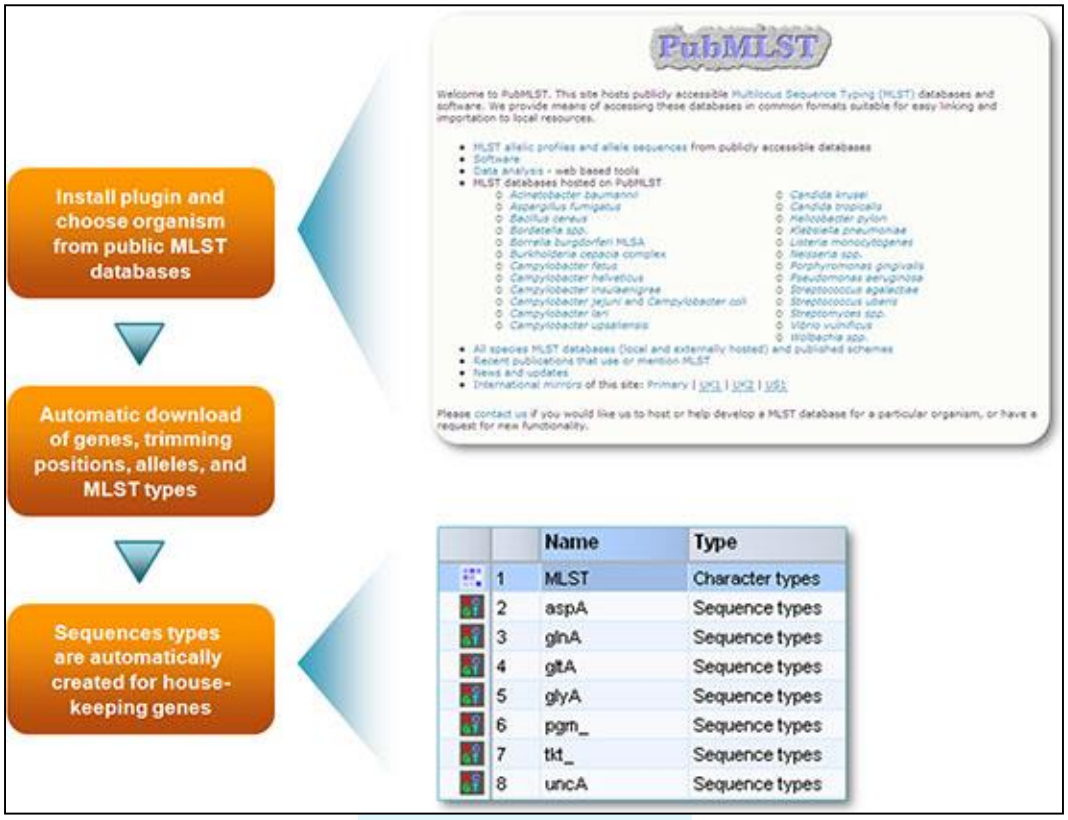
- Download BIONUMERICS software. BIONUMERICS software is widely used for the storage and analysis of MLST sequences.
- BIONUMERICS automatically analyses batches of sequence trace files, connects to online MLST databases, retrieves corresponding allele numbers, sequence types as well as available clonal complex information.
- BIONUMERICS can process hundreds of isolates in only seconds. Results are stored in the database and are available for statistical and population analysis, clustering, partitioning, identification using BIONUMERICS' impressive set of analysis tools.

Identification of bacteria

Multilocus Sequence Typing (MLST) Scheme

Analysis of MLST sequences in BIONUMERICS

- Setup scheme:



BIONUMERICS

Identification of bacteria

Multilocus Sequence Typing (MLST) Scheme

Analysis of MLST sequences in BIONUMERICS

- Workflow scheme:

Fully automatic processing workflow

•Automatic import and assembly of batches of sequencer trace files from various sources (AB, Beckman, Amersham, FASTA); file names are parsed into strain and gene information using a parsing definition.

•Consensus sequences are automatically trimmed using start and stop signatures and placed in the right direction.

•When the batch assembly is finished, an overview report is shown, listing status of each strain/gene combination.

•Double-click on a problem contig to display the detailed information window.

•Double-click on a particular problem to open the Assembler with the problem position selected.

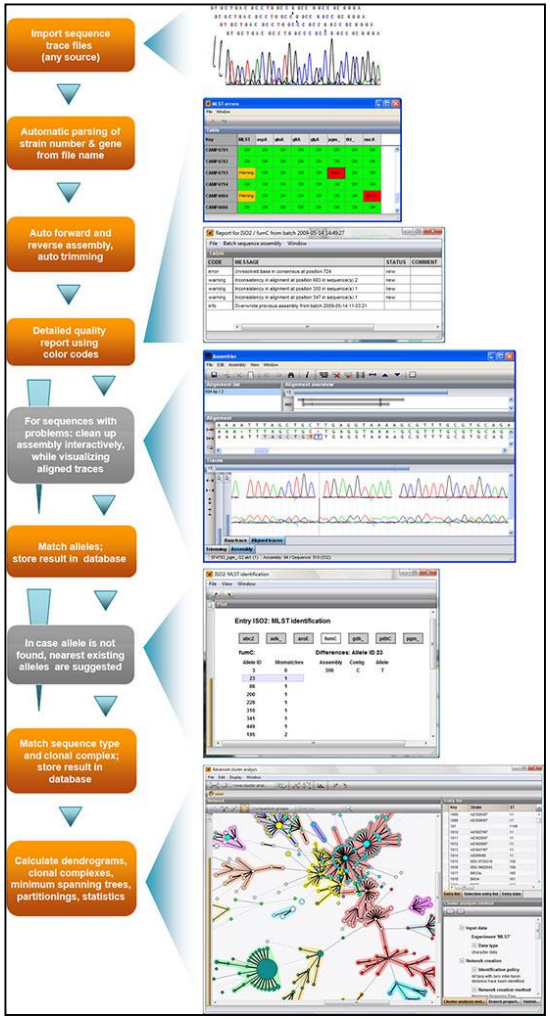
•For each problem position, show nearest existing alleles and suggested bases - easy verification with chromatograms.

•Alleles and MLST types can be identified by real-time connection to MLST server database, or by comparing to locally stored allele database (faster). In the latter case, local database can be updated automatically at startup.

•Allele and MLST type information for own strains is stored in the database and can be updated at any time for a selection of strains. BIONUMERICS will prompt you for any change in allele/MLST type definition that has occurred in the MLST server database.

•Calculate population modelling networks in the finest and most comprehensive cluster analysis application available today, using standard or custom priority rules and with branch significance support indication.

•Calculate and display partitioning for clonal complexes and use BIONUMERICS' rich set of statistics tools.



BIONUMERICS

Multilocus sequence Analysis (MLSA)
Using Environmentally Mediated Genes (MLSA-E)

- An alternative for discriminating between closely related *X. fastidiosa* isolates is to perform MLSA of genes influenced by environmental factors, termed environmentally mediated genes.
- Here multilocus sequence analysis of environmentally mediated genes (MLSA-E) was applied to identify *X. fastidiosa* isolate relationships.
- MLSA-E was used for genes related to processes important for establishing *X. fastidiosa* infections and colonization of the insect vector, such as surface attachment, biofilm formation, virulence, and nutrient transport and utilization.

Parker *et al.*,2012

Multilocus sequence Analysis (MLSA)
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Parker *et al.*,2012

Multilocus sequence Analysis (MLSA) Methods

Using Environmentally Mediated Genes (MLSA-E)

Genes selected for final *X. fastidiosa* MLSA-E analysis:

1. *acvB* (virulence);
2. *copB* (copper resistance);
3. *cvaC*[a] (toxin);
4. *fimA* (attachment);
5. *gaa*;
6. *pglA*[a] (cell wall degradation);
7. *pilA* (motility);
8. *rpfF* (pathogenicity);
9. xadA[a] (attachment);
10. etc.

Gene	Locus tag (Temecula)	Gene size (bp)	Category	Description	Forward primer (5′→3′)	Reverse primer (5′→3′)	Amplicon size (bp)	Reference(s)
acvB	PD_1902	900	Virulence	Virulence protein: suggested to regulate pathogenicity and disease symptoms	ACAGTA TCGCCG TC GAAG TGATGA	CATGCA TACRGC GATGYT TCCGAT	743	34
copB	PD_0101	930	Resistance	Copper resistance protein B precursor: encodes a copper resistance cation-translocating ATPase induced by copper exposure	ATGAAC ACCCGT AC CTGG TTCGTA	ATTTAG TCTCCA CCATGA GCCGCA	607–862	68, 82
cvaC[a]	PD_0215	309	Toxin	Colicin V precursor: encodes a bacteriocin precursor proposed to be a defense mechanism	TGCGTG AATTRA CA TTGA CCG	CCTAGT CTGCGG CTTAAG CAGATT	330	20, 62
fimA	PD_0062	555	Attachment	Fimbrial subunit precursor: component of type I pili important for biofilm formation and aggregation	CCCAGT GCGTCG TTATCG ATTATT GGT	TTTYGY ACTCTC AA GCAT CGCATC	557	18–20, 24, 25, 46
gaa	PD_0315	1,992	Toxin	Glutaryl-7-aminocephalosporanic acid acylase precursor: encodes a member of the newly described family of β-lactam antibiotic acylases	TGAGAG CTGCYG ATGTTC CAATGA	ACAGCT TCTGGC AAGAAC AAGCAC	1129	4, 5
pglA[a]	PD_1485	1,635	Cell wall degradation	Polygalacturonase precursor: needed for degrading host plant cell walls to allow colonization	TAGTGC TGGCCT AA CGAT GTYGGT	CCGTAT CAGCAA CCACAT GGAAGT	828/829	63, 71
pilA	PD_1924	447	Motility	Fimbrial protein: major structural protein of type IV pili used for twitching motility and upstream migration	ATCGCK CTGCCY AT GTAC CAAA	CAGCAT TGATCG TRTTGC TGTRTG	405	57
rpfF	PD_0407	873	Pathogenicity	Regulator of pathogenicity factors: involved in biosynthesis of diffusible signal factor (DSF) to mediate cell-to-cell signaling for biofilm formation	GCGCTC CATAGT TC GGAG TGATTT	ATGTCC GCTGTA CATCCC ATTCCT	825	8, 9, 60
xadA[a]	PD_0731	2,994	Attachment	Outer membrane afimbrial adhesin: may contribute to initial cell binding to surfaces	TGGGAG GTCAAA GYACTG CCATCA	GCATTG GCAGCA ACACTC GAATCA	1087/1108	25

[a] Genes selected on the basis of *dN/dS*.

dN/dS ratio: Nucleotide substitutions in genes coding for proteins can be either synonymous (do not change amino acid), alternatively called silent substitutions, or non-synonymous (changes amino acid). dN/dS measured across the whole protein sequence between two divergent species in theory tells you something about selection. If this ratio = 1, then the whole coding sequence evolves neutrally, when 0 < dN/dS < 1, it's under constraint, and when > 1 under positive selection.

Parker *et al.*,2012

Nucleic acid based tests
PCR (molecular kitchen) and plant pathogenic bacteria

- Nucleic- acid based tests offer greater sensitivity, specificity, reliability and may be quicker than many conventional methods used to detect plant-pathogenic bacteria in different plant hosts and environments.
- With the development of polymerase chain reaction (PCR), and especially real-time PCR, such high sensitivity is achieved, improving the accuracy of pathogen detection and identification.

Palacio-Bielsa *et al.*,2009

Comparison of sensitivity, specificity, feasibility, rapidness and cost of different techniques in detection of plant pathogenic bacteria and viruses

Technique	Sensitivity[a]	Specificity[b]	Feasibility[c]	Rapidness	Cost
Molecular hybridisation	+[d]	++++	++	+	+++
FISH	++	++	+++	+	++
Conventional PCR	+++	++++	+++	+++	+++
Nested PCR in a single tube	++++	++++	+++	++	+++
Cooperational-PCR[e]	++++	++++	+++	+++	+++
Multiplex PCR	+++	++++	+++	+++	+++++
Multiplex nested PCR	++++	++++	++	+++	++++
Real-time PCR[f]	+++++	+++++	++++	+++++	+++
NASBA[g]	+++++	++++	++++	++++	++
LAMP	++++	++++	+++	++++	++
Microarrays	+	+++++	+	++	+

[a] Sensitivity: probability of detecting true positives.

[b] Specificity: probability of detecting true negatives.

[c] Feasibility: practicability in routine analysis, execution and interpretation.

[d] The number of + symbols indicates how methods rate regarding each considered criterion, from acceptable (+) to optimum (+++++).

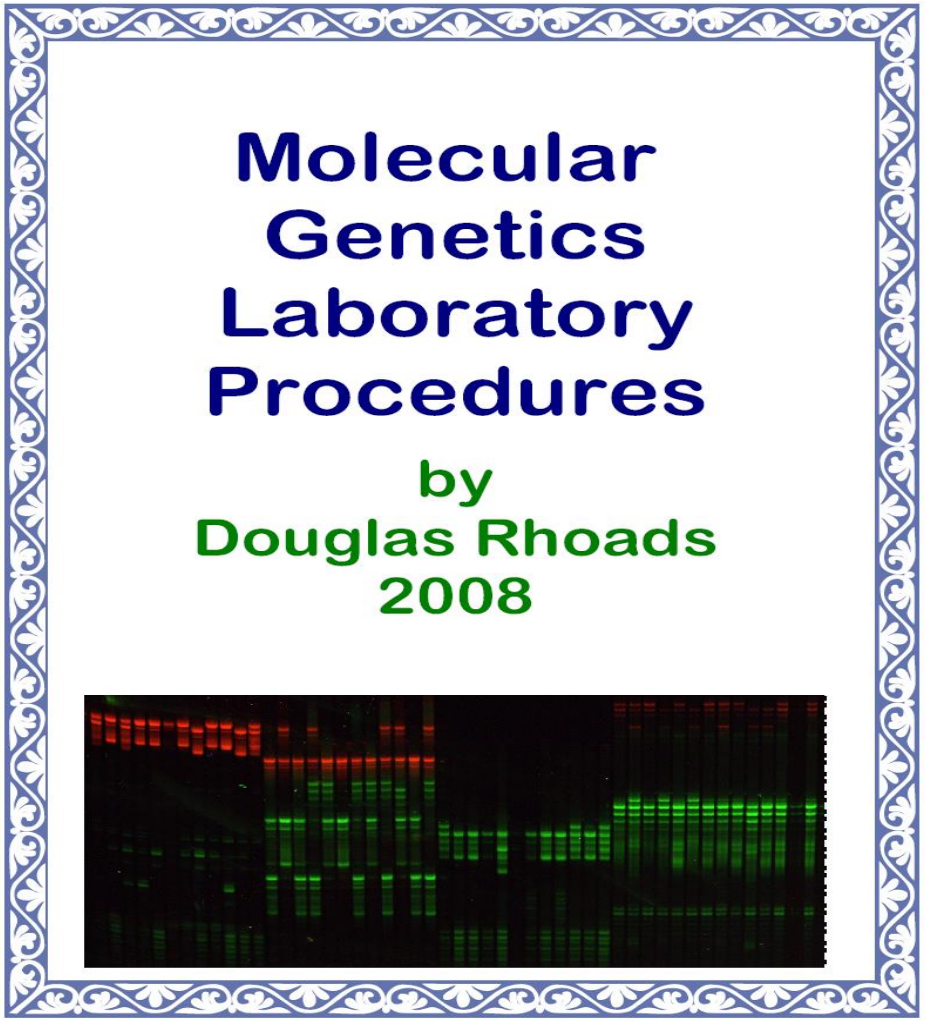
[e] Coupled with hybridisation and colorimetric detection.

[f] Using TaqMan probes.

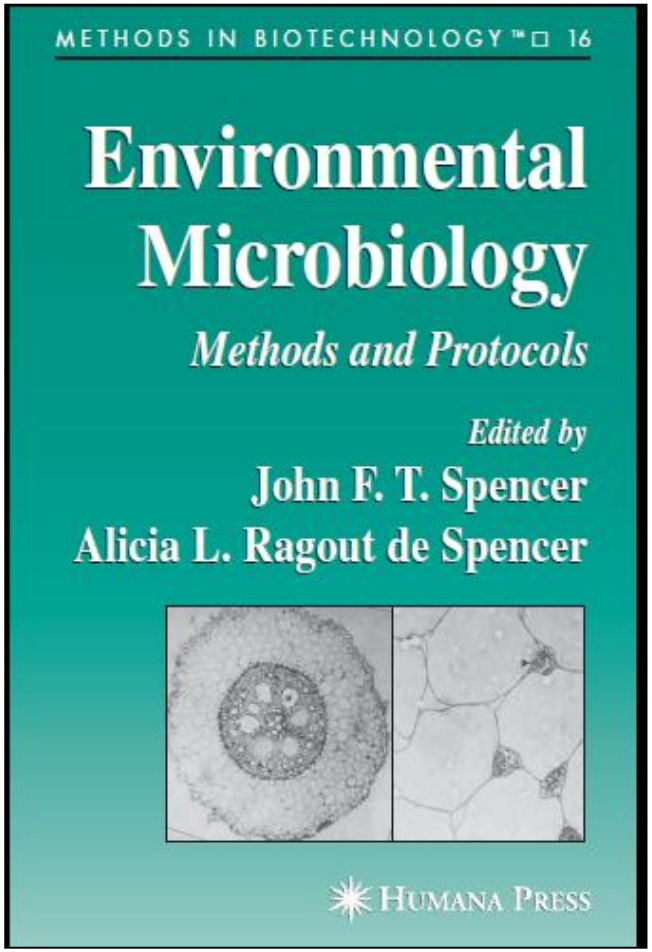
[g] Using Molecular Beacons probes.

López *et al.*,2011

Manual,2008


Molecular
Genetics
Laboratory
Procedures
by
Douglas Rhoads
2008

Environmental Microbiology: Methods and Protocols



Product Details

- Environmental Microbiology: Methods and Protocols.
- Spencer, J. F.T. and Ragout de Spencer, A. (eds.).
- Humana Press Inc., 2004.
- 423 pp.

Types of genetic markers

List of Markers	Acronym
Restriction Fragment Length Polymorphism	RFLP
Random Amplified Polymorphic DNA	RAPD
Amplified Fragment Length Polymorphism	AFLP
Variable Number Tandem Repeat	VNTR
Oligonucleotide Polymorphism	OP
Random Amplified Polymorphic DNA	RAPD
Single Nucleotide Polymorphism	SNP
Allele Specific Associated Primers	ASAP
Inverse Sequence-tagged Repeats	ISTR
Inter-retrotransposon Amplified Polymorphism	IRAP

Wikepedia,2017

Typing and target characteristics, electrophoresis platforms and references of several typing techniques

	Characterization of:	Target used for characterization	Electrophoresis platform
AFLP	Whole genome or plasmid	Restriction site and additional nucleotide	Polyacrylamide[a] or agarose
rep-PCR Genomic fingerprinting	Whole genome	Repetitive element oligonucleotide sequence	Agarose or polyacrylamide[a]
ARDRA	Ribosomal gene	Oligonucleotide sequence and restriction site	(MetaPhor) Agarose
ITS-PCR-FLP	Inter-transfer ribosomal gene spacer sequence	Oligonucleotide sequence and fragment length	Agarose or polyacrylamide[a]
IGS-PCR-FLP	Inter-ribosomal gene spacer sequence	Oligonucleotide sequence and fragment length	Agarose or polyacrylamide[a]
IGS-PCR-RFLP	Inter-ribosomal gene spacer sequence	Oligonucleotide sequence and restriction site	Agarose
T-RFLP	(Ribosomal) gene	Oligonucleotide sequence and restriction site	Polyacrylamide[a]
SSCP	(Ribosomal) gene	Specific oligonucleotide and total fragment sequence	Polyacrylamide[a]
T/DGGE	(Ribosomal) gene	Specific oligonucleotide and total fragment sequence	Polyacrylamide
RAPD/AP-PCR/DAF	Whole genome	Repetitive random oligonucleotide sequence	Agarose
PFGE	Whole genome	Low frequency restriction site	Agarose
MLST Plasmid profiling	Several genes Plasmid	DNA sequence Plasmid sizes	DNA-sequencer Agarose

Osborn and Smith,2005

Types of genetic markers

Characteristics of methods commonly used for molecular epidemiology

Data generation	Chromosomal target(s)	Data output	output Method examples
Restriction enzymes	Common restriction sites	DNA fragments visualised after agarose gel electrophoresis (AGE)	Restriction endonuclease analysis (REA)
Restriction enzymes	Common restriction sites	Ordered sequence scaffolds identified via instrument software	Optical mapping
Restriction enzymes	Rare restriction sites	DNA fragments visualised after AGE	Pulsed-field gel electrophoresis(PFGE)
Polymerase chain reaction (PCR)	Repetitive element or variable-number tandem repeat (VNTR) sequences	Amplified DNA fragments either visualised after AGE or via instrument software	Repetitive-element PCR (rep-PCR); VNTR typing; PCR ribotyping
DNA probes	Multiple genes	Hybridisation signal either identified visually or via instrument software	Microarray
DNA sequencing	Single or multiple genes	DNA sequence obtained via instrument software	Staphylococcus aureus protein A gene (spa) typing; multilocus sequence typing (MLST)
DNA sequencing	Whole genome	DNA sequence obtained via instrument software	Whole genome sequencing (WGS); next generation sequencing (NGS)

Goering *et al.*,2013

Fingerprint methods

1. PCR- and non-PCR-based fingerprinting techniques

- Some of current fingerprint methods based on both PCR- and non-PCR analyses:

1. RAPD(Random Amplification of Polymorphic DNA. pronounced rapid);
2. ARDRA(amplified ribosomal DNA restriction analysis);
3. AFLP(Amplified Fragment Length Polymorphism);
4. RFLP(restriction fragment length polymorphism);
5. BOX-PCR,
6. REP-PCR,
7. Sequencing,
8. MLST (Multilocus sequence typing);
9. Micro-arrays (also commonly known as DNA chip or biochip);
10. Oligonucleotide probes....

Elke,2010;..

Fingerprint methods
2. PCR-based fingerprinting techniques

- PCR-based fingerprinting techniques currently being exploited in strain discrimination.

1. Repetitive PCR fingerprinting (Rep-PCR).
2. rDNA PCR of the 16S-23S intergenic spacer region, ITS ('ribotyping').
3. Randomly amplified polymorphic DNA PCR (RAPD-PCR).
4. Arbitrary Primed (AP) PCR(AP-PCR).
5. Triplet Arbitrary Primed (TAP) PCR (TAP-PCR).
6. Multiplex PCR. Using specific primers have also been set up for simultaneous detection of two genes of the same bacterial pathogen, thus limiting false positives.

Fingerprint methods

Comparison of some useful PCR-based molecular fingerprinting techniques

	PFGE	Ribotyping	16S rRNA RFLP	Multiplex PCR	AP-PCR (RAPD)	TAP-PCR
Requires culturing	yes	yes	no	no	no	no
[1]Rapid technique	no	no	yes	yes	yes	yes
Discriminatory power	very high	medium	low	high	very high	very high
Reproducibility	very good	very good	very good	good	low	good
Labor intensive	yes	yes	no	no	no	no
Fingerprint surveys the entire genome rather than a sub-section	yes	no	no	no	yes	yes
Procedure has been automated	no	yes	no	no	no	no
Cost per sample	high	high	low	low	low	low
Requires use of restriction enzymes	yes	yes	yes	no	no	no
Single methodology applicable to majority of bacterial isolates	yes	yes	no	no	no	yes
Requires sequence knowledge of the organism	no	no	no	yes	no	no

[1] Indicates if technique can easily be completed in less than a day. Both PFGE and ribotyping take several days. However, the automated ribotyping procedure can be completed in one day.

PFGE, Pulsed-field gel electrophoresis;
Ribotyping is based on the analysis of sequences of a rRNA operone, a very important element of genomes of all bacteria;
Arbitrarily Primed PCR (AP-PCR) or Random Amplified Polymorphic DNA (RAPD).

O'Sullivan,2000

Fingerprint methods

Comparison of sensitivity, specificity, feasibility, rapidness and cost of different techniques in detection of plant pathogenic bacteria and viruses

Technique	Sensitivity[a]	Specificity[b]	Feasibility[c]	Rapidness	Cost
Molecular hybridisation	+[d]	++++	++	+	+++
FISH	++	++	+++	+	++
Conventional PCR	+++	++++	+++	+++	+++
Nested PCR in a single tube	++++	++++	+++	++	+++
Cooperational-PCR[e]	++++	++++	+++	+++	+++
Multiplex PCR	+++	++++	+++	+++	+++++
Multiplex nested PCR	++++	++++	++	+++	++++
Real-time PCR[f]	+++++	+++++	++++	+++++	+++
NASBA[g]	+++++	++++	++++	++++	++
LAMP	++++	++++	+++	++++	++
Microarrays	+	+++++	+	++	+

[a] Sensitivity: probability of detecting true positives.

[b] Specificity: probability of detecting true negatives.

[c] Feasibility: practicability in routine analysis, execution and interpretation.

[d] The number of + symbols indicates how methods rate regarding each considered criterion, from acceptable (+) to optimum (+++++).

[e] Coupled with hybridisation and colorimetric detection.

[f] Using TaqMan probes.

[g] Using Molecular Beacons probes.

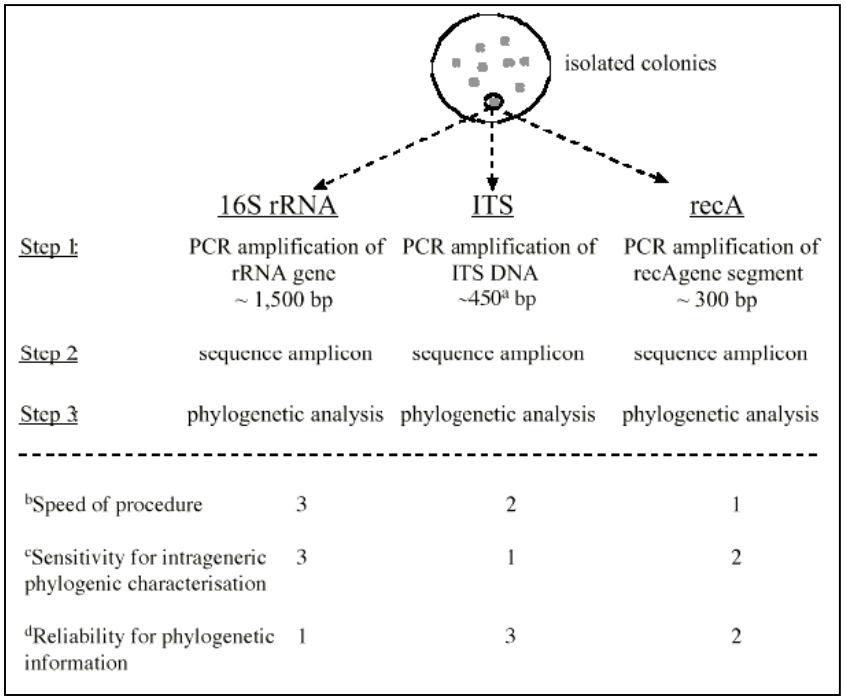
López *et al.*,

Fingerprint methods

Comparison of some useful molecular fingerprinting techniques

- Comparison of the three sequence-based typing and phylogenetic characterization approaches.
- The three sequences which have been used are:

1. 16S rRNA;
2. Sequence between the 16S and 23S rRNA genes, termed the internal transcribed spacer (ITS) aka the 16S-23S intergenic spacer region(non coding DNA); and
3. An internal portion of the *recA* gene.



	16S rRNA	ITS	recA
Step 1:	PCR amplification of rRNA gene ~ 1,500 bp	PCR amplification of ITS DNA ~450[a] bp	PCR amplification of recAgene segment ~ 300 bp
Step 2	sequence amplicon	sequence amplicon	sequence amplicon
Step 3	phylogenetic analysis	phylogenetic analysis	phylogenetic analysis
[b]Speed of procedure	3	2	1
[c]Sensitivity for intrageneric phylogenic characterisation	3	1	2
[d]Reliability for phylogenetic information	1	3	2

O'Sullivan,2000

Centrifuge application guide

Relative Centrifugal Force (RCF) or G-Force/revolutions per minute (rpm) conversion

Application for Pelleting	Low Speed (7000 rpm/ 7000 × *g*)	High Speed (30,000 rpm/ 100,000 × *g*)	Ultra (100,000 rpm/ 1,000,000 × *g*)
Cells	Yes	Yes	Feasible, but not recommended
Nuclei	Yes	Yes	Feasible, but not recommended
Membranous organelles	Some	Yes	Yes
Membrane fractions	Some	Some	Yes
Ribosomes	No	No	Yes
Macromolecules	No	No	Yes

Source: Data from Rickwood (1984).

Centrifuge application guide

Online tool for G-Force/RPM centrifuge conversion

G-Force/RPM Centrifuge Conversion Calculator

Enter the **radius of the rotor** in cm

Enter the **rotor speed** in rpm (RPM) and calculate RCF
or
Enter the **G-Force** in g (RCF) and calculate RPM

Rotor Radius 10.70 **cm**

RPM 10000 - + **rpm**

RCF 11952 **g** - +

Calculate RPM

Calculate G-Force

Choose the rotor:

Beckman | Sorvall | Thermo Scientific

Click on the radius:

Brand	Rotor	Min Radius (cm)	Max Radius (cm)	Max RPM (rpm)	Max RCF (g)
Beckman	A-100/18	0.95	1.47	95000	148322
Beckman	A-100/30	0.88	1.76	92000	166544
Beckman	A-110	0.95	1.47	110000	198859
Beckman	A-95	0.89	1.76	95000	177583
Beckman	ACR-90	0.32	1.35	90000	122253
Beckman	An-50Ti	7.20	7.20	50000	201240

www.geneinfinity.org/sp/sp_rotor.html

Centrifuge application guide

Relative Centrifugal Force (RCF) or G-Force/revolutions per minute (rpm) conversion

VALUABLE INNOVATIONS DESIGNED FOR YOU

		C 12	C 20	C 20-R ❄	C 48	C 48-R ❄	MF 20	MF 20-R ❄	MF 48	MF 48-R ❄
	Max. Capacity	4 x 100 ml	4 x 200 ml	4 x 200 ml	4 x 400 ml	4 x 400 ml	4 x 200 ml	4 x 200 ml	4 x 400 ml	4 x 400 ml
	Temp. Range			-9 / +40°C		-9 / +40°C		-9 / +40°C		-9 / +40°C
							AWELock	AWELock	AWELock	AWELock
PERFORMANCES	Max. RCF (xg)	3000	3500	3500	3820	3820	20350	20350	20350	20350
	Max. RPM	4000	4400	4400	4500	4500	13800	13800	13800	13800
Swing-out rotor	**Tubes**									
	5/7 ml collection tube	16	36	36	60	60	36	36	60	60
		4000 rpm 2862 xg	4400 rpm 3413 xg	4400 rpm 3413 xg	4500 rpm 3649 xg	4500 rpm 3649 xg	4400 rpm 3413 xg	4400 rpm 3413 xg	4500 rpm 3649 xg	4500 rpm 3649 xg
	10 ml collection tube	16	24	24	48	48	24	24	48	48
		4000 rpm 2862 xg	4400 rpm 3413xg	4400 rpm 3413xg	4500 rpm 3649 xg	4500 rpm 3649 xg	4400 rpm 3413xg	4400 rpm 3413xg	4500 rpm 3649 xg	4500 rpm 3649 xg
	15 ml conical bottom	4	12	12	28	28	12	12	28	28
		4000 rpm 2862 xg	4400 rpm 3500 xg	4400 rpm 3500 xg	4500 rpm 3763 xg	4500 rpm 3763 xg	4400 rpm 3500 xg	4400 rpm 3500 xg	4500 rpm 3763 xg	4500 rpm 3763 xg
	50 ml conical bottom	4	4	4	12	12	4	4	12	12
		4000 rpm 2862 xg	4400 rpm 3435 xg	4400 rpm 3435 xg	4500 rpm 3808 xg	4500 rpm 3808 xg	4400 rpm 3435 xg	4400 rpm 3435 xg	4500 rpm 3808 xg	4500 rpm 3808 xg
Angle rotor	**Tubes**									
	1,5-2 ml microtubes	-	-	-	-	-	24	24	24	24
		- -	- -	- -	- -	- -	13800 rpm 20350 xg	13800 rpm 20350 xg	13800 rpm 20350 xg	13800 rpm 20350 xg
	1,5-2 ml microtubes	-	-	-	-	-	30	30	30	30
		- -	- -	- -	- -	- -	12500 rpm 17990 xg	12500 rpm 17990 xg	12500 rpm 17990 xg	12500 rpm 17990 xg
	10 ml round bottom	-	-	-	-	-	10	10	10	10
		- -	- -	- -	- -	- -	13000 rpm 18130 xg	13000 rpm 18130 xg	13000 rpm 18130 xg	13000 rpm 18130 xg
	50 & 15 ml conical bottom	-	-	-	-	-	8	8	8	8
		- -	- -	- -	- -	- -	11300 rpm 15000 xg	11300 rpm 15000 xg	11300 rpm 15000 xg	11300 rpm 15000 xg
	100 ml	-	-	-	-	-	-	-	6	6
		- -	- -	- -	- -	- -	- -	- -	9300 rpm 10000 xg	9300 rpm 10000 xg
	15 ml	32	32	32	32	32	32	32	32	32
		4000 rpm 2500 xg	4400 rpm 3030 xg	4400 rpm 3030 xg	4400 rpm 3030 xg	4400 rpm 3030 xg	4400 rpm 3030 xg	4400 rpm 3030 xg	4400 rpm 3030 xg	4400 rpm 3030 xg
Microplates rotor	**Microplates**									
	96 well plate	-	-	-	-	-	6	6	6	6
		- -	- -	- -	- -	- -	4100 rpm 2380 xg	4100 rpm 2380 xg	4100 rpm 2380 xg	4100 rpm 2380 xg
	Cell culture plate	-	-	-	-	-	6	6	6	6
		- -	- -	- -	- -	- -	4100 rpm 2380 xg	4100 rpm 2380 xg	4100 rpm 2380 xg	4100 rpm 2380 xg
	Deep Well	-	-	-	-	-	2	2	2	2
		- -	- -	- -	- -	- -	4100 rpm 2380 xg	4100 rpm 2380 xg	4100 rpm 2380 xg	4100 rpm 2380 xg

Conversion between *g* and rpm
An Example: 5000g (~6200 rpm)

- The speed of a centrifuge is often described in revolutions per minute (RPM).
- Conversely, relative centrifugal force (RCF), commonly referred to as "g-force"
  is another universal unit for measuring the speed of a centrifuge.

$$RCF = 0.00001118 \cdot r \cdot RPM^2$$

RCF = relative centrifugal force
r = rotational radius in centimeters
RPM = rotations per minute

or

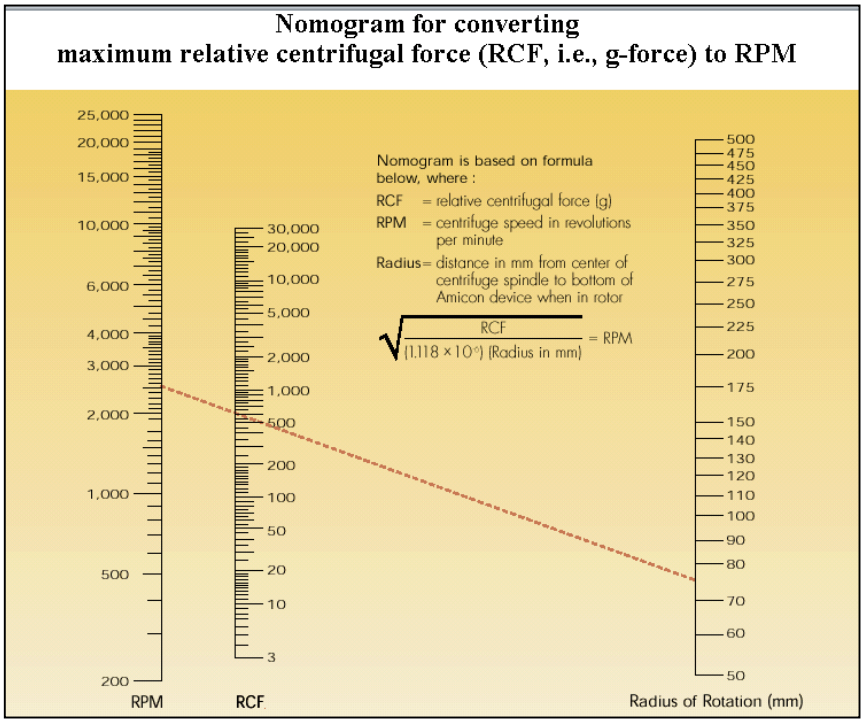
$$g\ \text{force} = 1.12 \times 10^{-5} \times r \times \text{rpm}$$

r = radius in cm

- RCF, will yield more reproducible results than selecting a rotor based on rpm characteristics.
- The *g* force is calculated as follows, and holds true for standard and microcentrifuges:

Conversion between *g* and rpm

- **To convert maximum relative centrifugal force (RCF or g) to RPM:**
- Determine centrifuge 's radius of rotation (in mm) by measuring distance from center of centrifuge spindle to bottom of device when inserted into rotor.
- Lay a ruler or draw a line from radius value in right-hand column value that corresponds to the device 's maximum rated g-force.
- Then read the maximum value from column at left.



Wikipedia,2008

Centrifuge models

Conversion between *g* and rpm

Model No.	Tube Volume (ml)	Max. RPM (rpm)	Max. RCF(g)
LAR 113	24 x 15	8,000	8,729 x g
LAR 114	10 x 50	10,000	12,857 x g
LAR 115	6 x 85	10,000	11,068 x g
WSR 2	6 x 750	5,000	6,149 x g

As an example, centrifugation of a sample at 5,000 RPM in a microcentrifuge that has a rotor with a radius of 7 cm will deliver acentrifugal force of 1,957 × g.

Useful numbers & conversions

Mega	M	10^{6}
kilo	k	10^{3}
milli	m	10^{-3}
micro	μ	10^{-6}
nano	n	10^{-9}
pico	p	10^{-12}
femto	f	10^{-15}
atto	a	10^{-18}

Isotopes

1 μCi = 2.2 x 10^{6} dpm = 37 kBq (for ^{32}P: 1 dpm=1 cpm)
1 Bq = 1 dps = 2.7 x 10^{-5} μCi Bequerel (Bq)
1 Gray (Gy) = 100 rad
1 Sievert (Sv) = 100 rem

Halflives:

β-emitters	^{32}P	14.29 days	^{35}S	87.44 days
	^{14}C	5730 years	^{3}H	12.43 years
	^{33}P	25.5 days		
γ-emitters	^{125}I	60 days	^{131}I	8.06 days

label	sp. act. Ci/mmole	μCi per μl	pmole per μl	% molecules labeled
^{32}P	800	10	12.5	10.7
^{32}P	3000	10	3.33	40.2
^{32}P	6000	100	16.6	80
^{35}S	1500	12.5	8.3	69

DNA Conversion Factors

1 bp = 660 g/mole
1 μg DNA = 1.515 nmoles bp
(nmoles bp)/(bp per molecule) = nmoles of DNA molecule
1 μg plasmid DNA= [1.515/(bp per plasmid)] nmoles of plasmid

Genome Sizes

Human	3 x 10^{9} bp
Yeast	2 x 10^{7} bp
E. coli	3 x 10^{6} bp

Douglas Rhoads,2008

Converting Kilograms to Milliliters

- 1 kilogram (kg) = 1000 milliliter (ml).
- Kilogram (kg) is a unit of Weight used in Metric system.
- Milliliter (ml) is a unit of Volume used in Metric system.
- Please note this is weight to volume conversion, this conversion is valid only for pure water at temperature 4 °C.

Conversion Chart

kilograms to milliliters Conversion Table:

1 **kg** = 1000 **ml**	51 **kg** = 51000 **ml**
2 **kg** = 2000 **ml**	52 **kg** = 52000 **ml**
3 **kg** = 3000 **ml**	53 **kg** = 53000 **ml**
4 **kg** = 4000 **ml**	54 **kg** = 54000 **ml**
5 **kg** = 5000 **ml**	55 **kg** = 55000 **ml**
6 **kg** = 6000 **ml**	56 **kg** = 56000 **ml**
7 **kg** = 7000 **ml**	57 **kg** = 57000 **ml**
8 **kg** = 8000 **ml**	58 **kg** = 58000 **ml**
9 **kg** = 9000 **ml**	59 **kg** = 59000 **ml**
10 **kg** = 10000 **ml**	60 **kg** = 60000 **ml**
11 **kg** = 11000 **ml**	61 **kg** = 61000 **ml**
12 **kg** = 12000 **ml**	62 **kg** = 62000 **ml**
13 **kg** = 13000 **ml**	63 **kg** = 63000 **ml**
14 **kg** = 14000 **ml**	64 **kg** = 64000 **ml**
15 **kg** = 15000 **ml**	65 **kg** = 65000 **ml**
16 **kg** = 16000 **ml**	66 **kg** = 66000 **ml**
17 **kg** = 17000 **ml**	67 **kg** = 67000 **ml**
18 **kg** = 18000 **ml**	68 **kg** = 68000 **ml**
19 **kg** = 19000 **ml**	69 **kg** = 69000 **ml**
20 **kg** = 20000 **ml**	70 **kg** = 70000 **ml**
21 **kg** = 21000 **ml**	71 **kg** = 71000 **ml**
22 **kg** = 22000 **ml**	72 **kg** = 72000 **ml**
23 **kg** = 23000 **ml**	73 **kg** = 73000 **ml**
24 **kg** = 24000 **ml**	74 **kg** = 74000 **ml**
25 **kg** = 25000 **ml**	75 **kg** = 75000 **ml**
26 **kg** = 26000 **ml**	76 **kg** = 76000 **ml**
27 **kg** = 27000 **ml**	77 **kg** = 77000 **ml**
28 **kg** = 28000 **ml**	78 **kg** = 78000 **ml**
29 **kg** = 29000 **ml**	79 **kg** = 79000 **ml**
30 **kg** = 30000 **ml**	80 **kg** = 80000 **ml**
31 **kg** = 31000 **ml**	81 **kg** = 81000 **ml**
32 **kg** = 32000 **ml**	82 **kg** = 82000 **ml**
33 **kg** = 33000 **ml**	83 **kg** = 83000 **ml**
34 **kg** = 34000 **ml**	84 **kg** = 84000 **ml**
35 **kg** = 35000 **ml**	85 **kg** = 85000 **ml**
36 **kg** = 36000 **ml**	86 **kg** = 86000 **ml**
37 **kg** = 37000 **ml**	87 **kg** = 87000 **ml**
38 **kg** = 38000 **ml**	88 **kg** = 88000 **ml**
39 **kg** = 39000 **ml**	89 **kg** = 89000 **ml**
40 **kg** = 40000 **ml**	90 **kg** = 90000 **ml**
41 **kg** = 41000 **ml**	91 **kg** = 91000 **ml**
42 **kg** = 42000 **ml**	92 **kg** = 92000 **ml**
43 **kg** = 43000 **ml**	93 **kg** = 93000 **ml**
44 **kg** = 44000 **ml**	94 **kg** = 94000 **ml**
45 **kg** = 45000 **ml**	95 **kg** = 95000 **ml**
46 **kg** = 46000 **ml**	96 **kg** = 96000 **ml**

Metric Length and Fluid Volume

- **centi (c) =** 10^{-2} or 1/100
  - centimeter (cm) = 10^{-2} m or 1/100 m
- **milli (m) =** 10^{-3} or 1/1000
  - millimeter (mm) = 10^{-3} m or 1/1000 m
  - milliliter (ml) = 10^{-3} l or 1/1000 l
- **micro (µ) =** 10^{-6} or 1/1,000,000
  - micrometer (m) = 10^{-6} m or 1/1,000,000 m
  - microliter (l) = 10^{-6} l or 1/1,000,000 l
- **nano (n)** = 10^{-9} or 1/1,000,000,000
  - nanometer (nm) = 10^{-9} m or 1/1,000,000,000 m

Kaiser,2009

Unit information

International System of Units

1 mg/mL = 1000 µg/mL

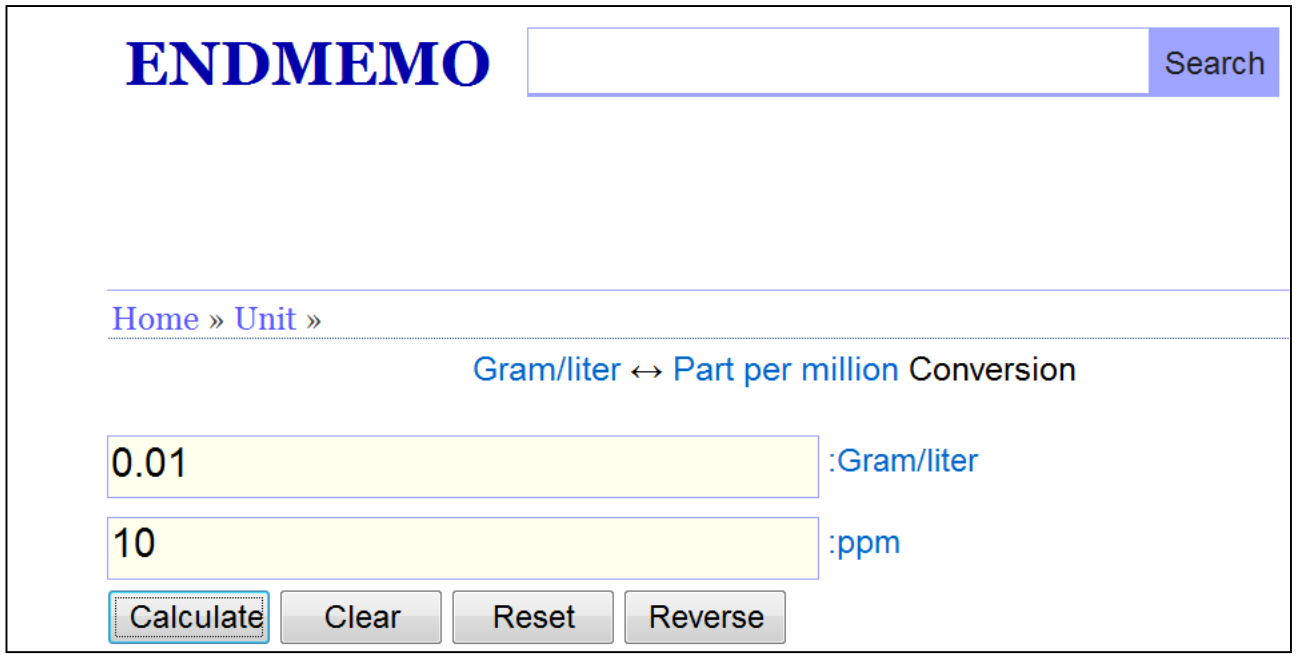
Submultiples				Multiples		
Value	**Symbol**	**Name**		**Value**	**Symbol**	**Name**
10^{-1} g	dg	decigram		10^{1} g	dag	decagram
10^{-2} g	cg	centigram		10^{2} g	hg	hectogram
10^{-3} g	mg	milligram		10^{3} g	kg	kilogram
10^{-6} g	µg	microgram (mcg)		10^{6} g	Mg	megagram (tonne)
10^{-9} g	ng	nanogram		10^{9} g	Gg	gigagram
10^{-12} g	pg	picogram		10^{12} g	Tg	teragram
10^{-15} g	fg	femtogram		10^{15} g	Pg	petagram
10^{-18} g	ag	attogram		10^{18} g	Eg	exagram
10^{-21} g	zg	zeptogram		10^{21} g	Zg	zettagram
10^{-24} g	yg	yoctogram		10^{24} g	Yg	yottagram
Common prefixes are in bold face.						

Wikipedia,2011

Endmemo

Online unit conversions

Gram/liter ↔ Part per million(ppm) Conversion



1mg/1000ml=1ppm
1mg/100ml=10ppm
1mg/10ml=100ppm

Endmemo

Gram/liter ↔ Part per million Conversion

1g/L=1000 ppm or 1 mg/L =1 ppm

1 mg/L =	1 ppm	2 mg/L =	2 ppm
3 mg/L =	3 ppm	4 mg/L =	4 ppm
5 mg/L =	5 ppm	6 mg/L =	6 ppm
7 mg/L =	7 ppm	8 mg/L =	8 ppm
9 mg/L =	9 ppm	10 mg/L =	10 ppm
11 mg/L =	11 ppm	12 mg/L =	12 ppm
13 mg/L =	13 ppm	14 mg/L =	14 ppm
15 mg/L =	15 ppm	16 mg/L =	16 ppm
17 mg/L =	17 ppm	18 mg/L =	18 ppm
19 mg/L =	19 ppm	20 mg/L =	20 ppm
21 mg/L =	21 ppm	22 mg/L =	22 ppm
23 mg/L =	23 ppm	24 mg/L =	24 ppm
25 mg/L =	25 ppm	26 mg/L =	26 ppm
27 mg/L =	27 ppm	28 mg/L =	28 ppm
29 mg/L =	29 ppm	30 mg/L =	30 ppm
31 mg/L =	31 ppm	32 mg/L =	32 ppm
33 mg/L =	33 ppm	34 mg/L =	34 ppm
35 mg/L =	35 ppm	36 mg/L =	36 ppm
37 mg/L =	37 ppm	38 mg/L =	38 ppm
39 mg/L =	39 ppm	40 mg/L =	40 ppm
41 mg/L =	41 ppm	42 mg/L =	42 ppm
43 mg/L =	43 ppm	44 mg/L =	44 ppm
45 mg/L =	45 ppm	46 mg/L =	46 ppm
47 mg/L =	47 ppm	48 mg/L =	48 ppm
49 mg/L =	49 ppm	50 mg/L =	50 ppm

Buffers

Tris buffer

TE buffer(Tris-EDTA buffer)

- Tris protects the DNA from pH Shifts.
- Tris, or tris(hydroxymethyl) aminomethane, is a common biological buffer, used throughout the DNA extraction process.
- During extraction from any number of sources, DNA is pH sensitive.

1. During cell lysis, removal of unwanted cellular components and precipitation, tris is used to maintain a stable pH.
2. Additionally, it plays a particularly important role in cell lysis.

Tan,2017

Buffers

TE buffer(Tris-EDTA buffer) (Continued)

- A typical recipe for making 1XTE buffer is:

1. 10 mM Tris, bring to pH 8.0 with HCl
2. 1 mM EDTA

- To make a 100 ml solution of TE Buffer:
- 1 ml of 1 M Tris base (pH 8.0) and 0.2 ml EDTA (0.5 M) are mixed and made up with double distilled water up to 100 ml.
- Add microliter amounts of high molarity HCl to lower the pH to 8.
- Autoclave to sterilize.
- It is then best to remove a working aliquot and not repeatedly access the stock.

TE Buffer is an autoclaved 100X concentrated solution of 1 M Tris-HCl, pH 8, with 0.1 M Na_2EDTA. This buffer is the standard for DNA and RNA purification, processing and storage.

Buffers

TE buffer(Tris-EDTA buffer) (Continued)

- To make the component solutions:
- **1M Tris (hydroxymethyl) aminomethane:**
- MWT of Tris is 121.4 g/mol.
- Dissolve 60.57 g in 500 ml water
- Adjust pH to 8.0 using HCl
- **0.5M EDTA (Ethylenediaminetetraacetic acid):**
- MWT of EDTA is 372.2 g/mol.
- 18.6 g in 500 ml ml water
- pH to 8.0 using NaOH
- EDTA will not be soluble until pH reaches 8.0.
- This will take time (hours)
- Use vigorous stirring, moderate heat (if desired) and time.

Alex Aitken; Wikipedia

Buffers

TE Buffer 10X Preparation and Recipe

TE Buffer 10X. Min. pH 7.5 and Max. pH 8.5

Component	Amount	Concentration
Tris-Cl (desired pH) (mw: 157.594 g/mol)	15.759 g	0.1 M
EDTA (pH 8) (mw: 292.24 g/mol)	2.92 g	0.01 M

- Prepare 800 mL of distilled water in a suitable container.
- Add 15.759 g of Tris-Cl (desired pH) to the solution.
- Add 2.92 g of EDTA (pH 8) to the solution.
- Add distilled water until volume is 1 L.

Buffers

TAE (1 M, pH 8.6) Preparation and Recipe

Min. pH 8 and Max. pH 8.5

Component	Amount	Concentration
Tris base (mw: 121.14 g/mol)	242 g	2 M
Disodium EDTA (mw: 336.21 g/mol)	18.61 g	1 M
Acetic Acid (mw: 60.05 g/mol)	59.955 g	1 M

- Prepare 800 mL of distilled water in a suitable container.
- Add 15.759 g of Tris-Cl (desired pH) to the solution.
- Add 2.92 g of EDTA (pH 8) to the solution.
- Add distilled water until volume is 1 L.

Buffers

How to make a 1X from a 10X?

- **Remember solutions are usually 1X when used.**
- Here, reagent is the TBE buffer.
- X refers to the concentration.
- It is a 10 times concentrated stock.
- You generally use reagents at 1X.
- How to make a 1X from a 10X?
- Answer:
- Assume you have 10X buffer. To make a 100ml final solution of 1X, use 10ml of 10X and add it to 90ml of water and you have your 100ml of 1X buffer.

Buffers

How to make 1xTAE from 50X TAE stock solution

- How to make a 1X from a 50X?
- Answer:
- Dilute 20 ml of stock into 980 ml of deionized water.

Buffers

How to make 1x TAE from a 5X TAE stock solution

- How to make a 1X from a 5X?
- Answer:
- 1:5 dilution = 1/5 dilution = 1 part sample and 4 parts diluent in a total of 5 parts.
- Dilute 20ml of stock into 80 ml of deionized water.
- Below is a useful formula for doing dilution calculations:

initial concentration x initial volume = final concentration x final volume

- For 100 ml:
- (5x) (X ml) = (1x) (100 ml)
- X ml = (1x) (100 ml)/(5x)
- X ml = 20 ml of a 5x (i.e. 20ml of stock into 80 ml of deionized water).

Buffers

How to make 500 ml of buffer solution when your stock solution is 5X

- **For 500 ml:**
- You need to make 500 ml of buffer solution and your stock solution is 5X.
- How would you make this solution? (Remember, solutions are usually 1X when used)
- **?(5X)=500ml(1X)**
- Answer:
- **100 mL**
- **The formula used for this calculation:**

Initial concentration x initial volume = final concentration x final volume

- ? Ml (5x) = (1x) (500 ml)
- ? ml = (1x) (500 ml)/(5x)
- ? ml = 100 ml of a 5x (i.e. 100ml of stock into 400 ml of deionized water).

Buffers

Tris Borate EDTA(TBE) buffer

- **Tris borate EDTA(TBE):**
- TBE Buffer is used for polyacrylamide and agarose gel electrophoresis.
- **A 1X solution** is obtained by adding 1 part of the 10XTBE buffer to 9 parts of deionized water.
- **For 0.5X TBE Electrophoresis Buffer for 1L:**
- 5X TBE 100 mL
- Distilled, deionized water 900 mL
- In an Erlenmeyer flask, add 100 mL of 5X TBE to 900 mL of distilled deionized water. Mix well and use.

Buffers

TBE Buffer Recipe

Used for agarose and polyacrylamide gel electrophoresis of nucleic acid

- **1x TBE (1 liter):**
- Dissolve 10.8 g Tris and 5.5 g Boric acid in 900 ml distilled water.
- Add 4 ml 0.5 M Na_2EDTA (pH 8.0) (alternatively use 9.3 g Na_2EDTA)
- Adjust volume to 1 Liter.
- Store at room temperature. Don't autoclave.

Note: EDTA will not go completely into a solution until the pH is adjusted to about 8.0. TBE Buffer is effective for the separation of fragments between 1 and 3000bp in length.

Buffers

Tris Acetate-EDTA (TAE) buffer

Used for agarose and polyacrylamide gel electrophoresis of nucleic acid

- **Tris acetate-EDTA (TAE) buffer 50X:**
- Tris base 242 g
- Glacial acetic acid 57.1 ml
- 0.5 M EDTA (pH 8.0) 100 ml
- pH was adjusted to 7.2 and the final volume was made to 1000 ml using distilled water.
- **1x TAE buffer (40 mM Tris-acetate/1 mM EDTA, pH 8).**

- **TAE Buffer Recipe 1 Liter of 10X TAE Running Buffer, pH 8.18-8.29**
- Tris-base 48.4 g
- Glacial Acetic Acid 10.9 g
- EDTA (free acid Molecular Weight: 292.24) 2.92 g
- DI Water to 1L.

TAE Buffer is used effectively for separating fragments which are larger than 4000bp and is also used to separate super coiled DNA.

Buffers
Loading buffer

- The loading buffer gives colour and density to the sample to make it easy to load into the wells.
- Also, the dyes are negatively charged in neutral buffers and thus move in the same direction as the DNA during electrophoresis.
- This allows you to monitor the progress of the gel.
- The most common dyes are bromophenol blue (Sigma B8026) and xylene cyanol (Sigma X4126).
- Density is provided by glycerol or sucrose.
- **Typical recipe:**
- 25 mg bromophenol blue or xylene cyanol
- 4 g sucrose
- H_2O to 10 mL
- The exact amount of dye is not important.
- Store at 4°C to avoid mould growing in the sucrose.
- 10 mL of loading buffer will last for years.

Lewis,2001

Phosphate buffer saline

PBS

Phosphate-buffered Saline 0.02M, pH 6.8

- Prepare 800 mL of dH_2O in a suitable container.
- Add 1.591 g of Potassium phosphate dibasic (K_2HPO_4) to the solution.
- Add 1.479 g of Potassium Phosphate Monobasic (KH_2PO_4) to the solution.
- Add dH_2O until the volume is 1 L.

Phosphate buffer saline

PBS

Phosphate-buffered Saline 10mM, pH 7.2

- There are many different ways to prepare PBS.
- Generally, PBS contains the following constituents:
- Phosphate-buffered Saline 10mM, pH 7.2
- NaCl 8 g
- KCl 0.2 g
- $Na_2HPO_4 .12H_2O$ 2.9 g
- KH_2PO_4 0.2 g
- Distilled H_2O 1 L
- Sterilized by autoclaving and stored in sealed container in cold room.

The buffer's phosphate groups help to maintain a constant pH.

Phosphate buffer saline

1X Phosphate Buffered Saline (PBS Buffer) Recipe

- There are many different ways to prepare PBS.
- Dissolve the following in 800ml distilled H_2O.
  - 8g of NaCl
  - 0.2g of KCl
  - 1.44g of Na_2HPO_4
  - 0.24g of KH_2PO_4
- Adjust pH to 7.4 with HCl.
- Adjust volume to 1L with additional distilled H_2O.
- Sterilize by autoclaving.

Phosphate buffer saline
PBS

- There are many different ways to prepare PBS.
- The buffer solution contains sodium chloride, sodium phosphate and/or potassium phosphate.
- There exist formulas for the use of either stock solutions or by weighing directly the solid components into distilled water.
- Here are given some examples:
- **(a) Phosphate buffer 0.01 M pH 7.4 containing 0.9% NaCl**
- Disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$)= 2.76 g
- Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)= 0.35 g
- Sodium chloride (NaCl)= 9.0 g
- Dissolve in 1000 ml distilled water and adjust pH to 7.4

Phosphate buffer saline
PBS

- **(b) Phosphate buffered saline (10x PBS) 0.1 M pH 7.2**
- Disodium hydrogen phosphate (Na_2HPO_4 anhydrous)= 10.9 g
- Sodium dihydrogen phosphate (NaH_2PO_4 anhydrous)= 3.2 g
- Sodium chloride (NaCl)= 90.0 g
- Dissolve in 1000 ml distilled water and adjust pH to 7.2.
- Dilute 1:10 with distilled water prior to use and adjust pH if necessary.

Phosphate buffer saline
PBS

- **c) Phosphate buffered saline (10x PBS) 0.1 M pH 7.4**
- Disodium hydrogen phosphate (Na_2HPO_4 anhydrous)= 14.4 g
- Sodium dihydrogen phosphate (KH_2PO_4 anhydrous)= 2.4 g
- Sodium chloride (NaCl)= 80.0 g
- Potassium chloride (KCl)= 2.0 g
- Dissolve in 1000 ml distilled water; pH is about 6.8, but when diluted to 1x PBS pH will change to 7.4; on dilution, the final 1x PBS will have a pH 7.4 and a final concentration of 10 mM phosphate, 137 mM NaCl and 2.7 mM KCl.

Phosphate buffer and phosphate buffered saline

- **(d) Phosphate buffer and phosphate buffered saline (PBS) 0.05 M pH 6.6-7.6**
- Stock solution A: 1 M KH_2PO_4(Potassium dihydrogene phosphate) in distilled water.
- Stock solution B: 1 M K_2HPO_4 (Dipotassium hydrogen phosphate) in distilled water.
- Stock solution C: 5 M NaCl (Sodium chloride) in distilled water.
- Example for a 0.05 M phosphate buffer pH 7.0:
- The solution is prepared by mixing 21.1 ml of stock A and 28.9 ml of stock B and dilution to one liter with distilled water; the desired pH should be confirmed by a pH meter.

Phosphate buffer and phosphate buffered saline

- **(d) Phosphate buffer and phosphate buffered saline (PBS) 0.05 M pH 6.6-7.6**

Preparation of 0.05 M phosphate buffer solution		
Stock solution A (mL)	**Stock solution B (mL)**	**pH-value**
32.0	18.0	6.6
29.8	20.2	6.7
26.5	23.5	6.8
24.0	26.0	6.9
21.1	28.9	7.0
18.4	31.6	7.1
16.8	34.2	7.2
13.4	36.6	7.3
11.2	38.8	7.4
9.4	40.6	7.5
7.8	42.2	7.6

Phosphate buffer saline

PBS

Phosphate buffer 0.1 M pH 5.3-8.04

- **Phosphate buffer 0.1 M pH 5.3-8.04**
- Stock solution A: 0.1 M potassium dihydrogen phosphate. Dissolve 13.61 g KH_2PO_4 in 1000 ml distilled water.
- Stock solution B: 0.1 M disodium hydrogen phosphate. Dissolve 17.8 g $Na_2HPO_4.2H_2O$ in 1000 ml distilled water.
- Preparation of the buffer solution: mix stock A and stock B according to the list below:

Phosphate buffer saline

Phosphate buffer 0.1 M pH 5.3-8.04

Adjust the final pH using a sensitive pH meter

Stock solution A (mL)	Stock solution B (mL)	pH-value
97.5	2.5	5.30
95.0	5.0	5.60
90.0	10.0	5.91
80.0	20.0	6.24
70.0	30.0	6.47
60.0	40.0	6.64
50.0	50.0	6.81
40.0	60.0	6.98
30.0	70.0	7.17
20.0	80.0	7.38
10.0	90.0	7.73
5.0	95.0	8.04

Phosphate buffer saline

PBS

PBS with 0.05 M phosphate buffer pH 7.0

- **Example for PBS with 0.05 M phosphate buffer pH 7.0:**
- The solution is prepared by mixing of 15.6 ml of stock A, 34.4 ml of stock B, 30.0 ml of stock C and dilution to one liter with distilled water; the desired pH should be confirmed by a pH meter.

- Be aware that sodium chloride lowers pH approximately 0.01 pH unit for each 0.01 increase in molarity.
- Furthermore, pH of phosphate buffers increases with decreasing temperature. Compared with a buffer at 25°C, buffers at 4°C will be 0.08 higher and a buffer at 37°C will be 0.025 lower.
- The concentration of phosphate also influences pH. The dilution value for phosphate, defined as the change of pH of a buffer when diluted with an equivalent volume of water is 0.08.
- To give an example, a 0.025 M phosphate buffer prepared with half of the volumes as indicated in the Table for a specific pH (see above), would be approximately 0.08 pH units higher than the expected pH.

Phosphate buffer saline

PBS

PBS with 0.05 M phosphate buffer pH 7.0

Preparation of 0.05 M phosphate buffer solution			
Stock A (mL)	Stock B (mL)	Stock C (mL)	pH-value
26.6		23.4	6.6
23.7		26.3	6.7
20.9		29.1	6.8
18.1		31.9	6.9
15.6		34.4	7.0
13.2		36.8	7.1
11.1		38.9	7.2
9.2		40.8	7.3
7.6		42.4	7.4
6.3		43.7	7.5
5.1		44.9	7.6

Phosphate buffer saline

PBS

Phosphate-buffered Saline 0.1M, pH 7.4

- **For 1L Phosphate-buffered Saline 0.1M, pH 7.4:**
- 8 gr NaCl
- 0.22 gr KCl
- 1.44 gr $Na_2HPO_4.2H_2O$
- 0.2 gr KH_2PO_4
- Adjust to pH 7.4 by mixing with necessary amounts of HCl or NaOH.
- Take 100 ml of the buffer and add it to 900 ml ddH_20.
- Sterilized by autoclaving and stored in sealed container in cold room.

Sterilization may not be necessary depending on its use. PBS can be stored at room temperature. Store up to 6 months at room temperature. Discard any reagent that shows evidence of contamination, precipitation, or discoloration.

Phosphate buffers
Potassium Phosphate Buffer

- Phosphate (0.05 mol/L pH 7.02)
- Na_2HPO_4 4.26 g
- KH_2PO_4 2.72 g
- Distilled water 1 L.
- Dissolve ingredients, Check pH and autoclave.

Phosphate buffers

Potassium Phosphate Buffer

Alternative method

- **Potassium phosphate buffer, 0.1M**
- Solution A: 27.2 g KH_2PO_4 per liter (0.2 M final) in water.
- Solution B: 34.8 g K_2HPO_4 per liter (0.2 M final) in water.
- Referring to Table A.2A.3 for desired pH, mix the indicated volumes of solutions and B, then dilute with water to 200 ml.
- Filter sterilize if necessary. Store up to 3 months at room temperature.

Phosphate buffers

Potassium Phosphate Buffer

Table A.2A.3 Preparation of 0.1M Sodium and Potassium Phosphate Buffers

Desired pH	Solution A (ml)	Solution B (ml)	Desired pH	Solution A (ml)	Solution B (ml)
5.7	93.5	6.5	6.9	45.0	55.0
5.8	92.0	8.0	7.0	39.0	61.0
5.9	90.0	10.0	7.1	33.0	67.0
6.0	87.7	12.3	7.2	28.0	72.0
6.1	85.0	15.0	7.3	23.0	77.0
6.2	81.5	18.5	7.4	19.0	81.0
6.3	77.5	22.5	7.5	16.0	84.0
6.4	73.5	26.5	7.6	13.0	87.0
6.5	68.5	31.5	7.7	10.5	90.5
6.6	62.5	37.5	7.8	8.5	91.5
6.7	56.5	43.5	7.9	7.0	93.0
6.8	51.0	49.0	8.0	5.3	94.7

[a] Adapted by permission from CRC (1975).

pH table for:

Potassium phosphate buffers & Sodium phosphate buffers

Alternative method: Calculation of concentration based on % which is actually more straightforward then molarity

- pH table for Potassium phosphate buffer at 25°C. At 0°C temperature, the solubility of solid potassium phosphate is 44 whereas in 20°C it increases to the level of 50.

pH	% K_2HPO_4 (dibasic)	% KH_2PO_4 (monobasic)
5.8	8.5	91.5
6.0	13.2	86.8
6.2	19.2	80.8
6.4	27.8	72.2
6.6	38.1	61.9
6.8	49.7	50.3
7.0	61.5	38.5
7.2	71.7	28.3
7.4	80.2	19.8
7.6	86.6	13.4
7.8	90.8	9.2
8.0	94.0	6.0

e.g. 8.5% means 8.5 g/100 ml $D.H_2O$.

- pH table for Sodium phosphate buffer at 25°C.

pH	% Na_2HPO_4 (dibasic)	% NaH_2PO_4 (monobasic)
5.7	6.5	93.5
5.8	8.0	92.0
5.9	10.0	90.0
6.0	12.3	87.7
6.1	15.0	85.0
6.2	18.5	81.5
6.3	22.5	77.5
6.4	26.5	73.5
6.5	31.5	68.5
6.6	37.5	62.5
6.7	43.5	56.5
6.8	49.0	51.0
6.9	55.0	45.0
7.0	61.0	39.0
7.1	67.0	33.0
7.2	72.0	28.0
7.3	77.0	23.0
7.4	81.0	19.0
7.5	84.0	16.0
7.6	87.0	13.0
7.7	89.5	10.5
7.8	91.5	8.5
8.0	94.7	5.3

Buffer and other solutions

- **Phosphate buffered saline – Tween 80 (PBST-80):**
- Tween 80 (0.5 ml) was added to 1 L PBS. The solution was stored at room temperature.
- **30% (v/v) Glycerol ($C_3H_5(OH)_3$):**
- Glycerol (30 ml) was added to 70 ml deionised water. The solution was sterilized by autoclaving and stored at room temperature.
- **0.5 M Ethylenediaminetetra acetic acid (EDTA) ($C_{10}H_{16}N_2O_8$):**
- EDTA (186 mg) was added to 1 L of deionised water. The pH was adjusted to pH 8. The solution was sterilized by autoclaving and stored at room temperature.

Piasecka,2010

Buffer and other solutions

- **1M Tris ($C_4H_{11}NO_3$):**
- Tris (121.14 g) was dissolved in 1 L deionised water. The pH was adjusted to pH 7.5. The solution was sterilized by autoclaving and stored at room temperature.
- **1M Tris HCl:**
- Dissolve 121g Tris Base (BPE152) in 800mL H_2O. Adjust solution to desired pH with concentrated HCl. Mix and add H_2O to 1 litre. The solution was sterilized by autoclaving and stored at room temperature.
- **Tris (10mM):**
- 0.12 g of tris was dissvoled in 100 ml of double distilled water and adjusted to pH 7.6 using HCl.

Note: Tris-HCl can be prepared using Tris base (molecular weight: 121.14 g/mol), or Tris-HCl (Tris base which is already combined with HCl in a 1:1 molar ratio, so the molecular weight is 157.6 g/mol).

Buffer and other solutions

- **TE buffer(details in next slide):**
- 10 ml 1M Tris (pH 7.5) and 2 ml 0.5 M EDTA (pH 8) to 800 ml deionised water. Mixed and adjust to 1 L with deionised water. The buffer was sterilized by autoclaving and stored at room temperature.
- Note: EDTA will not be soluble until pH reaches 8.0. Use vigorous stirring, moderate heat (if desired) and time

Piasecka,2010;..

Buffer and other solutions

- **20 mg/ml proteinase K (EC 3.4.21.64):**
- Proteinase K (20 mg) was added to 1 ml of deionised water.The solution was mixed and stored at -20°C.
- **6 M Sodium chloride (NaCl) (NaCl saturated H_2O):**
- NaCl (35 g) was added to 100 ml of deionised water. The solution was mixed, autoclaved and stored at room temperature. NaCl 6M is saturated salt solution stored at 37°C.
- **30% Polyethylene glycol (PEG) ($C_{2n}+_2H_{4n}+_6O_{n+2}$):**
- PEG (30 g) was added to 100 ml deionised water, and dissolved. The solution was stored at room temperature.
- **1.6 M Sodium chloride (NaCl):**
- NaCl (9.35 g) was added to 100 ml of deionised water. The solution was mixed, autoclaved and stored at room temperature.

Buffer and other solutions

- **7.5 M Potassium acetate (CH3CO2K):**
- CH_3CO_2K (73.65 g) was added to 100 ml of deionised water. The solution was mixed, autoclaved and stored at -20°C.
- **5 µg/µl Polyadenyli acid potassium salt (Poly A) (Carrier RNA):**
- Poly A (0.5 mg) was added to 100 µl of deionised water. The solution was mixed and stored at -20°C.
- **70% C_2H_6O (Ethanol Absolute, 100 % (200 proof):**
- Ethanol (15 ml) was added to 35 ml deionised water. The solution was stored at room temperature.

Piasecka,2010

Buffer and other solutions

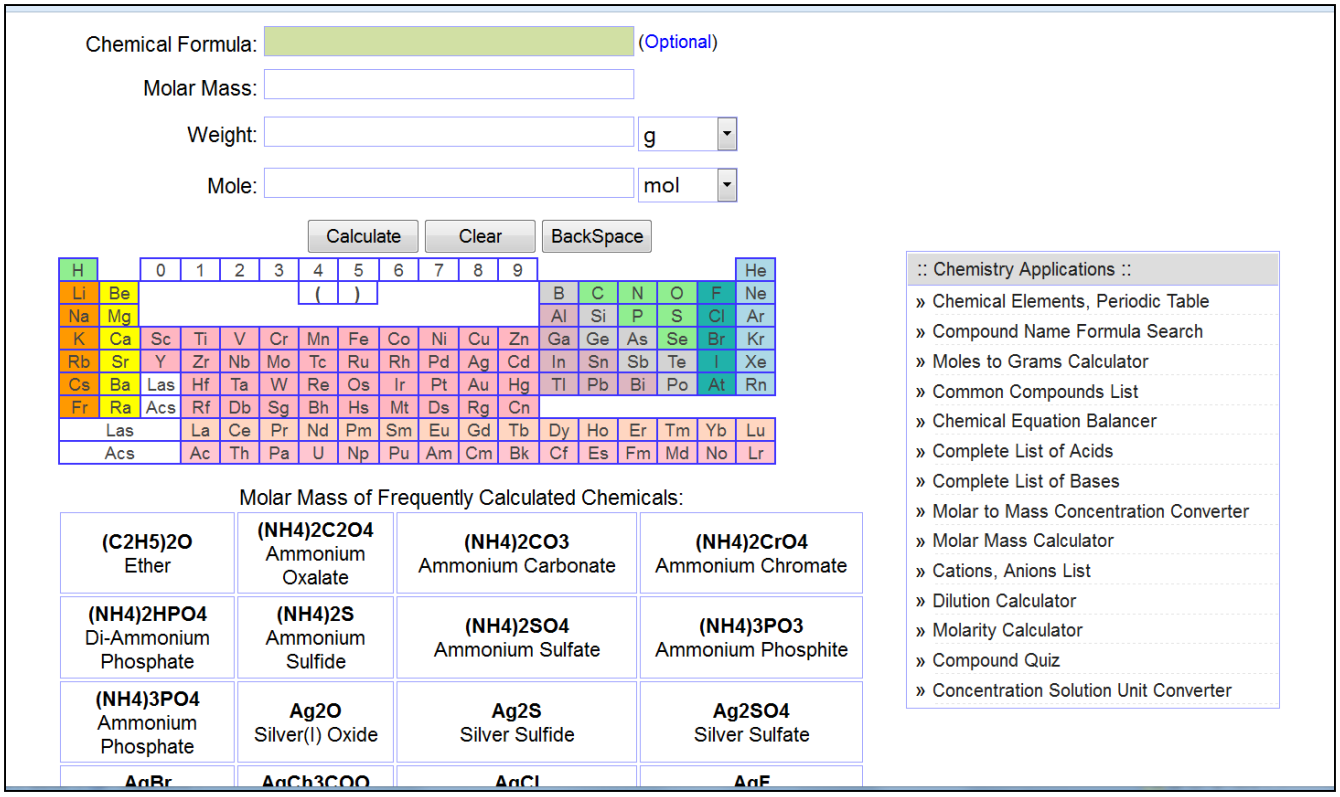
- **20% Sodium Dodecyl Sulphate (SDS) ($NaC_{12}H_{25}SO_4$):**
- SDS (20 g) was added to 100 ml deionised water, and dissolved.
- The solution was stored at room temperature. If SDS precipitated, the solution was incubated at 37°C until the SDS went back into solution.
- Store at room temperature. Sterilization is not necessary. Do not autoclave.
- **Sodium acetate (3M):**
- 10.2 g of sodium acetate was dissolved in 25 ml of double distilled water.
- **RNase A:**
- 2.5 mg of RNase A was dissolved in 1 ml of 10 mM Tris (pH 7.6) and boiled at 70°C for 10 min.

Piasecka,2010;..

Endmemo

Online unit conversions

Chemical Mole Gram Calculator



Molarity of NaCl
Molar and milimolar

- The molecular weight of a sodium chloride molecule (NaCl) is 58.44.
- The atomic mass (or weight) of Na is 22.99, the atomic mass of Cl is 35.45, so 22.99 + 35.45 = 58.44.

A. **Mole(M)=moles per liter. E.g.**

- 1M NaCl solution= If you dissolve 58.44 g of NaCl in a final volume of 1 liter, you have made a 1M NaCl solution, a 1 molar solution.
- 0.1M NaCl= To make a 0.1M NaCl solution, you could weigh 5.844 g of NaCl and dissolve it in 1 litre of water; or 0.5844 g of NaCl in 100 mL of water or make a 1:10 dilution of a 1M sample[take a 1mM stock solution, dilute it 1:10 (0.1 ml + 0.9 ml) to give O.1 M NaCl].
- 0.5M NaCl solution= 0.5 x 58.44 g of NaCl = 29.22 g/L
- 1.4M NaCl= 1.4 × 58.443 = 81.82 g/L
- 5M NaCl= 5 × 58.443 = 292.215 g/L

B. **Millimole=1/1000 of a mole. E.g.**

- 1mM= 58.44 g/1000= 0.05844 g NaCl. Dissolve 0.05844 g(58.44 mg) NaCl in 1 L water.

Molarity of NaCl
Molar and millimolar

- **Millimole=1/1000 of a mole. E.g.**
- 1mM= Dissolve 0.05844 g (58.44 mg) NaCl in 1 L water.

1. 70mM= 70 × 58.44 mg=4090 mg (4 g) NaCl in 1 L water.
2. 100mM= 100 × 58.44 mg=4090 mg (5.8 g) NaCl in 1 L water.
3. 200mM= 200 × 58.44 mg=11688 mg (11.6 g) NaCl in 1 L water.

Molarity of NaCl
Molar and milimolar

- **Millimole=1/1000 of a mole. E.g.**

1. A concentration of 70 mM is the same as 0.07 moles per liter. Take 0.07 moles of NaCl/liter. i.e. 58.44 grams ×0.07 =4 grams NaCl needed per liter.
2. To make 100 mM of your solution, take 0.1 moles/liter. i.e. 58.44 grams ×0.1= 5.84 grams NaCl in 1 L.
3. To make 200 mM of your solution, take 0.2 moles/liter. i.e. 11.6 grams NaCl (5.84 ×0.2= 11.6) per liter.
4. In this way, your solution multiply grams/liter by liters needed.

Molarity of NaCl

Millimolar to micromolar final concentration

- **Micromolar=1/1000,000 of a mole.**
- Question:
- I have a stock of 10 millimolar concentration. I want to use it at a final concentration of 5 micromolar.
- Answer:
- 10 millimolar (10mM) is 10 milli moles per litre. 5 micro moles per litre.
- Use the classical dilution equation: C1V1 = C2V2.
- Note that 10 mM = 10 000 µM
- V1 = (C2/C1) x V2 = (5 µM/10 000 µM) x 1 mL = 0.5 µL
- Take the smallest amount of stock you can measure with accuracy and dilute it to an amount which 2000 times that volume (the ratio of concentrations).

Endmemo

Online unit conversions

Molarity of $FeCl_3$ and $FeCl_3.6H_2O$

- The molecular weight of a Ferric chloride molecule ($FeCl_3$) is 162.2.
- The atomic mass (or weight) of Fe is 55.845, the atomic mass of Cl is 35.45, so 55.84+35.45= 162.2.
- 1M $FeCl_3$ solution= If you dissolve 162.2 g of $FeCl_3$ in a final volume of 1 liter, you have made a 1M $FeCl_3$ solution, a 1 molar solution.
- 0.5M $FeCl_3$ solution= 0.5 x 162.2 g of $FeCl_3$ = 81.1g/L
- 1.4M $FeCl_3$ = 162.2 × 1.4 = 227 g/L.
- 5M $FeCl_3$ = 162.2 × 5 = 811 g/L.
- 1mM $FeCl_3$ = Dissolve 0.1633 g $FeCl_3$ in 1 L water.

0.5M from $FeCl_3.6H_2O$: The molecular weight of $FeCl_3.6H_2O$ is 270.30 g/mol. Dissolve 135.2g of $FeCl_3.6H_2O$ in water containing 20ml of conc. HCl. Dilute to1 liter.
0.1M $FeCl_3.6H_2O$: 10mL conc HCl +27 g $FeCl_3.6H_2O$ per liter.

Endmemo

Online unit conversions

Molarity of HCl

- What is the difference between 1 molar and 0.1 molar?
- The molecular weight of a HCl is 36.5 g.

1. If you put 36.5 g of HCl into one litre of water, you would have 1.0 molar solution.
2. For 0.1 molar you use 3.65 g HCl.
3. OR make a 1:10 dilution of a 1M sample.

Calculating Molarity

Molarity of glucose sugar

Converting molarity to g/L

$$\text{Molarity (M)} = \frac{\text{moles solute}}{\text{liters solution}}$$

- The concentration of a solution of 1 mole dissolved in 1 L is 1 M ("one molar").
- The molecular weight of glucose ($C_6H_{12}O_6$) sugar (12x6+1x12+ 16x6) is 180 g/mole.

1. In the case of glucose, a 1 M glucose solution would have 1 mole = 180 g dissolved in 1 L.
2. So another way of saying that is 1 M glucose solution = 180 g/1000 ml = 0.18 g/ml
3. Alternatively you could call it 18% (w/v). i.e. 18g/100ml.

Calculating Molarity

Molarity of $KMnO_4$

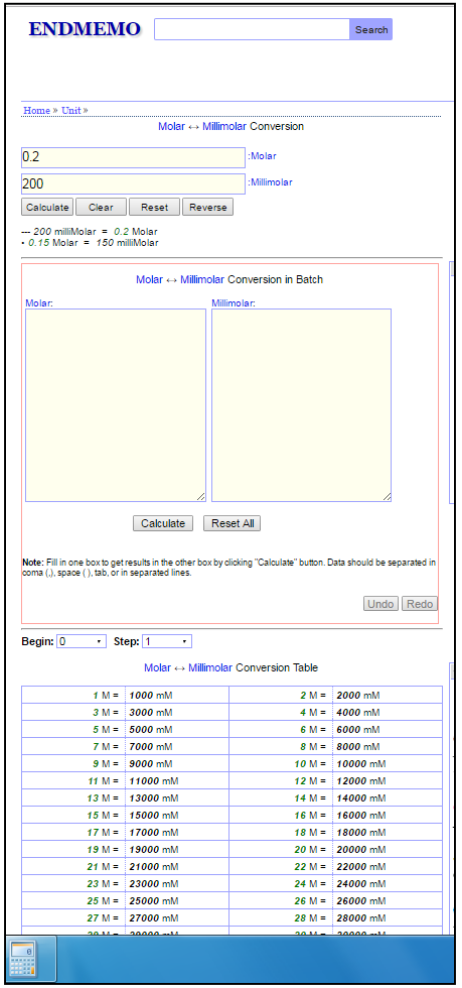
- Molar mass of each element:
- The atomic (molar) mass of K= 39.1 g
- mass of Mn= 54.9 g
- The atomic mass of O= 16.0 g
- The total atomic mass (formula mass) of $KMnO_4$ = 39.1 g + 54.9 g + (16.0 g x 4)= g.
- Thus, one mole of $KMnO_4$ weighs 158.0 grams.
- This is enough to calculate the molarity:
- Molarity= moles solute/Liter solution
- 1M solution of $KMnO_4$ =0.158 (158.0 g/1L D.H_2O).

$$\text{Molarity (M)} = \frac{\text{moles solute}}{\text{liters solution}}$$

Conversion of Measurement Units

Convert molar(M) to millimolar (mM)

1 mole is equal to 0.001 molar or 1 millimolar



Converting molarity to g/L

- You have a 1 M solution of NaCl, what is the concentration in % (w/v)?
- Molecular weight of NaCl= 58.44 grams/mole
- 1 M = 1 mol /1 L
- 1 mol of NaCl = 58.44 $g.mol^{-1}$
- 1 M of NaCl= 5.8%(w/v)

$$\text{Concentration (M)} = \frac{1\ \text{mol}}{1\ \text{L}} = \frac{58.44\text{g}}{1\ \text{L}} = \frac{5.844\text{g}}{100\ \text{mL}} = 5.8\%\ \text{(w/v)}$$

J. A. Koenig

Converting g/L molarity

- You have weighed out 20 g KCl (formula weight of KCl is 74.55 $g.mol^{-1}$) and dissolved it in 500 mL, what is the concentration in M?
- Work out how many moles are equivalent to 20 g.

Amount (in g) = Amount (in mol) x Molar Mass ($g.mol^{-1}$)

Rearranging:

$$\text{Amount (in moles)} = \frac{\text{amount (in g)}}{\text{molar mass (in } g.mol^{-1})}$$

$$= \frac{20g}{74.55g.mol^{-1}} = 0.268 \text{ mol}$$

Now work out the concentration by dividing the amount (in mol) by the volume (in L)

$$\text{Concentration (M)} = \frac{\text{amount (in mol)}}{\text{volume (in L)}}$$

$$= \frac{0.268mol}{0.5L} = 0.537 \text{ M}$$

Calculating Normality

- The normality of a solution is the number of equivalent weights of substance/liter.
- Normality takes into account both the:

1. Molarity of the solution and
2. The equivalent content of the acid or base, using the equation shown below:

Normality(N) = Molarity(M) x Equivalent(N/M) or

Gram equivalent=Mol. Wt/No. of H^+ ions involves in reaction

- E.g.
- H_2SO_4=98 g/1L=1M
- $H_2SO_4=\frac{98}{2}$ ⇒49g)/1L=1N. Valency or n factor for H_2SO_4 is 2.

Calculating Normality
Molarity and Normality of NaOH

- To make a 1 M solution of NaOH (23+16+1=40) in water, you will want to weigh out 40 gram(=1 mole) of NaOH, dissolve it in about 0.8 liters of water, and then add water to the solution to take the total volume up to exactly 1.0 liters.
- When the valence of the compound is one, Normality and Molarity are equal.

1. Molecular weight of NaOH = 40 grams/mole
2. Valence of NaOH = 1

- Therefore, for NaOH solutions, molarity and normality will be the same thing.
- NaOH=40 g/1L=1M
- NaOH=40 g/1L=0.04g (0.04g/1L)=1mM
- NaOH=40 g/1L=1N
- NaOH=20 g/1L=0.5N

Calculating Normality

Normality of HCl

- Generally the HCl that we get from manufacturer is 37%.
- It's concentration is 12N.
- So, Using the following formula, you can make the 1N solution.
- $N_1V_1 = N_2V_2$ or
  $V_1 = V_2 \times N_2/N_1$
- N1 = 12N (conc. HCl)
- N_2 = 1N
- $V_2(D.H_20)$ = 100 mL
- V1 = 100mL x 1N /12N V1 = 100mL
- For example
- 1N 100 ml HCl solution, x is volume of 37% HCl to be taken and diluted
- 12 X x = 1 X 100
- x = 100/12(= 8.3 ml)
- So, you can mix 8.3 ml of conc. HCl and 91.7 ml of dH_2O to make 1N 100 ml of HCl.

Calculating Normality

Normality of HCl

- Generally the HCl that we get from manufacturer is 37%.
- It's concentration is 12N.
- To make different normality of HCl in 100mL dH_2O:
- 0.5N= 4.15 mL of conc. HCl (37%) + 95.85 mL of dH_2O.
- 1N= 8.3 mL of conc. HCl (37%) + 91.7 mL of dH_2O.
- 6N= 49.8 mL of conc. HCl (37%) + 50.2 mL of dH_2O.
- 8N= 66.4 mL of conc. HCl (37%) + 33.6 mL of dH_2O.

Nucleotide composition

Base ratio or GC ratios

1. Guanine plus cytosine base composition of DNA has been used for a number of years in classical taxonomy.
2. GC ratios vary from about 20% to a high of about 80% in the Bacteria.

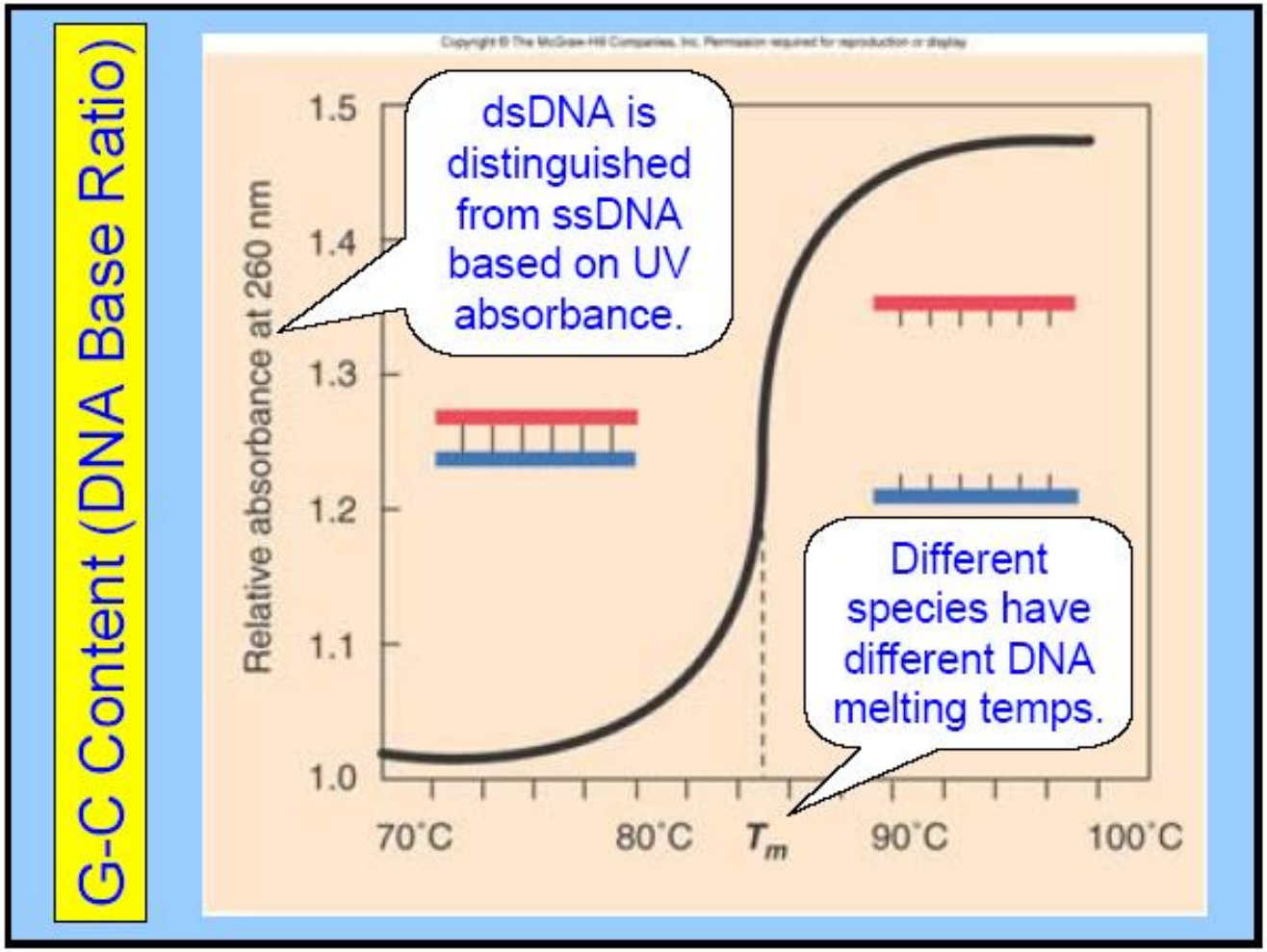
Nucleotide composition

Base ratio or GC ratios

- The relative amount of each base, reported as the G+C content, is characteristic of a genus.
- **Based on GC measurements:**
- The genera of certain plant pathogenic bacteria, such as *Erwinia*, are predicted to have moderate G+C contents.
- While others, such as *Clavibacter* and *Pseudomonas*, are predicted to have much larger G+C contents (upwards of 70%).
- Localized variations in the G+C content along the genome have significant implications for understanding the potential routes of genetic exchange between organisms.

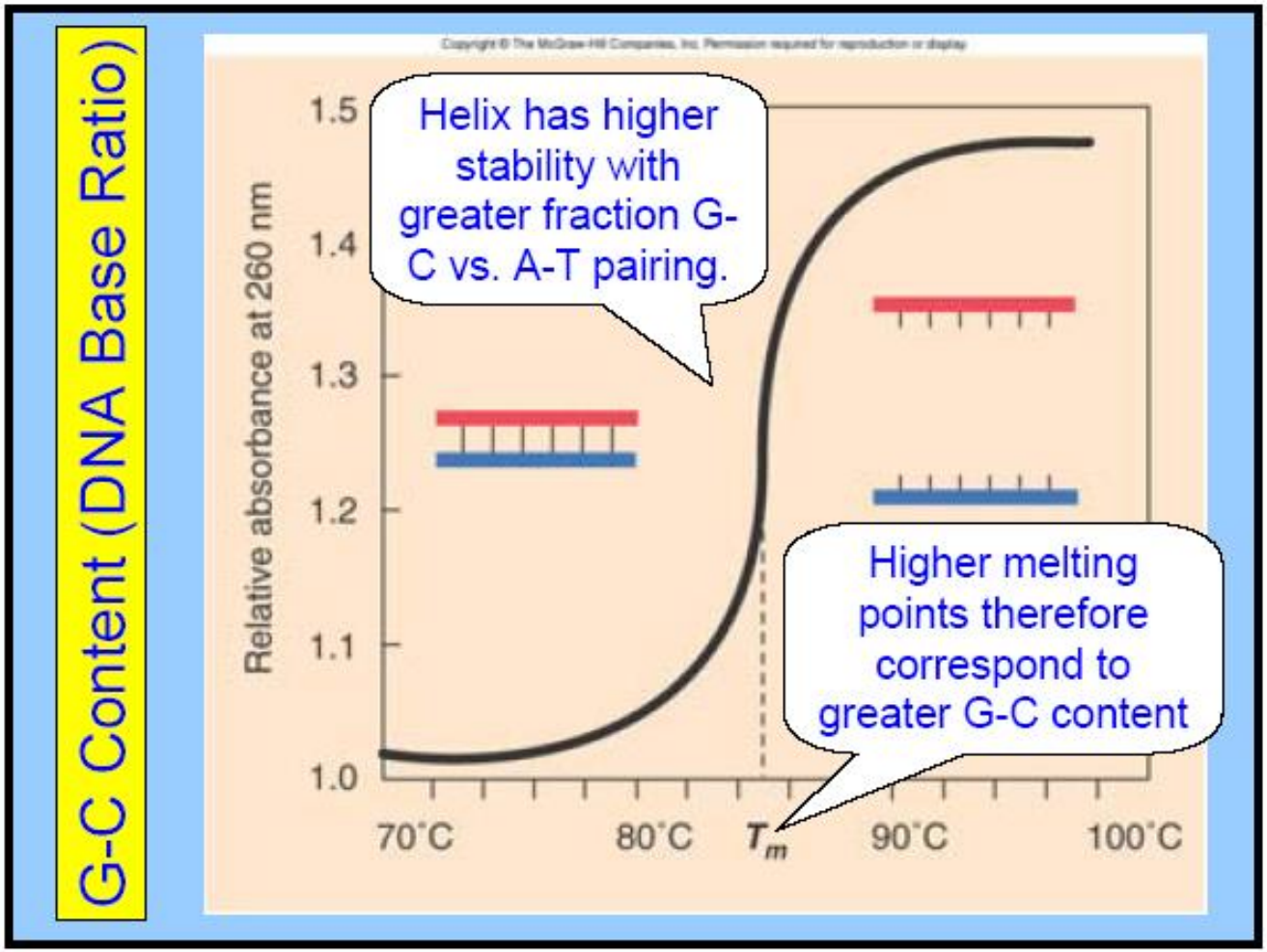
DNA Base Composition

Base ratio or GC ratios



DNA Base Composition

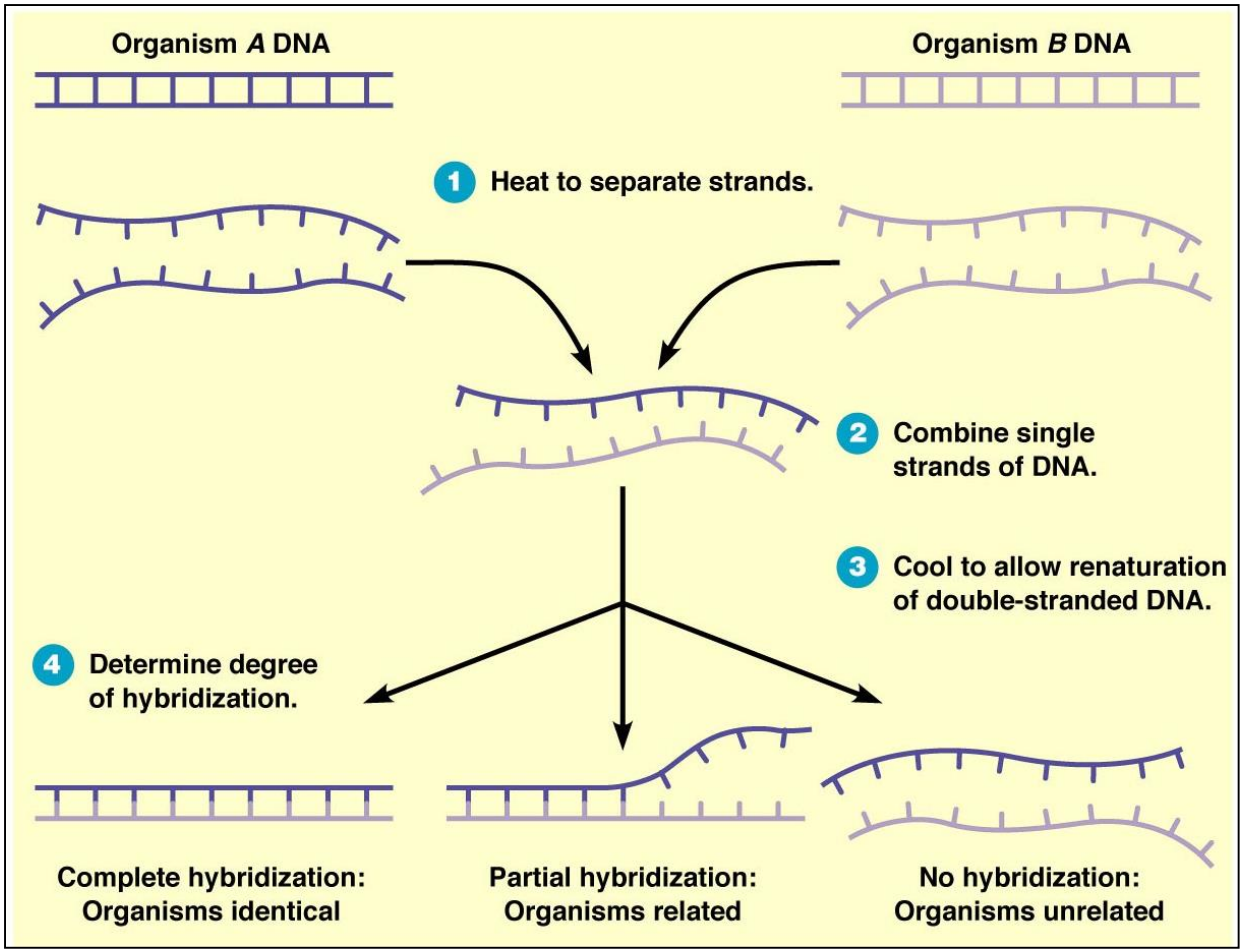
Base ratio or GC ratios



DNA:DNA hybridization
Experimental procedure

1. DNA(target or sample DNA) is extracted from the bacteria;
2. DNA strands are separated and fixed on a filter (nitrocellulose paper);
3. Labelled probe (probe DNA) is added;
4. Hybridization occurs with homologous sequences of target DNA;
5. After washing away nonhybridized probe, the hybridized DNA can be visualized on an X-ray film.
6. The value of DNA hybridization was that it provided a quantitative definition of what constituted a species, ~70% or greater DNA-DNA relatedness with a T_m of 5°C or lower.

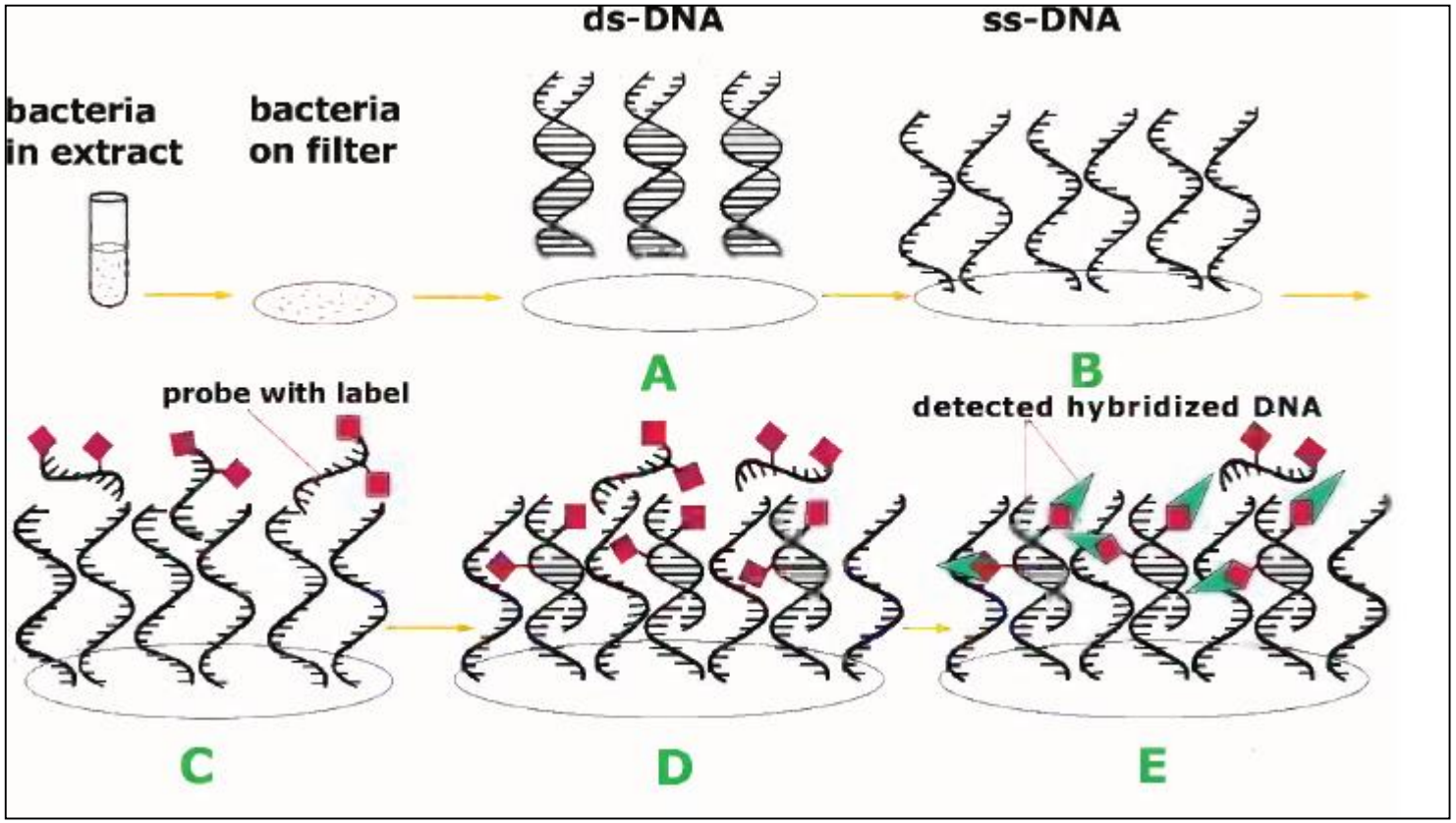
Nucleic Acid Hybridization



Karlm,2004

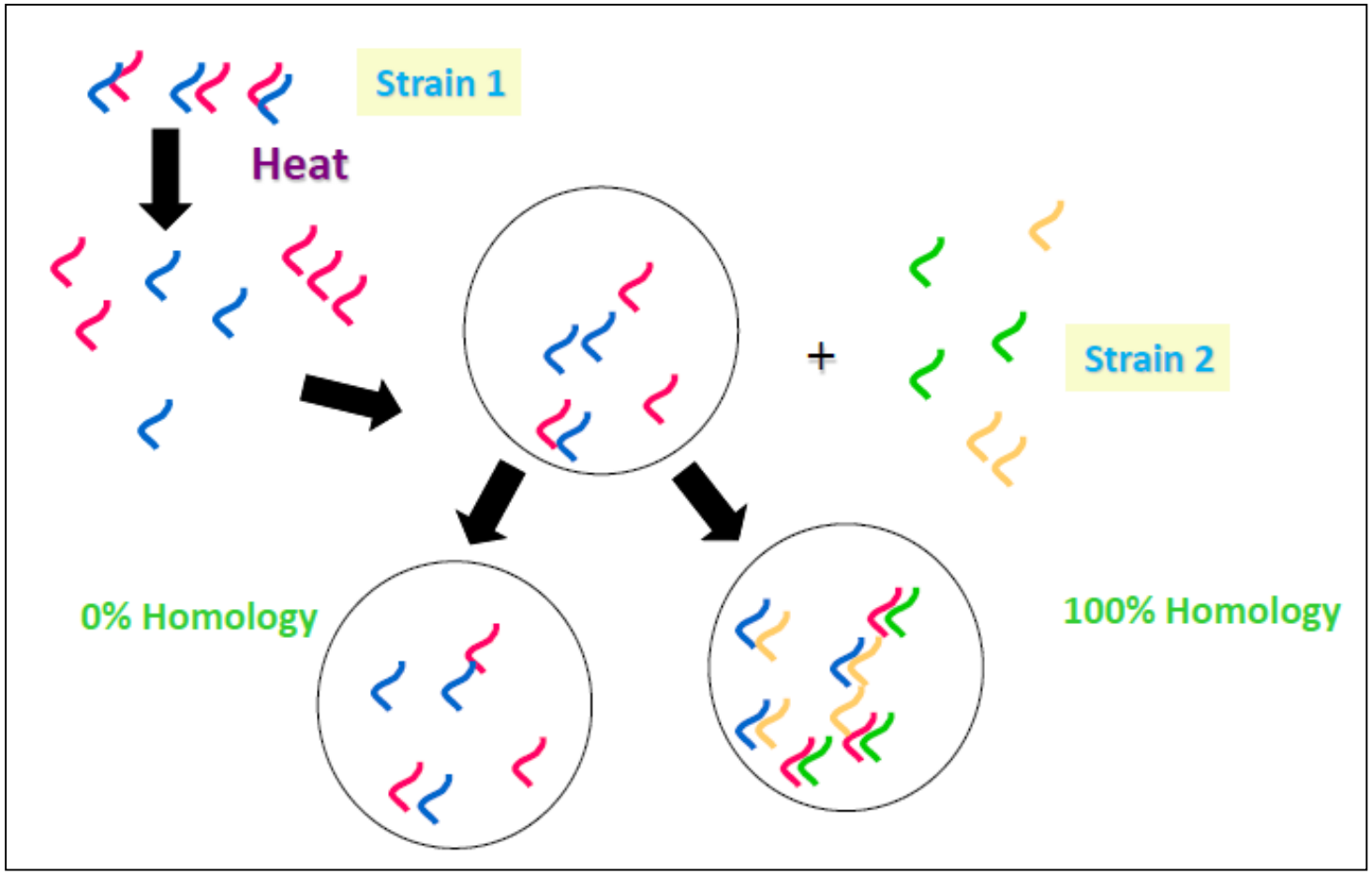
DNA:DNA hybridization

Experimental procedure



Janse,2006

DNA-DNA hybridization



Fox,2008

Hybridization

Types of hybridization/blotting techniques

- Blots are named for the target molecule.
- **Southern Blot**
  DNA cut with restriction enzymes - probed with radioactive DNA.
- **Northern Blot**
  RNA - probed with radioactive DNA or RNA.
- **Western Blot**
  Protein - probed with radioactive or enzymatically-tagged antibodies(e.g. alkaline phosphatase).
- Item 1& 2 are called nucleic acid hybridization.

A Southern blot (also called a Southern Transfer) is named after Ed Southern, its inventor. The other blotting techniques emerged from this method have been termed as Eastern (for post-translational protein modifications) and Southwestern (for DNA-protein interactions) blotting.

Hybridization

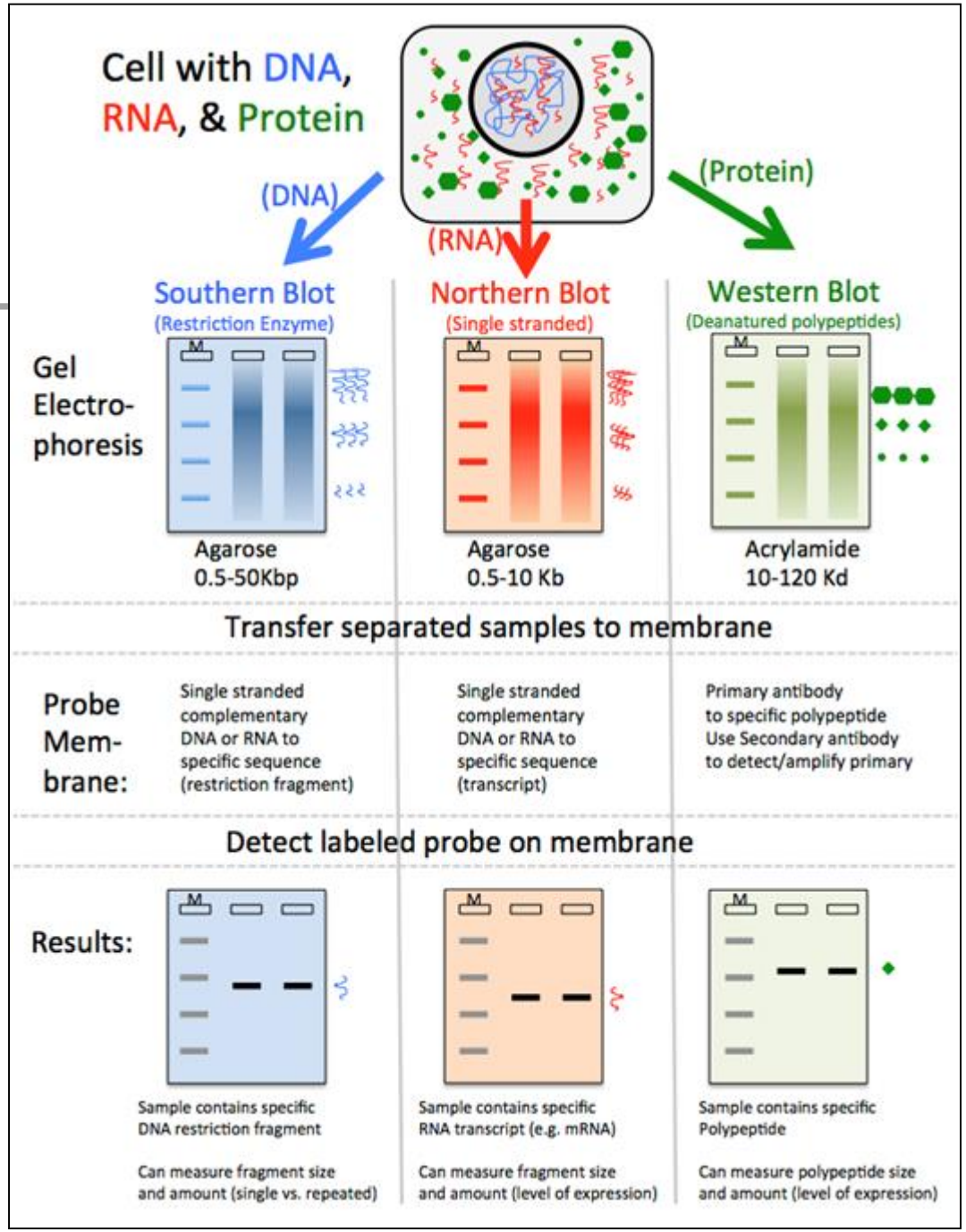
Comparison of Southern, Northern, and Western blots.

Size and amount of DNA, RNA, and polypeptides can be determined using similar blotting methods.

DNA is in blue, RNA in red, and polypeptides in green.

A marker lane is shown in the left of each gel to determine size.

A eukaryote cell is shown, but the same methods can be applied to prokaryotes, too. (Original-Locke-CC:AN)



Nucleic acid hybridization
Southern and Northern Blotting

- Nucleic acid hybridization, or reassociation, is a process by which complementary single-stranded (ss) nucleic acid anneals to double-stranded (ds) nucleic acid.
- This process occurs because of the hydrogen bonds between the two strands of the:

1. DNA:DNA,
2. DNA:RNA,
3. RNA:RNA duplex.

Hybridization

Types of hybridization/blotting techniques

Southern blot

- Southern blots are a method of detecting DNA and named after the surname of their inventor Ed Southern.
- A typical Southern blot experiment goes as follows:

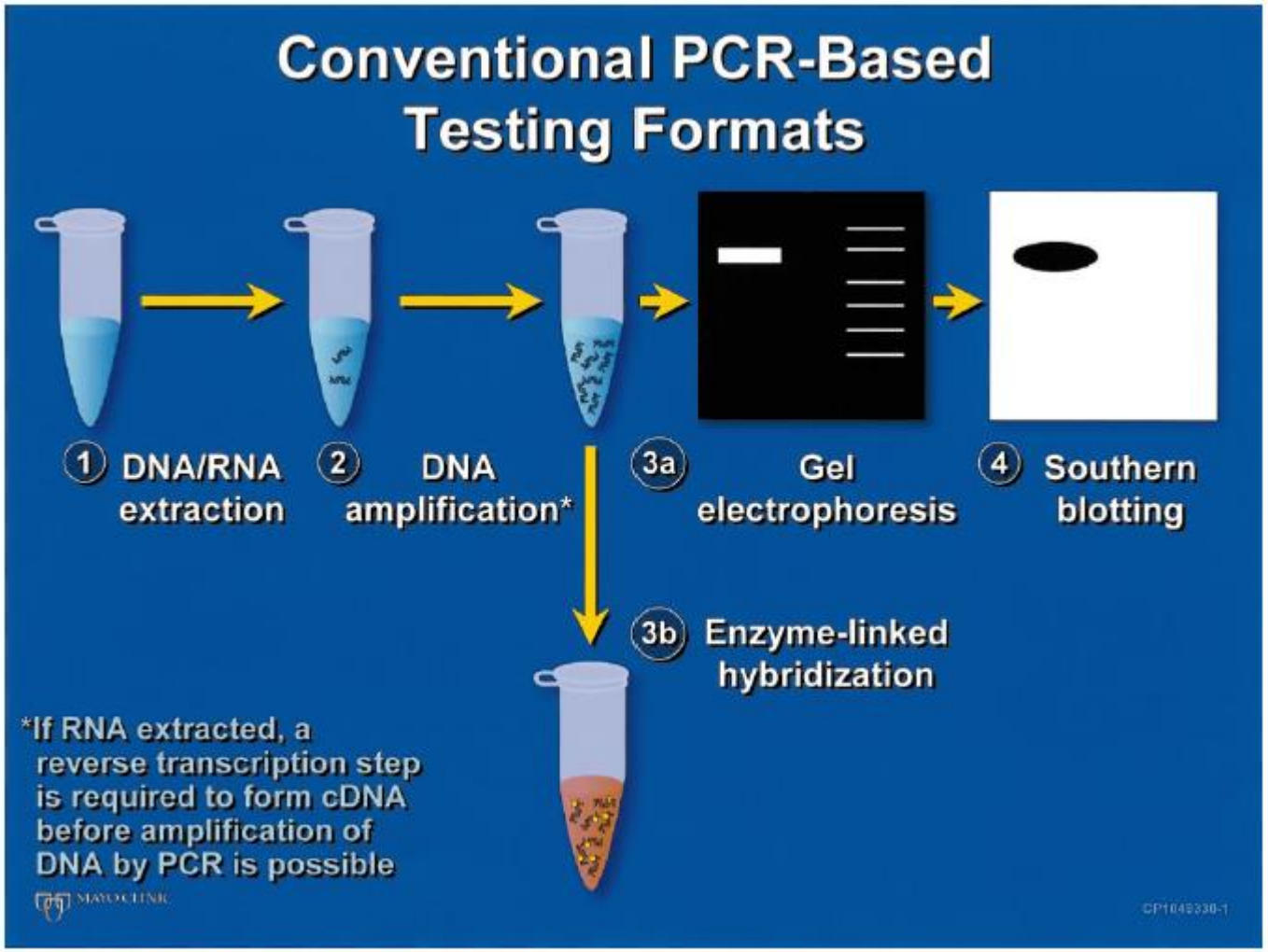
1. run a DNA gel,
2. transfer gel contents onto a membrane,
3. hybridize said membrane with radioactive DNA probe,
4. wash off unbound radioactive probe, and
5. detect radioactive probe.

Doronina,2016

Hybridization

Types of hybridization/blotting techniques

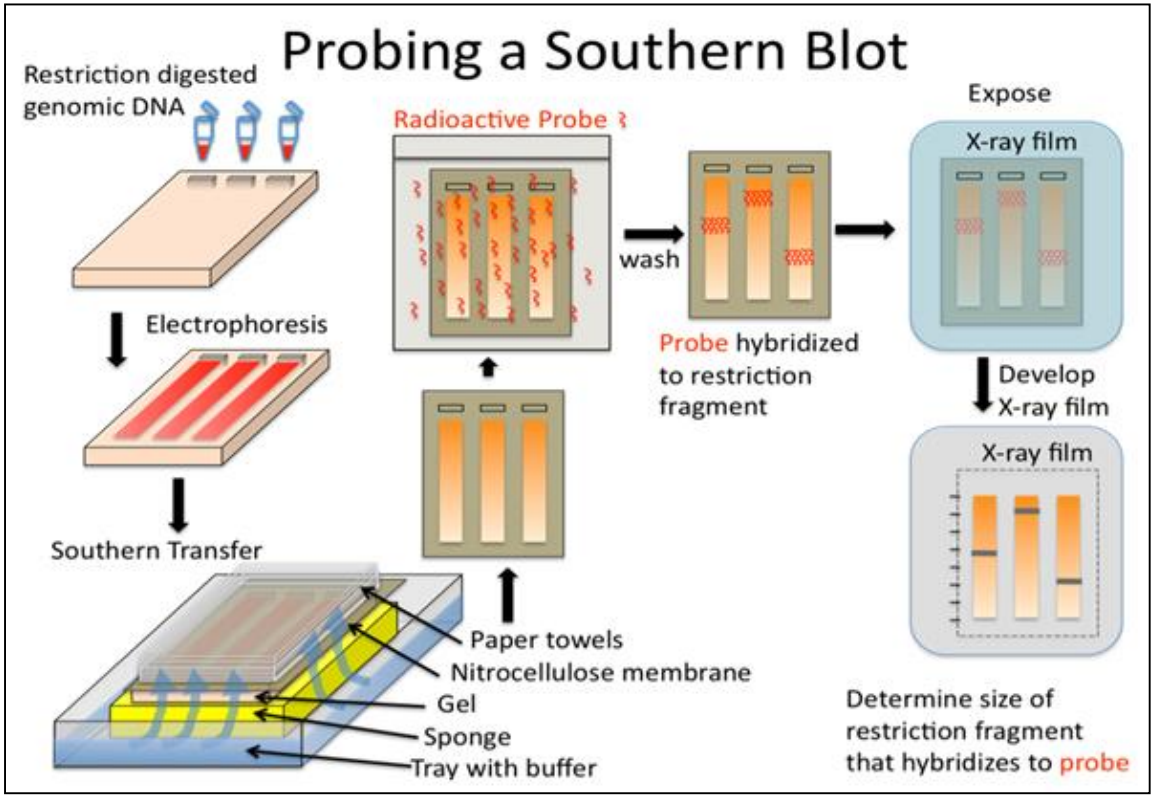
Southern Blot



Hybridization

Types of hybridization/blotting techniques

Southern Blot



A diagram of Southern blotting. Genomic DNA that has been digested with a restriction enzyme is separated on an agarose gel, then the DNA is transferred from the gel to a nylon membrane (grey sheet) by blotting. The DNA is immobilized on the membrane, then probed with a radioactively labeled DNA fragment that is complementary to a target sequence. After stringent washing, the blot is exposed to X-ray film to detect what size fragment the probe is bound. In this case, the probe bound to different-sized fragments in lanes 1, 2, and 3. In the last image the orange represent the position of the digested DNA, but it is not actually present on the X-ray film. (Original-J. Locke-CC:AN)

Hybridization

Types of hybridization/blotting techniques

Northern blot

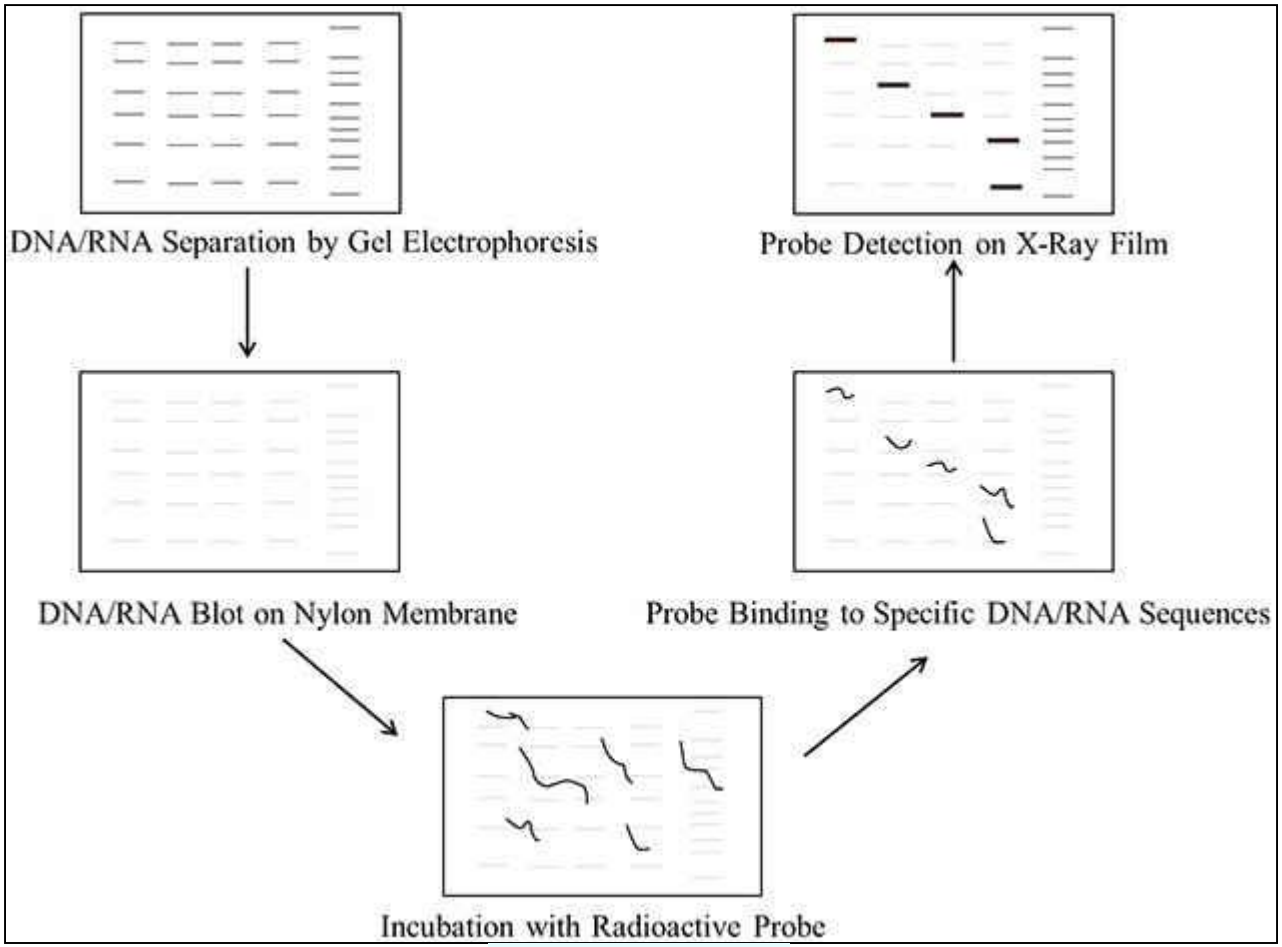
- Northern Blots are named after their big brother: The Southern blot.
- Northern Blots are done the same way as Southern blots but RNA is detected instead of DNA.
- So when invented the RNA method was named "Northern blot" as an homage to the original nucleic acid detection method.
- And the naming tradition just continued with Western blots.

Doronina,2016

Principles of hybridization

Southern and Northern Blotting

Steps involved in DNA/RNA blotting procedure



Sigma-Aldrich

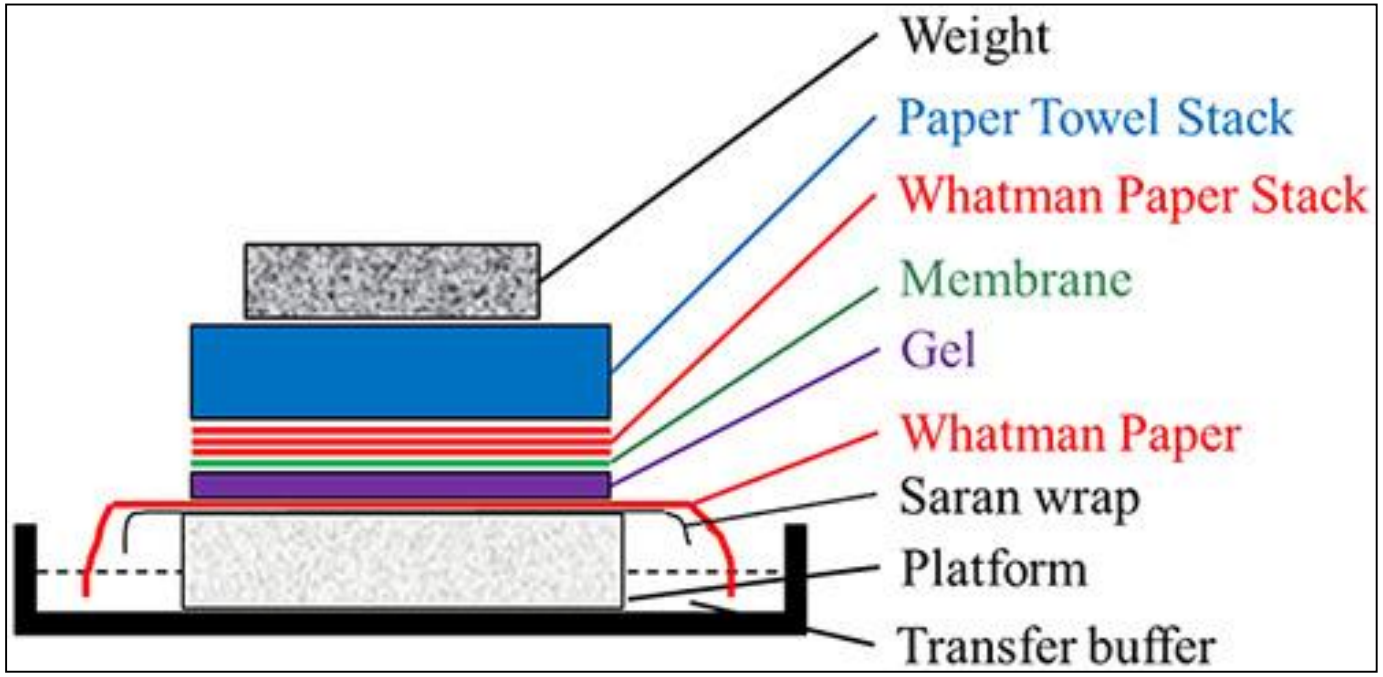
Principles of hybridization
Southern and Northern Blotting

- Southern and Northern blotting protocols involve the following major steps:

1. **Purification of DNA/RNA:** Extract and purify the DNA/RNA from either cells or tissue sources.
2. **Digestion of DNA:** Digest the DNA into fragments with restriction enzymes. This step is not required for RNA.
3. **Gel electrophoresis:** Separate the DNA fragments on agarose gel. The RNA samples can be separated on agarose gel with formaldehyde as the denaturing agent that limits secondary structures of RNA molecules.
4. **Transfer:** Transfer the DNA/RNA fragments from the gel onto a nylon membrane.
5. **Prehybridization (Blocking):** Wash the nylon membrane with a prehybridization solution containing salmon sperm DNA to block non-specific DNA interactions and reduce background noise. Alternatively, use the PerfectHyb™ Plus buffer, which doesn't require salmon sperm DNA for blocking.
6. **Preparation of probe:** Prepare fresh probe DNA and label with ^{32}P alpha-labeled dCTP.
7. **Hybridization:** Incubate the blot with labeled probe.
8. **Detection of probe:** Detect the probe and the DNA/RNA sequence of interest by exposure to film at -80°C.

Principles of hybridization

Southern/northern blot transfer assembly

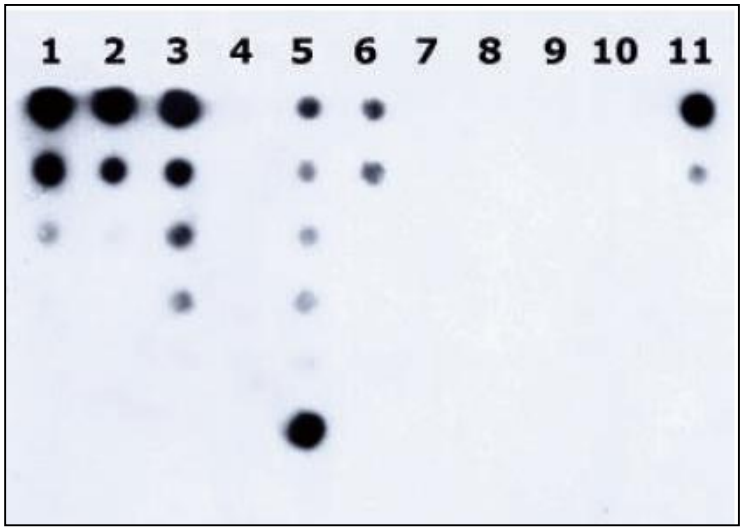


Sigma-Aldrich

Principles of hybridization

Southern/northern blot transfer assembly

- X-ray film showing dark spots (dots) where DNA of *Clavibacter sepedonicus* (*Cs*) is present in a dilution series of suspensions of pure cultures (1-3) and in potato extracts (5-9) hybridized with a probe that consists of a plasmid-derived sequence of this bacterium.
- Extracts 7-9 were negative, 4 and 10 are empty buffer rows, 11 is positive control DNA of *Cs*.
- The probe was labelled with biotin (to avoid radioactive label) and biotinylated probe detected with a PhotoGene (GIBCO BRL) detection kit.

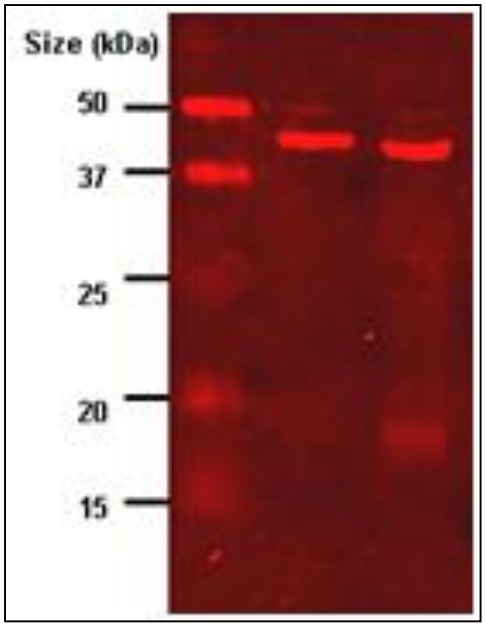


Janse,2006

Principles of hybridization

Western Blot

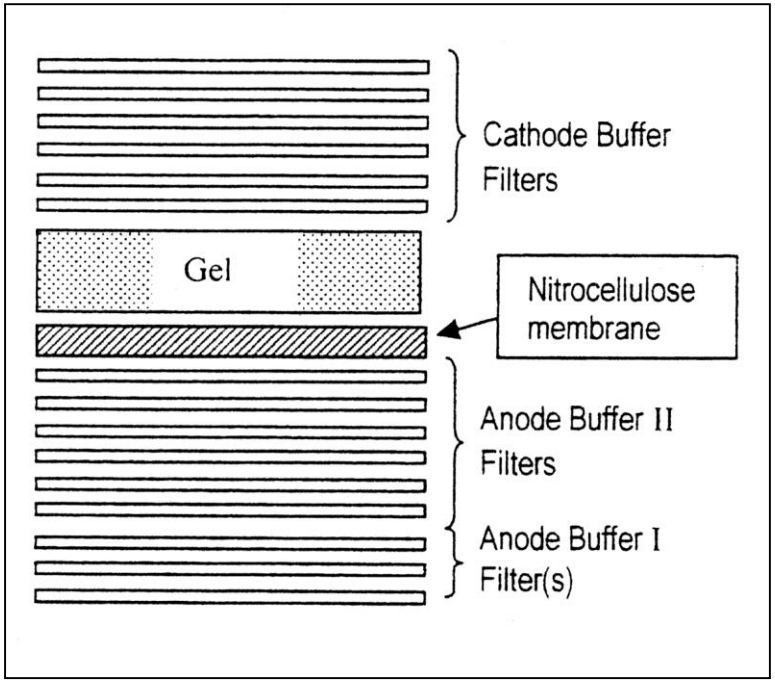
- In a Western blot, protein is size separated on a gel (usually an acrylamide gel) before transferring to a membrane, which is then probed with an antibody that specifically binds to an antigenic site on the target protein.
- This antibody is then detected by other antibodies with some fluorescent or color production marker system.
- It will also give bands proportional to the amount and size of the target protein.



Nickle and Barrette-Ng,2017

Hybridization
Western blot

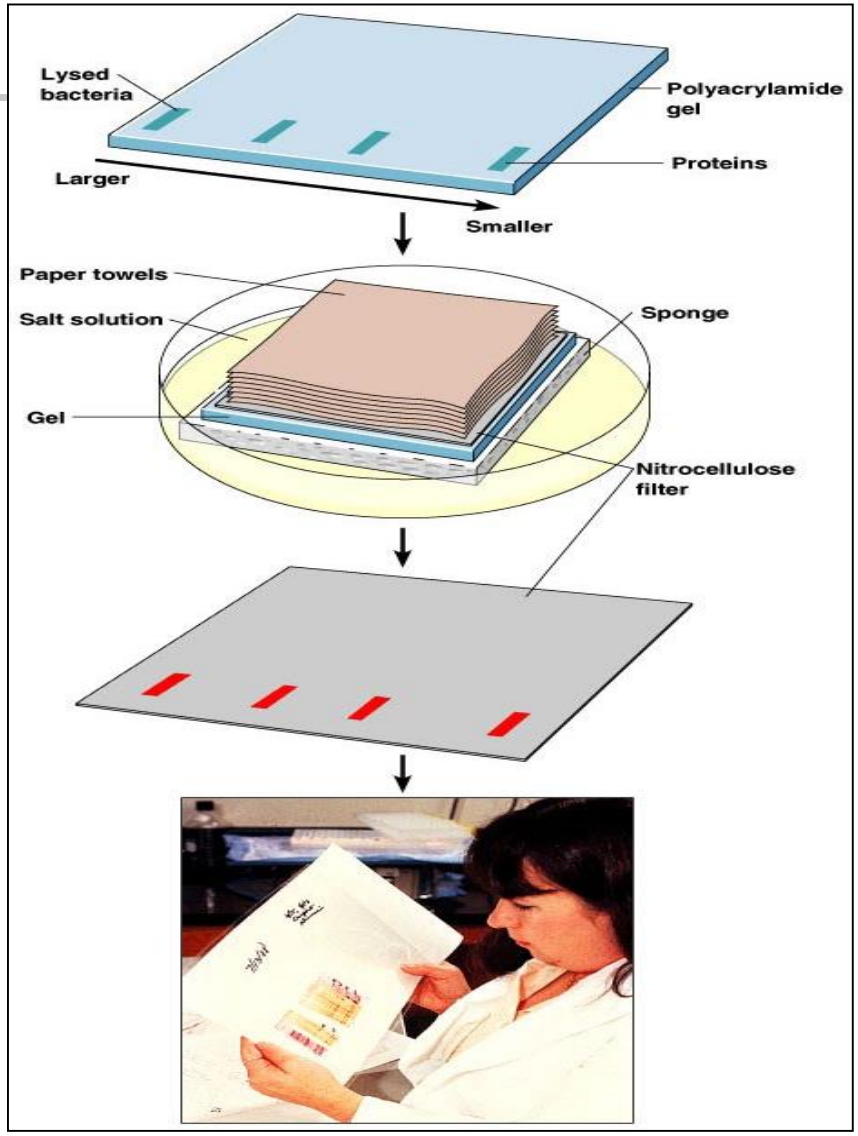
- Transfer electrophoretically separated proteins to a membrane and then identify target protein(BSA) with a labeled antibody.
- Primary antibody:
  - Antibody that binds directly BSA.
- Secondary antibody:
  - Antibody that binds to a primary antibody
  - Linked to a detectable label (alkaline phosphatase).



Allen,2011

Hybridization
Western blot

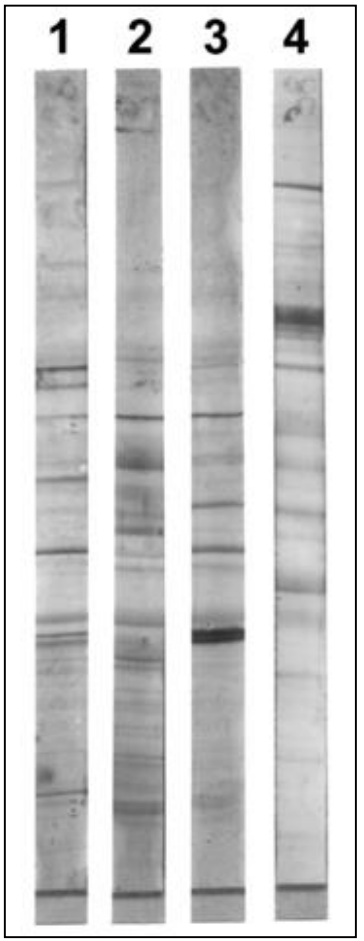
- Proteins run on gel and then transferred to a membrane:
  - Proteins bound to membrane.
  - Membrane probed with specific antibodies.



Karlm,2004

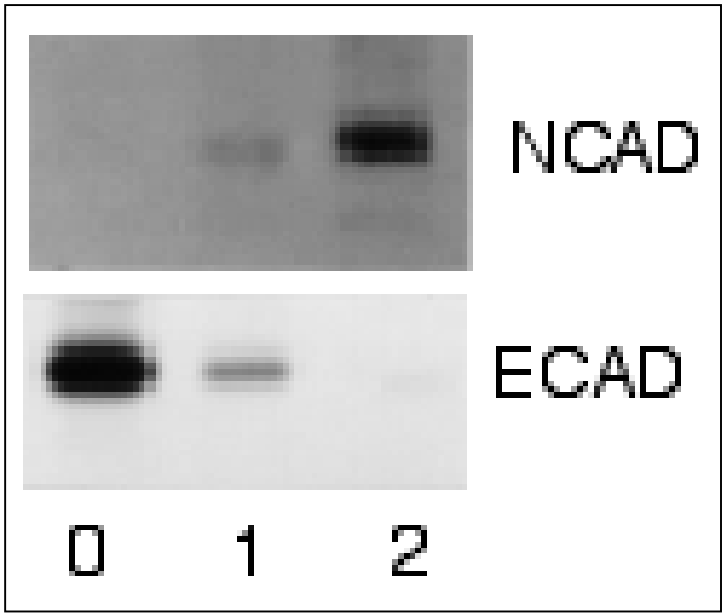
Western blot
Immunoblot

- The western blot (alternatively, immunoblot) is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract.
- It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/non-denaturing conditions).



Western blot
Immunoblot

- The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are probed (detected) using antibodies specific to the target protein.
- Western blot using radioactive detection system.



DNA microarrays

DNA hybridization principle

Gene expression studies

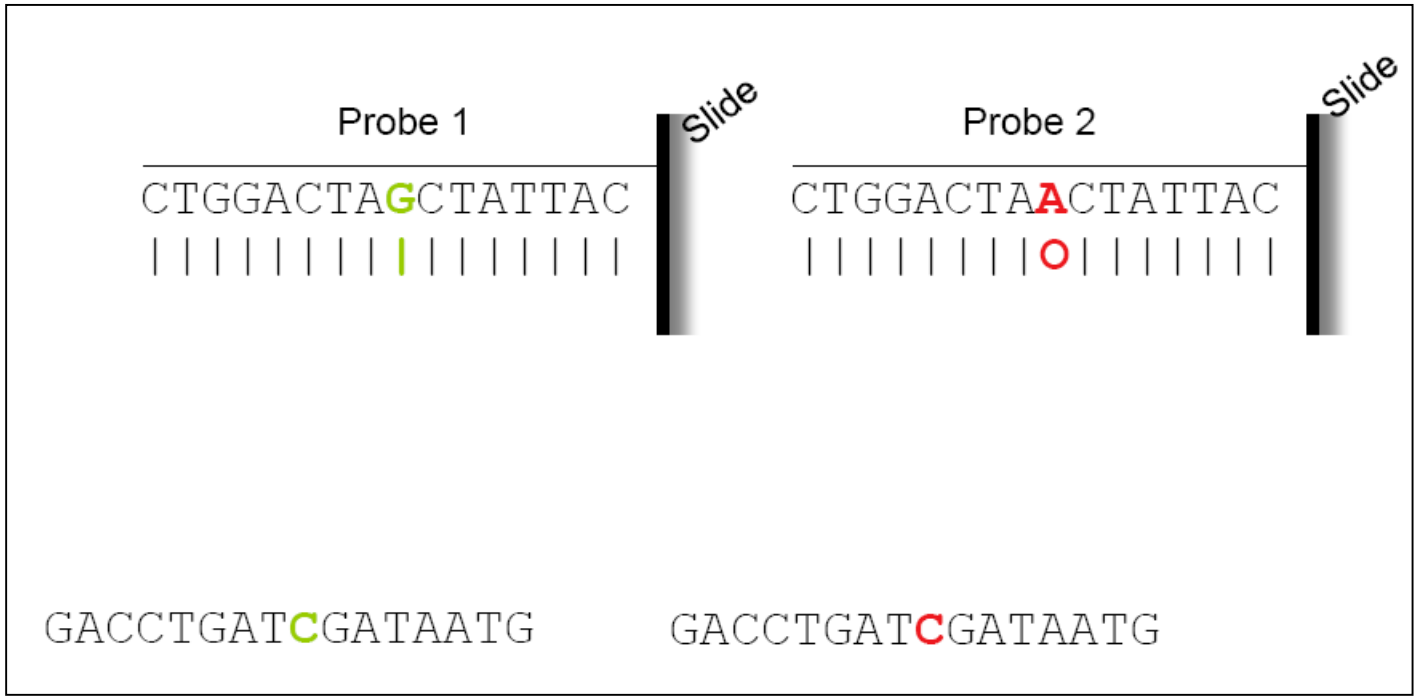
- Microarrays are generally composed of thousands of specific probes spotted onto a solid surface (usually nylon or glass).
- Each probe is complementary to a specific DNA sequence (genes, ITS, ribosomal DNA) and hybridisation with the labelled complementary sequence provides a signal that can be detected and analysed.

López *et al.*,2011

DNA microarrays

DNA hybridization principle

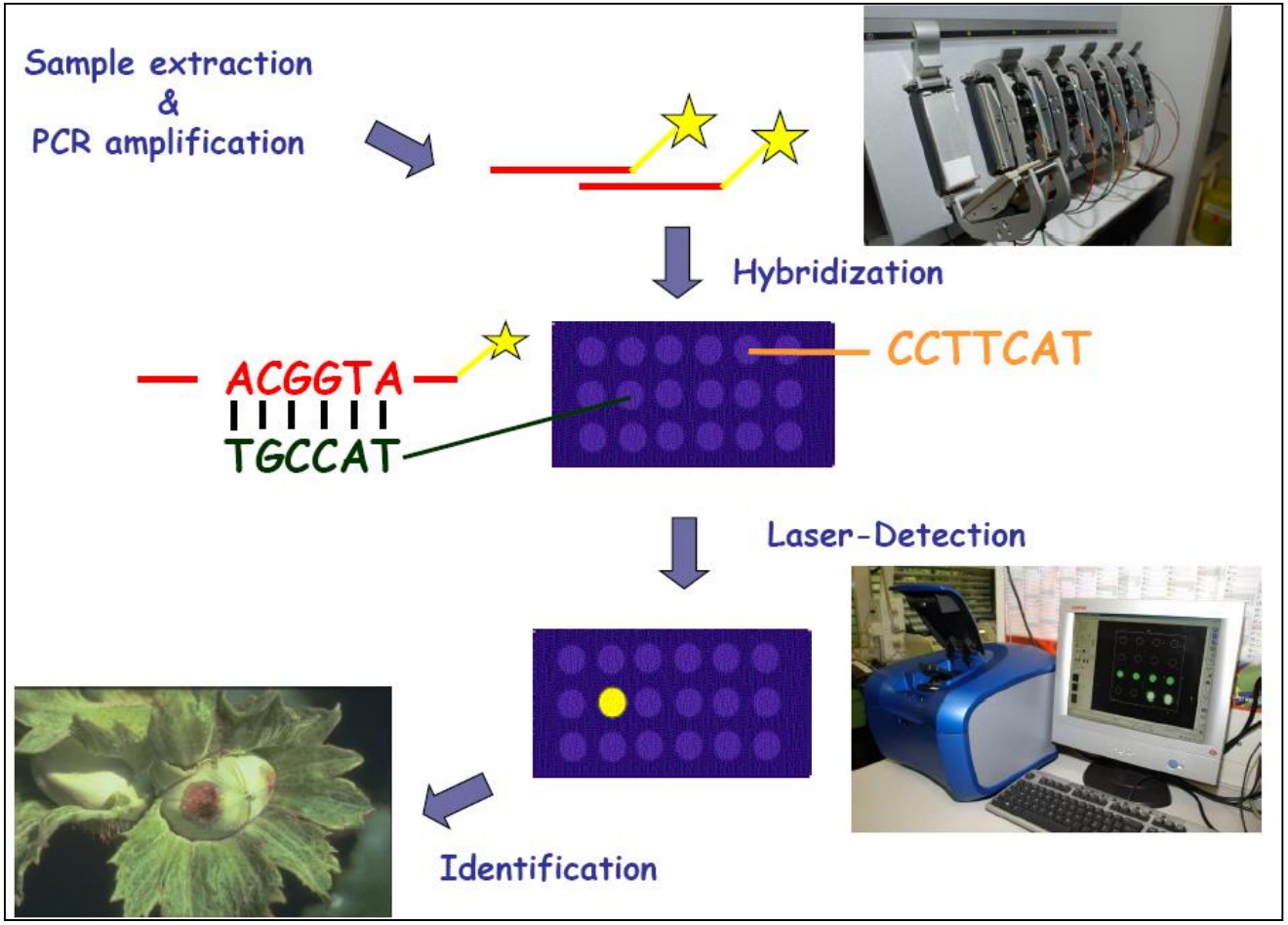
Gene expression studies



Duffy *et al.*,2008

DNA microarrays

DNA hybridization principle

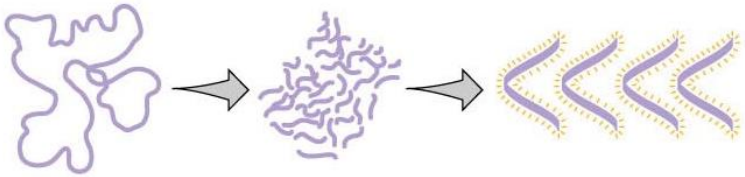


Duffy *et al.*,2008

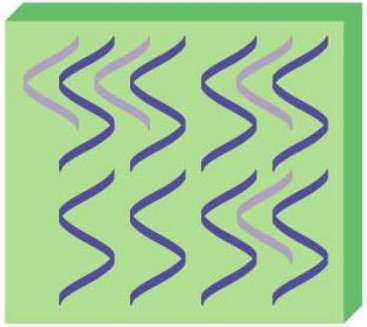
Nucleic Acid Hybridization: DNA Chip

Multiplex diagnostics can be achieved with microarrays

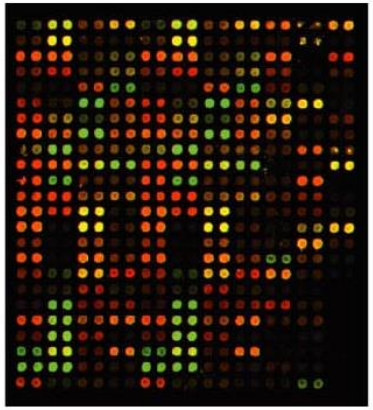
(a) A DNA chip can be manufactured to contain hundreds of thousands of synthetic single-stranded DNA sequences. Assume that each DNA sequence was unique to a different bacterial species.



(b) Unknown DNA from a patient is separated into single strands, enzymatically cut, and labeled with a fluorescent dye.



(c) The unknown DNA is inserted into the chip and allowed to hybridize with the DNA on the chip.



(d) The tagged DNA will bind only to the complementary DNA on the chip. The bound DNA will be detected by its fluorescent dye and analyzed by a computer. The red light is a gene expressed in normal cells; green is a mutated gene expressed in tumor cells; and yellow, in both cells.

Karlm,2004

Microarray technology
A modern method for detection

- DNA microarrays (which often also are called DNA or gene chips) offer the latest technological advancement for multi-gene detection and diagnostics.
- DNA microarrays were first described by Schena *et al.*, 1995 for the simultaneous analyses of large-scale gene expressions by a large number of genes.
- Some microarray experiments can contain up to 30,000 target spots.
- Usually chemically synthesized oligonucleotides 20-70 nucleotides in length, can be attached to a slide and the genes they represent can all be analyzed in a single experiment.

DNA microarrays

Application in plant pathogenic bacteria

- Despite the slow development of the microarray technology for detection of plant pathogenic bacteria, it shows some potential features that make it a very promising tool.
- The use of thousands of probes at the same time allows the possibility of detection and differentiation of several pathogens in only one analysis.
- Nevertheless, the need for a previous PCR reaction, the low level of sensitivity achieved, and the high cost of the equipment makes this technique still far from being used for routine analysis of plant pathogens (López *et al.*,2006) but it could be very useful for accurate identification of pure cultures.

López *et al.*,2008

DNA microarrays
Application in plant pathogenic bacteria

- A prototype DNA microarray for diagnosis has been developed for the rapid and simple identification of 22 European quarantine phytopathogenic bacteria (Pelludat *et al.*,2009).
- The microarray has 38 probes targeted to:
- The 16S rDNA, and
- The housekeeping genes *rpoB*, *groEL* and *ftsZ*.
- DNA microarrays differentiate the quarantine bacteria down to the species and, in some cases, subspecies level.

López *et al.*,2010

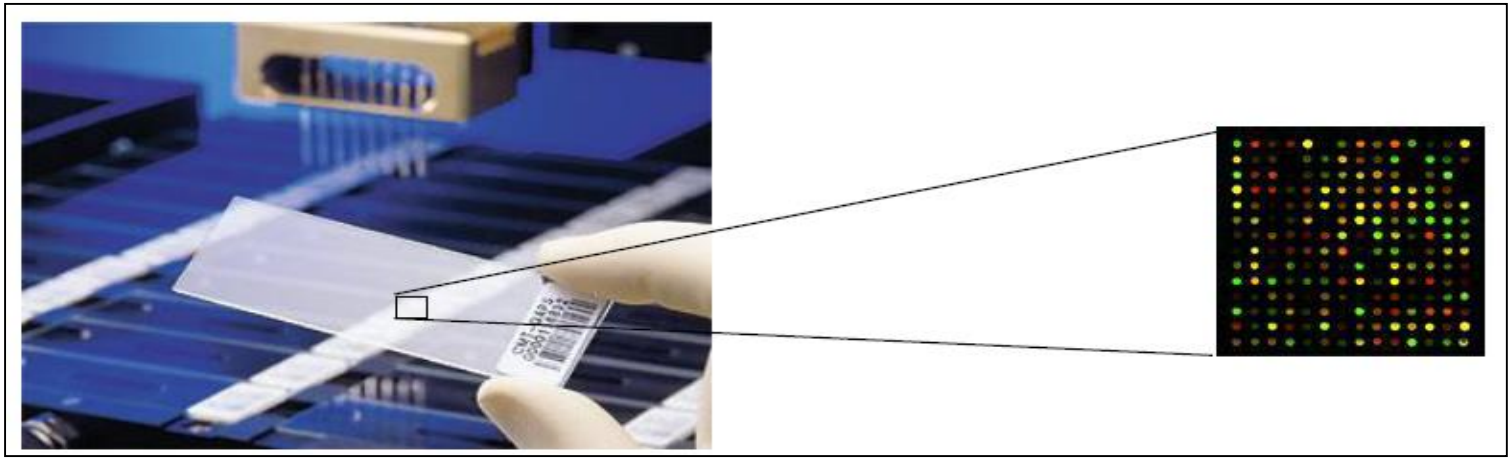
DNA microarrays
DNA or gene chips

- DNA microarray protocols normally rely on the principle of nucleic acid hybridization, with hundreds to thousands of probes arrayed as spots *en miniature* onto a solid support.
- The solid supports themselves are usually glass microscope slides, but can also be silicon chips or nylon membranes (chemically inert).
- The spots themselves can be DNA, cDNA, or oligonucleotides.

DNA microarrays

DNA or gene chips

- Glass slide with oligonucleotides spotted in an array on top.
- 10s, 100s, 1000s, 10‘000s, 100‘000s = number of probes spotted.



Duffy *et al.*,2008

Designing a Microarray Experiment
The basic steps

- Various aspects of microarray technology, including:

1. Probe development,
2. Array fabrication,
3. Assay target preparation,
4. Hybridization,
5. Washing,
6. Scanning, and
7. Interpretation

Designing a Microarray Experiment
The basic steps

- Spot oligos on to a specially coated slide using a robot (can be stored for several months).
- Extract sample DNA (same as with other PCR-based methods).
- Run standard PCR to amplify the probe target sequence (s) using fluorescent labels to mark the amplicon ends.
- Hybridize the PCR products with the microarray.
- Observe results using a fluorescent reader.

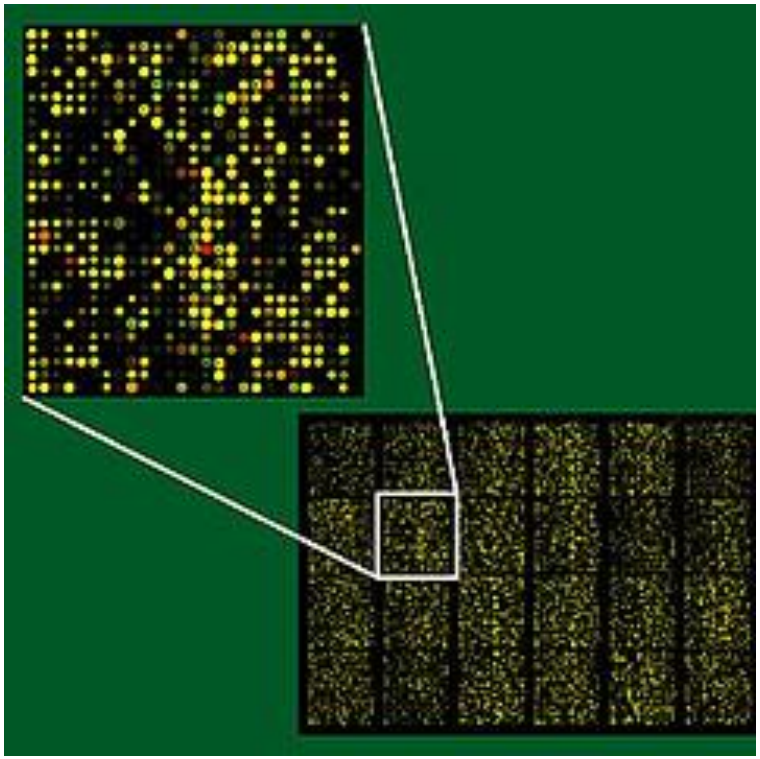
PCR product, sometimes called an amplicon: A portion of DNA amplified or copied during the polymerase chain reaction.

Duffy *et al.*,2008

Capture probe design

The sequence-specific fragment of DNA bound to the solid support in a microarray

- Oligonucleotide probes designed as with PCR primer design, a protocol called on-chip polymerase chain reaction (PCR).
- Identify specific DNA sequences in target organism and design a probe based on those sequences.
- Same challenges to find probes that capture all individuals within target organism, but exclude non-target organisms.



Duffy *et al.*,2008

DNA microarrays
Reading and calculation

- After this hybridization step is complete, place the microarray in a "reader" or "scanner" that consists of some lasers, a special microscope, and a camera.
- The fluorescent tags are excited by the laser, and the microscope and camera work together to create a digital image of the array.
- These data are then stored in a computer, and a special program is used either:

1. To calculate the red-to-green fluorescence ratio, or
2. To subtract out background data for each microarray spot by analyzing the digital image of the array.

DNA microarrays
Reading and calculation

- If calculating ratios, the program then creates a table that contains the ratios of the intensity of red-to-green fluorescence for every spot on the array.
- For example, using the scenario outlined above, the computer may conclude:
- Both cell types express gene A at the same level,
- Cell 1 expresses more of gene B,
- Cell 2 expresses more of gene C, and
- Neither cell expresses gene D.

The Colors of a Microarray

Reproduced with permission from the Office of Science Education, the National Institutes of Health.

In this schematic:

GREEN represents **Control DNA**, where either DNA or cDNA derived from normal tissue is hybridized to the target DNA.

RED represents **Sample DNA**, where either DNA or cDNA is derived from diseased tissue hybridized to the target DNA.

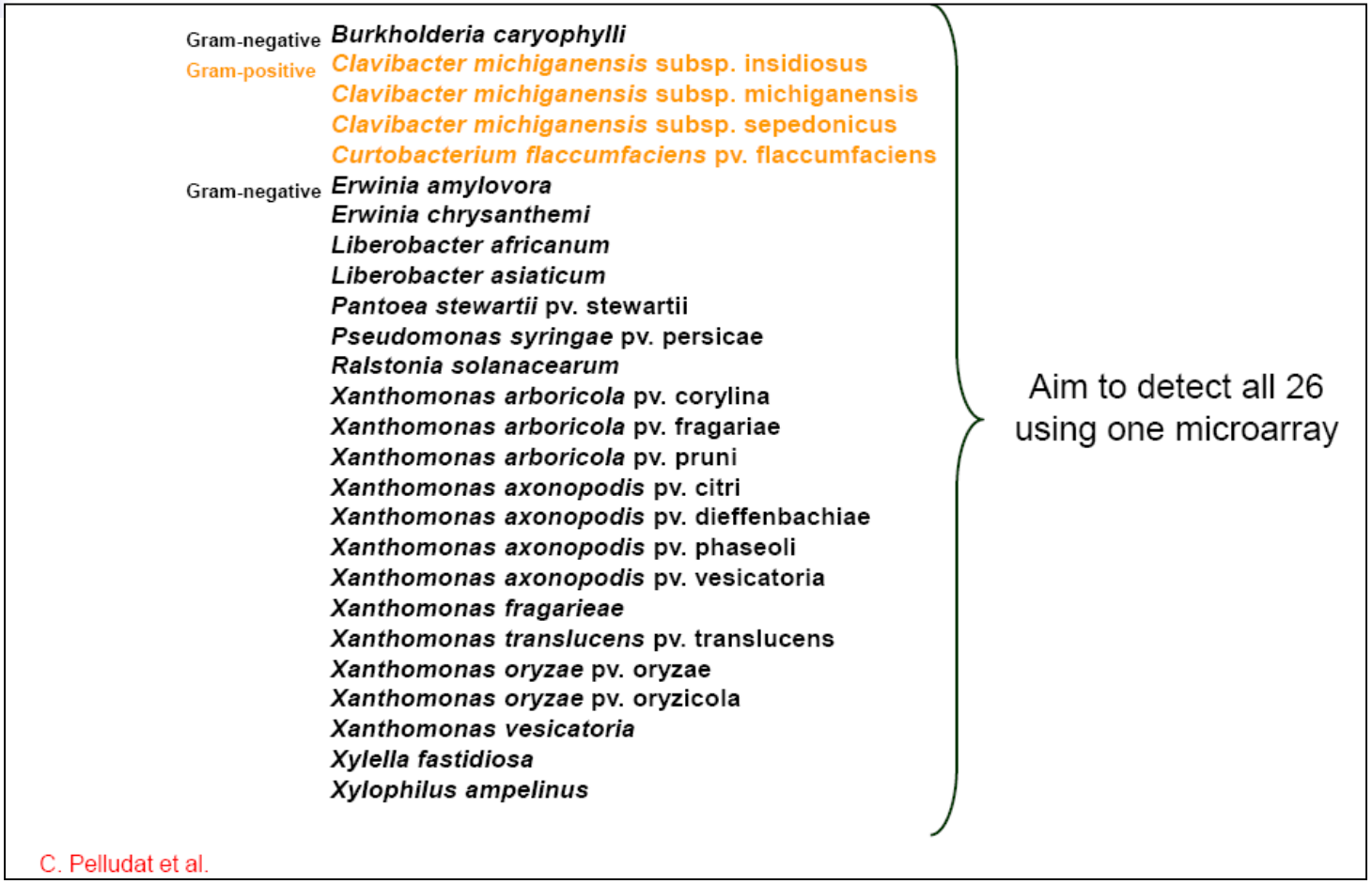
YELLOW represents **a combination of Control and Sample DNA**, where both hybridized equally to the target DNA.

BLACK represents areas where **neither the Control nor Sample DNA** hybridized to the target DNA.

Each spot on an array is associated with a particular gene. Each color in an array represents either healthy (control) or diseased (sample) tissue. Depending on the type of array used, the location and intensity of a color will tell us whether the gene, or mutation, is present in either the control and/or sample DNA. It will also provide an estimate of the expression level of the gene(s) in the sample and control DNA.

NCBI,2007

DNA microarrays
Quarantine bacteria chip



Duffy *et al.*,2008

DNA microarrays

A genome chip approaching universal diagnostics

Recent advances

- Several bacteria(including phytoplasmas), fungi, nematodes, weeds, insects, etc. can all be detected in a single method.
- No specialized training in these different targets is needed.
- Rapid results available.

DNA microarrays

A genome chip approaching universal diagnostics

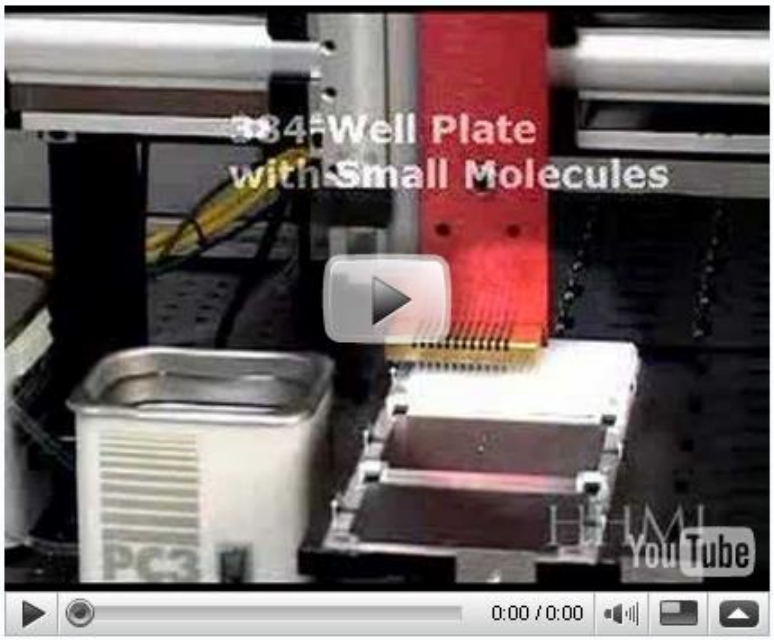
Recent advances

- So far this technique was confirmed for bacteria.
- Microarrays offer a virtually unlimited power for diagnostics, and many such chips have been designed for individual pests or groups of pests.
- Recent advances have made it technologically possible to produce diagnostic chips or sets of chips that can simultaneously detect all pest organisms.
- Preliminary data further indicates that this universal chip may have applications for discriminating between bacterial strains and thus present an alternative strain-fingerprinting approach.

Frey *et al.*,2009

Schematic overview of on-chip PCR for the detection of bacteria

- The glass chip contains two identical microarrays with several hundreds of covalently attached oligonucleotide primers.
- To achieve the very high density of DNA probes in the array, spotting or printing of the slides is carried out by specialized robots:

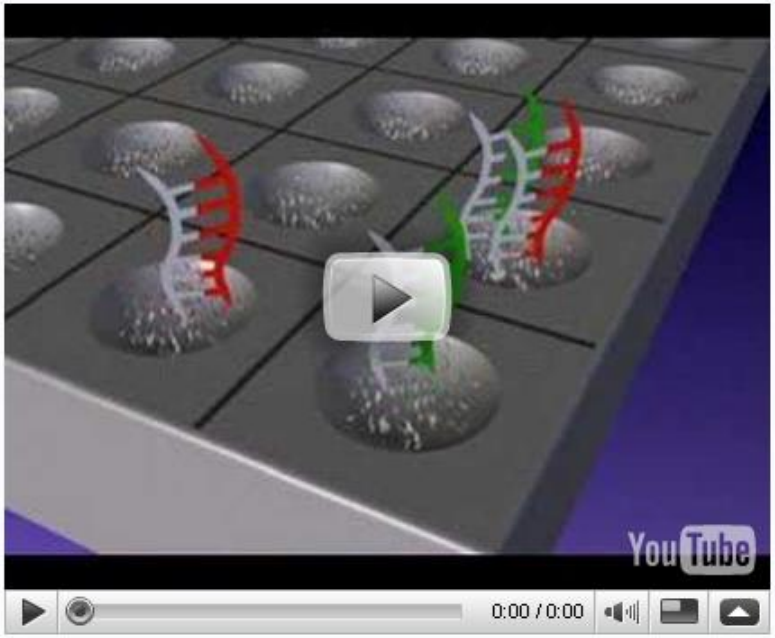


Video: Proneural

Ajcann,2007

Schematic overview of on-chip PCR for the detection of bacteria

- The usual method is to fluorescently label an RNA sample in an enzymic reaction, e.g. reverse transcription, during which the RNA is converted into complementary DNA (cDNA).
- Amplification of sequences by PCR is sometimes incorporated into this step:



Video: Proneural

Ajcann,2007

Schematic overview of on-chip PCR for the detection of bacteria

- **Detection by fluorescence scanning.**
- The cyanine dyes Cy3 and Cy5 which have excitation wavelengths of 635 nm and 532 nm respectively are most frequently used to label the RNA samples.
- Two-color labeling allows two samples, for example, two different tissues, treated and untreated, or infected and uninfected samples to be hybridized to the same array and their gene expression profiles compared via the difference in the fluorescence of the two samples.
- This complex mixture of sequences is then allowed to hybridize to the DNA capture probes on the array for up to 24 hours:

Video: Jung13

Ajcann,2007

Schematic overview of on-chip PCR for the detection of bacteria

- Unbound (non-complementary) sequences are washed away and the fluorescence of the individual DNA spots on the array is measured by laser excitation.

Video: Jung13

Ajcann,2007

Schematic overview of on-chip PCR for the detection of bacteria

- Statistical post-processing of the fluorescence data is usually necessary to eliminate artifacts and false results from the data obtained.

Video: Jung13

Ajcann,2007

Fluorescent *in situ* Hybridization

Using rRNA (rDNA) oligonucleotide probes

- Fluorescent *in situ* hybridization (FISH) is an attractive technique for the rapid detection and identification of bacteria or fungi directly from slide smears.
- FISH has been used to identify and quantify bacteria and fungi.
- This technology has the speed and ease-of-use of conventional staining methods combined with the specificity of molecular methods.

Fixed tissue preserved on a slide is "*in situ*" (in place/location).

FISH

Fluorescent *In Situ* Hybridization Test

- - FISH (Fluorescent *In Situ* Hybridization) test:
- Fluorescence in situ hybridization ("FISH") techniques, in which the nucleic acid probes are labeled with a fluorophor (i. e., a fluorescent tag or label that fluoresces when excited with light of a particular wavelength), represents a powerful tool for the analysis of numerical, as well as structural aberrations chromosomal aberrations.
- Advantages:
- It can detect pathogen concentration of 10^3 cfu/ml.
- It produced results within 4-6h.
- Disadvantages:
- Its reliable due to possible cross- reaction with some other harmless bacteria in soil, water and weed samples.
- Require a fluorescence microscope.

FISH technique

Using rRNA (rDNA) probes

- In this method short (20-30 mers) oligonucleotide probes against 16S or 23S rRNA/DNA are used.
- These oligonucleotide probes can be used for *in situ* hybridization, because they are able to diffuse through the cell wall of micro-organisms that are present:
- In thin tissue sections or in plant or soil extracts fixed on a microscopic slide.
- In the FISH technique, fluorescently labeled nucleic acid probes are hybridized to complementary rRNA targets located on ribosomes.
- When probes have been labelled with a fluorescent dye or a gold label the micro-organisms can be visualized by incident light (fluorescence) microscopy.

FISH technique
Using mRNA probes

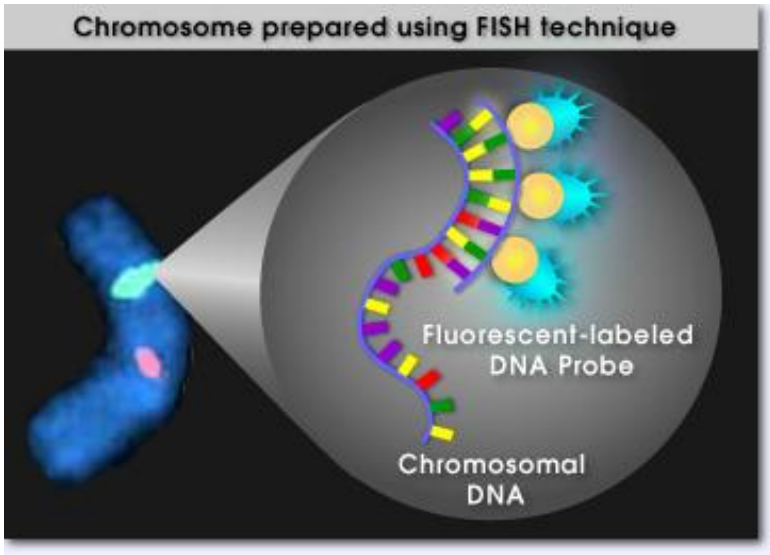
- Apart from rRNA, other forms of RNA (e.g., mRNA) can serve as a target for hybridizations.
- Recently, FISH-based methods have also been developed to detect low-copy-number targets on:

1. Plasmid (10^1 to 10^3 copies/cell) or
2. Chromosomal (<10 copies/cell) DNA).

Some rRNA-targeted oligonucleotide probes for fluorescent *in-situ* hybridization

Probe	Position	Probe sequence (5'→3')	Specificity	References
ALF1b	16S rRNA 19–35	CGTTCG(C/T)TCTGAGCCAG	"Alphaproteobacteria," but not exclusive	Manz et al., 1992
BET42a	23S rRNA 1027–1043	GCCTTCCCACTTCGTTT	"Betaproteobacteria"	Manz et al., 1992
GAM42a	23S rRNA 1027–1043	GCCTTCCCACATCGTTT	"Gammaproteobacteria," but not the deeply branching taxa	Manz et al., 1992
Delta 385	16S rDNA 385–402	CGGCGT(C/T)GCTGCGTCAGG	"Deltaproteobacteria" sulfate-reducers, but not exclusive	Rabus et al., 1996

FISH
Fluorescent *in situ* Hybridization



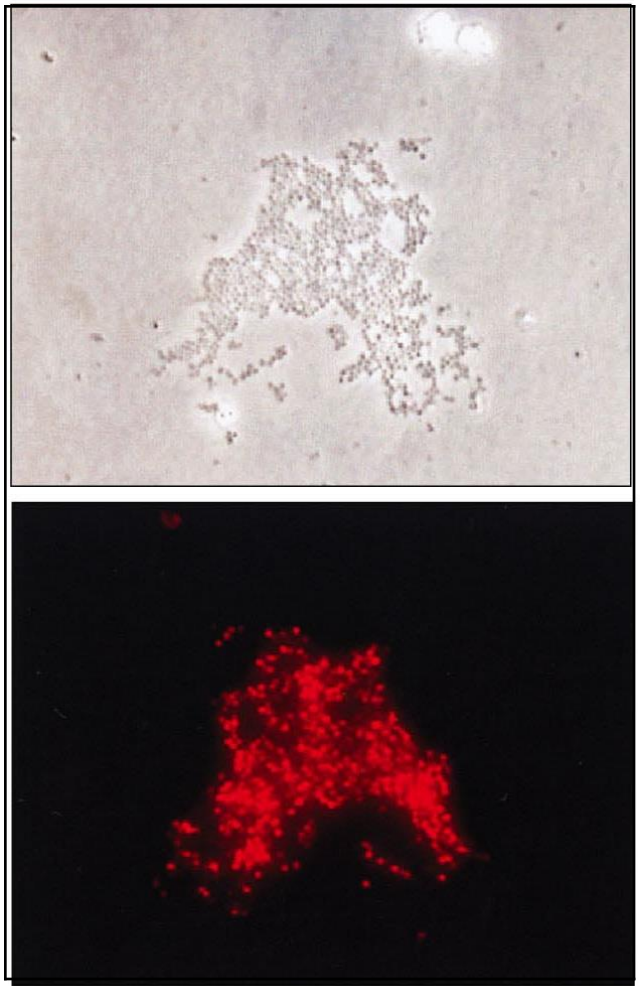
Fluoresces in the infra-red

One can use DNA & RNA probes for *in situ* hybridization.
In situ (in place) can use large DNA probes to identify the location of probe on a chromosome.

FISH

Fluorescent *in situ* Hybridization

Add DNA probe



Karlm,2004

FISH technique
Sensitivity/specificity of the method

- Sensitivity depends also on the metabolic activity of the cells, although killed cells show a positive reaction for a considerable period of time.
- It is quite high, usually much higher than in serological methods, but false-positive reactions are still possible due to homology of RNA of non-target organisms with the specific probe sequence.

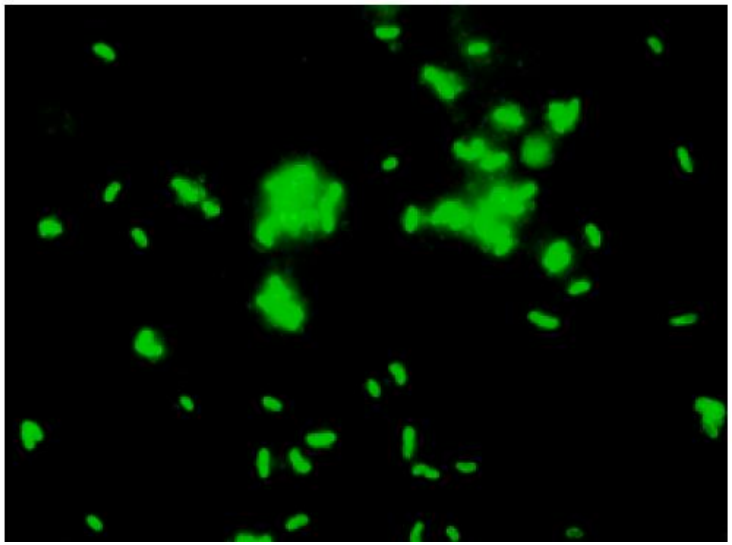
Janse,2006

Fluorescence *in situ* hybridization and immunofluorescence

- FISH and immunofluorescence have substantial overlap in their applications and benefits as single-cell detection techniques.
- Both are whole-cell methods.
- Apart from providing information on microbial identity, information about cell morphology, number, and distribution may also be collected for specific target cells.
- Neither FISH nor immunofluorescence approaches require that a cell be culturable.

Immunofluorescence (IF)

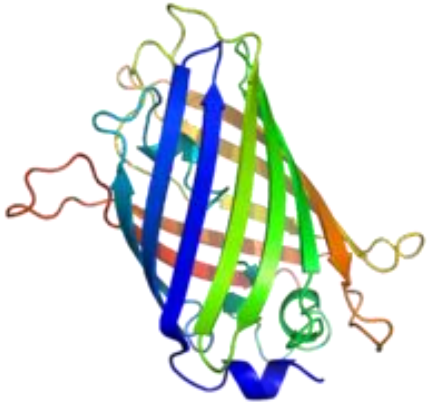
- Bacterial cells stained with fluorescent dye (fluorescence iosthiocyanate) labeled monoclonal antibody.



Green fluorescent protein (GFP)
Protein detection

- Green fluorescent protein:
- A protein that glows green under fluorescent light.
- GFP occurs naturally in jellyfish of the genus *Aequorea*.

Aequorea victoria



GFP ribbon diagram

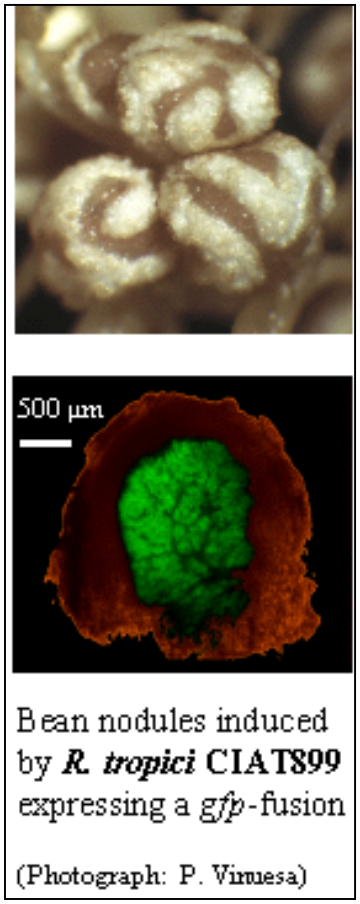
Green fluorescent protein (GFP)
Protein detection

- GFP has a sequence of three amino acids (serine-tyrosine-glycine) which is responsible for its fluorescence.
- When the gene of the target protein is fused to that of GFP, the fusion protein (i.e. target protein+ GFP) is detected by fluorescence microscopy.
- Therefore intracellular location of a specific protein was detected by monitoring protein-protein interactions.

Bacterial gene expression *in situ* on plants

GFP as a reporter of gene expression

- The combination of fluorescent markers with reporter gene technology has proven to be a powerful tool to study the behavior of bacteria on plants.
- The protocols make use of dual labeling with GFP as a reporter of transcriptional activity, and with rhodamine as a marker for FISH to identify specific bacterial cells among the natural plant microflora.



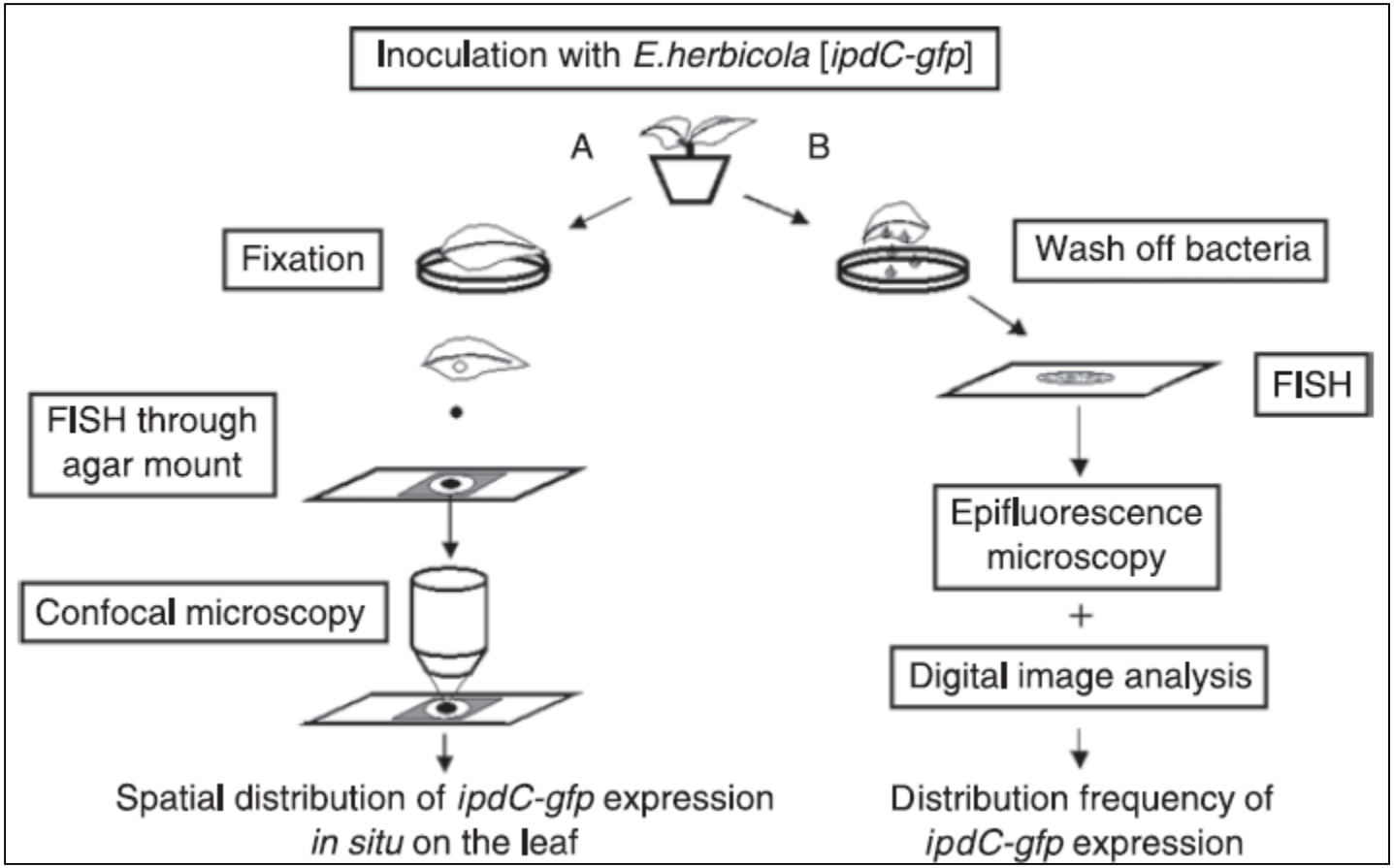
Bean nodules induced by ***R. tropici*** **CIAT899** expressing a *gfp*-fusion

(Photograph: P. Vinuesa)

Sapers *et al.*,2006;…

Schematic diagram of fluorescence microscopy strategies to investigate the distribution of gene expression at the bacterial cell level on plants

FISH



Sapers *et al.*,2006

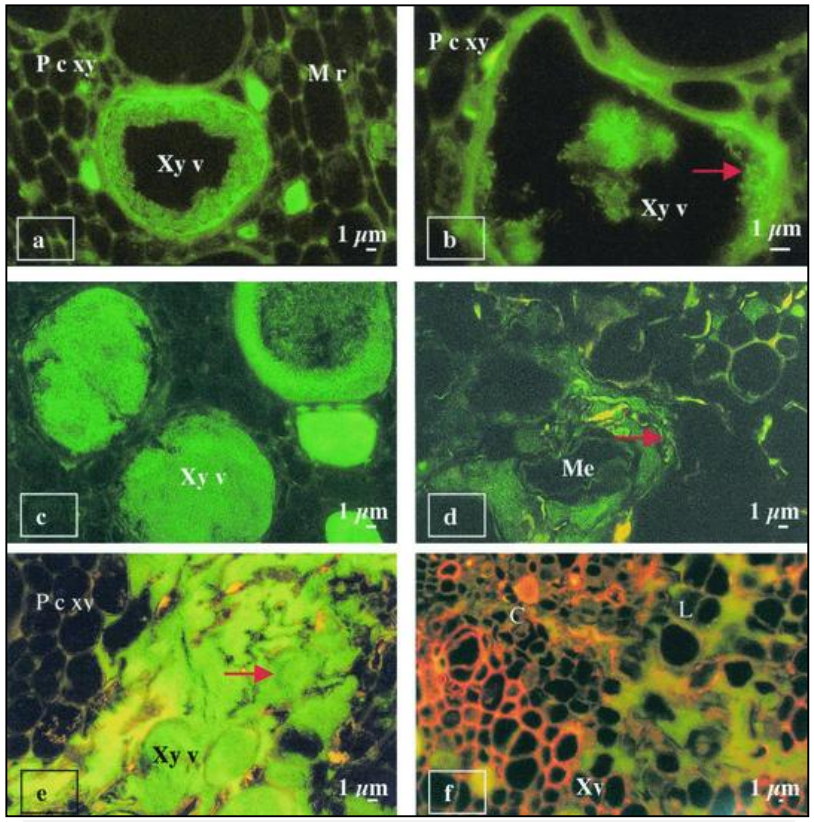
Green fluorescent protein (GFP)
Applications

- Because it retains its fluorescence after fixation with paraformaldehyde, GFP can be combined with fixation-dependent staining methods such as FISH.
- *In situ* monitoring of iron availability on leaf surfaces,
- Investigation of quorum-based interspecies communication,
- Measurement of the internal pH of bacterial cells,
- *In situ* analyses of individual cells within complex consortia such as biofilms.

Applications of GFP

Stem colonization of the bacteria

- Colonization of *Vitis vinifera* by a green fluorescence protein-labeled, *gfp*-marked strain of *Xylophilus ampelinus*, the causal agent of bacterial necrosis of grapevine.
- Micrographs of transversal stem slices of *V. vinifera* cv. Ugni blanc showing the location of *X. ampelinus* strain 2098::*gfp* 2 in the stem tissues under UV light.
- The bacterial cytoplasm fluoresces in green.

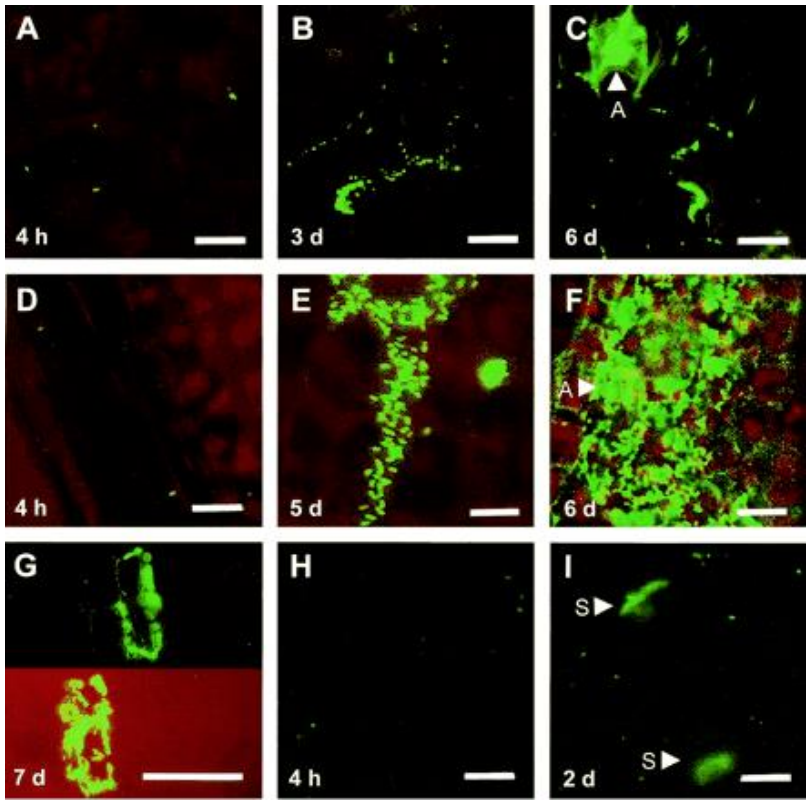


Micrographs of transversal stem slices.

Grall and Manceau,2003

Applications of GFP
Leaf colonization of the bacteria

- Fluorescence microscopy images of *P. agglomerans* BRT98-GFP on bean leaves (A to C),
- *P. agglomerans* BRT98-GFP on maize leaves (D to G),
- *P. syringae* pv. *syringae* B728a-GFP on bean leaves (H and I).
- The leaf cells appear red or black, and the GFP-labeled bacterial cells appear green.
- The bars represent 20 μm.
- Abbreviations:
- A, aggregate;
- S, stomatal opening.

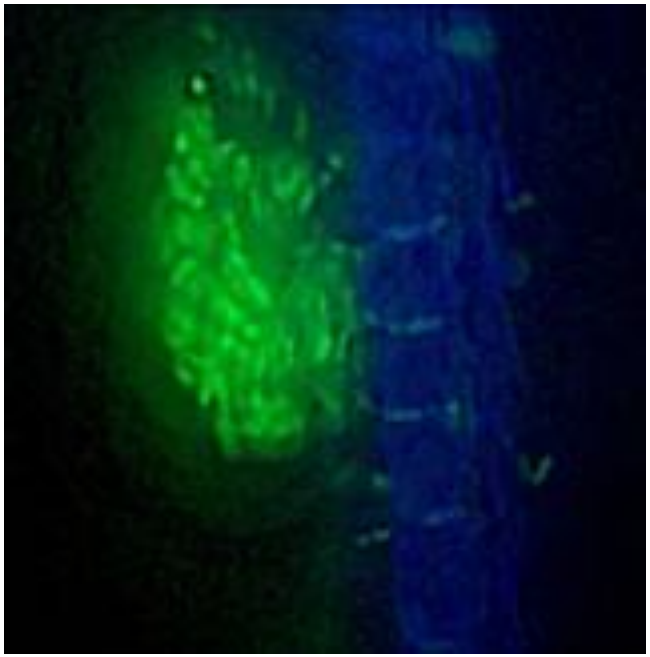


The transverse slices were 5 μm thick.

Applications of GFP

Movement of gfp-marked strains of *Xylella fastidiosa* across pit membranes

- Movement of gfp-marked strains of *Xylella fastidiosa* across pit membranes of a grape xylem vessel.



Lindow,2009

Plasmid analysis
Description

- Plasmids are circular, supercoiled DNA molecules present in many strains of bacteria.
- Most plasmids are small and are about 0.2 to 4 per cent of the size of the bacterial chromosome.
- As genetic material, all the plasmids fall into two groups:

1. The cryptic plasmids (which have no definite function except their own replication and dissemination), and
2. The plasmids which are responsible for definite phenotype.

Plant pathogenic bacteria
Plasmid genes

- Plasmid DNA is also widely employed in the design of primers for important bacterial diseases.
- The plasmid genes amplified may be associated to pathogenicity or be of unknown function.
- Examples:

1. Ti plasmid of *Agrobacterium* species. This plasmid was considered universal; nevertheless, recently some strains have been found without it (Llop *et al.*,2006) indicating the risk of false negatives.
2. pEA29 plasmid sequences for sensitive and specific detection of *E. amylovora*.

Palacio-Bielsa *et al.*,2009; López *et al.*,2011

Phytopathogenic strains of *P. syringae* containing plasmids

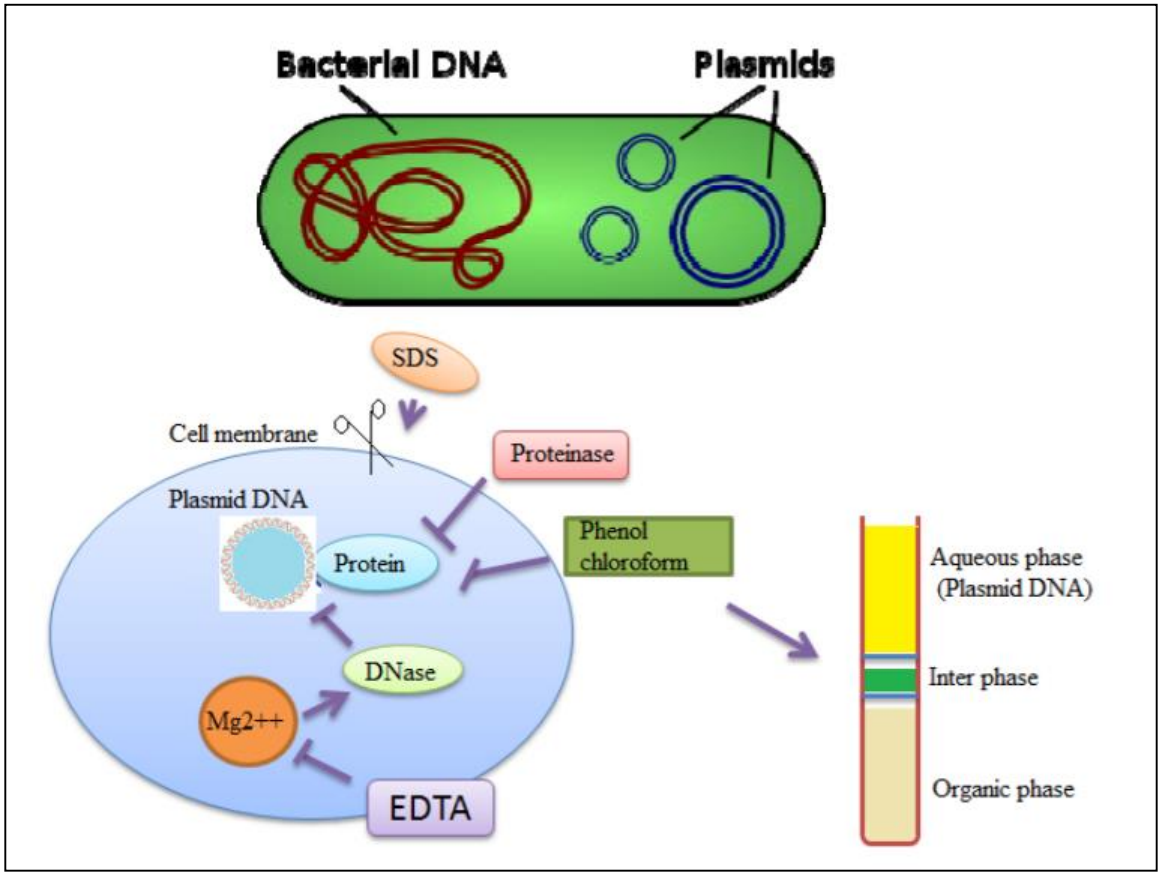
Pathovar	Reference
P. syringae pv. *angulata*	Piwowarski and Shaw,1982
P. syringae pv. *atrpurpurea*	Sato *et al.*,1983
P. syringae pv. *coronafaciens*	Piwowarski and Shaw,1982
P. syringae pv. *glycinea*	Curiale and Mills,1983
P. syringae pv. *lachrymans*	Coplin, 1989
P. syringae pv. *papulans*	Burr *et al.*,1988
P. syringae pv. *phaseolicola*	Quant and Mills,1984
P. syringae pv. *savastanoi*	Comai *et al.*, 1982
P. syringae pv. *striafaciens*	Beck-Von Bodmann and Shaw,1987
P. syringae pv. *syringae*	Gonzales *et al.*,1984
P. syringae pv. *tabaci*	Obukowicz and Shaw,1983;1985
P. syringae pv. *tomato*	Denny, 1988; Bender and Cooksey,1986

OECD,1997

Plasmid DNA Isolation

Schematic diagram of principle of Plasmid DNA Isolation

To isolate plasmid DNA from bacterial cells



Plasmid DNA Isolation

Schematic diagram of principle of Plasmid DNA Isolation

To isolate plasmid DNA from bacterial cells

- In prokaryotes, plasmid is double stranded, circular, and is found in the cytoplasm.
- The cell membranes must be disrupted in order to release the plasmid in the extraction buffer.
- Solution 1 contains glucose, Tris, and EDTA.
- Glucose provides osmotic shock leading to the disruption of cell membrane, Tris is a buffering agent used to maintain a constant pH8.
- Plasmid can be protected from endogenous nucleases by chelating Mg^{2++} ions using EDTA.
- Mg^{2++} ion is considered as a necessary cofactor for most nucleases.
- Solution II contains NaOH and SDS and this alkaline solution is used to disrupt the cell membrane and NaOH also denatures the DNA into single strands.

Plasmid DNA Isolation

Schematic diagram of principle of Plasmid DNA Isolation

To isolate plasmid DNA from bacterial cells

- Solution III contains acetic acid to neutralise the pH and potassium acetate to precipitate the chromosomal DNA, proteins, along with the cellular debris.
- Phenol /chloroform is used to denature and separate proteins from plasmid.
- Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and pure phenol layer.
- The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation.
- Once the plasmid DNA is released, it must be precipitated in alcohol.
- The plasmid DNA in the aqueous phase is precipitated with cold (0°C) ethanol or isopropanol.
- The precipitate is usually redisolved in buffer and treated with phenol or organic solvent to remove the last traces of protein, followed by reprecipitation with cold ethanol.

Alkaline lysis plasmid miniprep

Method 1

Preparation of reagents

1. **TE Buffer (pH 8.0):** 10 mm Tris HCl (pH 8.0) 1 mm EDTA (pH 8.0)
2. **Solution I:** Lysis solution
3. **Solution II:** Denaturing solution
4. **Solution III:** Neutralizing solution
5. **Phenol-chloroform Mixture:** Mix equal volume of phenol with chloroform. Keep the mixture on ice and add 20 ml TE buffer, extract by shaking for 15 minutes. Remove the dust on the surface layer using a pipette. Repeat 4-5 times. Add 30-40 ml of TE buffer and store it in dark.
6. **Isopropanol** (ethanol precipitation usually by ice-cold ethanol or isopropanol. Since DNA is insoluble in these alcohols, it will aggregate together, giving a pellet upon centrifugation.)

Alkaline lysis plasmid miniprep

Procedure (continued)

- Take 2 ml overnight culture and harvest cells by centrifugation for 5 minutes. Discard the supernatant carefully.
- Add 100 µl of solution I to the cell pellet and resuspend the cells by gentle mixing.
- Incubate the above mixture at room temperature for 5 minutes.
- Add 200 µl of solution II to the mixture and mix by inverting the tubes for 5 minutes.
- Incubate for 5-10 minutes at room temperature.
- Add 500µl of ice cold solution III to the mixture and mix by inverting the tube.
- Incubate on ice for 10 minutes.
- Centrifuge at 10,000 rpm for 5 minutes.
- Transfer the supernatant into fresh tube.
- Add 400 µl of phenol-chloroform mixture to the contents, mix well by inverting and incubate them at room temperature for 5 minutes.
- Centrifuge at 10000 rpm for 5 minutes.
- Collect the supernatant (viscous) using cut tips and transfer to a fresh tube.
- Add 0.8 ml of isopropanol and mix gently by inversion. Incubate for 30 min at room temperature.
- Centrifuge the contents at 10,000 rpm for 10 minutes.

Alkaline lysis plasmid miniprep

Procedure (continued)

- Discard the supernatant after centrifugation.
- After air drying for 5 minutes, add 100 µl of TE buffer or autoclaved distilled water to the pellet to resuspend the plasmid DNA. The contaminated salt in the DNA pellet can be removed with 70% ethanol washing.
- Take 10 µl of plasmid sample and dilute to 1 ml with distilled water for spectrometric analysis.
- The concentration of plasmid is determined using a spectrophotometer at 260/280 nm.
- An aliquot of plasmid DNA is used for agarose electrophoresis for quantitative and qualitative analyses.
- PRECAUTIONS:
- Cut tips should be used so that the plasmid is not subjected to mechanical disruption.
- The phenol chloroform extraction should be repeated depending on the source of plasmid to obtain pure plasmid.
- DNase free plastic wares and reagents should be used.

Alkaline lysis plasmid miniprep

Method 2

Solutions

1. 50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0);
   - Filter sterilized and stored at 4°C.
2. 0.2N NaOH (freshly diluted from 10N stock), 1% SDS.
   - This solution should be made up fresh the day of use.
3. 3 M potassium and 5 M acetate.
   - Store at 4°C.

5 mM potassium acetate	60 ml
Glacial acetic acid	11.5 ml
H_2O	28.5 ml

Bacterial cells were lysed with 200 µL lysis solution [0.2 N NaOH, 1% (wt/vol) sodium dodecyl sulfate (SDS)] for 4 min and neutralized with 150 µL of chilled 3 M potassium acetate, pH 4.8(Delaney *et al.*,2018).

Molecular Cloning: A Laboratory Manual, 2nd ed., pp.1.25-1.28.

Alkaline lysis plasmid miniprep (continued)

- Grow up a 5 ml culture overnight in the presence of the appropriate antibiotic.
- Harvest 1.5 ml of culture by centrifugation in an eppendorf tube.
- Resuspend the pellet in 100 µl of ice-cold solution 1.
- Store for 5 minutes at room temperature.
- Add 200 µl of solution 2 and mix the contents by inverting the tube rapidly two or three times.
- Do not vortex.
- Store for 5 minutes on ice.
- Add 150 µl of ice-cold solution 3 and mix by vortexing in an inverted position.
- Store for 5 minutes on ice.
- Centrifuge for 5 minutes in a Eppendorf centrifuge at 4^{o}C.

Molecular Cloning: A Laboratory Manual, 2nd ed., pp.1.25-1.28.

Alkaline lysis plasmid miniprep (continued)

- Transfer supernatant to a fresh tube and add an equal volume of phenol/chloroform to remove proteins.
- Mix by vortexing the tube and centrifuge for 2 minutes in an eppendorf centrifuge at room temperature.
- Transfer the top layer to a fresh tube and add two volumes of ethanol.
- Vortex gently and let stand at room temperature for 2 minutes.
- Centrifuge for 5 minutes in an Eppendorf centrifuge at 4°C.
- Remove supernatant and add 1 ml of 70% ethanol.
- Vortex briefly and recentrifuge.
- Remove supernatant and dry the pellet briefly in a vacuum dessicator.
- Add 50 µl of TE (pH 8.0) containing DNase-free pancreatic RNase (20 µl/ml).

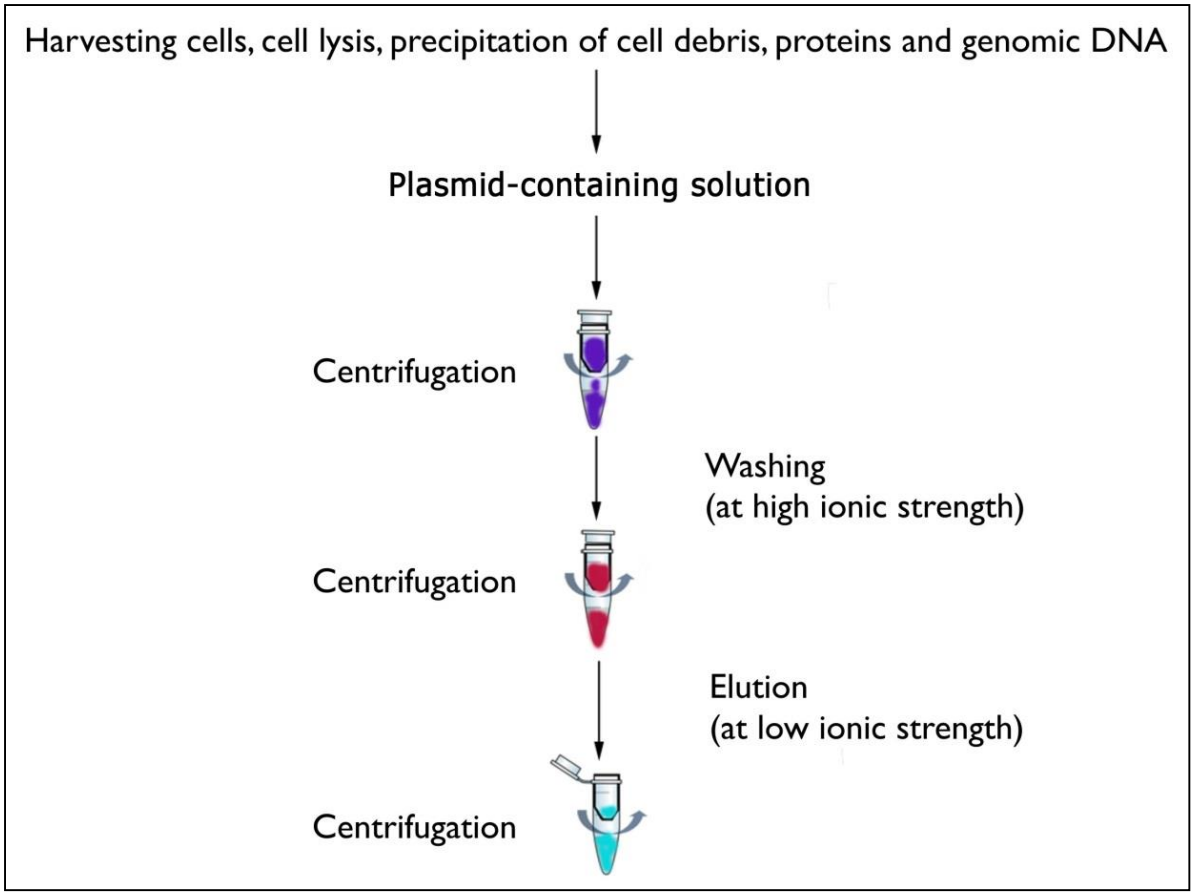
Molecular Cloning: A Laboratory Manual, 2nd ed., pp.1.25-1.28.

Alkaline lysis plasmid miniprep
Method 3

1. Grow bacteria overnight in 37° shaking incubator, with lids very loose and taped on. I normally use 5 ml of liquid medium in a 50 ml conical bottom tube. Be sure to include the appropriate selective antibiotic.
2. Remove 1 ml of culture from each tube and mix it with 1 ml of freeze down solution (65% glycerol, 0.1 M $MgSO_4$, 25mM Tris pH 8, autoclaved) in a cryo tube. Freeze at -85° C. (This is optional if you already have a freeze down of the clone).
3. Centrifuge the remainder of each culture at 1500 x g for 5 min. Pour off the supernatant and resuspend the bacterial cell pellet in 200 µl of GTE buffer (50 mM glucose, 25 mM Tris pH 8, 10 mM EDTA). If you don't want RNA, add 1 ul of 1 mg/ml RNAse to each suspension. Transfer the resuspended cells to a microcentrifuge tube.
4. After 5 min at room temperature, add 400 µl of freshly prepared alkaline solution (0.2 N NaOH, 1% SDS) and mix by inverting several times. (to prepare alkaline solution, mix 2 ml 1 N NaOH, 1 ml 10% SDS, and 7 ml H_2O).
5. After 5 min on ice, add 300 µl of 7.5 M ammonium acetate solution. Mix gently for a few seconds. Keep tube on ice for 10 min.
6. Centrifuge at 10,000 RPM for 3 minutes.
7. Transfer supernatant to a clean microcentrifuge tube, discard pellet.
8. Repeat steps 6 and 7.
9. Add 0.6 volumes of isopropanol (400 to 500 µl), mix, and incubate 10 min at room temperature.
10. Centrifuge at 21,000 RPM in high-speed centrifuge for 15 min.
11. Discard supernatant and add 1 ml of ice-cold 70% ethanol. Do not disturb the pellet; centrifuge at 21,000 RPM for 10 min
12. Discard supernatant and speed-vac about 3 min, or until no alcohol remains.
13. Dissolve pellet in 20 µl of TE (10 mM Tris pH 8, 1 mM EDTA).
14. Quantify spectrophotometrically.

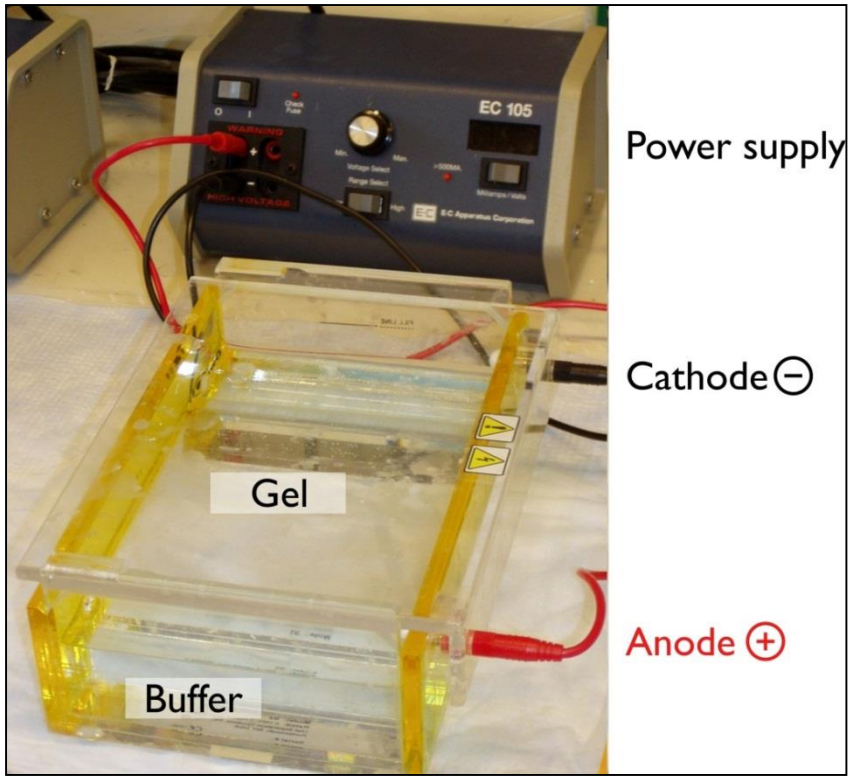
Morelle,1989

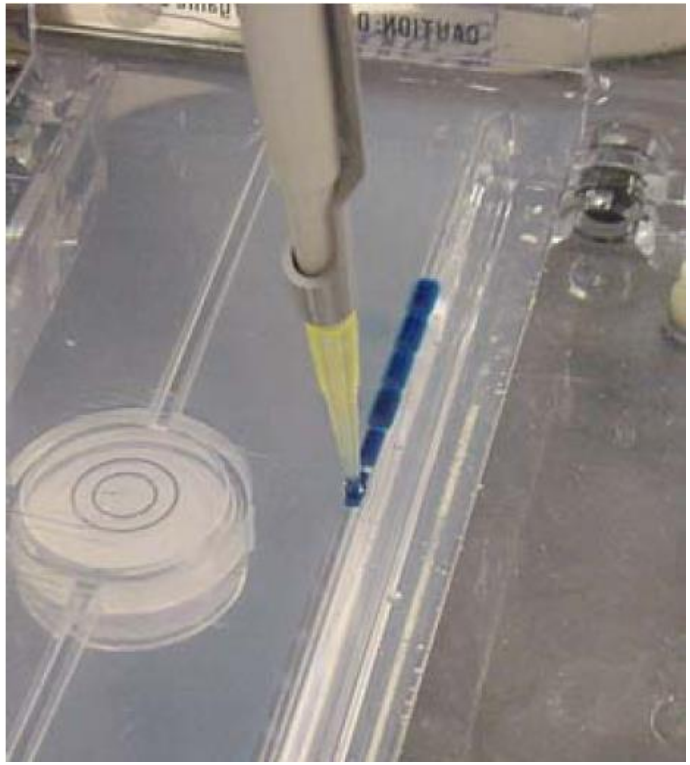
Rapid plasmid-screening method



Hegyi *et al.*,2013

Rapid plasmid-screening method





Hegyi *et al.*,2013

Agarose Gel profile

Gel electrophoretic image of plasmid DNA

- Plasmid DNA can exist in three conformations:

1. supercoiled,
2. open-circular (oc), and
3. linear.

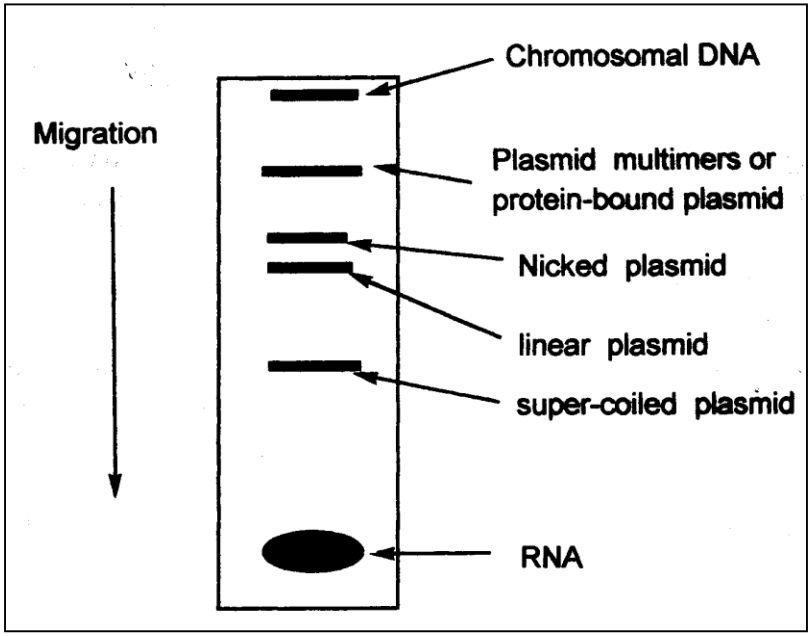
- *In vivo*, plasmid DNA is a tightly supercoiled circle to enable it to fit inside the cell.
- In the laboratory, following a careful plasmid prep,

1. most of the DNA will remain supercoiled,
2. but a certain amount will sustain single-strand nicks.
3. Given the presence of a break in only one of the strands, the DNA will remain circular.

Agarose Gel profile

Gel electrophoretic image of plasmid DNA

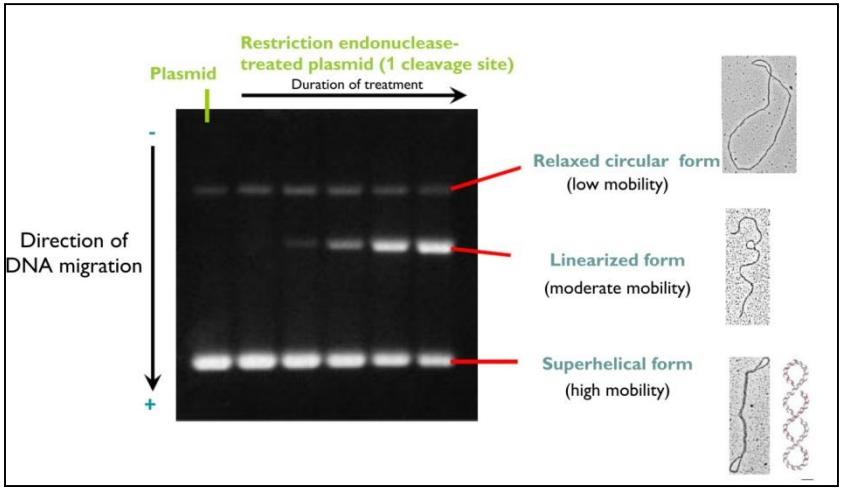
1. Supercoiled DNA is the most compact form of DNA and will consequently migrate more rapidly than other forms of DNA of the same or larger molecular weight.
2. Nicked relaxed Plasmid DNA has an intermediate mobility under our electrophoretic conditions.
3. Chromosomal DNA is large and will migrate the least distance.



Uncut plasmids can be in two forms: relaxed and supercoiled. Supercoiled plasmid DNA migrates faster in an agarose gel then a linearized plasmid does.

Gel electrophoretic image of plasmid DNA

- Besides their size, the electrophoretic mobility of DNA molecules is also significantly affected by their shape.
- Circular plasmid DNA has a compact structure, and its hydrodynamic size is much smaller—and its electrophoretic mobility is therefore greater—than that of linear DNA molecules of the same size, as the latter form a freely moving entropic chain.



Hegyi *et al.*,2013

Rapid plasmid-screening method

Characterization of the 1.8-kb plasmid pXV64 from *Xanthomonas vesicatoria*

- A small number of cells were picked by touching a colony with a toothpick.
- They were then inoculated into a microfuge tube containing 300 µl of L broth.
- After overnight growth, the cells were pelleted in a microfuge (10,000 rpm, 2 min).
- The cells were then suspended by vortexing in 20 µl of gel-loading mix (0.25% bromophenol blue and 30% glycerol in DNA sample buffer (5X).
- Then 40 µl each of chloroform and phenol (saturated with 1.0 M Tris-HCl, pH 8.0) was added (for cell lysis). Chloroform removes traces of phenol.
- The mixture was vortexed at full speed for 1 min followed by centrifugation for 10 min at 12,000 rpm (for DNA precipitation).

- Preparation of 1.0 M Tris-HCl, pH 8.0: Dissolve 121.14 g Tris in 800 ml dH_2O. Adjust pH to 7.0 with the appropriate volume of concentrated HCl. Bring final volume to 1 liter with deionized water. Autoclave and store at room temperature.
- DNA sample buffer (5X): 10mM Tris/HCl, pH 8.0 100mM NaCl 30% glycerol 0.25% bromophenol blue.

Rapid plasmid-screening method

Characterization of the 1.8-kb plasmid pXV64 from *Xanthomonas vesicatoria*

- 10 µl of the aqueous fraction was subjected to electrophoresis on 0.7% agarose minigel (5.2 × 6.0 cm) with TAE buffer (40 mM Tris-acetate, pH 8.0 containing 2 mM Na2-EDTA) at 100 volts for 30 min.
- The gel was stained with ethidium bromide (0.5 µg/ml) and the DNA bands were visualized under a UV transilluminator.

Weng *et al.*,1996

TAE (Tris-acetate-EDTA) DNA gel running buffer containing 40 mM Tris+20 mM Acetic Acid+1mM EDTA.

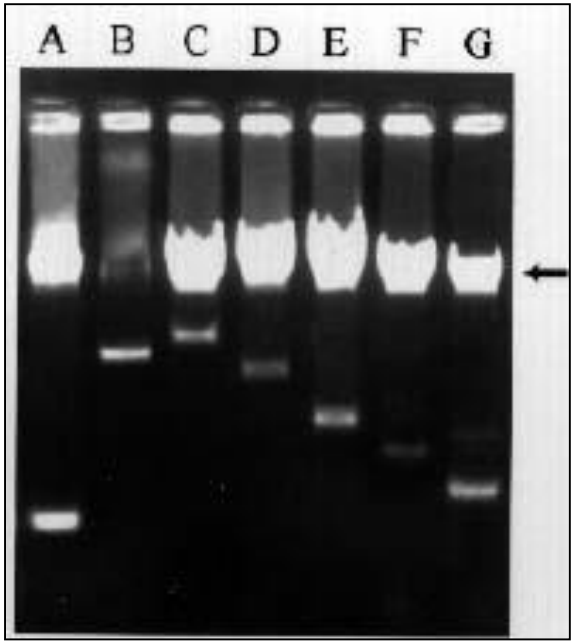
To prepare 1 L of 50x stock of TAE:
242 g Tris Base
18.6 g EDTA (or 100ml of 0.5M EDTA)
57.1 ml Glacial Acetic Acid

Rapid plasmid-screening method

A simple and rapid method for screening plasmids with sizes up to 15 kb has been developed

- Agarose gel electrophoresis of the plasmids extracted by the rapid method from Xv64 (A), Xv36 (B), Xc17 (C), and *E. coli* HB101 (D to G).
- Lanes:

A. pXV64 (1.8 kb);
B. pXV2 (14.6 kb)
C. pK701 (15.8 kb);
D. pRK415 (10.5 kb);
E. pBR325 (6.0 kb);
F. pACYC184 (4.2 kb) and
G. pUC18 (2.7 kb).



The chromosomal DNAs are indicated by an arrow. Plasmids with sizes up to 15 kb were detectable in *X. campestris* pv.*vesicatoria*, *X. campestris* pv.*campestris*, and *E. coli* as well, whereas some of the larger ones might have been masked by the smearing of the chromosome.

Weng *et al.*,1996

Total RNA isolation from bacterial cells

- Principle:
- Total RNA is isolated and separated from DNA and protein after extraction with a solution called as Trizol (Sigma).
- Trizol is an acidic solution containing guanidinium thiocyanate (GITC), phenol and chloroform.
- GITC irreversibly denatures proteins and RNases.
- This is followed by centrifugation.
- Under acidic conditions, total RNA remains in the upper aqueous phase, while most of DNA and proteins remain either in the interphase or in the lower organic phase.
- Total RNA is then recovered by precipitation with isopropanol.
- RNase enzymes can be inactivated by including diethyl pyrrocarbonate (DEPC).

Molecular Cloning: A Laboratory Manual, 2nd ed., pp.1.25-1.28.

Total RNA isolation from bacterial cells

Protocol

- Take 800 µL of bacterial culture in a fresh eppendorf. To this add 160 µL of Trizol (1/5th of culture volume).
- The solution was mixed well by pipetting several times. To this add 32 µl of chloroform (1/5th volume of trizol).
- Incubate for 2 to 5 minutes and centrifuge at 12000 rpm for 15 minutes at 4°C
- Transfer the aqueous phase into a new tube and add equal volume of isopropanol. Mix well.
- Centrifuge at 10000 rpm for 10 minutes at 4°C.
- Discard the supernatant and resuspend the pellet in 70% ethanol.
- Again centrifuge at 10000 rpm for 10 minutes at 4°C.
- Discard the supernatant. Air dry the pellet at 37°C for 10-15 minutes.
- Resuspend the pellet in 50 µL of TE buffer.
- Analyze the RNA sample quantitatively and qualitatively.

Molecular Cloning: A Laboratory Manual, 2nd ed., pp.1.25-1.28.

Total RNA isolation from bacterial cells

Quantitative Analysis of RNA

- To determine the amount and concentration of RNA sample isolated from bacterial cells.
- Remove a 10 µl aliquot of total RNA and dilute with 990 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.0).
- Read at A260 and A280 blanked against TE buffer and calculate the amount of RNA obtained.
- The RNA obtained may be deter mined by the formula:

  Total RNA (µg) = (A 260) (40 µg) (100) (0.05 ml)
- A260 is the absorbance of the solution at 260 nm
- 1 OD of RNA equals to 40 µg/ml/A 260
- 100 is the dilution factor, and 0.05 ml is the total volume.
- Concentration = Total µg/50 µl = µg/µl or mg/ml.

Molecular Cloning: A Laboratory Manual, 2nd ed., pp.1.25-1.28.

Polymerase chain reaction
PCR

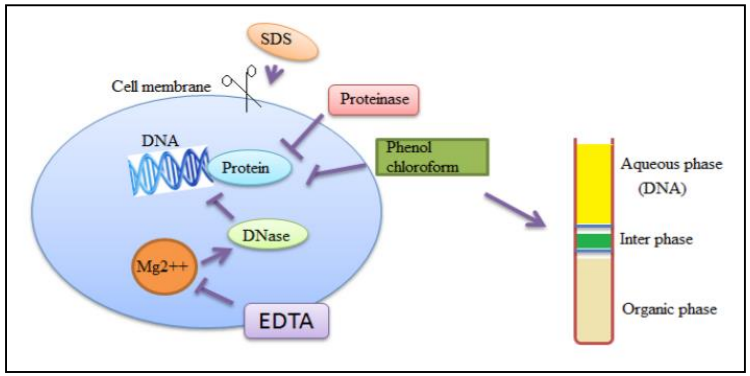
Standard to real-time PCR methods

Polymerase chain reaction(PCR)

Schematic diagram showing the principle of isolation of genomic DNA from bacteria

To isolate the genomic DNA from *E. coli* DH5α cells

- The genomic DNA isolation needs to separate total DNA from RNA, protein, lipid, etc. Initially the cell membranes must be disrupted in order to release the DNA in the extraction buffer.
- SDS (sodium dodecyl sulphate) is used to disrupt the cell membrane.
- DNA can be protected from endogenous nucleases by chelating Mg^{2++} ions using EDTA.
- Mg^{2++} ion is considered as a necessary cofactor for action of most of the nucleases.
- Nucleoprotein interactions are disrupted with SDS, phenol or proteinase K.
- Proteinase enzyme is used to degrade the proteins in the disrupted cell soup.



Polymerase chain reaction(PCR)
Introduction

- The polymerase chain reaction (PCR) is a technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA, generating thousands to millions of copies of a particular DNA sequence.
- Developed in 1983 by Kary Mullis, from America. Kary received a Nobel Prize in chemistry in 1993, for his invention of the polymerase chain reaction (PCR).
- PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications.

Polymerase chain reaction(PCR)
Function

- Portion of DNA amplified or copied during the polymerase chain reaction(logarithmic increase in target DNA)=amplicon or PCR product.
- PCR is based on the detection of intact DNA rather than intact viable cells and, therefore, a positive reaction may arise from either dead or live cells.

Polymerase chain reaction(PCR)

Standard, classical or traditional PCR methods

Direct-PCR and Bio-PCR

- **Standard-PCR:** Uses conventional *Taq* DNA polymerase or it's analogs to amplify targets from 100bp to 3kb long. After the completion of PCR, reaction products are analyzed by agarose gel electrophoresis and ethidium bromide staining.

1. **Direct-PCR** (Without enrichment and extracting DNA)**:**

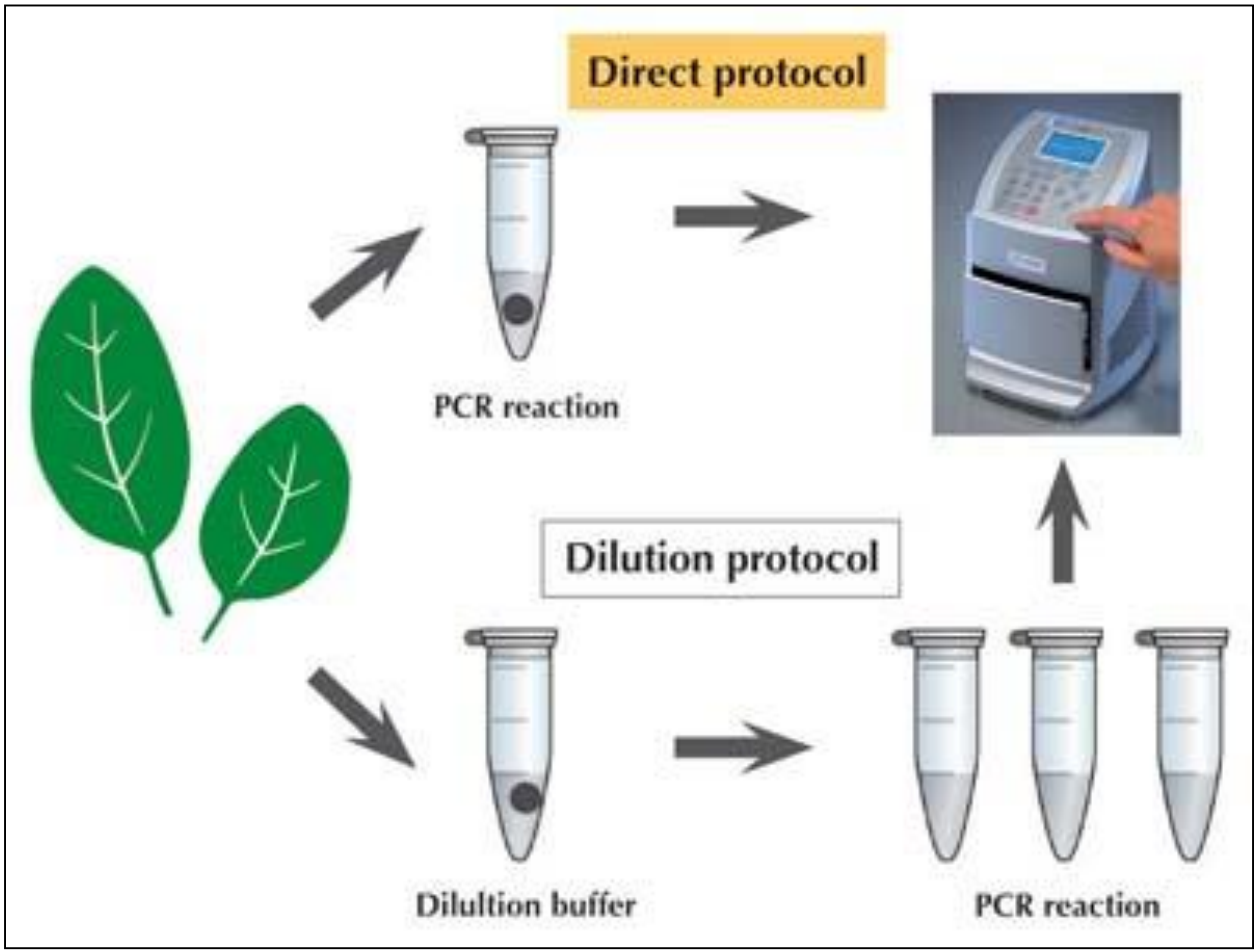
- Amplification of target DNA without any prior DNA purification step.
- A minimal amount of source material is applied directly in the PCR reaction without pretreatment or DNA isolation, resulting in a significant reduction of contamination risk, turnaround time and total costs.
- Detects whether the cell is dead or alive.

2. **Bio-PCR** (With enrichment and extracting DNA)**:**

- A refinement of the PCR method is known as bio-PCR detects living cells instead of dead cells.
- BIO-PCR involves plating samples onto agar media to enrich the target bacterium just prior to PCR.

See also direct real-time PCR for evolution of these traditional PCR methods

Polymerase chain reaction(PCR)
Direct PCR



Polymerase chain reaction(PCR)
Components and precautions

- Template DNA (genomic DNAs). Store working stocks of primers at -20°C.
- Primers (resuspended to a known concentration with sterile TE)
- Buffer (usually 10X) should always be kept at 4ºC.
- $MgCl_2$ (25mM is convenient)
- *Taq* polymerase (Taq DNA Polymerase 5 U/µl stored at -20°C).
- Deoxynucleotides(dNTPs) (2mM stock). A 2mM stock of dNTPs means that the final concentration of each dNTP (dATP, dCTP, dGTP, and dTTP) is 2mM -- NOT that all dNTPs together make 2mM. dNTPs come as 100mM stocks -- thaw and add 10µL of each dNTP to 460µL of ddH_20 to make 2mM. Store at -20°C.
- Sterile ddH_20
- Gloves
- PCR machine
- Aerosol tips, if desired.
- To reduce evaporation overlay the mix with a drop of mineral oil.
- PCR is very sensitive to contamination from outside DNAs. Steps should be taken to reduce the chance for contamination, such as wearing gloves, using aerosol tips (tips with a wad of cotton at the top), and not spitting in the tubes. I don't use aerosol tips and have dispensed with the gloves -- just be careful.
- Something that IS important is to assemble your reactions on ice.

Polymerase chain reaction(PCR)
Other components and precautions

- **An essential part of any PCR:**
- I can show you how to program it, or there's a manual.
- It takes 3-5 hours, depending on cycle conditions and thermolcycler. The thermalcycler has a four degree Celsius cycle at the end.
- This is basically a refrigerator. So if you see 4°C on the screen, it's a hold, and your PCR is done.
- Once the PCR is complete, the thermal cycler is set to 4°-10°C to maintain product integrity until such time as the tubes can be removed from the machine and stored at 4°C.
- It is perfectly acceptable to take their PCR out and put it in an agreed-upon spot in the refrigerator if you need the thermal cycler.
- If the tubes will left in the PCR machine overnight nothing will happen. Because the machine will remains at 4 degrees for many hours after completing the program.
- But it is sloppy and risky however, somebody may trip your samples, switch off the machine, etc.

Polymerase chain reaction(PCR)

Nucleoside triphosphates

- **Nucleoside triphosphates** are the building blocks of both DNA and RNA, which are chains of nucleotides made through the processes of DNA replication and transcription.
- For **DNA**, these are **dATP**, **dGTP**, **dCTP**, and **dTTP**.
- For **RNA**, these are **ATP**, **GTP**, **CTP**, and **UTP**.

	Nucleobase	Nucleosides	Nucleotides		
DNA	Adenine (A)	Deoxyadenosine	dAMP	dADP	dATP
	Guanine (G)	Deoxyguanosine	dGMP	dGDP	dGTP
	Cytosine (C)	Deoxycytidine	dCMP	dCDP	dCTP
	Thymine (T)	Deoxythymidine	dTMP	dTDP	dTTP
RNA	Adenine (A)	Adenosine	AMP	ADP	ATP
	Guanine (G)	Guanosine	GMP	GDP	GTP
	Cytosine (C)	Cytidine	CMP	CDP	CTP
	Uracil (U)	Uridine	UMP	UDP	UTP

Polymerase chain reaction(PCR)

Functions of the reagents

dNTPs

- The Deoxynucleoside triphosphates (dNTPs, sometimes called "deoxynucleotide triphosphates" are building blocks of new DNA strand.
- These are the four nucleotides(dATP, dCTP, dGTP, and dTTP), used by DNA polymerase to extend an annealed primer.
- Deoxynucleotides (dNTPs) may bind Mg^{2+} ions and thus affect the concentration of free magnesium ions in the reaction.

Nucleoside triphosphates are the building blocks of both DNA and RNA, which are chains of nucleotides made through the processes of DNA replication and transcription. For DNA, these are dATP, dGTP, dCTP, and dTTP. For RNA, these are ATP, GTP, CTP, and UTP.

Polymerase chain reaction(PCR)

Functions of the reagents

Taq polymerase

- Originally isolated from the hot-spring thermophylic bacterium *Thermus aquaticus*, but also available as an artificially synthesized product.
- **The Power of PCR:**

10 cycles	210	1,024
20 cycles	220	1,048,576
30 cycles	230	1,073,741,824

Taq polymerase (isolated from the bacterium *Thermus aquaticus*) often abbreviated to *Taq* Pol or simply *Taq*.

Polymerase chain reaction(PCR)

Functions of the reagents

dNTPs

- Standard PCRs contain equimolar amounts of all four dNTPs. Concentrations of 200-250μM of each dNTP recommended for Taq polymerase in reactions containing1.5 mM $MgCl_2$.
- High concentrations of dNTPs (>4mM) are inhibitory, perhaps because of sequestering of Mg^{2+}.
- However, a satisfactory amount of amplified product can be produced with dNTP concentrations as low as 20μM-0.5-1.0pM of an amplified fragment ~1 kb in length.
- Stocks of dNTPs should be stored at -20°C in small aliquots that should be discarded after the second cycle of freezing or thawing.

Polymerase chain reaction(PCR)

Functions of the reagents

$MgCl_2$

- $MgCl_2$: The polymerase requires a divalent cation (in this case Mg^{++} from the $MgCl_2$) in order to function. i.e. Mg^{++} increases Taq (DNA-dependent DNA polymerase) activity.
- The Mg^{++} also helps stabilize the two strands.
- A normal concentration of Mg^{++} in the reaction is 2.5μM.
- Higher concentrations yield greater promiscuity of the polymerase (i.e. false priming).
- Lower concentrations of Mg^{++} increase specificity of the target you're trying to amplify (i.e. more stringent and exact matching of bp's for priming and amplification to occur).
- So the trick is knowing how much (or how little) to use.

Polymerase chain reaction(PCR)

Functions of the reagents

The role of magnesium chloride

- Thermostable polymerase requires Mg^{++} as a cofactor to function during the PCR reaction.
- The Mg^{++} also helps stabilize the two strands.
- The more magnesium that is added to a PCR reaction, the quicker the reaction will proceed.

1. A normal concentration of Mg^{++} in the reaction is 2.5µM.
2. Higher concentrations yield greater promiscuity of the polymerase (i.e. false priming). i.e. DNA polymerase will work too quickly and often make errors in the copying process.
3. Lower concentrations of Mg^{++} increase specificity of the target you're trying to amplify. But it will not go as quickly as it should if at all. You may attempt to run a 40 cycle PCR but not get the amount of copies you intended.

PCR and plant pathogenic bacteria

PCR protocols

List of published PCR protocols

- Most of the available PCR protocols published between 1989 and 2007 for specific detection and identification of plant-pathogenic bacteria.
- **In this compilation, we found:**
- More than 50 protocols for species of the genus *Xanthomonas*,
- More than 40 for *Pseudomonas* spp.,
- 20 for *Ralstonia* spp.,
- 19 for *Clavibacter* and *Agrobacterium* spp.,
- 16 for *Erwinia* and *Xylella* spp.,
- 12 for *Pectobacterium* spp.,
- 11 for *"Candidatus* Liberibacter" spp.,
- 9 for *Burkholderia* spp.,
- 7 for *Streptomyces* and *Pantoea* spp.,
- 6 for *Dickeya* and *Xylophylus* spp.,
- 4 for *Leifsonia* spp.,
- 3 for *Acidovorax* spp., and
- Only one or two protocols for species of other genera.

Palacio-Bielsa *et al.*,2009

Programing PCR Reaction

PCR profile

In about 1 hour, 20 PCR cycles can amplify the target by a millionfold. But usually PCR reactions take 2-2.5 h. In general, 1 Kb of DNA takes 1 minute to amplify

- **Thermalcycler:**
- An essential part of any PCR. I can show you how to program it, or there's a manual. Just turn it on, wait a few seconds for the screen with the file to come up, press "file", type in your file name, press "enter", and "start", load your tubes, and wait.
- It takes 3-5 hours, depending on cycle conditions and thermalcycler.
- The thermalcycler has a four degree celsius cycle at the end. This is basically a refrigerator. So if you see 4°C on the screen, it's a hold, and your PCR is done.
- And, by intuition, if someone else's PCR is at 4°C, it is perfectly acceptable to take their PCR out and put it in an agreed-upon spot in the refrigerator if you need the thermalcycler.

PCR and plant pathogenic bacteria
Master mix preparation

- The Master mix buffer is often stored as a 10X stock solution (100 mM Tris-HCL, pH 8.3, 500 mM KCL, 1.5 mM $MgCl_2$) which is diluted to 1X for use.
- Both the Master mix buffer and the purified water can be stored at room temperature.
- Store deoxynucleotides, primers and Taq DNA polymerase enzyme at -20°C.
- Although 100 µl of master mix per reaction is generally used, it is possible to use as little as 25 or 50µl to save on cost of reagents.

PCR and plant pathogenic bacteria

Master mix preparation

The Master mix reagents include:

Final Purpose Conc.	Component	Purpose
	Water	
1 X	Buffer	keeps the master mix at the proper pH so the PCR reaction will take place.
200 µM	Deoxynucleotides	Provide both the energy and nucleosides for the cleotides synthesis of DNA. It is important to add equal amounts of each nucleotide (dATP, dTTP, dCTP, dGTP) to the master mix to prevent mismatches of bases.
0.2-1.0 µM	Primers	Short pieces of DNA (20-30 bases) that bind to the DNA template allowing Taq DNA polymerase enzyme to initiate incorporation of the deoxynucleotides. Both specific and universal primers can be used.
2.5U/100 µl	AmpliTaq polymerase	A heat stable enzyme that adds the polymerase deoxynucleotides to the DNA template.
0.05-1.0 µg	Template DNA	The which will be amplified by the PCR DNA

PCR and plant pathogenic bacteria
Master mix preparation

- Master mix reagents can be obtained from a variety of companies.
- Often the initial concentration of the reagent will differ depending on which company produced it.
- It is easy to figure out how much stock reagent to use by following a simple formula:

(initial concentration) X (volume needed) = (final concentration) X (volume of sample)

PCR and plant pathogenic bacteria
Master mix preparation

- For example: I have 10X buffer, 10 mM (10mM=10,000 µM) of each nucleotide, 0.5 mM primers and Taq DNA polymerase at 5 Units/µl.
- I want to make one 50 µl reaction.
- Calculations are as follows:

1. 10X buffer: (10X) X (5 µl) = (1X) X (50 µl mix reaction);
2. Nucleotides: (10,000 µM) X (1 µl) = (200 µM) X (50 µl mix reaction);
3. Primers: (500 µM) X (0.1 µl)= (1.0 µM) X (50 µl mix reaction).

- Since it is impossible to pipet 0.1 µl accurately, a dilution needs to be made first. Add 10 µl of stock primer solution to 990 µl of water to get 5 µM concentration of primers.

(initial concentration) X (volume needed) = (final concentration) X (volume of sample)

PCR and plant pathogenic bacteria
Master mix preparation

1. To make the master mix for one reaction of 50 µl add:
- 5 µl 10X buffer
- 4 µl each nucleotide (1 µl each of dATP, dCTP, dGTP, dTTP)
- 20 µl Each primer (10 µl of each)
- 1 µl Taq DNA polymerase (Total volume = 30 µl)
- Add 15 µl of water
- 5 µl of DNA template (Total volume = 50 µl)

2. If want to make 3 reactions, 3 X 50 µl = 150 µl.
- Use this number in the formula for "volume of sample."

PCR and plant pathogenic bacteria
Master mix preparation

- Thaw the PCR Master Mix at room temperature.
- Vortex the Master Mix and then spin it briefly in a microcentrifuge to collect the material in the bottom of the tube.
- Prepare one of the following reaction mixes (25µl, 50µl or 100µl reaction volume) on ice:
- For a 25µl reaction volume:

Component	Volume	Final Conc.
PCR Master Mix,2X	12.5µl	1X
upstream primer,10µM	0.25-2.5µl	0.1-1.0µM
downstream primer,10µM	0.25-2.5µl	0.1-1.0µM
DNA template	1-5µl	<250ng
Nuclease-Free Water to	25µl	N.A.

www.promega.com

PCR and plant pathogenic bacteria

Master mix preparation

Seven PCR enhancers: formamide, dimethyl sulfoxide (DMSO), glycerol, ethylene glycol, trehalose, BSA and Tween-20

- The optimization of the PCR protocol involved a gradient of annealing temperatures, as well as the application of seven PCR enhancers:
- formamide,
- dimethyl sulfoxide (DMSO),
- glycerol,
- ethylene glycol,
- trehalose,
- BSA, and
- Tween-20.

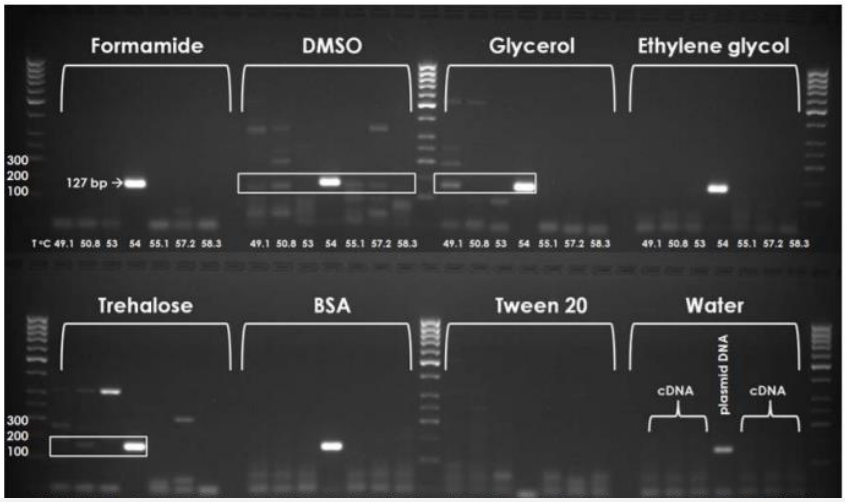
Simonovic *et al.*,2012

PCR and plant pathogenic bacteria

Master mix preparation

Seven PCR enhancers: formamide, dimethyl sulfoxide (DMSO), glycerol, ethylene glycol, trehalose, BSA and Tween-20

- PCR optimization of AtCKX1 gene amplification by gradient of annealing temperatures and seven different additives analyzed on a single gel for comparison.
- Either 100 ng of total cDNA or 2.5 ng of digested plasmid DNA were amplified in 25 µl-PCR reactions, of which 12.5 µl were loaded per lane.



Each enhancer was applied in one concentration, based on available literature data (Table 1), as follows: formamide 2% (final), DMSO 5%, glycerol 5%, ethylene glycol 1 M, trehalose 0.2 M, BSA 0.2 $\mu g \mu l^{-1}$ and Tween-20 0.5%.

Simonovic *et al.*,2012

PCR and plant pathogenic bacteria

Master mix preparation

Seven PCR enhancers: formamide, dimethyl sulfoxide (DMSO), glycerol, ethylene glycol, trehalose, BSA and Tween-20

- The 8 genes such as 16S rRNA, rpoB, gyrB, etc. were amplified under the same PCR conditions.
- Briefly, each PCR mixture contained 2 µL of extracted DNA (10 ng/µL), 1 µL of $MgCl_2$ (50 mM), 0.6 µL of dNTP (10 mM), 0.6 µL of each primer (10 ng/µL), 3 µL Bovine Serum Albumine (10 µg/µL) (BioLabs), 0.1 µL of Taq polymerase (SilverStar DNA polymerase, Eurogentec), 3 µL of PCR buffer (10X) and 19.7 µL of SDW in a total volume of 30 µL.

Abdeljalil *et al.*,2016

PCR and plant pathogenic bacteria

Master mix preparation

Seven PCR enhancers: formamide, dimethyl sulfoxide (DMSO), glycerol, ethylene glycol, trehalose, BSA and Tween-20

- What are the functions of DMS or bovine serum albumin(BSA) in PCR products of no amplification?

1. BSA like DMSO is recommended for difficult template (GC-rich or containing secondary structures) to improve its availibility for hybridization.
2. Prevent the loss of enzyme such as adhesion of enzymes to the reaction tubes and tip surfaces.
3. Used for the stabilization of salt concentration, pH or temperature so that the enzymes are not affected during DNA digestion.

https://www.researchgate.net

PCR and plant pathogenic bacteria

Master mix preparation

Functions of bovine serum albumin(BSA) and dimethyl sulfoxide(DMSO)

1. DMSO and BSA are adjuvants that make the DNA more accessible to the enzyme for amplification.
2. BSA significantly enhances PCR amplification yield when used in combination with organic solvents, DMSO or formamide.
3. BSA enhancing effects were obtained in several PCR applications, with DNA templates of high GC content and spanning a broad size range. When added to the reaction buffer, promoting effects of BSA were seen in the first cycles of the PCR, regardless of the size of the DNA to amplify.

https://www.researchgate.net

PCR and plant pathogenic bacteria
Primers (RNA or DNA)

- Primers (RNA or DNA), which are short, single-stranded DNA sequence generally about 20 nucleotides long, made in a laboratory.
- **RNA Primers:**
- The RNA primer is a short stretch of nucleic acid made up of the single-stranded RNA molecule.
- The RNA primers can not work efficiently because it is less stable than the DNA primers.

PCR and plant pathogenic bacteria
Primers (RNA or DNA)

- Primers (RNA or DNA), which are short, single-stranded DNA sequence generally about 20 nucleotides long, made in a laboratory.
- **DNA Primers:**
- The artificially synthesized DNA primers are used for the DNA amplification during the PCR reaction.
- It is a single-stranded molecule of DNA ranging from 12 nucleotides to 25 nucleotides.
- Notably, a pair of DNA primer, one for sense strand DNA called forward primer and one for antisense strand of DNA called reverse primer, is used for amplification of dsDNA.

PCR and plant pathogenic bacteria
Primers

- Different strategies have been developed to design PCR primers for specific detection.
- The DNA sequences from which the primers are designed for bacteria come from three main origins:

1. Pathogenicity/virulence genes,
2. Ribosomal genes, and
3. Plasmid genes.

López *et al.*,2011

PCR and plant pathogenic bacteria

Primers and target sequences utilized for primer design

Pathogenicity/virulence genes

- Multiple strategies being developed to design primers.
- e.g. sequences from pathogenicity-related genes which are specific to a pathogen, or to a group of pathogens:

1. The *pel* gene of soft-rot diseases caused by pectolytic species or subspecies of the genus *Pectobacterium*, or
2. A cluster of genes involved in the virulence systems of different bacterial families (*hrp*, *pth* and *vir* genes).

Palacio-Bielsa *et al.*,2009

PCR and plant pathogenic bacteria

Primers

Universal or specific primers

- A primer is a short segment of nucleotides which is complementary to a section of the DNA which is to be amplified in the PCR reaction.
- Primers are annealed to the denatured DNA template to provide an initiation site for the elongation of the new DNA molecule.
- Primers can either be:

1. Specific to a particular DNA nucleotide sequence, or
2. They can be "universal."

PCR and plant pathogenic bacteria

Primers

Universal or specific primers

1. Universal primers are complementary to nucleotide sequences which are very common in a particular set of DNA molecules. Thus, they are able to bind to a wide variety of DNA templates.
2. Bacterial ribosomal DNA genes contain nucleotide sequences that are common to all bacteria.

- Thus, bacterial universal primers can be made by creating primers which are complementary to these sequences.
- Examples of bacteria universal primer sequences are:
  Forward 5' GAT CCT GGC TCA GGA TGA AC 3' (20 mer)
  Reverse 5' GGA CTA CCA GGG TAT CTA ATC 3' (21 mer)

PCR and plant pathogenic bacteria
Characteristics of an ideal primer

- For broad-spectrum studies, primers of typically 18-30 nucleotides in length are the best.
- The optimal melting temperatures for primers in the range 52-58°C, generally produce better results than primers with lower melting temperatures (Kamel *et al.*,2003).
- Percentage of G and C content is an important characteristic of DNA and provides information about the strength of annealing.
- Primers should have GC content between 45 and 60 percent (Dieffenbach *et al.*,1995).

Wan Leng,2005

PCR and plant pathogenic bacteria
Characteristics of an ideal primer

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Wan Leng,2005

PCR and plant pathogenic bacteria
Programs for designing the primers

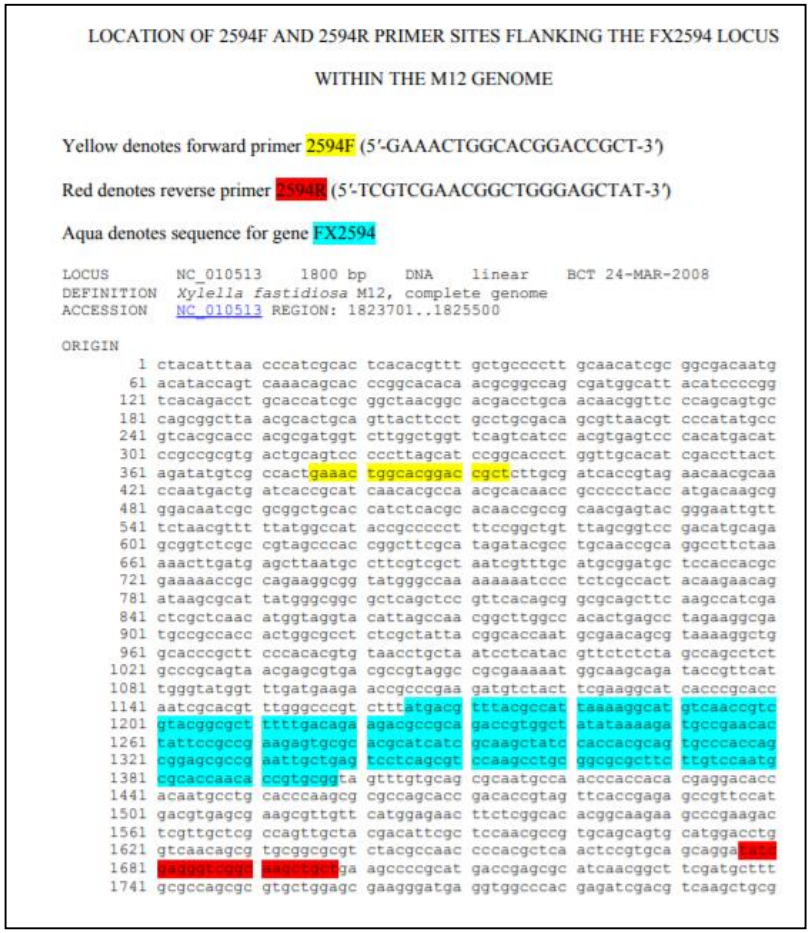
- However, the use of software in biological applications has given a new dimension to the field of bioinformatics.
- Many different programs for the design of primers are now available (Singh and Kumar,2001; Kamel *et al.*,2003).
- There are such as:

1. Gene Runner version 3.05,
2. Gene Fisher,
3. Primer3,
4. Primer Selection,
5. PCR Primer Design, 'Oligo' (National Biosciences, Inc, Plymouth MN), and so on.

Wan Leng,2005

Program for designing the primer pair of 2594F and 2594R for *Xylella fastidiosa*

OLIGO Primer Analysis Software was used to facilitate the design of 2594F and 2594R, which flank FX2594 in the M12 genome.



LOCATION OF 2594F AND 2594R PRIMER SITES FLANKING THE FX2594 LOCUS WITHIN THE M12 GENOME

Yellow denotes forward primer 2594F (5'-GAAACTGGCACGGACCGCT-3')

Red denotes reverse primer 2594R (5'-TCGTCGAACGGCTGGGAGCTAT-3')

Aqua denotes sequence for gene FX2594

LOCUS NC_010513 1800 bp DNA linear BCT 24-MAR-2008
DEFINITION *Xylella fastidiosa* M12, complete genome
ACCESSION NC_010513 REGION: 1823701..1825500

ORIGIN

```
   1 ctacatttaa cccatcgcac tcacacgttt gctgcccctt gcaacatcgc ggcgacaatg
  61 acataccagt caaacagcac ccggcacaca acgcggccag cgatggcatt acatccccgg
 121 tcacagacct gcaccatcgc ggctaacggc acgacctgca acaacggttc ccagcagtgc
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1741 gcgccagcgc gtgctggagc gaagggatga ggtggcccac gagatcgacg tcaagctgcg
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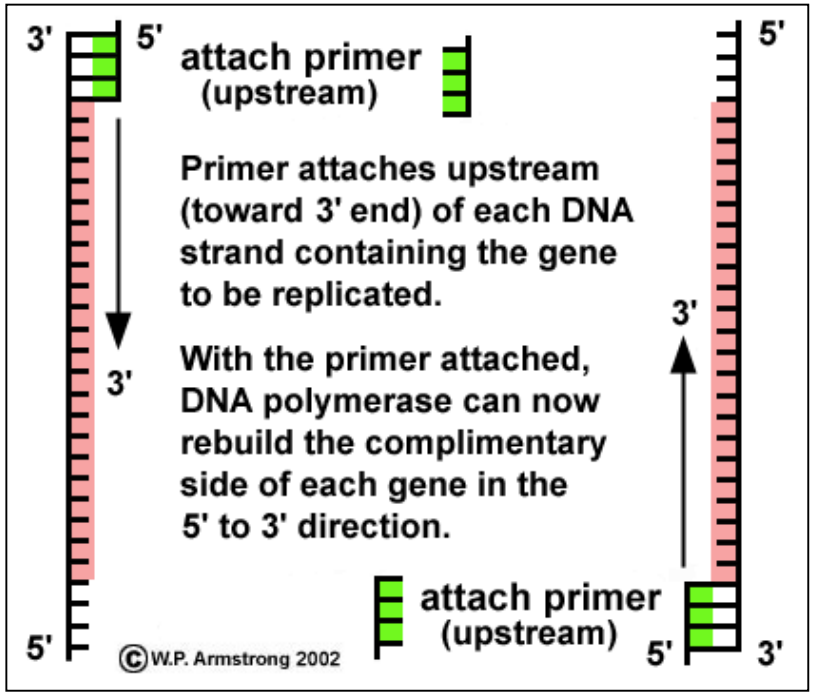
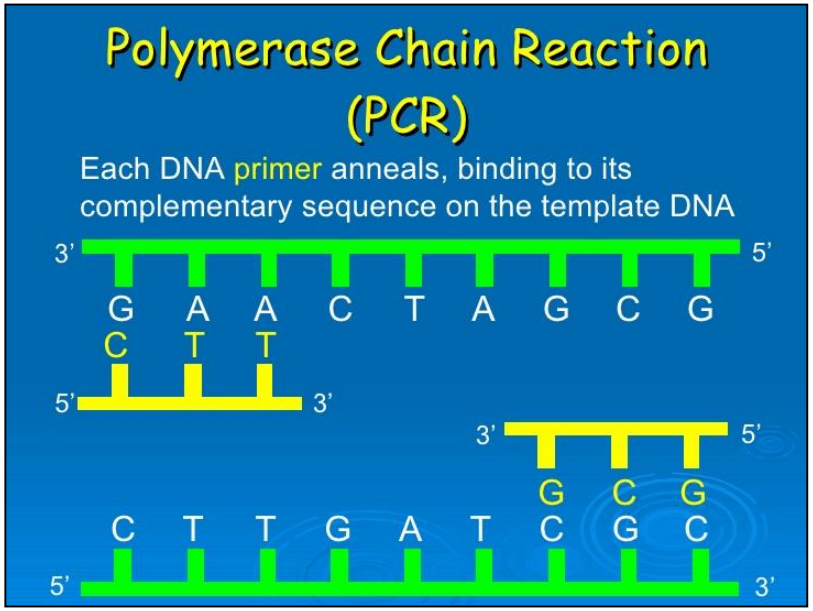
Nissen *et al.*,2010

Programing PCR Reaction

PCR profile

Denaturing, annealing and extending

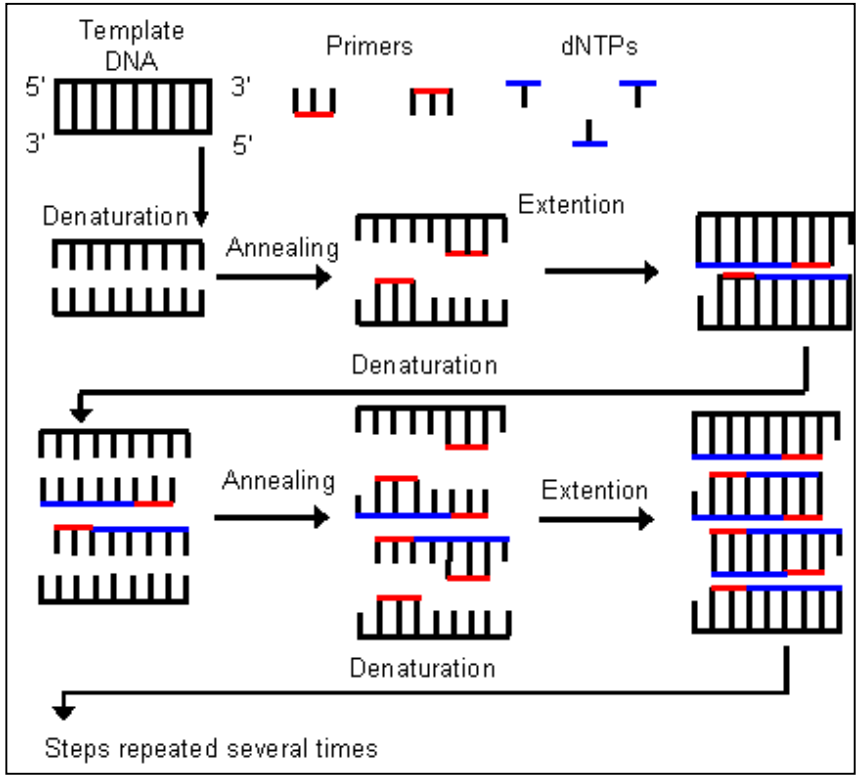


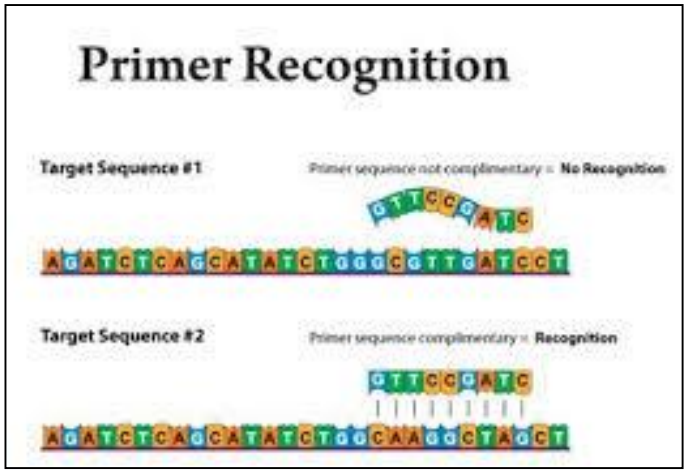


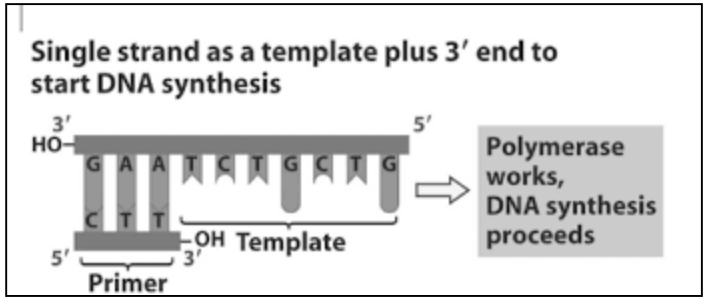
Programing PCR Reaction

PCR profile

Denaturing, annealing and extending



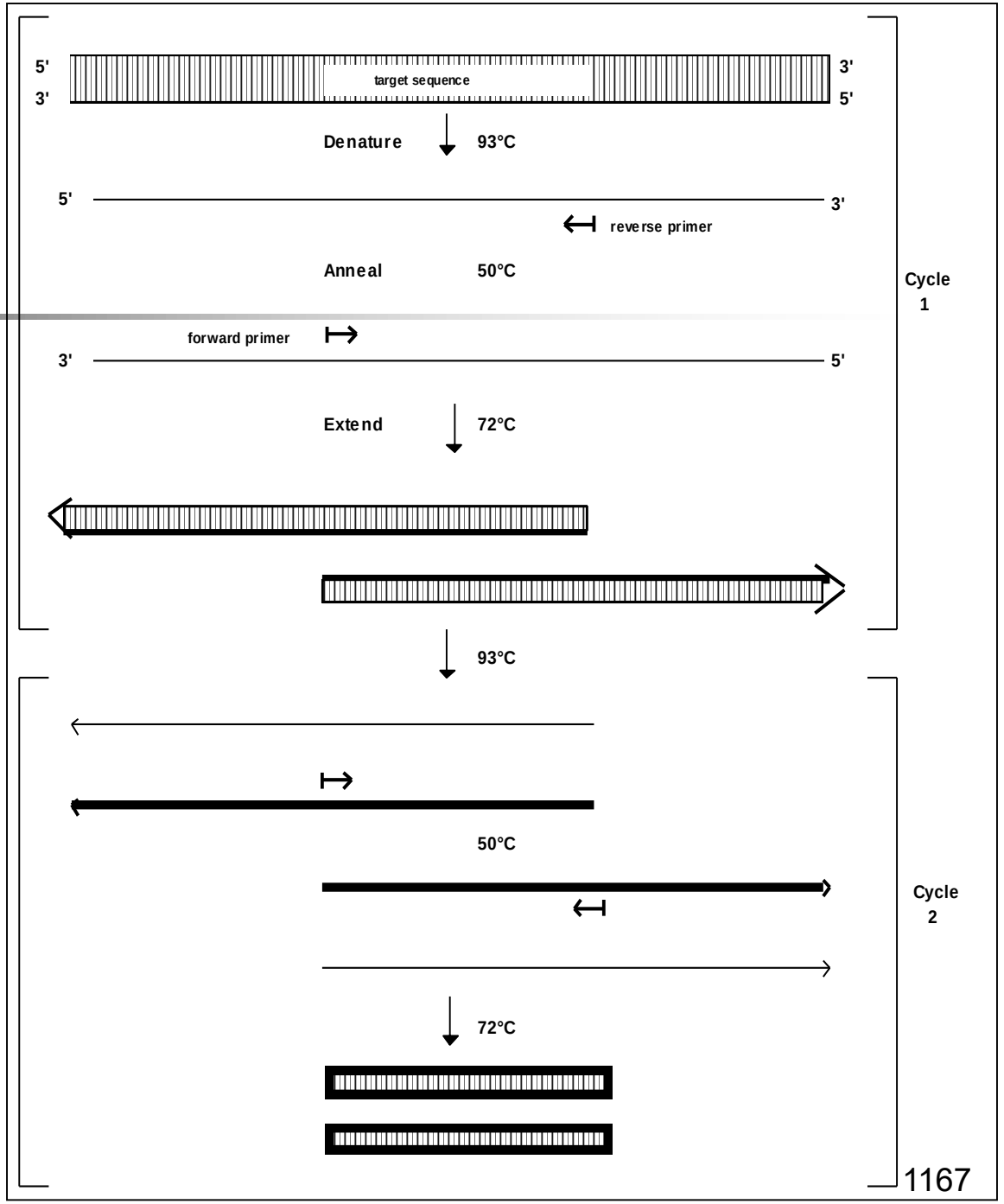




Programing PCR Reaction

PCR profile

In about 1 hour, 20 PCR cycles can amplify the target by a millionfold. In general, 1 Kb of DNA takes 1 minute to amplify. Usually PCR reactions take 2.5-3 h.



Programing PCR Reaction

PCR profile

In about 1 hour, 20 PCR cycles can amplify the target by a millionfold. But usually PCR reactions take 2-2.5 h. In general, 1 Kb of DNA takes 1 minute to amplify

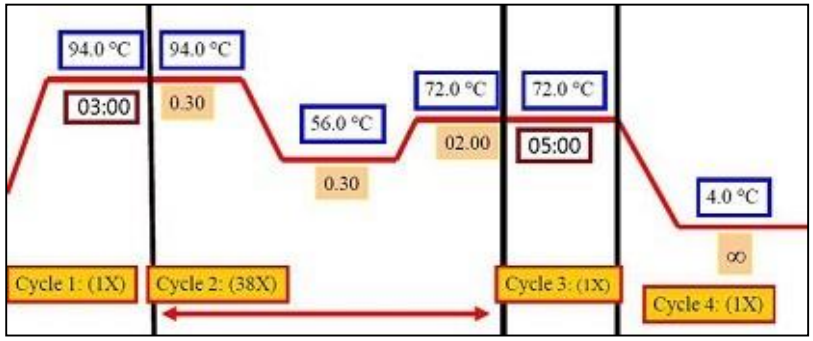
- **Three Typical Temperature Profiles:**
- 40 cycles of:

1. Denaturation at high temperature (92-94°C for 30-60 sec);
2. 93°C / 30 sec (DNA denaturation);
3. 50°C / 1 min (primer annealing);
4. 72°C / 2 min (DNA extension) then 4°C final holding temp.

- The amplification process takes 3-4 hours.

Example of thermocycler parameters used to amplify a particular gene of interest

Cycle step	Temperature	Time	Cycles
Initial denaturation	95°C	30 seconds	1
Denaturation	95°C	5-10 seconds	30 cycles
Annealing	*	10-30 seconds	
Extension	72°C	1 minute	
Final extension	72°C	5 minutes	1
	4°C	hold	



Programing PCR Reaction

PCR profile

PCR relies on three principles of molecular biology

- The process of synthesis of new stand of DNA involved three steps:

1. Denaturation at high temperature (92-94°C for 30-60 sec);
2. Annealing at low temperature (37-72°C for 30-60 sec) to attach specific primers to the single DNA complementary sequence.
3. Extension of nucleic acid strands at intermediate temperature (72°C for 30-60 sec) in the presence of free nucleotides and a *Taq* polymerase). The optimal temperature for DNA polymerase to act. DNA polymerase extends the primers, adding nucleotides onto the primer in a sequential manner, using the target DNA as a template.

These steps are repeated 20-35 times. In PCR, amplification is exponential because for each cycle, the DNA made in the previous cycles can also serve as template.

Programing PCR Reaction

PCR profile

PCR relies on three principles of molecular biology

- Taq polymerase has its optimum activity temperature at 75-80°C, and commonly a temperature of 72°C is used with this enzyme.
- DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction.
- The elongation temperature depends on the DNA polymerase.
- The time for this step depends both on the DNA-polymerase itself and on the length of the DNA fragment to be amplified.
- As a rule-of-thumb we use 1 minute per 1000 bp.

Taq DNA polymerase is a thermostable DNA polymerase that possesses a 5´→ 3´ polymerase activity.

Programing PCR Reaction

PCR profile

In about 1 hour, 20 PCR cycles can amplify the target by a millionfold. But usually PCR reactions take 2-2.5 h. In general, 1 Kb of DNA takes 1 minute to amplify

- During PCR, the sample is heated to 90°C (for 30 sec) to denature the DNA (separate the 2 strands). Then the sample is cooled down to 50°C (for 1 min) to allow the 2 primers to anneal to each DNA strand. Finally, the sample is heated to 72°C (for 2 min), which is the optimal temperature for *Taq* polymerase to add on nucleotides after each primer. This cycle of denaturing, annealing and extending is repeated 30-40 times.
- For each cycle, the targeted gene is doubled in number.
- **1** --> 2 --> 4 --> 8 --> 16 --> 32 --> 64 --> 128 --> 256 --> 512 --> 1024 --> 2048 --> 4096 --> 8192 --> 16,384 --> 32,768 --> 65,536 --> 131,072 --> 262,144 --> 524,288 --> **1,048,576**
- As shown above, after 20 cycles, over a million copies of a gene from one molecule of DNA is generated.

Programing PCR Reaction
The annealing temperature

- The annealing temperature chosen for a PCR depends directly on:

1. Length and
2. Composition of the primer(s).

- One should aim at using an annealing temperature (T_a) about 5°C below the lowest T_m of the pair of primers to be used (Innis *et al.*,1998).
- According to Blaber (1998), the annealing temperature is unique for each primer, it is a function of the length and base composition of the primer as well as the ionic strength of the reaction buffer.
- A simple formula for calculation of the T_m is:
- $T_m = 4(G + C) + 2(A + T)^o C$

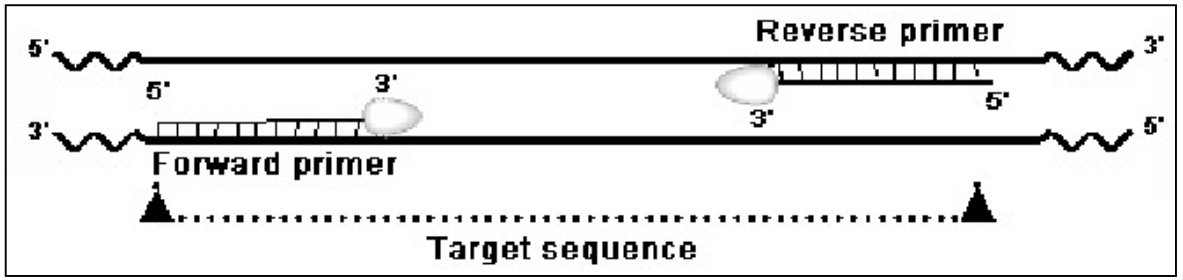
Wan Leng,2005

Programing PCR Reaction

Primers and their direction in PCR

Annealing

1. DNA synthesis is always from
2. Primers are always specified 5' to 3', left to right.
3. The primers anneal on opposite strands of the PCR template(3' to 5').
4. DNA polymerases will extend only from the 3' end of the primer, not the 5' end, and that when a primer is base-paired with the template, the primer and template strands are antiparallel.



Programing PCR Reaction

Primers and their direction in PCR

Annealing

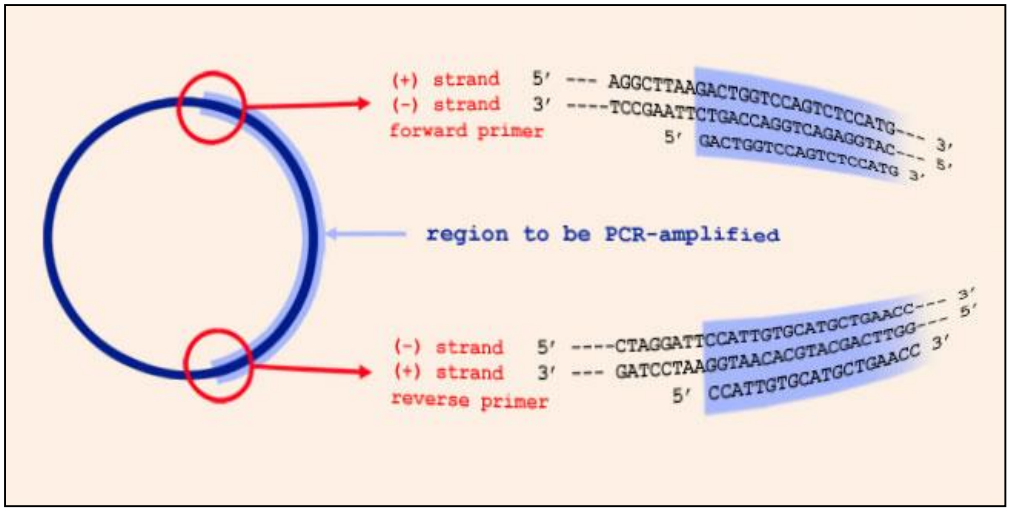
- The 3' end of the primer should be an exact match to the template DNA, because extension by DNA polymerase, during PCR, depends on a good match at the 3' end.
- The 3' ends of the two primers can base pair with each other.
- Thus, primers are often designed with the 3' end of the primer as a G or C.
- G-C base pairs have a stronger bond than A-T base pairs.
- Conversely, 5' tails can be readily added to primers without impacting primer annealing.

Programing PCR Reaction

Primers and their direction in PCR

Annealing

- In this picture, the forward primer anneals to the template (-) strand, and is identical to (a part of) the template (+) strand. And the reverse primer anneals to the template (+) strand, and is identical to (a part of) the template (-) strand.



SCL resources

Programing PCR Reaction

PCR profile

Annealing

- The polymerase attaches to the primer and attaches base pairs in a 3’ to 5’ direction. i.e. DNA polymerase extends each primer in the 5' to 3' direction.
- The terms 3’ and 5’ are in reference to the carbons of the sugar molecule in the sugar-phosphate backbone structure of the DNA strand.
- The mixture can be reheated to continue the cycle as long as there are enough primers and nucleotides to continue synthesizing new strands.

PCR amplification

Colony PCR

1. PCR without DNA extraction

- The colony PCR protocol is straightforward: simply add a small amount of a colony from a plate or dense bacterial liquid culture to the PCR master mix and proceed to thermocycling.
- Cell lysis occurs during the initial high-temperature incubation.
- The resulting PCR products can be analyzed with gel electrophoresis or other DNA detection methods.
- This time-saving method is perfect for screening just a few colonies or for high throughput screenings of many clones.

Merck,2023

PCR amplification

Colony PCR

PCR without DNA extraction

- Prepare PCR master mix (scaled based on the number of samples to analyze)
- Dispense PCR master mix (20 µL) into each PCR tube or plate.
- Using a sterile micropipette tip or a sterile toothpick transfer cells from each colony to a PCR tube and briefly stir to resuspend them in the PCR master mix.
- The mix may look slightly cloudy. Note: Don't pick too many cells. Overloading cells will interfere with the PCR.
- Amplify target DNA with the following thermocycling conditions:
- **Note**:
- The cell lysis step is longer than a typical initial denaturation to efficiently break the bacterial wall.

Merck,2023

PCR amplification

Colony PCR

PCR without DNA extraction

Step	Temperature	Time	Cycles
Cell lysis	94 °C	5 to 10 minutes	1
Denaturation	94 °C	0.5 minutes	30-40
Annealing	45 to 68 °C	0.5 minutes	
Extension	72 °C	1-2 minutes (~ 1 kb/min)	
Hold	4 °C	Indefinitely	

Merck,2023

PCR amplification
2. PCR with DNA extraction

- Direct amplification is not always successful because of the presence of phenolic compounds that inhibit the PCR reaction and other components that bind to the DNA after cell lysis.
- The application of DNA extraction procedures can resolve these problems.
- Numerous and complex reagents were used in DNA extraction procedures such as:

1. Boiling lysis method;
2. Digestion with proteinase enzymes, etc.
3. Phenol-chloroform;
4. CTAB.

Lolp *et al.*,1999

PCR amplification

DNA quantity of each method

DNA extraction methods

- Species and mean yield of DNA quantity (ng/μL) of each method.

Specie	No. of isolates (%)	Extraction method			
		Boiling	Phenol-chloroform	Extraction column	Salting out
S. epidermidis	107 (40.1)	1173.3	67.3	100.1	30.1
S. haemolyticus	91 (34.2)	1181.8	202.1	35.1	16.4
S. hominis	27 (10.1)	989.4	61.6	34.5	14.0
S. warneri	15 (5.6)	1002.2	NT	12.9	86.9
S. capitis	7 (2.6)	1016.5	89.2	15	21.1
S. saprophyticus	6 (2.2)	1052*	50.6	54.3	NT
S. cohnii	4 (1.5)	825*	28*	41.7	18.9*
S. caprae	3 (1.1)	NT	40*	28.8	NT
S. xylosus	3 (1.1)	NT	23	31.6	NT
S. sciuri	2 (0.7)	NT	NT	86.5	NT
S. lugdunensis	1 (0.4)	NT	NT	32*	NT
S. auricularis	1 (0.4)	NT	NT	41*	NT
Total	267 (100.0)	1018.2	87.8	65.5	28.2

Oliveira *et al.*,2014

Temperature

Temperature difference between the nucleus and the cytoplasm in mammalian cell

Nucleus temperature

- Temperature is a fundamental physical parameter related to many cellular functions, including gene expression, protein stabilization, enzyme-ligand interactions and enzyme activity.
- FLIM(fluorescence lifetime imaging microscopy) analysis confirmed:

1. a temperature difference between the nucleus and the cytoplasm, and
2. heat production near the mitochondria.

Hayashi *et al.*,2015

Temperature

Temperature difference between the nucleus and the cytoplasm in mammalian cell

Nucleus temperature

- The temperature difference between the nucleus and the cytoplasm changed depending on the cell cycle status, suggesting a correlation between cellular function and temperature.

1. The temperature in the nucleus was approximately 1°C higher than that in the cytosol, and
2. the temperature near the mitochondria was higher than that of the rest of the cytoplasm.

- Novel cellular thermometers are needed to evaluate the temperature distribution inside of a cell in the near future.

Hayashi *et al.*,2015

DNA extraction
Commercial Kits

- Bacterial DNA Extraction Kits provides a simple method for extracting DNA from Gram-positive and Gram-negative bacteria.
- As the demand for molecular tests increases, new automated methods of DNA extraction will have to be developed to handle larger numbers of samples.
- There are a number of DNA extraction kits available.
- DNA extractions were performed according to the manufacturer's instruction.
- All expected DNA were transferred into a labeled 1.5ml sterile eppendorf tube and stored at -20°C until use.
- Any unused DNA can be stored in the freezer usually at -20°C for future use.

DNA extraction

DNA preservation

Storage condition of extracted DNA

- As a general rule isolated DNA can be stored:

1. at 4°C for several weeks,
2. at -20°C for several months, and
3. at -80°C for several years.

- The most common method of storage of DNA is as a suspension in ethanol at -80°C.
- DNA stored in buffer is much less stable if denatured prior to storage i.e. single stranded is less stable.
- An alternative is to add an antioxidant/scavenger such as 1% ethanol.

DNA extraction

DNA preservation

Storage condition of extracted DNA

- Little is known about the effects of freezing long term and thawing on the DNA.
- The presence of sugars may protect the DNA by forming a glass structure. Therefor, the addition of sugars such as trehalose may aid preservation.
- Storage vessel is also a matter of note. Plastic microtubes have gained bad press in the past for spoiling or binding samples. DNA is charged and hydrophilic. Polypropylene is hydrophobic which should preclude any binding.

DNA extraction
Functions of different reagents

- Proteins, lipids, carbohydrates, and cell debris are removed with the organic mixture of phenol and chloroform.
- Phenol and chloroform are both protein removers.
- Chloroform and/or phenol to denature and separate the proteins from the DNA.
- Although phenol can denature proteins rapidly, it does not completely inhibit RNAse activity.
- This problem can be solved by using a mixture of phenol: chloroform: isoamyl alcohol (25:24:1).
- Isoamyl alcohol was added to chloroform to reduce foaming and stabilizes interphase between the aqueous and organic phase(proteins).

DNA extraction
Functions of different reagents

- CTAB being a cationic detergent while SDS is anionic. CTAB will help you to remove polysaccharides which are bind to DNA. This detergent simultaneously solubilizes the plant cell wall and lipid membranes of internal organelles and denatures proteins (enzymes and thus provides great advantage of to obtain good quality of DNA.
- Ethanol or isopropanol:
- The DNA will be precipitated by adding ethanol or isopropanol in 2:1 or 1:1 ratios. The isopropanol should be kept at 0˚C.
- Ammonium acetate use to precipitate DNA from water, or
- Sodium acetate (0.3M final conc, pH 5.2) which was also used for routine DNA precipitations.
- Tris buffer: resist changes in pH.

DNA extraction

Functions of different reagents

- Liquid nitrogen:
- Liquid Nitrogen freezes the tissue rapidly and allows fine grinding in mortar and pestle.
- It freezes the cell, easy to break cell wall, finer powder produced.
- It has an important role in obtaining good quality DNA.
- It also keeps harmful chemicals and natural enzymes (e.g. nucleases) deactivated due to its very low temperature.

DNA extraction
Functions of the reagents

- SDS (sodium dodecyl sulfate) is an anionic detergent which solubilizes the phospholipids and proteins of the cell membrane and release DNA.
- NaOH helps to break down the cell wall.
- EDTA (ethylene diamine tetraacetic acid is a chelating agent that binds metal cations such as magnesium, thereby preventing DNA degradation. EDTA will not be soluble until pH reaches 8.0.
- Note use of too much EDTA during DNA isolation (which alters Mg) may interfere with any PCR reaction you choose to do with the DNA.
- PVP (Polyvinyl pyrrolidone) included in the CTAB buffer to reduce polyphenolic compounds.
- Salt (NaCl) is for osmotic balance. NaCl also helps in solidifying and making DNA visible.

DNA extraction
Functions of the reagents

- RNase: RNAse is usually prepared by boiling at 100°C for 10 min because is quite thermoestable, whilst the DNAse gets quickly denatured.
- In RNAse-treat samples, the RNA degrades into nucleotides or small oligos, and depending on how you precipitate afterwards you might not recover that degraded RNA, resulting in a lower nucleic acid concentration in comparison with the non-RNAse treated sample.
- RNA contamination problems will be solved by 1) heating it at least 90 degrees for 5 minutes, and 2) making the DNase treatment as late as possible in the procedure.

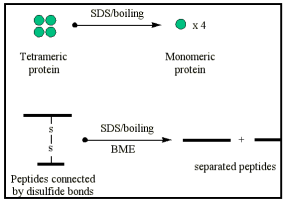
DNA extraction
Functions of the reagents

- Ethylenediaminetetraacetic acid (EDTA) is mainly used to sequester metal ions in aqueous solution.
- It is a powerful chelating agent causes dispersion of LPS molecules in Gram-negative bacteria. In molecular biology assays it was used to deactivate metal-dependent enzymes to suppress damage to DNA or proteins.
- To prepare EDTA at 0.5 M (pH 8.0):
- Add 186.1 g of disodium EDTA.$2H_2O$ to 800 ml of H_2O.
- Stir vigorously on a magnetic stirrer.
- Adjust the pH to 8.0 with NaOH (approx. 20 g of NaOH pellets).
- Dispense into aliquots and sterilize by autoclaving.
- The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approx. 8.0 by the addition of NaOH.

DNA extraction

Function of hot and cold water

- Excessive boiling can only denature DNA. Normally for DNA isolation we heat from 60-70 degree Celsius where it may not degrade the DNA.
- Cold water helps keep the DNA intact during the extraction process. How? Cooling slows down enzymatic reactions. This protects DNA from enzymes that can destroy it.



DNA extraction

1. Boiling lysis method(Method 1)

- Bacterial DNA was extracted by simple boiling method modified after Sambrook *et al.*,1989.
- Bacteria were grown in agar media at 28°C for 2 days.
- A loop full of cells was suspended in 25 µl of sterile distillated water and followed an addition of 25 µl of freshly prepared lysis buffer containing 0.1 N NaOH and 0.5% SDS. Store at room temperature. Sterilization is not necessary. Do not autoclave.
- The mixture was boiled in a water bath for 15 min.
- 200µl of TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) was added to the mixture which was then subjected to centrifugation for 15 min at 13,000 rpm.
- The supernatant formed by the aqueous phase that contains clear and suspended DNA was transferred to new sterile Eppendorf tube and stored at 4°C.

Sambrook *et al.*,1989;Elboutahiri *et al.*,2009

DNA extraction

1. Boiling lysis method(Modified method 1)

1. Pipet 500 µl of overnight culture of your unknown bacteria into a 1.5 ml microcentrifuge tube.
2. Centrifuge at 13,000 rpm for 1 minute.
3. Remove the liquid supernatant from the tube and discard in the biohazard waste container on your bench.
4. Add 250 µl of water to the cell pellet in the tube. Vortex well to resuspend the cells in the water.
5. Place the tube of cells in the dry ice bath for 3 minutes.
6. Transfer the tube to the 90°C water bath for 3 minutes.
7. Repeat steps 5 and 6 twice (The freeze/thaw cycle lyses the cells and releases the template DNA into solution).
8. Centrifuge at 13,000 rpm for 1 minute.
9. Supernatant contains DNA.

Kranz co-workers

DNA extraction

1. Boiling lysis method(Modified method 1) (Continued)

- Cells from a single bacterial colony growing on CVP, NA or LA using a sterile toothpick and resuspended in 500 µl of sterile double distilled water or 50 µl of 5 mM NaOH.
- Suspensions were boiled for 5 min at 95°C and immediately put on ice for 1-2 min.
- The cell homogenate then centrifuged at 10000 *g* for 5 min.
- A 100 µl aliquot of the supernatant was transferred to a sterile tube and stored at -20°C until PCR testing.
- One or 2 µl of the cell lysate was used as a template in PCR.

Czajkowski *et al.*,2009;..

DNA extraction

1. Boiling lysis method(Modified method 1) (Continued)

- Bacterial DNA for the amplification of the 16S rRNA gene was extracted using a boiling lysis method.
- Colonies on agar plates were suspended in lysis buffer (100 mM NaCl, 10 mM Tris/HCl, 1 mM EDTA and 1% Triton X-100) and then incubated at 90°C for 10 min.
- The mixture was then briefly centrifuged and the aqueous phase was used as template for a PCR.

Triton X-100 ($C_{14}H_{22}O(C_2H_4O)_n$) is a nonionic surfactant which can be used in DNA extraction as part of the lysis buffer (usually in a 5% solution in alkaline lysis buffer).

Ko *et al.*,2006;..

DNA extraction

1.2. Alkaline lysis

Phenol-chloroform extraction method

- 1.5 ml of nutrient broth containing isolated bacteria was transferred to an autoclaved 1.5 ml eppendorf tube.
- It was centrifugated at 5000 rpm for 5 minutes to harvest the cell.
- After that, the supernatant was discarded and this step was repeated to obtain high yield of cell harvest.
- The cell pellet was resuspended in 250µl of ice-cold TE buffer.
- To lyse the cell:

1. 10 µl of lysozyme (10 mg/ml) was added to the solution,
2. Followed by 5 µl of RNAse A and incubation was carried on for 45 minutes at 37^{o}C.
3. Last step of cell lysis, 10 µl of Proteinase K was added and the mixture was incubated at 37^{o}C for 45 minutes again.

Wan Leng,2005

DNA extraction

1.2. Alkaline lysis (continued)

- Then, 10µl of sodium dodecyl sulphate (SDS) was added to the suspension and it was gently inverted for 15-20 times.
- Incubation was carried out for 45 minutes at 37˚C.
- When the cell lysis process was completed; the total volume of the suspension became 285 µl.
- Phenol-chloroform extraction method was used after cell lysis process.

Addition of salts to interrupt hydrogen bonding between water and phosphates on the DNA. SDS ($C_{12}H_{25}SO_4Na$) is a detergent that is known to denature proteins.
The resulting mixture (NaOH and SDS) is incubated for a few minutes. During this time, the detergent disrupts cell membranes and allows the alkali to contact and denature both DNA and plasmid DNA.

Wan Leng,2005

DNA extraction

1.2. Alkaline lysis (Continued)

- Equal volume (285µl) of Phenol: Chloroform (1:1) was added to the mixture and it was inverted gently for 10 times.
- To harvest the cell, the suspension was centrifuged at 13000 rpm for 3 minutes.
- The supernatant was transferred to a new and clean eppendorf tube and again equal volume of chloroform: isoamyl alcohol (24:1) solution was added to the supernatant.
- The mixture was centrifuged at 13000 rpm for another 3 minutes.

Wan Leng,2005

DNA extraction

1.2. Alkaline lysis (continued)

- The supernatant was recovered from the tube and transferred to new eppendorf tube and at the same time, the volume of the supernatant was determined.
- 1/10 volume of sodium acetate 3M (pH 4.8) was added followed by 2 times volume of ice-cold 95% ethanol(addition of ethanol to pull DNA out of solution).
- After that, the solution was incubated at -70°C for 45 minutes.
- After incubation, the mixture was centrifuged at 13000 rpm for 15 minutes to get the DNA pellet.
- The supernatant was discarded carefully and the pellet remained in the tube.
- 700µl 70% ethanol was added and the tube was carefully and gently inverted for a few times.
- Centrifugation at 13,000 rpm for 15 minutes was done thereafter.
- Then, the supernatant was discarded and left to air dry for at least 30 minutes.
- DNA was finally resuspended in 100µl of double autoclaved deionized water.
- The DNA was then stored at 4˚C.

Wan Leng,2005

DNA extraction

1.3. CTAB extraction method

- The basic steps involved in extracting DNA from plant/animal cells and tissues is the same as for microbes.
- However, kits need to incorporate modifications to take into account the special features of each cells.
- Different kits are used for varied sources, including human tissues, blood, hair, rodent tissues, leaf, bacteria, yeast, fungi, insect, stool, body fluids, spores, soil, clinical samples (e.g. biopsy samples, fine needle aspirates), forensic samples (e.g. dried blood spots, buccal swabs), and finger prints.

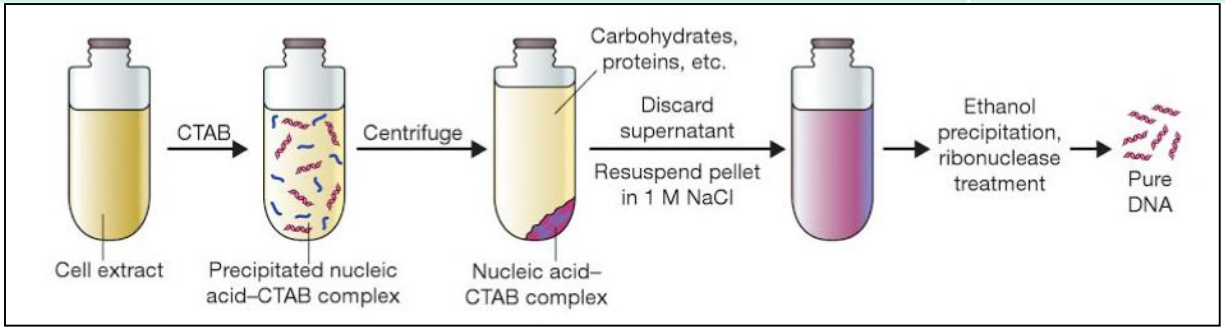
Dhaliwal,2016

DNA extraction

1.3. CTAB extraction method

Ingredients and notes

Extraction Buffer A (EBA)	Per 100 ml
2% (w/v) hexadecyltrimethylammonium bromide (CTAB)	2.0 g
100 mM Tris (pH 8.0) (Use 1 M stock) 10 mL	10 mL
20 mM EDTA (Use 0.5 M stock)	1 mL
1.4 M NaCl	8.2 g
4% (w/v) polyvinylpyrrolidone (PVP)	4.0 g
0.1% (w/v) ascorbic acid	0.1 g
10 mM β-mercaptoethanol (BME)* (Use 14.3 M stock)	70 µL



Keb-Llanes,2002;..

DNA extraction

1.3. CTAB extraction method

Ingredients and notes

- CTAB is Hexadecyltrimethylammonium bromide. Dissolve it before adding NaCl, with stirring and a little warmth(60°C), if necessary.
- When the NaCl is dissolved, lots of tiny bubbles come out of solution; they rise to the surface very slowly, simulating undissolved material.
- PVP of 40,000 average molecular weight makes the solution slightly translucent, but no large particles should be present after dissolving. relieves the effects of PCR inhibitors.
- Beta-mercaptoethanol should be kept in the refrigerator in a dry box.
- Isopropanol is an alcohol, so we can use it to precipitate DNA during extraction. Using ice-cold ethanol increases the yield of DNA.

DNA extraction

1.3. CTAB extraction method

Ingredients and notes

- Chloroform dissolves most of the impurities like protein, carbohydrate, cell debris etc.
- Using the phenol/chloroform-isoamyl alcohol procedure two phases will be obtained: the upper phase is aqueous and contains the nucleic acids and the lower one is organic and contains RNA and lipids; proteins. The latter(proteins) separate into the organic phase or lie at the phase interface.
- DNA can be further purified by precipitation of DNA with mix. of 7.5 M ammonium acetate and 95% ethanol(To make 475 ml, use 380 ml of 95% ethanol, 630 μl of 7.5 M NH4OAc, and bring to 475 ml with H_2O).1/3 volume of ammonium acetate and 2.5 volumes of ethanol(3.5:1.5).
- 7.5M ammonium acetate: Dissolve 57.81g of ammonium acetate in water to a final volume of 100ml. Sterilize by filtration (0.2μm filter). The final pH will be 5.5.

DNA extraction

1.3. CTAB+phenol-chloroform extraction method

A. For DNA extraction from plant tissues

- 1. Prepare CTAB buffer (see appendix), use within 2-3 days, store capped: Add polyvinylpyrrolidone mol. weight 40,000 (PVP-40) and β-mercaptoethanol and stir to dissolve before starting extractions:

CTAB	PVP-40	β-merc
0.5 ml	0.02g	2.5µl
5 ml	0.2g	25µl
20ml	0.8g	100µl

- 2. Weigh out 40-50 mg of frozen leaf tissue and place in a mortar.
- 3. Grind leaf tissue in a mortar with liquid N.
- 4. Add 500µl of CTAB buffer and grind samples with pestle.
- 5. Transfer solution to a 1.5 ml tube.

  (Optional) Add 4 µl of RNAse A and mix by inverting.

Doyle and Doyle, 1987; Doyle and Dickson, 1987; Cullings, 1992

DNA extraction

1.3. CTAB+phenol-chloroform extraction method

A. For DNA extraction from plant tissues

- 6. Incubate samples at 55°C for 1hr.
- 7. Add 500µl of 24:1 Chloroform-Isoamyl Alcohol(work in the fume hood) and mix well to form an emulsion by shaking tubes with hands.
- 8. Centrifuge for 8-10 minutes at maximum speed (13-15,000 rpm).
  a. Following centrifugation, you should have three layers: top: aqueous phase, middle: debris and proteins, bottom: chloroform.
  b. Proceed to the next step quickly so the phases do not remix
- 9. Pipette off the aqueous phase (top) containing DNA taking care not to suck up any of the middle or chloroform phases.
- 10. Place the aqueous phase into a new labeled 1.5 ml tube.
- 11. Estimate the volume of the aqueous phase.
  This should be approximately 350 µl.

Doyle and Doyle, 1987; Doyle and Dickson, 1987; Cullings, 1992

DNA extraction

1.3. CTAB+phenol-chloroform extraction method

A. For DNA extraction from plant tissues

- 13. Add 0.54 volumes (using the combined volume of aqueous phase and added AmAc) of cold isopropanol (=2-propanol).

  This should be approximately 204 µl.
- 14. Mix well.
- 15. Let sit in freezer for 45 min to an hour.
- Longer times (i.e. overnight) will tend to yield more DNA, but also more contaminants. (Herbarium material modification - leave in freezer for 2-4 days).
- 16. Centrifuge for 3 min at maximum speed (13-15,000 rpm).
- Orient tubes in an equal fashion to facilitate subsequent removal of supernatant without disturbing resultant DNA pellet.
- **17. Pipette off the liquid, being careful not to lose the pellet with your DNA.**

The DNA pellet at this stage is very loose and difficult to see.

Doyle and Doyle, 1987; Doyle and Dickson, 1987; Cullings, 1992

DNA extraction

1.3. CTAB+phenol-chloroform extraction method

A. For DNA extraction from plant tissues

- 18. Add 700 µl of cold 70% Ethanol and invert once to mix.
- 19. Centrifuge for 1 min at maximum speed (13-15,000 rpm).
- **20. Pipette off the liquid, being careful not to lose the pellet with your DNA.**
- 21. Add 700 µl of cold 95% Ethanol and invert once to mix.
- 22. Centrifuge for 1 min at maximum speed(13-15,000 rpm).
- **23. Pipette off the liquid, being careful not to lose the pellet with your DNA.**
- If you can't remove all the ethanol that is ok. It is better to leave some ethanol than risk sucking up your DNA!
- 24. Dry the pellet in a vacuum centrifuge or on a hot plate at 55°C.
- 25. Resuspend samples with 100 µl of water (ddH_2O). Allow to resuspend for 1hr at 55°C before using. Otherwise store in -20°C.

Doyle and Doyle, 1987; Doyle and Dickson, 1987; Cullings, 1992

DNA extraction

1.3. CTAB+phenol-chloroform extraction method

A. For DNA extraction from plant tissues

- **DNA quality and quantity assessment:**
- Assess the quality of the extracted DNA using a NanoDrop UV/Vis spectrophotometer and 0.7% (w/v) agarose gel, looking for a single absorbance peak at 260 nm, a 260/280 absorbance ratio of 1.8-2.0, and no evidence of substantial band shearing or contamination (either RNA or polysaccharide).

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA.
A ratio of ~1.8 is generally accepted as "pure" for DNA;
A ratio of ~2.0 is generally accepted as "pure" for RNA.

Healey *et al.*,2014;..

DNA extraction

1.3. CTAB+phenol-chloroform extraction method

A. For DNA extraction from plant tissues

- **Appendix:**
- **CTAB**: for 1L of CTAB buffer

  100 ml of 1 M Tris, pH 8.0

  280 ml of 5 M NaCl

  40 ml of 0.5 M EDTA

  20 g of CTAB (Cetyl Trimethyl Ammonium Bromide)

  to 1L with H_20
- **1 M Tris, pH 8.0**: for 1 L

  121.1 g Tris

  700 ml ddH_2O

  Dissolve tris and bring to 900 ml.

  pH to 8.0 with concentrated HCl (will need ~50ml)

  Bring to 1 L.

Doyle and Doyle, 1987; Doyle and Dickson, 1987; Cullings, 1992

DNA extraction

1.3. CTAB+phenol-chloroform extraction method

A. For DNA extraction from plant tissues

- **Appendix:**
- **0.5 M EDTA pH 8.0**: for 1 L

  186.12 g of EDTA

  750 ml ddH_2O

  Add about 20 g of NaOH pellets

  Slowly add more NaOH until pH is 8.0, EDTA will not dissolve until the pH is near 8.0.
- **5 M NaCl**: for 1 L

  292.2 g of NaCl

  700 ml ddH_2O

  Dissolve and bring to 1 L.

DNA extraction

1.3. CTAB+phenol-chloroform extraction method

A. For DNA extraction from plant tissues

- **Appendix:**
- **RNaseA (20 mg/ml) solution(optional):**

  RNaseA: 20 mg

  Tris-Cl (pII 7.5): 10mM

  NaCl: 15mM Sterile water was added to make the volume to 1 ml. The solution was heated at 100°C for 15 minutes to inactivate any Dnase present and then stored in aliquotes at -20°C.
- **7.5 M Ammonium acetate**: for 250 ml

  144.5 g ammonium acetate

  Bring to volume with ddH_20

DNA extraction

1.3. CTAB+phenol-chloroform extraction method

For DNA extraction from plant tissues

4. DNA quality and quantity assessment:

- Assess the quality of the extracted DNA using a NanoDrop UV/Vis spectrophotometer and 0.7% (w/v) agarose gel, looking for a single absorbance peak at 260 nm, a 260/280 absorbance ratio of 1.8-2.0, and no evidence of substantial band shearing or contamination (either RNA or polysaccharide).

Healey *et al.*,2014

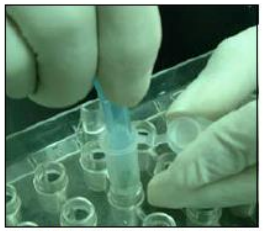
DNA extraction using CTAB method

DNA extraction using DNA trap

DNA Extraction (small scale) using CTAB method

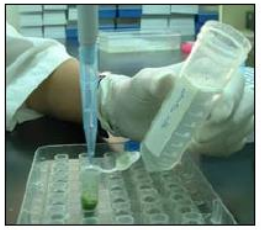
DNA Extraction using DNA Trap

Collect 1 g of leaf tissue and put in 1.5 ml tube.



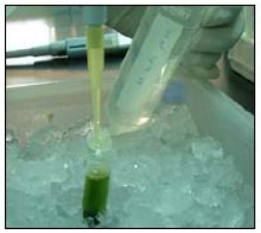
Freeze the tissue using liquid nitrogen,

grind it to find powder.



Add 1,000 ul of Extraction buffer and mix well.

Incubate for 1 hour at 65 °C.



Put sample for 5 minutes on ice.

Centrifuge for 5 minutes at 14,000 rpm.

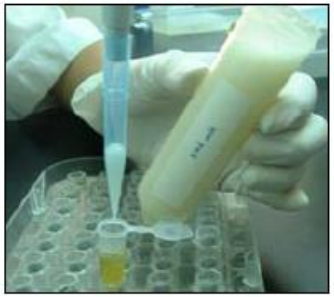
DNA extraction using CTAB method

DNA extraction using DNA trap

DNA Extraction (small scale) using CTAB method

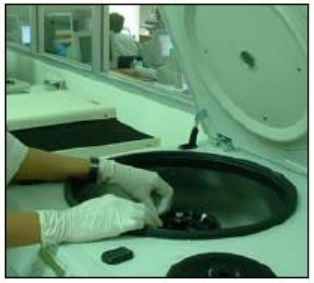
Add 100 ul of neutralizing buffer and mix well.

Put sample for 5 minutes on ice.



Transfer for upper phase into a new 1.5 ml tube.

Add 500 ul of trapping buffer and mix gently.



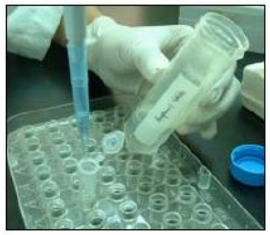
Centrifuge for 1 minutes at 2,200 rpm.

Discard the supernatant solution.

DNA extraction using CTAB method

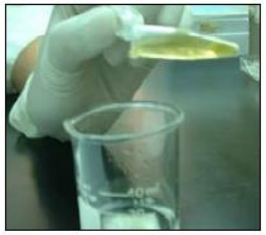
DNA extraction using DNA trap

DNA Extraction (small scale) using CTAB method

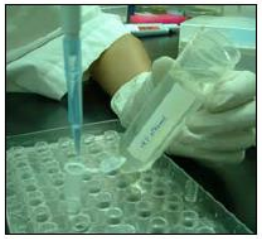


Add 500 ul of washing buffer I and mix well.

Centrifuge for 1 minutes at 2,200 rpm.



Discard the supernatant solution.



Add 500 ul of washing buffer II

Centrifuge for 30 sec. at 14,000 rpm.

Discard the supernatant solution.

DNA extraction using CTAB method

DNA extraction using DNA trap

DNA Extraction (small scale) using CTAB method

Dry in an incubator for 1 hour at 65 °C

or until all of the liquid has evaporated.

Rehydrate the DNA in 100 ul of

elution buffer and mix well.

Incubate for 30 minutes at 65 °C.

Centrifuge for 30 sec. at 14,000 rpm.

Transfer the supernatant solution into

a new 0.2 ml tube.

DNA extraction

1.3. CTAB extraction method

B. For bacterial DNA extraction

- The isolation of prokaryotic nucleic acid is much less work-intensive than those described for plants and macroalgae.
- Most Bacteria and Archaea have cell walls that can be easily broken through and lysed for the isolation of DNA and RNA.
- Ideally the material used should be grown from pure culture.
- Depending on the type of organism used for genetic material, either agar or liquid cultures can yield similar results, as long as there is no contamination in the process.
- Extra care must be taken when using nutrient rich media, since most airborne bacteria can grow in this as well.

Nishiguchi *et al.*,2002

DNA extraction

1.3. CTAB extraction method

For bacterial DNA extraction

- In case of plant sample, crush leaves with help of mortar and pestle using PVP to remove phenolic contamination, add adequate amount of CTAB into leaves to make fine slurry, take 1.5ml of sample into 2ml eppendorf tube.
- Add 10µl of β-mercaptoethanol into each eppendorf tube, shake well to mix properly. Incubate at 65°C for 1 hour in water bath.
- Centrifuge at 13000rpm for 15mins, collect supernatant in new eppendorf tube and add equal amount of CIA to the supernatant.
- Three layers appear, collect the upper layer in a fresh eppendorf tube then add equal volume of chilled isopropanol.
- Incubate for 20mins at 20°C so as to precipitate the DNA.
- Centrifuge at 13000 rpm for 15 mins.
- Discard the supernatant and add 500µl of 70% ethanol to the pellet obtained for washing.
- Mix well and centrifuge at 8000 rpm for 5 mins.
- Discard the supernatant and add 70µl of TE buffer and proceed for gel electrophoresis and quantification.
- **NOTE:**

1. The above protocol will be used for DNA isolation from FUNGI, ALGAE, PLANT, BLOOD & BACTERIA.
2. In case of bacterial DNA isolation, the incubation after adding CTAB will be at room temperature for 30 minutes.
3. In case of blood CTAB buffer is used in place of WBC buffer.

Kumar *et al.*,2012

Quantification of nucleic acids
DNA concentration

- Most experiments depend on accurate quantitation of the particular nucleic acid, RNA or DNA, which you are manipulating or analyzing.
- Several methods exist with different levels of accuracy and sensitivity.
- UV Spectrophotometry- best for quantifying oligos, single stranded RNA and DNA.

Ayyadurai,2006

Quantification of nucleic acids

Quantification of DNA

UV Spectrophotometry

- Ten microliters of DNA sample was mixed with 990µl of TE buffer and was read at 260 nm in a spectrophotometer.
- An optical density (OD) value of one corresponds to approximately 50 µg/ml of double stranded DNA (Sambiook *et al*., 1989).
- Based on the OD value, DNA samples were quantified.

Ayyadurai,2006

Quantification of DNA
UV Spectrophotometry

1. Allow the spectrophotometer and UV source lamp to warm up (>30 min.).
2. Determine the minimum volume which can be measured in your cuvettes (dependent on the height of the beam and cross section of the cuvette. Add that volume of 10 mM NaOH to the cuvette (include the reference cuvette if double beam spec). Wipe the faces of the cuvette(s) with a Kimwipe to remove smudges or fingerprints and place in respective® 2 holders. Zero the machine. **Note**: some people use H_2O for these measurements but these determinations may be more sensitive to base composition and 'double stranded-ness' of the sample nucleic acid. Therefore, NaOH is recommended.
3. Add some of your sample to the sample cuvette (usually 1/10th to 1/100th of the volume in the cuvette) and mix. This should be an amount which will give a significant reading (see below) and that you don't mind not getting back.
4. Read the absorbance value. Significant readings are those in the range between 0.05 and 0.5, determinations based on readings outside of this range are less precise.
5. Calculate the nucleic acid concentration in the original sample according to the formula.

Equation 1 DNA Concentration from A_{260}

$$\mu g/ml\ nucleic\ acid = \frac{50\ \mu g/ml * A_{260}\ reading}{dilution\ factor}$$

$$where:\ dilution\ factor = \frac{sample\ added}{total\ volume\ in\ cuvette}$$

See other slides on quantification or optical density(OD) of DNA.

Measurement of nucleic acid using spectrophotometry

Quantity and quality of DNA preparations

- Quantity and quality of DNA preparations were checked by standard spectrophotometry and gel electro-phoresis and all the samples were diluted to 10 ng DNA per microlitre.
- Use dH_2O to calibrate the spectrophotometer.
- Better results with regard to DNA quality (mean ratio of A260/280 =1.95) and average concentration of DNA (= 1018.2 ng/µL), respectively.

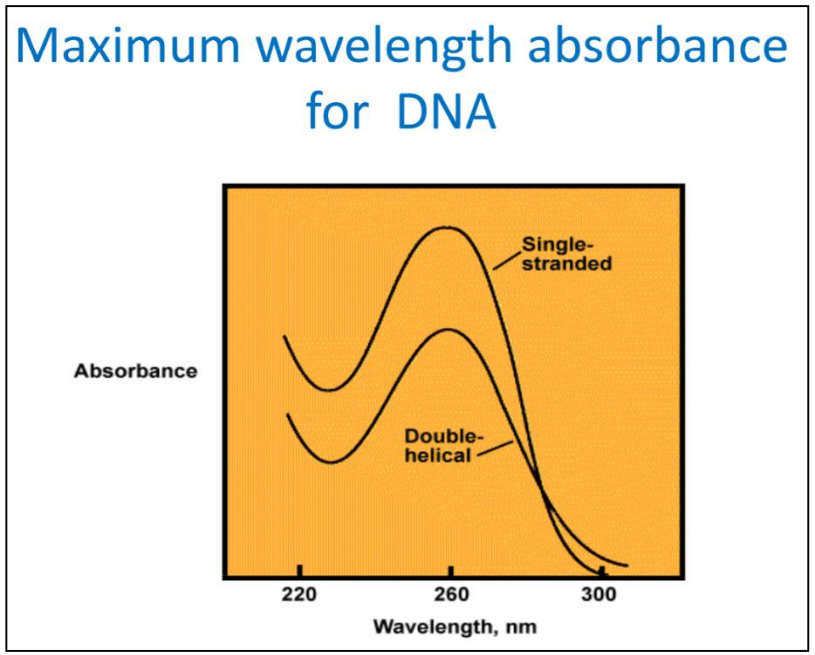
Note: Pure preparations of DNA or RNA have the OD_{260}/OD_{280} values of 1.8 and 2.0 respectively. If there is contamination with proteins, the ratio will be significantly less than the values given above.

Grover *et al.*,2012; Oliveira *et al.*,2014;..

Measurement of nucleic acid using spectrophotometry

Maximum wavelength absorbance for DNA

- Purity of DNA samples was estimated based on ratio between OD at 260 and 280 nm.
- Pure DNA sample has an OD value 260/280 between 1.8 and 2.0.
- Contamination with protein or phenol reduces the value (Sambrooke *et al*.,1989)



Ayyadurai,2006; Kalendar,2014

Measurement of nucleic acid using spectrophotometry

Quantity and quality of DNA preparations

- The bases in nucleic acids have max. absorption at 260 nm.
- Proteins have a max. absorption at 280 nm.
- Polyphenols/Polysaccharides have a max. absorption at 230 nm
- A solution which has 50 µg/ml of dsDNA has an absorption of 1 at 260 nm;
- A solution which has 40 µg/ml of ssDNA has an absorption of 1 at 260 nm.

- OD_{260}/OD_{280} = 1.8 (protein-free DNA);
- >1.8 - probably contaminated with RNA.

- OD_{260}/OD_{230} > 2.0 (polysaccharide compounds - free DNA);
- <1.8- contaminated polysaccharide, poor quality DNA.

Kalendar,2014

DNA extraction

DNA concentration (Quantification)

- The amount of DNA present in the extracted fraction(DNA concentration) was quantified using a Gene Quant spectrophotometer at two different wavelengths 260 and 280 nm.
- An OD of 1 corresponds to approximately 50 µg/ml for double stranded DNA.
- The ratio between the reading at 260 nm and 280 nm (OD_{260}/OD_{280}) will give the purity of the nucleic acid.
- The DNA samples were appropriately diluted in sterile double distilled water or 1 X TE.
- DNA purity was measured according to the following equation.
- The samples were stored at 4°C or were frozen at -20°C for long term storage.

$$\text{Amount of DNA} = \frac{\text{O.D at 260 nm}}{\text{O.D at 280 nm}}$$

See other slides on quantification or optical density(OD) of DNA.

DNA extraction

DNA concentration (Quantification)

- DNA concentrations and qualities are calculated using A_{260} and A_{280} spectrophotometric measurements.
- Adjust to a final concentration of 0.5 µg/µl using the calculation:

$$50 \times 200 \times OD_{260} = \text{DNA concentration (µg/ml)}$$

- Where
- '50' is the correction coefficient for double-stranded DNA,
- '200' is the dilution factor (1:200), and
- OD_{260} is the observed reading at 260 nm.
- DNAs are stored at 4°C until needed.

Eric B. Brown

See other slides on quantification or optical density(OD) of DNA.

DNA extraction

DNA concentration (Quantification)

- Sometimes primer units are listed in optical density reading (OD).
- If this is a problem you will need to convert to molarity using the following equations:
- Change optical density reading of primer to molarity (μM units)

1. N = # of primer bases
2. SIGMA 260 =~ 10,000 X N/ m X cm
3. Molecular weight =~ 330 X N
4. OD_{260} / SIGMA 260 X 10^6 = Concentration (μM).

- For example: A primer is 20 bases long/OD_{260} = 10.

1. N = 20
2. SIGMA 260 =~ 10,000 X 20/m X cm = 20,000/m X cm
3. Molecular weight =~ 330 X 20 = 6,600
4. Thus, 10 OD_{260}/20,000 $m^{-1}cm^{-1}$ X 10^6 =50 μM.

DNA extraction

Measure concentration of DNA with the Nano Drop

NanoDrop spectrophotometer

- The quantity and quality of DNA obtained was evaluated using a NanoDrop spectrophotometer with absorbance of 260/80 nm.
- Two µl of DNA extracted from each sample were placed directly on the spectrophotometer (NanoDrop,2000) and measures in 230, 260 and 280 nm were performed.
- The system software provides the DNA concentration in ng/µl and automatically calculates the absorption ratio 260/280 (A260/280) and 260/230 (A260/230).

Oliveira *et al.*,2014;..

DNA extraction

Measure concentration of DNA with Nano Drop

NanoDrop instruments

1. NanoDrop 2000c UV-Vis spectrophotometer
2. NanoDrop 3300 Fluorespectrometer

- All NanoDrop instruments utilize a patented sample retention system that allows the quantification of DNA and RNA from 1-2 µl samples.

	NanoDrop 2000c	NanoDrop 3300
How does it work?	Absorbance of UV-Visible light	Fluorescence
What is the usable concentration range?	0.4 - 15,000 nanograms /µL	0.05 - 2,000 picograms /µL
Can it detect contaminants?	Yes - spectral data and purity ratios indicate sample purity	No - quantification only
Can I selectively measure dsDNA/ssDNA/RNA even when the others are present?	No – measures the total absorbance of the sample	Yes – assays are selective for dsDNA/ssDNA/RNA
Which is faster?	No specific sample preparation needed. Measurement takes 5 seconds.	Requires reagent preparation and mixing with samples and measurement of standards
Do I need a standard curve?	No	Yes

20 picomoles/µl means 20 micromolar (20µM).

DNA extraction

Measure concentration of DNA with the Nano Drop

NanoDrop Protocol

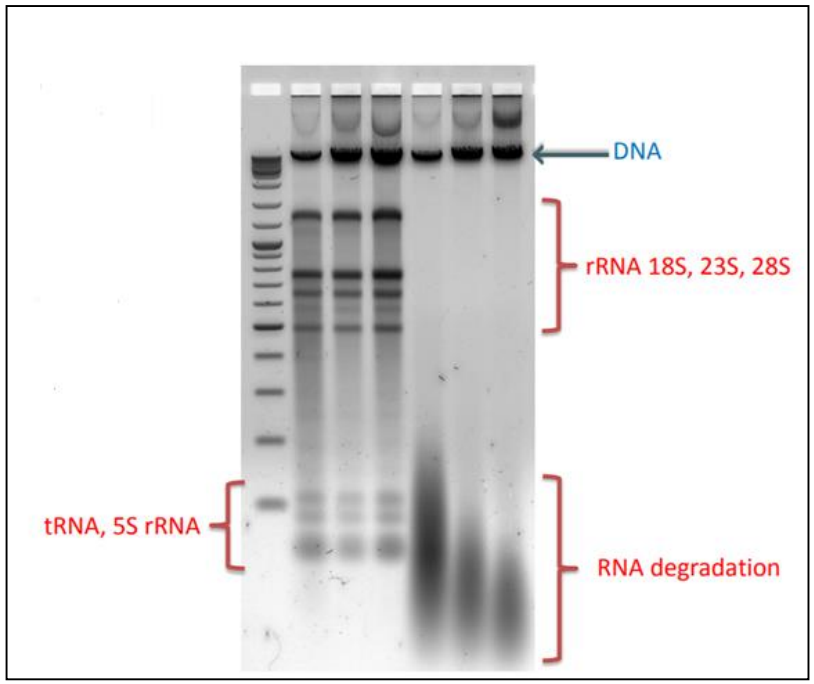
- Blank with 2 µl of buffer (whatever DNA was eluded with in the miniprep).
- Run 2 µl of each sample. After each sample rinse with 2 µl of buffer.
- Clean with 2 µl of deionized (DI) water after use.
- Rinse with a few drops of water to completely cover the reader.

1. There should be a large peak at 260 nm where DNA absorbs.
2. Proteins absorb at 280 nm and the 260/280 ratio measures the protein contamination.
3. Other contaminants absorb at 230 nm and the 260/230 ratio measures other contaminants.

DNA extraction

Agarose gel DNA verification

- Agarose gel electrophoresis can be used to separate DNA fragments of different sizes.
- Different forms of a DNA molecule of the same size can also be separated by agarose gel electrophoresis.
- Larger molecules migrate more slowly than smaller ones through the matrix of the gel.

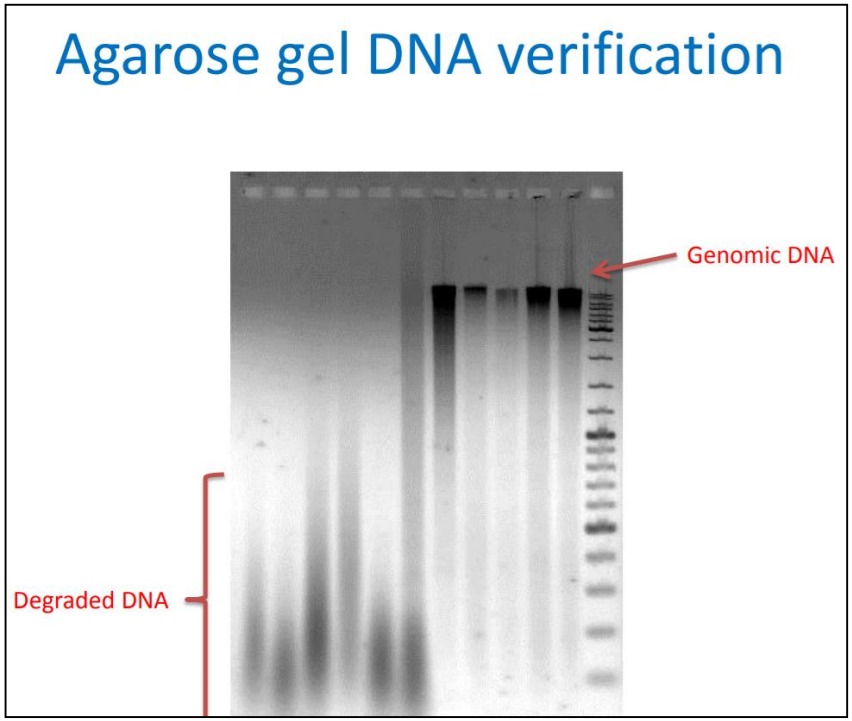


Kalendar,2014

DNA extraction

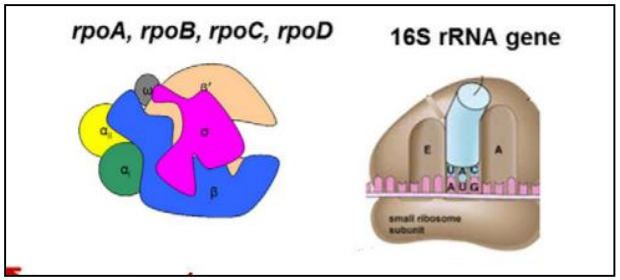
Agarose gel DNA verification

- Agarose is polysaccharide which is extracted from seaweed.
- It is used to separate DNA fragments of 300-10,000 bp at 0.5-2%.
- Because of its compact size supercoiled DNA will move faster than relaxed or nicked circular forms.

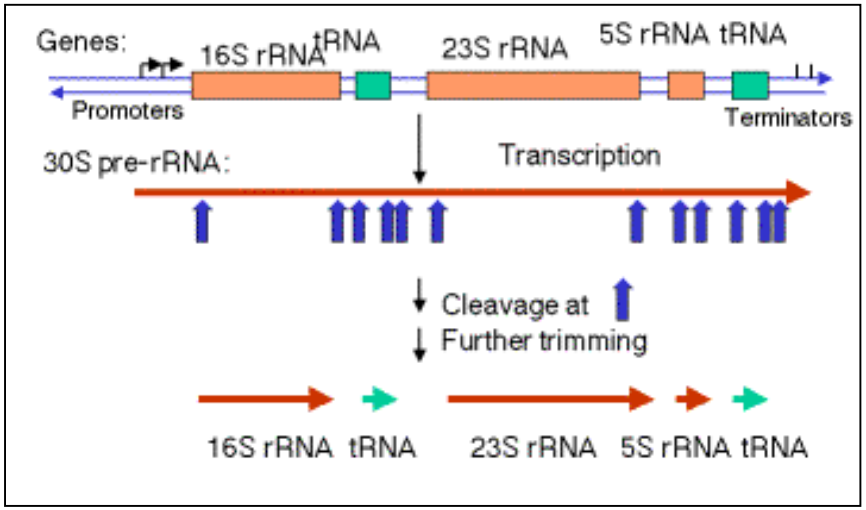


Kalendar,2014

Target sequences
2. Ribosomal RNA operon



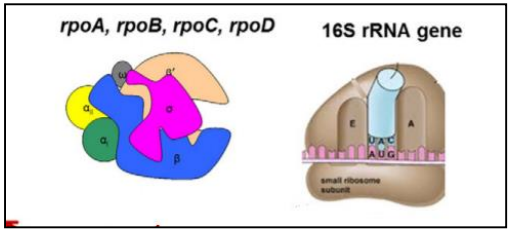
- The ribosomal operon (cluster of co-transcribed genes) has also been employed as a source of primers in many models.
- The advantage of this target is the universality of the ribosomes in all bacteria, and a size (1,600-2,300 bp), which enables the whole operon to be sequenced quickly and suitable primers to be selected.



Palacio-Bielsa *et al.*,2009; López *et al.*,2011;..

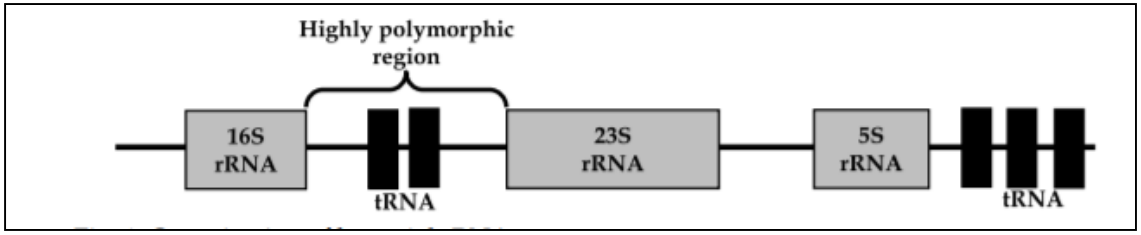
Target sequences

Ribosomal RNA operon(rrn)



Organization of bacterial rRNA operon

- rRNA molecules were found throughout nature and carry out the same function and their structure changes very little over time.
- The rRNAs are not identical between species. The actual sequence of the nucleotides in rRNAs (and in the rDNA genes) does vary between species.
- Similarities and dissimilarities in rRNA nucleotide sequences are a good indication of how related or unrelated different cells and organisms are.



Target sequences
Ribosomal RNA operon(rrn)

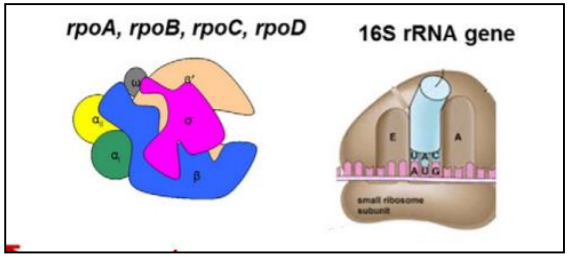
- Among genotyping methods, analyses of multigene families such as *rrn* operons and tRNA genes have been demonstrated to be highly useful.
- The rrn operon is a multigene family that frequently presents more than one copy in most bacteria.
- The rDNA genetic loci in eubacteria include, in 5' to 3' order: 16S, 23S, and 5S rRNA genes, which are separated by intergenic transcribed spacer (ITS) regions.
- Sequence evaluations of the 16S rRNA have been used frequently as a powerful and accurate method for determining inter- and intraspecific relationships.

Bacterial rRNA operon starts with the promotor (p).

Bacterial rRNA operon ending at terminator(t).

Spencer and Spencer,2004;..

Target sequences
Ribosomal RNA operon(rrn)

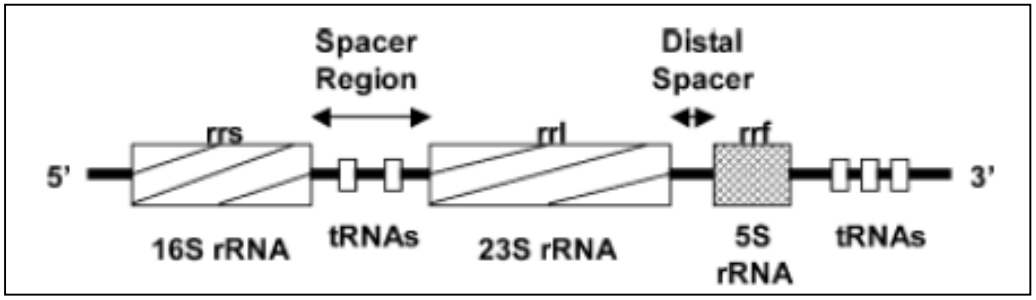


rrs, small ribosomal genes encode 16SrRNA, *rrl*, large genes encode 23S rRNAs and *rrf*, ribosome release factor genes encode 5S rRNAs.

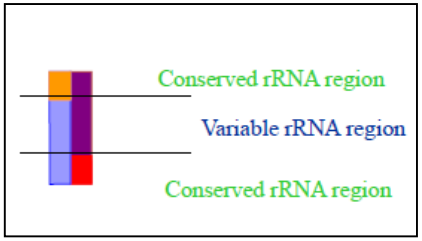
- The rrn locus consisted of:

1. A 16S rRNA gene (rrs), followed by
2. An intergenic transcribed spacer (ITS) containing two genes of $tRNA^{Ile}$ (isoleucyl tRNA synthetase)and $tRNA^{Ala}$, a 23S rRNA gene (rrl), an ITS devoid of tRNA genes and a 5S rRNA gene (rrf).

- The internally transcribed spacer region (ITS) between the 16S and 23S rRNA genes appears to be more variable than 16S and 23S rRNA genes.



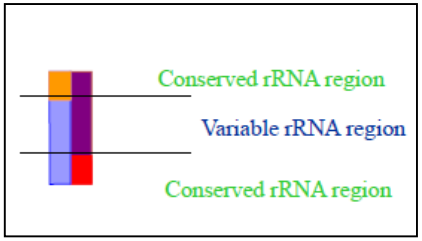
Schematic diagram of a typical ribosomal rRNA operon.



Ribosomal RNA operon(rrn)

Key features of rRNAs for phylogenetics

- When a single species splits into two distinct evolutionary lineages, differences can accumulate in the sequence of the rRNAs between the two lineages.
- Biologists call the process "sequence divergence."
- The divergence of rRNA sequences generally occurs very slowly (they are among the most slowly evolving of genes, but changes still happen).
- In addition, the structure and function of the rRNAs generally stay the same between species (and changes in the actual structure are usually lethal).



Ribosomal RNA operon(rrn)

Key features of rRNAs for phylogenetics

- Some regions of rRNAs evolve (i.e., diverge) slowly and others diverge rapidly.
- Some regions are basically the same across most or all taxa.
- This shared sequence has allowed researchers to use a specialized laboratory method known as PCR to help read the sequences of rRNAs from different (and even unknown) species.

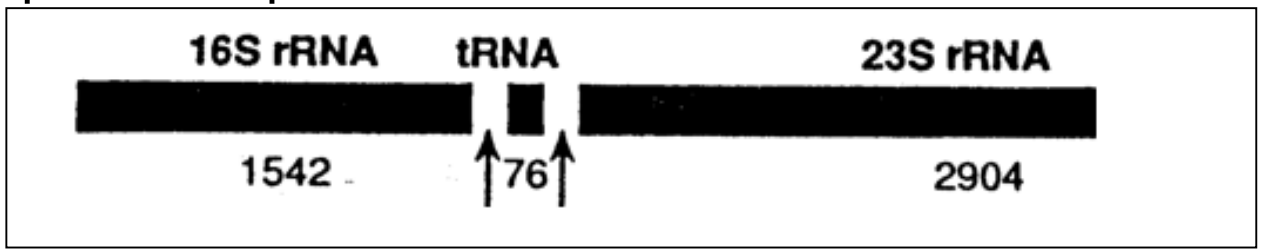
Target sequences

Single locus sequence analyses

Other molecules

- Other molecules have been tested but not suitable for determining evolutionary relationships:

1. tRNA is too constrained and contains too few nucleotides (70-90 nucleotides),
2. 5S rRNA is larger than tRNA (approximately 120 nucleotides) but still too small for statistical reasons while
3. Cytochromes are not universally present in all cells, making comparison impossible.



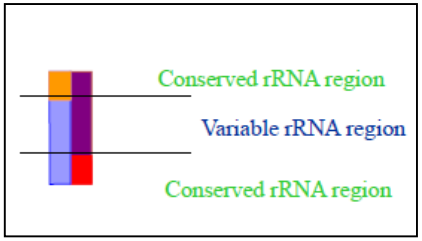
Sequence analysis of the spacer region between the 16S and 23S rRNA genes reveals the presence of genes for $tRNA^{Ile}$ and $tRNA^{ALa}$

Wan Leng,2005;..

PCR amplification

Single locus sequence analyses

Target sequences: 16S rRNA gene (rDNA)



- 16s rRNA is the RNA product of the DNA gene.
- In prokaryotes, the 16S ribosomal RNA (rRNA) genes are essential and occur in at least one copy in a genome.
- But there is more than one copy of the 16s rRNA gene in most bacteria. i.e. multiple sequences of 16S rRNA can exist within a single bacterium.
- Several pieces of RNA are important for proper ribosome function.
- This RNA is not translated to protein.
- It can be refer to the "rRNA gene" or "rDNA" to designate the DNA in the genome that produces the ribosomal RNA.

PCR amplification

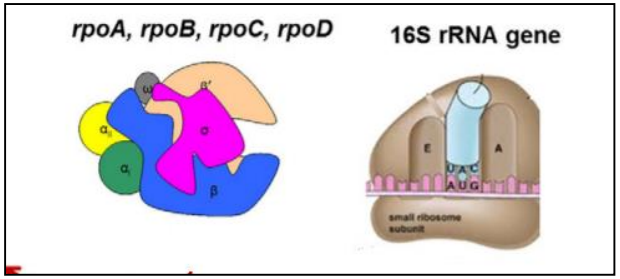
Single locus sequence analyses

Target sequences: 16S rRNA gene (rDNA)

- The 16Sr DNA which codes for the small subunit of ribosomal RNA, is now the most widely used informational macromolecule for bacterial systematic studies at the family, genus, species, and subspecies levels.
- Such a 16S rDNA sequence based identification technique will substantially facilitate the ecological study and the control of difficult to culture microorganisms.

Tariq *et al.*,2008

Ribosomal RNAs
Functions



- Ribosomal RNAs (rRNA) perform critical functions in the ribosome that allow protein synthesis to occur.
- The key catalytic activity of the ribosome – the creation of a chemical bond between two amino acids (known as a peptide bond) – comes from the RNA component of the ribosome.
- This, and other catalytic roles for RNA, were discovered relatively recently and were a bit of a surprise, since for many years it had been thought that all catalytic activities in cells were from proteins.

Ribosomal RNAs
Functions

1. Among these molecular markers, 16S rRNA, an ~1500 base pair gene coding for a catalytic RNA that is part of the 30S ribosomal subunit, has desirable properties that allowed it to become the most commonly used such marker.
2. Ribosomal RNA (rRNA) associates with a set of proteins to form ribosomes.
3. These complex structures, which physically move along an mRNA molecule, catalyze the assembly of amino acids into protein chains.
4. They also bind tRNAs and various accessory molecules necessary for protein synthesis.

Ribosomal RNA (rRNA) is a component of the ribosomes, the protein synthetic factories in the cell.

Ribosomal RNAs

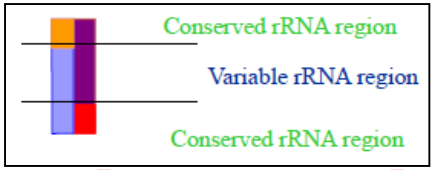
16S rDNA

Functions

- Like the large (23S) ribosomal RNA, it has a structural role, acting as a scaffold defining the positions of the ribosomal proteins.
- The 3' end contains the anti-Shine-Dalgarno sequence, which binds upstream to the AUG start codon on the mRNA.
- The 3'-end of 16S RNA binds to the proteins S1 and S21 known to be involved in initiation of protein synthesis.
- Interacts with 23S, aiding in the binding of the two ribosomal subunits (50S+30S).
- Stabilizes correct codon-anticodon pairing in the A site, via a hydrogen bond formation between the N1 atom of Adenine residues 1492 and 1493 and the 2'OH group of the mRNA backbone.

Wikipedia,2015

16S rDNA gene



Consists of 8 highly conserved regions and 9 variable regions

- The 16S rRNA gene contains regions that are:

1. Highly 'conserved' (have the same sequence in all bacteria and archaea), and

- Conserved regions of the gene can be used to bind primers for PCR and sequencing.
- For 16S rDNAs, the primers (15-20 nucleotides (nt) are located in the conservative regions that flank a target region used for phylogenetic analysis.

2. Highly 'variable' (have sequences that are unique at the genus or species level).

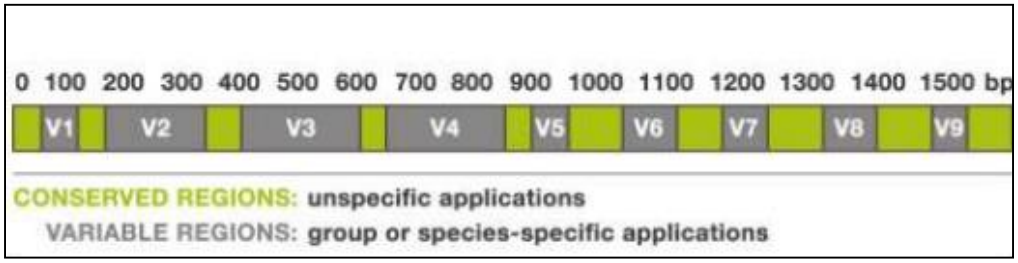
- Variable regions to determine the identity of the organism.

Moran,2010

16S rDNA gene

1.5 kb in length, consists of 8 highly conserved regions and 9 variable regions

- 16S ribosomal DNA (or 16S ribosomal RNA) is a component of the 30S subunit of prokaryotic ribosomes.
- It is approximately 1.5kb (or 1500 nucleotides) in length.
- The genes coding for it are referred to as 16S rDNA and are used in reconstructing phylogenies.

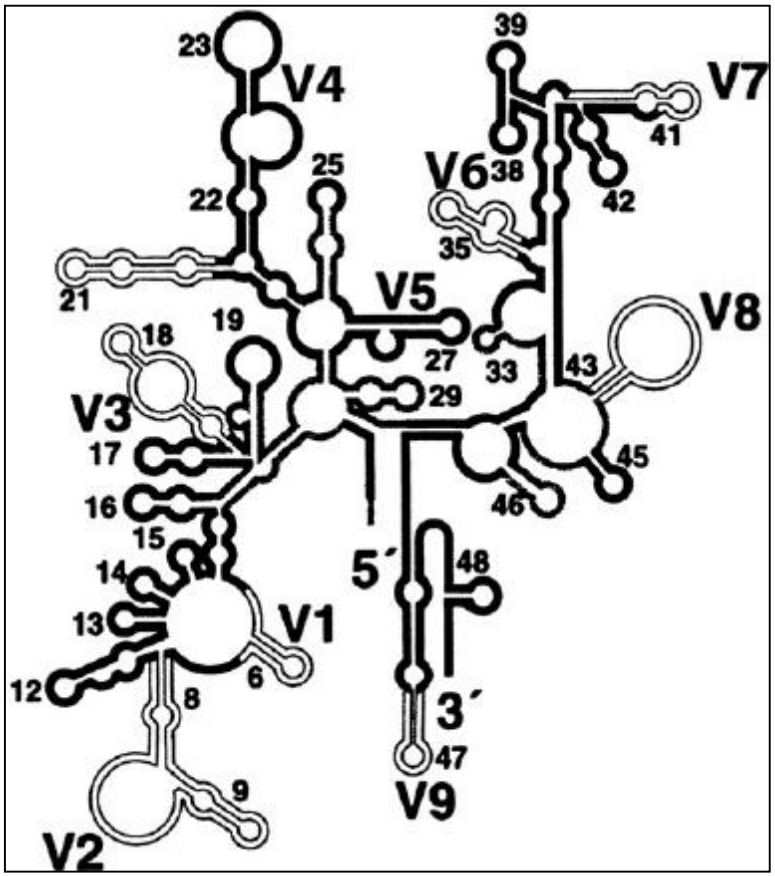


Wikipedia,2012; Sophie Arbefeville; Hudson *et al.*,2010

16S rDNA gene

1.5 kb in length, consists of 8 highly conserved regions and 9 variable regions

- Since the invention of the polymerase chain reaction (PCR) technique, the variant regions, V1–V9, of the 16S rRNA genes (rDNAs) have been used for species identification. e.g. v3 (variable region) of the 16S rDNA gene.



16S rDNA gene

Evaluation of different 16S rRNA gene V regions for exploring bacterial diversity

- Massive partial sequencing of 16S rRNA genes has become the predominant tool used for studying microbial ecology.
- However, determining which V regions and primer sets should be used for screening microbial communities.
- V1–V2 and V1–V3 regions were the most reliable regions in the full-length 16S rRNA sequences, while most V3 to V6 regions (including V3, V4, V3–V4, V5, V4–V5, V6, V3–V6, V4-V6, and V5–V6) were more closely aligned with the SILVA SSU Ref 123NR database.
- Overall, V4 was the most prominent V region for achieving good domain specificity, higher coverage and a broader spectrum in the Bacteria domain, as confirmed by the validation experiments.

Zhang *et al.*,2018

PCR amplification

3. Primers used to analysis 16S rRNA sequences

A. Universal primers

- Sequence analysis of the 16S rRNA sequences is done with the help of several primers, called "universal primers".
- When a small piece of this sequence is used as a primer in a PCR assay, it acts as a "universal primer" to nonselectively amplify any bacterial DNA in the DNA sample.
- These primers target the conserved region of 16S rRNA gene and amplify the target in parts.
- Several amplified parts could be assembled together to have the entire sequence of the complete 16S rRNA.

Wikipedia,2012; Goh *et al.*,2011; Universal Bacterial PCR Fact Sheet, 2004

DNA Molecules

The length of a single/double-stranded DNA molecules is measured in units of purine or pyrimidine base

DNA	Sequence	Length
Single-stranded	A-C-G-T-C	5 bases
Double-stranded	A-C-G-T-C T-G-C-A-G	5 base pairs

A kilobase (kb) is a unit of length of nucleic acids equal to 1000 base pairs of DNA or RNA.
kb (= kbp) = kilo base pairs = 1,000 bp
Mbp = mega base pairs = 1,000,000 bp or one million base pairs.
Gb = giga base pairs = 1,000,000,000 bp.
Daltons: One hydrogen weighs 1 dalton (Da).
14,000,000=14×10^6 or 1.4×10^7

PCR amplification

Primers used to analysis 16S rRNA sequences

Universal primers

- Primer Length: It is generally accepted that the optimal length of PCR primers is 18-22 bp.
- Thus, the conventional PCR usually requires 20-25 nucleotide (nt) primers.
- The length of the oligonucleotide is usually denoted by mer (from Greek *meros*, "part").
- For example, a fragment of 25 bases would be called a 25-mer.

1 kilobase is 1000 base pairs. So 3000kb = 3,000,000 base pairs. Remember that each base pair is two nucleotides. i.e. base pair is the combination of two nucleotides whose bases are bonded with each other and lie on opposing strands of a DNA molecule. The base pairs are adenine and thymine or guanine and cytosine in DNA. A DNA sequence, generally PCR products of 150-1000 bp are produced.

PCR amplification

Primers used to analysis 16S rRNA sequences

B. Bacterial primer specificity

- None of the primers in current use are truly "universal" and no single set of primers can be recommended that are guaranteed to amplify all prokaryotes.
- Here we re-assess the specificity of commonly used 16S rRNA gene primers and present these data in tabular form designed as a tool to aid simple analysis, selection and implementation.

Baker *et al.*,2003

PCR amplification

Primers used to analysis 16S rRNA sequences

Bacterial primer specificity

- Over 10% of bases in the 16S rRNA gene are totally conserved (within a sample of 500 bacterial sequences).
- Regions of differing variability in the 16S rRNA variability map are colour-coded to facilitate the analysis of primer variability:

1. totally conserved positions(brown);
2. highly conserved positions (red);
3. variable positions (blue), and
4. nucleotides present in *E. coli* (green);

- nucleotide positions with variability… (black).

Baker *et al.*,2003

PCR amplification

Primers used to analysis 16S rRNA sequences

Bacterial primer specificity

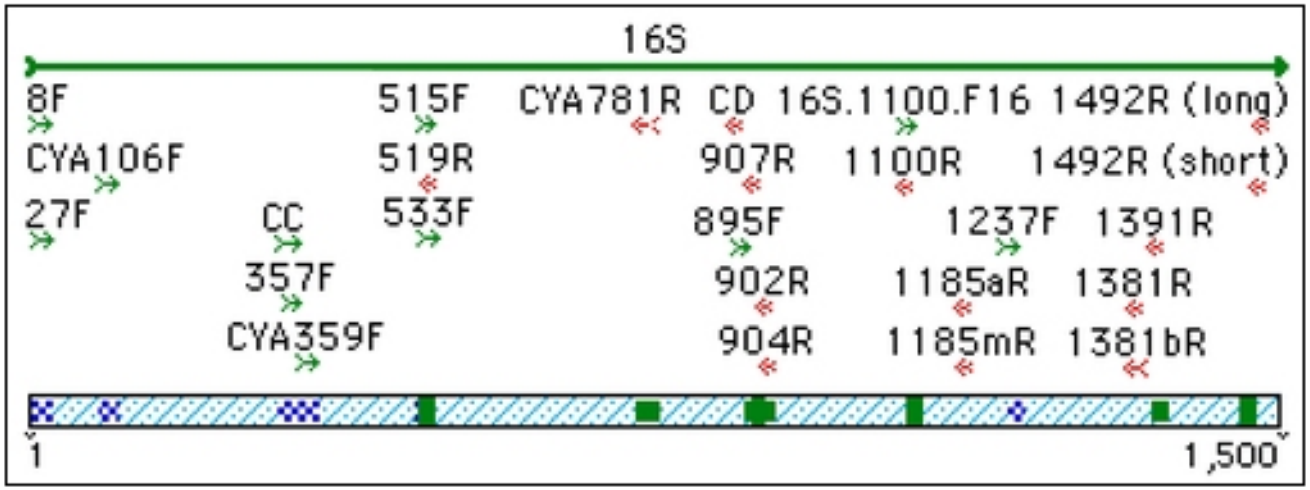
Baker *et al.*,2003

Primers for the prokaryotic 16S rDNA

A set of 16S rRNA gene universal and specific primers

- The 16S rRNA gene is approximately 1500 bases in length.
- The primer map for the 16S SSU rRNA gene includes both:

1. Universal (8F, 27F,515F, 907R, 1492R...), and
2. Specific primers (902R,904R,…).



Lutzoni Lab; Bélisle,2010

Primers for the prokaryotic 16S rDNA

The first set of 16S rDNA PCR universal primers

- The first sets of primers were designed by using conservative regions of 16S rDNA sequences from different species and were named according to their positions on *Escherichia coli* 16S rDNA.
- This has become the protocol for subsequent primer design.
- For example, primer E685 corresponds to eubacterial P4 region and primer A344 targets the archaeal H339 region.

Wang and Qian,2009

Primers for the prokaryotic 16S rDNA

Single locus sequence analyses

Oligonucleotide primers used for 16s rDNA amplification and sequencing of *Xanthomonas* spp.

Primer[a]	Sequence	Target positions[b]	Application
16F27	5′ AGAGTTTGATCMTGGCTCAG 3′	8-27	Amplification
16R343	5′ ACTGCTGCCTCCCGTA 3′	358-343	Sequencing
16F355	5′ ACTCCTACGGGAGGCAGC 3′	337-355	Sequencing
16R519	5′ GTATTACCGCGGCTGCTG 3′	536-519	Sequencing
16F530	5′ TTCGTGCCAGCAGCCGCGG 3′	512-530	Sequencing
16R685	5′ TCTACGCATTTCACCGCTAC 3′	704-685	Sequencing
16F704	5′ GTGTAGCGGTGAAATGCGTAGA 3′	685-704	Sequencing
16F946	5′ CCCGCACAAGCGGTGGA 3′	930-946	Sequencing
16R1087	5′ CTCGTTGCGGGACTTAACCC 3′	1206-1087	Sequencing
16F1195	5′ AGGAAGGTGGGGATGACGTC 3′	1195-1214	Sequencing
16R1389	5′ ACGGGCGGTGTGTACAAG 3′	1389-1372	Sequencing
16R1525	5′ TTCTGCAGTCTAGAAGGAGGTGWTCCAGCC 3′	1525-1496	Amplification

[a] F, forward primer; R, reverse primer.

[b] The numbering of target positions is based on the numbering of the *E. coli* 16s rRNA sequence.

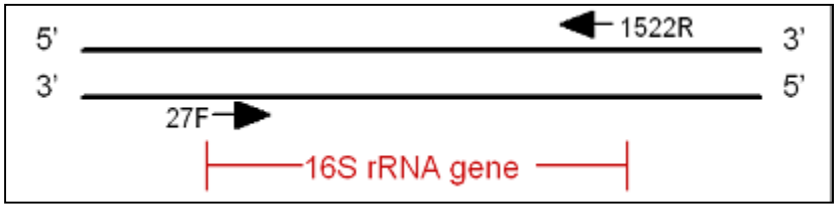
Hauben *et al.*,1997

PCR amplification

A set of 16S rDNA PCR universal primers

27F and 1492R or 27F and 1522R primers

- The most common universal primer pair was devised by Weisburg *et al.*,1991 and are currently referred to 27F and 1492R. This high purity grade universal primers were used for the conventional PCR approach.
- 27F, 5'-AGAGTTTGATCMTGGCTCAG-3’
- 1492R, 5'-GGTTACCTTGTTACGACTT-3’
- Schematic of the bacterial and archaeal 16S rRNA gene (approximately 1500 bp in length) and the PCR primers (primers 27F and 1522R) to conserved regions that are used in gene amplification.



Moran,2010;…

PCR amplification

A set of 16S rDNA PCR universal primers

27F and 1495R primer

- The 16S rDNA genes were amplified from the total DNA extracted from the biogas slurry sample with a bacterial primer set 27f/1495r and amplicons were ligated to pMD-18 T vector to construct a library.
- In total, 310 clones were randomly selected and sequenced.
- Bacterial and archaeal clone libraries were generated from polymerase chain reaction (PCR)-amplified 16S rDNA using bacterial primers
- 27f (5′-GAG AGT TTG ATC CTG GCT CAG-3′) and
- 1495r (5′-CTA CGG CTA CCT TGT TAC GA-3′).

Bianciotto *et al.*,1996; Liu *et al.*,2009

PCR amplification

A set of 16S rDNA PCR universal primers

8F and 1492R primers

- 16 s_8F
- AGAGTTTGATCMTGGC 'Universal' forward primer designed to amplify/sequence diverse bacterial 16s rDNA sequences;
- 16 s_1492rev
- TACCTTGTTAYGACTT 'Universal' reverse primer designed to amplify/sequence diverse bacterial 16s rDNA sequences.

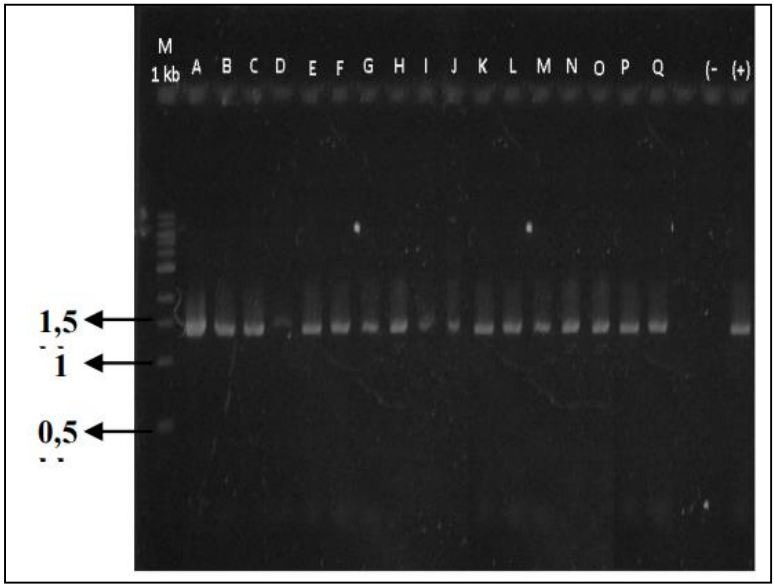
Saxon *et al.*2014

PCR amplification

A set of 16S rDNA PCR universal primers

63F and 1387R primers

- PCR analyses of near-full-length 16S rRNA:
- For identification of ice bacteria *Pseudomonas* sp. and *Xanthomonas campestris*.
- 63f (5'-CAGGCCTAACACATGCAAGTC-3') primer, and
- 1387r primer (5'-GGGCGGWGTGTACAAGGC-3') (Marchesi *et al.*, 1998).
- The best choice for amplification of nearly full-length 16S rRNA sequences.
- Size of PCR product is 1300 bp.



The amplification of 16S rRNA gene uses primers 63F and 1387R. The product of this 16S rRNA gene amplification is approximately 1300 bp.

Waturangi *et al.*,2008; Sherafati *et al.*,2014; Fitriani *et al.*,2013

Cross-kingdom amplification

Commonly used Bacteria-specific primers 8F and 27F paired with the universal primer 1492R amplify both eukaryotic and prokaryotic rRNA genes

- The amplification of coral 16S rRNA genes by using primer sets that had previously been described as *Bacteria* specific.
- Primer sets 8F/1492R and 27F/1492R were used in separate reactions to amplify extracted rRNA genes.

Primer	Sequence	*Tm* (°C)	Target
8F	5′-AGAGTTTGATCCTG GCTCAG	52	Bacteria
27F	5′-AGAGTTTGATCMTG GCTCAG	52-50	Bacteria
63F	5′-CAGGCCTAACACAT GCAAGTC	54	Bacteria
1492R	5′ -GGTTACCTTGTTAC GACTT 3′ -AAGTCGT AACAAGGTAACC*	47	Universal
1542R	5′ -AAGGAGGTGATCC AGCCGCA 3′ -TGCGG CTGGATCACCTC CTT*	56	Universal

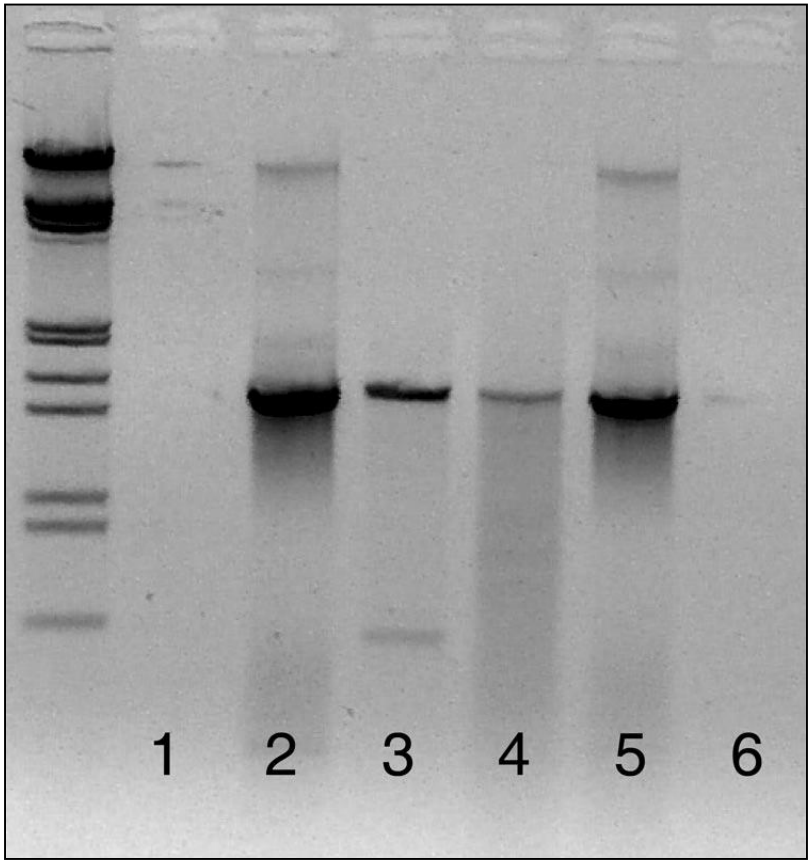
*, reverse complement found on positive strand. Primers are shown in 5′-3′ orientation, unless noted otherwise. Degeneracies, M=A/C.
-The specific melting temperatures(T_m) of all primers are mentioned above. The annealing or specific melting temperatures(T_m) of PCR reaction should be calculated. Tm value of one of some primer is 52.8 (50mM NaCl) and another one is 54.5 (50 mM NaCl) as given by the primer making company. Simple rule(formula) for calculation of melting temperature (Tm) = 4 * Number of G or C + 2 * Number of A or T (°C).

Galkiewicz and Kellogg,2008;..

Cross-kingdom amplification

Commonly used bacteria-specific primers 8F and 27F paired with the universal primer 1492R amplify both eukaryotic and prokaryotic rRNA genes

- Lanes 1, 2, and 3 were amplified using 63F/1542R.
- Lane 1 is the negative control,
- lane 2 is the positive control containing only bacterial DNA,
- lane 3 is the coral tissue sample.
- Lanes 4, 5, and 6 were amplified using 8F/1492R.
- Lane 4 is amplified coral tissue, indistinguishable from the positive control in lane 5.
- Lane 6 is the negative control.



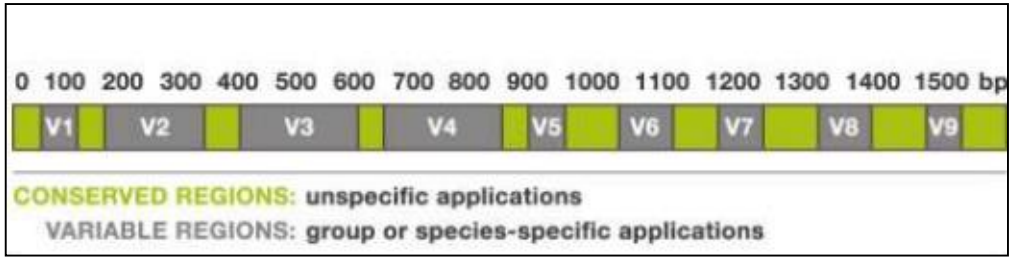
Galkiewicz and Kellogg,2008

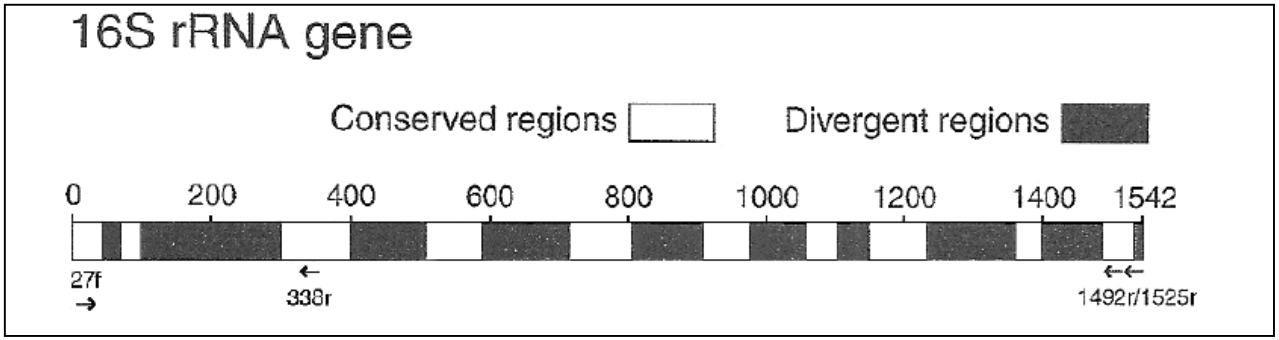
PCR amplification

A set of 16S rDNA PCR universal primers

27f/338r and 1492r/1525r primers

- Conserved and variable regions of 16S rRNA gene with universal primers (27f,338r and 1492r/1525r) attached to the conserved regions (source: Maiwald,2004).





Sophie Arbefeville; Marta *et al.*,2011

PCR amplification

A set of 16S rDNA PCR universal primers

27F and 1525R or G3PDHF and G3PDHR primers

Primer type	Primer sequence	Expected size of product
Forward universal bacterial primer (27f), Reverse universal bacterial primer (1525r)	5′-AGAGTTTGATCMTGGCTCAG-3′ 5′-AAGGAGGTGWTCCARCC-3′	1,500 bp
Forward G3PDH primer, Reverse G3PDH primer	5′-ACCACAGTCCATGCCATCAC-3′ 5′-TCCACCACCCTGTTGCTGTA-3′	452 bp

However, for some applications shorter amplicons may be necessary for example for 454 sequencing with Titanium chemistry (500-ish reads are ideal) the primer pair 27F-534R covering V1 to V3.

Universal Bacterial PCR Fact Sheet 2004

PCR amplification

A set of 16S rDNA PCR universal primers

P0 and P6 primers

- To amplify the 16S rDNA of endophytic potentially pathogenic *P. syringae* (pseudomonads), the primers:
- P0 (5‘-GAGAGTTTGATCCTGGCTCAG-3') and
- P6 (5‘-CTACGGCTACCTTGTTACGA-3') (Grifoni *et al.*,1995) were used.
- Also the 16S rRNA primer pair:
- P0 (5’-GAGAGTTTGATCCTGGCTCAG-3’)/
- P6(5’-CTACGGCTACCTTGTTACGA-3’) were used for PCR and sequencing analysis of *P. reactans*.

Scortichini and Loreti,2007;Ansari Dezfooli *et al.*,2013

PCR amplification

A set of 16S rDNA PCR universal primers

P0 and P6 primers

- PCR was assembled with GoTaq® polymerase (Promega, Italy) and Master Mix 5× (Promega, Italy) and the amplification of a region of 16S rRNA gene was performed by using the primers P0 (5′-GAGAGTTTGATCCTGGCTCAG-3′) and P6 (5′-CTACGGCTACCTTGTTACGA-3′).
- PCR amplification was carried out in 50 µL reaction mixtures using 5 µL of 10× buffer, 5 µL of $MgCl_2$ (25 mM final concentration), 5 µL of dNTPs (2 mM final concentration), 1 µL of each primer (10 µM final concentration), 0.5 µL of Taq DNA polymerase at 5 U/µL, 20 µL of DNA extract by microLYSIS® method and dH_2O to the final volume of 50 µL.

The thermal cycling conditions were 94°C for 30 s, 30 cycles of 94°C for 30 s denaturation, 58°C for 30 s annealing, 72°C for 2 min extension, and a final extension step of 72°C for 2 min.

Cappa *et al.*,2014

PCR amplification

A set of 16S rDNA PCR universal primers

P1 and P6 primers

- Total genomic DNA of antagonistic bacterial strain (*Pseudomonas* sp.) were isolated by the alkaline lysis method.
- The primers used for amplification of full length 16S rRNA gene were universal primer:
- P1 (forward primer, 5′-CGGGATCCAGAGTTTGATCCTGGTCAGAACGAACGCT-3′), and
- P6 (reverse primer, 5′-CGGGATCCTACGGCTACCTTGTTACGACTTCACCCC-3′), which correspond to *E. coli* positions 8-37 and 1479-1506, respectively and amplifies 1500 bp fragment.

Reaction mixture (25 µl), prepared for 16S rRNA gene amplification was initially denatured at 94°C for 2 min followed by 25 cycles consisting of 1) denaturation at 94°C for 60 s, 2) primer annealing at 52°C for 60 s and 3) primer extension at 72°C for 3 min and finally, extension at 72°C for 20 min in a thermal cycler.

Tariq *et al.*,2009

PCR amplification

A set of 16S rDNA PCR universal primers

P1 and P6 primers

- A large fragment of the 16S rRNA gene was amplified in a 100-ml reaction mixture by using:

1. Universal forward primer P1 (5′-CGggatccAG AGTTTGATCCTGGTCAGAACGAACGCT-3′, corresponding to positions 8 to 37 in *Escherichia coli* 16S rDNA and
2. Universal reverse primer P6 (5′-C GggatccTACGGCTACCTTGTTACGACTTCACCCC-3′, positions 1479 to 1506).

- The lowercase letters in the primer indicate the restriction site of *Bam*HI.

Forward primer was designed to bind the 8-37 base region, and the reverse primer to bind 1479 to 1506 base region of the 16srDNA gene.

Tan *et al.*,1997

PCR amplification

A set of 16S rDNA PCR universal primers

P1 and P6 primers

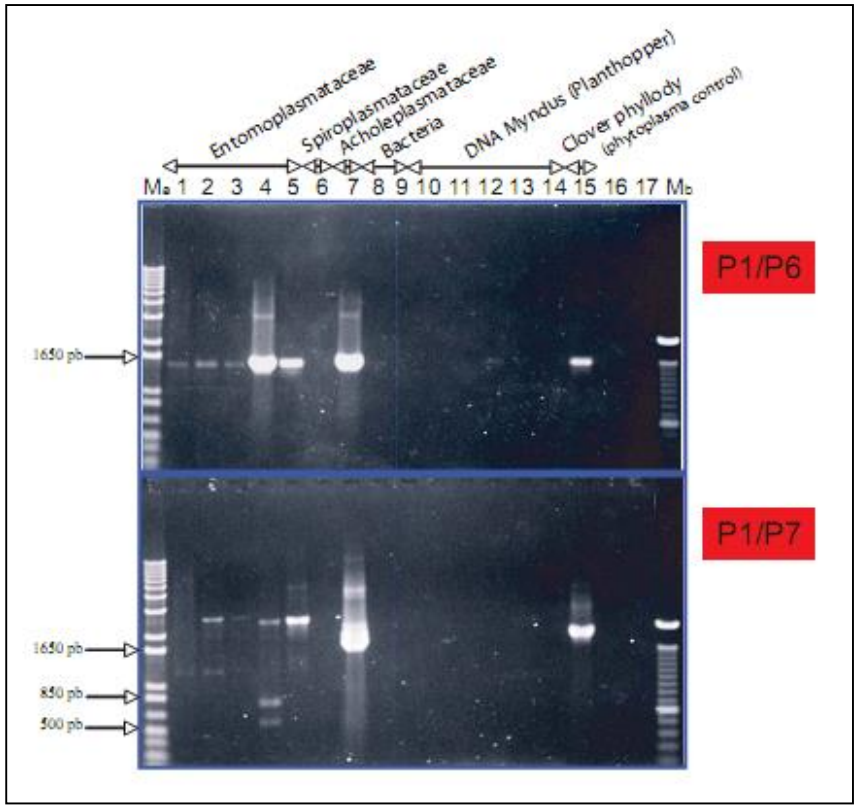
- The P1/P6 universal primers to amplify nearly complete 16S rRNA gene sequence of bacteria such as *Pseudomonas* and *Bacillus* spp., permitted the amplification of an approximate 1,500-bp fragment.
- 16S P1= 5′ AGAGTTTGATCCTGGTCAGAACGCT-3′
- 16S P6= 5′ TACGGCTACCTTGTTACGACTTCACCCC-3′.
- For phytoplasma DNAs, *Leifsonia xyli* and some gram-positive bacteria (Actinomycetales), the sequence of the forward primer (P1) 5‘-AAGAGTTTGATCCTGGCTCAGGATT-3' and the reverse primer (P6) 5'-TGGTAGGGATACCTTGTTACGACTTA-3' was designed.

Tan *et al.*,1997; Chung and Jeong,2003; Tian *et al.*,2009; Wang *et al.*,2011

PCR/nested PCR analyses
Phytoplasma universal primers
P1/P7 and P1/P6

- When using primers P1/P7 and P1/P6

1. not only phytoplasma DNA could be amplified but also
2. some mollicutes, or
3. Some bacterium DNAs.



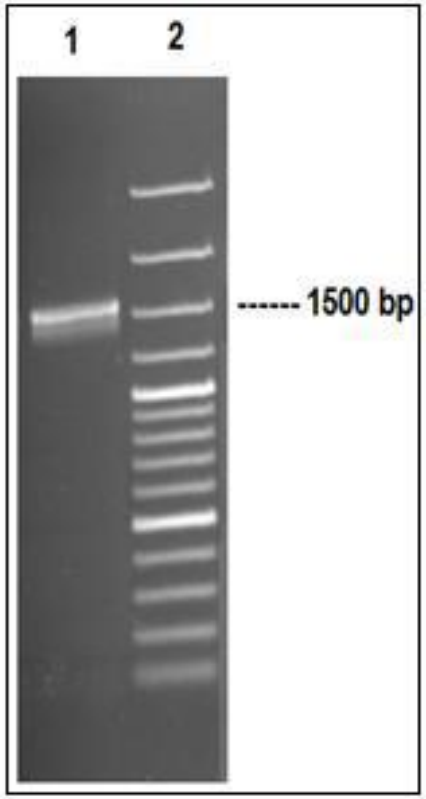
Dollet and co-workers

PCR amplification

A set of 16S rDNA PCR universal primers

8F and 1541R primers

- Bacterial 16S rDNA was amplified by using the universal forward primer
- 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and
- reverse primer 1541R (5'-AAGGAGGTGATCCAGCCGCA-3').
- Agarose gel electrophoresis of 16S rDNA gene PCR product.
- Lane 1: 16S rDNA gene PCR product of bacterial strain Y-2,
- Lane 2: DNA size maker (Gene Ruler 100bp DNA ladder plus, Fermentas).



Soltani Nezhad and Rahbarian,2014

PCR amplification

A set of 16S rDNA PCR universal primers

8F and 1541R primers

- PCR amplification of 16S rRNA gene sequencing of isolated cellulose degrading bacteria was done using universal primer pair consisting of:
- 8F (AGAGTTTGATCCTGGCTCAG) and
- 1541R (AAGGAGGTGGATCCANCCRCA).

Singh *et al.*,2015

PCR amplification

A set of 16S rDNA PCR universal primers

PA and PH primers

- The almost complete 16S rRNA gene sequence was determined for fluorescent pseudomonad strains of clinical origin as well as rain bacteria.
- DNA was amplified using the 16S rRNA universal primers:
- pA (5'-AGAGTTTGATCCTGGCTCAG-3') and
- pH (5'-AAGGAGGTGATCCAGCCGCA-3'), which are complementary to *E. coli* 16S rRNA positions 8-27 and 1544-1525, respectively.
- Amplify 1.6 kb of the small subunit ribosomal RNA (SSU rDNA gene) (Edwards *et al.*,1989).

Dabboussi *et al.*,2002; Laurine Blanchard,2013

PCR amplification

A set of 16S rDNA PCR universal primers

Fd1 and Rd1 primers

- For *Stenotrophomonas*, *Microbacterium*, *Burkholderia*, *Bacillus* and *Pseudomonas*.
- Ribosomal 16S rDNA genes were amplified using the universal bacterial primers:
- Fd1, 5'-CAGAGTTTGATCCTGGCTCAG-3' (forward) and
- Rd1, 5'-AAGGAGGTGATCCAGCC-3' (reverse), corresponding to positions 8 to 28 and 1526 to 1542 from the *Escherichia coli* 16S rDNA gene, respectively (Weisburg *et al.*,1991).

Marquez-Santacruz *et al.*,2010

Primer map for the 16S SSU rRNA gene

The target group of each primer sequences and targets for some of the above primers are forthcoming; the missing primer data can be found in Turner *et al.*,1999.

Primer*	Sequence (5'-3')	Target Group	Reference
8F	AGAGTTTGATCCTGGCTCAG	Universal	Turner et al. 1999
27F	AGAGTTTGATCMTGGCTCAG	Universal	Lane et al. 1991
CYA106F	CGGACGGGTGAGTAACGCGTGA	Cyanobacteria	Nübel et al. 1997
CC [F]	CCAGACTCCTACGGGAGGCAGC	Universal	Rudi et al. 1997
357F	CTCCTACGGGAGGCAGCAG	Universal	Turner et al. 1999
CYA359F	GGGGAATYTTCCGCAATGGG	Cyanobacteria	Nübel et al. 1997
515F	GTGCCAGCMGCCGCGGTAA	Universal	Turner et al. 1999
533F	GTGCCAGCAGCCGCGGTAA	Universal	Weisburg et al. 1991
895F	CRCCTGGGGAGTRCRG	Bacteria exc. plastids & Cyanobacteria	Hodkinson & Lutzoni 2009
16S.1000.F16	CAACGAGCGCAACCCT	Universal	Turner et al. 1999
CYA781R	GACTACWGGGGTATCTAATCCCWTT	Cyanobacteria	Nübel et al. 1997
CD [R]	CTTGTGCGGGCCCCCGTCAATTC	Universal	Rudi et al. 1997
902R	GTCAATTCITTTGAGTTTYARYC	Bacteria exc. plastids & Cyanobacteria	Hodkinson & Lutzoni 2009
904R	CCCCGTCAATTCITTTGAGTTTYAR	Bacteria exc. plastids & Cyanobacteria	Hodkinson & Lutzoni 2009
907R	CCGTCAATTCMTTTRAGTTT	Universal	Lane et al. 1991
1185mR	GAYTTGACGTCATCCM	Bacteria exc. plastids & Cyanobacteria	Hodkinson & Lutzoni 2009
1185aR	GAYTTGACGTCATCCA	Lichen-associated Rhizobiales	Hodkinson & Lutzoni 2009
1381R	CGGTGTGTACAAGRCCYGRGA	Bacteria exc. *Asterochloris* sp. plastids	Hodkinson & Lutzoni 2009
1381bR	CGGGCGGTGTGTACAAGRCCYGRGA	Bacteria exc. *Asterochloris* sp. plastids	Hodkinson & Lutzoni 2009
1492R (l)	GGTTACCTTGTTACGACTT	Universal	Turner et al. 1999
1492R (s)	ACCTTGTTACGACTT	Universal	Lane et al. 1991

primer, an equimolar mix of all of the constituent primers implied by the degenerate sequence is recommended, since machine mixes are generally not guaranteed to approximate equimolarity. Primers developed by members of the Lutzoni Lab are in bold.

Lutzoni Lab; Bélisle,2010

PCR amplification

A set of 16S rRNA gene universal primers

Primer name	Sequence (5'-3')
B27F	AGA GTT TGA TCC TGG CTC AG
U1492R	GGT TAC CTT GTT ACG ACT T
928F	TAA AAC TYA AAK GAA TTG ACG GG
336R	ACT GCT GCS YCC CGT AGG AGT CT
1100F	YAA CGA GCG CAA CCC
1100R	GGG TTG CGC TCG TTG
337F	GAC TCC TAC GGG AGG CWG CAG
907R	CCG TCA ATT CCT TTR AGT TT
785F	GGA TTA GAT ACC CTG GTA
805R	GAC TAC CAG GGT ATC TAA TC
533F	GTG CCA GCM GCC GCG GTA A
518R	GTA TTA CCG CGG CTG CTG G

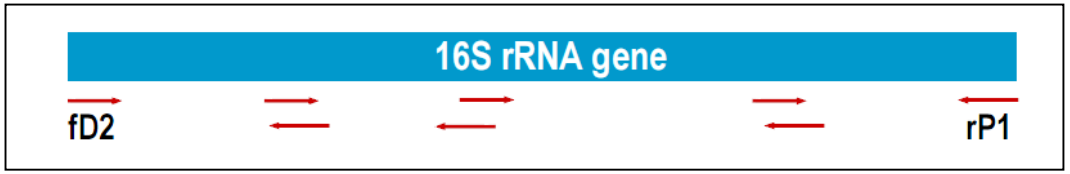
Wikipedia,2012

PCR amplification

16S rRNA sequencing

A set of universal bacterial primers

- The universal prokaryote 16S rRNA primer pair fD2/rP1 amplifies plant and bacteria (Weisburg *et al.*,1991).
- fD2/rP1 also used in combination with range of specific 16S rRNA PCR primers. E.g. primer pair LSg2f/rP1 was used to amplify the 16S rRNA gene from syndrome "basses richesses" BLO (Liberibacter) of sugar beet.
- A 16S rDNA fragment of about 1.5 Kb was amplified by PCR using universal primers fD2 and rP1 and sequenced.
- fD2/rP1 also amplifies 16S rRNA in *Xyllela fastidiosa*.



Name	Sequence (5´-3´ direction)	Purpose[a]
fD2	AGAGTTTGATCATGGCTCAG	Amplification of the 16S rRNA gene (T_A: 42°C)
rP1	ACGGTTACCTTGTTACGACTT	
16S-3	ATATTGCACAATGGGCGC	Sequencing of the amplified 16S rRNA gene
16S-5	GCCATTGTAGCACGTGTGTAG	

[a] T_A: Annealing temperature used for PCR.

Liefting *et al.*,2009; Rozhon 2010;

PCR amplification

Comparative evaluation of four bacteria-specific primer pairs for 16S rRNA gene surveys

16S rRNA gene V regions

- The primer pair 341f/785r had the highest coverage for Bacteria representing 96.51% of the 159,615 bacterial sequences in the SILVA v123 database that were expected to be amplified.
- The second best Bacterial domain coverage is expected from 799f/1193r (85.55%), followed by 68f/518r (78.59%), and 967f/1391r (66.54%).

Primer pair[a]	Sequence (5′-3′)	Position in *E. coli*[a]	V-regions	Amplicon size	Domain coverage (%)[b]			References
					Bact.	Archaea	Eukaryota	
68f	TNANACATGCAAGTCGRRCG	49–68	V1–V3	450	78.59	1.37	0.001	McAllister et al., 2011
518r	WTTACCGCGGCTGCTGG	518–534						Lee et al., 2010
341f	CCTACGGGNGGCWGCAG	341–357	V3–V4	444	96.51	82.96	0.14	Klindworth et al., 2013
785r	GACTACHVGGGTATCTAATCC	785–805						
799f	AACMGGATTAGATACCCKG	781–799	V5–V7	394	85.55	0	0	Chelius and Triplett, 2001
1193r	ACGTCATCCCCACCTTCC	1177–1194						Bodenhausen et al., 2013
967f	CAACGCGAAGAACCTTACC	967–985	V6–V8	424	66.54	0.007	0.03	Sogin et al., 2006
1391r	GACGGGCGGTGWGTRCA	1391–1407						Walker and Pace, 2007

Primer sequence, 16S rRNA gene variable region, amplicon size, domain coverage rate and literature reference.
[a]*Primer position according to E. coli numbering (Baker et al., 2003) umulative-sum-scal.*
[b]*Primer pair coverage was calculated using PrimerProspector 1.0.1 according to the non-redundant SILVA SSU r123 database and the weighted score method (Walters et al., 2011).*

Thijs *et al.*,2017

PCR amplification

Comparative evaluation of four bacteria-specific primer pairs for 16S rRNA gene surveys

16S rRNA gene V regions

- Primer pair 341f/785r produced the highest number of bacterial OTUs, phylogenetic richness, Shannon diversity, low non-specificity and most reproducible results, followed by 967f/1391r and 799f/1193r.
- Primer pair 68f/518r showed overall low coverage and a bias toward Alphaproteobacteria.
- All primer sets were found to be highly specific for the domain Bacteria as almost no Archaea and Eukaryotic reads were amplified, and the theoretical coverage for the domains Archaea and Eukaryota was low (<1.37%).

Thijs *et al.*,2017

PCR amplification

16S/23S rRNA sequencing

A set of universal bacterial primers

- 27f 5'AGAGTTTGATCMTGGCTCAG
- 342r 5'CTGCTGCSYCCCGTAG
- 357f 5'CTCCTACGGGAGGCAGCAG
- 519r 5'GWATTACCGCGGCKGCTG
- 530f 5'GTGCCAGCMGCCGCGG
- 1100r 5'GGGTTGCGCTCGTTG
- 1114f 5'GCAACGAGCGCAACCC
- 1392r 5'ACGGGCGGTGTGTRC
- 1406f 5'TGYACACACCTCCCGT
- 1492r 5'TACGGYTACCTTGTTACGACTT
- 1525r 5'AAGGAGGTGWTCCARCC

Lane,1991;Wikipedia,2009

PCR amplification

PCR master mix and reaction condition

Using the universal primers

- Label the top of a sterile 0.2 mL microcentrifuge tube with your initials.
- Pipet 49 µl of the "PCR mix" in your ice bucket into the 0.2 mL microcentrifuge tube.
- The PCR mix contains the forward and reverse primers, dNTPs, Taq polymerase, $MgCl_2$ and PCR reaction buffer.
- To the PCR mix add 1 µL of the cell solution from step 7 above. (Note how little of the template solution we are adding.)
- Your sample is now ready for amplification. Your instructor or TA will collect your reaction tube for amplification with the rest of the section.
- The reaction is conducted in a PCR machine (also known as a thermal cycler). The reaction will proceed as follows:
  - 1 cycle 94°C for 3 minutes (Denature);
  - followed by 40 cycles 94°C for 1 minute (Denature);
  - 50°C for 1 minutes (Anneal);
  - 72°C for 1 minutes (Elongation).

Bednarski,2006

PCR amplification

Using the universal primers of 27F/1492R

- **For the eubacterial domain, 16S rDNA:**
- 27F, 5'-AGAGTTTGATCMTGGCTCAG-3';
- 1492R, 5'-GGTTACCTTGTTAC GACTT-3

1. Primers (final concentration 0.5 mM).
2. Taq polymerase and 10X STR buffer (Promega).
3. Thermal cycler (PCR).
4. TAE buffer (1X): 0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0.
5. 1% and 2% agarose gel in 1X TAE buffer.
6. Ethidium bromide staining solution in 1X TAE buffer.
7. Molecular weight markers: 1 kb and 100 bp DNA Ladders (Promega).
8. Loading buffer (Promega).
9. Sterile-distilled water.

Spencer and Spencer,2004

PCR amplification

Using the universal primers of 27F/907R

- 16S rRNA gene sequence determination:
- Primer 27F : 5′-AGAGTTTGATCMTGGCTCAG-3′
- Primer 907R : 5′-CCGTCAATTCMTTTGAGTTT-3′

	Initial concentration	Final concentration	
Buffer	10 ×	1 ×	2.50 µl
$MgCl_2$	50 mM	2 mM	1.00 µl
Primer 27F	20 µM	1 µM	1.25 µl
Primer 907R	20 µM	1 µM	1.25 µl
dNTPs	4 mM	200 µM	1.25 µl
Taq	5 U	2 U	0.40 µl
DNA sample			5.00 µl
Water			12.35 µl
Total			25 µl

Thermal cycling consists of an initial denaturation at 95°C for 10 min to allow activation of the Taq polymerase, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min and extension at 72°C for 2 min, and a final extension at 72°C for 10 min.
The amplification of PCR product in 2.0 % (w/v) agarose gel electrophoresis is 900 bp.
Used this PCR product for direct sequencing.

Nazerian,2010

PCR amplification

Degenerate primers

Primer modifications

- Primers can also be designed to be 'degenerate' – able to initiate replication from a large number of target locations.
- A PCR primer sequence is called degenerate if some of its positions were several possible bases.
- For example, in the primer GG{CG}A{CTG}A, the third position in C or G and the fifth is C,T or G.
- Thus, degenerate primers having a number of nucleotide options at several positions in the internal primer sequence which allow annealing to and amplification of a variety of related sequences.

Ben-Dov *et al.*,2006;..

PCR amplification

Degenerate primers vs. non-degenerate primers

Applications

1. Non-degenerate primers (ordinary primers) don't amplify many of unknown genes, whereas
2. Degenerate primers were designed to amplify a set of DNA sequences.
3. Degenerate primers are widely used and extremely useful in the field of microbial ecology.

- They allow for the amplification of genes from thus far:

1. Uncultivated microorganisms, or
2. Allow the recovery of genes from organisms where genomic information is not available.

Ben-Dov *et al.*,2006; Wikipedia,2012

PCR amplification

Degenerate primers

Utility of inosine at the 3′-terminus of 16SrRNA universal primers

- 8F and 907R are one of the known universal 16S rDNA primers.
- Here the 3′-terminus of both primers were substituted by inosine (8F-I and 907R6).
- The degenerate base inosine can pair with all four natural nucleotides (bases) of T, A, G or C.
- In this way, degenerate primers (containing degenerate bases) enhance universality of the primers for amplification of related sequences of 16S rRNA genes from different microorganisms.
- And greatly reduce the specificity of the PCR amplification.

PCR amplification

Degenerate arbitrary primers

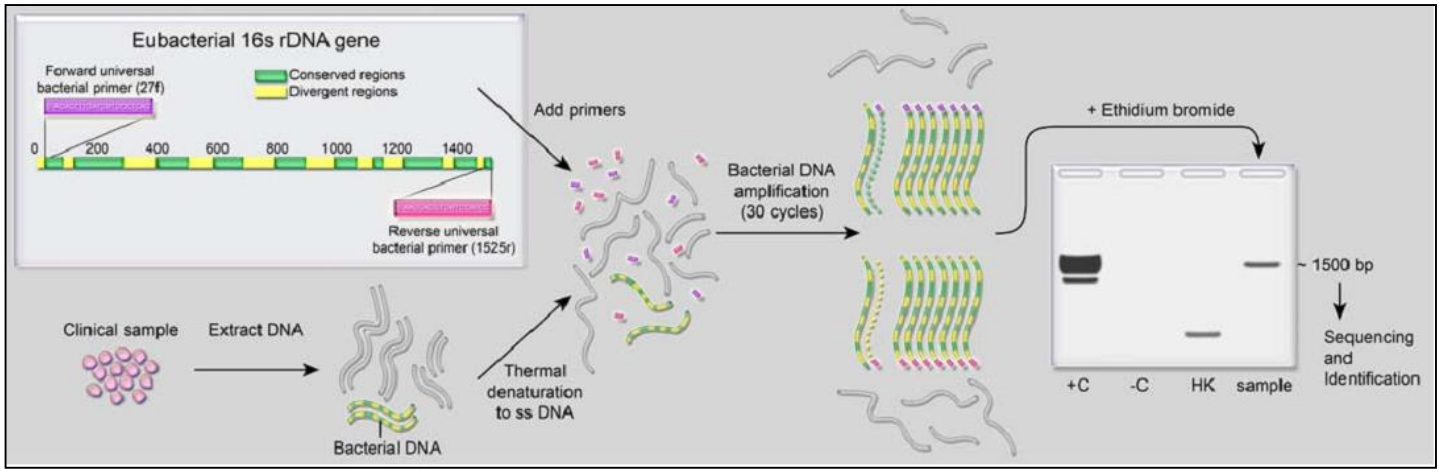
Inosine-containing degenerate primers

- The ability of inosine to act as a degenerate base makes it particularly useful as a way to reduce the overall degeneracy of degenerate PCR primer sets.
- Since inosine is capable of base-pairing with any natural nucleotide, it can be used to substitute I for any natural nucleotide or "N" (A,C,G,T) degenerate position.
- An example of degenerate arbitrary primers:

  5’-TCTTICGNATCTTNGGA-3’

PCR amplification

4. Optimization of PCR condition for 16S rRNA genes



Universal Bacterial PCR Fact Sheet, 2004

PCR amplification

Optimization of PCR condition for 16S rRNA genes

dNTPs, $MgCl_2$, annealing temperatures

- Various concentrations of dNTPs, $MgCl_2$ at different annealing temperatures were optimized to get best results of gene amplification using mastercycler gradient.
- DNA and annealing temperatures were optimized for the specific amplification full length 16S ribosomal gene.

Tareq *et al.*,2008

PCR amplification

Optimization of PCR condition for 16S rRNA genes

Characteristics of ideal primers

- The ideal primer generally has the following characteristics:

1. Usually a 20-24 nt primer works well.
2. Melting temperature (T_m) between 55 and 65°C (usually corresponds to 45-55% G+C for a 20-mer). A T_m of 55-65°C works best in most applications.

- Remember that you are dealing with TWO primers in PCR.
- Their T_m's should be within 5°C of each other; the closer the better! If T_m's are mismatched, amplification will be inefficient: the primer with the higher T_m will misprime at lower temperatures, while the primer with the lower T_m may not work at higher temperatures.

Primer guidelines, Howard Judelson,2006

PCR amplification

Optimization of PCR condition for 16S rRNA genes

Characteristics of ideal primers

3. The annealing temperature (T_a) for a primer pair is generally calculated as 5°C lower than the estimated melting temperature.
4. Avoid primers with long polyG or polyC stretches that can promote non-specific annealing.
5. Avoid polyA and polyT as these will "breath" and open the primer-template complex.
6. Avoid polypyrimidine (T, C) and polypurine (A, G) stretches, which may lead to an odd shape of the double helix.

Primer guidelines, Howard Judelson,2006

PCR amplification

Other house keeping genes rather than 16S rRNA gene

- Non protein-coding housekeeping:

1. 16S rRNA gene. Unable to separate some species.

- Some protein-coding housekeeping genes:

1. *gyrB* (gene) and its protein(DNA gyrase, beta subunit),
2. *rpoD* (RNA polymerase, σ^{70} factor),
3. *rpoD* (RNA polymerase β-subunit gene)
4. *dnaK* (Chaperone protein DnaK),
5. *trpB* (tryptophan synthase, beta subunit), and
6. *recA* (recombinase A).

Like the 16S rRNA gene, housekeeping genes such as rpoD, rpoB,.. are essential and ubiquitous genes universally present in the bacterial kingdom. DNA gyrase is a type II topoisomerase found in all bacteria and is essential for forming negative supercoils.

PCR amplification

Other house keeping genes rather than 16S rRNA gene *rpoD* (σ^{70} factor)

- Different bacterial species exhibit large differences in the number of alternative sigma factor encoding genes and consequently huge flexibility in their transcriptional regulatory patterns.
- The rpoD is the gene encoding the sigma 70 subunit of RNA polymerase.
- The rpoB gene, encoding the beta-subunit of RNA polymerase, has been postulated to be a good candidate for phylogenetic analysis and identification of bacteria.

Mittenhuber,2002;..

PCR amplification

Other house keeping genes rather than 16S rRNA gene *rpoD* (σ^{70} factor)

- Distribution pattern of sigma factors belonging to the s^{70}-family, the ECF (extracytoplasmic stimuli) subfamily and the s^{54}-family within 31 bacterial species.
- Additionally, information on the phylogenetic position, habitat and the genome size of each species is given.
- Two quotients "genome size divided by number of sigma factors in genome" (quotient 1) and "total number of genes divided by number of sigma factors in genome divided by 100" (quotient 2) were calculated.

Mittenhuber,2002

PCR amplification

Other house keeping genes rather than 16S rRNA gene

rpoD (σ^{70} factor)

Name	Abbreviation	Phylogenetic position	Habitat and features	free living	Genome size; number of genes	Total number of sigma factors	Number of sigma factors in σ^{70} family	Number of sigma factors in σ^{54} family	Number of ECF sigma factors	Quotients 1; 2
Aquifex aeolicus	Aae	Aquificales	marine, deep sea vents; chemolithoautotroph, hyperthermophilic (96°C)	Yes	1.55 Mb; 1522	4	3	1	0	0.39; 0,38
Bacillus halodurans	Bha	Low GC gram positive	alkaliphilic organism from deep sea sediment	Yes	4.2 Mb; 4066	19	8	1	10	0.22; 0.21
Bacillus subtilis	Bsu	Low GC gram positive	soil, association with plants	Yes	4.21 Mb; 4221	18	10	1	7	0.23; 0.23
Borrelia burgdorferi	Bbu	Spirochaetales	arachnid and mammalian hosts	No	0.91 Mb; 903	3	2	1	0	0.30; 0.30
Buchnera sp. APS	Bsp	α-subdivision of proteobacteria	symbiont of pea aphid	No	0.64 Mb; 564	2	2	0	0	0.32; 0.28
Campylobacter jejuni	Cje	ε-subdivision of proteobacteria	involved in food poisoning, especially in contaminated poultry; causes inflammatory enterocolitis	No	1.64 Mb, 1731	3	2	1	0	0.55; 0.58
Caulobacter crescentus	Ccr	α-subdivision of proteo-bacteria	nutrient poor aquatic habitats	Yes	4.02 Mb; 3794	17	2	1	14	0.24; 0.22
Chlamydia trachomatis MoPn	Ctr	Chlamydiales	mouse pathogen (pneumonitis)	No	1.04 Mb; 825	3	2	1	0	0.35; 0.28
Clostridium acetobutylicum	Cac	Low GC grampositive	soil, anaerobic spore former	Yes	3.94 Mb; 4023	14	10	1	3	0.28; 0.29
Deinococcus radiodurans	Dra	*Thermus/ Deinococcus* group	isolated from environments rich in organic nutrients; as well as from nutrient poor habitats	Yes	2.65 Mb; 3174	2	1	0	1	1.33; 1.59
Escherichia coli K12	Eco	γ-subdivision of proteobacteria	colonizes lower gut of animals, survives release into environment	Yes	4.64 Mb; 4290	7	4	1	2	0.66; 0.61

Mittenhuber,2002

PCR amplification

Other house keeping genes rather than 16S rRNA gene *gyrA* and *gyrB* genes

- DNA topoisomerases(enzymes/proteins) are divided into two classes:

1. type I enzymes (topoisomerases I, III and V), and
2. type II enzymes (topoisomerases II, IV and VI).

- Type II topoisomerases DNA gyrase (encoded by genes *gyrA* and *gyrB*) and topoisomerase IV (encoded by genes *parC* and *parE*).
- Topoisomerase II(called gyrase in bacteria) an essential topoisomerase found in all bacteria and responsible for forming negative supercoils.

See also the phylogeny file

PCR amplification

Other house keeping genes rather than 16S rRNA gene

gyrA and *gyrB* genes

- DNA gyrase consists of two subunits, GyrA and GyrB in bacteria.
- *gyrA* gene produces DNA gyrase protein subunit A (protein GyrA).
- *gyrB* gene produces DNA gyrase protein subunit B (protein GyrB).
- DNA gyrase was initially discovered in *E. coli* in 1976 (Gellert *et al.*,1976a).
- These genes (*gyrA* and *gyrB*) are organized differently in different bacteria.

See also the phylogeny file

PCR amplification

Protein-coding housekeeping genes

gyrB gene

- The *gyrB* gene provides accurate and faster results than 16S rRNA gene sequence to identify bacterial species and subspecies (Yamamoto *et al*.,1999).
- The gyrB gene sequences from different bacterial groups such as *Pseudomonas* (Yamamoto *et al*.,1999), *Acinetobacter* (Yamamoto *et al*.,1999), *Mycobacterium* (Kasai *et al*., 2000), *Bacillus thuringiesis* (La Duc *et al*.,2004), and *E. coli* (Fukushima *et al*.,2002) have been used for phylogenetic tree analysis.

PCR amplification

Protein-coding housekeeping genes

gyrB gene

- However, 16S rRNA gene sequence is also helpful for the identification of bacterial species (Joung, 2002).
- Screening of *Bacillus* species using gyrB gene sequence is an initial process to identify bacteria at the subspecies level.

PCR amplification

Protein-coding housekeeping genes

gyrB gene

- Identification of PGPR *Bacillus amyloliquefaciens* subsp. *plantarum* with gyrB universal primers (UP-1 & UP-2r).
- PCR amplification program:
- Denaturation at 95°C for 30 seconds. The second step was lowering the temperature at 65°C to allow primers to anneal to complementary sequences for 30 seconds, and the third step was to synthesize of DNA strand through DNA polymerase enzymes to create double strand called primer extension at 72°C for 40 seconds. These steps were repeated 15 times. In the second step, the annealing temperature was changed to 50°C. The remaining temperatures were the same and were repeated 30 times. The final stage was a primer extension at 72°C for 5 minutes.

PCR amplification

Protein-coding housekeeping genes

gyrB gene

- **gyrase B gene sequences:**
- The PCR DNA product size was ~1.2kb, and gyrB gene amplified successfully using UP-1 and UP-2r universal primers.

PCR amplification

Primer G3 and G4 were used to detect *gyrB* gene in *P. syringae* pv. *aesculi*

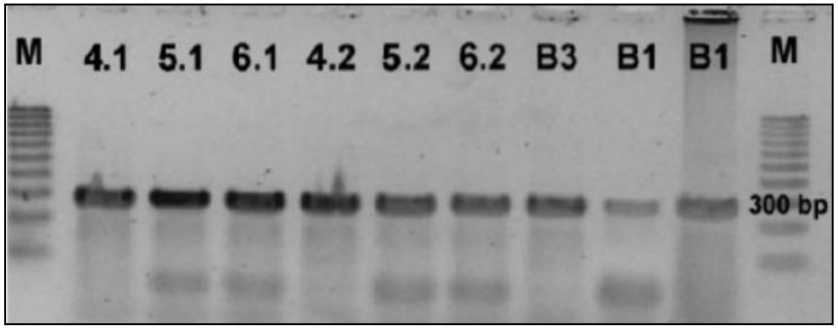
- There is a great similarity in the gyrase B gene sequences of the numerous closely related *P. syringae* pathovars.
- Polymerase chain reaction (3 h) was performed with the Qiagen Tag Core Kit.
- PCR primers G3 and G4 were designed from the gyrase B gene sequence of *P. syringae* pv. *aesculi*, the causal agent of stem bleeding on horse chestnut trees.
- Prior to primer design, available gyrase B gene sequences were aligned with ClustalX to detect DNA regions of preference for the pathovar *aesculi*.
- The G3 forward primer is 5'-CGTCGGTGAAAGTGATACCAC-3' and G4 reverse primer is 5'-CAGCGCAATCTCGACACCAAT-3'.
- Primers enclose a DNA region of 256 bp. The total length of the PCR product should be 298 bp.

Schmidt *et al.*,2009

PCR amplification

Primer G3 and G4 were used to detect *gyrB* gene in *P. syringae* pv. *aesculi*

- **Partial gyrase B gene sequences:**
- Gel with DNA bands of the partial gyrase B gene from *Pseudomonas syringae* pv. *aesculi* obtained with primers G3 and G4.
- Lanes 4 to 6 = tree number (cf. Table 1).
- Lanes 1 and 2 = first and second DNA elution.
- B1 = unpurified bacteria outgrown from sawdust of tree 4 using different template amounts.
- B3 = pure culture of *P. syringae* pv. *aesculi* from the isolation in 2007.
- M = Marker.



Schmidt *et al.*,2009

PCR amplification

Protein-coding housekeeping genes

gyrB and *rpoD* genes in PGPR

- Pairs of primers used for identification of tomato-associated rhizobacteria and detection of antibiotic biosynthesis genes.
- The PCR DNA product size was 500 bp for both:
- gyrB gene, and
- rpoD (also known as sigma 70) gene.

Primer name	Sequence	Amplicon size (pb)	Annealing Temperature (°C)
799f	AACMGGATTAGATACCCKG	400	52
1492r	GTTACCTTGTTACGACTT	400	52
rpoB-f	GACGATCATYTWGGAAACCG	500	52
rpoB-r	GGNGTYTCRATYGGACACT	500	52
gyrB-f	AAAACAACCRATTCATGAAG	500	52
gyrB-r	TCGCTTCACTATTYCCAAGT	500	52
FENA1F	GACAGTGCTGCCTGATGAAA	757	54
FENA1R	GTCGGTGCATGAAATGTACG		
FENB1F	CAGCCGCTGTCAACAAGATA	950	54
FENB1R	ACACGACATTGCGATTGGTA		
FEND1F	TTTGGCAGCAGGAGAAGTTT	964	53
FEND1R	GCTGTCCGTTCTGCTTTTTC		
FENE1F	GCCAAAAAGAAACGAGCAAG	756	53
FENE1R	GTCGGAGCTAACGCTGAAAC		
BACC1F	GAAGGACACGGCAGAGAGTC	814	60
BACC1R	CGCTGATGACTGTTCATGCT		

Abdeljalil *et al.*,2016

PCR amplification

Optimization of PCR condition for 16S rRNA genes

Sensitivity and specificity of the primers

- When we use a pair of particular primers e.g. Psf/Psr (*Pseudomons* genus specific primer pair) and get different reactions to the primers, we need to determine the specificity and sensitivity of the used primers by following equations(De Bore and Beuman,1999;Maráz *et al.*,2006):

$$\text{Sensitivity (\%)} = \frac{\text{number of true positives (p)}}{\text{p + number of false negatives}} \times 100$$

$$\text{Specificity (\%)} = \frac{\text{number of true negatives (n)}}{\text{n + number of false positives}} \times 100$$

- Imagine 14 *Pseudomons* spp. were analyzed by PCR, of which 2 were negative in PCR reaction. On the other hand, 6 non-*Pseudomons* strains were amplified by the same primer(*Pseudomons* genus specific primer pair).
- It means the sensitivity of the primers was 87.5% while the specificity was only 66.6%. Such primers with low specificity values were not further used for preliminary screening of *Pseudomonas* spp.

Márta *et al.*,2011

PCR amplification

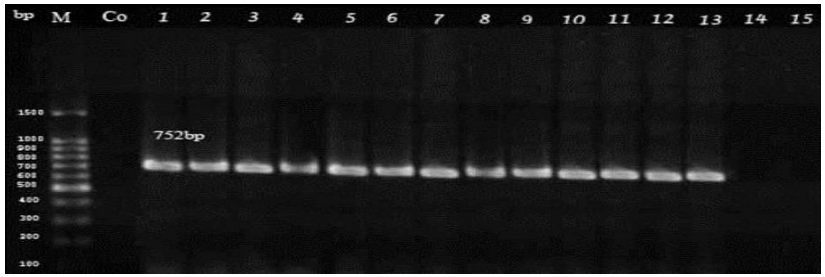
Molecular detection of syringomycin related genes

Primer B1 and B2 were used to detect *syrD* or *syrB* gene

- The primers B1 and B2 locate into the open reading frame of the *syrB* gene and yield a 752-bp product (Sorensen *et al.*,1998).

Components	Volume of one reaction	n x Reaction
Deionise distil water	15.8µl	n x15.8µl
10x buffer with $Mgcl_2$	2.5µl	n x 2.5µl
dNTP mixture	2.5µl	n x 2.5µl
Reverse primer	1µl	n x 1µl
Forward primer	1µl	n x 1µl
Taq polymerase	0.2µl	n x 0.2µl
Made a master mix and then sub-divided as 23µl aliquots to n eppendorf tubes		
Sample DNA	2µl to each respective tube	
Total	25µl final reaction volume each	

The component required for specific primers syrB1 and syrB2 amplification.



Agarose gel Electrophoresis of PCR with primers B1 and B2 corresponding to gene *syrB*. Co: Negative control, 1-13: *Pseudomonas syringae* pv. *syringae* strains 752bp, 14 &15 strains of *P. syringae* , M: 100-1500bp DNA molecular marker.

Wazeer *et al.*,2014

PCR amplification
Detecting PCR products

- Take 1/10th-1/3rd of the reaction mix **CAREFULLY** from under the oil or from under the Vaseline or solidified wax, using a micropipette with plugged tip.
- Mix this with some gel loading buffer (1:1- 1:5 mix: loading buffer): This is TBE containing 10-20% glycerol or sucrose and a dash of bromophenol blue (BPB) tracking dye.
- Load 5-30 µl of sample into wells of 0.8-3.0% submarine agarose gel made up in TBE, preferably containing 5 µg/ml ethidium bromide.
- Run at 80-120 volts (not too slow or small products diffuse; not too fast or bands smear) until BPB reaches end of gel (large products) or 2/3 down gel (small products).
- Use DNA markers going from 2kb down to 100 bp or less (recommend BM PCR markers).
- View on UV light box at 254-300 nm, photo 1-5 sec.

Agarose gel electrophoresis of DNA
How much DNA should be loaded?

- For PCR reactions, it depends on the PCR but in routine applications 10-20 µL should be plenty to see the product on the gel.
- Typically, a band is easily visible if it contains about 20 ng of DNA.
- **Too much DNA** loaded onto a gel is a bad thing.
- The band appears to run fast (implying that it is smaller than it really is) and in extreme cases can mess up the electrical field for the other bands, making them appear the wrong size also.
- **Too little DNA** is only a problem in that you will not be able to see the smallest bands because they are too faint.

Lewis,2001

Gel electrophoresis

Staining dyes

- Different stains and staining procedures are used for different classes of macromolecules:
- Staining DNA
  - DNA is stained with ethidium bromide (EtBr), which binds to nucleic aids. The DNA-EtBr complex fluoresces under UV light.
- Staining RNA
  - RNA is stained with ethidium bromide (EtBr), which binds to nucleic aids. The RNA-EtBr complex fluoresces under UV light.
- Staining Protein
  - Protein is stained with Coomassie Blue (CB). The protein-CB complex is deep blue and can be seen with visible light.

Gel electrophoresis

Nucleic acid gel stains

1. Silver nitrate solution

- Silver nitrate solution (for electrophoresis staining):
- Dissolve 0.15 grams of NaOH in 150 ml of water.
- Add 3.5 ml of concentrated NH_4OH (Ammonium Hydroxide)and bring to a volume of 200 ml.
- Separately, dissolve 2.0 grams of silver nitrate in a final volume of 10 ml.
- With constant stirring, add 8.0 ml of the silver nitrate to the 200 ml of $NaOH/NH_4OH$.
- This solution should be prepared immediately prior to use, and used within 30 minutes.

Spencer and de Spencer,2004

Gel electrophoresis

Nucleic acid gel stains

2. Ethidium bromide

- The most convenient method to visualize DNA in gel electrophoresis is staining with the fluorescent dye ethidium bromide.
- Ethidium bromide is usually prepared as a stock solution of 10 mg/ml in water, stored at room temp and protected from light.
- The dye is usually incorporated into the gel and running buffer, or conversely, the gel is stained after running by soaking in a solution of ethidium bromide (0.5 µg/ml for 30 min).
- The stain is visualized by irradiating with a UV light source (i.e. using a transiluminator) and photgraphing with polaroid film.
- The usual sensitivity of detection is better than 0.1 µg of DNA.

Gel electrophoresis

Nucleic acid gel stains

3. Gel red

- Because ethidium is a DNA intercalating agent, it is a powerful mutagen.
- Incorporation of ethidium in the DNA of living organisms (i.e. you and I) can cause (unwanted) mutations.
- Gel Red is an innovative, stable, non-hazardous, and environmentally safe fluorescent nucleic acid dye designed by Biotium as an alternative to toxic ethidium bromide (EtBr) for staining dsDNA, ssDNA and RNA in agarose gels andpolyacrylamide gels.

Agarose gel electrophoresis of DNA
What percentage gel?

- Agarose gel electrophoresis can be used for the separation of DNA fragments ranging from 50 base pair to several megabases (millions of bases) using specialized apparatus.
- Increasing the agarose concentration of a gel reduces the migration speed and enables separation of smaller DNA molecules.
- The distance between DNA bands of a given length is determined by the percent agarose in the gel.

Agarose gel electrophoresis of DNA
What percentage gel?

- In general lower concentrations of agarose are better for larger molecules because they result in greater separation between bands that are close in size.
- The disadvantage of higher concentrations is the long run times (sometimes days).
- Instead high percentage agarose gels should be run with a pulsed field electrophoresis (PFE), or field inversion electrophoresis.
- Prepared agarose gel should be stored in an airtight container (e.g., a screw-topped bottle) to prevent desiccation.

Agarose gel electrophoresis of DNA
What percentage gel?

1. 1% gels are common for many applications.
2. 0.7%: good separation or resolution of large 5-10kb DNA fragments;
3. 2% good resolution for small 0.2-1kb fragments;
4. Up to 3% can be used for separating very tiny fragments but a vertical polyacrylamide gel is more appropriate in this case.

- Shorter molecules move faster and migrate farther than longer ones.

Agarose gel electrophoresis of DNA
What percentage gel?

- Most agarose gels are made between 0.7% and 2%.
- A 0.7% gel will show good separation (resolution) of large DNA fragments (5-10 kb) and a 2% gel will show good resolution for small fragments (0.2-1 kb).
- Some people go as high as 3% for separating very tiny fragments but a vertical polyacrylamide gel is more appropriate in this case.
- Low percentage gels are very weak and may break when you try to lift them.
- High percentage gels are often brittle and do not set evenly.
- I usually make 1% gels.

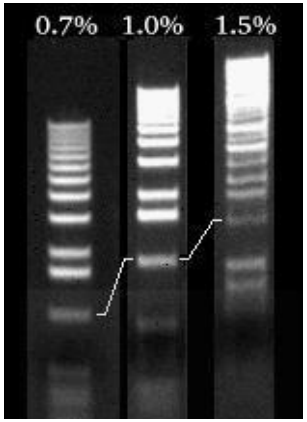
Lewis,2001

Agarose gel electrophoresis of DNA
What percentage gel?

Recommended % Agarose	Optimum Resolution for Linear DNA
0.5	1,000–30,000bp
0.7	800–12,000bp
1.0	500–10,000bp
1.2	400–7,000bp
1.5	200–3,000bp
2.0	50–2,000bp

Agarose gel electrophoresis of DNA
What percentage gel?

- Typical value for running an agarose gel is 5 volts per cm (length of gel).



Agarose (%)	Range of separation of linear DNA (in kilobases)
0.3	60 - 5
0.6	20 - 1
0.7	10 - 0.8
0.9	7- 0.5
1.2	6 - 0.4
1.5	4 - 0.2
2.0	3 - 0.1

zampbioworld.org.,2011;..

Gel electrophoresis

Molecular weight standards

DNA/RNA markers (molecular ladders) or simply marker

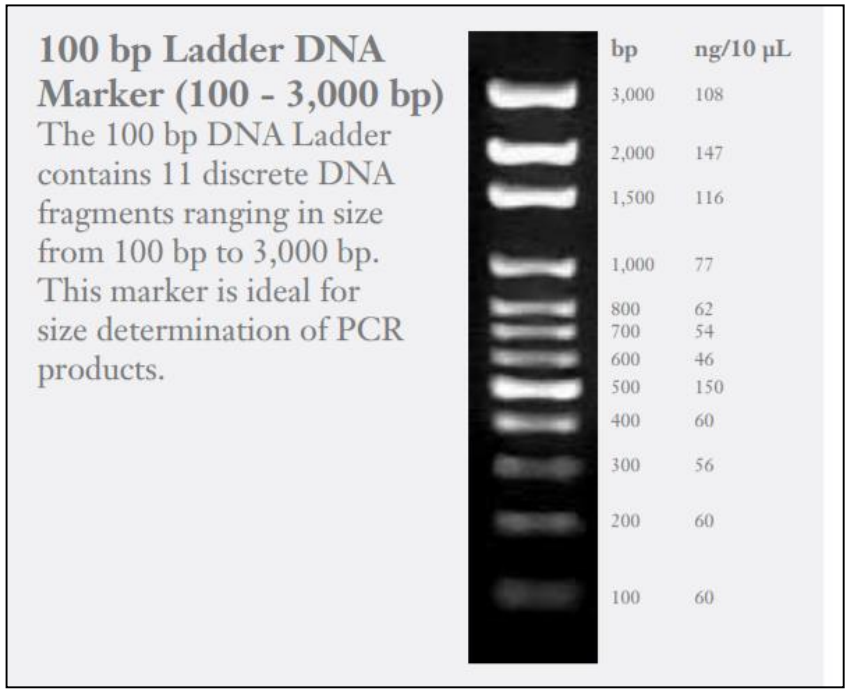
- A DNA ladder usually contains a set of known DNA fragments with different sizes in base pairs (bp) or kilo bases (kb).
- These DNA fragments are separated and visualized as DNA bands on a gel.
- DNA ladders, DNA markers, nucleic acid ladders, and nucleic acid markers are interchangeable phrases.
- These separated DNA bands together look like a ladder on the gel and therefore it is called DNA ladder.
- DNA ladders are used in gel electrophoresis to determine the size and quantity of testing DNA fragments of genomic, plasmid, and PCR DNA.

Gel electrophoresis

Molecular weight standards

DNA/RNA markers (molecular ladders) or simply marker

- Most DNA ladders are in liquid form.
- A few microliters of a DNA ladder is usually loaded on a gel with testing DNA fragments.
- The 100 bp DNA Ladder contains 11 discrete DNA fragments ranging in size from 100 bp to 3,000 bp.
- This marker is ideal for the size determination of PCR products.

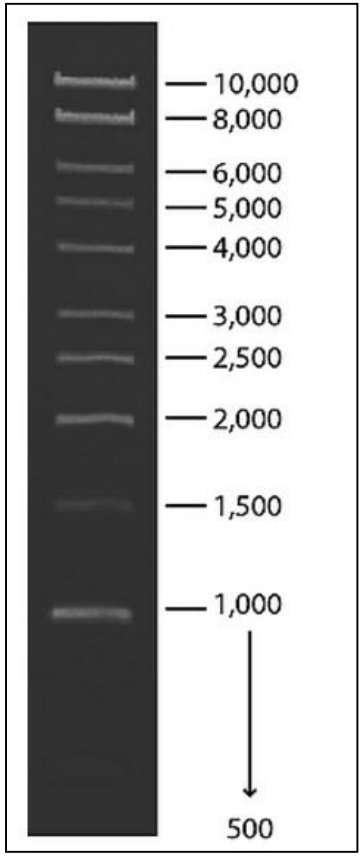


Gel electrophoresis

Molecular weight standards

DNA/RNA markers (molecular ladders) or simply marker

- Sigma's 1 kb Ladder contains 11 fragments consisting of 500 bp repeats from 0.5 to 3 kb, 1 kb repeats from 3 to 6 kb, and 2 kb repeats from 6 to 10 kb.
- Suitable for use in Northern and Southern blotting.
- 1 kb DNA Ladder has been used as a molecular marker to determine the molecular weight and size of double stranded DNA during electrophoresis.

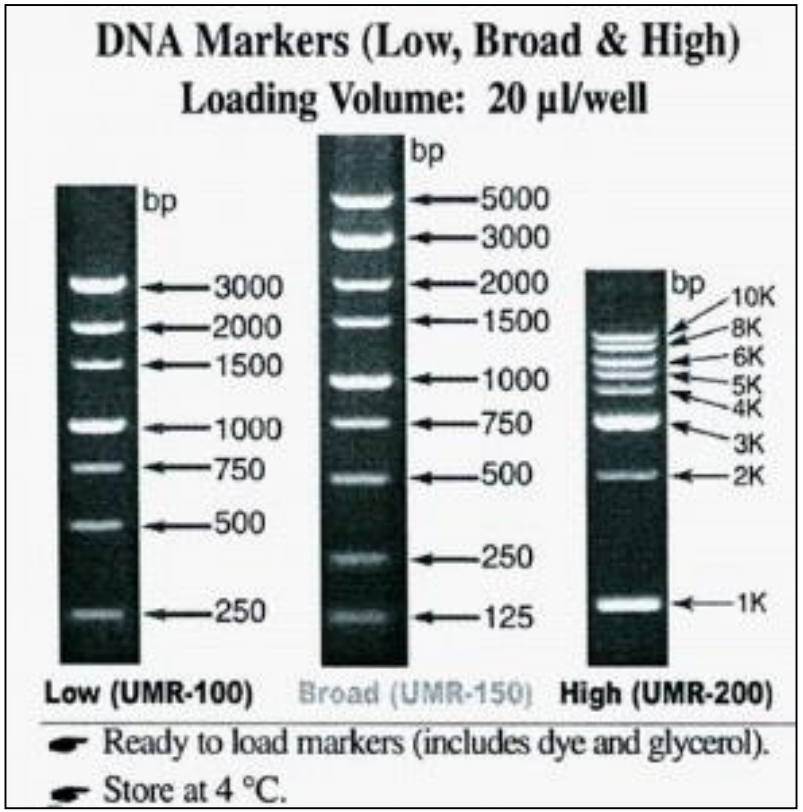


Gel electrophoresis

Molecular weight standards

DNA/RNA markers (molecular ladders) or simply marker

- The Low, High and Broad Range Markers offer a wide sizing range from 125 bp - 10,000 bp.
- Low Range DNA Marker: e.g. 25-500 bp Low Range DNA Marker A,(9 DNA fragments: 25, 50, 75, 100, 150, 200, 300, 400, 500bp).
- Wide (broad) range DNA ladder (8 DNA fragments: 5, 3, 2, 1.5, 1, 0.5, 0.25 and 0.125 kb).



Gel electrophoresis

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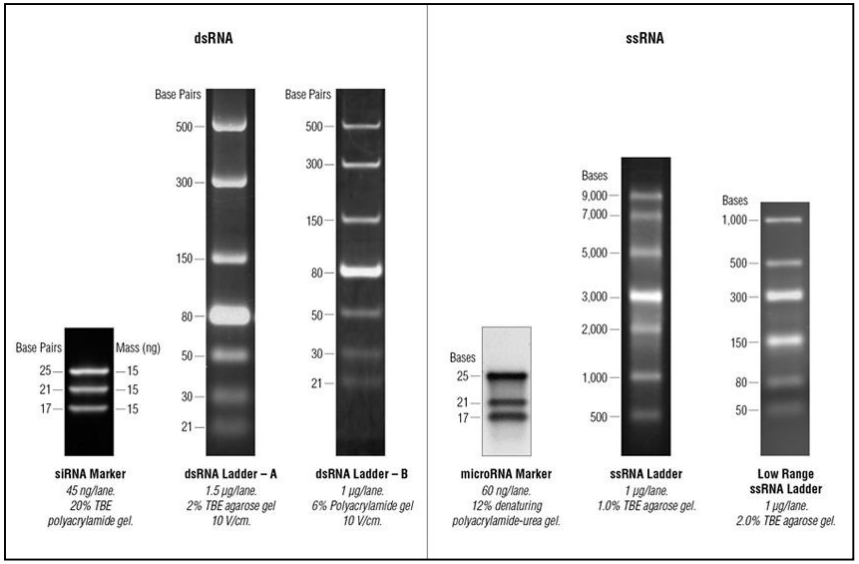
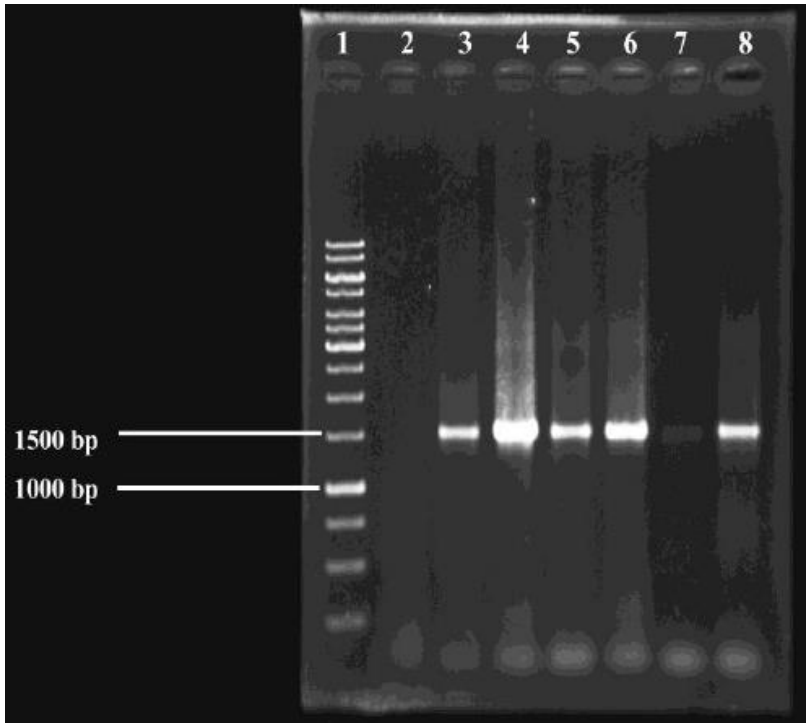


Gel electrophoresis

Molecular weight standards

DNA/RNA markers (molecular ladders) or simply marker

- Agarose gel electrophoresis after 16S rDNA amplification:
- Lane 1: GeneRuler 1 kb DNA ladder
- Lanes 2 to 8: 16S PCR products of DNA.



Tina *et al.*,2014

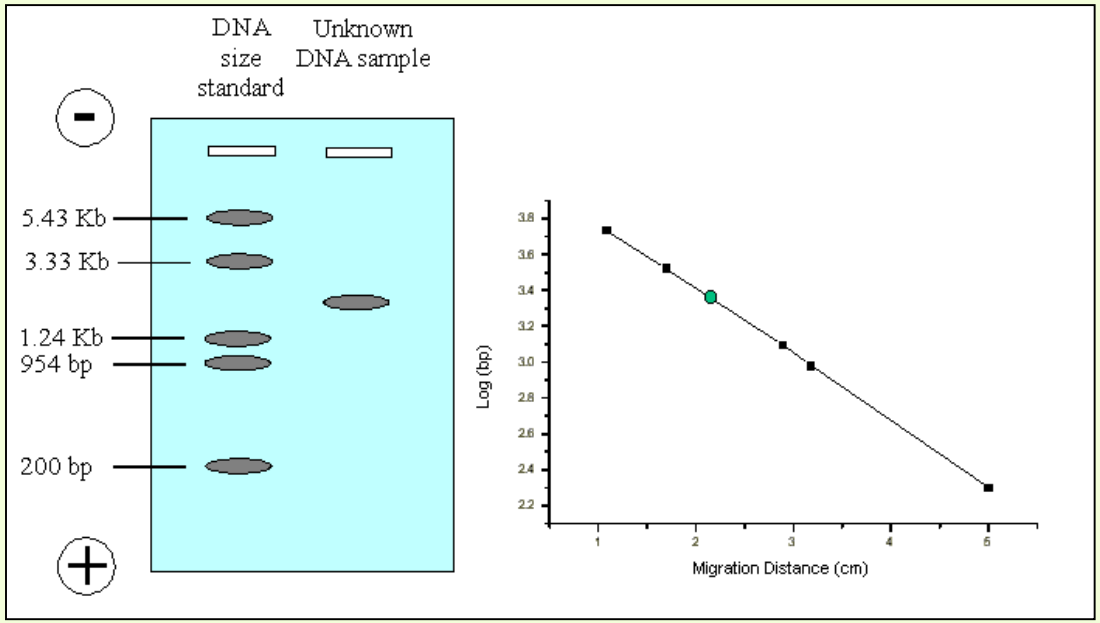
Molecular weight standards
Size markers

- Molecular weights are measured with different units for DNA, RNA, and protein:
- DNA: Molecular weight is measured in base-pairs, or bp, and commonly in kilobase-pairs (1000bp), or kbp.
- RNA: Molecular weight is measured in nucleotides, or nt, and commonly in kilonucleotides (1000nt), or knt. [Sometimes, bases, or b and kb are used.]
- Protein: Molecular weight is measured in Daltons (grams per mole), or Da, and commonly in kiloDaltons (1000Da), or kDa.

DNA Molecular Weight Markers

Running the marker in a lane at one end of the gel

- In both DNA and protein gels, molecular weight markers are run in a lane at one end of the gel.



zampbioworld.org,2011

Molecular weight standards

Running the marker in the two lanes: in a lane at one end of the gel and in the middle lane

- More recently, companies have started producing ladder markers with bands at defined intervals, e.g. 0.5, 1, 1.5, 2, 2.5 kb and so on up to 10 kb.
- If you know the total amount of DNA loaded into a marker lane, and you know the sizes of all the bands, you can calculate the amount of DNA in each band visible on the gel.
- This can be very useful for quantifying the amount of DNA in your sample bands by comparison with the marker bands.
- It is good to load two markers lanes, flanking the samples.

Lewis,2001

Agarose gel electrophoresis of DNA

Staining the gel

Ethidium bromide as gel loading dye

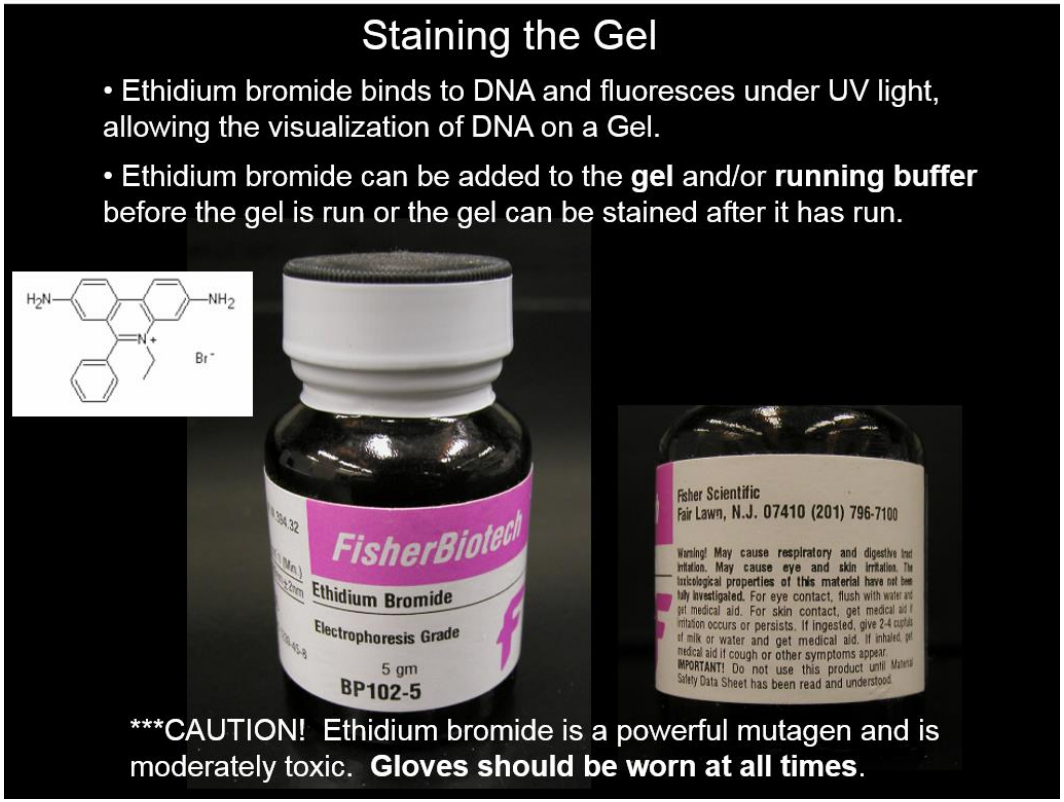
- Weigh out 0.5 g of agarose into a 250 mL conical flask. Add 50 mL of 0.5 X TBE buffer, swirl to mix.
- Microwave for about 1 minute to dissolve the agarose.
- Leave it to cool on the bench for 5 minutes down to about 60°C (just too hot to keep holding in bare hands).
- Add 1 µL of ethidium bromide (10 mg/mL) and swirl to mix.
- Pour the gel slowly into the tank. Push any bubbles away to the side using a disposable tip. Insert the comb and double check that it is correctly positioned.
- Leave to set for at least 30 minutes, preferably 1 hour, with the lid on if possible.
- Pour 0.5 X TBE buffer into the gel tank to submerge the gel to 2-5 mm depth.
- This is the running buffer.

Ethidium bromide(now replaced with new safe fluorescent nucleic acid dyes)

Agarose gel electrophoresis of DNA

Staining the gel

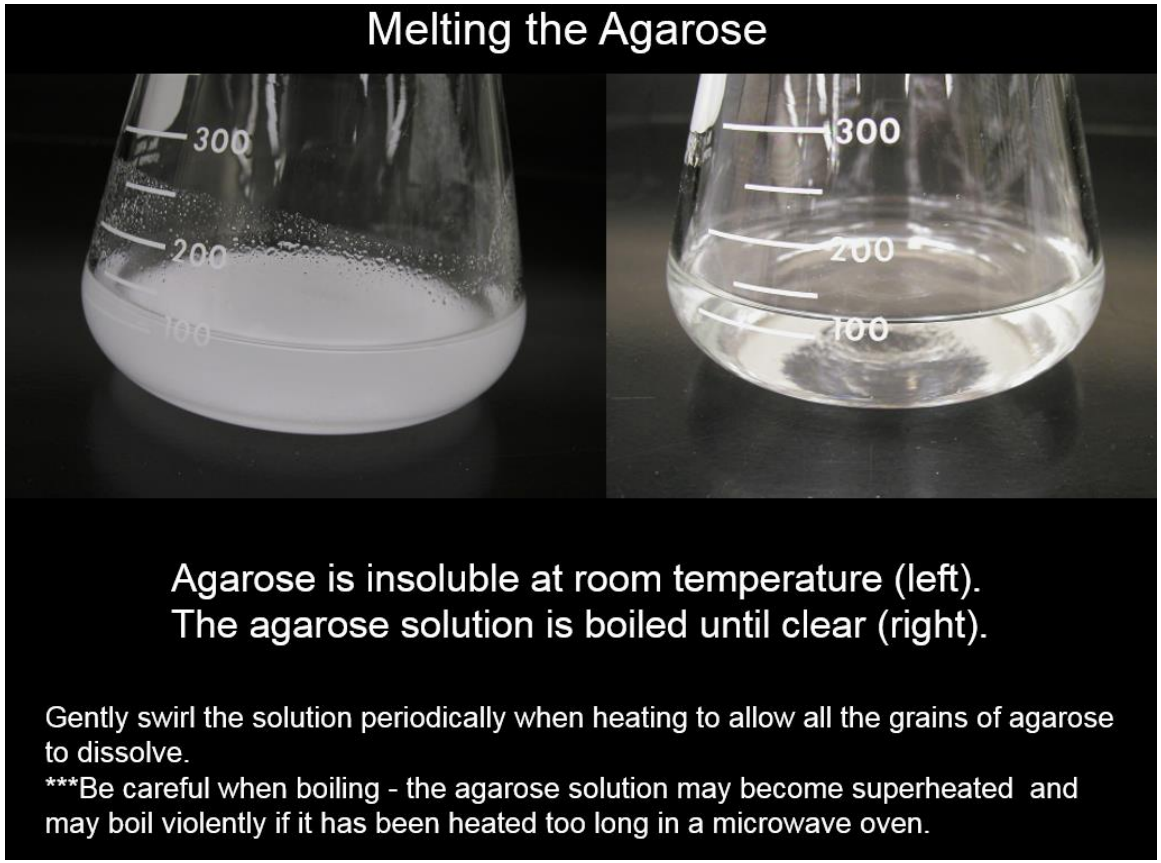
Gel loading dye



Ethidium bromide (now replaced with new safe fluorescent nucleic acid dyes)

Agarose gel electrophoresis of DNA

The agarose solution is boiled until clear (right)



Electrophoresis of PCR Products

Gel electrophoresis equipment

The gel should be run at ~80-100 volts for 45-75 minutes

- Electrophoresis equipment includes cells and power supplies.
- Electrophoresis equipment applies an electric charge to molecules, causing them to migrate towards their oppositely charged electrode.
- Load all your samples, then hook up the electrodes and turn on the power source.
- Set the voltage to the desired level, typically 1 to 10 V/cm of gel(the distance in cm refers to the distance between electrodes).
- Run the gel from 80-100 volts(ampere), but not above 120, because you run the risk of melting the gel.

Lee,1998;..

Electrophoresis of PCR Products

Gel electrophoresis equipment

The gel should be run at ~80-100 volts for 45-75 minutes

- The concentration of gel affects the resolution of DNA separation.
- For a standard agarose gel electrophoresis:

1. a 0.8% gel gives good separation or resolution of large 5-10kb DNA fragments,
2. while 2% gel gives good resolution for small 0.2-1kb fragments.
3. 1% gels is often used for a standard electrophoresis.

Wikipedia,2019

Electrophoresis of PCR Products

Gel electrophoresis equipment

The gel should be run at ~80-100 volts for 45-75 minutes

- The general recommended voltage are:
- 5-10 V/cm for DNA size <1 kb
- 4-10 V/cm for DNA size between 1-12 kb
- 1-3 V/cm for DNA greater than 12 kb.
- 5-10V/cm (the distance in cm refers to the distance between electrodes,).
- The higher the voltage, the faster the DNA moves. T
- Run the samples about 2/3 the way down the gel (you'll gain experience at telling when to stop the gel).

Electrophoresis of PCR Products

The electrophoresis

Run the gel appoximately 35 min (80V) or 25 min (100V)

- After all the DNA samples have been loaded into the wells, the electrodes are connected to the power supply.
- When the power is turned on, the negatively charged molecules move through the gel toward the positive pole.
- Run the gel at 80-150 V until the dye line is approximately 75-80% of the way down the gel.
- Both the DNA fragments and the dye molecules have a negative charge.

Electrophoresis of PCR Products

Gel electrophoresis

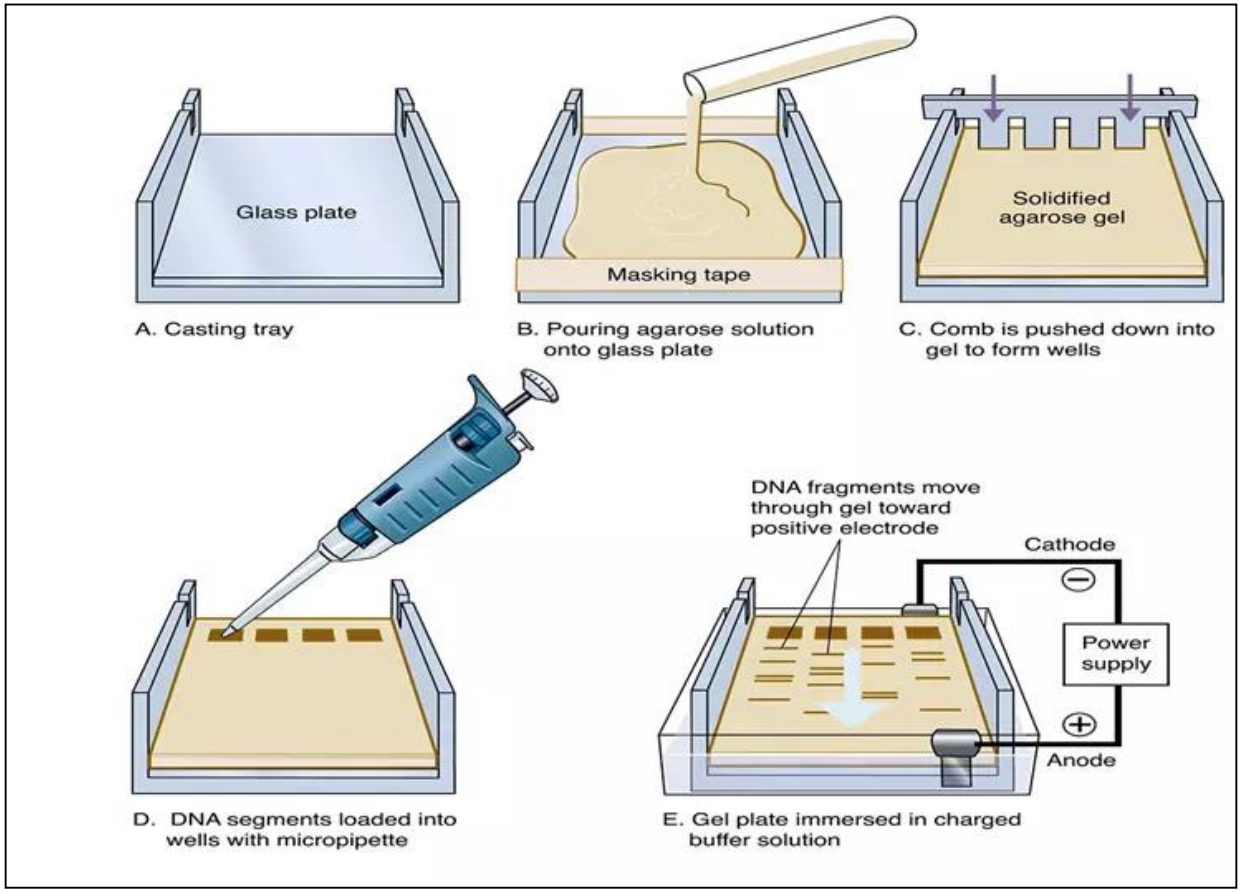
The gel should be run at ~80-100 volts for 45-75 minutes

- **Staining**
- There is a gel staining area in the fume hood. Put your gel in with minimal splashing. Add 20-50 uL ethidium bromide (toxic, hazard, cancerous!!!!). I will show you how to do this properly.
- In 15 minutes, check to see how far the dye has migrated in the gel.
- If it is midway between wells (or from the end for the second set of wells), then turn the power off.
- Place your gel inside the photodocumentation system using the U.V. box.
- Print out your gel image using the photodoc system.

Lee,1998;..

Agarose gel electrophoresis of DNA

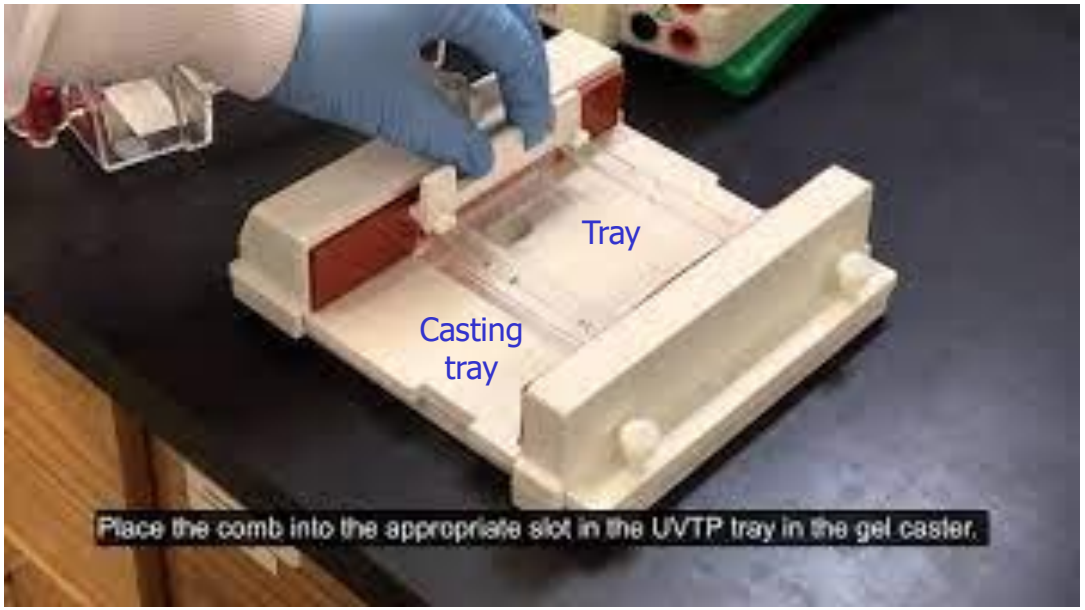
Instrumentation of electrophoresis



Agarose gel electrophoresis of DNA

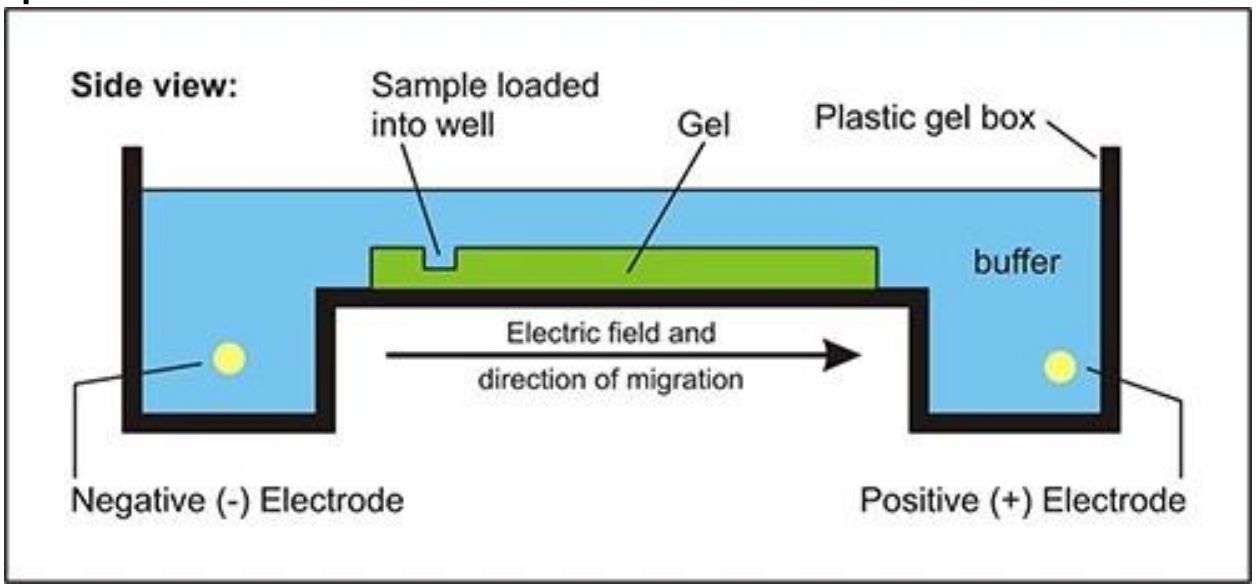
Cool slightly (~60ºC). Carefully pour. Avoid air bubbles

- Allow the agrose solution to cool (~60°C) and then carefully pour the melted agrose solution into tray in the the casting tray. Avoid air bubbles.



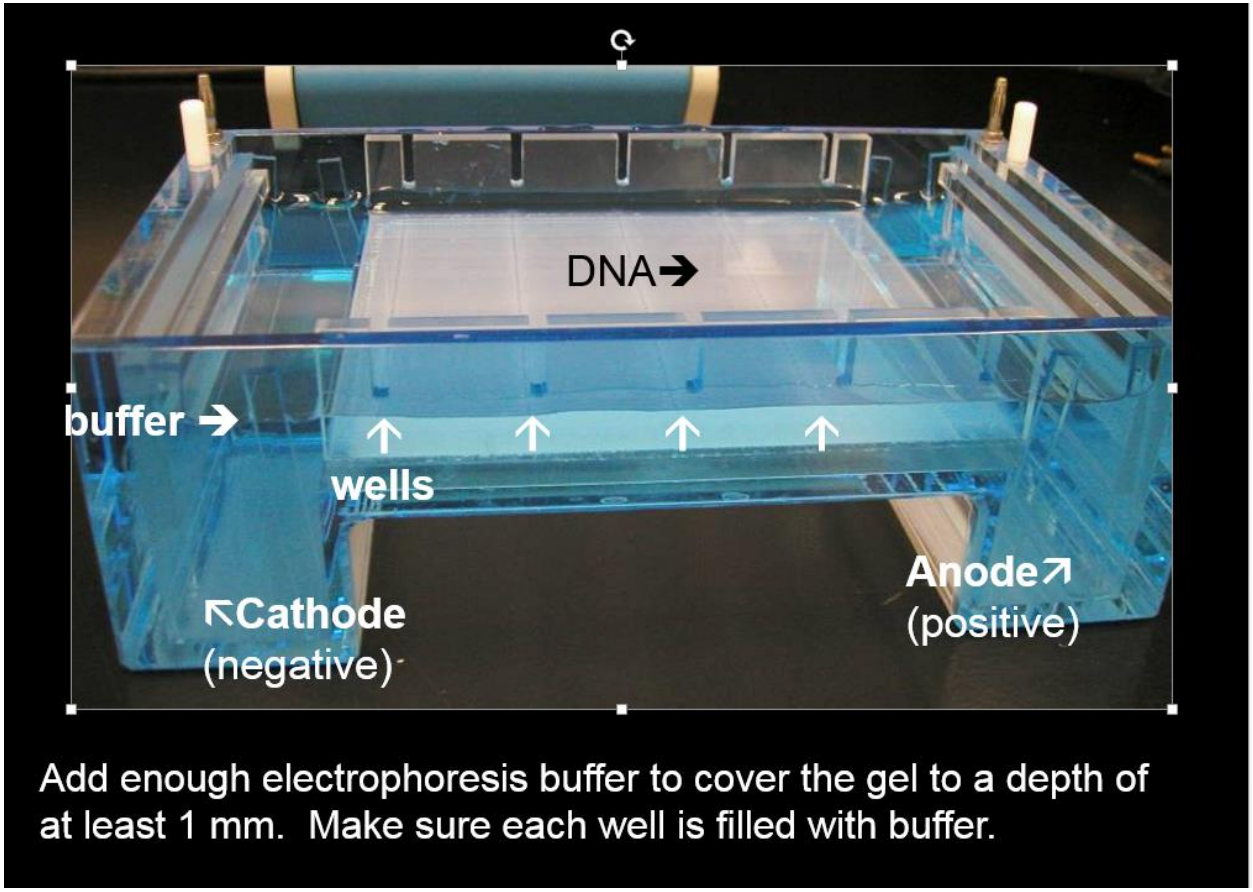
Agarose gel electrophoresis of DNA
Instrumentation of electrophoresis

- **Electrophoresis Chamber**
- It is a plastic box or tank, which is filled with a buffer for the migration of biomolecules. It has a transparent cover, which facilitates easy viewing of the migration process. It is connected to a power source.



The electrophoresis

Run the gel appoximately 35 min (80V) or 25 min (100V)



Agarose gel electrophoresis of DNA

DNA Loading Dye

Mix the samples of DNA with the 6X sample loading(Bromophenol Blue (for color) and Glycerol buffer (for weight)

- Add loading dye to the DNA samples to be separated.
- DNA loading dye is typically made at 6X concentration (0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol).
- Loading dye helps to track how far your DNA sample has traveled, and also allows the sample to sink into the gel.

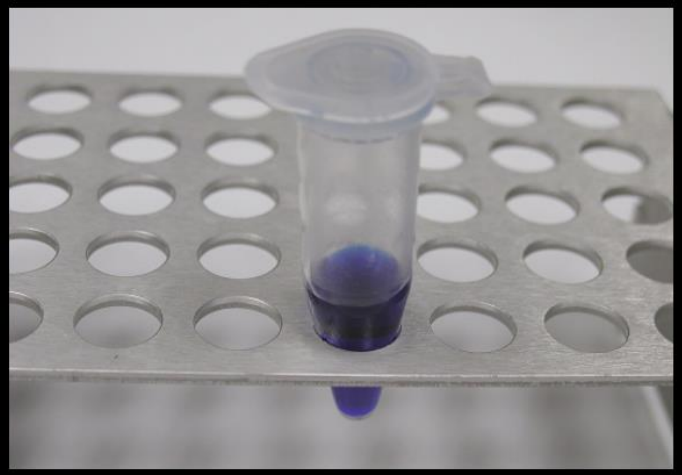
Lee *et al.*,2012

Agarose gel electrophoresis of DNA

Mix the samples of DNA with the 6X sample loading(Bromophenol Blue (for color) and Glycerol buffer (for weight)

Sample Preparation

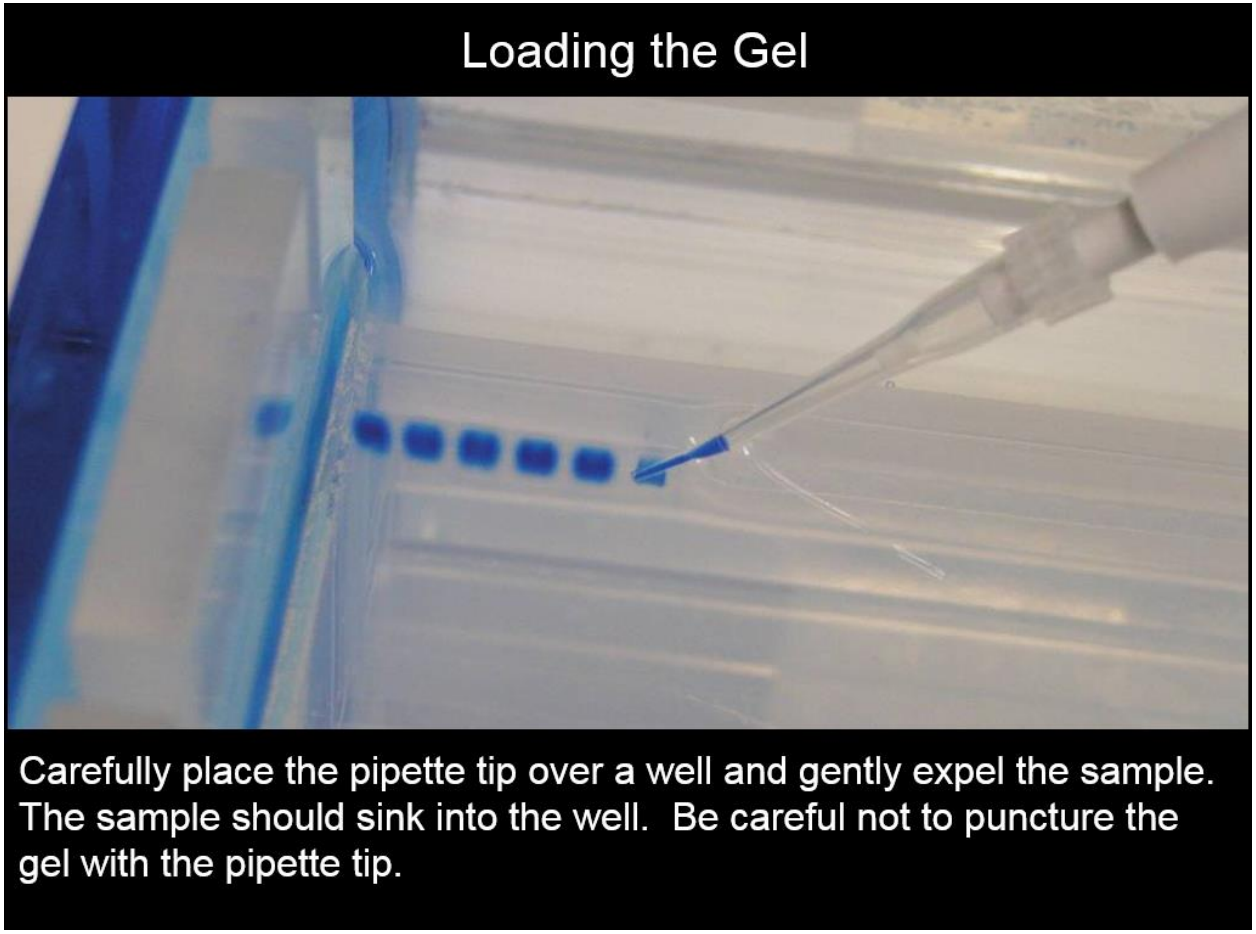
Mix the samples of DNA with the 6X sample loading buffer (w/ tracking dye). This allows the samples to be seen when loading onto the gel, and increases the density of the samples, causing them to sink into the gel wells.



6X Loading Buffer: →

- Bromophenol Blue (for color)
- Glycerol (for weight)

Agarose gel electrophoresis of DNA
Loading the gel

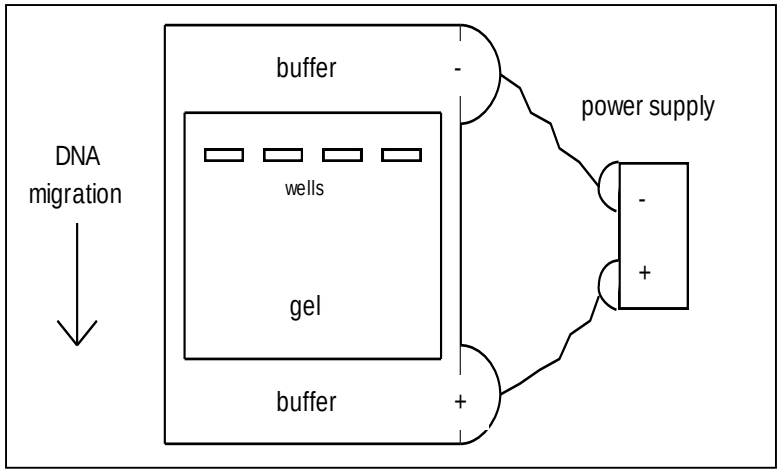


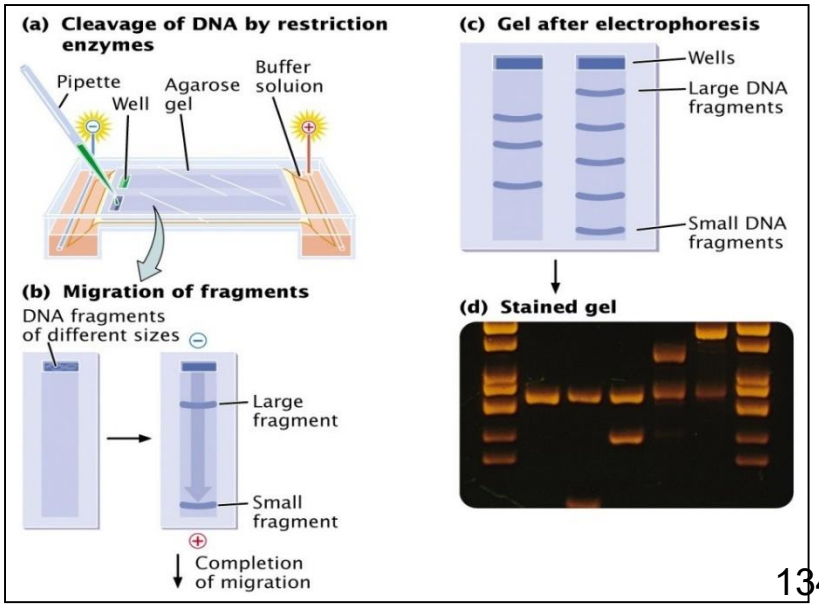
Gel Electorphoresis Lecture 2006

Electrophoresis of PCR Products

Viewing DNA fragments

- Upon completion of PCR, hold samples at 4°C and then kept at -20°C until usage.
- Prepare the DNA for loading by addition of 1/10 volume stop-loading buffer (contains EDTA, glycerol, and bromphenol blue).
- Analyze by gel electrophoresis and be sure to include size markers in at least one well on the same gel.

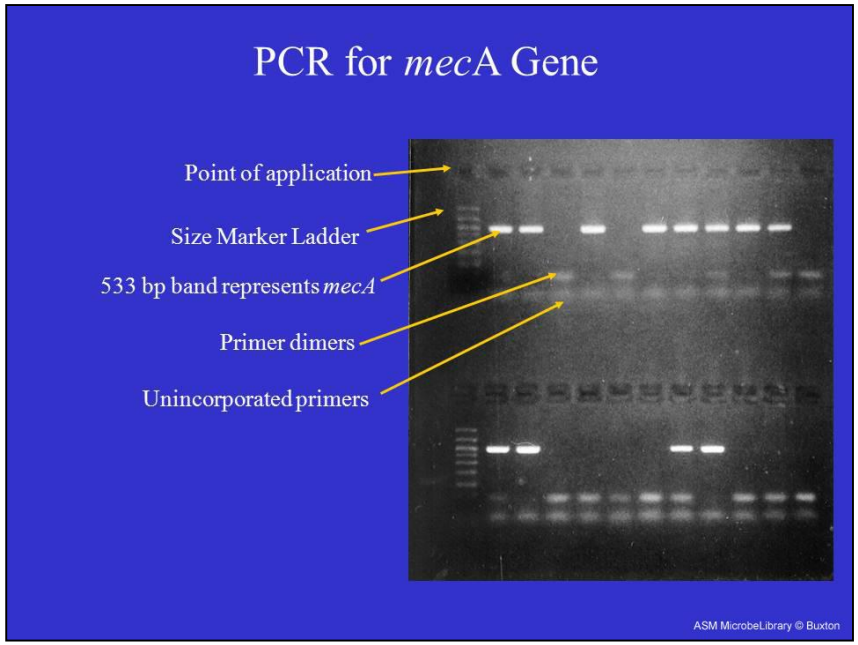




Electrophoresis of PCR Products

Viewing DNA fragments

- Example PCR gel electrophoresis agarose gel demonstrating a 533 bp amplicon as well as primer dimers and unincorporated primers.
- Rebecca Buxton, University of Utah.



Electrophoresis of PCR Products

Gel electrophoresis

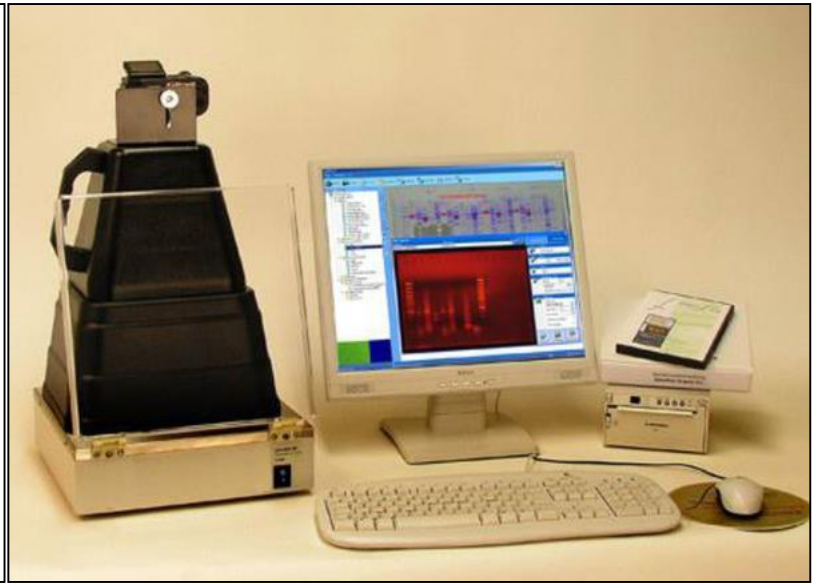
Gel documentation system

- In 15 minutes, check to see how far the dye has migrated in the gel.
- If it is midway between wells (or from the end for the second set of wells), then turn the power off.
- Place your gel inside the photodocumentation system using the U.V. box.
- Print out your gel image using the photodoc system.

Lee,1998;..

Gel electrophoresis

Gel documentation system for all fluorescent & white light applications



Transilluminators

MiniBIS model without transilluminator can be used with these transilluminators

	
Pro D-Transilluminator Can be used stand alone or integrated with the MiniBIS gel documentation system.	**Standard Transilluminator**

Gel electrophoresis
Storing stained gels

- After the experiment is finished, the resulting gel can be stored in a plastic bag in refrigerater.
- Unlike the bands on gels stained with methylene blue, those stained by CarolinaBLU™ should not fade for many months, provided that the gel is stored out of the light and in distilled or deionized water.
- We recommend that gels are stored, tightly-wrapped in a plastic bag, in a refrigerator, with a small amount of distilled water.

Gel electrophoresis

Storing stained gels

Questions and answers

- For long storage you can keep it in 4°C.
- Store in room temperature for 1-3 days and it works good.
- You can leave it as is at 4 degrees for a week at the very least.
- Overnight is okay at 4°C, but if I was going to store it for longer than that I would just freeze the slices at -20°C to be safe.

In silico(virtual) PCR

Synonyms: digital PCR, virtual PCR, electronic PCR, e-PCR

- Computer simulation of PCR amplification, AFLP-PCR and restriction digest of complete bacterial genomes.
- There are several currently online and offering instant PCR in silico on the web.
- *in silico* (virtual) PCR searches a sequence database with a pair of PCR primers, using an indexing strategy for fast performance.

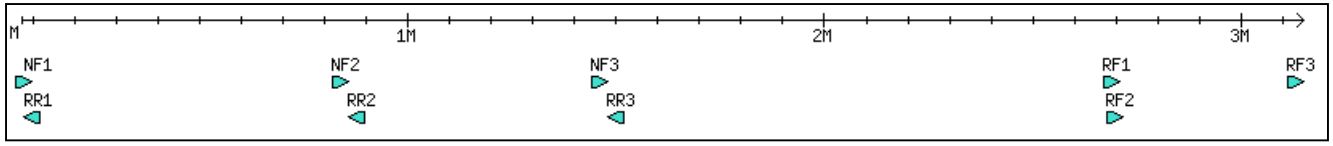
Wikipedia,2014;..

In silico(virtual) PCR

Synonyms: digital PCR, virtual PCR, electronic PCR, e-PCR

- Some goals regarding PCR product prediction:

1. The design of appropriate short or long primer pairs
2. Determining primer location, orientation, length of each amplicon,
3. Simulation of electrophoretic mobility,
4. Identification of open reading frames, and
5. Links to other web resources.



Display of primer locations and orientations within the genome of *Corynebacterium glutamicum* ATCC 13032 *Kitasato*. Visual primer locations are helpful in determining different possible bands produced from multiple primers.

Wikipedia,2014; Heuermann and Bastola

In silico(virtual) PCR

Synonyms: digital PCR, virtual PCR, electronic PCR, e-PCR, isPCR

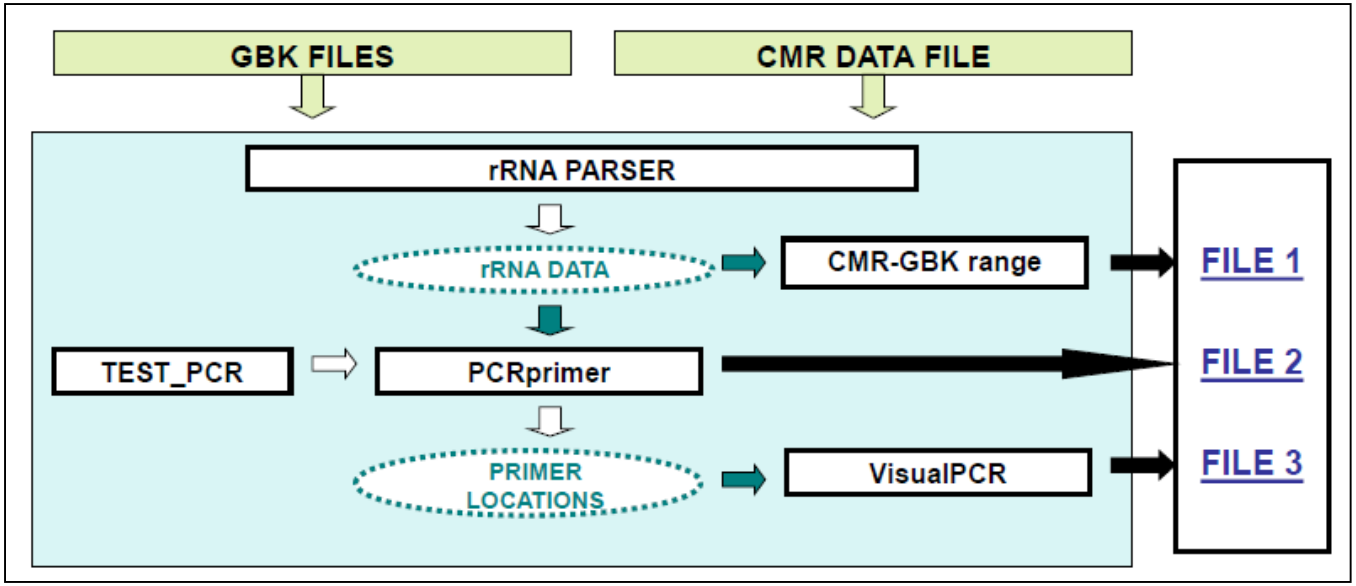
- The *in silico* PCR (is*PCR*) is a tool written in Perl using the Bioperl module.
- The program consists of multiple steps and uses sequence data from two publicly available databases, namely GenBank at NCBI and Comprehensive Microbial Resources (CMR).
- If genome sequences for organism are available, computational tool such as isPCR can be used to comprehensively search any genome to quickly determine possible PCR products and assist in the experimental design.

Heuermann and Bastola

In silico(virtual) PCR

Synonyms: digital PCR, virtual PCR, electronic PCR, e-PCR, isPCR

- An overview of in silico PCR (isPCR) showing work flow and associations between various input/output files, parser and visualization tools.

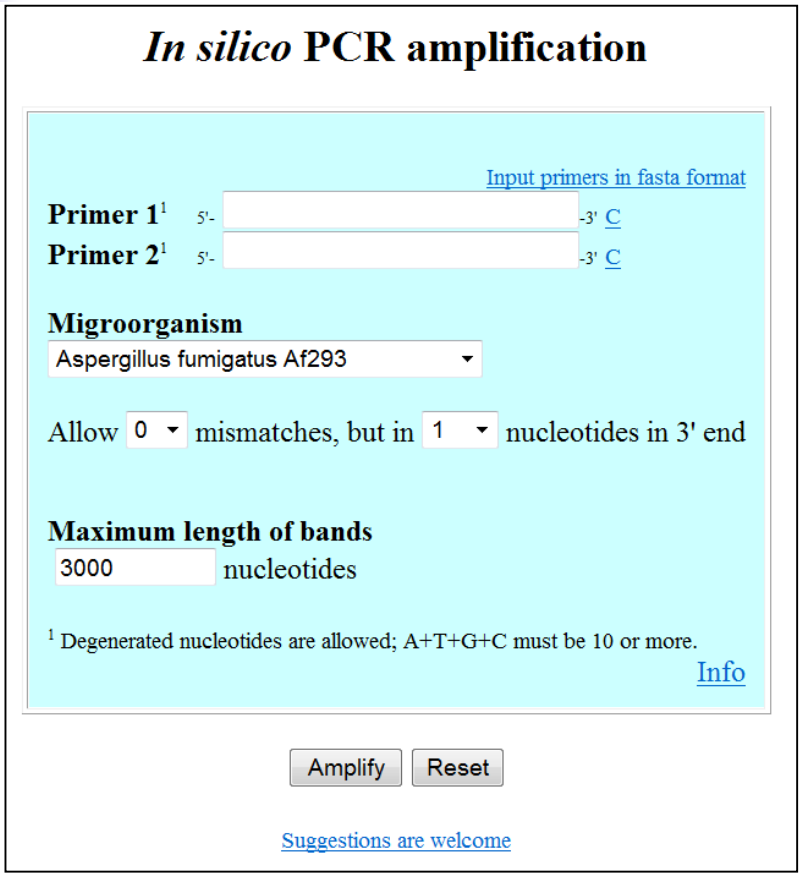


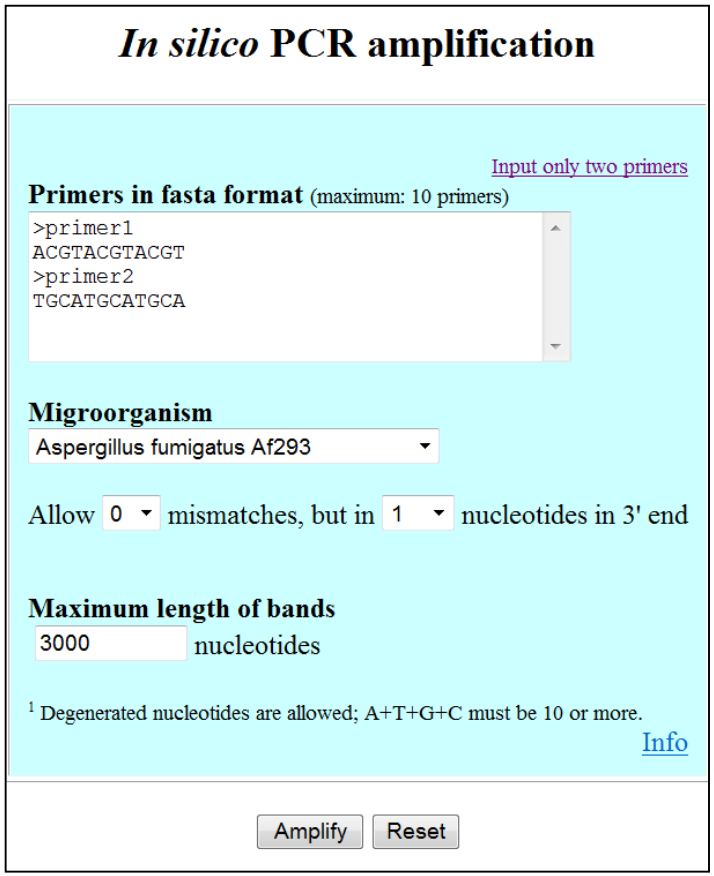
Heuermann and Bastola

In silico(virtual) PCR

PCR simulated against fungal genomes

First(left) and second (right) steps





In silico(virtual) PCR

PCR simulated against fungal genomes

Final step

In silico PCR amplification
Primer -> 5'-ACGTACGTACGT-3'
Primer -> 5'-TGCATGCATGCA-3'
No mismaches allowed.

G+C:

Aspergillus fumigatus Af293 chromosome 1 (0 bp)

No bands amplified
A. fumigatus Af293 chromosome 2 (0 bp)

No bands amplified

Computed in 0 seconds

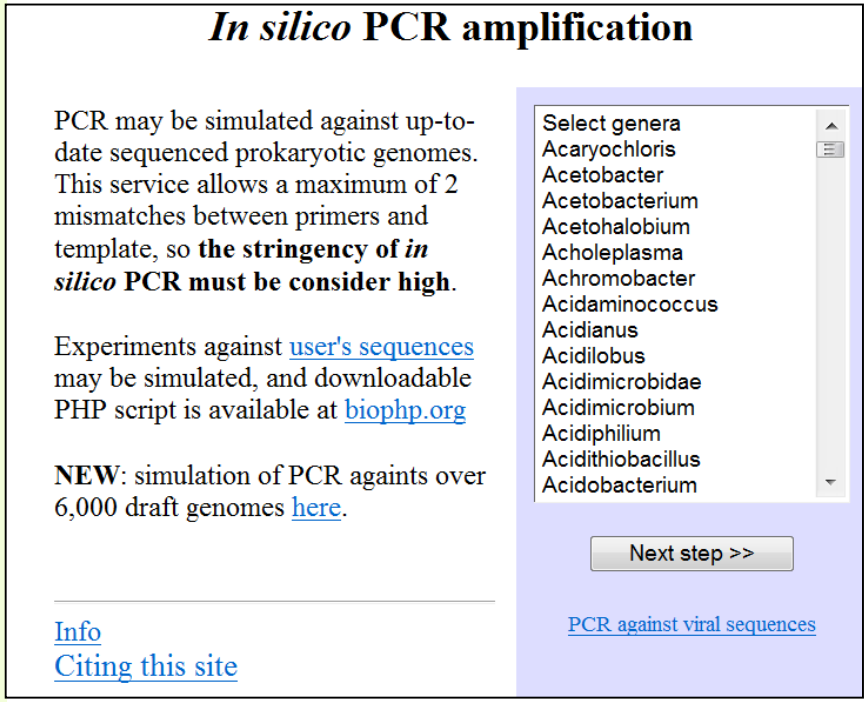
Please help us improve this resource by reporting problems, suggestions etc.

In silico(virtual) PCR

PCR simulated against prokaryotic genomes

First step

- PCR may be simulated against up-to-date sequenced prokaryotic genomes.
- This service allows a maximum of 2 mismatches between primers and template, so the stringency of *in silico* PCR must be consider high.



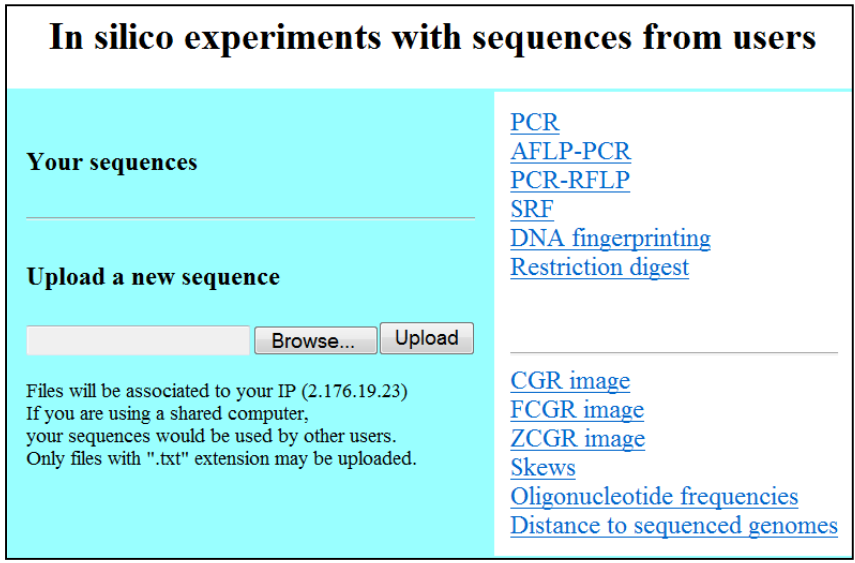
insilico.ehu.es/PCR/

In silico(virtual) PCR

PCR simulated against prokaryotic genomes

Second step

- This service allows uploading to insilico.ehu.es.com up to 5 sequences per user.
- Each sequence is limited to 5 million nucleotides.
- Before uploading your sequence, save it to a text/plain file (".txt" extension) with Fasta format.
- No information is recorded from users or uploaded sequences, but for statistics of our website.
- Do not use the file in the other services but PCR, PCR-RFLP and AFLP-PCR amplification (aberrant results will be obtained).



insilico.ehu.es/PCR/

Probes or PCR?

- In principle, there is no difference between an oligo (nucleotide) probe or a PCR primer.
- Both are oligonucleotides (from Greek prefix *oligo-*, "having few, having little") of somewhere between 18 and 25 bp (approx), however, it is more difficult to make a good primer than a probe the probe just has to hybridize.
- The primer also has to initiate an enzyme reaction and therefore the end has to be high in GC.
- However a PCR reaction is a more sensitive method, and faster compared to a hybridization.

BIO-PCR

Biological amplification followed by PCR

Bacteria cultured on agar media prior to PCR

- This technique improves sensitivity of PCR pathogen detection.
- The enrichment of the bacteria in semi selective liquid medium prior to PCR (BIO-PCR) is an important initial step for the success in PCR detection extremely low number of the target bacteria from infected plant tissues such as potato tubers.
- In BIO-PCR technique viable cells of *Erwinia* from potato peel extract are enriched in liquid medium and detected in low levels in seed potato without DNA extraction.

Hadas *et al.*,2006; Degefu *et al.*,2006

BIO-PCR

Biological amplification followed by PCR

Bacteria cultured on agar media prior to PCR

- 100 µl of supernatant of the potato peel extract was transferred to 15 ml Sartedt tubes containing 5 ml of PEM (liquid sodium polypectate medium) and incubated at 27 °C for 48 hrs shaking at 150 rpm.
- The resulting bacterial cells were collected by centrifugation at 7000 rpm for five minutes and washed twice by resuspending in 2 ml sterile ultra pure water.
- The washed bacterial cells were then resuspended in 250 µl of sterile ultra pure water and used as PCR template or stored in -20°C until analyzed which is only for a couple of days.

Degefu *et al.*,2006

BIO-PCR

Biological amplification followed by PCR

Bacteria cultured on agar media prior to PCR

- In classical PCR (direct PCR without DNA extraction), 10 µL of the seed extract were used as template DNA.
- Whereas for Bio-PCR, 100 µL of the seed extract were spread onto NBY medium and incubated at 28°C for 48 h.
- The culture was harvested by adding 1 mL of water and suspending the cells with glass-loop.
- A 10 µL aliquot of the bacterial suspension was added to the PCR reaction as template DNA.

Hadas *et al.*,2006

BIO-PCR

Biological amplification followed by PCR

Bacteria cultured on agar media prior to PCR

- Besides increasing sensitivity by 100- to 1,000-fold over conventional PCR methods, the enrichment technique stops substances called inhibitors from interfering with the action of a key enzyme, Taq polymerase.
- Bio-PCR works best with fast-growing bacteria such as *Ralstonia solanacearum* (bacterial wilt of potato and tomato), and *Acidovorax avenae*, which causes bacterial fruit blotch of watermelon.
- However, Bio-PCR also improves detection of slow-growing pathogens such as *Xylella fastidiosa*, responsible for Pierce's disease of grapes and leaf scorch of shade trees.

BIO-PCR

Biological amplification followed by PCR

Bacteria cultured on agar media prior to PCR

- Success of BIO-PCR is highly dependent upon the timing of washing the target colonies from the agar medium.
- Normally pin-point size colonies contain 1,000 or more cells each.
- This is normally the minimum number of cells per ml needed for detection by PCR using 1 µl samples.
- Direct PCR without enrichment on EBB or EBBA media was positive for only two of five samples.

Direct PCR	BIO-PCR	
	EBB	EBBA
38.74[d]	26.85	25.71
39.58	26.99	23.71
0	29.81	25.10
0	25.80	25.74
0	24.94	23.52

Zaho *et al.*,2007

BIO-PCR

Bacteria cultured on agar media prior to PCR

- BIO-PCR was 100 times more sensitive than classical(direct) PCR.
- Whereas BIO-PCR was positive for samples containing $2x10^2$ cfu mL^{-1}, classical PCR failed to detect samples containing $3x10^3$ cfu mL^{-1}(per ml).
- Neither classical PCR nor BIO-PCR were positive for undiluted samples containing very high numbers ($1x10^{-5}$ cfu mL^{-1} or greater.

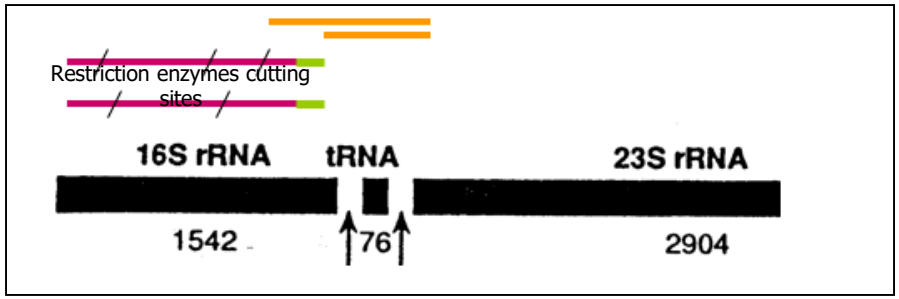
	X. albilineans[a]	
Isolation CFU[b]	PCR	BIO-PCR[c]
ND	+	+
133000[d]	+	+
14000[d]	+	+
4900[d]	+	+
2900[e]	+	+
620[e]	+	+
120[e]	–	+
10[f]	–	+
0	–	–

Wang *et al.*,1999

Microbial diversity

Quick Assessment of Diversity "One band = One bug" (?) (little phylogeny information)

- **RISA (ribosomal intergenic spacer analysis):**
  - Often get overlapping bands (on band = > 1 bug)
  - Phylogenetic information limited by 16SrDNA overlap
- **ARDRA (amplified ribosomal DNA restriction analysis):**
  - ARDRA is an RFLP on the 16SrDNA amplified by PCR.
  - Good for identification of isolates; esp. with multiple restriction enzymes.
  - Too many bands makes it hard to interpret mixed populations.
- **T-RFLP (terminal restriction fragment polymorphism):**
  - Steps like ARDRA, but terminal 3' end of gene is fluorescent
  - Multiple restriction enz. Give best results; maybe used to query RDP.



Microbial diversity

Genotyping of bacterial isolates by ARDRA

P. tolaasii

- Amplified ribosomal DNA restriction analysis (ARDRA) was performed on the PCR-amplified 16S rDNA products from each of the isolates using four specific restriction enzymes *Cfo*I, *Hae*III, *Hinf*I and *Sac*I.
- Five microliters of each PCR product was digested for 2 h at 37°C with 1.5 U of each restriction endonuclease.
- Aliquots (5 µL) of each digested product were analyzed by gel electrophoresis in an 8% nondenaturing acrylamide gel (acrylamide: N,N'-Methylenebisacrylamide, 29:1) and by silver nitrate staining, as described previously.
- Fragment sizes were estimated using a low range, 50 bp DNA ladder and a final grouping of isolates was performed by a visual comparison of the restriction patterns.

ARDRA

Amplified ribosomal DNA restriction enzyme analysis

P. tolaasii

- The high degree of polymorphism cause problems in the identification of the *P. tolaasii*.
- Therefore, DNA samples were isolated from *P. tolaasii* strains and PCR reactions were performed with special primers.
- So called ARDRA was performed with *Cfo*I, *Hae*III, *Hinf*I and *Sac*I restriction enzymes, respectively.

1. The *Cfo*I *enzyme* provided 4 groups, but
2. The Hinf*I* and *Sac*I digestion resulted 8 different patterns.

Sajben et al

ARDRA

Amplified Ribosomal DNA restriction enzyme analysis

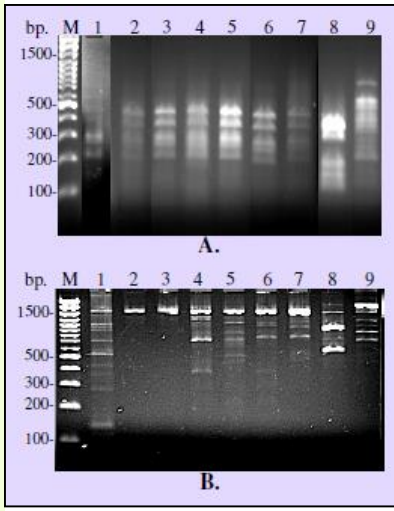
P. tolaasii

- The ARDRA pattern of the strains of *P. tolaasii*.

A. Digestion with *Cfo*I enzyme.

B. Digestion with *Sac*I enzyme.

- The strains in the lines as the following: 1: 6, 2: 7, 3: 8, 4: 29, 5: 17,6: 15, 7: 37, 8: 25, 9: 56.
- M: GeneRuler 100bp DNA Ladder Plus



Pseudomonas strains	*Cfo*I	*Hae*III	*Sac*I	*Hin*fI
6	1	1	1	1
7	2	2	2	2
8	2	2	2	2
29	2	3	3	3
17	2	3	4	4
15	2	4	5	5
37	2	4	6	6
25	3	5	7	7
56	4	6	8	8

Table 2. Classification of the strains on the basis of ARDRA results

Sajben et al

RFLP analysis

Restriction fragment length polymorphisms

RFLP without PCR (Southern blotting)

- RFLP without PCR (Southern blotting):
- The old fashioned RFLP is based on Southern blot.
- You cut the genomic DNA (usually one 6-cutter and one 4-cutter REs, depending on the genome size), run a long gel, transfer the DNA to a membrane and hybridize with a marked cloned fragment (nowadays, you could do it with a PCR fragment), the probe.
- Depending on the number of sequences of interest in the genome Southern blotting may need 3-10 µg of DNA template per sample probed.

See more slides in the following slideShare.

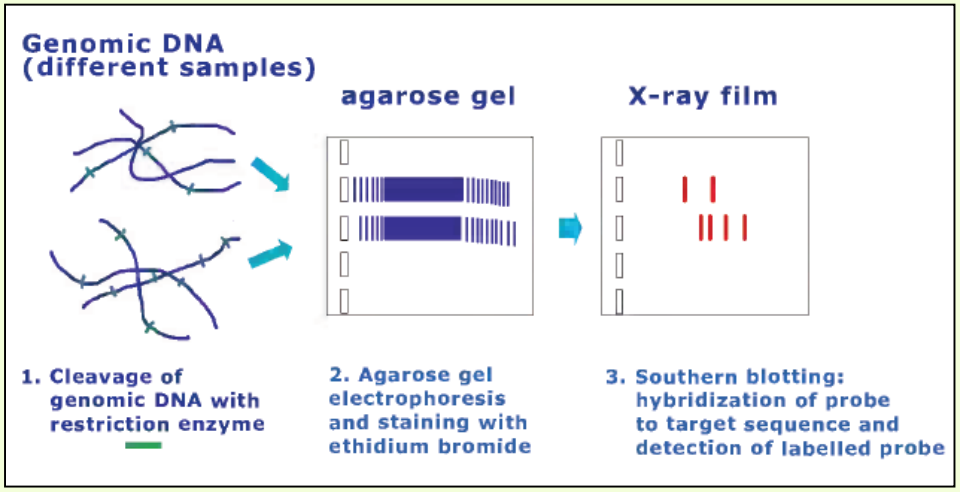
Goicoechea,2016

RFLP analysis

Restriction fragment length polymorphisms

RFLP without PCR (Southern blotting)

- In RFLP analysis, the amplified DNA fragments considered to be specific to *R. solanacearum* (single fragment 281 bp) were double digested with *Hae*III and *Msp*I restriction endonucleases.
- *Msp*I and *Hae*III recognize and cleave at CCGG and CCG sites, respectively. The resulting restriction fragments are separated according to their lengths by gel electrophoresis.



Janse,2006;..

RFLP analysis

Restriction fragment length polymorphisms

RFLP with PCR

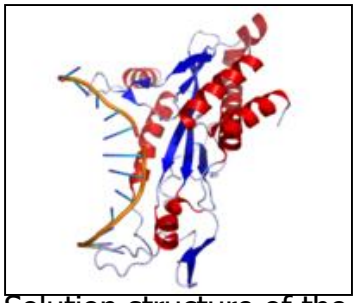
- RFLP with PCR:
- In PCR-RFLP you amplify some specific fragment of the genome,
- Cut it with a restriction enzyme (usually 4-cutter because the size of the PCR product is small) and
- Run a gel to see the fragments.

Goicoechea,2016

RFLP analysis

Restriction enzymes

Restriction endonucleases



Solution structure of the restriction enzyme EcoRI.

- The procedures involve:

1. Isolation of DNA,
2. Digestion of total genomic DNA with restriction endonucleases.

- For example, *EcoR*I always cuts DNA at GAATTC as indicated below), size fractionation of the resulting DNA fragments by electrophoresis.

```
G|AATTC
CTTAA|G
```

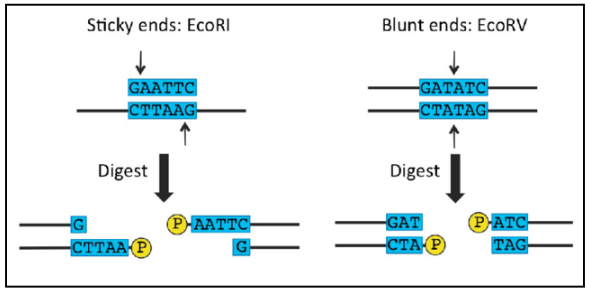
RFLP analysis

Restriction enzymes(Restriction endonucleases)

Hundreds of different restriction enzymes have been isolated. Each one cuts DNA at a specific base sequence

- Restriction enzymes are generally classified into three types:
- Type I: cleave at sites remote from recognition site;
- Type II: cleave within the site or at short specific distances from recognition site.
- Type III: cleave at sites a short distance from recognition site;
- Some examples are listed below:

Enzyme	**Cutting site**
*Bam*HI	GGATCC
*Hae*III	GGCC
*Pst*I	CTGCAG
*Hinf*I	GANTC
*Msp*I	CCGG



Restriction endonucleases

Examples of restriction enzymes

Cutting sites

Enzyme	Source	recognized sequence	Cleavage sites	Cleavage	Type of end
AluI[1]	*Arthrobacter luteus*	5'AGCT 3'TCGA	5'AG↓CT 3'TC↑GA	5'---AG CT---3' 3'---TC GA---5'	Blunt end
BamHI[1]	*Bacillus amyloliquefaciens*	5'GGATCC 3'CCTAGG	5'G↓GATCC 3'CCTAG↑G	5'---G GATCC---3' 3'---CCTAG G---5'	Sticky end
EcoRI[1]	*Escherichia coli*	5'GAATTC 3'CTTAAG	5'G↓AATTC 3'CTTAA↑G	5'---G AATTC---3' 3'---CTTAA G---5'	Sticky end
EcoRII	*Escherichia coli*	5'CCWGG 3'GGWCC	5'↓CCWGG 3'GGWCC↑	5'--- CCWGG---3' 3'---GGWCC ---5'	Sticky end
EcoRV	*Escherichia coli*	5'GATATC 3'CTATAG	5'GAT↓ATC 3'CTA↑TAG	5'---GAT ATC---3' 3'---CTA TAG---5'	Blunt end

CreationWiki,2015

Restriction endonucleases

Examples of restriction enzymes

Cutting sites

EcoP15I	*Escherichia coli*	5'CAGCAGN_{25}NN 3'GTCGTCN_{25}NN	5'CAGCAGN_{25}↓NN 3'GTCGTCN_{25}NN↑	5'---CAGCAGN_{25} NN---3' 3'---GTCGTCN_{25}NN ---5'	Frayed end
HaeIII[1]	*Haemophilus aegyptius*	5'GGCC 3'CCGG	5'GG↓CC 3'CC↑GG	5'---GG CC---3' 3'---CC GG---5'	Blunt end
HhaI[1]	*Haemophilus haemolyticus*	5'GCGC 3'CGCG	5'GCG↓C 3'C↑GCG	5'---GCG C---3' 3'---C GCG---5'	Sticky end
HindII[8]	*Haemophilus influenzae*	5'GTGGAC 3'CAGCTG	5'GTG↓GAC 3'CAG↑CTG	5'---GTG GAC---3' 3'---CAG CTG---5'	Sticky end
HindIII	*Haemophilus influenzae*	5'AAGCTT 3'TTCGAA	5'A↓AGCTT 3'TTCGA↑A	5'---A AGCTT---3' 3'---TTCGA A---5'	Sticky end
HinfI	*Haemophilus influenzae*	5'GANTC 3'CTNAG	5'G↓ANTC 3'CTNA↑G	5'---G ANTC---3' 3'---CTNA G---5'	Frayed end

CreationWiki,2015

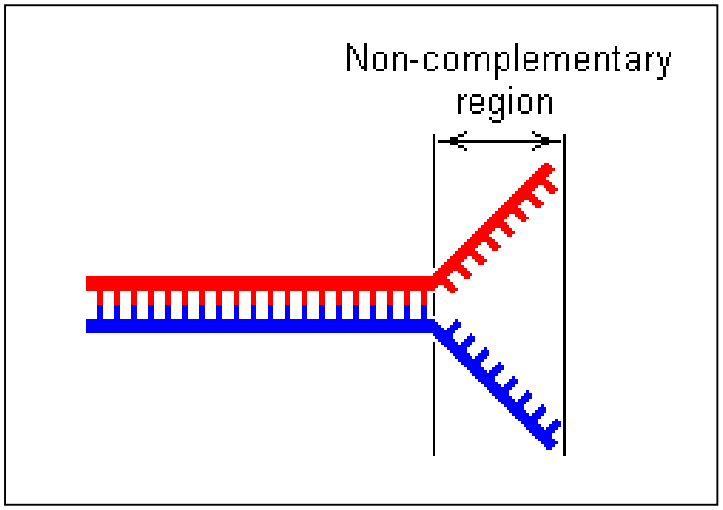
Restriction endonucleases

Examples of restriction enzymes

Cutting sites(Frayed end)

- A frayed end refers to a region of a double stranded (or other multi-stranded) DNA molecule near the end with a significant proportion of non-complementary sequences; that is, a sequence where nucleotides on the adjacent strands do not match up correctly:

5′ -ATCTGACTAGGCA-3′
3′-TAGACTGACTACG–5′



The term "frayed" is used because the incorrectly matched nucleotides tend to avoid bonding, thus appearing similar to the strands in a fraying piece of rope.
Although non-complementary sequences are also possible in the middle of double stranded DNA, mismatched regions away from the ends are not referred to as "frayed".

Wikipedia,2019

PCR-RFLP analysis

Restriction fragment length polymorphism analyses of bacterial 16S rDNA

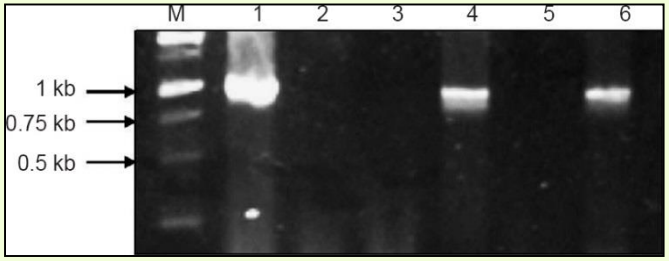
- **RFLP of 16SrRNA gene PCR product:**
- The 996 base pair product of 16S rRNA gene was purified (Qiagen, USA) and digested with *Hae*III restriction enzyme (Fermentas, USA) according to manufacturer's instructions to generate fragments, band patterns of which were characteristic for each organism.
- The amplified products from all the bacterial strains used as controls were also digested with *Hae*III according to manufacturer's instructions. Digestions were carried out in a total volume of 40 µl. The reaction mixture consisted of 10-15 µl of PCR product, 5 U of restriction enzyme and volume adjusted with sterile distilled water.
- The digest was electrophoresed in 2 per cent agarose with 0.5 µg/ml ethidium bromide and photographed using a gel documentation system (Herolab, Weisloch, Germany).
- The bands obtained from PCR-RFLP were analyzed using the GelCompare II version 2.5.

Rohit *et al.*,2016

PCR-RFLP analysis

Restriction fragment length polymorphism analyses of bacterial 16S rDNA

- Representative gel picture of PCR amplification of 16S rRNA gene (996 bp) from different samples.
- M-1 kb DNA ladder.
- Lane 1-Positive control; Lane 2-Negative control; Lanes 3-6, different blood samples.



- The specific identification of the organism was by subjecting the amplicon to RFLP. A typical gel with reference cultures and samples is shown in Fig. 2 A and 2B, respectively.

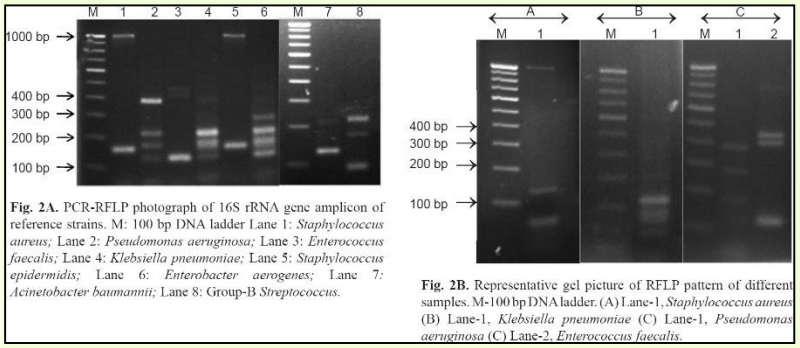


Fig. 2A. PCR-RFLP photograph of 16S rRNA gene amplicon of reference strains. M: 100 bp DNA ladder Lane 1: *Staphylococcus aureus;* Lane 2: *Pseudomonas aeruginosa;* Lane 3: *Enterococcus faecalis;* Lane 4: *Klebsiella pneumoniae;* Lane 5: *Staphylococcus epidermidis;* Lane 6: *Enterobacter aerogenes;* Lane 7: *Acinetobacter baumannii;* Lane 8: Group-B *Streptococcus.*

Fig. 2B. Representative gel picture of RFLP pattern of different samples. M-100 bp DNA ladder. (A) Lane-1, *Staphylococcus aureus* (B) Lane-1, *Klebsiella pneumoniae* (C) Lane-1, *Pseudomonas aeruginosa* (C) Lane-2, *Enterococcus faecalis.*

Rohit *et al.*,2016

PCR-RFLP analysis

Restriction fragment length polymorphism analyses of bacterial 16S rDNA

1. Obtain bacterial 16S rDNA PCR products (Qiagen Qiaquick PCR clean-up columns). There will be approximately 55 µl of DNA available for restriction digest analyses.
2. Label 3 different 1.5 ml eppendorph tubes-1 each for the enzyme and suspect DNA sample. Note that as a group you will need to digest 3 samples for the unknown (crime scene) sample that you prepared. Label them with your initials and the number for each bacterium.
3. Place 15 µl of purified PCR product DNA into each of the three tubes. BE SURE TO USE A NEW PIPETTE TIP EVERY TIME. Also, place 15 µl of unknown (crime scene) into the corresponding tube. Bump the liquid to the bottom of each of the 1.5 ml tubes by brief centrifugation.
4. Make a restriction enzyme digestion master mix(es) in a 1.5 ml eppendorph tube-make enough for more reactions than you have planned. Digest each sample with 5 units of the appropriate enzyme(s).
5. Use the chart below as a guide and have an instructor check your math before proceeding:

RCR-RFLP analysis

Restriction fragment length polymorphism analyses of bacterial 16S rDNA

Mix for reactions:

	Per Reaction
10X Buffer 2:	2.0 µl
100 X BSA:	0.2 µl
10 units enzyme #1:	_____ µl
10 units enzyme #2:	_____ µl
Sterile dH_2O:	_____ µl
Total Volume:	**5 µl**

5. Upon completion of making the master mixes, vortex and briefly centrifuge the master mixes down to the bottom of the tube.

6. Add 5 µl of restriction enzyme master mix to each appropriate DNA sample tube. At this point you are mixing 5 U of enzyme with 15 µl of DNA.

7. Vortex samples briefly to mix and then briefly centrifuge down to the bottom of the tube.

PCR-RFLP analysis

Restriction fragment length polymorphism analyses of bacterial 16S rDNA

8. Incubate all digest tubes overnight at 37°C (either in water bath or incubator)

DAY 2:

1. Pour a 1.2% agarose gel using 100 ml of 1X TAE buffer (don't forget to add 2 µl of ethidium bromide after gel is cooled). Use the narrow-width 10 well combs.
2. Without opening your tube, place tubes directly into centrifuge and briefly centrifuge solution down to the bottom of the tube.
3. Add 5 µl of 5X gel-loading dyes to each digest sample, vortex briefly, and briefly centrifuge solution down to the bottom of the tube.
4. When gel is solidified, load 3 µl of KB+ DNA ladder, 25 µl of each digested sample, and then 3 µl of KB+ DNA ladder. Run gel until lower gel dye reaches bottom of gel. Document your gel using the Fotodyne Imaging System and PC image and analyze the DNA fragment sizes using Total-Lab analysis software.

PCR-RFLP analysis

Restriction fragment length polymorphism analyses of *Pseudomonas gyrB* sequences

- Restriction analysis of the *gyrB* sequences (the DNA gyrase B subunit gene) was set up in silico with Serial Cloner v2.6.1 49 using the amplicon sequences obtained by a virtual PCR reaction run with the designed primers on a group of representative strains as templates.
- A set of classical enzymes was tested to select those ones producing differential RFLP patterns for each reference *Pseudomonas* species.
- To confirm the in silico results, in vitro PCR-RFLP assays were carried out in a final volume of 20 µl, containing 10 µl of PCR mix and 2 U of the endonuclease *TaqI* (type-2 restriction enzyme).
- Reactions were incubated at 62°C for 3 h. The restriction products were separated by electrophoresis in 2% agarose gels in 0.5 TBE at 6 V/cm for 2 h. Gels were stained with ethidium bromide and DNA banding patterns were visualized under UV light.

Agaras and Valverde,2018

PCR-RFLP analysis

Restriction fragment length polymorphism analyses of *Pseudomonas gyrB* sequences

- Novel oligonucleotides targeting the *Pseudomonas* spp. gyrB gene.

Gene	Amplicon Size (bp) [a]	Primer	Sequence (5′ → 3′)	Melting Temp. (°C) [b]	Annealing Temp. (°C)	
					Recommended [c]	Optimal
gyrB	1461–1467	gyrB-F gyrB-R	AGCATYAARGTGCTGAARGG GGTCATGATGATGATGTTGTG	55.3 53.1	55–59	57

- The size of the gene amplicons(1461-1467 bp) was estimated from the sequence of available Pseudomonas spp. genomes in GenBank on September 2017.

Agaras and Valverde,2018

PCR-RFLP analysis

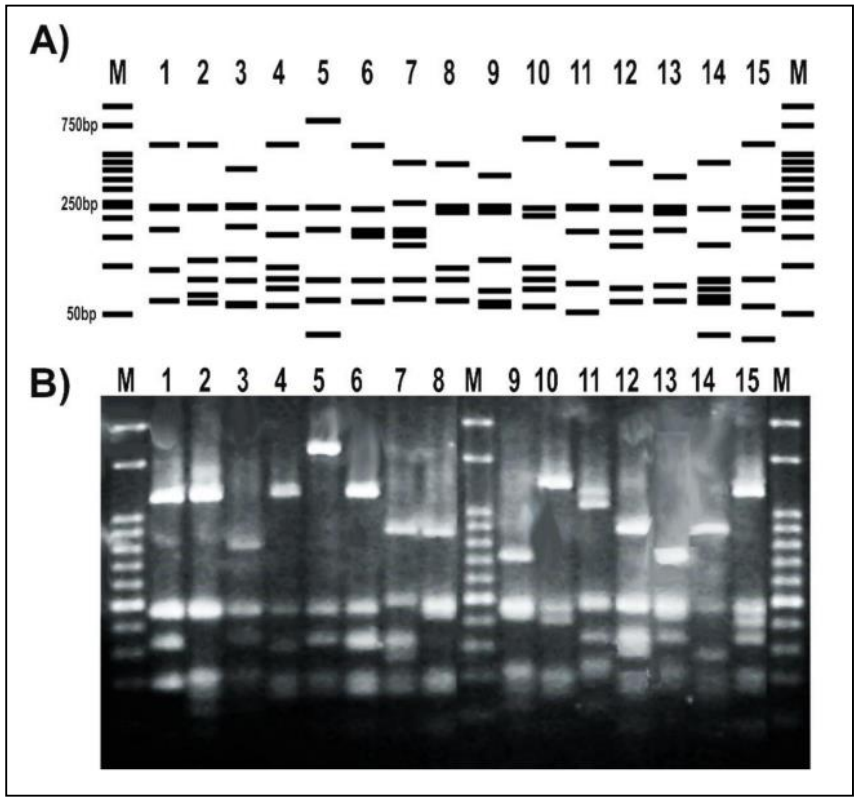
Restriction fragment length polymorphism analyses of *Pseudomonas gyrB* sequences

- Set up of a PCR-RFLP protocol targeting *Pseudomonas* gyrB gene.

A. RFLP patterns obtained from the in silico analysis of the amplicons sequenced at Macrogen S.A. and digested with *TaqI*;

B. RFLP patterns of representatives from our pseudomonads collection.

C. Strains were as follows: 1, *P. fluorescens* 2-79; 2, P. sp. 1008; 3, *P. fluorescens* SBW25; 4, *P. protegens* CHA0; 5, *P. simiae* WCS417; 6, *P. chlororaphis* SMMP3; 7, *P. syringae* pv. *syringae* DC3000; 8, *P. putida* KT2440; 9, *P. aeruginosa* PA01; 10, *P. alkylphenolica* KL28; 11, *P. stutzeri* ATCC 17588; 12, *P. stutzeri* 2018; 13, P. sp. CF5; 14, *P. sihuiensis* N23; 15, *P. donghuensis* SVBP6. Markers (M) are 50-bp DNA ladder (Embiotec).



Agaras and Valverde,2018

PCR and sequencing primers used in this study

Burkholderia spp.

- UP-1E/Apru for amplification of gyrB gene.
- 70F2/70R2 for amplification of rpoD gene (also known as sigma 70).

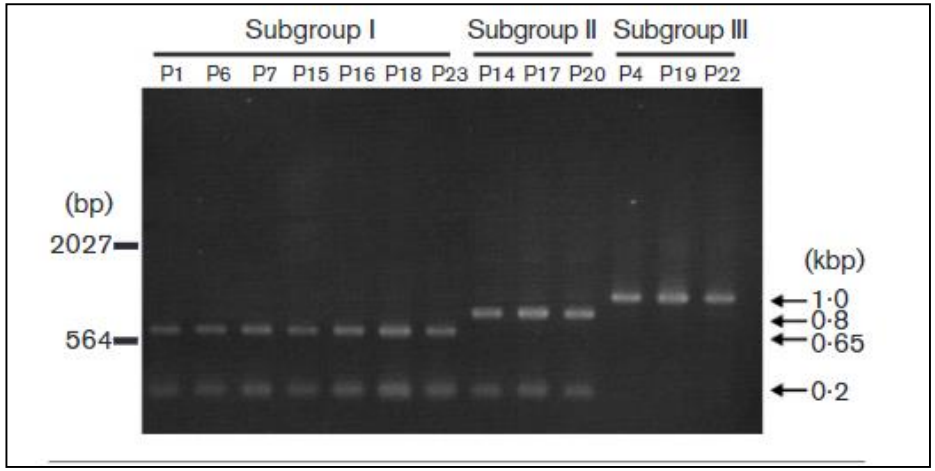
Primer	Target gene	Sequence (5′–3′)	Length (nt)	Reference
UP-1E	*gyrB*	CAGGAAACAGCTATGACCAYGSNGGNGGNAARTTYRA	37	Yamamoto *et al.* (2000)
AprU		TGTAAAACGACGGCCAGTGCNGGRTCYTTYTCYTGRCA	38	Yamamoto *et al.* (2000)
70F2	*rpoD*	ACGACTGACCCGGTACGCATGTAYATGMGNGARATGGG	38	This study
70R2		ATAGAAATAACCAGACGTAAGTTNGTRTAYTTYTTNGCDAT	41	This study
glu-FW	*gyrB* of *B. glumae*	GAAGTGTCGCCGATGGAG	18	This study
glu-RV		CCTTCACCGACAGCACGCAT	20	This study
pla-FW	*gyrB* of *B. plantarii*	TCGAGCTGGCTGCGCCTC	18	This study
pla-RV		GTCGTCGCCCGAGGTCTCG	19	This study
gla-FW	*gyrB* of *B. gladioli*	CTGCGCCTGGTGGTGAAG	18	This study
gla-RV		CCGTCCCGCTGCGGAATA	18	This study

N, any; R, A or G; S, C or G; Y, C or T; M, A or C.

Maeda *et al.*,2006

Phylogenetic relationships among *B. plantarii* strains *Burkholderia* spp.

- 23 rice strains were divided into three subgroups.
- Nucleotide sequences of the gyrB PCR products from strains in subgroup I showed one restriction site for both *Hae*II and *Sac*I (data not shown).
- One restriction site for *Hae*II was located in the gyrB PCR products from strains in subgroup II.
- No *Hae*II and *Sac*I restriction sites were present in the gyrB PCR products from strains in subgroup III.
- *Sac*I and *Hae*II digestion of the gyrB PCR products allowed discrimination of strains among subgroups I, II and III.



Ethidium bromide-stained gel of *Hae*II- and *Sac*I-digested partial fragments of gyrB amplified by PCR from *B. plantarii* strains using primers UP-1E and AprU.

Maeda *et al.*,2006

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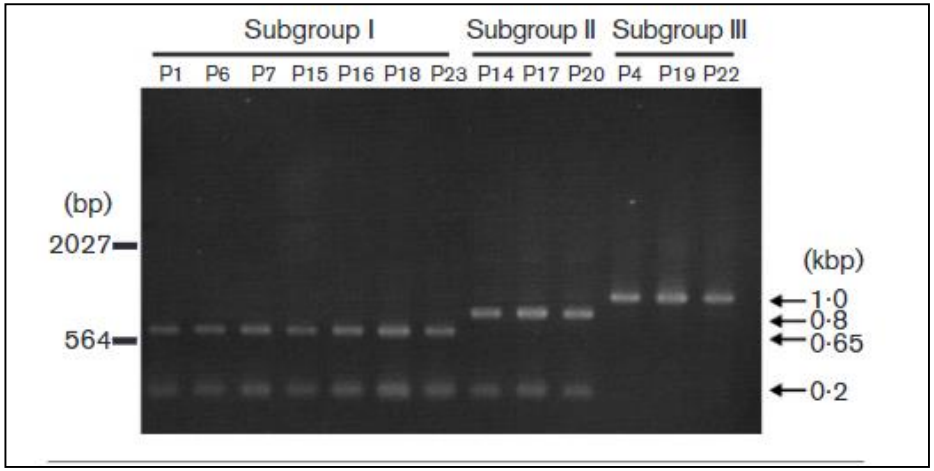

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Maeda *et al.*,2006

RFLP analysis

RFLP without PCR

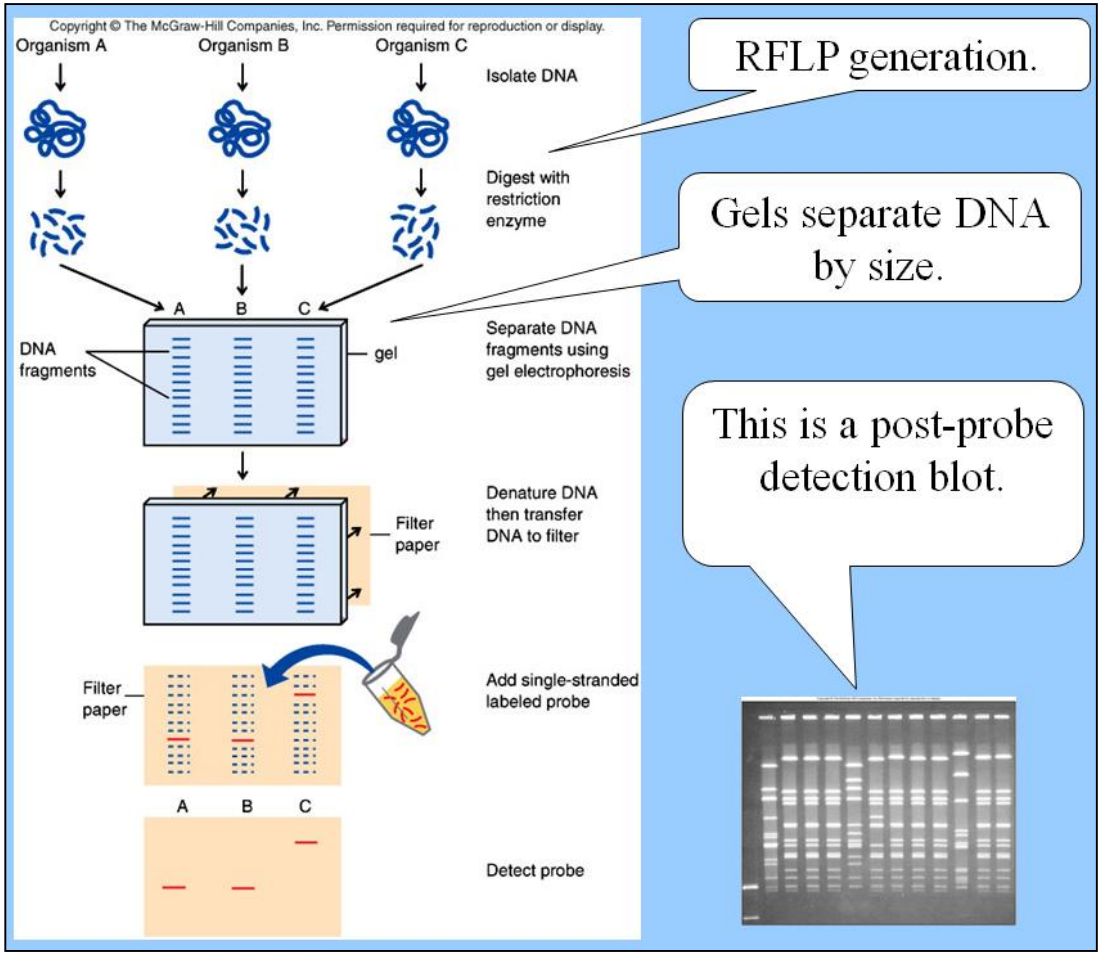
Post-probe detection blot

- Since RFLP without PCR makes a smear on the gel, better hybridize the cut DNA on membrane with known DNA of interest and then make autoradiographies
- To obtain a pattern, DNA is blotted after agarose gel electrophoresis onto a filter (so-called Southern blotting) and hybridized.
- After hybridization visualization of the pattern can be achieved as described under DNA slot/blot hybridization (DNA-DNA/DNA-RNA hybridization).

RFLP analysis

RFLP without PCR

Post-probe detection blot



Abedon,2011

RFLP analysis

RFLP without PCR

Restriction fragment length polymorphisms

RFLP

Restriction Fragment Length Polymorphisms

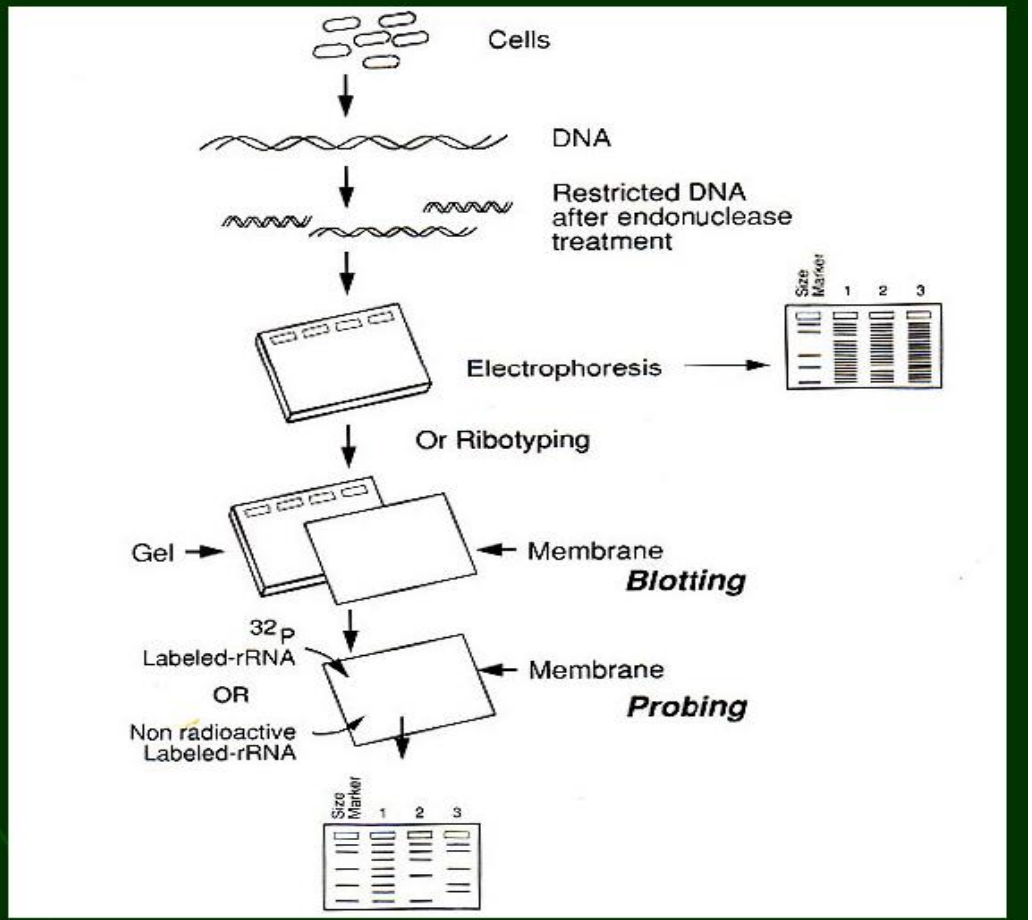
few Kb of DNA

Electropherogram

Southern Blot

Probe

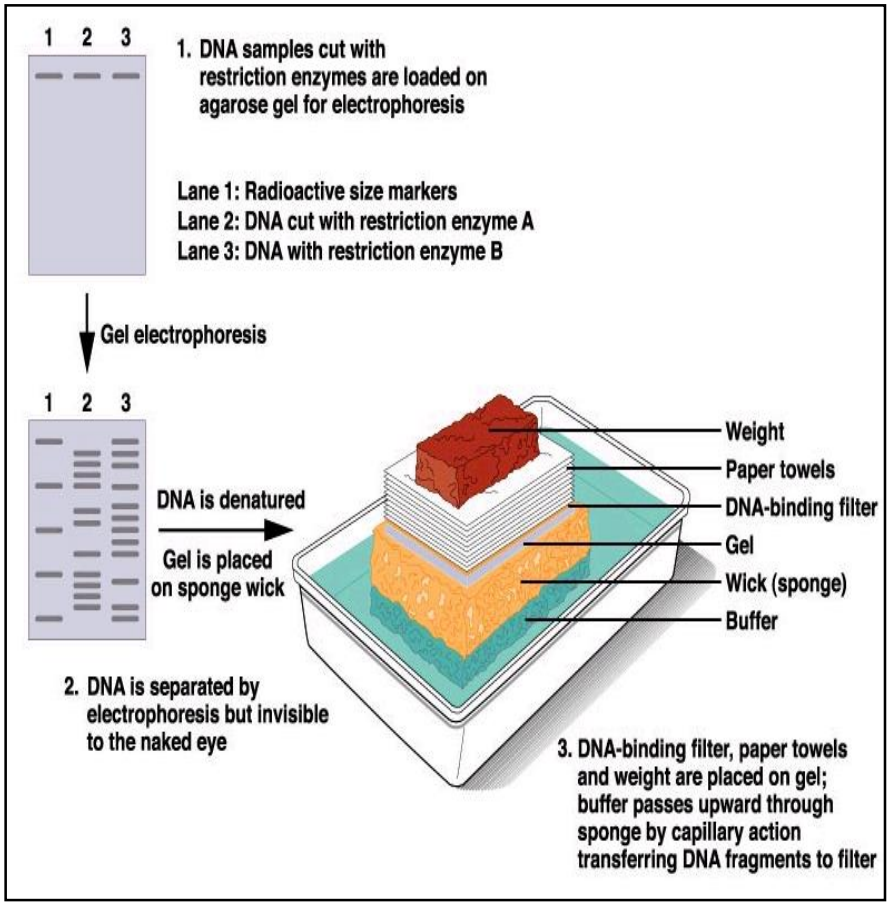
Detection

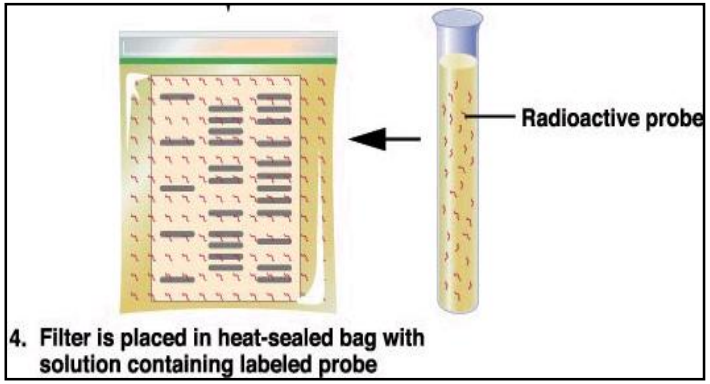


RFLP analysis

RFLP without PCR

Southern blotting

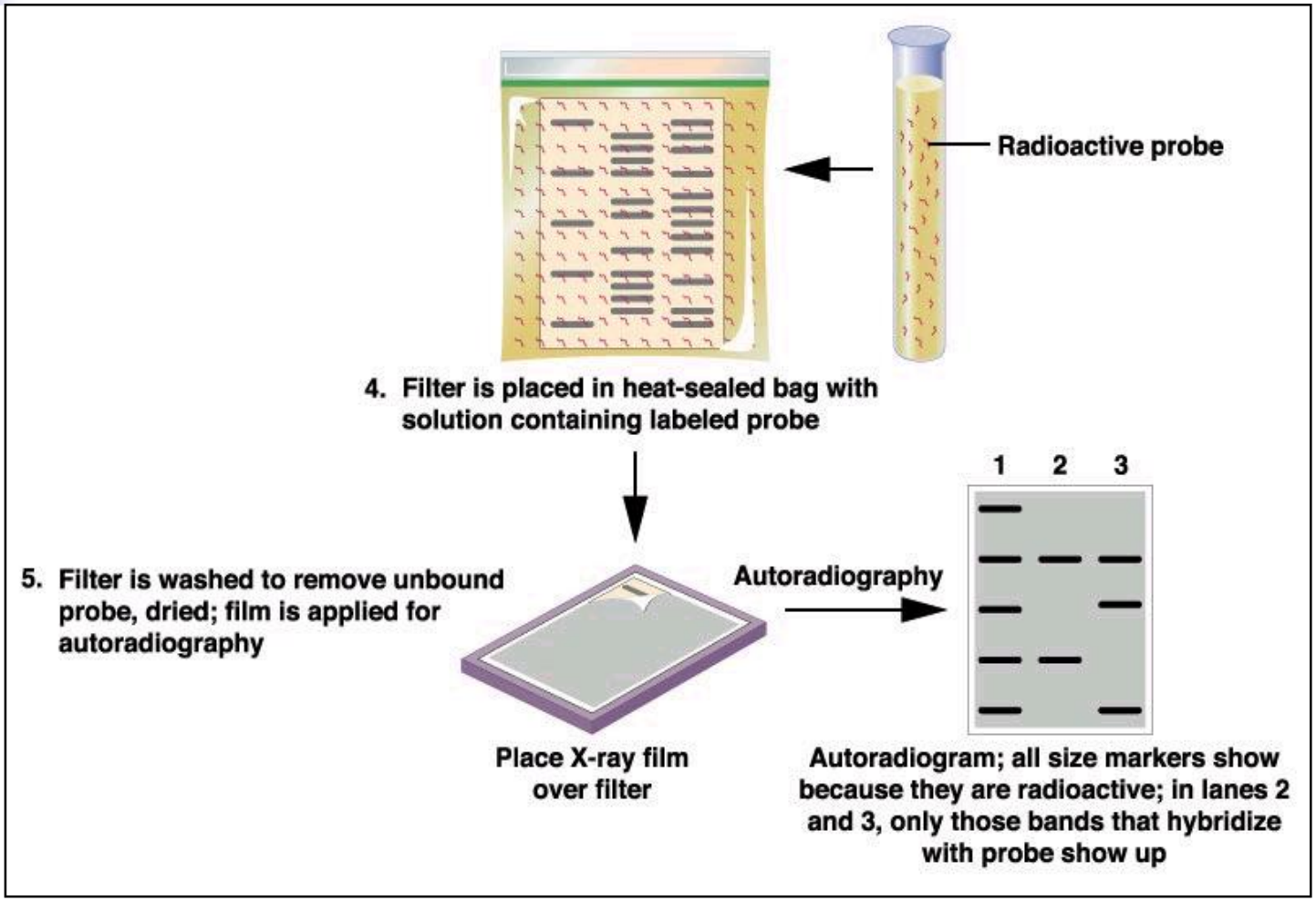




RFLP analysis

RFLP without PCR

South blotting



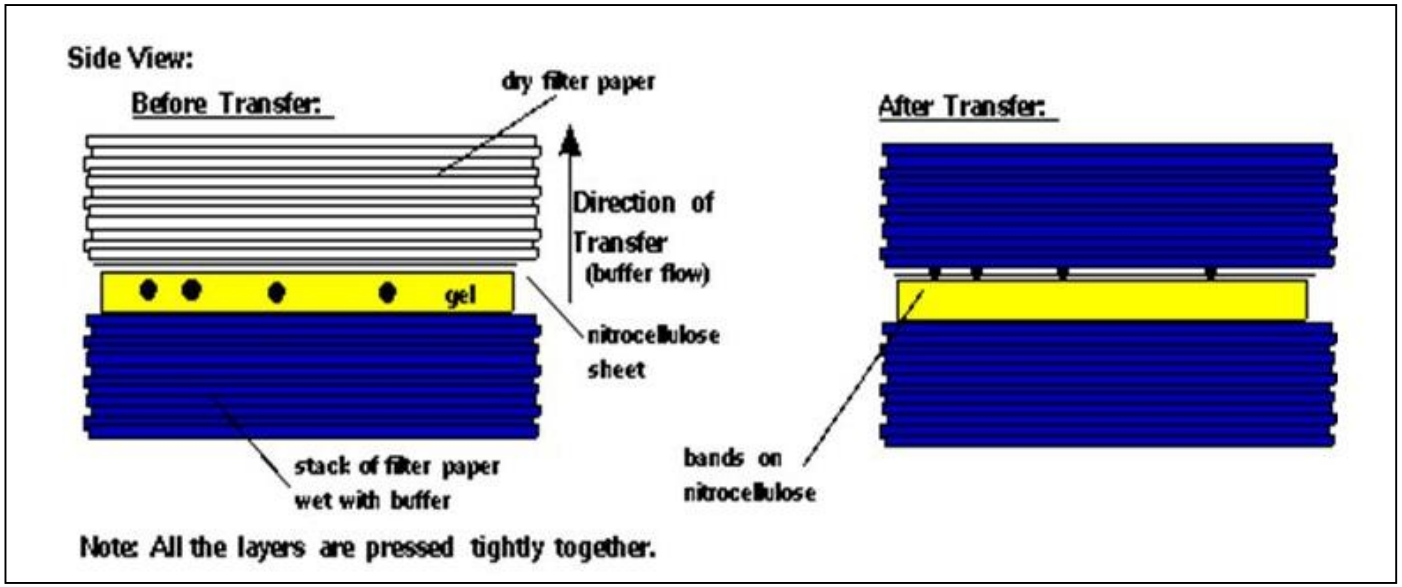
Methods of blotting

- The DNA, RNA, or protein can be transferred to nitrocellulose in one of two ways:

1. Electrophoresis, which takes advantage of the molecules' negative charge.
2. Capillary blotting, where the molecules are transferred in a flow of buffer from wet filter paper to dry filter paper.

Southern blotting
Capillary blotting

- Capillary blotting-fragments are eluted from the gel and deposited onto the membrane by buffer that is drawn through the gel by capillary action.

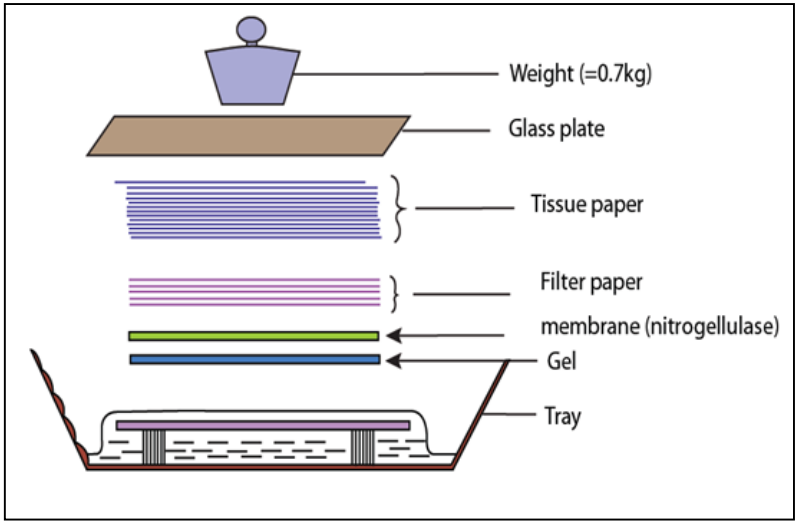


Usually for southern blotting

Southern blotting

Transfer stack

- The blotting paper acts like a wick.
- Driven by capillary action, fluid is drawn from the reservoir up though the gel and into the stack of dry paper.
- As the fluid migrates up through the gel, it carries the denatured DNA fragments up with it out of the gel.
- When the fragments reach the membrane, they stick to the membrane and remain there.
- Because the wick, gel, membrane, and stack of paper lay directly on top of each other, when the DNA fragments are transferred up onto the membrane, they form the same pattern that they formed in the gel.



Blocking before adding probe

- In all three blots (Southern, Northern and Westerns), the labeled probe is added to the blocked filter in buffer and incubated for several hours to allow the probe molecules to find their targets.
- Because no molecules have yet bound on the surface of the filter, the filters are soaked in a blocking solution which contains a high concentration of DNA, RNA, or protein.
- This prehybridization buffer coats the filter and prevents the probe from sticking to the filter itself.
- During hybridization, we want the probe to bind only to the target molecule.

Important point

- In a Southern Blot, the DNA molecules in the gel are double-stranded, so they must be made single stranded in order for the probe to hybridize to them.
- To do this, the DNA is transferred using a strongly alkaline buffer, which causes the DNA strands to separate - this process is called denaturation - and bind to the filter as single-stranded molecules.
- RNA and protein are run in the gels in a state that allows the probe to bind without this pre-treatment.

Labelling PCR products with radioactive ^{32}P or Digoxigenin

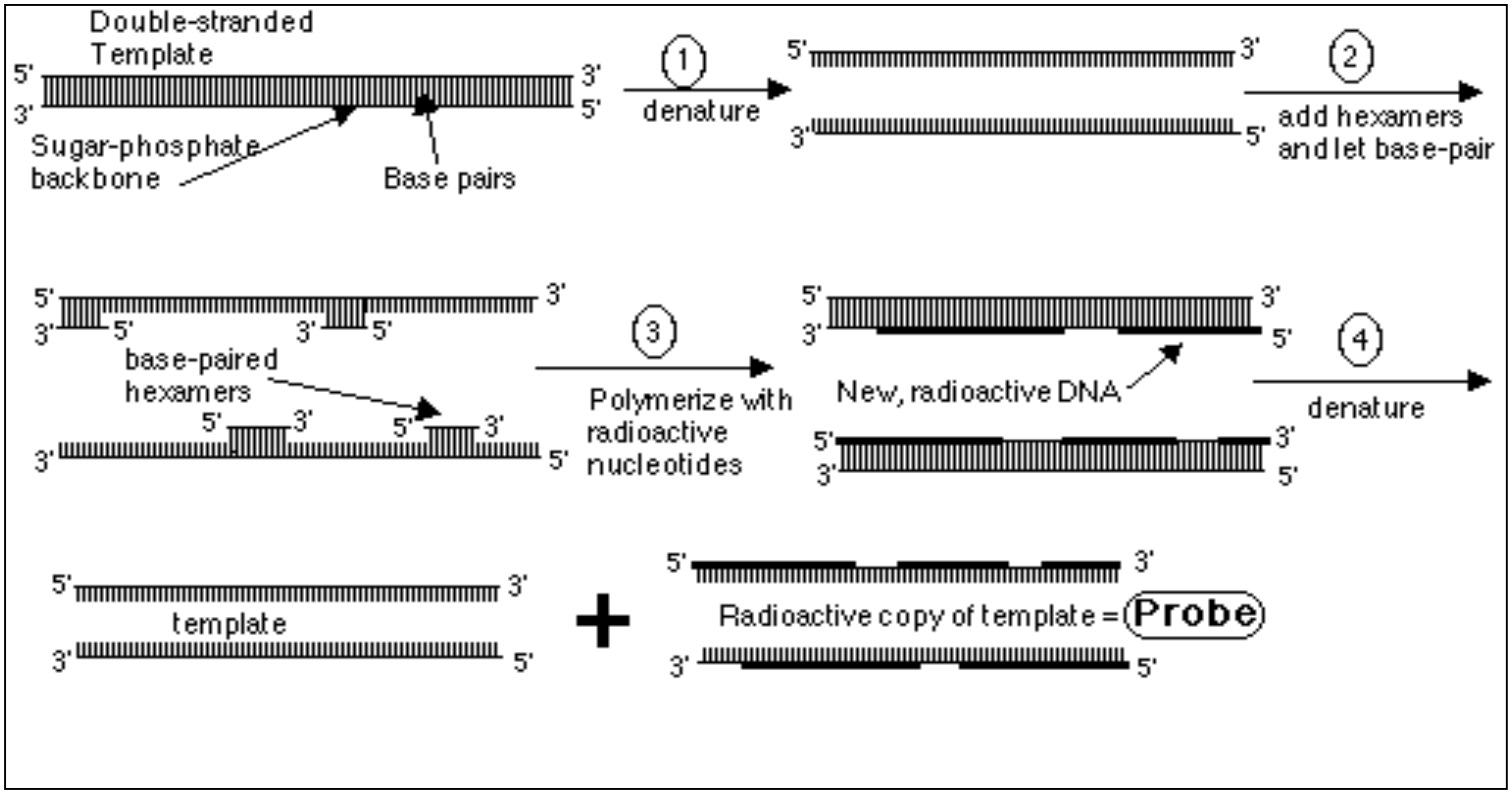
- Digested DNA separated on agarose gels can be transferred as nylon or nitrocellulose membranes using a Southern blot.
- These fragments are hybridized with a complementary single-strand DNA or RNA probe, labeled with:

1. Radioactive ^{32}P (which is detected by autoradiography),
2. Chemicals such as Biotin or Digoxygenin which are two ligands that are linked to nucleotide bases for nonradioactive labelling.

- The radioactively labelled DNA can be detected with X ray film.

Labeling the probe

Radioactive DNA probes for Southern blots



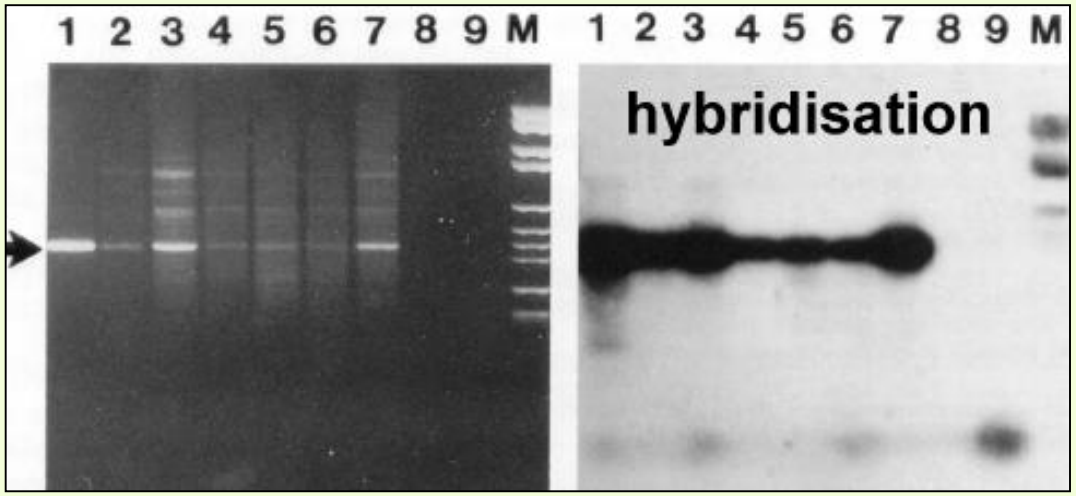
labeled DNA shown in gray

Detecting the probe

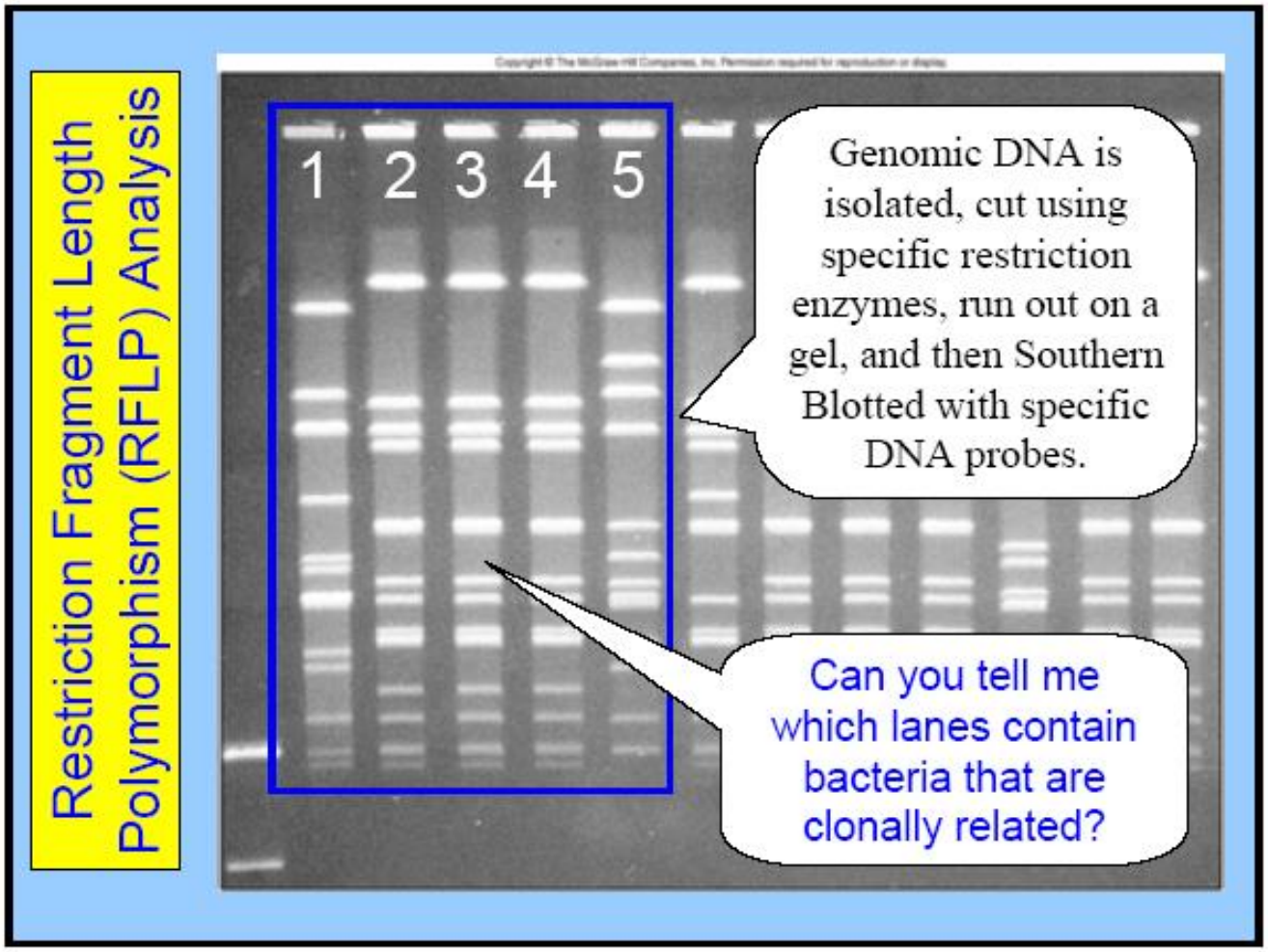
- The filter is rinsed repeatedly in several changes of buffer to wash off any un-hybridized probe.
- The filter now looks like a blank sheet of paper - you must now detect where the probe has bound:
- The radioactively labelled DNA can be detected with X ray film (Autoradiography).
- If you press the filter up against X-ray film and leave it in the dark for a few minutes to a few weeks, the film will be exposed wherever the probe bound to the filter.
- After development, there will be dark spots on the film wherever the probe bound.

Gel electrophoresis and Southern blotting

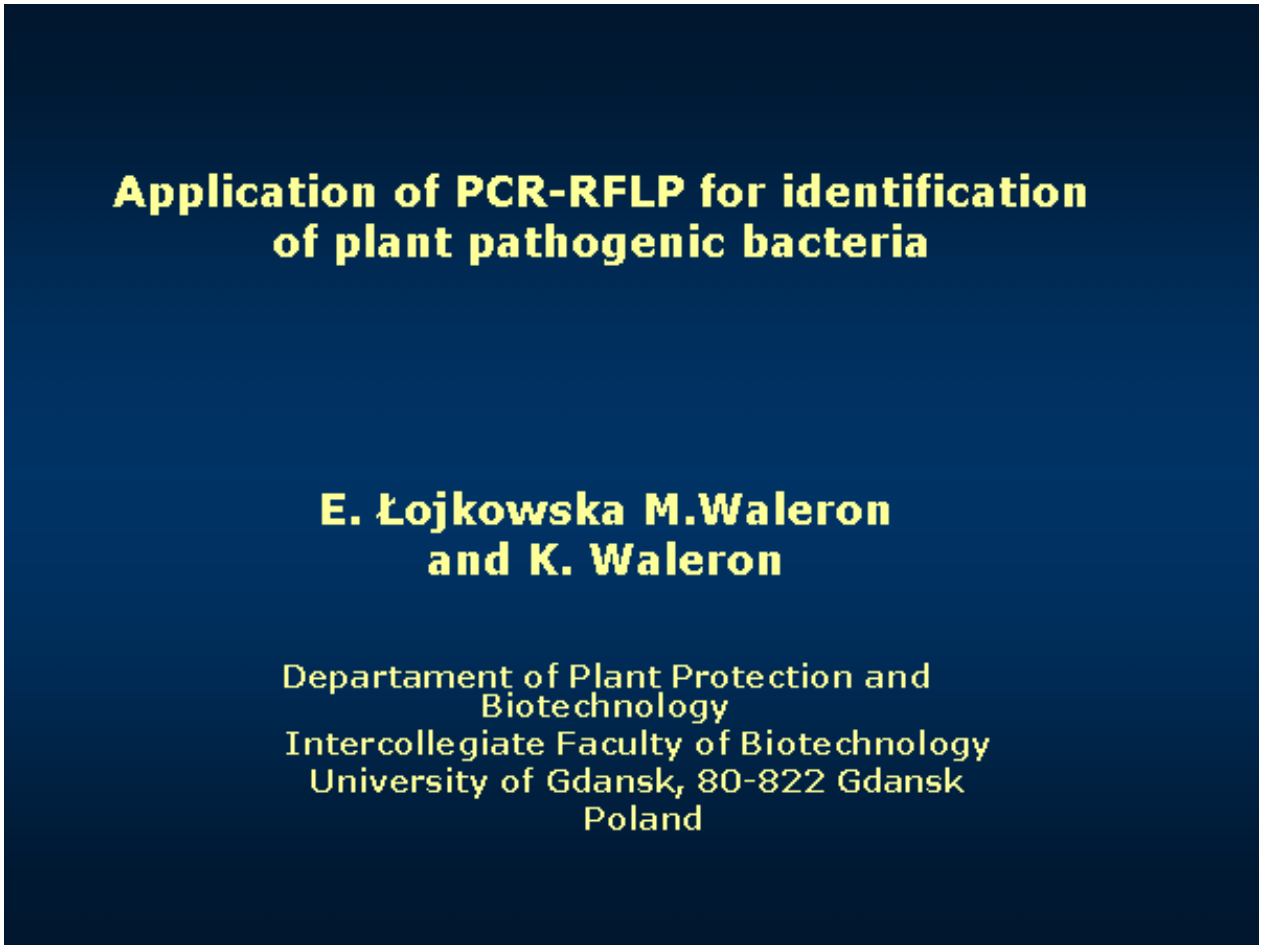
- The left panel is a photo of an EthBR-stained 2% agarose gel; the right is an autoradiograph of a Southern blot probed with ^{32}P-labelled HPV-16 DNA. Note how much more sensitive blotting is, and how much more specific the detection is.
- EthBr=ethidium bromide.



RFLP
Southern blotting



PCR-RFLP analysis
Identification of plant pathogenic bacteria

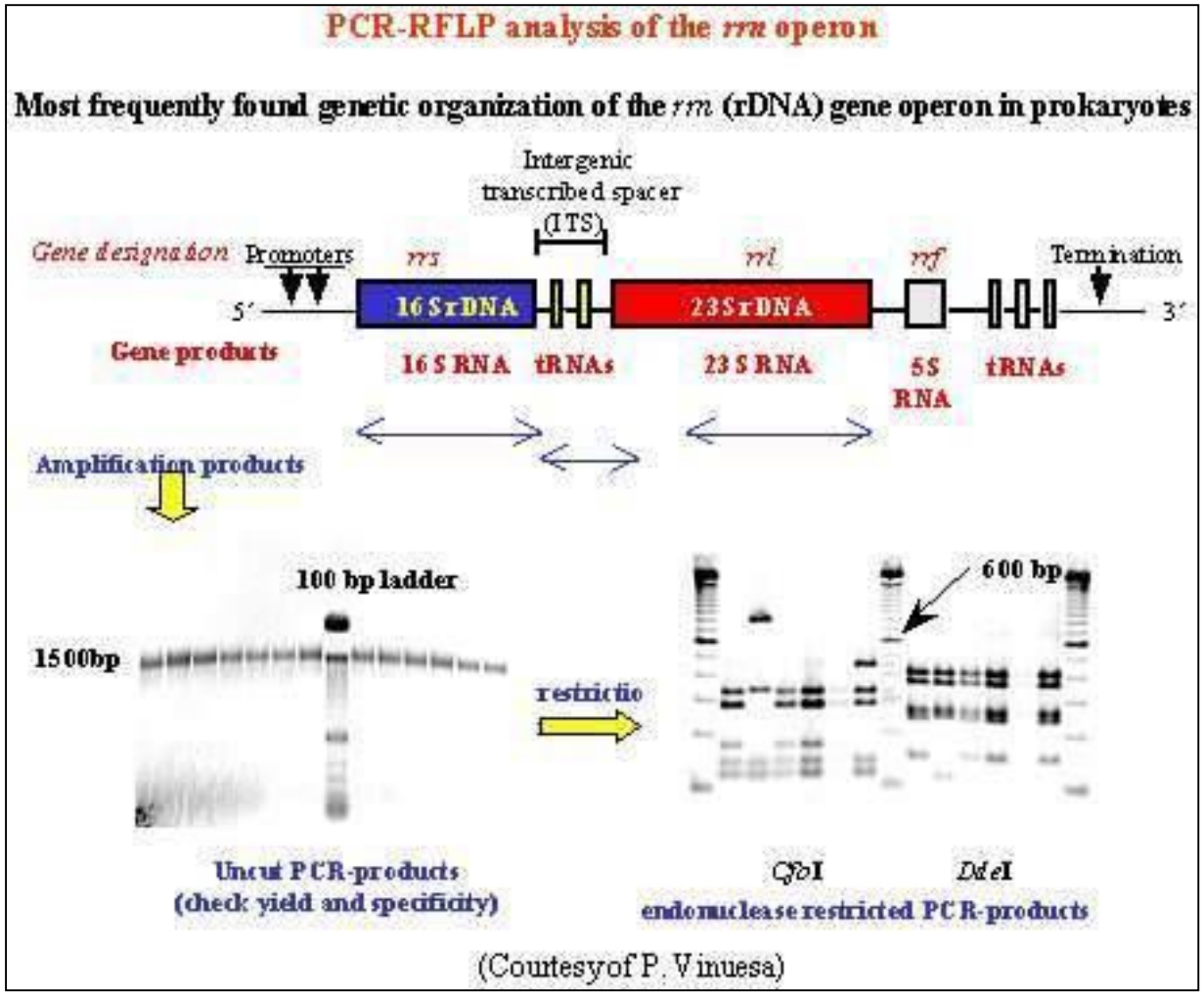


Lojkowska *et al.*,2004

PCR-RFLP

- PCR-RFLP is the faster and easer way method.
- RFLP analysis is discriminative at low taxonomic level, often strain level and is now often combined with PCR, e.g. digestion after PCR of the only some loci such as:
- 16S rRNA,
- *gyrB*,
- *rpoD* (also known as sigma 70),
- *Irp* (iron receptor protein), and
- *recA* (Recombinase A)sequences.

PCR-RFLP analysis
Detecting rRNA molecules



PCR-RFLP
recA gene

- RecA is a 38 kilodalton protein essential for the repair and maintenance of DNA.
- It is a ubiquitous protein, present in nearly all bacteria.
- It has multiple activities, all related to DNA repair during DNA recombinations.

PCR-RFLP analysis

Based on *recA*(Recombinase A) gene

The design of primers for the *recA* gene

The sequence of *Erwinia carotovora* recA gene was described by Zhao & McEntee (1990)

The sequence of designed primers (Waleron *et al.*, 2002):

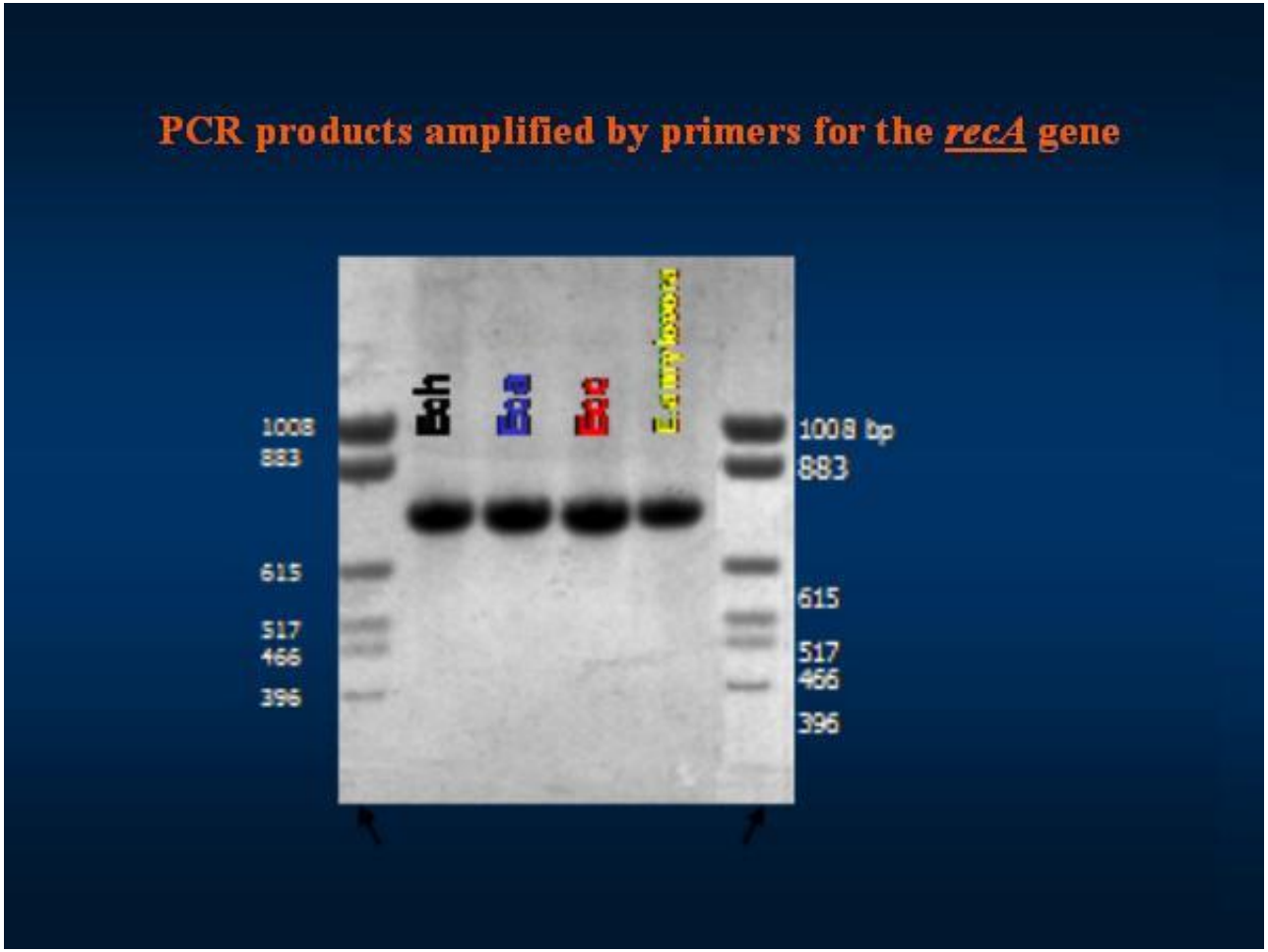
5′-GGTAAAGGGTCTATCATGCG-3′
5′-CCTTCACCATACATAATTTGGA-3′

was checked in the complete GeneBank and EMBL databases using the BLAST-n program

Lojkowska *et al.*,2004

PCR-RFLP analysis

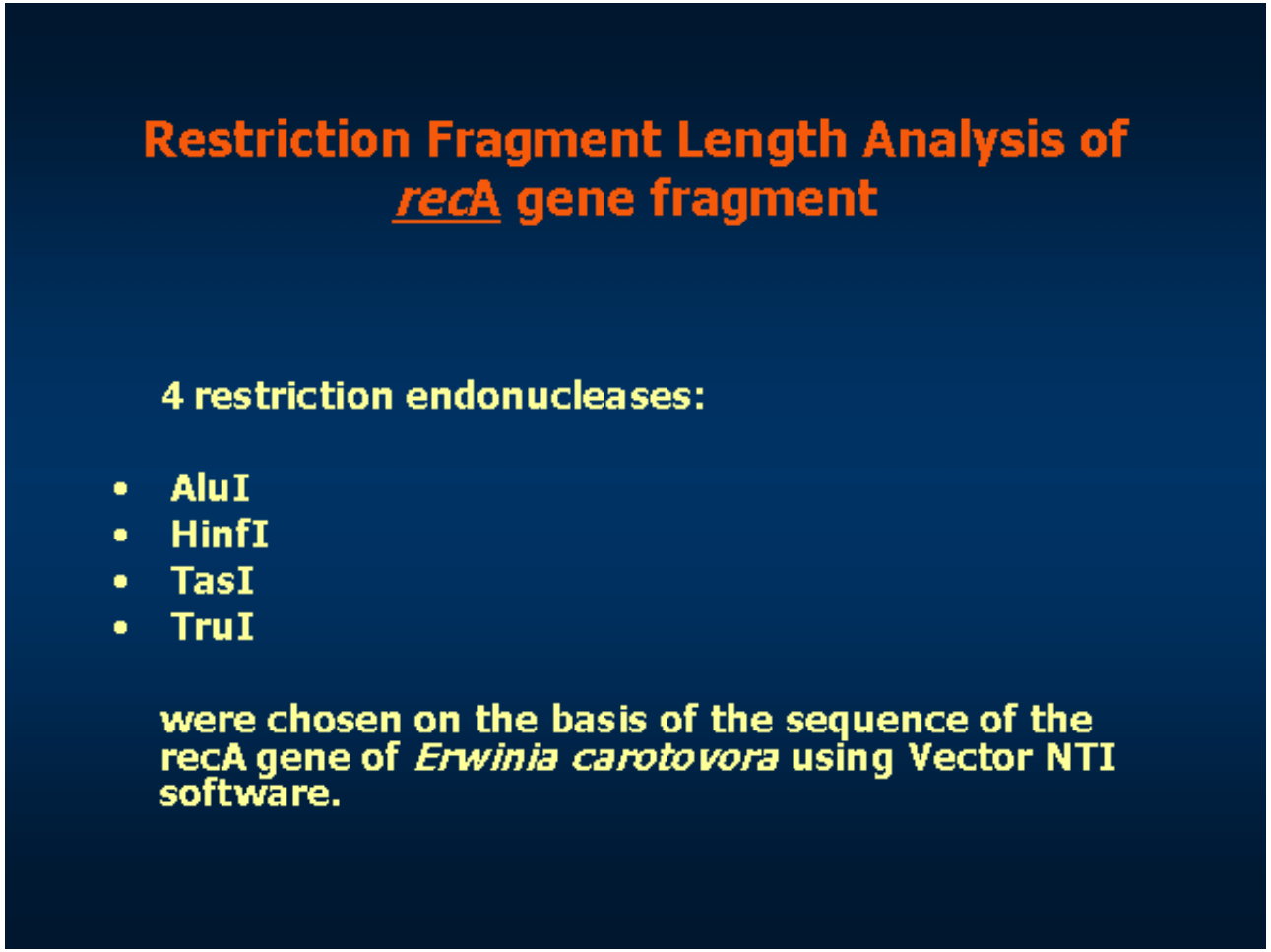
Based on *recA* gene



Lojkowska *et al.*,2004

PCR-RFLP analysis

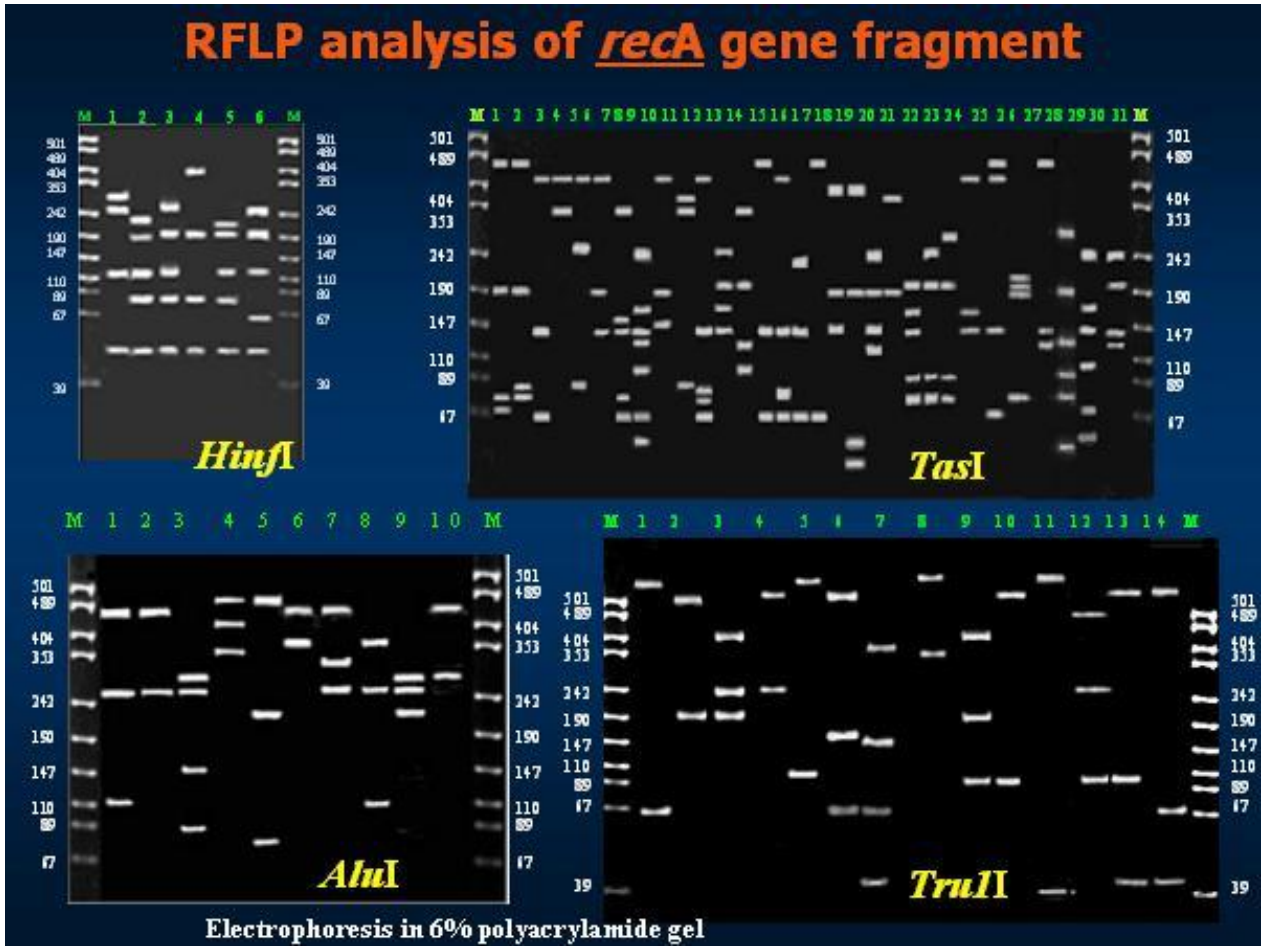
Based on *recA* gene



Lojkowska *et al.*,2004

PCR-RFLP analysis

Based on *recA* gene



Lojkowska *et al.*,2004

PCR-RFLP analysis

Based on *recA* gene

Number of profiles described for the species from the genus *Erwinia based on* RFLP analysis of *recA* gene fragment

Species	Number of strains	Amount of profils	Number of profis
Eca	106	2	1, 2
Ecc	158	18	3-20
Ecb	12	1	21
Eco	12	1	22
Ecw	5	1	23
E. amylovora	39	1	32
E. ananas	5	2	25, 26
E. cacticida	16	3	29, 30, 31
E. carnegiena	1	1	31
E. chrysanthemi	93	15	33-46
E. cypripedi	5	1	24
E. herbicola	6	2	27, 28
E. mallotivora	1	1	55
E. milletiae	1	1	28
E. nigrifluens	3	1	51
E. persicinum	3	1	49
E. psidii	1	1	54
E. pyrifoliae	6	1	58
E. quercina	1	1	56
E. rhapontici	6	1	53
E. rubifaciens	3	1	50
E. salicis	2	1	52
E. stewartii	2	1	48
E. tracheiphila	1	1	57

Lojkowska *et al.*,2004

T-RFLP

Terminal restriction fragment length polymorphism analyses

PCR methods of microbial community analysis

- (T-RFLP) is one of the newest tools for evaluating microbial communities.

1. Target gene is amplified by PCR
1. Restriction enzymes are used to cut the PCR products

- T-RFLP analysis is a method for rapid profiling of mixed populations of an homologous amplicon (i.e., diverse sequences of a single gene).

T-RFLP

Terminal restriction fragment length polymorphism analyses

PCR methods of microbial community analysis

- Assessment of the diversity, structure, and dynamics of complex microbial communities with T-RFLP has mainly been based on PCR-amplified 16S rRNA genes (16S rDNA).
- PCR assays for genes other than 16S rDNA have also been used to generate T-RFLP community patterns for particular functional groups of bacteria.

Spencer and Spencer,2004

T-RFLP

Terminal restriction fragment length polymorphism analyses

Community analysis

- The technique involves:

1. nucleic acid extraction,
2. PCR amplification with fluorescently labeled primers,
3. restriction digestion, and
4. automated sizing of labeled terminal restriction fragments (TRFs).

- T-RFLP has advantages over other community analysis methods in that phylogenetic information can be obtained without direct sequencing of individual 16S rRNA gene fragments.

T-RFLP

Amplification of 16S rRNA genes from plate community DNA

Primers 8-27F and 907-926R

- Bacterial 16S rRNA genes were amplified by PCR using primers 8-27F and 907-926R.
- PCR-amplification targeted the universal eubacterial 16S rDNA portion of about 880 bp using:
- Forward primer 27f (5'-AGA GTT TGA TCC TGG CTC AG-3'), and
- Reverse primer 907r (5'-CCG TCA ATT CCT TTG AGT TT-3') following the approach described by So and Young,1999.
- The former oligonucleotide was labeled at its 5 end with 6-carboxyfluorescein to facilitate fluorescence-based detection of the products.

Rodrı´guez *et al.*,2006

T-RFLP

Primers (8F, 907R, 11F, 226F, 1111R or EF4) and restriction enzymes (*Msp1*, *Hha1*, *Rsa1*, *HaeIII*, and *Bfa1*)

- T-RFLP FRAGSORT is a computer program that compares the TRFs obtained from samples processed with widely used primers (8F, 907R, 11F, 226F, 1111R or EF4) and restriction enzymes (*Msp1*, *Hha1*, *Rsa1*, *HaeIII*, and *Bfa1*) to TRFs from corresponding *in silico* amplification and digestions of RDP II alignments (version 8.0).
- Fragment sizing error can be either a constant value or a variable value based on fragment size.
- Output is a list of microorganisms and TRF sizes that correlate with multiple experimentally generated TRFLP profiles in descending order from the greatest to the least normalized TRF peak areas.

T-RFLP

Terminal restriction fragment length polymorphism analyses

Community analysis

Figure 2. Primers and Restriction Enzymes

The following primer sequences and restriction enzymes are currently supported by tRFLP FragSort. Numbers in brackets [#,#,#] denote mismatches, insertions and deletions allowed in the target of the primer sequence.

Primer	Target	Sequence
8f	universal bacterial	agagtttgatcatgg [3,0,0]
11f	universal bacterial	gtttgatcmtggctcag [3,0,0]
907r	universal bacterial	ccgtcaattcmtttragtt [3,0,0]
226f	Pseudomonas	atgagcctaggtcggattagct [3,0,0]
1111r	Pseudomonas	gtgctggtaactaaggacaaggg [3,0,0]

Restriction Enzymes and Restriction sites

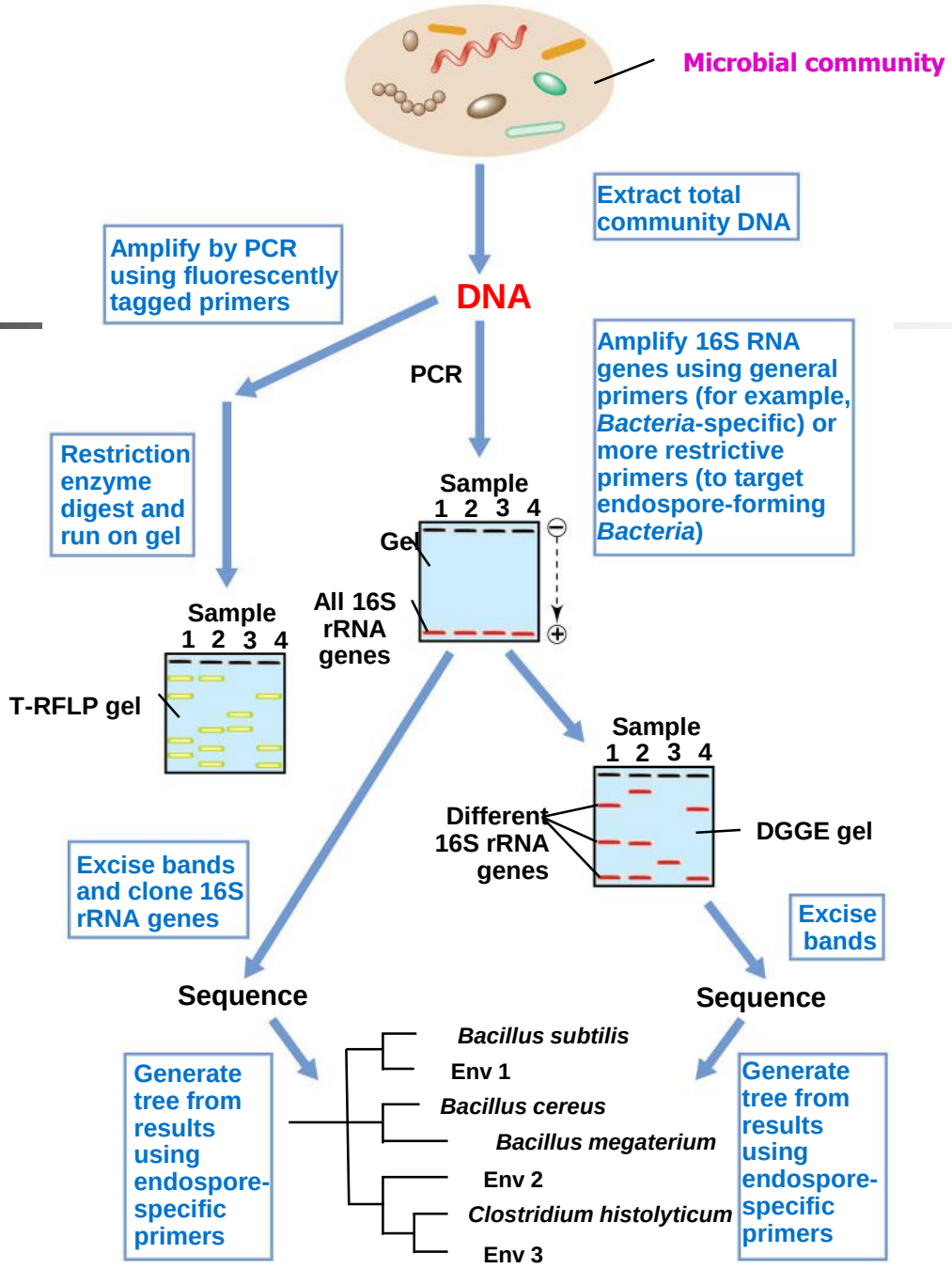
BfaI	c^thg
HaeIII	gg^cc
HhaI (CfoI)	gcg^c
MspI	c^cgg
RsaI	gt^ac

Frederick C. Michel, Jr. and Stephen Sciarini

T-RFLP
Community analysis

- Validation experiments showed that at least three different digestions must be used to accurately identify pure cultures and members of defined bacterial communities.
- Analysis of samples from six agricultural soils showed that a majority of individual TRF sizes obtained from environmental samples correspond to sizes predicted by *in silico* digestion.
- However, the TRF areas corresponding to organisms consistent with three different digestions comprised less than 40% of the total PCR product.
- In conclusion, FRAGSORT is a useful tool for rapidly analyzing multiple digestion T-RFLP data.

Michel and Sciarini



T-RFLP
Community analysis

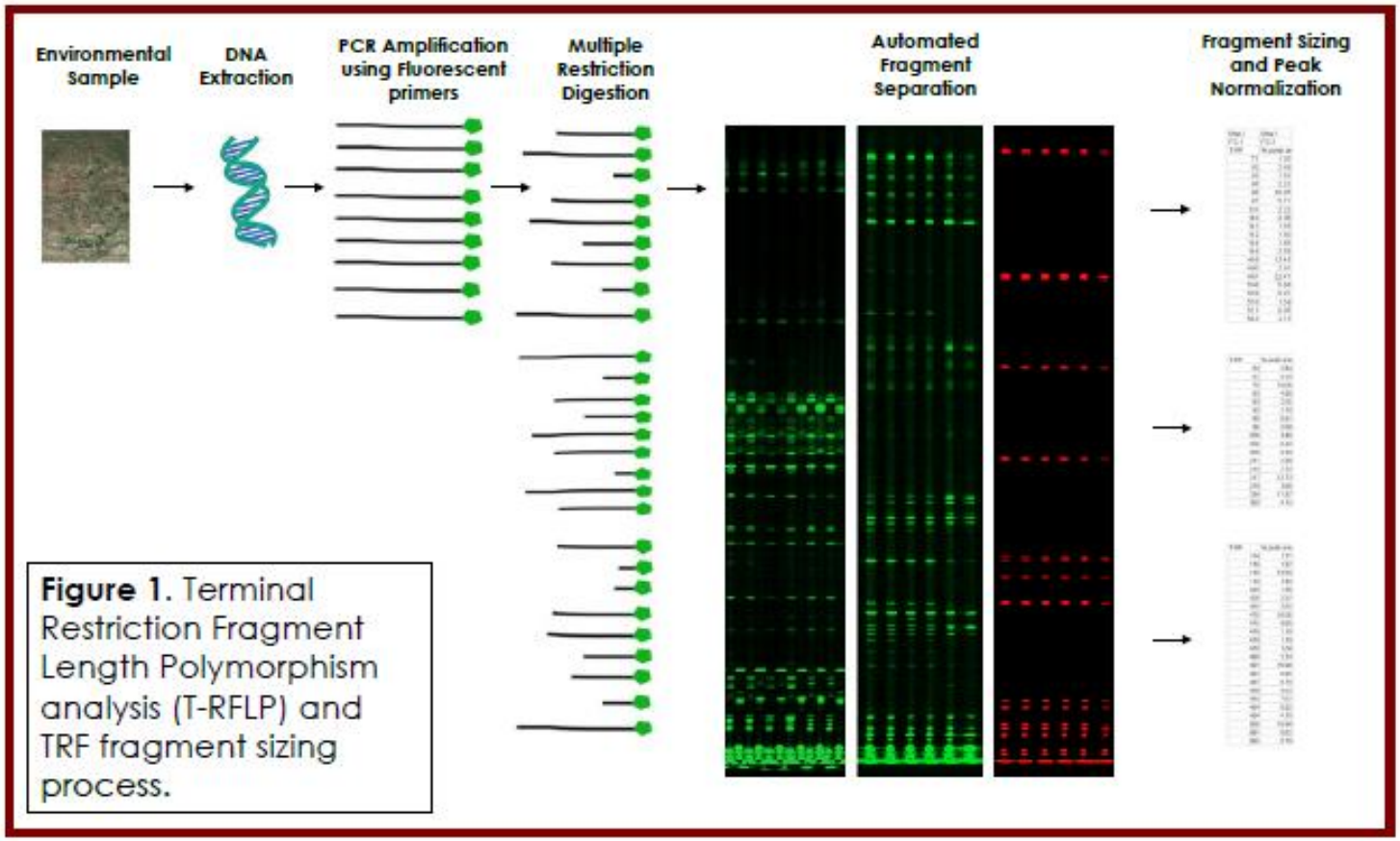


Figure 1. Terminal Restriction Fragment Length Polymorphism analysis (T-RFLP) and TRF fragment sizing process.

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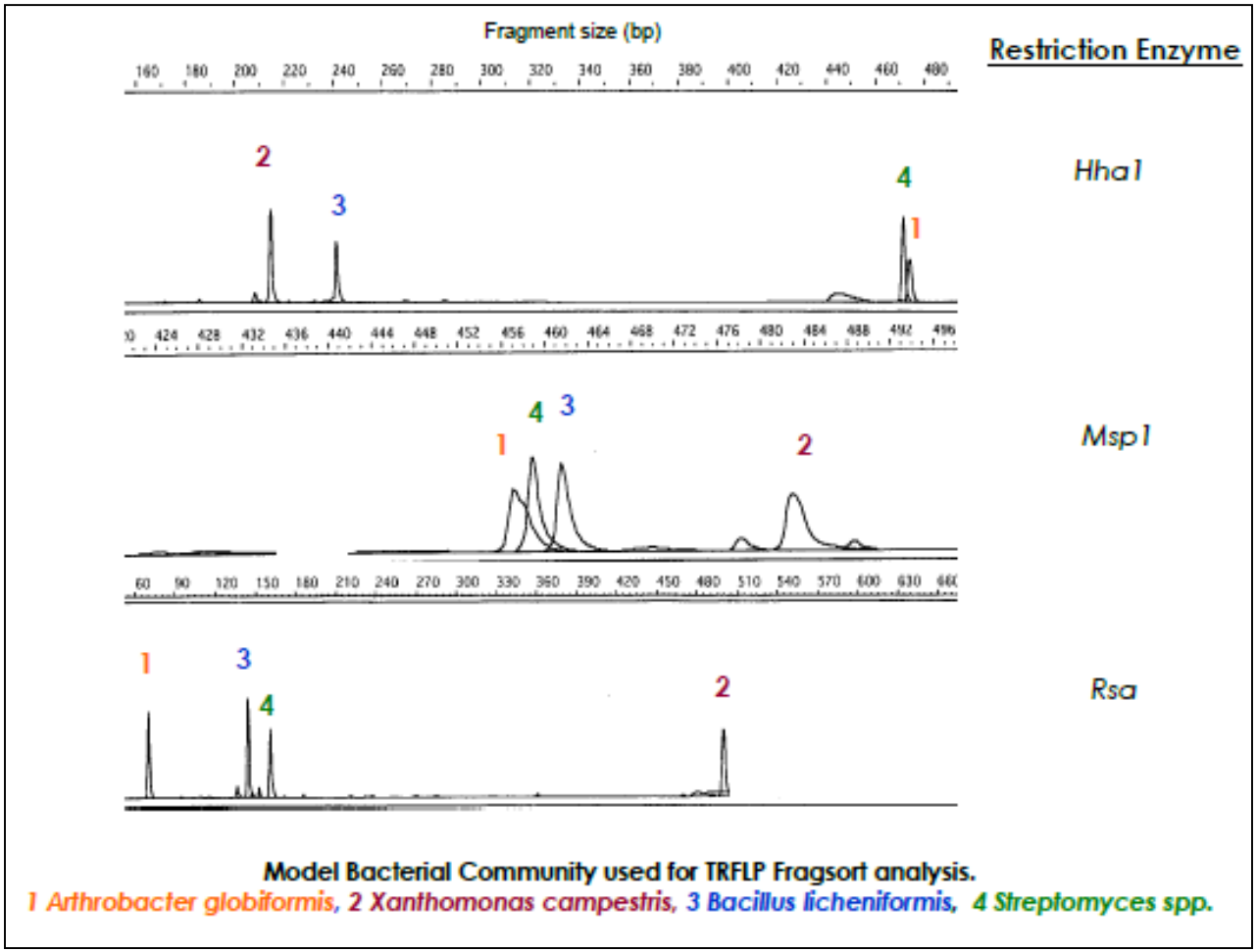
T-RFLP
Community analysis



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T-RFLP
Community analysis

Table 1. Effect of TRF fragment sizing error and strains with similar TRF sizes on identification of six pure cultures and an organic soil.

Strain	TRF Size (bp)			Number of Strains Matching 3 TRFs (same genus)					
				Fragment Sizing Error					
	Hha1	*Msp1*	*Rsa1*	1 bp	2 bp	3 bp	5 bp	Eqn A	Eqn B
Xanthomonas oryzae LMG 5047	214	497	480	7 (6)	7 (6)	9 (8)	9 (8)	8 (6)	6 (5)
Streptomyces sampsonii ATCC 25495	468	160	452	5 (5)	49(49)	55(55)	55 (55)	5 (5)	5 (5)
Bacillus licheniformis DSM 13	240	144	456	29(26)	36(33)	41(38)	45 (42)	32 (3)	8 (1)
Escherichia coli [gene=rrnB operon	372	495	426	85(27)	91(27)	98(27)	101 (27)	95 (27)	85 (27)
Arthrobacter globiformis str. 168	471	66	455	1 (1)	3 (1)	3 (1)	3 (1)	3 (1)	3 (1)
Mycobacterium avium STR IIIB	366	160	79	13(13)	18(18)	23(22)	25(24)	15(15)	12(12)
Agricultural Soil	-	-		99	380	610	905	165	95

a- Equation A = 1.0×10^{-4} bp^3 + 1×10^{-4} bp^2 – 4×10^{-4} bp +1.12.
b- Equation B = 0.5×10^{-4} bp^3 + 7×10^{-4} bp^2 – 2×10^{-4} bp +0.56
c- The forward primer was 8f (see text for sequence).
d- Ribosomal database version 8.0 containing 34,531 strains was used.

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T-RFLP
Community analysis

Table 2. Analysis of Agricultural Soil[1] using T-RFLP Fragsort

Restriction Enzyme	Soil 1	2	3	4	5	6[a]
Hha1						
Number of TRFs	82	30	68	65	43	78
TRFs in Database	77	28	63	63	40	75
Peak Area Sum of Identifiable TRFs	98%	98%	98%	98%	92%	96%
Msp1						
Number of TRFs	59	52	61	62	49	66
TRFs in Database	32	35	31	46	34	50
Peak Area Sum of Identifiable TRFs	54%	81%	55%	64%	53%	61%
Rsa1						
Number of TRFs	27	40	45	34	18	28
TRFs in Database	19	20	20	14	11	15
Peak Area Sum of Identifiable TRFs	41%	34%	32%	19%	30%	32%
Strains that correlate to TRFS from *Hha1*, *Msp1* and *Rsa1* digestions	445	231	266	290	269	318
Distinct 3 fragment ribotypes	67	43	54	62	39	65
%of PCR product in 3 fragment ribotypes	116%	64%	73%	91%	66%	82%

1- Samples are replicates from an organic transition plot. The primers used were 8f and 1492r. A variable fragment sizing error was used (Eqn A).

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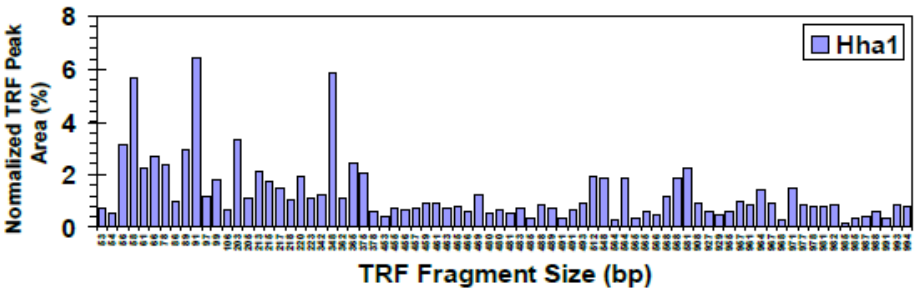
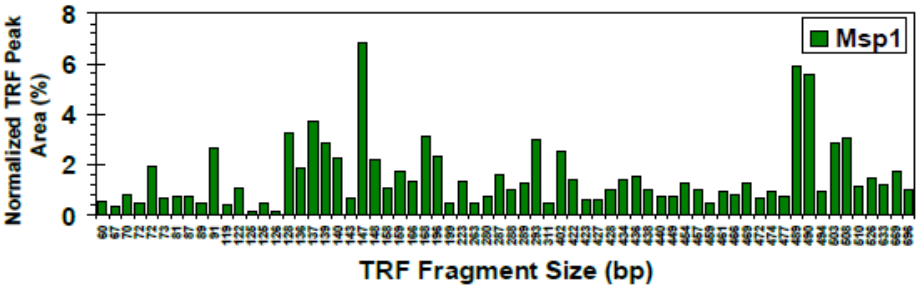
T-RFLP
Community analysis





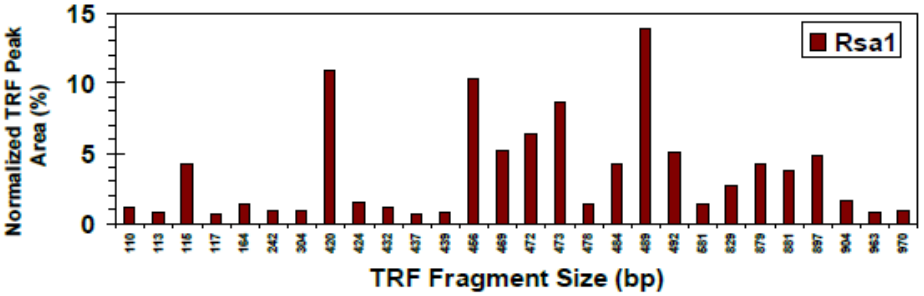


Figure 3. Terminal Restriction Fragment lengths and normalized peak areas determined for an agricultural soil (Soil 6) using primer 8f and 3 digestion by three different restriction enzymes.

AFLP analysis

Amplification fragment length polymorphism

- AFLP is a PCR-based technique that uses selective amplification of a subset of digested DNA fragments to generate and compare unique fingerprints for genomes of interest.
- The DNA is cut with the two enzymes (e.g. *MseI* and *EcoRI*).
- The use of two enzymes results in the generation of a large number of relatively small fragments which can be amplified.
- PCR and sequencing gels are used for display.
- Polymorphisms are amplified and visualized on polyacrylamide sequencing gel using autoradiography.

AFLP PCR

AFLP is a highly sensitive PCR-based method for detecting polymorphisms in DNA. AFLP can be also used for genotyping individuals for a large number of loci

•Genomic DNA is digested with one or more restriction enzymes. tetracutter (MseI) and a hexacutter (EcoRI).

↓

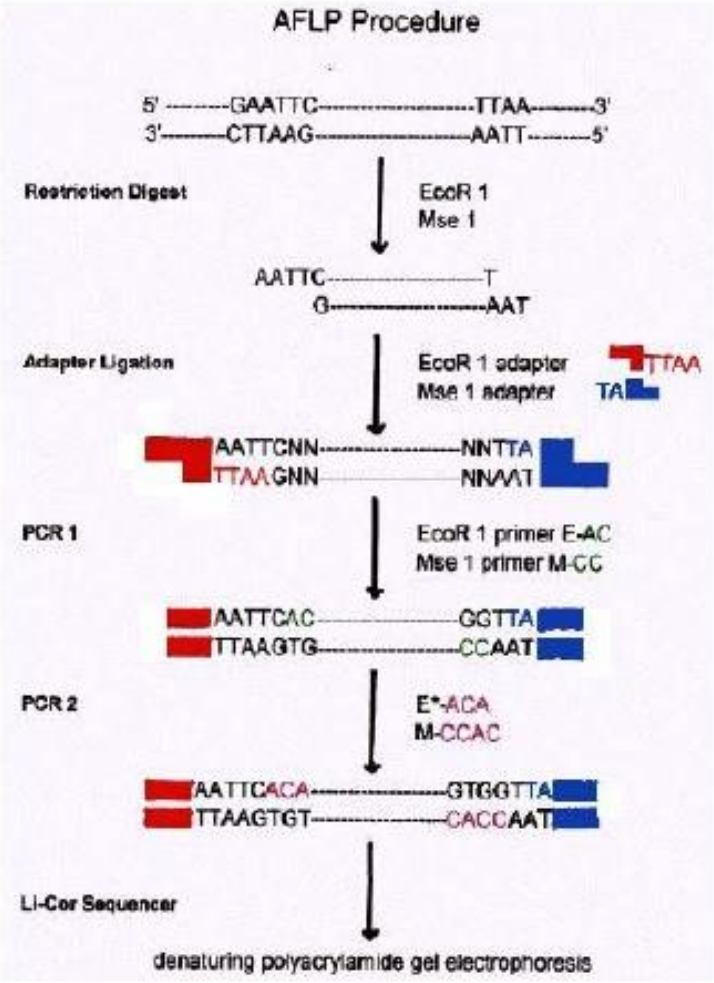
•Ligation of linkers to all restriction fragments.

↓

• Pre-selective PCR is performed using primers which match the linkers and restriction site specific sequences.

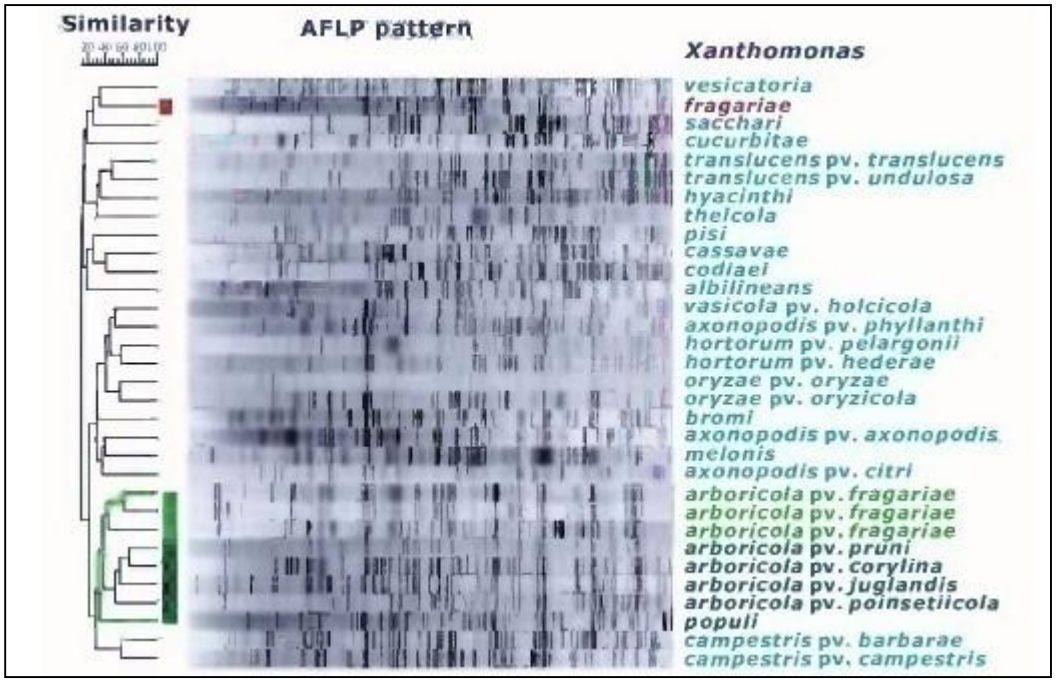
↓

•Electrophoretic separation and amplicons on a gel matrix, followed by visualisation of the band pattern.



AFLP analysis
Xanthomonas species

- UPGMA (= unweighted pair group method with arithmetic mean) cluster analysis of 33 AFLP® fingerprints of *Xanthomonas* species, including three strains (PD 2696, 2780, 3164) of the strawberry blight pathogen *X. arboricola* pv. *fragariae*, showing the taxonomic position of these strains and their relatedness to other pathovars of *X. arboricola* (light and dark green boxes) and no relatedness to the usual strawberry pathogen, namely *X. fragariae* (red box).



Janse *et al.*,2001

AFLP analysis

Difficulty with scoring the bands with the AFLP method

- Scoring the bands can be a difficulty with the AFLP technique.
- One way around this is to use GeneScan.
- Here one of the primers is labeled with a fluorochrome and a fluorescent ladder is included in the samples when they are separated by gel electrophoresis.
- The gel is read by a laser and the data computerized to give sequencing electropherogram (electrogram or chromatogram) and precise read outs of the size of the fragments.

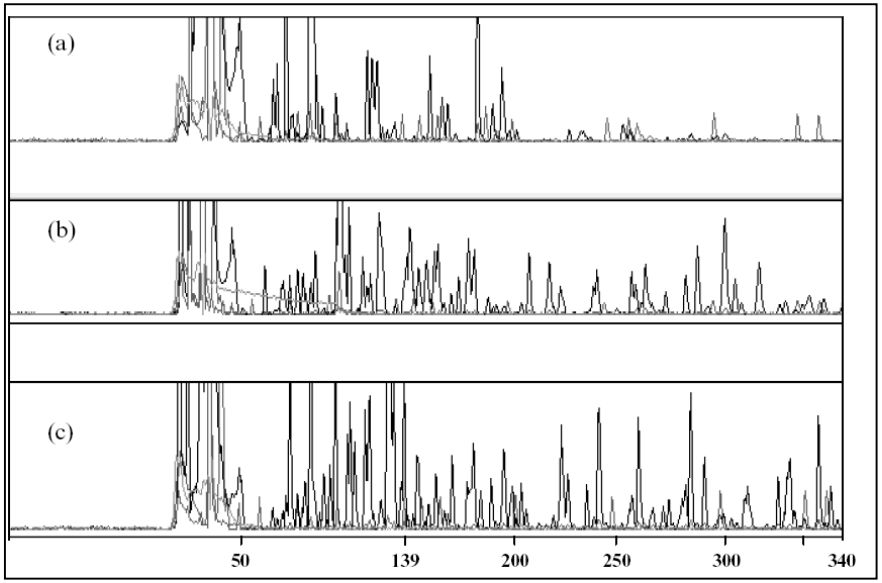
Electropherograms of AFLP fragments

- Electropherograms of AFLP fragments, ranging in size from 0 to 350 nucleotides, obtained from three isolates from different geographic locations:

a) South Pacific

b) Hainan Island, China

c) Ghana.



Strange,2003

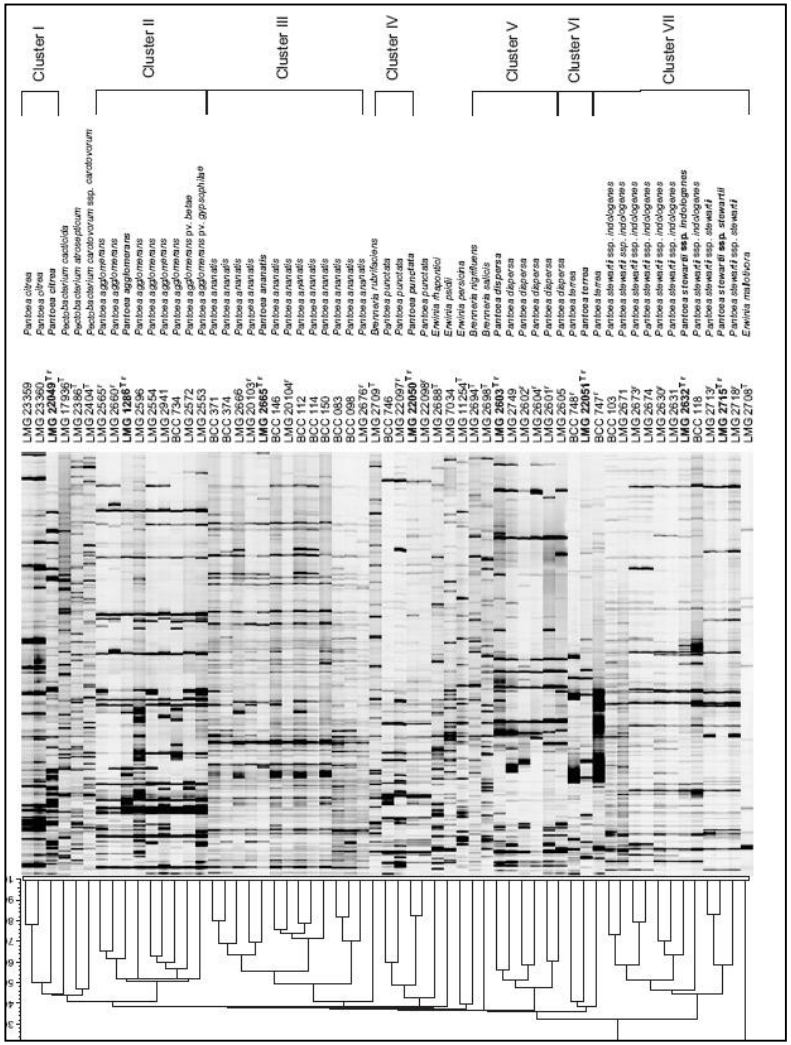
Fluorescent amplified fragment length polymorphism
FAFLP

- Fluorescent amplified fragment length polymorphism (FAFLP) analysis was investigated for use as a rapid, molecular-based identification technique to the species level of the genus *Pantoea*.
- FAFLP has proven to be a rapid, reproducible identification technique for all species of the genus *Pantoea*.

Brady *et al.*,2006

FAFLP analysis of *Pantoea* species

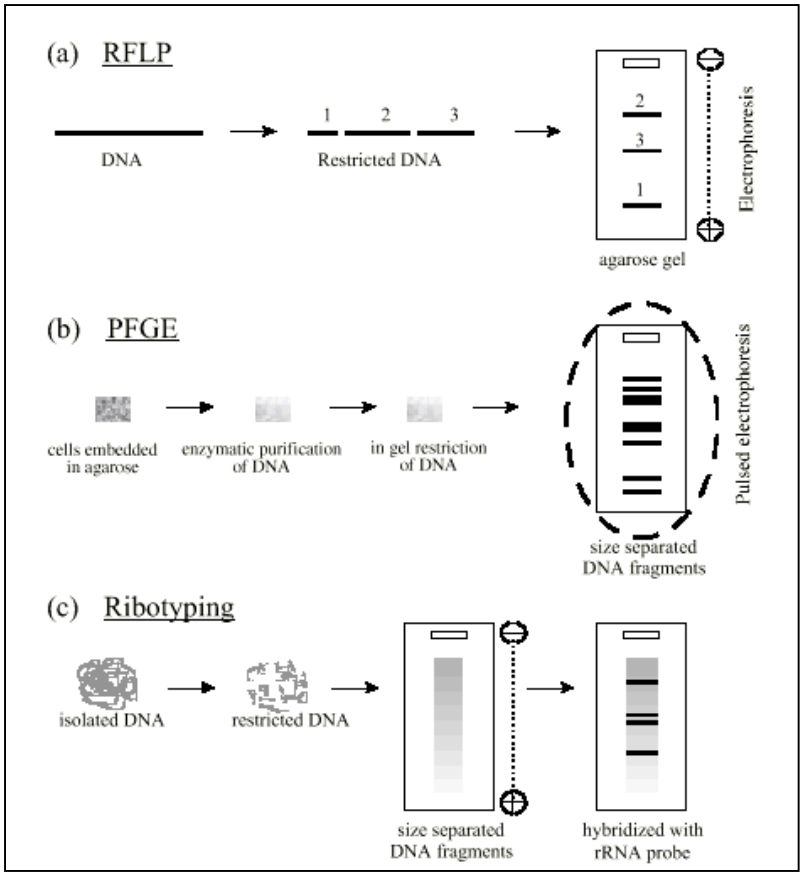
- In the UPGMA dendrogram produced from FAFLP analysis of the *Pantoea* strains, seven distinct clusters, one for each species of *Pantoea*, were identified.



Brady *et al.*,2006

Illustration of commonly used fingerprinting techniques
RFLP, PFGE and ribotyping

- **PFGE:**
- Pulse field gel electrophoresis (PFGE) to separate large restriction fragments (50-1,000 kb) into a characteristic RFLP.
- Whole cells are embedded in agarose and DNA is enzymatically purified and restricted by rare cutting enzymes *in situ*.
- The agarose plug is then inserted into a well in an agarose gel and the restricted fragments are separated by an electric current which pulses from different angles.



O'Sullivan,2000

Pulsed field gel electrophoresis
PFGE

- The procedure for this technique is relatively similar to performing a standard (normal/conventional)gel electrophoresis except:

1. instead of constantly running the voltage in one direction, the voltage is periodically switched among three directions.
2. DNA fragments from 100 to 200 base pairs (bp) up to 50 kilobase pairs (kb) are routinely separated by standard gel electrophoresis. Whereas DNA molecules larger than 50 kb (from a few kb to over 10 megabase pairs (Mb) can be separated by PFGE.
3. DNA molecules in PFGE move together in a size-independent manner. The PFGE procedure takes longer than normal gel electrophoresis due to the size of the fragments.

Pulsed field gel electrophoresis
Applications

- PFGE may be used for genotyping or genetic fingerprinting.
- It is commonly considered a gold standard in epidemiological studies of pathogenic organisms.

Pulsed-field gel electrophoresis of agarose-embedded DNA

Method

- A 0.8% (w/v) chromosomal grade agarose gel was prepared with 0.5xTBE buffer.
- Agarose-embedded DNA plugs were placed in the wells using an alcohol-flamed spatula.
- The wells were then sealed with molten 08% (w/v) chromosomal grade agarose and allowed to set and cool for 1 h.
- The gel was then placed into the electrophoresis cell of a CHEF DR II PFGE system(Bio Rad) filled with 0.5xTBE maintained at 9°C.
- Gels were typically run for 50h at 3 Vcm^{-1}, initial pulse time 250 s rising to 900 s, although the run time and final pulse time can be extended to improve the separation of close bands.
- Gels were stained with ethidium bromide (0.5 µg ml^{-1}, 30 min), destained for 30min under running water and photographed under u.v. transillumination with a CU5 land camera.

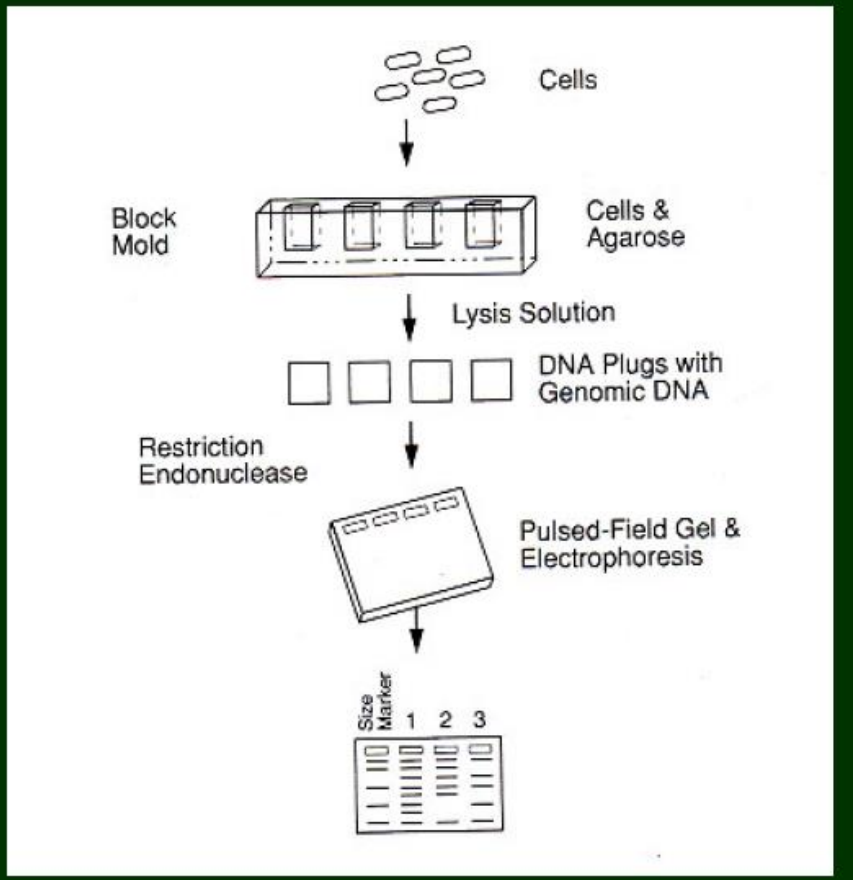
Wigley and Burton,2000

PFGE

Pulsed field gel electrophoresis

PFGE

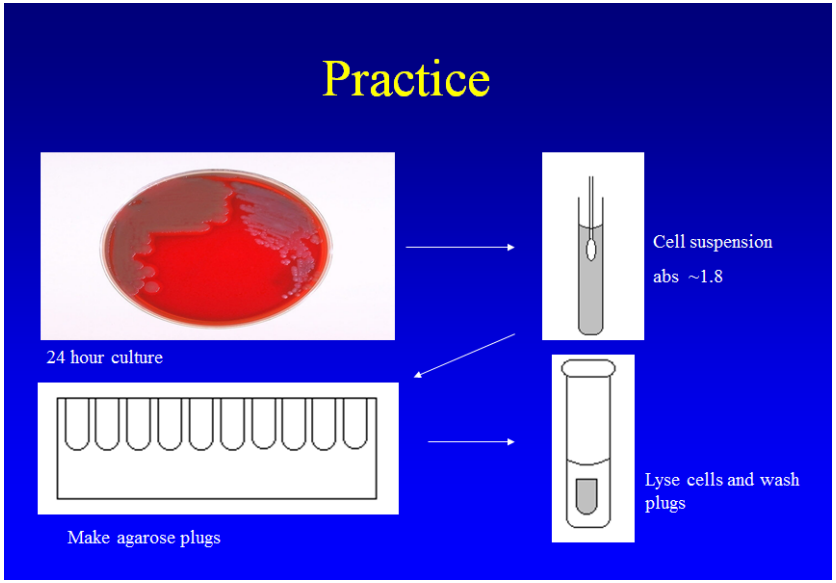
- Pulsed Field Gel Electrophoresis (PFGE)
- Genomic fingerprint
- Fragments maybe several hundred Kb



PFGE

Pulsed field gel electrophoresis

1. A cell suspension is made and adjusted spectrophotometrically.
2. The cells are mixed with agarose and stay embedded in the semi-porous material.
3. The DNA extraction steps remove the other cellular components (proteins, lipids etc.) while leaving the DNA in the agarose plug.

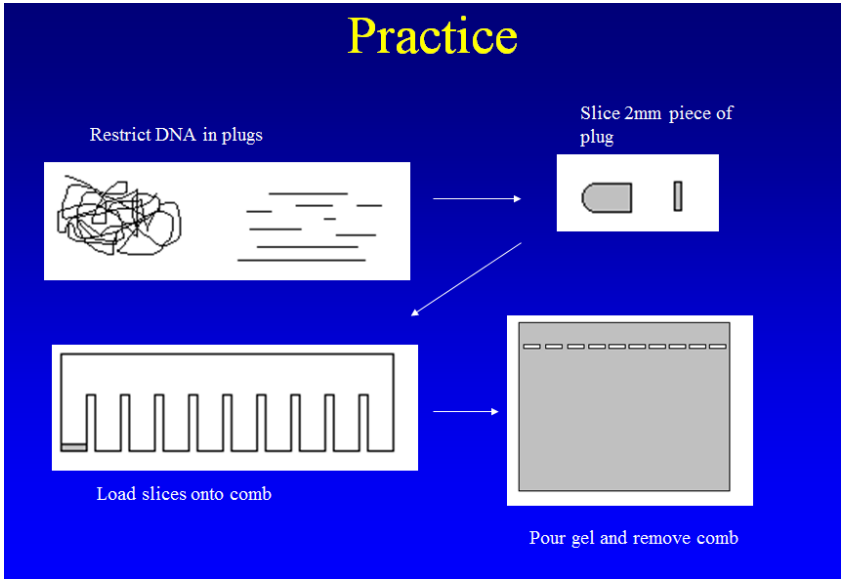


Bob Wickert

PFGE

Pulsed field gel electrophoresis

4. The DNA is cut into various sized fragments by restriction enzymes.
5. The DNA fragments are separated by size through gel electrophoresis.

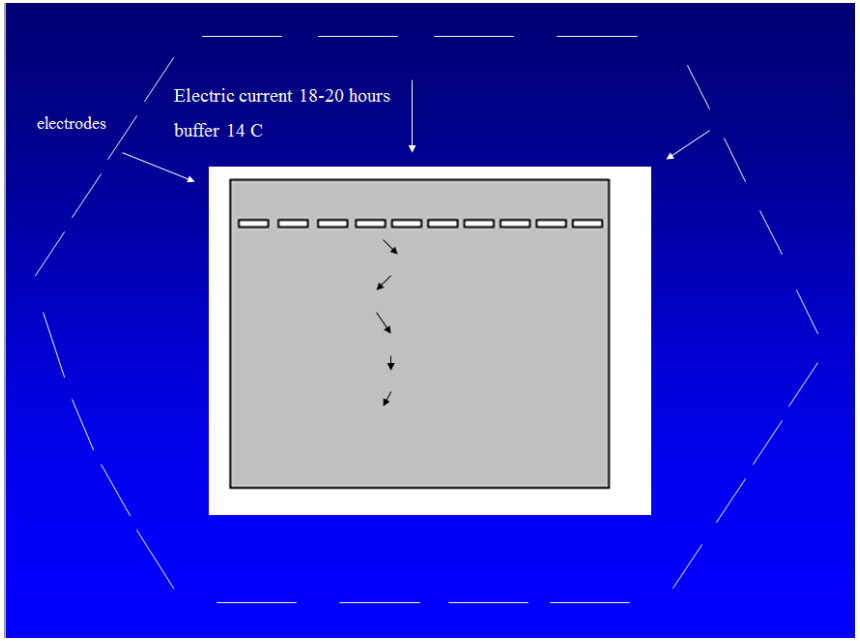


Bob Wickert

PFGE

Pulsed field gel electrophoresis

- The term "pulse field" comes from this step.
- Here the DNA is subjected to an electrical current from various angles.
- The DNA takes a serpentine route through the gel with the smaller fragments traveling faster and the larger fragments slowed by the agarose matrix.



Bob Wickert

PFGE
Pulsed field gel electrophoresis

- The term "pulse field" comes from this step.
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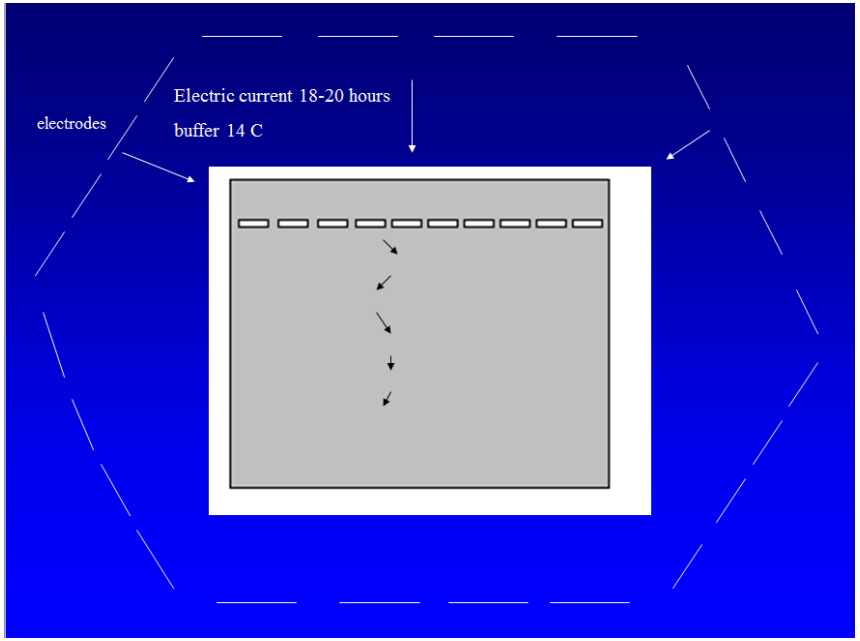
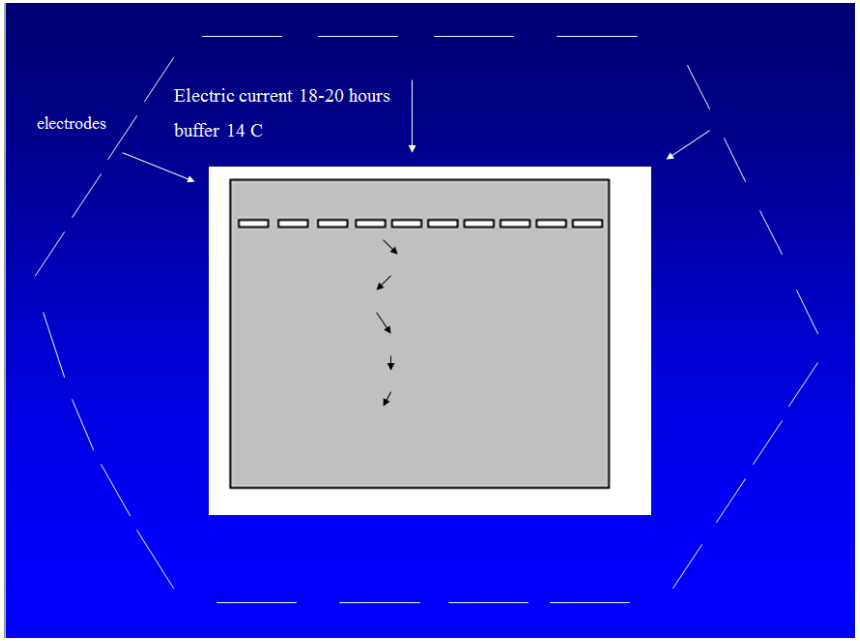


Bob Wickert

PFGE

Pulsed field gel electrophoresis

- The term "pulse field" comes from this step.
- Here the DNA is subjected to an electrical current from various angles.
- The DNA takes a serpentine (snake) route through the gel with the smaller fragments traveling faster and the larger fragments slowed by the agarose matrix.

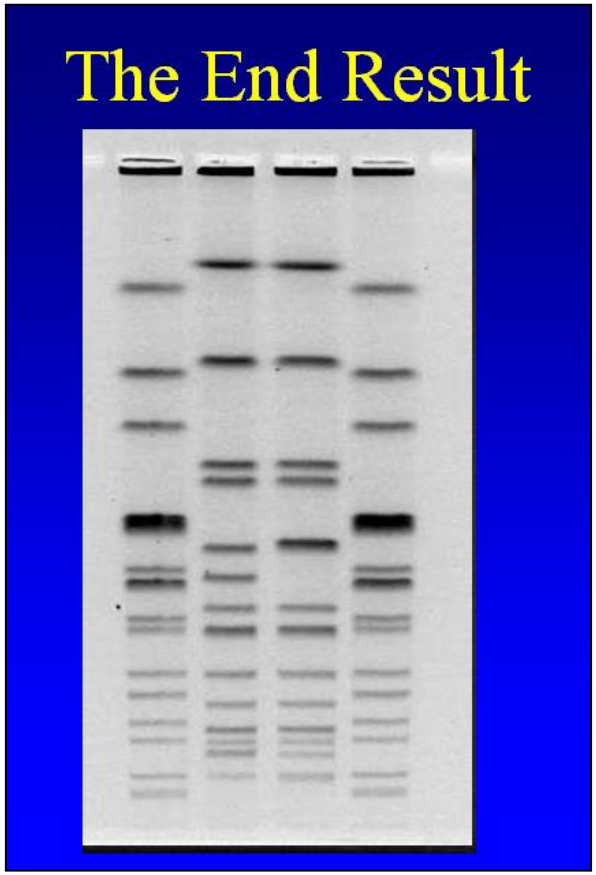


Bob Wickert

PFGE

Pulsed field gel electrophoresis

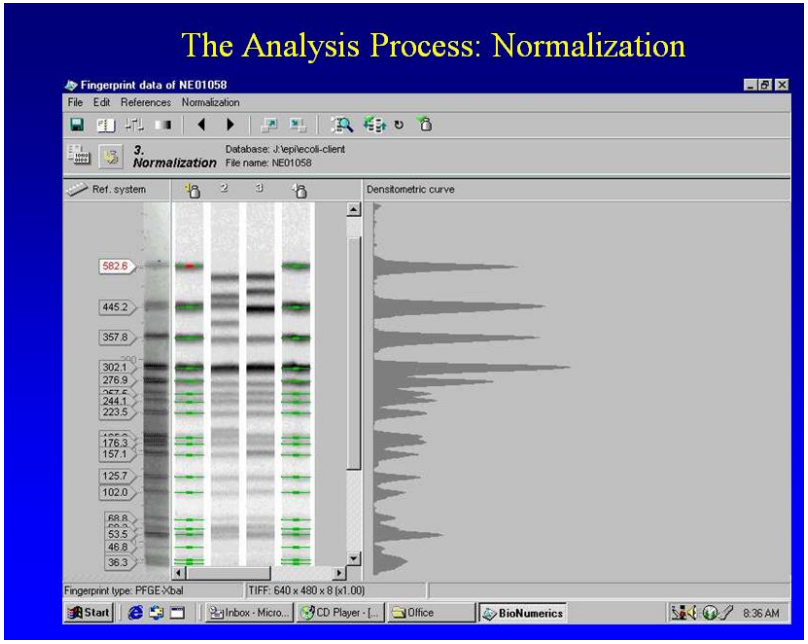
- After the electrophoresis step, the DNA is visualized by a fluorescent stain specific for DNA.
- Lanes 1 and 4 are standards (they are identical).
- Lanes 2 and 3 are two *E. coli* isolates (the pattern is similar but not identical).



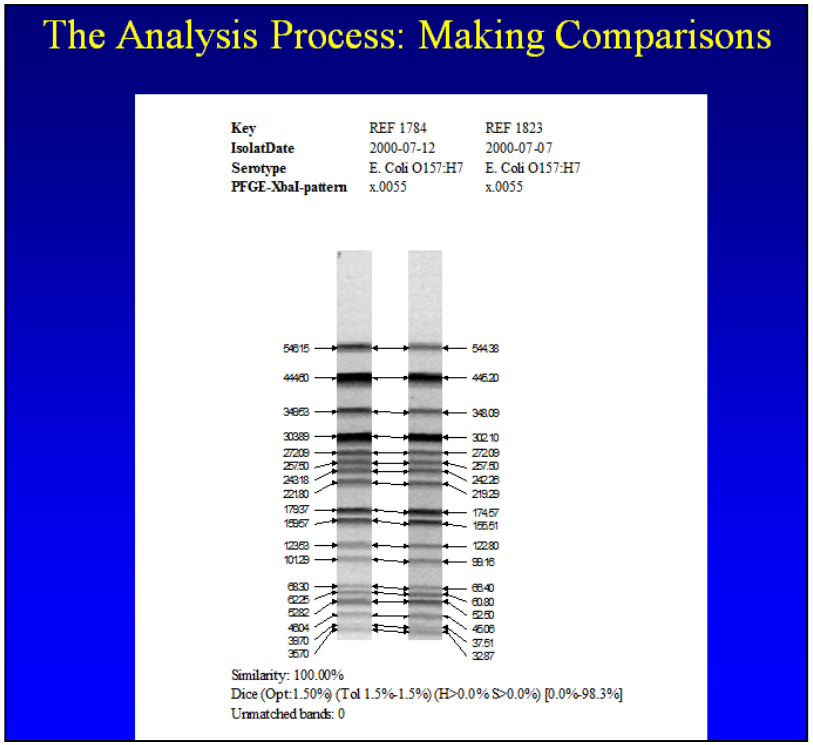
Bob Wickert

PFGE

Pulsed field gel electrophoresis



The software used to analyze pfge patterns normalizes differences in gel conditions to a global reference.

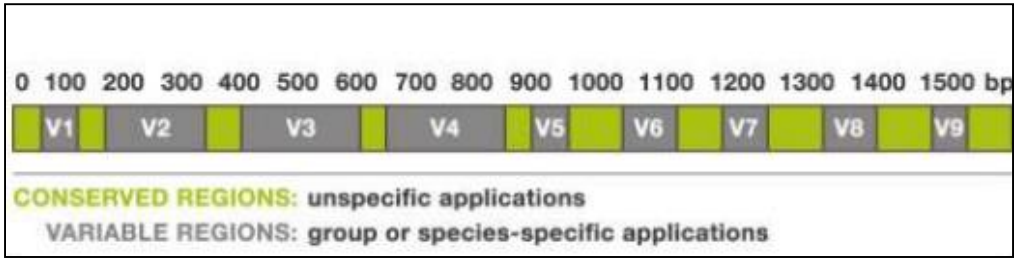


Here we see a pfge pattern match.

Bob Wickert

DNA-based PCR-denaturing gradient gel electrophoresis
DGGE

- The 16S rRNA gene consists of highly conserved and highly variable regions.
- One of the most efficient methods used to exploit 16S rDNA sequence divergence that is frequently observed in various species is denaturing gradient gel electrophoresis (DGGE).
- DNA fragments of the same size but with different sequence compositions are separated in denaturing gradient gels that contain a linear gradient of DNA denaturants (urea and formamide).
- PCR-DGGE has great potential to identify closely related species based on 16S rRNA gene sequence divergence.



Abu Al-Soud *et al.*,2003;..

DNA-based PCR-denaturing gradient gel electrophoresis

DGGE

- A PCR-DGGE was used for detection and identification of bacteria.
- PCR-DGGE and 16S rRNA gene libraries as an alternative to improve the detection and identification rate of bacterial species from many samples.
- DGGE is a rapid method for analyzing microbial diversity from dozens of samples.

Navarro-Noya *et al.*,2012

DNA-based PCR-denaturing gradient gel electrophoresis
DGGE

- Denaturing gradient gel electrophoresis (DGGE) is a commonly used molecular technique for:

1. rapid fingerprint analysis of microbial community composition,
2. diversity, and
3. dynamics.

- The method is rapid and affordable, allowing multiple samples to be processed simultaneously.
- The protocol outlines steps for preparing gel solutions, pouring gels, operating the DGGE apparatus, and excising fingerprint bands of interest for sequencing.

Green *et al.*,2010

DNA-based PCR-denaturing gradient gel electrophoresis
DGGE

- **Conformation separation:**
- DNA fragments of same length but different base composition can be separated based on their **three-dimensional conformation**.
- One of the conformation separation methods is DGGE.
- Bacterial and fungal communities can be analyzed by DGGE after PCR amplification of 16S rDNA and 18S rDNA genes, respectively.
- Primers are designed to specifically hybridize to conserved regions of the bacterial 16S rDNA or fungal 18S rDNA genes.

Spencer and Spencer,2004;..

Denaturing Gradient Gel Electrophoresis

DGGE

- Separated DNA of same size based on sequence differences.
- Different sequences "behave differently at different amounts of denaturing chemical (or heat; see TGGE)
- At some point 16SrRNA DNA strands completely separates.
- Complete separation of PCR amplicon is hindered by GC-clamp added to one of the PCR primers.

Hinder: stop someone or something from making progress

Primers used for DGGE

A common set of primers used to amplify the rDNA of bacterial and fungal communities

Pair of primers[a]	Size of the PCR product (bp)[b]	Target	Sequence	Reference
P63f- P518r	511	*Bacteria*	5'-CAGGCCTAACACATGCAAGTC-3' 5'-ATTACCGCGGCTGCTGG-3'	*(31)*
P338f- P518r	236	*Bacteria*	5'-ACTCCTACGGGAGGCAGCAG-3' 5'-ATTACCGCGGCTGCTGG-3'	*(2)*
Arch340f- Arch519r	233	*Archaea*	5'-CCCTACGGGG(C/T)GCA(G/C)CAG-3' 5'-TTACCGCGGC(G/T)GCTG-3'	*(6)*
Act243f- Act513r	342	*Actinomycetes*	5'-GGATGAGCCCGCGGCCTA-3' 5'-CGGCCGCGGCTGCTGGCACGTA-3'	*(5)*
NS2f- Fungr5r	230	Fungi	5'-GGCTGCTGGCACCAGACTTGC-3' 5'-GTAAAAGTCCTGGTTCCC-3'	*(32)*
GC clamp[c]			5'-CGCCCGCCGCGCGCGGCGGGC GGGGCGGGGGCACGGGGGG-3'	*(2)*

[a]f, forward primer; r, reverse primer.
[b]GC clamp included.
[c]GC clamp attached to the 5' end of the reverse primer.

Spencer and Spencer,2004

DNA-based PCR-denaturing gradient gel electrophoresis

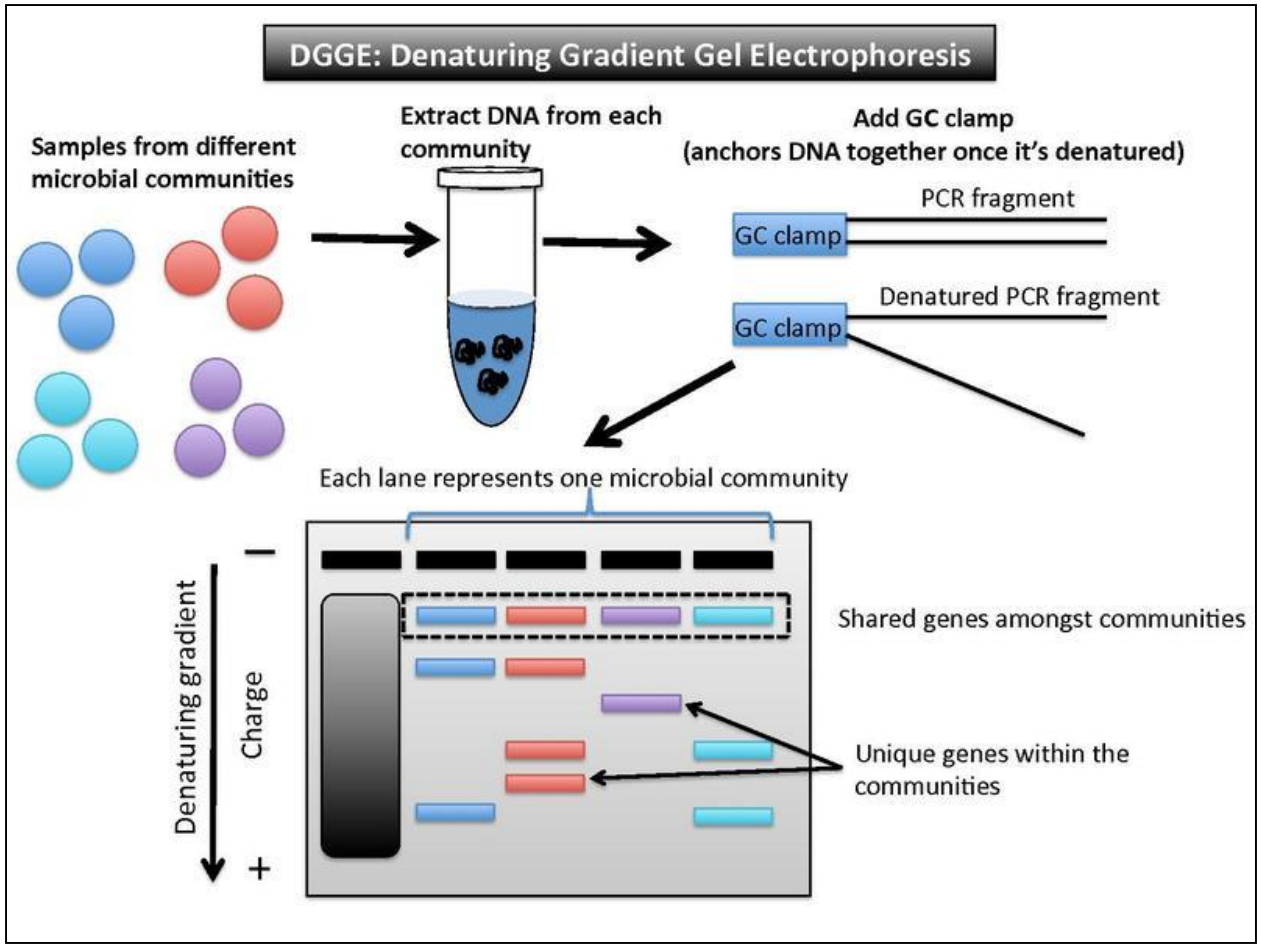
DGGE

- In DGGE systems, PCR products that have nucleotide substitutions will migrate to a different position on DGGE in comparison to the original PCR products, and thus allow the detection of mutations in this particular gene.
- In this way, DGGE separate DNA fragments of the same size but differing according to their base-pair sequences.

Spencer and Spencer,2004

DNA-based PCR-denaturing gradient gel electrophoresis

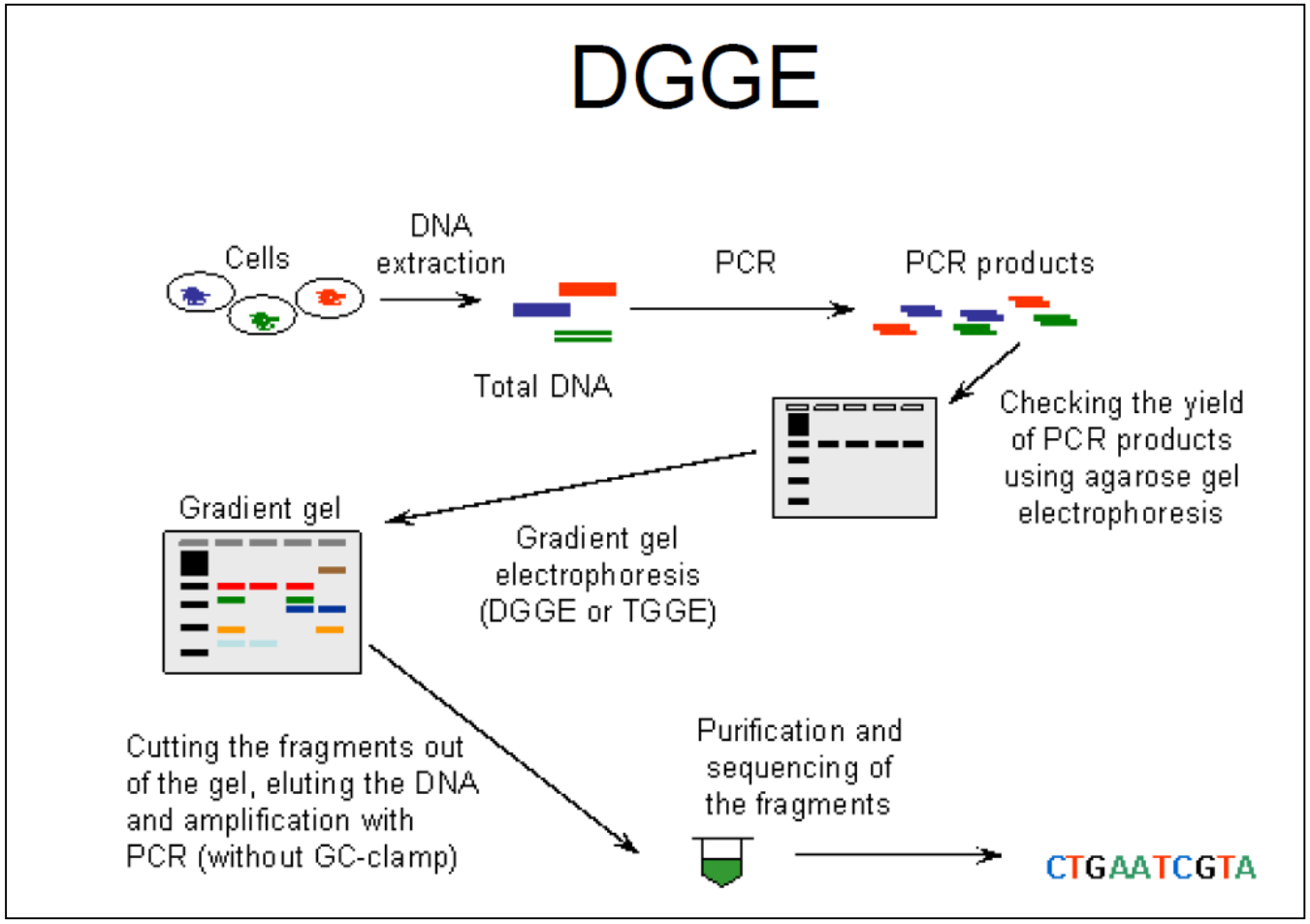
Step-by-step procedure of using DGGE analysis in microbiology



Ilyanassa,2012

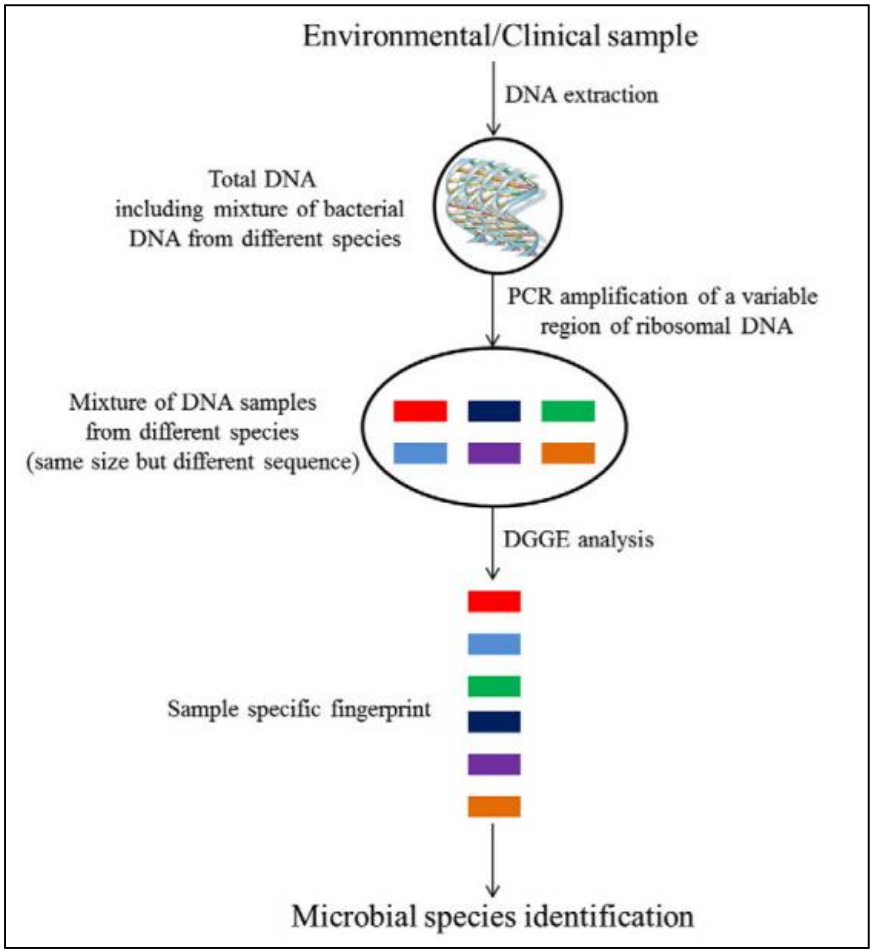
DNA-based PCR-denaturing gradient gel electrophoresis

Step-by-step procedure of using DGGE analysis in microbiology



DNA-based PCR-denaturing gradient gel electrophoresis

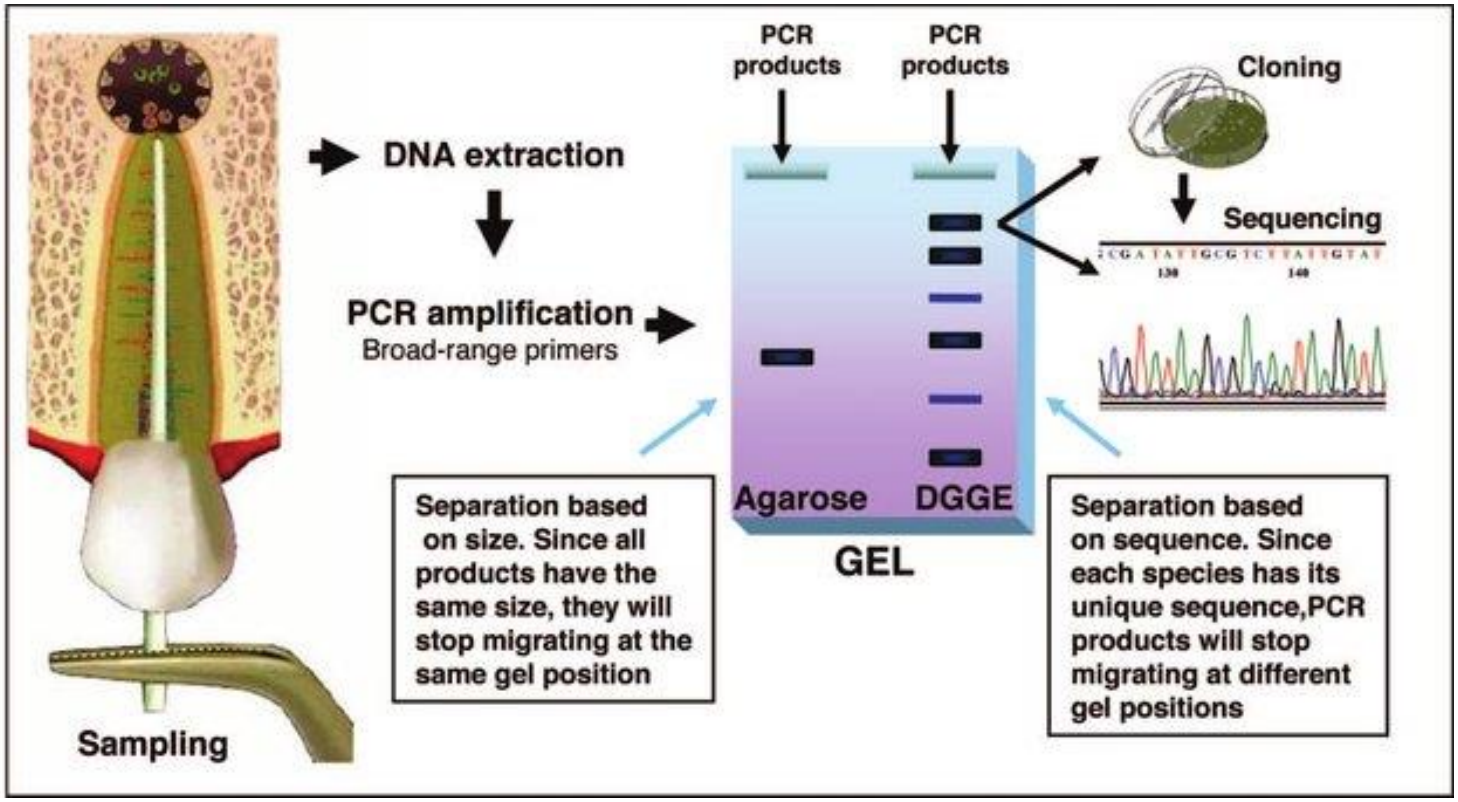
Flow diagram of the application of DGGE to environmental samples



Das *et al.*,2014

DGGE

Step-by-step procedure of using DGGE analysis in microbiology



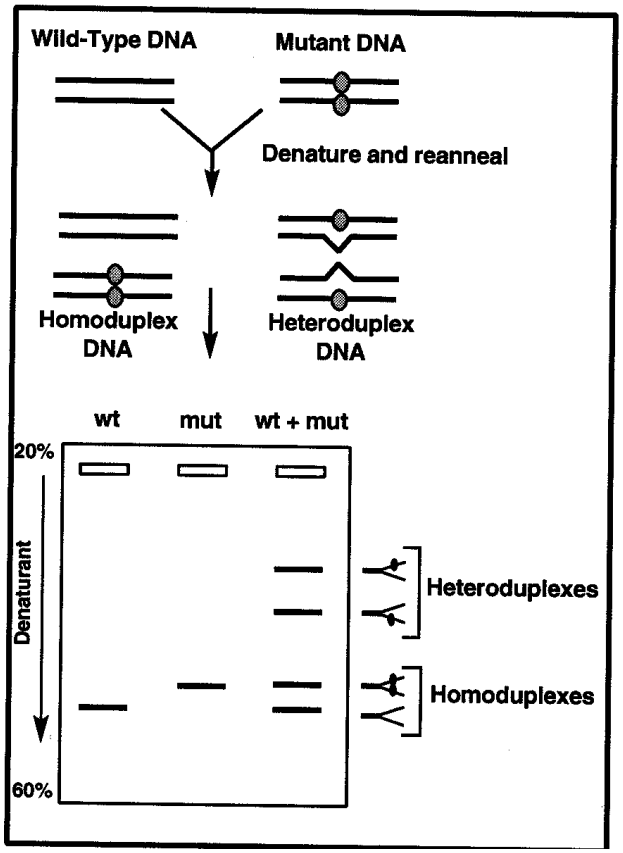
Bands of interest can be cut out and sequenced directly to identify bacterial species.

Siqueira *et al.*,2005

DNA-based PCR-denaturing gradient gel electrophoresis

Step-by-step procedure of using DGGE analysis in microbiology

- Gradient Parallel for Analysis:
- Conditions change mostly due to size of amplicon.
- May be applied at different taxa (groups).
- Bands may be cut out and DNA cloned for phylogenetic analysis.
- Larger bands more information.



DNA-based PCR-denaturing gradient gel electrophoresis
DGGE

a) Illustration of density gradient gel electrophoresis (DGGE) of a PCR derived rRNA amplicon from four different bacteria.

- Gel electrophoresis in a non-denaturing gel results in a single band, whereas electrophoresis through a gel containing increasing concentrations of a denaturant results in the separation of the four different products, based on their sequence dependent melting patterns.
- Note, to maintain the double stranded integrity of the products, a G/C clamp is generally included in one of the primers used in the amplification.

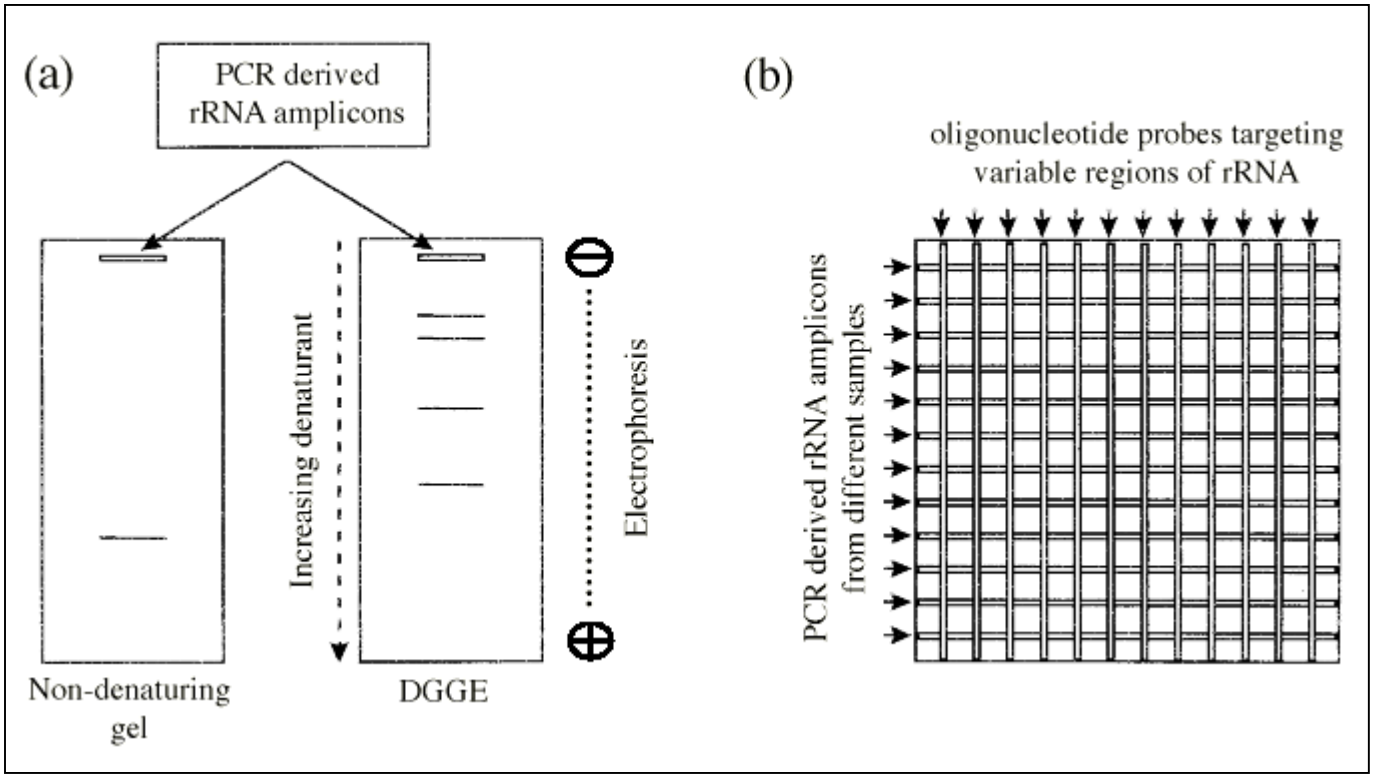
b) Illustration of the checkerboard hybridization format for analyzing large numbers of samples with multiple probes.

- Each vertical channel contains a different bound probe and each horizontal channel contains a PCR derived rRNA amplicon from a different individual.
- In this example, 144 different hybridizations can be carried on one membrane.

O'Sullivan,2000

DNA-based PCR-denaturing gradient gel electrophoresis

DGGE

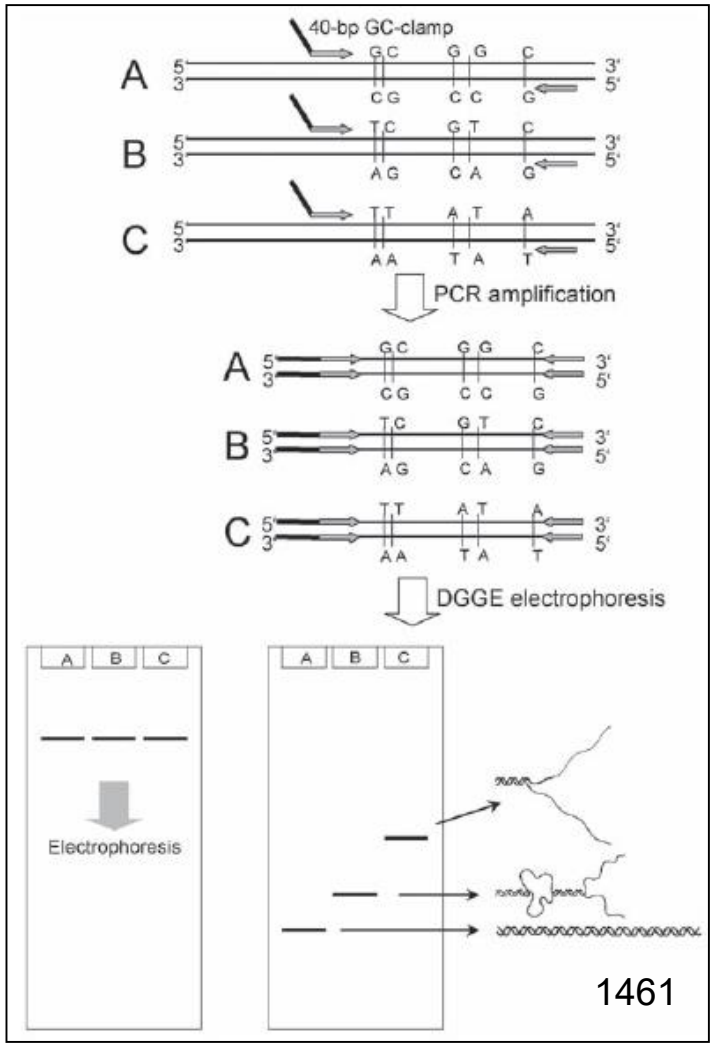


O'Sullivan,2000

DNA-based PCR-denaturing gradient gel electrophoresis

DGGE

- Schematic diagram showing the formation of GC-clamped(a GC-rich region) PCR products from genes A, B, and C which have single base substitutions.
- Because PCR product A has a higher melting temperature than PCR products B and C, it migrates further in the DGGE gel.
- On complete melting of the gene product, a branched structure is formed, which migrates much more slowly through the DGGE gel.



Spencer and Spencer,2004

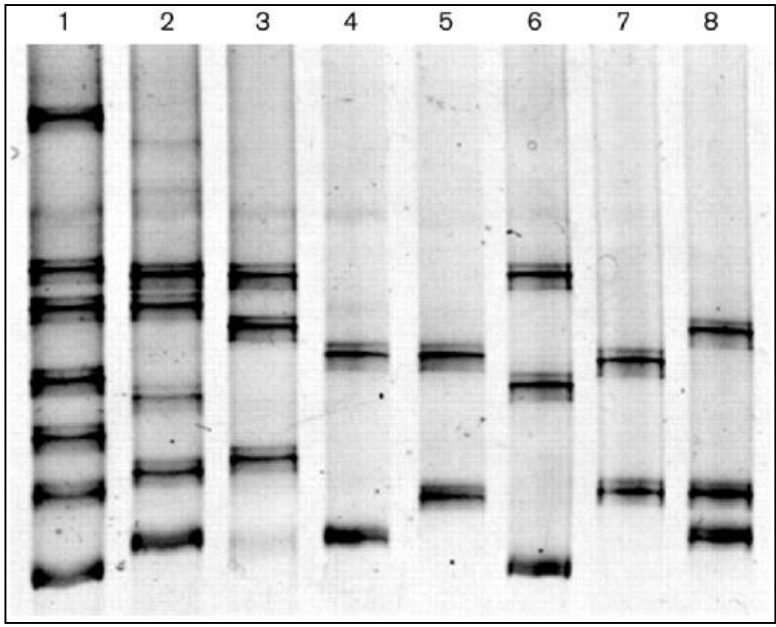
DNA-based PCR-denaturing gradient gel electrophoresis
DGGE

- 16S rDNA sequences of different human/animal bacterium *Helicobacter* species were analysed by Melt94 (http://web.mit.edu/osp/www/melt.html) to assist in primer selection and to determine DGGE conditions.
- DNA extracted from reference *Helicobacter* strains and faeces of zoo animals was used as a template to amplify the V6-7 region of 16S rDNA by using primers 1F-GC and 2R.
- DGGE analysis of amplicons (15 µl) was performed on 9% polyacrylamide (37.5:1 acrylamide/bisacrylamide) gels that contained a 15-30% urea plus formamide gradient [100% denaturing solution contains 7 M urea and 40% (v/v) formamide].
- Electrophoresis was performed in 0.5×TAE at 200 V at 60°C for 4 h by using a DCode System for DGGE (Bio-Rad).
- Gels were stained with ethidium bromide (0.2 µg ml^{-1} in 0.5×TAE) for 15 min and visualized by using a GelFotoSystem (Techtum Lab).

Abu Al-Soud *et al.*,2003

DNA-based PCR-denaturing gradient gel electrophoresis
DGGE

- Separation of PCR products amplified by genus-specific primers from different *Helicobacter* species in a 9% polyacrylamide 15-30% denaturing gradient gel (increasing gradient of denaturant from top to bottom).
- Lanes: 1, *H. muridarum*, *H. canis*, *H. pullorum*, *H. pylori*, *Helicobacter cholecystus* *H. fennelliae* and *H. bizzozeronii*.
- Lanes: 2, *H. bilis*, *H. pametensis*, *H. mustelae*, *'H. rappini* and *H. felis*.
- Lanes: 3, *H. ganmani*, *H. suncus*, *H. felis* and *H. acinonychis*.
- Lanes: 4, *H. cinaedi* and *H. salomonis*.
- Lanes: 5, *H. cinaedi* and *H. hepaticus*.
- Lanes: 6, *H. rodentium*, *H. cinaedi* and *H. salomonis*.
- Lanes: 7, *H. cinaedi* and *H. cinaedi*.
- Lanes: 8, *H. trogontum*, *H. cinaedi* and *H. bizzozeronii*.



Abu Al-Soud *et al.*,2003

DNA-based PCR-denaturing gradient gel electrophoresis

Burkholderia species

- DGGE analyses of the PCR products obtained using specific primers for the 16S region showed that there were sufficient differences in migration behavior to distinguish the majority of the *Burkholderia* species tested.

Gitaitis and Nischwitz,2006

Genomic fingerprinting

Repetitive mobile elements

Repetitive PCR(Rep-PCR)

- rep-PCR approach is a valuable tool for determination of genetic variation among an extensive range of bacterial species.
- It offers a highly sensitive level of discrimination at the species level, and even to some extent at the:
- Pathovar,
- Biovar, or
- Subspecies levels.
- REP-, ERIC- and BOX-PCRs were used on both the Gram –ve and Gram+ve bacteria.

Genomic fingerprinting

Instability mediated by specialized genetic elements

Repetitive mobile elements

- There are several kinds of specialized genetic elements playing a role in genomic instability.
- We first describe a number of mobile elements:

1. Miniature inverted-repeat transposable elements [MITEs],
2. repetitive extragenic palindromic [REP] sequences,
3. insertion sequences [ISs],
4. bacterial interspersed mosaic elements [BIMEs],
5. transposons,
6. transposable bacteriophages, and
7. genomic islands.

Darmon and Leach,2014

Genomic fingerprinting

Instability mediated by specialized genetic elements

Repetitive mobile elements

- PCR Based- Amplification of a single target specific to a pathogen-Targeting known repetitive sequences:

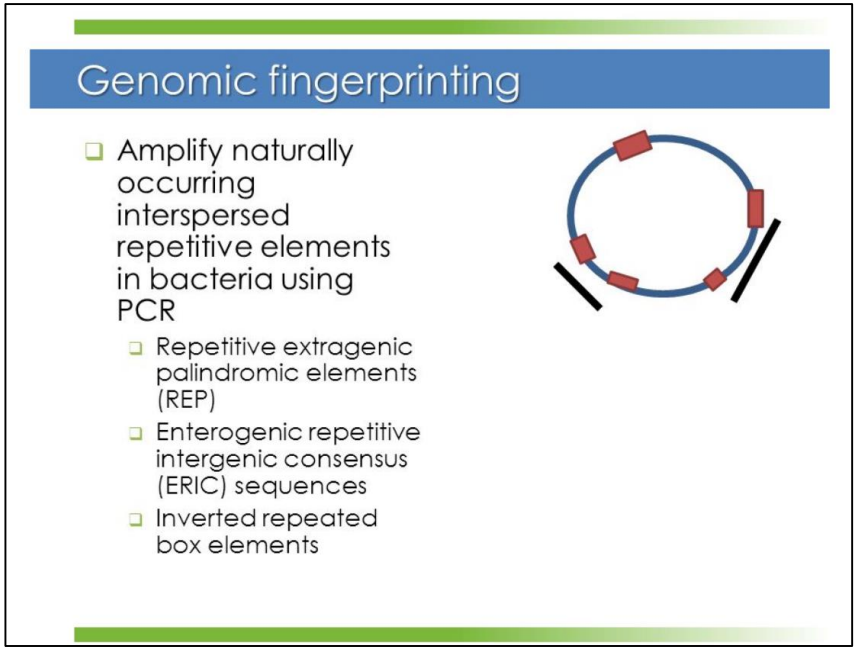
1. enterobacterial repetitive intergenic consensus sequences (ERIC),
2. repetitive extragenic palindromic sequences (REP),
3. double repetitive element (DRE),
4. inverted repeated box elements(BOX),
5. insertional sequence (IS),
6. polymorphic guanine/cytosine-rich repetitive sequences (PGRS).

Singh,2018

Genomic fingerprinting
Repetitive elements (regions)

- Amplify naturally occurring interspersed repetitive elements in bacteria using PCR:

1. Repetitive extragenic palindromic elements (REP);
2. Enterogenic repetitive intergenic consensus (ERIC) sequences;
3. Inverted repeated box elements (BOX).



Genomic fingerprinting

Repetitive mobile elements

A Repetitive Extragenic Palindrome (REP) element

- A Repetitive Extragenic Palindrome (REP) element is a short base pair sequence capable of producing a stem-loop structure.
- REP elements are located in different orientations and arrays between genes within an operon or at the end of an operon.
- They are always found outside of structural genes.
- Groups of REP elements are also known as BIMEs (bacterial-interspersed mosaic elements).
- The actual function of these elements is unclear.
- They may act as mRNA stabilizers, targets for DNA gyrase binding, or hot spots for transposition and recombination events.

Genomic fingerprinting

Instability mediated by specialized genetic elements

Repetitive mobile elements

- A distinct locations are present in prokaryotic genomes, referred as repetitive element sequence based-polymerase chain reaction (rep-PCR).
- The initial discovery of repetitive extragenic palindromic (REP) elements occurred in the genomes of enteric bacteria *Escherichia coli* and *Salmonella*.
- The family of REP elements is generally between 33 and 40 bp in length, has 500 to 1,000 copies per genome, and comprises about 1% of the bacterial genomes of *E. coli* and *Salmonella*.

Hiett and Seal,2019

Genomic fingerprinting

Genomic identification and characterization

Repetitive elements (regions)

- Several families of short repetitive DNA sequences, widely distributed in the genome, have been identified in bacteria.
- REP sequences have been located between genes within an operon or at the end of an operon, in different orientations and in tandem arrays, and in operons distributed throughout the genome.
- The REP sequence has been identified in intergenic regions within operons from different bacterial species.

The repetitive elements may be present in both orientations.

Ridley,1998

Genomic fingerprinting

rep-PCR

Three families of repetitive sequences

- Three families of repetitive sequences have been identified, including:

1. the 35-40 bp repetitive extragenic palindromic (REP) sequences,
2. the 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and
3. the 154 bp BOX element (Versalovic *et al.*,1994)

Palindromes: The characters read the same backward as forward. Some examples of palindromic words are noon, civic, radar, level, rotor, kayak, madam, and refer.

BOX elements enhance genetic diversity. The BOX primer is generally recommended since it generates robust fingerprints, and yields a highly complex fragment pattern.

Rademake and Bruijn; EPPO, 2010-09

rep-PCR

Primer and PCR conditions

BOX-PCR; REP-PCR and ERIC-PCR

PCR conditions BOX-PCR

Primers
Reference: Smith *et al.* (2001) and Versalovic *et al.* (1994)
Forward: BOXA1R 5′-CTA.CGG.CAA.GGC.GAC.GCT.GAC.G-3′
Reverse: Forward = Reverse
Product: 100–3500 bp

BOX-PCR conditions: initial denaturation at 95°C 7 min followed by 30 cycles (94°C 1 min, 53°C 1 min, 65°C 8 min) and one final step at 65°C 16 min before cooling at 4°C.

PCR conditions REP-PCR

REP-PCR

Primers
Reference: Smith *et al.* (2001); Versalovic *et al.*, 1994.[1]
Forward: REP1R-1 5′-III ICG ICG ICA TCI GGC-3′
Reverse: REP2-1 5′-ICG ITT ATC IGG CCT AC-3′

I = Inosine.

PCR conditions ERIC-PCR

Primers
Reference: Smith *et al.* (2001) and Versalovic *et al.* (1994)
Forward: ERIC1R 5′-ATG TAA GCT CCT GGG GAT TCA C-3′
Reverse: ERIC2 5′-AAG TAA GTG ACT GGG GTG AGC G-3′

ERIC-PCR conditions: initial denaturation at 95°C 7 min followed by 30 cycles (94°C 1 min, 52°C 1 min, 65°C 8 min) and one final step at 65°C 16 min before cooling at 4°C.

EPPO, 2010-09

rep-PCR

Primers

BOX-PCR; REP-PCR and ERIC-PCR

- REP:
- REP 1 R-I [5′-IIIICGICGICATCIGGC-3′], and
- REP 2-I [5′-ICGICTTATCIGGCCTAC-3′]
- ERIC:
- ERIC 1 R [5′-ATGTAAGCTCCTGGGGATTCAC-3′], and
- ERIC 2 [5′- AAGTAAGTGACTGGGGTGAGCG-3′])
- BOX:
- BOXA1R [5′-CTACGGCAAGGCGACGCTGACG-3′])

Venkatesh,2002

rep-PCR
Primers
REP-PCR

- Universal primers based on REP sequences were used for REP-PCR (Versalovic *et al.*,1991).
- The REP primers were modified by inserting a base (A, T, G or C) at one of three N positions in the reverse Rep-2-Dt primer (Table 2).
- We examined five primer sets that included different combinations of REP primers:
- (Rep-1R-Dt/Rep-2-Dt, Rep-1RDt/Rep-2A, Rep-1R-Dt/Rep-2T, Rep-1R-Dt/Rep-2G and Rep-1R-Dt/Rep-2C).

Primer	Primer sequence (5'-3')
Rep-1R-Dt	IIINCGNCGNCATCNGGC (forward)
Rep-2-Dt	NCGNCTTATCNGGCCTAC
Rep-2A	ACGACTTATCAGGCCTAC
Rep-2T	TCGTCTTATCTGGCCTAC
Rep-2G	GCGGCTTATCGGGCCTAC
Rep-2C	CCGCCTTATCCGGCCTAC

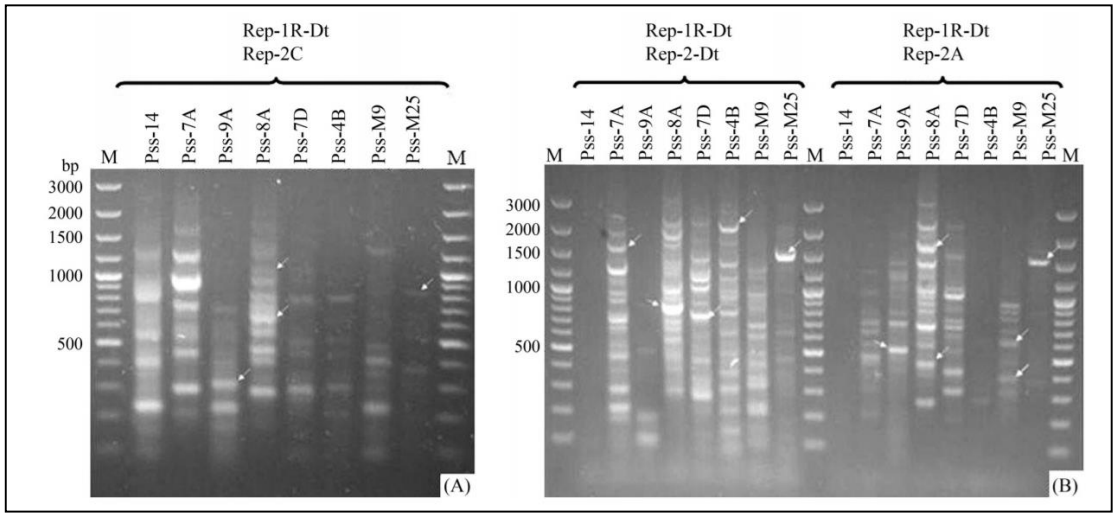
Letters in bold type indicate the bases that were altered in each primer at the positions indicated by N (underlined) in the first primer.

Universal and modified primers used for REP-PCR.

Çepni and Gürel,2012

rep-PCR
Primers
REP-PCR

- Repetitive-PCR fragments of *P. savastanoi* pv. *savastanoi* isolates obtained with different REP primer combinations.
- (A) Rep-1R-Dt and Rep-2C,
- (B) Rep-1R-Dt and Rep-2-Dt, and Rep-1R-Dt and Rep-2A.
- M - DNA ladder.



Çepni and Gürel,2012

rep-PCR
Primers
ERIC-PCR

- ERIC-PCR primer pairs ERIC 1R:
- ERIC 1R primer (5´-TGT AAG CTC CTG GGG ATT CAC-3´), and
- ERIC 2 primer (5´-AAG TAA GTG ACT GGG GTG AGC G-3´) (Versalovic *et al.*,1991).
- More primer sets against different repetitive elements have been developed.

rep-PCR

Primers

BOX-PCR; REP-PCR and ERIC-PCR

- REP:
- REP1 R-I [5′-IIIICGICGICATCIGGC-3′], and
- REP2-I [5′-ICGICTTATCIGGCCTAC-3′]
- ERIC:
- ERIC 1R [5′-ATGTAAGCTCCTGGGGATTCAC-3′], and
- ERIC 2 [5′- AAGTAAGTGACTGGGGTGAGCG-3′])
- BOX:
- BOXA1R [5′-CTACGGCAAGGCGACGCTGACG-3′])

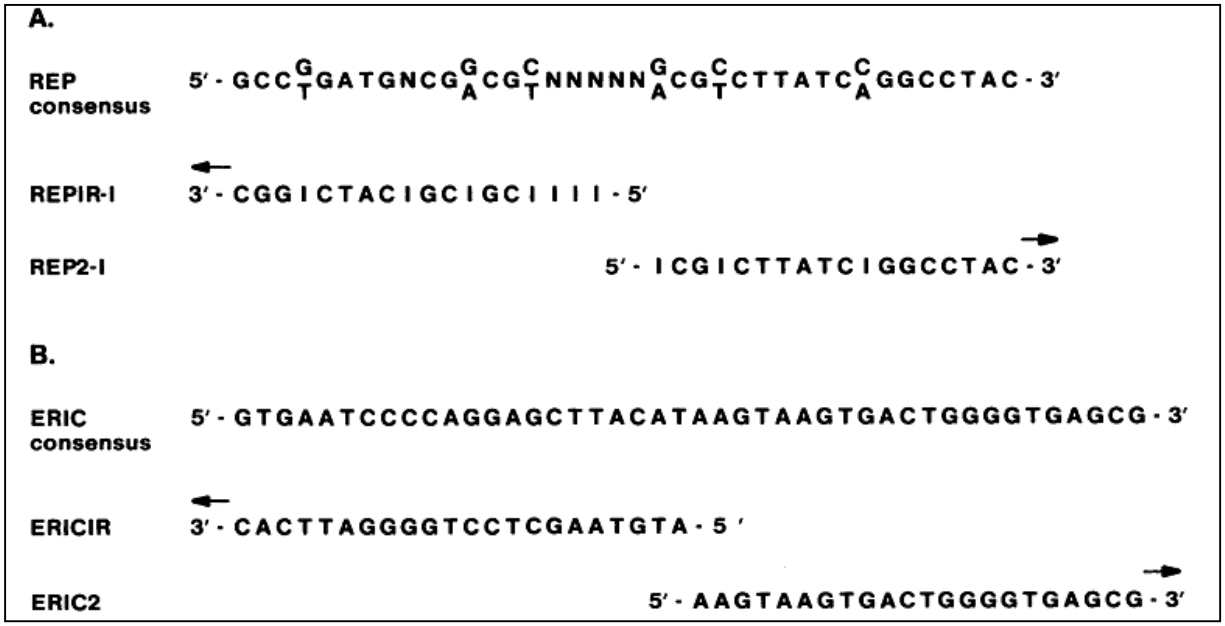
Venkatesh,2002

rep-PCR

Primers

REP-PCR and ERIC-PCR

- The I's denote inosines.
- The arrows denote the direction of *Taq* polymerase extension.



Bruijn,1992

BOX-PCR
Conserved subunits

- BOX elements consist of differentially conserved subunits, namely boxA, boxB, and boxC (Martin et al.,1992).
- Only the boxA-like subunit sequences appear highly conserved among diverse bacteria.
- BOX elements were the first repetitive sequences identified in a Gram-positive organism (*Streptococcus pneumoniae*).

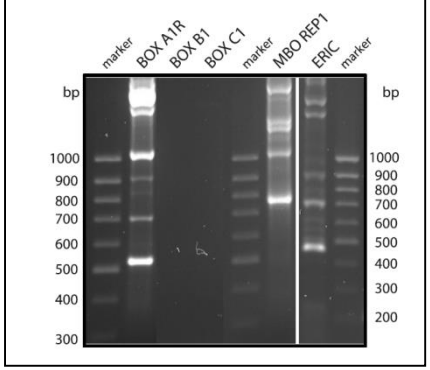
Genersch and Otten,2003

BOX-PCR

Primers

1. BOX elements enhance genetic diversity. e.g. more than 100 BOX elements are randomly distributed in intergenic regions of the pneumococcal genome.
2. Therefore, BOX primer is generally recommended since it generates robust fingerprints, and yields a highly complex fragment pattern.
3. BOX-PCR, carried out with single primer PCR primer.
4. The amplification of a PCR product using either forward/reverse primer is possible but you will get extremely low amount of product in each cycle.

BOX-PCR
Primers



- The most suitable rep-PCR in the case of Gram-positive bacteria was BOX-PCR, carried out with single primer PCR primer.
- The DNA sequences of the primers used for DNA fingerprinting were as follows (Versalovic *et al.*,1994):

1. 5´-CTACGGCAAGGCGACGCTGACG-3(BOX A1R),
2. 5´-TTCGTCAGTTCTATCTACAACC-3´(BOX B1),
3. 5´-TGCGGCTAGCTTCCTAGTTTGC-3´(BOX C1).

- The 22-mer BOX A1R oligonucleotide was used to generate BOX-PCR profiles from boxA-like subunit sequences.
- Primers BOX B1 and BOX C1 generated no bands at all, making these two primers not suitable for DNA fingerprinting of bacteria.

Genersch and Otten,2003;..

PCR conditions

REP,ERIC & BOX

BOX	ERIC	REP
94°C for 1 min.	94°C for 1 min.	94°C for 1 min.
53°C for 1 min.	52°C for 1 min.	40°C for 1 min.
65°C for 8 min.	65°C for 8 min.	65°C for 8 min.

Rademake and Bruijn

PCR conditions

REP,ERIC & BOX

Alternative PCR conditions

PCR	*Initial Denaturation*	Amplification cycles			*Final elongation*
		Denaturation	*Annealing*	*Elongation*	
REP-PCR	95 °C, 7 min	94 °C, 1 min	94 °C, 1 min	94 °C, 1 min	65 °C, 15 min
ERIC -PCR	95 °C, 7 min	44 °C, 1 min	52 °C, 1 min	53 °C, 1 min	65 °C, 15 min
BOX- PCR	95 °C, 7 min	65 °C, 8 min	65 °C, 8 min	65°C, 8 min	65 °C, 15 min

Venkatesh,2002

Preparation of master mix

Per one 25 µl reaction:	For ... 25 µl reaction:	Stock solution
5 µl	... µl	5 x Gitschier Buffer
0.2 µl	... µl	BSA, 20 mg/ml
2.5 µl	... µl	DMSO (dimethylsulfoxide), 100%
12.65 µl	... µl	ddH_2O, use 13.65 µl for BOX
1.25 µl	... µl	mix of dNTP's (1:1:1:1)
1 µl	... µl	Primer 1
1 µl	... µl	Primer 2, not applicable for BOX
0.4 µl	... µl	Taq DNA Polymerase, 5 U/l

Rademake and Bruijn

Preparation and stocking the components of master mix

- Mix master mix
- Collect by centrifugation.
- Aliquot 24 µl master mix to each tube.
- To reduce evaporation overlay the mix with a drop of mineral oil (mineral oil can be applied in sample tubes in earlier stage in order to prevent cross contamination of the samples).
- Autoclaved ddH_2O, 2.5 ml aliquots in 5 ml screw cap vials are stored at RT.
- BSA, 20 mg/ml nuclease free (Boehringer, #711454),the solution is divided in 20 ml aliquots and stored at -20°C.

Preparation and stocking the components of master mix

- DMSO 100%, the solution is divided in 0.5 ml aliquots and stored at 20C, Mix gently.
- Ultra pure dNTP set, 100 mM each.
- 100 mM solutions are mixed 1:1:1:1 to obtain a solution with 25 mM of each nucleotide, the solution is divided in 100 ml aliquots and stored at -20C.
- Add 1 µl of the samples to be fingerprinted to the reaction mix.
- Taq DNA Polymerase 5 U/µl stored at -20C.

Preparation of Gitschier buffer

1. Prepare the following stock solutions and autoclave them separately:

- 1 M $(NH4)_2SO_4$
- 1 M Tris-HCl (pH 8.8)
- 1 M MgCl
- 0.5 M EDTA (pH 8.8)

2. To prepare 200 ml of 5 x Gitschier combine:

- 16.6 ml of 1M $(NH4)_2SO_4$
- 67 ml of 1 M Tris-HCl (pH 8.8)
- 6.7 ml of 1 M $MgCl_2$
- 1.3 ml of a 1:100 dilution of 0.5 M EDTA (pH 8.8)
- 2.08 ml of a 14.4 M commercial stock of b-mercapto-ethanol, stored at 4°C.
- Adjust finally to 200 ml with approximately 106 ml water and mix. Store at -20°C in 1 ml aliquots.

Rademake and Bruijn

Rep-PCR

Procedure of REP and ERIC-PCR

- REP and ERIC-PCR were carried out with the same kind of mix but at 0.3 µg/µl of each primer (REP1R-I and REP2-I, or ERIC 1R and ERIC2).
- DNA amplification consisted of an initial denaturation step at 95°C for 5 min followed by cycles of 94°C (60 s), 40°C (60s) for REP-PCR and 52°C (60s) for ERIC-PCR, 65°C (5 min).
- After 30 cycles, a final extension was done at 65° C for 15 min.
- PCR fragments were separated by electrophoresis on agarose gel (1.5%) at 6 V per cm, stained with ethidium bromide, and DNA bands in the gels photographed under UV light, using a gel documentation system.

Primer solutions are divided in 200 ml aliquots and stored at -20C.

Poliakoff *et al.*,2005; Rademake and Bruijn

Rep-PCR analysis
E. nigrifluens

- **Procedure:**
- Bacterial isolates were subjected to rep-PCR analysis using:

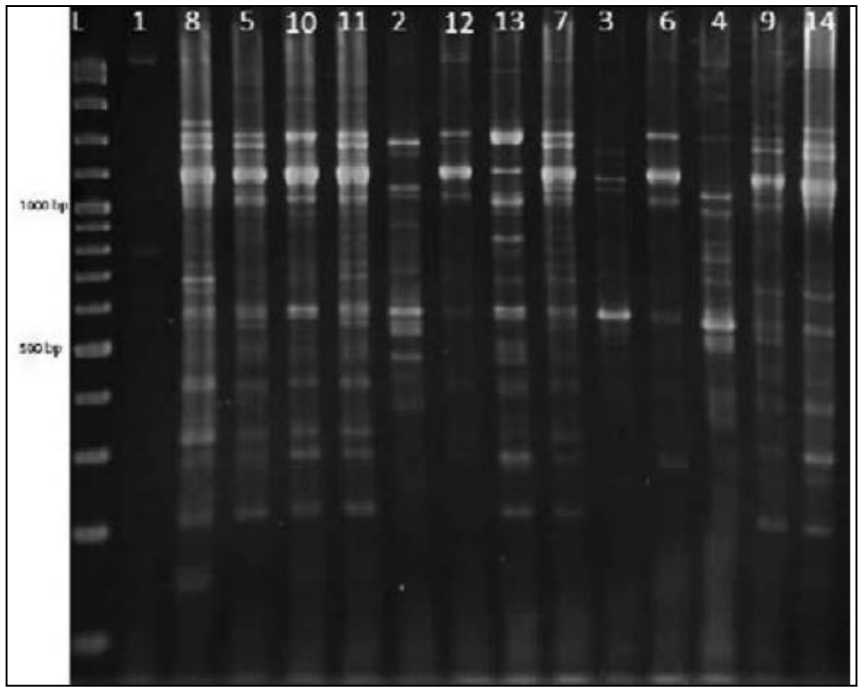
1. The primers:
2. REP 1R, and
3. REP 2I.

- DNA was extracted from bacterial cells grown on Luria-Bertani broth for 16 h at 27±1°C in an orbital shaker at 200 rpm.
- Amplicons were separated by electrophoresis on 1.5% agarose gels in 0.5xTAE buffer (20 mM Tris-acetate, 0.5 mM EDTA, pH 8.0) at 50 V and 4°C for 14 h.
- DNA fingerprints were visualised with a UV transilluminator and their images captured with the EuroClone Photoprint camera system.

Moretti *et al.*,2007

REP-PCR
Xanthomonas spp.

- Patterns of ERIC-PCR on agarose gel electrophoresis.
- Lanes 1-15 include 15 isolates of *Xanthomonas* , and
- Lane L indicates DNA ladder 100bp plus.



Asgarani *et al.*,2014

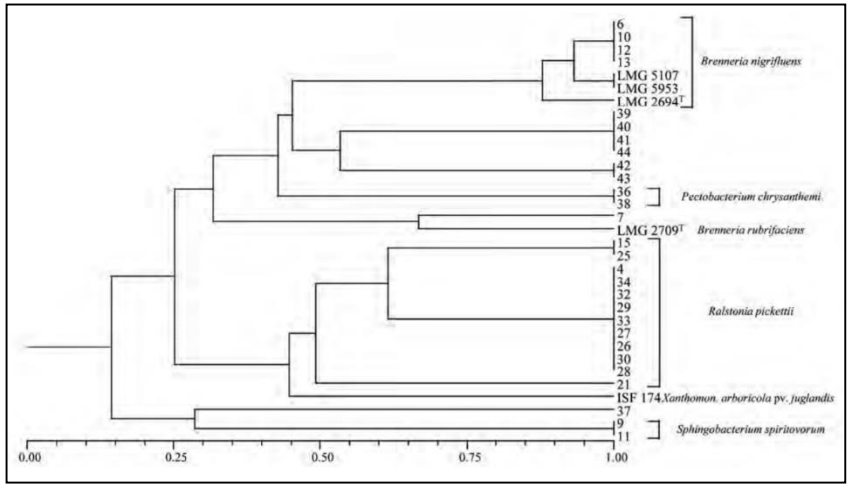
REP-PCR
NTSYSpc software

- ERIC-PCR amplification products were listed as discrete character states per strain (presence or absence of band).
- Jaccard's similarity coefficients between each strain were calculated using the SIMQUAL program in NTSYSpc, version 2.02e, and these data were subjected to UPGMA cluster analysis by use of the SAHN program of NTSYS.

REP-PCR analysis

Brenneria (ex. *Ewinia*) *nigrifluens*

- The genomic REP-PCR profiles consisted of bands ranging in size from 300 to 2000 bp.
- Seventeen reproducible, clearly resolved bands were scored for UPGMA analysis.
- Dice's coefficient and Jaccard's similarity index yielded very similar dendrograms (data not shown).



Dendrogram derived from REP-PCR fingerprint data, obtained by cluster analysis (UPGMA) and Dice's coefficient.

Moretti *et al.*,2007

BOX-PCR-based identification of bacterial species belonging to *Pseudomonas syringae* - *P. viridiflava* group

- Following the visual inspection, the patterns of all of the isolates were analyzed more rigorously using the Bio-Profil software (Vilber Lourmat, France).
- Band sizes were assigned by direct comparison to concurrently run DNA standards (1 kb).
- This information was used to construct a matrix table where each isolate was matched with a notation +/-, where (+) represents the identical presence and position of a band in the fingerprints to be compared.

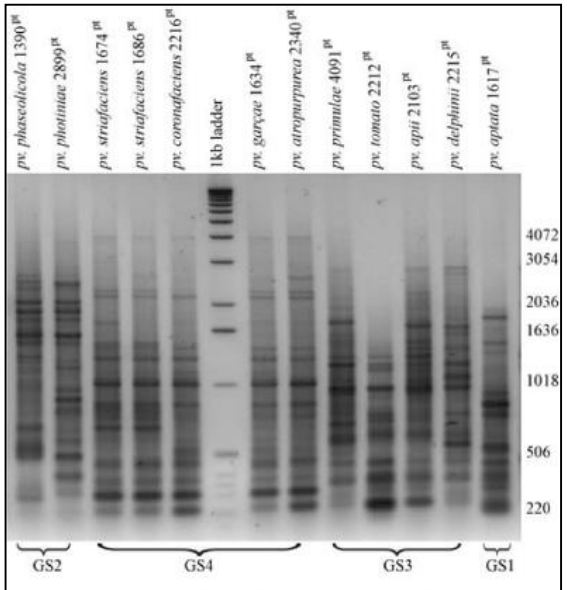


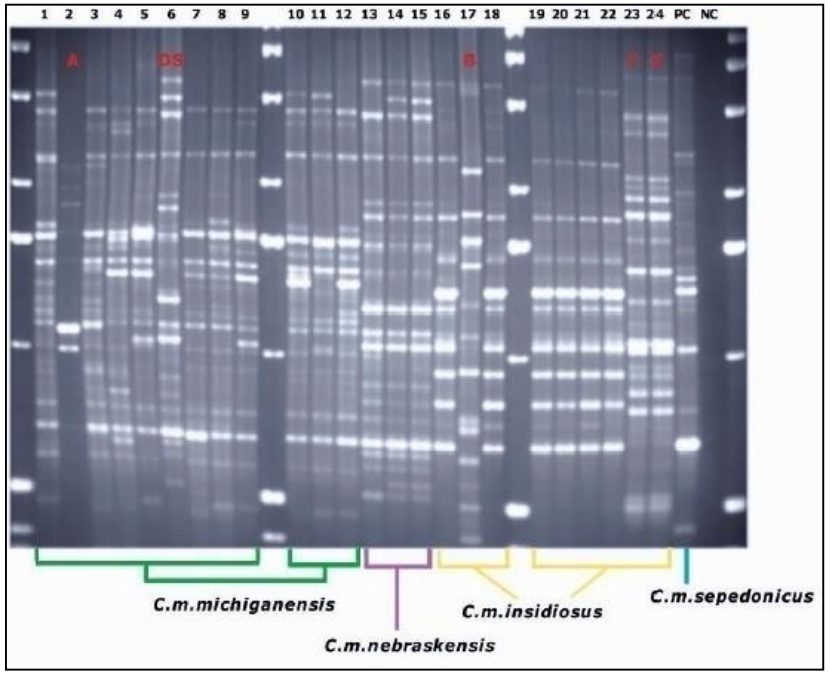
Figure 4 - Agarose gel electrophoresis of BOX-PCR fingerprinting patterns from genomic DNA of different pathovars belonging to *Pseudomonas syringae* (pathovars *photiniae*, *striafaciens*, *coronafaciens*, *garçae*, *atropurpurea*, *primulae*, *tomato*, *apii*, *delphinii* and *aptata*) and *P. savastanoi* pv. *phaseolicola*, which represent some of the nine known genomic species (GS), assigned after Gardan *et al.* (1999). Isolates obtained from the "Collection Française des Bactéries Phytopathogènes" (CFBP, Angers, France). The molecular size marker is a 1-kb ladder (Gibco BRL Life Technologies Inc.) and the sizes are indicated in base pairs. Shown is a negative image of an ethidium bromide gel. pt: pathotype strain.

Marques *et al.*,2008

BOX-PCR analysis

Used to discriminate several ex. subsp. of *Clavibacter michiganensis*

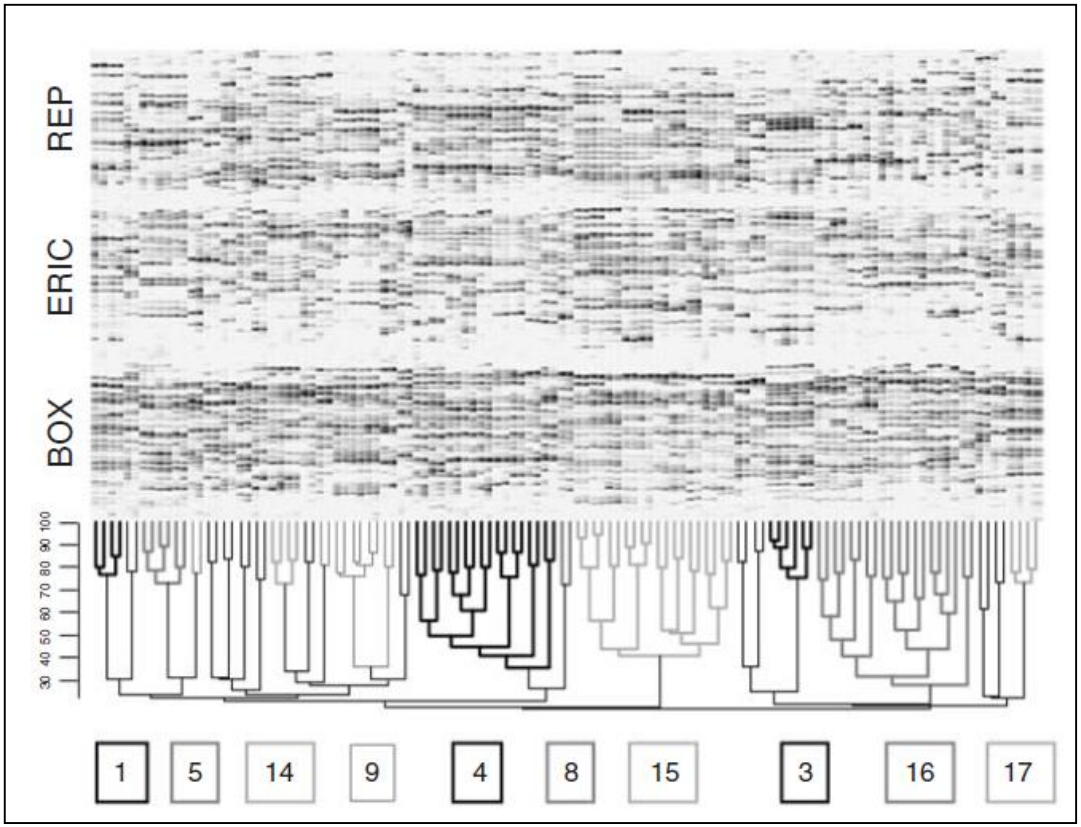
- BOX-PCR results for several subspecies of *Clavibacter michiganensis*.
- Non-numbered lanes 1 kb DNA ladder;
- lanes 1-9 and 10-12 *C. m.* subsp. *michiganensis*;
- lanes 13-15 *C. m.* subsp. *nebraskensis*;
- lanes 16-18 and 19-24 *C. m.* subsp. *insidiosus*;
- lane 25 positive control *C. m.* subsp. *sepedonicus*;
- lane 29 negative control, ultra pure water.
- A, B and C = misidentified strains, strain C tested twice; DS = deviating strain of *C. m.* subsp. *michiganensis*, probably a mislabelled strain of *C. m.* subsp. *nebraskensis* (compare profiles!).



Janse,2006

BOX-, ERIC and REP-PCR genomic fingerprinting as applied for the classification and identification of *Xanthomonas*

Clusters of 59 *Xanthomonas* strains correspond to DNA-DNA homology groups, as indicated in squares, or species using combined BOX-, ERIC and REP-PCR genomic fingerprints.



Osborn and Smith,2005

Rep-PCR analysis
X. fastidiosa

- Repetitive sequence based-PCR technology (REP-PCR) was used to identify unique PCR products for *X. fastidiosa* causing Pierce's Disease (PD).
- The DNA from *X. fastidiosa* strains were amplified with primers:
- REP1-R (5'-IIIICGICGIATCCIGGC-3‘),and
- REP 2 (5'-ICGICTTATCIGGCCTAC-3').
- The overall reactions were adjusted to a final volume of 20 µl, containing:
- 1 X PCR buffer (50 mM KCl, 200 mM Tris-HCl, pH 8,4),
- 2 mM $MgCl_2$,
- 200 µM of each dNTP,
- 1 U of *Taq* DNA polymerase,
- 30 ng of each primer (REP1-R and REP 2) and
- 50 ng of template DNA.

Travensolo *et al.*,2005

Rep-PCR
Procedure of BOX-PCR

- BOX-PCR was carried out in 25-µL volumes containing:
- 1 × DNA polymerase buffer,
- 1.5 mM $MgCl_2$,
- 0.3 µg/µl BOX A1R primer,
- 0.8 mM dNTP
- 2 U of DNA polymerase (Taq Goldstar, Eurogentec), and as template DNA, 5 µL of a bacterial cell suspension at 10^8 cfu mL^{-1}.
- With an initial denaturation step at:
- 95°C for 7 min followed by cycles of 94°C (60 s), 53°C (60 s), 65°C (8 min).
- After 30 cycles, a final extension was done at 65°C for 16 min.
- PCR fragments were separated by electrophoresis on agarose gel (1.5%) at 6 V per cm, stained with ethidium bromide, and photographed under UV light.

Poliakoff *et al.*,2005; Rademake and Bruijn; Marques *et al.*,2008

Rep-PCR

Procedure of BOX-PCR

In details

1. Grow overnight bacterial cultures and transfer 0.75 ml of each bacterium to labeled 1.5 ml microcentrifuge tubes.
2. Pellet cells by centrifugation for 1 minute.
3. Discard the supernatant and wash cells in 0.5 ml of deionized sterile water.
4. Repeat steps 2 and 3 so that the cells are washed a total of 2 times.
5. Resuspend cells in 0.5 ml deionized sterile water.
6. Use 1 µl of the cell suspension for PCR.

Wang,2012

Rep-PCR

Procedure of BOX-PCR

Continued

1. Count the number of samples to amplify and prepare master PCR mix. Multiply the following by the number of samples and combine in order, on ice:

	Stock Conc	Volume	Final Conc
Water		18.1 ul	
10X Buffer	10X	2.5 ul	1 X
$MgCl_2$	25 mM	3 ul	4.5 mM*
dNTPs	25 mM ea	0.25 ul	0.25 mM ea
Primer BOX A 1R	100 uM	0.5 ul	2 uM
JumpStart DNA Polymerase	2.5U/ul	0.4 ul	1U/reaction
Total		24 ul	

* The 10X buffer contains 15 mM $MgCl_2$; other components include 100 mM Tris (pH 8.3 at 25/C), 500 mM KCl

Wang,2012

Rep-PCR

Procedure of BOX-PCR

Continued

2. Add 24 µl of the master mix to 1 µl of cells in 0.2 ml PCR tubes. Cap and then mix well by tapping gently.
3. Spin tubes briefly to pellet liquid to the bottom and use the following thermal cycle program to amplify DNA:

- Single step at 95°C for 2 min.
- 35 cycles of: 94°C for 3 sec 92°C for 30 sec 50°C for 60 sec 65°C for 8 min
- Single step at 65°C for 8 min.
- The sequence of the PCR primer (BOX A 1R) is CTA CGG CAA GGC GAC GCT GAC G.

Wang,2012

BOX-PCR analysis

Used to discriminate to identify *Pseudomonas syringae - P. viridiflava* group at species level

- BOX-PCR, independent from the other rep-PCR techniques, has revealed the possibility of delineating *P. syringae* genomospecies.
- BOX-PCR-based identification of bacterial species belonging to *Pseudomonas syringae - P. viridiflava* group.

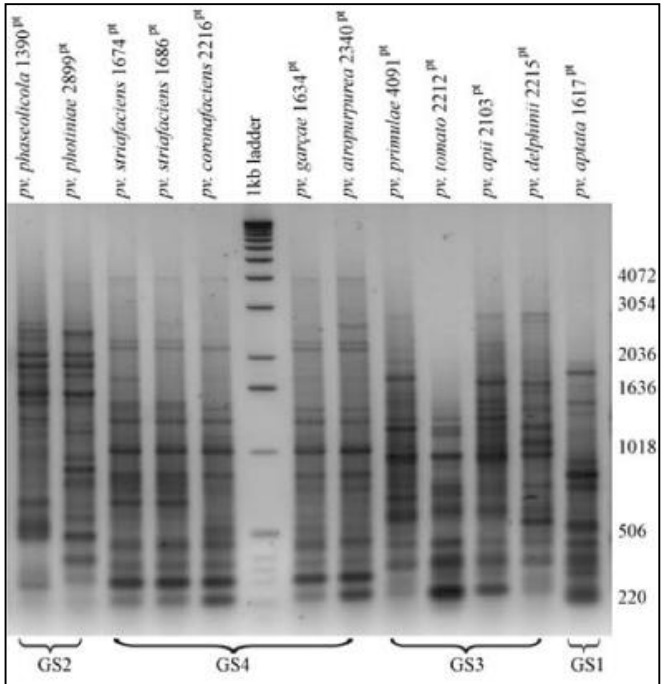


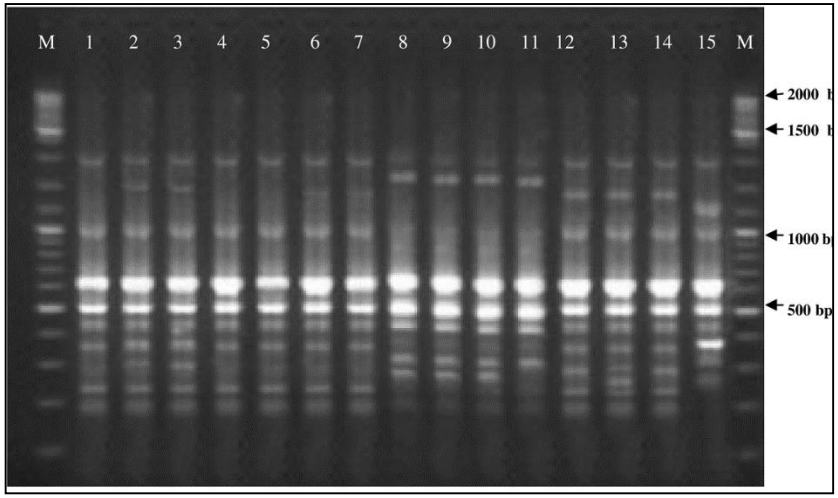
Figure 4 - Agarose gel electrophoresis of BOX-PCR fingerprinting patterns from genomic DNA of different pathovars belonging to *Pseudomonas syringae* (pathovars *photiniae*, *striafaciens*, *coronafaciens*, *garçae*, *atropurpurea*, *primulae*, *tomato*, *apii*, *delphinii* and *aptata*) and *P. savastanoi* pv. *phaseolicola*, which represent some of the nine known genomic species (GS), assigned after Gardan *et al.* (1999). Isolates obtained from the "Collection Française des Bactéries Phytopathogènes" (CFBP, Angers, France). The molecular size marker is a 1-kb ladder (Gibco BRL Life Technologies Inc.) and the sizes are indicated in base pairs. Shown is a negative image of an ethidium bromide gel. pt: pathotype strain.

Marques *et al.*,2008

BOX-PCR analysis

Used to discriminate to identify *Pseudomonas syringae* strains

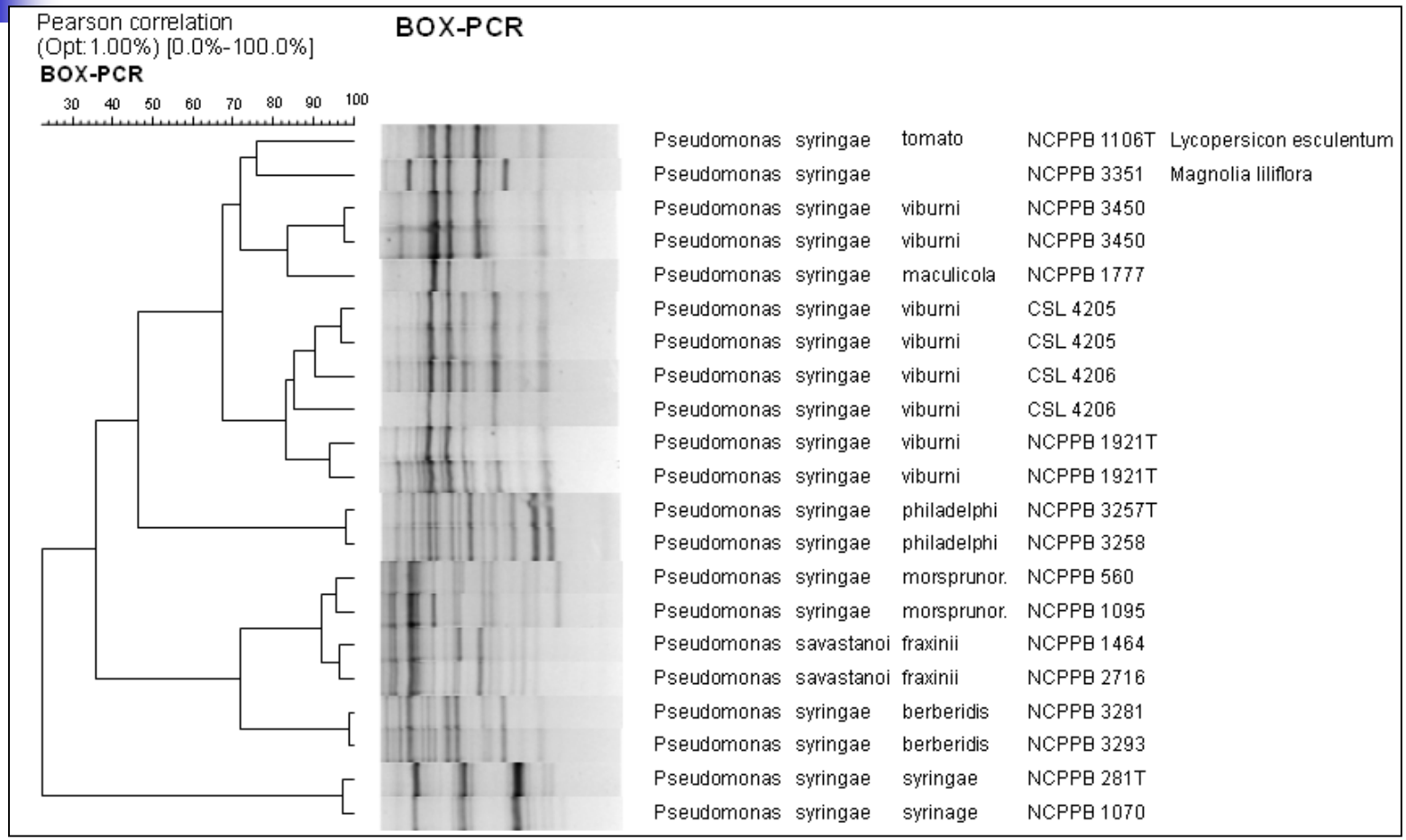
- BOX-PCR fingerprint patterns of Pss strains isolated from different hosts.
- The BOX-PCR was carried out with BOX A1R primer (Versalovic *et al.*, 1991).
- PCR products, ranging from 200 to 2500 bp in size, were amplified with BOX-PCR primer.



BOX A1R primer (5'CTACggCAAggCgACgCTgACg-3') was purchased from Metabion Co., Germany.

Najafi Pour and Taghavi,2011

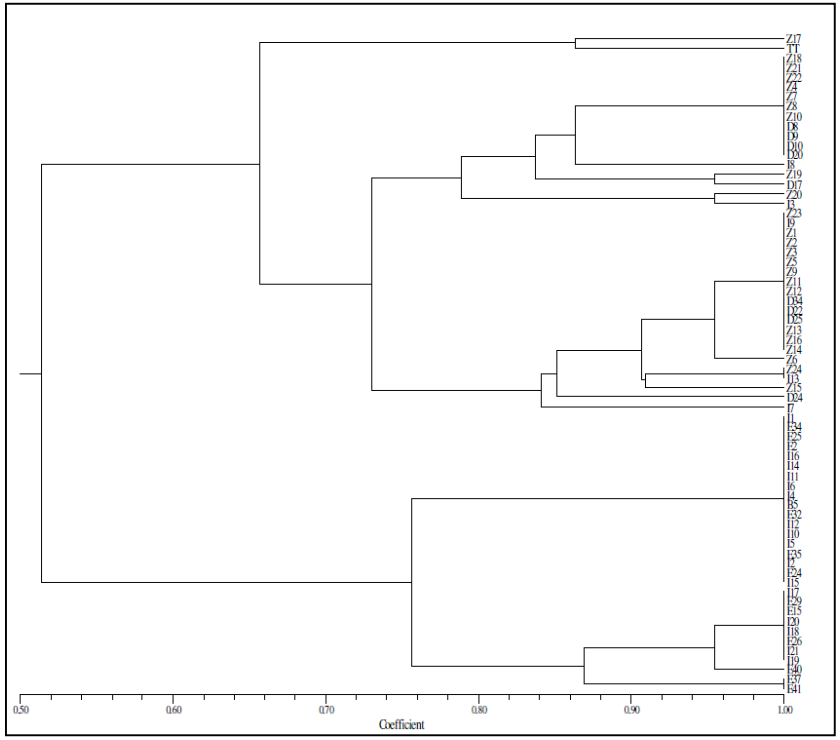
BOX-PCR fingerprints showing unique pattern within *P.s.* pv. *viburni*



Stead *et al.*,2005

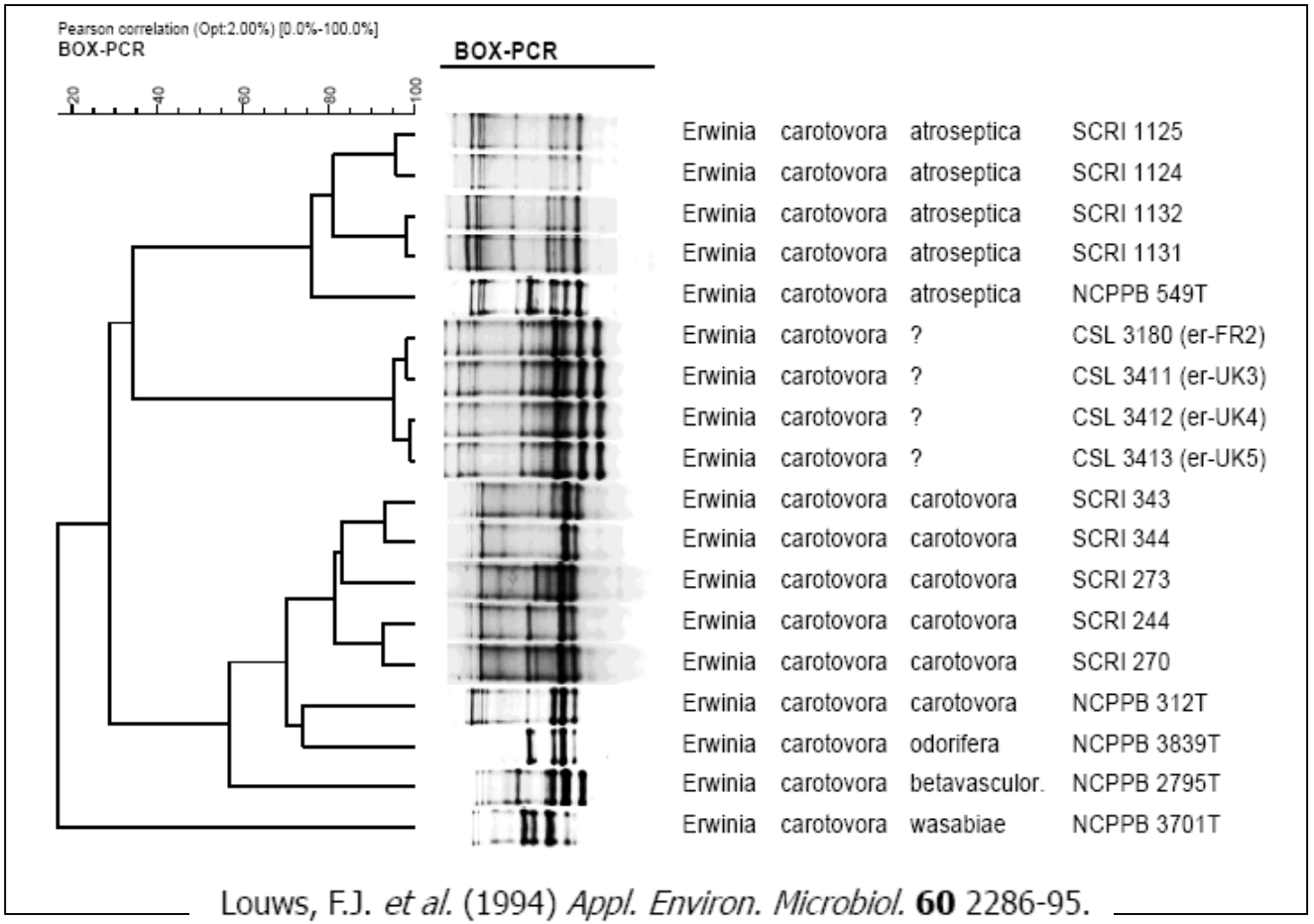
BOX-PCR analysis
Pectobacterium spp.

- Similarity dendrogram of *Pectobacterium* strains based on BOX-PCR.
- Results revealed two main clusters among the pathogen strains and their similarity value, based on UPGMA was about 51.5%.
- The first group was divided into two subgroups at 65.5% similarity.
- The second group was also divided into two subgroups at 75.5% similarity.



Tavasoli *et al.*,2011

BOX-PCR fingerprints showing unique pattern within ex. *Erwinia carotovorum*



Louws, F.J. *et al.* (1994) *Appl. Environ. Microbiol.* **60** 2286-95.

Richard Thwaites

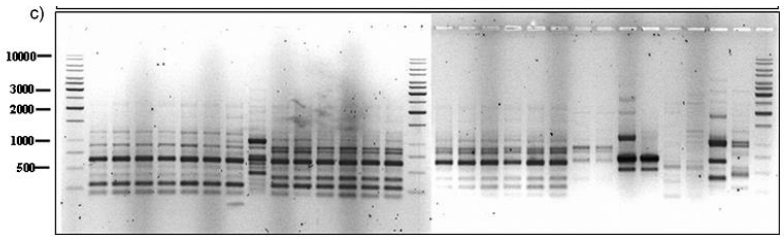
PCR for detection of numerous pathovars of *Pseudomonas syringae* pv. *actinidiae* Insertion sequence (IS) elements

- IS50-PCR reactions were performed witha single 20 bp oligonucleotide primer (IS50 GGT TCC GTT CAG GAC GCT AC) complementary to the IS50 portion of Tn5, as described by Sundin & Murillo (1999).
- Following the amplification step, 10 µL of the products were separated on 1% agarose gel at 4 V/cm for 4-5 h.

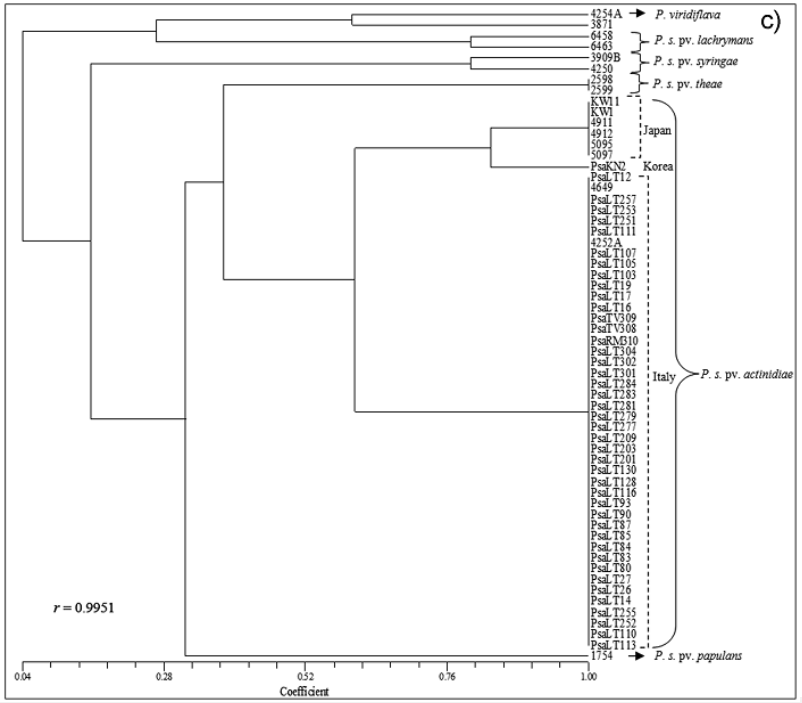
Transposable elements (TEs), or transposons, are a large group of mobile genetic elements with ability to actively transfer themselves into new locations in their host´s DNA. Insertion sequences (ISs) present the simplest examples of TEs.

Mazzaglia *et al.*,2011;..

PCR for detection of numerous pathovars of *Pseudomonas syringae* pv. *actinidiae* Insertion sequence (IS) elements



Examples of PCR fingerprints of *Pseudomonas syringae* pv. *actinidiae* and other *P. s.* pathovars obtained with IS50(c) primer.



Computer generated dendrogram of genetic similarity resulting from the data set of IS50-PCR amplifications, using UPGMA analysis and Dice's coefficient. The values of the cophenetic correlation coefficient (r) are reported.

Mazzaglia *et al.*,2011

Ribotyping
Molecular fingerprinting

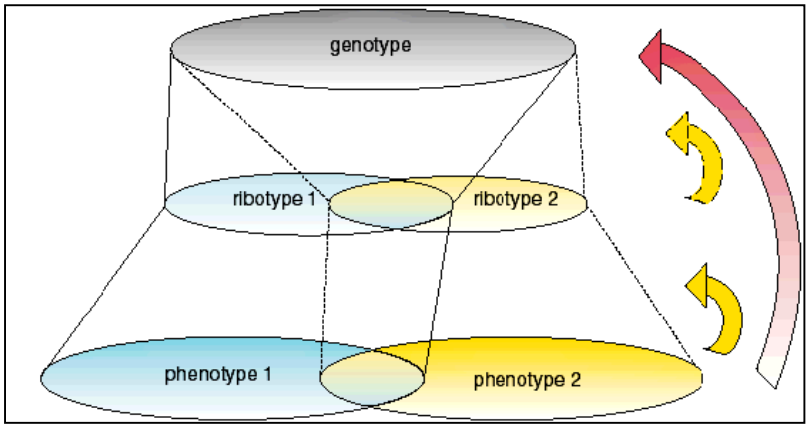
- Ribotyping is based on the unique pattern of restriction enzyme digests of DNA encoding the 16S ribosomal RNA molecule.
- Recently, the more variable intergenic spacer (IGS, ITS) region which lies between the 16S and 23S rRNA transcription units, was targeted for differentiation of bacterial species.
- Ribotyping is very specific, rapid (sequencing of nucleotides is not involved), and is increasingly used in clinical laboratories as well as in food/water microbiology.
- This technique is also powerful for the differentiation of many plant pathogenic including enterobacterial species and strains.

Ribotyping
Advantages & Disadvantages

- The ribotype is defined as the ribonucleoprotein system of any cell.
- Ribotype theory substitutes the genotype-phenotype duality with the trinity genotype-ribotype-phenotype, and proposes that life on earth originated with the ancestors of today's ribotypes ribonucleoprotein system.

Ribotyping

Advantages	Disadvantages
• Easy to type • Highly reproducible • Easy to perform • Easy to interpret • Easy to automate	• Tedious • Time-consuming • Expensive



A ribosome is composed of rRNA and ribosomal proteins known as a ribonucleoprotein.

Ribotyping

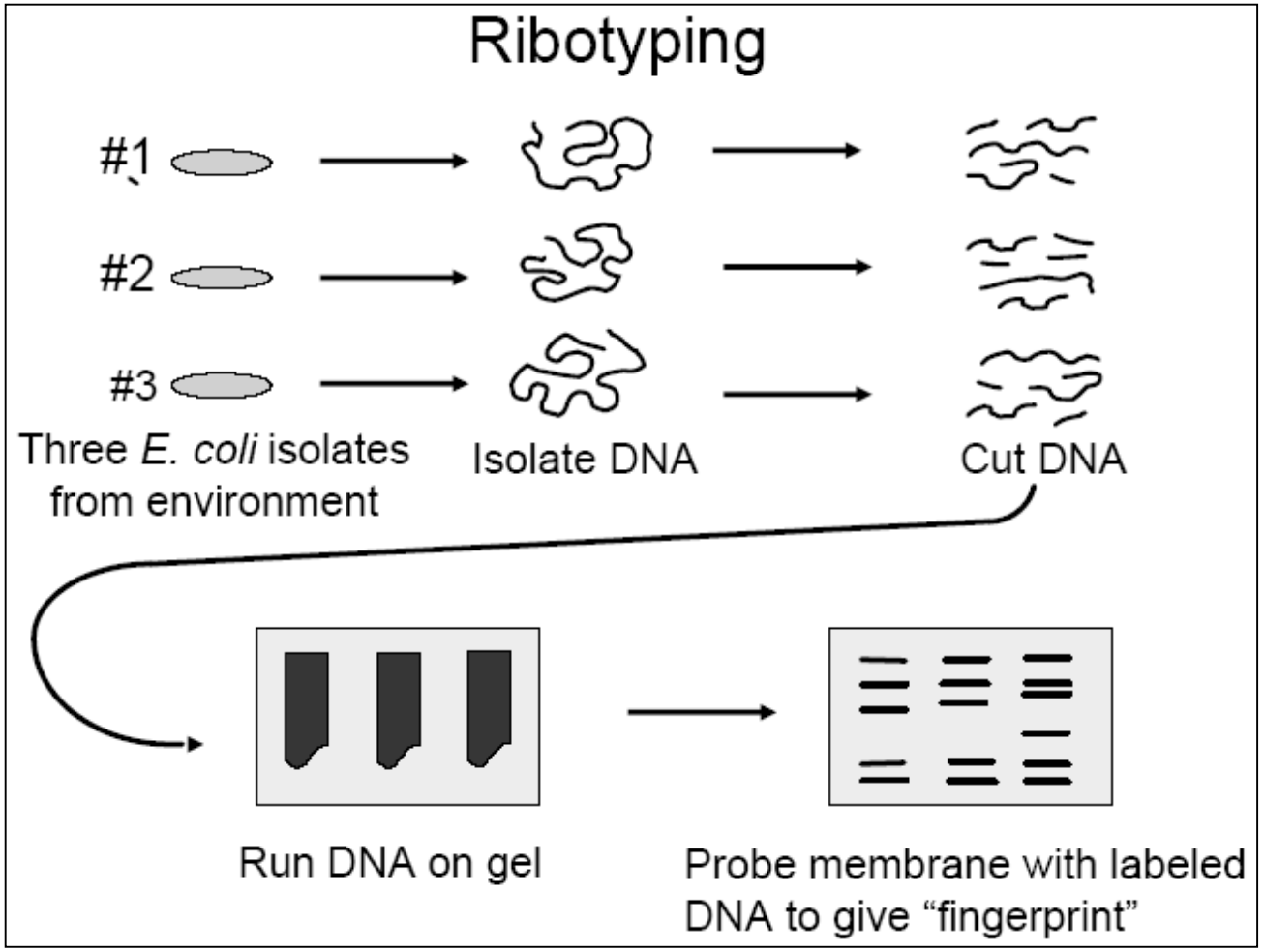
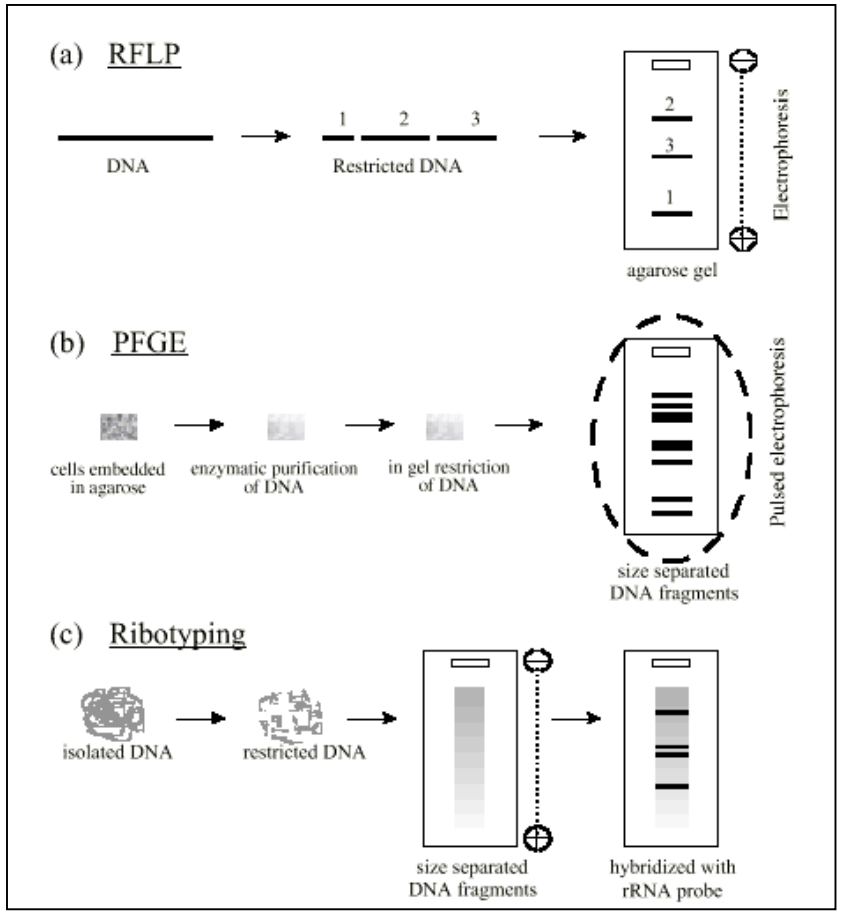

Ribotyping
#1
#2
#3
Three E. coli isolates from environment
Isolate DNA
Cut DNA
Run DNA on gel
Probe membrane with labeled DNA to give "fingerprint"

Illustration of commonly used fingerprinting techniques
Ribotyping compared with RFLP, PFGE

- **Ribotyping**
- DNA is isolated from a cultured isolate, restricted and size separated in an agarose gel.
- The gel is then hybridized with labeled rRNA probe, which binds to fragments containing copies of the rRNA operon.
- Following probe detection, fragments with bound probe are visualized, forming a characteristic RFLP.



O'Sullivan,2000

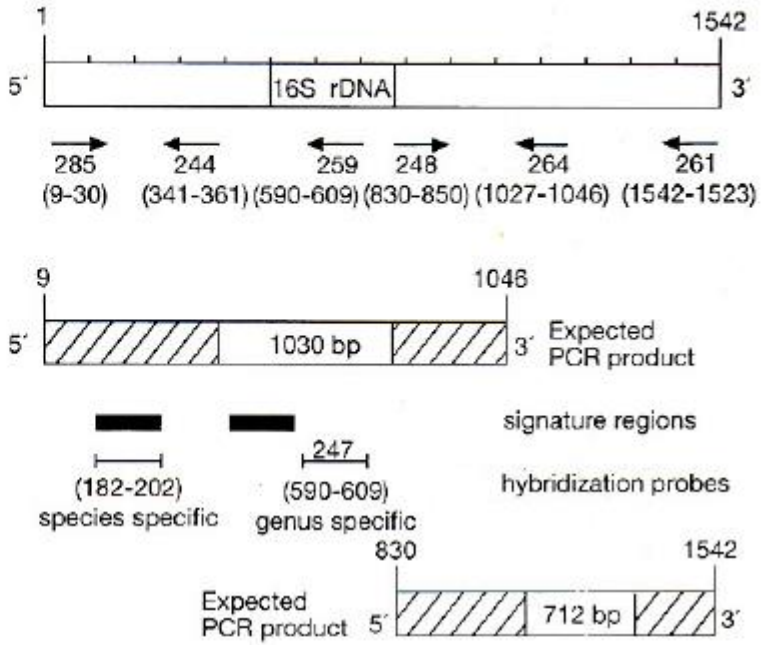
Ribotyping

Riboprinting®

- Genomic restriction fragment digestion
- Probes specific for 16S to 23S rRNA gene sequences
- DuPont and user data base comparisons

Mycobacterium speciation using 16S rRNA gene

- Species specific vs genus specific regions of 16S rRNA gene
- Examine sequence alignment



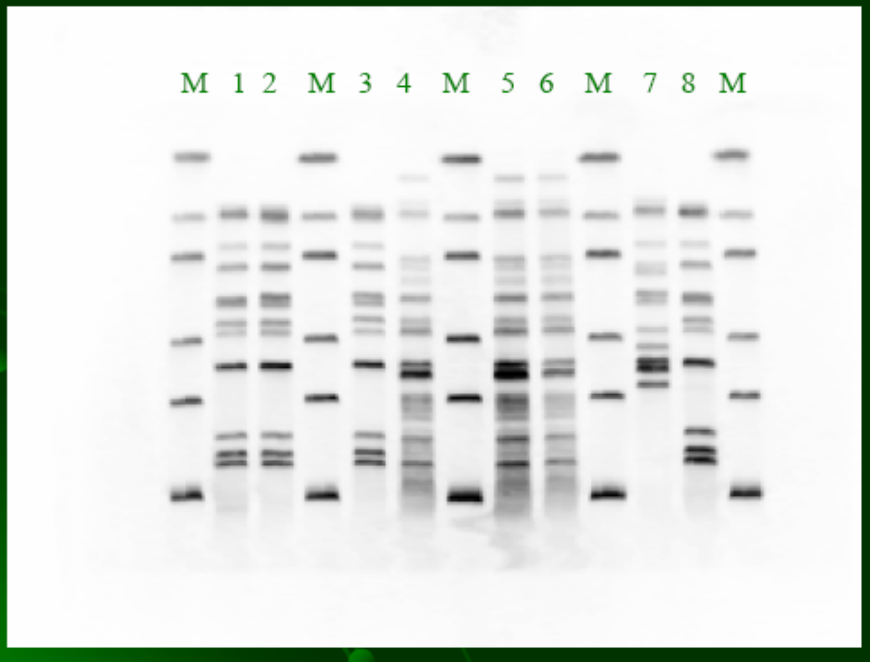
285: 5′ GAGAGTTTGATCCTGGCTCAG 3′
244: 5′ CCCACTGCTGCCTCCCGTAG 3′
259: 5′ TTTCACGAACAACGCGACAA 3′
248: 5′ GTGTGGGTTTCCTTCCTTGG 3′
264: 5′ TGCACACAGGCCACAAGGGA 3′
261: 5′ AAGGAGGTGATCCAGCCGCA 3′

species-specific

M.tb complex:	5′ ACCACAAGACATGCATCCCG	3′
M.avium:	5′ ACCAGAAGACATGCGTCTTG	3′
M.intracellulare:	5′ ACCTAAAGACATGCGCCTAA	3′
M.leprae:	5′ ATAGGACTTCAAGGCGCATG	3′
M.genavense:	5′ CCACAAAACATGCGTTCCGTG	3′

The analysis was done with the RiboPrinter Microbial Characterization System (Qualicon)

Ribotyping output from the Qualicon™ Riboprinter

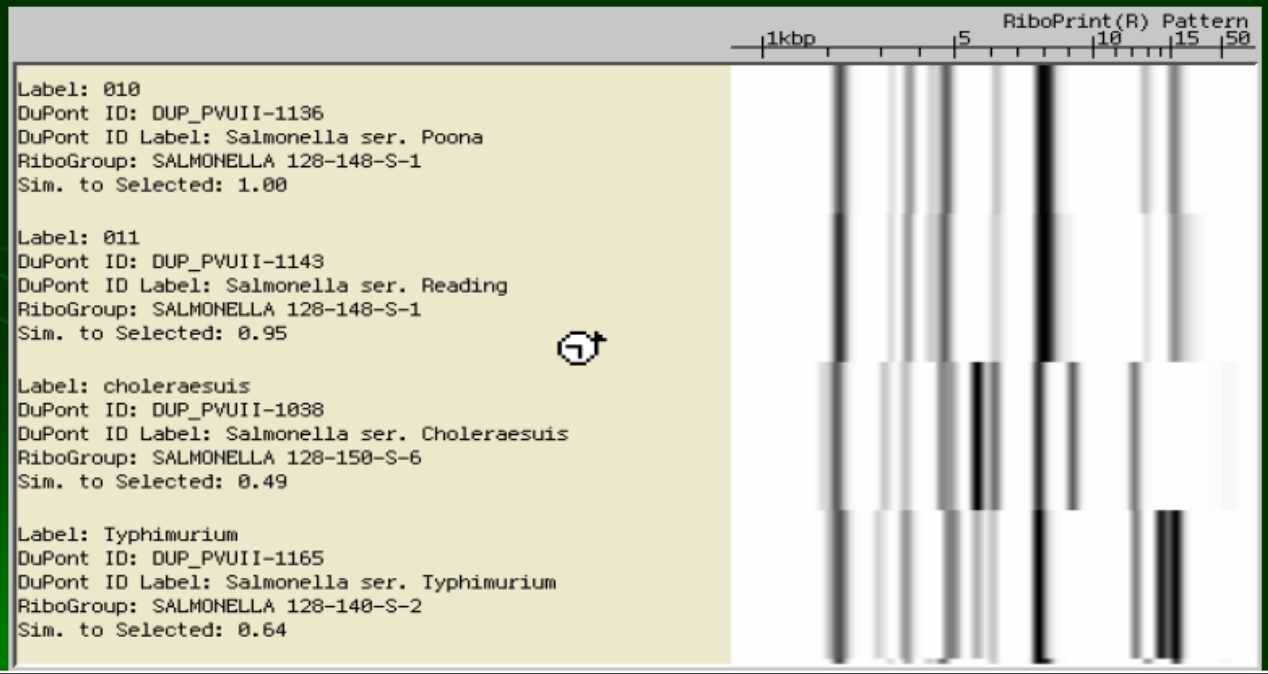


Raw image of the blot displays the 8 sample and 5 molecular mass marker lanes used to standardize the Riboprint pattern from multiple gels, or various instruments

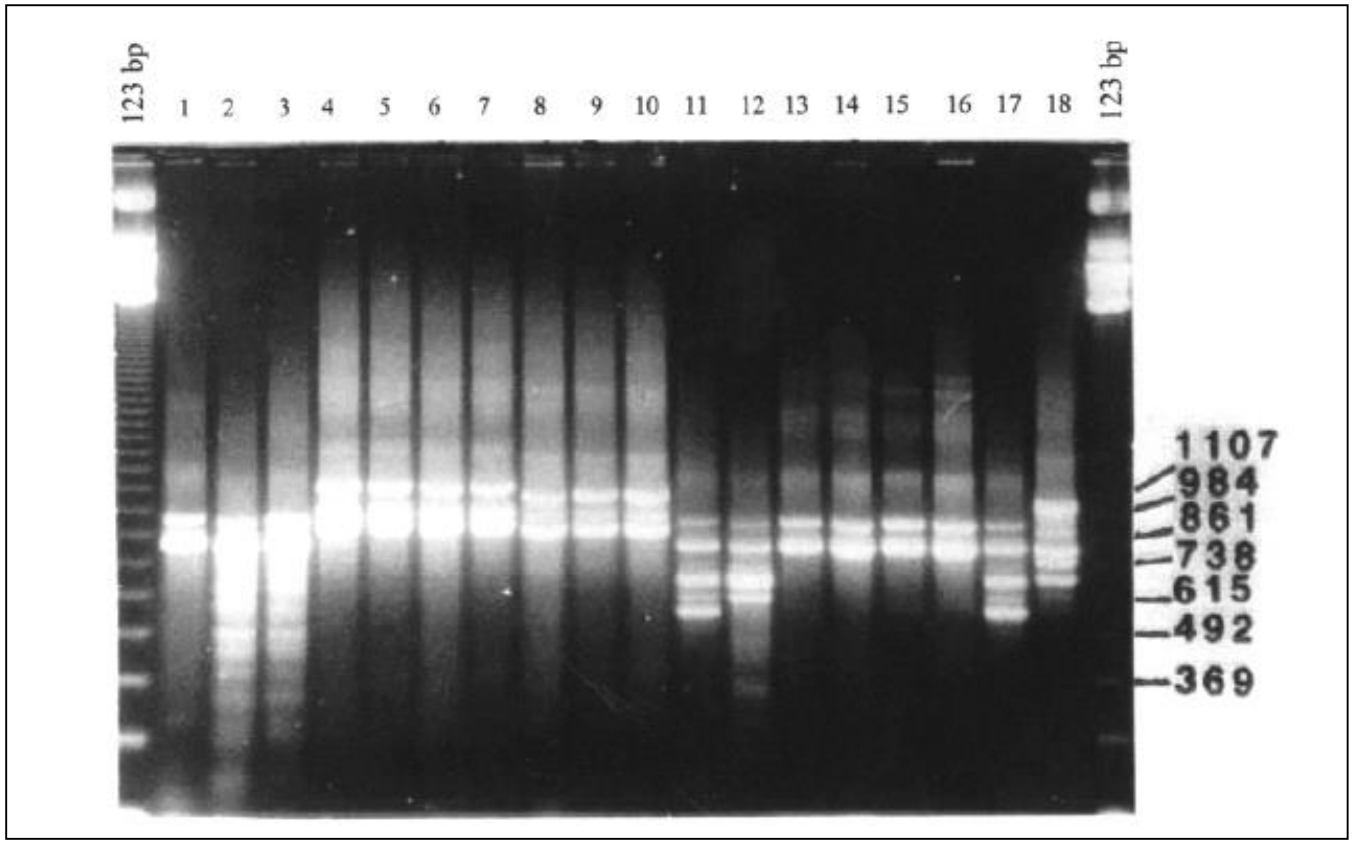
Ribotyping

Riboprinting®

- Multiple enzymes may be used to increase the power of discrimination



Ribotyping within the genus *Erwinia*



Intergenic spacer (IGS) region

RAPD

Random amplification of polymorphic DNA

- RAPD(pronounced as "rapid") is a type of PCR, but the segments of DNA that are amplified are random.
- The scientist performing RAPD creates several arbitrary, short primers (8-12 nucleotides).
- Unlike traditional PCR analysis, RAPD does not require any specific knowledge of the DNA sequence of the target organism;
- RAPD technique has the potential to detect polymorphism throughout the entire genome.

RAPD
Random amplification of polymorphic DNA

- RAPD analysis has been instrumental in the:

1. Genetic mapping of the plant genome and is
2. Key to understanding the divergence of races among fungal pathogens.
3. RAPD-PCR holds its greatest promise in the genetic differentiation of bacterial strains and has been employed to differentiate both Gram negative and Gram positive organisms.

Eric W. Brown;..

RAPD

Random amplification of polymorphic DNA

1. In recent years, RAPD has been used to characterize, and trace, the phylogeny of diverse plant and animal species.
2. Therefore, if a mutation has occurred in the template DNA at the site that was previously complementary to the primer, a PCR product will not be produced, resulting in a different pattern of amplified DNA segments on the gel.

NCBI,2017;..

RAPD PCR

Amplify segments of DNA which are essentially unknown to the scientist (random)

- However, in RAPD analysis, the target sequence(s) (to be amplified) is unknown.
- The scientist will design a primer with an arbitrary sequence.
- RAPD-PCR reactions require the addition of a single oligomer (primer) usually between 8-10 bp in length.
- Decamer primers(10 nucleotides long) were applied for RAPD-PCR analyses.
- A large number of decamer primers were available for RAPD-PCR(10-120 in numbers).

Arbitrary primers: Generally, short (7 to 12 base pairs) segments of DNA that do not correspond to known genomic sequences and thus may pair with (anneal to) many sites (arbitrary). They are used in PCR methods such as RAPD and DAF.

RAPD

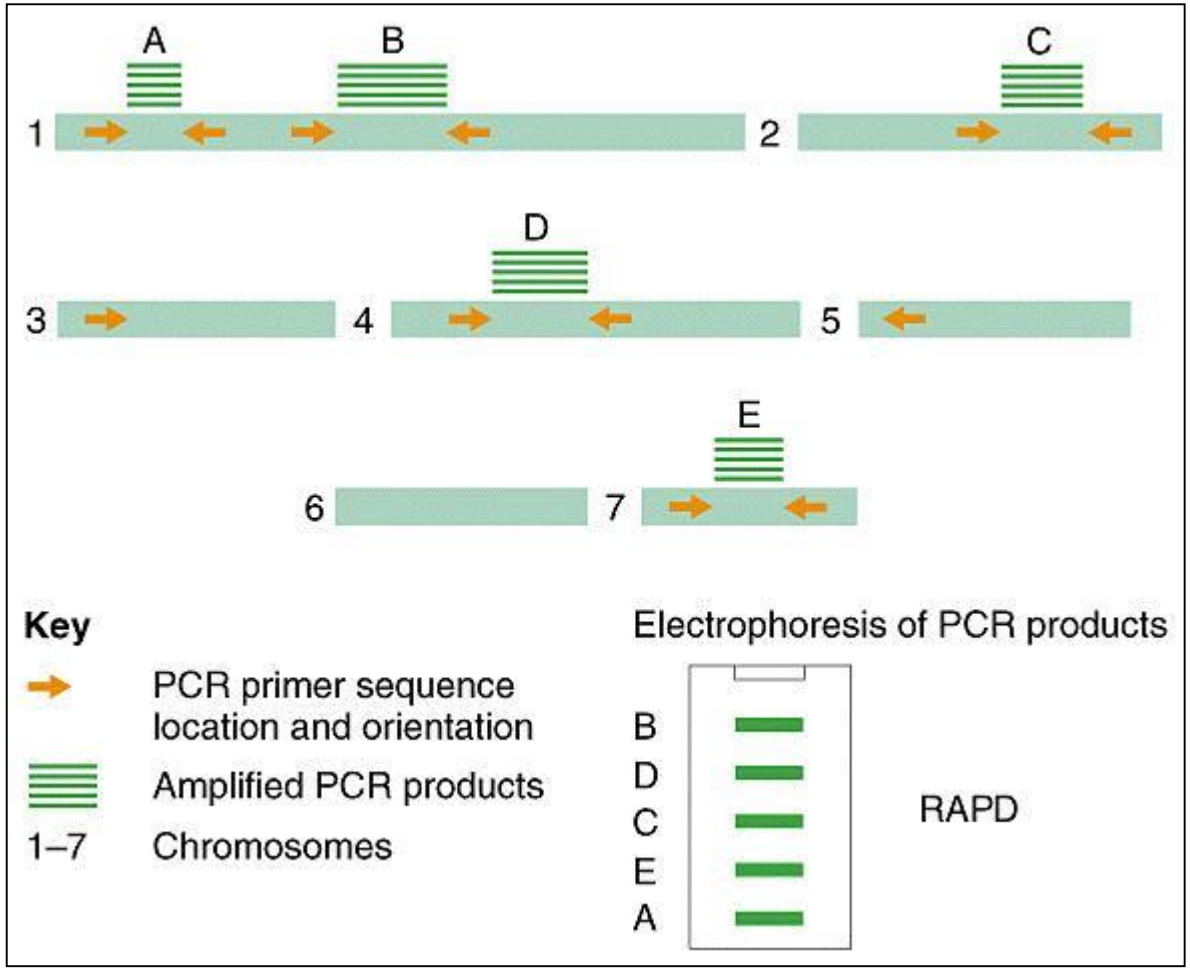
Primers

- The arbitrary, short (c. 10 bp) primers (nonspecific primers) will bind at arbitrary places on the DNA strand at a permissive annealing temperature of 36-45º C.
- Amplification gives 3-4 bands which can be separated by standard DNA electrophoresis on agarose gels.
- The pattern of amplification products is usually discriminative at low taxonomic level, often strain level.

Janse,2006; Eric W. Brown

RAPD

Random amplification of polymorphic DNA



RAPD

Oligonucleotides and related cycling conditions often employed in PCR-based fingerprint analyses of bacteria

Oligo name / sequence (5′–3′)	Method	Annealing temp (°C)	Extension temp (°C)	Extension time (min)	Number of cycles
7. OPA-1 / CAGGCCCTTC	RAPD-PCR	35	72	2.0	35
8. Bc3 / CCGGCGGCG	RAPD-PCR	35	72	2.0	35
9. Bc4 / CGGCCCCTGT	RAPD-PCR	35	72	2.0	35
10. Bc5 / CGGCCACTGT	RAPD-PCR	30	72	2.0	35

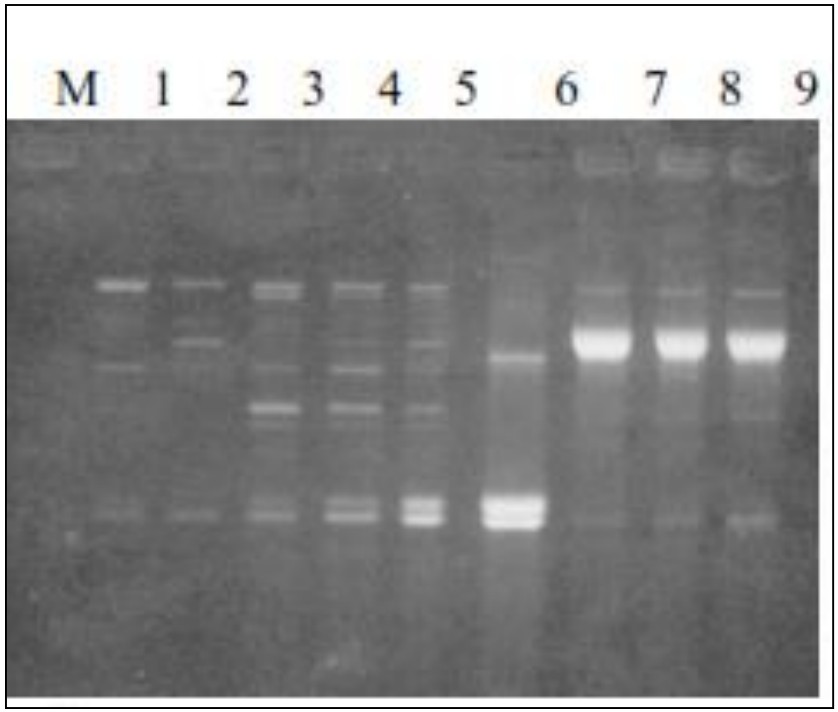
RAPD analysis
Xanthomonas phaseoli

- PCR reaction was performed in 20 µl volume containing RAPD buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin), 0.2 mM of each dNTP (Boehringer Mannheim), 15 ng of ten mer primer, 17.5 ng of DNA, 1.5 units Taq Polymerase (Gibco).
- Nine primers were used for RAPD analysis: OPG-03, OPG-06, OPH-01, OPH-04, OPH-06, OPH-07, OPH-08, OPH-09 and OPH-12.
- The amplifications were performed in a MJ Research thermocycler, programmed for 35 cycles of 1 min at 94°C, followed by 1 min at 35°C, 1.5 min at 72°C and a final extension at 72°C for 5 min.
- Aliquots of the final amplified products were visualized in 1.4% agarose gels containing 0.5 µg/ml of ethidium bromide.

Mário de Carvalho Nunes *et al.*,2008

RAPD analysis
Xanthomonas phaseoli

- RAPD polymorphism of nine isolates of *Xanthomonas axonopodis* pv. *phaseoli*, with primer OPG-06.
- Lane M: molecular size marker (1 Kb ladder Gibco BRL);
- lane 1: Davis isolate; lane 2: W33 isolate; lane 3: W43 isolale; lane 4: W44 isolate; lane 5: W45 isolate; lane 6: W63 isolate; lane 7: W67 isolate; lane 8: W68 isolate; lane 9: W69 isolate.



Mário de Carvalho Nunes *et al.*,2008

RAPD
P. syringae pv. *actinidiae*

- One hundred and twenty random primers, including 40 commercial 10-mer oligonucleotide primers and 80 commercial 12-mer oligonucleotide primers (Sangon Biotech, Shanghai, China), were used to select primers for screening the specific fragment of *P. syringae* pv. *actinidiae*.
- Amplification was performed in a total volume of a 25 μl reaction mixture with 50 pmol of primer, 100 ng of genomic DNA, 1 Unit of Taq DNA polymerase, 10× reaction buffer (500 mM KCl, 100 mM of Tris-HCl pH 8.3, 25 mM of $MgCl_2$, 0.01% of gelatin), 10mM of dNTP (dCTP, dGTP, dATP, dTTP), and extra sterilized distilled water.

Liu *et al.*,2012

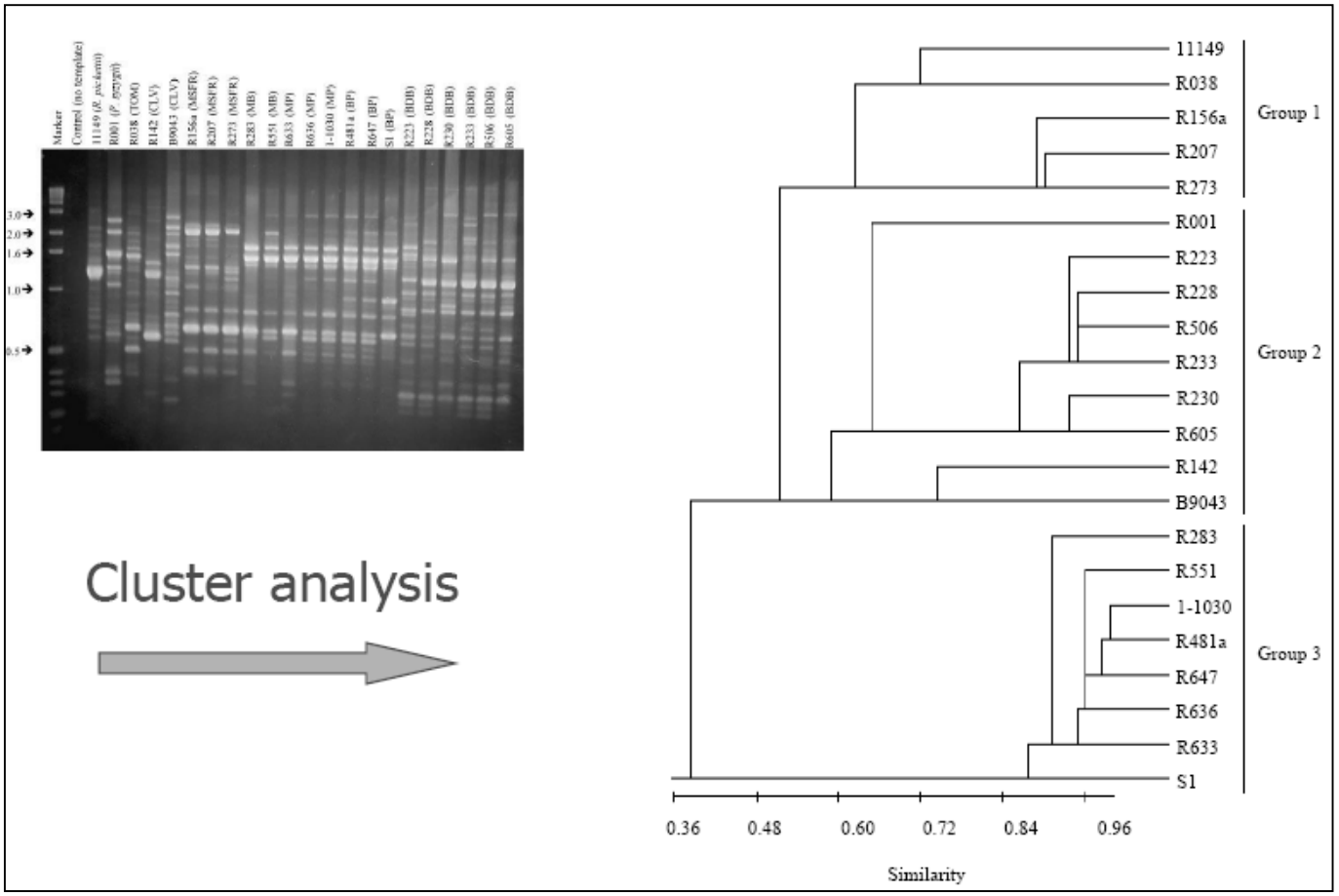
RAPD

P. syringae pv. *actinidiae*

- The conditions of DNA amplification were optimized and followed the procedure of Williams *et al*., 1990 with few modifications.
- The PCR program consisted of an initial denaturation of 5 min at 94°C, followed by 40 cycles at 94°C for 60 s (denaturation), 36°C for 60 s (annealing) and 72°C for 2 min (extension), with an additional extension period of 10 min at 72°C.
- A negative control, without genomic DNA, was run with every set of samples to confirm that no contaminating DNA was present in the reaction.
- Amplifications were carried out in a Mastercycler (Mastercycler Gradient, Eppendorf, Germany).
- The amplified PCR products were separated by electrophoresis on 1.5% agarose gels in 1×TAE buffer, visualized and imaged by gel documentation system (GelDoc 2000, Bio-Rad, USA) after staining with ethidium bromide.

Liu *et al*.,2012

RAPD
Data analysis



Richard Thwaites

RAPD
Drawbacks

- RAPD appears to be sensitive to colony age and standardization is difficult (Clerc *et al.*, 1998).
- Also, this method is unsuitable for the direct detection of pathogens *in situ* in plants or soil.
- RAPD's are going out of favour as they are difficult to reproduce.
- Instead AFLP's are gaining increasing ground.

Illustration of three PCR fingerprinting techniques

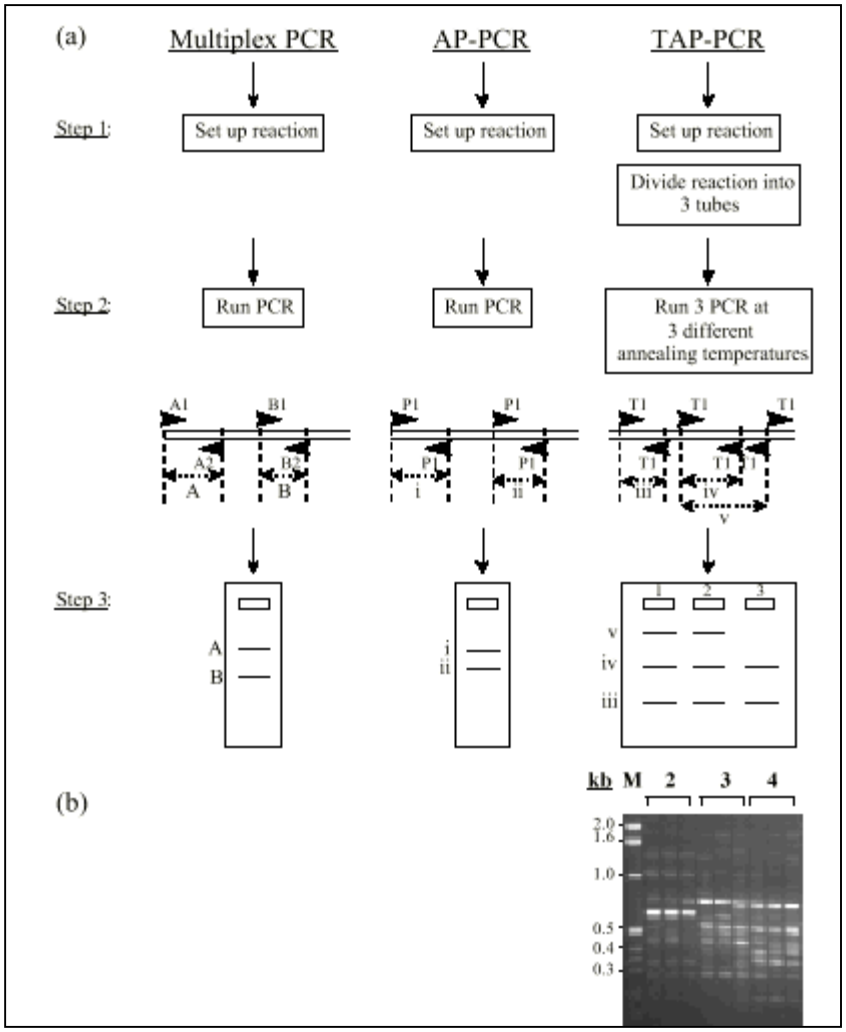
AP-PCR, TAP-PCR and Multiplex PCR

- **Arbitrary Primed (AP) PCR:**
- Differs from conventional PCR in that only a single short primer (usually 10-12 bases), whose sequence is arbitrarily chosen, is used.
- **Triplet Arbitrary Primed (TAP) PCR:**
- It maintains the significant advantages of AP-PCR, but increases the confidence limits of the fingerprinting result.
- **The multiplex PCR procedure:**
- Uses multiple pairs of primers in a single PCR mixture; the primer pairs being specific to different DNA sequences. By targeting multiple genes at once, a single PCR can provide the information that otherwise would require several times the amount of reagents and take longer to perform.

O'Sullivan,2000;...

Illustration of three PCR fingerprinting techniques

AP-PCR, TAP-PCR and Multiplex PCR



O'Sullivan,2000

Multiplex PCR

A variant of PCR enabling amplification of many targets

The simultaneous detection of two or more DNA

- The simultaneous detection of two or more DNA or/and RNA targets can be afforded by duplex or multiplex PCR in a single reaction with several specific primers included in the PCR cocktail.
- Multiplex PCR is very useful in plant pathology because different bacteria or viruses frequently infect a single crop or host.
- This methodology has demonstrated to be a valuable tool for detection and identification purposes (López *et al.*,2006).

López *et al.*,2011

Multiplex PCR

A variant of PCR enabling amplification of many targets

The simultaneous detection of two or more DNA

- Types of Multiplex PCR:

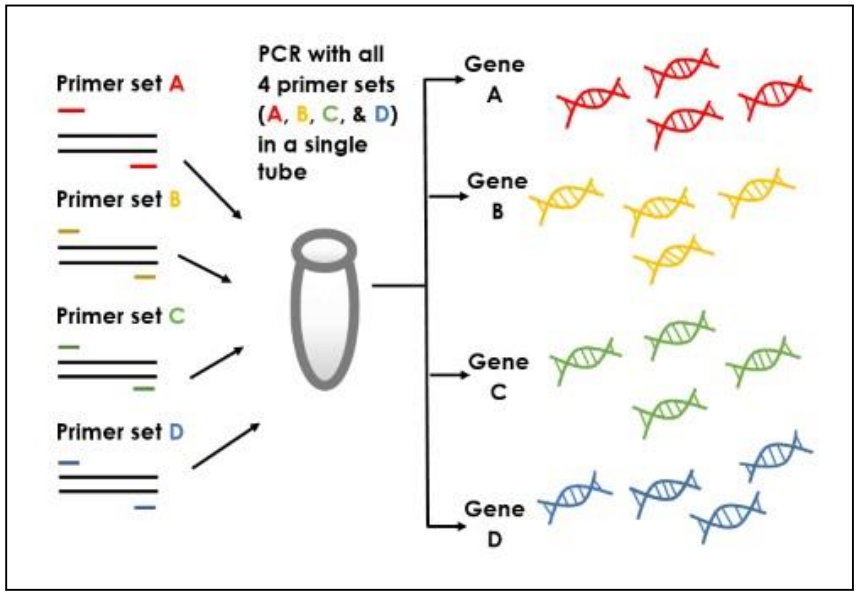
1. Single template PCR reaction; this technique uses a single template which can be a genomic DNA along with several pairs of forward and reverse primers to amplify specific regions within a template.
2. Multiple template PCR reaction; this technique uses multiple templates and several primer sets in the same reaction tube.

PCR fingerprinting techniques

Multiplex PCR

Set up of the mPCRs

- To check the specificity of the primers, single PCRs were performed, containing only the forward and the reverse primer for detection of target gene individually.
- Presence of multiple primer may lead to cross hybridization with each other and the possibility of mis-priming with other templates.



Polymerase chain reaction(PCR)

Variants of PCR

- Often only a small modification needs to be made to the standard PCR protocol to achieve a desired goal:

Technique	Abbreviation
Polymerase chain reaction	PCR
Reverse transcription polymerase chain reaction	RT-PCR
Real-time polymerase chain reaction	qPCR
RT-PCR/qPCR combined technique	qRT-PCR

Wikipedia,2015

Polymerase chain reaction(PCR)

Variants of PCR

- **Conventional PCR:**
- Amplify a specific target DNA sequence.
- Taq DNA Polymerase possesses a 5′→ 3′ polymerase activity.
- Set PCR machine for 40 to 50 cycles.
- Amplify targets (amplicon, product of a PCR from 100bp to 3kb long.
- Requires post-PCR procedures.
- PCR products resolved by observing DNA stained on agarose gel electrophoresis.

- **Nested PCR:**
- Used to increase the specificity of DNA amplification.
- Nested polymerase chain reaction involves two sets of primers, used in two successive runs of polymerase chain reaction, the second set intended to amplify a secondary target within the first run product.
- The products from the first PCR are then used as template in a second PCR, using one ('hemi-nesting') or two different primers whose binding sites are located (nested) within the first set, thus increasing specificity.

Richard Thwaites; Wikipedia,2015

Polymerase chain reaction(PCR)

Variants of PCR

- **Nested PCR:**
- In nested PCR you use outside primers for first round(s) of amplification and inside primers for second round of amplification.

- **Semi-nested PCR:**
- In semi-nested PCR you use outside primers for first round(s) and one inside primer and the other previously used outside primer for the second round of amplification.

Richard Thwaites; Wikipedia,2015

Polymerase chain reaction(PCR)

Variants of PCR

Nested PCR

- Nested PCR uses two sequential sets of primers.
- The first primer set (termed "inner" or internal primer pairs) binds to sequences outside the target DNA, as expected in standard PCR, but it may also bind to other areas of the template.
- The second primer set (termed "outer" or external primer pairs) was added to the product of the first reaction as the amplification target which in turn, binds to sequences in the target DNA that are within the portion amplified by the first set (that is, the primers are nested).
- The primary advantage of nested PCR is that if the first primers bind to and amplify an unwanted DNA sequence, it is very unlikely that the second set of primers will also bind within the unwanted region.

Polymerase chain reaction(PCR)

Variants of PCR

Nested PCR

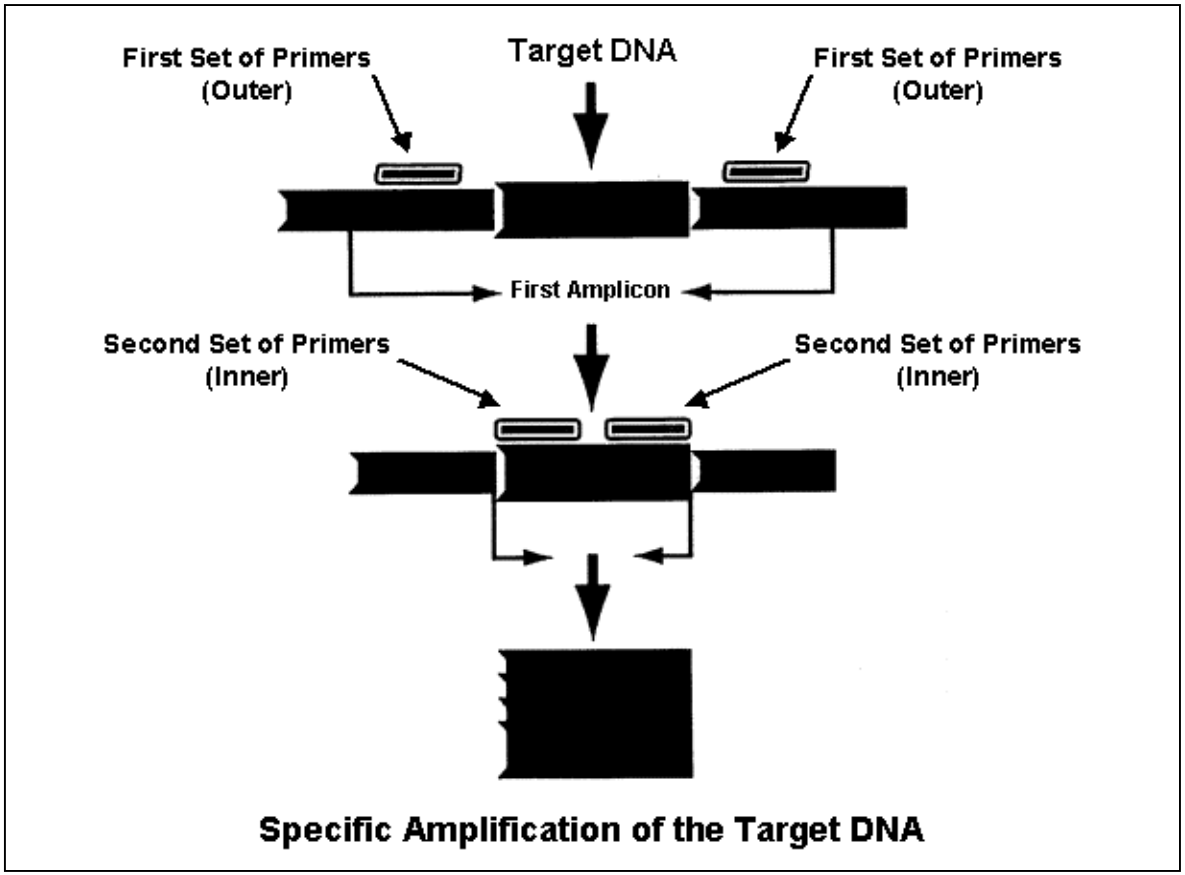
- The external primer pair should amplify a fragment large enough to permit the design of an appropriate internal couple.
- This first round of PCR was followed in the same thermocycler by a second denaturation step of 94°C for 4 min and 40 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s.
- The PCR products were visualized after electrophoresis on 1.5% agarose gels.
- Also, the nested PCR in a single closed tube was compared to standard one-round and two-tube nested procedures.
- The nested PCR in a single closed tube is its greater specificity and thus a higher reliability for diagnosis.

Llop *et al.*,2000

Polymerase chain reaction(PCR)

Variants of PCR

Nested PCR



The specificity and sensitivity were greater than those of standard PCR procedures that used a single primer pair.

Polymerase chain reaction(PCR)

Variants of PCR

Nested PCR vs. Semi-nested PCR

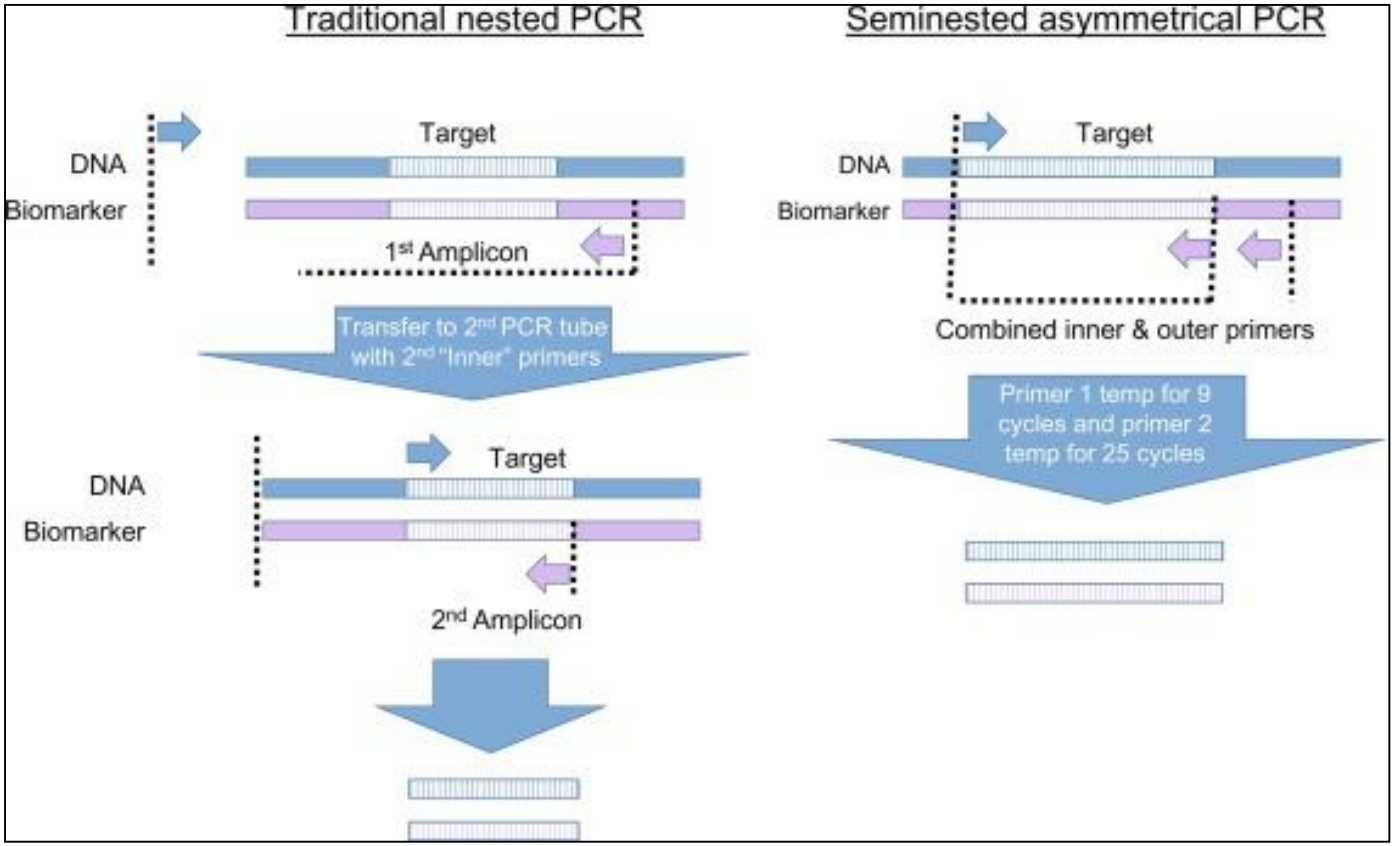
- **Nested PCR:**
- In nested PCR you use outside primers for first round(s) of amplification and inside primers for second round of amplification.

- **Semi-nested PCR:**
- In semi-nested PCR you use outside primers for first round(s) and one inside primer and the other previously used outside primer for the second round of amplification.

Polymerase chain reaction(PCR)

Variants of PCR

Nested PCR vs. Semi-nested PCR

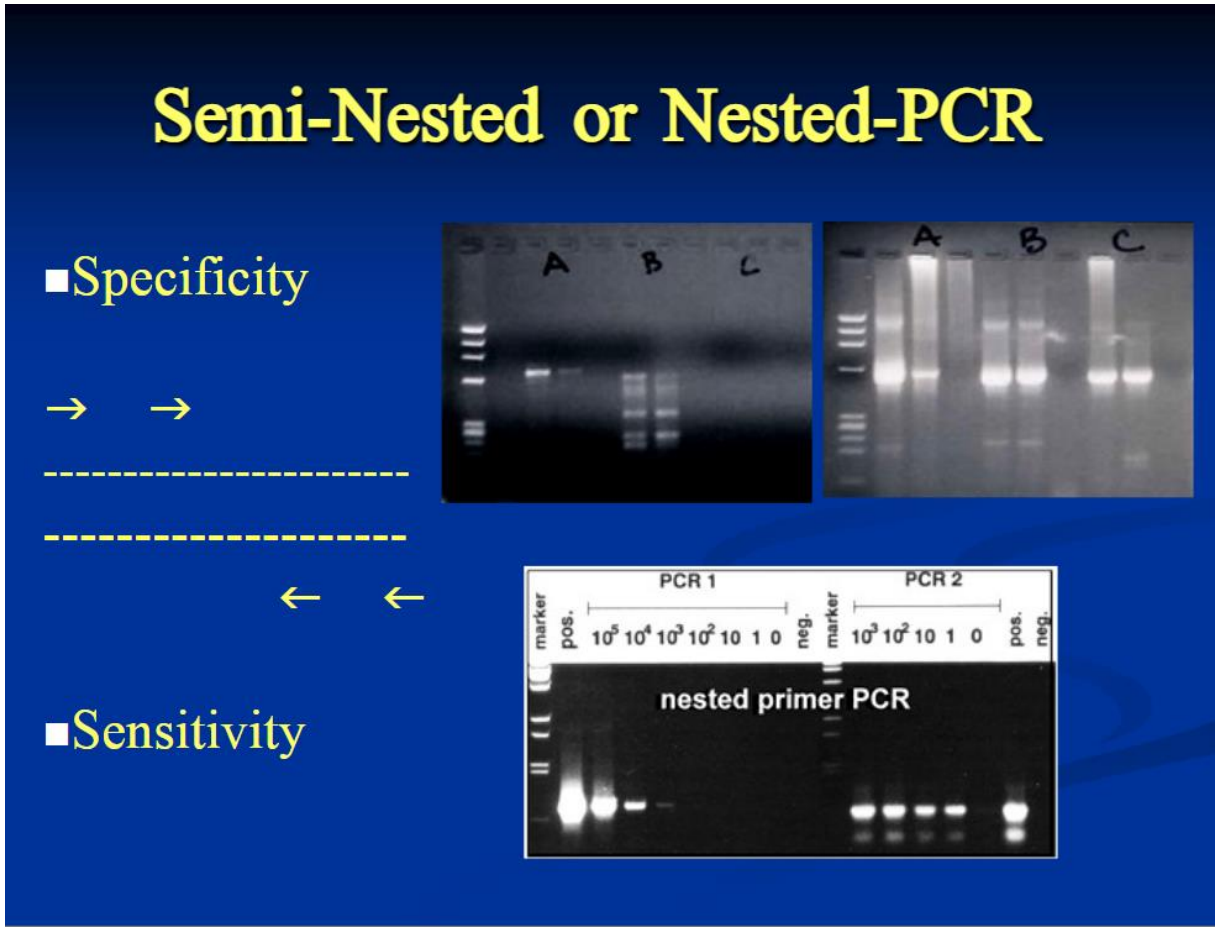


Shen,2019

Polymerase chain reaction(PCR)

Variants of PCR

Nested PCR vs. Semi-nested PCR



Wells,2009

Polymerase chain reaction(PCR)

Variants of PCR

PCR-SSCP technique

- After PCR amplification of the target sequence(16SrRNA), the amplified product is denatured into single stranded DNAs (ssDNA) and subjected to non-denaturing polyacrylamide gel electrophoresis.
- Under non-denaturing conditions, ssDNA has a secondary structure that could be determined by the nucleotide sequence.
- The mobility of the ssDNA depends on the secondary structure of the amplified product.
- The different positions of the bands of ssDNA on the gel indicate different sequences.
- PCR-SSCP is capable of detecting >90% of all single-base substitutions in 200 bp fragments.

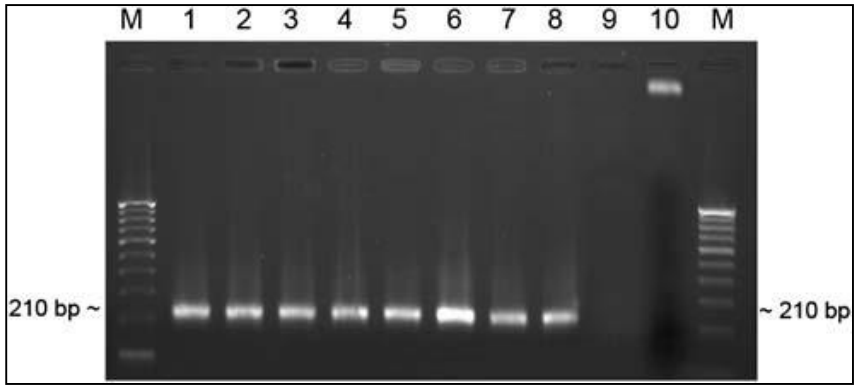
Srinivasa *et al.*,2012

Polymerase chain reaction(PCR)

Variants of PCR

PCR-SSCP technique

- 16S rRNA primers (FP 50-TGGTAGTCCACGCCTAAAC-30 and 50-CTGGAAAGTTCCGTGGATGT-30) were used to amplify the tested bacterial DNA in all the respective positive isolates to confirm the pathogens.
- The PCR product of 210 bp was obtained in all the isolates including the *E. coli* bacteria.



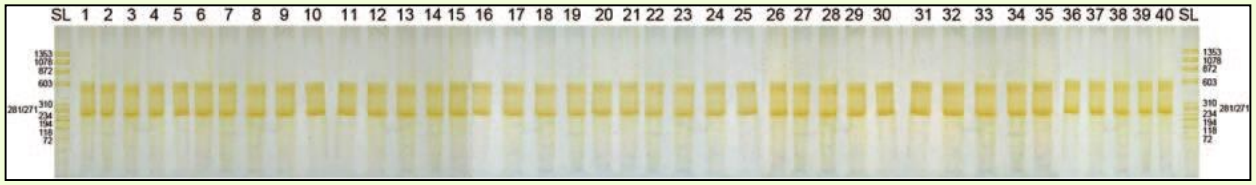
Srinivasa *et al.*,2012

Polymerase chain reaction(PCR)

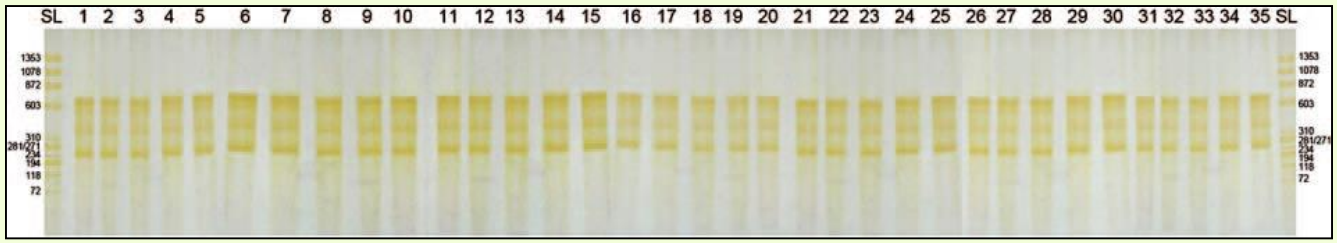
Variants of PCR

PCR-SSCP technique

- The motilities of the ssDNA of all tested bacteria had species-specific pattern:



PCR-SSCP banding pattern of *R. solanacearum* isolates.



PCR-SSCP banding pattern of *X. axonopodis* pv. vesicatoria.

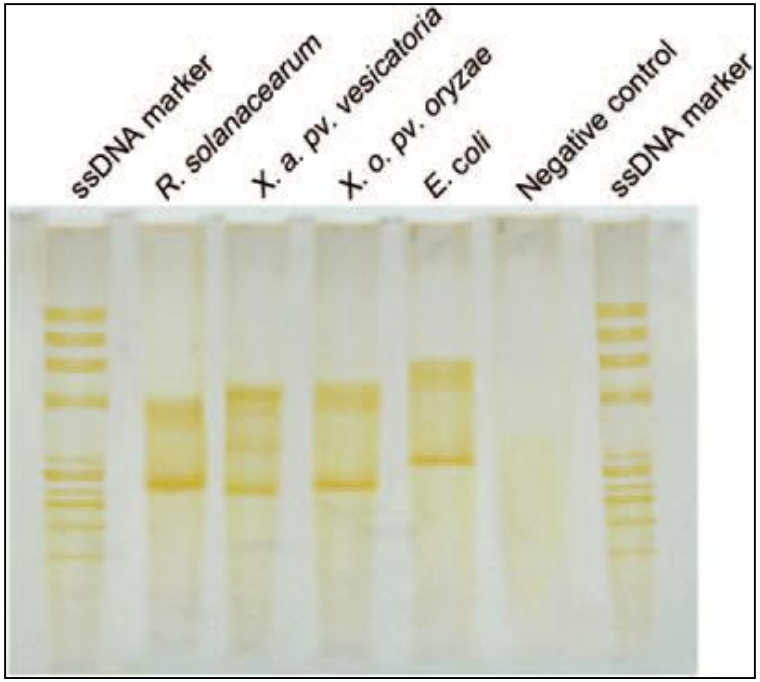
Srinivasa *et al.*,2012

Polymerase chain reaction(PCR)

Variants of PCR

PCR-SSCP technique

- PCR-SSCP banding pattern of different phytopathogenic bacteria(*Ralstonia solanacearum*, *Xanthomoans axonopodis* pv. *vesicatoria*, and *Xanthomonas oryzae* pv. *oryzae*) along with the non-relevant bacteria and negative control.



Srinivasa *et al.*,2012

Polymerase chain reaction(PCR)

Variants of PCR

Conventional multiplex and uniplex PCR

- **Conventional multiplex PCR:**
- Amplify several different DNA sequences (loci) simultaneously in the same reaction.
- This process amplifies DNA in samples using multiple primers.
- The primer design for all primers pairs has to be optimized so that all primer pairs can work at the same annealing temperature during PCR.
- PCR products were distinguishable by agarose gel electrophoresis.

- **Conventional uniplex PCR:**
- Uniplex PCR uses only one starting point (primer) for amplification and multiplex PCR uses multiple primers.
- PCR products were distinguishable by agarose gel electrophoresis.

Polymerase chain reaction(PCR)

Real-time PCR (multiplex and uniplex) and RT-PCR

The cDNA products were used as templates in semi-quantitative PCR or real-time PCR analysis

- **Real-time PCR(QPCR):**
- (Quantitative real time polymerase chain reaction).
- A method that allows to follow in real time the amplification of a target.
- The majority of real-time PCR assays are run for 40 cycles.
- Measures the products generated during each cycle of PCR process.
- Fluorescence is used as a measure of amplicon production.
- At cycle 27 maybe 4,000,000 amplicons.
- Rapid, no post-amplification processing steps.
- Quantification of mRNA using real-time RT-PCR.

- **RT-PCR(reverse transcription polymerase chain reaction):**
- RT-PCR is a PCR test that is designed to detect and measure RNA.
- RT-PCR differs from conventional PCR by first taking RNA and converting the RNA strand into a DNA strand.
- The enzyme (reverse transcriptase) was used to convert RNA to cDNA.
- The amplified cDNA was then analyzed by conventional PCR (requires post-amplification processing steps).
- For quantification of RT-PCR products, quantitative real-time PCR(QPCR) was used.

Polymerase chain reaction(PCR)

Variants of PCR

Conventional PCR vs. RT-PCR

- Reverse transcription PCR, or RT-PCR, allows the use of RNA as a template.
- An additional step allows the detection and amplification of RNA.
- The RNA is reverse transcribed into complementary DNA (cDNA), using reverse transcriptase.
- The sensitivity of RT-PCR targeting abundant transcripts could detect quantities as low as one bacterium, which was not possible using PCR.
- The quality and purity of the RNA template is essential for the success of RT-PCR.

Neidler,2017; Wang *et al.*,2016

Polymerase chain reaction(PCR)

Variants of PCR

Conventional PCR vs. RT-PCR

- RT-PCR(reverse transcription polymerase chain reaction) is used to detect and quantify messenger RNA (mRNA).
- RT-PCR is also used to make cDNA libraries, which can aid the study of gene expression and sequences.
- RT-PCR shows a higher sensitivity compared to conventional PCR.

Wang *et al.*,2016

Polymerase chain reaction(PCR)

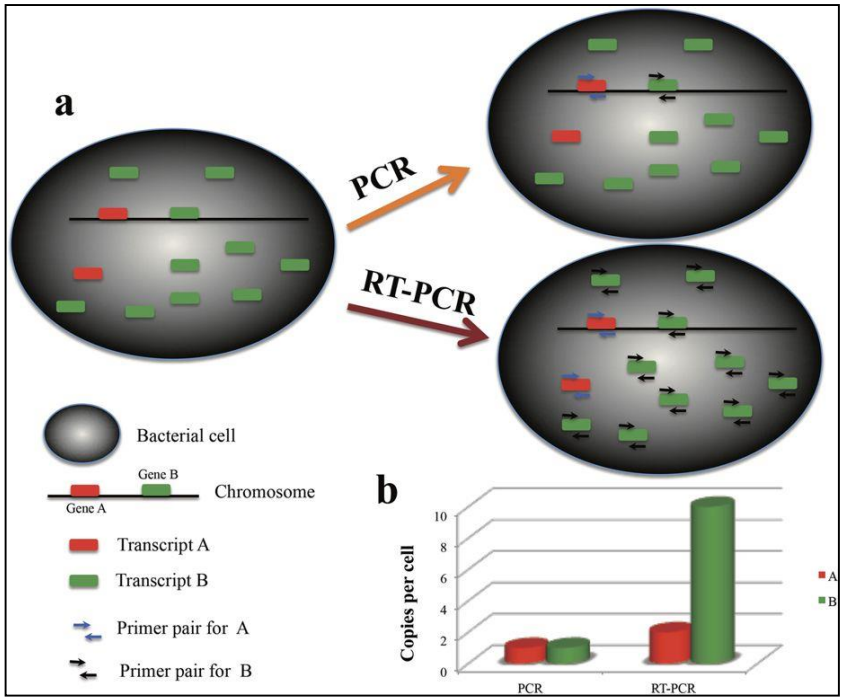
Variants of PCR

Conventional PCR vs. RT-PCR

a. In a bacterial cell, one copy of gene A and nine copies of gene B are transcribed. PCR amplifies only genomic DNA, while RT-PCR could amplify both genomic DNA plus the transcripts.

b. The detected copies of gene A and B by PCR and RT-PCR.

c. PCR would detect only one nucleic acid molecule, while RT-PCR would detect two and 10 nucleic acid molecules for A and B, respectively.



RT-PCR: reverse transcription polymerase chain reaction

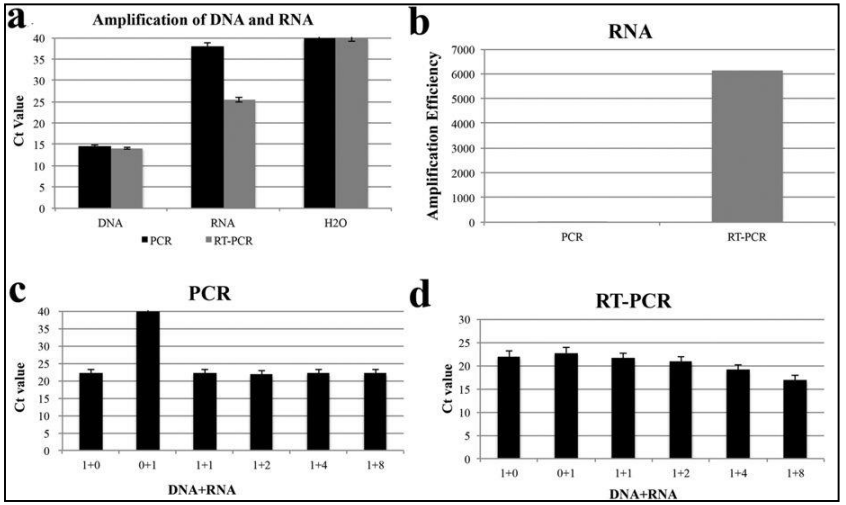
Wang *et al.*,2016

Polymerase chain reaction(PCR)

Variants of PCR

Conventional PCR vs. RT-PCR

- DNA and RNA were extracted from *Brucella* culture and detected by PCR and RT-PCR (a);
- Efficiencies of RNA amplification by PCR and RT-PCR were compared (b);
- DNA was mixed with increasing quantities of RNA and detected by
- PCR (c), and
- RT-PCR (d).



Wang *et al.*,2016

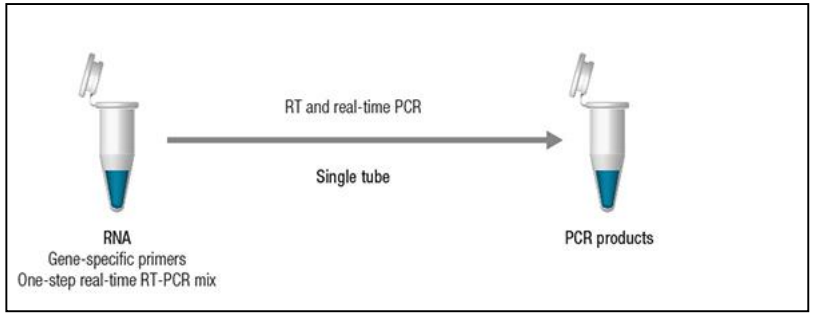
RT-PCR

Two-step reverse transcription polymerase chain reaction (RT-PCR) or real time PCR

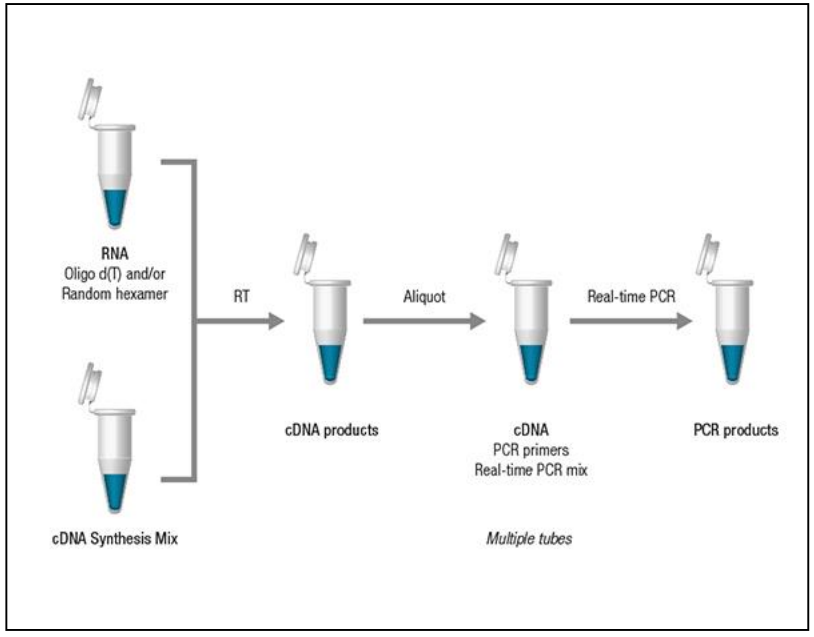
- Real-Time PCR has become an increasingly popular technique for analysis of gene expression.
- There are two primary methods of real-time PCR that can be performed.
- The first involves including the reverse transcriptase step in the same tube as the PCR reaction (one-step).
- The second method involves creating cDNA first by means of a separate reverse transcription reaction and then adding the cDNA to the PCR reaction (two-step).

RT-PCR

Two-step reverse transcription polymerase chain reaction (RT-PCR) or real time PCR



One-step RT-PCR



Two-step RT-PCR

One-step real-time RT-PCR is generally less sensitive than two-step RT-PCR.

Bioline

RT-PCR

One-step vs Two-step reverse transcription polymerase chain reaction (RT-PCR)

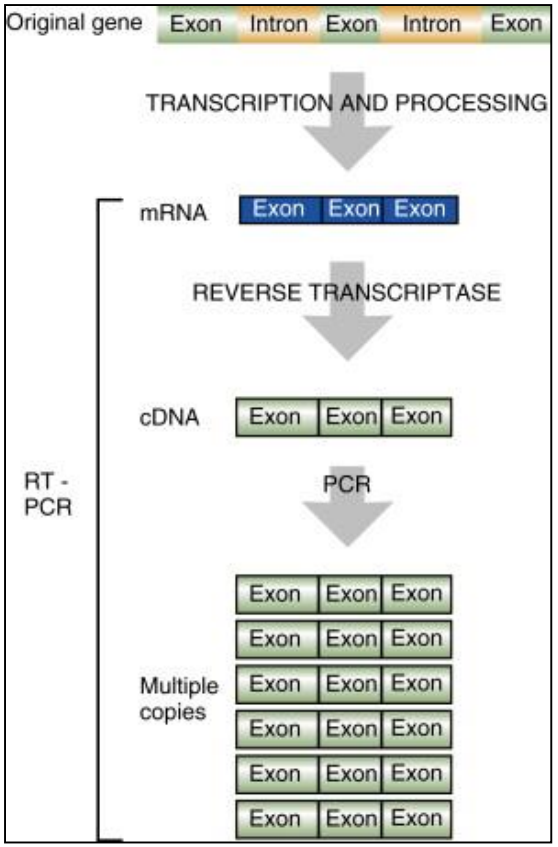
- Reverse transcription PCR (RT-PCR) uses mRNA rather than DNA as the starting template.
- In two-step reverse transcription polymerase chain reaction (RT-PCR):
- First step: RNA molecules were converted into their complementary DNA (cDNA) sequences by reverse transcriptase enzymes.
- Second step: followed by amplification of the cDNA by standard PCR.
- Second step in RT-PCR is simply amplification of newly synthesized cDNA by traditional or real-time PCR.

RT-PCR

Two-step reverse transcription polymerase chain reaction (RT-PCR)

- First step:
- A RNA strand (template) is reverse transcribed into its complementary DNA copy(cDNA) using reverse transcriptase, and
- Second step:

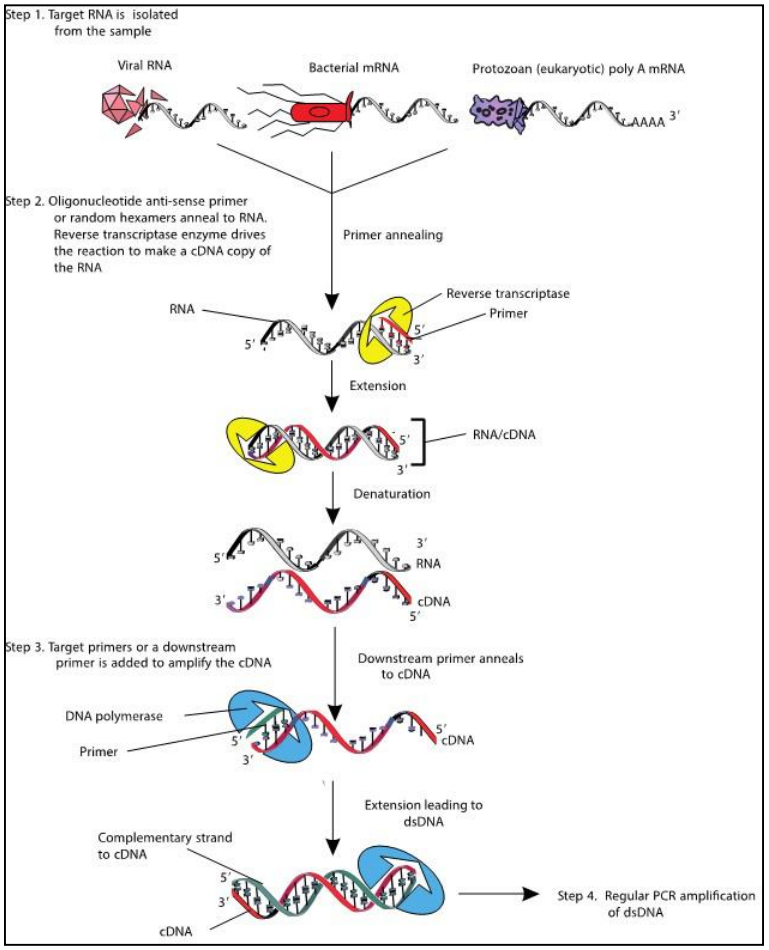
1. cDNA is amplified using traditional PCR.



RT-PCR

Step-by-step process of RT-PCR analysis of environmental RNA samples

- RT-PCR provides a means for using RNA to produce cDNA using specialized enzymes, known as reverse transcriptase (RT).
- This cDNA can then be used as the starting template for subsequent amplification with conventional PCR.



JoVE Science Education Database,2019

RT-PCR

One and two-Step RT-PCR Kit/protocols

CONTENTS

COMPONENTS OF THE KIT

The solutions included in the Two-Step RT-PCR Kit have been carefully prepared to yield the best possible results. Each reagent has been tested extensively and its concentration adjusted to meet USB® standards. It is strongly recommended that the reagents supplied in the kit be used as directed.

The following solutions are included in the kit:

M-MLV Reverse Transcriptase (25X): M-MLV Reverse Transcriptase in 50% glycerol storage buffer.

Taq DNA Polymerase (50X): Taq DNA polymerase (1.25 units/µl); 20mM Tris-HCl, pH 8.5, 0.1mM EDTA, 100mM potassium chloride, 1mM dithiothreitol (DTT), 50% glycerol and stabilizers.

RT Reaction Buffer (5X): Buffer including 7.5mM magnesium chloride (1.5mM final concentration in reaction) and optimized for two-step RT-PCR.

PCR Reaction Buffer (10X): 100mM Tris-HCl, pH 8.6, 500mM potassium chloride, 15mM magnesium chloride.

PCR Nucleotide Mix, Ultrapure: 10mM each of dATP, dCTP, dGTP, dTTP

Ribonuclease Inhibitor: Recombinant ribonuclease inhibitor (4 units/µl) in 50% glycerol storage buffer.

Magnesium Chloride (25mM)

Water, RNase-Free (DEPC-Treated)

This kit and all the enclosed reagents should be stored at -15°C to -30°C (NOT in a frost-free freezer). Keep all reagents on ice when removed from storage for use.

The USB® Two-Step RT-PCR Kit

RT-PCR

Step-by-step process of RT-PCR analysis of environmental RNA samples

- **RNA Extraction:**
- Extract RNA from target microorganism using a commercial extraction kit according to manufacturer's instructions.
- Briefly, first mix the samples with lysis buffer supplemented with ethanol, then add the samples into spin columns.
- Centrifuge the columns at approximately 12,000 x g, discard flowthrough.
- Add wash buffer to the columns and centrifuge again.
- Add RNase-free water to the columns and centrifuge for 30s to elute RNA into sterile 1.5-mL low-adhesion microfuge tubes.

RT-PCR

Step-by-step process of RT-PCR analysis of environmental RNA samples

- **Reverse Transcription – PCR (continued):**
- Retrieve the following reagents from the -20°C freezer: dNTP, concentrated (e.g., 10x) reverse transcription buffer, primers (in this example, random primers). Thaw these on ice or at room temperature inside a clean hood, and keep them on ice once thawed. Also retrieve the reverse transcriptase and RNase inhibitor and keep them on ice.
- Calculate the reagent volumes needed to make a "master mix" that combines all the reagents constant among every reaction (see Table 1 for a sample reaction). Prepare enough master mix for every sample, as well as a positive control (using a known transcript template) and negative control (e.g., without reverse transcriptase, with only water as template, etc.) reactions. Include an extra 10% in volume.
- Assemble the master mix in 1.5-mL low-adhesion microfuge tubes. This minimizes the binding of reagent molecules to the tubes' plastic walls.
- When reagents are thawed, add calculated volumes to the microfuge tube. Gently vortex and centrifuge each tube before addition. Make sure to change pipette tips between adding each reagent to prevent contamination.

RT-PCR

Step-by-step process of RT-PCR analysis of environmental RNA samples

- **Reverse Transcription – PCR (continued):**
- After all reagents have been added, vortex and centrifuge the master mix to ensure a homogeneous mixture. Put reagents back into storage at -20°C.
- Prepare and label 8-tube PCR strips, designating one tube for each sample or control reaction.
- Aliquot an equal volume of master mix into each tube. Then, add reaction-specific components, such as the RNA extracts.
- Place the strip cap securely onto the PCR strip, and centrifuge in a minicentrifuge with a strip tube adaptor to ensure all liquid is collected at the bottom of each tube (Figure 1).
- Place PCR strip securely in thermocycler. Press down to ensure tubes are secured.
- Set the thermocycler to run the program appropriate for the RT being used (see Table 2 for a sample protocol). When the program is complete, the tubes will contain cDNA products, which can then be subjected to PCR amplification. Store cDNA at -20°C until use.

RT-PCR

Step-by-step process of RT-PCR analysis of environmental RNA samples

- **Reverse Transcription – PCR (continued):**

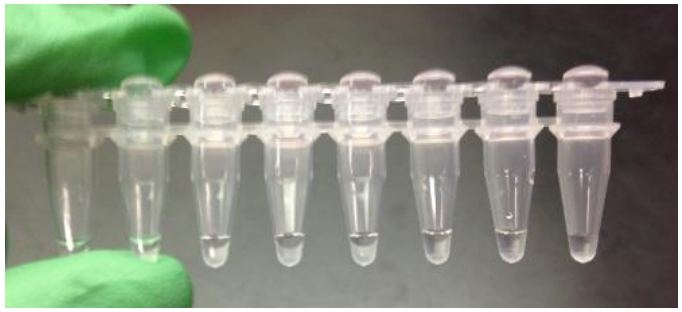


Figure 1. Capped 8-tube strip containing master mix and extract.

Reagent	Volume per 1 reaction (µL)
10x RT Buffer	2
25x dNTPs	0.8
10x Random Primer	2
Multiscribe	1
Rnase Inhibitor	1
Molecular Grade H_2O	3.2
Total Volume	10

Table 1. RT Master Mix Ingredients.

Step 1	Step 2	Step 3	Step 4
25°C , 10 min	37°C , 120 min	85°C , 5 min	4°C , ∞

Table 2. RT Reaction Thermocycler Program.

RT-PCR

Step-by-step process of RT-PCR analysis of environmental RNA samples

- When RT- PCR is complete, some of the PCR product can be separated and visualized on an agarose gel (Figure 3). In this example, a gene-specific primer was used to detect for the presence of an RNA virus. Bands of the expected size are obtained from two of the samples and the positive control reaction, but not from the negative control, indicating the presence of this virus in two of the water samples being tested.

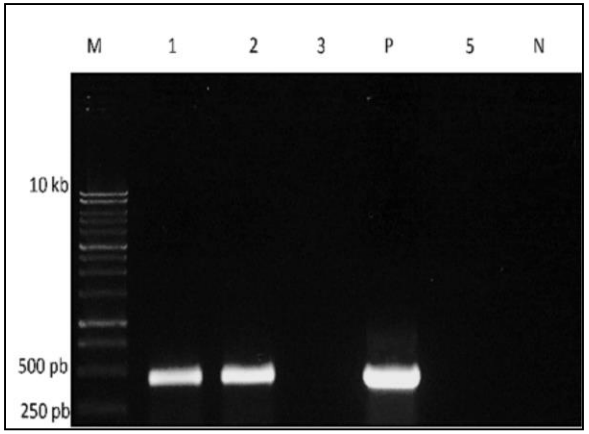
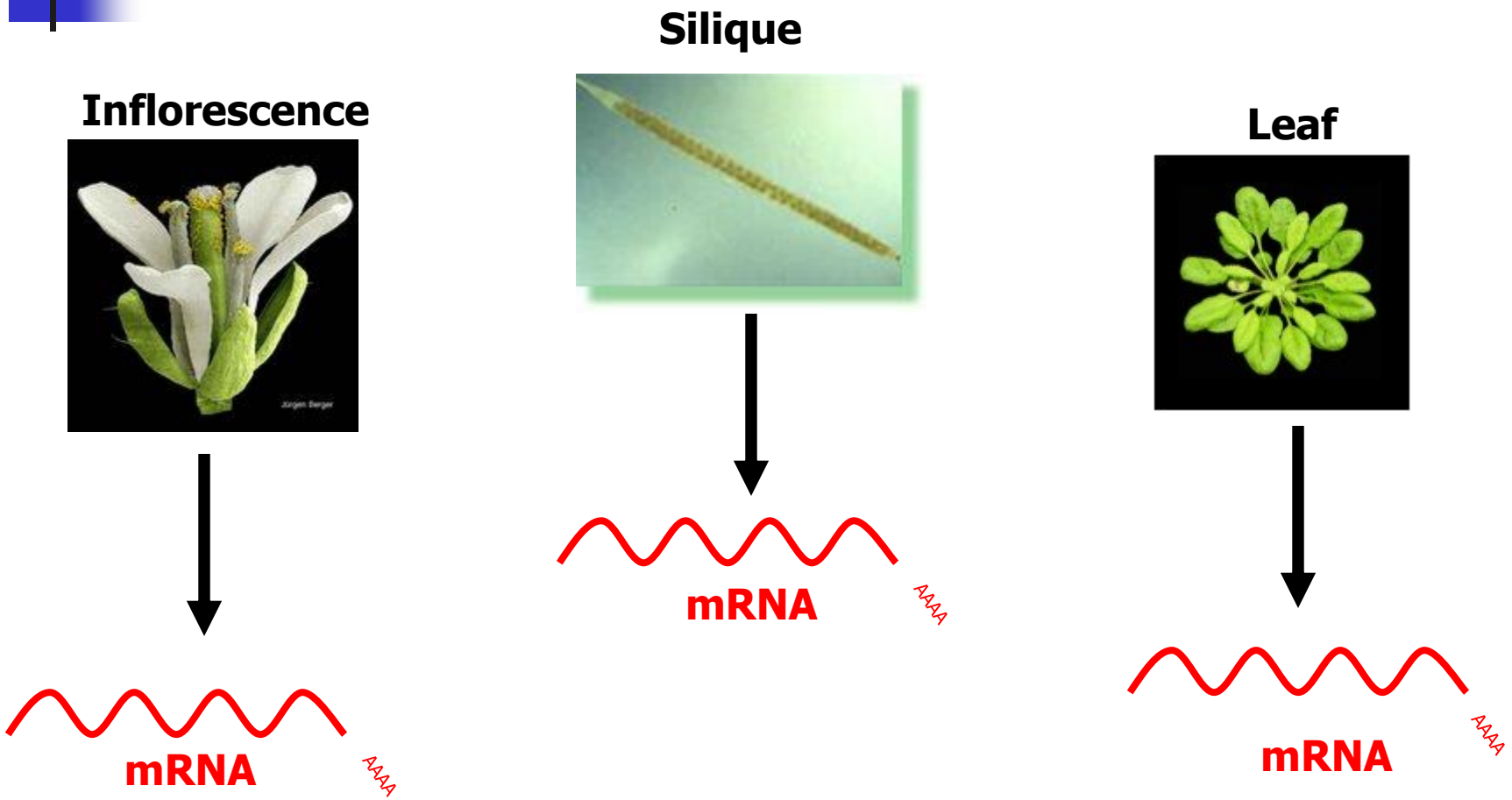


Figure 3. Gel electrophoresis of RT-PCR products. M: DNA size marker; P: positive control; N: negative control. Reactions using RNA from four water samples were run in lanes 1, 2, 3, and 5.

RT-PCR protocol in brief

Step 1

Isolation of RNA (e.g. mRNA)from different sources and conversion of RNA to cDNA by reverse transcription

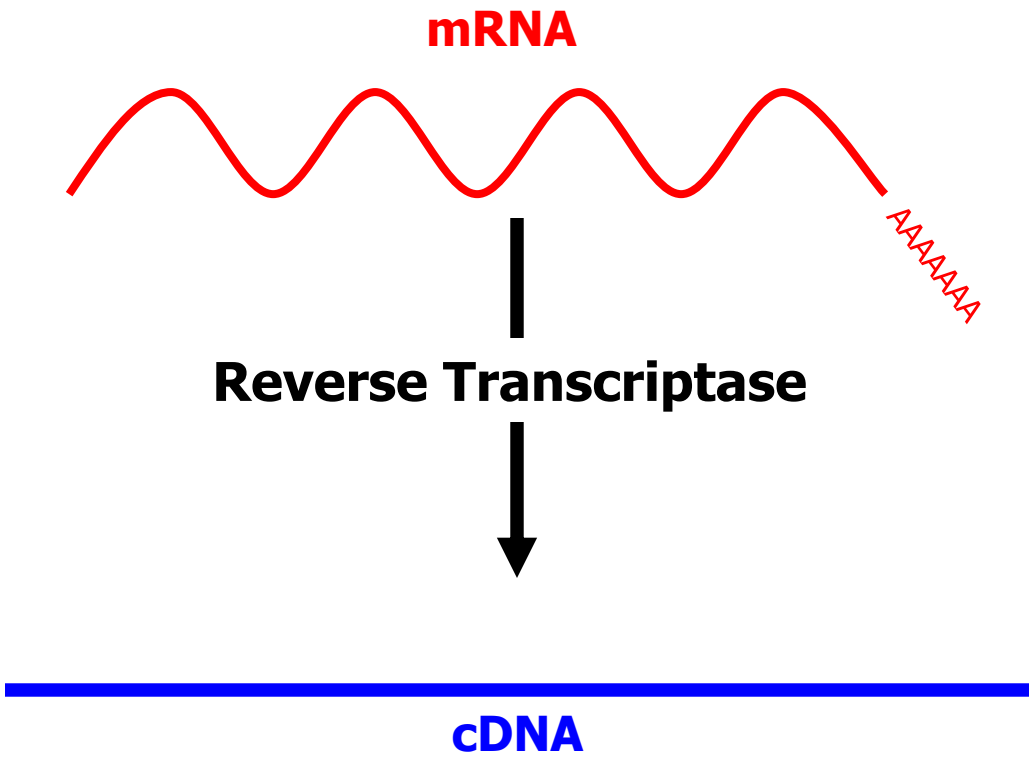


Jordan, Jennifer, and Brian

RT-PCR protocol in brief

Step 2

Synthesize cDNA using the enzyme reverse transcriptase (RT)

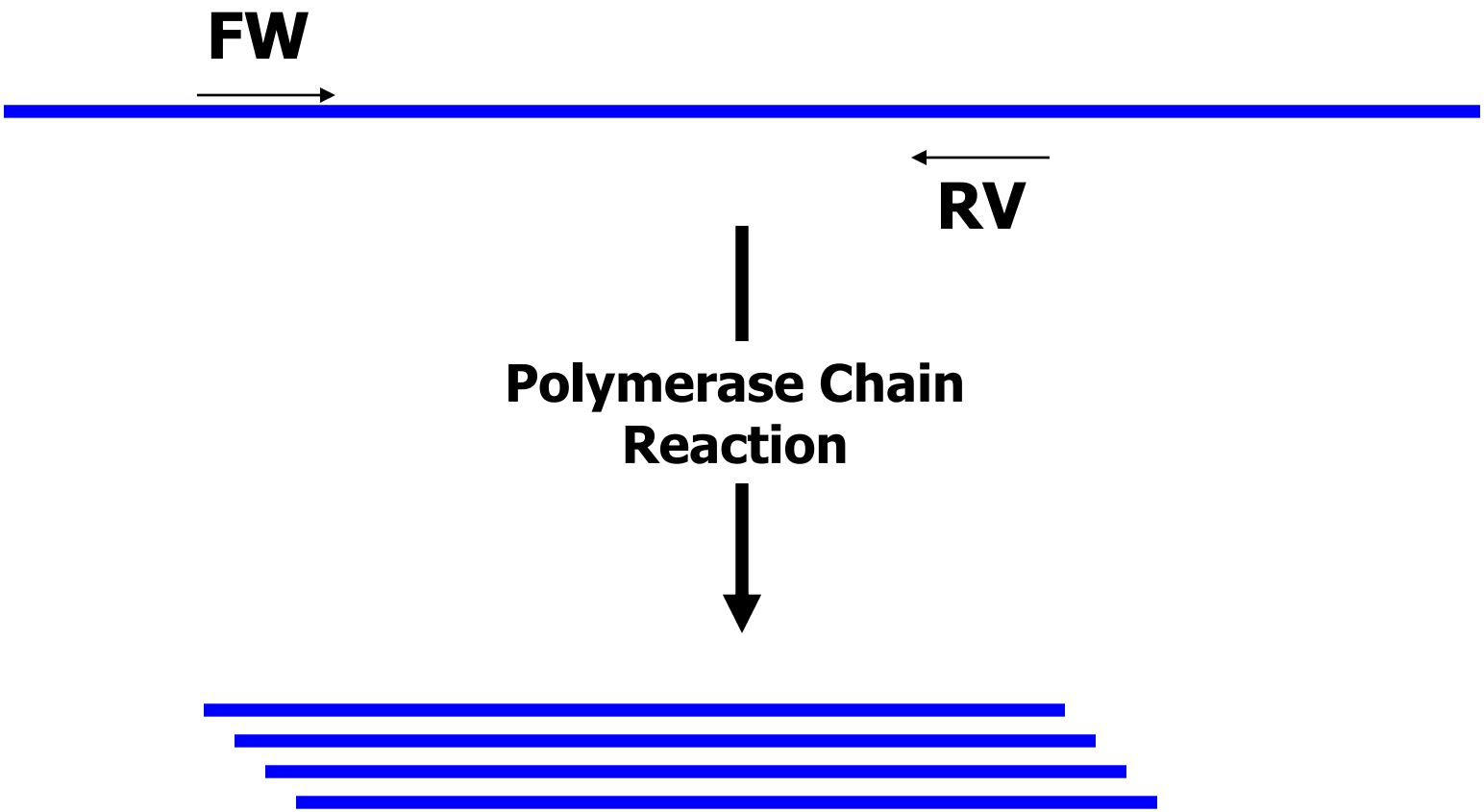


Jordan, Jennifer, and Brian

RT-PCR protocol in brief

Step 3

Amplify cDNA by traditional PCR using primers



Jordan, Jennifer, and Brian

RT-PCR protocol in brief

Step 4

RT-PCR product was analyzed either by standard PCR or real-time PCR reaction

- Once mRNA is extracted and converted to cDNA, the amplified product can be further analyzed either by:

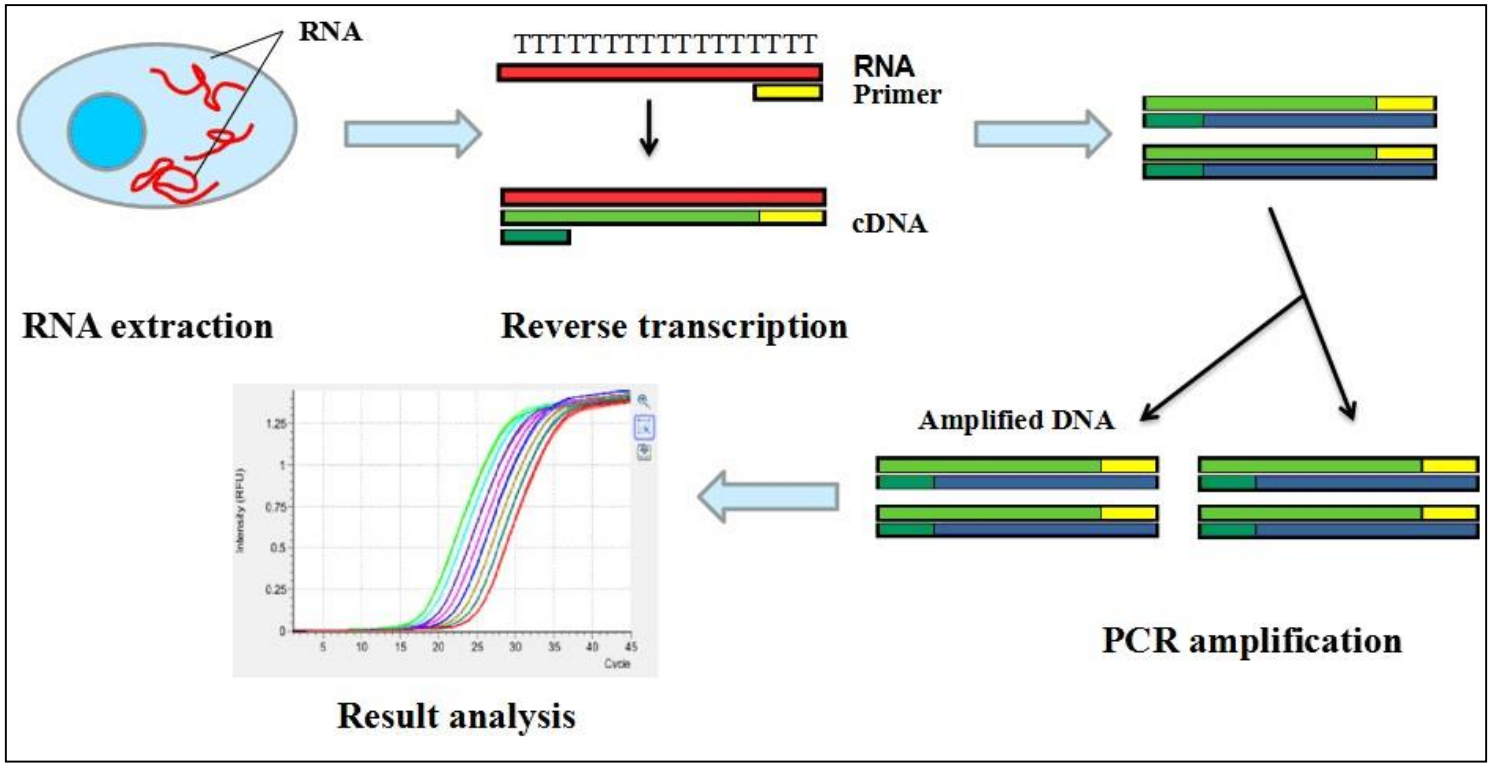
1. standard PCR, or
2. real-time PCR reaction.

- Real-time PCR reaction is performed with either SYBR green or TaqMan fluorescence to quantify RT-PCR products.
- The combined technique (RT-PCR+Real-time PCR), described as quantitative RT-PCR or real-time RT-PCR (sometimes even quantitative real-time RT-PCR), is often abbreviated as qRT-PCR, RT-qPCR,or RRT-PCR.

RT-PCR protocol in brief

Step 4

Visualize with quantitative RT-PCR(qRT-PCR)



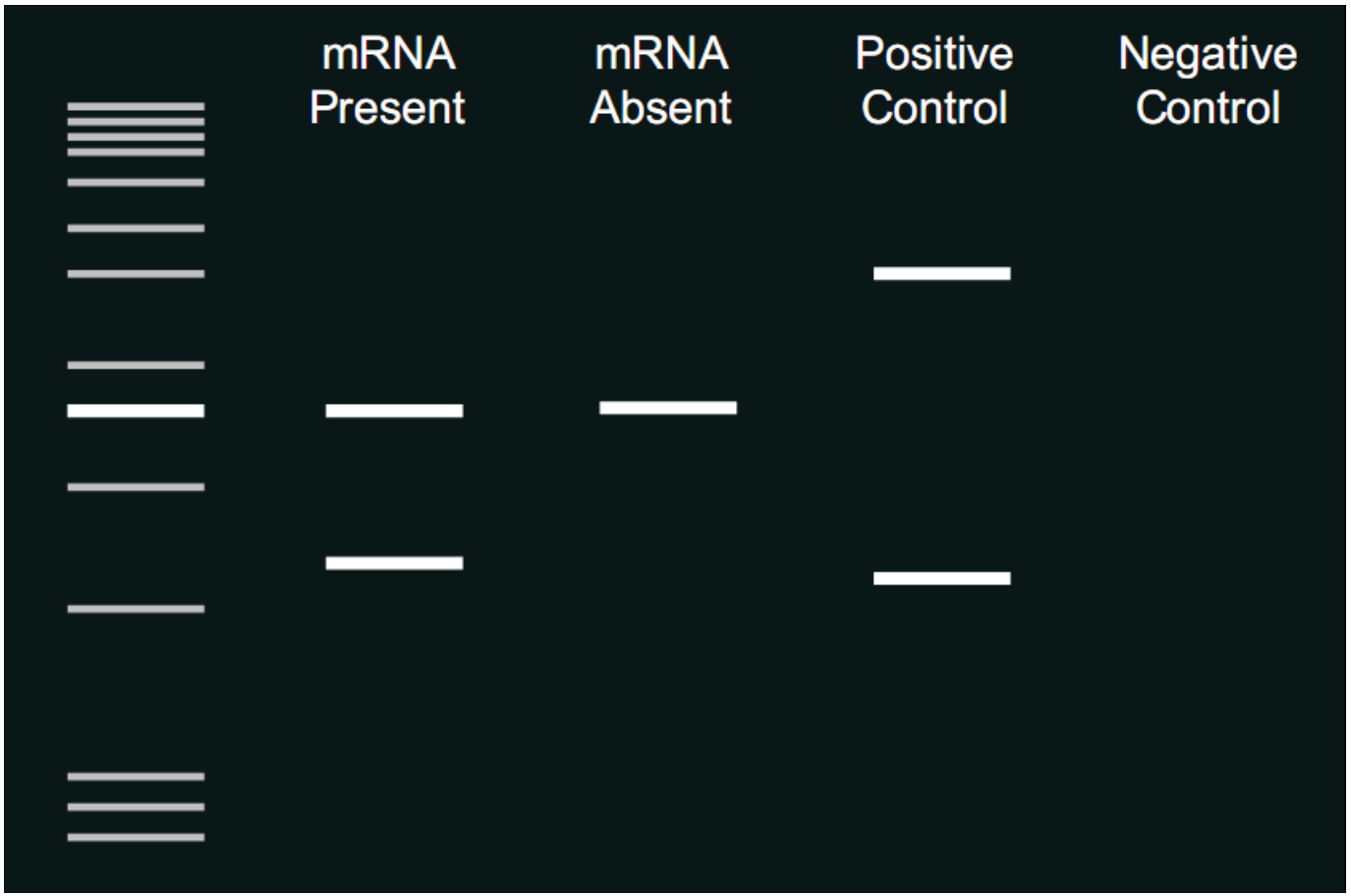
RT-PCR can be used to quantify mRNA levels from much smaller samples. In fact, this technique is sensitive enough to enable quantitation of RNA from a single cell.

Note: Taq polymerase does not work on RNA samples, so standard PCR cannot be used to directly amplify RNA molecules.

RT-PCR protocol in brief

Step 4

Visualize with gel electrophoresis



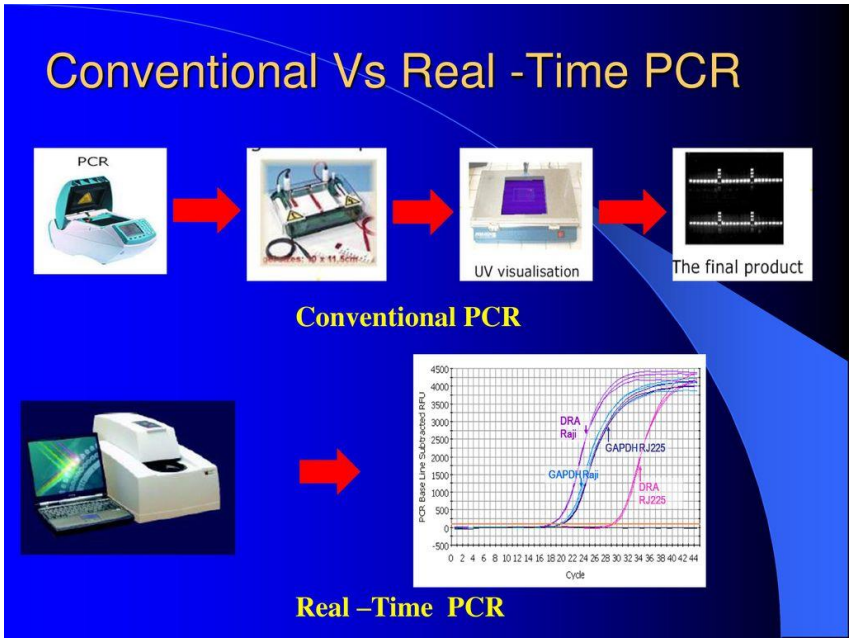
Jordan, Jennifer, and Brian

Polymerase chain reaction(PCR)

Variants of PCR

Conventional PCR vs. Real-time PCR

- Conventional PCR: The amplified product is detected by an end-point analysis i.e. by running DNA on an agarose gel after the reaction has finished.
- Real-time PCR: allows the accumulation of amplified product to be detected and measured as the reaction progresses, that is, in "real time".



Real-time PCR can be used quantitatively (quantitative real-time PCR), and semi-quantitatively, i.e. above/below a certain amount of DNA molecules (semi quantitative real-time PCR).

Polymerase chain reaction(PCR)

Variants of PCR

Multiplex and uniplex real-time PCR

- **Multiplex real-time PCR:**
- Based on the TaqMan technology.
- Multiplex real-time PCR can detect no more than 4 to 5 targets depending on available instruments.

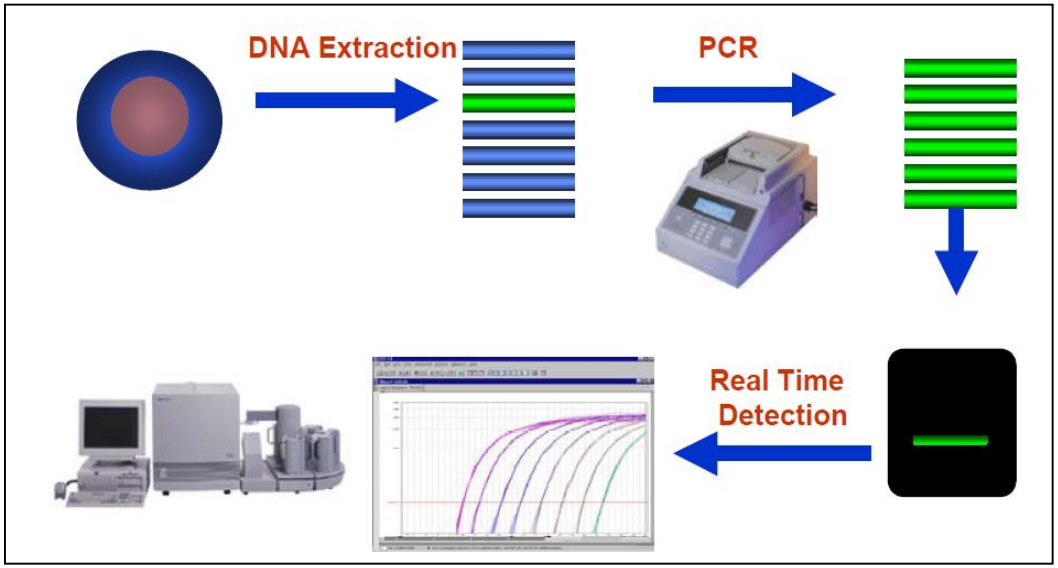
- **Uniplex real-time PCR:**
- Uniplex real-time PCR can determine the presence of only 1 target.
- Fluorescence was recorded at the annealing steps during the 2nd 40 cycles on an Mx3000P detection system.

Real-time PCR

Real-time reverse-transcription PCR

The evolution of PCR to Real-Time

- A major development of PCR technology. The gold standard for detection and quantification of DNA/RNA. Requirement of 1000-fold less RNA than conventional assays(3 picogram= one genome equivalent). No post-PCR processing of products (high throughput, low contamination risk).

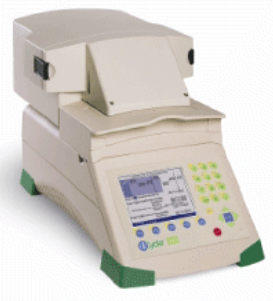


Applied biosystems;..

Real-time PCR
The advantages

- By now it is clear that the preceding steps are problematic and give inaccurate readings on RNA concentrations.
- In contrast, the real-time PCR method is precise and repeatable.
- Traditional PCR methods use agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction.
- Whereas Real-Time chemistries allow for the detection of PCR amplification during the early phases of the reaction.
- Real-Time PCR makes quantitation of DNA and RNA easier and more precise than past methods.

Real-time PCR
The advantages


The real-time machine

- Sensitive, specific and high dynamic range;
- Simple, rapid and reproducible;
- Real time detection and quantification of pathogen load;
- Useful for qualitative end-point data;
- Closed system no need for post amplification processes;
- Low risk of contamination;
- Automation (96 well format) high-throughput analysis;
- Reliable instrumentation and appropriate chemistries;
- Pathogen detection assays, SNP genotyping,...

SNP genotyping is the measurement of genetic variations of single nucleotide polymorphisms (SNPs) between members of a species. It is a form of genotyping, which is the measurement of more general genetic variation.

Elke,2010

Real-time PCR

The advantages and major disadvantage

1. High-throughput screening of plant samples is facilitated by shorter PCR run times and also by the fact that no post-PCR processing steps are required.
2. As different reporter dyes exist, which fluoresce at different wavelengths.
3. It is also possible to include more than one reaction in one tube.

- Perhaps the major disadvantage of real-time PCR is the initial cost of the equipment.

Weller *et al.*,2006

Real-time PCR
Quantitative PCR(qPCR)

- Real-Time PCR allows us to measure minute amounts of DNA sequences in a sample!
- In order to monitor level of expression of the target gene, real-time PCR is used.
- It is also referred to as quantitative PCR(qPCR). Good for quantitation of gene expression and pathogen detection.
- This involves the use of fluorescent primers.
- The PCR machine monitors the incorporation of the primers and display an amplification plot which can be viewed continuously thru the PCR cycle.
- Real time PCR yields immediate results.

Real-time PCR

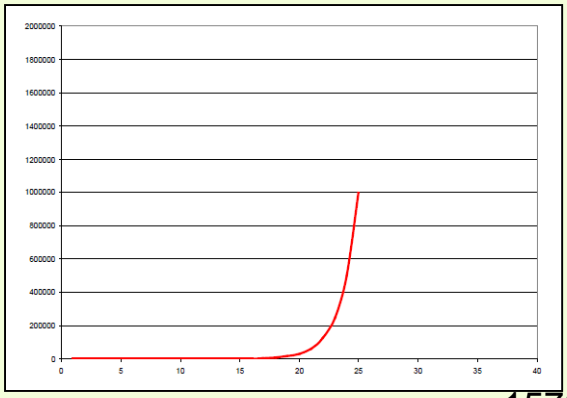
How does Real-Time PCR work?

Tubes with cycle No. 22-25

- **What about cycle 22?**
- Not a whole lot different. 125,000 copies of the amplicon.
- **And the cycle before that, 23?**
- Almost the same, but only 250,000 copies of the amplicon.
- **What was it like last cycle, 24?**
- Almost exactly the same, except there were only 500,000 copies of the amplicon.

- **What's is in our tube, at cycle 25?**
- A soup of nucleotides, primers, template, amplicons, enzyme, etc.
- 1,000,000 copies of the amplicon right now.

- **If we were to graph the amount of DNA in our tube, from the start until right now, at cycle 25, the graph would look like this:**

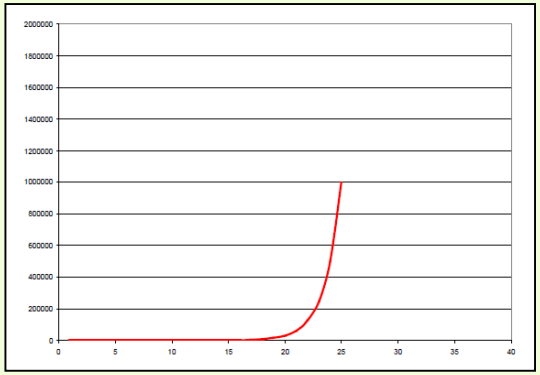


Real-time PCR

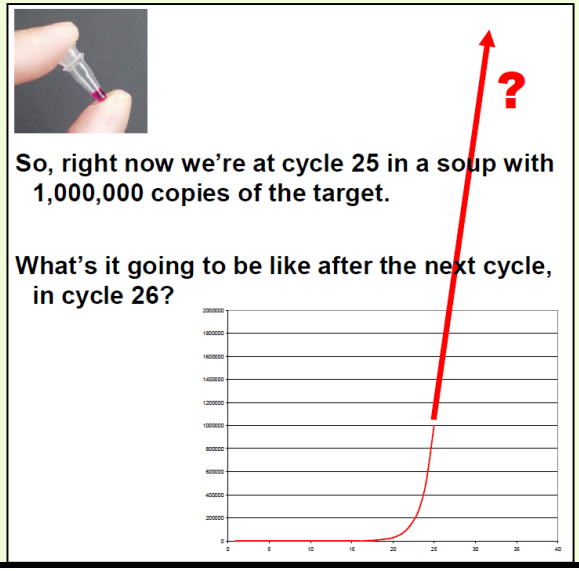
How does Real-Time PCR work?

Tubes with cycle No. 25-26

- So, right now we're at cycle 25 in a soup with 1,000,000 copies of the target.



- What's it going to be like after the next cycle, in cycle 26?



David Palmer

Real-time PCR

How does Real-Time PCR work?

Tubes with cycle No. 25-200

- So, right now we're at cycle 25 in a soup with 1,000,000 copies of the target.
- What's it going to be like after the next cycle, in cycle 26?
- Probably there will be 2,000,000 amplicons.
- And cycle 27?
- Maybe 4,000,000 amplicons.
- And at cycle 200?
- In theory, there would be 1,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000 amplicons… Or 10^35 tonnes of DNA…
- To put this in perspective, that would be equivalent to the weight of ten billion planets the size of Earth!!!!

David Palmer

Real-time PCR

How does Real-Time PCR work?

Tubes with cycle No. 25-200

- So, right now we're at cycle 25 in a soup with 1,000,000 copies of the target.
- What's it going to be like after the next cycle, in cycle 26?
- Probably there will be 2,000,000 amplicons.
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- To put this in perspective, that would be equivalent to the weight of ten billion planets the size of Earth!!!!

David Palmer

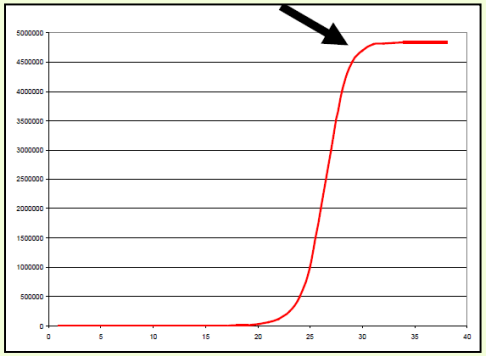
Real-time PCR

How does Real-Time PCR work?

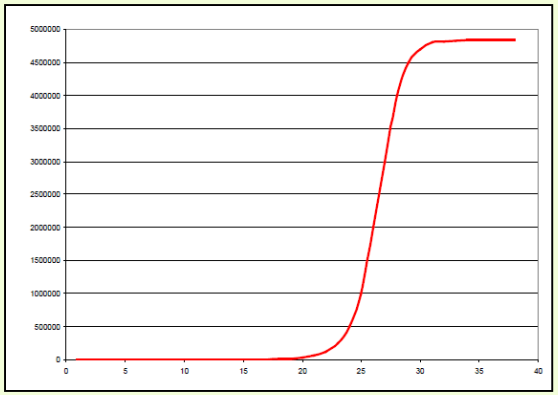
Tubes with cycle No. 22-25

- A clump of DNA the size of ten billion planets won't quite fit in our PCR tube anymore.
- Realistically, at the chain reaction progresses, it gets exponentially harder to find primers, and nucleotides. And the polymerase is wearing out.
- So exponential growth does not go on forever!

- If we plot the amount of DNA in our tube going forward from cycle 25, we see that it actually looks like this:



- How can all this be used to measure DNA quantities??

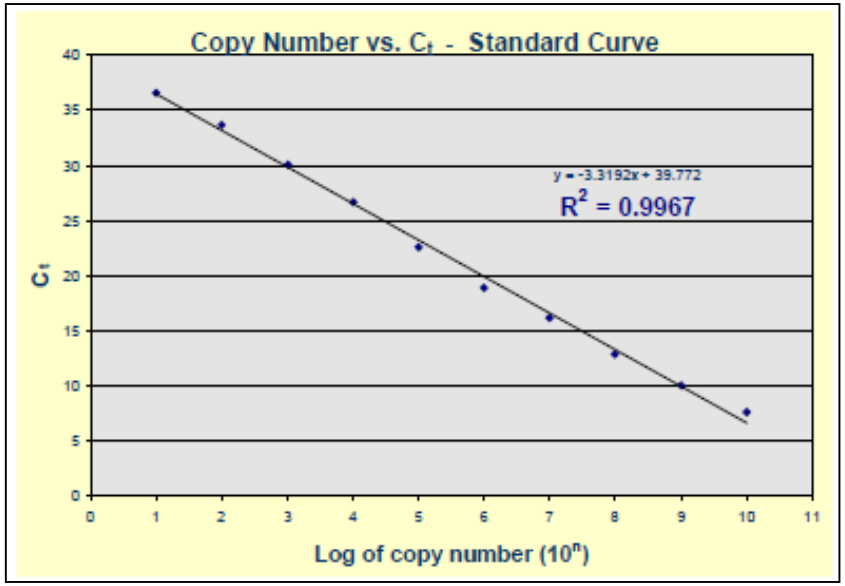


Real Time PCR

Measuring Quantities

How sensitive is Real-Time PCR?

- Ultimately, even a single copy can be measured! In reality, typically about 100 copies is around the minimum amount.
- One hundred copies of a 200-bp gene is equivalent to just twenty attograms (2 x 10^{-17} g) of DNA!



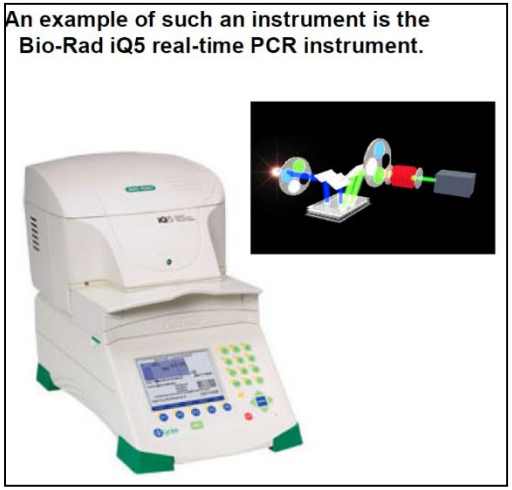
Attogram: An SI(International System of Units) unit of mass equal to 10^{-18} grams.

Real-time PCR

What type of instruments are used with Real-time PCR?

- Real-time PCR instruments consist of three main components:

1. Thermal Cycler (PCR machine);
2. Optical Module (to detect fluorescence in the tubes during the run);
3. Computer (to translate the fluorescence data into meaningful results).



David Palmer

Real-time PCR

Reagents used to fluoresce in the presence of amplified DNA

SYBR green and ethidium bromide dyes

- The most commonly used non-specific DNA binding dye is a DNA intercalating agent, SYBR green.
- Although ethidium bromide was the first dye to be used as a DNA-binding fluorophore (Higuchi *et al.*, 1992).
- SYBR green is the preferred dye because its binding affinity to double-stranded DNA (dsDNA) is more than 100 times higher than that of ethidium bromide and it does not interfere with the amplification process since it binds to the minor groove of the dsDNA.

Real-time PCR

Reagents used to fluoresce in the presence of amplified DNA

SYBR green and ethidium bromide dyes

- SYBR Green I dye and ethidium bromide are two reagents used to monitor DNA synthesis.
- They bind to double-stranded DNA and emit light when illuminated with a specific wavelength.

1. SYBR Green I dye is the most widely used double-strand DNA-specific dye reported for real time PCR. It fluoresces much more brightly than ethidium.
2. Ethidium bromide can also be used for detection but its carcinogenic nature renders its use restrictive.

Real-time PCR

Reagents used to fluoresce in the presence of amplified DNA

Phenomenon of fluorescence

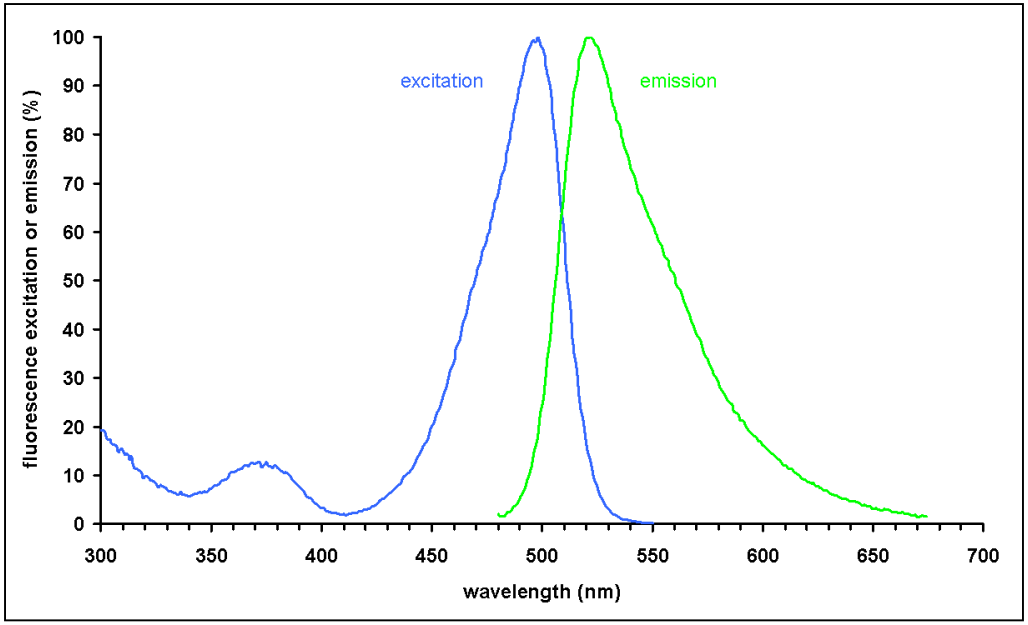
- During amplification, a double stranded amplicon accumulates, which results in a net increase in the amount of dye binding and fluorescence.
- When DNA is denatured, SYBR Green I dye is released and the fluorescence signal is reduced.
- Difference between peak absorption and peak emission wavelength/color:
- A dye absorbs photons of light at one wavelength and re-emit them at another wavelength.
- Lower energy, toward red colors;
- Higher energy, toward blue colors.

Real-time PCR

Reagents used to fluoresce in the presence of amplified DNA

SYBR Green (double-stranded DNA binding dye)

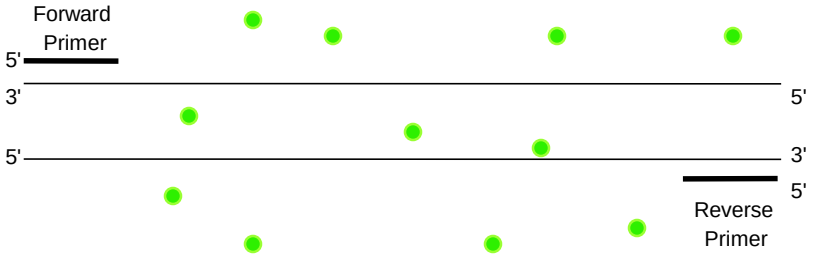
- SYBR Green I binds to DNA.
- The resulting DNA-dye-complex absorbs blue light (λ_{max}=497 nm) and emits green light (λ_{max}=520 nm).



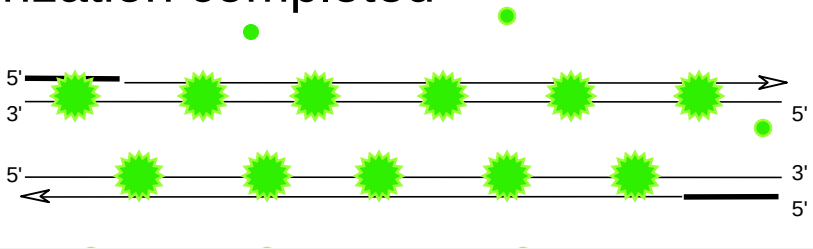
Real-time PCR

SYBR Green (double-stranded DNA binding dye)

Polymerization



Polymerization completed



The dye only fluoresces when bound to the dsDNA (i.e., the PCR product). Emits a strong fluorescent signal upon binding to double-stranded DNA.

Real-time PCR

What type of software is used with Real-time PCR?

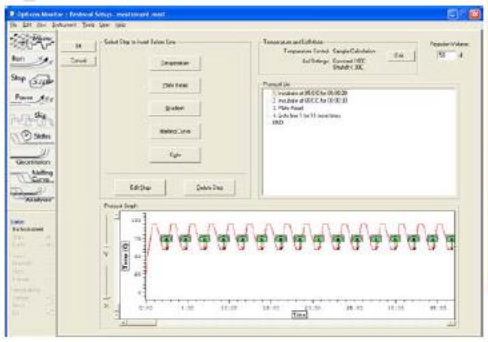
The real-time software converts the fluorescent signals in each well to meaningful data

What Type of Software is used with Real-Time PCR?

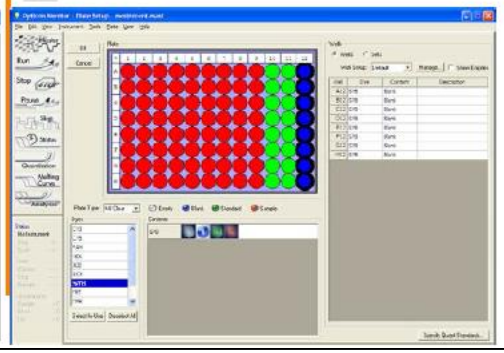
The real-time software converts the fluorescent signals in each well to meaningful data.

1. Set up PCR protocol.
2. Set up plate layout.
3. Collect data.
4. Analyze data.

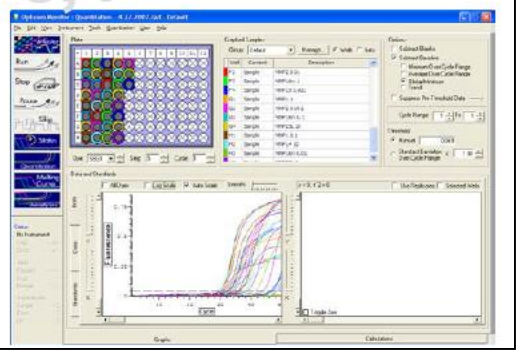
1



2



3,4



Real-time PCR

Types of real-time PCR

Specific and non-specific fluorescence detection

- At present, real-time fluorescent detection chemistries are sub-divided into two groups:

1. Specific detection methods (probe-based real-time PCR, also known as TaqMan PCR);
2. Non-specific detection methods (intercalator-based method, or SYBR Green method.

Animation 1: http://www.sigmaaldrich.com/life-science/molecular-biology/pcr/learning-center/sybr-green-animation.html).
Animation 2: http://www.sigmaaldrich.com/life-science/molecular-biology/pcr/learning-center/probed-based-qpcr-animation.html).

Real-time PCR

Types of real-time PCR

Non-specific fluorescence detection

- Initially, intercalator dyes such as SYBR® Green and ethidium bromide were used to measure real-time PCR products.
- These dyes are used as a nucleic acid stain in molecular biology.
- These double-stranded DNA dye in the PCR reaction binds to newly synthesized double-stranded DNA and gives fluorescence.
- The primary disadvantage to these dyes is that they detect accumulation of both specific and nonspecific PCR products.

Intercalation means to insert. Intercalator dyes are used to interpose between the stacked bases of DNA.

Real-time PCR

Types of real-time PCR

Non-specific fluorescence detection

- The fluorogenic dye, binds non-specifically to dsDNA; it does not bind to single-stranded DNA (ssDNA).
- Because SYBR green molecules bind non-specifically to any dsDNA, they will also bind to non-specific products if present in a reaction.
- Therefore, SYBR green PCR assays need to be properly optimized to avoid amplification of non-specific products or production of primer dimers, and thus reporting of false positive results.
- The unbound form of SYBR green exhibits very little fluorescence but emits a strong fluorescent signal once bound to dsDNA.

Real-time PCR

Non-specific fluorescence detection

- Non-specific detection using DNA binding dyes. e.g. SYBR® Green, PicoGreen and ethidium bromide.
- Due to its non-specific annealing, the SYBR Green dye is subject to produce false quantification readings should there be any contamination or non-targeted priming.
- However, The SYBR Green dye is less costly and it is has highly useful application in melt-curve analysis and strain identification.

Real-time PCR

Types of real-time PCR

Specific fluorescence detection

- Specific detection methods use fluorogenic-labeled oligonucleotide probes, in addition to primers.
- These probes are designed to bind to a specific sequence on the target DNA, thus increasing the specificity of the PCR.
- When using the specific detection methods, post PCR processing is not necessary because the fluorogenic probes only allow detection of a specific amplification product, consequently eliminating detection of non-specific PCR products.
- The following are the two commonly used probe-based real-time PCR chemistries:

1. Hydrolysis probes (or 5'-exonuclease oligonucleotide probes);
2. Hybridization probes (or FRET probes).

Real-time PCR

Development of TaqMan® chemistry

Specific fluorescence detection

- Probe-based real-time PCR, also known as TaqMan PCR, requires a pair of PCR primers as regular PCR does, an additional fluorogenic probe which is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached.
- The availability of these fluorogenic probes enabled the development of a real-time method for detecting only specific amplification products.
- TaqMan method is more accurate and reliable than SYBR green method, but also more expensive.

Real-time PCR

Development of TaqMan® chemistry

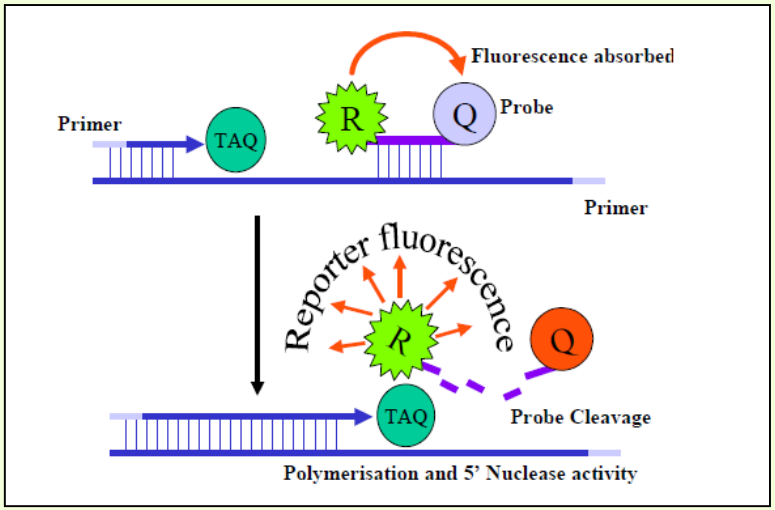
2. Specific binding probes using two dyes: fluorecent reporter dye and quencher dye

- Fluorogenic-labeled probes use the 5' nuclease activity of *Taq* DNA polymerase.
- The probe is a linear oligonucleotide with a fluorogenic dye attached to the 5' end and a quencher molecule attached to the 3' end.
- While the probe is intact, the proximity of the quencher dye greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET).

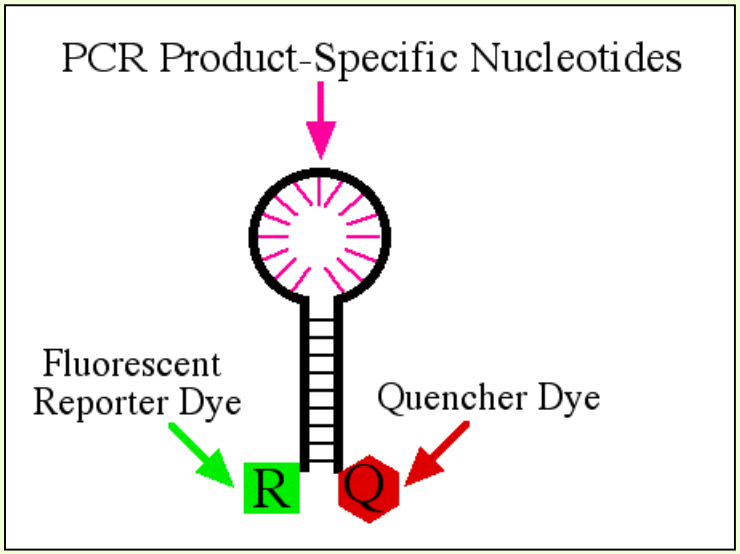
Real-time PCR

Types of real-time PCR

- Intercalator-based method, also known as SYBR Green method.



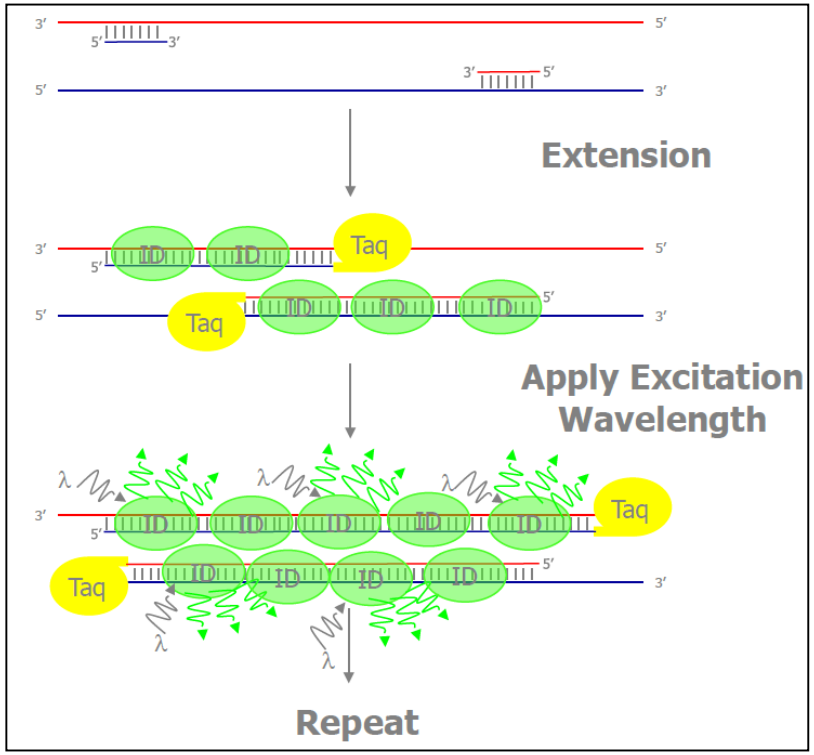
- Probe-based real-time PCR, also known as TaqMan method.



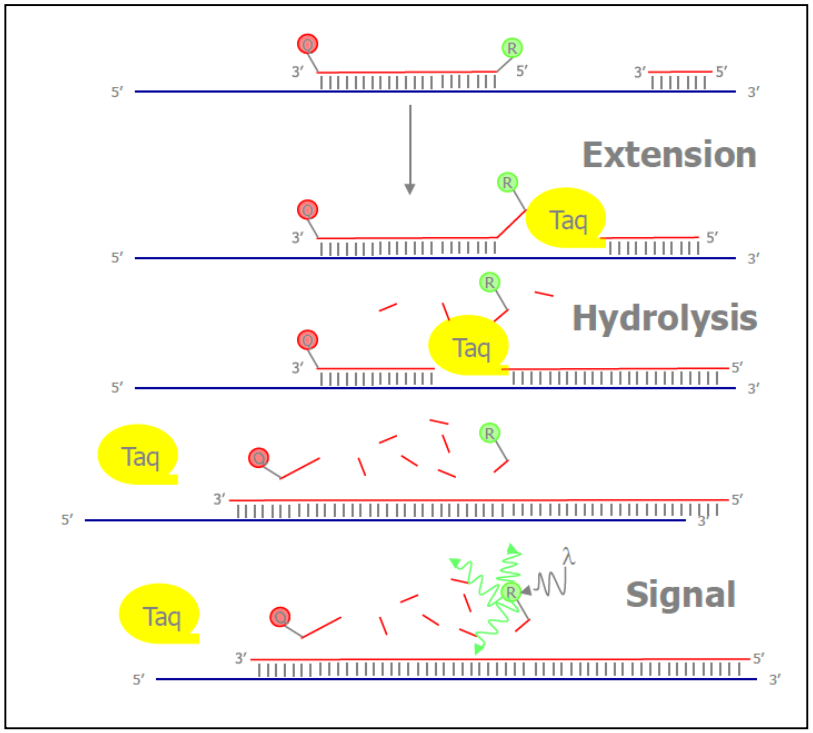
Real-time PCR

Reagents used to fluoresce in the presence of amplified DNA

Fluorescent Dyes in PCR Intercalating Dyes

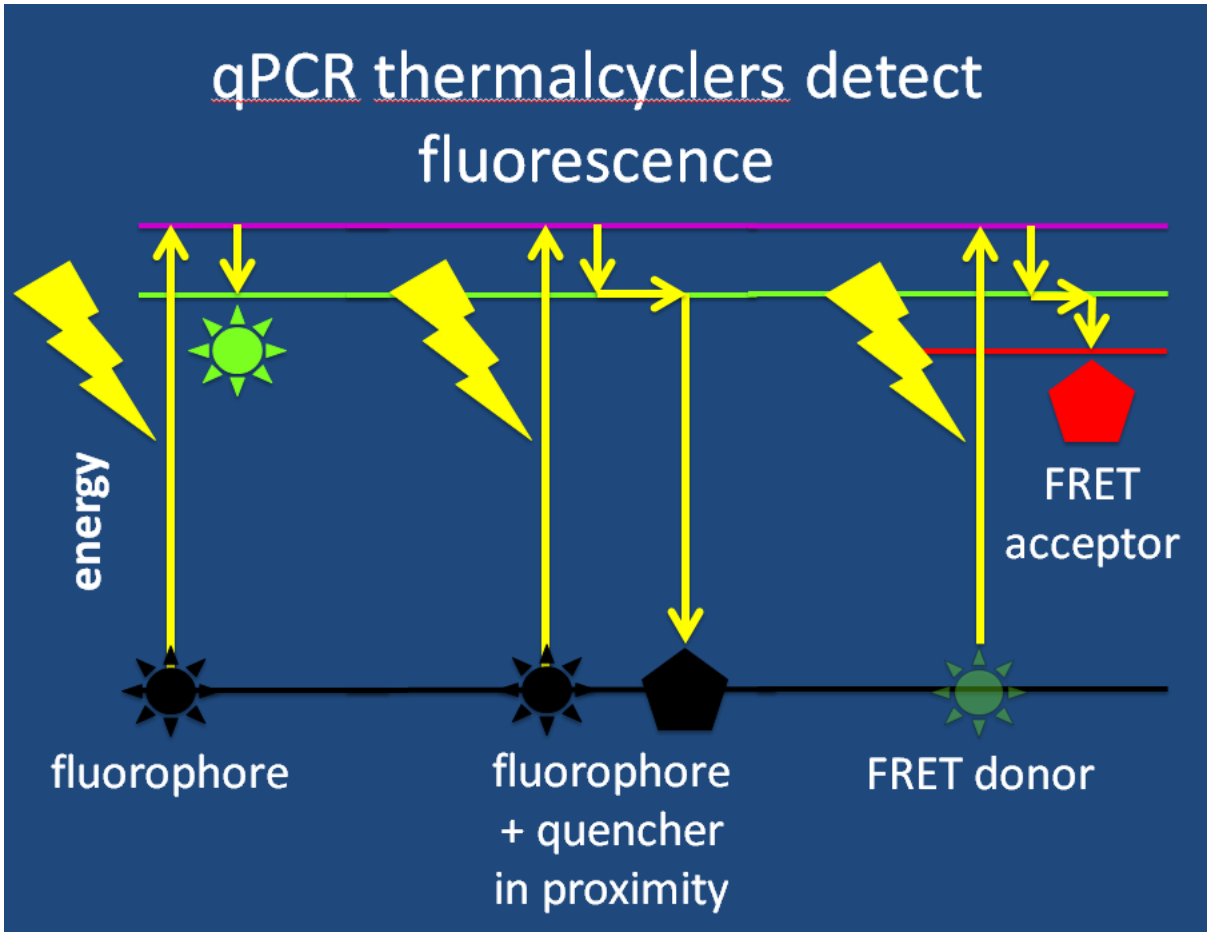


Fluorescent Dyes in PCR Probes



Real-time PCR

Reagents used to fluoresce in the presence of amplified DNA



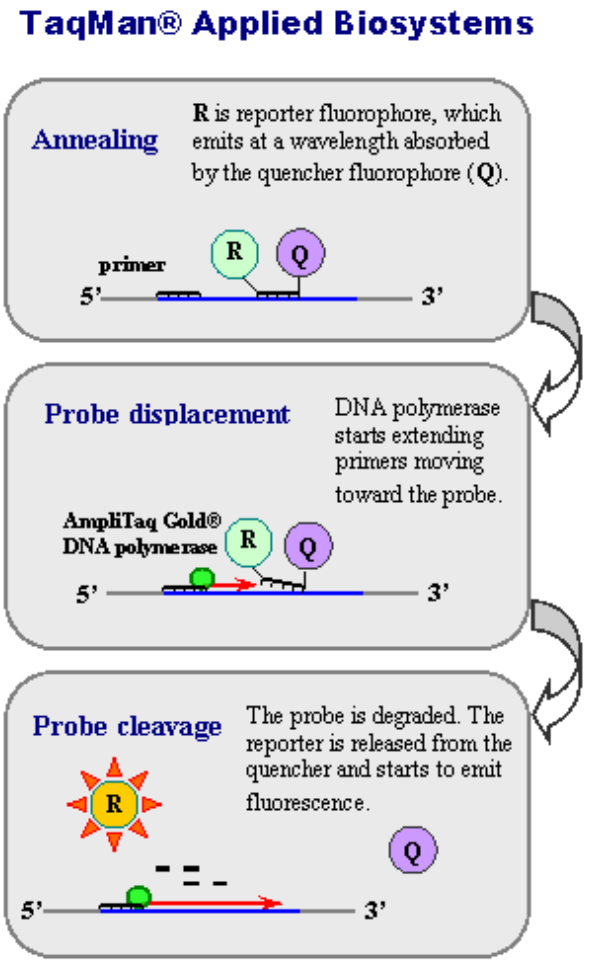
Real-time PCR
The TaqMan® Method

- In this method a special thermocycler equipped with a sensitive camera that monitors the fluorescence in each well of the 96-well plate at frequent intervals during the PCR Reaction.
- The amount of fluorescent intensity usually measured by a laser present within real-time PCR machine.
- The amount of fluorescence, monitored at each amplification cycle, is proportional to the log of concentration of the PCR target.

Weintraub and Jones,2010;..

Real-time PCR
The TaqMan® Method

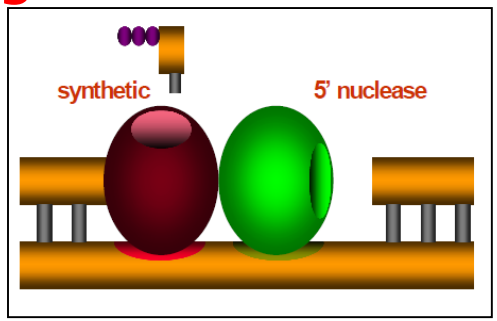
- TaqMan assay (named after *Taq* DNA polymerase) was one of the earliest methods introduced for real time PCR reaction.
- This technique became possible after introduction of an oligonucleotide probe, which was designed to hybridize within the target sequence.
- Cleavage of the probe during PCR because of the 5' nuclease activity of *Taq* DNA polymerase can be used to detect amplification of the target-specific product.



Heid *et al.*,1996

Real-time TaqMan RT-PCR
The TaqMan® Method

- Quantitative real-time PCR has become possible by the development of detectors that can measure fluorescence that is emitted during the PCR cycle.
- The method is based on the 5'-->3' exonuclease activity of the *Taq* DNA polymerase, which results in cleavage of fluorescent dye-labelled TaqMan® probes during PCR.



Taq polymerase activity

Janse,2006; Weintraub and Jones,2010; Applied biosystems

Real-time TaqMan RT-PCR

Standard rules to be followed when designing realtime (TaqMan) PCR primers and probes

- **Primer Rules:**

1. Primers with melting temperature (Tm) of 58-60°C.
2. Primers 15-30 bp in length
3. Run of identical nucleotide should not be allowed, especially G
4. The total no. of Gs and Cs in the last 5 nucleotides at the 3' end should not exceed 2
5. Amplicon length should ideally be 50-150 bp in length and not exceed 400 bp.

- **Probe Rules:**

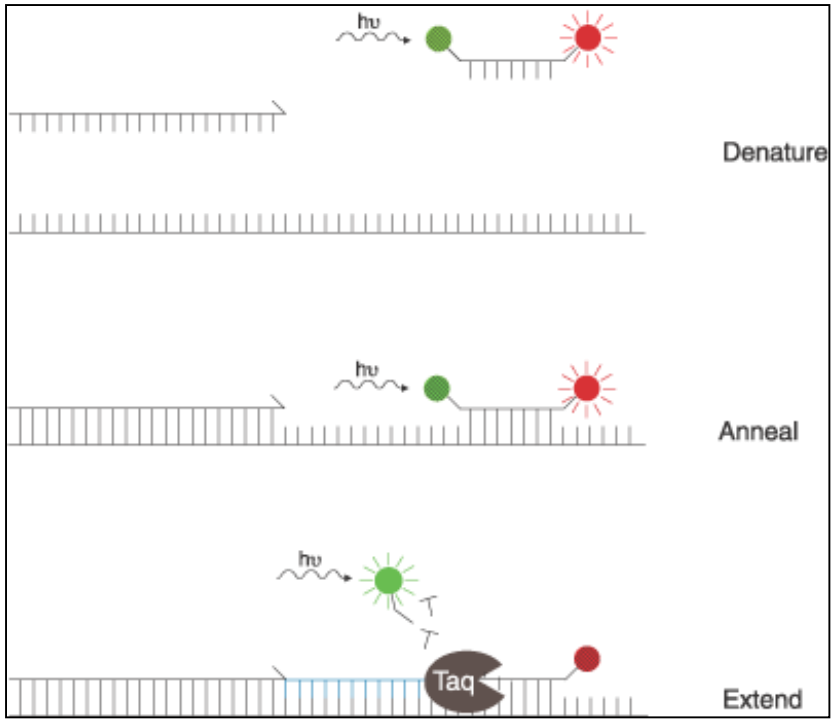
1. Tm of 10°C higher than primers
2. Should not be runs of identical nucleotides, especially Gs
3. GC content 30-80%
4. More Cs than Gs produces a higher DRn
5. No G at the 5' end.

Weller *et al.*,2006

Principle of TaqMan® PCR

Other Images of TaqMan® in Action

- Normalize the primer concentrations and mix gene-specific forward and reverse primer pair.
- Each primer (forward or reverse) concentration in the mixture is 5 pmol/μl.



Davidson College, Davidson,2003;..

Real-time PCR
Nomenclature commonly used

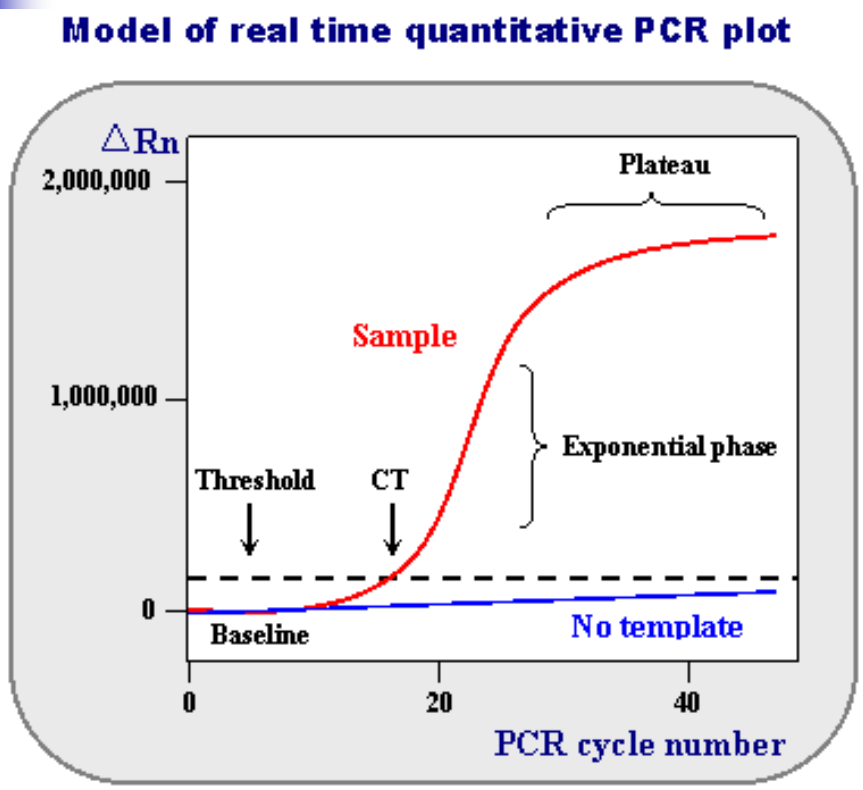
- Baseline is defined as PCR cycles in which a reporter fluorescent signal is accumulating but is beneath the limits of detection of the instrument.
- ΔRn is an increment of fluorescent signal at each time point. The ΔRn values are plotted versus the cycle number.
- Threshold is an arbitrary level of fluorescence chosen on the basis of the baseline variability.
- A signal that is detected above the threshold is considered a real signal that can be used to define the threshold cycle (Ct) for a sample. Threshold can be adjusted for each experiment so that it is in the region of exponential amplification across all plots.
- Ct is defined as the fractional PCR cycle number at which the reporter fluorescence is greater than the threshold. Lower Ct values indicate higher amounts of target DNA. The Ct is a basic principle of real time PCR and is an essential component in producing accurate and reproducible data.

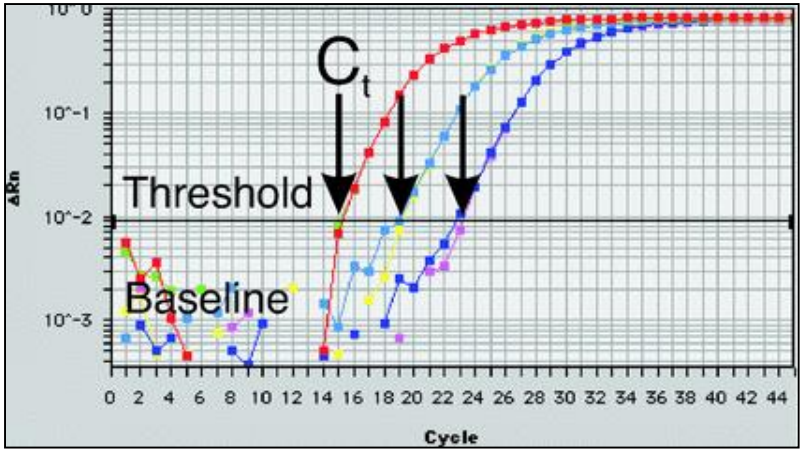
Heid *et al.*,1996

Real-time PCR

Model of QRT-PCR (Quantitative real time-PCR)

Nomenclature commonly used



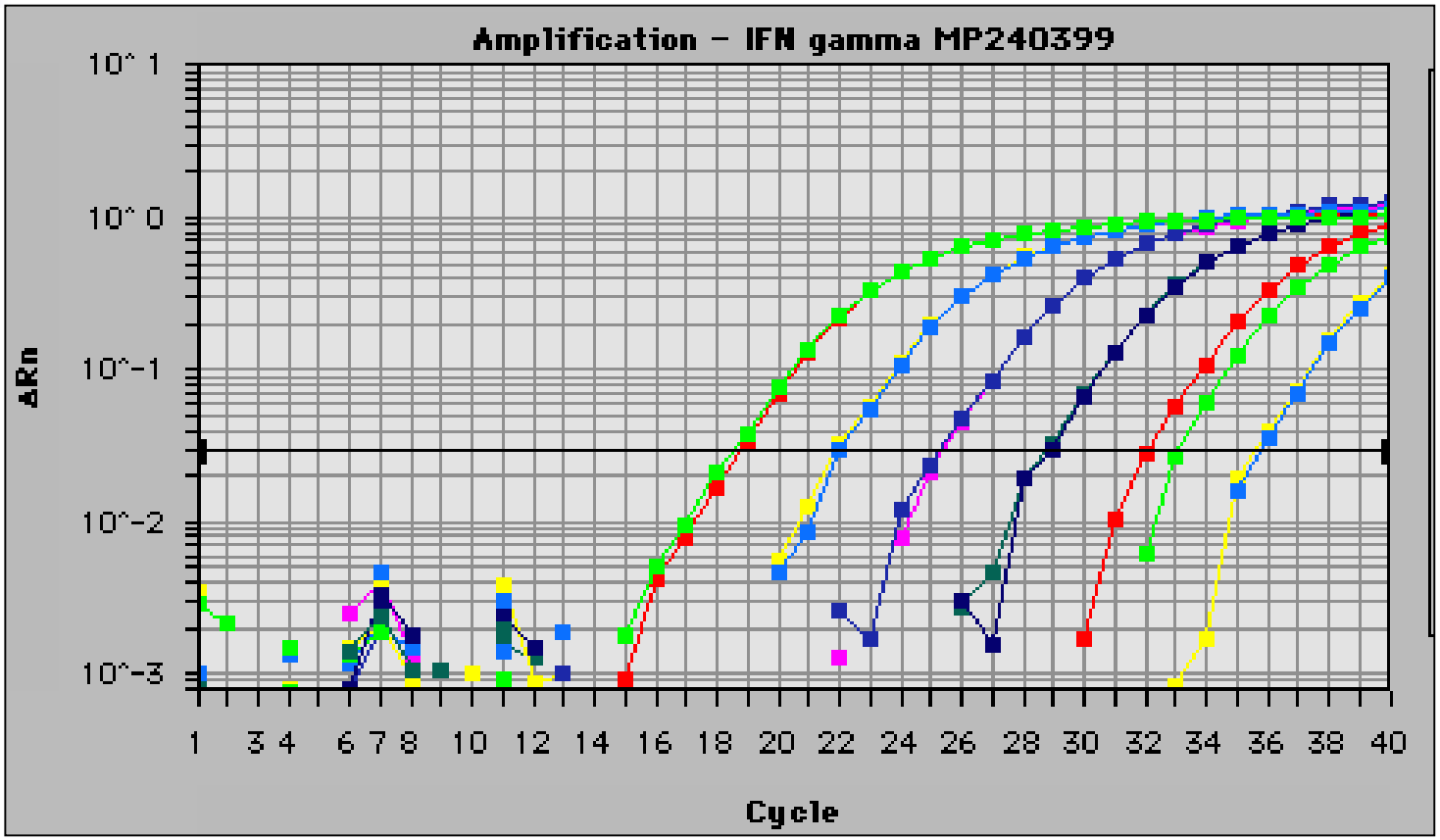


DNA amplification with detection of the products in a single tube.
Fluorescence monitoring during each PCR cycle.

Heid *et al.*,1996;..

TaqMan® PCR

A graph printiout of actual data found using the TaqMan® probe



Davidson College, Davidson,2003

Real-time PCR

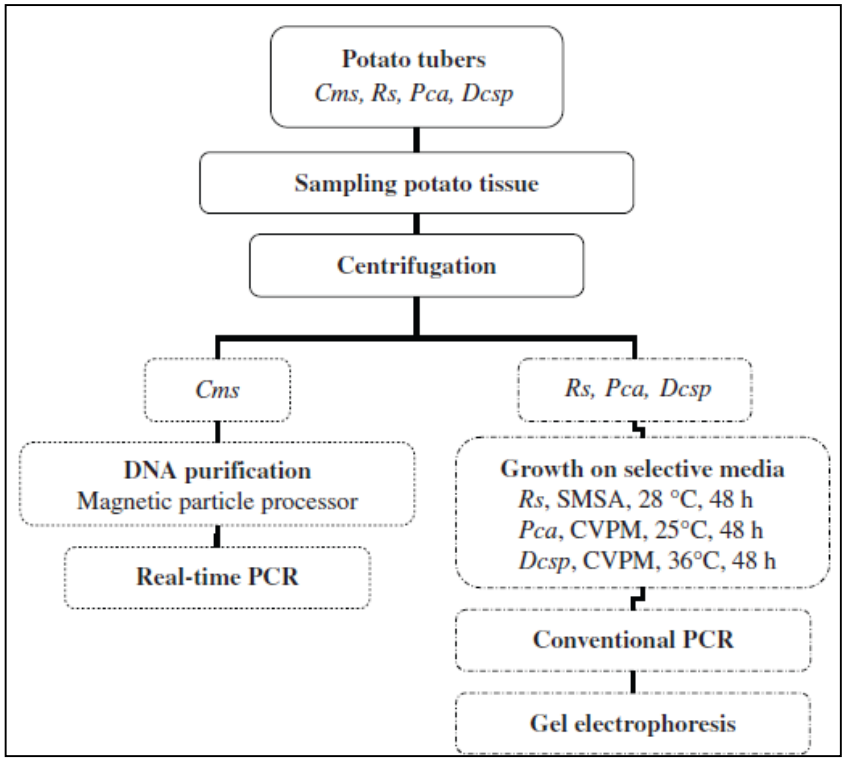
Used to detect and quantify plant pathogenic bacteria

- A real-time PCR assay has been developed for the detection and quantification of many bacteria.
- e.g. real time RT-PCR was developed to detect and quantify the agent of apple proliferation phytoplasma group.
- It is less laborious than nested PCR/RFLP.
- Real-time PCR assay is equally sensitive and allows to estimate the concentration of phytoplasmas in infected tissues or insects.
- In case of *Clavibacter sepedonicus* (*Cs*) purification step coupled with real-time PCR, using a TaqMan ® assay, results in a rapid and reliable method of detection.

Real-time PCR

For detection of *Cms*, *Rs*, *Pca*, and *Dcsp*

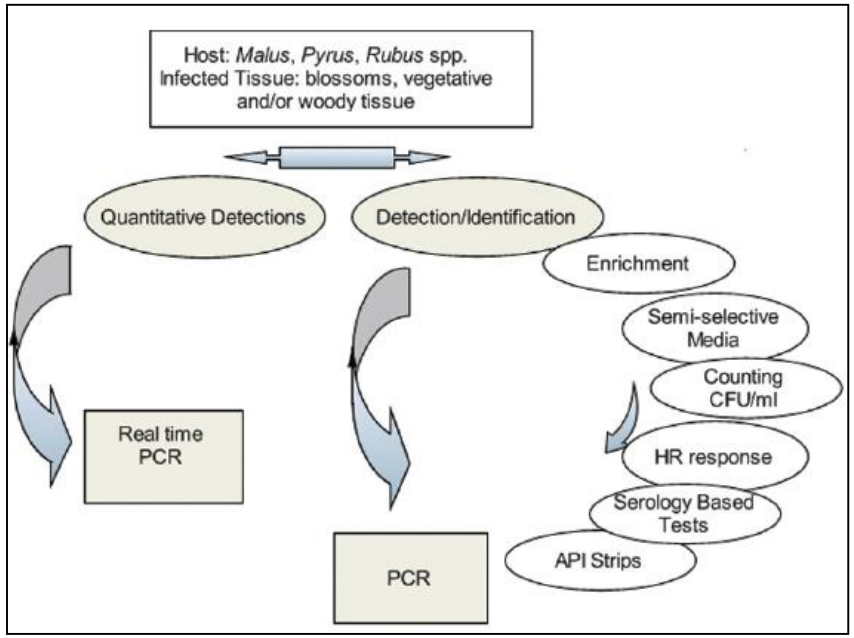
- Real-time PCR is used as an additional confirmatory test.
- DNA amplification and analysis by either conventional PCR and subsequent detection using gel electrophoresis (*Pca*, *Dcsp*, previously *Erwinia chrysanthemi* and *Ralstonia solanacearum*, *Rs*) or detection using real-time PCR (*Cms* only).



Burns,2009

Real-time PCR
For detection of *E. amylovora*

- Schematic representation of the PCR-based methods for the quantitative and traditional based PCR identification of *E. amylovora*.
- The protocols described on the extreme right hand side (clear semi-circles) represent the multitude of techniques that are often used in literature.



Burns,2009

Real-time PCR

Detection of *X. arboricola fragariae* (*Xaf*)

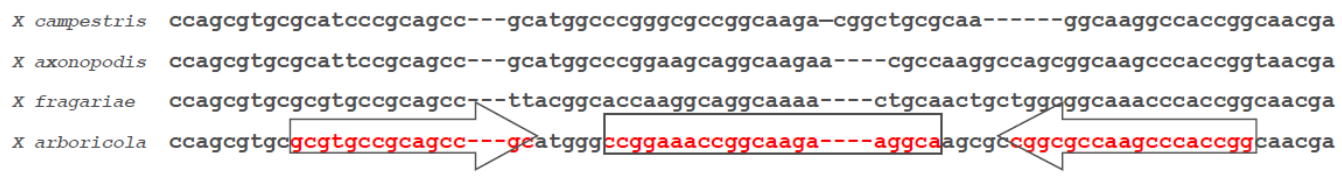
- Designed within *Xaf prolyl endopeptidase (pep1)* gene sequence.
- Specifically detects all *Xaf* strains and other *X. arboricola* pvs.
- Detects as few as 10^3cells *Xaf* in 0.7 cm strawberry leaf disc DNA extract.

Parkinson and co-workers

Real-time PCR

Detection of *X. arboricola fragariae* (*Xaf*)

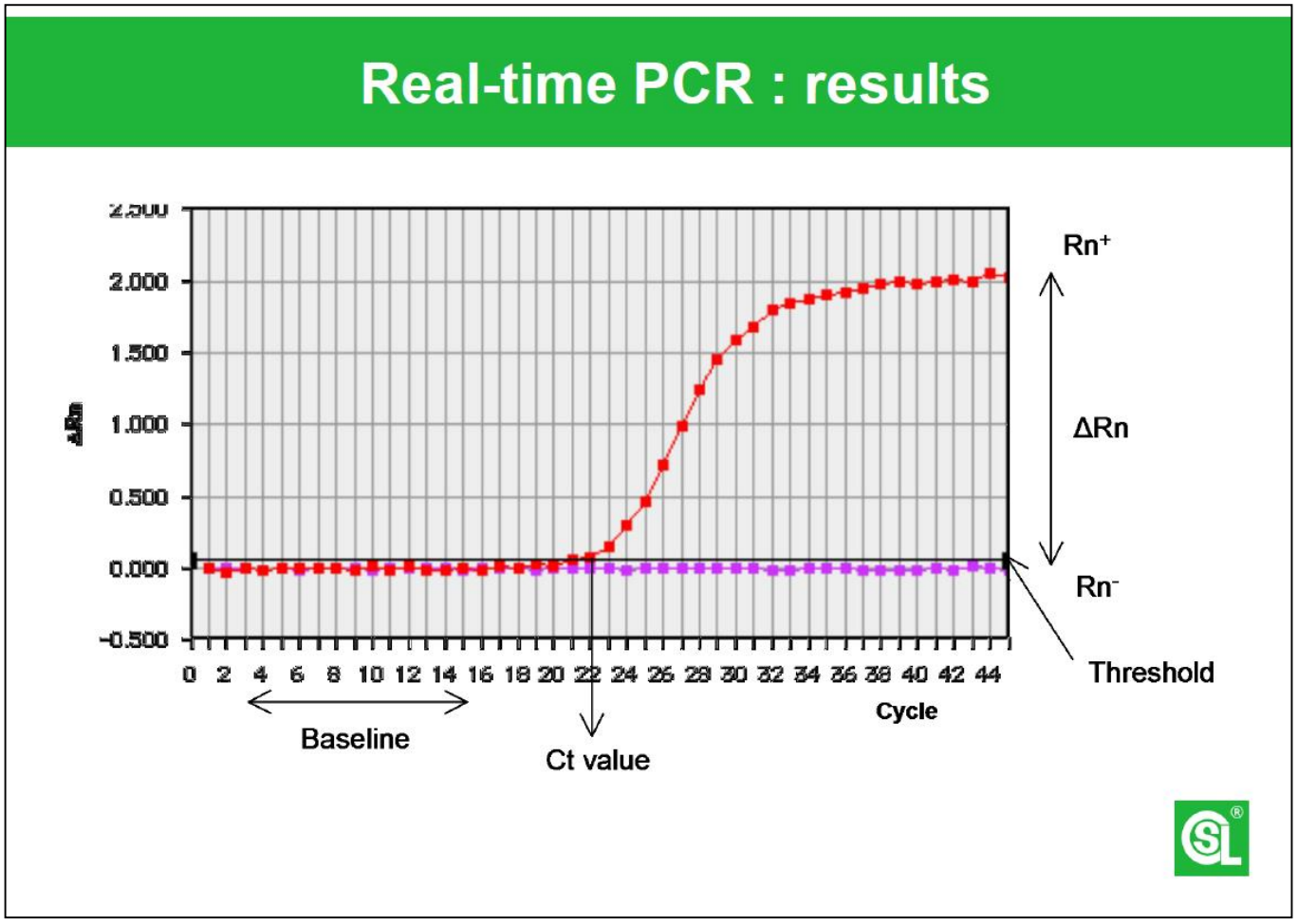
Alignment of partial *pep*1 with TaqMan primers & probe for *X. arboricola* detection



Parkinson and co-workers

Real-time PCR

Detection of *X. arboricola fragariae* (*Xaf*)



Parkinson and co-workers

Real-time PCR

Detection of *X. arboricola fragariae* (*Xaf*)

Specificity of *Xaf pep1* assay

Bacterial Strain	TaqMan CT value
Xanthomonas arboricola pv. *fragariae*	15
Xanthomonas arboricola pv. *fragariae*	16
Xanthomonas arboricola pv. *fragariae*	19
Xanthomonas arboricola pv. *fragariae*	16
Xanthomonas arboricola pv. *fragariae*	17
Xanthomonas arboricola pv. *fragariae*	16
Xanthomonas arboricola pv corylina	18
Xanthomonas arboricola pv pruni	18
Xanthomonas arboricola pv pruni	16
Xanthomonas arboricola pv juglandis	16
Xanthomonas arboricola pv celebensis	16
Xanthomonas arboricola pv populi	40
Xanthomonas fragariae	40
Xanthomonas fragariae	40
Xanthomonas fragariae	40
Xanthomonas fragariae	40
Xanthomonas fragariae	40
Xanthomonas fragariae	40
Xanthomonas fragariae	40
Xanthomonas fragariae	40
Xanthomonas fragariae	40
Xanthomonas albilineans	40
Xanthomonas axonopodis	40
Xanthomonas axonopodis pv begoniae	40
Xanthomonas axonopodis pv phaseoli	40
Xanthomonas campestris pv campestris	40
Xanthomonas campestris pv vesicatoria	40
Xanthomonas hortorum pv pelargonii	40
Xanthomonas hortorum pv. hederae	40
Xanthomonas melonis	40
Xanthomonas oryzae pv oryzae	40
Xanthomonas pisi	40
Xanthomonas vesicatoria	40
Erwinia herbicola	40
Pseudomonas syringae pv syringae	40

Parkinson and co-workers

Multicolor combinational probe coding technology in a single real-time
Identification of 8 foodborne pathogens

- Real-time PCR is becoming the gold standard for detection and quantification of DNA/RNA in diagnostics laboratories.
- However, Real-time PCR assays have been widely used for detecting foodborne pathogens but have been much less frequently applied in species identification, mainly because of the low number of species they can distinguish in 1 reaction.

Haung *et al.*,2007

Multicolor combinational probe coding technology in a single real-time
Identification of 8 foodborne pathogens

1. A uniplex real-time PCR can determine the presence of only 1 target.
2. Triplex real-time TaqMan PCR assay that can simultaneously identify *Acanthamoeba* spp., *B. mandrillaris*, and *N. fowleri* in the same PCR vessel.
3. Multiplex real-time PCR can detect no more than 4 to 5 targets depending on available instruments.

- Therefore, to determine the existence of a specific species among a large number of suspected pathogens, multiple, uniplex or multiplex real-time PCR reactions have to be performed.

Haung *et al.*,2007; Qvarnstrom *et al.*,2006

Multicolor combinational probe coding technology in a single real-time
Identification of 8 foodborne pathogens

- To be more practical for species identification, a real-time PCR platform must cover more targets that can be distinguished in a single reaction.
- The present study used a new probe coding/labeling strategy, termed multicolor combinational probe coding (MCPC), to increase the number of targets that can be distinguished in a single real-time PCR for rapid and reliable species identification.

Haung *et al.*,2007

Multicolor combinational probe coding technology in a single real-time
Identification of 8 foodborne pathogens

- The multicolor combinational probe coding (MCPC) uses fluorophore combinations in addition to single fluorophores to label probes.
- The combination rule allows n types of fluorophores to label $N = C_n^1 + C_n^2 + \ldots + C_n^n = 2^n - 1$ different probes in a combinatorial manner.
- Thus, up to 15 probes can be labeled using 4 different fluorophores, and 15 targets can be detected on a 4-color real-time PCR machine.
- Using MCPC, 8 foodborne pathogens can be accurately identified at the species level in a single real-time PCR.

Haung *et al.*,2007

Multicolor combinational probe coding technology in a single real-time

Fig. Illustration of the principle of MCPC for pathogen identification

A. Flow chart of MCPC. Genomic DNA is amplified initially by the matched tagged primer pair. Further amplification is mediated by the universal primer pair. The accumulation of the amplicon is detected by the matched molecular beacons probes, producing a corresponding real-time PCR profile.

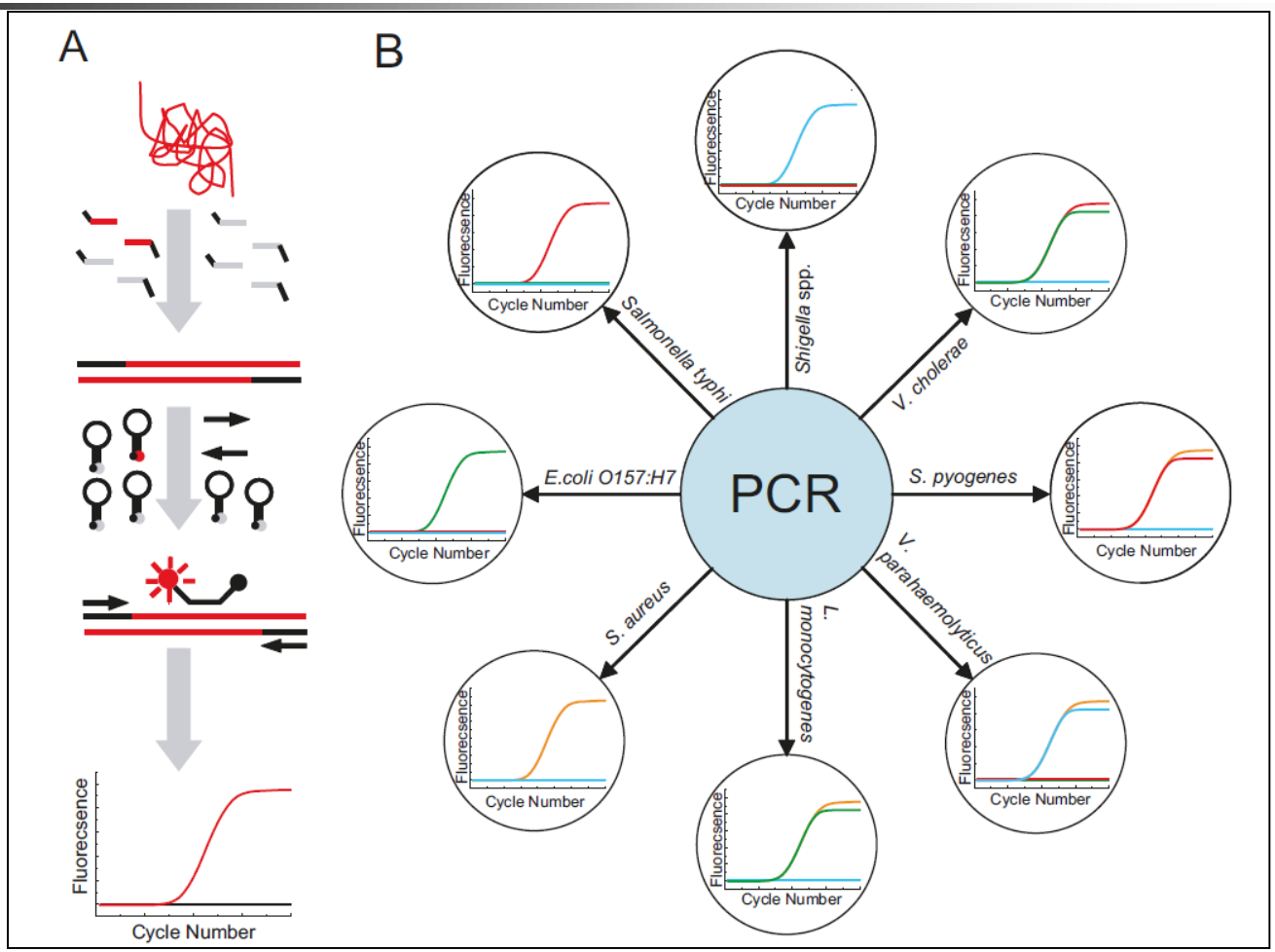
B. Eight possible PCR profiles corresponding with the 8 pathogens according to the MCPC design in this study.

- Each unique PCR profile is indicated by the appearance of the characteristic fluorescence increase.
- The color of the curves stands for the different fluorophore labels:
- FAM (orange), HEX (green), ROX (red), and Cy5 (blue).

Haung *et al.*,2007

Multicolor combinational probe coding technology in a single real-time

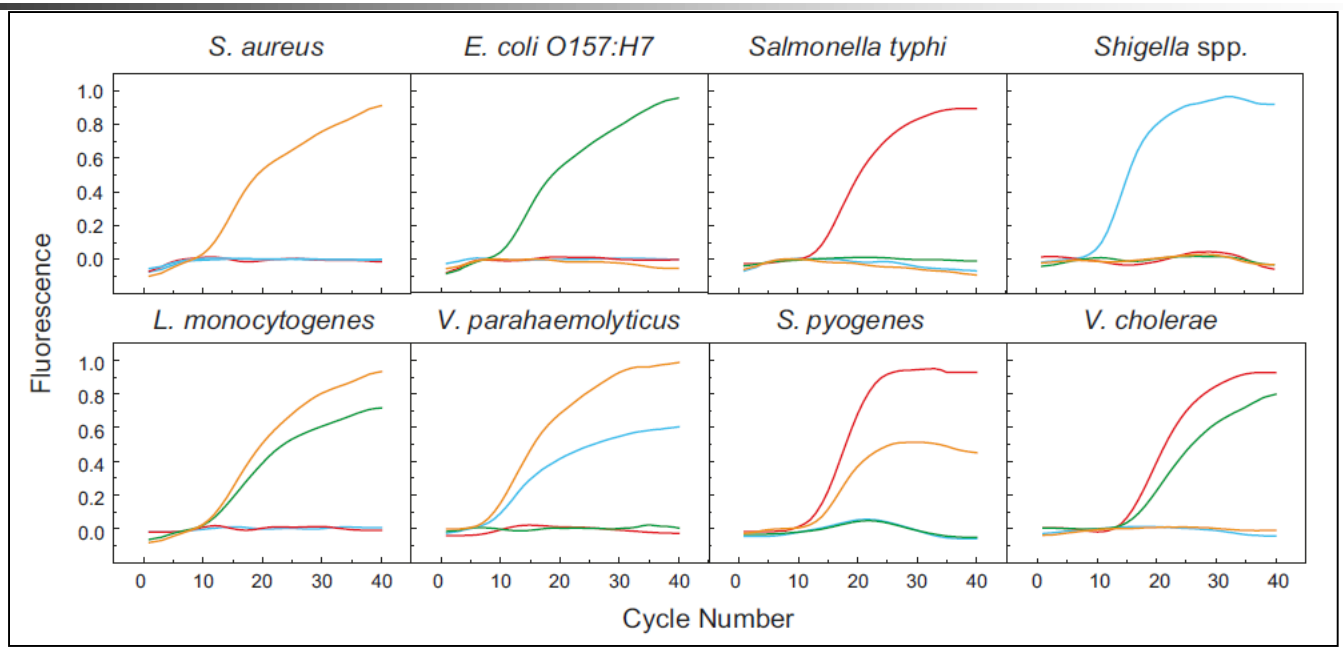
Fig. Illustration of the principle of MCPC for pathogen identification



Haung *et al.*,2007

Multicolor combinational probe coding technology in a single real-time

Fig. Identification of 8 bacterial species with a single MCPC assay



According to the designs in this study, the probes for *S. aureus*, *E. coli* O157:H7, *S. typhi*, *Shigella* spp., *L. monocytogenes*, *V. parahaemolyticus*, *S. pyogenes*, and *V. cholerae* were labeled with FAM, HEX, ROX, Cy5, FAM HEX, FAM Cy5, FAM ROX, and HEX ROX, respectively. For each of the 8 pathogens, FAM fluorescence (orange), HEX fluorescence (green), ROX fluorescence (red), Cy5 fluorescence (blue), both FAM and HEX fluorescence, both FAM and Cy5 fluorescence, both FAM and ROX fluorescence, and both HEX and ROX fluorescence were increased correspondingly.

Haung *et al.*,2007

Microsatellites

Simple sequence repeats (SSRs) or short tandem repeats

- Two different classes of molecular markers exist:

1. Inter simple sequence repeats (ISSRs), and
2. Simple sequence repeats (SSRs) or 'microsatellites.

- Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs), are repeating sequences of 2-5 base pairs of DNA.
- ISSR (for inter-simple sequence repeat) is a general term for a genome region between microsatellite loci.
- Since an ISSR may be a conserved or nonconserved region, sequence diversity in ISSR-PCR is lower than in SSR-PCR.

Inter Simple Sequence Repeats
ISSR-PCR
Primer modifications

- Sequences amplified by ISSR-PCR can be used for DNA fingerprinting.
- ISSRs are DNA fragments of about 100-3000 bp located between adjacent, oppositely oriented microsatellite regions.
- ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18 bp).
- About 10-60 fragments from multiple loci are generated simultaneously, separated by gel electrophoresis and scored as the presence or absence of fragments of particular size.
- Techniques related to ISSR analysis are

1. Single Primer Amplification Reaction (SPAR) that uses a single primer containing only the core motif of a microsatellite, and
2. Directed Amplification of Minisatellite-region DNA (DAMD) that uses a single primer containing only the core motif of a minisatellite.

van Treuren

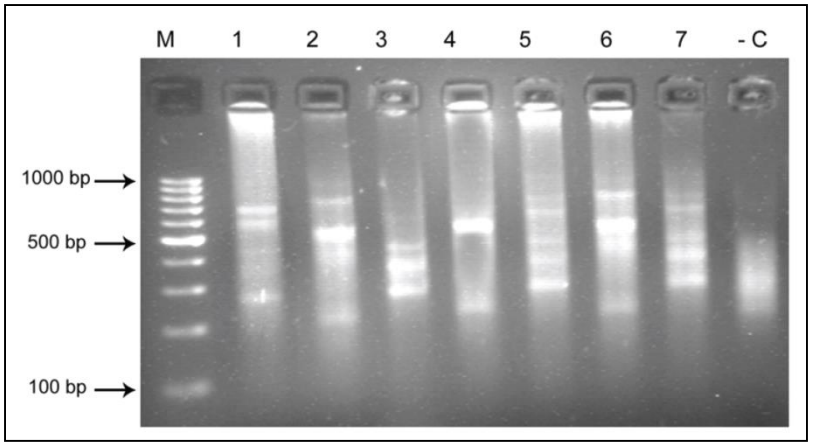
Microsatellite ISSR analysis

ISSR-PCR analysis of different isolates of *Xanthomonas* by using primer A-31

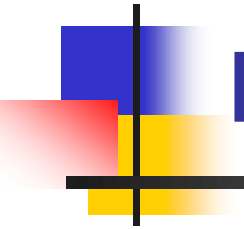
- **M:** DNA marker (100bp)
- **1:** *X. axonopodis* (citrus fruit)
- **2:** *X. axonopodis* (Rhizospheric soil)
- **3:** *X. campestris* (*Mangifera indica*)
- **4:** *X. maltophilia* (*Brassica campestris*)
- **5:** *X. maltophilia* (*Pisum sativum*)
- **6:** *X. nematophilus* (Root nodules)
- **7:** *X. nematophilus* (*Brassica campestris*)
- **-C:** Negative control.

S. #	Primer	Sequence
1	A-16	5'CACACACACACAR3'
2	A-31	5'AGCAGCAGCAGC3'
3	D-3	5'GACAGACAGACAGACA3'

List of primers and their sequences used in ISSR analysis.



Fatima *et al.*,2012



DNA sequence analysis

Steps of sequence analysis

See also bacterial classification file

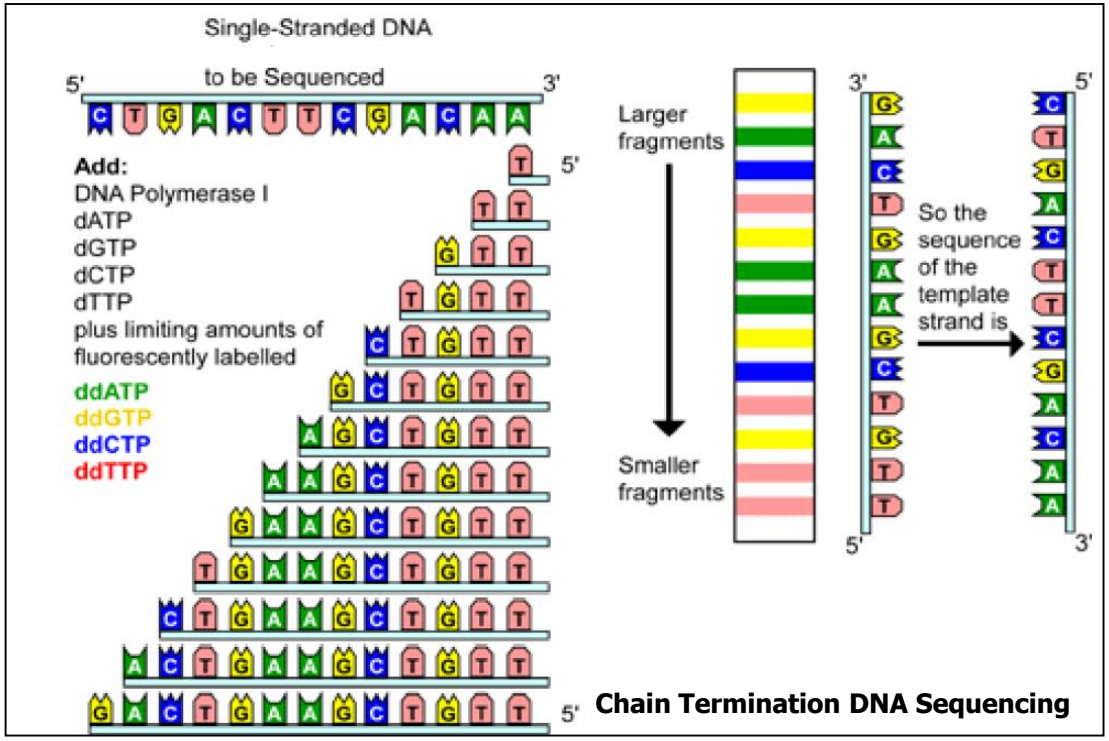
DNA sequence analysis

Principles of DNA sequencing

Chain Termination DNA Sequencing

DNA Sequencer (any brand)

- The process of determining the order of the nucleotide bases along a DNA strand is called sequencing.



NCBI

DNA sequence analysis

Principles of DNA sequencing

Chain Termination DNA Sequencing

- Chain termination sequencing involves:
- The synthesis of new strands of DNA complementary to a single-stranded template (step I).
- The template DNA is supplied with a mixture of all four deoxynucleotides, four dideoxynucleotides--each labeled with a different color fluorescent tag, and DNA polymerase (step II).
- As all four deoxynucleotides are present, chain elongation proceeds until, by chance, DNA polymerase inserts a dideoxynucleotide. The result is a new set of DNA chains all of different lengths (step III).
- The fragments are then separated by size using gel electrophoresis (step IV).
- As each labeled DNA fragment passes a detector at the bottom of the gel, the color is recorded. The DNA sequence is then reconstructed from the pattern of colors representing each nucleotide sequence (step V).

DNA Sequencer (any brand)

DNA sequence analysis

- Fast, accurate and of acceptable costs;
- Next (-Next) generation sequencing = new automatic, high-througput DNA and low cost sequencing technologies providing qualitative and quantitative sequence data;
- Continued technical developments will probably make sequencing part of diagnostic bacteriology as culture is today;
- However, technologies remain rather complex → successful adoption in bacteriological lab will require expertise in both molecular biology and bioinformatics.

Elke,2010

DNA Sequencer (any brand)

DNA sequence analysis

- In this exercise, you will set up the reaction to generate the DNA sequence of the amplified DNA.
- The amplified fragment will be used as template in a DNA sequencing reaction.
- The DNA sequencing reaction you will be using is actually quite similar to a PCR reaction.
- The DNA of interest will be used as a template, and DNA polymerase will copy the template to produce a new strand of DNA.

Bednarski,2006

DNA Sequencer (any brand)

DNA sequence analysis

- The main difference will be the inclusion in the DNA replication reaction of special nucleotides called 2', 3'-dideoxynucleotides.
- As the name implies, these nucleotides do not have an -OH group on either the 2' or 3' carbon.
- Whenever they are incorporated into a growing DNA strand, they are the last nucleotide incorporated, because there is no 3' -OH available for the next phosphodiester bond.
- If one has a way of identifying the different dideoxynucleotides (A or G or T or C), one can know the last base incorporated in any newly replicated strand of DNA.

Bednarski,2006

DNA Sequencer (any brand)

DNA sequence analysis

- In our sequencing reactions, we will use dideoxynucleotides labeled with different colored fluorescent tags.
- Also, in DNA sequencing only one primer is used, so only one of the two strands is used as a template in the sequencing reaction.
- Once the results of the DNA sequencing are known, we will be able to search the database of known sequences for a match to your sequence.

Bednarski,2006

DNA sequencing

16S rDNA and 16S-23S intergenic spacer region (ISR)

- 16S rDNA is the normal target for sequencing.
- Some of the rDNA regions are well conserved throughout the evolutionary process and other parts are variable even within a species.
- Sequence information has also become available on the 16S-23S intergenic spacer region (ISR) and suggests that considerable variation can occur between species in both:

1. The length and
2. The sequence of this region.

DNA sequencing

Conserved regions in the microbial genome

- Some of these conserved regions in the microbial genome are:
- 16S rDNA genes
- *hrp* genes,
- *rpo*B gene,
- *gyr*B gene, or
- elongation factor Tu.
- These can be sequenced as alternative targets to differentiation below the species level (pathovar level).

DNA sequencing

Minimal standards

Limitations of 16S rDNA gene sequencing

- Although this technique relies on sequencing of the DNA that encodes the 16S rRNA subunit, like phenotypic tests, it surveys only a small portion of the microbial genome.
- Since ribosomal genes are highly conserved, sequence variation between strains belonging to different species from distinct genera is less evident with rDNA gene sequencing than with DNA pairing studies, where DNA relatedness values are used to compare strains.

Janda and Abbott,2002

DNA sequencing

Minimal standards

Limitations of 16S rDNA gene sequencing

- It is generally accepted that an unidentified isolate whose rDNA gene sequence is <97% similar to those of the isolate's closest phylogenetic neighbors constitutes a new taxon.
- Many attempted were made to establish cutoff values for strain relatedness based upon sequencing of 16S rDNA.

Janda and Abbott,2002

DNA sequencing

Minimal standards

Limitations of DNA sequences deposited in microbial genome databases

- More and more sequences deposited in the INSDC (International. Nucleotide Sequence Database Consortium) such as GenBank@NCBI.
- The accuracy of a partial or complete sequence of a 16S rDNA gene or of any other bacterial gene(s) deposited in GenBank or other databases is dependent upon how extensively the bacterial strain from which the sequences were derived has been characterized.
- Since biochemical characteristics are still the touchstone for bacterial identifications, the choice in the use of commercial versus conventional methodologies for the identification of strains is important.

Janda and Abbott,2002;..

DNA sequencing

16S rRNA sequence identity may not be sufficient to guarantee species identity

- There are many more examples in the literature of the misidentification of bacteria by commercial systems.
- Thus, while the sequence data may be correct, if it is incorrectly associated with the wrong taxa, major errors in the literature can result.
- Sequence heterogeneity in the literature can result from strain misidentification.

See also bacterial classification file.

Janda and Abbott,2002

DNA sequencing

16S rRNA sequence identity may not be sufficient to guarantee species identity

- Although 16S rRNA gene sequencing is:

1. highly useful in regards to bacterial classification,
2. it has low phylogenetic power at the species level, and
3. poor discriminatory power for some genera, and DNA relatedness studies are necessary to provide absolute resolution to these taxonomic problems.

- The genus *Bacillus* is a good example of this.
- The type strains of *B. globisporus* and *B. psychrophilus* share >99.5% sequence similarity with regard to their 16S rRNA genes, and yet at the DNA level exhibit only 23 to 50% relatedness in reciprocal hybridization reactions.

Janda and Abbott,2007

DNA sequencing

16S rRNA sequence identity may not be sufficient to guarantee species identity

- These groups include (not exclusively),
- the family *Enterobacteriaceae* (in particular, *Enterobacter* and *Pantoea*),
- rapid-growing mycobacteria, the *Acinetobacter baumannii-A. calcoaceticus* complex,
- *Achromobacter*,
- *Stenotrophomonas*, and
- *Actinomyces*.
- Some of these problems are related to bacterial nomenclature and taxonomy while others are related to different issues cited in next slides.

Janda and Abbott,2007

DNA sequencing

16S rRNA sequence identity may not be sufficient to guarantee species identity

- Unfortunately, no universal definition for species identification via 16S rRNA gene sequencing exists, and authors vary widely in their use of acceptable criteria for establishing a "species" match.
- In none of these studies does the definition of a species "match" ever exceed 99% similarity (1% divergence).
- Based on the data listed above, even this threshold value may not be sufficient in all instances to guarantee an accurate identification.

Janda and Abbott,2007

DNA sequencing

16S rRNA sequence identity may not be sufficient to guarantee species identity

- In the case of *Aeromonas veronii* the genome can contain up to six copies of the 16S rRNA gene that differ by up to 1.5% among themselves.
- This implies intragenomic heterogeneity of the 16S rRNA gene among aeromonads and would preclude the use of this technology alone for species identification.
- The collective data described above strongly suggest that any microbial identifications using 16S rRNA distance scores of 1% are unsatisfactory for a diagnostic or public health reference laboratory.

Janda and Abbott,2007

Limits of 16S rRNA sequences in identification of bacteria at species level

Recommended guidelines for use of 16S rRNA gene sequencing for microbial identification

Category	Guidelines
Strain to be sequenced	• Phenetic profile of strain is not known by general grouping to present difficulties for identification by 16S rRNA gene analysis. • For such strains requiring molecular identification, another housekeeping gene is required (e.g., *rpoB*)
16S rRNA gene sequencing	• Minimum: 500 to 525 bp sequenced; ideal: 1,300 to 1,500 bp sequenced • <1% position ambiguities
Criteria for species identification	• Minimum: >99% sequence similarity; ideal: >99.5% sequence similarity; • Sequence match is to type strain or reference strain of species that has undergone DNA-relatedness studies; • For matches with distance scores <0.5% to the next closest species, other properties, including phenotype, should be considered in final species identification.

Janda and Abbott,2007

Species Concept in Prokaryotes

Tips for writing a taxonomic paper describing new species

- Anyone who is working on isolation of bacteria should have a good chance of finding a new species. Using genome sequencing, I would say that your chance of finding new species from one isolation study from soil or water should be almost 100%.
- It is our duty, as good microbiologists, to make them known to the scientific world by describing them as new species.
- Assuming that you have isolated multiple strains, **designate the type strain of your new species**. If you have a single strain, that strain will become the type strain of the new species.

Species Concept in Prokaryotes

Tips for writing a taxonomic paper describing new species

Prove it is a new species by 16S rRNA gene (16S) and genome sequencing

- Start with 16S sequencing and obtain a full-length, high-quality sequence. Don't trust the assembled sequence from sequencing firms, as these service companies almost never edit the final sequence.
- Always check the chromatograms (Sanger *ab1* files) manually.
- I have seen terrible 16S sequences used for creating a new species, which are not really new species.
- Search the sequence against the EzBioCloud 16S database at **www.ezbiocloud.net**.
- If you find any hit lower than 98.7%, you probably have a new species given that your sequence is of good quality [Learn more].

Species Concept in Prokaryotes

Tips for writing a taxonomic paper describing new species

Prove it is a new species by 16S rRNA gene (16S) and genome sequencing

- Let's say you have ~99% 16S similarity, don't be disappointed.
- You still have a very good chance of finding a new species.
- I suggest you go for whole genome sequencing.
- For the taxonomic purposes, a guideline was published here.
- Calculate Average Nucleotide Identity (ANI) values between your isolate and type strains of closely related species in the 16S searches using EzBioCloud database.
- The ANI cutoff is 95~96% for species.

Steps of sequence analysis

The amplified fragment will be used as template in a DNA sequencing reaction

Amplification and sequencing of rRNA genes

1. Extract nucleic acid from isolates or amplify sequence in the polymerase chain reaction.
2. Purify pure culture or amplified DNA using a kit.
3. Send to sequencing facility for PCR based sequencing with an appropriate primer.
4. Edit sequence.
5. Using the web based database (The Ribosomal Database Project (RDP) find closest relative to the primary structure of this sequence with Sequence Match.
6. Align sequences with aligned sequences of the closest relatives from the RDP database using alignment software. Proof read the alignment: base the alignment of conserved and variable areas on secondary structure of a closely related organism (available from your instructor or specified web pages.
7. Create a distance matrix.
8. Test sequence for potential chimeric structures.
9. Export the aligned product and the aligned sequences of the closest relatives into software able to create a tree of phylogenetic relationship based on specific algorhythms.

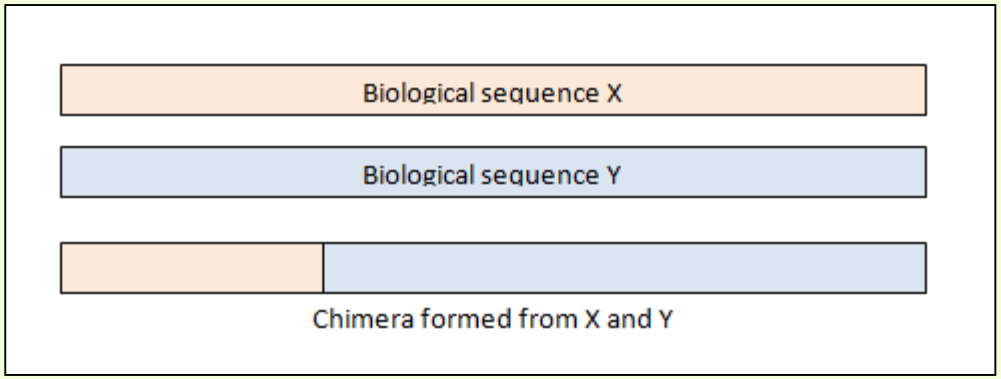
- Now enough is known to design a probe for the analyzed sequence.

Steps of sequence analysis

Amplification and sequencing of rRNA genes

Chimeric structures

- Chimeras are sequences formed from two or more biological sequences joined together. Amplicons with chimeric sequences can form during PCR.
- Chimeras are common in amplicon sequencing when closely related sequences are amplified.
- The majority of chimeras are believed to arise from incomplete extension.
- During subsequent cycles of PCR, a partially extended strand can bind to a template derived from a different but similar sequence. This then acts as a primer that is extended to form a chimeric sequence.
- In 16S sequencing, we typically find that only a small fraction of reads is chimeric, perhaps of the order of 1% to 5%.



Steps of sequence analysis

Amplification and sequencing of rRNA genes

Removing chimeric sequences from DNA sequence

- Many online tools are available to detect and remove chimeras:
- Decipher:
- http://decipher.cee.wisc.edu/FindChimeras.html
- Bellerophon:
- http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl
- Pintail:
- http://www.mybiosoftware.com/pintail-1-1-analyse-16s-rrna-chimera-anomalies.html
- Mallard:
- http://greengenes.lbl.gov/cgi-bin/nph-index.cgi

Steps of sequence analysis

Purification of PCR Product

Qualitative and Quantitative Analysis of Purified PCR Product

- **Purification of PCR Product:**
- PCR amplified product which was cooled to 4°C and stored at -20°C was taken out from the freeze.
- Purification of PCR products was carried out using QIAquick™ Purification Kit (QIAGEN, Cat No. 28104).
- The purified PCR products were electrophoresed through agarose gel.
- Then, quantitation of the purified PCR was done by reading the absorbance of DNA at 260nm by using spectrophotometer Perkin Elmer MBA 2000 version 1.04.
- Purity of the PCR product was obtained by reading the absorbance at wavelength of 260/280nm.

Wan Leng,2005

DNA sequencers

MicroSeq™

MicroSeq™ 16S rRNA gene sequencing

- Genomic DNA is extracted directly from dead or alive bacterial colonies grown under any conditions.
- The 16S rRNA gene is amplified using universal primers and thermalcyclers.
- The amplified 16S rRNA gene product is sequenced using dye terminator cycle sequencing chemistry.
- The sequence reactions are analyzed using automated DNA sequencers and software.
- Unknown bacteria samples are identified using the MicroSeq™ microbial identification software and 16S rRNA gene sequence database, containing over 1,500 entries.
- Dependant upon the level of confidence, speed and cost desired, either the entire 1540 base pair 16S rRNA gene, or a smaller portion of the gene can be (500 bp) sequenced.
- A printout of the actual sequence data and the identification is included in each report.

See also classification file.

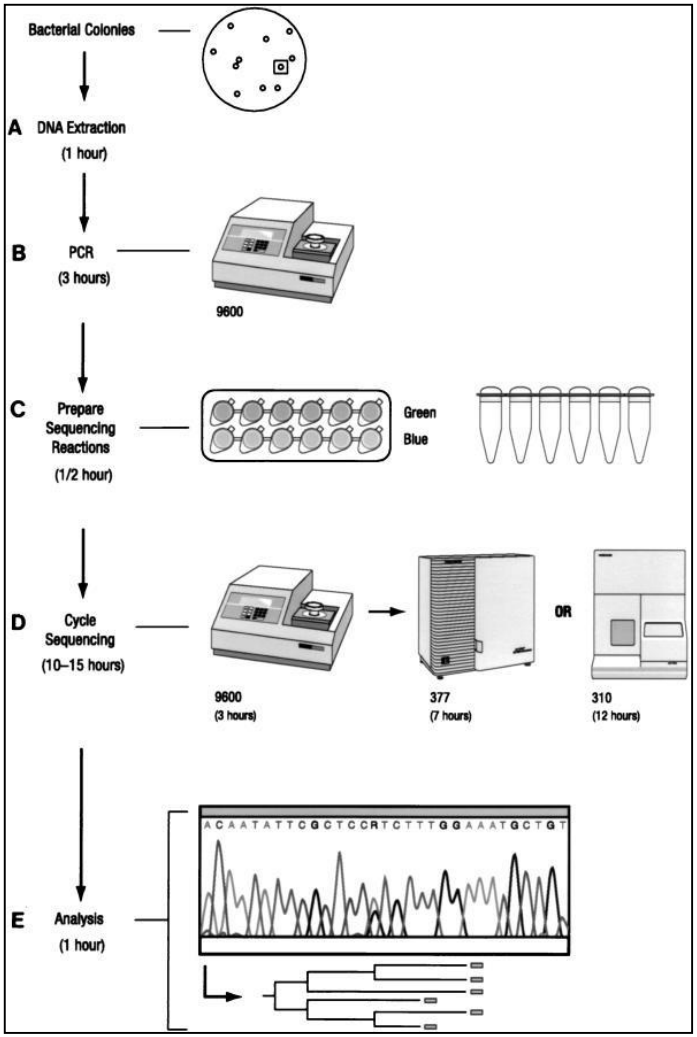
Sequence analysis of 16S rRNA gene
MicroSeq system

- Flowchart of the MicroSeq process from culture to sequence
- The total elapsed time was 15.5 to 18.5 h, comprising:

A. Bacterial DNA extraction,
B. PCR,
C. Sequencing reaction preparation,
D. Cycle sequencing,
E. Analysis.

- The time required for each step is indicated.

Tang *et al.*,1998



Steps of sequence analysis

Amplification and sequencing of 16S rRNA genes

Extract DNA and set up a PCR reaction

1. Scrape a small blob of cells from the agar plate.
2. Stir the loop into a microcentrifuge tube containing 500 ml of sterile water. Close the tube and vortex very well (about 1 minute or until the cell clump is dispersed).
3. Place the tubes into a boiling water bath for 10 min. Use the special tube holder to prevent the tops of the tubes from opening during boiling.
4. Remove from the bath and centrifuge the tube contents for 10 min at 13,000 rpm in the microcentrifuge.
5. Transfer approximately half the supernatant to clean, labeled microcentrifuge tubes using an automatic pipettor.

- This will serve as the DNA template for your PCR reaction.
- If you need to stop at this point, you can store the DNA preparation (i.e., supernatant) in the freezer.

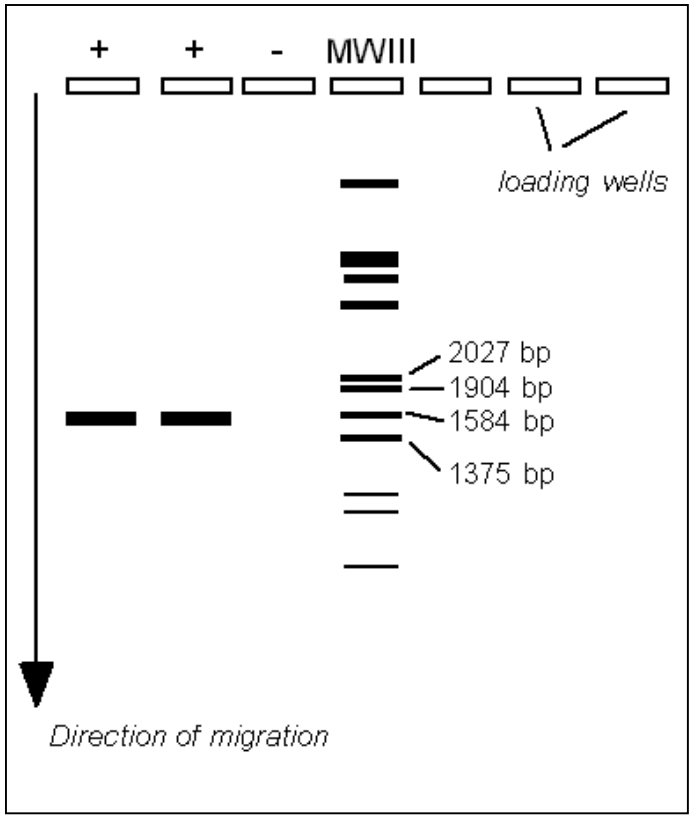
Moran,2010

Steps of sequence analysis

Sequence analysis of 16S rRNA gene

Examine PCR product on agarose gel

- Agarose gel showing ~1500 bp PCR product from the bacterial 16S rRNA gene.
- The first two lanes (labeled '+') are PCR products from two bacterial isolates.
- The third lane (labeled '-') is the negative control.
- The fourth lane (labeled 'MWIII') is the molecular weight marker.



Moran,2010

DNA sequencing
Purified PCR products

- The purified PCR products together with the universal or specific primers were sent for sequencing.
- Sequence analysis software compares genes analyzed from unknown bacteria to a proprietary 16S rDNA sequence library.

DNA sequencing

Purified PCR products

More than one band on the gel

1. If you have a specific band after running your sample on the gel you can clean your PCR product using a cleaning kit and then you send the clean PCR product for sequencing with your specific primers.
2. But if you have more than one band on the gel you'll need a different strategy like:

i. cloning into competent cells;

ii. cut the band on the proper size (your band expected size) then clean it and send it for sequencing with the specific primers.

Cut out the slice of agarose containing the DNA fragment of interest, and store it at 4°C in an Eppendorf tube sealed with parafilm.

DNA sequencing

Purified PCR products

More than one band on the gel need to cut the band

- You need to be sure there are no byproducts/more bands on your PCR product (gel).
- If so, for more safe you cut out specific band from gel and purify it prior the sequencing.
- To do so, cut the band by clean scalpel then weigh my band and jump directly to QIAquick Gel Extraction Kit for further purify.
- After elution step: Just send 20µl of what you have for sequencing.
- For your condition it is enough to send just one primer of the two primers you already did use for your PCR reaction.

DNA sequencing

Purified PCR products


QIAquick Gel Extraction Kit

More than one band on the gel need to cut the band

- The QIAquick Gel Extraction Kit provides:

1. spin columns,
2. buffers, and
3. collection tubes for silica-membrane-based purification of DNA fragments from gels (up to 400 mg slices) or enzymatic reactions.

- DNA ranging from 70 bp to 10 kb is purified using a simple and fast bind-wash-elute procedure and an elution volume of 30–50 µl.
- An integrated pH indicator allows easy determination of the optimal pH for DNA binding to the spin column.
- The procedure can be fully automated on the QIAcube.

DNA sequencing

Purified PCR products

More than one band on the gel need to cut the band

- Disadvantages of this procedure(gel cutting) include low yields, exposure of the DNA to ethidium and UV light, which damages it, and contamination from other bands.
- This procedure seems to cause the most problems among our users-unless you're expecting multiple bands, your time might be better spent adjusting the PCR conditions to give one, or at least a dominant, band.
- You might also consider cloning the products and sequencing individual plasmid clones if several attempts at sequencing a gel-extracted band fails.

Steps of sequence analysis

Sequence analysis of 16S rRNA gene

Take your PCR product for sequencing

- When you have a PCR product of the appropriate size and concentration, take the product for DNA sequencing.
- Choose your isolate with the best PCR product (i.e., strongest band) to continue working with.
- Fill out the "DNA Sequence Request Form" (provided in the laboratory handout) with the required information.
- To keep rest of your PCR product cold during transport, put the tube in a plastic cup filled with ice.
- In approximately 1 week, DNA sequence result will received by e-mail.
- Save a copy of the sequence in a text file on a floppy disk or save the e-mail so you can access the file from another computer.

Moran,2010

Steps of sequence analysis

Sequence Report

Success result

BIONEER

Sequencing Report

Register date : MAR 11th 2011
Register No. : Q20110328-15
Orderer : UPM, plant protection
Name : EISA NAZERIAN

Bioneer messages for customers

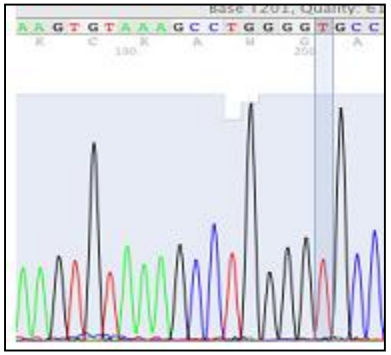
Data Information					
No.	sample name	primer	Result	data group	comment
1	1sh	F	SUCCESS		
2		R	SUCCESS		

Data group	
A	reading length is short or noisy. Re-reaction is recommended.
B	Failed at analysing. Re-reaction is recommended.
C	There are some problem at analysis because of template structure. Re-reaction is not recommended.
D	Re-reaction is not recommended.
Re-reaction result is released after one day.	

Steps of sequence analysis

Sequence Report

Nucleotide sequences and Electropherogram



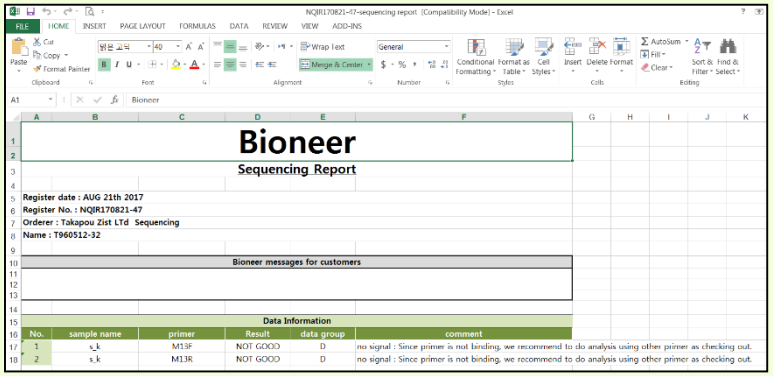
- Sequence data are unambiguous;
- Increased reliablilty and reproducibility;
- Easy to compare with other laboratories;
- Culturables and non-culturables;
- Public databases (GenBank, EMBL,..) available
- Data generated by newer sequencing methods remain the same;
- The availability of accurate software packages and the quality and completeness of database will affect the identification;
- Interpretation of results require some skills (careful attention for sequence alignments and analysis).

Elke,2010

Sequence Report

Nucleotide sequences and electropherogram
Failed at analysing. Re-reaction is recommended

- no signal: Since primer is not binding, we recommend to do analysis using other primer as checking out.



Bioneer

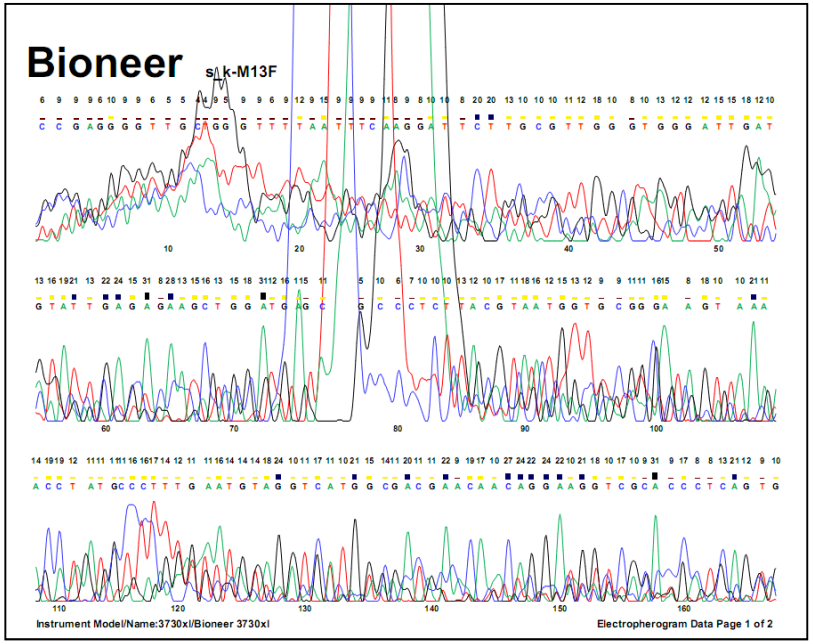
Sequencing Report

Register date : AUG 21th 2017
Register No. : NQIR170821-47
Orderer : Takapou Zist LTd Sequencing
Name : T960512-32

Bioneer messages for customers

Data Information

No.	sample name	primer	Result	data group	comment
1	s_k	M13F	NOT GOOD	D	no signal : Since primer is not binding, we recommend to do analysis using other primer as checking out.
2	s_k	M13R	NOT GOOD	D	no signal : Since primer is not binding, we recommend to do analysis using other primer as checking out.



Base sequence: adenine (A, green), thymine (T, red), cytosine (C, orange), and guanine (G, blue).

Sequence analysis of 16S rDNA

BLAST analysis of 16S rRNA gene sequence

- 16S rDNA amplified products are recovered from agarose gel using a Wizard PCR Preps DNA Purification System (Promega).
- Sequencing is carried out in a DNA sequencer (e.g., ABI 373 Stretch).
- Retrieved sequences can be compared to bacterial rDNA sequences present in the GeneBank, and similarities searched using BLAST tools.
- Phylogenetic trees can be constructed with the programs available at http://rdp.cme.msu.edu.

Spencer and Spencer,2004

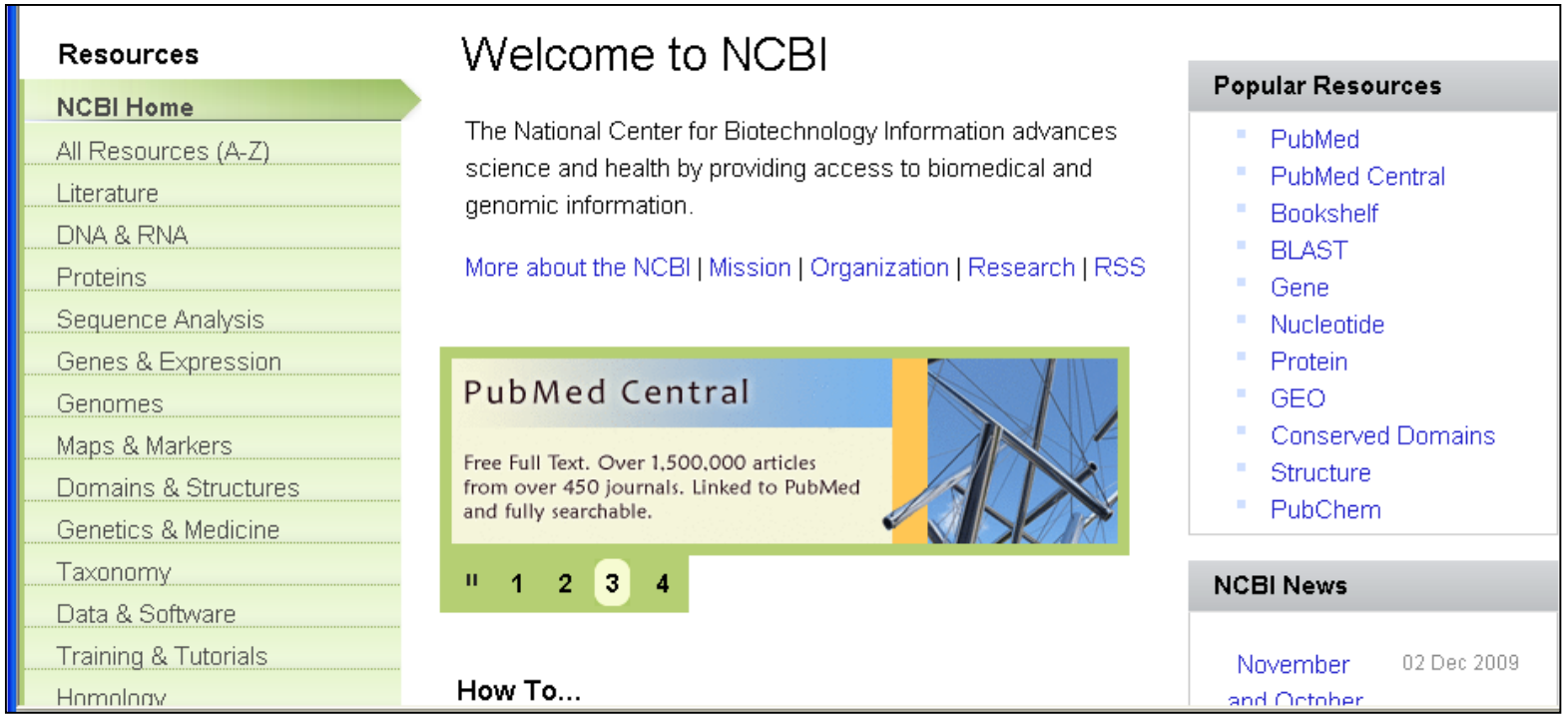
Steps of sequence analysis
BLAST search at GenBank

- To explore biodiversity you will work with the 16S rRNA sequence of your bacterium using databases and programs available on the Internet.
- This exercise will tell you whether your isolate corresponds to a previously characterized species, or whether it is something new.
- If it is new, you can determine which organisms are its closest relatives, and perhaps predict some of the physiological properties of your unknown based on what is known about its relatives.

Moran,2010

NCBI

National Center for Biotechnology Information



Protein and gene sequence comparisons are done with BLAST program on the NCBI website.

Steps of sequence analysis

Nucleotite BLAST

BLAST search at GenBank or other servers

- Choose one of the public servers
  - NCBI www.ncbi.nlm.nih.gov/blast
  - EBI www.ebi.ac.uk/blast
  - EMBNet www.expasy.ch/blast
- Select a database to search:
  - NR to find any protein sequence
  - Swiss-Prot to find proteins with known functions
  - PDB to find proteins with known structures
- Cut and paste your sequence
- Click the **BLAST** button

Spiegel,2007

Steps of sequence analysis
BLAST search at GenBank

- GenBank has approximately 13,543,000,000 bases in 12,814,000 sequence records as of August 2001, derived from viruses, bacteria, and complex eukaryotic organisms.
- Included among these many sequences are a number of 16S rRNA gene sequences from Bacteria and Archaea.
- The current number of 16S rRNA sequences is approximately 12,000.
- Some of these are 'complete' (they cover the entire 1500+ bases of the 16S rRNA gene), while other are 'partial' (like yours will be) and cover only a portion of the gene (your sequence includes only ~400 bases near the beginning of the gene).

Moran,2010

Steps of sequence analysis
BLAST search at GenBank

- A sophisticated computer program called BLAST compares the query sequence you submit to all of the sequences previously deposited in the GenBank database.
- BLAST finds sequences in the database that are most similar to the submitted sequence, and then returns the names and a brief description of the matching sequences in the output from the program.

Query sequence vs. subject sequence
Nucleotide or protein sequence, referred to as a "query" and nucleotide or protein sequences within a database, referred to as "subject" sequences.

Moran,2010

Steps of sequence analysis

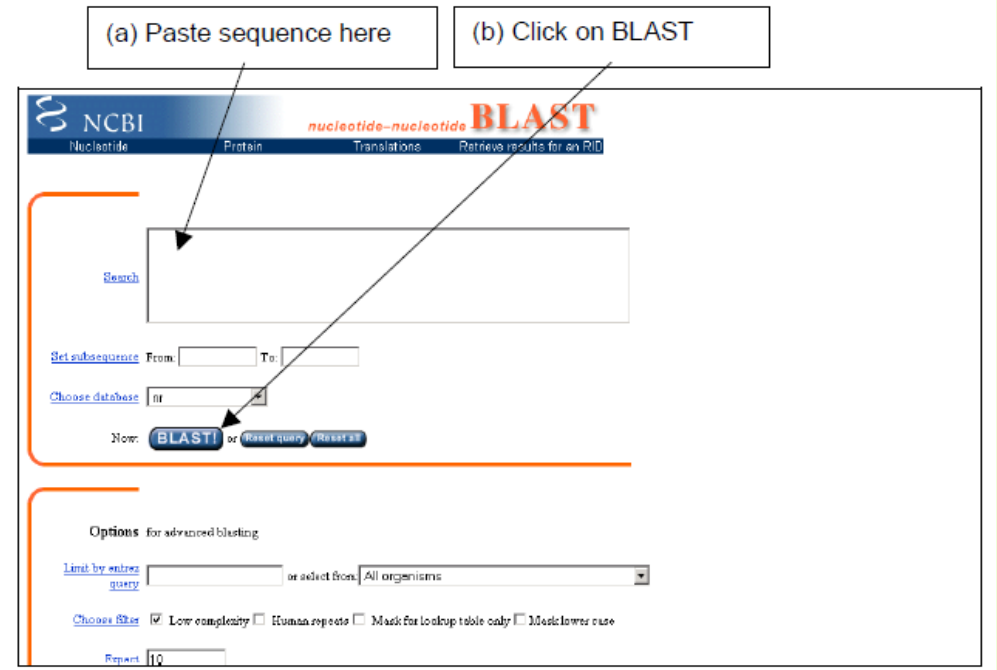
BLAST search at GenBank

- BLAST(Basic Local Alignment Search Tool) is a tool for comparing one sequence with all the other sequences in a database.
- BLAST can compare:
  - DNA sequences
  - Protein sequences
- BLAST is more accurate for comparing protein sequences than for comparing DNA sequences.

Spiegel,2007

Steps of sequence analysis
BLAST search at GenBank

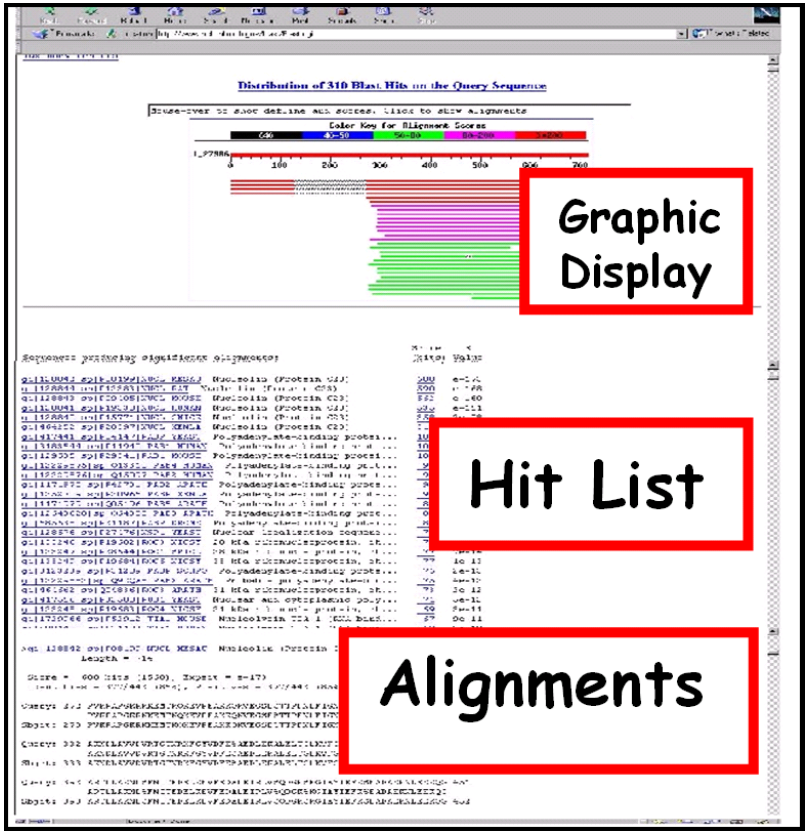
- View of NCBI BLAST page.
- Paste your sequence into the top box labeled 'Search'.
- Start the query by clicking the 'BLAST!' button.



Moran,2010;Boonham,2008

Interpreting BLAST result
Reading BLAST Output

- Graphic Display
  - Overview of the alignments
- Hit List
  - Gives the score of each match
- Alignments
  - Details of each alignment



Spiegel,2007

Interpreting BLAST result

Reading BLAST Output

- Graphic Display
  - Overview of the alignments
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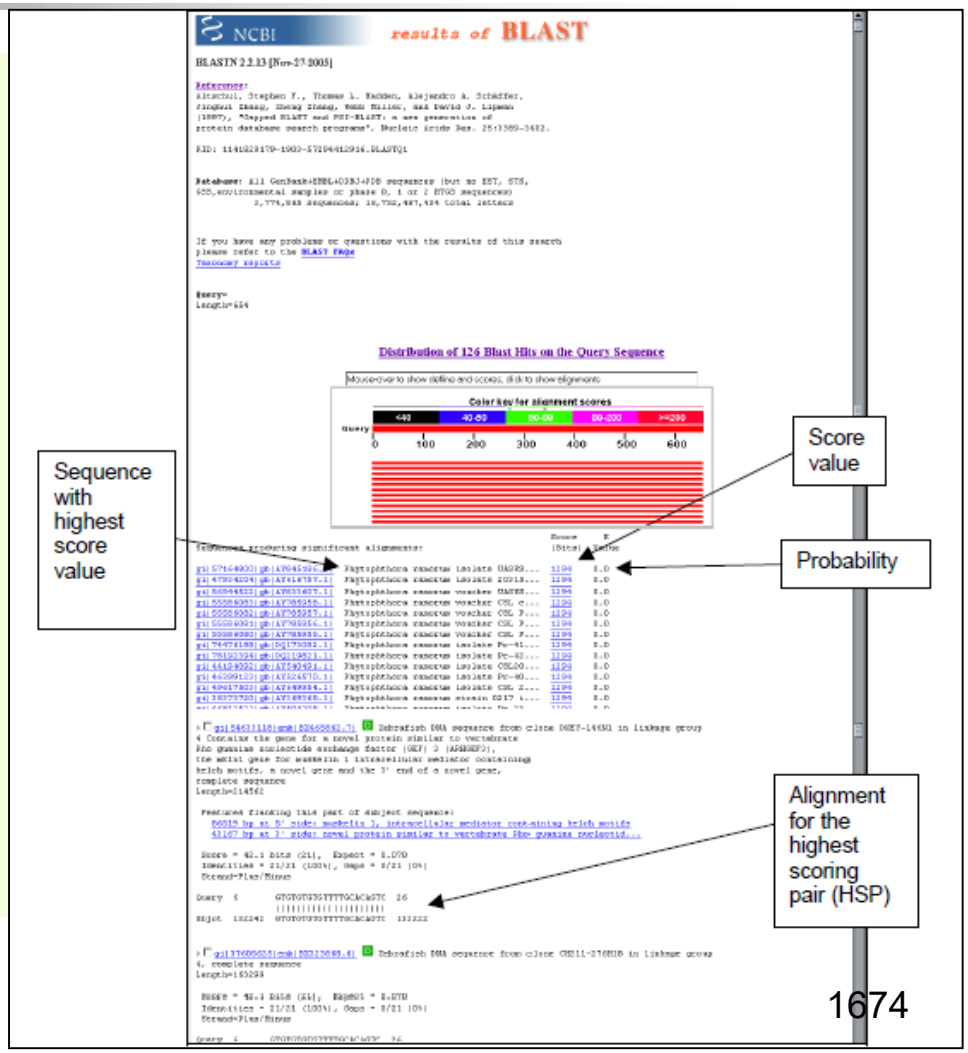


Spiegel,2007;Boonham,2008

Interpreting BLAST result

Interpreting the score values

- The first format (under the heading "Distribution of Blast Hits on the Query Sequence") is a graphical representation of the results, showing the matches to your input sequence as a stack of colored bars with the best matches at the top.
- The color of the bar indicates the match score (i.e., degree of similarity) between your sequence and the database matches.
- **Red indicates the best matches.**
- Black indicates the poorest.
- The length of the bar shows the length of the sequence that was noticeably similar to the query.

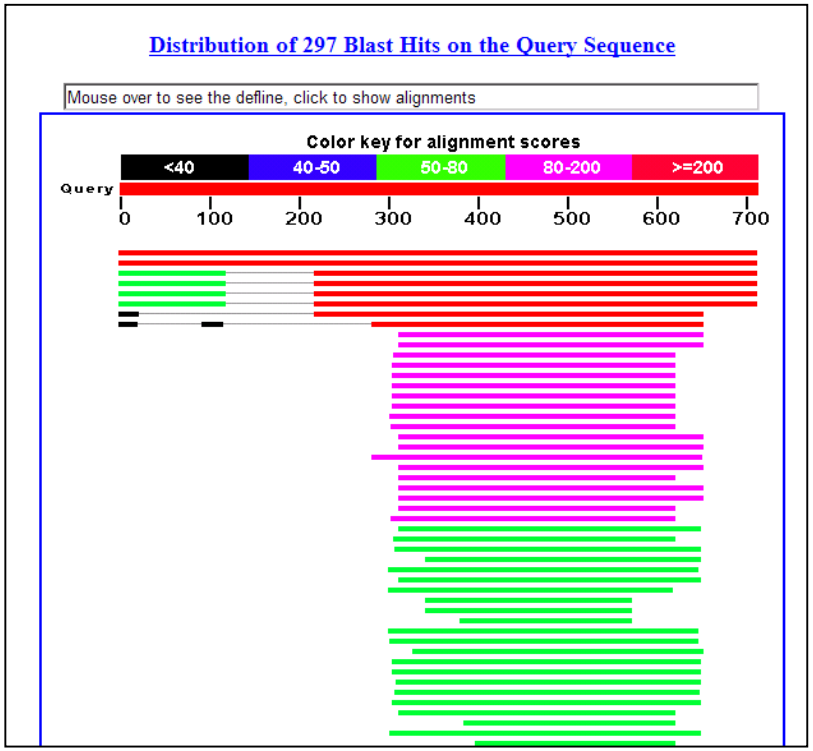
Moran,2010

Interpreting BLAST result

Reading BLAST Output

The Graphic Display

- The Horizontal Axis (0-700) corresponds to your protein (query).
- Color codes indicate that match's quality
  - Red: very good
  - Green: acceptable
  - Black: bad
- Thin lines join independent matches on the same sequence.



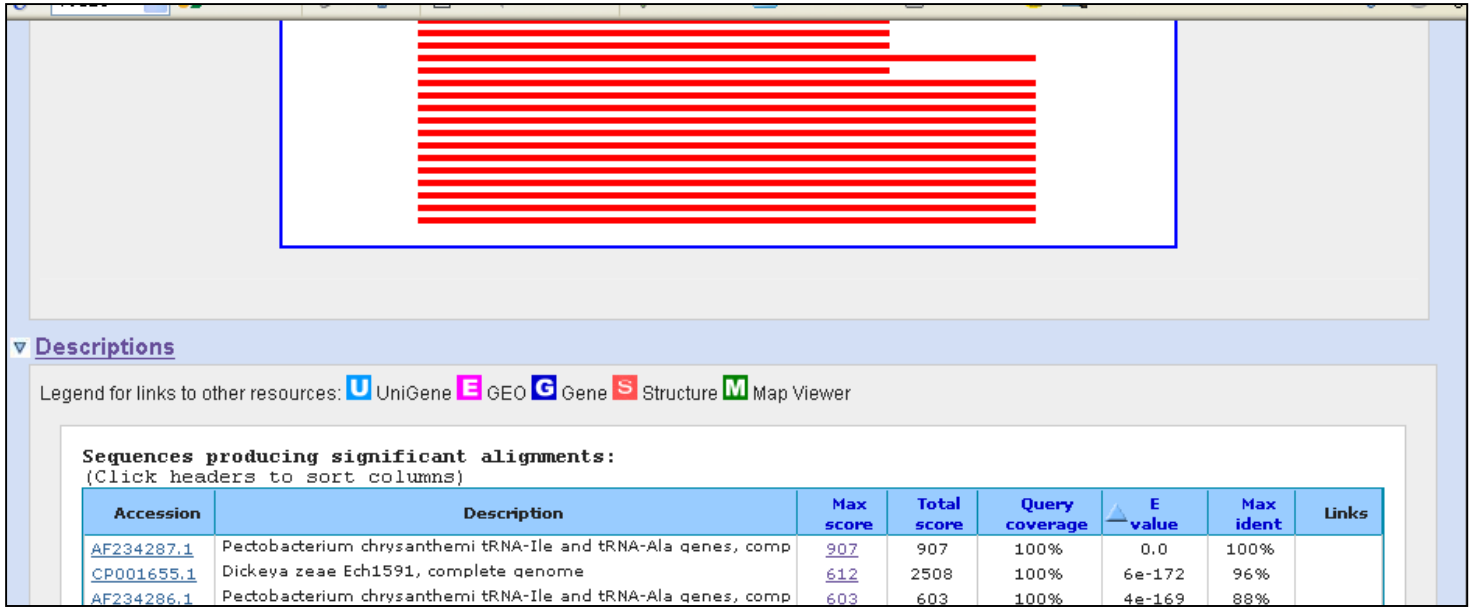
Spiegel,2007

Interpreting BLAST result

The Graphic Display

16S rDNA sequence of *Pectobacterium* spp.

- 10 sequences of bacteria with the highest identities against the query were used.
- BLAST analysis of the 16S rDNA consensus sequences showed 99.9% identity to NCBI database 16S rDNA sequences.



Descriptions

Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer

Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
AF234287.1	Pectobacterium chrysanthemi tRNA-Ile and tRNA-Ala genes, comp	907	907	100%	0.0	100%	
CP001655.1	Dickeya zeae Ech1591, complete genome	612	2508	100%	6e-172	96%	
AF234286.1	Pectobacterium chrysanthemi tRNA-Ile and tRNA-Ala genes, comp	603	603	100%	4e-169	88%	

Steps of sequence analysis

GenBank

Depositing sequences in GenBank

- Before submitting a request, remove any numerical digits in the query sequence or replace them with the appropriate letter codes (e.g., N for an unknown nucleotide residue or X for an unknown amino acid residue).

A	Adenine
G	Guanine
C	Cytosine
T	Thymine
U	Uracil
R	Purine (A or G)
Y	Pyrimidine (C or T)
Z	2-Aminoadenine
N	**Any nucleotide**
W	Weak (A or T)
S	Strong (G or C)
M	Amino (A or C)
K	Keto (G or T)
B	Not A (G or C or T)
H	Not G (A or C or T)
D	Not C (A or G or T)
V	Not T (A or G or C)

Steps of sequence analysis

GenBank

Depositing sequences in GenBank

- GenBank is the National Institutes of Health (NIH) genetic sequence database, a collection of all publicly available DNA sequences.
- Scientists who determine the sequences of genes (or sometimes of entire genomes) deposit the sequence information in GenBank.
- Aside from doing this as a service to the scientific community, many journals require submission of new sequence data to GenBank as a precondition to publishing a manuscript referring to the sequence.

Moran,2010

Steps of sequence analysis

GenBank

Depositing sequences in GenBank

- All sequence entries at GenBank are identified by a pair of identifiers:

1. An accession, and
2. A numeric identifier, and this number is frequently called a GI number (GenInfo Identifier).

- The accession is stable, but each new version of the sequence entry for the accession receives a new GI number.

ACCESSION - The primary accession number is a unique, unchanging code assigned to each entry. Used often when citing sequence in journals.
VERSION - The primary accession number and a numeric version number associated with the current version of the sequence data in the record. This is followed by an integer key (a "GI") assigned to the sequence by NCBI.

Steps of sequence analysis

GenBank

Depositing sequences in GenBank

- Identification of free-living N-fixing bacterial isolates using BLAST analysis.
- *DNA sequences for NC4 and NC10 were deposited in Genbank with the following accession numbers:
- KT008116.1 and
- KT008115.1, respectively

Isolate (s)	Accession no.	Bacteria ID	Sequence similarity (%)
NC2	JN834034	*Bacillus cereus*	96
*NC4	EU910254	*Lysinibacillus* sp.	98
*NC10	HM037179	*Klebsiella* sp.	99
NC11	HM037179	*Klebsiella* sp.	99
NFC1	JF514501	*Bacillus cereus*	97
NFC4	KC250221	*Bacillus subtilis*	100
NFC6	JQ831466	*Bacillus cereus*	96
NFC7	JX649951	*Bacillus licheniformis*	99

Emmyrafedziawati and Stella,2018

Steps of sequence analysis

GenBank

GenBank release notes

- LOCUS: GT222015 402 bp mRNA linear EST 22-JUN-2010.
- DEFINITION: gh1521 *Candidatus* Phytoplasma aurantifolia cDNA AFLP Citrus aurantiifolia/*Candidatus* Phytoplasma aurantifolia mixed EST library cDNA clone gh1521, mRNA sequence.
- ACCESSION number: GT222015
- VERSION: GT222015.2
- AUTHORS: Ghayeb Zamharir, M., Hasanzadeh, N., ZamaniZadeh, H.R., AliZadeh AliAbadi, A., Mardi, M. and Hoseini Salekdeh, G.
- Oganism: bacteria
- On Oct 19, 2009 this sequence version replaced gi:261597083.

Steps of sequence analysis

GenBank

Depositing sequences in GenBank

- So, the two types of sequence identification numbers are:

1. GI (GI/gi number), and
2. VERSION,

- These have different formats and were implemented at different points in time.

1. A GI/gi number (genInfo identifier) is a unique integer which identifies a particular sequence; the gi number will change every time the sequence changes. Thus, GI number has been used for many years by NCBI to track sequence histories in GenBank and the other sequence databases it maintains.
2. The VERSION system of identifiers was adopted in February 1999 by the International Nucleotide Sequence Database Collaboration (GenBank, EMBL, and DDBJ).

Steps of sequence analysis

GenBank

Depositing sequences in GenBank

1. **GI number** (sometimes written in lower case, "gi") is simply a series of digits that are assigned consecutively to each sequence record processed by NCBI.

- The GI number bears no resemblance to the Accession number of the sequence record.
- Nucleotide sequence GI number is shown in the VERSION field of the database record.
- Protein sequence GI number is shown in the CDS/db_xref field of a nucleotide database record, and the VERSION field of a protein database record.

Steps of sequence analysis

GenBank

Depositing sequences in GenBank

- The gi number provides a unique identifier that specifies an exact sequence.
- If a sequence in GenBank is modifed, even by a single base pair, a new gi number is assigned to the updated sequence (example: from gi 5868931 to gi 6358754).
- However, the accession number stays the same (example: AC009999).
- This ensures that anyone who analyzes gi 5868931 and stores the analysis can be sure it will be valid as long as AC009999 has gi 5868931 attached to it.

Steps of sequence analysis

GenBank

Depositing sequences in GenBank

- The gi number is stable and retrievable.
- NCBI keeps the last version of every gi number and includes this history in the record.
- For example, anyone can examine this history and determine that gi 5868931 was replaced by gi 6358754.
- Upon aligning gi 5868931 to gi 6358754 to determine their relationship, a researcher may decide to remap the former analysis to gi 6358754, or perhaps reanalyze the data.

Steps of sequence analysis

GenBank

Depositing sequences in GenBank

2. **VERSION** is made of the accession number of the database record followed by a dot and a version number (and is therefore sometimes referred to as the "**accession.version**").

- Nucleotide sequence version contains two letters followed by six digits, a dot, and a version number (or for older nucleotide sequence records, the format is one letter followed by five digits, a dot, and a version number).
- Protein sequence version contains three letters followed by five digits, a dot, and a version number.

Steps of sequence analysis

GenBank

Preferred method of citing sequences

- The preferred method of citing sequences is with the Accession.Version number.

1. The Accession identifies a particular sequence record while
2. The version number tracks changes to the sequence itself.

- From the above example, AC009999.1 refers to gi 5868931 while AC009999.2 refers to the updated version of the sequence, gi 6358754.
- If you are trying to find the most recent version of a sequence, use the accession number as your query, since using accession. version will return a specific version of the sequence.

Software analysis
Phylogenetic Tree Building at RDP

- Use a browser to access the RDP analysis page (http://rdp.cme.msu.edu/html/analyses.html);
- Find the 'Phylip Interface' row and click on the 'run' triangle under the Small Subunit heading.
- You are now on the 'Start' page for the Phylip analysis tools.
- Brings your sequence into RDP and formats it for tree building……
- Choose the 'Distance Matrix' button from the top of the page. This takes you to a tool that will compare the sequences.
- Click on the 'Calculate Matrix' button to generates a table showing relatedness for all pairs of sequences, and these are the values that will be used to make the tree.
- Next click on 'Phylogenetic Tree'…..
- Your phylogenetic tree is generated in the main window.

Moran,2010

Ribosomal Database Project
A large collection of rRNA sequences

- Ribosomal database project (RDP) Classifier was used to assign taxonomic lineage to the genus level (http://rdp.cme.msu.edu).
- Ribosomal database project (RDP):

1. Provides a variety of analytical programs.
2. RDP Release 10, Update 32*:* May 14, 2013: 2,765,278 16S rRNAs.

- There are several technological and scientific advantages for using 16S rRNA genes sequences for studying the phylogeny of bacteria. The main assets are:

1. The availability of a near-universal database;
2. The availability of highly conserved 16S rRNA primers.

Vandamme,2013;..

PHYLIP
Version 3.6

- It includes programs to carry out parsimony, distance matrix methods, maximum likelihood, and other methods on a variety of types of data, including DNA and RNA sequences, protein sequences, restriction sites, 0/1 discrete characters data, gene frequencies, continuous characters and distance matrices.
- It is the most widely-distributed phylogeny package, with over 20,000 registered users, some of them satisfied.
- It competes with PAUP* to be the program responsible for the most published trees.
- It has been distributed since October, 1980 and is now celebrating its 25th anniversary, as the oldest distributed phylogeny package.
- PHYLIP is distributed at the PHYLIP web site at http://evolution.gs.washington.edu/phylip.html.

See phylogeny file

Software analysis
Phylogenetic Tree Building at RDP

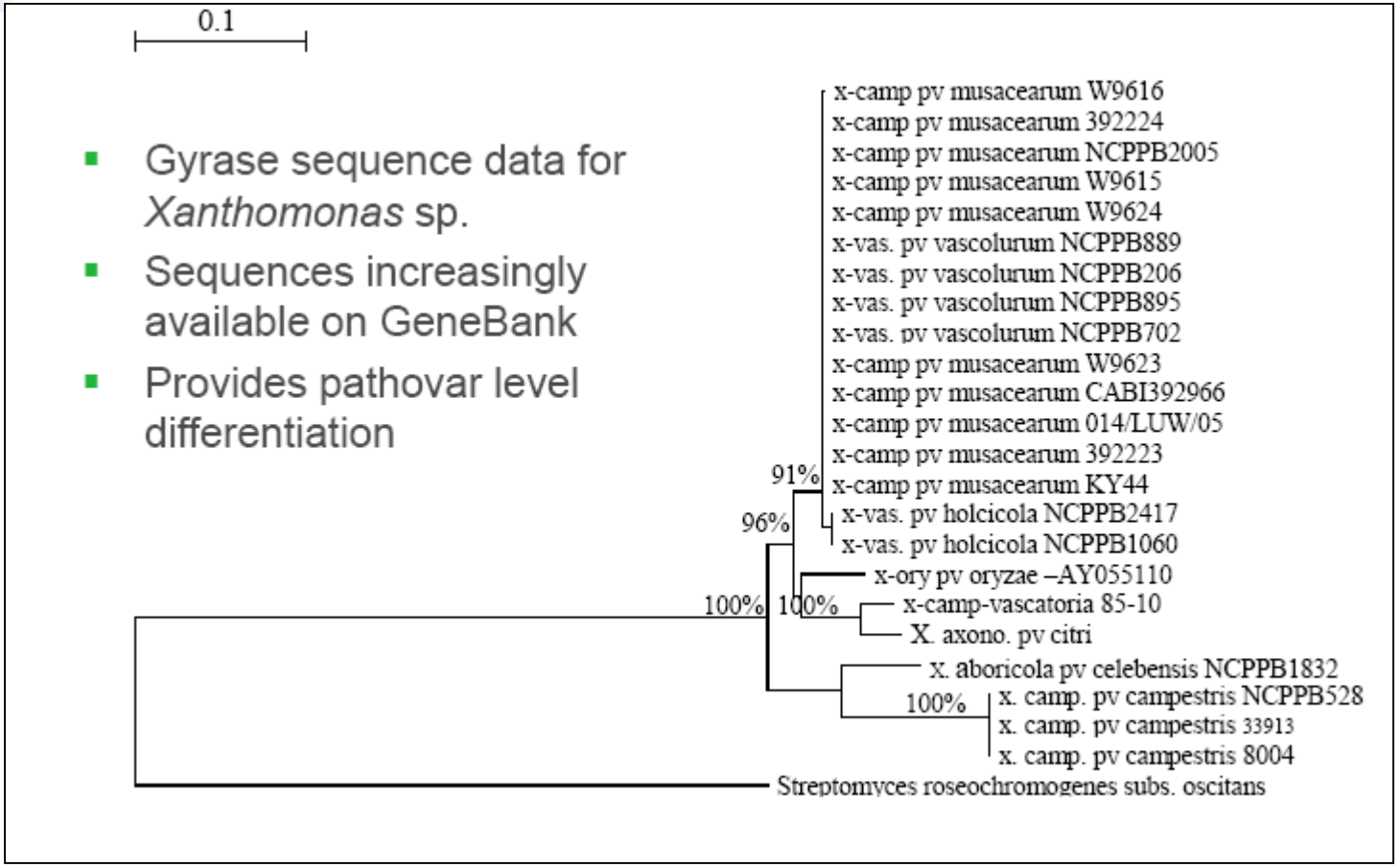
- Each bacterial sequence was subjected to software analysis (www.ebi.ac.uk and http://itol.embl.de/,....) to draw phylogenic tree.
- The Ribosomal Database Project (RDP) hosts a web site dedicated to the analysis of bacterial 16S rRNA sequences.
- You will use the tools at the RDP site to build a phylogenetic tree showing the relationship of your bacterium to some better-known and better-studied bacteria.
- The tree will group 16S rRNA genes according to sequence similarity, providing information on how your bacterium fits into the existing taxonomic framework for prokaryotes.

Large number of 16S rDNA sequences were generated and deposited in public databases, such as the Ribosomal Database Project (RDP) database.

Moran,2010;…

Sequence alignment

Gyrase sequence data for *Xanthomonas* sp.

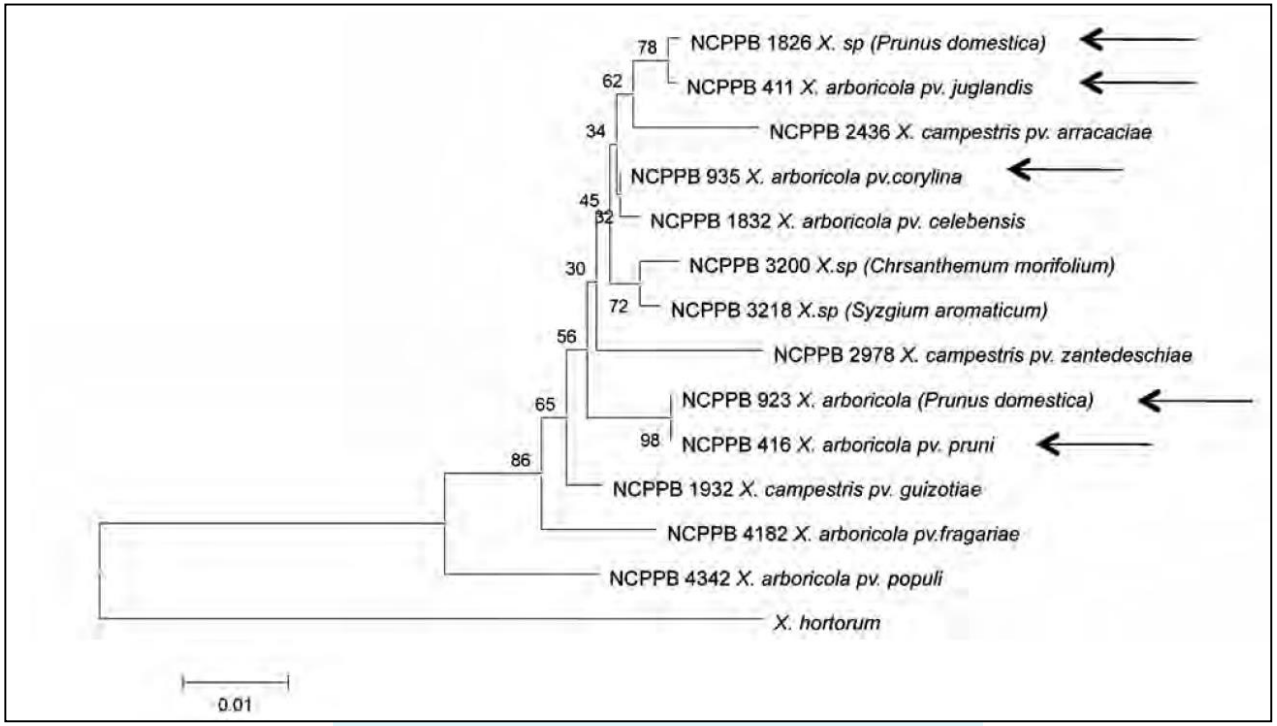


Julian Smith

Sequence alignment

Gyrase sequence data for *Xanthomonas* sp.

- Phylogenetic discrimination *X. arboricola* strains by neighbour-joining analysis of the *gyrB* locus (Parkinson *et al.*,2009).
- Arrows indicate stone fruit and nut pathogens.

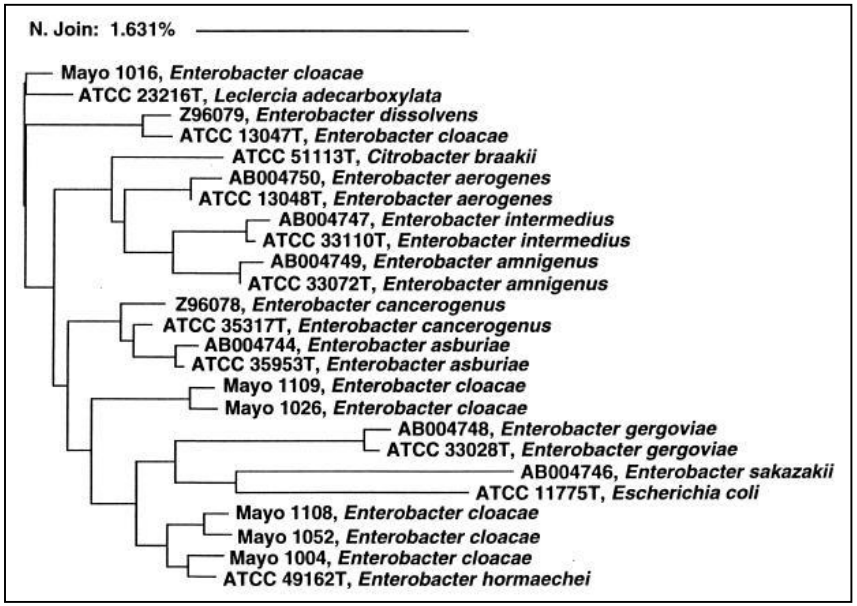


Parkinson and Elphinstone,2010

Identification of *Enterobacter* spp.

Based on sequence analysis of different regions of the 16S rRNA gene

- Neighbor-joining analysis of DNA sequences from several *Enterobacter* spp.
- Phylogenetic analysis was based on full 16S rRNA gene sequences, and the scale reflects relative phylogenetic distance.
- Isolates with names beginning with Mayo were evaluated in this study.
- Isolates with names beginning with accession numbers were retrieved from GenBank.
- The remaining isolates, whose names begin with ATCC numbers, were type strains stored in the MicroSeq database.



Tang *et al.*,1998

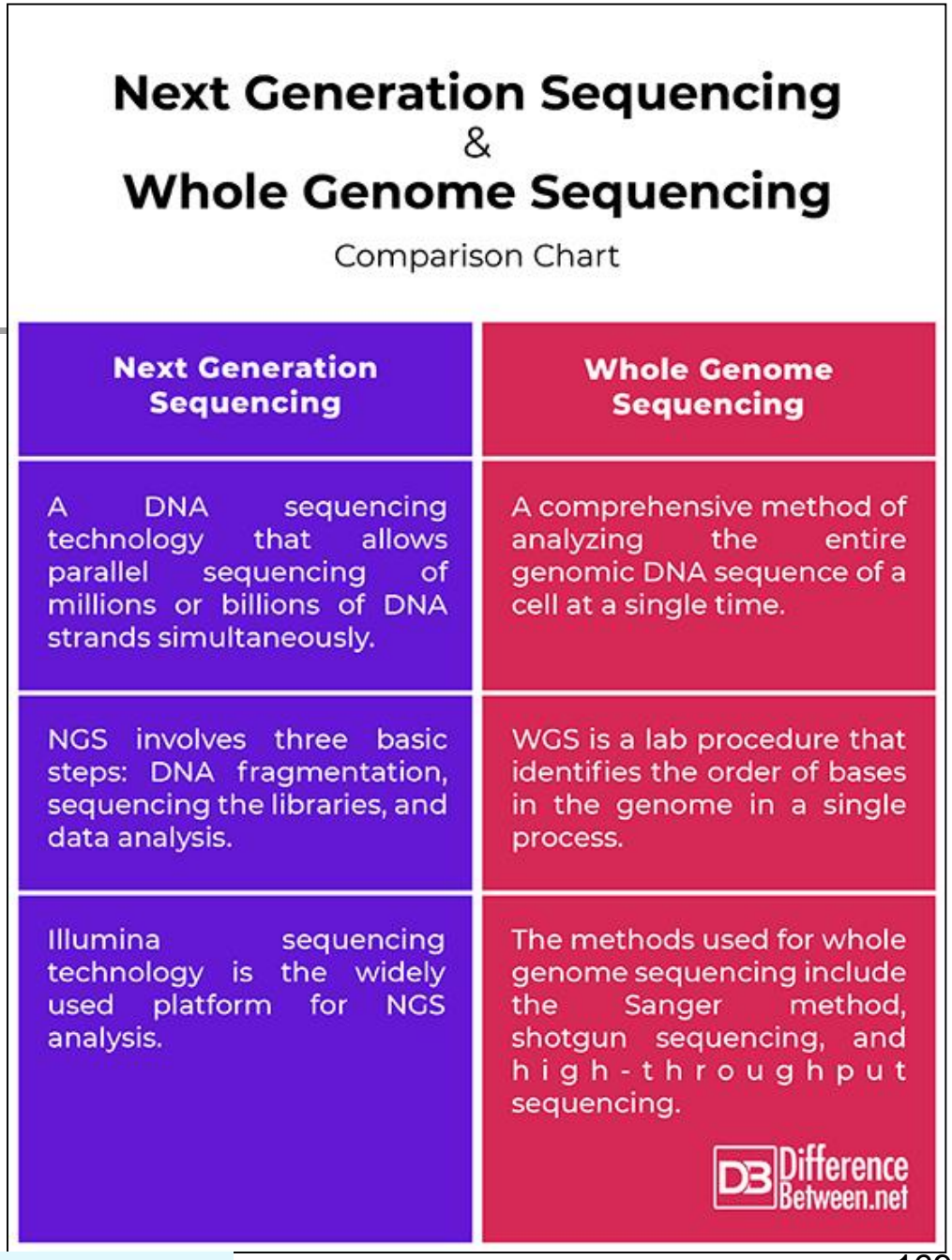
Next-generation sequencing(NGS) Whole genome sequencing(WGS)

- Next-generation sequencing (NGS) is a massively parallel sequencing technology that offers ultra-high throughput, scalability, and speed.
- **It determines the order of nucleotides in entire genomes or targeted regions of DNA or RNA.**

- Whole genome sequencing (WGS) provides the most comprehensive data about a given organism.
- **It determines the entire DNA sequence all at once.**

See also the Bacterial Diagnosis part1 ppt.

The conventional Sanger sequencing method which is still considered as the gold standard for sequencing has its limitations.
With the ability to sequence more than a million DNA fragments at a time, the next-generation sequencing (NGS) has revolutionized the ability to generate large volumes of sequence data at an extremely low cost.



Khillar, 2021

Next-generation sequencing(NGS)
Massively parallel sequencing technology

- Next-generation sequencing (NGS) or high-throughput sequencing, is a massively parallel sequencing technology have exponentially increased the quantity of sequences generated, producing up to several million bases (Gb) in a single run.
- The main concept behind them is similar (library preparation).
- The technology is used to determine the order of nucleotides in:

1. entire genomes, or
2. targeted regions of DNA or RNA.

Natalie Robertson; Pritchard *et al.*,2016;..

Next-generation sequencing(NGS)
Massively parallel sequencing technology
Starting materials(DNA preparation)

- In general, the core steps in preparing RNA or DNA for NGS analysis are:

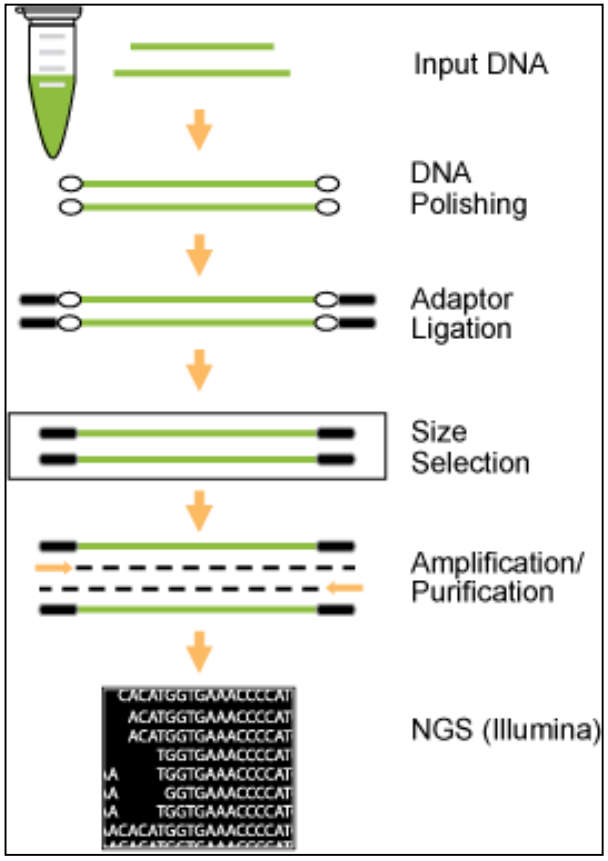
1. fragmenting and/or sizing the target sequences to a desired length,
2. converting target to double-stranded DNA,
3. attaching oligonucleotide adapters to the ends of target fragments, and
4. quantitating the final library product for sequencing.

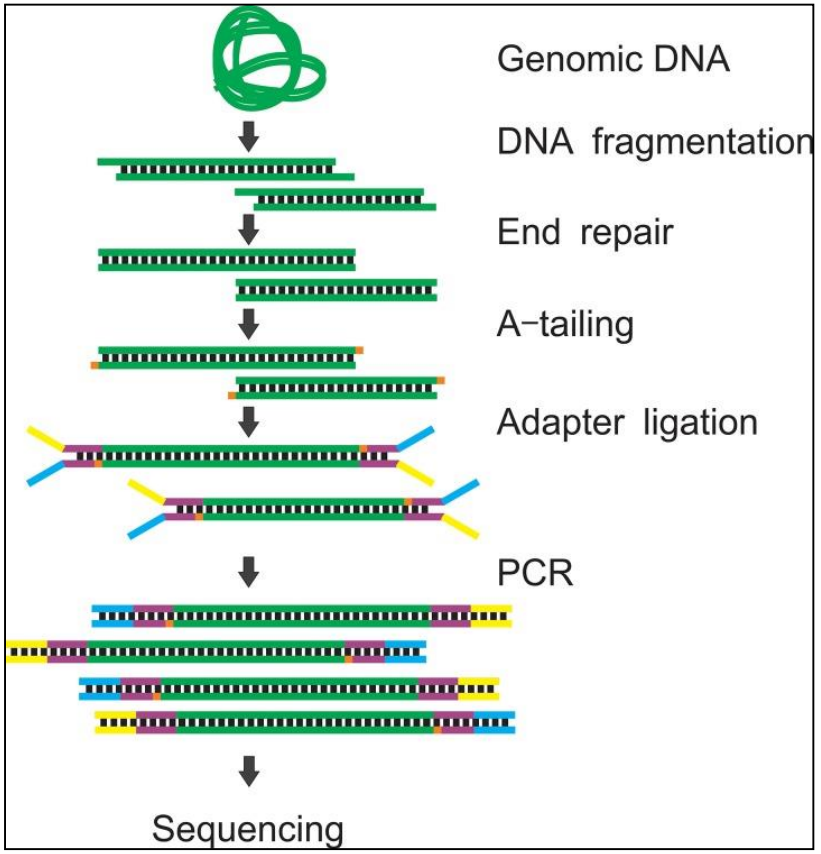
Head *et al.*,2018

Next-generation sequencing(NGS)

Massively parallel sequencing technology

Workflow from different points



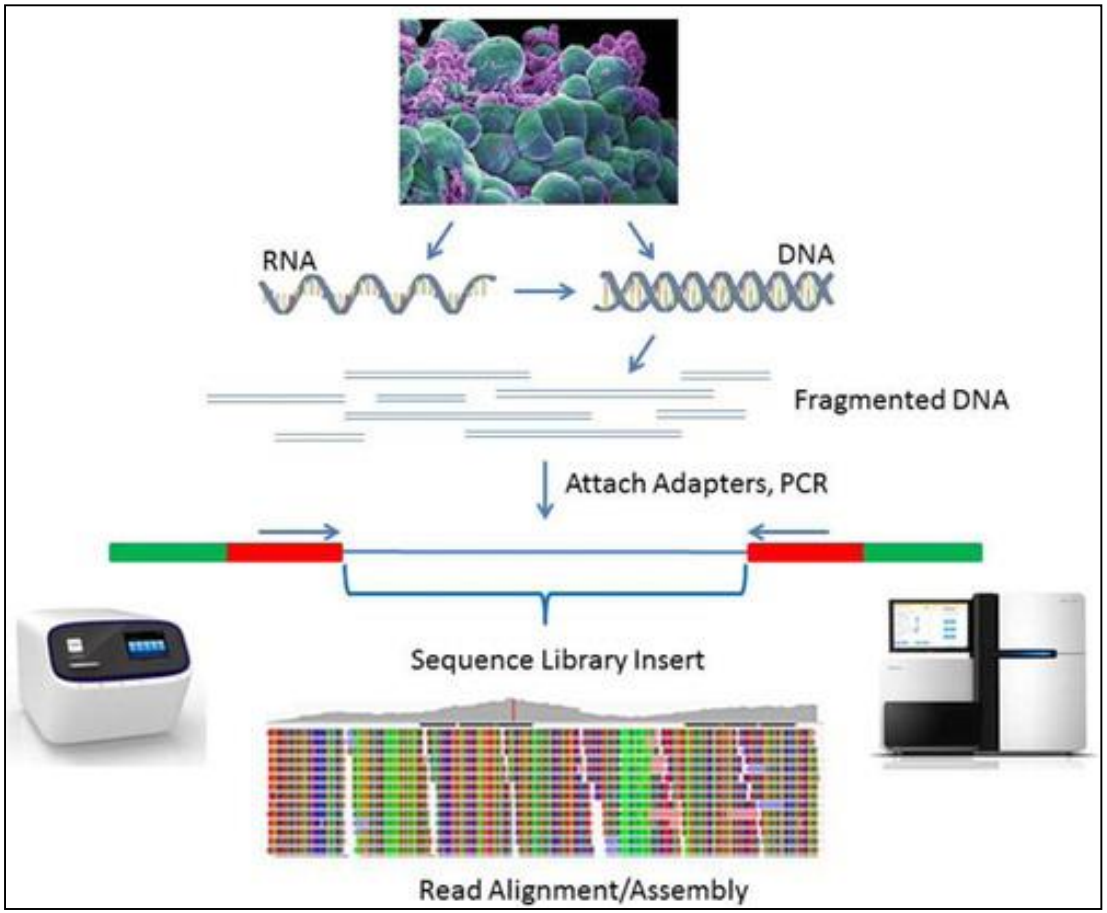


EpiGentek Group Inc.; Ma *et al.*,2017

Next-generation sequencing(NGS)

Massively parallel sequencing technology

Basic workflow for NGS library preparation

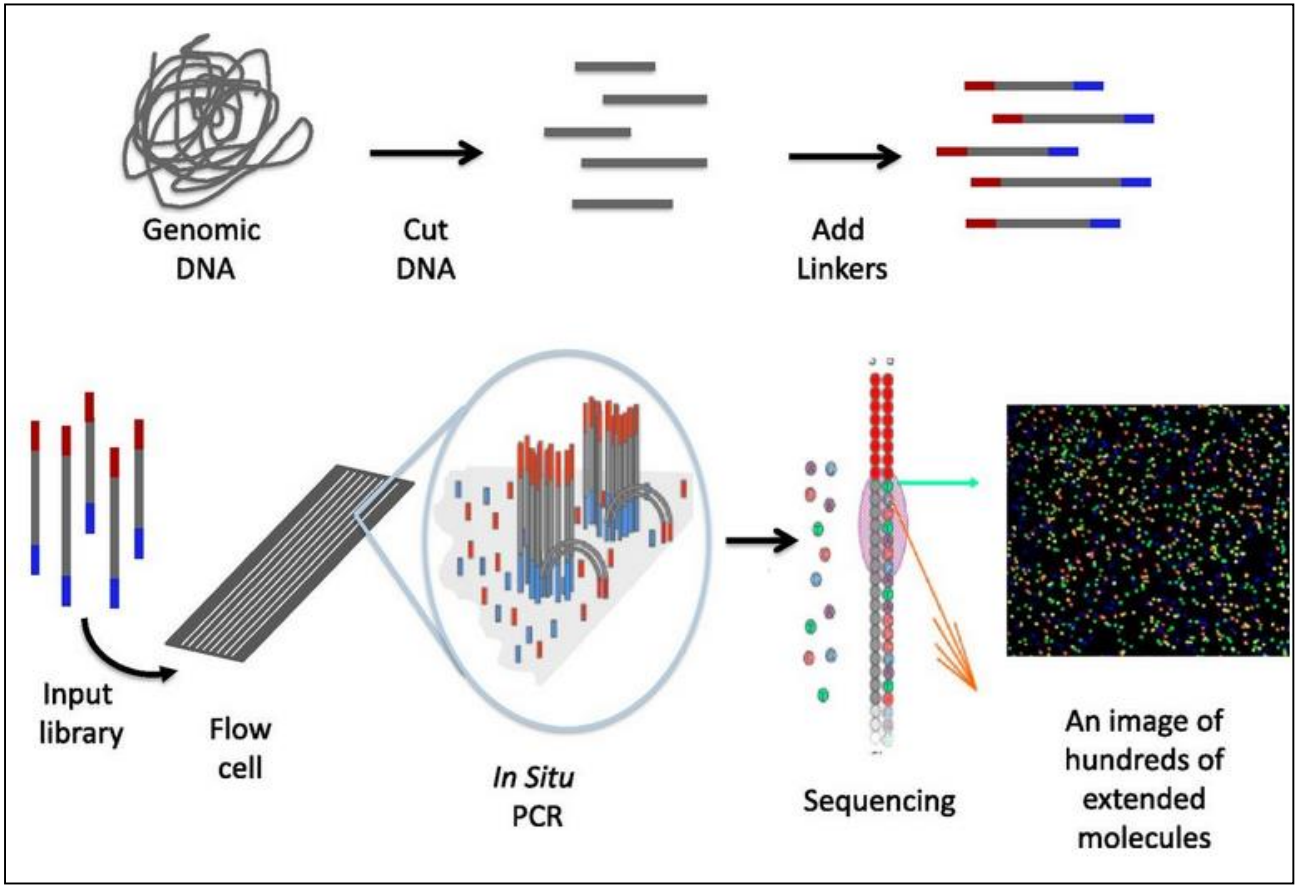


Head *et al.*,2018

Next-generation sequencing(NGS)

Massively parallel sequencing technology

Basic workflow for NGS library preparation



Johnsen *et al.*,2013

Next-generation sequencing(NGS)
Massively parallel sequencing technology
Starting materials(DNA preparation)

- Starting materials can include fragmented dsDNA isolated from various tissue or cell samples, dsDNA enriched from a ChIP reaction, MeDIP/hMeDIP reaction, or exon capture.
- DNA should be relatively free of RNA because large fractions of RNA will impair end repair and dA-tailing, resulting in reduced ligation capabilities.
- The input amount of DNA can be from 0.2 ng to 100 ng. For optimal preparation, the input amount should be 10 ng to 50 ng.

Next-generation sequencing(NGS)
Massively parallel sequencing technology
DNA library preparation

- The EpiNext High-Sensitivity DNA Library Preparation Kit (Illumina) includes all reagents required at each step of the workflow to carry out a successful DNA library preparation.
- In the library preparation, DNA is first fragmented to an appropriate size (about 300 bps in peak size).
- The end repair/dA tailing (end polishing) of the DNA fragments are performed simultaneously.
- Note: because DNA fragmentation does not result in homogeneous, blunt-ended fragments, end repair is needed to ensure that each molecule is free of overhangs. The DNA dA-Tailing Kit efficiently adds a non-template dAMP (dA) to the 3' end of a blunt-ended DNA fragment.
- dAMP: damage-associated molecular pattern.

Next-generation sequencing(NGS)
Massively parallel sequencing technology
DNA library preparation

- Adaptors are then ligated to both ends of the polished DNA fragments for amplification and sequencing.
- Ligated fragments are size selected and purified with MQ beads, which allows for quick and precise size selection of DNA.
- Size-selected DNA fragments are amplified with a high-fidelity PCR Mix that ensures maximum yields from minimum amounts of starting material and provides highly accurate amplification of library DNA with low error rates and minimum bias.

Next-generation sequencing(NGS)
Multiplex sequencing
Multiple library preparation/indexes (barcodes)

- To save resources, multiple libraries can be pooled together and sequenced in the same run—a process known as multiplexing.
- During adapter ligation, unique index sequences, or barcodes, are added to each library.
- These barcodes are used to distinguish between the libraries during data analysis.
- A de-multiplexing algorithm sorts the reads into different files according to their indexes.
- Each set of reads is aligned to the appropriate reference sequence.

Next-generation sequencing(NGS)
Multiplex sequencing
Multiple library preparation/indexes (barcodes)

- Illumina adapters typically contain either:

1. one (i7 index), or
2. two index sequences (i7 and i5 indices) for each sample library.

- Prepared libraries may then be pooled (multiplexed), sequenced on the same flow cell lane, and subsequently deconvoluted (de-multiplexed) computationally, resulting in significant cost savings and experiment scalability.

MacConaill *et al.*,2018

Next-generation sequencing(NGS)

Multiplex sequencing

Multiple library preparation/indexes (barcodes)

- The resulting PCR amplicons are then used as templates within the second-step PCR for further amplification, but also to include the indexes (barcodes) as well as the Illumina adaptors.
- The Illumina indexing strategy for the second-step PCR consists of 16 forward primers and 24 reverse primers.
- The combinatorial use of these primers (16 x 24) defines the maximal number of 384 samples which can be pooled and sequenced on one Illumina MiSeq/NextSeq run.

Next-generation sequencing(NGS)

Multiplex sequencing

Multiple library preparation/indexes (barcodes)

- Indexed forward primers for the second-step PCR.

Primer Name	Sequence (5′-3′)	Index Name	Index Sequence
NGS_i5_S502	AATGATACGGCGACCACCGAGATCTACAC**CTCTCTAT**TCGTCGGCAGCGTC	S502 **(set A/B)**	CTCTCTAT
NGS_i5_S503	AATGATACGGCGACCACCGAGATCTACAC**TATCCTCT**TCGTCGGCAGCGTC	S503 (set A/B)	TATCCTCT
NGS_i5_S505	AATGATACGGCGACCACCGAGATCTACAC**GTAAGGAG**TCGTCGGCAGCGTC	S505 (set A/B)	GTAAGGAG
NGS_i5_S506	AATGATACGGCGACCACCGAGATCTACAC**ACTGCATA**TCGTCGGCAGCGTC	S506 (set A/B)	ACTGCATA
NGS_i5_S507	AATGATACGGCGACCACCGAGATCTACAC**AAGGAGTA**TCGTCGGCAGCGTC	S507 (set A/B)	AAGGAGTA
NGS_i5_S508	AATGATACGGCGACCACCGAGATCTACAC**CTAAGCCT**TCGTCGGCAGCGTC	S508 (set A/B)	CTAAGCCT
NGS_i5_S510	AATGATACGGCGACCACCGAGATCTACAC**CGTCTAAT**TCGTCGGCAGCGTC	S510 (set A/B)	CGTCTAAT
NGS_i5_S511	AATGATACGGCGACCACCGAGATCTACAC**TCTCTCCG**TCGTCGGCAGCGTC	S511 (set A/B)	TCTCTCCG
NGS_i5_S513	AATGATACGGCGACCACCGAGATCTACAC**TCGACTAG**TCGTCGGCAGCGTC	S513 **(set C/D)**	TCGACTAG
NGS_i5_S515	AATGATACGGCGACCACCGAGATCTACAC**TTCTAGCT**TCGTCGGCAGCGTC	S515 (set C/D)	TTCTAGCT
NGS_i5_S516	AATGATACGGCGACCACCGAGATCTACAC**CCTAGAGT**TCGTCGGCAGCGTC	S516 (set C/D)	CCTAGAGT
NGS_i5_S517	AATGATACGGCGACCACCGAGATCTACAC**GCGTAAGA**TCGTCGGCAGCGTC	S517 (set C/D)	GCGTAAGA
NGS_i5_S518	AATGATACGGCGACCACCGAGATCTACAC**CTATTAAG**TCGTCGGCAGCGTC	S518 (set C/D)	CTATTAAG
NGS_i5_S520	AATGATACGGCGACCACCGAGATCTACAC**AAGGCTAT**TCGTCGGCAGCGTC	S520 (set C/D)	AAGGCTAT
NGS_i5_S521	AATGATACGGCGACCACCGAGATCTACAC**GAGCCTTA**TCGTCGGCAGCGTC	S521 (set C/D)	GAGCCTTA
NGS_i5_S522	AATGATACGGCGACCACCGAGATCTACAC**TTATGCGA**TCGTCGGCAGCGTC	S522 (set C/D)	TTATGCGA

- Indexed reverse primers for the second-step PCR.

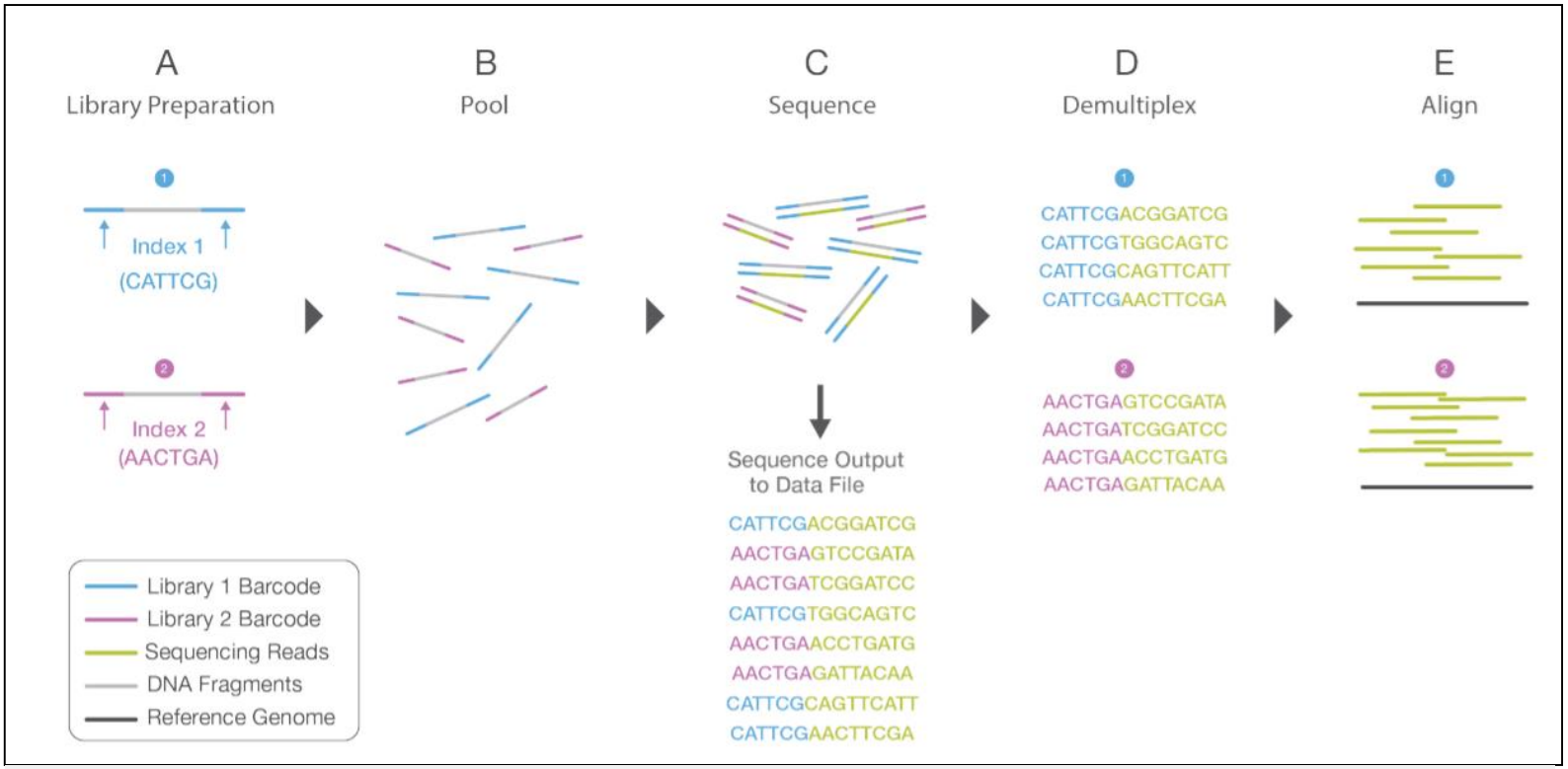
Primer Name	Sequence (5′-3′)	Index Name	Index Sequence
NGS_i7_N701	CAAGCAGAAGACGGCATACGAGAT**TCGCCTTA**GTCTCGTGGGCTCGG	N701 **(set A/C)**	TCGCCTTA
NGS_i7_N702	CAAGCAGAAGACGGCATACGAGAT**CTAGTACG**GTCTCGTGGGCTCGG	N702 (set A/C)	CTAGTACG
NGS_i7_N703	CAAGCAGAAGACGGCATACGAGAT**TTCTGCCT**GTCTCGTGGGCTCGG	N703 (set A/C)	TTCTGCCT
NGS_i7_N704	CAAGCAGAAGACGGCATACGAGAT**GCTCAGGA**GTCTCGTGGGCTCGG	N704 (set A/C)	GCTCAGGA
NGS_i7_N705	CAAGCAGAAGACGGCATACGAGAT**AGGAGTCC**GTCTCGTGGGCTCGG	N705 (set A/C)	AGGAGTCC
NGS_i7_N706	CAAGCAGAAGACGGCATACGAGAT**CATGCCTA**GTCTCGTGGGCTCGG	N706 (set A/C)	CATGCCTA
NGS_i7_N707	CAAGCAGAAGACGGCATACGAGAT**GTAGAGAG**GTCTCGTGGGCTCGG	N707 (set A/C)	GTAGAGAG
NGS_i7_N710	CAAGCAGAAGACGGCATACGAGAT**CAGCCTCG**GTCTCGTGGGCTCGG	N710 (set A/C)	CAGCCTCG
NGS_i7_N711	CAAGCAGAAGACGGCATACGAGAT**TGCCTCTT**GTCTCGTGGGCTCGG	N711 (set A/C)	TGCCTCTT
NGS_i7_N712	CAAGCAGAAGACGGCATACGAGAT**TCCTCTAC**GTCTCGTGGGCTCGG	N712 (set A/C)	TCCTCTAC
NGS_i7_N714	CAAGCAGAAGACGGCATACGAGAT**TCATGAGC**GTCTCGTGGGCTCGG	N714 (set A/C)	TCATGAGC
NGS_i7_N715	CAAGCAGAAGACGGCATACGAGAT**CCTGAGAT**GTCTCGTGGGCTCGG	N715 (set A/C)	CCTGAGAT
NGS_i7_N716	CAAGCAGAAGACGGCATACGAGAT**TAGCGAGT**GTCTCGTGGGCTCGG	N716 **(set B/D)**	TAGCGAGT
NGS_i7_N718	CAAGCAGAAGACGGCATACGAGAT**GTAGCTCC**GTCTCGTGGGCTCGG	N718 (set B/D)	GTAGCTCC
NGS_i7_N719	CAAGCAGAAGACGGCATACGAGAT**TACTACGC**GTCTCGTGGGCTCGG	N719 (set B/D)	TACTACGC
NGS_i7_N720	CAAGCAGAAGACGGCATACGAGAT**AGGCTCCG**GTCTCGTGGGCTCGG	N720 (set B/D)	AGGCTCCG
NGS_i7_N721	CAAGCAGAAGACGGCATACGAGAT**GCAGCGTA**GTCTCGTGGGCTCGG	N721 (set B/D)	GCAGCGTA
NGS_i7_N722	CAAGCAGAAGACGGCATACGAGAT**CTGCGCAT**GTCTCGTGGGCTCGG	N722 (set B/D)	CTGCGCAT
NGS_i7_N723	CAAGCAGAAGACGGCATACGAGAT**GAGCGCTA**GTCTCGTGGGCTCGG	N723 (set B/D)	GAGCGCTA
NGS_i7_N724	CAAGCAGAAGACGGCATACGAGAT**CGCTCAGT**GTCTCGTGGGCTCGG	N724 (set B/D)	CGCTCAGT
NGS_i7_N726	CAAGCAGAAGACGGCATACGAGAT**GTCTTAGG**GTCTCGTGGGCTCGG	N726 (set B/D)	GTCTTAGG
NGS_i7_N727	CAAGCAGAAGACGGCATACGAGAT**ACTGATCG**GTCTCGTGGGCTCGG	N727 (set B/D)	ACTGATCG
NGS_i7_N728	CAAGCAGAAGACGGCATACGAGAT**TAGCTGCA**GTCTCGTGGGCTCGG	N728 (set B/D)	TAGCTGCA
NGS_i7_N729	CAAGCAGAAGACGGCATACGAGAT**GACGTCGA**GTCTCGTGGGCTCGG	N729 (set B/D)	GACGTCGA

www.illumina.com

Next-generation sequencing(NGS)

Multiplex sequencing

DNA library preparation/indexes (barcodes)



Gains in throughput from multiplexing come with an added layer of complexity, as sequencing reads from pooled libraries need to be identified and sorted computationally in a process called de-multiplexing before final data analysis.

www.illumina.com

Whole genome sequencing(WGS)

Also known as WGS, full genome sequencing, complete genome sequencing, or entire genome sequencing

- Whole genome sequencing is the process of determining the complete DNA sequence of an organism's genome at a single time.
- The widespread use of whole-genome sequencing(WGS) enable us to detect and identify bacterial plant pathogens in support of legislative and policy efforts in food security.
- Routine whole-genome sequencing offers future potential for even more precise classification and identification of virulence determinants in novel pathogens.

Whole genome sequencing(WGS)

BAC-by-BAC (clone-by-clone shotgun strategy) and Whole Genome Shotgun (WGS) sequencing

- There are essentially two ways to sequence a genome:

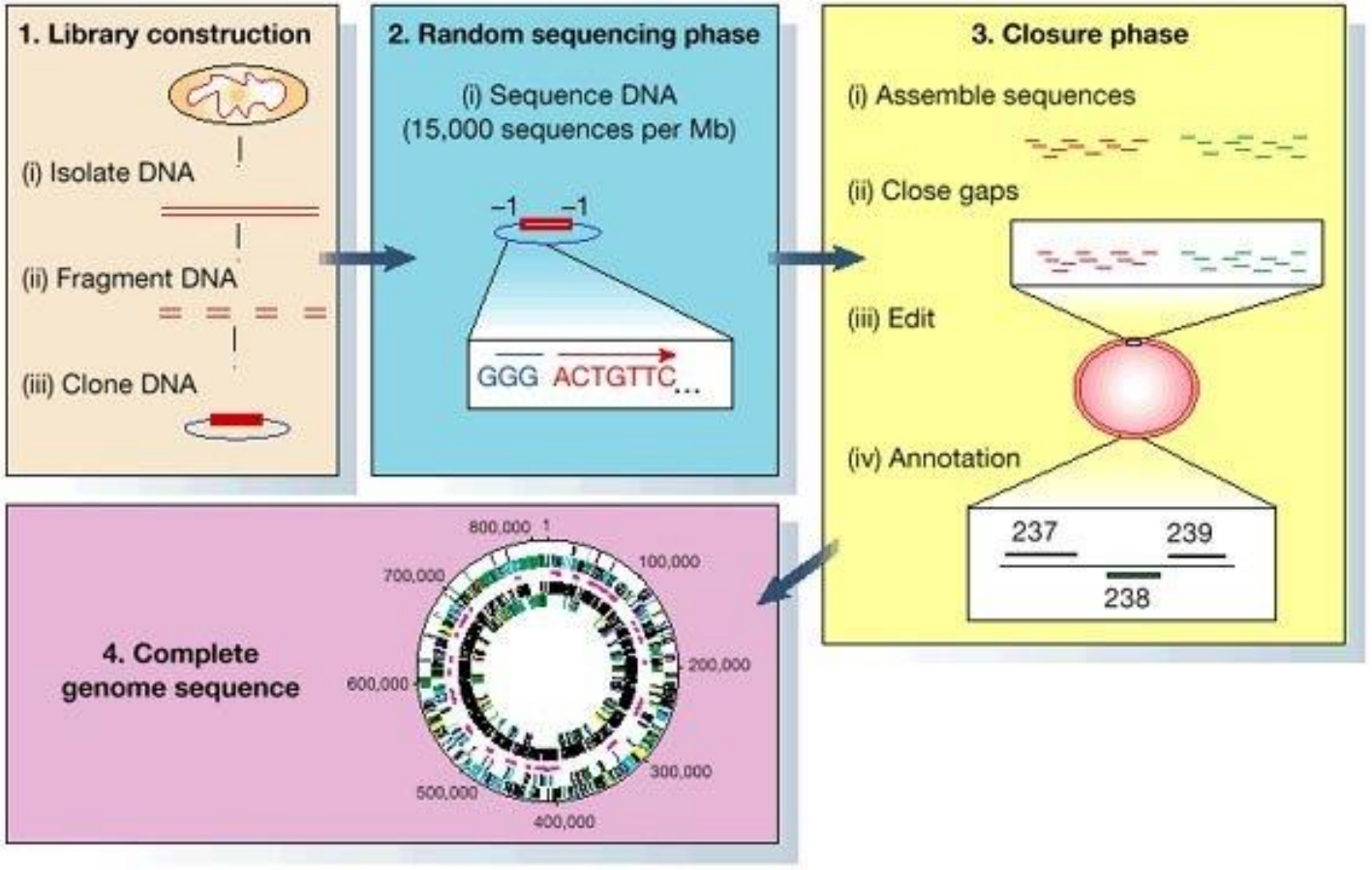
1. BAC-by-BAC strategy (also known as hierarchical shotgun sequencing or a clone-by-clone shotgun strategy);
2. Whole Genome Shotgun (WGS) sequencing.

See also Bacterial Management-Part I and Bacterial genetics files.

Trivedi,2000;..

Whole genome sequencing(WGS)

Diagram depicting the steps in a whole-genome shotgun sequencing project



Fraser *et al.*,2000

Isothermal amplification methods

Isothermal= Equal or constant temperatures

1. NASBA(Nucleic acid sequence based amplification): An isothermal amplification method used to detect RNA targets.
2. LAMP(Loop-mediated isothermal amplification): An isothermal amplification method used to detect DNA targets.

- Note: Although LAMP was initially developed for DNA it can be adapted to amplify RNA (RT-LAMP).

López *et al.*,2011; Putnam,2011

LAMP

LAMP vs. PCR

LAMP Assays are Faster, Simpler Once Developed

	PCR	LAMP
1.	Requires temperature cycling	Isothermal – single temperature
2.	Requires 2 primers	Requires 6 primers
3.	Slow: Typically >1hr	Rapid: Typically <30 min
4.	Typical yield ~ 0.2 µg	Typical yield ~ 10–20 µg
5.	Not amenable to visual detection	Amenable to visual detection based on turbidity etc.
6.	Sensitive to sample matrix inhibitors	Tolerant to sample matrix inhibitors
7.	Can be multiplexed	Difficult to multiplex

LAMP

Loop-mediated isothermal amplification

New generation of gene amplification assay

- **Plant pathogen's detection quicker than a coffee make.**
- LAMP detects pathogen's specific genes in less then 30 minutes.
- Loop-mediated isothermal amplification (LAMP) is another type of isothermal amplification that it is being increasingly used in the diagnostic field offering sensitivity and economic costs (Notomi *et al.*,2000).
- The method has since been adapted for use with fungi, nematodes, viruses and phytobacteria.
- This method was used for detection of *Rhodococcus fascians*, *Ralstonia solanacearum*, *Xanthomonas*, *E. amylovora*, *Xylella*, *Ca.* Liberibacter, *Ca.* Phytoplasma with high specificity, efficiency, and speed without thermal cycling.

López *et al.*,2011; Putnam,2011;Lenarcic *et al.*,2012

LAMP
A novel nucleic acid amplification method
Advantages

- LAMP' is characterised by the use of 4-6 different primers.
- The amplification proceeds at a constant temperature using strand displacement reaction.
- Amplification and detection of a gene can be completed in a single step, by incubating the mixture of samples, primers,
- DNA polymerase with strand displacement activity and substrates at a constant temperature (about 63C).
- Compared to PCR and real-time PCR, LAMP has the advantages of reaction simplicity and higher amplification efficiency.

Parida *et al.*,2008;..

LAMP

Loop-mediated isothermal amplification

Advantages

- All the reagents were incubated in a single tube.
- The whole procedure is very simple and rapid wherein the amplification can be completed in less than 1 h.
- LAMP can quickly generate large quantities of amplicon from low abundance template.
- Gene amplification can be visualised by the naked eye either as turbidity or in the form of a colour change when SYBR Green I, a fluorescent dsDNA intercalating dye, is employed.
- Unlike PCR which requires one pair of primers, LAMP requires a minimum of two nested primer pairs.
- Generally, primers should be 15-25 bases long with 40-60% GC content (annealing 55-65°C).

Parida *et al.*,2008; Salinas and Little,2012

LAMP

Loop-mediated isothermal amplification

Advantages

- LAMP takes place in a single microfuge tube and requires only a water bath or heat block.
- Results are evaluated visually without extensive manipulation of product.
- A microfuge tube containing:

1. A small amount of extracted sample DNA, and
2. A set of four primers (termed inner and outer primers) that are specific to six regions of your target DNA.
3. A DNA polymerase with strand displacement activity.

López *et al.*,2011; Putnam,2011

LAMP

Primer design for LAMP

A set of six or eight primers

- LAMP is characterized by the use of 4-8 different primers.

1. Four different primers recognize 6 distinct regions of the target(DNA) gene.
2. Six different primers specifically designed to recognize eight distinct regions on the target gene.

- Combinations of six primer regions for basic LAMP signatures, or combinations of eight primer regions for LAMP signatures with loop primers, which can be used as LAMP signatures.

LAMP

Primer design for LAMP

1. Set of four primers

- The four primers used are as follows:

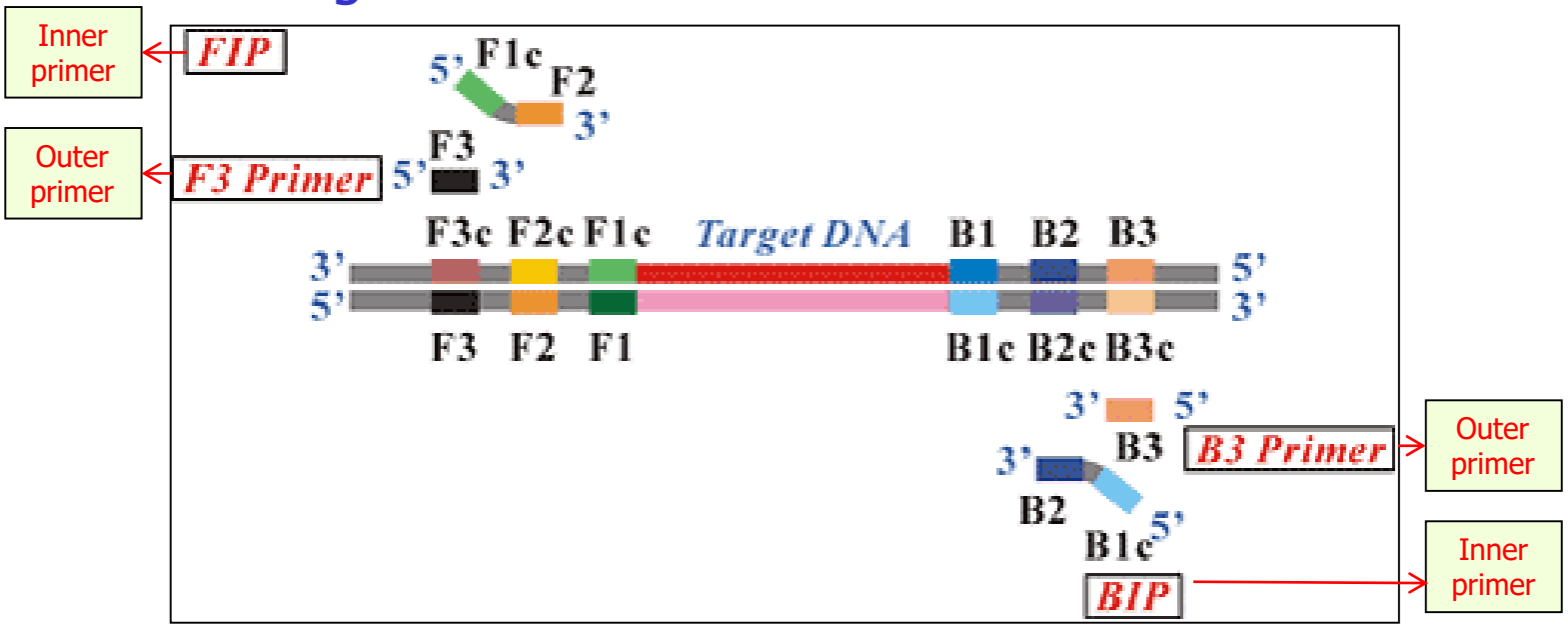
1. Forward Inner Primer (FIP)
2. Forward Outer Primer (FOP or F3)
3. Backward Inner Primer (BIP)
4. Backward Outer Primer (BOP or B3)

LAMP

A set of four primers

Two outer primers (F3 and B3) and two inner primers (FIP, BIP)

- These primers(outer and inner primers) recognize a total of six distinct sequences in the target DNA. i.e.
- The F3c, F2c and F1c regions at the 3' side and the B1, B2 and B3 regions at the 5' side.



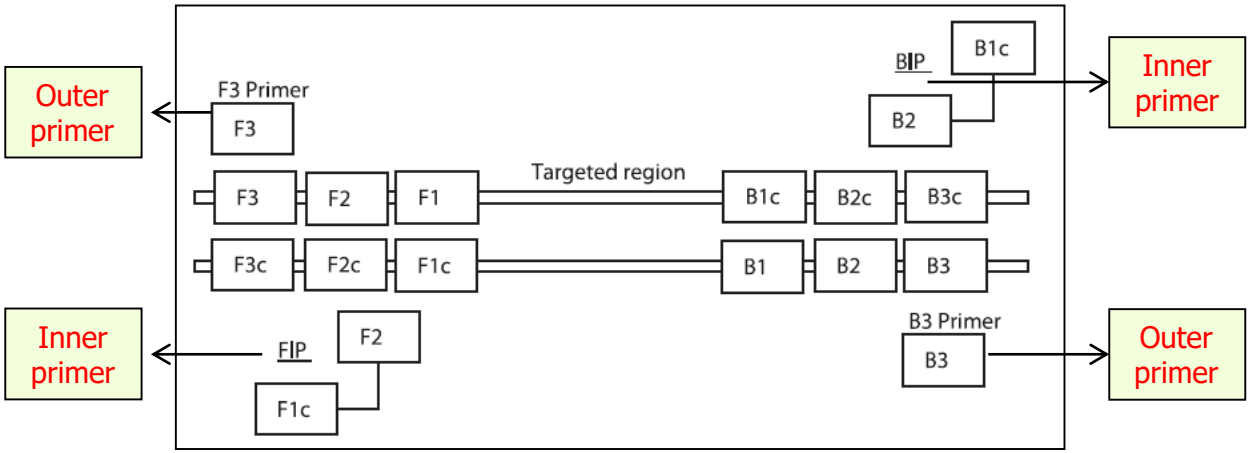
View animation in Eiken Genome Site

LAMP

A set of four primers

Two outer primers (F3 and B3) and two inner primers (FIP, BIP)

- FIP and BIP are complementary to sequences from both strands of the targeted DNA that have close location to each other (designated as F1 and F2c for FIP and B1 and B2c for BIP, respectively).
- The other two oligonucleotides, termed outer primers (F3 and B3) are like ordinary PCR primers and are complementary to the sequences F3c and B3c.



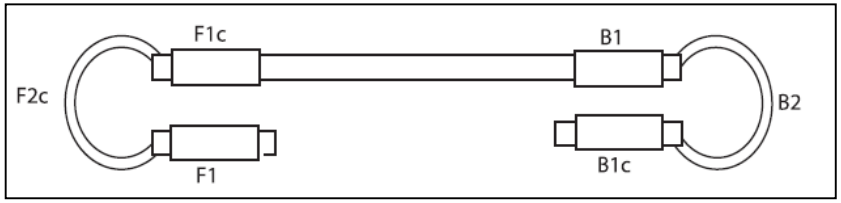
Kalvatchev *et al.*,2011

LAMP

Structure of amplification products

Dumb-bell form produced in the initial stages of LAMP

- Initially, all four primers are necessary for formation of a specific double-stranded structure with loops at each end.
- DNA polymerase is used at 60-65°C (the specific temperature used depends on system optimization) and a series of looped structures result.
- The amplification products are:
- Stem-loop DNA structures with several inverted repeats of the target and
- Cauliflower-like structures with multiple loops, yielding >500 µg/ml.



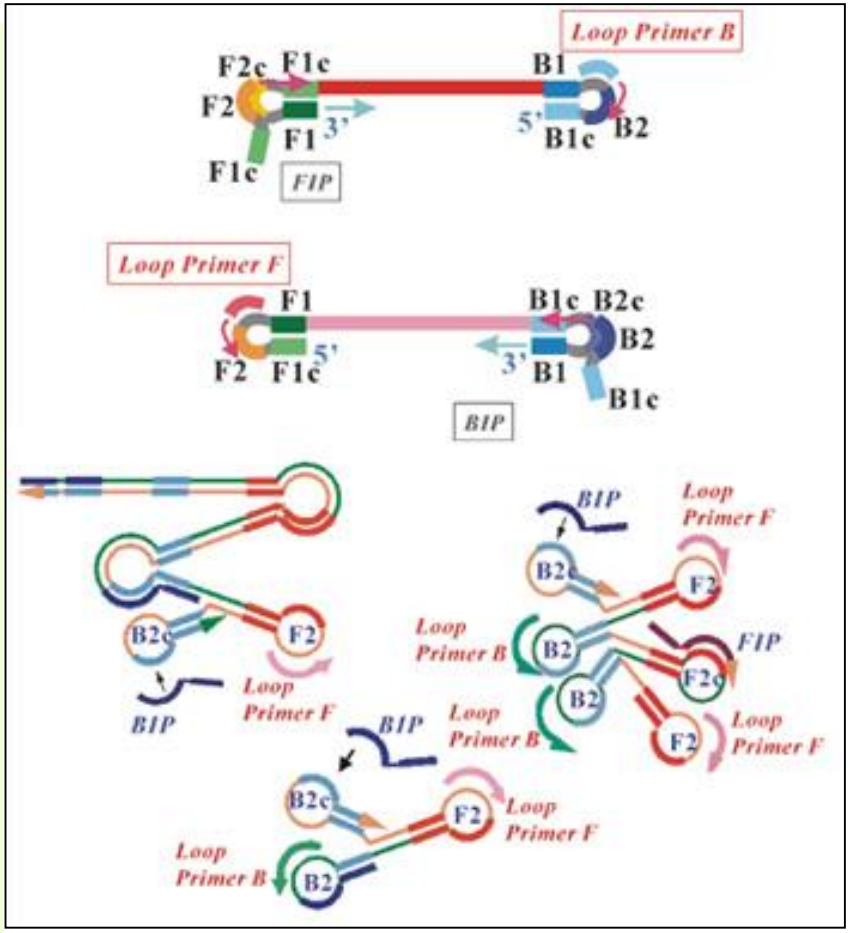
López *et al.*,2011; Kalvatchev *et al.*,2011

LAMP

Structure of amplification products

An example is shown in the figure where there is an amplified product containing six loops

- The Loop primers (either loop primer B or loop orimer F), containing sequences complementary to the single stranded loop region (either between the B1 and B2 regions, or between the F1 and F2 regions) on the 5' end of the dumbbell-like structure, provide an increased number of starting points for DNA synthesis for the LAMP method.



Eiken Genome Site

LAMP

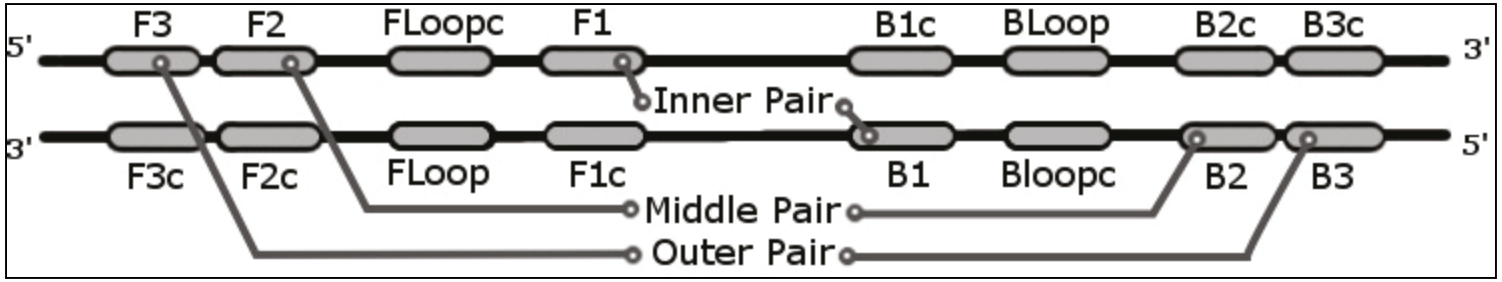
Primer design for LAMP

2. Set of four primers

- The four primers used are as follows:

1. Two outer primers(F3 and B3), and
2. Two loop primers (FLoop and BLoop),
3. Two middle primers (B2 and F2) and
4. Two inner primers (FIP, BIP).

- These pairs represent the location and orientation of the primers with respect to the target template during each extension in which they participate.



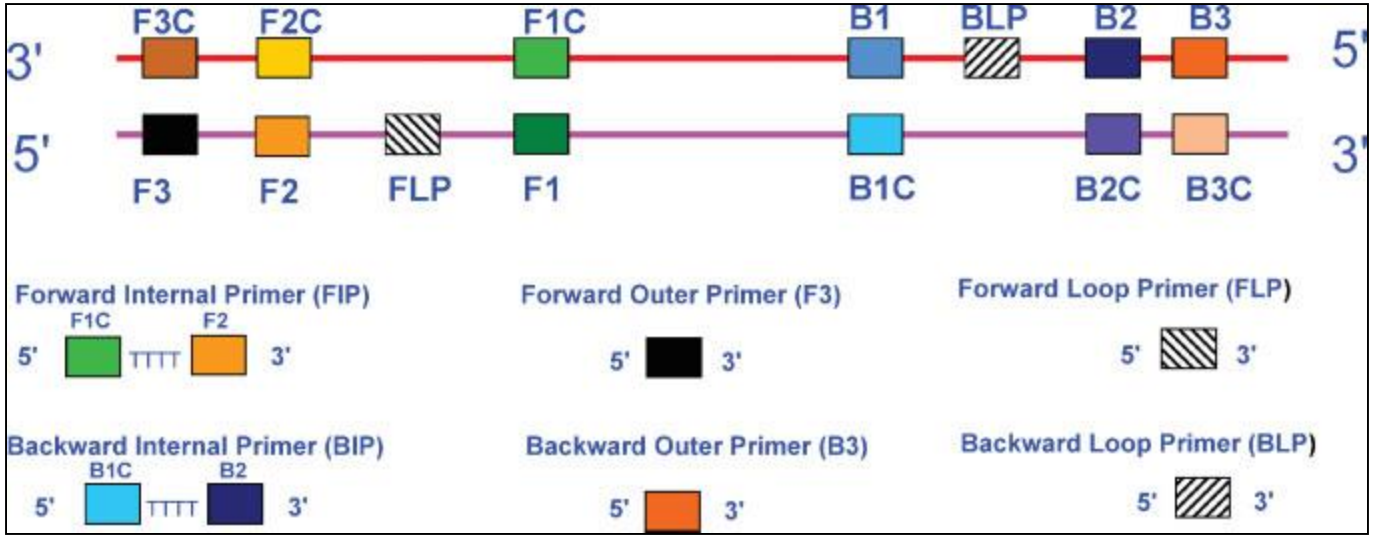
Torres *et al.*,2011

LAMP

A set of eight primers

Two inner, two loop, two middle and two outer primers

- Schematic representation of primer design for RT-LAMP(reverse transcription loop mediated isothermal amplification) assay showing the position of the six primers spanning the target gen.



Parida *et al.*,2008

LAMP reaction

- The LAMP reaction was carried out in a 25 µl volume containing:
- 1.6 M each FIP and BIP, 0.2 M each F3 and B3, 0.8 M each LF and LB, 1.4 mM each deoxynucleoside triphosphate, 0.8 M betaine, 20mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.2% Tween 20, 8 U of the *Bst* DNA polymerase large fragment and 5 µl of target DNA.
- The mixture was incubated at 65°C using a conventional heating block and was heated at 80°C for 2 min to terminate the reaction.

Yoshida *et al.*,2005

View animation of the reaction
(http://loopamp.eiken.co.jp/e/lamp/index.html).

LAMP
Procedures

1. If using a pure culture, simply boil the bacteria briefly to extract DNA.
2. An aliquot is then added to the reaction mixture, the tubes are incubated at 60°C, and
3. Visualization is with PicoGreen (1.0 µl of 1/10-diluted original dye) or any other fluorescent dyes such as SYBR Green I.

- The amplification takes place at 60-65ºC for 60 min. and entire process is completed in less than 3 hours.
- Importantly, this method does not require denaturation of a DNA template.

López *et al.*,2011; Putnam,2011

LAMP
Procedures

1. Samplings: Sample preparation takes 5 min.
2. Running the test: Max 30 min.
3. Results: Use friendly equipment, easy to read.
4. Average testing times: 20 min.

Lenarcic *et al.*,2012

LAMP
Procedures

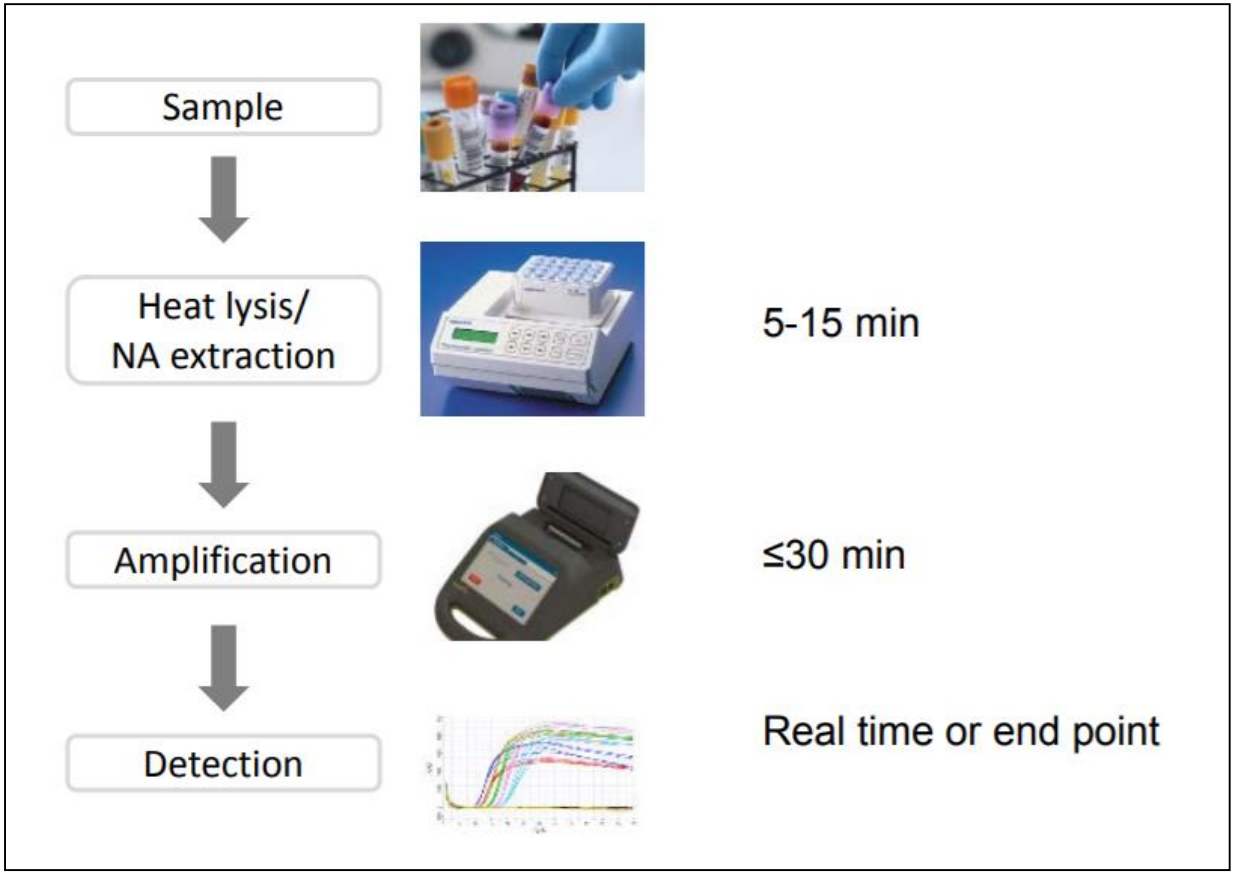
- The turbidity derived from the white precipitate of magnesium pyrophosphate in the mixture was detected by the naked eye.
- If it is difficult to see this turbidity, a DNA binding dye may be used, which will integrate with the amplified product.
- This dye-DNA complex will fluoresce under UV light, which makes it easier to distinguish between positive and negative reactions.

López *et al.*,2001; Putnam,2011

LAMP

LAMP Workflow

Easy with Low Complexity Instrumentation

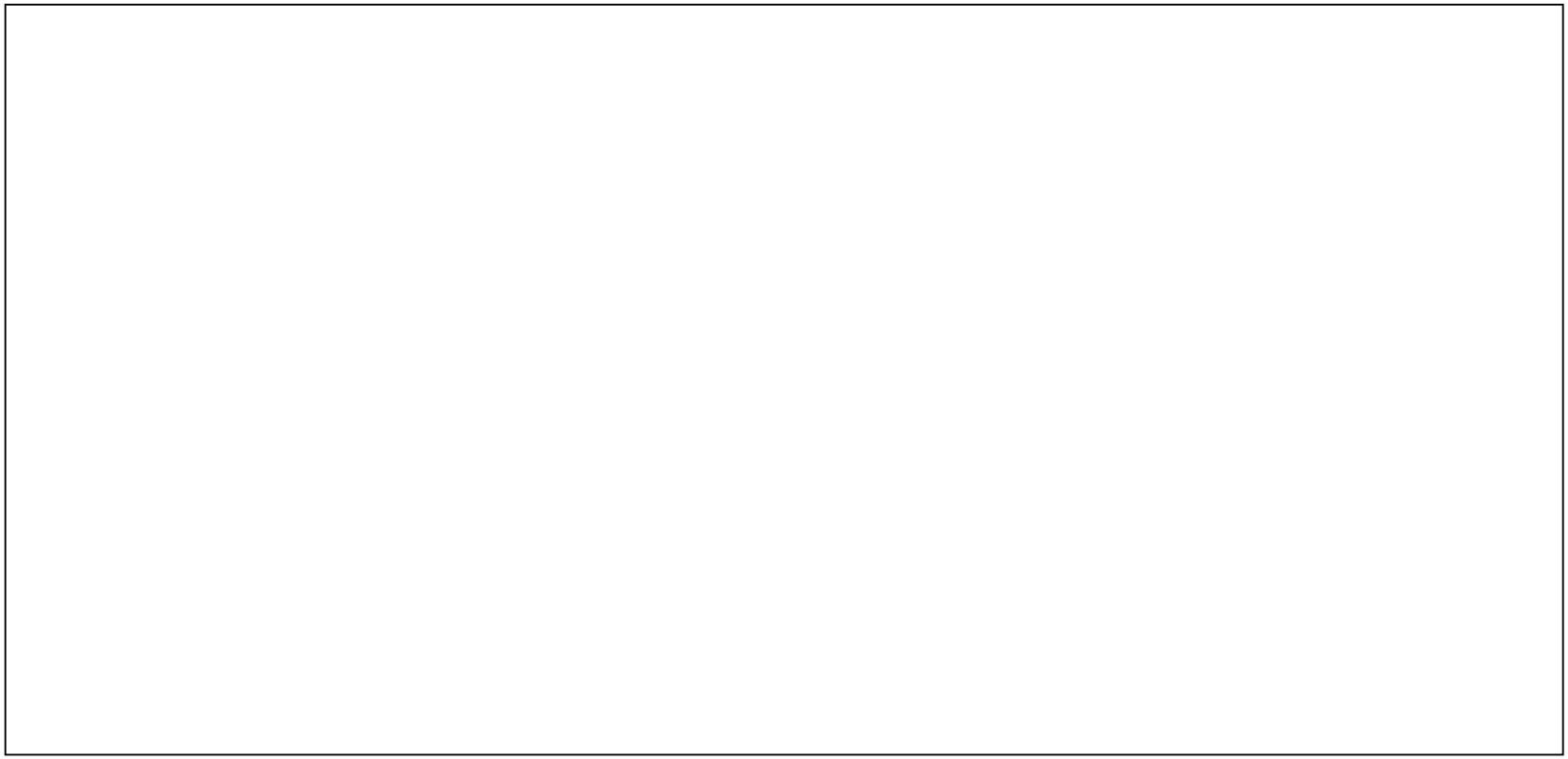


Lucigen

LAMP

Detection of the LAMP products

Procedures

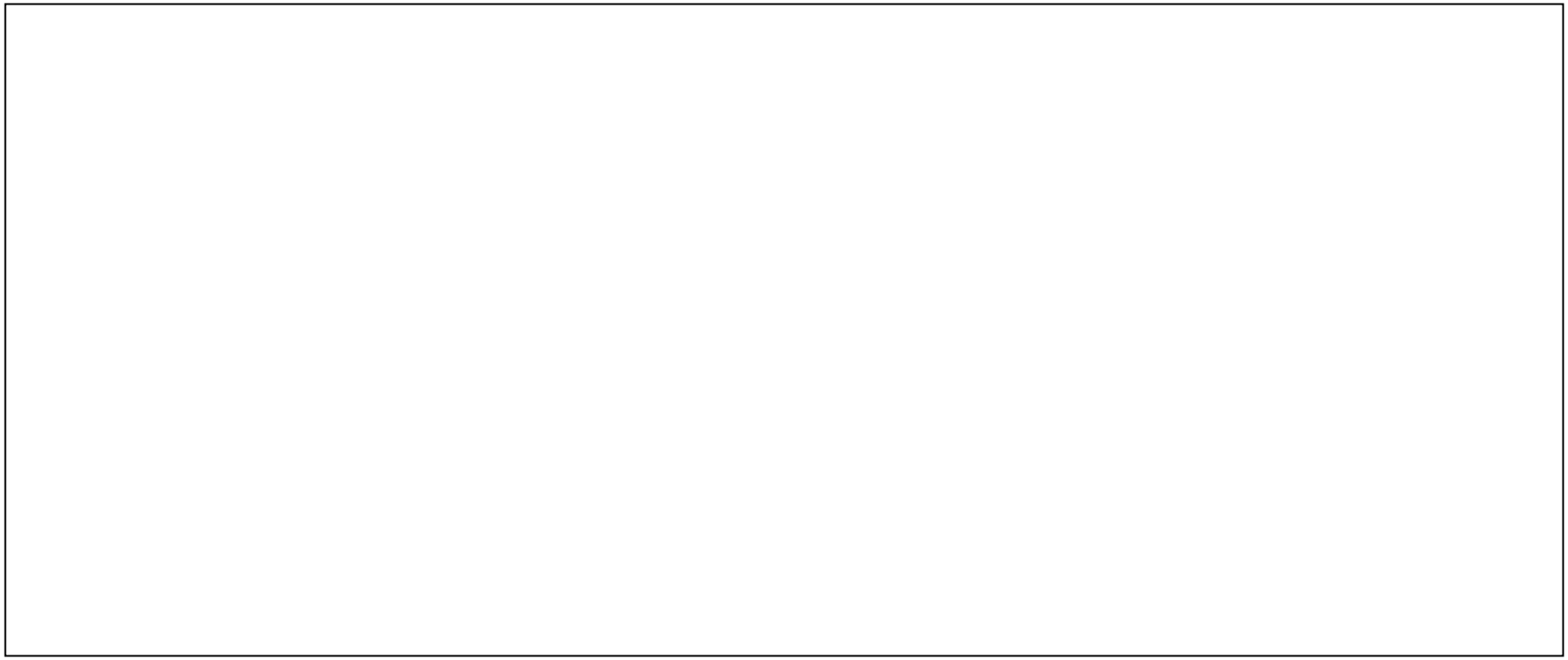


Lenarcic *et al.*,2012

LAMP

Detection of the LAMP products

Procedures

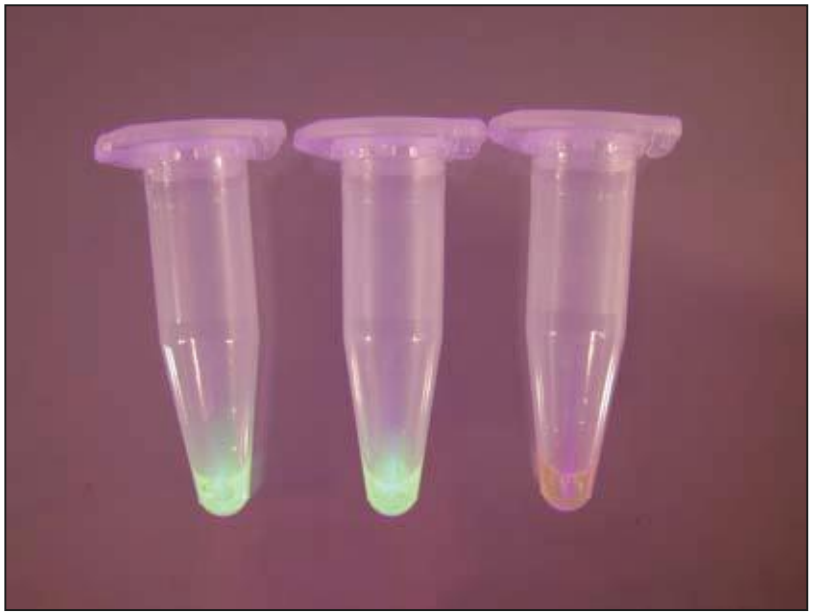


Lenarcic *et al.*,2012

LAMP

Detection of the LAMP products

- Positive LAMP reactions (green) are shown in the two tubes on the left.
- The tube on the right is a negative reaction(orange).
- The image was taken under UV illumination and fluorescence is due to addition of PicoGreen to all tubes.
- Orange color indicate no DNA amplification. Same as in no DNA (water only).



Putnam,2011

LAMP

Eight genes assessed and five were selected *Xylella fastidiosa*

- A total of eight ORF or gene targets were assessed for the design of *Xylella fastidiosa* specific LAMP primers.
- Of these, three (16S rRNA, gyrB, hypothetical protein) were rejected due to low sequence (<98 percent) conservation, and similarity to related genera such as *Xanthomonas*.

Gene	Length (bp)	Location on genome (9a5C strain)	% Nucleotide Identity between strains
16S rRNA	1536	65886-67422	96.7%
gyrB	?	4634-7078	?
pdiA	465	1751131-1750670	99.1%
rimM	513	106499-107011	99.3%
hicB	336	1603673-1604032	99.7%
gltA	853	933968-935257	99.4%
FtsH	642	94878-94237	98.7%
Hypothetical Protein (Francis *et al.* 2006)	801	?	97.9

Ward *et al.*,2010

LAMP

Five candidate genes

Xylella fastidiosa

- Primers for LAMP amplification were designed against the five candidate genes (*pdiA*, *rimM*, *hicB*, *gltA* and *FtsH*) using the standard default parameters of the online PrimerExplorer V4 software (Eiken Chemical Co.,Tokyo).
- Outer (F3/B3) and loop (FIP/BIP) primer pairs were designed for all five gene targets.

Ward *et al.*,2010

LAMP

Primer sets designed for LAMP amplification of *Xylella fastidiosa*

Three (16S rRNA, gyrB, hypothetical protein) were rejected

Gene target	Primer name	Sequence(5'--3')
Disulphide Isomerase (*pdiA*)	XF-DIS-FIP	CGTGGGCAATTTTGACCGCAATTTTTCGGAATGCCATCCTGGATT
	XF-DIS-BIP	GTGCTTTGCAATCAGCGCCGTTTTAACTGGTGCACCGACTGT
	XF-DIS-F3	GCCATCCGAATTCACCACAA
	XF-DIS-B3	ACACACAAACCAACCCTCTT
	XF-DIS-LF	AGTCAGGCCTATGGCGAC
	XF-DIS-LB	TACGCAATGATTTATCGAGTGCTCG
16S rRNA processing protein (*rimM*)	XF-RIMM-LF	TGCAAGTACACACCCTTGAAG
	XF-RIMM-LB	TTCCGTACCACAGATCGCT
	XF-RIMM-F3	CCGTTGGAAAACAGATGGGA
	XF-RIMM-B3	GAGACTGGCAAGCGTTTGA
	XF-RIMM-FIP	ACCCCGACGAGTATTACTGGGTTTTTCGCTACCGAGAACCACAC
	XF-RIMM-BIP	GCGCTGCGTGGCACATAGATTTTTGCAACCTTTCCTGGCATCAA
Citrate synthase (*gltA*)	XF-GLTA-LF	ATCGGATAGCCGCGGTACAACA
	XF-GLTA-LB	GACGAGTTTGCCAAGTTTGATG
	XF-GLTA-F3	GCAAGCTGTAAGTCTGCCAT
	XF-GLTA-B3	ACCGCCAAGAAAGTTCTTCA
	XF-GLTA-FIP	GGAGTGCTCAGCTAACTGCTCATTTTTGATGGTGAAAAGGGTGTGT
	XF-GLTA-BIP	ATGAACGGTGAGTTGCCGAGAATTTTATCATCGTGTGATGCGTGAT

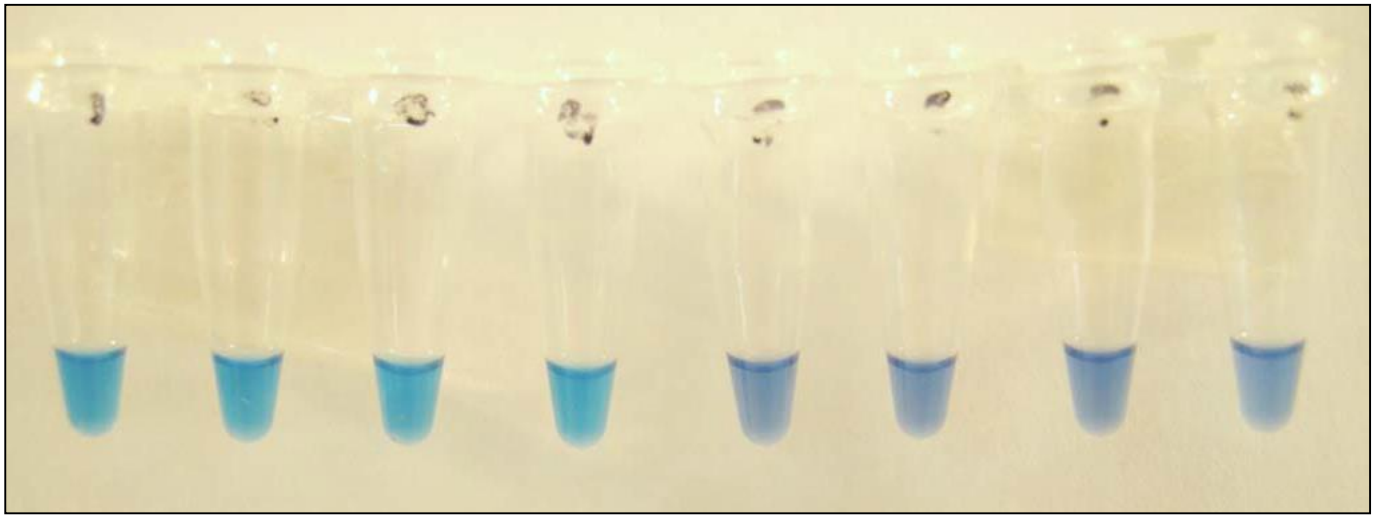
Ward *et al.*,2010

LAMP

Detection of the LAMP products

Procedures

- An example of positive (blue, tubes 1-4) and negative (purple, tubes 5-8) loop mediated isothermal amplification using hydroxynapthol dye as an indicator.



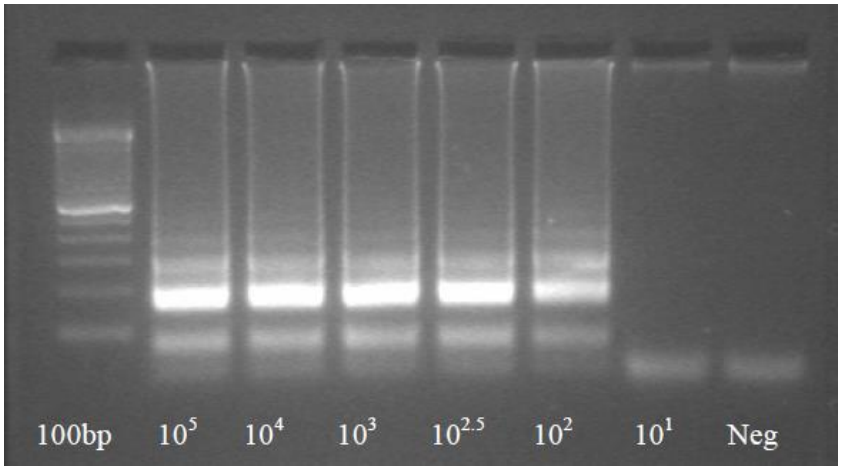
Ward *et al.*,2010

LAMP

Comparison of the specificity

LAMP vs. PCR and real-time PCR

- A comparison of the specificity of the LAMP assay was made to published *Xylella*-specific standard PCR and real-time qPCR assays.
- *Xylella*-free host species and non-target bacterial species were also examined (negative control).
- A dilution series of *Xylella fastidiosa* total DNA diluted in water amplified by LAMP using primers targeting the *rimM* gene.
- Initial copy number per µl of loaded total DNA is given.



Ward *et al.*,2010

Four primers recognize six distinct region of the 439bp specific ITS gene of *Lxx*

Leifsonia Xyli subsp. *xyli* (*Lxx*)

A. Detection of LAMP products by yellow green color change.

B. Agarose gel electrophoresis visualization of the LAMP.

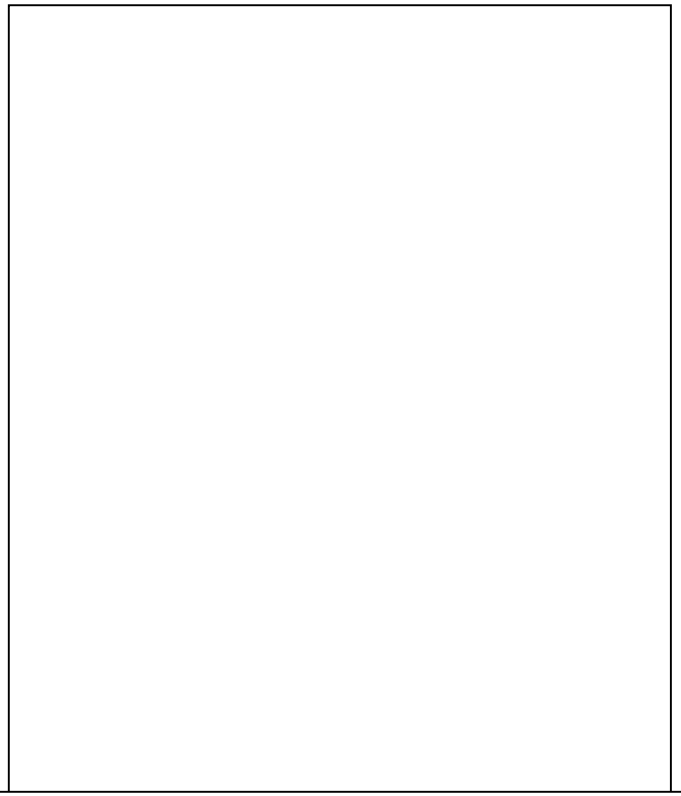
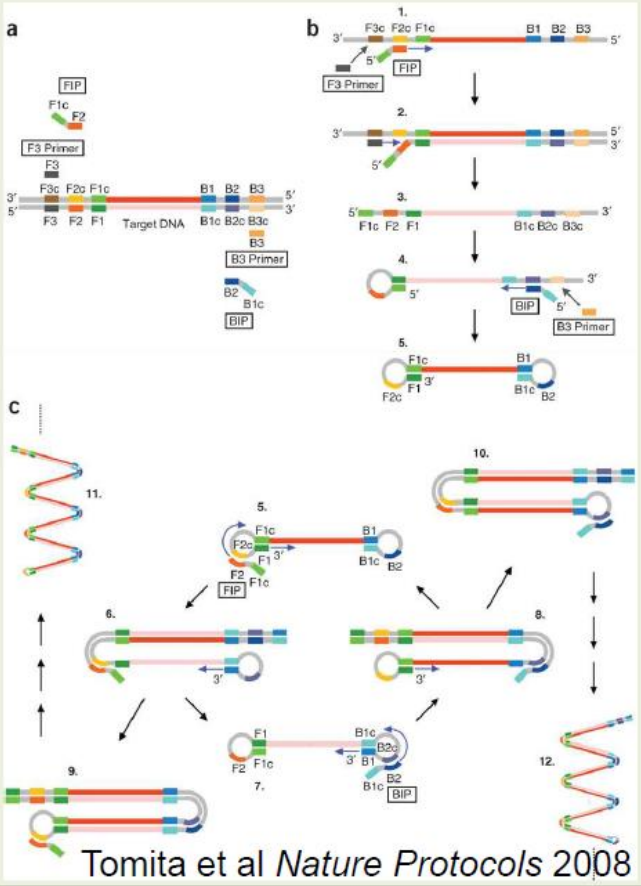


FIGURE 3: Products amplified by LAMP reactions with different Mg^{2+} concentrations. (a) Detection of LAMP products by color change. Samples turning yellowish green were considered positive, while the ones remaining orange were considered negative. (b) Agarose gel electrophoresis visualization of the LAMP products. Lane M, 15000 + 2000 bp marker; lanes 1, 2: the sterilized ddH_2O; lanes 3, 4: 20 ng/μL negative DNA extracted from the internode juice without *Lxx*; lanes 5, 6: 20 ng/μL positive DNA extracted from the *Lxx*-infected juice; lanes 7, 8: 20 ng/μL positive plasmid.

Liu *et al.*,2013

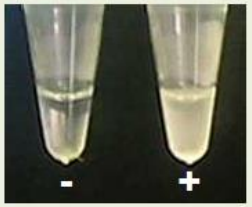
LAMP
R. solanacearum

- LAMP: Loop-mediated isothermal AMPlification



Tomita et al *Nature Protocols* 2008

- **A simple rapid PCR-like way to generate large quantities of DNA**
- Highly specific (3 primer sets)
- Only need 2 temperatures
- No thermocycler required



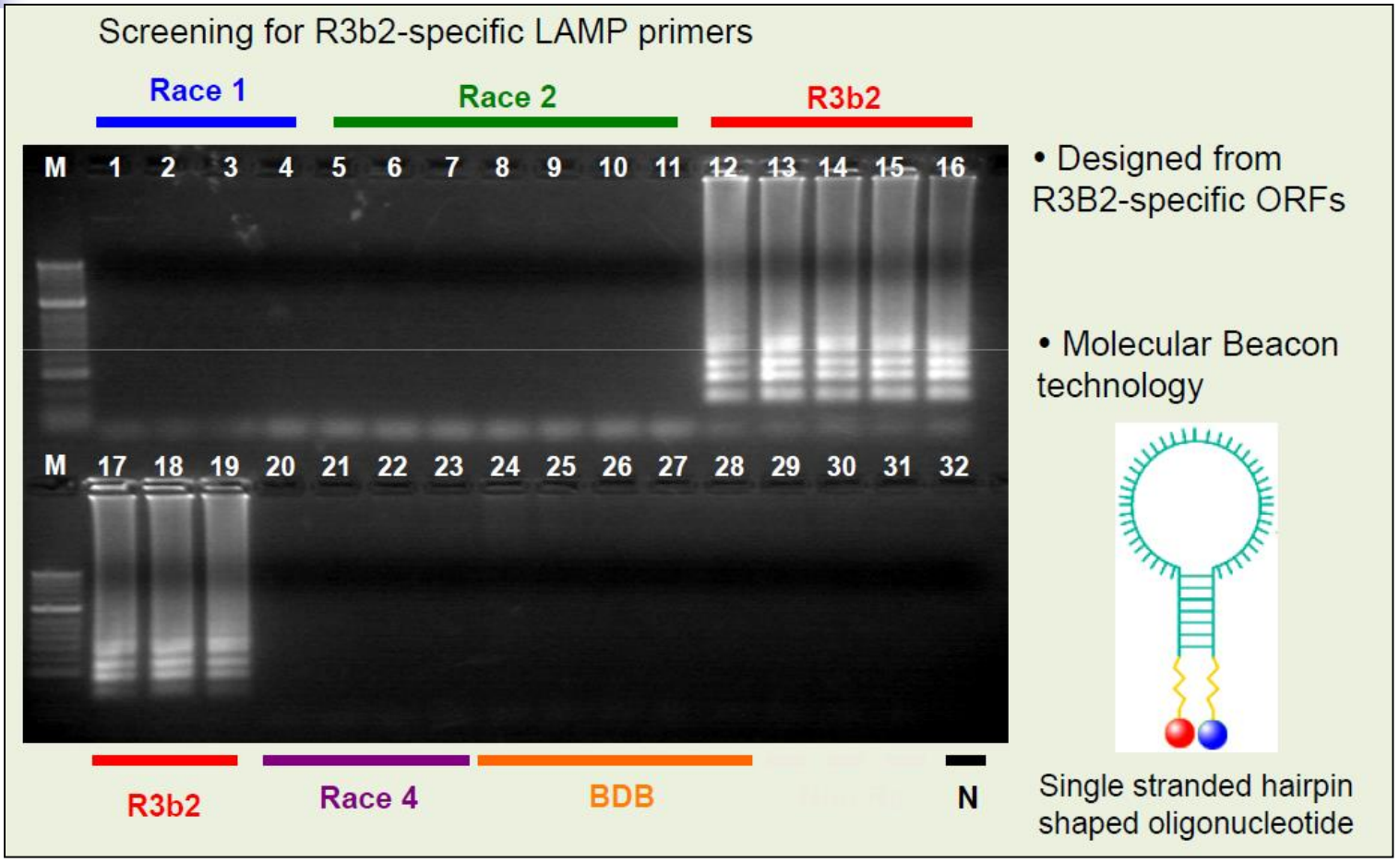
Rapid Visual Assessment

- Sensitive to PCR inhibitors

Champoiseau,2009

Diagnostic methods

LAMP: Loop-mediated isothermal Amplification



Champoiseau,2009

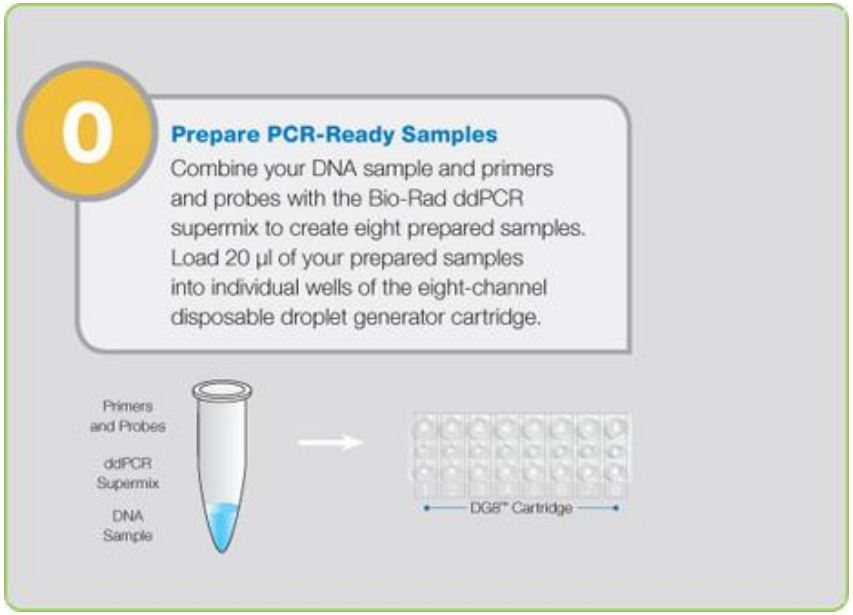
ddPCR
Droplet Digital polymerase chain reaction

- ddPCR is a novel technique that quantify the nucleic acids target without the need of an external calibrator.
- This method is a robust approach for diagnosis of plant pathogens also with a low target concentration.
- Among the advantages of the ddPCR the authors reports the potential for the quantitative detection of:
- *Xanthomonas citri* subsp. *citri*, and
- *Xylella fastidiosa.*

Scala *et al.*,2018

ddPCR
Droplet Digital polymerase chain reaction

- Droplet Digital PCR (ddPCR) is a method for performing digital PCR that is based on water-oil emulsion droplet technology.
- A sample is fractionated into 20,000 droplets, and PCR amplification of the template molecules occurs in each individual droplet.



Bio-Rad

ddPCR

Droplet Digital polymerase chain reaction

How Does Droplet Digital PCR Work?

- ddPCR technology uses a combination of microfluidics and proprietary surfactant chemistries to divide PCR samples into water-in-oil droplets.
- The droplets support PCR amplification of the template molecules they contain and use reagents and workflows similar to those used for most standard TaqMan probe-based assays.
- Following PCR, each droplet is analyzed or read to determine the fraction of PCR-positive droplets in the original sample.
- These data are then analyzed using Poisson statistics to determine the target DNA template concentration in the original sample.

ddPCR

Droplet Digital polymerase chain reaction

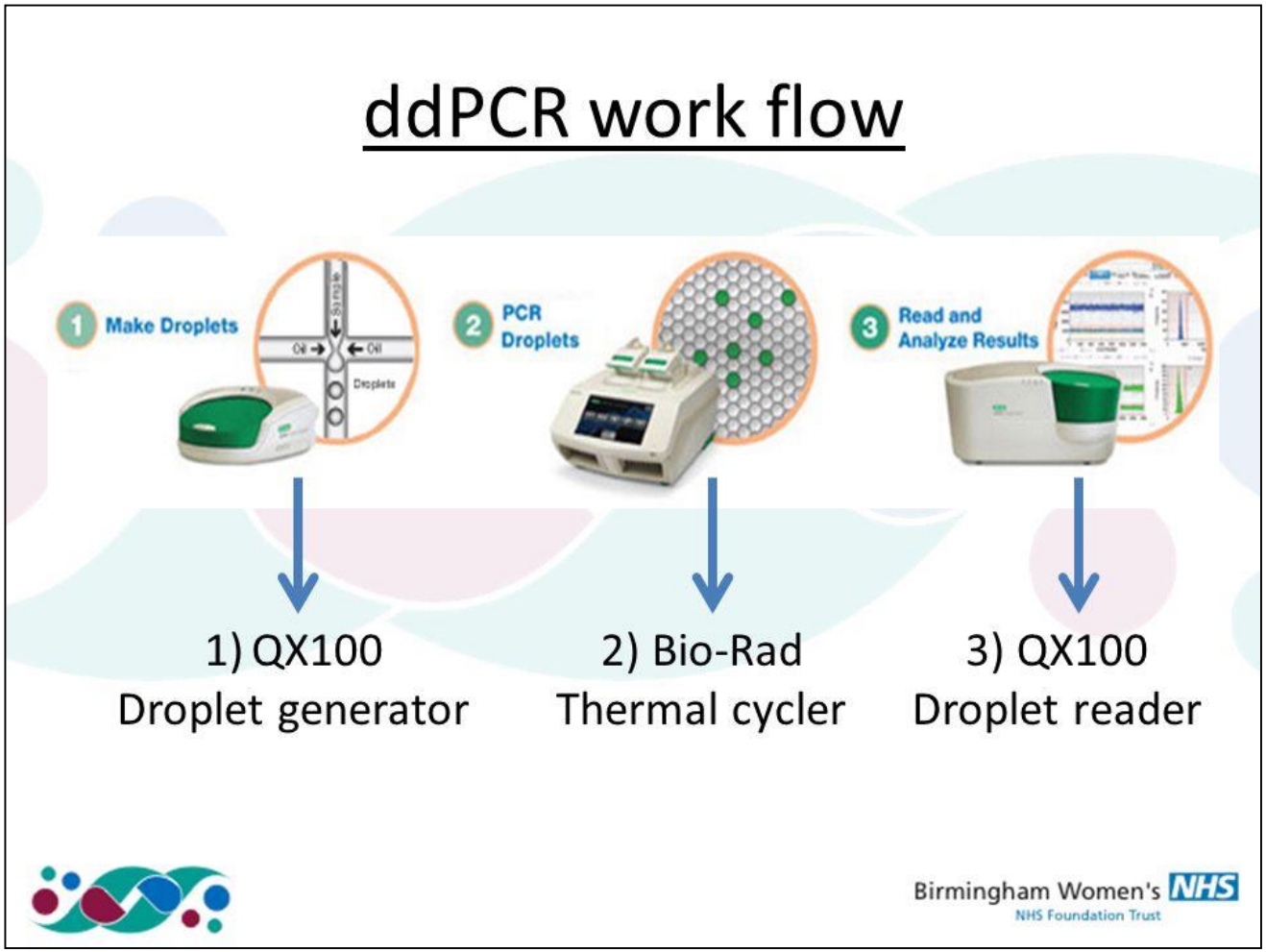
QX200™ Droplet Digital™ PCR System

- The QX200 Droplet Generator partitions samples (20 µl into 20,000 nanoliter-sized droplets) for PCR amplification.
- Following amplification using a thermal cycler, droplets from each sample are analyzed individually on the QX200 Droplet Reader, where PCR-positive and PCR-negative droplets are counted to provide absolute quantification of target DNA in digital form.

ddPCR

Droplet Digital polymerase chain reaction

How Does Droplet Digital PCR Work?



ddPCR

Droplet Digital polymerase chain reaction

QX200™ Droplet Digital™ PCR System

- Bio-Rad's Droplet Digital™ PCR System consists of two instruments:

1. the QX200 Droplet Generator, and
2. the QX200 Droplet Reader,
3. plus their associated software and consumables.

Bio-Rad

ddPCR

Droplet Digital polymerase chain reaction

QX200™ Droplet Digital™ PCR System

- The ddPCR System can be used to:

1. Detect rare DNA target copies with unmatched sensitivity;
2. Determine copy number variation with unrivaled accuracy;
3. Measure gene expression levels with exquisite precision.