Lecture Supplements

Plant Bacteriology

Bacterial Pathogenesis-Part 1

Compiled by N. Hassanzadeh

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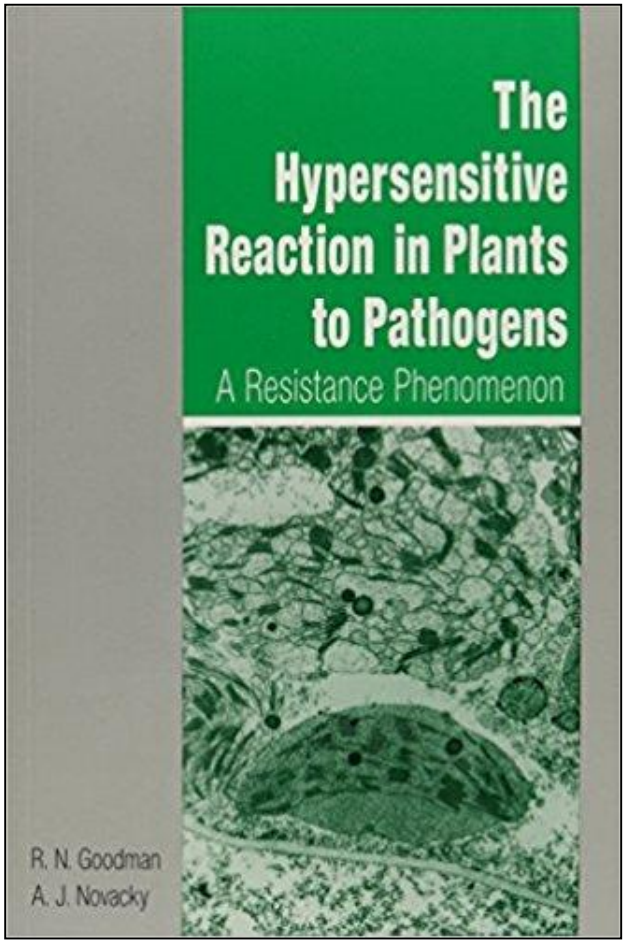

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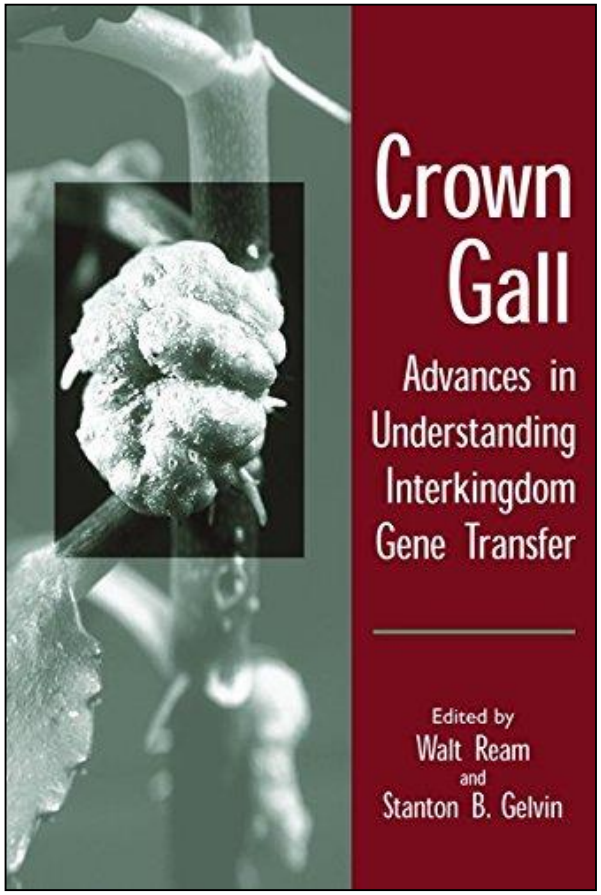

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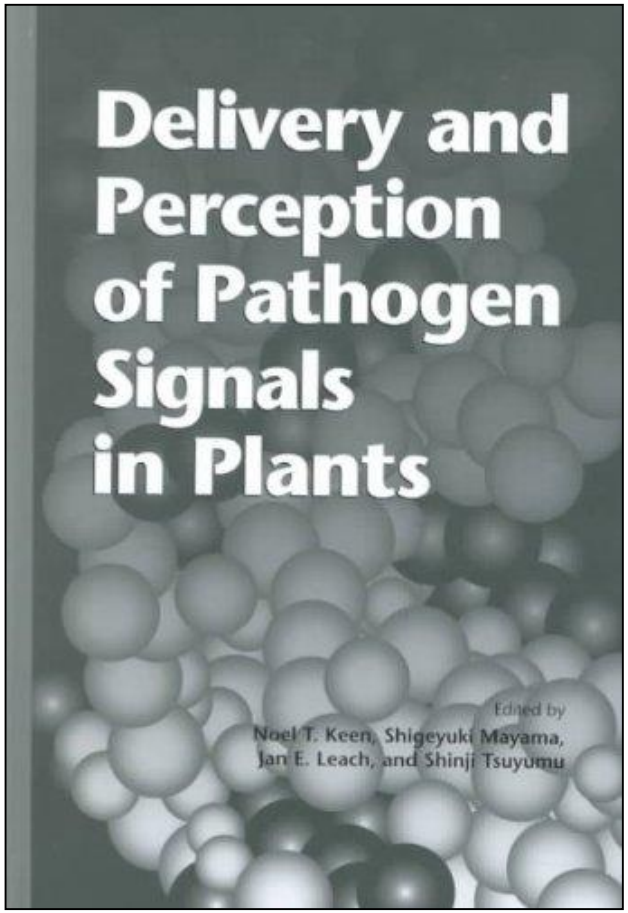

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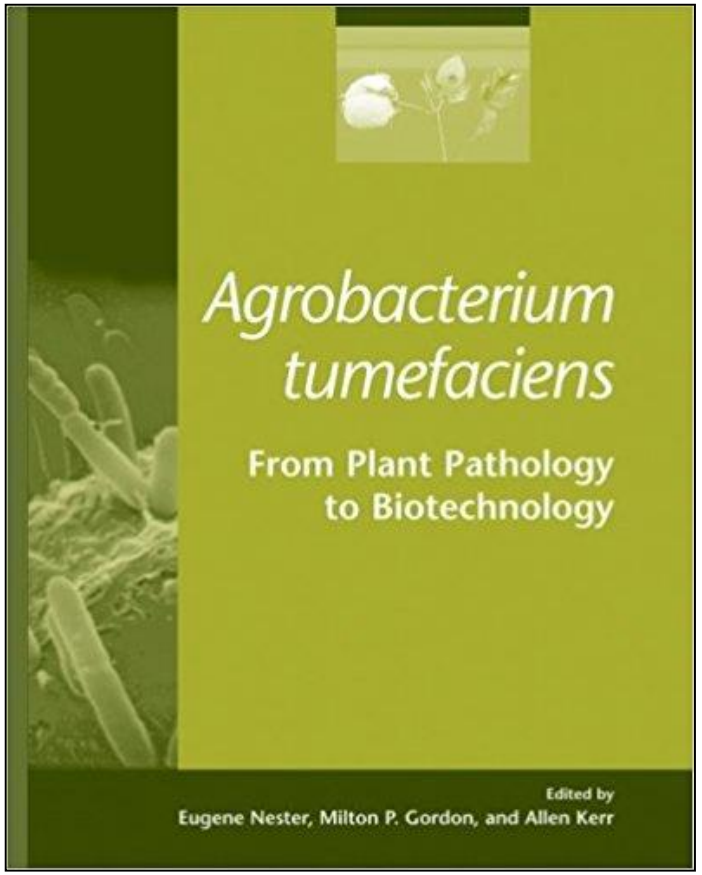

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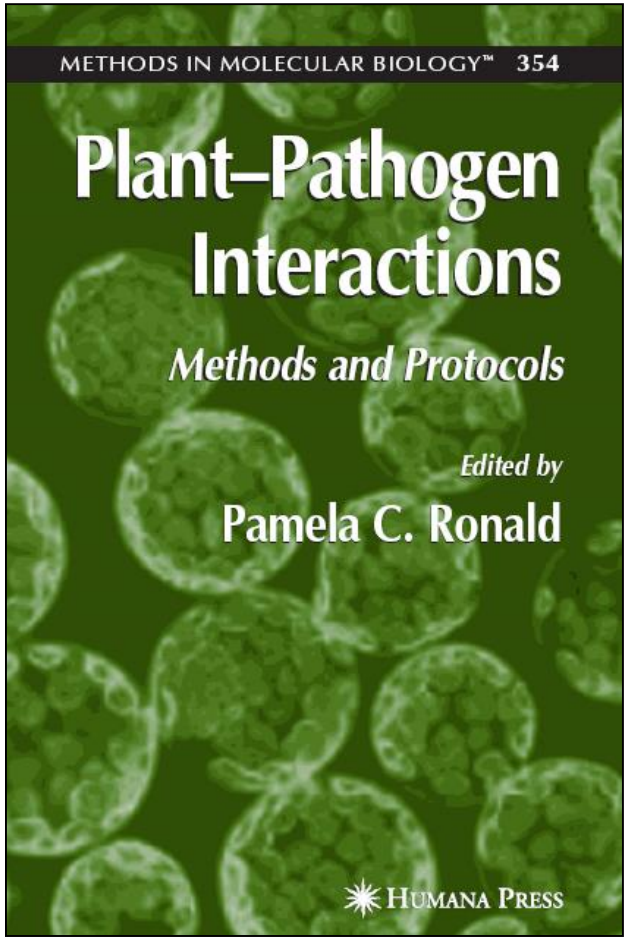

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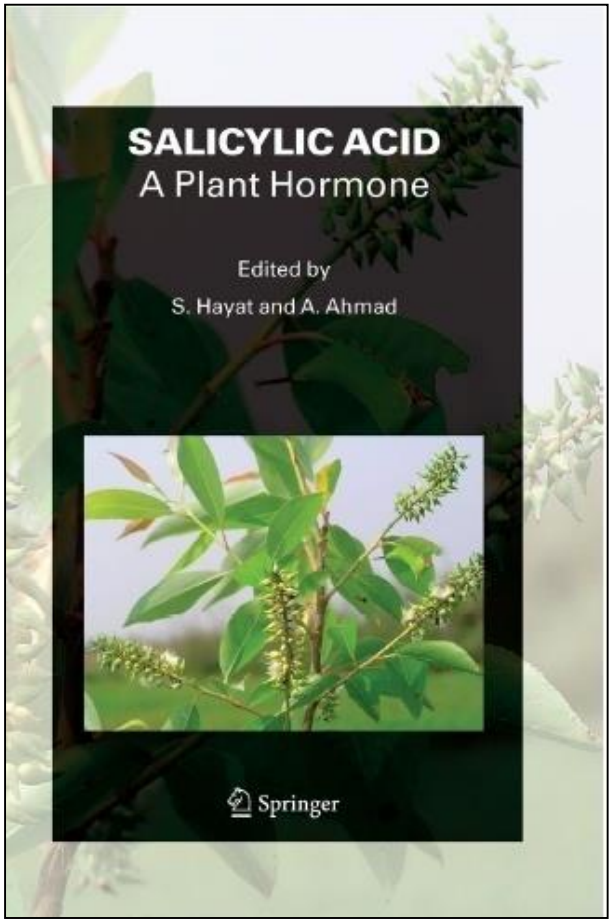

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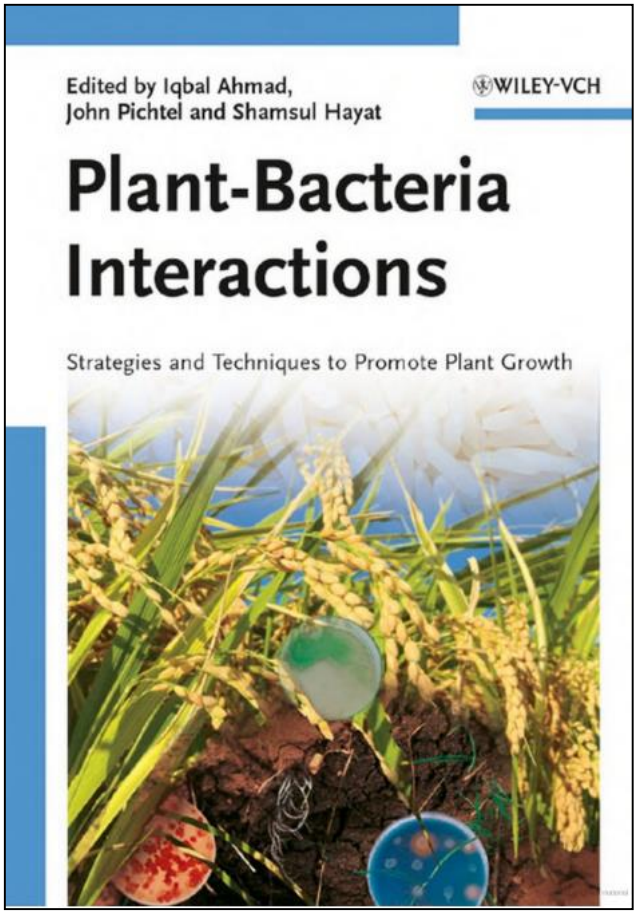

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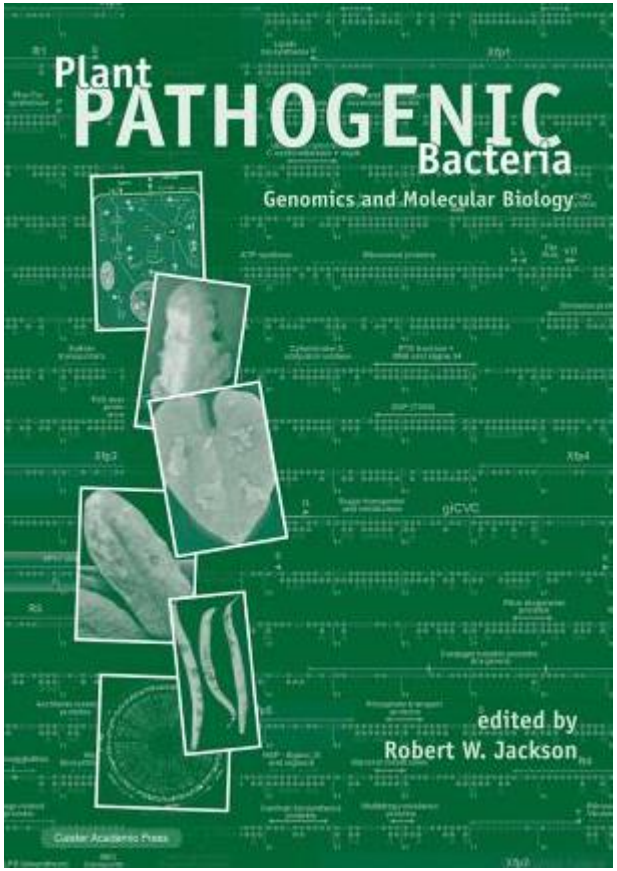

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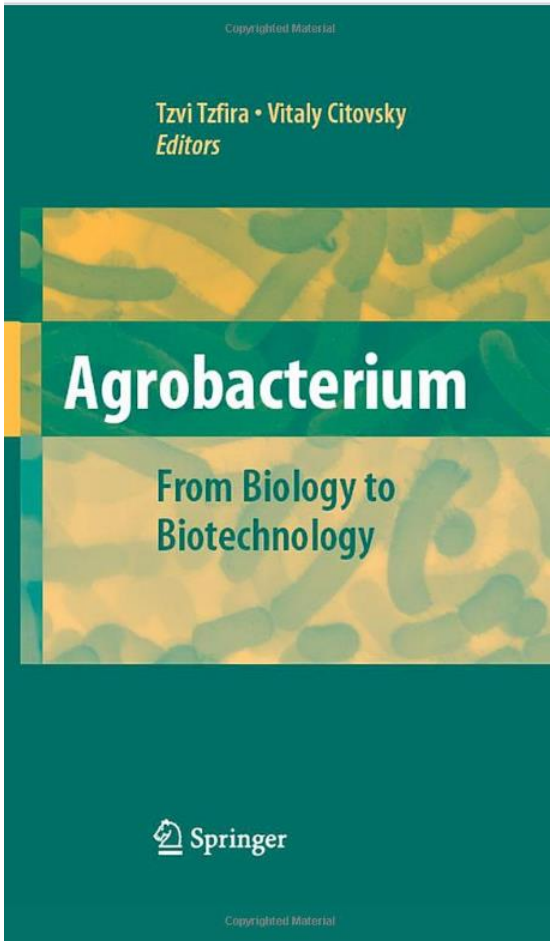

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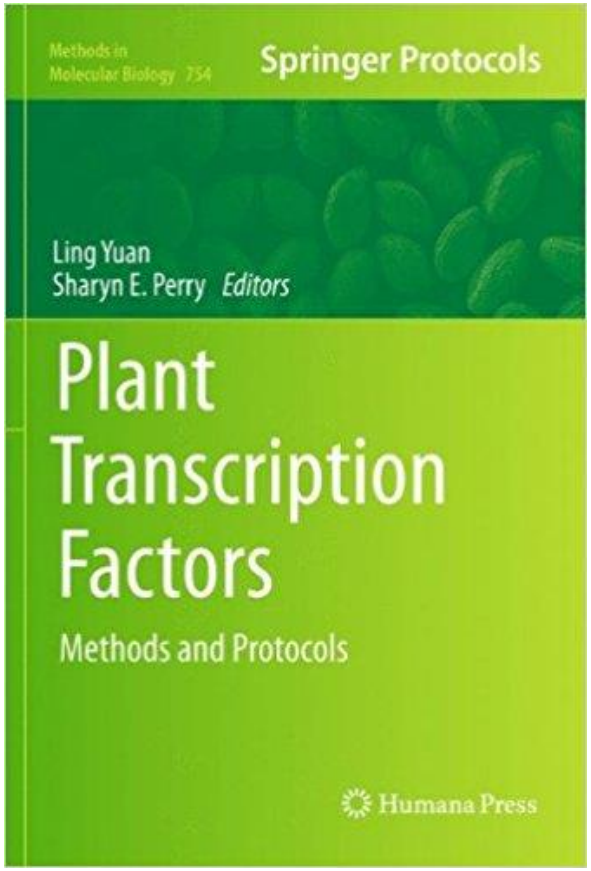

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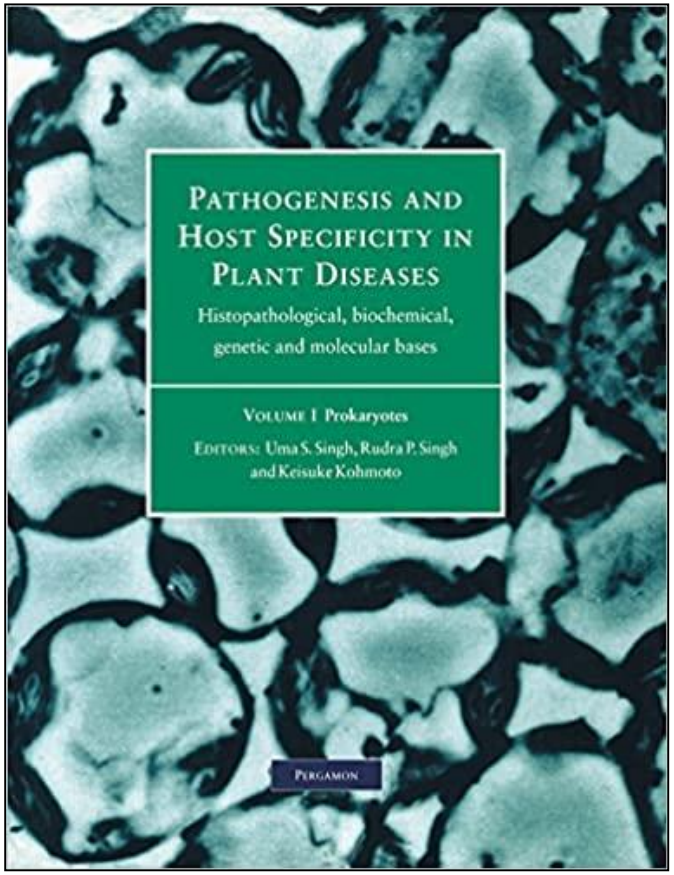

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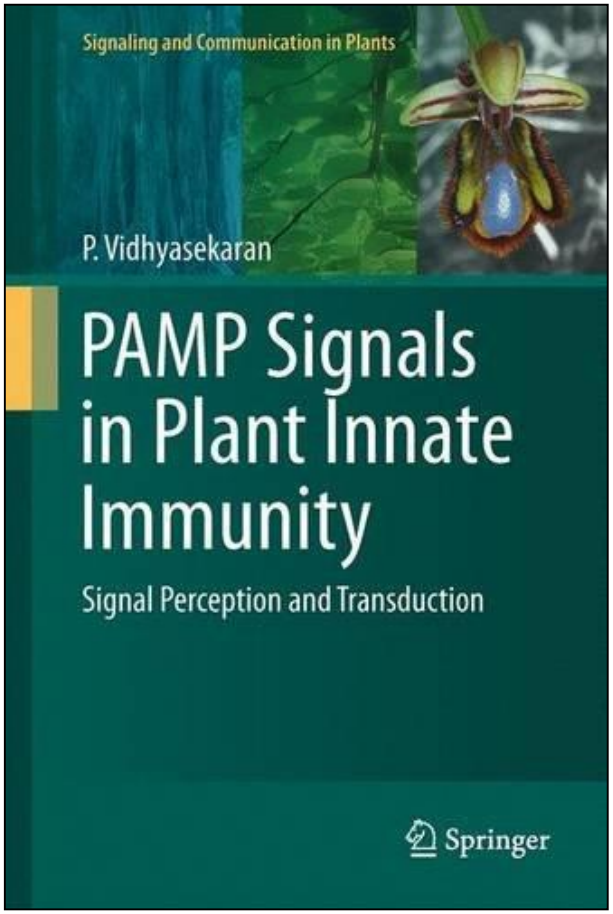

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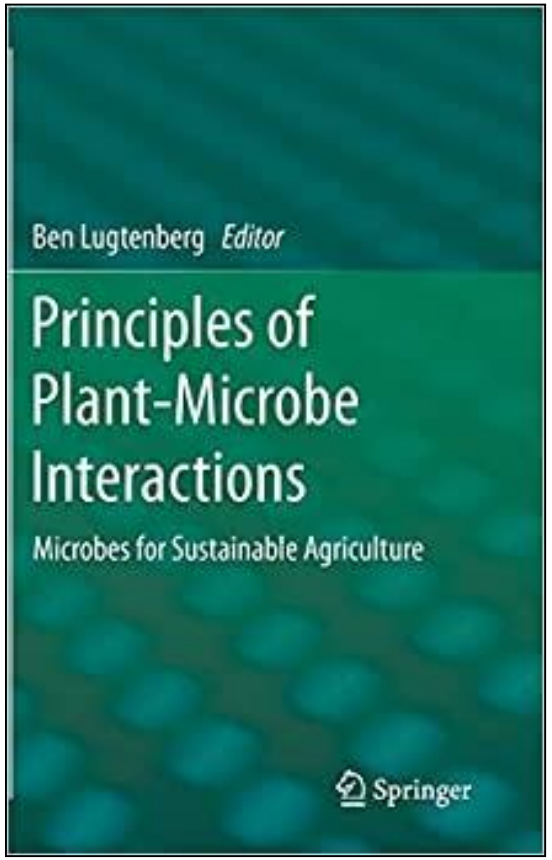

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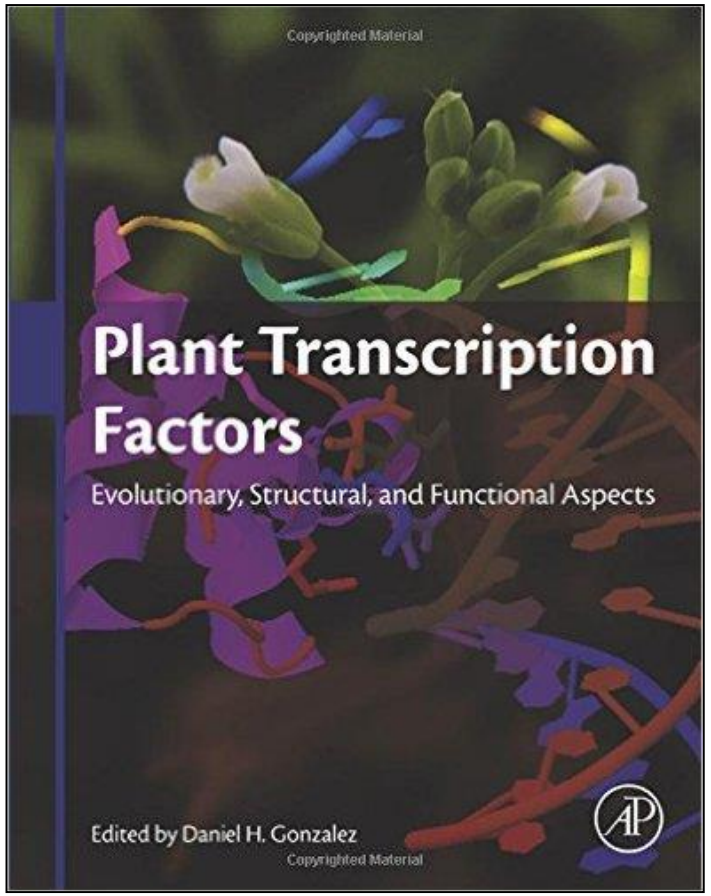

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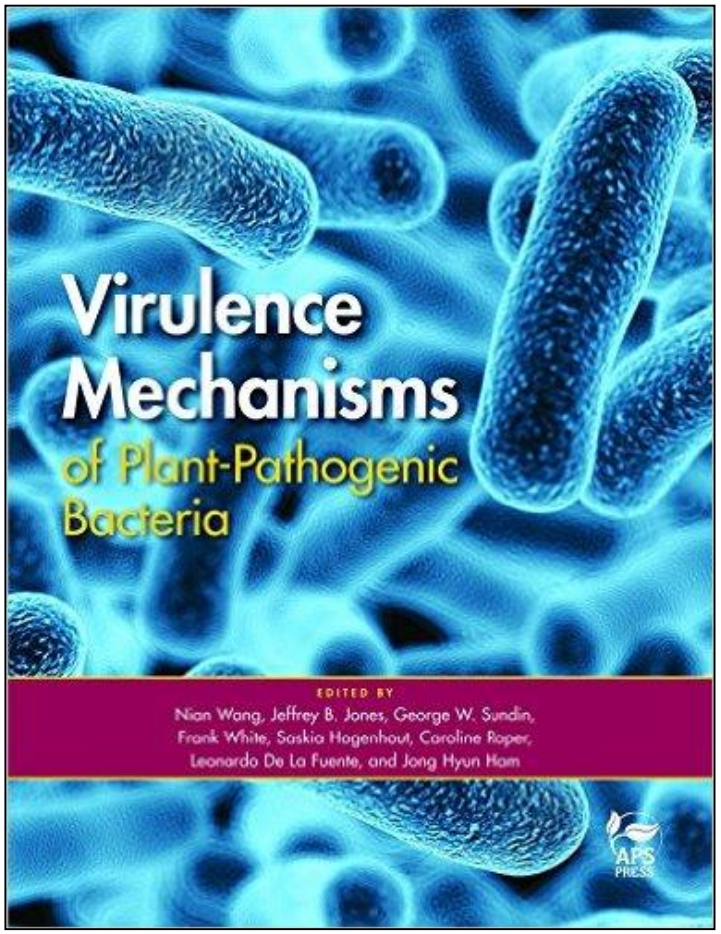

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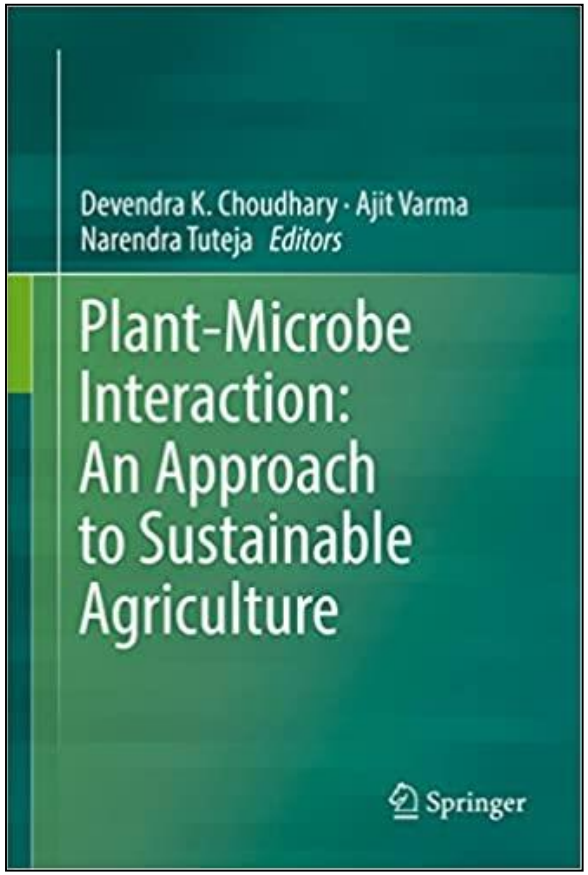

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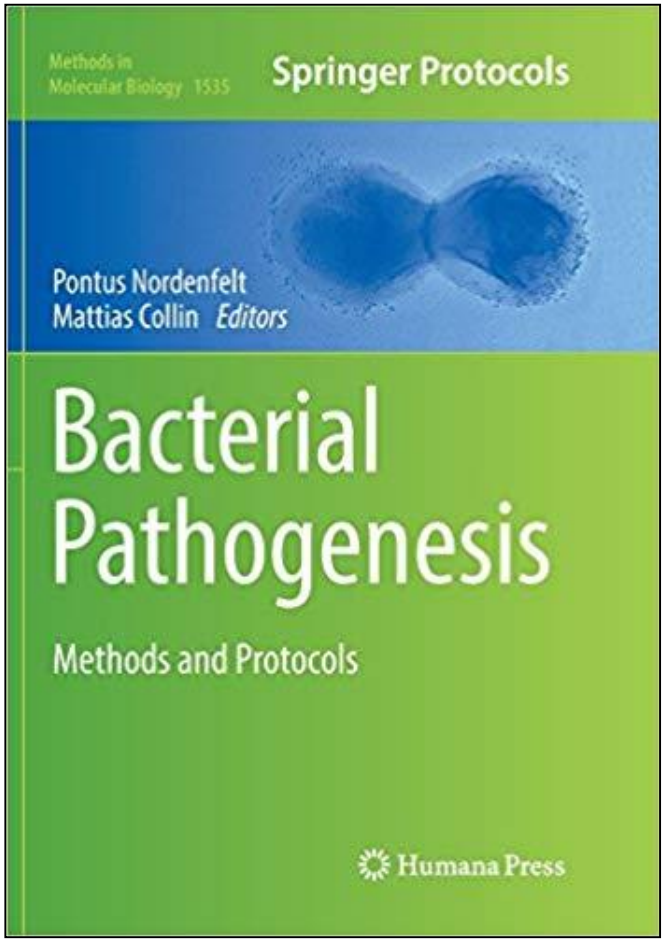

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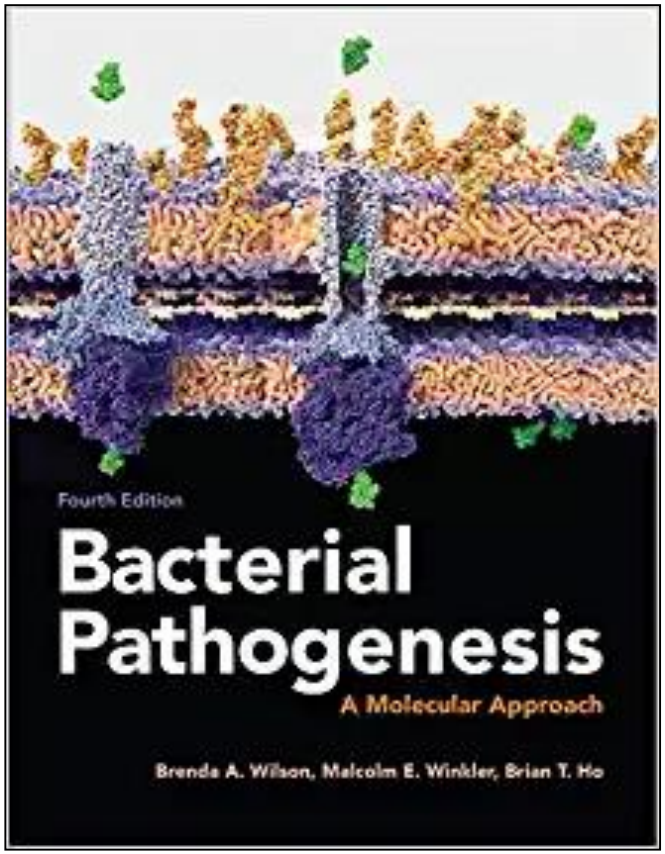

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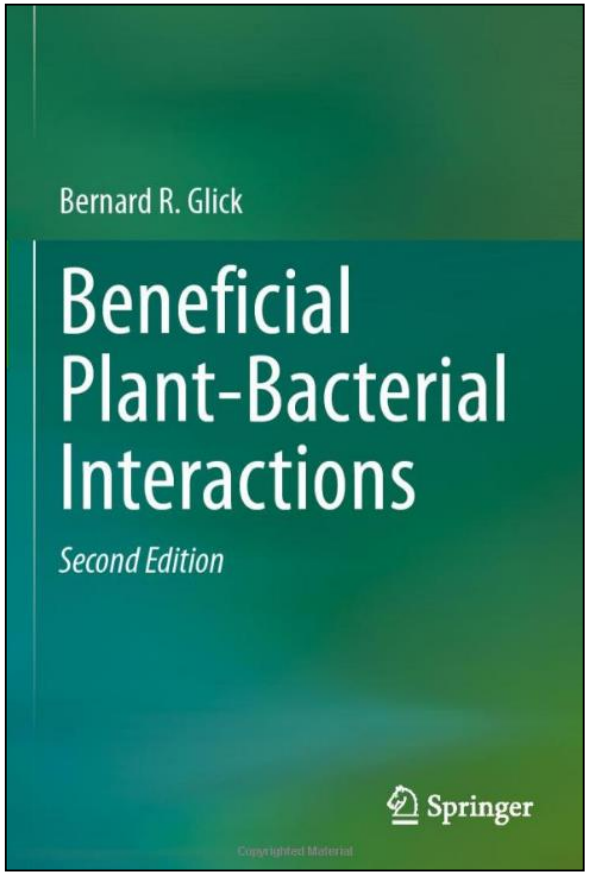

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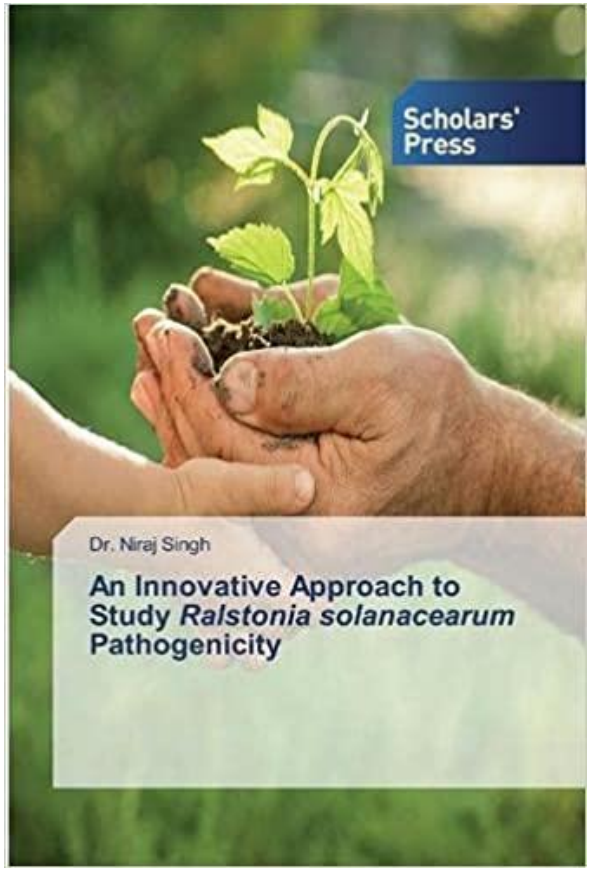

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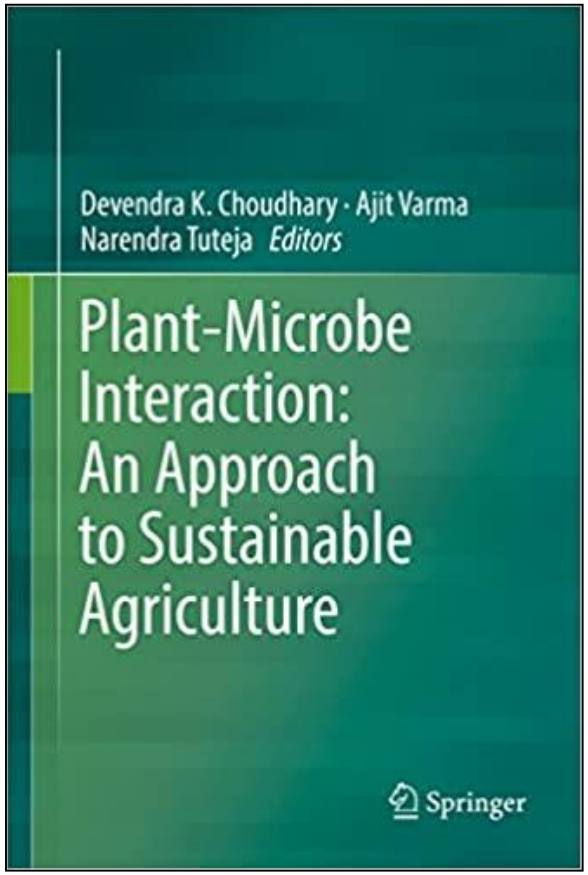

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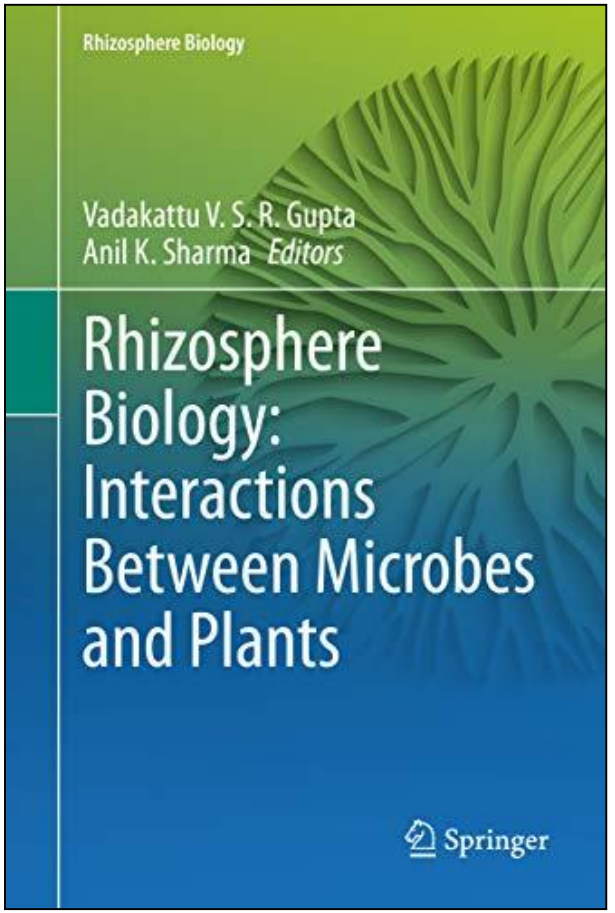

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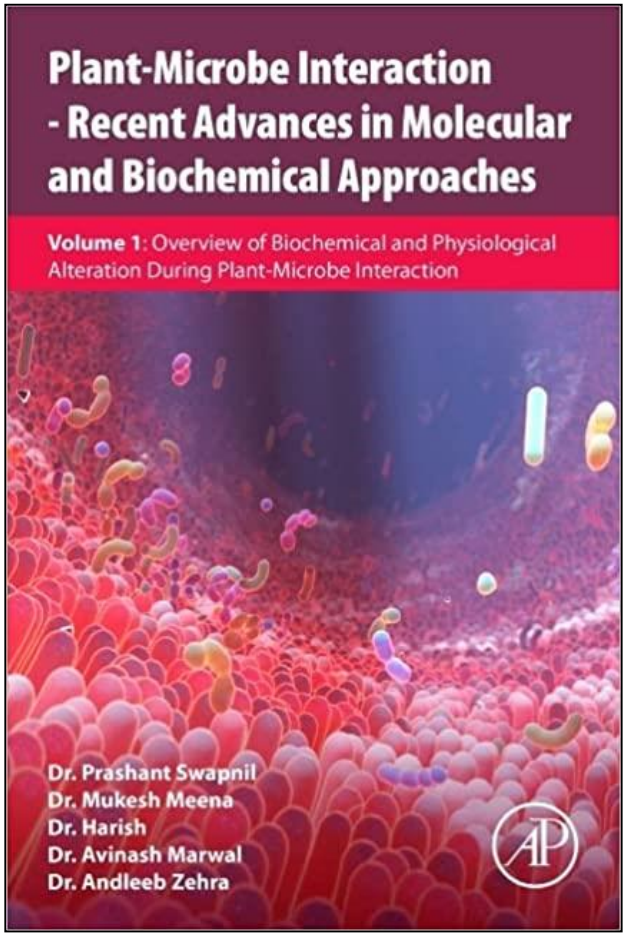

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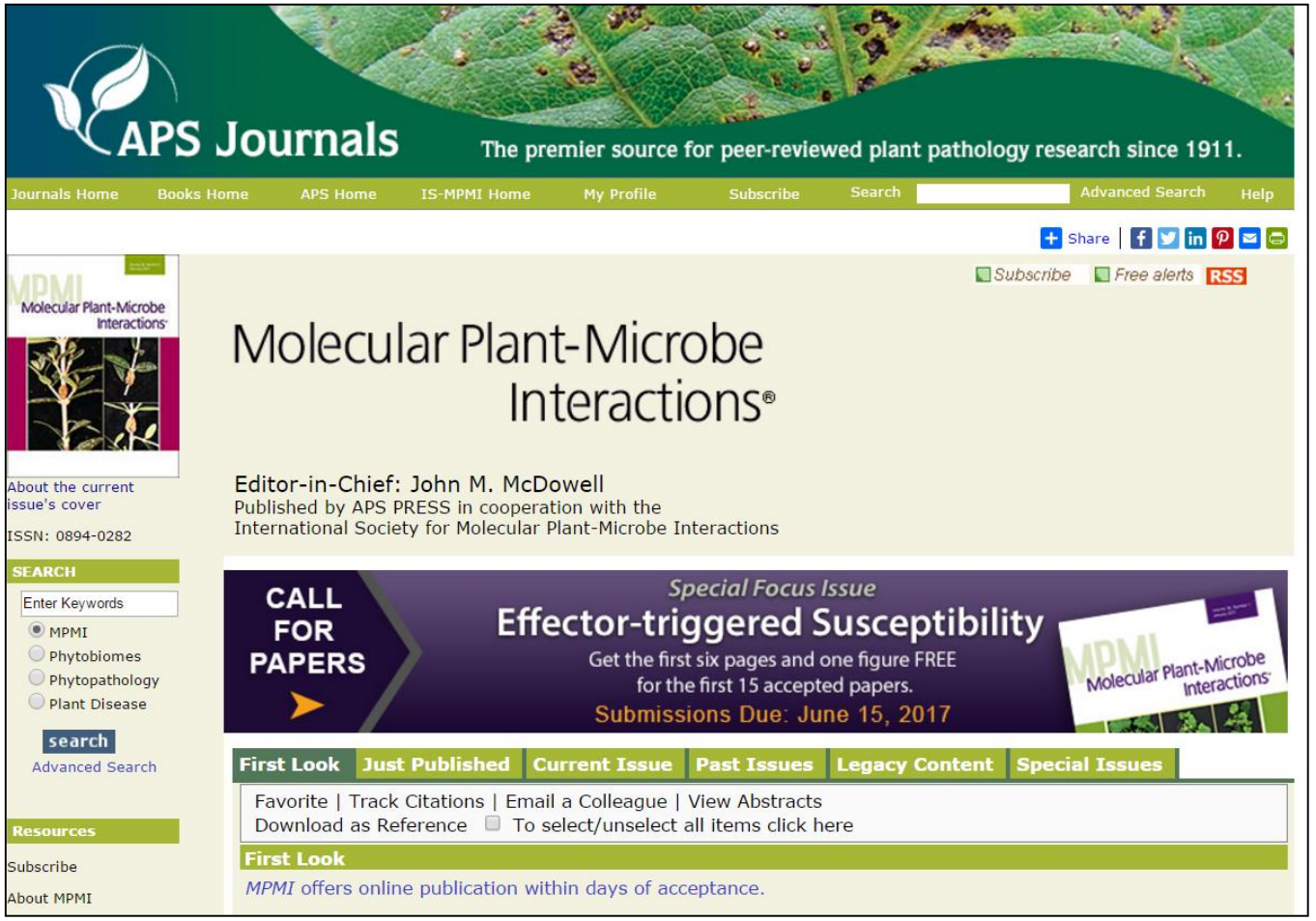

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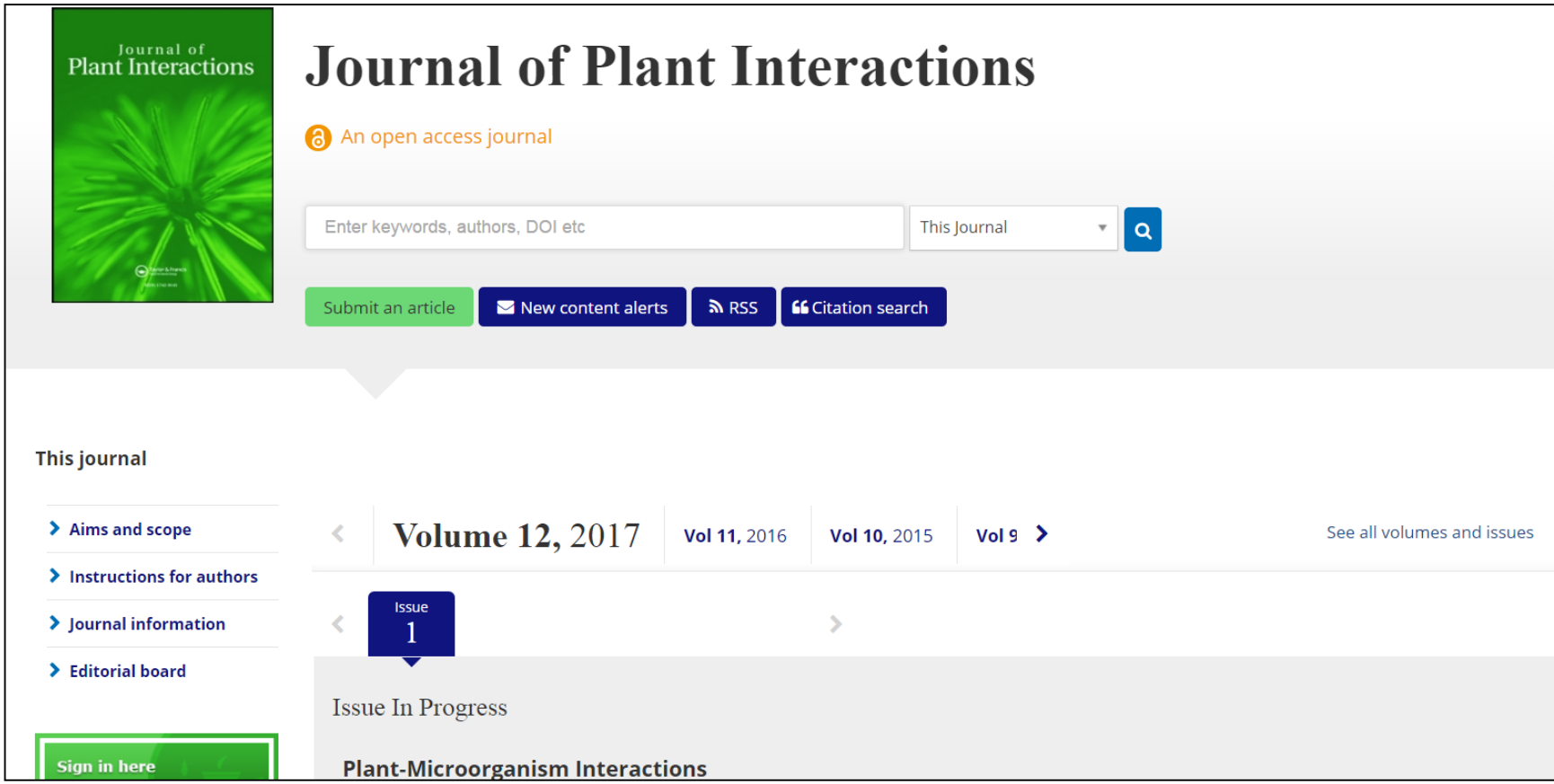


Bacterial Pathogenesis

Definition of pathogenesis

1. The origination and development of a disease.
2. The manner of development of a disease.
3. The development of a disease and the chain of events leading to that disease.
4. Host-bacterial interactions at cellular and molecular levels.

Bacterial Pathogenesis

Synonym Names

- Bacterial-Plant Interactions
- Host-Parasite Relations
- Genetics of Host-Parasite Interactions
- Mechanisms of Pathogenesis
- Molecular Biology of Plant-Pathogen Interactions
- Molecular Mechanisms of Plant-Pathogen Interactions
- Molecular Plant-Microbe Interaction
- Molecular Mechanisms of Plant Disease Resistance
- Physiology of Parasitism in Plant Diseases
- Plant-Microbe Interactions
- Plant-(phytopathogenic) Prokaryotes Interactions.

Present era of microbiology as its third golden age

- Essentially all microbiologists now speak a common language.
- So that the boundaries that previously separated subdisciplines from each other have faded:
- Physiology has become indistinguishable from Pathogenesis;
- Ecologists and molecular geneticists speak to each other;
- Biochemistry is spoken by all; and
- Mirabile dictu (wonderful to say)! Molecular biologists are collaborating with taxonomists.

Martin Dworkin

Editor-in-Chief of The Book Prokaryotes

Bacterial Pathogenesis
The background

- Progress in understanding type III secretion and pathogenicity in mammalian pathogens has already inspired plant pathologist, and will most probably continue to do so (Huguet,2004).
- Many important components that define the outcomes of plant-microbe interaction have been isolated and characterized through:

1. Classical genetic,
2. Newly developed genomic and biochemical approaches.

Note: Facultative genes (regulated gene): Genes that are transcribed only when needed. Turned on/off in response to change in the cell's environment. e.g., bacterial virulence genes.

Common bacterial pathogens of plants and animals

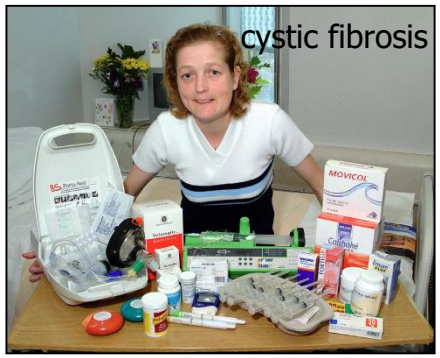
- Modern science has shown by biochemical, genetic and molecular biological analyses that bacteria are quite heterogeneous.
- Some are related to and grouped with animal and human pathogens.
- There are even a few strains of bacteria that cross kingdoms:
- They can infect both plants and humans.
- The genetic basis for such novelty is of immense interest and significance regarding the basis of infectious disease.

Common bacterial pathogens of plants and animals

Examples of cross-kingdom pathogens

Bacteria that can attack both plants and animals:

1. *Erwinia* spp.: A well-known cause of a variety of wilt diseases in plants, including bacterial fire blight of apples and pears.
2. *Burkholderia cepacia* (the causal agent of soft rot in onion) can cause life-threatening infections CF disease (cystic fibrosis) as human wounds and abscesses)patients.
3. But by far the best studied cross-kingdom pathogen is *P. aeruginosa*.



Common mechanisms of humans and plants bacterial pathogens

- They use common means of attachment, secretion and genetic regulation.
- They share many virulence factors, such as:

1. Extracellular polysaccharides,
2. Pathogenicity islands, and
3. Some type III secreted effectors.

- There are homologous and analogous toxins and virulence molecules.
- There are pathogens that are quite adept (highly skilled) at attacking both plants and animals.

Analogous: An organ or structure that is similar in function to one in another kind of organism but is of dissimilar evolutionary origin.

Guttman,2004

Common mechanisms of humans and plants bacterial pathogens

Common cell killing mechanism

- Plant pathogens and human pathogens have more in common than one would think.
- e.g. The mechanism by which *Yersinia pestis*, the cause of plaque (deadly plaque) kills the host's cells, is very similar to the mechanism used by plant pathogens.
- Due to remarkable similarities between:

1. The plant and human pathogens,
2. Their relative innate immune systems,
3. Disease-associated genes,

- There must be some compelling reason to use plant pathogens as models for human pathogenesis.
- Maybe some days plant diseases will help us understand human diseases.

Common mechanisms of humans and plants bacterial pathogens

- Given these similarities, and the many experimental advantages of plant biology, including:

1. Ease of replication,
2. Stringent(tight) genetic and reproductive control, and
3. High throughput(output)with low cost, it is proposed that plants would make excellent models for the study of human pathogenesis.

Guttman,2004

Bacterial Pathogenesis

Important concepts in phytobacteriology

- **Bacteria:**

1. Plant pathogenic bacteria act in groups, power in numbers.
2. Bacterial pathogens gain access to plants through wounds or by exploiting natural openings.
3. Bacterial pathogens can acquire new virulence genes via horizontal transfer.
4. Bacterial pathogens can manipulate plant cells by injecting DNA or proteins into plant cells or with toxins.

- **Plants:**

1. Plants can fight back by evolving ability to recognize these effectors and launch resistance/defense mechanisms.

Plant bacterial pathogens

Specialized properties

- Compared to the large number of bacterial species, relatively few are capable of infecting plants.
- This suggests that specialized properties are needed for bacteria to interact with plants.
- The bacteria generally gain access to the apoplast of plant cell through:

1. Stomata and other natural openings, or
2. Wounds (e.g. *A. tumefaciens*).

- Host compromised or not, pathogens "have distinct patterns of interaction with the host that enable them to have the upper hand in the relationship and elude at least some of the plant's defenses."

Apoplast or intercellular space is a free diffusional space outside the plasma membrane.

Plant bacterial pathogens
Specialized properties

- During their life span, pathogenic microorganisms encounter different types of environments.
- They successively alternate saprophytic and pathogenic phases that could be assimilated to "diet" and "copiotrophic"(environments which are rich in nutrients) periods.
- The success of pathogenic pathogens depends on their ability to:

1. Colonize host tissues, and
2. Counter and defense against host defense mechanisms such as plant-generated H_2O_2,organic peroxides, and superoxides.

Reverchon *et al.*,2002

PR proteins
Apoplast and vacuoles

- The plant apoplast plays a critical role in the:

1. transport of water and nutrients and
2. interactions between plants and pathogens.

- **Most of pathogenesis-related (PR) proteins are located in the apoplast.**
- In general,

1. acidic PR proteins are located in the apoplast, and
2. basic PR proteins, in the vacuole.

Naz *et al.*,2014; Enoki and Suzuki,2016

Apoplast
PR proteins functions

- These proteins have been implicated in active defense, potentially restricting pathogen development and spread.
- Some PR proteins possess antimicrobial activity.
- Also they can directly affect pathogen integrity, and/or generate signal molecules through their enzymatic activity that act as elicitors to induce other plant defense related pathways.
- One response of plants to attempted infection by pathogens is to accumulate apoplastic proteins, including pathogenesis related (PR) proteins.
- The expression of defense or PR proteins in apoplast of plant cells enhance plant resistance to pathogens.

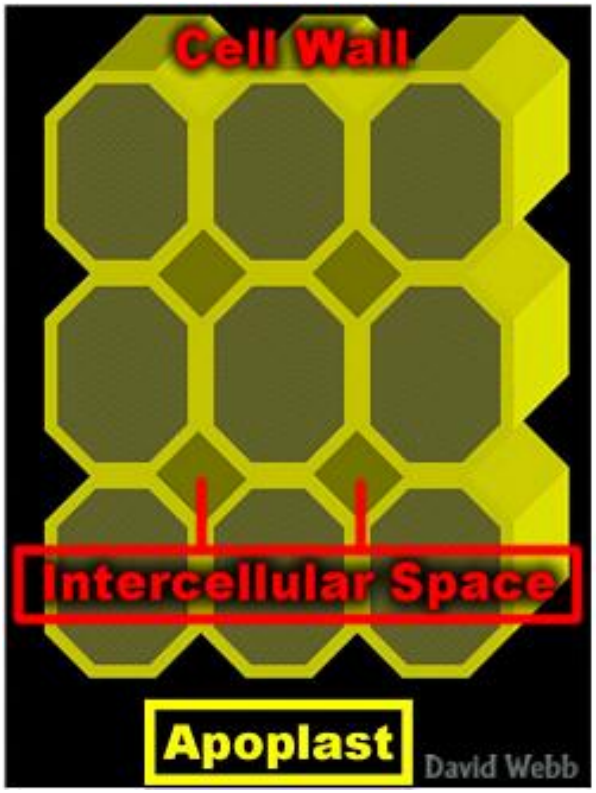
Naz *et al.*,2014; Enoki and Suzuki,2016

Apoplast

The apoplast is the portion of the plant cell outside the cell membrane. This region includes the cell walls and intercellular space of the plant

Adaptation to the plant apoplast by plant pathogenic bacteria

- The apoplast consists of:
1. The cell walls, and
2. Intercellular spaces.
- If all of the protoplasts are destroyed, the apoplast remains like a honey-comb.
- The apoplast is a nutrient-limited environment that is guarded by plant defenses (including antimicrobial compounds and other plant defese responses).



Jackson,2009; David Webb

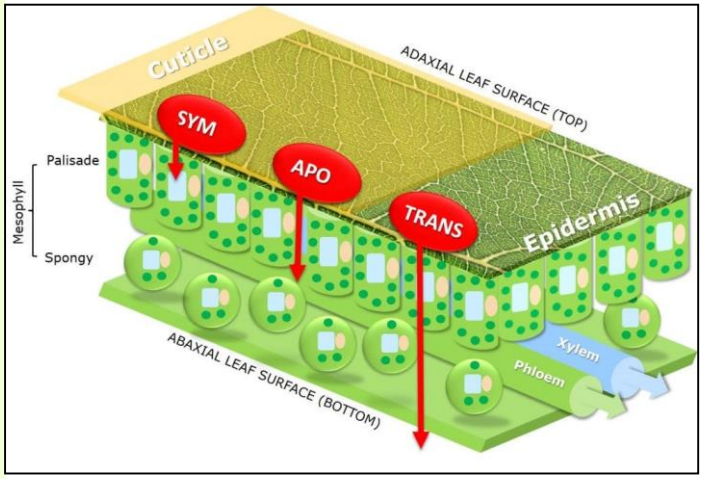
Apoplast

The apoplast is the portion of the plant cell outside the cell membrane. This region includes the cell walls and intercellular space of the plant

Adaptation to the plant apoplast by plant pathogenic bacteria

- Many plant pathogenic bacteria spend most of their parasitic life in the apoplast (intercellular space) of plant tissues and cause symptoms.
- Plant pathogenic bacteria (biotrophs and necrotrophs) have evolved several strategies to successfully colonize this niche, which include:

1. The type III secretion system and its effectors,
2. Toxins and cell wall degrading enzymes, among others.



Jackson,2009; David Webb; BioScience Solutions

Apoplast

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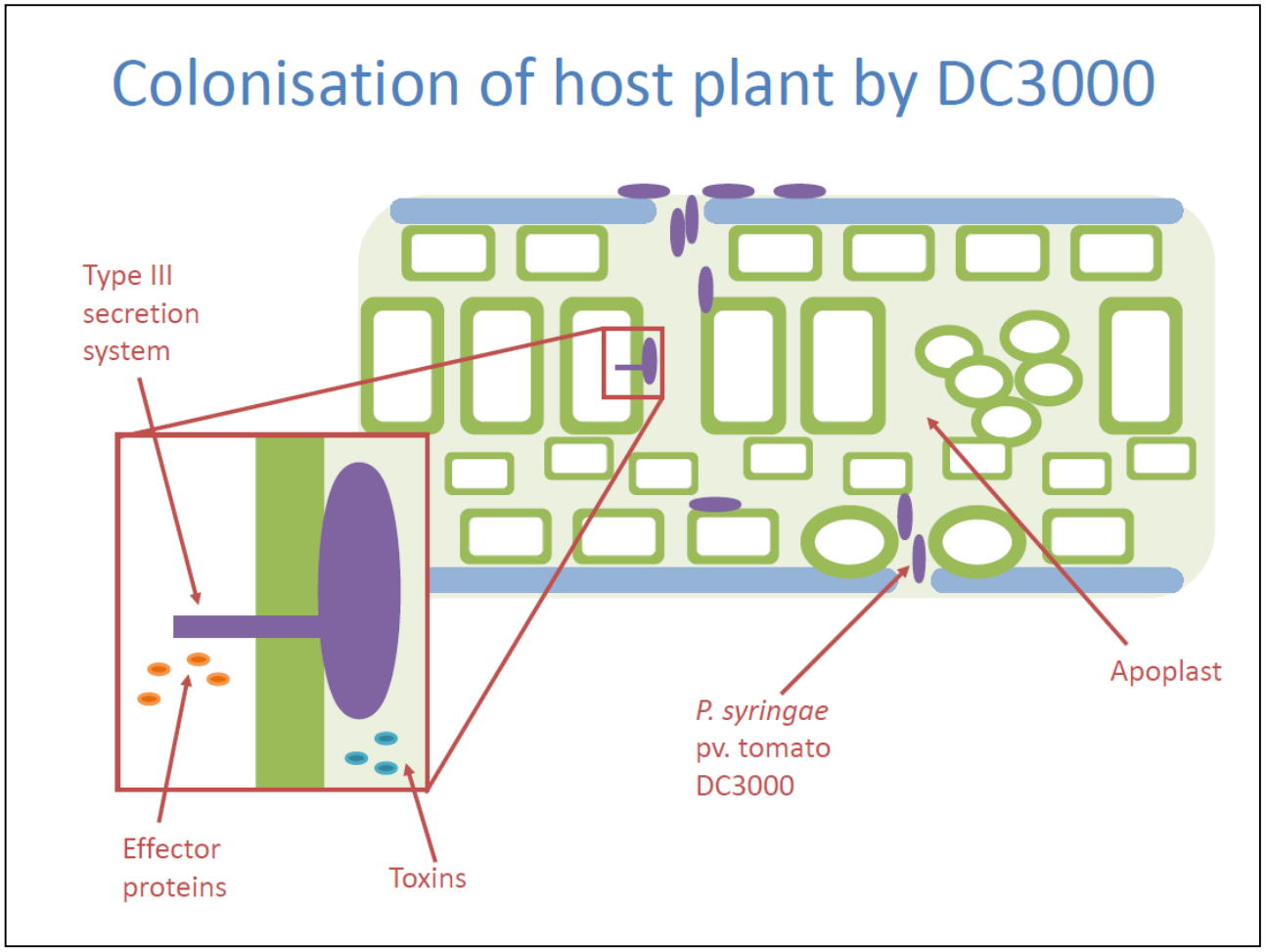
Adaptation to the plant apoplast by plant pathogenic bacteria



L. McCraw

Plant-Pathogen Interactions

Types, nature, goals and approaches

Mechanisms of interactions at the cellular and molecular levels

Plant-Microbe interaction

Types of microorganisms

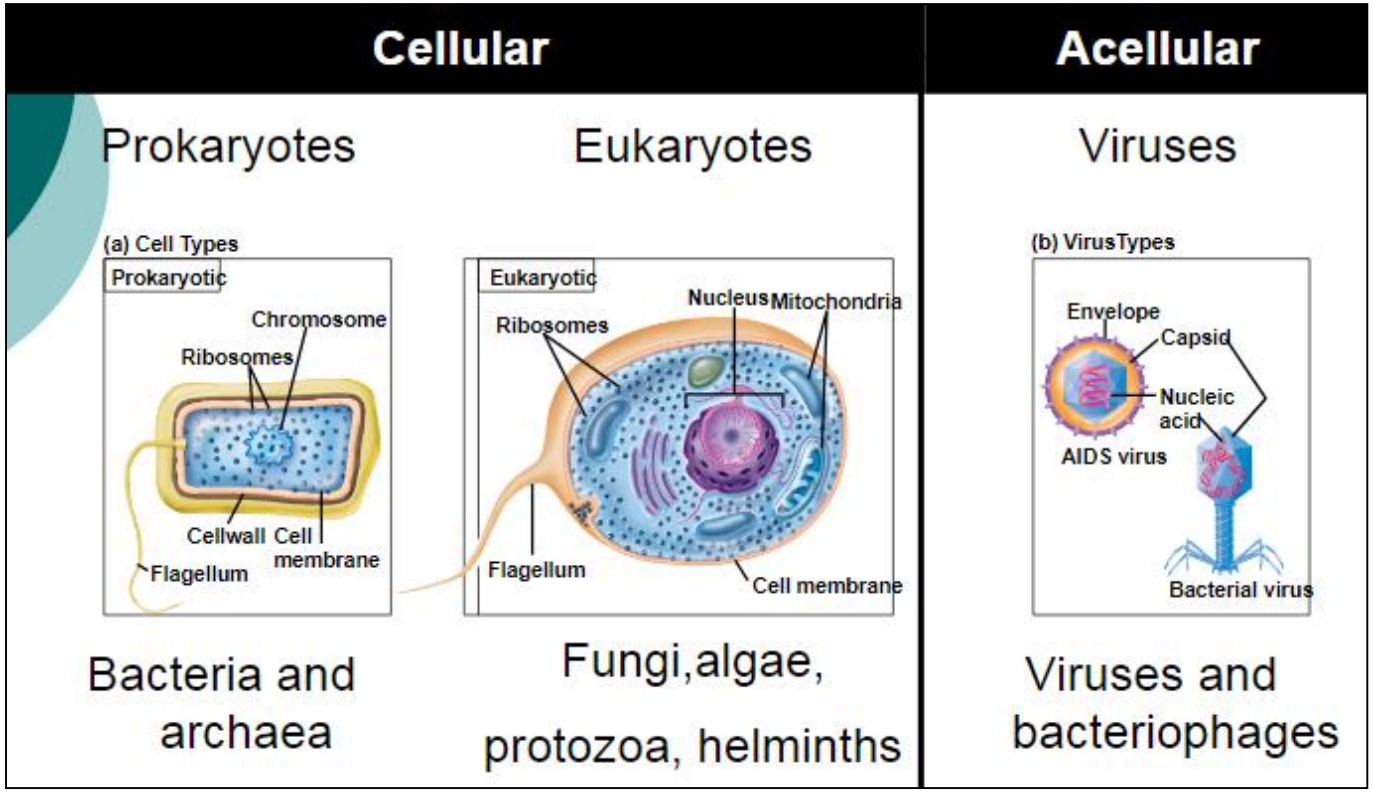
- Microbiology is the study of living organisms of microscopic size.
- Microbes consist of a variety of organisms:

1. Bacteria
2. Algae
3. Fungi
4. Viruses
5. Protozoa

- Less than 1% of known microorganisms cause disease.

Plant-Microbe interaction

Types of microorganisms



Plant-Microbe interaction

Factors affecting plant-microbe interactions

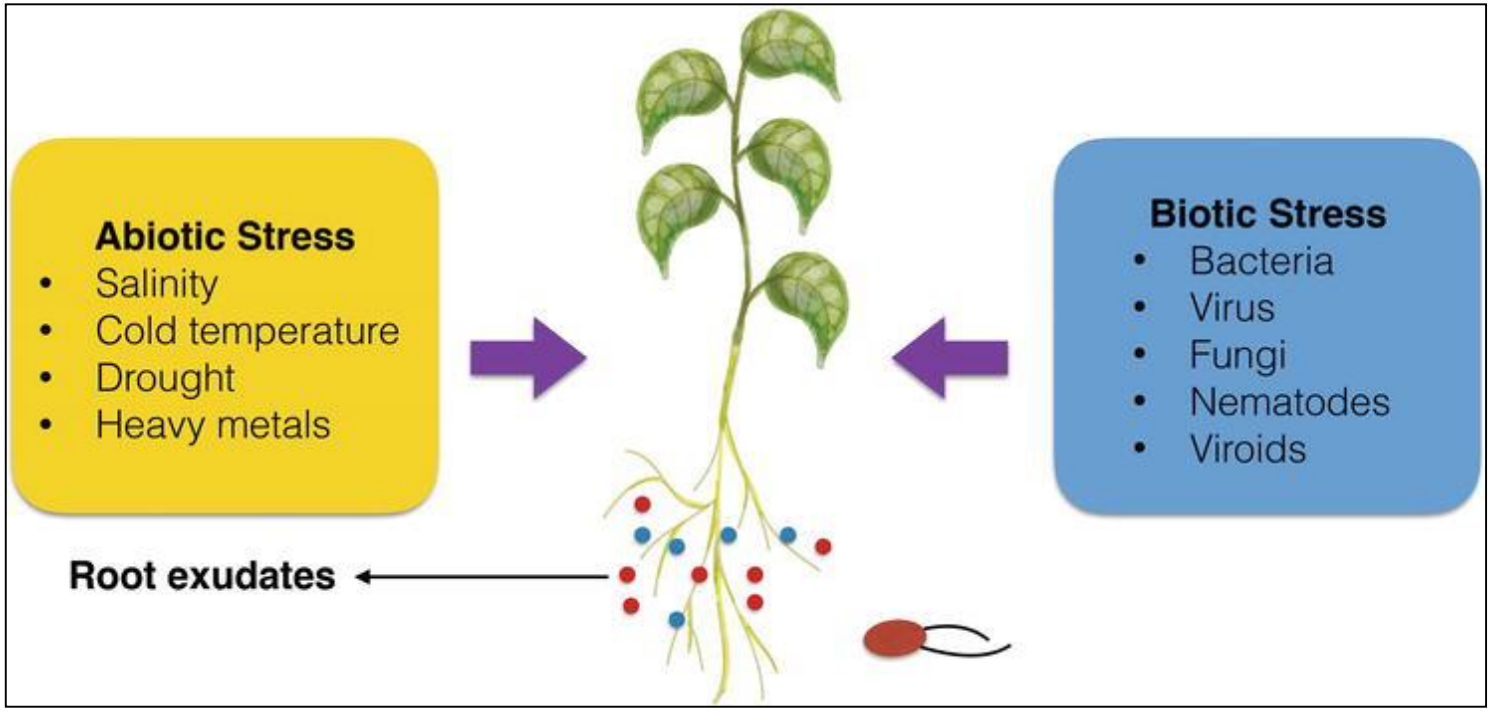
- Plants secrete various organic compounds resulting in a nutritionally enriched environment favorable for microbial growth.
- As a result, plants are heavily colonized with a diversity of microbes whose reservoir is primary the soil.
- Microbes that colonize plants are called either:

1. epiphytes (colonize plant surface), or
2. endophytes (colonize plants interior).

Thakur,2018

Plant-Microbe interaction

Factors affecting plant-microbe interactions

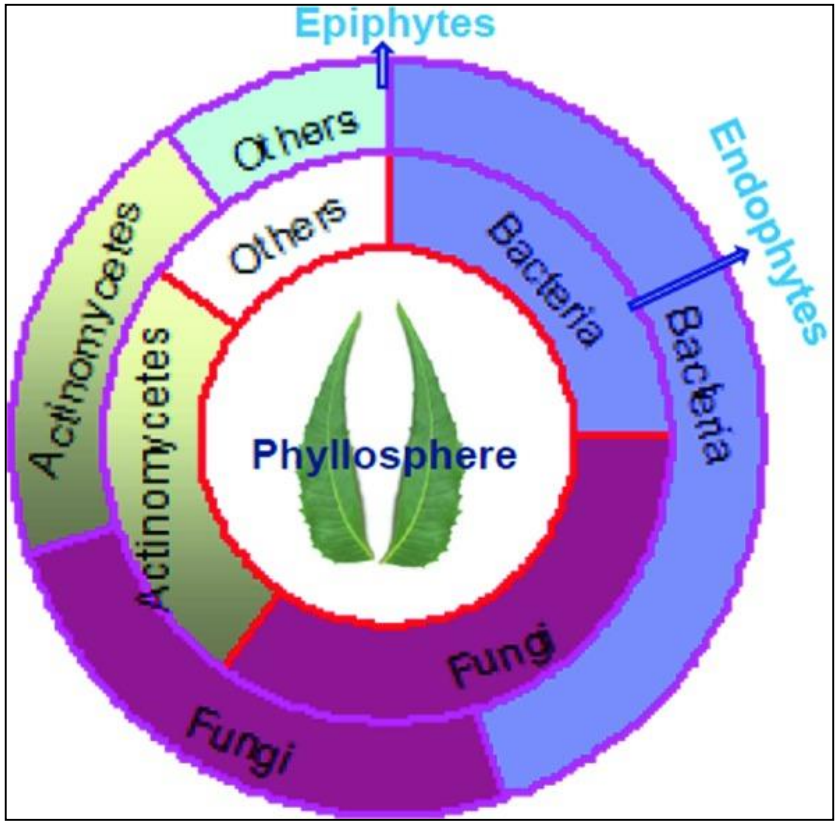


Ho *et al.*,2017

Plant-Microbe interaction

Epiphytic and endophytic microbes in phyllosphere

- The nature of various microorganisms (epiphytic and endophytic) associated with phyllosphere.
- Among the diverse community of microbes, bacteria are the predominant community on leaves and its range is between 10^2 and 10^{12} g^{-1} of the leaf.
- Thompson *et al.*,1993 identified 78 bacterial species from the sugar beet.

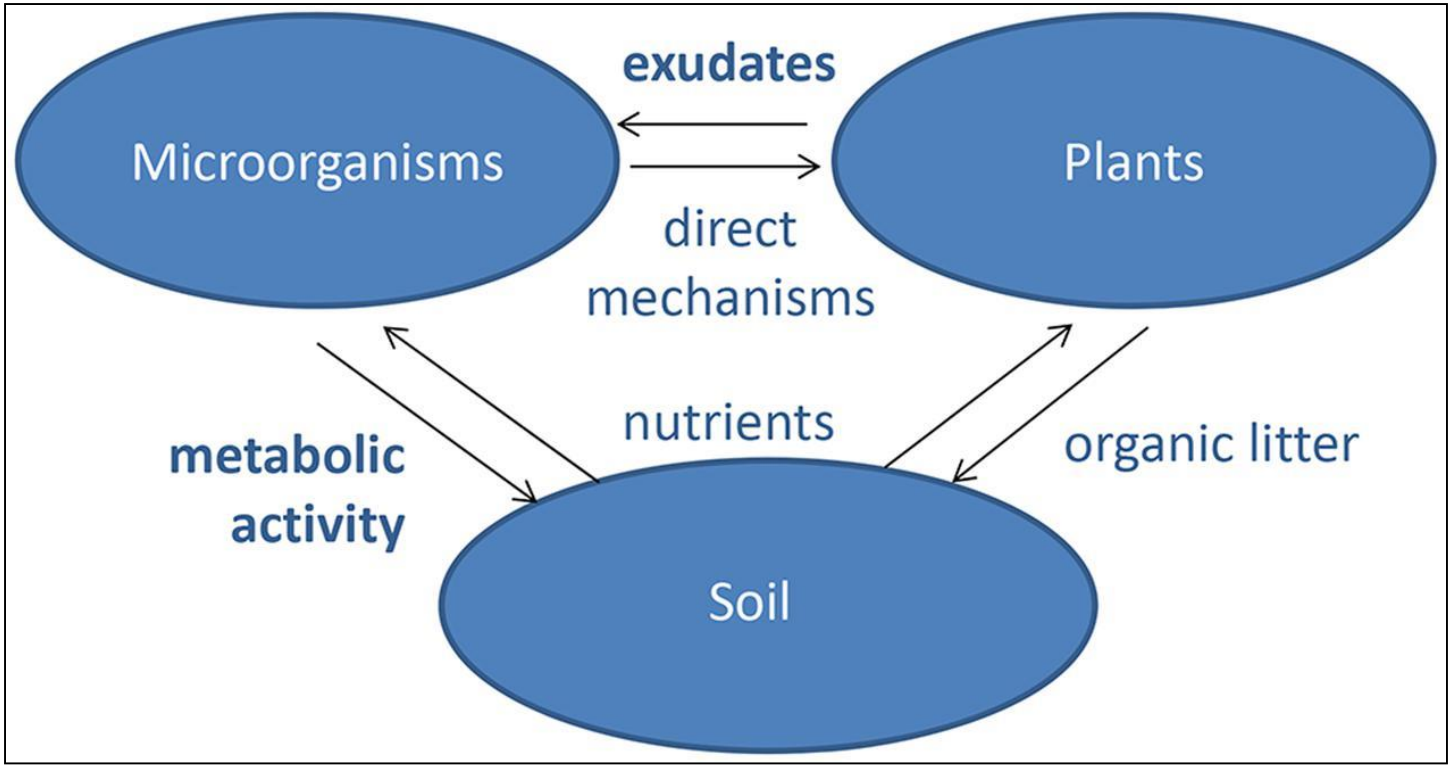


Sivakumar *et al.*,2020

Plant-Microbe interaction

Soil microorganisms

Interactions between plants, microbiota, and soil



Microbial communities influence plants in direct and indirect ways.

Jacoby *et al.*,2017; Thakur,2018

Plant-Microbe interaction

Plant-microbe interactions

- Plants and microbes can have variety of interactions including pathogenic, symbiotic and associative.
- In brief, these are:

1. Pathogenic Relationship
2. Symbiotic relationships
3. Mutualism
4. Commensalism
5. Parasitism.

Thakur,2018

Plant-Microbe interaction

Symbiotic bacteria

Mutual relationships

- **In mutualism, both organisms, bacteria and host, benefit**.
- Bacteria benefit from the plant nutrients provided by the roots, but plants can benefit from their rhizobacteria as well.
- E.g.
- Bacteria known as Plant Growth-Promoting Rhizobacteria (PGPR).

Plant-Microbe interaction

Symbiotic relationships

Commensalism

- **In commensalism, one organisms, bacteria or host, benefit.**
- In commensalism, one organism benefits while the other is unaffected.
- This occurs when the bacterium *Staphylococcus epidermidis* uses the dead cells of the human skin as nutrients.

Plant-Microbe interaction

Symbiotic bacteria

Competition relationships

- **In competition, both organisms, compete with their neighbors for space and resources.**
- Competition has a negative effect on both of the species (-/- interaction).

Plant-Microbe interaction

Symbiotic bacteria

Parasitism relationships

- **A type of symbiosis in which one organism benefits while harming the other is called parasitism.**
- Most pathogenic bacteria are obligate parasites; that is, they are found only in association with their hosts.
- Pathogenic parasites, which cause disease, do so by resisting the host's defenses and growing at the expense of the host.

The goals

1. Studying plant-pathogen relationships

- **Virulence of pathogenic bacteria:**
- Plant-pathogen interaction is a multifaceted process, mediated by the pathogen- and plant-derived molecules which mainly include:

1. proteins,
2. sugars, and
3. lipopolysaccharides.

Boyd *et al.*,2013

The goals
Studying plant-pathogen relationships

- **Virulence of pathogenic bacteria:**
- Better understand how a pathogenic bacterium invades the host and causes disease by comparing genes that code for:

1. Bacterial surface proteins,
2. Secreted proteins (secretomes) of these organisms, and
3. by the process of elimination.

- One can conclude that the proteins are involved in pathogenesis and other virulence roles.

The goals

1. Studying plant-pathogen relationships

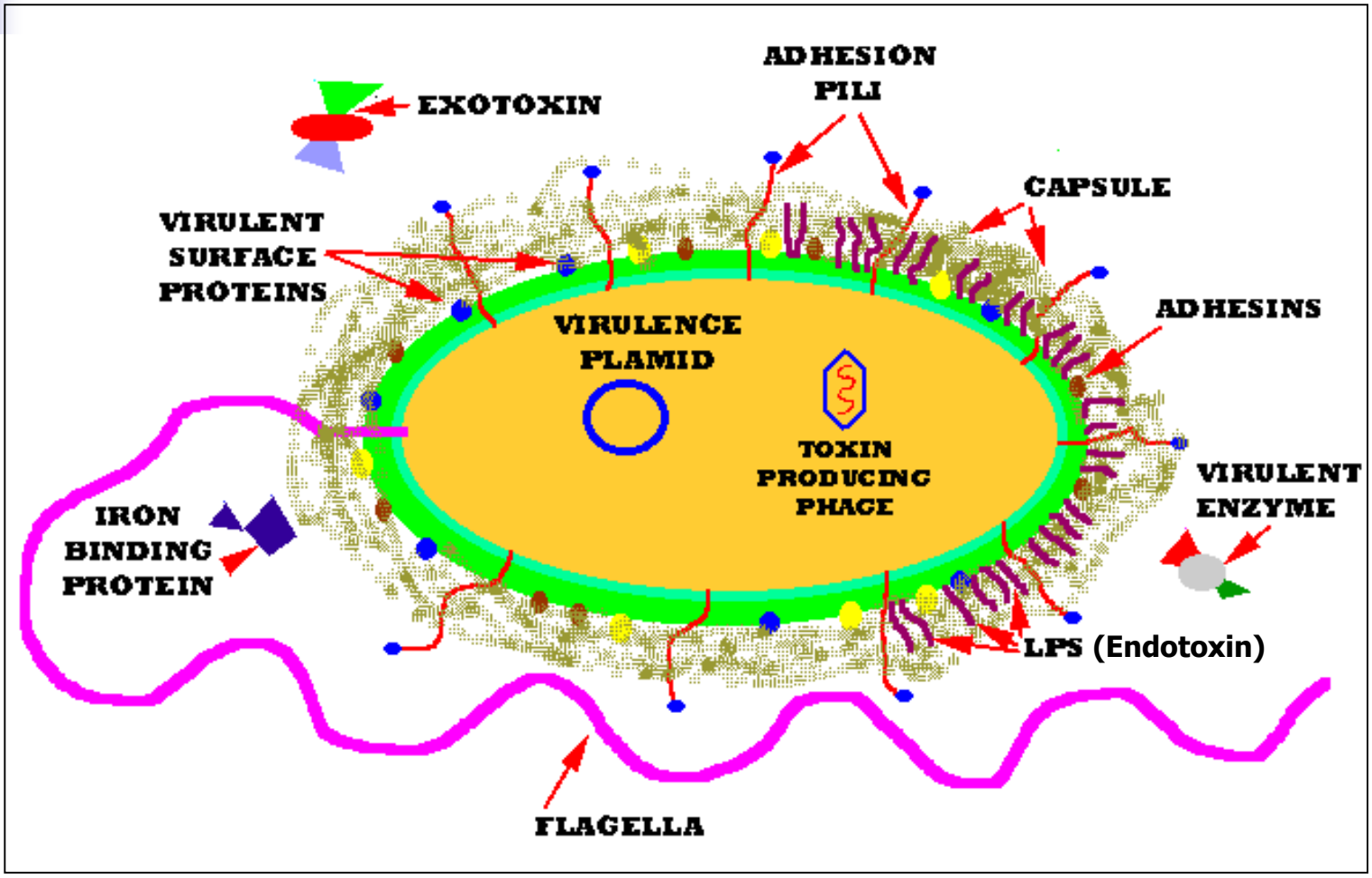
- **Virulence of pathogenic bacteria:**
- Plant-bacterial interactions have provided one of the best systems for studying plant-pathogen relationships and the molecular basis of disease resistance in plants.
- Better understand how a pathogenic bacterium invades the host and causes disease by comparing genes that code for:

1. Bacterial surface proteins,
2. Secreted proteins (secretomes) of these organisms and by the process of elimination.

- One can conclude that the proteins are involved in pathogenesis and other virulence roles.

The goals

Studying plant-pathogen relationships



The goals

2. Antipathogenesis approach

- The antipathogenesis approach (novel disease control approaches) involves the:
- identification of weaknesses in a pathogenesis strategy, as targets for the development of effective disease control measures (Birch,2001).
- Novel knowledge gained from these front lines undoubtedly will be translated into practical applications in control of crop diseases.

The goals

3. To identify and design the novel genes

- We now have hundreds of millions of DNA sequences from all sorts of organisms deposited in databases.
- However, our abilities to translate raw genomic data into useful knowledge are still very limited.
- Because the navigation system of bacteria has been studied by scientists employing the latest advances in genetics, biochemistry, biophysics, structural biology and other traditional biological disciplines, the system has become one of the best understood molecular systems in nature.

The goals

To identify and design the novel genes

- Plant biotechnology as a new era for plant pathology and plant protection is directed at the design of novel genes that will enable transformed plants to inactivate or resist key pathogenicity factors.

1. These genes can be obtained from different sources, and
2. Engineered into plants to enhance resistance to plant pathogens.

Fermin-Muñoz *et al.*,2000

The goals

To identify and design the novel genes

- The four major areas of research and application of plant genetic engineering for resistance to plant pathogens consist of:

1. Enhancing resistance with plant genes;
2. Enhancing resistance with pathogen derived genes (derived from the pathogens themselves);
3. Enhancing resistance with antimicrobial proteins;
4. Enhancing resistance with plantibodies.

A plantibody is an antibody produced by genetically engineered crops.

Fermin-Muñoz *et al.*,2000

The goal

4. Engineering resistance to pathogenic bacteria

- The first avirulence genes were cloned from bacteria (Staskawicz *et al.*,1984) and the very first plant resistance genes defined and cloned in:

1. Tomato, and
2. Arabidopsis.

- conferred resistance specifically to strains of *Pseudomonas syringae*.

Pseudomonas syringae -affected Arabidopsis.

Future challenges
In plant-microbe interactions

- Future challenges in this field are to:
- Further elucidate the molecular basis of:

1. R-Avr recognition,
2. Signal initiation, and
3. Transduction during the establishment of *R*-dependent immunity,
4. Dissect the genetic constitution of nonhost resistance,
5. Reveal its molecular links with the multifaceted host resistance.

Future challenges

In plant-microbe interaction

Continued

- These practical aspects include:

1. Facilitated cloning and transfer of important *R* genes in crop species;
2. Genetic engineering of broad-spectrum, and
3. Durable plant disease resistance.

Advances in plant microbe interactions

Research areas

- Plant microbe interactions are at the forefront of plant science research and have shaped the way we approach the study and exploration of pro- and eukaryotic organisms and their environment.
- Advances in plant microbe interactions at the molecular, physiological and ecological levels have improved our understanding of how plants interact with bacteria, fungi and other organisms.

Allen *et al.*,2009

Advances in plant microbe interactions

Research areas

- These interactions are at the heart of our biological understanding of model organisms that are currently being translated into agricultural applications including but not limited to the biological control of crop protection and performance.

1. Pathogen colonization of plant surface or vasculature;
2. System has unique biology (e.g. *Agrobacterium tumefaciens*);
3. Plant-associated human pathogen;
4. Pathosystem has potential impact on medical biology.

Allen *et al.*,2009

Advances in plant microbe interactions

Future perspective

- The understanding of plant-microbe and microbe-microbe interactions will provide a helpful future perspective as a modulating microbiome for:

1. minimizing disease incidence, and
2. enhancing gross plant productivity.

- Further, beneficial plant-associated bacteria could act as counterparts against pathogens within the microbial ecosystem, as well as stabilize the ecosystem, enhance biodiversity, avoid pathogen outbreaks, and increase plant productivity.

Ho *et al.*,2017

Advances in plant microbe interactions

Future perspective

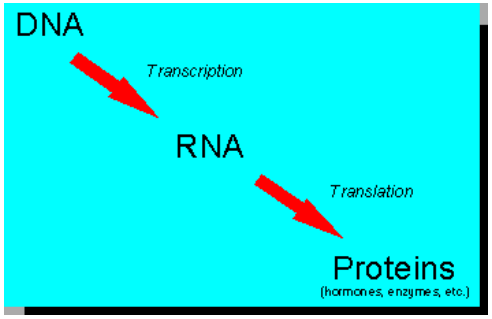
- A well-studied plant-microbe partnership in the future will also help increase crop productivity at little expense and could, in turn, lead to another "Green Revolution".

Ho *et al.*,2017

Protein-Protein Interactions

Proteins

Structure and functions



In prokaryotes, the process of transcription and translation occur simultaneously. The translation of mRNA starts even before a mature mRNA transcript is fully synthesized. This simultaneous transcription and translation of a gene is termed coupled transcription and translation.

Genetic codes

Required for proteins but not sugars and lipids

- Almost all of the major molecules(macromalocules) inside any living thing can be classified as either:

1. carbohydrate,
2. protein,
3. Lipid, or
4. nucleic acid.

- Genetic code controls the structure of proteins.
- Genetic code is not required for carbohydrates and lipids.

Genetic codes

Required for proteins but not sugars and lipids

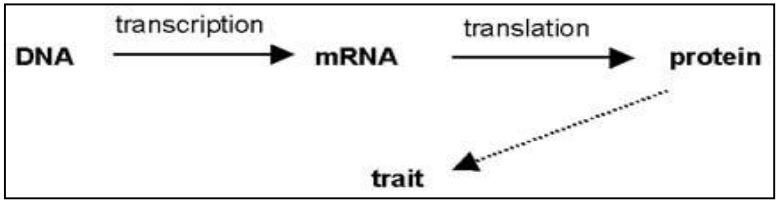
- It is true that the genetic code controls the structure of proteins.
- Lipids and carbohydrates are small molecules not proteins.
- Therefore they cannot be coded by DNA.
- However, proteins that are coded by DNA are used by cells in the body to synthesize lipids and carbohydrates as well as most small molecules used in an organism.
- Carbohydrates and lipids are either modified or synthesized by enzymatic reactions.
- In turn, enzymes are proteins.

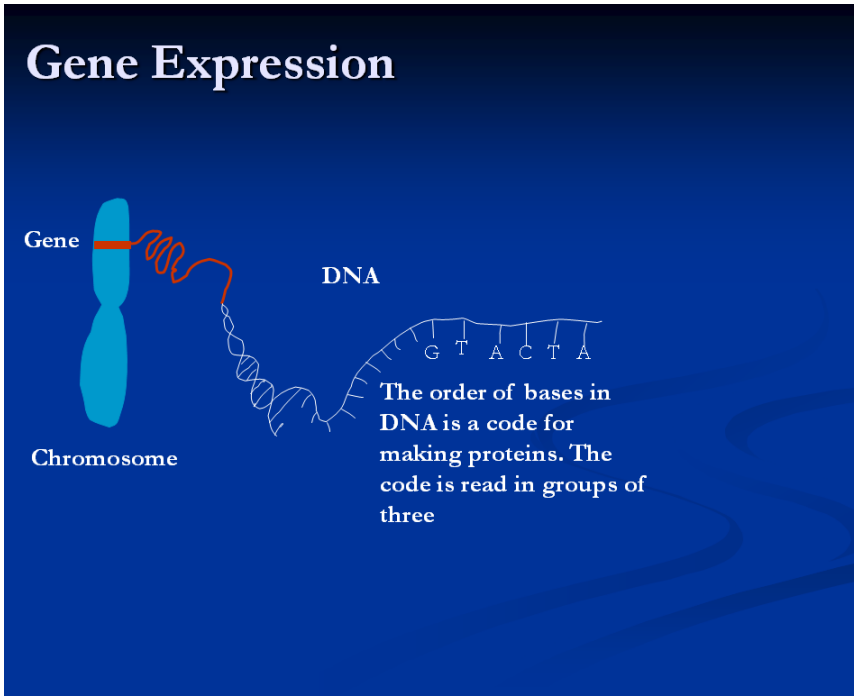
Gene expression

For protein synthesis

Proteins are the building blocks of life

- Generally speaking, proteins do everything in the living cells.
- All functions of the living organisms are related with proteins.
- Each protein or group of proteins are responsible for they own specific function.





Gene expression

With many exceptions

Campbell and co-workers

Biology is based on evolutionary changes

Why does biology have so many exceptions?

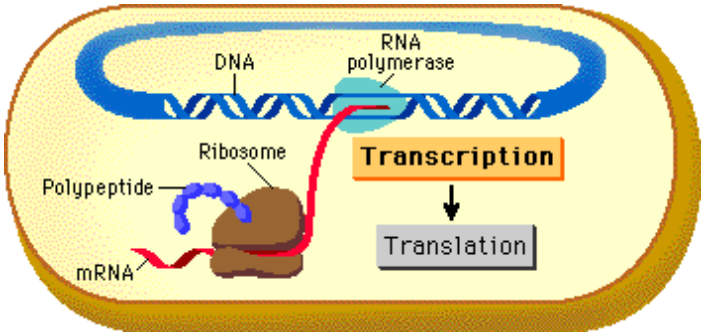
In physics/chemistry, properties don't change

BUT

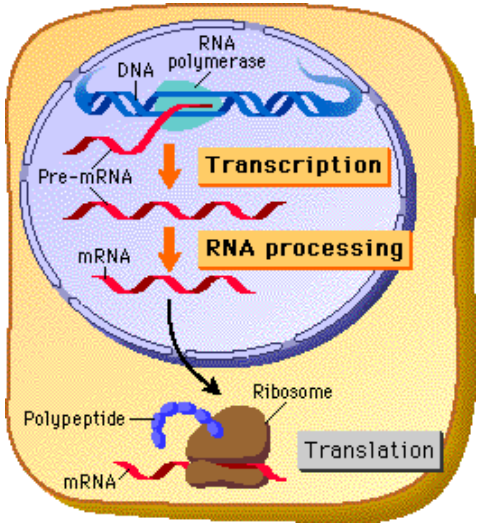
biology is based on evolutionary change.

Campbell and co-workers

Prokaryotic vs. Eukaryotic transcription



Pearson Education



In a prokaryotic cell, transcription and translation are coupled; that is, translation begins while the mRNA is still being synthesized. This is because there is no nucleus to separate the processes of transcription and translation, when bacterial genes are transcribed, their transcripts can immediately be translated.

In a eukaryotic cell, transcription occurs in the nucleus, and translation occurs in the cytoplasm. This is because transcription occurs in the nucleus to produce a pre-mRNA molecule.
The pre-mRNA is typically processed to produce the mature mRNA, which exits the nucleus and is translated in the cytoplasm.

Protein synthesis
Transcription and Translation
Central Dogma

- The production of protein takes place in two steps.
- In the first step, called transcription, the permanent DNA message (1) is copied into a temporary messenger RNA (the gene) by an enzyme RNA polymerase (2).
- This mRNA message can be read by a complex cellular "machine" called a ribosome (3).
- In this second step, called translation, the ribosome assembles amino acids in an order specified by the mRNA to create a specific protein (4).

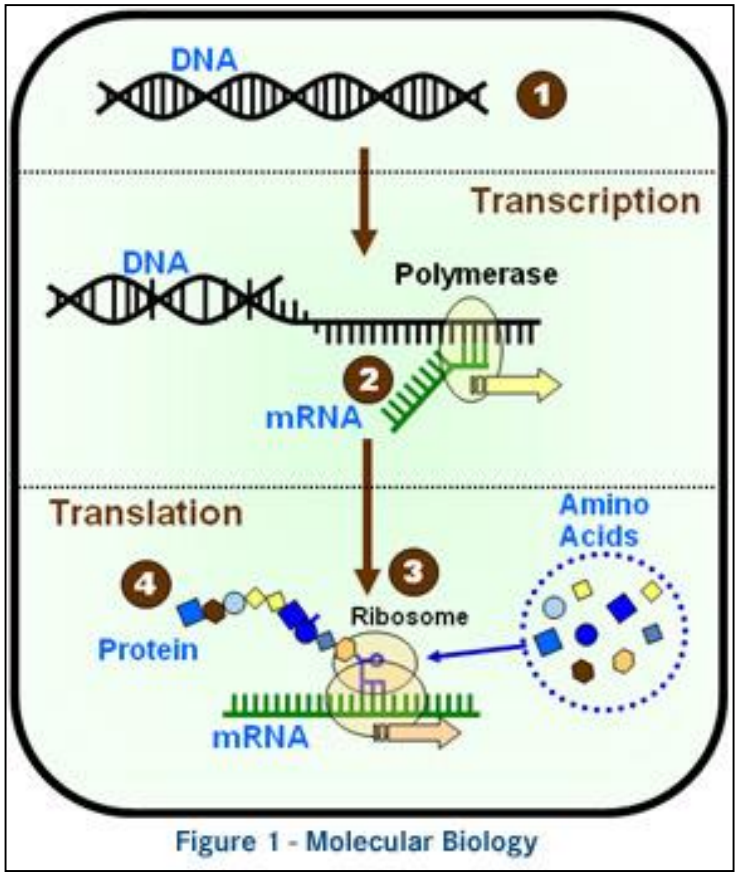
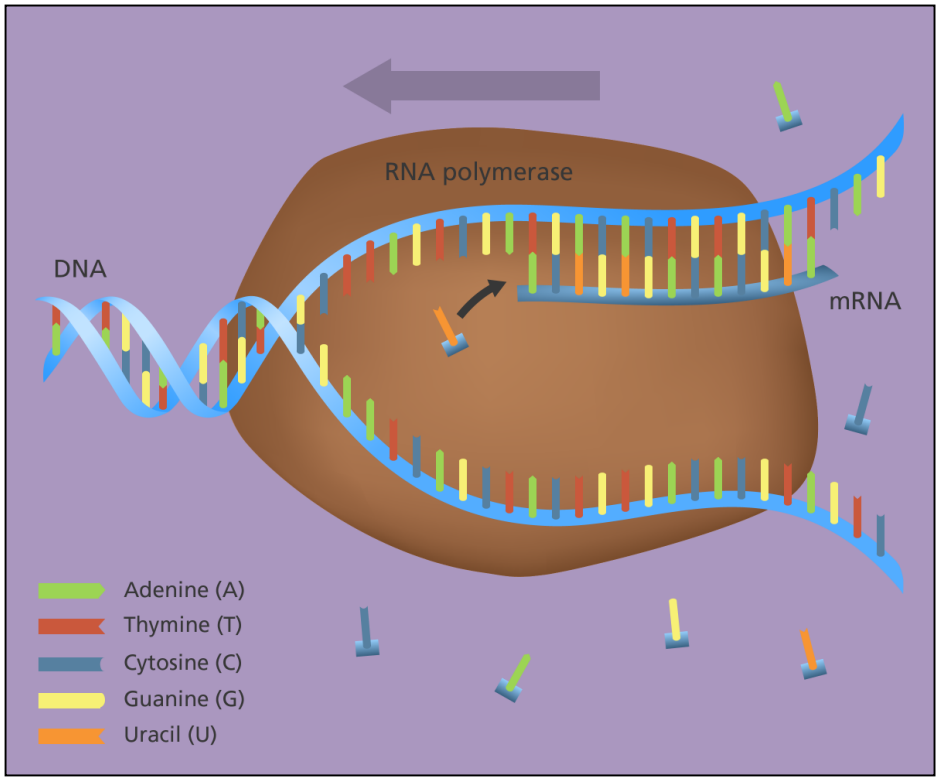


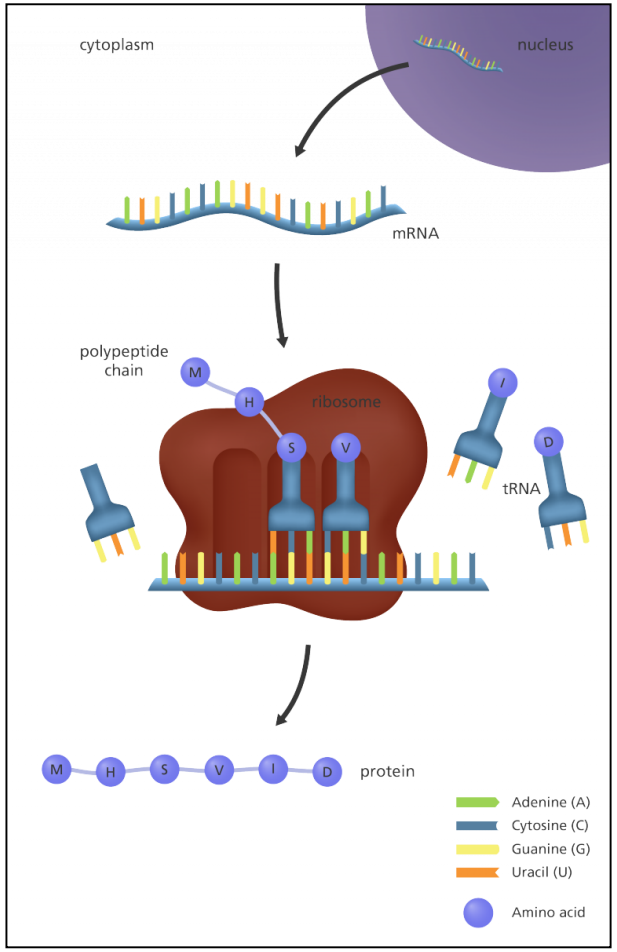
Figure 1 - Molecular Biology

Monsanto Co.,2010

Protein synthesis

Transcription and Translation



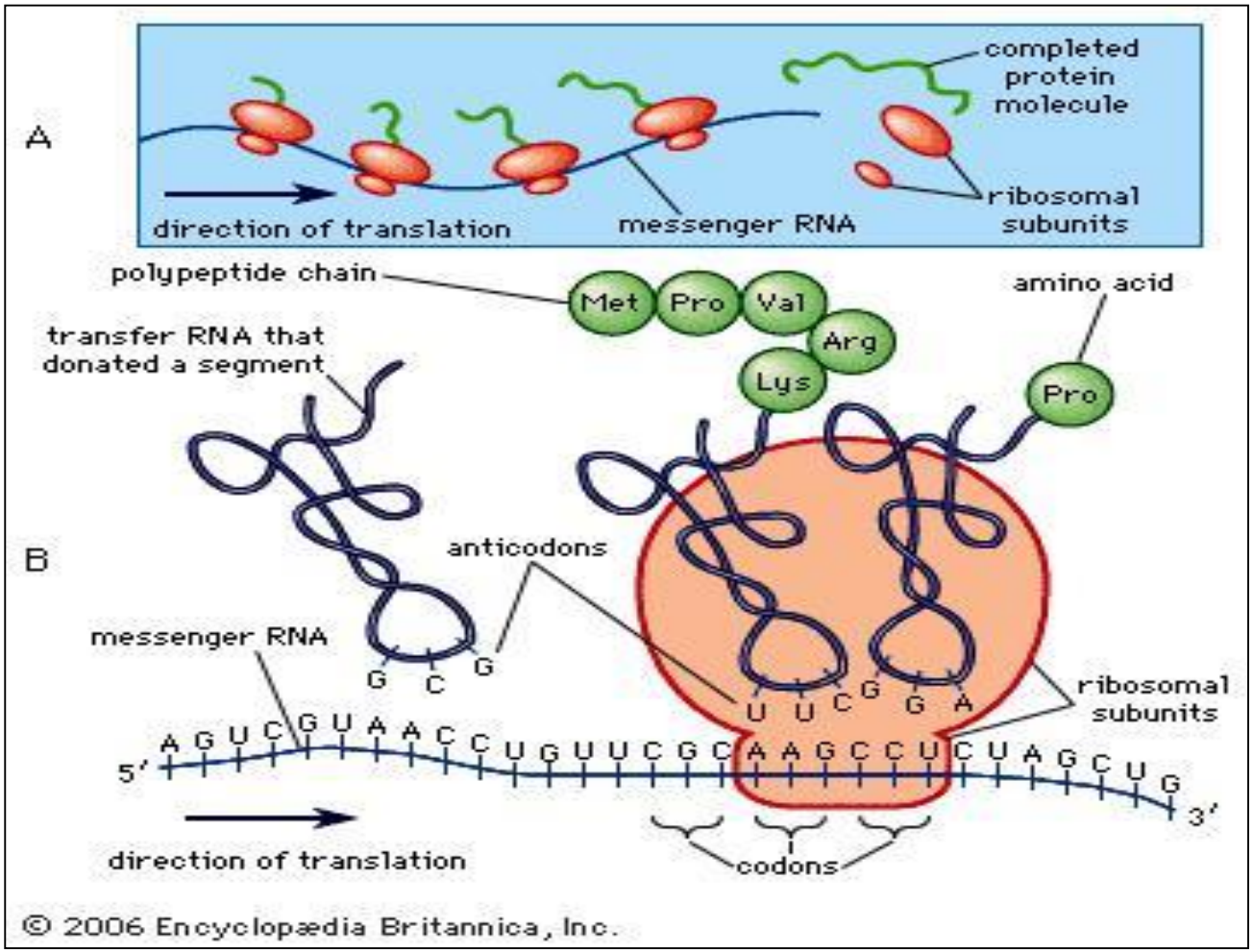


Facts yourgenome_org.htm

Protein synthesis

Translation

Initiation, elongation and termination



Molecular structures of proteins
Four levels of protein structure

- The variety of protein structures may be INFINITE...
- Because a protein of 300 amino acids made with 20 different kinds of amino acids can have 20^{300} different linear arrays of aa's [10^{390} different proteins].
- It is almost impossible to estimate the total number of different proteins in the nature.

primary - linear sequence of aa's

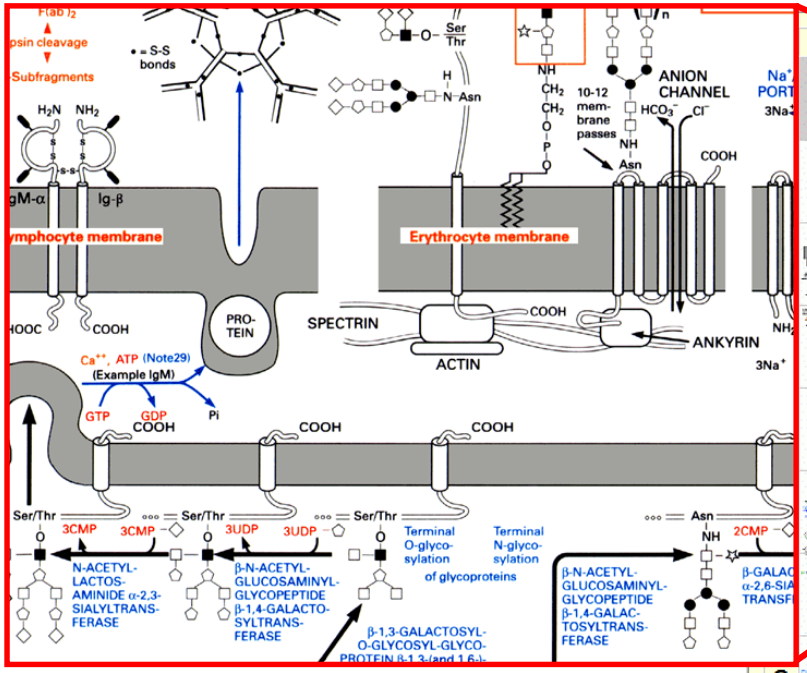
secondary - regular, recurring orientation of aa in a peptide chain due to H-bond

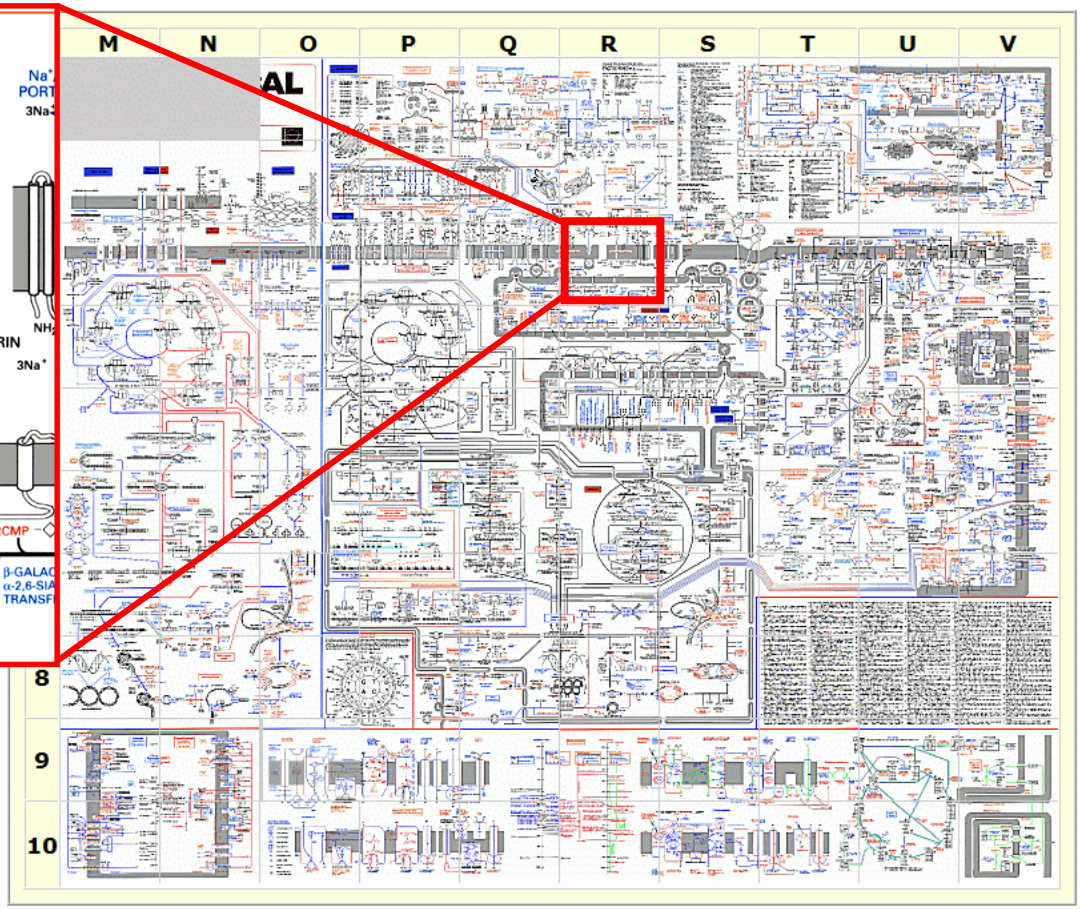
tertiary - complete 3-D shape of a peptide due to weak electrostatic forces

quaternary - spatial relationships between different polypeptides or subunits

Our life is maintained by molecular network systems

Proteins play key roles in a living system





Molecular network system in a cell

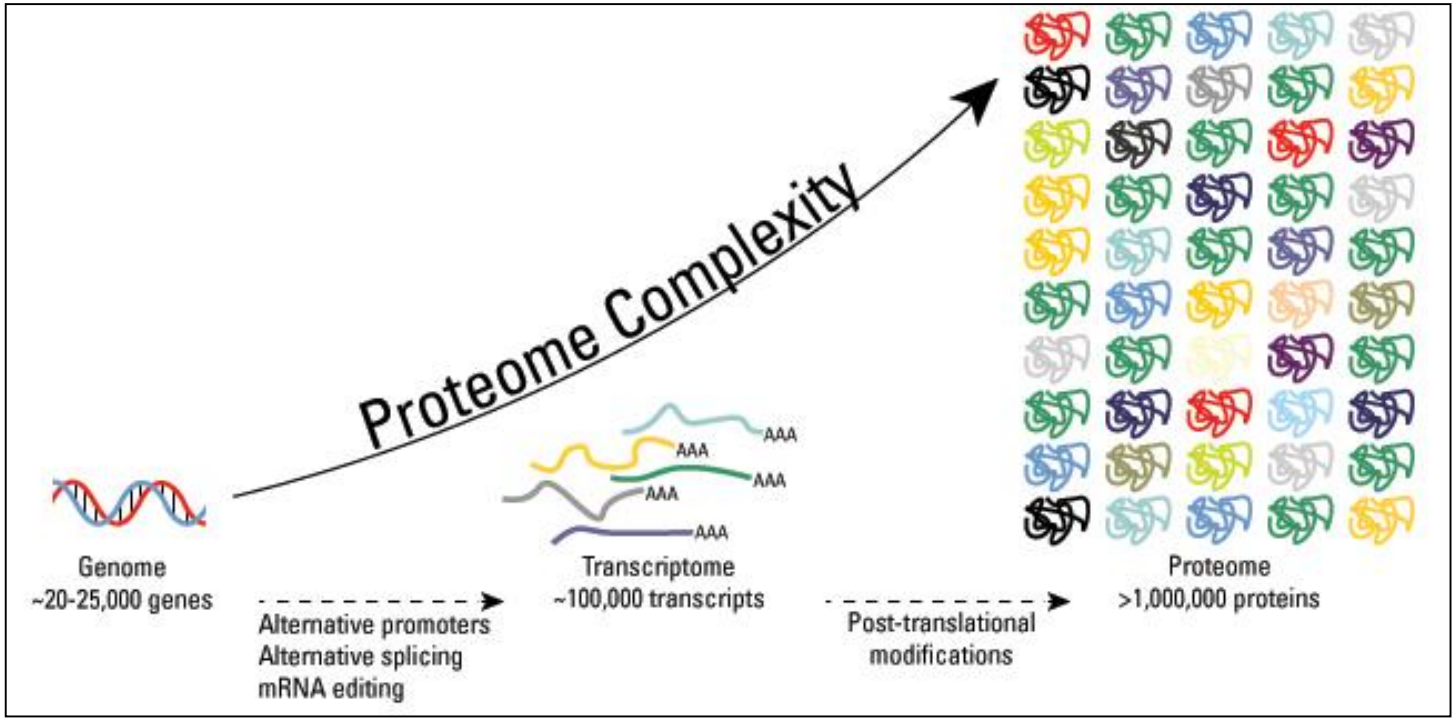
(From ExPASy Biochemical Pathways; http://www.expasy.org/cgi-bin/show_thumbnails.pl?2)

Gene expression
For protein synthesis

- Simplest bacterial genomes contain 500 to 600 genes.
- *E. coli* genomes contain 4,288 genes.
- The human genome comprises 20-25,000 genes.
- The proteome (entire complement of proteins expressed by a cell, organism, or tissue type) is estimated to encompass over 1 million proteins.
- Most eukaryotic mRNAs encode single gene product.
- A mRNA found in prokaryotes that encodes more than one protein(multiple proteins).

Snider,2013;..

Gene expression
For protein synthesis



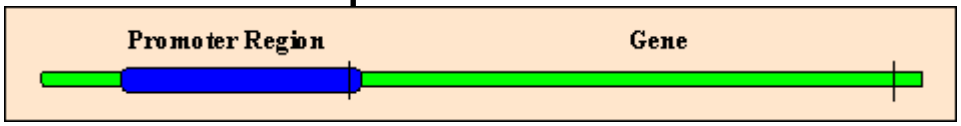
Snider,2013;..

Operon

Structure and functions

mRNA

- Common in bacteria but rare in eukaryotes.
- In bacteria, genes with related functions are often (but not always) located together in a group known as a operon.
- Enzymes in many biosynthetic pathways of bacteria and viruses are encoded by operons.
- An operon has a single promoter and is transcribed into a single polycistronic (cistron=equivalent to a gene) mRNA molecule, which carries the information for several proteins.
- Promoter: Region of DNA where RNA polymerase attaches and initiates transcription.



Dale and Park,2004;..

Polycistronic mRNA

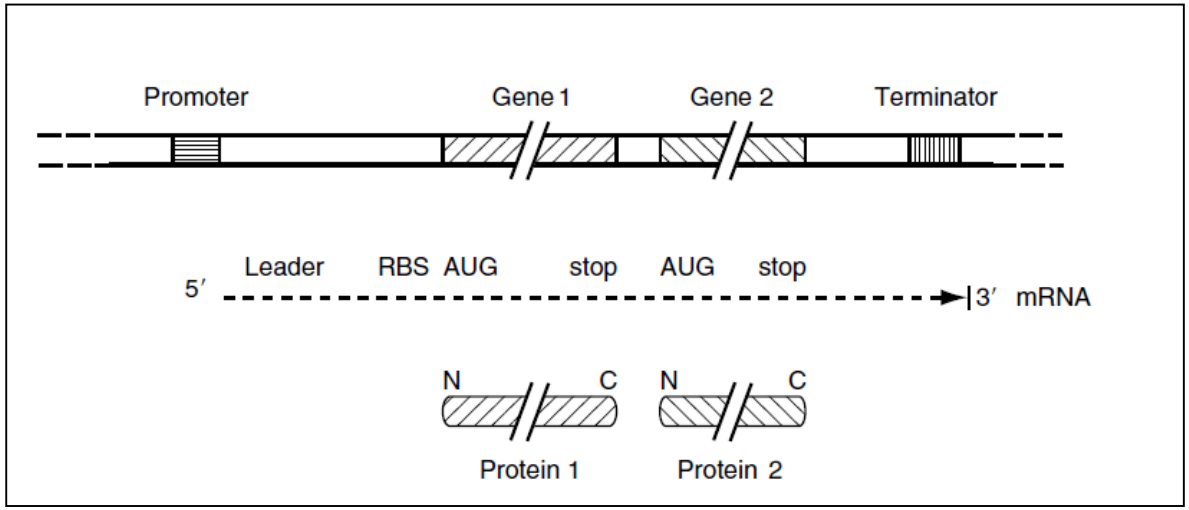
Monocistronic mRNAs vs. polycistronic mRNAs

Prokaryotic mRNA encodes more than one protein

- A mRNA found in prokaryotes that encodes more than one protein.
- Each eukaryotic mRNA contains information coding for only one protein, hence monocistronic, whereas prokaryotic mRNAs may encode more than one protein and are said to be polycistronic.
- Most eukaryotic mRNAs encode single gene product.

A typical operon

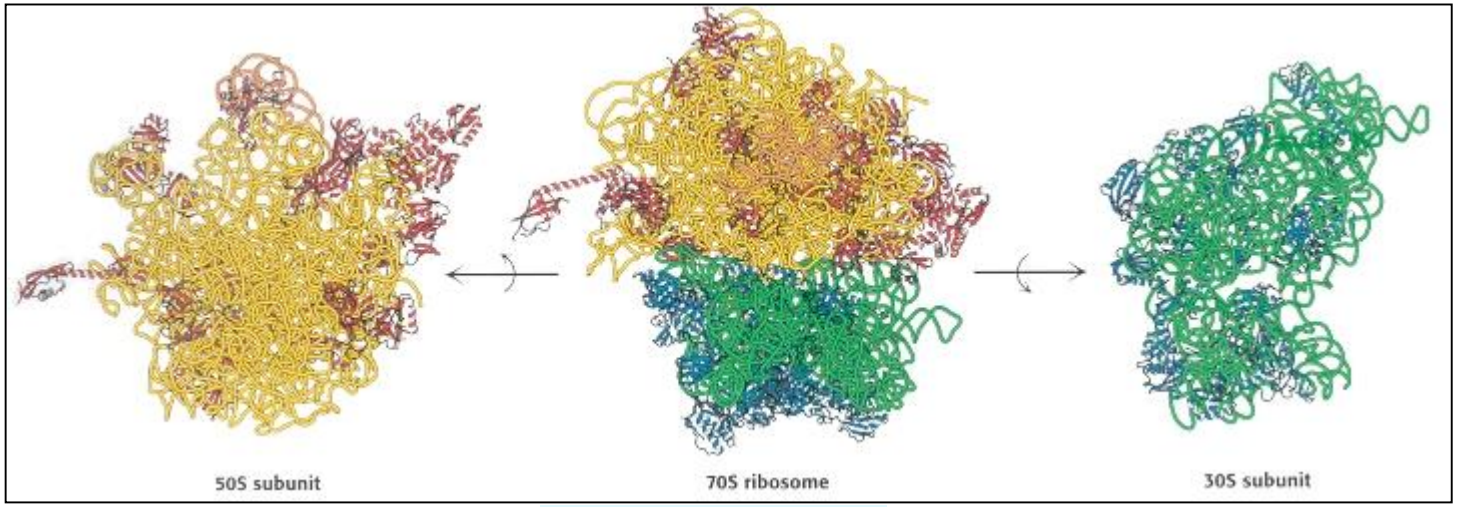
- A typical operon is transcribed from a single promoter into a polycistronic mRNA from which several independent polypeptides can be translated.
- Promoter region: Region of DNA usually upstream from the gene which regulates gene activity.



Dale and Park,2004;..

Protein synthesis
1. Bacterial ribosomes

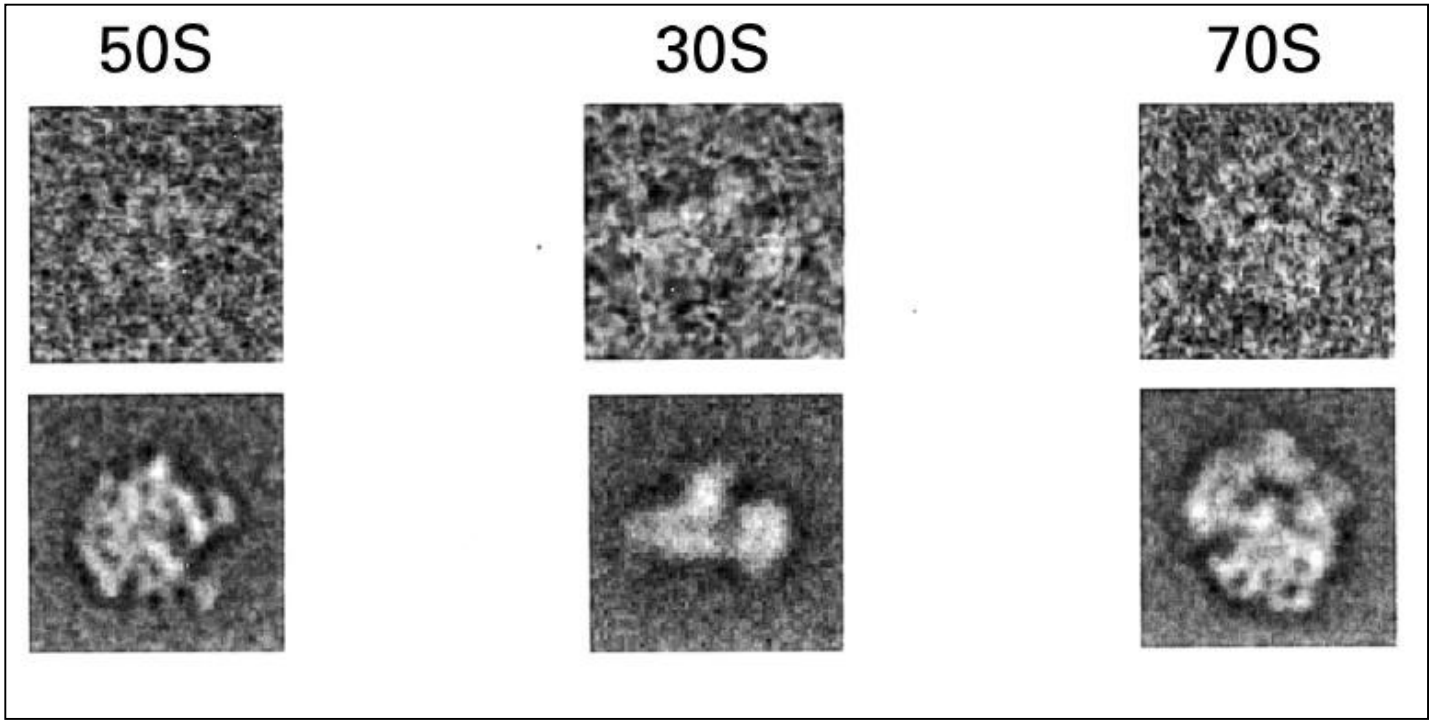
- Detailed models of the ribosome based on the results of x-ray crystallographic studies of the 70S ribosome and the 30S and 50S subunits.
- 23S RNA is shown in yellow, 5S in orange, 16S RNA in green, proteins of the 50S subunit in red, and proteins of the 30S subunit in blue.



Berg *et al.*,2002

Bacterial ribosome

Ribosomal subunits



Complete structures of both ribosomal subunits have been determined using X-Ray crystallography.

Bacterial ribosome
RNA and protein components

- Each subunit is composed of:

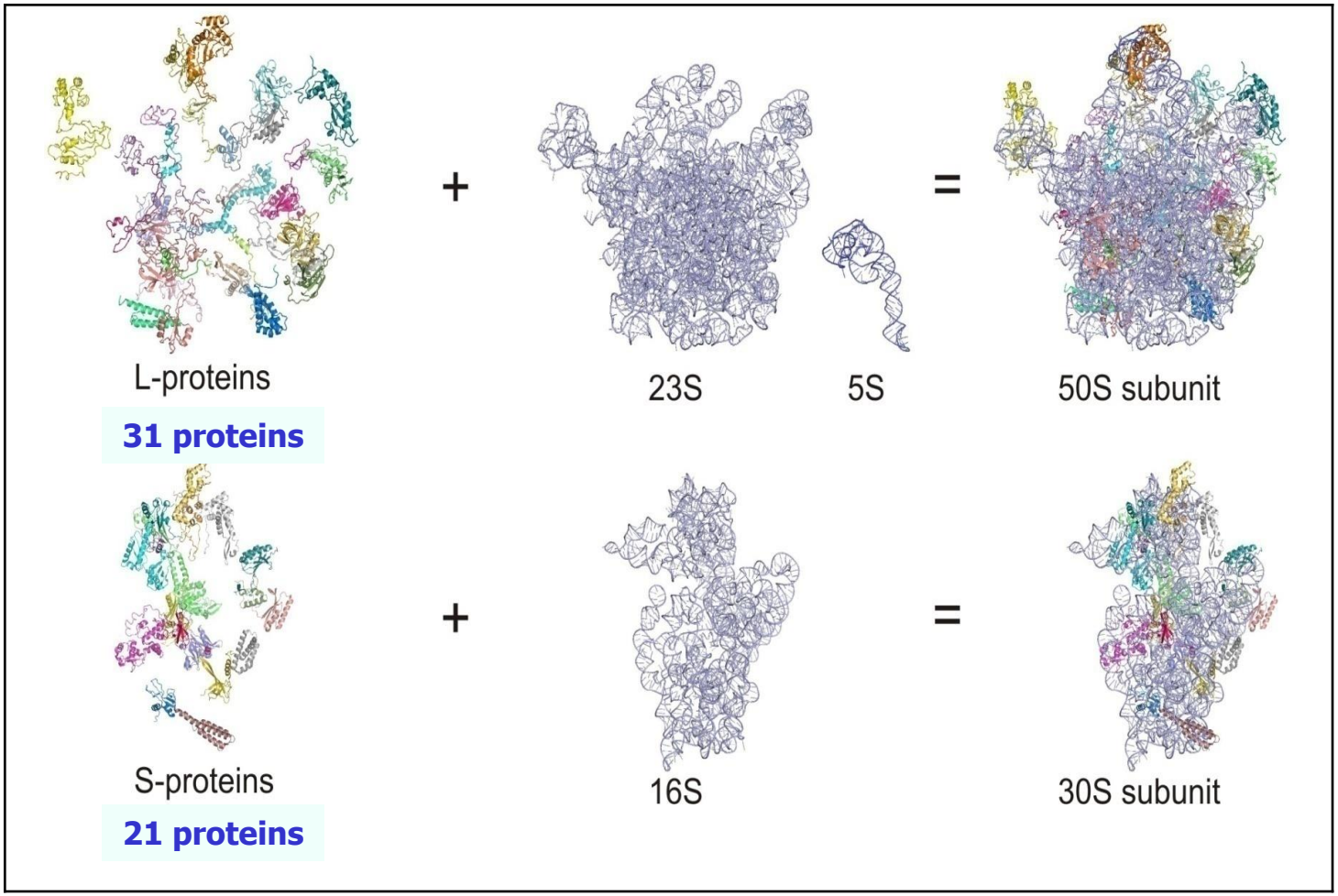
1. Ribosomal RNA (rRNA), and
2. Ribosomal proteins (r-proteins).

- The larger (50S) subunit has two RNA molecules (23S and 5S) plus 31 different polypeptides(large r-proteins or L-proteins).
- The smaller one (30S) contains a single RNA molecule (16S) and 21 polypeptides (small r-proteins or S-proteins).

30S subunit		50S subunit
rRNA	16S	23S and 5S
Proteins	21	31

Ribosomal subunits

Part protein and Part RNA



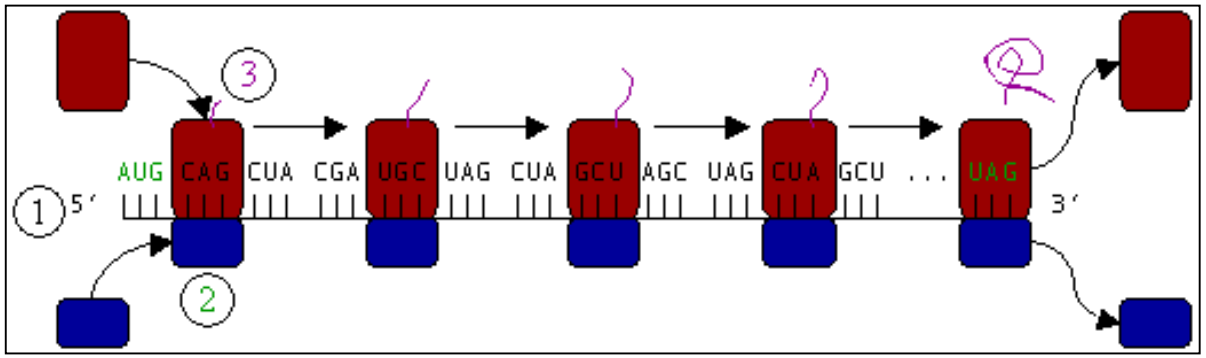
Ribosomes
The protein factory

- The ribosome is a protein-synthesizing machine has a mass of about 2.5 Mda.
- A single cell of *E. coli* contains about 20,000 ribosomes and this accounts for about 25% of the total cell mass.

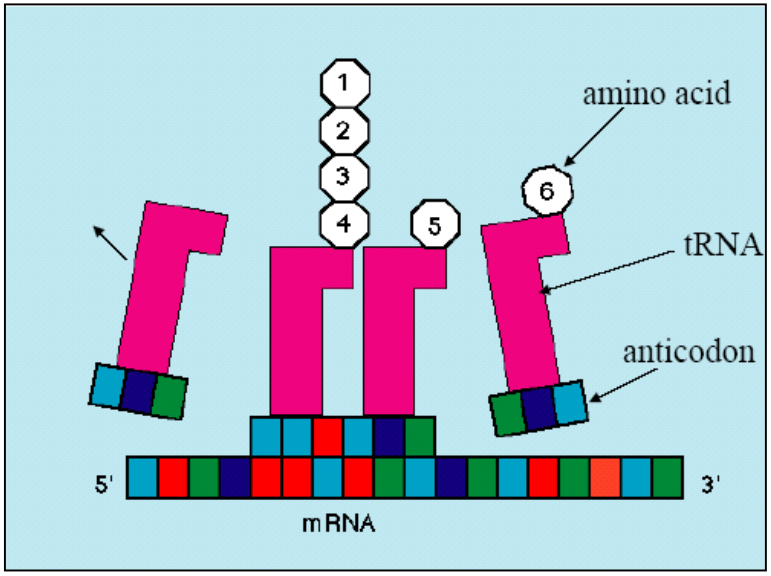
The ribosomes are cytoplasmic nucleoproteins composed of rRNAs plus a number of proteins.
Chromosomes are also nucleoproteins, formed mainly of DNA and basic proteins.

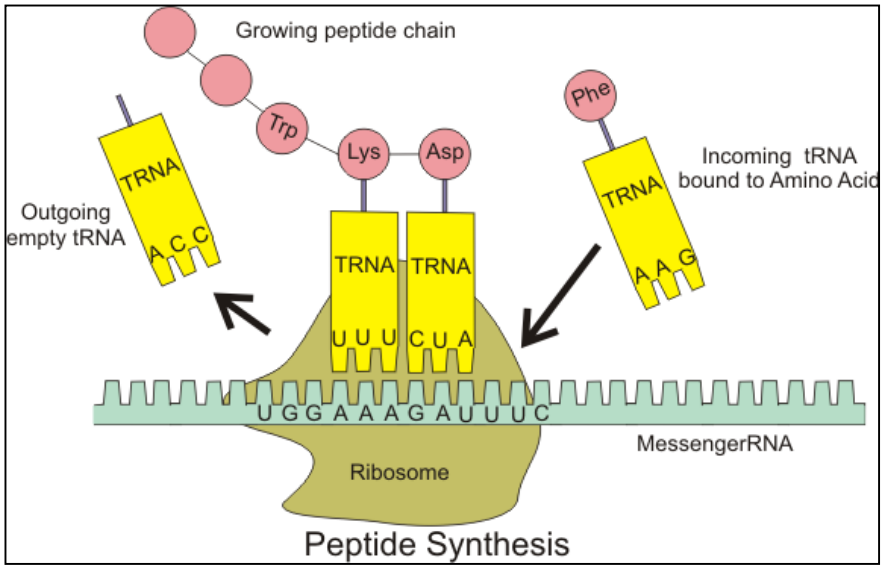
Ribosomal structure and function
Protein synthesis in ribosomes

- A ribosome moves along an mRNA from 5′ to 3′.
- mRNA is also made in the 5' to 3' direction.
- The mRNA is a complementary copy of the DNA gene but RNA uses uracil instead of thymine.
- It carries genetic information from DNA.



Protein synthesis
2. mRNA, tRNA, rRNA





Anticodon in tRNA complementary to a codon on mRNA. Transfer RNA (tRNA) binds to both mRNA and amino acids (the building blocks of proteins) and brings the correct amino acids into the growing polypeptide chain during protein formation.

Types of RNA in bacterial cells

RNA content of *E. coli* cells

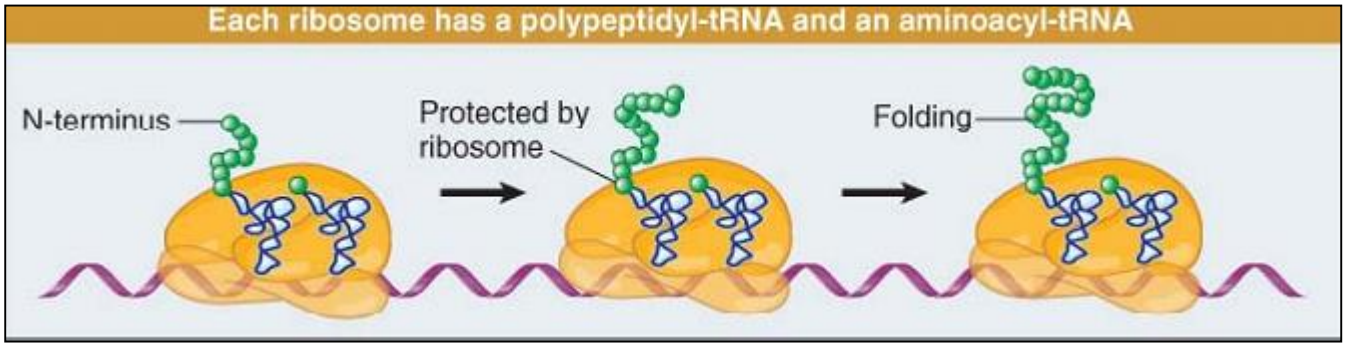
tRNA, rRNA, and mRNA

Type	Steady state levels	Synthetic capacity	Stability
rRNA	83%	58%	High
tRNA	14%	10%	High
mRNA	3%	32%	Very low

A stable condition that does not change over time or in which any one change is continually balanced by another. Expression level of each gene in these types are in steady(continuous) state.

Protein synthesis
mRNA, tRNA, rRNA

- The majority of RNA molecules are tRNAs and rRNAs.
- Ratio of rRNA to mRNA (~97:3).
- Many ribosomes bind to one mRNA.
- An mRNA is simultaneously translated by several ribosomes.



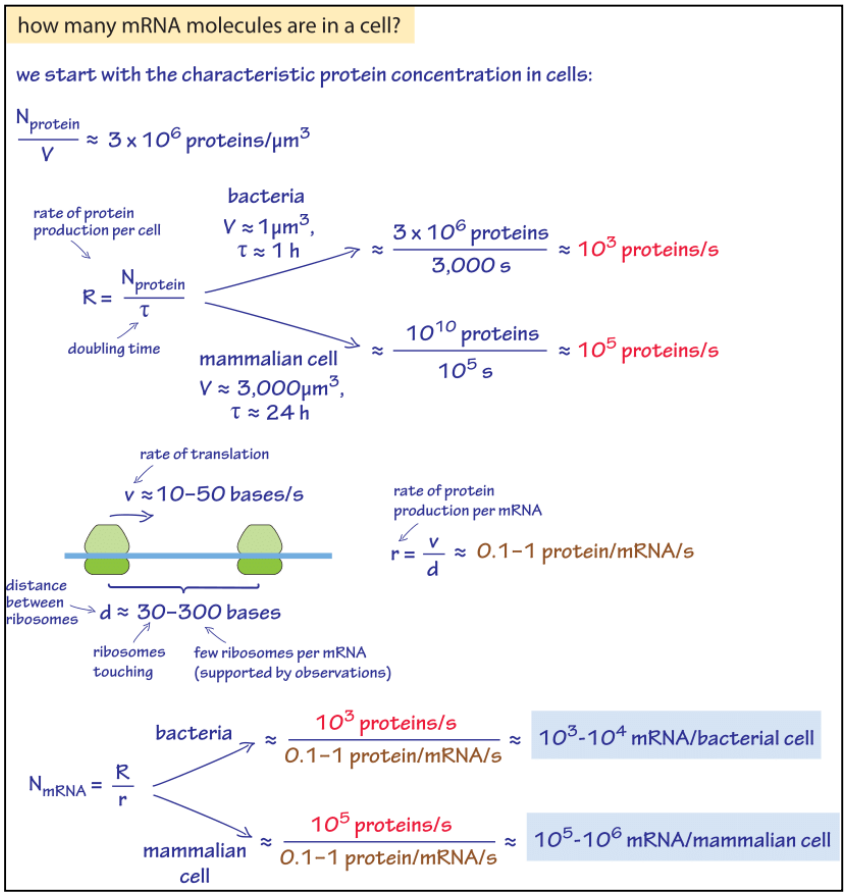
Protein synthesis
mRNA

- mRNA accounts for only 1-5% of the total cellular RNA although the actual amount depends on the cell type and physiological state.
- Approximately 360,000 mRNA molecules are present in a single mammalian cell.

1. 10^3-10^4 mRNA per bacterial cell, and
2. 10^5-10^6 mRNA per the 3000 μm^3 characteristic size of a mammalian cell.

Protein synthesis
mRNA

- As shown in this back of the envelope calculation we can derive an estimate for rapidly dividing cells of 10^3-10^4 mRNA per bacterial cell and 10^5-10^6 mRNA per the 3000 μm^3 characteristic size of a mammalian cell.



Protein synthesis
mRNA

- Bacterial mRNA is unstable and has a half-life of only a few minutes.
- mRNA life in prokaryotic cell is short (few seconds to two minutes) as mRNA is unstable.
- mRNA in eukaryotic cell has a life of few hours to few days; it is quite stable.
- In prokaryotes translation is a faster process, each mRNA adds about 20 amino acids per second.
- In eukaryotic cell, mRNA adds one amino acids per second, thus a slower process.

Protein synthesis

Initiation-the first phase of translation

tRNA

- The adaptor molecule for translation is tRNA.
- A charged tRNA has an amino acid at one end, and at the other end it has an anticodon for matching a codon in the mRNA; i.e. it "speaks the language" of nucleic acids at one end and the "language" of proteins at the other end.
- The machinery for synthesizing proteins under the direction of template mRNA is the ribosome.

Protein synthesis

Strands and directions of synthesis

1. All strands are synthesized from the 5' ends > > > to the 3' ends for both DNA and RNA.
2. Protein chains are synthesized from the amino ends > > > to the carboxy ends.
   Color mnemonic (memory techniques): the old end is the cold end (blue); the new end is the hot end (where new residues are added) (red).

DNA	Coding Strand (Codons)	**5'** > > > - - - - - - T T C - - - - - - > > > **3'**
	Template Strand (Anti-codons)	**3'** < < < - - - - - - A A G - - - - - - < < < **5'**
mRNA	Message (Codons)	**5'** > > > - - - - - - U U C - - - - - - > > > **3'**
tRNA	Transfer (Anti-codons)	**3'** < < < A A G < < < **5'**
Protein	Amino Acid	**Amino** > > > Phenylalanine > > > **Carboxy**

All DNA strands are read from the 5' to the 3' end where the 5' end terminates in a phosphate group and the 3' end terminates in a sugar molecule.

Sandler and Nüsslein

Proteins
Importance

- Proteins are macromolecules composed of amino acids linked together through peptide bonds.

1. The most widely distributed biomolecules.
2. The most abundant biomolecules (45% of human body). In bacterial cells, proteins make about a half of the dry weight of cells.
3. The most complex biomolecules.
4. The most diversified biological functions.

For example only in *E. coli* cell about 3000 different proteins are known.

Components of proteins

- Major elements:
- C (50~55%),
- H (~7%),
- O (19~20%),
- N (13~19%),
- S (~4%)
- Trace elements:
- P, Fe, Cu, Zn, I, …

The C atom is an optically active center.

Components of proteins

The average nitrogen content

- The average nitrogen(N) content of proteins was found to be about 16 percent (0.16), which led to use the calculation N x 6.25 (1/0.16 = 6.25) to convert nitrogen(N) content into protein content.

$$\left(\frac{1}{0.16}\right)=6.25$$

- The 6.25 content factor is used for most proteins.
- The protein quantity can be estimated:

protein in 100g sample (g%) = N per gram x 6.25 x 100

History of protein study

- The word protein comes from Greek language (prota) which means "of primary importance".
- This name was introduced by Jons Jakob Berzelius in 1838 for large organic compounds with almost equivalent empirical formulas.

Proteome and Proteomics

- "Proteins are central to our understanding of cellular function and disease processes, and without a concerted effort in proteomics, the fruits of genomics will go unrealized."

Ian Humphery-Smith

Proteome and Proteomics
Definitions

- The proteome has been defined as the entire complement of proteins expressed by a cell, organism, or tissue type, and accordingly,
- Proteomics is the study of this complement expressed at a given time or under certain environmental conditions.
- Proteomics is a new frontier explore a comprehensive knowledge about all the proteins of a cell at a specific given time.

Proteomics
Objectives

- Biological process:
  - The overall process toward which this protein contributes.
- Molecular function:
  - The biological activity the protein accomplishes.
- Cellular component:
  - The location of protein activity.

Proteomics
Approaches

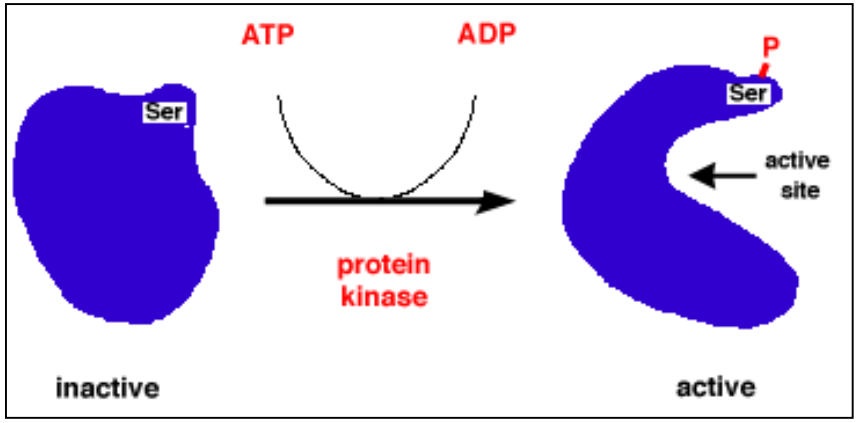
1. Separation
2. Sequence determination
3. 3D-structure
4. Functionality
5. Expression regulation
6. Post-translation modification(such as phosphorylation and glycosylation, modify the structure and function of proteins).

Proteomics

Approaches

Protein post-translation modification(PTMs)

- Phosphorylation (the covalent attachment of a phosphate group to either serine, threonine or tyrosine) is the most common modification, and is catalyzed by enzymes known as protein kinases.



Shuttleworth,1997

Proteomics

Approaches

Protein post-translation modification(PTMs)

- **Types of post-translational modification include:**
- Polypeptide folding into a globular protein with the help of chaperone proteins to arrive at the lowest energy state;
- Modifications of the amino acids present, such as removal of the first methionine residue;
- Disulfide bridge formation or reduction.
- Protein modifications that facilitate binding functions:
  - Glycosylation
  - Prenylation of proteins for membrane localization
  - Acetylation of histones to modify DNA-histone interactions
- Addition of functional groups that regulate protein activity:
  - Phosphorylation
  - Nitrosylation
  - GTP(Guanosine-5'-triphosphate) binding.

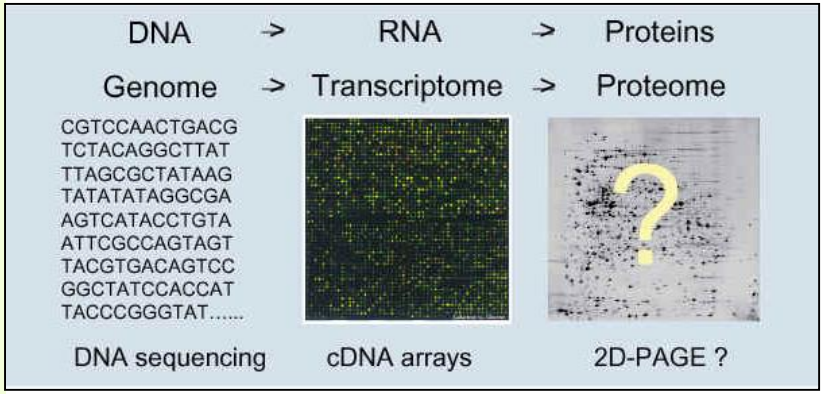
Hayworth,2013

Proteome and Proteomics

Three main steps in proteome research

- There are three main steps in proteome research:

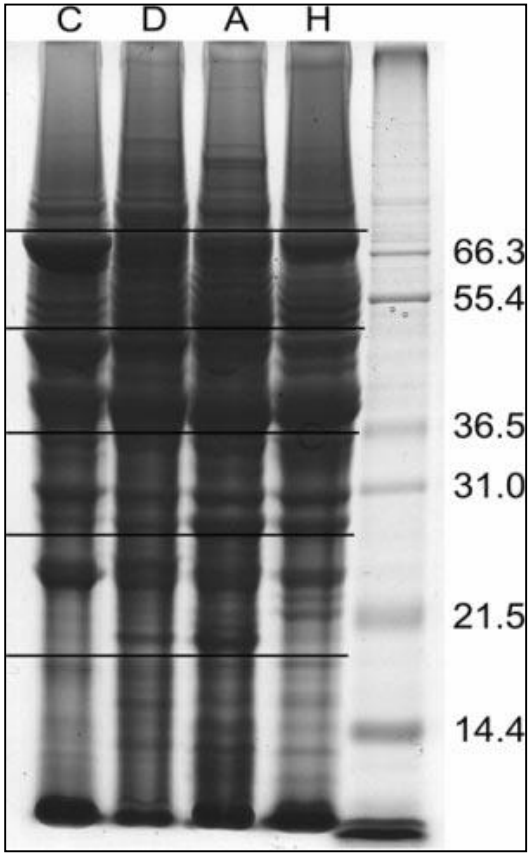
1. Separation of individual proteins by 2-D polyacrylamide gel electrophoresis (2-D PAGE).
2. Identification by mass spectrometry or N-terminal sequencing of individual proteins recovered from the gel.
3. Storage, manipulation, and comparison of the data using bioinformatics.



Proteome and Proteomics

Separation of extracellular proteins by SDS-PAGE

- To investigate potential mechanisms of disease resistance and susceptibility, we analyzed changes in the extracellular proteome, or secretome, using the *Arabidopsis-Pseudomonas syringae* pathosystem.
- Proteins from the extracellular medium were collected after no infection (C) or infection with *Pst* DC3000 (D), *Pst* DC3000 expressing *avrRpm1* (A), or *Pst* DC3000 lacking a functional type III secretion system (H).
- After separation, the gel was stained with colloidal Coomassie.
- Lines indicate where the gel was cut to obtain gel slices.



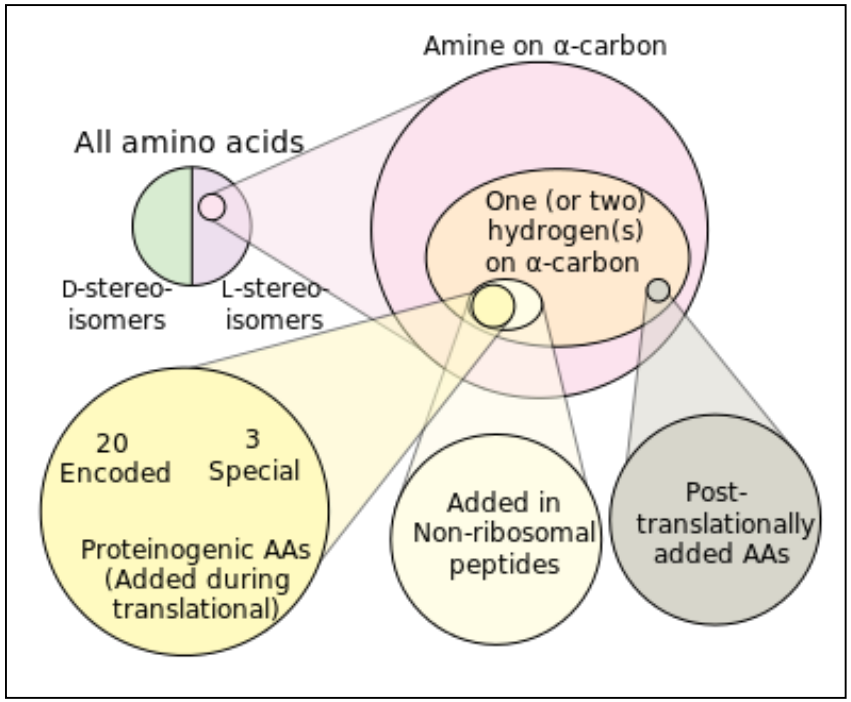
Kaffarnik *et al.*,2009

Various groups of amino acids

Natural amino acids vs. Unnatural amino acids

- There are various groups of amino acids:

1. 20 standard amino acids.
2. 23 proteinogenic amino acids.
3. over 80 amino acids created abiotically in high concentrations.
4. about 900 are produced by natural pathways.
5. over 118 engineered amino acids have been placed into protein.



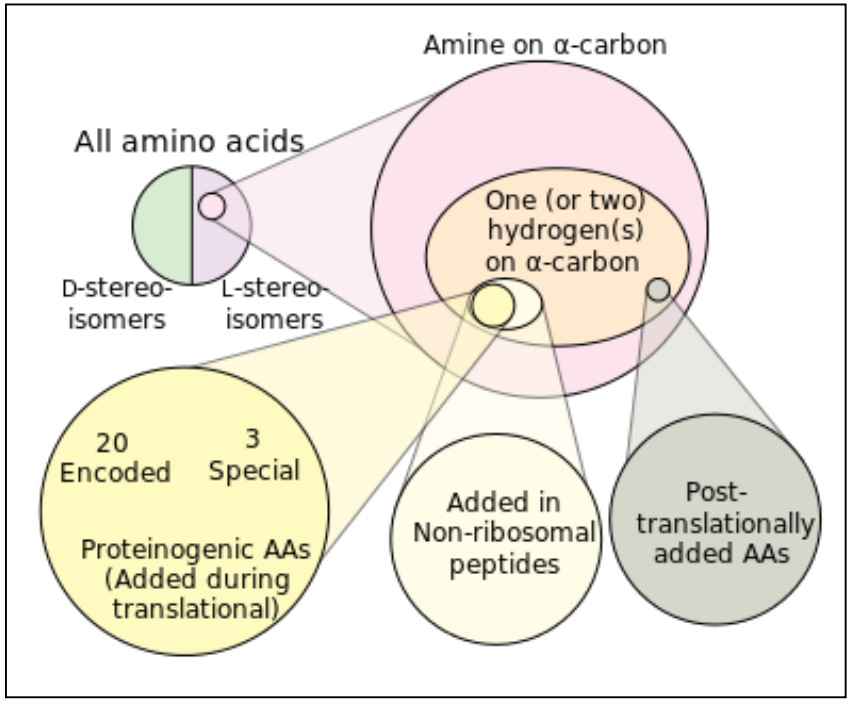
Proteinogenic amino acids are a small fraction of all amino acids.

Weikipedia

Various groups of amino acids

Natural amino acids vs. Unnatural amino acids

1. Only 23 natural amino acids (20 in all organisms)are synthesized by the translational machinery(ribosomes).
2. Whereas non-coded, non-proteinogenic, or "unnatural" amino acids are enormous(hundreds) and are not naturally encoded or found in the genetic code of any organisms.



Proteinogenic amino acids are a small fraction of all amino acids.

Weikipedia

General classification of amino acids
Standard (natural)amino acids

- **Standard (natural, proteinogenic, coded)amino acids:**
- These amino acids join together to form short polymer chains called peptides or longer chains called either polypeptides or proteins.
- These polymers are linear and unbranched, with each amino acid within the chain attached to two neighboring amino acids.
- There are 23 proteinogenic amino acids in prokaryotes, but only 21 are encoded by the nuclear genes of eukaryotes.

1. Of these, 20 are encoded by the universal genetic code.
2. The remaining 3 (special amino acids i.e. selenocysteine, pyrrolysine and N-formylmethionine are incorporated into proteins by unique post-translational synthetic mechanisms.

- N-formylmethionine is often the initial amino acid of proteins in bacteria.

General classification of amino acids
Non-standard (unnatural)amino acids

- **Non-standard (unnatural, non-proteinogenic, non-coded)amino acids:**
- Aside from the 23 standard amino acids, there are many other amino acids that are called non-proteinogenic or non-standard.
- They are either not found in proteins (for example carnitine, GABA), or are not produced directly.
- Non-standard amino acids that are found in proteins are formed by post-translational modification, which is modification after translation during protein synthesis.
- Nonstandard amino acids often occur as intermediates in the metabolic pathways for standard amino acids — for example, ornithine and citrulline occur in the urea cycle.

LinkedIn Corporation, 2015

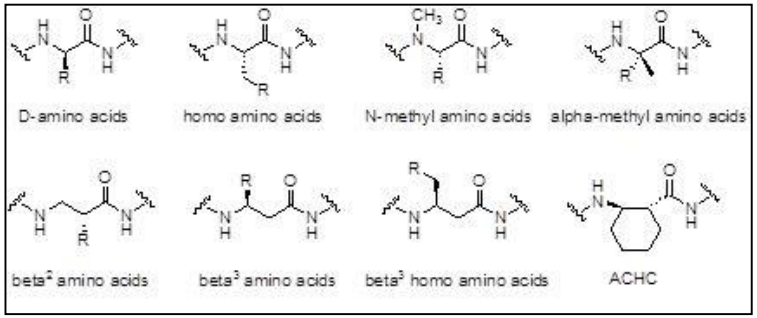
Unnatural amino acids

- Unnatural(unusual amino acids, non-coded) are the non-proteinogenic amino acids that either occur
1. Naturally, or
2. are chemically synthesized.
- These are becoming more and more important as tools for modern drug discovery research.
- Due to their unlimited structural diversity and functional versatility, they are widely used to increase the activity or selectivity and plasma stability of peptides in drug discovery projects.

Junkers, Aldrich ChemFiles 2008;...

Unnatural amino acids

- Another example is the use of non-natural amino acids for the induction or stabilization of secondary structures (helices, sheets, turns).
- E.g.
- derivatives of glycine, phenylglycine, alanine, phenylalanine, and innovative α,α-disubstituted amino acids, as well as a comprehensive listing of proline derivatives and β-amino acids.



Junkers, Aldrich ChemFiles 2008;...

Natural amino acids

The 20 proteinogenic natural amino acids

The structure of an amino acid

- An amino acid is a molecule containing two functional groups:

1. An amine group ($-NH_2$),
2. A carboxylic acid group (-COOH), and
3. There is an additional group called the side chain, designated with an R-group.

- A side-chain that is specific to each amino acid.
- Variation seen in naturally occurring amino acids arises from differences in this side chain.
- Only the R groups change.

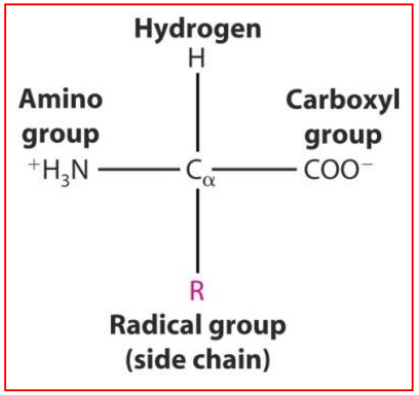
Natural amino acids

The 20 proteinogenic natural amino acids

The structure of an amino acid

- Proteins are long polymers made up of 20 different amino acid monomers.
- All 20 natural (proteinogenic) amino acids have the similar basic structure.
- The key elements of amino acid are:

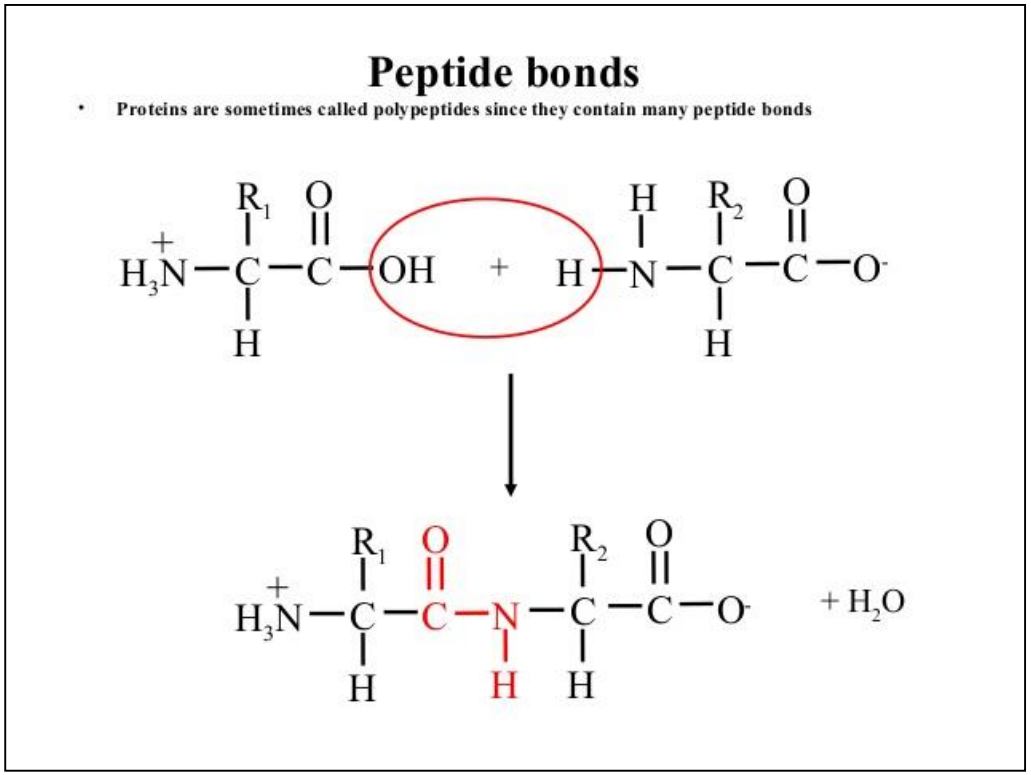
1. carbon,
2. hydrogen,
3. oxygen, and
4. nitrogen.



The α-carbon is where the different substituents attach to each different amino acid. R is a carbon containing side chain or branch. This carbon side chain may also contain sulfur, nitrogen or oxygen.

Protein or peptides
Peptide bond

- Peptide bond that links amino acids to form polypeptides and proteins.



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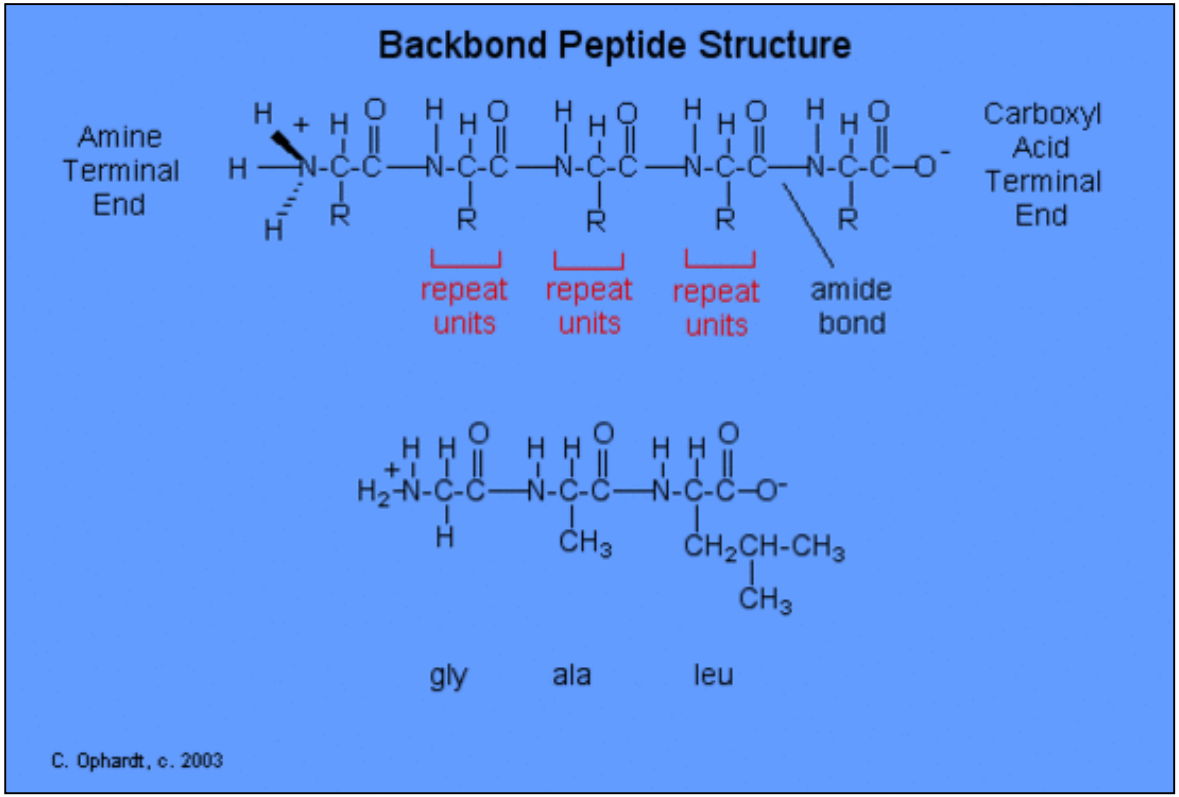

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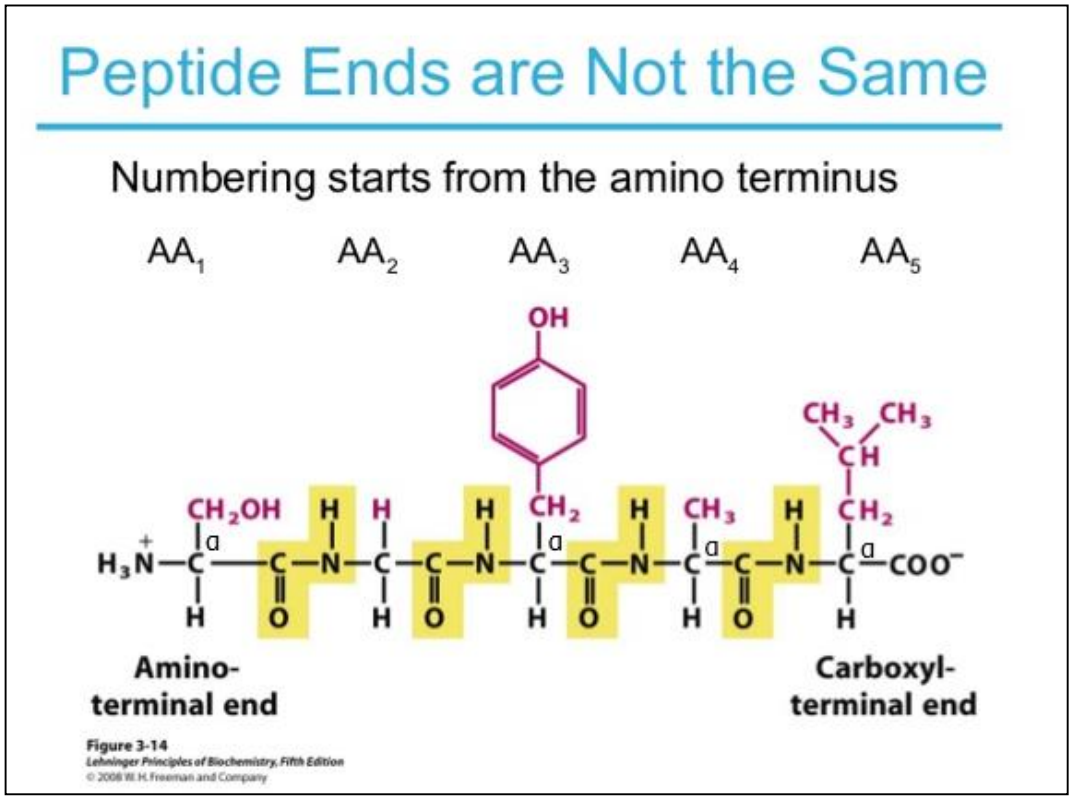
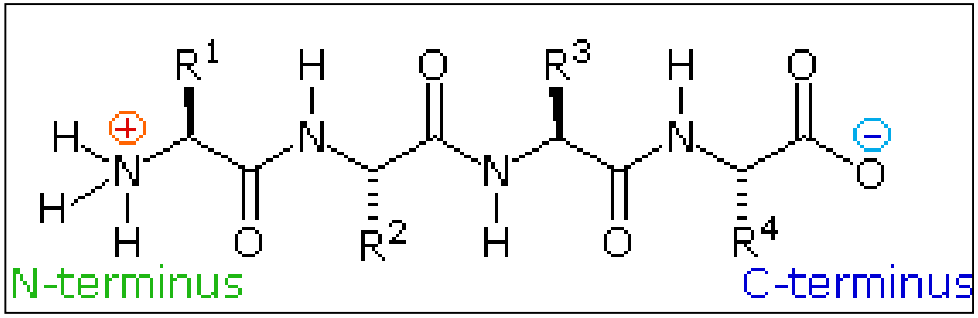


Protein or peptides
Basic structure

- The structures build from 2 to 100 amino acids with molecular weight up to 10 kDa are usually called peptides.
- A one hundred residue (amino acid) protein weighs ~11,000 Da, or 11 kilodaltons (kD).



Dalton= A very small unit of mass, about the mass of a hydrogen atom(the atomic weight of hydrogen atom is about one dalton). Dalton was used to express the molecular weight of proteins. 1Da is about 1.660 54 × 10^{-24} gram. Alanine is 89 Da.

Natural amino acids

The 20 proteinogenic natural amino acids

Protein synthesis

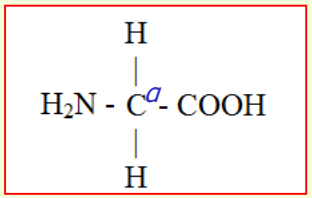
- The key chemical characteristic of amino acids is that they link together to form proteins.

1. Amine groups are basic (proton acceptor),
2. carboxylic acid groups are acidic (proton donator).

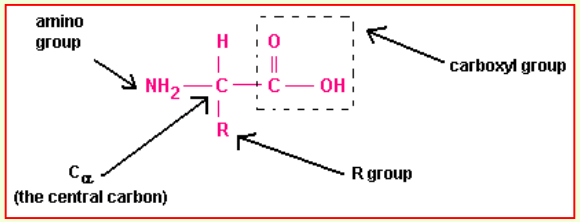
- Because:
- the COOH functional group is an acid, and
- the NH_2 functional group is a base,
- the two ends of amino acids can readily react with each other.

The structure of a natural amino acid
α-amino acids

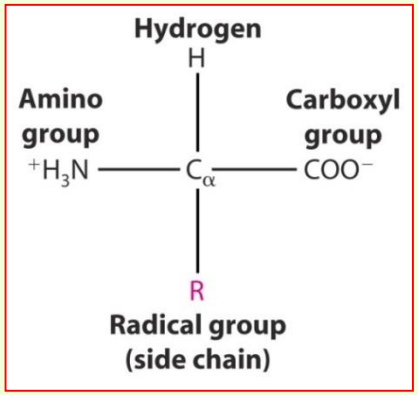
- The carbon atom to which the amino group, carboxyl group, and side chain (R-group) are attached is the alpha carbon (C^a).
- The first carbon atom after the carbon that attaches to the functional group is called the alpha carbon; the second, beta carbon, the third, gamma carbon, etc.
- *α*-amino acids or 2-amino acids, are amino acids in which both functional groups are attached to the same carbon atom.



Glycine: simplest *α*-amino acid in which R = hydrogen.



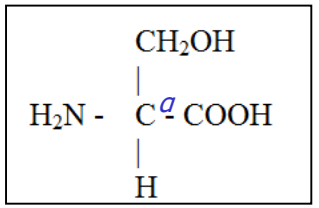
Alanine: R methyl group (CH_3) attach to central carbon.



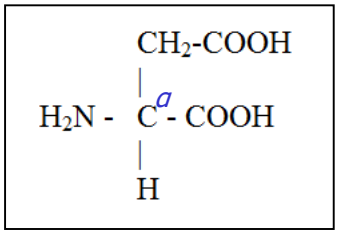
Structure of alpha amino acids

α-amino acids

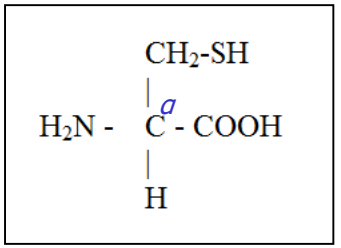
- Serine (Ser) is an amino acid in which R contains an alcohol functional group.



- Aspartic acid (Asp) contains a carboxylic acid functional group.

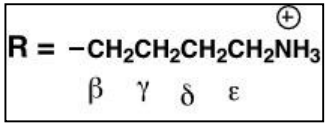


- Cysteine (Cys) contains sulfur.



The structure of a natural amino acid

Basic unit of protein



- Based on the site of attachment of the functional R group the amino acids are classified as:

1. Alpha amino acids: CH_2. It starts from the central carbon. Glycine is the simplest *α*-amino acid in which R = hydrogen.
2. Beta amino acids: CH_2CH_2 (e.g. beta-alanine H2N-CH2-CH2-COOH).
3. Gamma amino acids: COOH-CH2-CH2-CH2-NH2 (e.g. γ-Aminobutyric acid).

- Alpha-amino acids are the building blocks of proteins.
- The alpha amino acids (free amino acids) are the most common form found in nature.
- Only alpha amino acids involve in protein synthesis.
- Beta and gamma amino acids cannot form protein molecules.
- But may form part of some peptides.

Amino Acids

The weight and other general properties of natural amino acids

- One hydrogen weighs 1 dalton (Da).
- The average amino acid weighs 110 daltons.
- A one hundred residue (amino acid) protein weighs ~11,000 Da, or 11 kilodaltons (kD).

1. **Solubility:** Amino acids are soluble in water, acids, alkalis, but sparingly soluble in organic solvents.
2. **Color:** Amino acids are colorless, white solids.
3. **State:** Amino acids are solid crystalline compounds.
4. **Melting points:** Amino acids have high melting points.

A dalton is unit of mass equal to one twelfth ($^1/12$) the mass of the most abundant isotope of carbon, carbon-12, which is assigned a mass of 12.

Abbreviations for amino acids

Each amino acid has standard 3-letter and 1-letter abbreviations

Amino Acid	3-letter	1-letter	Amino Acid	3-letter	1-letter
Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic Acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamic Acid	Glu	E	Serine	Ser	S
Glutamine	Gln	Q	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

Gene expression for protein synthesis
Genetic codes

- The genetic code is said to be universal because a codon represents the same amino acids in almost all organisms.
- There are more than one codon for the same amino acid.
- The magic number in the 'case of codons' is 3.
- Putting 3 nucleotides together will provide for 64 possible amino acids.
- Many of these 3 groups code for the same amino acid.
- Arginine and leucine are encoded by 6 triplets, isoleucine by 3, methionine and tryptophan by 1, and all other amino acids by 4 or 2 codons.

Genetic code

DNA codon table(64 codons)

Standard genetic code

1st base	2nd base T		2nd base C		2nd base A		2nd base G		3rd base
T	TTT	(Phe/F) Phenylalanine	TCT	(Ser/S) Serine	TAT	(Tyr/Y) Tyrosine	TGT	(Cys/C) Cysteine	T
	TTC		TCC		TAC		TGC		C
	TTA	(Leu/L) Leucine	TCA		TAA	Stop (*Ochre*)	TGA	Stop (*Opal*)	A
	TTG		TCG		TAG	Stop (*Amber*)	TGG	(Trp/W) Tryptophan	G
C	CTT		CCT	(Pro/P) Proline	CAT	(His/H) Histidine	CGT	(Arg/R) Arginine	T
	CTC		CCC		CAC		CGC		C
	CTA		CCA		CAA	(Gln/Q) Glutamine	CGA		A
	CTG		CCG		CAG		CGG		G
A	ATT	(Ile/I) Isoleucine	ACT	(Thr/T) Threonine	AAT	(Asn/N) Asparagine	AGT	(Ser/S) Serine	T
	ATC		ACC		AAC		AGC		C
	ATA		ACA		AAA	(Lys/K) Lysine	AGA	(Arg/R) Arginine	A
	ATG[A]	(Met/M) Methionine	ACG		AAG		AGG		G
G	GTT	(Val/V) Valine	GCT	(Ala/A) Alanine	GAT	(Asp/D) Aspartic acid	GGT	(Gly/G) Glycine	T
	GTC		GCC		GAC		GGC		C
	GTA		GCA		GAA	(Glu/E) Glutamic acid	GGA		A
	GTG		GCG		GAG		GGG		G

Wikepedia,2015

Genetic code

RNA codon table(64 codons)

Standard genetic code

1st base	2nd base U		2nd base C		2nd base A		2nd base G		3rd base
U	UUU	(Phe/F) Phenylalanine	UCU	(Ser/S) Serine	UAU	(Tyr/Y) Tyrosine	UGU	(Cys/C) Cysteine	U
	UUC		UCC		UAC		UGC		C
	UUA	(Leu/L) Leucine	UCA		UAA	Stop (*Ochre*)	UGA	Stop (*Opal*)	A
	UUG		UCG		UAG	Stop (*Amber*)	UGG	(Trp/W) Tryptophan	G
C	CUU		CCU	(Pro/P) Proline	CAU	(His/H) Histidine	CGU	(Arg/R) Arginine	U
	CUC		CCC		CAC		CGC		C
	CUA		CCA		CAA	(Gln/Q) Glutamine	CGA		A
	CUG		CCG		CAG		CGG		G
A	AUU	(Ile/I) Isoleucine	ACU	(Thr/T) Threonine	AAU	(Asn/N) Asparagine	AGU	(Ser/S) Serine	U
	AUC		ACC		AAC		AGC		C
	AUA		ACA		AAA	(Lys/K) Lysine	AGA	(Arg/R) Arginine	A
	AUG[A]	(Met/M) Methionine	ACG		AAG		AGG		G
G	GUU	(Val/V) Valine	GCU	(Ala/A) Alanine	GAU	(Asp/D) Aspartic acid	GGU	(Gly/G) Glycine	U
	GUC		GCC		GAC		GGC		C
	GUA		GCA		GAA	(Glu/E) Glutamic acid	GGA		A
	GUG		GCG		GAG		GGG		G

Wikepedia,2015

Amino Acids

DNA Codon table

20 Amino acids, their single-letter data-base codes (SLC), and their corresponding DNA codons

- In this table, the twenty amino acids found in proteins are listed, along with the single-letter code used to represent these amino acids in protein data bases.
- The DNA codons representing each amino acid are also listed.
- All 64 possible 3-letter combinations of the DNA coding units T, C, A and G are used either to encode one of these amino acids or as one of the three stop codons that signals the end of a sequence.
- While DNA can be decoded unambiguously, it is not possible to predict a DNA sequence from its protein sequence.
- Because most amino acids have multiple codons, a number of possible DNA sequences might represent the same protein sequence.

Amino Acid	SLC	DNA codons
Isoleucine	I	ATT, ATC, ATA
Leucine	L	CTT, CTC, CTA, CTG, TTA, TTG
Valine	V	GTT, GTC, GTA, GTG
Phenylalanine	F	TTT, TTC
Methionine	M	ATG
Cysteine	C	TGT, TGC
Alanine	A	GCT, GCC, GCA, GCG
Glycine	G	GGT, GGC, GGA, GGG
Proline	P	CCT, CCC, CCA, CCG
Threonine	T	ACT, ACC, ACA, ACG
Serine	S	TCT, TCC, TCA, TCG, AGT, AGC
Tyrosine	Y	TAT, TAC
Tryptophan	W	TGG
Glutamine	Q	CAA, CAG
Asparagine	N	AAT, AAC
Histidine	H	CAT, CAC
Glutamic acid	E	GAA, GAG
Aspartic acid	D	GAT, GAC
Lysine	K	AAA, AAG
Arginine	R	CGT, CGC, CGA, CGG, AGA, AGG
Stop codons	Stop	TAA, TAG, TGA

The 20 Amino Acids

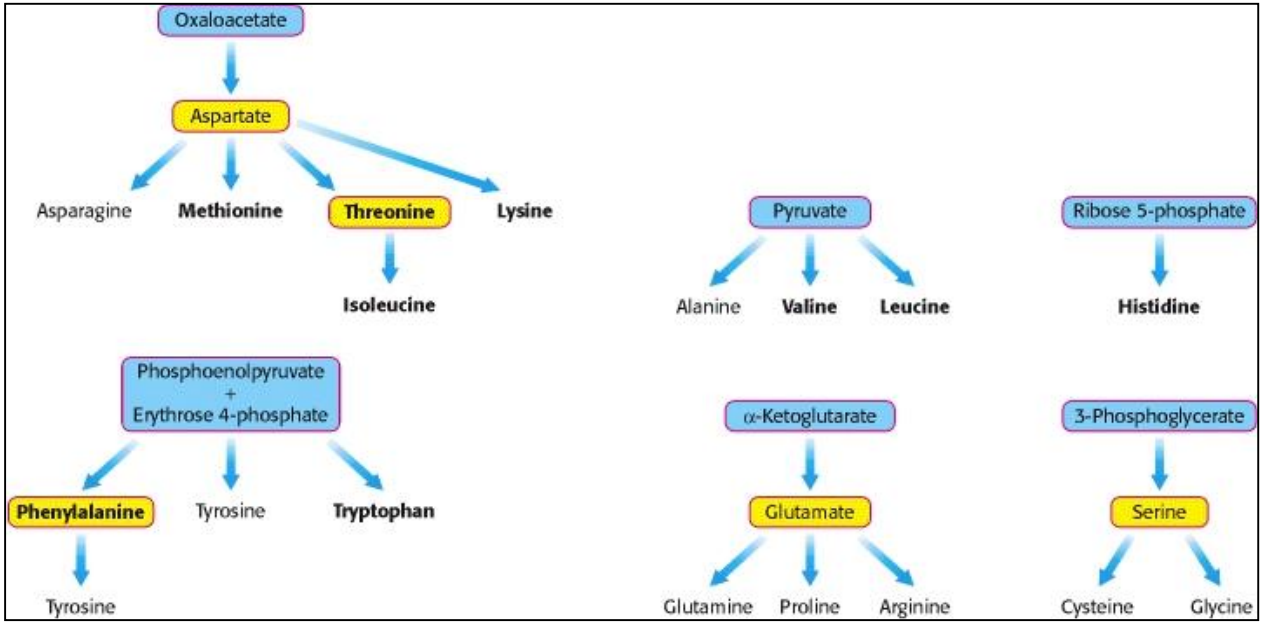
Name, abbreviation and linear structures

Name	Abbreviation	Linear Structure
Alanine	**ala** A	CH3-CH(NH2)-COOH
Arginine	**arg** R	HN=C(NH2)-NH-(CH2)3-CH(NH2)-COOH
Asparagine	**asn** N	H2N-CO-CH2-CH(NH2)-COOH
Aspartic Acid	**asp** D	HOOC-CH2-CH(NH2)-COOH
Cysteine	**cys** C	HS-CH2-CH(NH2)-COOH
Glutamic Acid	**glu** E	HOOC-(CH2)2-CH(NH2)-COOH
Glutamine	**gln** Q	H2N-CO-(CH2)2-CH(NH2)-COOH
Glycine	**gly** G	NH2-CH2-COOH
Histidine	**his** H	NH-CH=N-CH=C-CH2-CH(NH2)-COOH
Isoleucine	**ile** I	CH3-CH2-CH(CH3)-CH(NH2)-COOH
Leucine	**leu** L	(CH3)2-CH-CH2-CH(NH2)-COOH
Lysine	**lys** K	H2N-(CH2)4-CH(NH2)-COOH
Methionine	**met** M	CH3-S-(CH2)2-CH(NH2)-COOH
Phenylalanine	**phe** F	Ph-CH2-CH(NH2)-COOH
Proline	**pro** P	NH-(CH2)3-CH-COOH
Serine	**ser** S	HO-CH2-CH(NH2)-COOH
Threonine	**thr** T	CH3-CH(OH)-CH(NH2)-COOH
Tryptophan	**trp** W	Ph-NH-CH=C-CH2-CH(NH2)-COOH
Tyrosine	**tyr** Y	HO-Ph-CH2-CH(NH2)-COOH
Valine	**val** V	(CH3)2-CH-CH(NH2)-COOH

Amino Acids

Biosynthetic families of amino acids in bacteria and plants

- Major metabolic precursors are shaded blue.
- Amino acids that give rise to other amino acids are shaded yellow.
- Essential amino acids are in **boldface type**.



Berg, Tymoczko and Stryer,2002

Amino Acids
Essential and Nonessential Amino Acids

- The twenty amino acids can be divided into two groups of 10 amino acids.

1. Ten are essential(must be obtained from the diet) and
2. 10 are nonessential(not specifically required in the diet).

- Most microorganisms such as *E. coli* can synthesize the entire basic set of 20 amino acids, whereas
- Human beings cannot make 9 of them e.g. histidine, lysine, tryptophan, leucine, methionine, phenyalanine.

Amino Acids

Essential and Nonessential Amino Acids

The Ten "Nonessential" Amino Acids
Alanine
Asparagine
Aspartate
Cysteine (requires sulfhydryl group from methionine)
Glutamate
Glutamine
Glycine
Proline
Serine
Tyrosine (synthesized from phenylalanine)

The Ten "Essential" Amino Acids
Arginine (see below)
Histidine
Isoleucine
Leucine
Lysine
Methionine
Phenylalanine
Threonine
Tryptophan
Valine

Human beings cannot make 9 of them e.g. histidine, lysine, tryptophan, leucine, methionine, phenyalanine .Thus 11 amino acids that the human body makes are called non-essential amino acids.

Natural amino acids

Three different structures based on the nature of the side chain

- Amino acids are categorized into three groups based on the nature of the side chain.

1. Nonpolar side chains: Nine of the amino acids have side chains that are nonpolar.
2. Polar side chains: The second category of amino acid contains six different molecules that have polar side chains.
3. Polar but not charged: A group of five amino acids have side chains that are not only polar, but charged.

The structure of amino acids

Classification based on amino acid side chains

Polar(acidic, basic and neutral), Polar(charged and uncharged) and nonpolar(neutral)

- The R group is often referred to as the amino acid side chain.
- Some amino acid side chains are positively charged;
- Some are negatively charged;
- Some are neutral;
- Some are hydrophobic;
- Some are hydrophilic.

Amino Acids

Classification of amino acid side chains

Polarity of the amino acid side chains

- Polar (or hydrophilic) amino acids have side chains that interact with water (prefer to reside in an aqueous environment).
- They dissolve in the water.
- Nearly always found on the outside of proteins.
- Nonpolar (or hydrophobic) amino acids have side chains that do not interact with water.
- They do not dissolve.
- Always found on the outside of proteins.

Kuang,2010;IMGT,2013;..

The structure of amino acids

Classification of amino acid side chains

Polar(acidic, basic and neutral), Polar(charged and uncharged) and nonpolar(neutral)

- Based on polarity of side chains, amino acids are classified into four groups as follows:

1. Non-polar amino acids (neutral)
2. Polar amino acids with no charge(neutral)
3. Polar amino acids(basic) with positive charge
4. Polar amino acids(acidic) with negative charge

Polar (or hydrophilic) amino acids have side chains that interact with water side chains that interact with water. To identify nonpolar, polar, electrically charged amino acids of proteins you basically need to focus on the side chain of the amino acid.

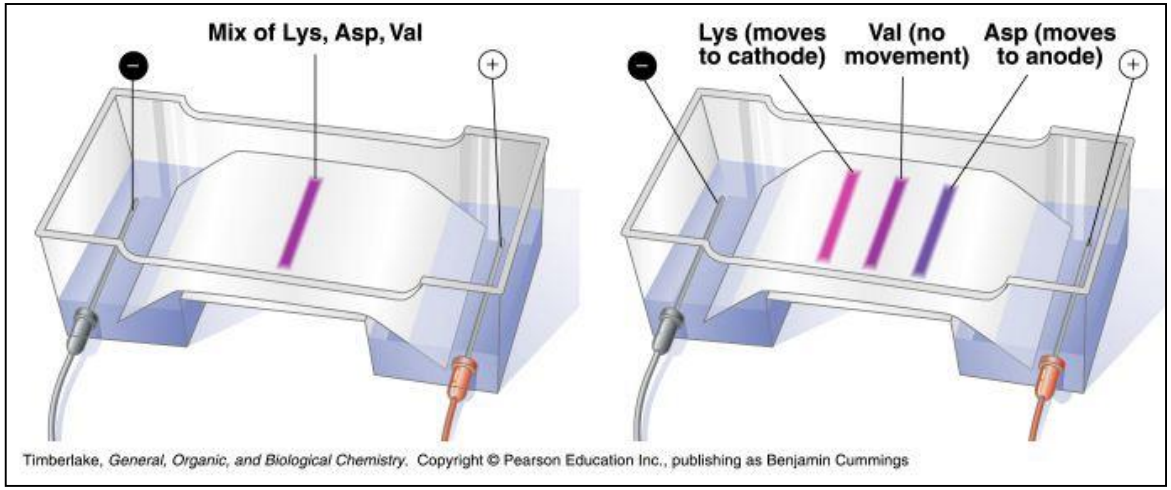
Ophardt,2003;TutorVista.com;..

Electrophoresis of amino acids

At pH7, most amino acids are neutral (no net charge)

- Electrophoresis is a technique used to separate charged molecules with an electric field.
- A buffered solution is used to conduct the charge and allow the charged molecules to move:

1. Negatively charged amino acids move towards the anode (+).
2. Positively charged amino acids move towards the cathode (-).



lysine: positively charged polar; Valine: nonpolar; Asparagine: uncharged polar. Asparagine in basic medium, it exists in the form of negatively charged ion and move towards anode.

Amino Acids

Classification of amino acid side chains

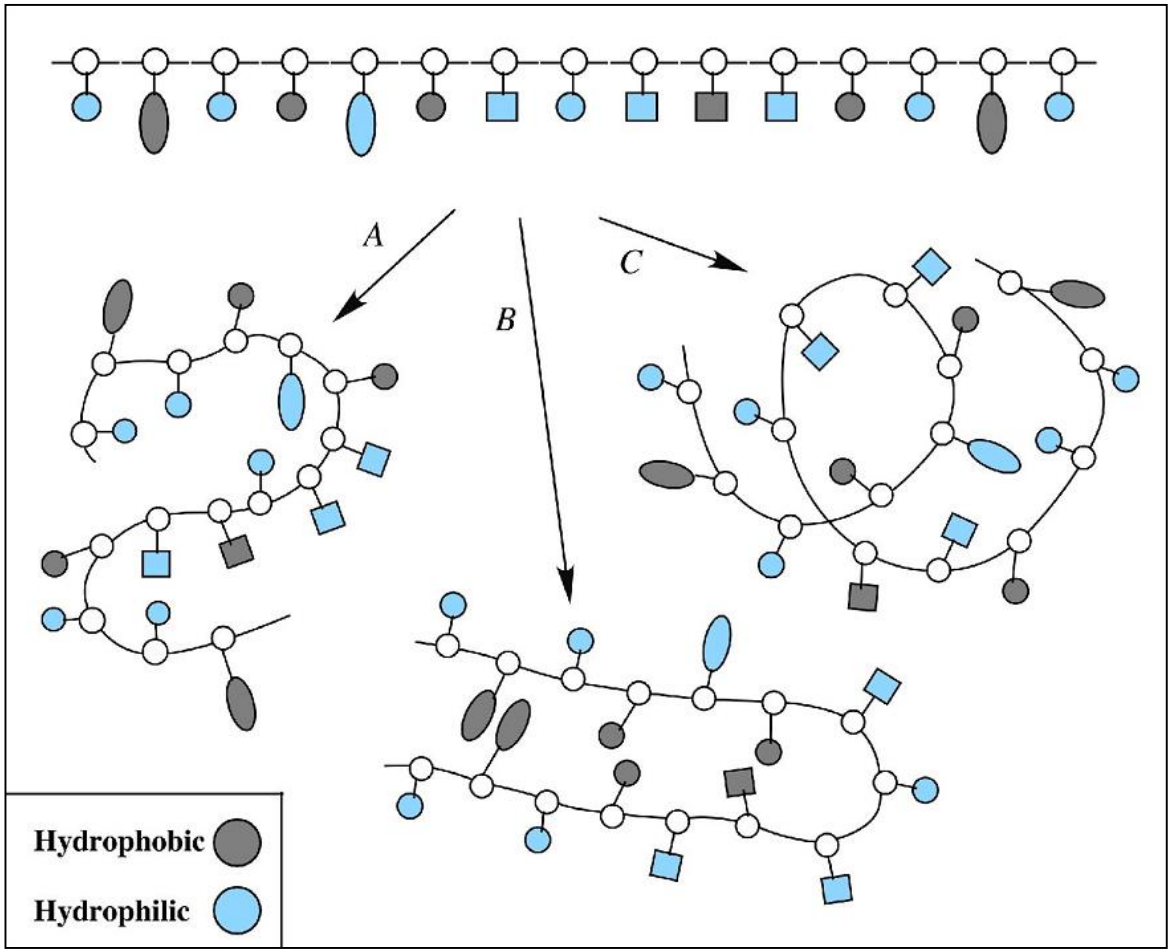
Polarity of the amino acid side chains

- Polar (or hydrophilic) amino acids have side chains that interact with water (prefer to reside in an aqueous environment). They dissolve in the water.
- Nearly always found on the outside of proteins.
- Nonpolar (or hydrophobic) amino acids have side chains that do not interact with water (do not like to reside in an aqueous i.e. water environment). They do not dissolve.
- Tend to cluster their side chains together in the inside of proteins.

Kuang,2010;IMGT,2013;..

Amino Acids

Polarity of the amino acid side chains



www.indiana.edu

Charge on a protein

What makes a protein positively or negatively charged?

Acidic and basic proteins

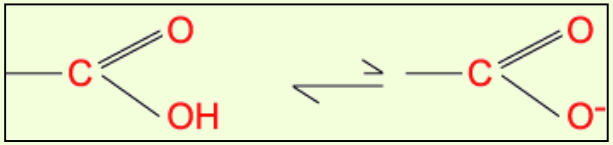
1. The basic amino acid residues (e.g. glutamine, asparagine) exhibit positive charges,
2. Whereas acidic amino acid residues (e.g. glutamic acid, aspartic acid) exhibit negative charges.

- If the protein is made up of mostly basic amino acids, the net charge of the protein will be positive, and the protein is said to be a basic protein.
- Likewise, if the protein contains a lot of acidic amino acid residues, the net charge will be negative, and the protein is acidic.

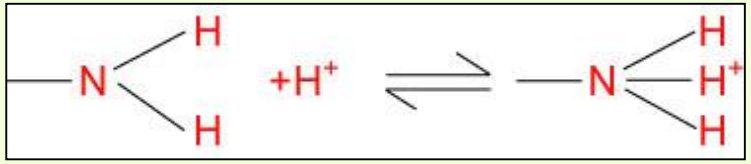
Amino acids

Polar acidic (negative charged) amino acids
Polar basic (positive charged) amino acids

- **Negative charged (acidic side chains)**
- If the side chain contains an acid functional group, the whole amino acid produces an acidic solution. The side chains lose an H^+ ion (proton) and becoming negatively charged.



- **Positive charged (basic side chains)**
- If the side chain contains an amine functional group, the amino acid produces a basic solution. The side chains accept an H^+ ion (proton) and becoming positively charged.



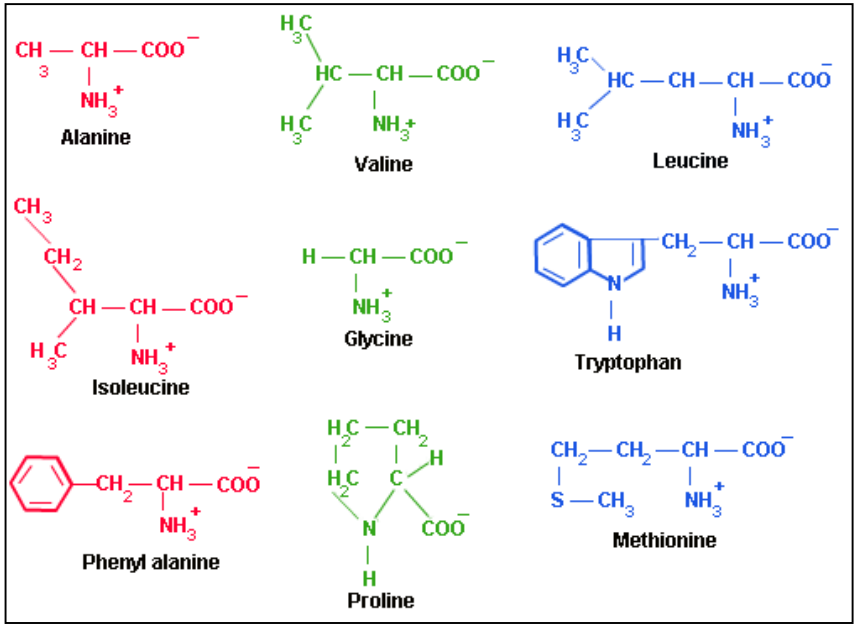
IMGT,2013;..

Non-Polar or Hydrophobic Amino Acids

Non-polar amino acids have equal number of amino and carboxyl groups and are neutral

Nonpolar amino acids with no charge(neutral)

- Non polar amino acids have equal number of amino and carboxyl groups and are neutral.
- These amino acids are hydrophobic and have no charge on the 'R' group.
- The amino acids in this group are:
- alanine, valine, leucine, isoleucine, phenyl alanine, glycine, tryptophan, methionine and proline.



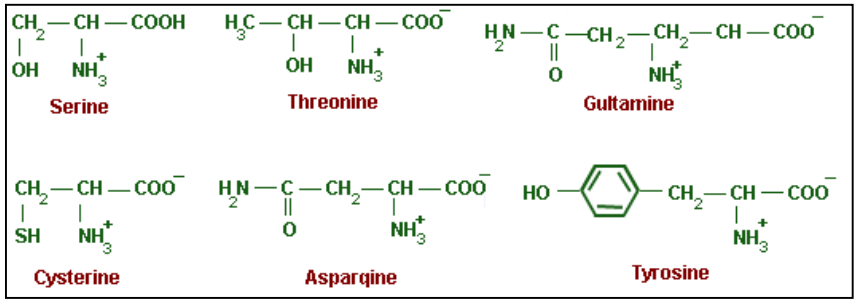
If the side chain contains a hydrocarbon alkyl group (having no double or triple bond functional groups), or a benzene ring, it's going to be nonpolar.

Polar or Hydrophilic Amino Acids

Amino acids with hydrophilic side groups

Polar amino acids with no charge(neutral)

- These amino acids do not have any charge on the 'R' group (neutral).
- These amino acids participate in hydrogen bonding of protein structure.
- The amino acids in this group are:
- serine, threonine, tyrosine, cysteine, glutamine and asparagine.

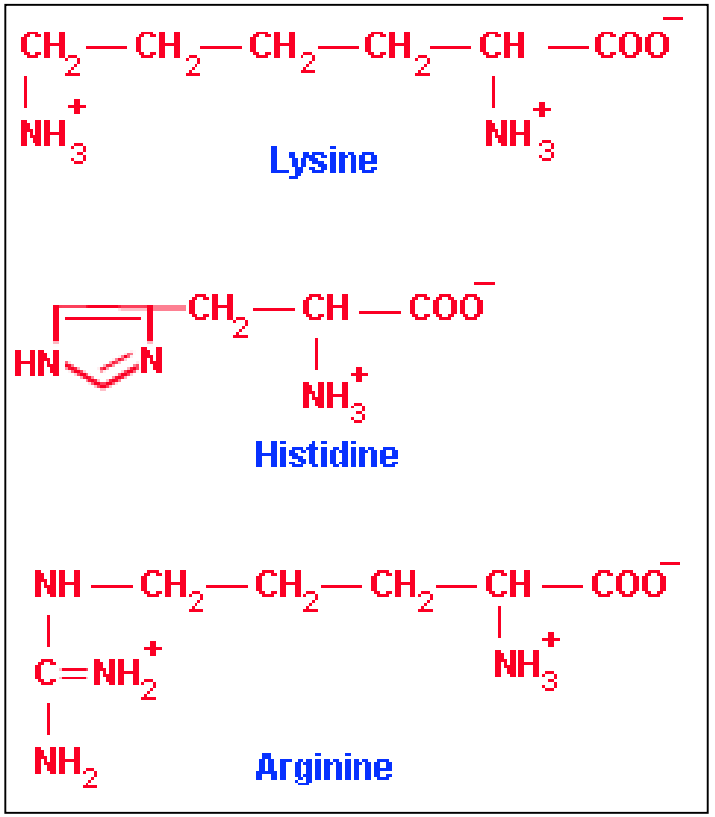


Polar or Hydrophilic Amino Acids

Polar amino acids with positive charge have more amino groups as compared to carboxyl groups

Polar amino acids(basic) with positive charge

- Amino acids with:
1. hydrophilic side groups;
2. with positive charge having more amino groups as compared to carboxyl groups making it basic.
- The amino acids which have positive charge on the 'R' group are placed in this category.
- lysine, arginine, histidine (positively charged at pH 7).



Any side chain that contains an acid, amide (-NH-CO-), alcohol, or amine will be polar.

Polar or Hydrophilic Amino Acids

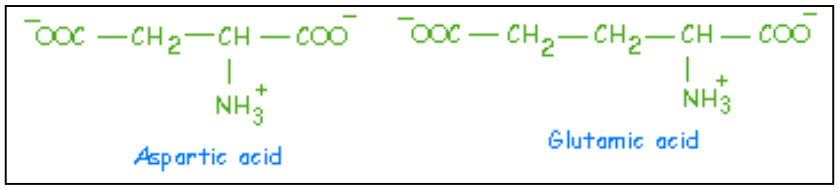
Polar amino acids with negative charge have more carboxyl groups than amino groups

Polar amino acids(acidic) with negative charge

- Amino acids with:

1. hydrophilic side groups;
2. with negative charge having more carboxyl groups than amino groups making them acidic.

- The amino acids which have negative charge on the 'R' group are placed in this category.
- They are aspartic acid and glutamic acid.



They are called as dicarboxylic mono-amino acids.
Aspartate, glutamate
(negatively charged at pH 7)

The structure of amino acids

Polar (charged) and nonpolar (uncharged) functional groups

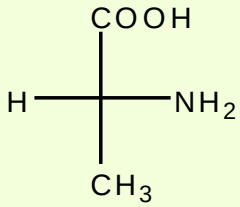
Name	3-letter symbol	1-letter symbol	Type of side chain
Alanine	Ala	A	nonpolar
Arginine	Arg	R	pos. charged polar
Asparagine	Asn	N	uncharged polar
Aspartic acid	Asp	D	neg. charged polar
Cysteine	Cys	C	uncharged polar
Glutamic acid	Glu	E	neg. charged polar
Glutamine	Gln	Q	uncharged polar
Glycine	Gly	G	nonpolar
Histidine	His	H	pos. charged polar
Isoleucine	Ile	I	nonpolar
Leucine	Leu	L	nonpolar
Lysine	Lys	K	pos. charged polar
Methionine	Met	M	nonpolar
Phenylalanine	Phe	F	nonpolar
Proline	Pro	P	nonpolar
Serine	Ser	S	uncharged polar
Threonine	Thr	T	uncharged polar
Tryptophan	Trp	W	nonpolar
Tyrosine	Try	Y	uncharged polar
Valine	Val	V	nonpolar

The structure of amino acids

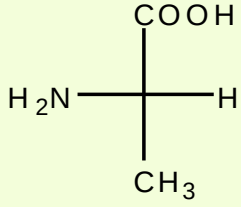
D-and L-amino acids

- Each amino acid has two possible enantiomers(opposite forms).
- These are classified as D or L as with sugars.
- Amino acids in nature are almost exclusively L-amino acids.
- When a Fischer projection is written with the acid at the top, and the R group at the bottom:

  - if the amine group is on the right hand, it's a D-amino acid.
  - if the amine group is on the left hand, it's an L-amino acid.



D-Alanine



L-Alanine

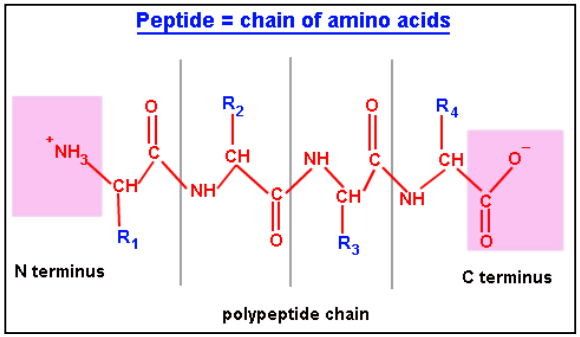
D form of Amino acids hard to break down

The structure of amino acids
D-and L-amino acids

1. D-form amino acids (also known as Dextro, Dextrorotatory) tend to taste sweet,
2. L-forms (also known as Levo, Levorotatory)are usually tasteless.

- There are a few more complex labels that you might see in product manufacture.
- One of them is DL.
- These compound mixes are often referred to as DL forms, meaning they are 50% by weight the D form of the compound and 50% by weight of the L form.

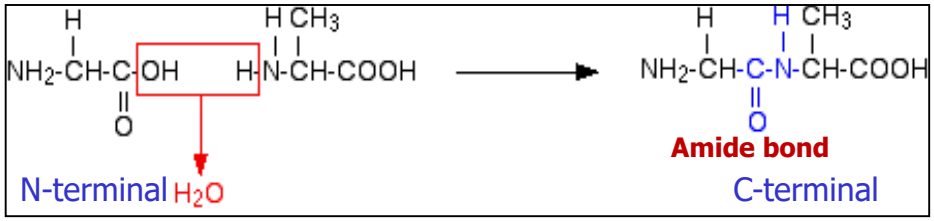
Proteins
Peptides



- Each amino acid carries:

1. The peptide chain is known as the backbone, and
2. The "R" groups are known as side chains.

- The side chain or R group is not part of the backbone or the peptide bond.
- A peptide bond (amide bond) is a covalent chemical (intra) bond formed between two molecules e.g. two amino acids.
- When two amino acids are joined together, a dipeptide is formed.



Amide bond /covalent/ peptide bond
C=O Bond formation

Proteins

The covalent and non-covalent bonds

1. The covalent bonds:

- Connect the atoms of a single amino acid.
- Covalent bonds are the sharing of electrons between two non-metal elements.
- Covalent bonds are the strongest chemical bonds contributing to protein structure.

2. The non-covalent bonds:

- A non-covalent interaction differs from a covalent bonds in that it does not involve the sharing of electrons, but rather involves more dispersed variations of electromagnetic interactions between molecules or within a molecule.

Proteins
The non-covalent bonds

- Non-covalent interactions are the dominant type of interaction between supermolecules.
- Non-covalent interactions are critical in maintaining the three-dimensional structure of large molecules, such as proteins and nucleic acids.
- The non-covalent bonds help shape individual molecules and groups of molecules and ions, but are weak enough to be continually broken and re-formed in the dynamic molecular interplay that is life.
- For cells, both covalent bonds and noncovalent interactions are important.

Proteins

Some proteins with non-covalent bonds

H, ironic and hydrophobic bonds

- The non-covalent bonds are weak interactions between ions, molecules, and parts of molecules.
- There are at least three principle kinds of noncovalent bonds:

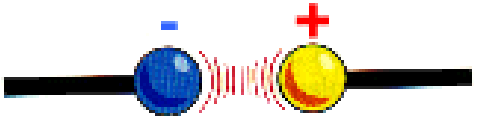
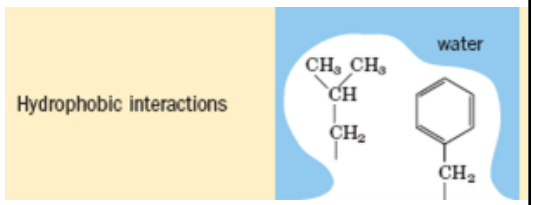
1. ionic interactions;
2. hydrogen bond;
3. hydrophobic interactions.

- As stated above, non-covalent bonds are not as strong as covalent bonds, but the additive effect of many non-covalent bonds can stabilize a molecule.
- Non-covalent bonds are very important in the structure of proteins.

Proteins

Some proteins with non-covalent bonds

Hydrogen, ironic and hydrophobic bonds

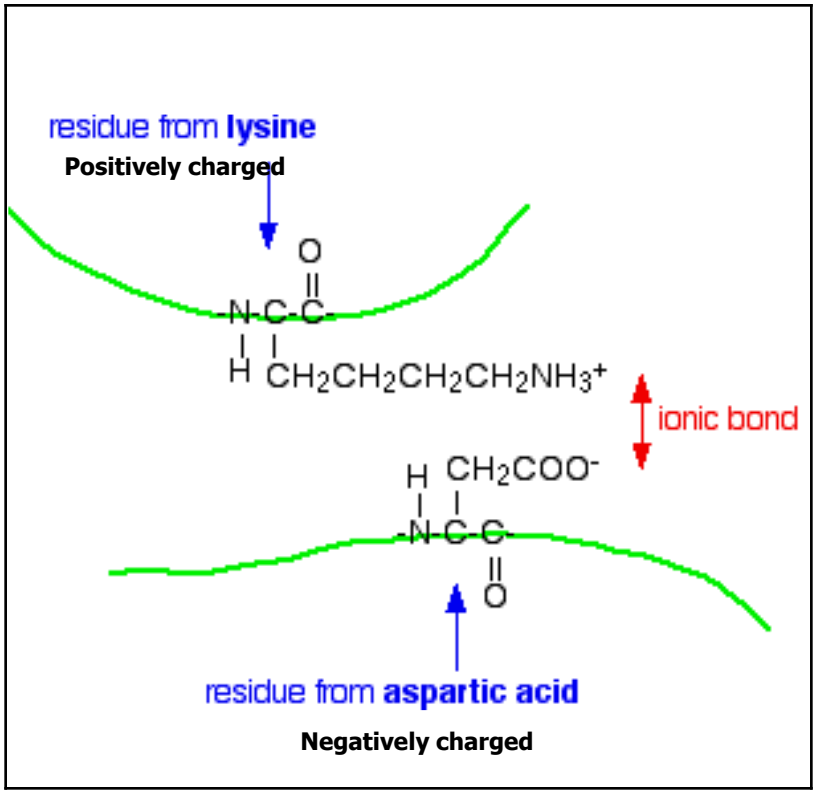
	Ionic bonds involve interactions between the oppositely charged groups of a molecule. When electrons are transferred, the "bond" is said to be "ionic". E.g. aspartic acid (Asp) and glutamic acid (Glu) residues, salt bridges; protein side chains - DNA backbone interactions.
	Hydrogen bonds are formed by "sharing" of a hydrogen atom between two negative charged atoms such as N and O. E.g. DNA binding domains which have particular hydrogen-bonding patterns with specific bases.
	Hydrophobic interactions result when non-polar molecules are in a polar solvent, e.g. H_2O. The non-polar molecules group together to exclude water (hydrophobic means water fearing). By doing so they minimize the surface area in contact with the polar solvent.

Proteins

Some proteins with non-covalent bonds

Ironic bonds

- **Interactions between the oppositely charged (+ve and –ve)groups of a molecule:**
- In ionic interactions, the negatively-charged carboxyl groups on aspartic acid (Asp) and glutamic acid(Glu) residues may be attracted by the positively-charged free amino groups on lysine (Lys) and arginine (Arg) residues.



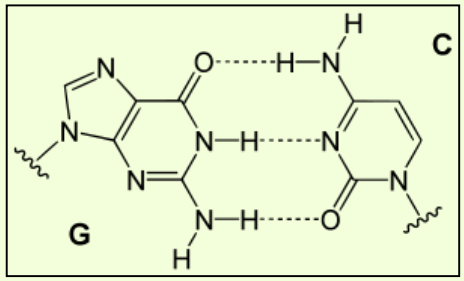
Clark,2004;..

Proteins

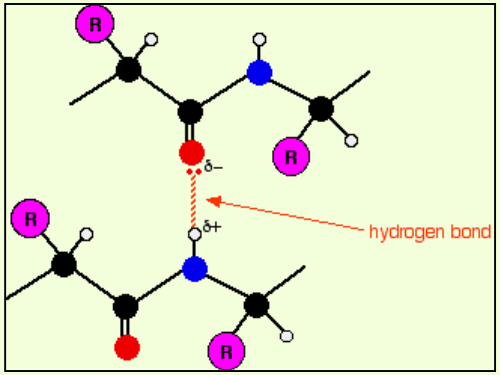
The non-covalent bonds

Hydrogen bond

- Cytosine and Guanine in DNAs are held together by three hydrogen bonds.
- Hydrogen bonds are responsible for specific base-pair formation in the DNA double helix and a major factor to the stability of the DNA double helix structure.



- These secondary structures of proteins are held together by hydrogen bonds.
- An oxygen atom and the hydrogen attached to a nitrogen atom.
- The hydrogen has the partial positive charge.

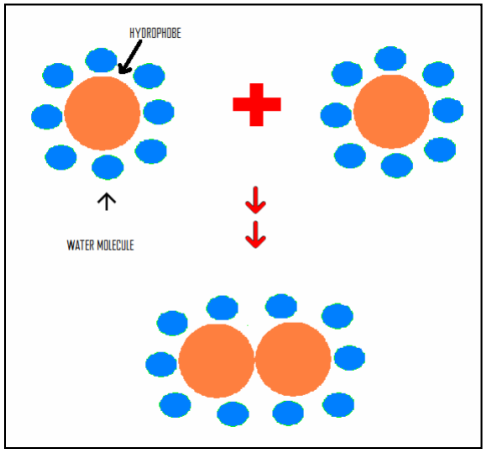


Proteins

The non-covalent bonds

Hydrophobic interactions

- Hydrophobes are nonpolar molecules and usually have a long chain of carbons that do not interact with water molecules.
- The mixing of fat and water is a good example of this particular interaction.
- When a hydrophobe is dropped in an aqueous medium, hydrogen bonds between water molecules will be broken to make room for the hydrophobe; however, water molecules do not react with hydrophobe.



ChemWiki

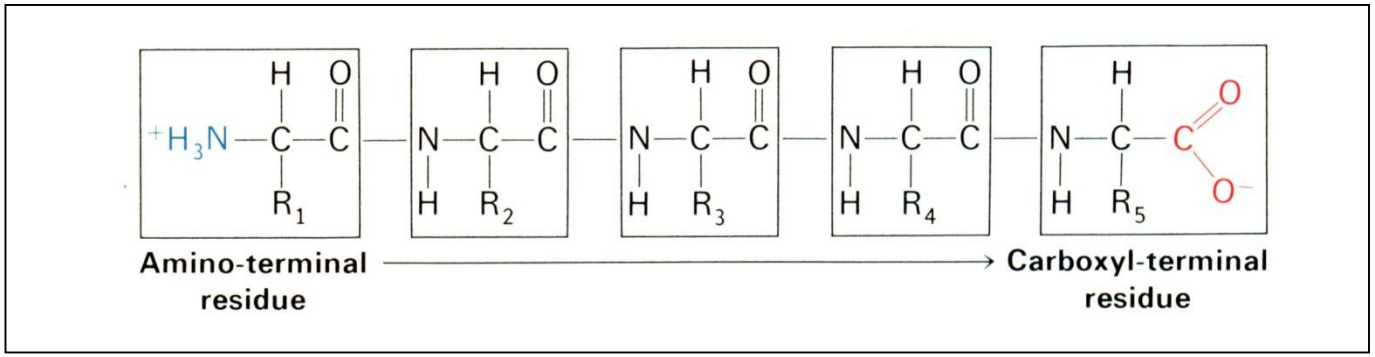
Proteins

Residues

AAs in peptides are called as residues

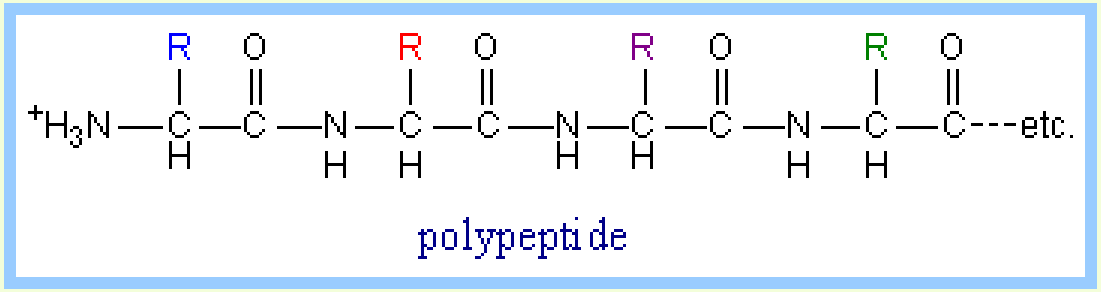
- Residue: an amino acid in a oligo- or polypeptide, protein; a peptide unit.
- The amino acids once linked in the protein chain:

1. An individual amino acid is called a residue, and
2. The linked series of carbon, nitrogen, and oxygen atoms are known as the main chain or protein backbone.



Types of proteins
Monomeric and oligomeric

- Protein molecule that consists of a single polypeptide chain is said to be monomeric (e.g. proteins with primary structures).
- Proteins made up of more than one polypeptide chain, as many of the large ones are, are called oligomeric.



Proteins
Polypeptides or proteins

- Oligopeptide: short polymer of residues; up to 10-20 residues.
- Polypeptide: longer polymer of residues; larger sizes
- Protein: one or more polypeptide chains.
- Proteins (also known as) are organic compounds:

1. Made of amino acid;
2. Arranged in a linear chain, and
3. Folded into a globular form(the side chains will help determine the conformation in an aqueous solution).

Protein Folding

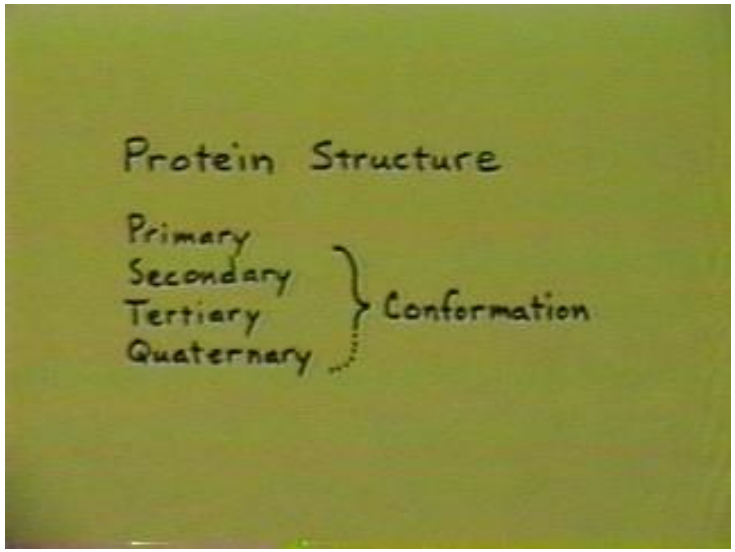
Conformation or fold

Biologically active native state

- Each protein folds into a unique three-dimensional structure defined by its amino acid sequence.
- The peptide bond allows for rotation around it and therefore the protein can fold and orient the R groups in favorable positions.

1. Many protein chains spontaneously fold into the native state,
2. Others require the assistance of:

- Enzymes, or
- Other proteins called chaperones.



Amino acid sequence → 3-D structure

Protein Folding
What determines fold?

- The shape into which a protein naturally folds is known as its native conformation.

1. Some proteins require the aid of molecular chaperones(multisubunit proteins) to fold into their native states (conformations) or quaternary structures.
2. Many others can fold unassisted, simply through the chemical properties of their amino acids.

- Some molecules have multiple conformations.

Native proteins - These proteins are not changed after translation.

Protein Folding

Co-tranlsational folding of a protein

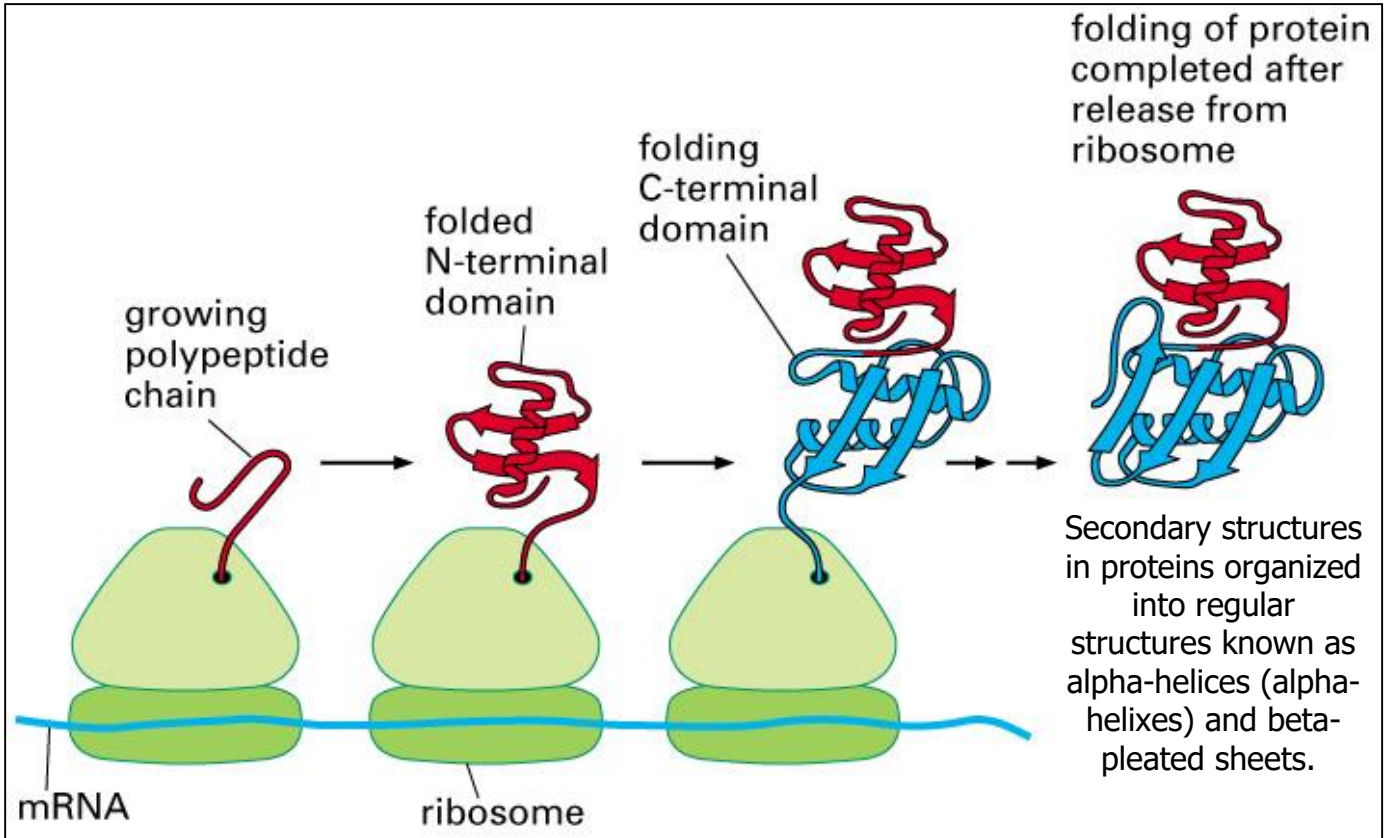
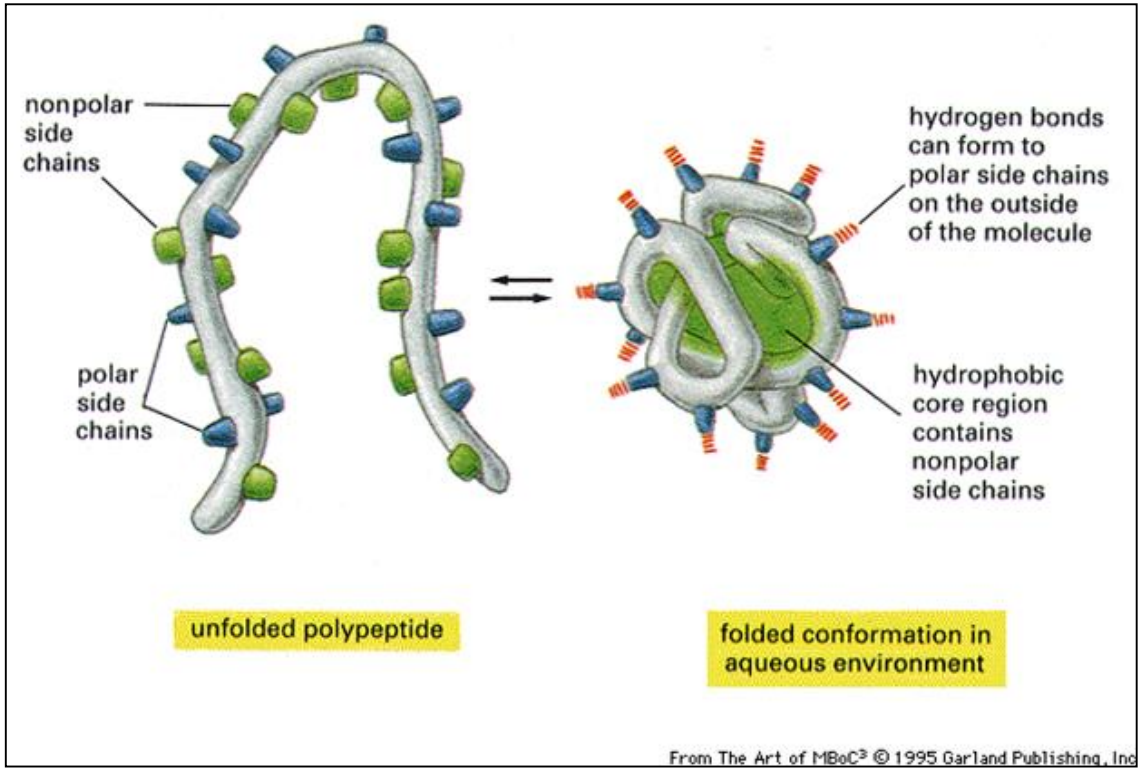


Figure 6–81. Molecular Biology of the Cell, 4th Edition.

Protein Folding

Conformation or fold

Globular proteins



The side chains will help determine the conformation in an aqueous solution.
Polar (or hydrophilic) amino acids are dissolve in the water and always found on the outside of proteins.

Protein Folding

Heat-shock proteins (HSPs)

Quality control of protein folding

- Heat shock proteins (HSP) are a family of proteins that are produced by cells in response to exposure to stressful conditions.
- HSPs are found in virtually all living organisms, from bacteria to humans.
- Similar phenomena in prokaryotes and other eukaryotes.

1. In eukaryotes this regulation is performed by heat shock factor (HSF), and
2. In bacteria by sigma factor (σ^{32}).

Wikipedia,2015

Protein Folding

Heat-shock proteins (HSPs)

Quality control of protein folding

- The Hsp70s are an important part of the cell's machinery for protein folding, and help to protect cells from stress.
- They were first described in relation to heat shock but are now known to also be expressed during other stresses including:

1. exposure to cold,
2. UV light, and
3. during wound healing or tissue remodeling.

Ron *et al.*,2000;..

Protein Folding

Heat-shock proteins (HSPs) in bacteria

Quality control of protein folding

- The bacterial heat-shock response is not limited to changes in temperature and is a general stress response, but also induced by other environmental changes, such as:

1. the addition of ethanol,
2. heavy metals,
3. high osmolarity,
4. pollutants,
5. starvation or
6. interaction with eukaryotic hosts (for diseases).

Note: heat-shock proteins have been shown to play an important role in pathogenesis.

Protein Folding

Heat-shock proteins (HSPs)

Quality control of protein folding

- Heat-shock proteins are named according to their molecular weight.
- For example,

1. Hsp40 (heat shock protein kilodaltons in size). This is also known as chaperone DnaJ.
2. Hsp60 (heat shock protein 60 kilodalton in size)
3. Hsp70 (heat shock protein 70 kilodaltons in size)
4. Hsp90 (heat shock protein 90 kilodaltons in size).

Protein Folding

Heat-shock proteins (HSPs)

Quality control of protein folding

- The heat-shock response controls the expression of more than 20 genes that code for:

1. chaperones,
2. proteases and
3. regulatory proteins.

Ron *et al.*,2000

Protein Folding

Heat-shock proteins (HSPs)

DnaK-DnaJ-GrpE chaperone system+σ^{32}

- The heat-shock response is the mechanism by which cells react to increases in temperature to prevent damage, and it involves the expression of the almost universally conserved heat-shock genes.
- Many heat-shock proteins (HSPs) are molecular chaperones or proteases and function by:

1. Facilitating refolding of damaged proteins, or
2. Eliminating proteins that cannot be repaired.

- σ^{32} (RpoH) - the heat shock sigma factor, it is turned on when the bacteria are exposed to heat. Due to the higher expression, the factor will bind with a high probability to the polymerase-core-enzyme.
- σ^{32} (RpoH) directly controlled by the DnaK-DnaJ-GrpE chaperone system.

Sigma 32 (sigma RpoH) factor
Heat shock control

- σ^{32} has greater affinity for RNA polymerase, core enzyme than σ^{70} at high temperatures.
- σ^{32} is also responsible for genetic responses to other environmental insults.
- σ^{32} regulation is a multivalent process consisting of transcriptional, translational and postranslational controls.

Protein Folding

Heat-shock proteins (HSPs)

DnaK-DnaJ-GrpE chaperone system+σ^{32}

- σ^{32} (RpoH) is turned on when the bacteria are exposed to heat.
- This heat-shock sigma factor (σ^{32}) is coded by the *rpoH* gene and binds to specific heat-shock promoters located upstream of heat-shock genes.

dnaK gene codes for ⟶ DnaK chaperone (HSP70) ⟶ DnaK-DnaJ-GrpE chaperone system regulates activity of the bacterial heat shock transcription factor σ^{32} (RpoH).

Protein Folding

Heat-shock proteins (HSPs)

The DnaK chaperone system

- In *Escherichia coli* the genes encoding HSPs form a regulon that is positively controlled by:

1. the *rpoH* gene product;
2. the heat shock promoter-specific σ^{32} subunit of RNA polymerase.

- The level and activity of σ^{32} are limiting for heat shock gene transcription.

Gamer *et al.*,1996

Protein Folding

Heat-shock proteins (HSPs)

DnaK-DnaJ-GrpE chaperone system+σ^{32}

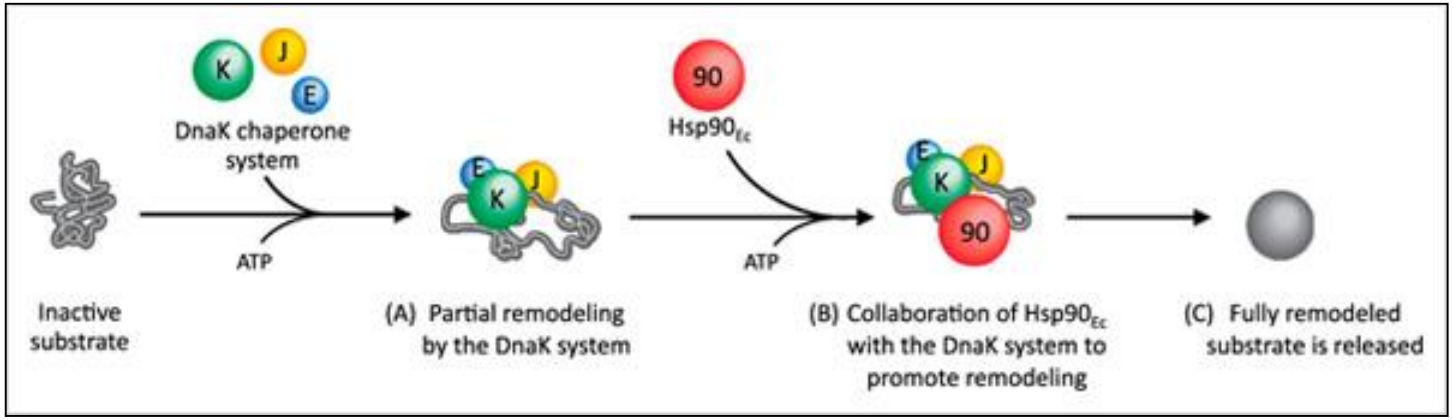
1. During heat shock (positive response), the intracellular concentration of RpoH increases, due to slightly increased transcription, increased synthesis and stabilization of the protein.
2. In the absence of heat shock, or after heat shock(negative response), activity is inhibited by transient association with DnaK-DnaJ-GrpE system, which reduces the amounts of free active RpoH, makes it unstable and mediates its degradation by the FtsH protease.

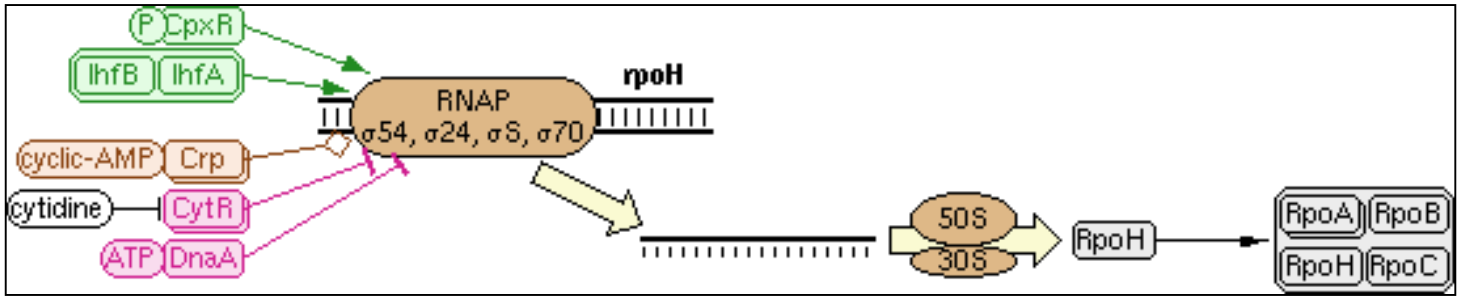
The DnaK chaperone system mediates inactivation and degradation of σ^{32} probably through association with the heat shock promoter specific σ^{32} subunit of RNA polymerase.

Protein Folding

Heat-shock proteins (HSPs)

The DnaK chaperone system





Gamer *et al.*,1996;Biocyc.org;..

Protein Folding

Heat-shock proteins (HSPs)

DnaK-DnaJ-GrpE chaperone system+σ^{32}

- Induction occurs mainly at the post-transcriptional level, via translational thermoregulation:

1. At low temperature, the structure of the rpoH mRNA blocks its translation,
2. while at high temperature, melting of the mRNA secondary structure facilitates ribosome binding and synthesis of the RpoH protein.

UniProt

Prokaryotic DNA transcription

Anti-sigma factors

Anti-sigma factor 70 Rsd against sigma factor 70

- Anti-sigma factors bind to sigma factors and inhibit transcriptional activity.
- Anti-sigma factors are antagonists to the sigma factors, which regulate numerous cell processes including flagellar production, stress response, transport and cellular growth.
- For example, anti-sigma factor 70 Rsd protein in *E. coli* is present in the stationary phase and blocks the activity of sigma factor 70 which in essence initiate gene transcription.

Note: Anti-sigma factors inhibit the function of sigma factors whereas, anti-anti-sigma factors restore sigma factor function.

Protein Folding

Heat-shock proteins (HSPs)

Quality control of protein folding

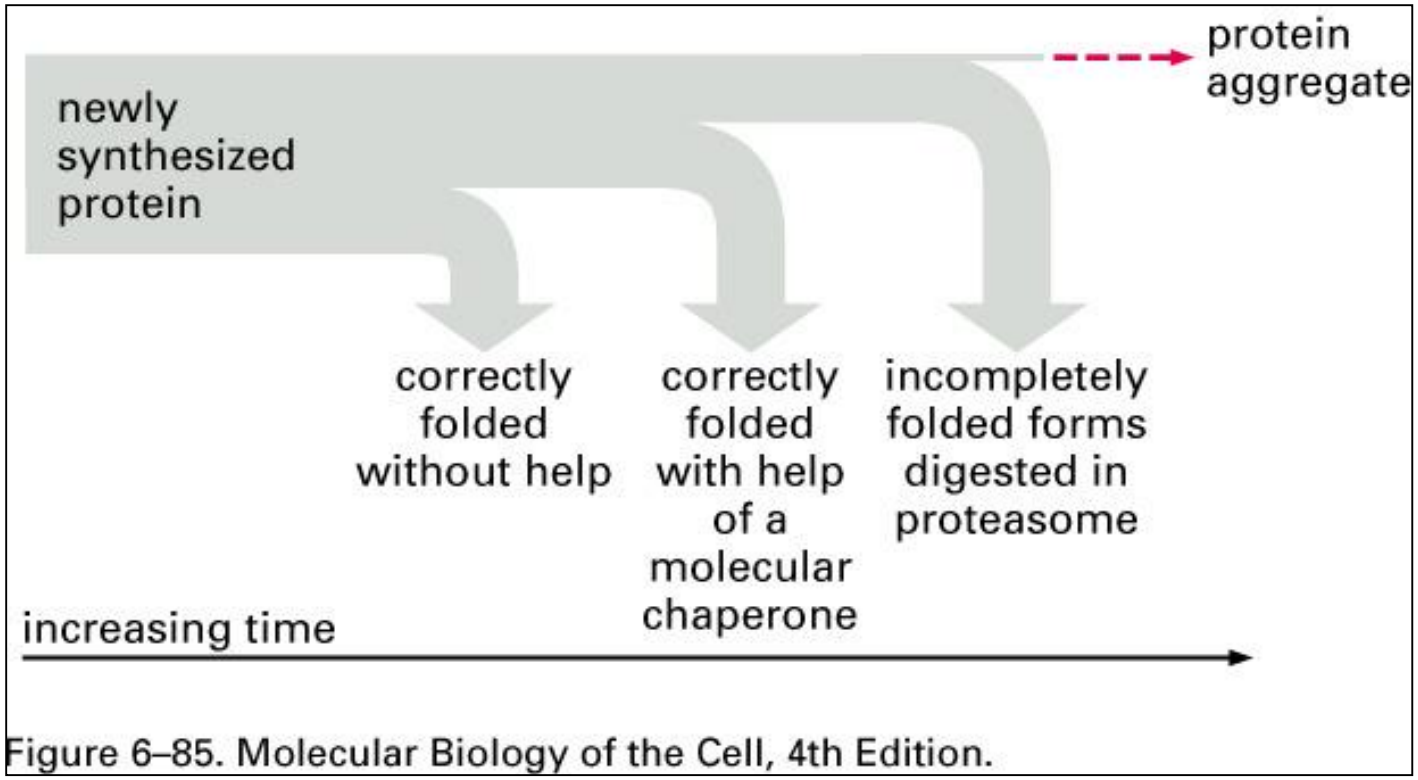


Figure 6–85. Molecular Biology of the Cell, 4th Edition.

Protein Folding

Heat-shock proteins (HSPs)

Protease action on unneeded/damaged proteins

- The main function of the proteasome is to degrade unneeded or damaged proteins by proteolysis, a chemical reaction that breaks peptide bonds.

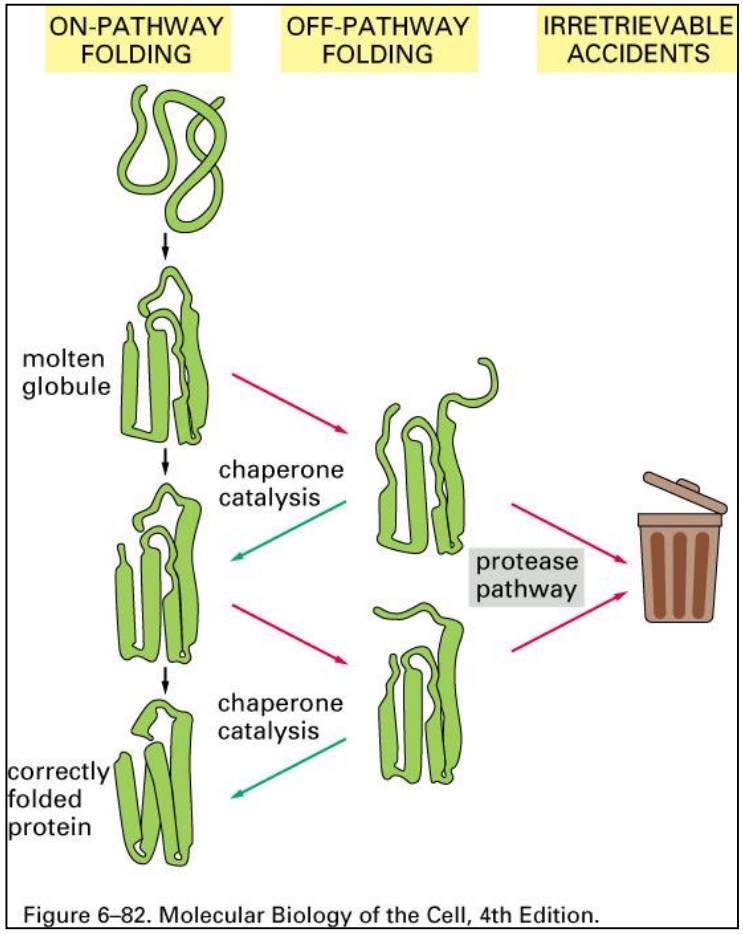


Figure 6–82. Molecular Biology of the Cell, 4th Edition.

Chaperones

Heat-shock proteins (HSPs)

Help misfolded proteins refold properly

- There are two main families of molecular chaperones – hsp70 and hsp60.

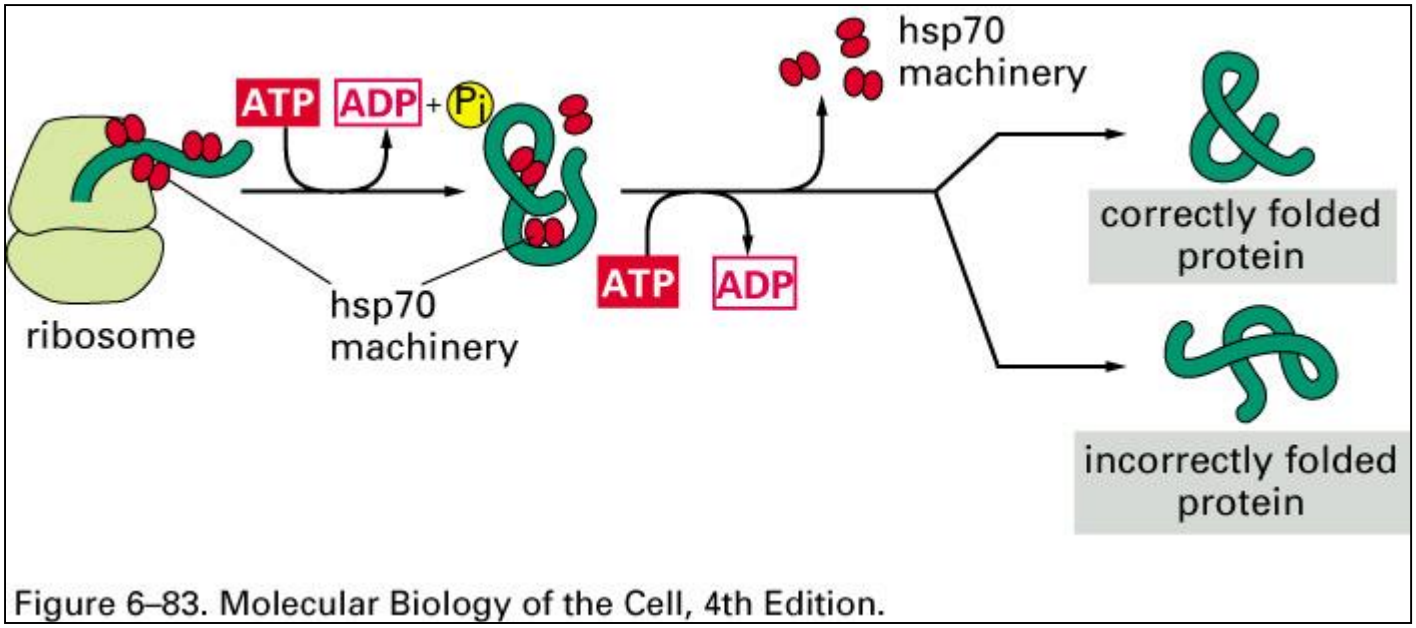


Figure 6–83. Molecular Biology of the Cell, 4th Edition.

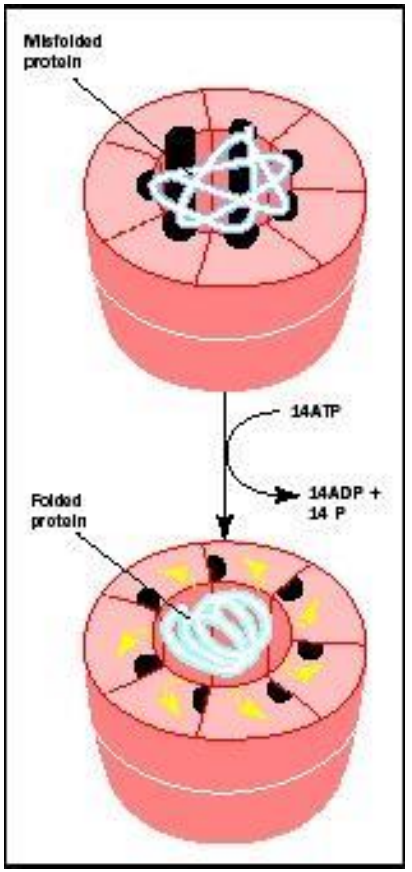
Misfold (of a protein or nucleic acid): To fold into an unusual or incorrect tertiary structure; often a cause of Alzheimer's and similar diseases.

Chaperones

Heat-shock proteins (HSPs)

Help misfolded proteins refold properly

- **Molecular Chaperones** are families of proteins to help "properly fold" a new protein under temperature elevation (25°C --> 32°C) cells make heat shock proteins (HSPs).
- To refold properly, chaperones use energy from ATP.

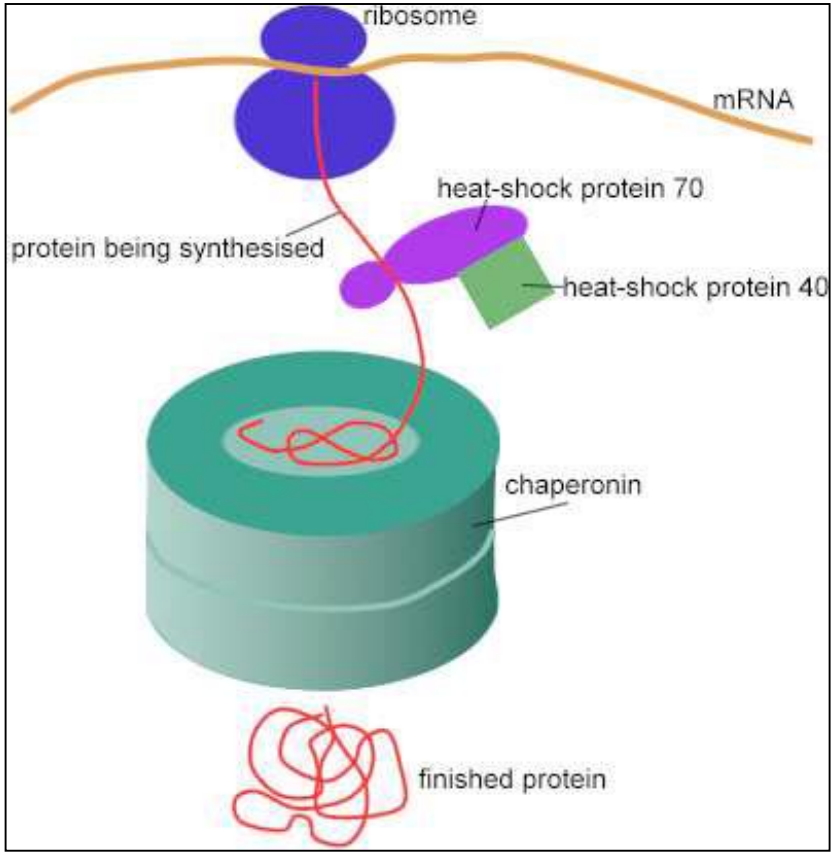


Chaperones

Heat-shock proteins (HSPs)

Help misfolded proteins refold properly

- As the ribosome moves along the molecule of mRNA, a chain of amino acids is built up to form a new protein molecule.
- The chain is protected against unwanted interactions with other cytoplasmic molecules by heat-shock proteins and a chaperon molecule until it has successfully completed its folding.
- Hsp60 chaperones are also called chaperonins.

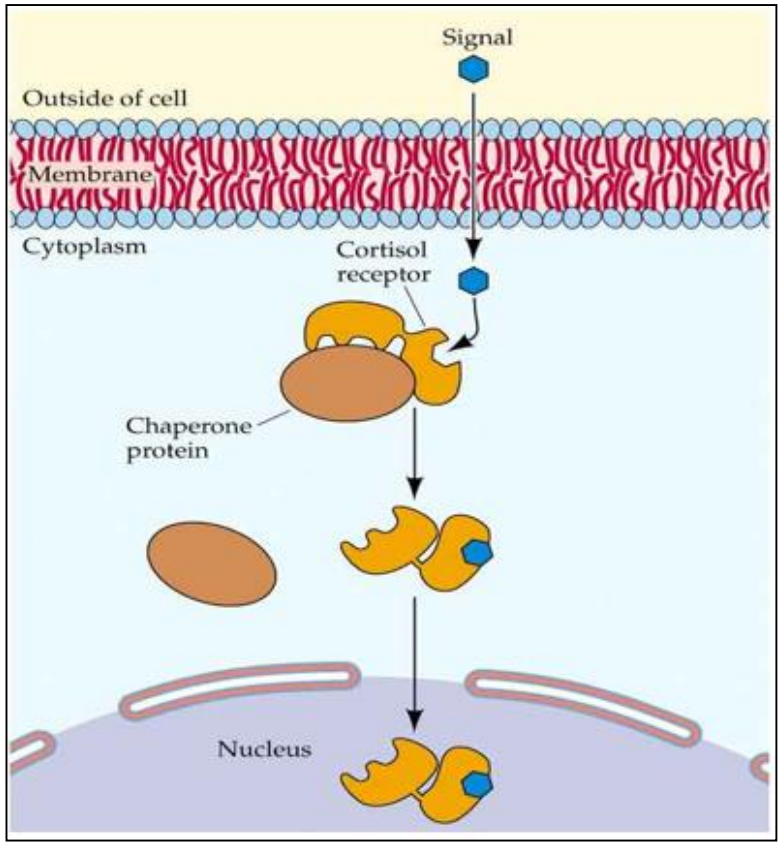


Source: (http://www.cs.stedwards.edu/chem/Chemistry/CHEM43/CHEM43/HSP/FUNCTION.HTML)

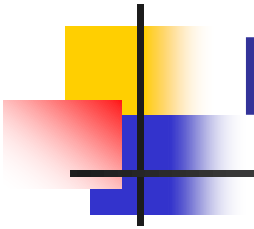
Chaperones
Other functions

1. Chaperon proteins stabilize folded proteins.
2. It may protect the effector protein from coming in contact with other proteins.
3. Chaperon protein is attached to and to facilitate the export of an effector protein secreted through a type III secretion system of bacteria.
4. Unfold them for:

- translocation across membranes, or
- degradation.



Cortisol is a steroid hormone



Protein structure

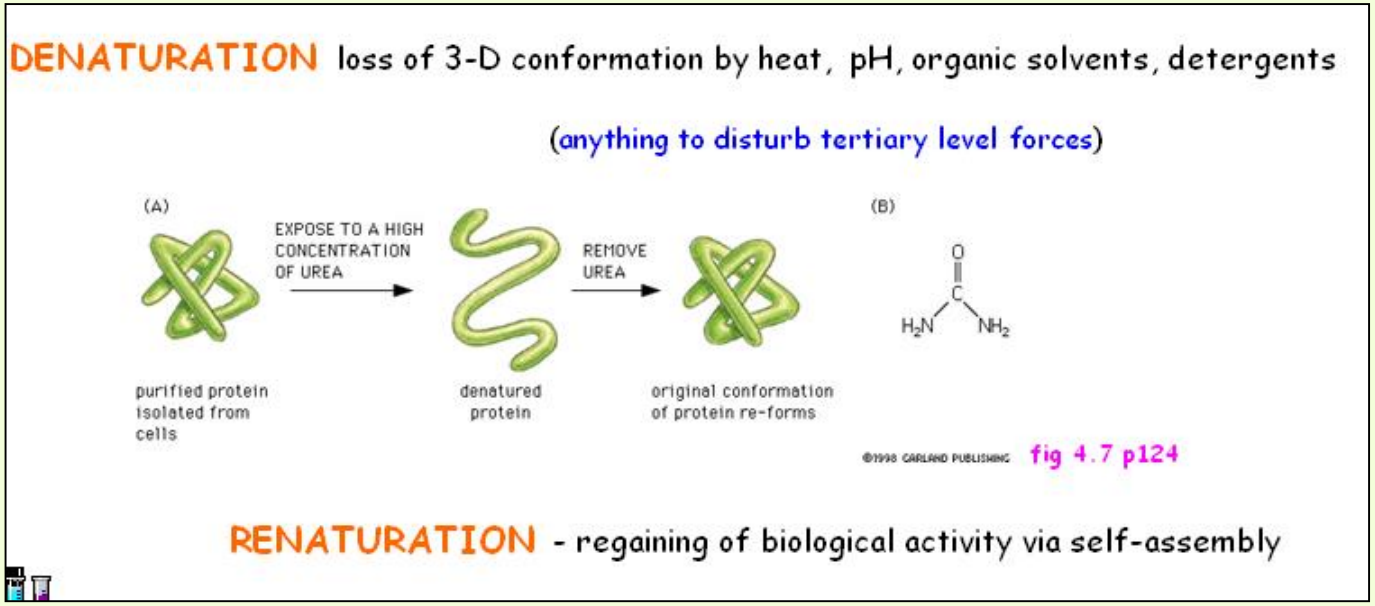
1. **What affects fold?**
2. **What determines fold?**

Protein structure

What affects fold?

Denaturation

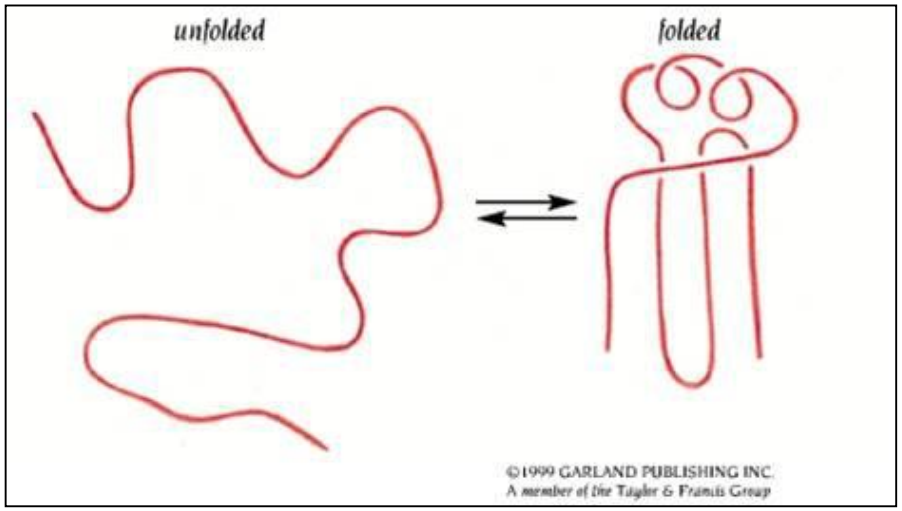
- Prions (proteins that multiply themselves; an infectious agent smaller than a virus, composed primarily of protein) can change the conformation of other proteins.
- Denaturation is the process of unfolding the protein.



Protein denaturation

pH, temperature or chemical compounds

- Slight changes in pH or temperature or chemicals such as strong acids, bases, organic solvents, detergents can convert a solution of biologically active proteins in their native state to a biologically inactive denatured state.



Protein structure

What determines fold?

Name	Type of side chain
Alanine	nonpolar
Glutamate	neg. charged polar
Isoleucine	nonpolar
Leucine	nonpolar
Lysine	pos. charged polar
Threonine	uncharged polar
Valine	nonpolar

- A simple theoretical model for polypeptides consists of hydrophilic and hydrophobic beads on a string.
- This contrasting distribution of polar and nonpolar residues reveals a key facet of protein architecture.

1. Alanine and leucine (nonpolar), lysine and glutamate (polar) favor alpha helix.
2. Whereas, beta-branched amino acids threonine, valine, and isoleucine (nonpolar), favor beta sheet.

Polar (or hydrophilic) amino acids have side chains that interact with water.

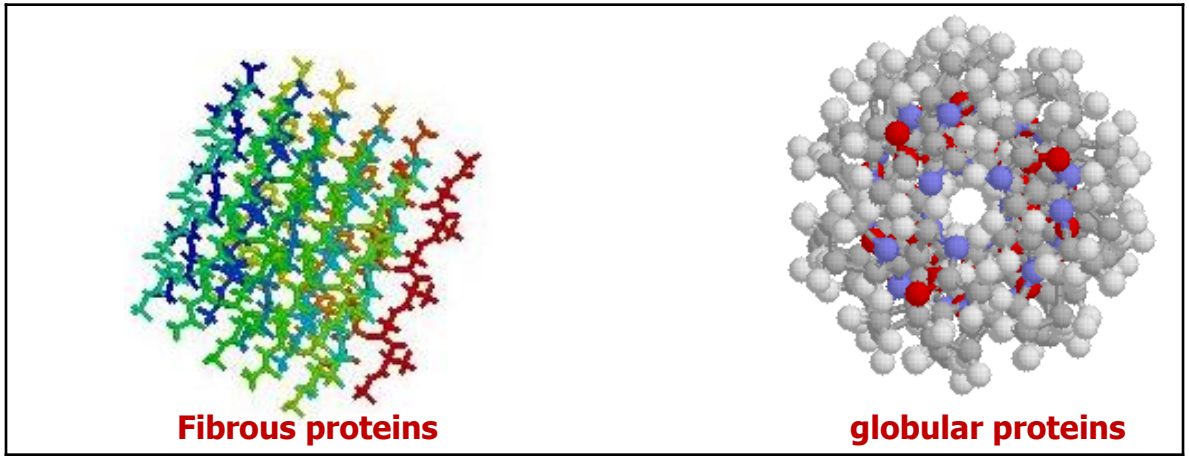
Protein Structure

Two regular folding patterns

α-helix and β-sheet

- Two regular folding patterns have been identified – formed between the bonds of the peptide backbone:

1. α-helix: Protein turns like a spiral – fibrous proteins (hair, nails, horns).
2. β-sheet: Protein folds back on itself as in a ribbon – globular protein.

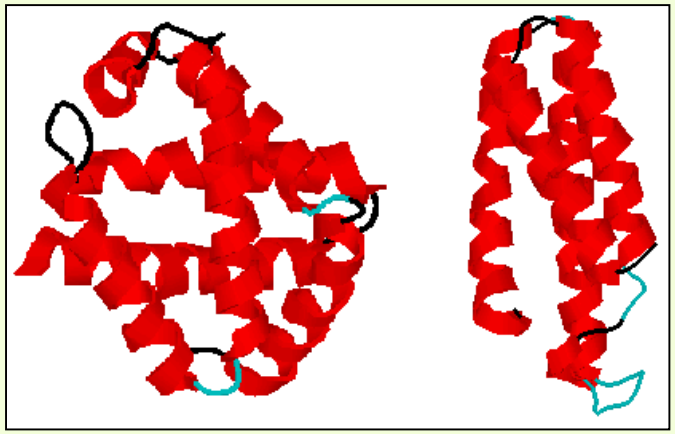


Protein structures

What determines fold?

α-helix and β-sheet

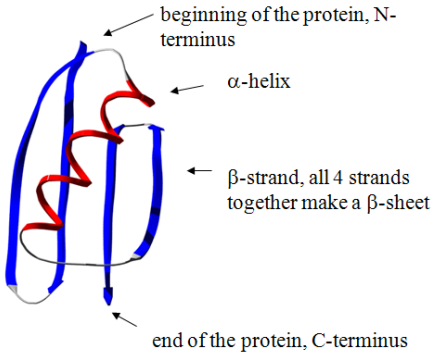
- Some proteins are made up of mostly alpha helicies.



- Some are mostly beta sheet.

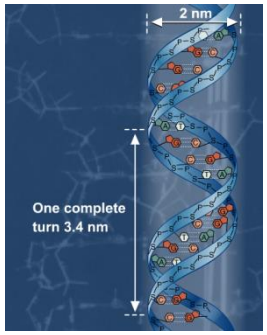
Wampler,1996

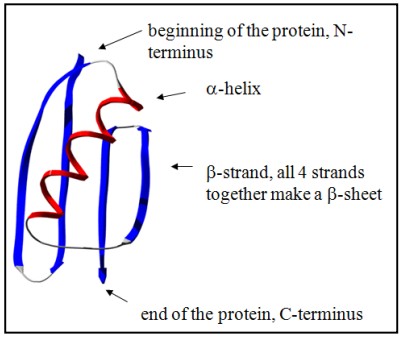
Protein Folding
α-helix



- In the alpha helix, the polypeptide chain is coiled tightly in the fashion of a spring.
- The "backbone" of the peptide forms the inner part of the coil while the side chains extend outward from the coil.
- The surface of an a-helix largely consists of the R-groups of amino acid residues.
- When side chains were removed from backbone, this short chain of amino acids appear helical, resembling the shape of a spiral staircase.
- Therefore the simplest representation of an alpha-helix is a trace of the alpha carbons.

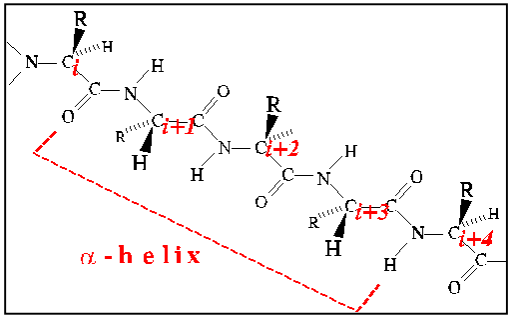
Protein Folding
α-helix



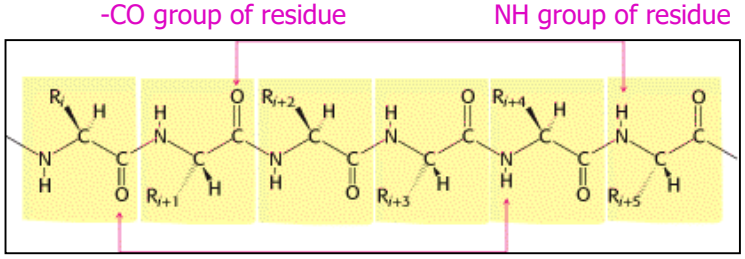


One complete 360 degree turn of the helix in DNA is equal to 3.4 nanometer

- The alpha helix is formed when the amino acid backbone turns around at 3.6 amino acids per turn. i.e. one "turn" of the coil requires 3.6 amino acid units.
- In an alpha helix 18 residues will make complete turn or about 3.6 per one turn
- The helix is stabilized by hydrogen bonds between the >N-H of one amino acid and the >C=O on the 4th amino acid away from it.

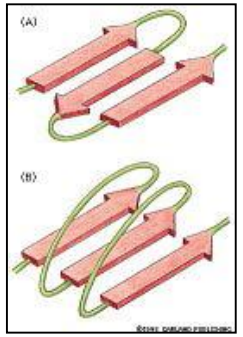


3.6 amino acids per turn



The -CO group of residue n is H-bonded to the -NH group of residue (n+4).

Protein Folding
β-sheets



- β sheets formed when two or more lengths of a protein chain lie next to each other so as to form hydrogen bonds between their respective backbones.
- Each length that participates in a β sheet is called a β strand.
- β sheets are compact and stable structures(form rigid structures with the H-bond).
- It is core of many proteins is the β sheet.
- Can be of 2 types:

a) Anti-parallel: run in an opposite direction of its neighbor.

b) Parallel: run in the same direction with longer looping sections between them.

Protein Folding

Branched-chain amino acids

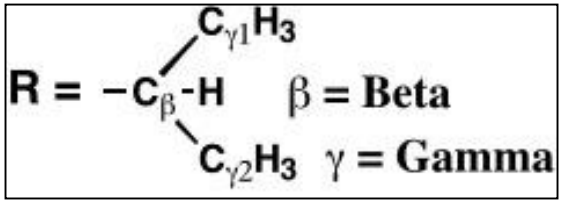
β-sheets

- C-beta branched hydrophobic amino acids contain only C and H in their R groups.

1. Valine, Isoleucine and Theronine branched at C-beta.
2. Leucine branched at C-gamma.

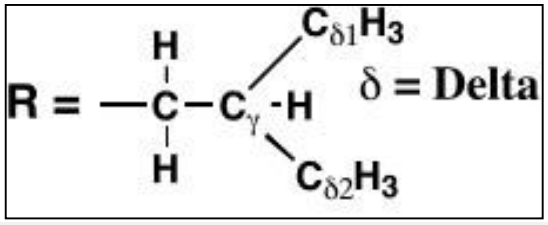
- Leucine and Isoleucine both have a 4 carbon R group.
- There is a lot more bulkiness near to the protein backbone.
- It means that these amino acids are more restricted in the conformations the main-chain.

1. It is more difficult for these amino acids to adopt an alpha-helical conformation, but
2. It is easy and even preferred for them to lie within beta-sheets.



Valine -Branched at C-beta.

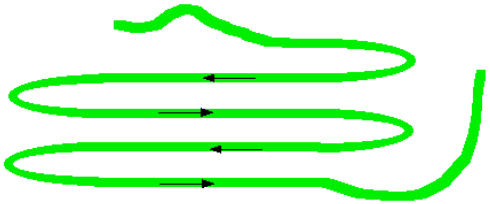
The alpha and beta carbons of valine are sensitive.



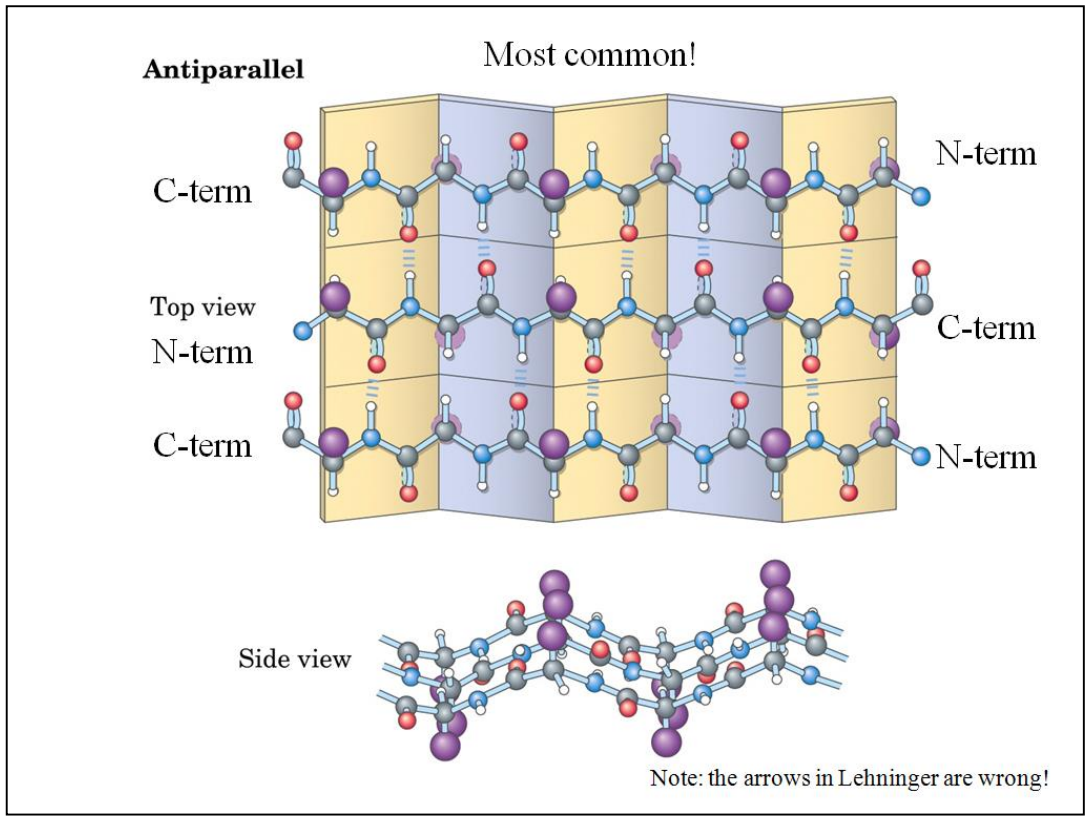
Leucine -Branched at C-gamma.

The β (Beta) sheet

Antiparallel arrangement-Most common

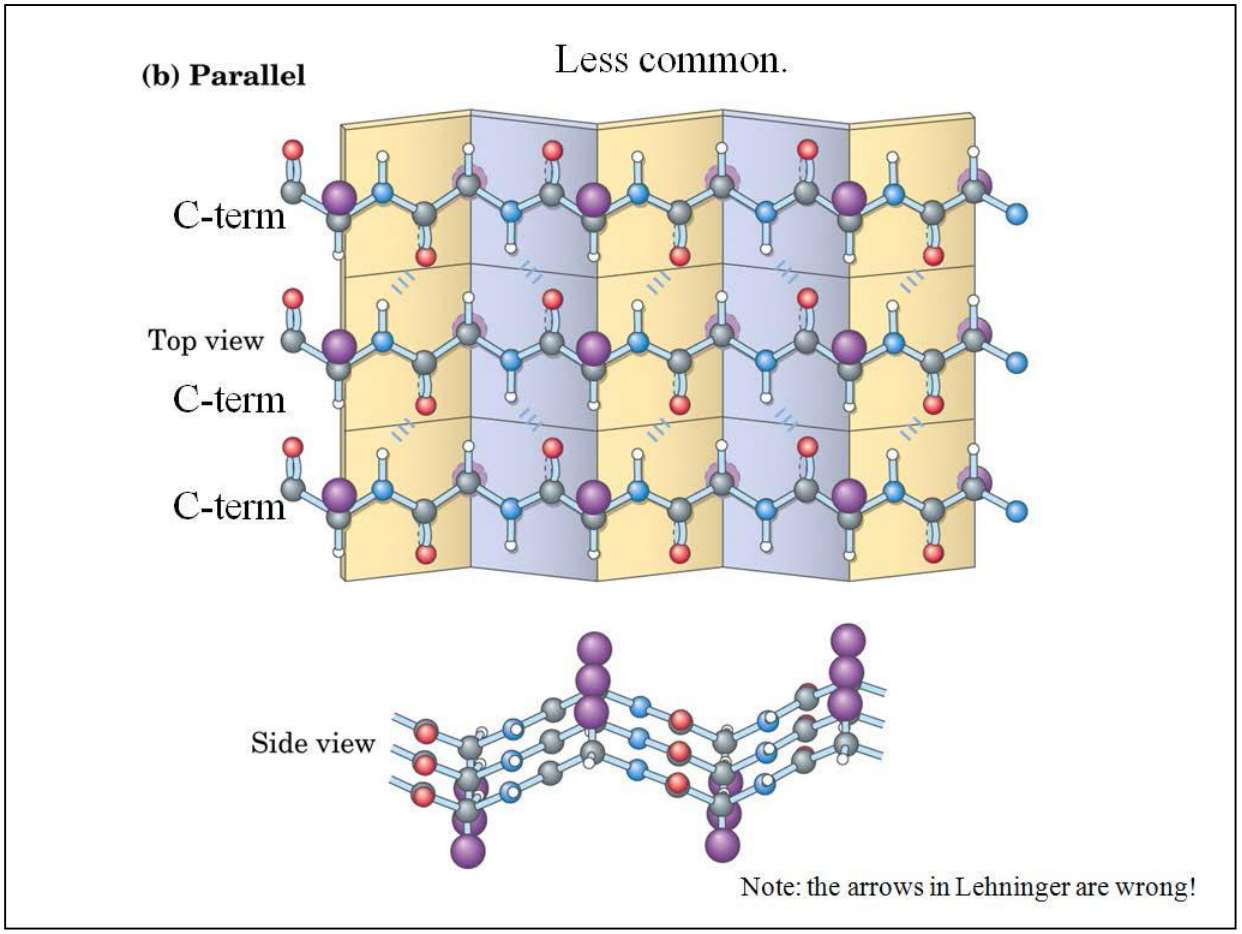


- Beta-pleated sheets consist of peptide chains side-by-side, held together by backbone H-bonding( ).



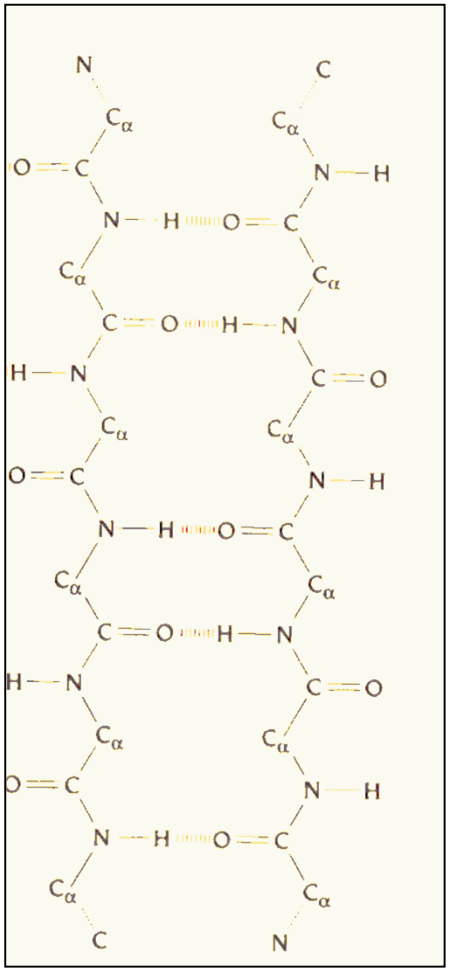
The ß (Beta) sheet

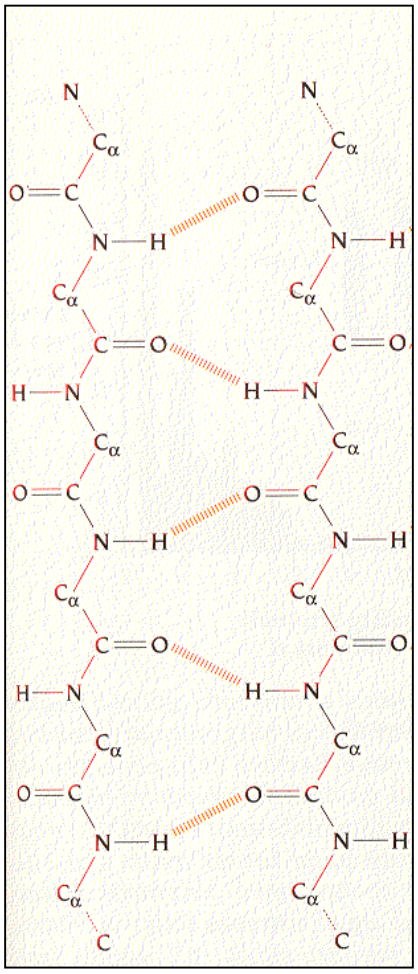
Parallel arrangement-Less common



The ß (Beta) sheet

Anti-parallel vs. parallel b-sheet





Stephen Sontum

Protein strucuture

Domains, motifs, and folds

- Proteins are frequently described as consisting of several structural units.
- These units include:

1. domains,
2. motifs, and
3. folds.

Protein Domains

Compact three-dimensional tertiary structure

- A protein domain is a conserved part of a given protein sequence and (tertiary) structure exist independently of the rest of the protein chain.
- Protein domains are a structural entity, usually meaning a part of the protein structure which folds and functions independently.
- Many proteins consist of several structural domains.
- A domain is a compact, semi-independent region of 100-150 amino acids that has:

1. a hydrophobic core, and
2. hydrophilic exterior.

1. Motifs are short sequences and domains are longer ones.
2. Motifs are structural characteristics and domains are functional regions.

Protein motifs

Super secondary structure

- Secondary structures often group together to form a specific geometric arrangements known as motifs.
- Since motifs contain more than one secondary structural element, these are referred to as super secondary structures.
- In addition to secondary structural elements, protein structural motifs often include loops of variable length and unspecified structure.
- The zinc finger is a common structural motif in many protein DNA binding domains.

1. Motifs are short sequences and domains are longer ones.
2. Motifs are structural characteristics and domains are functional regions.

Domain and motif

Differences between motif and domain

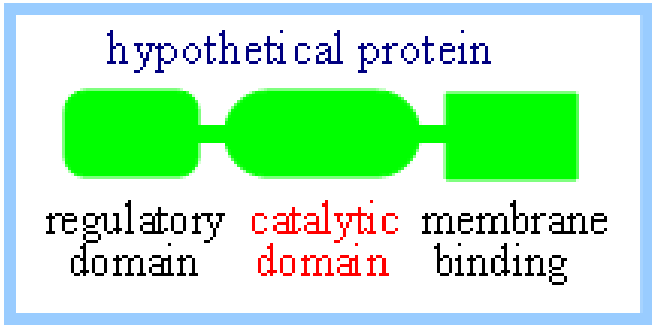
1. Domains can contain a motif. A domain could contain multiple motifs.
2. Domains are more stable, folded, complex and larger.
3. Domain is larger than motif. Motifs are short sequences and domains are longer ones. Domains vary in length from between about 25 amino acids up to 500 amino acids in length.
4. Motifs are structural characteristics and domains are functional parts of a protein(functional regions).
5. Both domains and motifs can be functional or just mere structural.

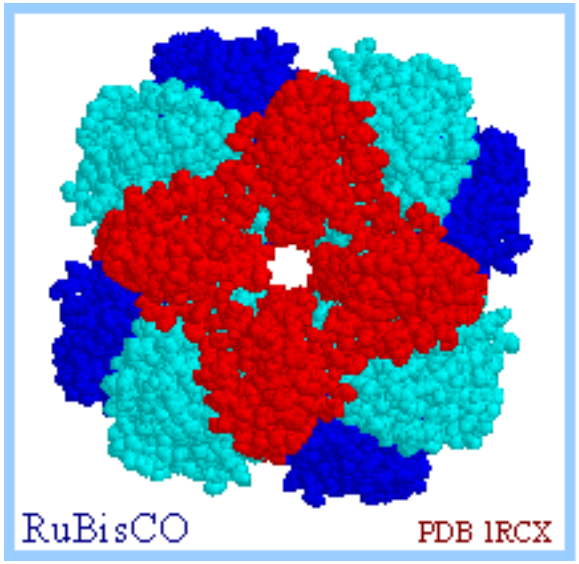
Protein Domains

Structurally independent folding units looking like separate
Ribulose bisphosphate carboxylase, a multimeric enzyme

- For example, an enzyme's primary structure may include a segment that folds to produce:

1. an active site with particular catalytic activity,
2. other segments that may mediate regulation of the enzyme or
3. binding of the enzyme to a membrane.





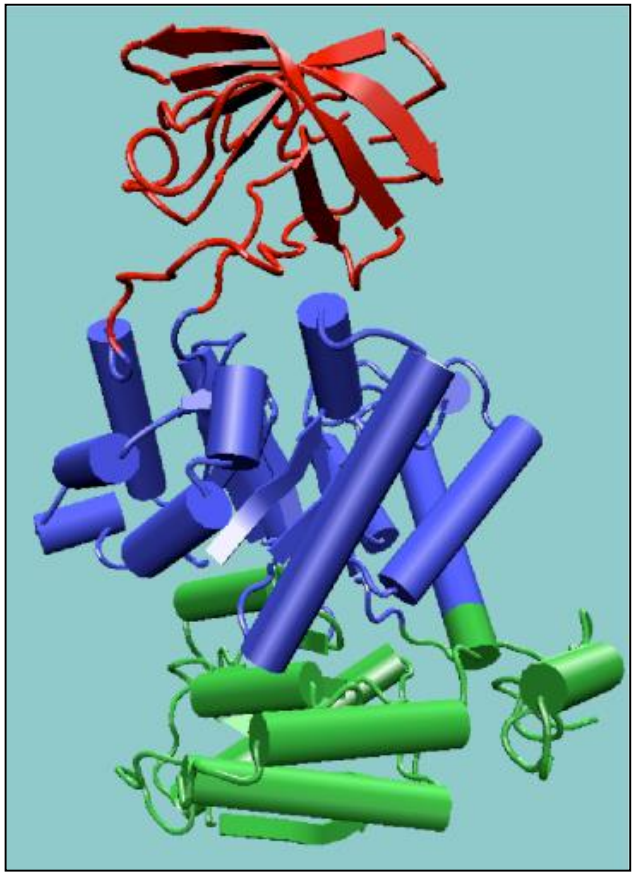
Diwan,2003

Protein Domains

Structurally independent folding units looking like separate Tertiary structure

- Domains are functional regions of the proteins.
- Four main classes of protein structures:

1. α-domains
2. β-domains (antiparallel β)
3. α/β domains
4. α+β domains



Pyruvate Kinase (1pkn) has three domains.

Stephen Sontum;..

Protein Domains

Membrane proteins

Tertiary structure

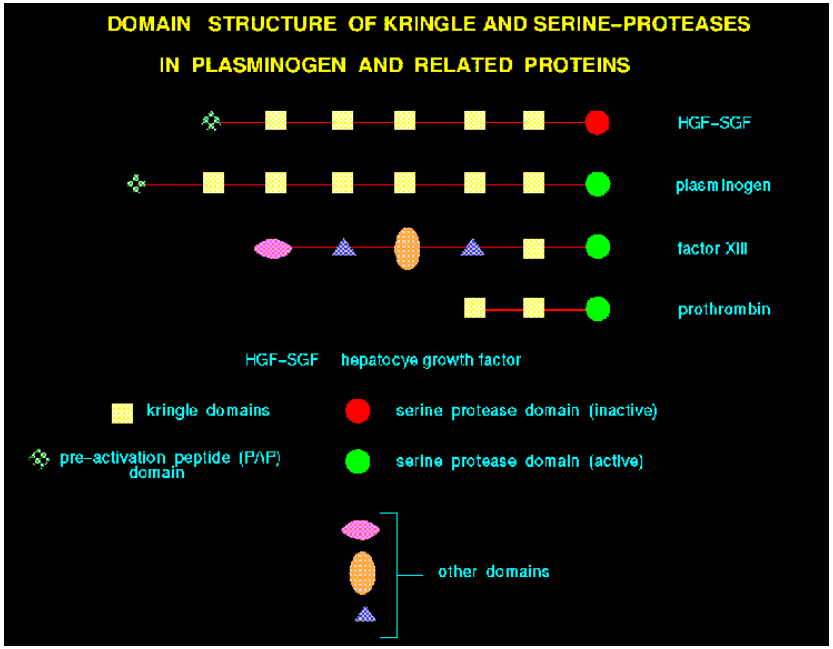
- Protein domains on extracellular surface: cell-cell signalling and interactions.
- Protein domains within membrane: move molecules across the membrane.
- Protein domains on the inner cell membrane: anchor proteins/intracellular signalling.

Protein Domains

Kringle=Danish pastry

Structurally independent folding units looking like separate

- Proteins can have one to many domains depending on protein size.
- e.g. Kringle Domains, proteins domains that fold into large loops stabilized by 3 disulfide linkages.
- The name of this structural proteins domains derives from its resemblance to Danish pastries known as kringlers.
- Generally, larger proteins (proteins with more than about 200 amino acid residues) are multi-domain (2 or more) folding domains.



Sowdhamin,1995

Determining protein structures

Three procedures commonly used to determine peptide structures

1. Circular dichroism spectroscopy
2. X-ray crystallography
3. Nuclear magnetic resonance spectroscopy
4. Prediction based on the protein sequence homology
5. Computer simulation

- X-ray crystallography: use diffraction pattern to obtain structure.
- Cryoelectron microscopy: low dose of electrons used to scan image.
- NMR: spacial relation of certain atoms.

Jim Bidlack,..

Determining protein structures

The procedures commonly used to determine peptide structures

- Protein structures can be determined experimentally (in most cases) by:

1. X-ray crystallography

- The interaction of x-rays with electrons arranged in a crystal can produce electron-density map, which can be interpreted to an atomic model. Crystal is very hard to grow.

2. Nuclear magnetic resonance (NMR)

- Some atomic nuclei have a magnetic spin. Probed the molecule by radio frequency and get the distances between atoms. Only applicable to small molecules. But this is very expensive and time-consuming can we predict structures by computational means instead?

Craven,2002; Kuang,2010

Levels of organization
Classification of protein structures

Primary structure

- Amino acid sequence of the protein.

Secondary structure

- Local folding patterns, mostly alpha helix and beta sheet.

Tertiary structure

- The overall folding pattern of the entire polypeptide.

Quaternary structure

- Interaction between 2 polypeptide chains.

Protein structure has a hierarchical nature

Hierarchical nature of protein structure

Primary structure (Amino acid sequence)

↓

Secondary structure (α-helix, β-sheet)

↓

Tertiary structure (Three-dimensional structure formed by assembly of secondary structures)

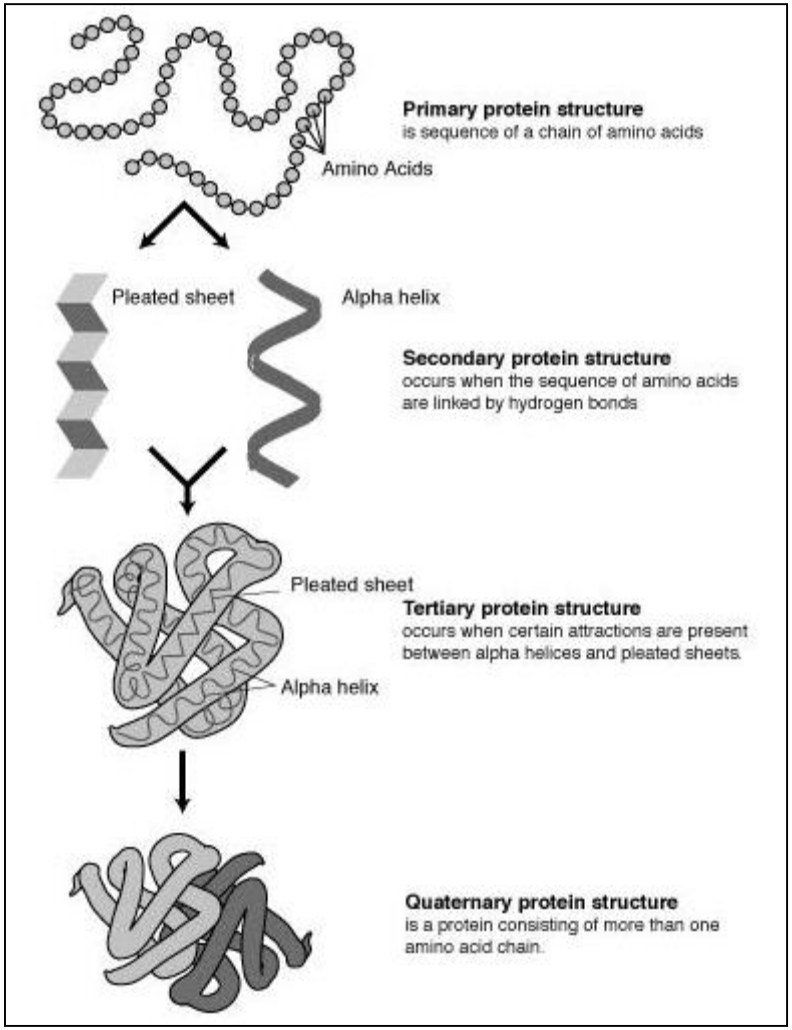
↓

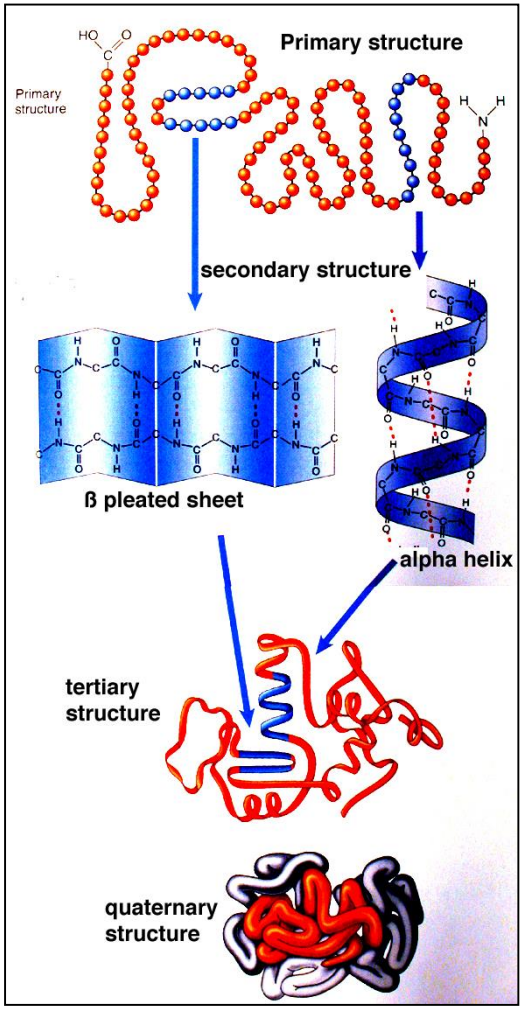
Quaternary structure (Structure formed by more than one polypeptide chains)

Tadashi Ando,2006

Levels of organization

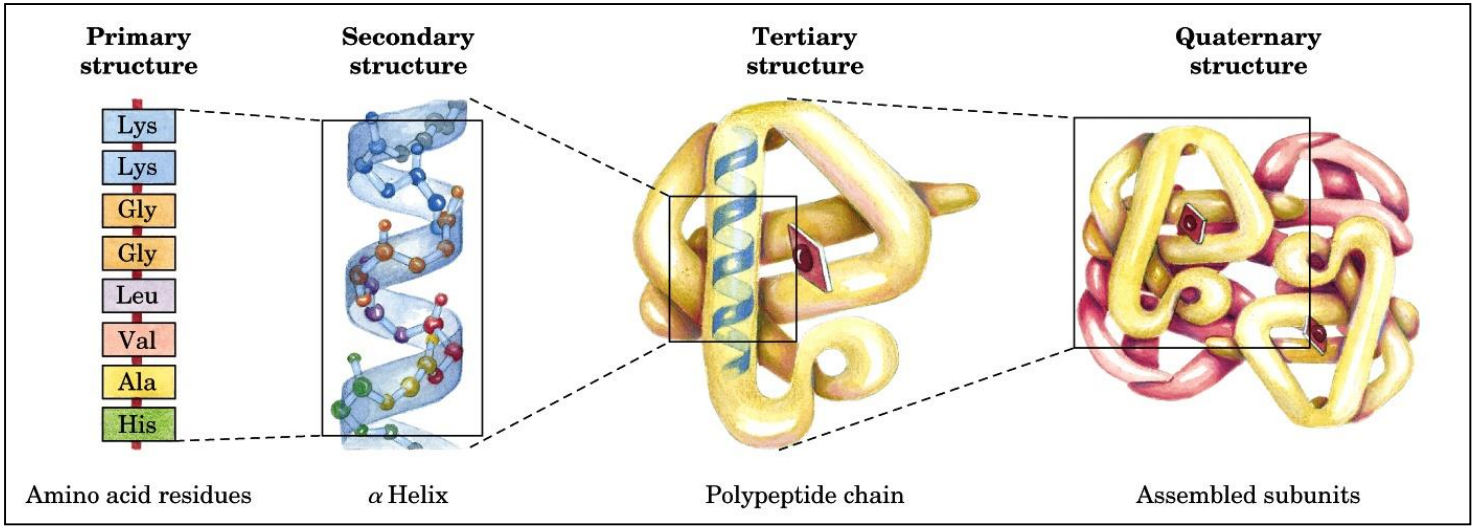
The four levels of protein structure





Levels of organization

The four levels of protein structure

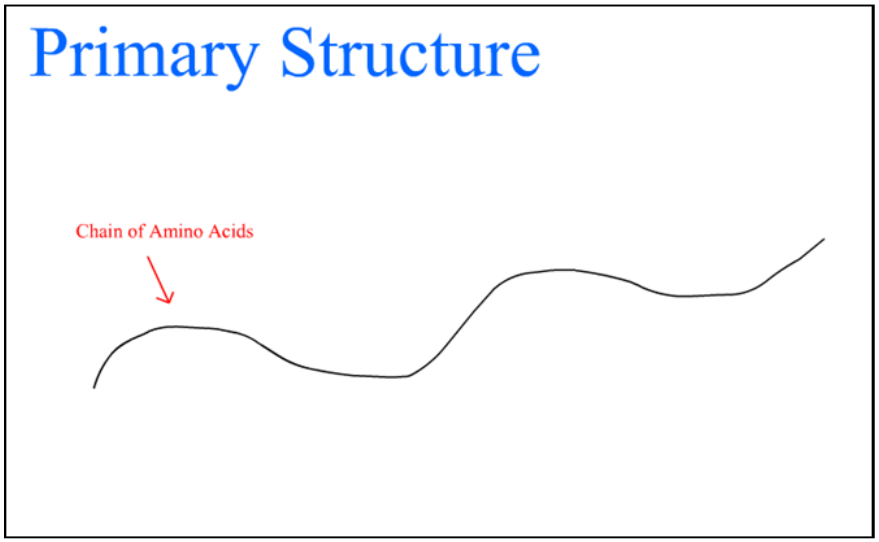


Siegal,2010

Protein synthesis
Primary structure

- The amino acid sequence is called as primary structure.

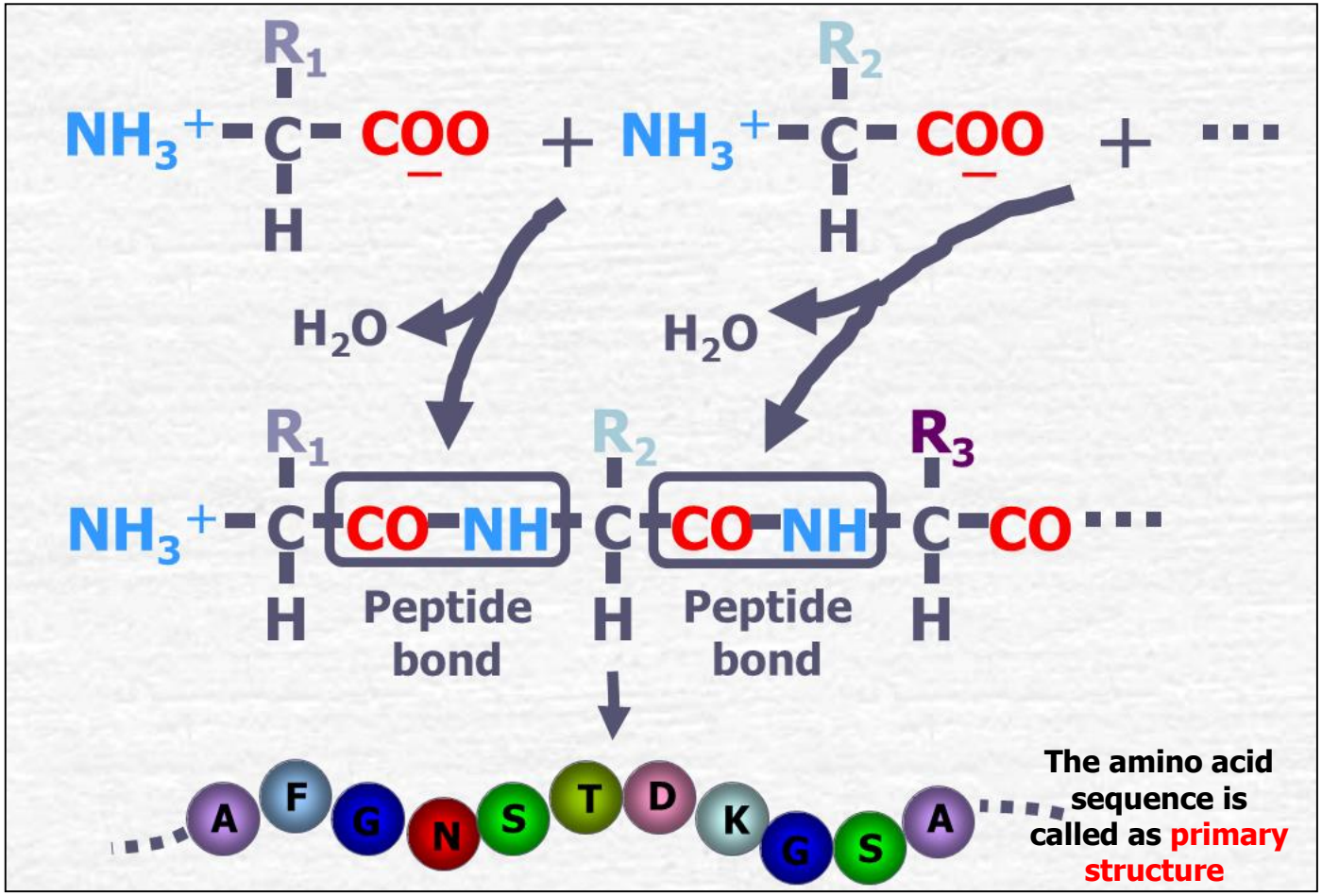
H_3N^+-A_1-A_2-A_3-A-A-A-A-A-A-A-A-A-A-A-A-A-A-A_{98}-A_{99}-A_{100}-COO^-



Protein synthesis

A polypeptide

Primary structure

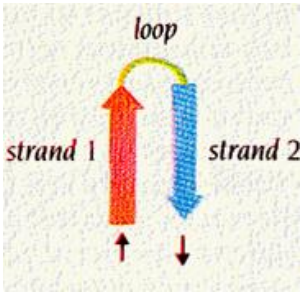


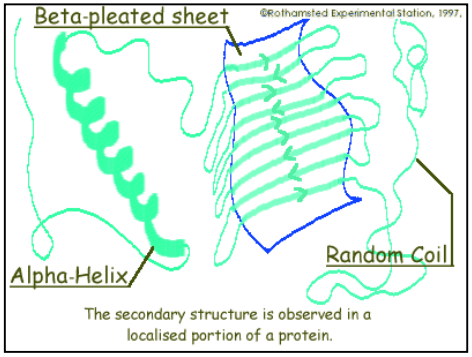
Tadashi Ando,2006

Protein structures

Secondary protein structure

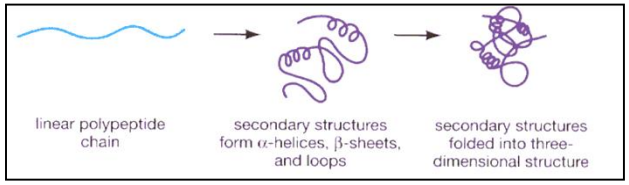
α-helix and β-sheet





- Common secondary structures are:
  1. α-helices
  2. β-strands, and
  3. Loops or turn random coil.
- This gives a protein functional properties such as flexibility and strength.
- About 60% of polypeptide chains exist as alpha helices and beta sheets, whereas the remaining include random coils and turns.

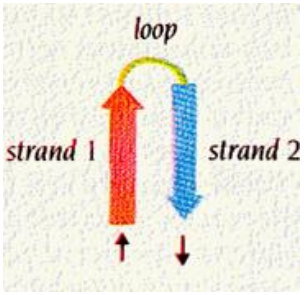
Loops, unlike alpha helices or beta sheets, are an irregular secondary structure in proteins. Loops may be considered a diverse class of secondary structures comprising turns, 'random coils', and strands which connect the main secondary structures (alpha helices and beta strands).



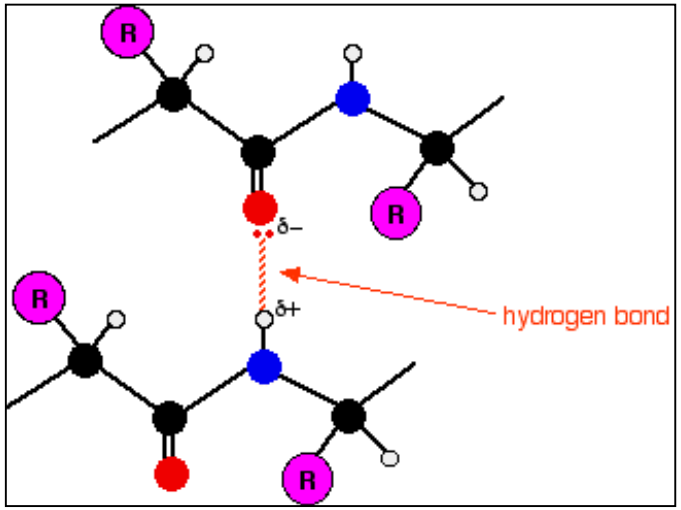
Protein structures

Secondary protein structure

α-helix and β-sheet



- These secondary structures are held together by hydrogen bonds.
- These form as shown in the diagram between one of the lone pairs on an oxygen atom and the hydrogen attached to a nitrogen atom.

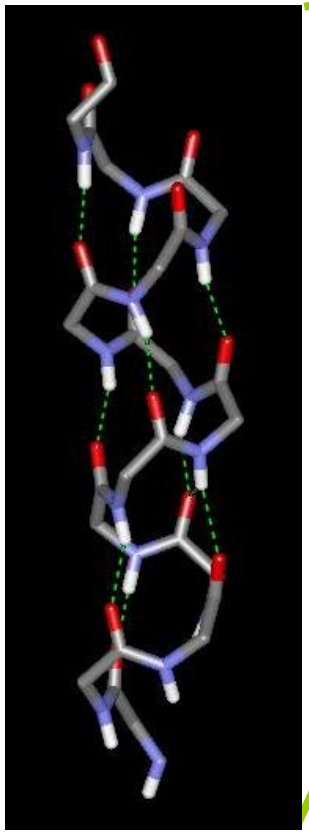


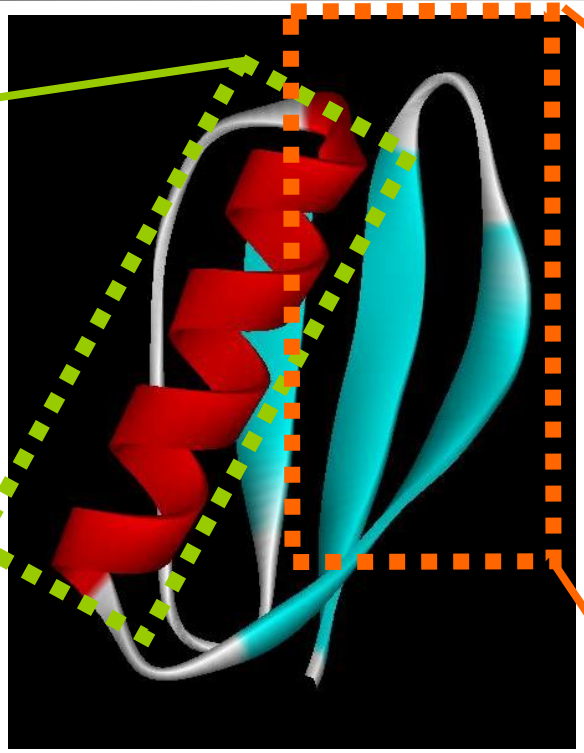
Clark,2004

Protein synthesis

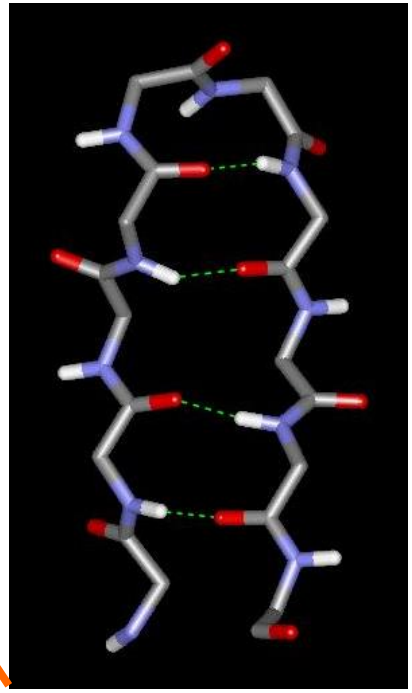
Basic structural units of proteins: Secondary structure

α-helix





β-sheet



Secondary structures, α-helix and β-sheet. H-bonds are responsible for stabilizing the secondary structure.

Tadashi Ando,2006;..

Protein secondary structure

Random coils

- Different kinds of turns in the chain are also classified as secondary structures.
- Random coils refer to folds of the protein usually form the binding and active sites of proteins.
- Random coil do not fit into a classification.
- About 50% of all protein structure comes under this category.

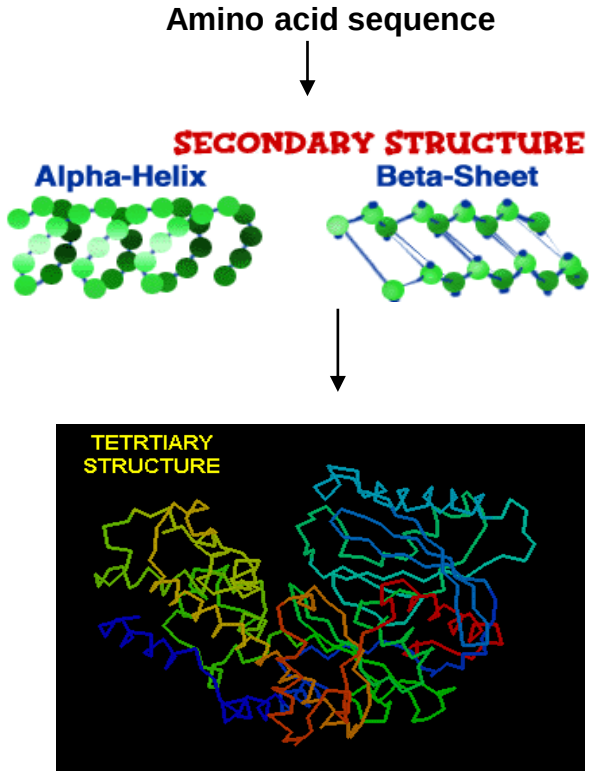
Binding site – where proteins interact with one another.

Protein synthesis
Tertiary structure

- The tertiary structure of a protein is a description of the way the whole chain (including the secondary structures) folds itself into its final 3-dimensional shape.
- The tertiary structures are formed in combinations of:

1. α helices,
2. β sheets, and
3. loops and turns.

- Amino acid sequence → secondary structure → tertiary structure



Protein synthesis

Tertiary structure

The final shape of a protein

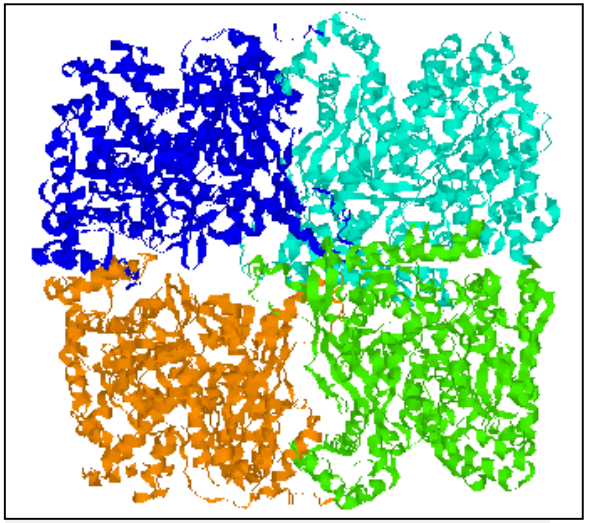
1. 3-D organization of a polypeptide chain.
2. Compacts proteins.
3. Interior is mostly devoid of water or charged groups.

Protein synthesis

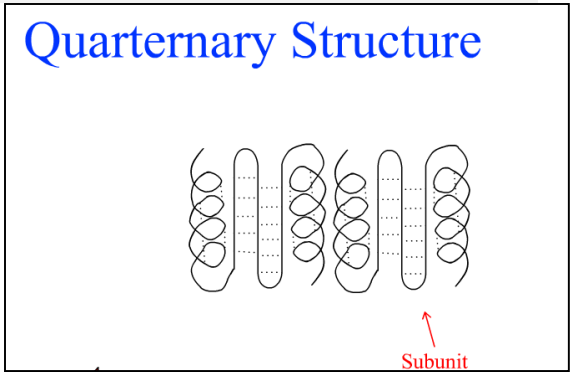
Quaternary structure

The highest level of organisation is the quaternary structure

- Quaternary structure is the combination of two or more polypeptide chains(subunits), to form a complete unit.
- **The interactions between the chains are not different from those in tertiary structure**, but are distinguished only by being **interchain rather than intrachain**.
- Subunit: Each polypeptide chain of large protein.



The four subunit arrangement



A dimer protein is made of two subunits

Protein synthesis

Quaternary structure

Made of two or multiple subunits

- Some proteins are composed of two folded subunits (dimer) or more polypeptide chains (multimer).
- It includes organizations from simple dimers to large homooligomers and complexes with defined or variable numbers of subunits.
- Quaternary structure from:

1. simple dimers
2. large homooligomers, and
3. complexes with defined or variable numbers of subunits.

Protein synthesis

Quaternary structure

Nomenclature of quaternary structures

- The number of subunits in an oligomeric complex is described using names that end in -mer (Greek for "part, subunit").
- Higher order complexes are usually described by the number of subunits, followed by -meric.

•1 = monomer/subunit •2 = dimer •3 = trimer •4 = tetramer •5 = petamer •6 = hexamer	•7 = heptamer •8 = octmer •9 = nonamer •10 = decamer •11 = undecamer •12 = dodecamer	•13 = tridecamer •14 = tetradecamer •15 = pentadecamer* •16 = hexadecamer •17 = heptadecamer* •18 = octadecamer	•19 = nonadecamer •20 = eicosamer •21-mer •22-mer •23-mer* •etc.

*No known examples

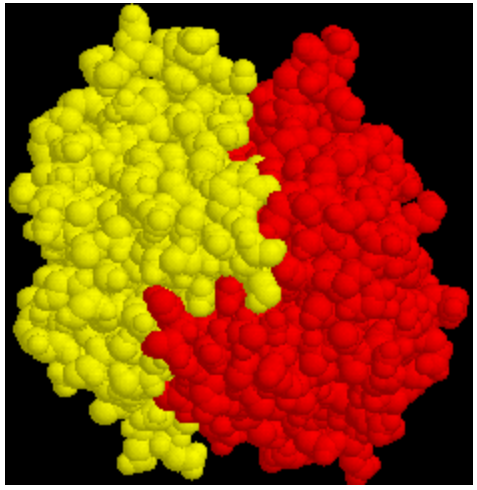
Wikipedia

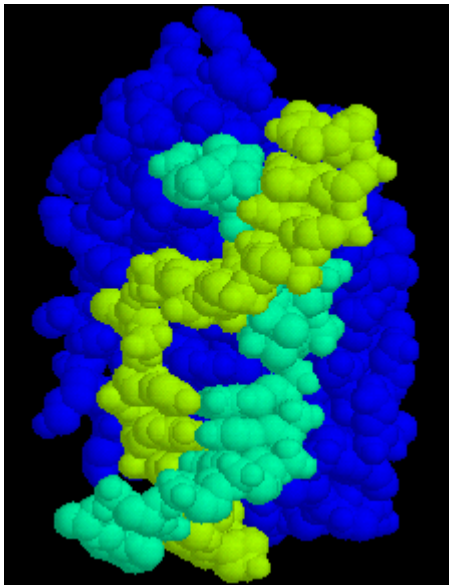
Protein synthesis

Quaternary structure

The highest level of organisation is the Quaternary structure

- The arrangement of separate molecules, such as in protein-protein or protein-nucleic acid interactions.
- Some proteins work as multi-complex machines and have to undergo a quaternary level of folding.





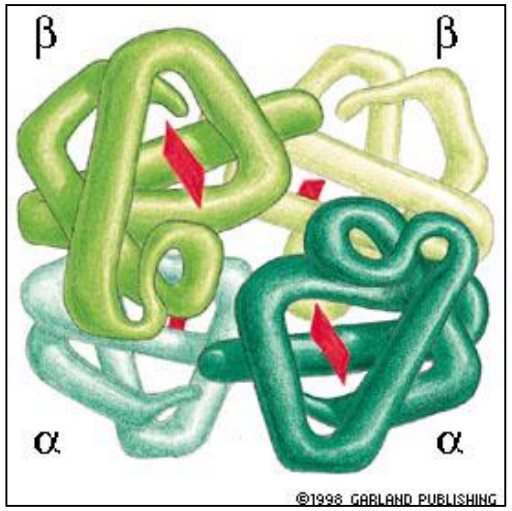
Protein quaternary structure

Multi subunit proteins

The final shape of a protein

- If a protein consists of more than one chain polypeptide chains, the shape is referred to as the quaternary structure.
- The quaternary structure is the final shape of a protein.
- It involves the combination of multiple chains of amino acids, along with their:

1. alpha helices,
2. beta sheets,
3. hydrogen bonding,
4. ionic bonding,
5. disulfide bridges, and
6. hydrophobic/hydrophilic interactions.

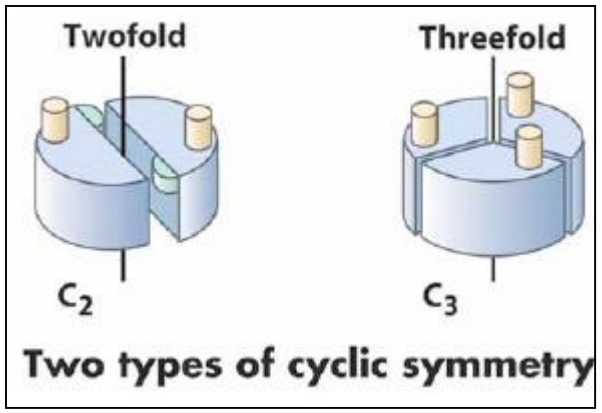


Hemoglobin tetramer:
2 α globin subunits
2 β globin subunits

Protein quaternary structure

Arrangement of multiple folded protein or coiling protein molecules in a multi-subunit complex

- The structure formed by several protein molecules (polypeptide chains), usually called protein subunits in this context, which function as a single protein complex.
- Arrangement of polypeptides in multimeric proteins is generally symmetrical.
- Quaternary structure can play important functional roles for multi subunit proteins, especially in regulation.



Ziegler,2008

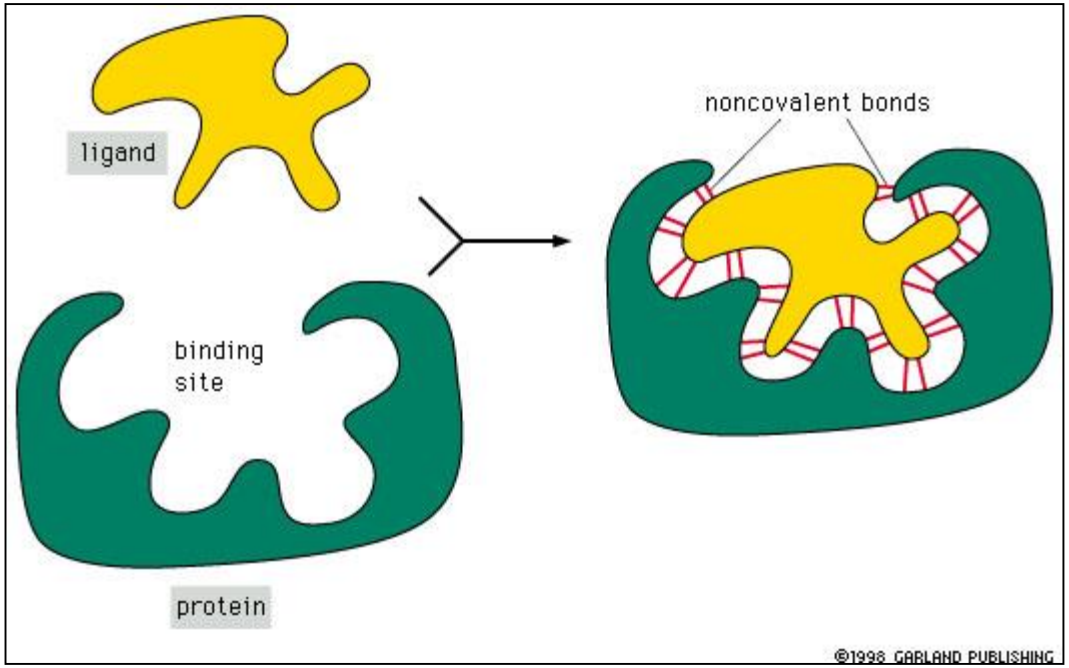
Protein quaternary structure
Terminology

- Each polypeptide chain in a multi chain protein (large proteins) is called a subunit.
- A dimer protein: Protein made of 2 subunits. Can be same subunit or different subunits.
- A trimeric protein: Protein made of 3 subunits.
- A tetrameric protein: Protein made of 4 subunits.
- Some proteins produce protein complexes of homo- and hetero-nature:
- **homo**(dimer or trimer etc.): identical subunits.
- **hetero**(dimer or trimer etc.): more than one kind of subunit (chains with different amino acid sequences).
- Different subunits designated with Greek letters. e.g., subunits of a heterodimeric protein= the "α subunit" and the "β subunit".

Ziegler,2008

Protein quaternary structure
Terminology

- Ligand: The molecule that a protein can bind.
- Binding site: Part of the protein that interacts with the ligand. It consists of a cavity formed by a specific arrangement of amino acids.



Structural classification of proteins
Homology comparison

- Hand-curated hierarchical taxonomy of proteins based on:

1. Their structural, and
2. Evolutionary relationships.

Class

Fold Level

Superfamily

Family

Root: scop

Classes:

1. All alpha proteins [46456] (218)
2. All beta proteins [48724] (144)
3. Alpha and beta proteins (a/b) [51349] (136)
   Mainly parallel beta sheets (beta-alpha-beta units)
4. Alpha and beta proteins (a+b) [53931] (279)
   Mainly antiparallel beta sheets (segregated alpha and beta regions)
5. Multi-domain proteins (alpha and beta) [56572] (46)
   Folds consisting of two or more domains belonging to different classes
6. Membrane and cell surface proteins and peptides [56835] (47)
   Does not include proteins in the immune system
7. Small proteins [56992] (75)
   Usually dominated by metal ligand, heme, and/or disulfide bridges
8. Coiled coil proteins [57942] (6)
   Not a true class
9. Low resolution protein structures [58117] (24)
   Not a true class
10. Peptides [58231] (116)
    Peptides and fragments. Not a true class
11. Designed proteins [58788] (42)
    Experimental structures of proteins with essentially non-natural sequen

hand-curated, meaning I find them by hand and add them by hand

Chothia, Murzin

Structural classification of proteins

Homology comparison

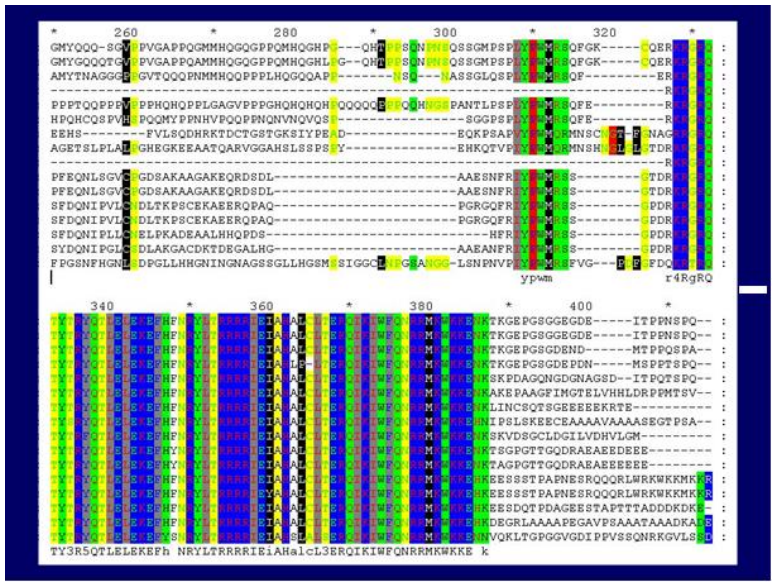
Protein families

1. Have similarities in amino acid sequence and 3-D structure.
2. Have similar functions such as breakdown proteins but do it differently.

Alignment of amino acid sequences is a very useful tool for evolutionary studies and provide more information than structure does

Homology comparison

- Proteins from different species have similar but not identical sequences. This fact implies that they have similar but not identical protein structures
- The pattern of variation at the amino acid level give clues of the selective constraints operating in the sequence or even in the protein structure.



Alignment of DNA and amino acid sequences

Nucleotide sequence alignment vs. Protein alignment

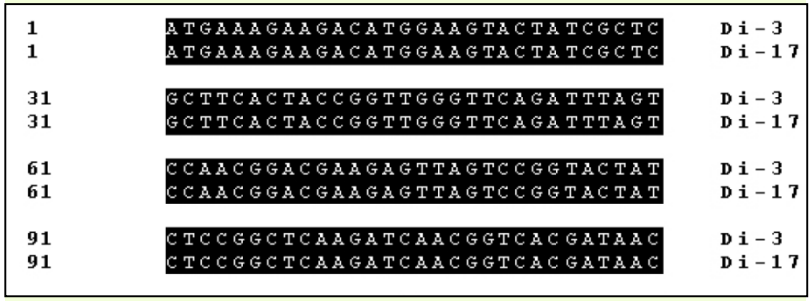
- These amino acid differences could potentially create a significant change or changes in the protein, including protein structure and overall protein charge.
- There are many on-line programs available to test these possibilities.
- These programs are capable of converting nucleic acid sequences (DNA) to amino acid sequences (protein),

1. Calculating the predicted secondary structure of proteins from the amino acid sequences,
2. Calculating protein hydrophobicity, etc.

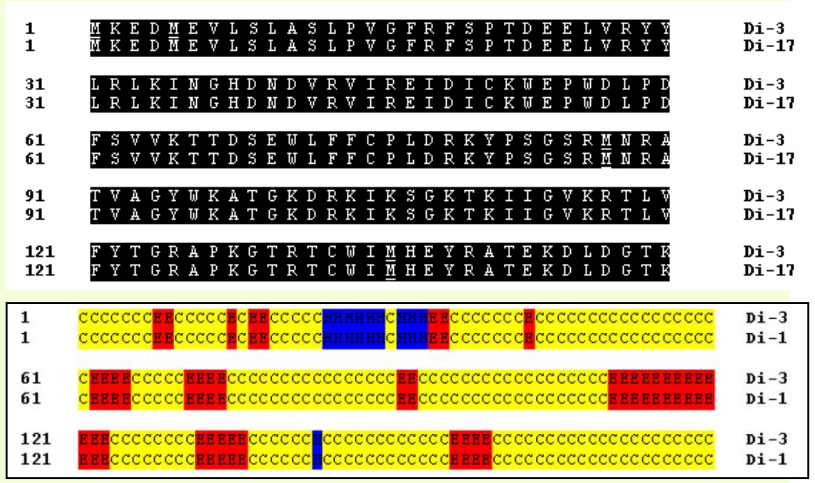
Alignment of DNA and amino acid sequences

Nucleotide sequence alignment vs. Protein alignment

- Nucleotide sequence alignment



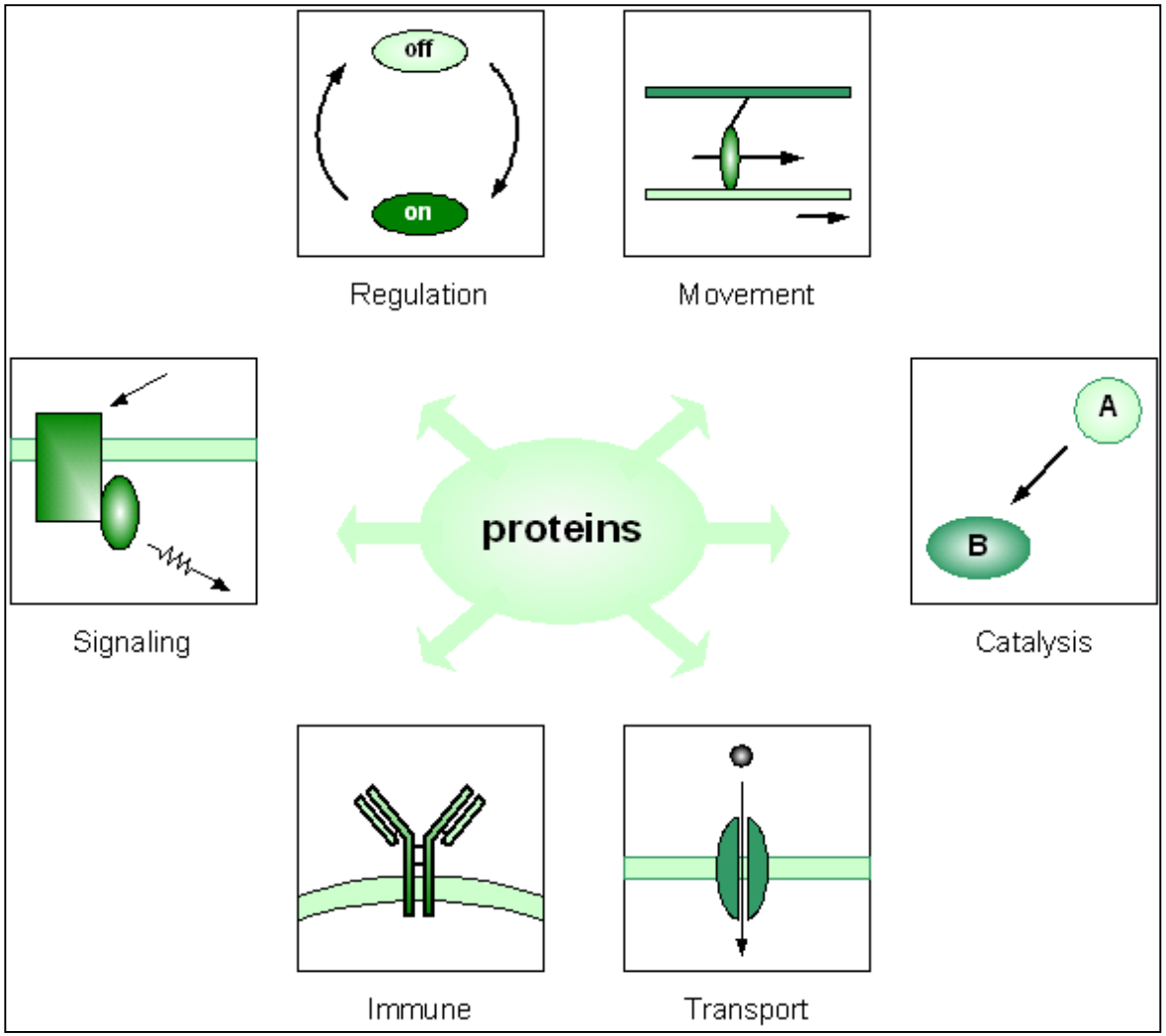
- Protein alignment



The predicted secondary structure of the resistant proteins (Di) is depicted in colored figure.
C corresponds to coils, **E** corresponds to beta-strands, and **H** corresponds to alpha-helices.
Areas in **bold** (unshaded areas) denote a lack of consensus between the two sequences.

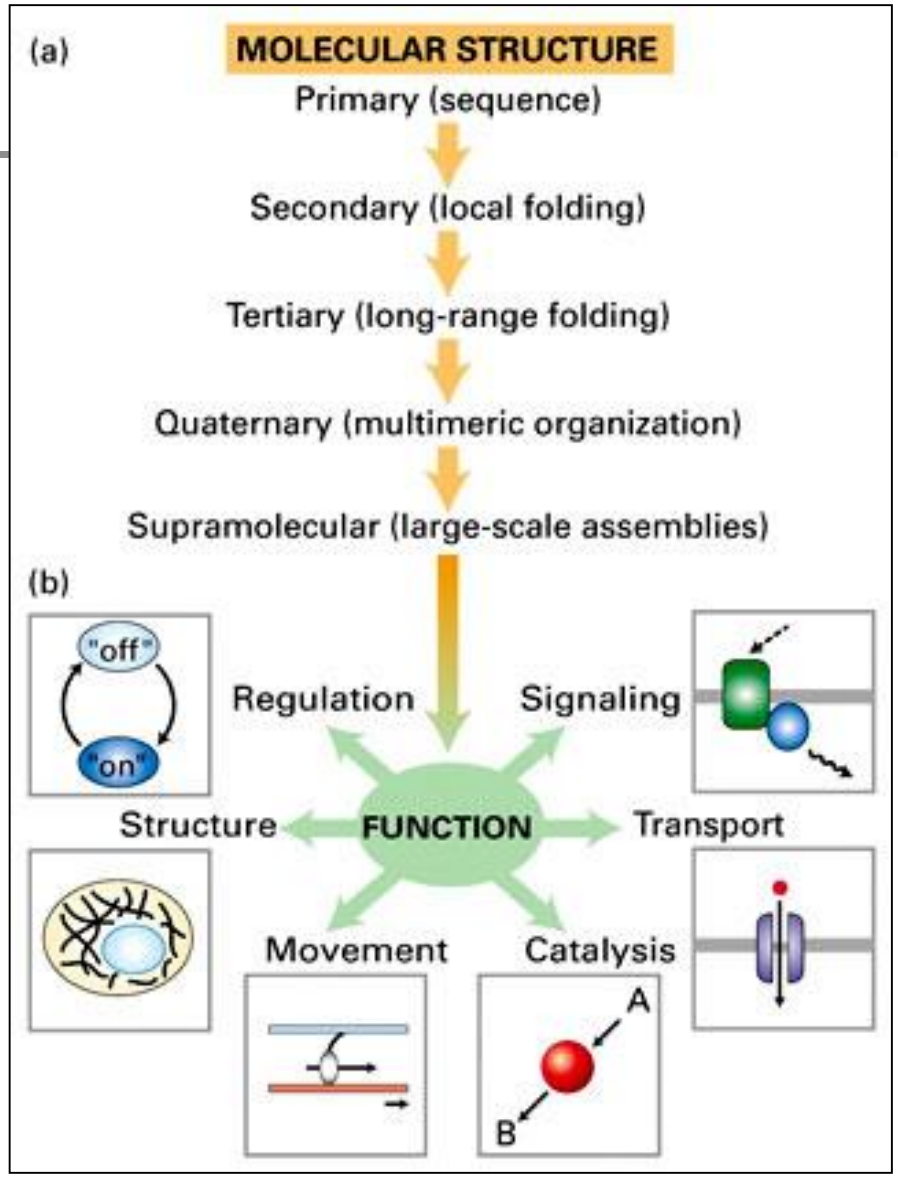
What do proteins do?

The most diversified biological functions



Biological functions of proteins

Protein structure is closely related to its function.



The proteins
Functions

- Proteins have a number of biologically important roles in the cell.
- They include:

1. Enzymes
2. Structural
3. Transport
4. Motor
5. Storage
6. Signaling: This group of proteins is involved into signalling translation process.
7. Receptors: These proteins are responsible for signal detection and translation into other type of signal.
8. Gene regulation
9. Special functions.

The proteins
Functions

1. Cell motility: Proteins link together to form filaments(e.g. flagella) which make movement possible.
2. Structural proteins: Actin fibers in muscles and cytoskeleton– is made from thousands of actin molecules as a helical fiber (actin-like in bacteria).
3. Membrane proteins: Outer membrane proteins (OMPs) in Gram-negative bacteria, protein channels, gap junctions.
4. Organic catalysts in biochemical reactions: Enzymes are responsible for catalyzing the thousands of chemical reactions of the living cell.
5. Transport and storage: Hemoglobin and iron storage proteins(ferritins).

The proteins
Functions

6. Regulatory proteins: Hormones, transcription factors. Protein hormones which regulate metabolism.
7. Protein machines: Complexes of 10 or more proteins that work together such as DNA replication, RNA or protein synthesis, trans-membrane signaling. Ribosome, the molecular machine that translates RNA into proteins.
8. Defense against pathogens: Poisons/toxins, PR proteins, antibodies, complement inhibitory proteins. Antibodies which are molecules of the immune system.
9. Chaperones: Participate in a large variety of cellular functions. They assist in protein folding, stabilize proteins under stress conditions and maintain polypeptide chain components in a loosely folded state for translocation across organelle membranes.

The proteins
Functions

- **Signal transducting proteins:**
- G proteins
- MAP kinase
- Olfactory receptor(proteins capable of binding odour molecules that plays a central role in the sense of smell (olfaction). Responsible for the detection of odor molecules form insects,…)
- Receptor tyrosine/threonine-serine kinases

Proteins classification based upon chemical composition

Simple and conjugated proteins

- Based upon chemical composition, proteins are divided into two major classes:

1. Simple proteins, which are composed of only amino acids.
2. Conjugated (complex) proteins, which are composed of amino acids and additional organic and inorganic groupings.

- Conjugated proteins include:
  - Glycoproteins, which contain carbohydrates.
  - Lipoproteins, which contain lipids.
  - Nucleoproteins, which contain nucleic acids.

Proteins classification based upon chemical composition

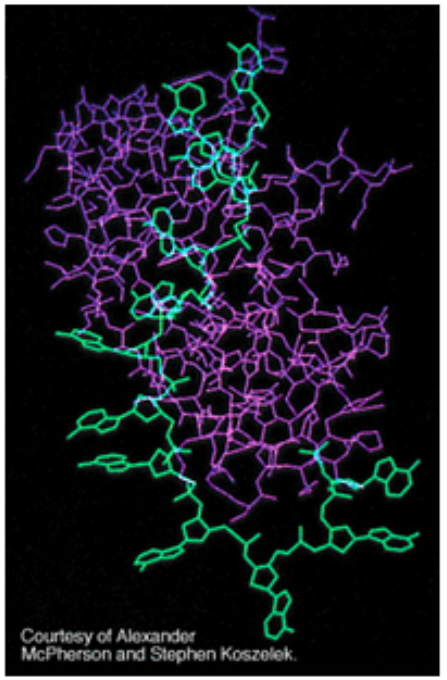
Conjugated (Complex) proteins

Complex Proteins:

lipoproteins - (+ lipids)
blood, membrane, & transport proteins

glycoproteins - (+ carbohydrates)
antibodies, cell surface proteins

nucleoproteins - (+ nucleic acids)
ribosomes & organelles



Common terminology:

dipeptide = 2 amino acids tripeptide = 3 amino acids
peptide = short chain of amino acids (20-30)
polypeptide = many amino acids (up to 4,000)
protein = polypeptide with well defined 3D structure

Structural plant proteins

The plant cell walls

- Proteins, which may have structural or enzymatic functions, can account for as much as 15 per cent of the cell wall.
- These are different from pathogenesis-related proteins.
- Most of those that have been characterized are from dicotyledonous species.
- They consist of extensins, which are:
- Hydroxyproline-rich glycoproteins (HRGPs);
- Glycine-rich proteins (GRPs);
- Proline-rich proteins (PRPs);
- Arabino-galactan proteins (AGPs), and
- Solanaceous lectins.
- Variants of HRGPs are found in monocotyledonous species, which contain threonine and histidine hydroxyproline-rich glycoproteins (THRGPs and HHRGPs, respectively).

Structural bacterial proteins
Cell wall and other cell structures

1. About half of the dry mass of a bacterial cell consists of carbon, and also
2. About half of it can be attributed to proteins.

- The bacterial cell consists mostly of water.

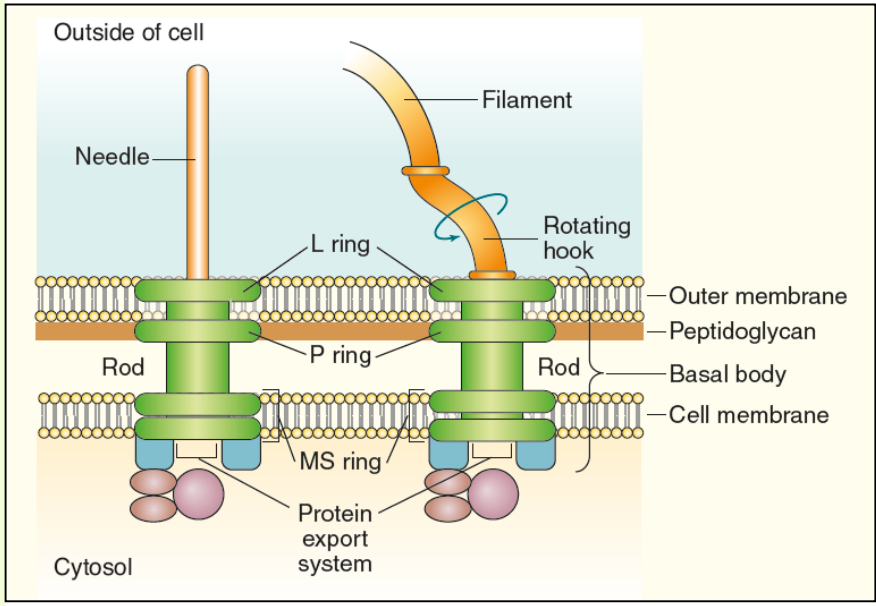
1. Wet mass of the cell is ca. 1 pg.
2. The dry mass of a single cell can be estimated as 20% of the wet mass, amounting to 0.2 pg.

Structural bacterial proteins

Flagellum structure

Divergence of quaternary structures among bacterial flagellar filaments

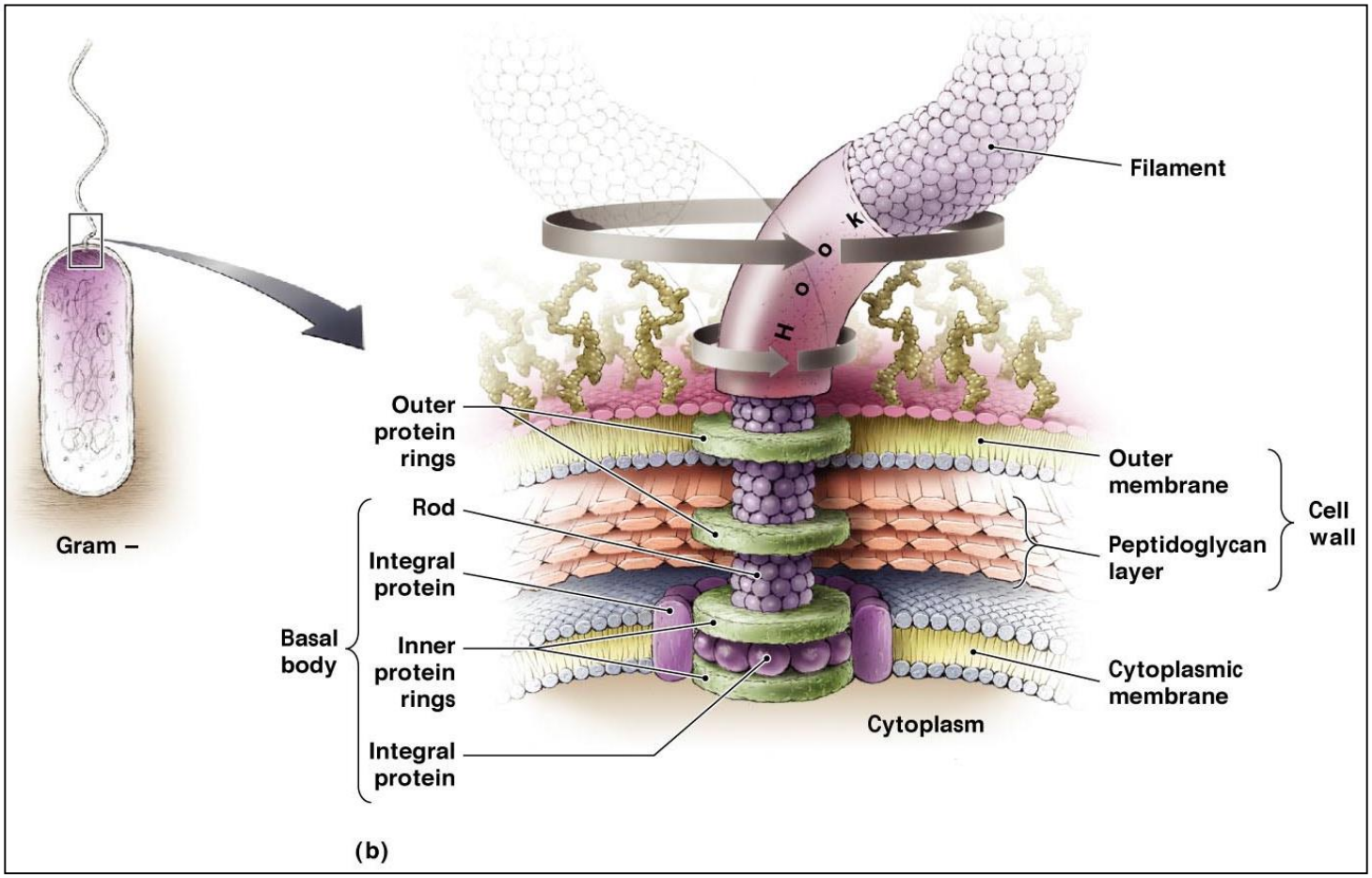
- Composed of three parts:
- Filament: Flagella are mostly composed of flagellin (a protein) that is bound in long chains and wraps around itself in a left handed helix.
- The number of units, the wavelength and diameter of a single helix of the flagella are determined by the protein subunits.
- Hook: Transition between filament and motor.
- Basal body: Anchor in cell wall and motor.



Jones and Bartlett publishers;..

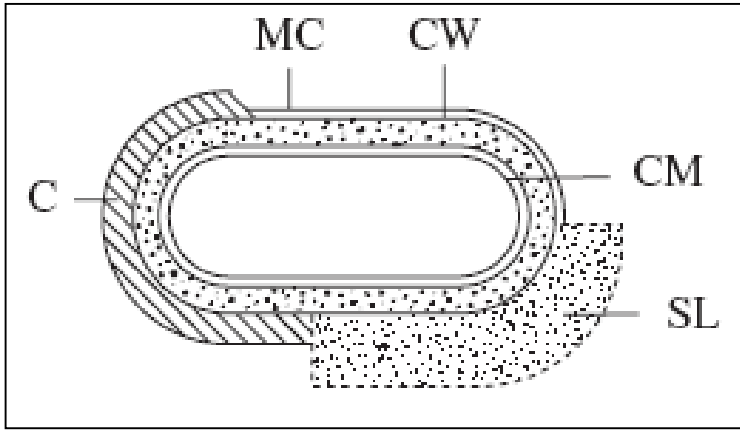
Bacterial flagella structure

Proteins



Structural bacterial proteins
Capsule & S-Layers

- Diagram of the surface structure of a prokaryotic cell showing the capsule and slime layer.
- C, capsule (made up of mainly EPS);
- CM, cytoplasmic membrane;
- CW, cell wall;
- MC, micro-capsule;
- SL, slime layer (made up of glycoproteins).
- The S-Layer is directly attached to the outer membrane, rather than the peptidoglycan.



Kim and Gadd 2008

S-Layer

A surface protein layer

Glycoproteins

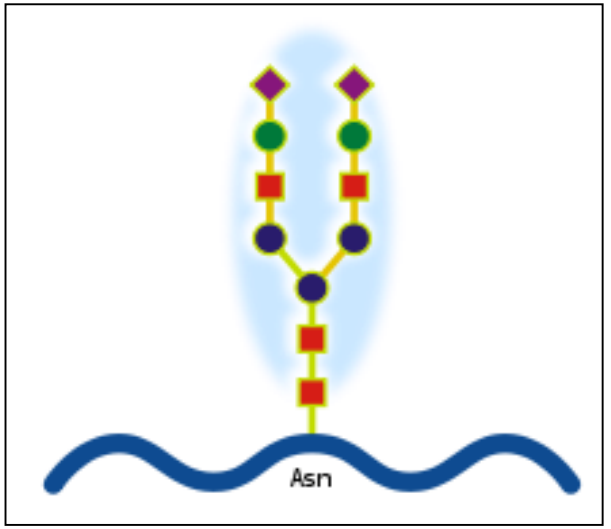
- Glycoproteins are ubiquitous in nature, although they are relatively rare in bacteria.
- In addition to forming these s-layers, glycoproteins also function as bacterial flagella.
- These are made up of bundles of glycoproteins protruding from the cell's surface.
- Their rotation provides propulsion.
- In plants, glycoproteins have roles in cell wall formation, tissue differentiation, embryogenesis, and sexual adhesion (certain algal species).

Glycoproteins

Glycoproteins are proteins with attached sugars

Occurs in eukaryotes and widely in archeae, but very rarely in bacteria

- Glycoprotein is a compound in which carbohydrate (sugar) is covalently linked to protein.
- *N*-linked glycosylation is the attachment of a sugar molecule (a process known as glycosylation) to a nitrogen atom in an amino acid residue in a protein.
- N-linked protein glycosylation at Asn(Asparagine) residues is Asn-any AA- Ser or Thr.
- The middle amino acid (X) can not be proline (Pro).



Bacterial capsules

Structural bacterial proteins

Chemical composition of some bacterial capsules

Bacterium	Capsule composition	Structural subunits
Gram-positive Bacteria		
Bacillus anthracis	polypeptide	D-glutamic acid
Bacillus megaterium	polypeptide and polysaccharide	D-glutamic acid, amino sugars, sugars
Gram-negative Bacteria		
Pseudomonas aeruginosa	polysaccharide	mannuronic acid
Agrobacterium tumefaciens	polysaccharide	(glucan) glucose

Composed usually of polysaccharide (dextran and xanthan gums are derived from these), or sometimes simple amino acid repeats, often with D-amino acids.

Bacterial cell wall structures

Bacterial cell wall proteins

Gram-positive (a) and Gram-negative (b) cell walls

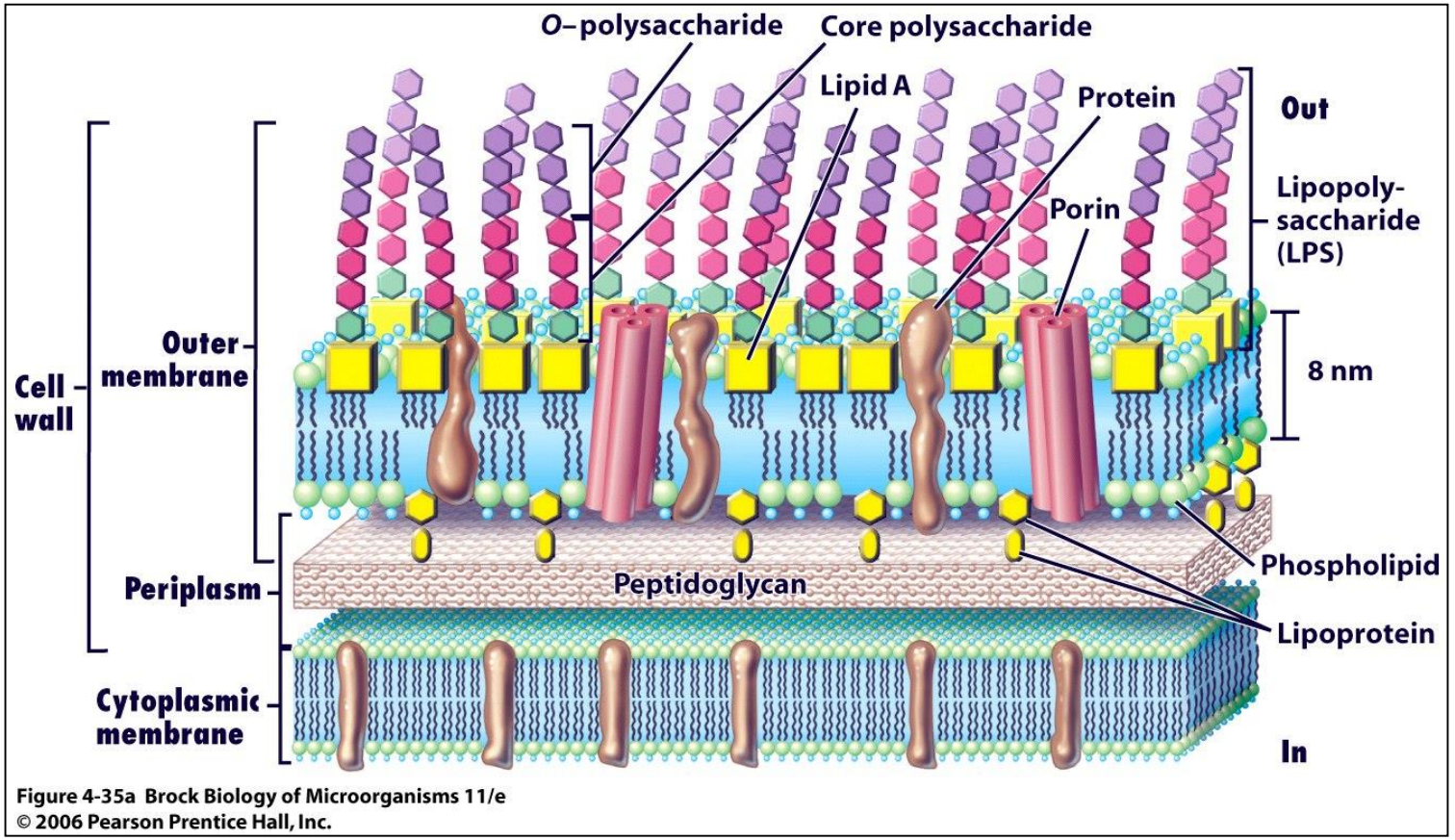


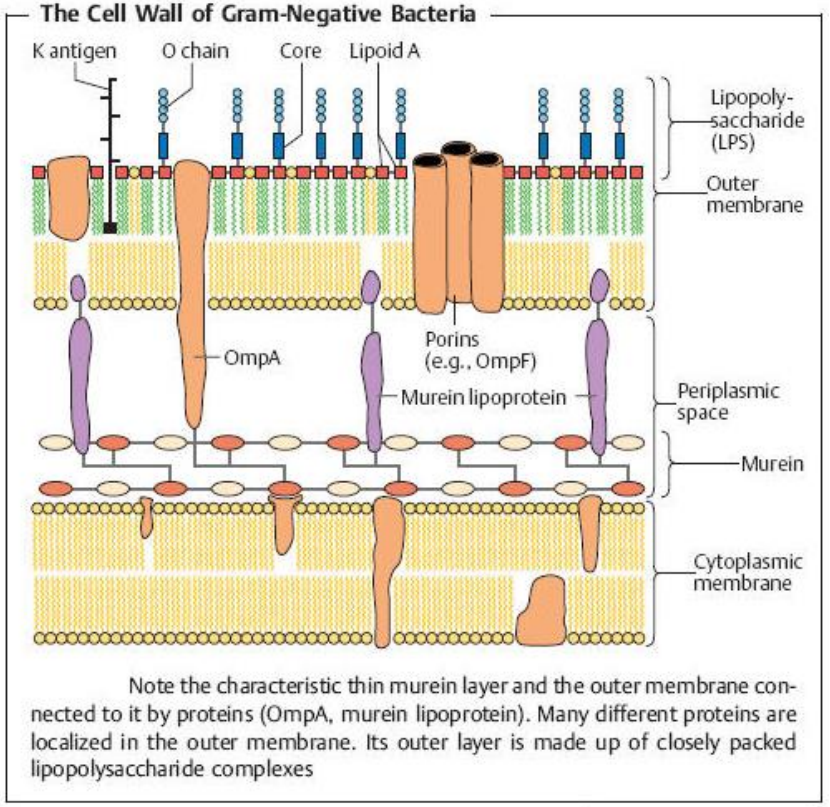
Figure 4-35a Brock Biology of Microorganisms 11/e

Bacterial cell wall composition

Gram-negative (b) cell wall

Porins and lipoproteins

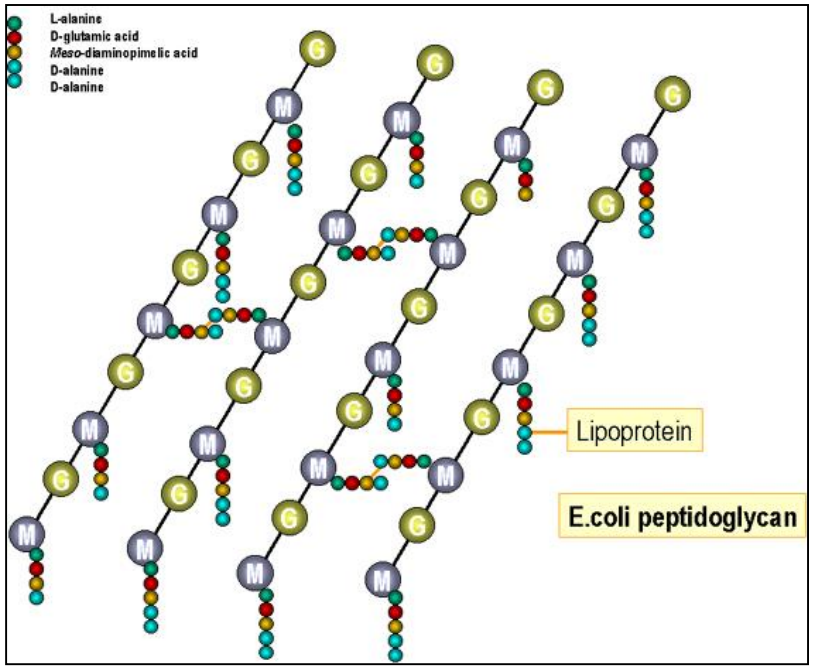
- **Braun lipoprotein:**
- Bacterial lipoproteins having a lipid-modified cysteine at the N-terminus are important components of the cell envelope and responsible for various cellular activities.
- **OmpC and OmpF porins:**
- Proteins that form pores or channels through outer membrane for passage of useful molecules (nutrients) but not harmful substances from the environment.
- **OmpA protein:**
- Provides receptor for some viruses and bacteriocins; stabilizes mating cells during conjugation.

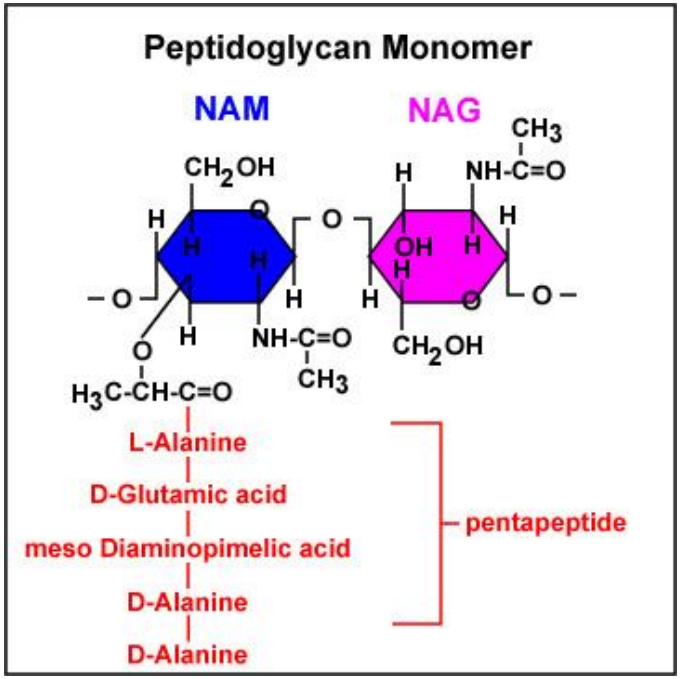


Peptidoglycan monomer

Gram-ve cell

Lipoproteins

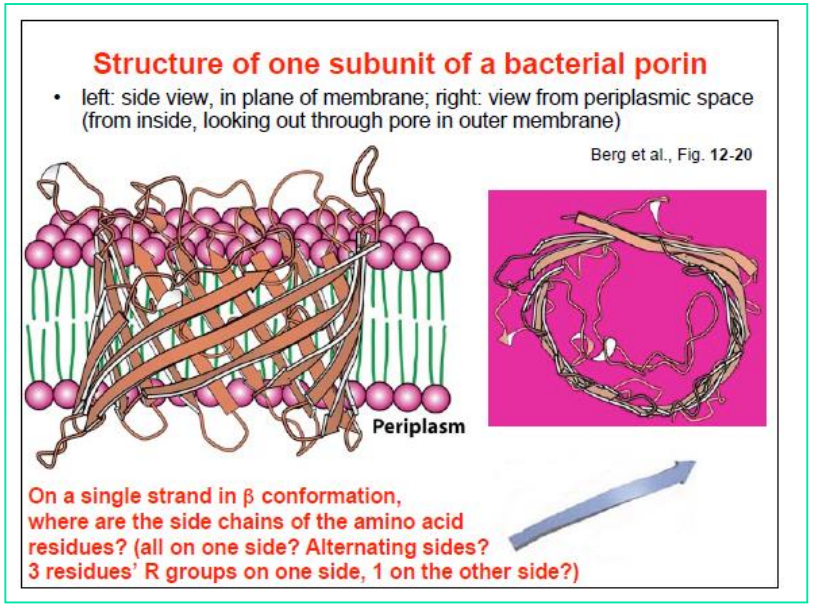




Structural bacterial proteins

Structure of one subunit of a bacterial porin

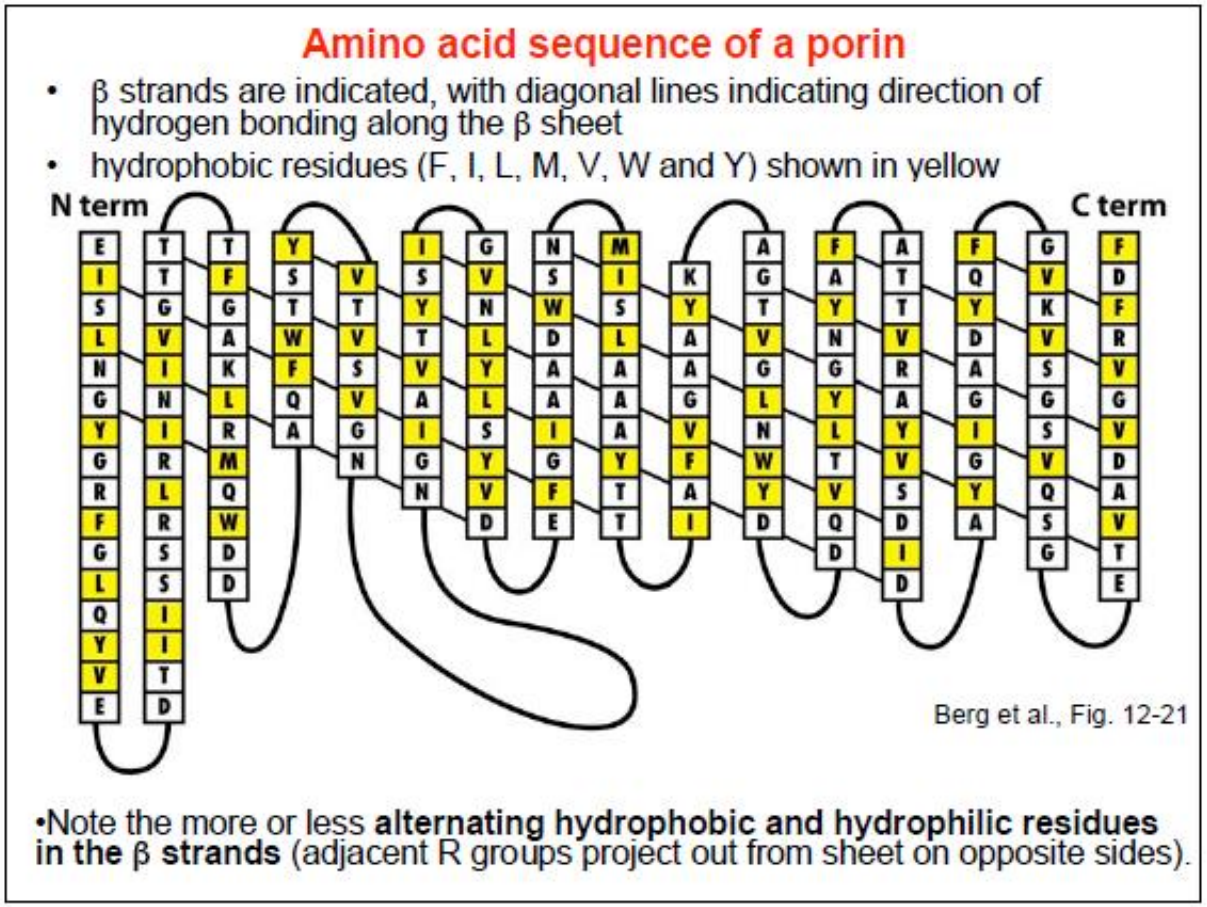
- Porins exist in the outer membrane, which act like pores for particular molecules.
- High permeability to nutrients, wastes, solutes, ions, and antibiotics.
- So it is a route of antibiotic influx.
- Antibiotic resistance caused by loss or modification of porins. e.g. losing of OmpC.



Ziegler,2008;..

Structure of one subunit of a bacterial porin

Amino acid sequence of a porin



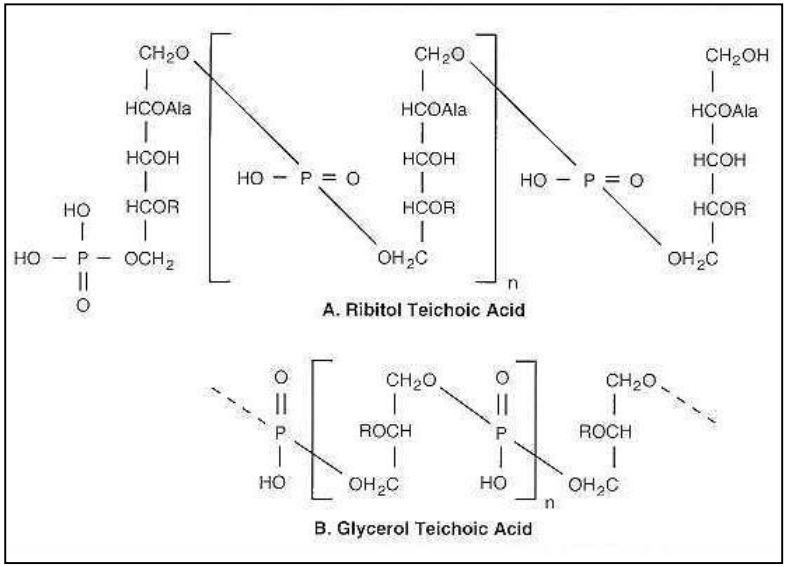
Ziegler,2008

Teichoic acids

- They consist of phosphate-linked backbones of sugar alcohol residues, to which are attached various sugars and D-alanine residues.
- The sugar alcohol may be:

1. Glycerol(glycerol teichoic acids) or
2. Ribitol (ribitol teichoic acids).

- The first type occurs in both the cell wall and cell membrane, the second only in the cell wall.



Sugar alcohol is neither a "sugar" nor an "alcohol"

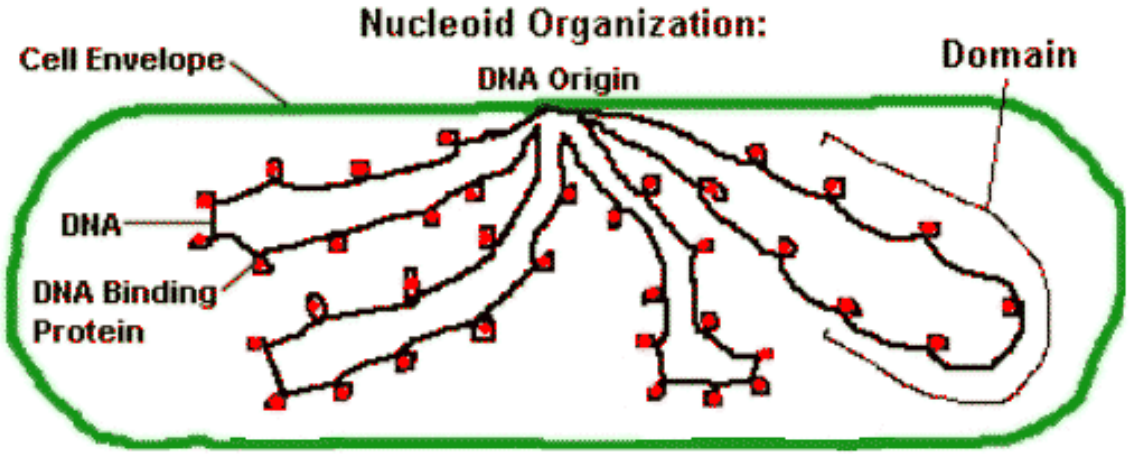
Teichoic acids
Function

- The main function of teichoic acids is to provide rigidity to the cell-wall by attracting cations such as magnesium and sodium.
- Teichoic acids also assist in regulation of cell growth by limiting the ability of autolysins to break the β(1-4) bond between the *N*-acetyl glucosamine and the *N*-acetylmuramic acid.
- Teichoic acids serve as an attachment site for some parasites and bacteriophages.

Bacterial DNA

Structural bacterial proteins

- Bacterial chromosome is not enclosed inside of a membrane-bound nucleus but confined to in an area referred to as the nucleoid.
- It is attached to the cell wall(plasma membrane).
- Though it isn't bounded by a membrane, it is visibly distinct (by transmission microscopy) from the rest of the cell interior.
- The DNA is not free but associated with many proteins.



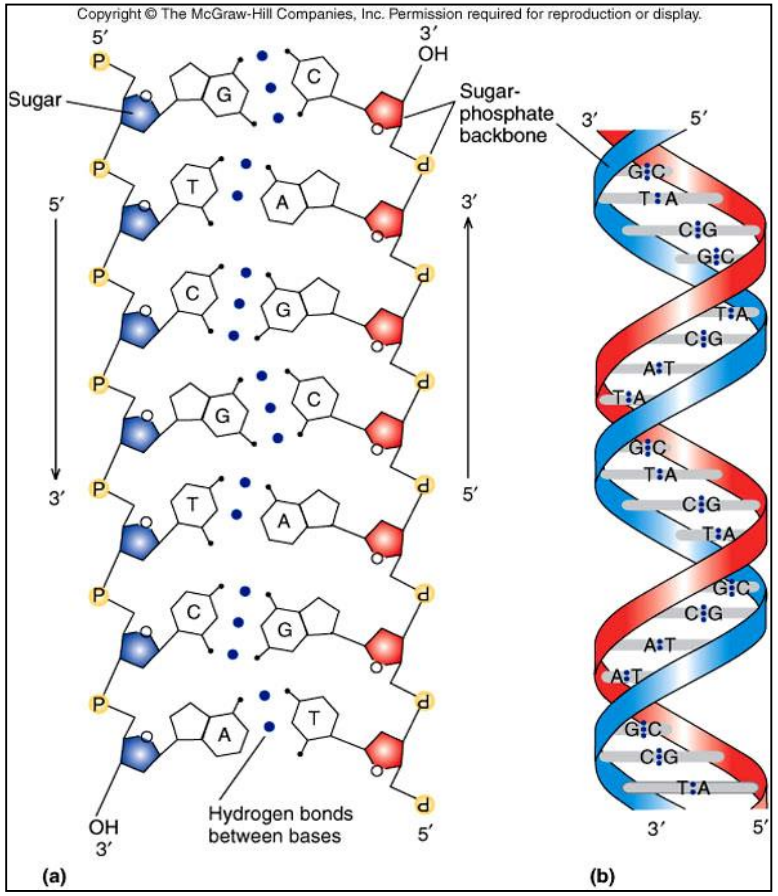
Bacterial DNA

Structural bacterial proteins

- Nucleotides consist of:

1. A five-carbon sugar ribose (RNA) or deoxyribose (DNA);
2. A nitrogenous base(ring structure)
3. One or more phosphates.

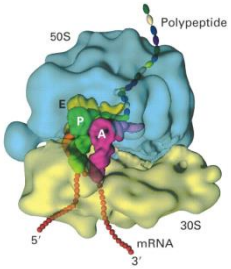
- In DNA, four different bases are found: two purines, called **adenine** (**A**) and **guanine** (**G**) and two pyrimidines, called **thymine** (**T**) and **cytosine** (**C**).
- RNA contains:
- The same purines, **adenine** (**A**) and **guanine** (**G**).
- RNA also uses the pyrimidine **cytosine** (**C**), but instead of thymine, it uses the pyrimidine **uracil** (**U**).

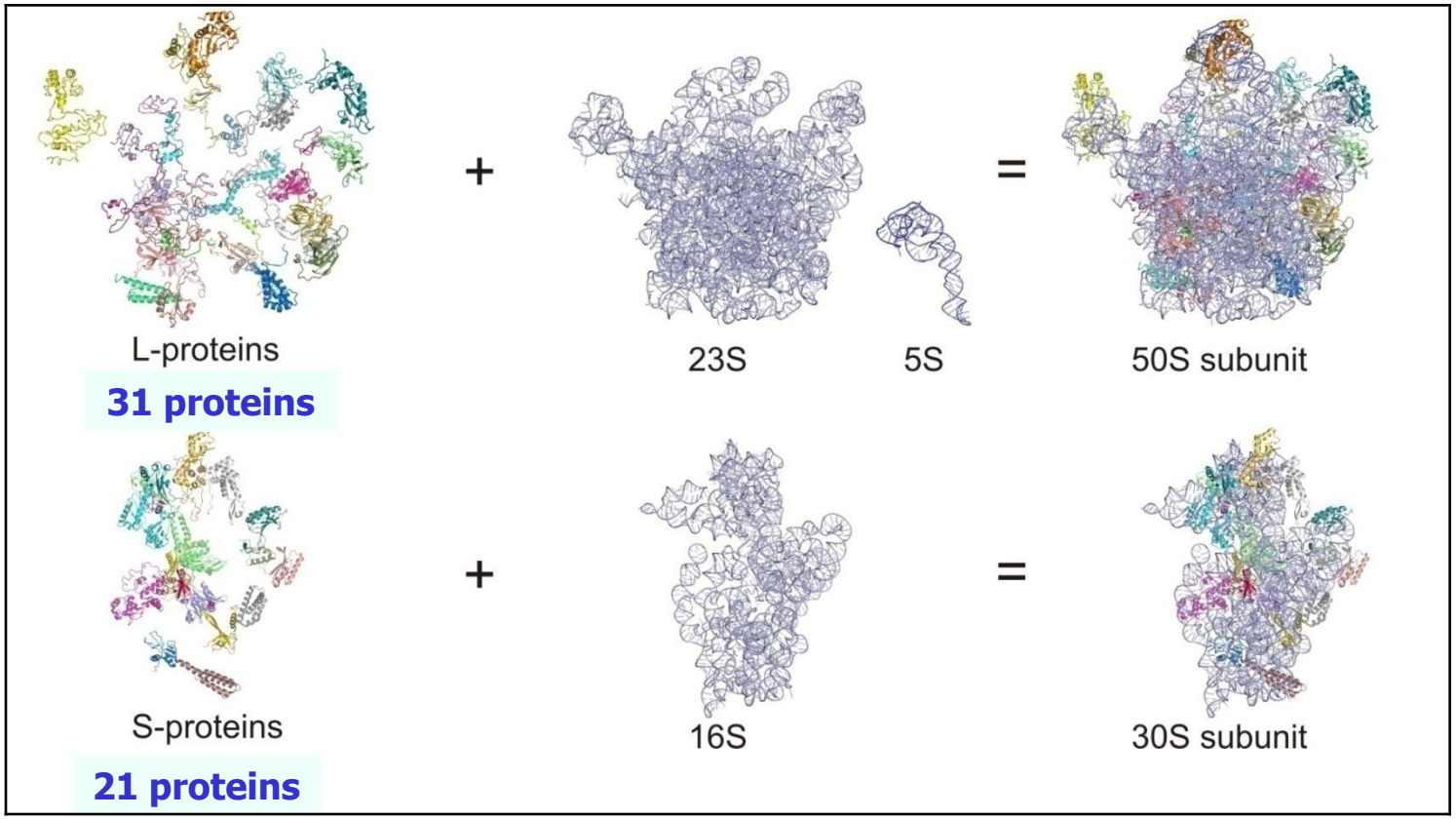


Structural bacterial proteins

Nucleoproteins

Ribosomal subunits: Part protein and Part RNA





Nucleoproteins: nucleic acids(DNA or RNA)+protein.

The protein is successively removed from the nucleoproteins by pepsin and trypsin.

Nucleoproteins —Pepsin, trypsin→ **nucleic acid + protein**

Secreted bacterial proteins

Quorum sensing

Signal molecules

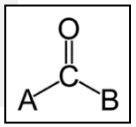
- **LuxI protein:**
- Acyl-HSL signals have

1. acylated homoserine lactone ring,
2. Chain lengths vary from 4 to 18 carbon atoms and in the substitution of a carbonyl at the third carbon.

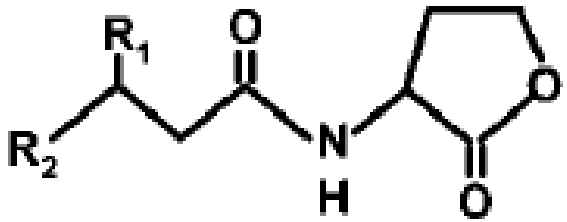
- **LuxR proteins:**
- Consist of two domains:

1. N-terminal acyl-HSL-binding domain,
2. C-terminal DNA-binding domain.

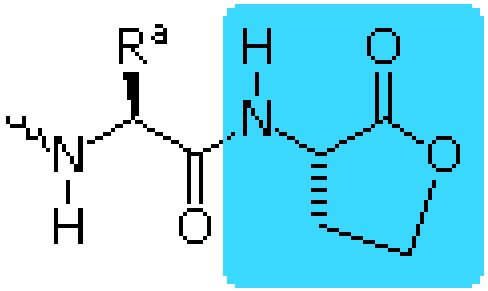
In organic chemistry, a carbonyl group is a functional group composed of a carbon atom double-bonded to an oxygen atom: C=O.



N-Acylhomoserine lactones (AHLs)



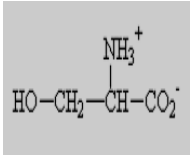
lactone (AHL) homoserine Acyl



Lacton moiety of AHL

Acyl homoserine lactones are lactone rings with a carbonyl tail (C_4-C_{16}) with varying functional lengths (Nagy,2010).

Lactones are cyclic esters, a ring of two or more carbon atoms and a single oxygen atom with a ketone group at one of the carbons adjacent to the other oxygen.



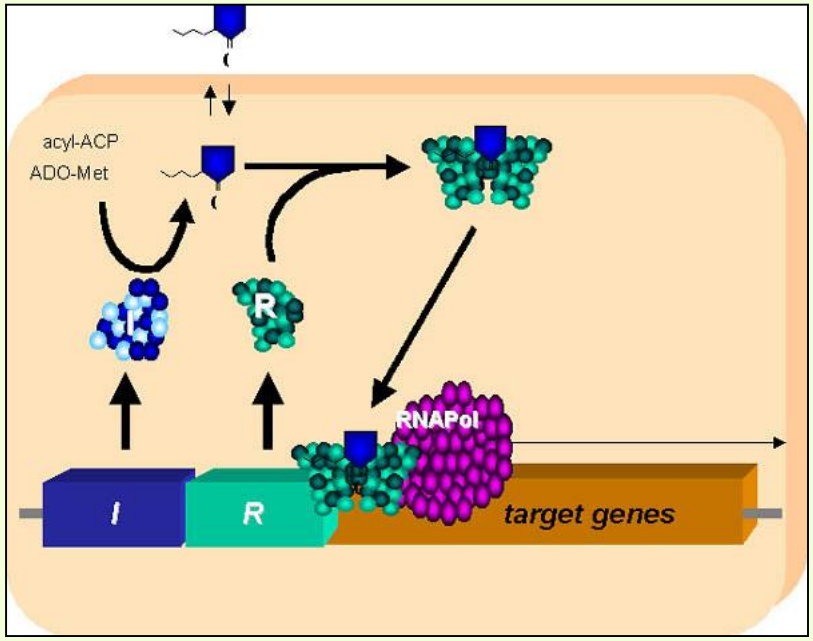
L- serine(ser)

N-Acylhomoserine lactones (AHLs)

- Acyl homoserine lactones are lactone rings with a carbonyl tail (C_4-C_{16}) with varying functional lengths.
- Lactones are cyclic esters, a ring of two or more carbon atoms and a single oxygen atom with a ketone group at one of the carbons adjacent to the other oxygen.
- Homoserine (also called isothreonine) is an α-amino acid with the chemical formula $HO_2CCH(NH_2)CH_2CH_2OH$.
- L-Homoserine is not one of the common amino acids encoded by DNA.
- It differs from the proteinogenic amino acid serine by insertion of an additional -CH_2- unit into the backbone.

Quorum sensing
I and R proteins

- AHL receptors (R proteins) are typically transcriptional activators that form dimers or multimers and bind to "receptor box" promoter sequences in the bacterial DNA, thus enhancing expression of sets of genes with these promoter sequences.

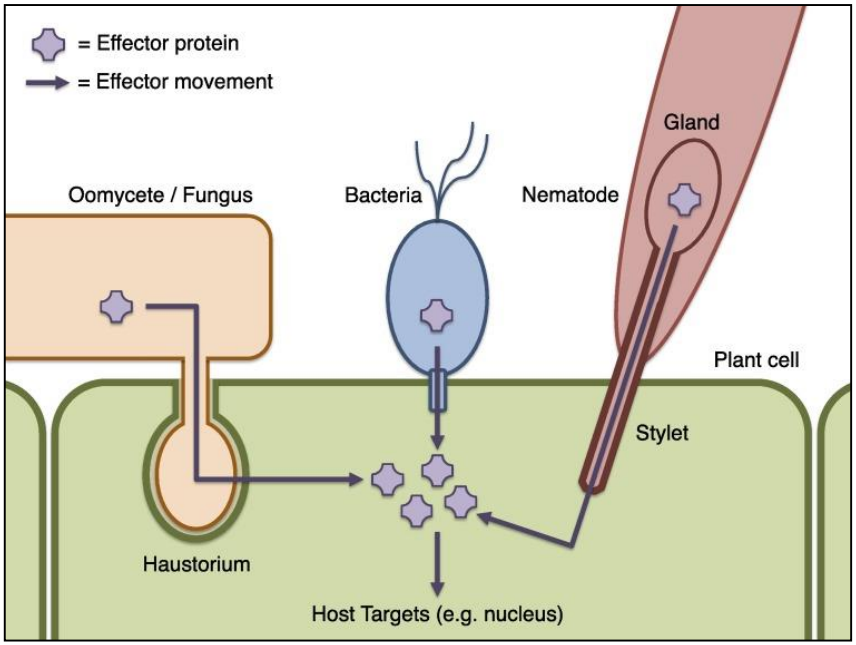


Pierson III,2005

Pathogen-generated secreted proteins **Effectors**

- Effectors are produced by:

1. all the major species of pathogenic bacteria infecting plants and animals and humans.
2. fungi,
3. Viruses, and
4. nematodes.



Surico,2013; Cock *et al.*,2013

Pan-genome and Core genome

Pan-genome (or supra-genome) describes the full complement of genes in a species

- Pan genome: global gene repertoire of a bacterial species:

  Core genome + Dispensable genome

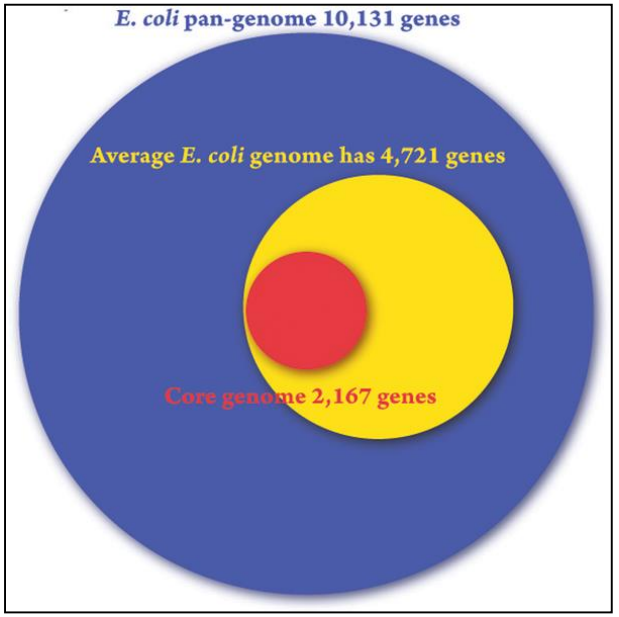
1. Core genome: genes shared by all strains of the same species.
2. Dispensable genome: consisting of partially shared and strain-specific genes, i.e. genes present in some but not all of the same species.

Campbell and co-workers

The Bacterial Pan-Genome

Relevance of the pan-genome model

1. Evolution of Prokaryote,
2. Ecology,
3. Pathogenicity,
4. Biotechnology.



Venn diagram of the pan-genome (blue), average genome (yellow), and core genome (red) of the sequenced *E. coli* strains according to Touchon *et al.*,2019. *E. coli* repertoire(full genome) includes 10,131 genes. Individual strains have an average of 4,721 genes, and it is estimated that only 2,167 of these will be the "core genes" that (nearly) all *E. coli* strains have.

Hendrickson,2009; Microgen,2013

Pathogen derived molecules - Part 1

Pathogen-generated secreted proteins

Bacterial effectors

- Effectors are proteins expressed by plant pathogens to aid infection of specific plant species.
- These molecules can alter plant processes and are central to understanding the complicated interplay between plants and their pathogens.
- Effector proteins are virulence factors that are produced in the bacteria and translocated into infected host cells using different type of secretion systems such as:
- Type 3 secretion system (TTSS/T3SS),
- Type 4 secretion system (TFSS/T4SS),
- Type VI secretion system (T6SS).

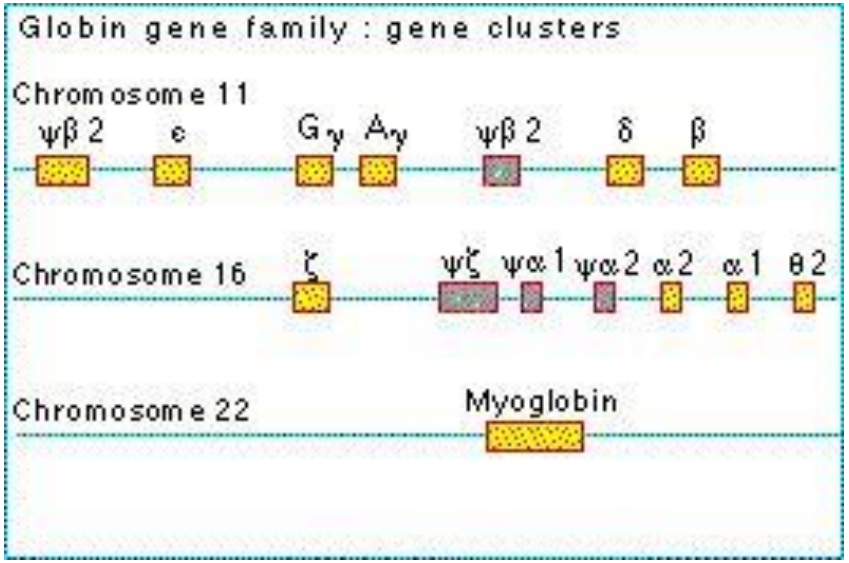
Wikepdia,2021;..

Gene family

Gene family vs gene cluster

What is a gene family?

- A gene family is a set of several similar genes, formed by duplication of a single original gene, that generally have similar biochemical functions.
- E.g.
- One such family are the genes for human haemoglobin subunits.
- The 10 genes are in two clusters on different chromosomes(16 and 22), called the α-globin and β-globin loci.



Gene family

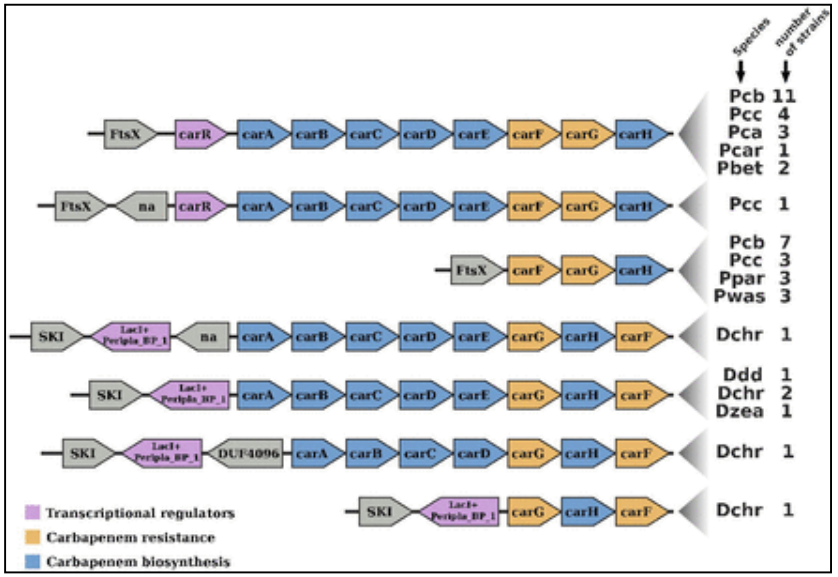
Gene family vs gene cluster

What is a gene cluster?

- Gene clusters are the genetic building blocks of bacteria and archaea.
- Prokaryotic genomes are:

1. highly organized,
2. the genes associated with a particular function, and
3. often occur near each other.

Yutefar Shyntum *et al.*,2018



***car* gene cluster in Soft-Rot-Enterobacteriacea (SRE):**

A cluster of nine carbapenem (car) genes has been identified on the chromosome of *P. carotovora*.

Carbapenem is a class of very effective antibiotic agents most commonly used for the treatment of severe bacterial infections.

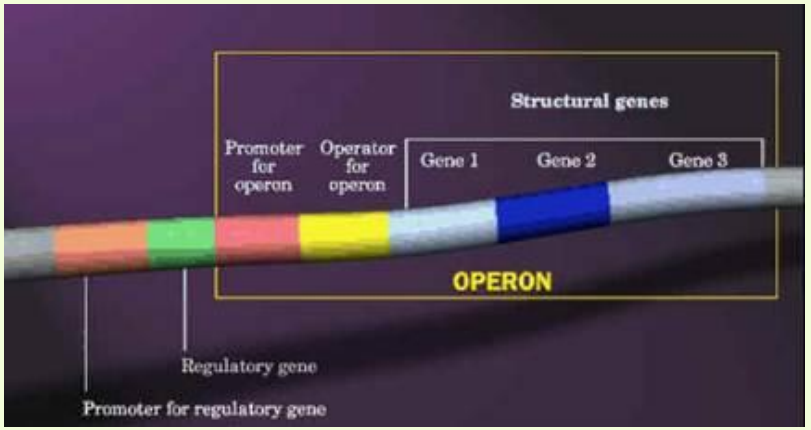
Gene family

Gene operon vs gene cluster

What is the difference between a gene cluster and an operon?

- A gene is one part of a genome. Each gene can be broken down into important parts:

1. A promoter,
2. coding region, and
3. terminator.



The coordinated transcription and translation of the lac operon structural genes is supported by a shared promoter, operator, and terminator.

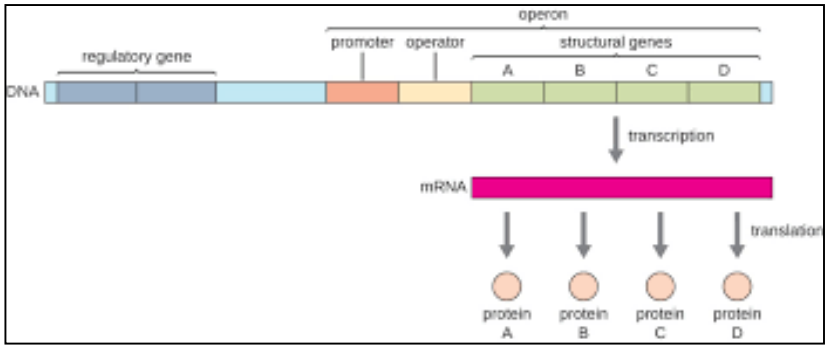
Nature Education,2014;..

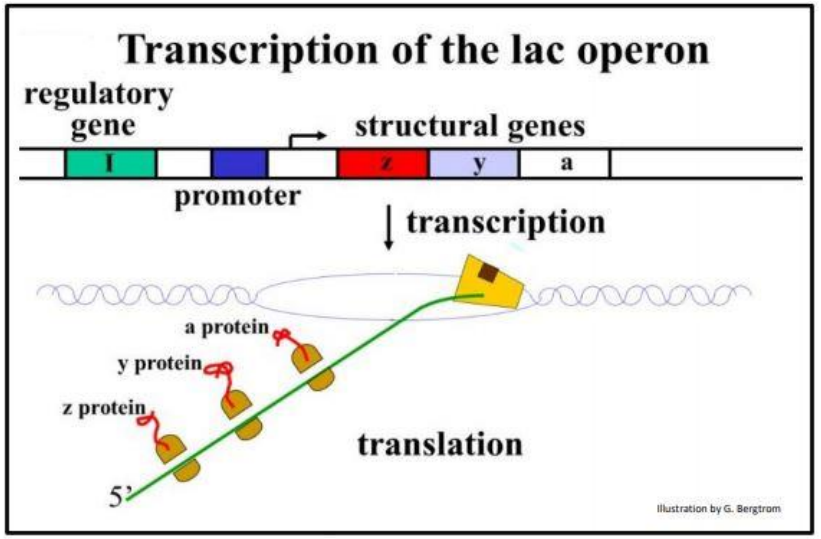
Gene family

Gene operon vs gene cluster

What is the difference between a gene cluster and an operon?

1. A gene cluster as a set of functionally related genes located in close physical proximity in a genome.
2. The term operon refers to a set of genes under common regulatory control, that are transcribed into a single mRNA and are all co-directional in orientation on the chromosome. E.g. lac operon. Three lactose metabolism genes (lacZ, lacY, and lacA) are organized together in a cluster called the lac operon.





Lumen waymaker; Biology LibreTexts

Gene family

A gene family is a group of genes that are related in structure and often in function

- A gene family is a set of homologous genes (from a few genes to several hundred genes) within one organism.
- A gene cluster is part of a gene family.
- A gene cluster is a group of two or more genes found within an organism's DNA that encode for similar polypeptides, or proteins, which collectively share a generalized function and are often located within a few thousand base pairs of each other.
- In bacteria, gene clusters may be similar to an operon in which all genes are controlled by a single promoter and operator.

Wikipedia,2022

Gene family

What is the significance of the gene family?

- Classifying individual genes into families helps researchers describe how genes are related to each other.
- Classification systems for genes continue to evolve as scientists learn more about:

1. the structure and function of genes, and
2. the relationships between them.
3. Additionally, gene families may provide clues for identifying genes that are involved in particular diseases.

Genetics Home Reference,2015

Gene family

A group of genes that are related in structure and often in function

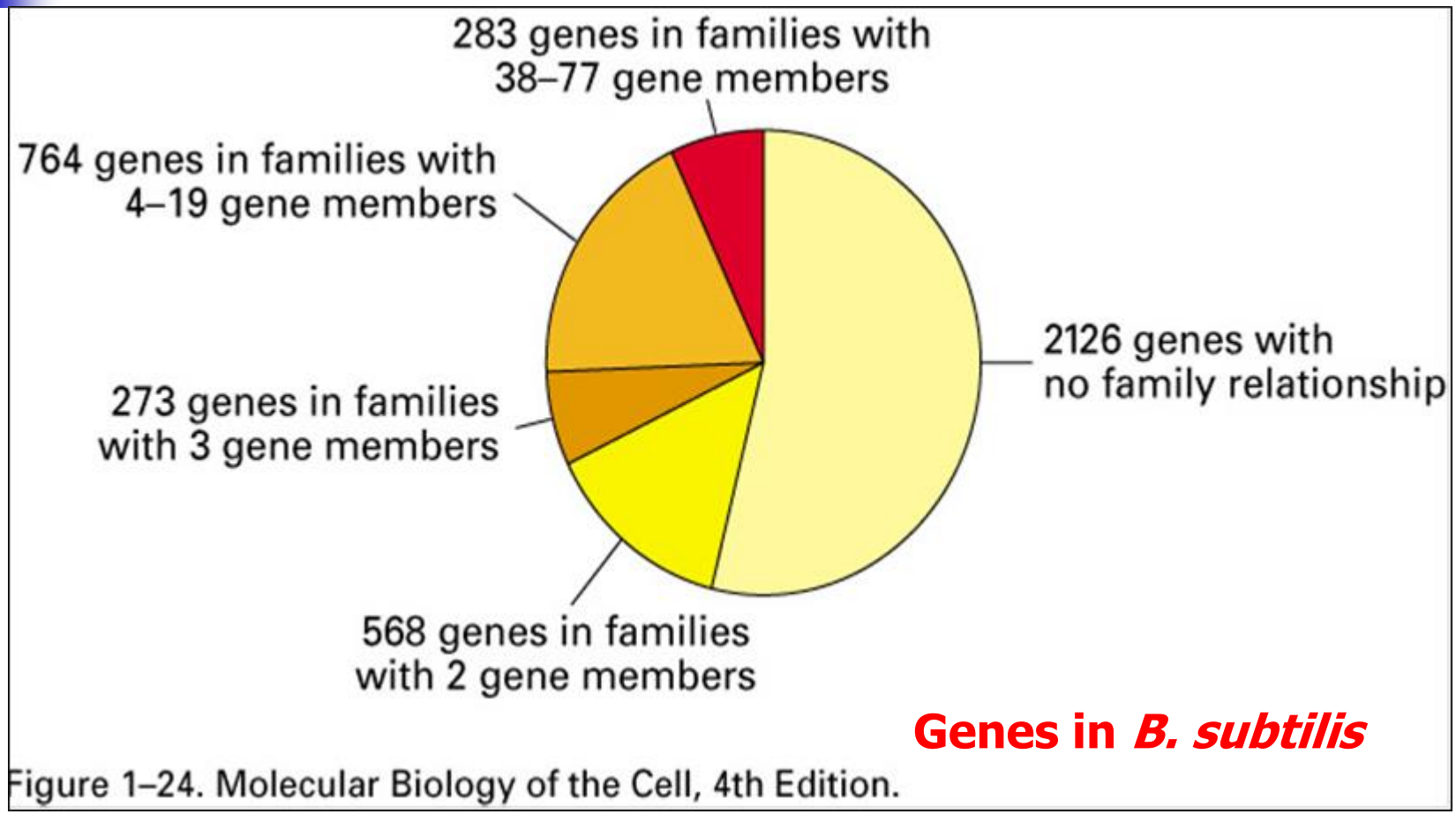


Figure 1–24. Molecular Biology of the Cell, 4th Edition.

Campbell and co-workers

Secreted bacterial proteins

Pathogen-generated secreted proteins

Effector families

- The pangenome of *P. syringae* encodes 57 families of effectors injected by the type III secretion system.
- Each bacterial strain of *Pseudomonas syringae* expressing about 15-30 of these 57 families of effectors.

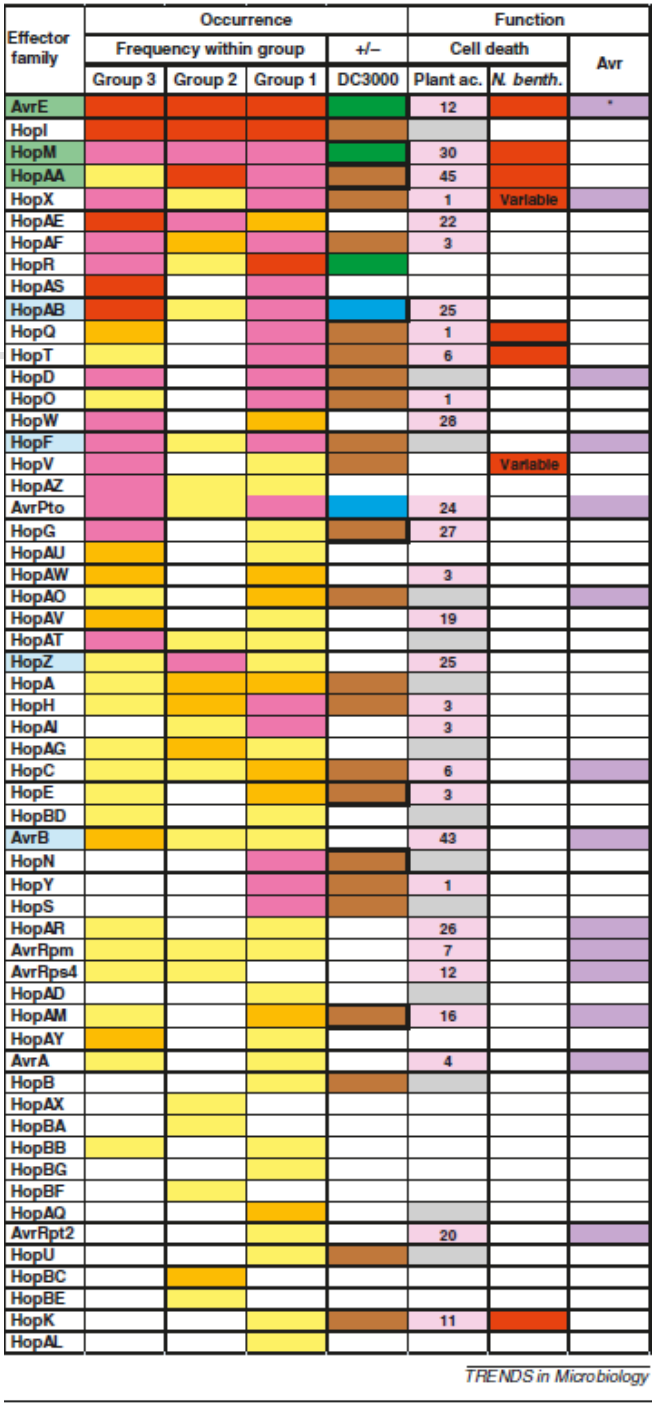
Hrp outer protein (Hop):

A generic designation for *P. syringae* type III effectors

The phylogenetic distribution of effector families in the *Pseudomonas syringae*.

Plant accessions (Plant ac.)

Nicotiana benthamiana (colloquially known as benth or benthi) is a close relative of tobacco and species of *Nicotiana indigenous* to Australia.



Lindeberg *et al.*,2012

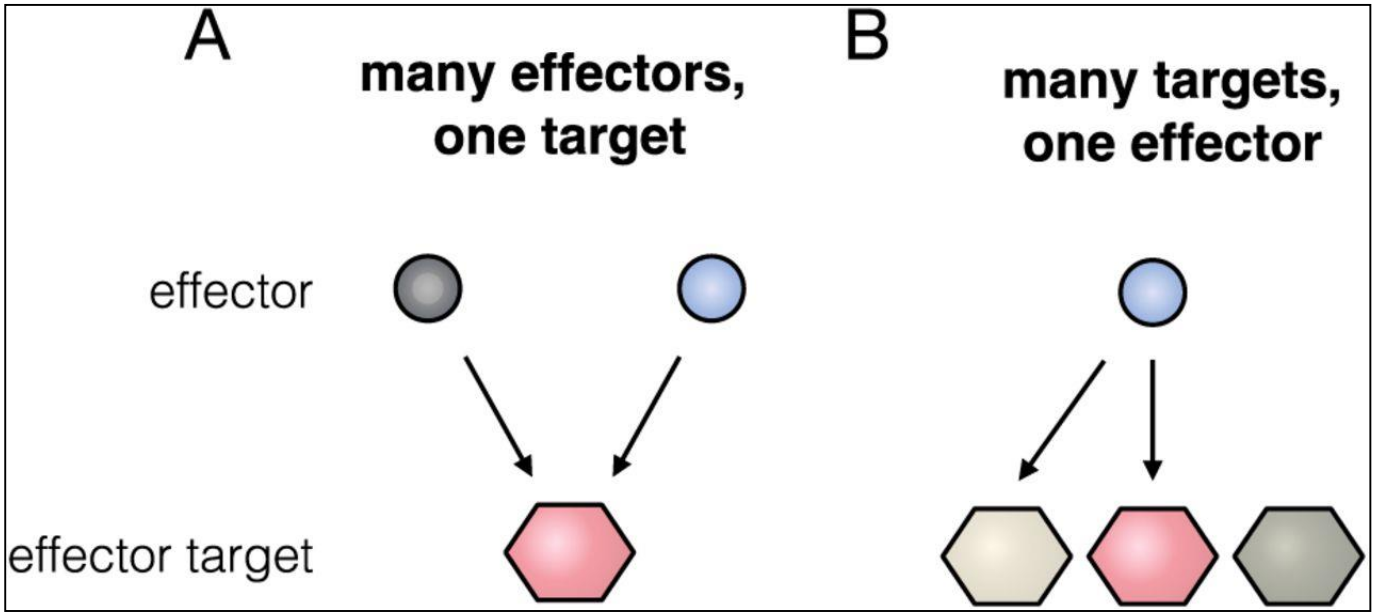
Pathogen-generated secreted proteins
Bacterial protein effectors
Types and numbers

Species	number of effectors
Chlamydia (multiple species) sexually transmitted diseases in humans	+16
E. coli EHEC (O157:H7)	40-60
E. coli (EPEC)	>20
Legionella pneumophila	>330 (T4SS)
Pseudomonas aeruginosa	4
Pseudomonas syringae	14 (>150 in multiple strains)
Salmonella enterica	+60
Yersinia (multiple species) *Y. pestis* is the causative agent of the plague	14

Wikipedia,2021;..

Pathogen-generated secreted proteins
Bacterial effectors

- Effector proteins are usually critical for virulence.
- Some bacteria inject only a few effectors into their host's cells while others may inject dozens or even hundreds.



Bialas *et al.*,2017; Wikepdia,2021

Pathogen-generated secreted proteins
Bacterial effectors
Functions

- Effectors are pathogen molecules that:

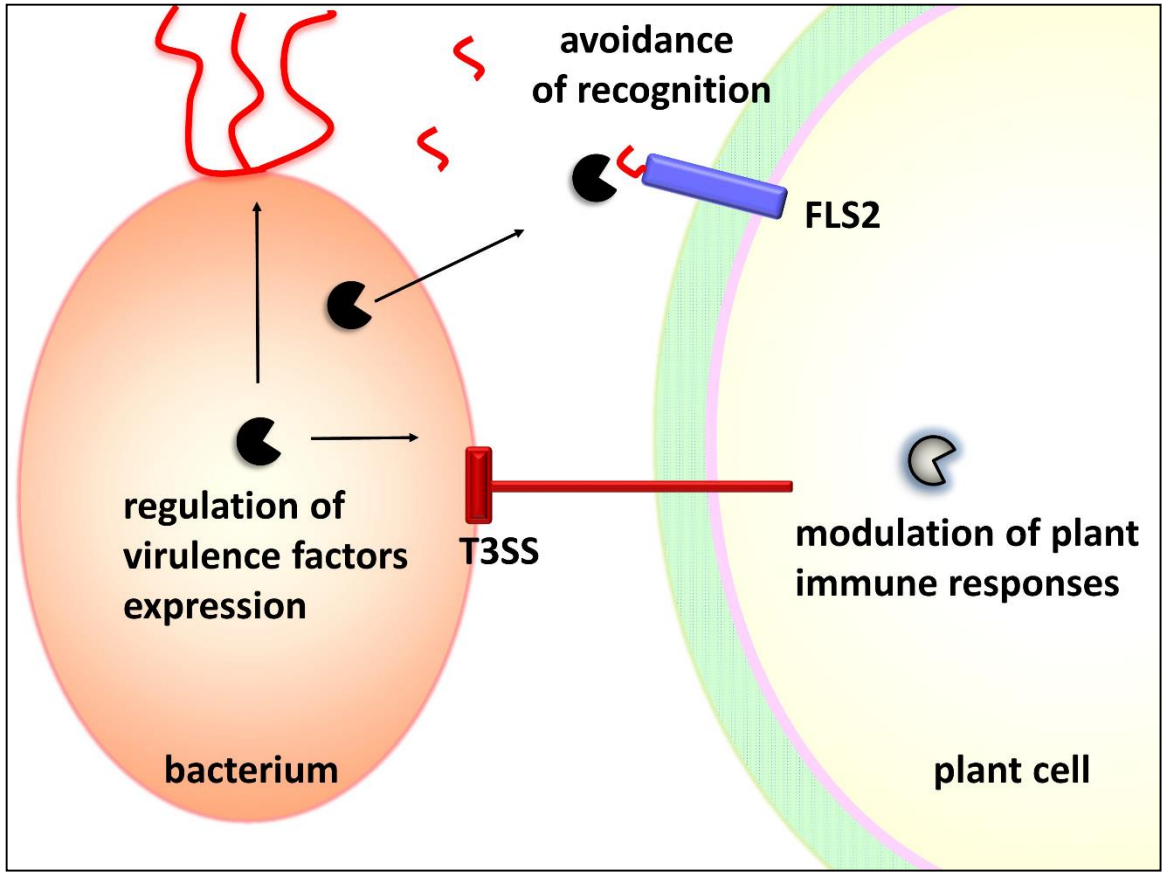
1. help the pathogen to invade host tissue,
2. suppress its immune system,
3. help the pathogen to survive,
4. manipulate host cell structure, in order to cause/facilitate the formation of symptoms.
5. often contribute quantitatively to pathogen aggressiveness.

Wikepdia,2021;..

Pathogen-generated secreted proteins
Bacterial effectors
Functions

FLS2 (Leu-rich repeat receptor kinase) is the PRR for flagellin recognizes.

PRR: pattern recognition receptors in plants.



Figaj,2019

Secreted bacterial proteins

Pathogen-generated secreted proteins

TAL and non-TAL effectors

- What determinants allow a bacterium to be a pathogen?
- TALes (Transcription Activator-Like effectors) represent the largest family of type III effectors among pathogenic bacteria and play a critical role in the process of infection.
- TAL effectors are proteins secreted by bacterial pathogens into plant cells, where they enter the nucleus and activate expression of individual genes.
- Strains of *Xanthomonas oryzae* pv. *oryzae* (Xoo) and some strains of other *Xanthomonas* pathogens contain large numbers of TAL genes.

Li *et al.*,2009;..

Secreted bacterial proteins

Pathogen-generated secreted proteins

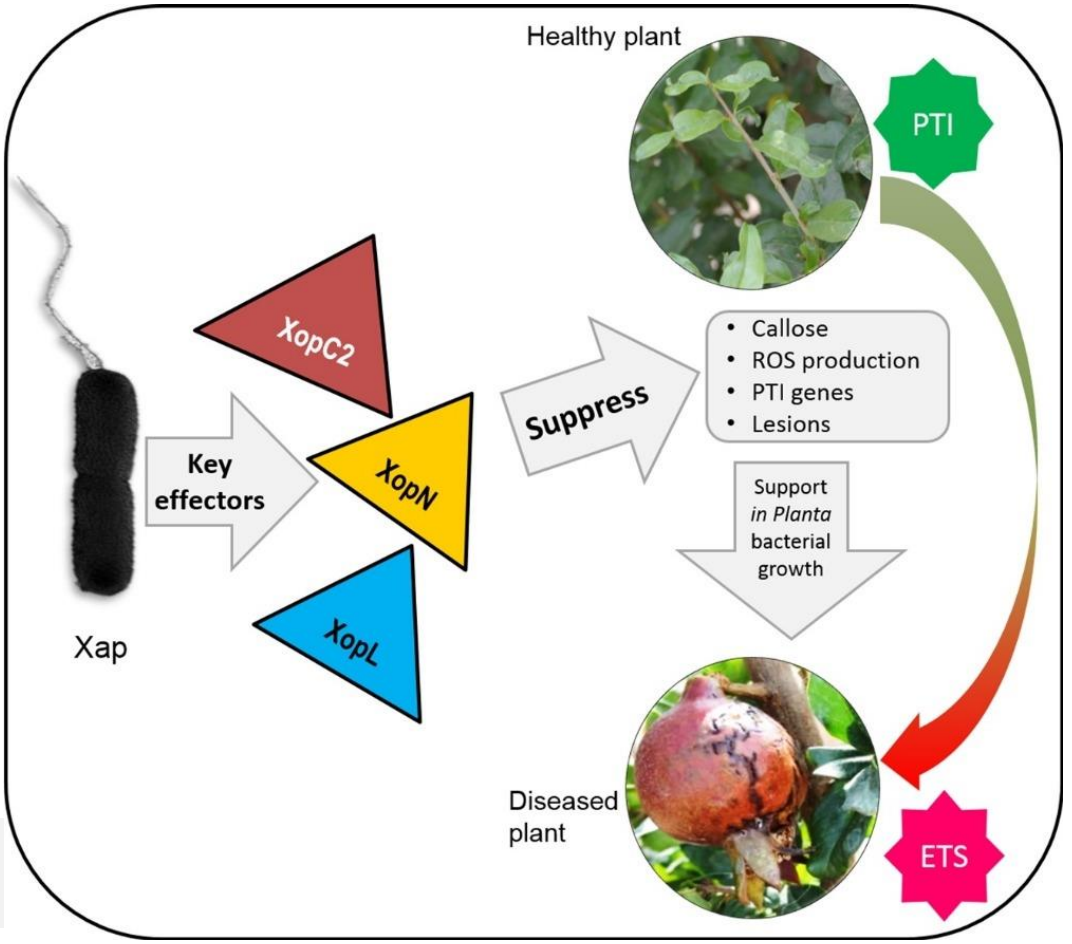
TAL and non-TAL effectors

- TALEs are key virulence factors in numerous *Xanthomonas* spp.
- *Xanthomonas* TALEs induce the expression of host susceptibility (S) genes to cause disease.
- The number of TALEs in different *Xanthomonas* bacteria varies from 0 (the majority of pepper and tomato infecting strains) to close to 30 (as found in *X. oryzae* pv. *oryzicola*).
- While most non-TALE effectors of *Xanthomonas* are usually associated with disruption and manipulation of host defense signaling.

Teper *et al.*,2021

Xop (*Xanthomonas* outer protein)
Non-TAL (Xop) effectors

During infection, the *Xanthomonas axonopodis* pv. *punicae* (Xap), the bacterial blight pathogen of pomegranate, secretes six non-TAL (Xop) effectors into the pomegranate cells through a specialized type three secretion system (T3SS).



PAMP-triggered immunity (PTI)
Effector-triggered susceptibility (ETS).

Mondal *et al.*,2020

Secreted bacterial proteins

Pathogen-generated secreted proteins

Bacterial effectors

- Two major sets of pathogenicity-related genes/proteins:

1. *avr* genes/Avr
2. *hrp* genes/Hrp proteins

- Other genes/proteins are:

1. *hrc*/Hrc
2. *dsp*/Dsp (The major effector gene *in E. amylovora*)
3. *pth*/Pth
4. *hop*/Hop

- *hrp* (for hypersensitive reaction and pathogenicity) genes; *hrc* (for *hrp* genes conserved). disease-specific (*dsp*) gene. Almost all *hrp* genes are homologous. Since these genes are also found in animal bacteria, these were termed *hrc* genes(*hrp* and conserved).
- The *hpa* (*hrp*-associated) genes encode harpin-like proteins.
- *Xop*, *Xanthomonas* outer protein.
- Hop (Hrp outer protein) in *Pseudomonas* and many other bacteria.
- *Pth*, pathogenicity gene necessary to condition pathogenicity on a given host.

Pathogen-generated secreted proteins **Effectors**

- The proteins secreted by Hrp TTSSs have been given a variety of names including:

1. Avr (for avirulence).
2. Hop (Hrp outer protein) in *Pseudomonas* and many other bacteria such as *P. syringae*, *Erwinia*, *Pantoea* spp., etc.
3. Xop (*Xanthomonas* outer protein).
4. Pop (*Pseudomonas* outer protein, which actually are *R. solanacearum* proteins based on its earlier genus name),

- Hop, Xop and Pop proteins were originally identified based on their property of limiting the host range of the pathogen.

Espinosa and Alfano,2004

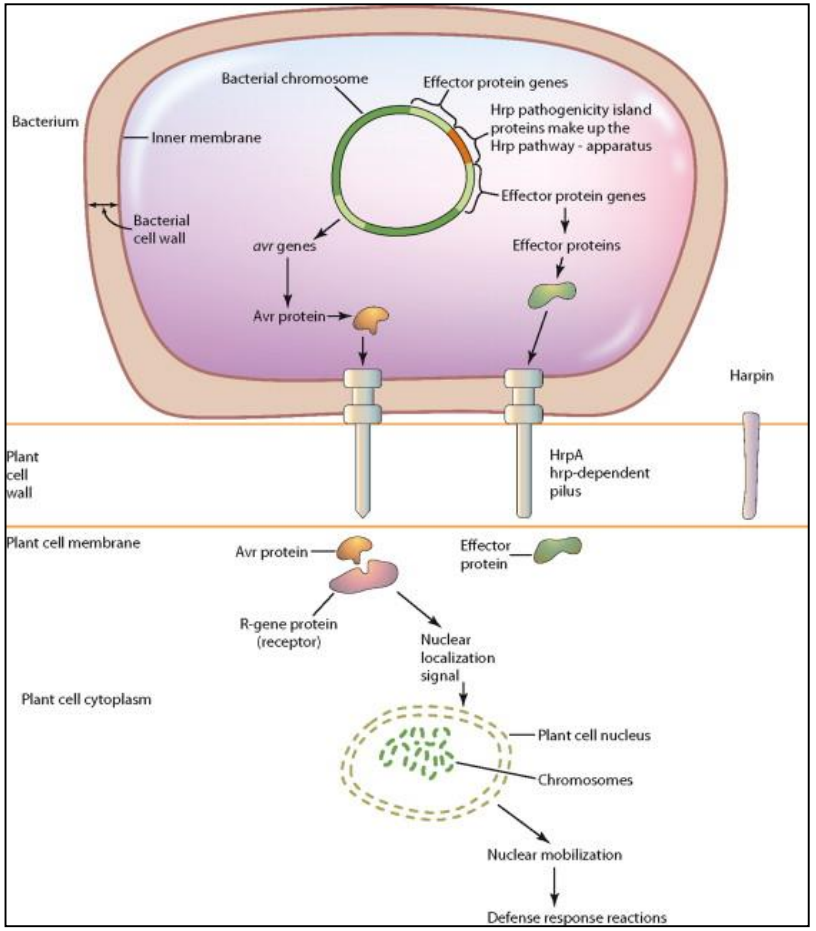
Bacterial *avr* genes

Dual-acting genes

As long as one pair of *avr* / *R* genes matches in pathogen and host, an HR results, while in the absence of this matching, disease is the outcome.

Pathogen-generated secreted proteins
Effectors
Avr genes and type III secretion system

Diagrammatic representation of the hrp or type III secretion system in bacteria.



Agrios,2005

The *avr* genes

Avirulence genes

- The first avirulence gene was isolated from *Pseudomonas* bacteria infecting soybean.
- Over 50 bacterial avirulence genes have been isolated and sequenced.
- More than 30 bacterial *avr* genes in pathovars of *Pseudomonas syringae* and *Xanthomonas* spp. have been described.
- They are:

1. Chromosomal, or
2. plasmid-borne.

Function of *avr* genes

Mutation of *avr* genes

- Mutations in *avr* genes make the bacterium invisible to the host defense recognition system.
- Therefore, plant fails to develop an HR and the bacterium is able to cause disease.
- Because the lack of *avr* or *R* genes usually results in a compatible (disease) interaction.

Function of *avr* genes
In plant pathogenic bacteria

- Some of *avr* genes which play a role in pathogenicity on susceptible plants are:
- *avrBs2* from *X. vesicatoria*,
- *avrRpm1* from *P. syringae* pv. *maculicola*,
- *avrBs3* family of *avr* genes in *Xanthomonas campestris*,
- *avrA* and *avrE* in *Pseudomonas syringae* pv. *tomato*,
- *X. oryzae* pv. *oryzae* has more than 12 avrBs3/pthA members, and two of them have been subcloned and well-characterized, avrXa7 and avrX10.

Bacterial avirulence genes

Bacterial avirulence genes with virulence or virulence-associated properties

Gene or protein	Organism	possible function	Related proteins or alleles
avrBs2	*X. vesicatoria*	Cytoplasmic	Agrocinopine synthase, glycerol-phosphodiesterase
avrXa7	*X. o.* pv. *oryzae*	NLS, AD, DNA binding, nucleus	*avrBs3* family
avrb6	*X. c.* pv. *malvacearum*	Nucleus	*avrBs3* family
avrPto	*P. s.* pv. *tomato* (T1)	MYM, kinase binding, IM	-
avrRpm1	*P. s.* pv. *maculicula*	MYM, inner membrane?	AvrPpiA1 (*P.s.* pv. *pisi*)
avrE	*P. s.* pv. *tomato* (PT23) *E. amylovora*	Unknown	DspA (DspE) (*E. amylovora*)
avrF	*P. s.* pv. *syringae* *E. amylovora*	Chaperone	DspB (DspF) (*E. amylovora*)
avrA	*P. s.* pv. *tomato* (PT23)	Cytoplasmic	AvrA (*P. s.*pv. *glycinea*), AvrBs1

White *et al.*,2000

The *avr* genes

Structure of *avrBs3* gene products

Xanthomonas *AvrBs3* family-type III effectors

- TAL (transcription activator-like) effectors (often referred to as TALEs).
- The central domain of these genes contains a series of 102 bp, directly repeated DNA sequences.
- Each avrBs3-homolog may contain different numbers of the 102-bp repeat. For example,

1. the avrBs3, avrBs3-2, and pthA genes contain 17.5 copies;
2. avrXa10 contains 15.5 copies, and
3. avrB6 contains 13.5 copies.

See also TAL effectors (Transcription activator-like) effectors and Plant Bacterial pathogensis-Part2.

Structure of *avrBs3* gene products

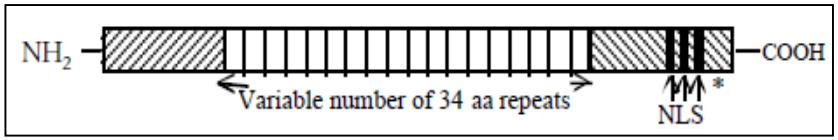
Xanthomonas *AvrBs3* family-type III effectors

- Molecular characterization of several members of the *avrBs3* family reveals that three key structures of the gene products:

1. A central region of tandem 33-35 residue repeats;
2. Three nuclear localization signal (NLS) sequences, and
3. An acidic activation domain (AAD) have roles in avirulence and/or virulence.

(b)

Repeat	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
AvrXa10	NI	HG	NI	HG	NI	NI	NN	HD	NI	HD	NN	HG	NS	NG	HD	NG		
AvrBs3	HD	NG	NS	NG	NI	NI	NI	HD	HD	NG	NS	NS	HD	HD	HD	NG	HD	NG
Avrb6	HD	NI	NG	HD	HD	NI	HD	NI	NS	HD	HD	HD	NN	NG				
PthA	NI	NG	NI	NI	NG	HD	NG	HD	NG	NG	NG	NG	NS	HD	HD	NG	NG	
PthN	NI	HD	HD	NI	HD	NI	NG	NI	NN	HD	NI	NG	NG	NN				



Leucine-nonpolar amino acids with neutral charge

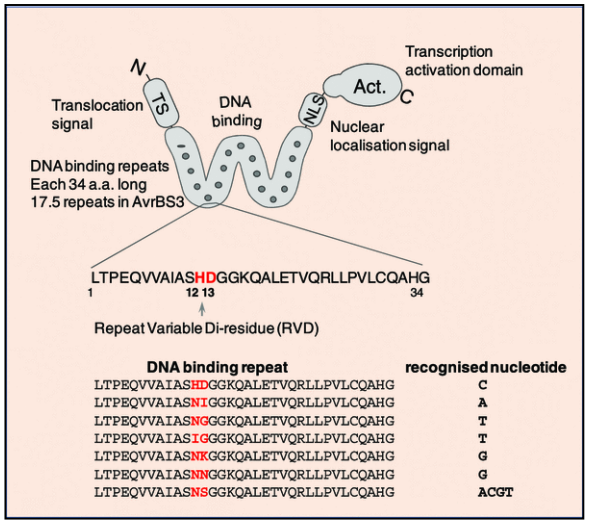
The central domain is composed of 17.5 nearly identical 34-amino-acid repeats.
The number and order of the repeats determine:

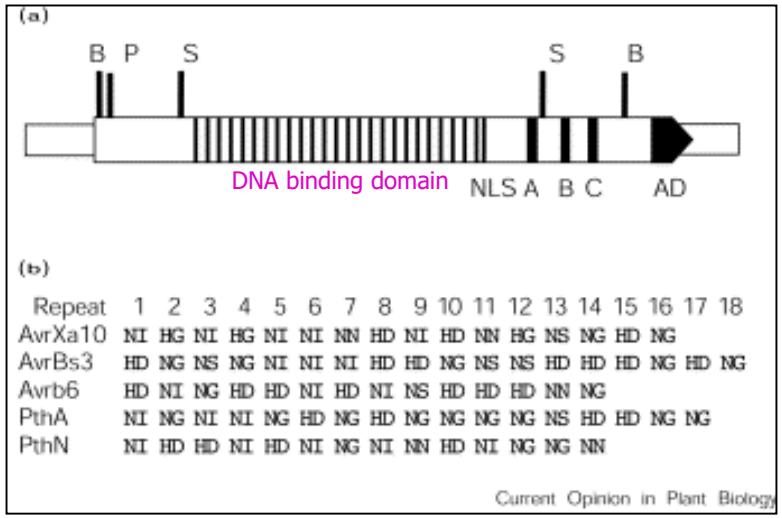
1. **the specificity of recognition in resistant plants, and**
2. **the virulence function in susceptible plants.**

Structure of bacterial *avr* genes

Map of the phenotype gene in the *avrBs3* family

- AvrBs3 has a central domain that is highly repetitive, containing 17.5 nearly identical 34-amino-acid repeats in selected members of *avrBs3* family.
- Members of the TAL effector family differ differing in the 12th and 13th a.a. (Repeat Variable Di-residue, RVD) in the DNA binding domain.
- Functional nuclear localization signals (NLSs) are required for the avirulence activity of *avrBs3* (*X. vesicatoria*).
- AD=acidic transcriptional domain.

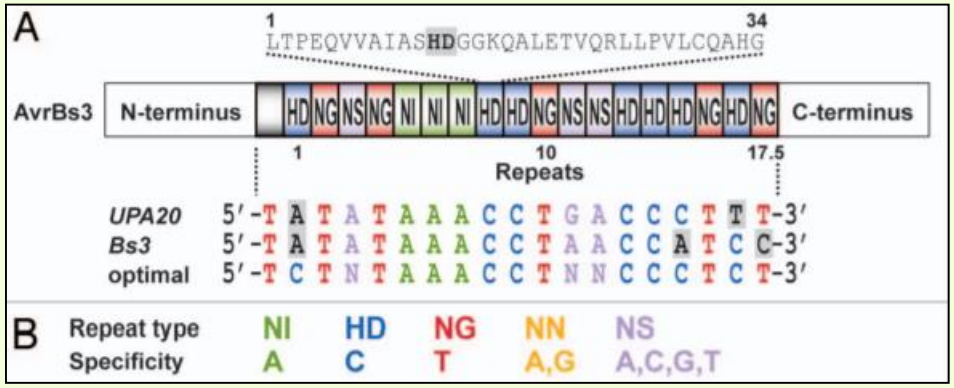




Domain architecture of the AvrBs3 effector

Xanthomonas campestris pathovars

- The amino acid sequence of the AVRBs3 protein is characterized by a central region consisting of 17.5 nearly identical repeats of 34 amino acids.
- The 17.5 repeats of AvrBs3 were found to be essential for recognition by the Bs3 resistance gene.
- The target box of AvrBs3 (17.5 repeats) is 19 bp long, differ mainly at positions 12 and 13.



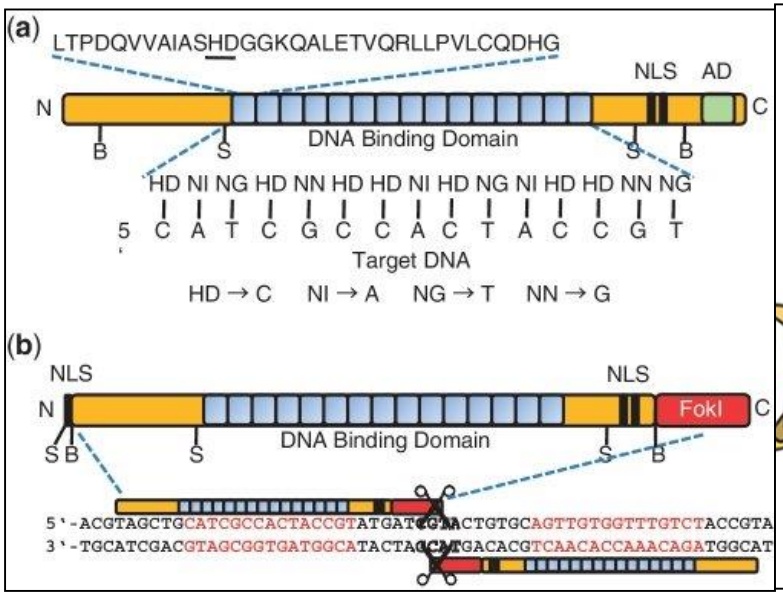
Scholze & Boch,2010;…

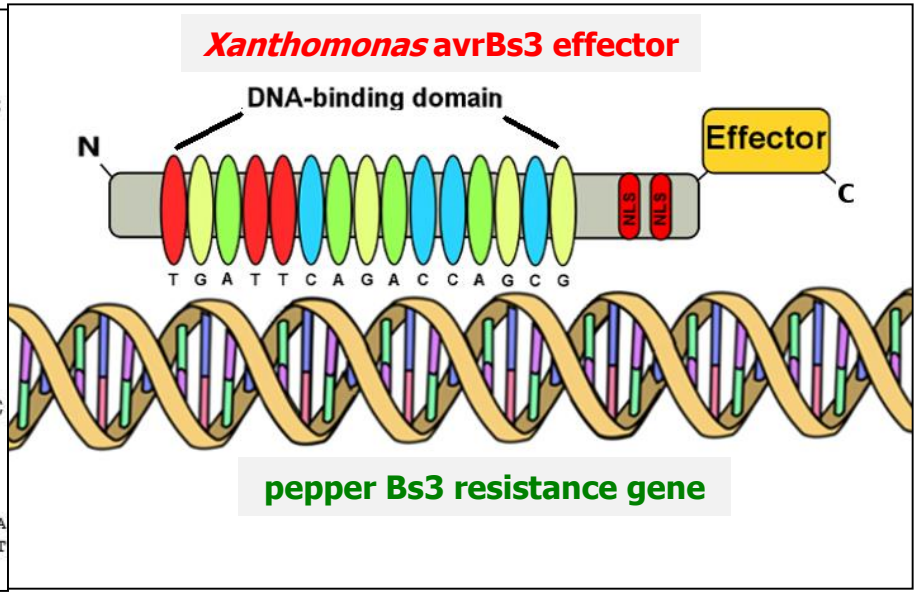
Structure of bacterial *avr* genes

Map of the phenotype gene in the *avrBs3* family

Domains of TAL effector AvrBs3

- One repeat from pathogen effector(underlined HD in figure a) recognizes one base pair on the host plant DNA (A, C, G, T) according to the repeat specificity and aligned.

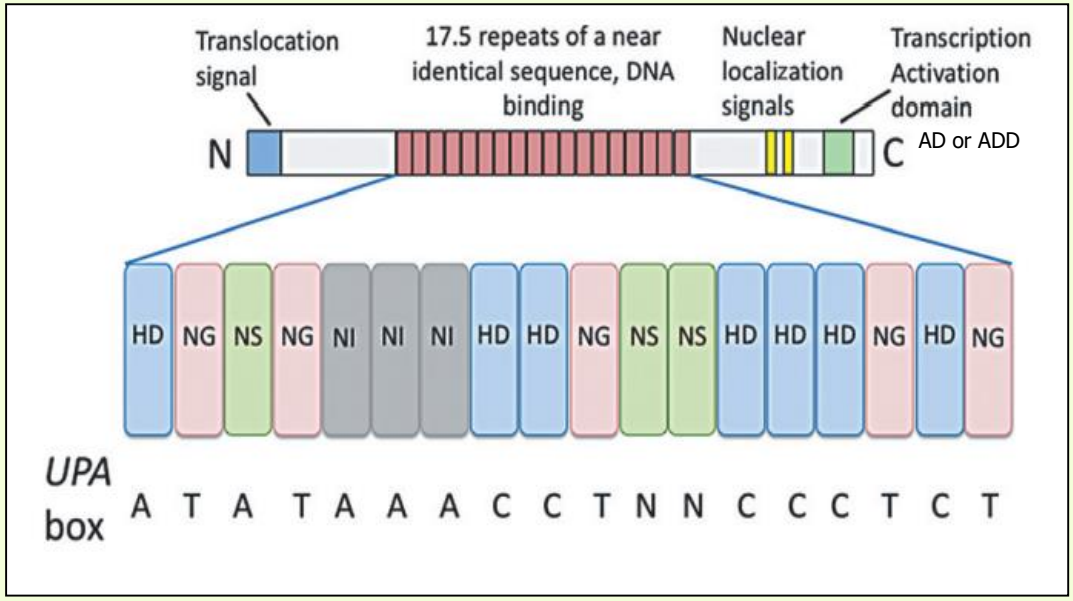




Domain architecture of the AvrBs3 effector

Xanthomonas campestris pathovars

- Domain architecture of the AvrBs3 effector showing the variations at positions 12 and 13 in the repeats and the nucleotides recognized in the consensus UPA (upregulated by AvrBs3) box.



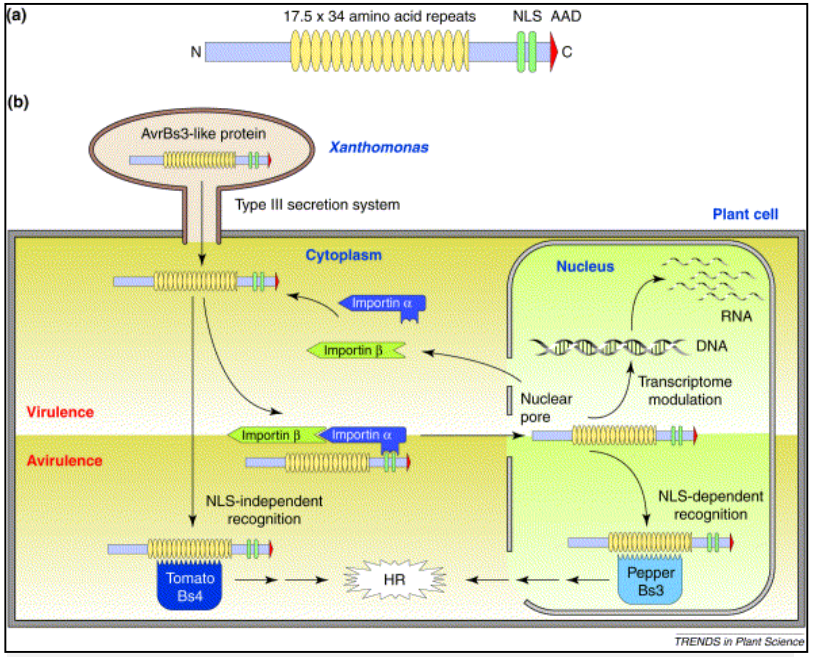
Target DNA base can be expressed as NI = A, HD = C, NG = T, NN = G or A, and NS = A, C, G, or T.

Mansfield *et al.*,2012

AvrBs3 from *X. vesicatoria*
Localization of avirulence proteins

- Structure of AvrBs3 from *X. vesicatoria*, the prototype of the AvrBs3 protein family.
- The central domain is composed of 17.5 nearly identical 34-amino-acid repeats.
- The C-terminus contains:

1. Two functional nuclear localization signals (NLS), and
2. An acidic transcriptional activation domain (AAD).



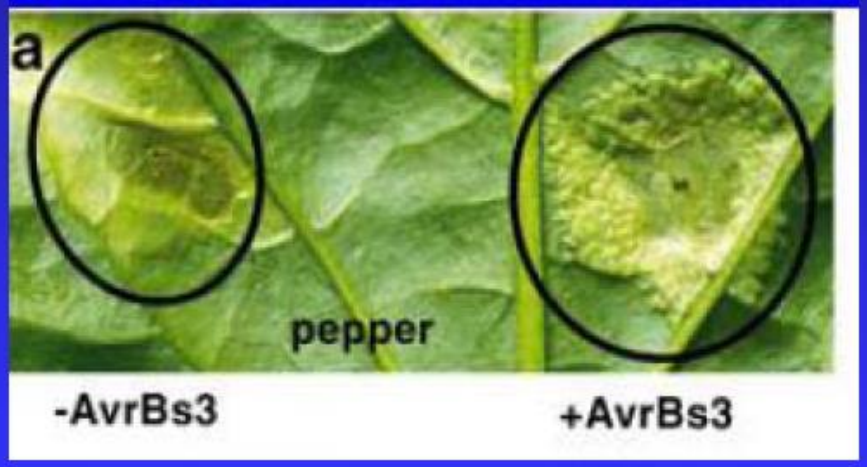
Arrival of AvrBs3 localizes to the plant cell nucleus.

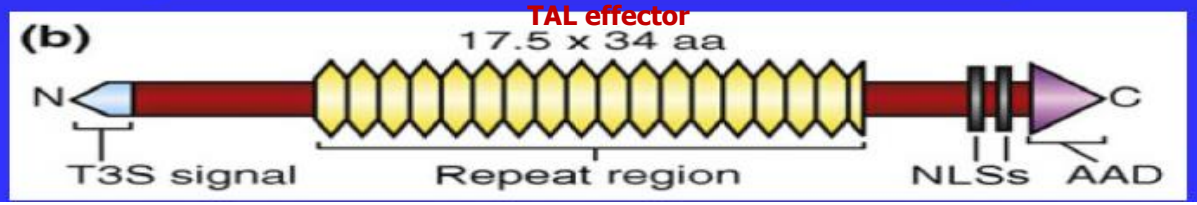
Importin α which, together with importin β, mediates nuclear protein import.

Pathogenicity of AvrBs3

known as TAL effectors (TALEs) in *X. vesicatoria*

AvrBs3 specifically induces a hypertrophy in susceptible pepper plants and other solanaceous species. In field studies, it has indeed been shown that *AvrBs3* promotes bacterial spreading.





Zhaokaijun

Transcription activator-like effector (TALE) led to invention of Transcription activator-like effector nucleases (TALEN)

TALE vs. TALEN

- Transcription activator-like (TAL) effectors are DNA binding proteins produced by *Xanthomonas* bacteria when they infect plants.
- These proteins can activate the expression of plant genes by recognizing and binding host plant promoter sequences through a central repeat domain consisting of a variable number of ~34 amino acid repeats.
- The residues at the 12th and 13th positions of each repeat are hyper-variable. There appears to be a simple one-to-one code between these two critical amino acids in each repeat and each DNA base in the target sequence, e.g. NI = A, HD = C, NG = T, and NN = G or A.

- TAL effectors have been utilized to create site-specific gene-editing tools by fusing target sequence-specific TAL effectors to nucleases (TALENs), transcription factors (TALE-TFs) and other functional domains.
- These fusion proteins can recognize and bind chromosome target sequences specifically to execute their gene editing functions, such as:
- gene knockout, knockin (with donor plasmid), mutagenesis, activation, repression and more.
- Unlike zinc fingers' nucleotide triplet recognition, TAL effector domains recognize single nucleotides, which allows researchers to be able to specifically target whatever sequence they want.

TALEN is a fusion protein consisting of a TALE DNA-binding domain with a FokI nuclease domain that is often used in zinc finger nuclease (ZFN) technology.

Transcription activator-like effector (TALE) led to invention of Transcription activator-like effector nucleases (TALEN)

TALE vs. TALEN

- Transcription activator-like effector (TALE) led to invention of Transcription activator-like effector nucleases (TALEN) used for gene editing and gene therapy.
- Engineered TAL effectors can also be fused to the cleavage domain of FokI to create TAL effector nucleases (TALEN) or to meganucleases (nucleases with longer recognition sites) to create "megaTALs.
- Such fusions share some properties with zinc finger nucleases and may be useful for genetic engineering and gene therapy applications.

GeneCopoeia,2021;Wikipedia,2021

Transcription activator-like effector (TALE) led to invention of Transcription activator-like effector nucleases (TALEN)

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GeneCopoeia,2021;Wikipedia,2021

Transcription activator-like effector (TALE) led to invention of Transcription activator-like effector nucleases (TALEN)

TALE vs. TALEN

- **Advantages**

1. Target any sequence in any cell;
2. Highly sequence-specific genome editing;
3. For gene knockout, knockin, mutagenesis, activation, repression and more;
4. Flexible TAL effector design of binding and functional domains, such as TALEN and TALE-TF.

Engineered TALE engineered transcription factors

Transcription activator-like effector (TALE) led to invention of Transcription activator-like effector nucleases (TALEN)

TALE vs. TALEN

- **TALE-TF services:**
- A key application for TALEs is the targeted activation and repression of target genes in cells by fusing transactivation domains to TALE DNA binding domains.
- The TALE-TF construct is a powerful tool to selectively modulate gene expression in eukaryotic cells with exquisite specificity.
- The TALE-TF contains a TALE DNA binding domain fused to the VP64 transcription activator.

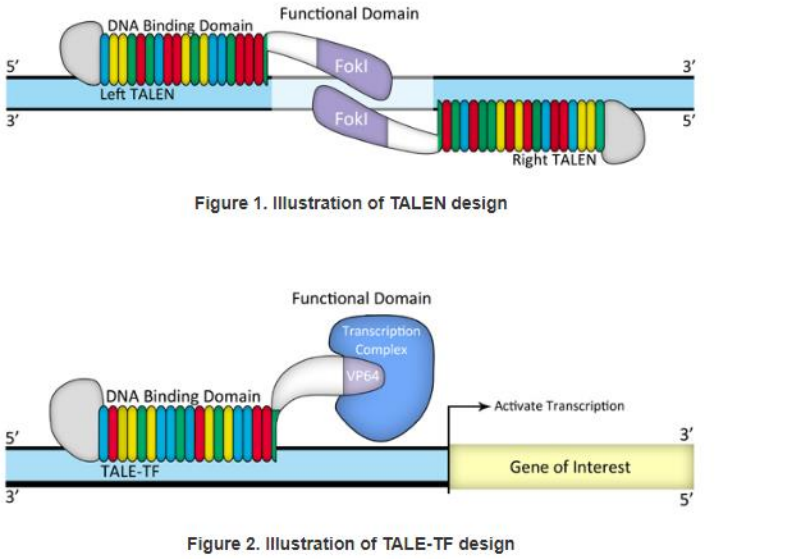


Figure 1. Illustration of TALEN design

Figure 2. Illustration of TALE-TF design

Engineered TALE engineered transcription factors

TALE-TF: Transcription Activator-Like Effector-Transcription Factor.

GeneCopoeia,2021

Engineered TALE engineered transcription factors

Transcription activator-like effector (TALE) led to invention of Transcription activator-like effector nucleases (TALEN)

TALE vs. TALEN

- **TALEN services**
- A TAL effector nuclease (TALEN) contains a TALE DNA binding domain fused to the FokI nuclease. Two TALENs must bind on each side of the target site for FokI to dimerize and cut. TALENs induce a site-specific double-strand break (DSB), which is repaired by non-homologous end joining (NHEJ), which is error-prone and frequently causes small insertions or deletions ("indels") near the DSB site.
- These indels often cause frameshift mutations that can efficiently knock a gene out. Alternatively, an exogenous double-stranded donor DNA fragment can be introduced into the genome at the DSB by homologous recombination (HR).
- TALENs have been used to generate stably modified human embryonic stem cell and induced pluripotent stem cell (IPSCs) clones, and to generate knockout organisms such as mice, worm(*C. elegans*), and zebrafish.

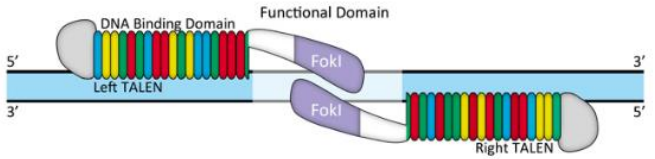


Figure 1. Illustration of TALEN design

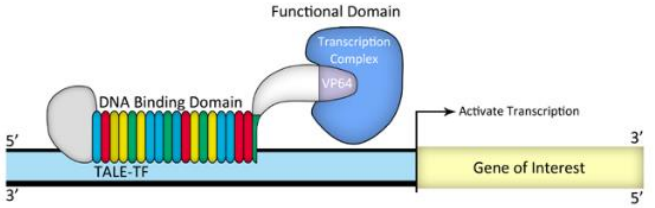


Figure 2. Illustration of TALE-TF design

GeneCopoeia,2021

The *hrp* genes

Dual-acting genes

Hypersensitive response and pathogenicity (hrp) genes control the ability of major groups of plant pathogenic bacteria to elicit the hypersensitive response (HR) in resistant plants and to cause disease in susceptible plants.

Pathogen-generated secreted proteins
Effectors
hrp/hrc genes and type III secretion system

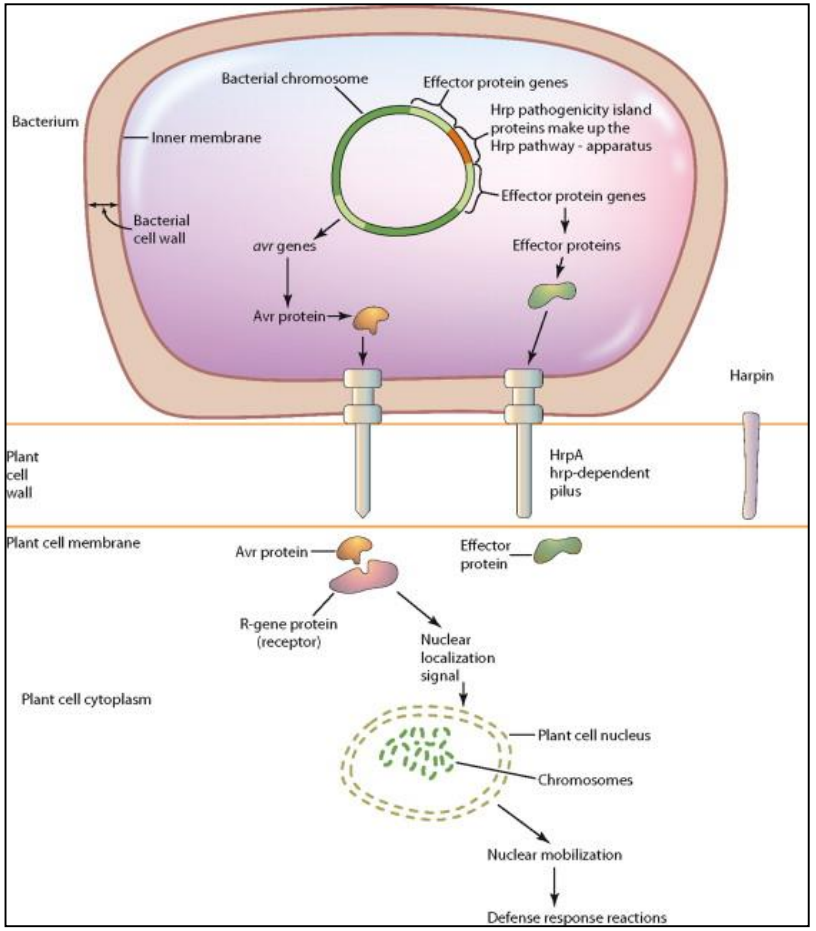
- In *P. syringae*,

1. *hrp*/*hrc* genes encode the Hrp (type III secretion) system, and
2. avirulence (*avr*) and Hrp-dependent outer protein (*hop*) genes encode effector proteins.

Collmer *et al.*,2000

Pathogen-generated secreted proteins
Effectors
hrp/hrc genes and type III secretion system

Hrp/Hrc-encoded proteins assemble an apparatus (TTSS) spanning the inner and outer bacterial membranes that enables the bacterium to deliver bacterial proteins (effectors), directly into the host cytoplasm.



Agrios,2005; Rodríguez-Puerto *et al.*,2021

Pathogen-generated secreted proteins
Effectors
hrp/hrc genes and type III secretion system

- The *P. syringae* TTSS is encoded by the hrp-hrc gene cluster.
- One of the genes within this cluster, hrpJ, which is a type III secreted protein that is required for:

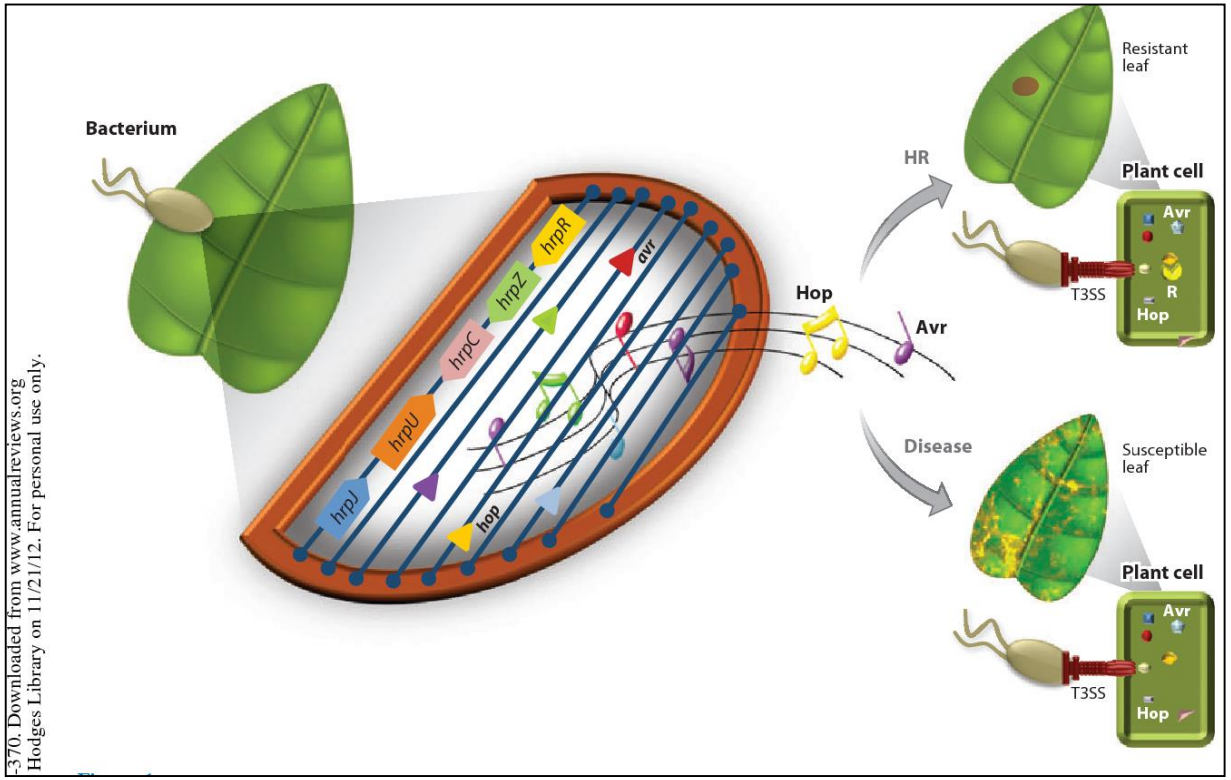
1. plant pathogenesis,
2. injection of effectors, and
3. secretion of the HrpZ1 Harpin.

- In the absence of hrpJ, specific accessory proteins, like HrpZ1, may not be extracellularly localized, resulting in disabled translocation of effectors into plant cells.

ZQ *et al.*,2006

Pathogen-generated secreted proteins
Effectors
hrp/hrc genes and type III secretion system

- HrpJ in hrp-hrc gene cluster is a type III secreted protein that is important for pathogenicity and the translocation of effectors into plant cells.



hrp gene organization

- The *hrp* (pronounced harp) genes in plant-pathogenic bacteria are present as clusters spanning roughly 25 kb.
- *hrp* genes are always clustered and are localized either:

1. on the bacterial chromosome, or
2. on a plasmid.

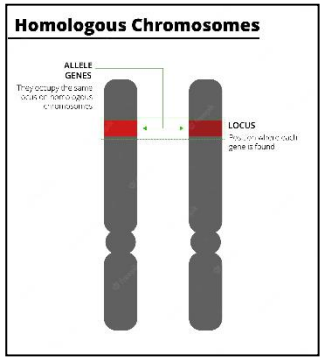
hrp/ hrc genes
Chromosome or megaplasmid borne

- In all genomes that have been sequenced to date, the *hrp/ hrc* genes are found clustered:

1. In a single region of the chromosome, or
2. On a 2.1-Mb megaplasmid in the case of *R. solanacearum*, or
3. A 150-kb plasmid in *Pantoea agglomerans* pv. *gypsophilae*.

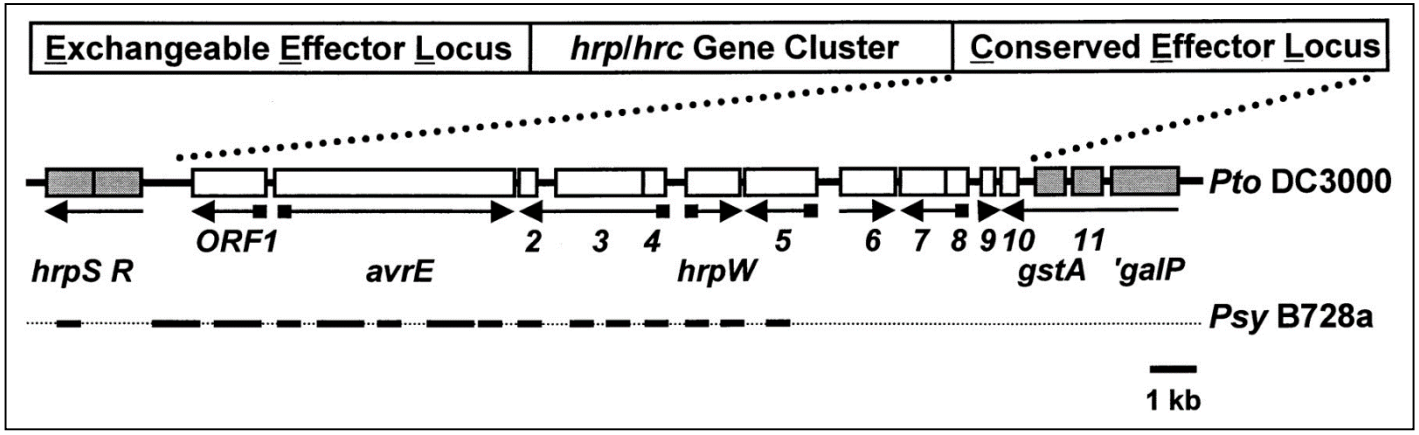
- These clusters of *hrp/hrc* genes are typically flanked by regions that contain different effector genes in different bacterial species or pathovars.

Alfano and Collmer,2004



hrp gene organization

- In *Pseudomonas syringae* the *hrp* cluster is flanked on both sides by effector genes.
- Several other *avr* genes are also located in the regions flanking the large pathogenicity gene (*hrp*) cluster.



Locus is a term that we use to tell us where on a chromosome a specific gene is. So it's really the physical location of a gene on a chromosome. In contrast to conserved hrp genes, the precise role of non-conserved hrp genes remains to be investigated.

Alfano *et al.*,2000

hrp/hrc genes
Functions

I. Assembling Hrp system/Hrp pilus:

- At least 9 *hrp* genes (termed *hrc* for *hrp* conserved) are conserved which encode components of the TTS system.
- The TTSS pathway is encoded by:

1. hrp (HR and pathogenicity) and
2. hrc (HR and conserved) genes.

II. Secretion or translaocation of proteins:

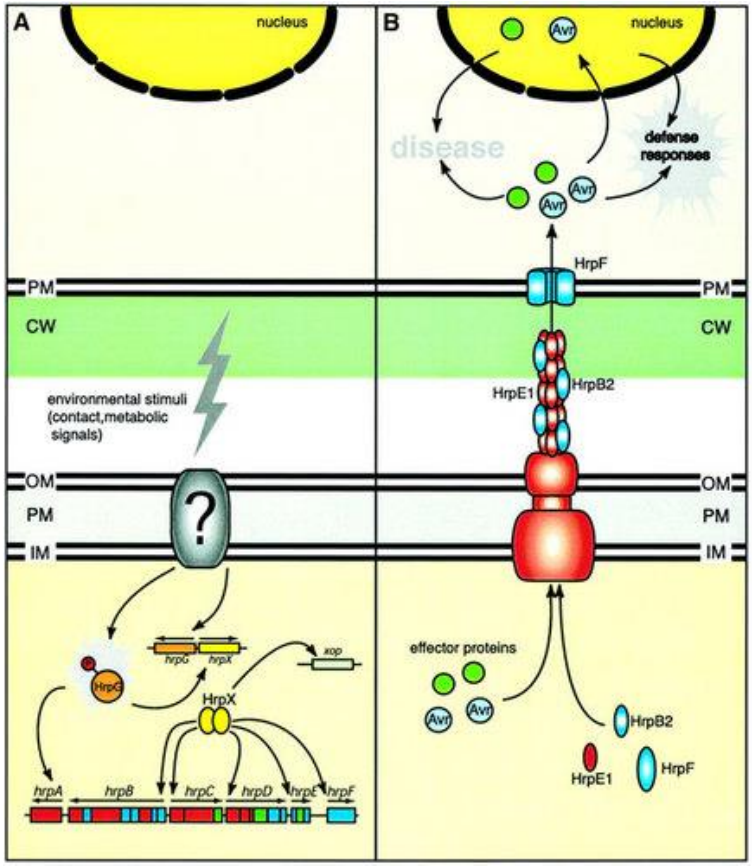
- Translocates effector proteins (e.g. Harpins) into the plant cell.
- Harpins are glycine-rich and heat-stable proteins that are secreted through type III secretion system in Gram-negative plant-pathogenic bacteria.

hrp/hrc genes

Model for *hrp* gene regulation and type III secretion in *X. vesicatoria*

A. HrpG activates the expression of *hrpA* and, via HrpX, the expression of *hrpB–hrpF* as well as of a number of *xop* genes.

B. Expression of *hrp* genes is essential for the formation of the TTS apparatus, which spans both bacterial membranes and mediates secretion of Hrp and effector proteins.



Büttner and Bonas,2002

The *hrp* genes
In plant bacterial pathogens

- The *hrp* gene cluster itself encodes and controls the secretion of a glycine-rich protein called harpin:
- HrpZ from *P. syringae*,
- HrpN from *E. amylovora*,
- PopA from *R. solanacearum*.
- In *Erwinia amylovora*, this protein was given the name harpin and the corresponding gene designated *hrpN*.
- This was the first example of such a protein and gene identified from any bacterial species.

Harpins
Functions

- Harpin is a single protein was identified that:

1. Elicited HR in non-host plants;
2. Necessary for pathogenesis.

- Mutations in *hrp* genes cause loss of the ability of the bacteria to:

1. Not elicit the HR in resistant plants;
2. Not cause disease symptoms.

- The mutants thus behave like non-pathogenic bacteria.

hrp genes
Physical characteristics of harpins

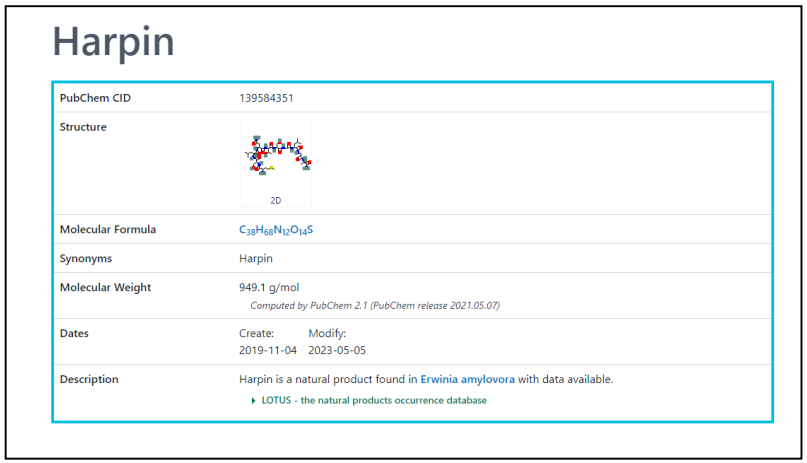
1. 403 amino acids in length;
2. Approximately 44 kD in molecular mass(small);
3. Heat stable: 100°C for 10 minutes does not eliminate biological activity;
4. Rich in glycine but lacks cysteine;
5. Water soluble;
6. Acidic;
7. With no known enzymatic activity.

- GenBank accession number for the amino acid sequence: AAC31644.

hrp genes

Physical characteristics of harpins

E. amylovora



Harpin is a natural product found in *Erwinia amylovora* with data available.

PubChem

hrp genes
Harpin Pro

- Protein isolated from a naturally occurring bacteria.

1. Harpin Pro stimulates the sugarcane to grow faster through increased photosynthesis.
2. The harpin protein triggers the plant into thinking it's under a bacterial attack.

- This false alarm creates a multi-faceted sequence of physiological and biochemical reactions that increase the plant's natural defenses and place the plant on high alert.

DuxAgri

Hrp/hrc genes

TTSSs and the proteins they secrete have been given a variety of names depending on the species of origin.

- Those *hrp* genes that are broadly conserved in:

1. Plant pathogenic bacteria (*Pseudomonas*, *Erwinia*, *Ralstonia*, *Xanthomonas*), and
2. Animal pathogenic bacteria (*Yersinia*, *Salmonella*, *Shigella* spp.).

- The TTSSs and the proteins they secrete have been given a variety of names depending on the species of origin.
- For example,

1. The TTSS from *Yersinia* spp. is called the Ysc system (Yop secretion), and
2. The effector proteins it secretes are Yops (*Yersinia* outer protein).

Hrc proteins of plant pathogenic bacteria and their animal pathogen and flagellar homologs

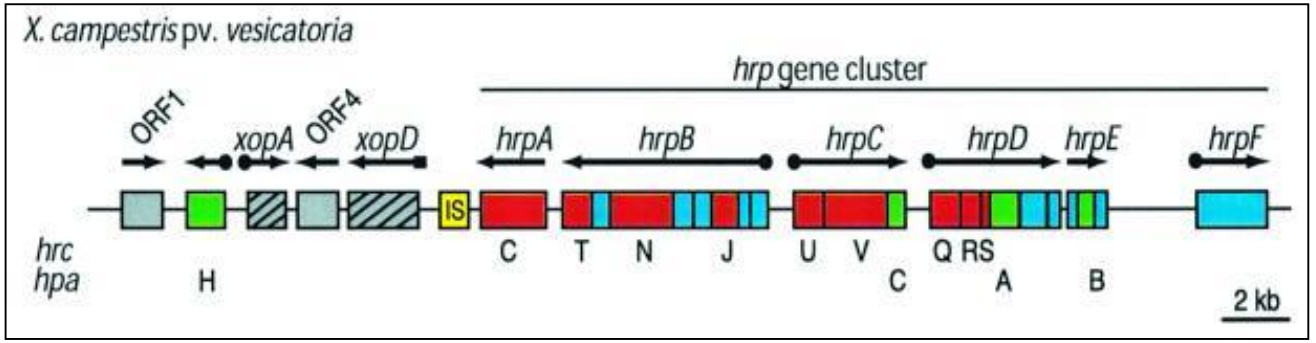
- The designations for Hrc (HR and conserved) homologs in various bacteria outside of the plant pathogen group.

Plant pathogen Protein	*Yersinia* protein	*Salmonella* protein	*Shigella* protein	Flagellar protein(s)
HrcC	YscC	InvG	MxiD	
HrcJ	YscJ	PrgK	MxiJ	FliF
HrcN	YscN	SpaL	Spa47	FliL
HrcQ	YscQ	SpaO	Spa33	FliN, -Y
HrcR	YscR	SpaP	Spa24	FliP
HrcS	YscS	SpaQ	Spa9	FliQ
HrcT	YscT	SpaR	Spa29	FliR
HrcU	YscU	SpaS	Spa40	FlhB
HrcV	LcrD	InvA	MxiA	FlhA

Alfano and Collmer,1997

Organization of the *hrp/hrc* gene cluster *hrp*-associated (*hpa*) genes *Xanthomonas vesicatoria*

- The *hrp* region also contains so-called *hrp*-associated (*hpa*) genes, which are not essential for bacterial pathogenicity but contribute to the interaction with the host plant. Hrp and *hpa* genes encode harpin and harpin-like proteins, respectively.



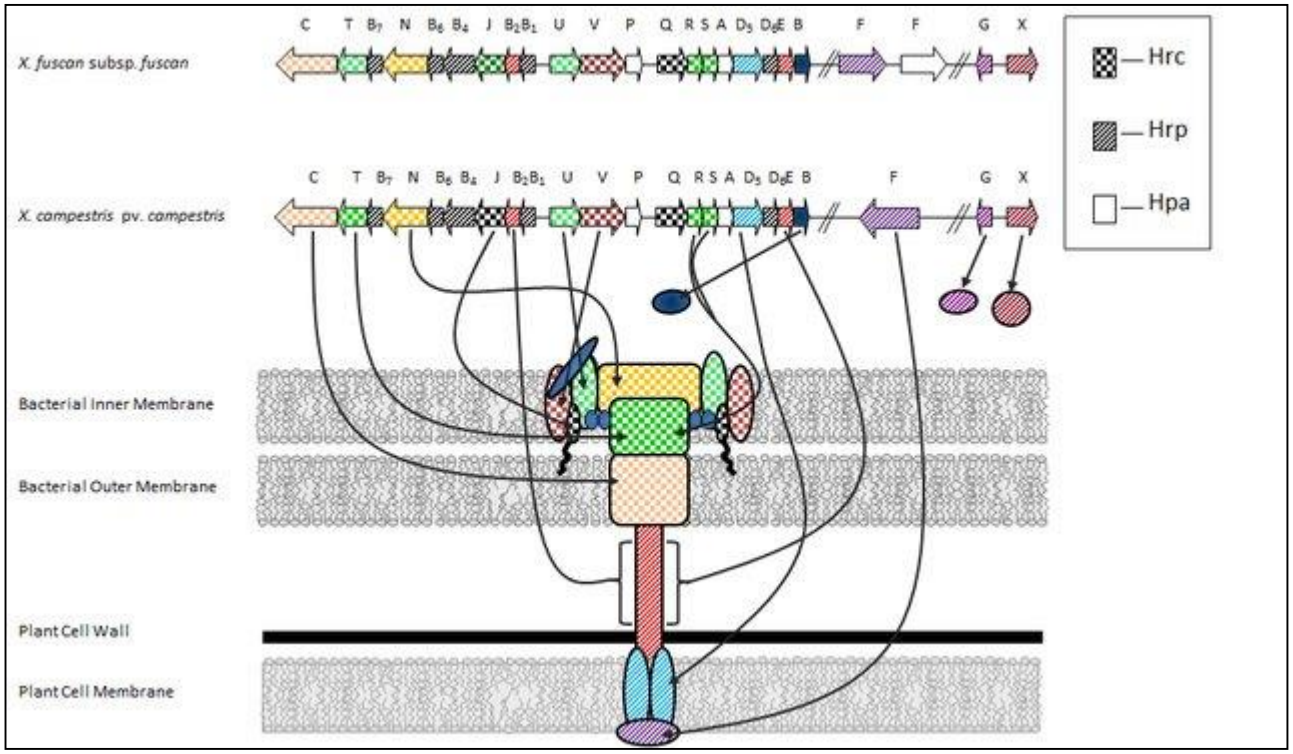
The regions contain hrp, hrc and hpa genes (represented in blue, red and green, respectively). Arrows indicate the direction of transcription. Black dots and squares refer to the presence of PIP(plant-inducible promoter) and hrp boxes, respectively. Hatched regions correspond to sequences with low G+C content; yellow regions refer to mobile genetic elements.

Büttner and Bonas,2002

Organization of the *hrp/hrc* gene cluster

hpa (hrp-associated) genes encode harpin-like proteins

Xanthomonas citri pv. *fuscans*



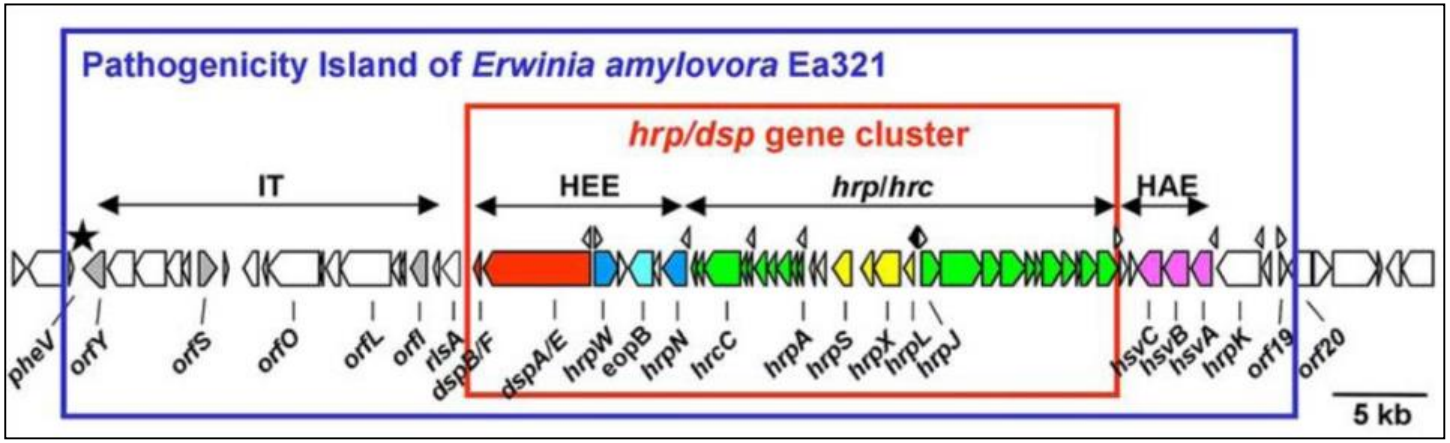
Overview of the Hrc, Hrp and Hpa genes from the Hrp cluster in *X. citri* pv. *fuscans*, the common bacterial blight (CBB) of dry bean along with a structural model of the TTSS from *X. campestris* pv. *campestris*. The genes in the Hrp cluster are associated with the type III secretion system which is used to inject effector molecules into the host cell.

Perry and Pauls,2011

Pathogen-generated secreted proteins Effectors

hrp or type III secretion system

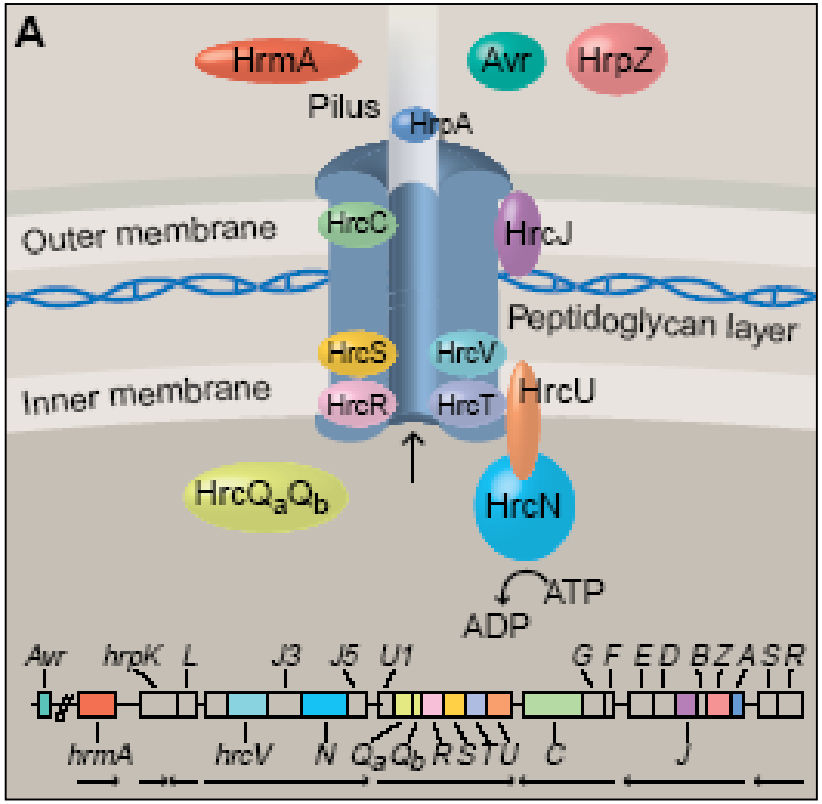
- Schematic map of the hrp pathogenicity island in *Erwinia amylovora* strain Ea321.
- IT, Island transfer region; HEE, Hrp effectors and elicitors region; hrp/hrc, hypersensitive reaction and conserved region; HAE, Hrp-associated enzyme region (From Oh and Beer,2005)



Lee,2014

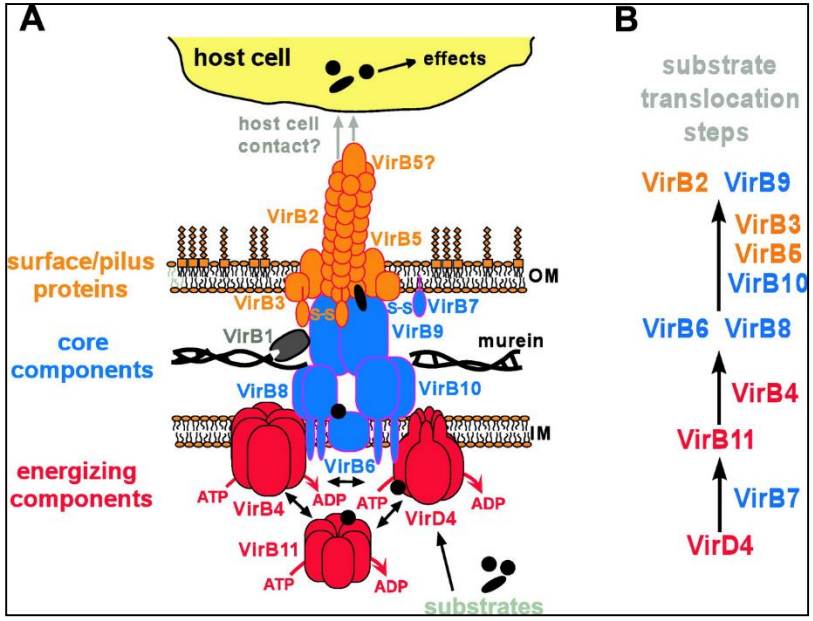
Organization of the *hrp/hrc* gene cluster
Hrp secretion system of *P. syringae*

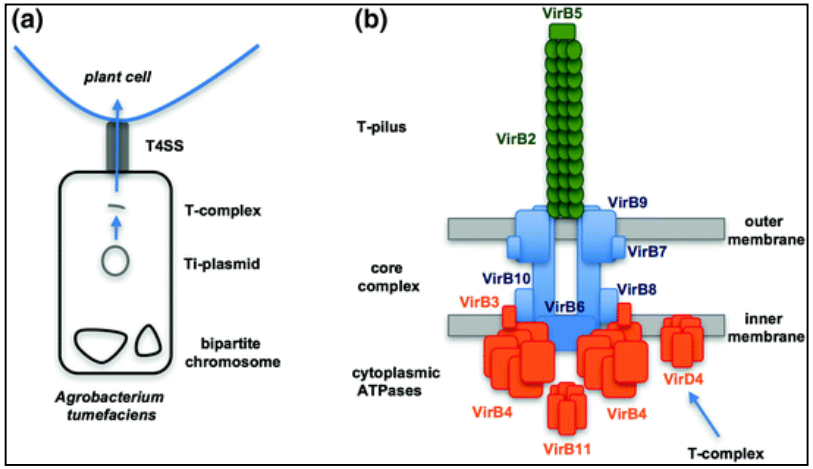
- The genomic organization of the *hrp/hrc* genes is presented in the lower section of the panel.
- HrcN has a conserved ATPase domain, which suggests that it may play a role in providing the energy needed to actively transport these proteins into the plant cell.
- *Avr* genes are often located in or adjacent to the *hrp* gene cluster.



A unified nomenclature for *P. syringae* T3SS-secreted proteins was established. The detailed naming system can be found in the website: www.pseudomonas-syringae.org.

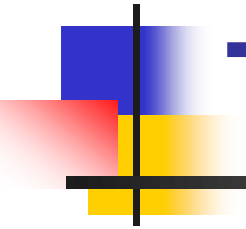
Pathogen-generated secreted proteins
Effectors
Type IV Secretion in *Agrobacterium tumefaciens*





The *Agrobacterium tumefaciens* VirB/D4 type IV secretion system (T4SS) comprises 12 membrane-bound proteins, and it assembles a surface-exposed T-pilus.

Baron,2006;Sharifahmadian and Baron,2018



The *R* genes

Induced plant proteins

Induced plant proteins
R proteins

- *R*-gene-encoded proteins have to fulfill two tasks:

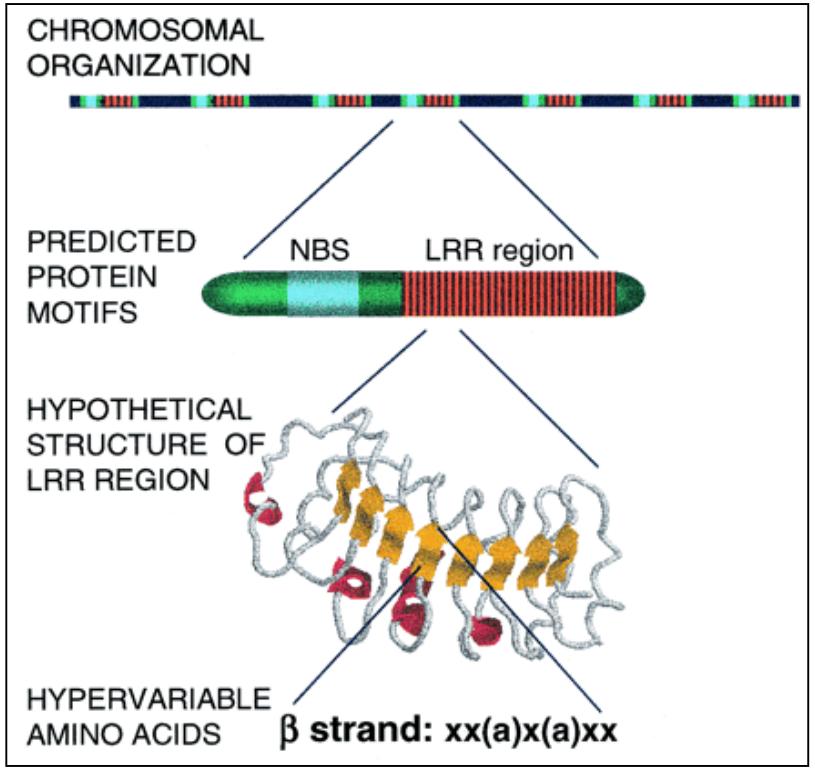
1. To recognize a pathogen-derived signal,
2. To initiate a coordinated plant defense reaction.

- In another word:

a. Effector recognition- Enable plants to detect *avr* gene specified pathogen molecules.
b. Signal transduction functions in order to activate plant defenses.

Induced plant proteins
R proteins

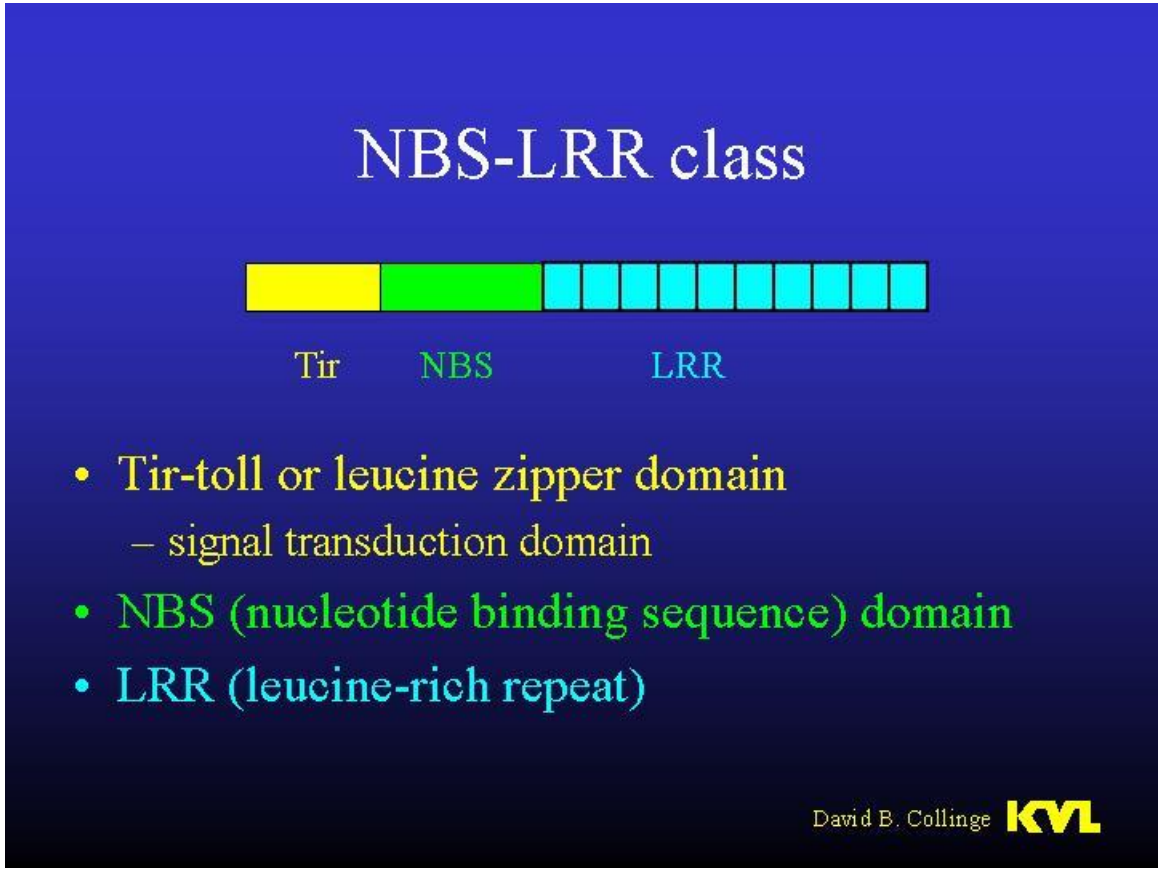
- Most *R* proteins contain:
  1. Leucine-rich repeats (LRRs),
  2. A central nucleotide-binding site (NBS), and
  3. A variable amino-terminal domain.
- The LRRs are mainly involved in recognition, whereas the amino-terminal domain determines signalling specificity.
- NBS functions as a molecular switches of plant defense.



Each LRR comprises a core of about 26 amino acids containing the Leu-xx-Leu-xx-Leu-x-Leu-xx-Cys/Asn-xx motif (where x is any amino acid), which forms a β-sheet).

Common structural features of *R* genes products

The structure of a NBS-LRR protein



The-Tir toll or leucine zipper domain

Induced plant proteins

Pathogenesis-related proteins (PRPs)

PR genes/PR proteins

- Pathogenesis-related proteins (PR proteins) play crucial roles in the plant defense system.
- PRPs as plant-derived molecules are induced in tissue infected by pathogens as well as systemically and are associated with the development of systemic acquired resistance (SAR).
- Infection of plants with pathogens often leads to an increase in the amounts of proteins belonging to several classes of PR proteins with antimicrobial activity.
- Toxicity of PRs can be generally accounted for by their hydrolytic, proteinase-inhibitory and membrane-permeabilizing ability.
- A special class of PRs inducers are hormones such as ethylene, jasmonates, abscisic acid, kinetin and auxins.

Induced plant proteins
Pathogenesis-related proteins(PRPs)

Family	Type member	Properties	Gene symbols
PR-1	Tobacco PR-1a	Unknown	*Ypr1*
PR-2	Tobacco PR-2	β-1,3-glucanase	*Ypr2*, [*Gns2* (‘*Glb*’)]
PR-3	Tobacco P, Q	Chitinase type I, II, IV, V, VI, VII	*Ypr3*, *Chia*
PR-4	Tobacco ‘R’	Chitinase type I, II	*Ypr4*, *Chid*
PR-5	Tobacco S	Thaumatin-like	*Ypr5*
PR-6	Tomato Inhibitor I	Proteinase-inhibitor	*Ypr6*, *Pis* (‘*Pin*’)
PR-7	Tomato P_{69}	Endoproteinase	*Ypr7*
PR-8	Cucumber chitinase	Chitinase type III	*Ypr8*, *Chib*
PR-9	Tobacco “lignin-forming peroxidase”	Peroxidase	*Ypr9*, *Prx*
PR-10	Parsley “PR1”	Ribonuclease-like	*Ypr10*
PR-11	Tobacco “class V” chitinase	Chitinase, type I	*Ypr11*, *Chic*
PR-12	Radish Rs-AFP3	Defensin	*Ypr12*
PR-13	Arabidopsis THI2.1	Thionin	*Ypr13*, *Thi*
PR-14	Barley LTP4	Lipid-transfer protein	*Ypr14*, *Ltp*
PR-15	Barley OxOa (germin)	Oxalate oxidase	*Ypr15*
PR-16	Barley OxOLP	Oxalate-oxidase-like	*Ypr16*
PR-17	Tobacco PRp27	Unknown	*Ypr17*

Further details can be found at http://www.bio.uu.nl/~fytopath/PR-families.htm.

Van Loon *et al.*,2006

DNA-binding proteins
Required for expression of new proteins

- The expression of proteins responsible for moment-to-moment chemical and structural tasks is critical to the functioning of living cells.
- This requires that the cell regulate the expression of new proteins.
- DNA-binding proteins play an important role in this process.
- These proteins interact with DNA by means of various structural motifs, and can stimulate or repress transcription of messenger RNA, depending on the properties and location of the DNA sequence to which they bind.

DNA-binding proteins

DNA-binding domains

DNA-binding motif

- Protein-protein and protein-nucleic acid interactions are essential functions of many proteins.
- DNA-binding proteins are proteins that are composed of DNA-binding domains.
- The role of the DNA-binding domain is to bring the transcription-activation domain into the vicinity of the promoter.

1. A domain can contain several motifs.
2. Motifs are short sequences and domains are longer ones.

- Only a few structural motif are responsible for binding DNA in a large number of different DNA-binding proteins.

DNA-binding proteins

DNA-binding domains

Transcription factors

- Any protein that is needed for the initiation of transcription is defined as a transcription factor.
- General transcription factors (GTFs), also known as basal transcriptional factors, are a class of protein transcription factors that bind to specific sites (promoter) on DNA to activate transcription of genetic information from DNA to messenger RNA.

In bacteria, transcription initiation requires:
1. an RNA polymerase, and
2. a single general transcription factor (GTF): sigma factor.

DNA-binding proteins

DNA-binding domains

Transcription factors

- Transcription factor (TF) binding sites or motifs (TFBMs):
- Structural motifs such as helix-turn-helix proteins are common types of motifs that are

1. found in different transcription factors, and
2. responsible for binding to DNA.

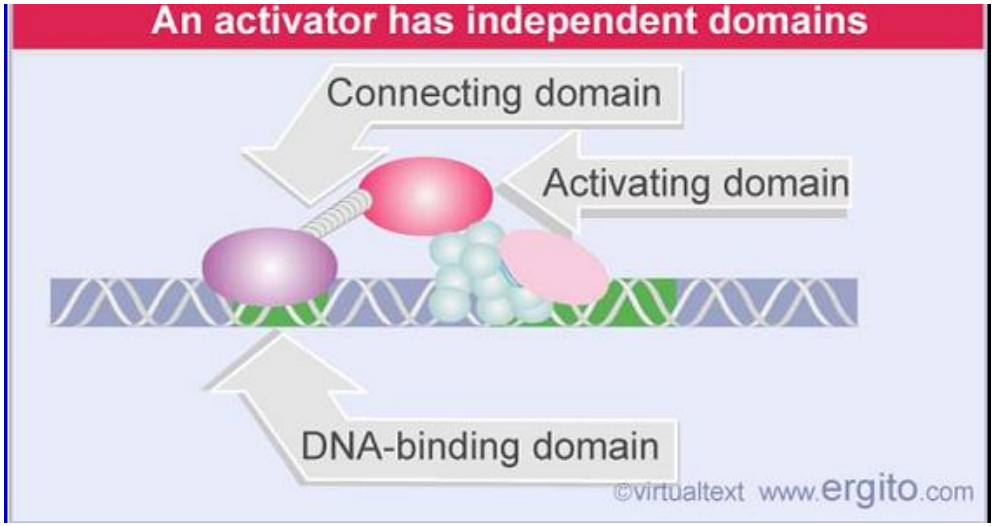
DNA-binding proteins

DNA-binding domain

DNA-binding motifs

- All transcription factors have two domains that are necessary for their function.

1. A DNA-binding domain (motifs that binds DNA such as helix-turn-helix, helix-loop-helix, leucine zipper, zinc finger,..);
2. A domain that activates transcription.

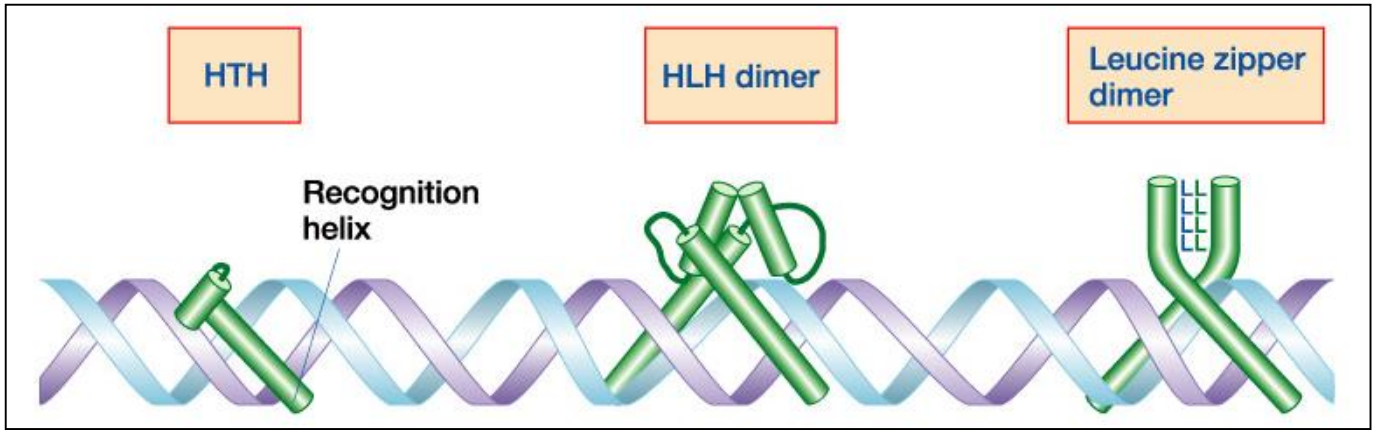


DNA-binding proteins

DNA-binding motifs

DNA-binding motifs

- A DNA-binding domain (DBD) contains at least one motif that recognizes double- or single-stranded DNA.
- This specific binding region is called a promoter.
- The transactivation domain (TAD) is where other proteins (co-regulatory proteins) bind to the transcription factor(in bacteria sigma factor).



M. F. Kusie; Wiki,..

DNA-binding proteins

DNA-binding domains

DNA-binding motif

- The word motif can sometimes mean the same thing as domain - for example, someone might refer to a "DNA-binding motif" in a protein.
- However, a motif is typically smaller than a domain, can occur in DNA, RNA, and proteins, and has to do with the specific sequence.
- As an example, the zinc finger motif is found in protein domains that bind DNA, RNA, and other proteins.

Motifs
DNA-binding motif

- A structural motif in a protein is something like a helix-loop-helix or a beta-hairpin turn that

1. Can appear in multiple different kinds of protein domains, and
2. doesn't necessarily have the same exact function in those different domains,
3. but typically has a fairly conserved sequence that is very similar.

DNA-binding proteins

DNA-binding domains

DNA-binding motif

- The three-dimensional structure of a single polypeptide chain is termed its tertiary structure.
- Tertiary structures are different combinations of the secondary structures (α helices, β strands, and loops).
- Tertiary structure is subdivided into certain portions that are termed:

1. Motifs, and
2. domains.

- Motifs are simple combinations of secondary structure that occur in:

1. many different proteins and which
2. carry out a similar function.

Motifs

Conserved regions of protein or DNA sequences

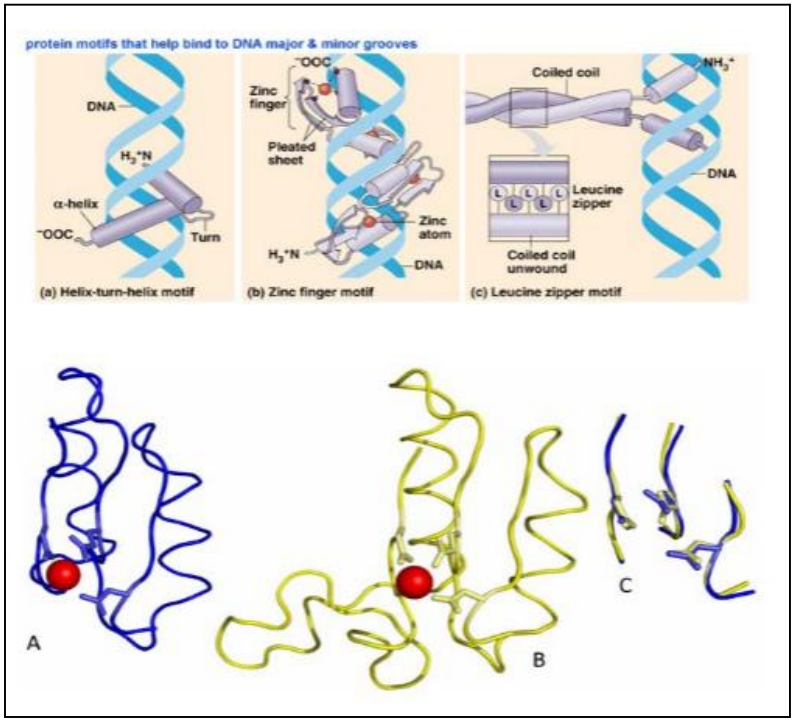
- In genetics, a sequence motif is:

1. a nucleotide, or
2. amino-acid sequence pattern.

Protein motifs

An amino-acid sequence pattern

- Protein motifs such as helix-turn-helix,.. helps RNA polymerase binds to DNA for transcription.
- helix-turn-helix motif was found both in eukaryotes and in prokaryotes.



A motif can occur in DNA, RNA, and proteins, and has to do with the specific sequence. E.g. the zinc finger motif is found in protein domains that bind DNA, RNA, and other proteins.

Prat Thiru;..

S-Layer

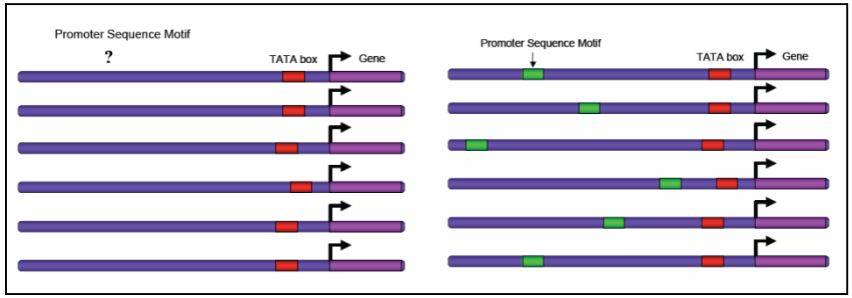
A surface protein layer

Glycoproteins

- S-layer is a surface protein layer found in many different bacteria and in some archaea where it serves as the cell wall.
- *Pseudomonas* and *Streptococcus* have slime layer.
- It is somewhat looser structures, more easily deformed layer.
- In certain bacteria the slime layer that surrounds the outermost components of cell walls are made up of glycoproteins of high molecular weight.
- Glycoprotein is a compound in which carbohydrate (sugar) is covalently linked to protein.

DNA motifs
A nucleotide sequence pattern

- In eukaryotes: the TATA promoter sequence is an example of a highly conserved DNA sequence motif.
- In prokaryotes: Either
- -10 box 5'-TATAAT-3 or
- -35 box 5'-TTGACA-3' promoter motifs/sequences.
- These sequences are usually found in the 5' promoter region of a gene.

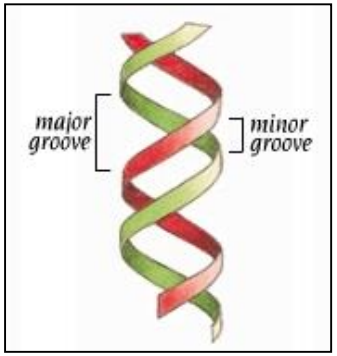


Prat Thiru;..

DNA-binding proteins

DNA-binding domains

Structural DNA-binding motif



- Each structural motif contains features that are highly conserved among many organisms.

1. Some proteins bind DNA in its major groove (e.g. the zinc finger).
2. some other in the minor groove (e.g. HMG-I(Y)–DNA complex), and
3. some need to bind to both (e.g. the Leucine Zipper Motif).

The major groove, being wider than the minor groove, can accommodate larger structural motifs.

Atlas Genet Cytogenet Oncol Haematol, 2003

DNA-binding proteins

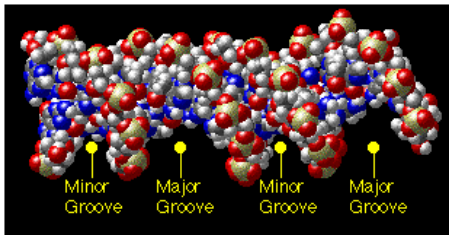
DNA-binding domains(DBDs)

Specific and non-specific DNA-binding motifs

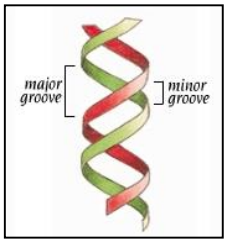
- **Sequence-specific DNA-binding motifs**
- Many DNA-binding domains must recognize specific DNA sequences, such as DBDs of transcription factors that activate specific genes.
- Sequence-specific DNA-binding proteins generally interact with the major groove of DNA.
- **Non-specific DNA-binding motifs**
- Nonspecific DNA binding generally involves simple attachment through either of the DNA grooves.
- E.g. structural proteins around DNA. These proteins organize the DNA into a compact structure.

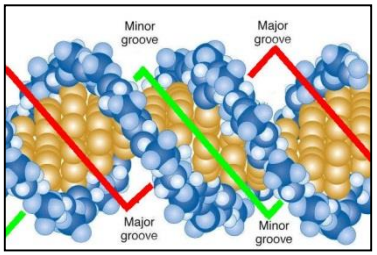
DNA

Major and minor grooves of DNA



- The sugar-phosphate backbones spiral around the outer surface of DNA.
- The strand backbones are closer together on one side of the helix than on the other.
- The major groove occurs where the backbones are far apart, the minor groove occurs where they are close together. The grooves twist around the molecule on opposite sides.
- The major groove is approximately 50% wider than the minor.
- The major groove, is 22 Å wide and the minor groove, is 12 Å wide.
- The major and minor grooves are opposite each other.

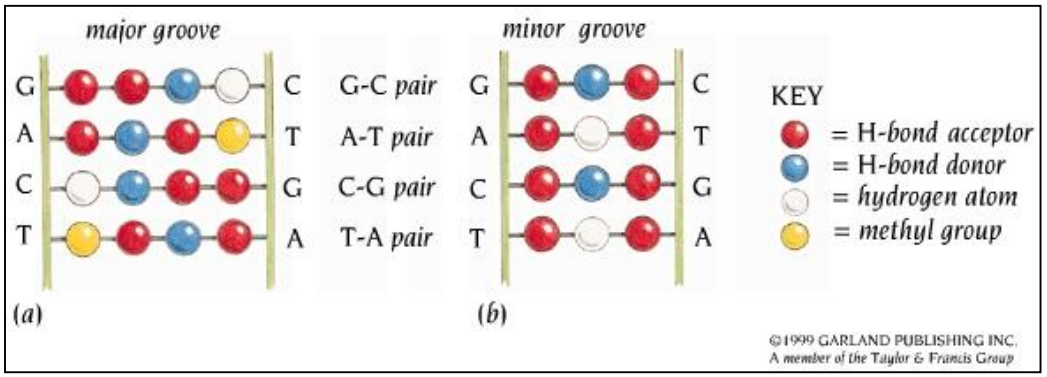




DNA

Major and minor grooves of DNA

- The major groove has twice the information content of the minor groove.
- A-T base pairs can be differentiated from G-C pairs via either groove.
- But minor groove could not discriminate between A-T and T-A base pairs, or G-C and C-G.
- Hence the major groove has a four-symbol code, whereas the minor groove has only a two-symbol code, and only half information content.



DNA-binding proteins

DNA-binding domains

DNA-binding motif

Motif	Examples of proteins with this motif
1. Sequence-specific DNA-binding motifs	
Helix-turn-helix family	
Standard helix-turn-helix	*Escherichia coli* lactose repressor, tryptophan repressor
Homeodomain	*Drosophila* Antennapedia protein
Paired homeodomain	Vertebrate Pax transcription factors
POU domain	Vertebrate regulatory proteins PIT-1, OCT-1 and OCT-2
Winged helix-turn-helix	GABP regulatory protein of higher eukaryotes
High mobility group (HMG) domain	Mammalian sex determination protein SRY
Zinc-finger family	
Cys_2His_2 finger	Transcription factor TFIIIA of eukaryotes
Multi-cysteine zinc finger	Steroid receptor family of higher eukaryotes
Zinc binuclear cluster	Yeast GAL4 transcription factor
Basic domain	Yeast GCN4 transcription factor
Ribbon-helix-helix	Bacterial MetJ, Arc and Mnt repressors
TBP domain	Eukaryotic TATA-binding protein
β-Barrel dimer	Papillomavirus E2 protein
Rel homology domain (RHB)	Mammalian transcription factor NF-κB
2. Non-specific DNA-binding motifs	
Histone fold	Eukaryotic histones
HU/IHF motif(a histone-like motif)	Bacterial HU and IHF proteins
Polymerase cleft	DNA and RNA polymerases

DNA-binding proteins

DNA-binding domains

DNA-binding motif

- DNA-binding motifs are regions of regulatory proteins which bind to DNA. e.g.

1. helix-turn-helix motif (originally discovered in bacteria. Common to many prokaryotes);
2. Helix-loop-helix motif (eukaryotic transcription factors);
3. zinc finger motif (common DNA-binding motifs found in eukaryotic transcription factors, and have also been identified in prokaryotes);
4. leucine zipper motif (eukaryotic transcription factors).

Leucine zippers are a dimerization domain of the bZIP (Basic-region leucine zipper) class of eukaryotic transcription factors.

DNA-binding proteins

DNA-binding domains

Structural DNA-binding motif

1. **Zinc finger motif:** It contains one or more zinc ions which are crucial for the structural stability.
2. **Helix-turn-helix motif(HTH):** It consists of two α helices and a short extended amino acid chain between them (composed of three elements). Not to be confused with the basic **helix-loop-helix domain(HLH)**.
3. **Leucine zipper motif:** It is formed by two α helices, which are held together by hydrophobic interactions between leucine residues. The leucine zipper generally appears as a dimer of a helices that form a coiled coil.
4. **Helix-loop-helix motif(HLH):**
   It is characterized by two α helices connected by a loop.

DNA-binding proteins

DNA-binding domains

DNA-binding motifs

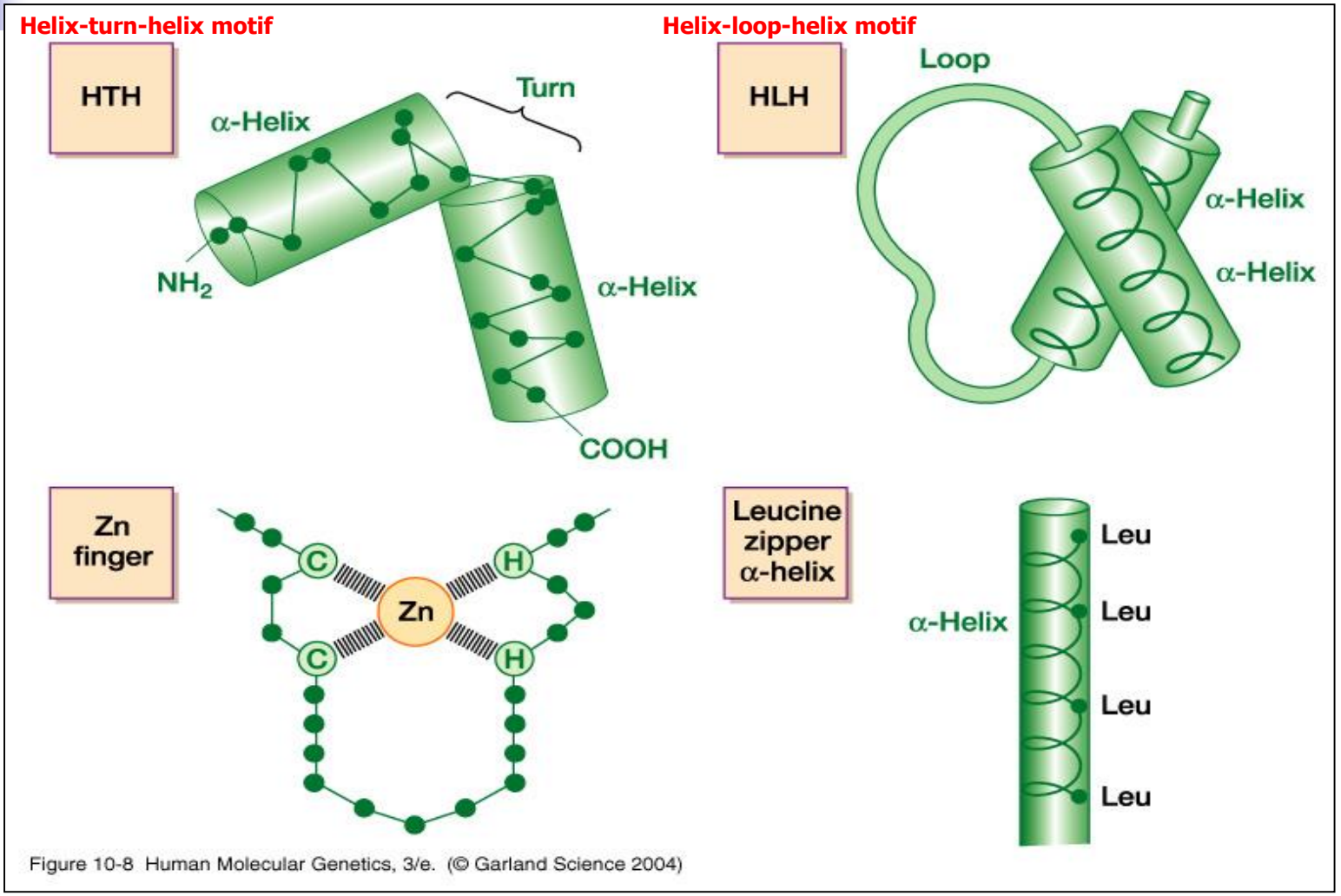
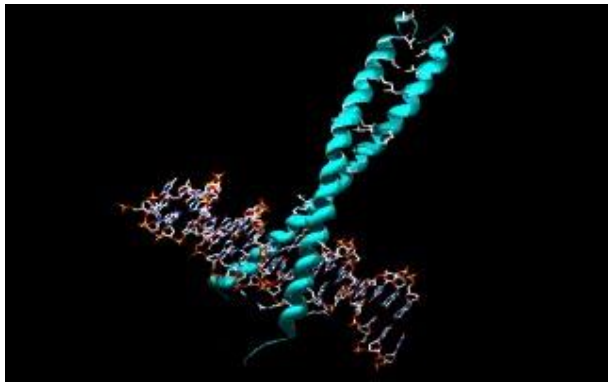


Figure 10-8 Human Molecular Genetics, 3/e. (© Garland Science 2004)

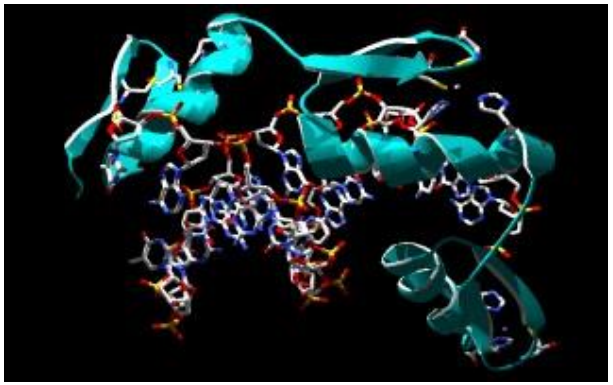
DNA-binding proteins

DNA-binding domains

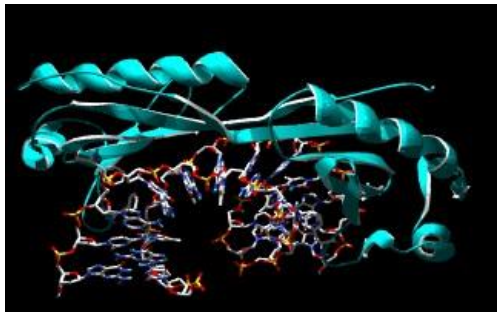
Structural DNA-binding motifs



Leucine zipper



Zif 286 protein (zinc finger)



TATA-binding Protein

It binds a highly conserved sequence (TATAAAAG) in the promoter of eukaryotes.

Sequence-specific DNA-binding motifs
Helix-turn-helix motif
Interacts with the major groove of DNA

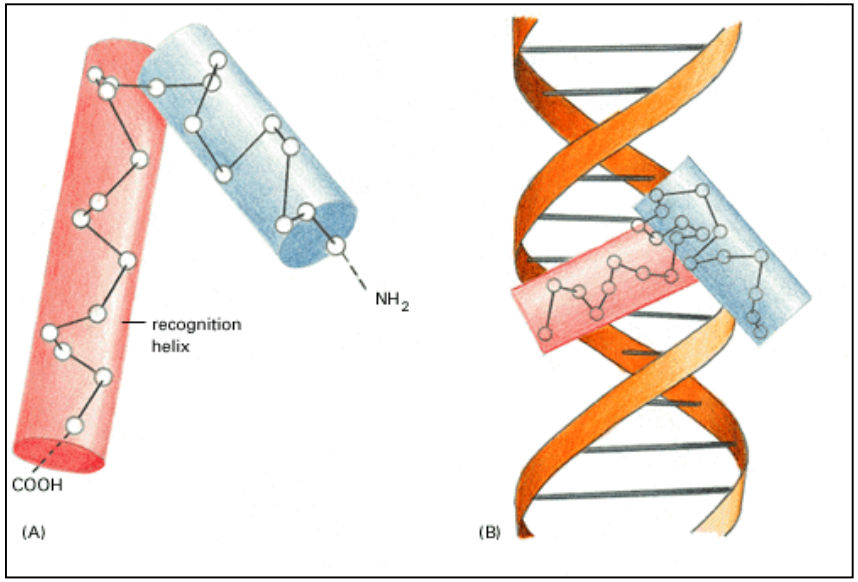
- The helix-turn-helix motif was first recognized in prokaryotic DNA-binding proteins.
- It is composed of two α-helices joined by a short strand of amino acids (turn).
- The recognition and binding to DNA by helix-turn-helix proteins is done by the two α helices, one occupying the N-terminal end of the motif, the other at the C-terminus.
- C-terminus recognition helix interacts with the major groove of DNA, and hence it is often called the "recognition helix".
- The other α helix stabilizes the interaction between protein and DNA, but does not play a particularly strong role in its recognition.

Sequence-specific DNA-binding motifs
Helix-turn-helix motif
Interacts with the major groove of DNA

A. Each white circle denotes the central carbon of an amino acid.

B. The carboxyl-terminal alpha helix (red) is called the recognition helix because it participates in sequence-specific recognition of DNA.

- As shown in (B), this helix fits into the major groove of DNA, where it contacts the edges of the base pairs.

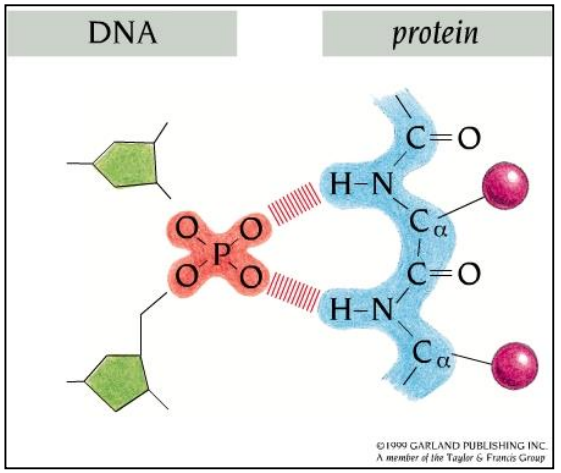


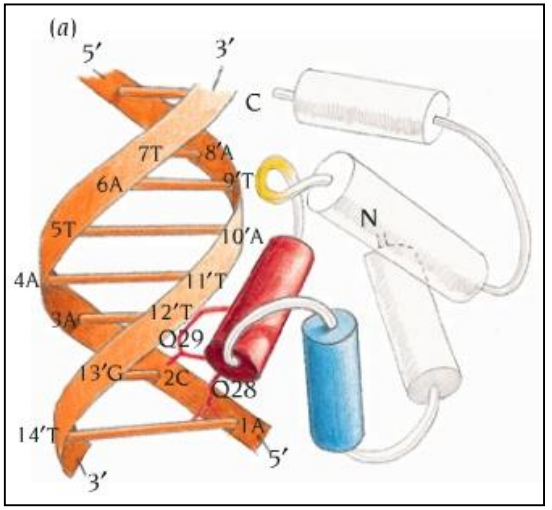
HTH consists of two α helices.

Alberts *et al.*,1994

Sequence-specific DNA-binding motifs
Helix-turn-helix motif
Interacts with the major groove of DNA

- Helix-turn-helix motif is the most common DNA-binding motif in prokaryotes.
- One of the helices, DNA recognition helix, gets inserted in the major groove of DNA
- Helix-turn-helix proteins are often dimeric.
- Why dimeric? Dimer binds to DNA stronger than monomer.
- By changing the relative positions of monomers, the dimer activity can be easily turned on and off.

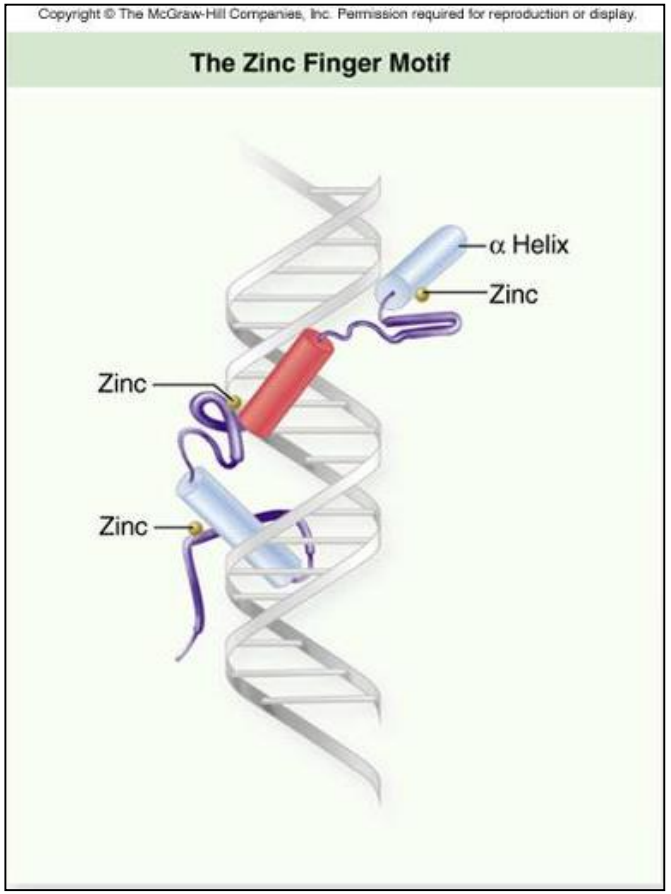




Sequence-specific DNA-binding motifs
The Zinc Finger motif
Interacts with the major groove of DNA

- The zinc-finger motif comprises a DNA-binding domain.
- A zinc finger is a small protein structure motif that is characterized by the coordination of one or more Zinc ions.

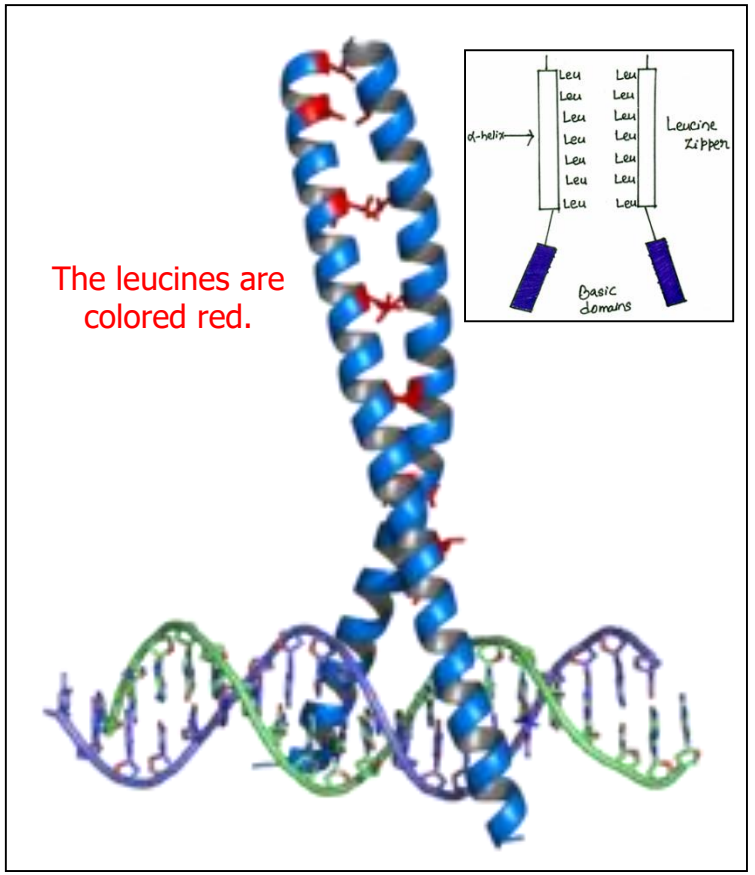
1. The C-terminal part of each finger forms helices that bind DNA ;
2. The N-terminal part form sheets.



Wikipedia;..

Sequence-specific DNA-binding motifs
The Leucine Zipper motif
Interacts with the major groove of DNA

- Leucine Zipper (blue) bound to DNA.
- The leucine residues that represent the 'teeth' of the zipper are colored red.
- A leucine zipper is formed by two α helices, one from each monomer.
- The two leucine zippers in effect form a Y-shaped structure, in which the zippers comprise the stem, and the 2 basic regions bifurcate symmetrically to form the arms that bind to DNA.



Sequences specific DNA-binding motifs

Transcriptional factors (TFs)

Bind to either enhancer or promoter regions of DNA

- DNA-binding proteins that regulate transcription by binding DNA are called transcription factors (TFs).
- Transcriptional factors (TFs) are site-specific transcription factors that recognize and bind specific DNA sequences.
- The whole mechanism of transcription depends highly on the cooperation of TF proteins.

Wikipedia;..

Sequences specific DNA-binding motifs
Transcriptional factors (TFs)
Bind to either enhancer or promoter regions of DNA

- Transcription factor (sometimes called a sequence-specific DNA-binding factor) is a protein that binds to specific DNA sequences.
- TFs acting as flags on DNA and regulating the transcription of genes.
- In general, transcription factors are dimeric, each with one helix containing basic amino acid residues that facilitate DNA binding.

Transcription

RNA polymerase in different cells

Promoters and transcription factors

- The proteins that sit on the promoter are called transcription factors (TFs).
- TFs and promoters work together:

1. to turn a gene on (and make a protein), or
2. to turn a gene off (and stop making a protein), or even
3. to just make more or less protein.

The Tech Museum of Innovation,2013

Transcription

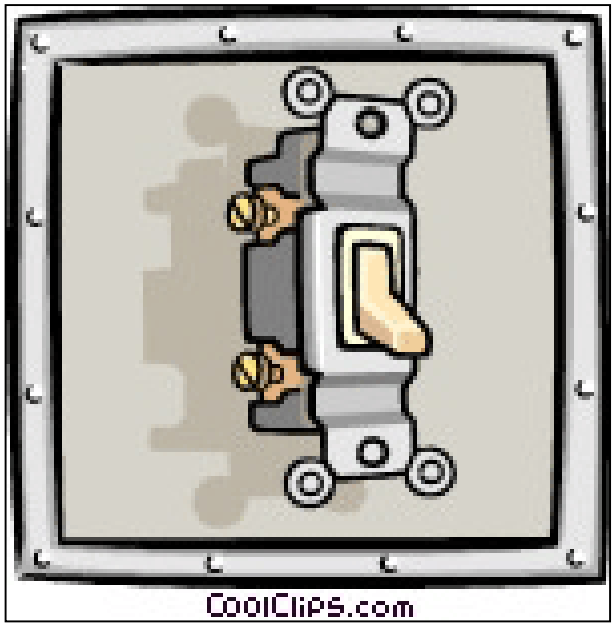
RNA polymerase in different cells

Gene promoters

- Let's think of turning on a gene, like turning on a light bulb in a room.
- Our gene is the light bulb and its promoter is the light switch.

1. TFs are called activators when they turn genes on.
2. TFs are called repressors when they turn off a gene.

- Note: the light can't be turned on at all.



A gene's light switch is called a promoter. TFs can either turn genes on or off.

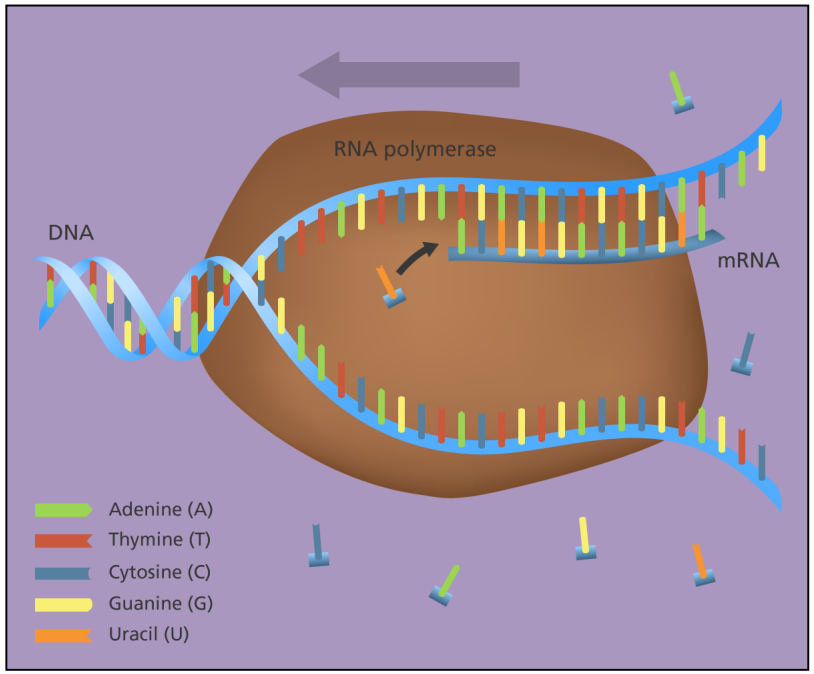
The Tech Museum of Innovation,2013;..

Prokaryotic DNA transcription

RNA polymerase copy DNA to RNA(mRNA)

Phases of transcription

1. Initiation: Binding of RNA polymerase to promoter, unwinding of DNA, formation of primer.
2. Elongation: RNA polymerase catalyzes the processive elongation of RNA chain, while unwinding and rewinding DNA strand.
3. Termination: termination of transcription and disassemble of transcription complex.



A ρ factor (Rho factor) is a prokaryotic protein involved in the termination of transcription.

Prokaryotic vs. Eukaryotic RNA Polymerase

RNA polymerases come in different forms, but share many features

- In eukaryotic cells, there are three different RNA polymerases (RNA Pol).
- Prokaryotic cell has one.
- Each RNA Pol is responsible for a different class of transcription:

1. PolI transcribes rRNA (ribosomal RNA),
2. PolII mRNA (messenger RNA), and
3. PolIII tRNA (transfer RNA) and other small RNAs.

TABLE 12-1 The Subunits of RNA Polymerases

Prokaryotic		Eukaryotic		
Bacterial	**Archaeal**	**RNAP I**	**RNAP II**	**RNAP III**
Core	**Core**	**(Pol I)**	**(Pol II)**	**(Pol III)**
β'	A′/A″	RPA1	RPB1	RPC1
β	B	RPA2	RPB2	RPC2
α^{I}	D	RPC5	RPB3	RPC5
α^{II}	L	RPC9	RPB11	RPC9
ω	K	RPB6	RPB6	RPB6
	[+6 others]	[+9 others]	[+7 others]	[+11 others]

Note: The subunits in each column are listed in order of decreasing molecular weight.

Source: Data adapted from Ebright R.H. 2000 *J. Mol. Biol.* **304:** 687–698, Fig. 1, p. 688. © 2000 Academic Press.

The eukaryotic RNAPs recognize different promoters. RNA pol II can bind to a DNA sequence within the promoter of many genes, known as the TATA box, to initiate transcription.

Prokaryotic vs. Eukaryotic RNA Polymerase

RNA polymerases come in different forms, but share many features

- Bacteria have only a single RNA polymerases.
- This one RNA polymerase (with the help of sigma factor) synthesizes all classes of RNA:

1. mRNA,
2. rRNA,
3. tRNA.

TABLE 12-1 The Subunits of RNA Polymerases

Prokaryotic		Eukaryotic		
Bacterial	**Archaeal**	**RNAP I**	**RNAP II**	**RNAP III**
Core	**Core**	**(Pol I)**	**(Pol II)**	**(Pol III)**
β'	A′/A″	RPA1	RPB1	RPC1
β	B	RPA2	RPB2	RPC2
α^{I}	D	RPC5	RPB3	RPC5
α^{II}	L	RPC9	RPB11	RPC9
ω	K	RPB6	RPB6	RPB6
	[+6 others]	[+9 others]	[+7 others]	[+11 others]

Note: The subunits in each column are listed in order of decreasing molecular weight.

Source: Data adapted from Ebright R.H. 2000 *J. Mol. Biol.* **304:** 687–698, Fig. 1, p. 688. © 2000 Academic Press.

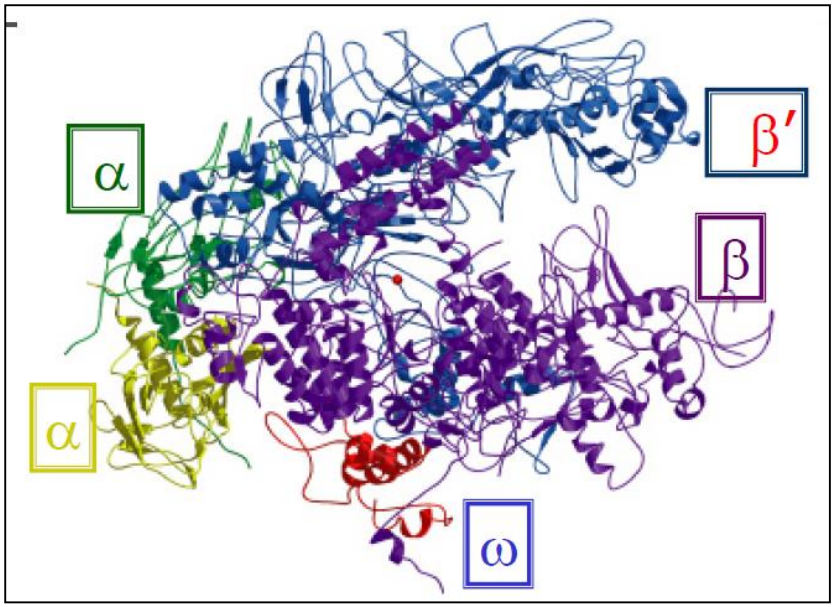
The role of ω(omega) has been unclear.

Note: the sigma factors are a class of proteins which are needed for recognizing the core promoter region for transcriptional initiation.

Prokaryotic RNA polymerase

The enzyme without the sigma subunit is called the 'core' enzyme and with sigma subunit is called the holoenzyme

- One type exists;
- The core enzyme has five subunits ($\beta\beta'\alpha_2\omega$):
- Two alpha, beta, beta', sigma and omega subunits.
- Only the first three subunits (alpha, beta, beta') are required for polymerase activity and RNA synthesis.
- A sigma factor (σ factor) is a protein needed only for initiation of RNA synthesis.
- It is a bacterial transcription initiation factor (equivalent of transcription factors in eukaryotes).



RNA polymerase core enzyme is a multimeric protein α, β, β′, ω.
The β′ subunit is involved in DNA binding. The β subunit contains the polymerase active site.
The two α subunits acts as scaffold on which the other subunits assemble.
ω may facilitates assembly of RNAP and stabilizes assembled RNAP.

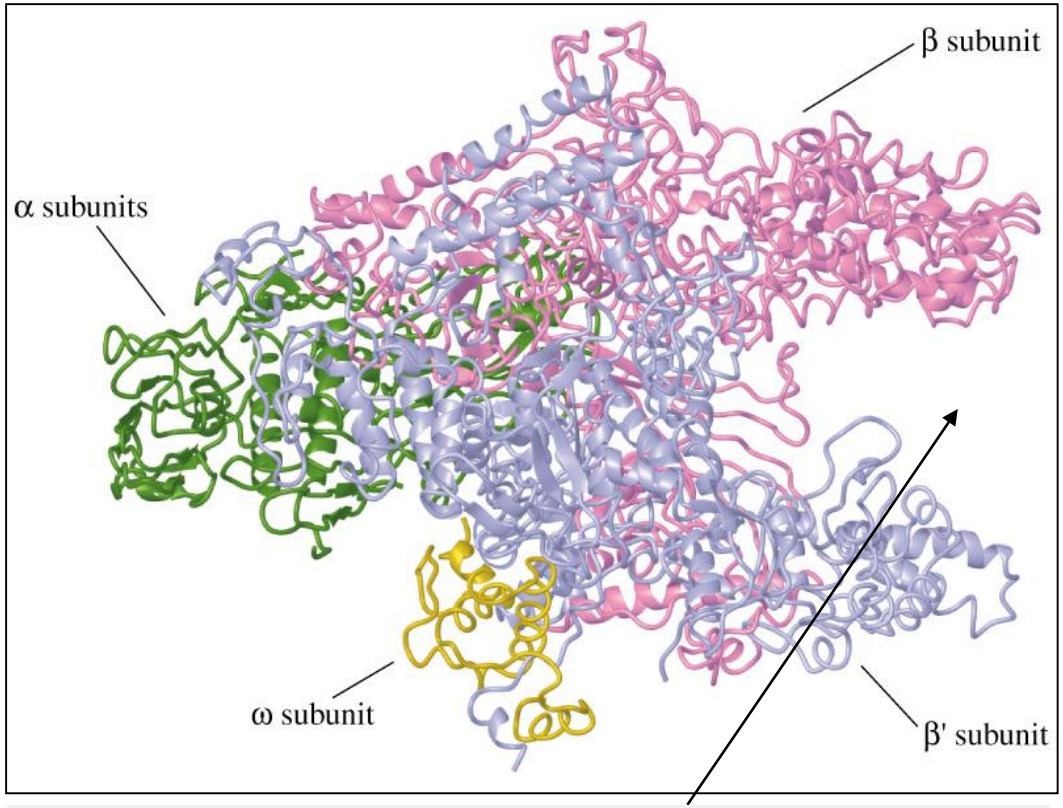
Prokaryotic RNA polymerase

The enzyme without the sigma subunit is called the 'core' enzyme

- **β′ Subunit:**
- The β′ subunit is involved in DNA binding.
- Its Mol. wt is 155-160 KD, coded for by rpo-C gene.
- This is perhaps one of the largest proteins produced by the bacteria.
- The strong affinity for DNA stems from its COO-terminal, which has a zinc finger motif, this region provides strong DNA binding forces.
- Beta and beta′ together bind to both strands of DNA, but catalytic activity is located in β subunit.

Prokaryotic RNA polymerase

The enzyme without the sigma subunit is called the 'core' enzyme



Site of DNA binding and RNA polymerization.

Transcription

Bacterial promoters

Zinc finger motif

- Both zinc ions are bound to β′ of RNA polymerase.
- The β′ residues coordinating zinc are conserved throughout eubacteria and chloroplasts.
- Four cysteine residues in the *Escherichia coli* RNAP largest subunit, β′ was locate that one of the two zinc ions tightly associated with the RNA polymerase enzyme.
- In the absence of zinc, or when zinc binding is prevented by mutation, the RNAP gets non-functional.

Marko *et al.*,1999

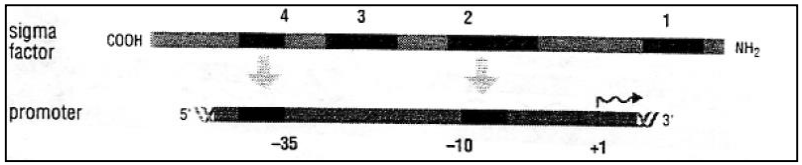
Transcription by RNA polymerase proceeds in a series of steps

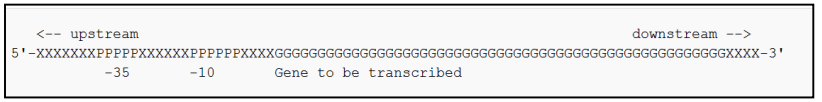
Bacterial promoters

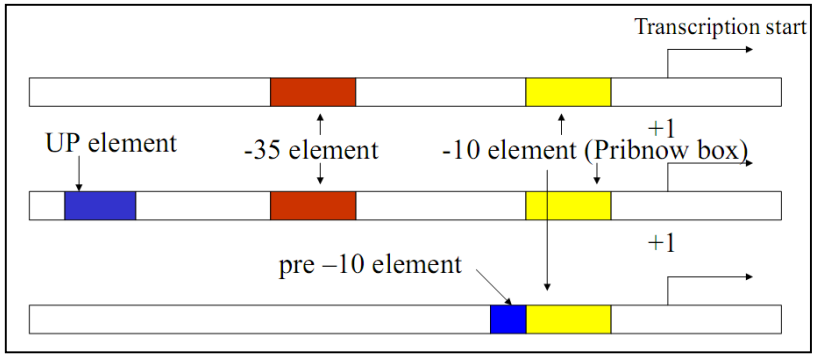
- Most bacterial promoters have –35 and –10 elements (regions).

1. Some have UP element (upstream promoter element).
2. Some lack –35 element, but have extended –10 region.

- Note also that molecular biologists use a numbering system which has no zero!
- The first nucleotide of the RNA transcript is numbered +1;
- Position +1 is the transcription start site.
- The nucleotide immediately upstream from that is numbered -1.



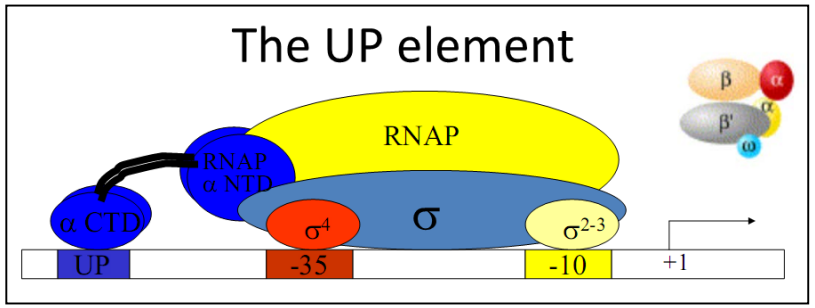




- **Pribnow Box: A region of DNA to which RNA polymerase binds before initiating the transcription of DNA into RNA.**
- **The -35 region and the -10 ("Pribnow box") region comprise the basic prokaryotic promoter.**
- **The DNA is unwound and becomes single-stranded ("open") in the vicinity of the initiation site (defined as +1).**

Transcription by RNA polymerase proceeds in a series of steps
Bacterial promoters- UP element

- Some bacterial promoters have UP element (upstream promoter element).
- UP element is an AT rich motif present in some strong promoters (e.g. rRNA).
- UP element interacts directly with C-terminal domain of RNA polymerase a subunits.



Transcription
Bacterial promoters

- In bacteria, the promoter contains two short sequence elements approximately -10 and -35 nucleotides upstream from the transcription start site.
- A combination of approaches shows that the -10 TATAAT and -35 TTGACA sequences are the essential DNA sequences in most *E. coli* promoters.

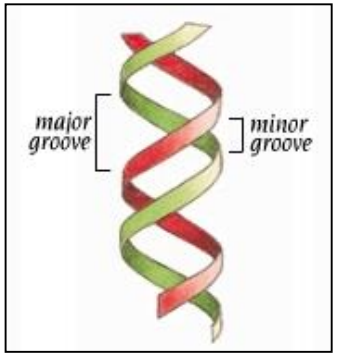
1. The sequence at -10 has the consensus sequences/motif TATAAT.
2. The sequence at -35 has the consensus sequences TTGACA/motif.

Wikipedia,2015;..

Transcription

Bacterial promoters

DNA binding motifs



1. The helix-turn-helix motif(HTH), used by most σ-factors, maintains its specificity and accuracy by binding in the major groove of DNA, where it can interact with the base pairs in the DNA double-helix.

- One such DNA-binding motif, the helix-turn-helix motif helps specifically recognize DNA promoters at both the -35 and -10 positions.
- The HTH motif is commonly used for transcriptional repression. HTH motifs are observed in the *lac* repressor.

2. As mentioned earlier the β' subunit of RNA polymerase has a zinc finger motif, provides strong DNA binding forces.

Transcription

Bacterial promoters

σ^{70}(RpoD) is the housekeeping sigma factor or also called as primary sigma factor, transcribes most genes in growing cells

- Every cell has a "housekeeping" sigma factor that keeps essential genes and pathways operating.
- In the case of *E. coli* and other gram-negative rod-shaped bacteria, the "housekeeping" sigma factor is σ^{70}.
- Promoter sequences are recognized only by RNA polymerase holoenzyme containing Sigma-70.
- The sigma 70 subunit of RNA polymerase recognize and contacts both the -35 and the -10 boxes.

```
  <-- upstream                                                    downstream -->
5'-XXXXXXXPPPPPXXXXXXPPPPPPXXXXGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGXXXX-3'
          -35       -10       Gene to be transcribed
```

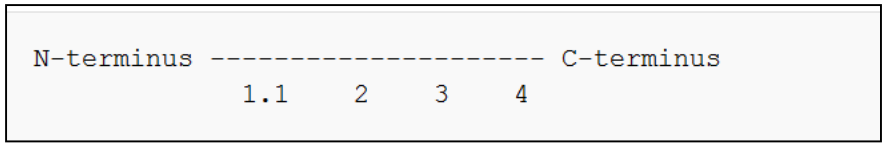
Wikipedia,2015

Transcription

Bacterial promoters

Promoter for mRNA transcription is recognized by sigma subunit of RNA polymerase

- Sigma factors have four main regions that are generally conserved:



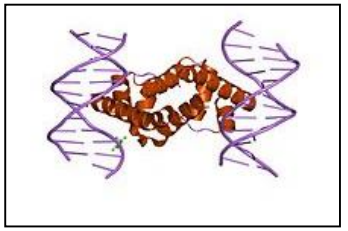
- The regions are further subdivided (e.g. 2 includes 2.1, 2.2, etc.)
- Region 1.1 is found only in "primary sigma factors" (RpoD σ^{24}, σ^{28}), RpoS or σ^{38} in *E. coli*). It is involved in ensuring the sigma factor will only bind the promoter when it is complexed with the RNA polymerase.
- Region 2.4 recognizes and binds to the promoter -10 element (formerly called pribnow box).
- Region 4.2 recognizes and binds to the promoter -35 element.

Wikipedia,2015

Transcription

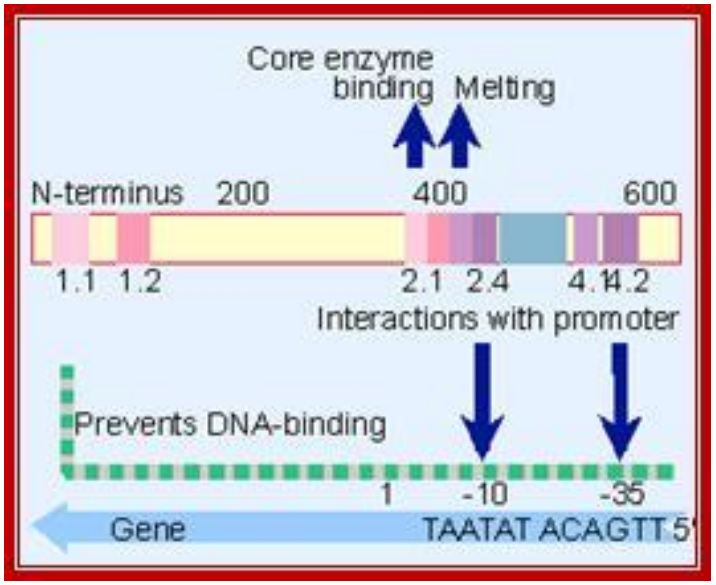
Bacterial promoters

35 and -10 sequences

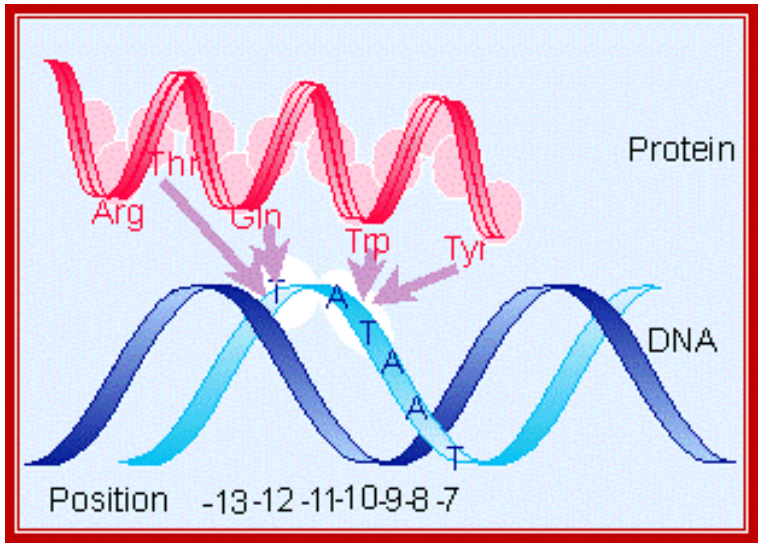


Crystal structure of *Escherichia coli* sigma70 region 4 bound to its -35 element DNA.

- The sigma subunit of RNA polymerase contacts both the -35 and the -10 boxes.
- Region 2.4 recognizes and binds to the promoter -10 element.
- Region 4.2 recognizes and binds to the promoter -35 element.



The sig-70 has four domains, called, from the N-terminal, as 1, 2, 3 and 4. The C- terminal 4th domain has helix turn helix motifs and recognizes -35 sequences and bind.



Sigma protein specific amino acids contact specific DNA sequences.

Prokaryotic DNA transcription

mRNA transcription is recognized by the sigma protein

RNA polymerase copy DNA to RNA(mRNA)

- In initiation process, RNA polymerase holo-enzyme (RNA polymerase+sigma factor) binds to DNA and scans for promoter sequences (start sequences).
- The sigma factor is the subunit of the RNA polymerase complex that recognizes the specific promoter sequence of DNA that the RNA polymerase complex should bind to.
- Scanning occurs in only one dimension, 100 times faster than diffusion limit.
- During scanning enzyme is bound non-specifically to DNA.
- Can quickly scan 2000 base pairs.

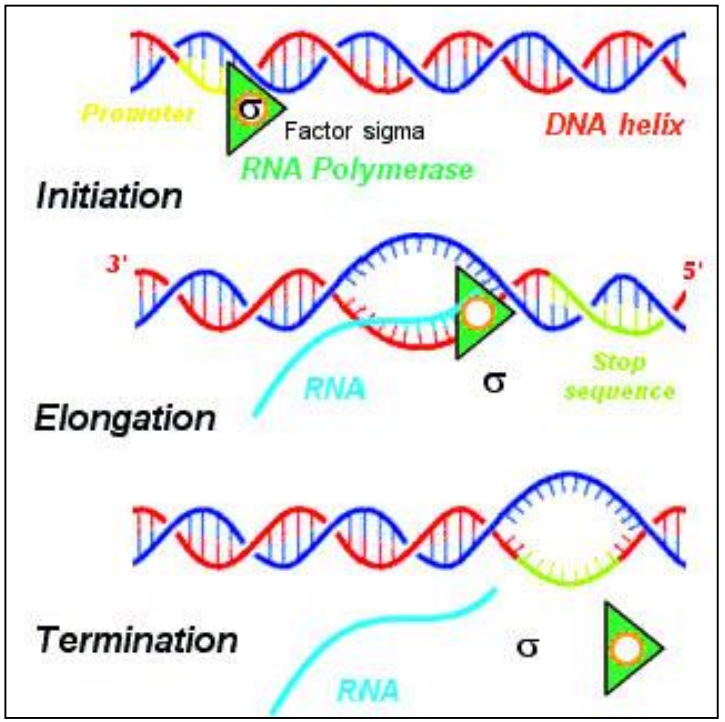
Holoenzyme is the core enzyme saturated with sigma factor 70. A biochemically active compound formed by the combination of an enzyme (core enzyme) with a coenzyme or cofactor(sigma factor).

Prokaryotic DNA transcription

mRNA transcription is recognized by the sigma protein

Transcription proceeds in one direction

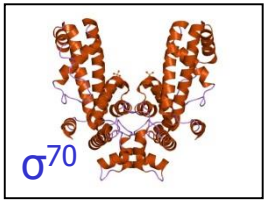
- In bacteria, all transcription is performed by a single type of RNA polymerase.
- The sigma subunit dissociates from the RNA polymerase core enzyme shortly after transcription begins.
- Nucleotides are added onto the 3' end of the growing RNA chain.



However, a recent study has shown that σ^{70} can remain attached in complex with the core RNA polymerase, at least during early elongation. It was indicates that sigma plays roles during early elongation.

Sigma factors

Sigma factors or regulons



- Sigma factor (also known as the regulon) is a protein needed only for initiation of RNA synthesis.
- Sigma factors are a major regulator of prokaryotic gene expression.
- It is well known that bacteria use different sigma factors to control the initiation specificity at different promoters, including those promoters whose genes encode virulence factors.

Paget *et al.*,2003; Wilson *et al.*,2015

Sigma factors

Different numbers and specialized sigma factors

- Including both:

1. Major or abundant primary (housekeeping) sigmas, and
2. Minor alternative (secondary) sigmas from diverse organisms.

- Every molecule of RNA polymerase holoenzyme contains exactly one housekeeping" sigma factor (primary sigma factor), transcribes most genes in growing cells.
- Primary sigma factor is present in all growth conditions.

1. The number of sigma factors varies between bacterial species.

- Thus there are members of the σ^{70} family of sigma factors.

Paget *et al.*,2003;..

Sigma factors

Different numbers and specialized sigma factors

- Every molecule of RNA polymerase holoenzyme contains exactly one sigma factor subunit.
- The number of sigma factors varies between bacterial species.
- *E. coli* has 7 sigma factors.
- The 10 sigma factors thus far identified in *B. subtilis*.
- Sigma factors are distinguished by their characteristic molecular weights.
- For example, σ^{70} refers to the sigma factor with a molecular weight of 70KDa.

Sigma factors

Different numbers and specialized sigma factors

- Every molecule of RNA polymerase holoenzyme contains exactly one sigma factor subunit.
- Sigma factors allow sequence-specific binding of RNA polymerase to bacterial promoters.
- The number of sigma factors varies between bacterial species.
- *E. coli* has seven sigma factors.
- The 10 sigma factors thus far identified in *B. subtilis*.
- *Streptomyces avermitilis* and *Streptomyces coelicolor* with 13 and 14 σ^{70} genes, respectively.
- Sigma factors are distinguished by their characteristic molecular weights.
- For example, σ^{70} refers to the sigma factor with a molecular weight of 70KDa.

Sigma factors

Major and minor sigma factors

Name	Function
σ^{70}(RpoD) (major)	Housekeeping sigma factor transcribes most genes in growing cells
σ^{19} (FecI)	the ferric citrate sigma factor, regulates the fec gene for iron transport
σ^{24}(RpoD)	the extracytoplasmic/extreme heat stress sigma factor
σ^{28}(RpoD)	the flagellar sigma factor
σ^{32} (RpoH)	the heat shock sigma factor, it is turned on when the bacteria are exposed to heat
σ^{38} (RpoS)	the starvation/stationary phase sigma factor
σ^{54} (RpoN)	the nitrogen-limitation sigma factor

Note: There are also anti-sigma factors that inhibit the function of sigma factors and anti-anti-sigma factors that restore sigma factor function.

Sigma factors
Alternative sigma factors
Minor alternative (secondary) sigmas

- Bacteria and especially those capable of persisting in diverse environments, such as *Escherichia coli* provide particularly valuable models for exploring how single-celled organisms respond to environmental stresses.
- Bacteria have developed sets of specific response genes that are regulated by a subset of the σ^{70}-like sigma factors in order to respond to a changing environment. E.g.

1. When the cell is put under stress by high temperature.
2. Sporulation sigma factors in *Bacillus subtilis*.

- Because it is the sigma subunit that is responsible for promoter recognition, different sigma subunits may allow different promoters to be recognized.

Sofer,1999;..

Sigma factors

Alternative sigma factors

Minor alternative (secondary) sigmas

- Different sigma subunits may be made by the cell under special circumstances (for example, when the cell is put under stress by high temperature).

Factor	Gene	Use	-35	Separation	-10
σ^{70}	*rpoD*	General	TTGACA	16 - 19 bp	TATAAT
σ^{32}	*rpoH*	Heat Shock	CCCTTGAA	13 - 15 bp	CCCGATNT
σ^{28}	*fliA*	Flagella	CTAAA	15 bp	GCCGATAA
σ^{54}	*rpoN*	Nitrogen starvation	CTGGNA	6 bp	TTGCA

Sigma factors

Alternative sigma factors

Minor alternative (secondary) sigmas

1. σ^B (SigB), and
2. σ^S(RpoS)

- identified as general stress responsive alternative sigma factors in Gram-negative and in Gram-positive bacteria, respectively.

Boor,2006

Sigma factors

Alternative sigma factors

Minor alternative (secondary) sigmas

- σ^B, a group III sigma factor encoded by *sigB*, was initially identified and characterized in *B. subtilis*, but has also been identified in other Gram-positive bacteria.
- The *B. subtilis* σ^B-dependent general stress regulon is large: over 200 genes are expressed following bacterial exposure to heat, acid, ethanol, salt stress, entry into stationary phase, or starvation for glucose, oxygen, or phosphate.
- *sigB* mutants are sensitive to oxidative stress.

Boor,2006

Alternative sigma factors and their roles in bacterial virulence

The stationary phase sigma factor RpoS

- The alternative sigma factors RpoS (σ^{38}) has been shown to regulate the expression of genes in response to stationary phase, nutrient deprivation, and oxidative and osmotic stress.
- These are environments which are physiologically relevant to those encountered by many microbial pathogens during the natural course of infection.
- The RpoS sigma factor has been shown to be important for virulence in a number of plant and animal bacterial pathogens including *P. aeruginosa*.

Heat-shock proteins are also essential for stationary phase.

Kazmierczak *et al.*,2008

Alternative sigma factors and their roles in bacterial virulence

The stationary phase sigma factor RpoS

- Among the pathogenic bacteria, *Pseudomonas aeruginosa* is perhaps the best understood in terms of the virulence factors regulated and the role the Quorum sensing plays in pathogenicity.
- Regulation of Quorum sensing by RpoS in *Pseudomonas aeruginosa*.
- RsmA, RpoS, QocR all negatively regulate the Rhl or Las Quorum sensing systems, thus preventing early activation of these systems.

Whiteley *et al.*,2000; Deep *et al.*,2000

Alternative sigma factors and their roles in bacterial virulence

PvdS of *Pseudomonas aeruginosa*

- Some bacterial pathogens are known to express virulence genes in low iron environments.
- In *P. aeruginosa*, the siderophore pyoverdine is a virulence factor.
- PvdS is required for virulence and appears to regulate only virulence-related genes.
- The genes involved in pyoverdine synthesis are located in three clusters on the *P. aeruginosa* chromosome and regulated by ECF sigma factor PvdS.

Extracytoplasmic function (ECF) sigma factors, σ^E

Kazmierczak *et al.*,2008; Brooks and Buchanan,2008

Alternative sigma factors involved in virulence

- As alternative sigma factors have been shown to regulate expression of both virulence and virulence-associated genes, these sigma factors can contribute both directly and indirectly to bacterial virulence.

Family, class, and sigma factor[a]	Bacterial species[b]
σ^{70} family	
Stress response	
σ^{B}	*B. anthracis*, *L. monocytogenes*, *M. tuberculosis*, *S. aureus*, *S. epidermidis*
σ^{S}	*E. coli*, *P. aeruginosa*, *S. enterica* serovar Typhimurium, *S. enterica* serovar Typhi
σ^{F}	*M. tuberculosis*
ECF	
RpoE	*H. influenzae*, *S. enterica* serovar Typhimurium, *V. cholerae*
AlgU	*P. aeruginosa*
PvdS, FpvI	*P. aeruginosa*
σ^{C}	*M. tuberculosis*
σ^{D}	*M. tuberculosis*
σ^{E}	*M. tuberculosis*
σ^{H}	*M. tuberculosis*
HrpL	*Erwinia* spp., *P. syringae*
σ^{28}	
FliA	*C. jejuni*, *H. pylori*, *S. enterica* serovar Typhimurium, *V. cholerae*, *Y. enterocolitica*
σ^{54} family	
σ^{N}	*C. jejuni*, *H. pylori*, *L. monocytogenes*, *P. aeruginosa*, *P. syringae*, *V. cholerae*, *V. parahaemolyticus*

Kazmierczak *et al.*,2008

Cell starvation
(p)ppGpp production

- Guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) – collectively known as (p)ppGpp, is accumulated rapidly when bacterial cells encounter with nutritional stress(starvation) conditions.
- (p)ppGpp, is synthesized rapidly when bacterial cells starved for:

1. Amino acids;
2. Other stress conditions, including deprivation of phosphorus, iron, carbon source or fatty acids.

- This is in order to reactivates translation.

Guanosine: A nucleoside consisting of guanine and ribose. It is a component of RNA.

(p)ppGpp and starvation signaling
Presence and absence of (p)ppGpp

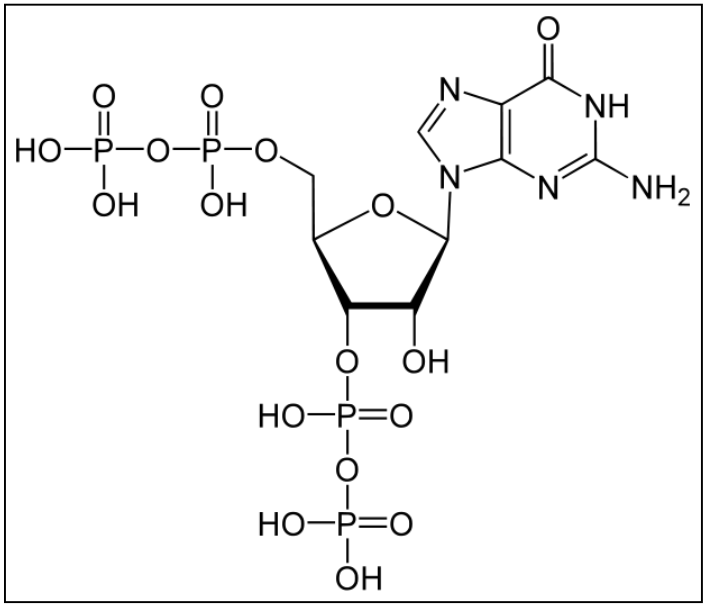
- **Presence of (p)ppGpp:**
- (p)ppGpp as a global regulator is important during starvation and stress signalling in many bacteria.
- (p)ppGpp pool increases with any types of nutrient limitation.
- **Absence of (p)ppGpp:**
- A complete absence of (p)ppGpp causes multiple amino acid requirements, poor survival of aged cultures, aberrant cell division, morphology, and immotility, as well as being locked in a growth mode during entry into starvation.

Wikipedia,2016

(p)ppGpp

A hyperphosphorylated guanosine nucleotide

- Guanosine tetraphosphate (ppGpp)
- Guanosine pentaphosphate (pppGpp).
- ppGpp has about 8-fold greater efficiency than that of pppGpp.



Guanosine: A nucleoside consisting of guanine and ribose.
Adenosine: Composed of a molecule of adenine+ a ribose sugar molecule.
ATP (adenosine triphosphate): Consists of adenosine and three phosphate groups.

Wikipedia,2016

(p)ppGpp and starvation signaling

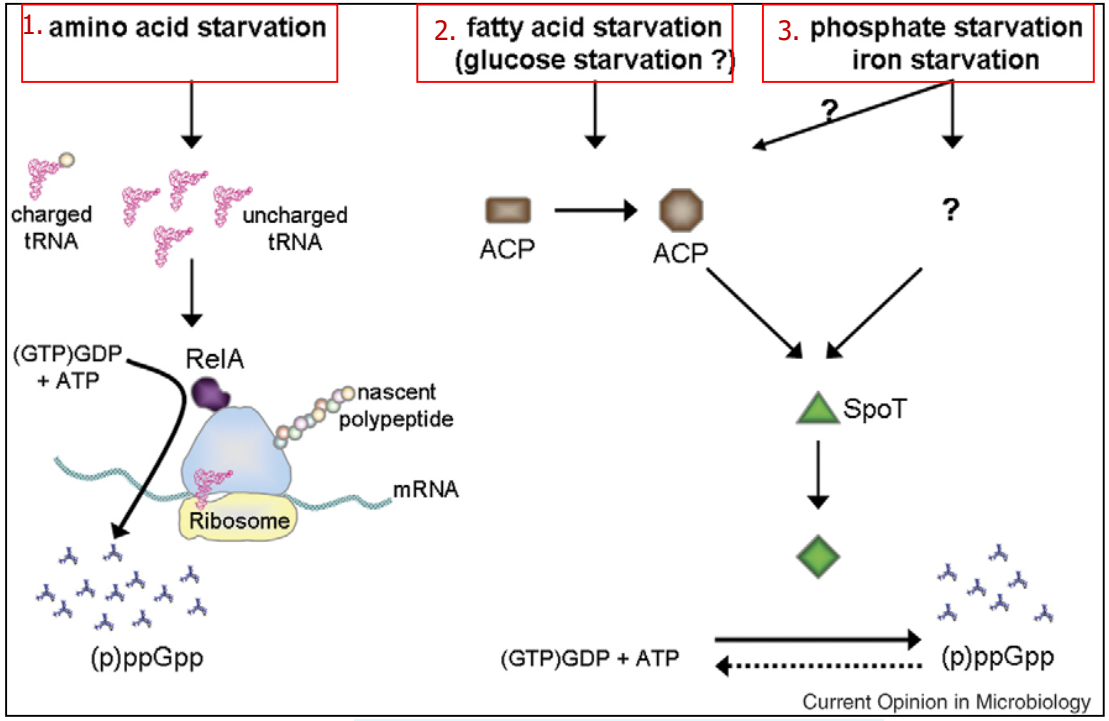
- (p)ppGpp is synthesized by two stringent response genes *relA* and *spoT* in *E. coli* and different bacteria.
- p)ppGpp is not restricted to prokaryotes because a growing number of relA-spoT homologues, designated *RSH* genes:
- At-RSH1 in *Arabidopsis thaliana*, and
- Nt-RSH2 of *Nicotiana tabacum* have been identified in plants.

Braeken *et al.*,2006

Control of bacterial transcription, translation and replication

By (p)ppGpp, a hyperphosphorylated guanosine nucleotide

- The enzymes RelA and SpoT are triggered by different starvation signals (amino acid, fatty acid and phosphate starvations) to produce (p)ppGpp for reactivates translation.



1. RelA synthesizes pppGpp/ppGpp from GTP/GDP. GTP/GDP act as a substrate for the synthesis of RNA. Uncharged tRNAs changed into charged tRNAs by RelA.

2. Acyl carrier protein (ACP) binds to SpoT and shifts the balance of its activity towards (p)ppGpp synthesis.

Srivatsan and Wang,2005

Control of bacterial transcription, translation and replication

By (p)ppGpp, a hyperphosphorylated guanosine nucleotide

1. Mechanism of RelA-mediated (p)ppGpp synthesis:

- Under conditions of amino acid starvation, large pools of uncharged tRNAs are generated that can bind to the A-site of the ribosome with low affinity and block the ribosome as a result.

I. RelA detects a blocked ribosome.

i. RelA binds to the ribosome and mediates (p)ppGpp synthesis.

ii. RelA synthesizes pppGpp/ppGpp from GTP/GDP, respectively, using ATP.

II. Concomitantly with (p)ppGpp synthesis, RelA is released.

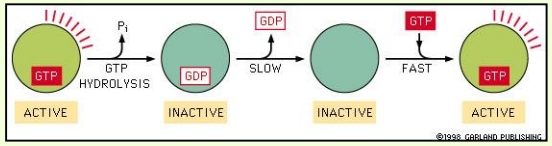
III. RelA hops to the next ribosome and the process is repeated, leading to levels of (p)ppGpp required to activate the stringent response.

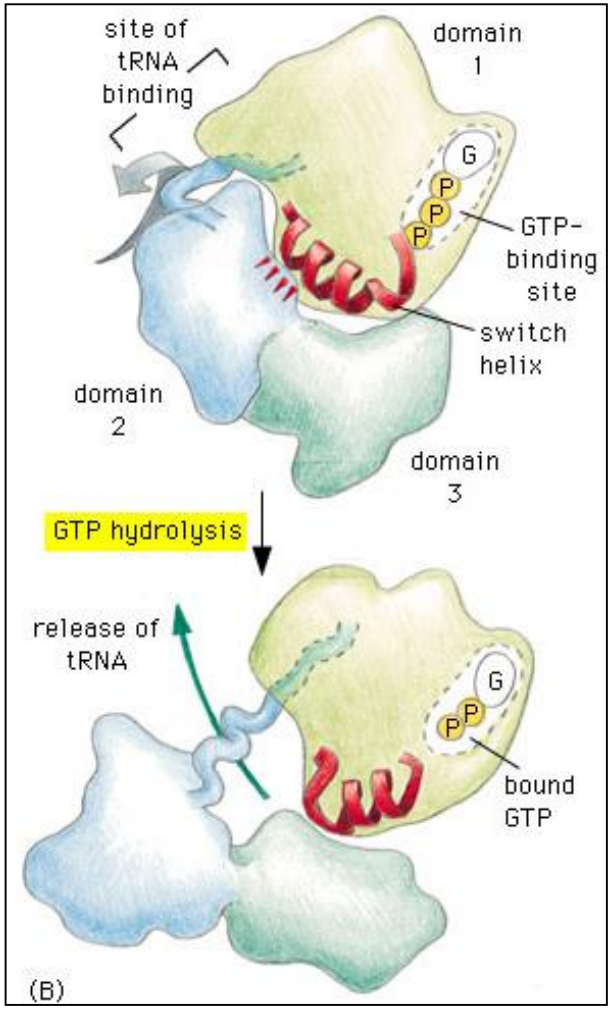
- Thus, a high levels of (p)ppGpp required to activate the stringent response and reactivates translation.

Braeken *et al*.,2006

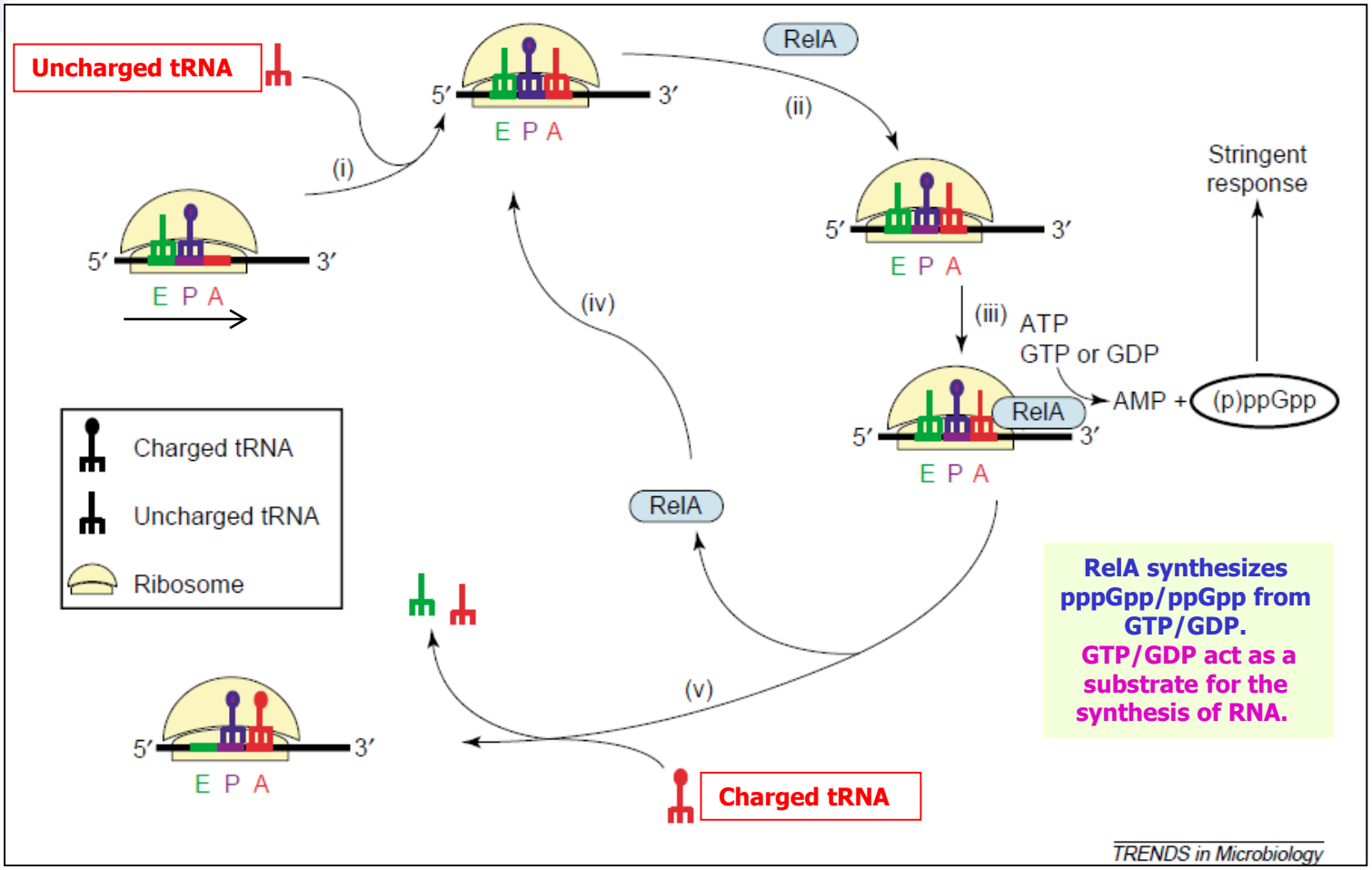
GTP/GDP-binding protein regulators
Regulate G proteins

- During the elongation stage of translation, GTP is used as an energy source for the binding of a new amino-bound tRNA to the A site of the ribosome.
- GTP is also used as an energy source for the translocation of the ribosome towards the 3' end of the mRNA.
- GTP/GDP can act as a substrate for the synthesis of RNA during the transcription process.





Mechanism of RelA-mediated (p)ppGpp synthesis



Braeken *et al.*,2006

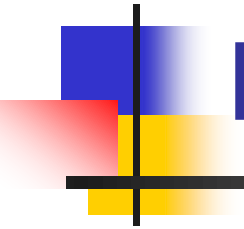
Control of bacterial transcription, translation and replication

By (p)ppGpp, a hyperphosphorylated guanosine nucleotide

2. Mechanism of SpoT-mediated (p)ppGpp synthesis:

- SpoT synthesizes and hydrolyzes (p)ppGpp through distinct active sites.
- Fatty acid starvation or potentially, glucose starvation, triggers a conformational change in the acyl carrier protein (ACP), which binds to SpoT and shifts the balance of its activity towards (p)ppGpp synthesis.
- Phosphate or iron starvation also results in (p)ppGpp accumulation through modulation of SpoT activity.

Srivatsan and Wang,2005; Braeken *et al.*,2006



Model systems

Model pathosystems

Model systems

- Sometimes its easier to study a process in a simple organism that it is to study it in a more complicated organism.
- Movement, responding to the environment, reproduction, respiration, etc.
- A MODEL system is a simple organism that is studied to understand a complicated activity.
- We used bacteria to figure out:

1. How DNA is replicated,
2. How genes are turned on and off,
3. How cells respond to their environmental changes.

- Other examples: mice, fruit flies, nematode worms.

Seelker,2010

Model systems

New insights for disease management

- Studies of pathogen interactions in model systems, particularly *Arabidopsis thaliana*, are enabling clearer understanding of susceptibility and resistance applicable to more complex plants (Heath,2002).
- Sequencing of major plant genomes is underway as well, with rice being completed.
- Multiple alleles and chromosomes, as well as complex traits are challenges in understanding and managing host resistance.
- Compiling information from sequencing and functional analysis of both pathogens and major crop plants is expected to bring new insights useful for sustained disease management.

An allele is a variant form of a given gene.

Vidaver and Lambrecht,2004

Model pathosystems

- Since it would be impossible to address all plants and pathogens of interest, we have focused, where possible, on several specific model pathosystems.
- Systems inclusive of the:

1. Pathogen,
2. Plant host,
3. Insect vector (if applicable),
4. Environment.

Psedoumonas syringae

Model pathosystems

With variable virulence and resistance phenotypes

- Individual strains of the plant pathogenic bacterium *Pseudomonas syringae* vary in their ability to produce:

1. Toxins, enzymes
2. Phytohormones,
3. Resist antimicrobial compounds,
4. Nucleate ice.

- These phenotypes with similar evolutionary origin enhance virulence.

Application to Biotechnology: *Pseudomonas syringae* is perhaps the best studied bacterial plant pathogen to date. This is because it serves as a model organism for studying the interaction between plants and bacterial pathogens.These microbes hold a number of special characteristics and produce a wide variety of potentially useful compounds. For example, they show a high resistance to copper and antibiotics. In fact, they encode genes that bestow resistance to cationic antimicrobial peptides and antibiotics. *Pseudomonas syringae* is also a microbe of great interest for its ice nucleation properties.

Hwang *et al.*,2005;MicrobeWiki,2011

Model pathosystems

Bacterial pathosystems

Bacteria-*A. thaliana* pathosystem

- All classes of phytopathogens cause disease in *Arabidopsis*.
- *A. thaliana* pathosystem provides a unique opportunity to unravel the molecular interactions underlying plant pathogenesis.
- *Arabidopsis thaliana*, and certain strains exhibit race-cultivar specificity on this host, thus providing a model pathosystem for studying both compatible and incompatible host–pathogen interactions.
- e.g.

1. *P. s.* pv. *tomato*/*A. thaliana* system
2. *P. s.* pv. *phaseolicola*/*A. thaliana* system
3. *P. s.* pv. *maculicola* (leaf spot of cauliflower)/*A. thaliana* system
4. *X. campestris* pv. *campestris*/*A. thaliana* pathosystem.

Model pathosystems

Arabidopsis pathosystems

Disease	Pathogen
Fungal diseases:	
Downy mildew	*Peronospora parasitica*
Dark leaf spot	*Alternaria brassicae*
Powdery mildew	*Erysiphe cruciferarum*; *E. cichoracearum*
Leaf mold and leaf spot	*Cladosporium* sp.
Bacterial diseases:	
Black rot on crucifers	*Xanthomonas campestris* pv. *campestris*
Bacterial leaf spot of Brassica	*Pseudomonas syringae* pv. *maculicula*
Bacterial speck of tomato	*P. syringae* pv. *tomato*
Viral diseases:	
Mild stunting	Tobacco mosaic virus
Mild stunting and desiccation	Turnip crinkle virus
Vein clearing and chlorotic spots	Cauliflower mosaic virus

Adapted from Baker *et al.*,1997

Model pathosystems

Arabidopsis pathosystems

A. thaliana - *R. solanacearum* pathosystem

- According to Quirino and Bent (2003), establishing an *A. thaliana-R. solanacearum* pathosystem requires the following criteria:

1. Optimization of the conditions growth of *A. thaliana*.
2. Development of a successful pathogen interaction protocol.
3. Establishment of disease scoring methods.
4. A source of virulent and avirulent *R. solanacearum* strains.
5. Identification of susceptible and resistant ecotypes of *A. thaliana*.

Weich,2004

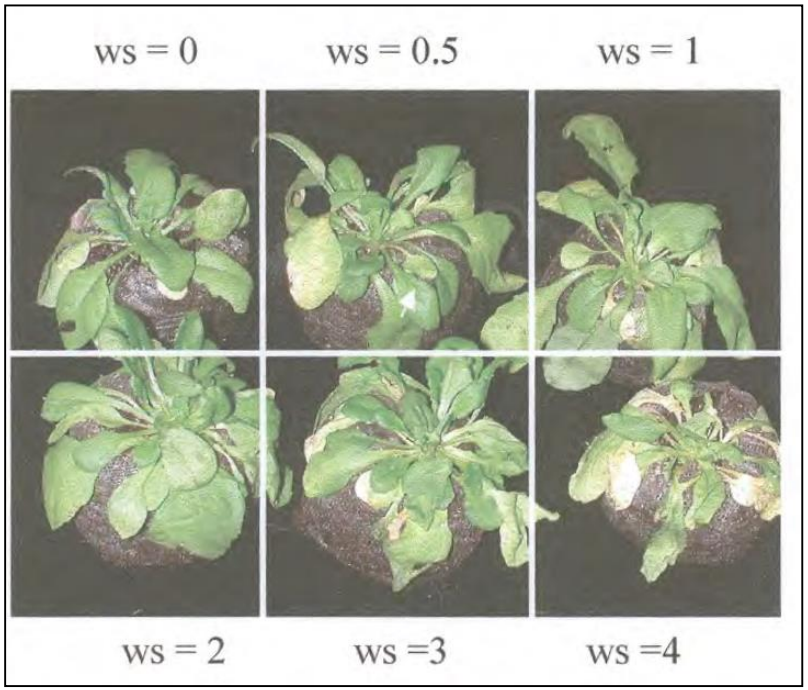
Model pathosystems

Arabidopsis pathosystems

A. thaliana - *R. solanacearum* pathosystem

- Rated scale of wilted *A. thaliana* plants inoculated with *R. solanacearum*.

Scale	Description
0	No Disease
0.5	First leaf wilting
1	2 or more leaves wilted, less than 25% of the leaves wilted
2	Less than 50% of the leaves wilted
3	More than 50% to 75% of the leaves wilted
4	76-100% wilt, the plant yellows and dies



Weich,2004

Genome sequencing

In all organisms

Plants and animals

- One of the most significant developments in biology in recent years is the world-wide commitment to fully sequence a number of genomes, including:

1. Mammals such as human and rat,
2. Plants such as *Arabidopsis thaliana* and rice, and
3. Microbial genomes such as bacteria and yeast.

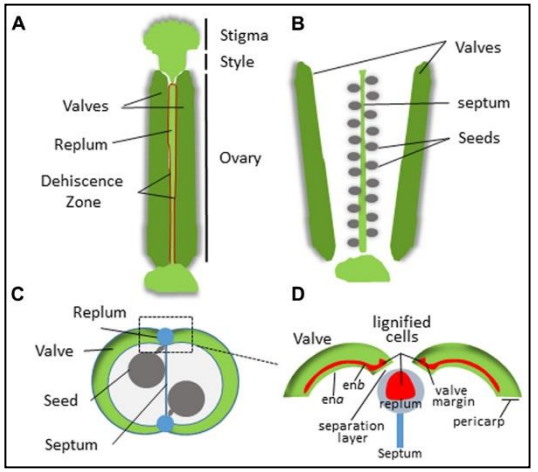
- One important focus of such genome studies in both plants and animals is to gain an understanding of resistance to invading pathogens.
- It is now possible to better characterize signal transduction pathways involved in disease resistance.

The whole-genome sequenced model plants

- *Arabidopsis thaliana*
- Rice
- Tomato

The whole-genome sequenced model plants

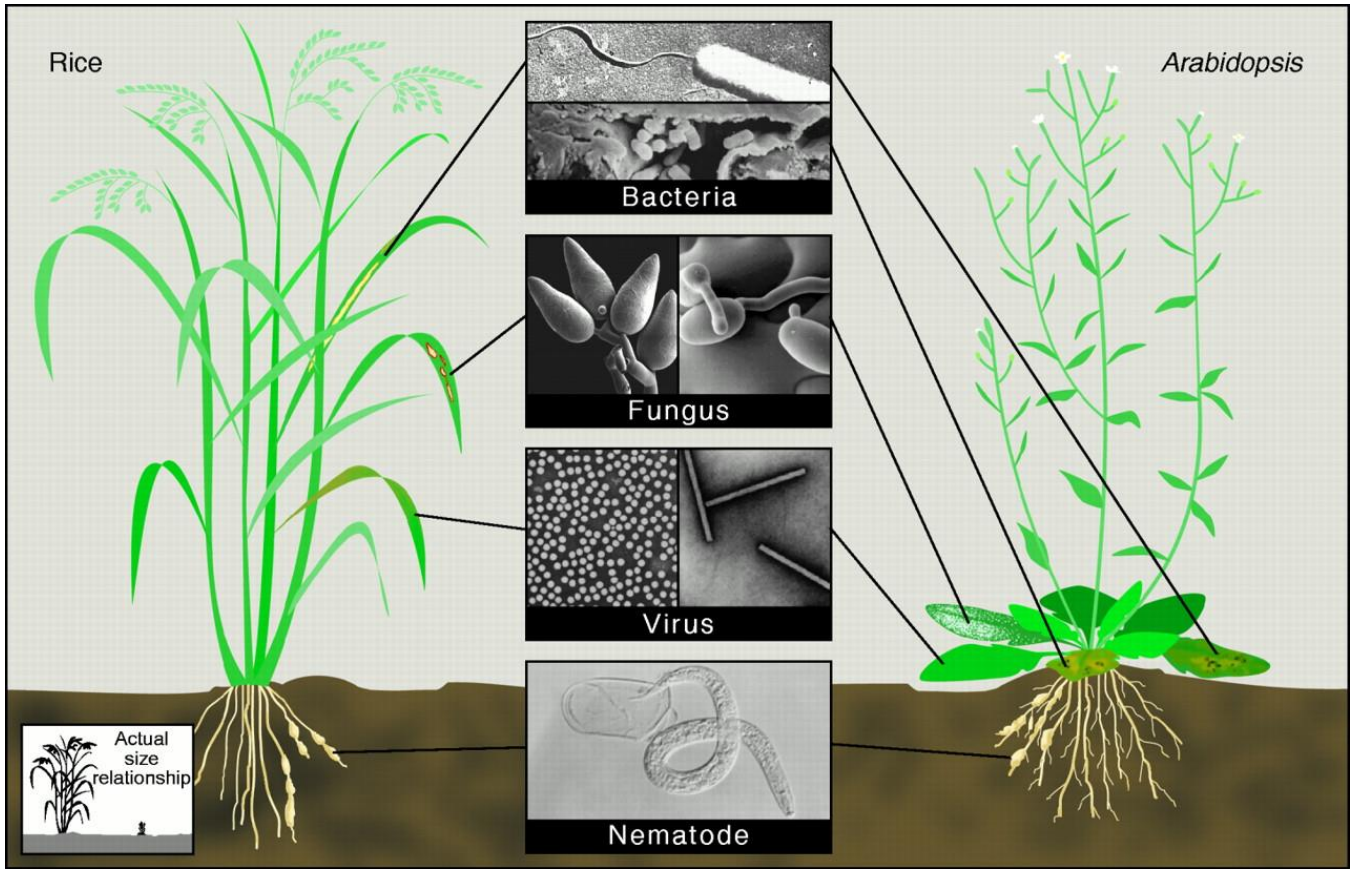
Arabidopsis thaliana life cycle and flower architecture



Arabidopsis thaliana life cycle and flower architecture
Mouse-ear cress (*Arabidopsis thaliana*) seed pods are called siliques. They are cigar shaped, 1 to 2 cm long, and form just below open flowers.

Rice and *Arabidopsis thaliana*

Model plants for host-pathogen interactions



A rice plant (left) and *Arabidopsis thaliana* (right), a model plant for host-pathogen interactions(Baker *et al.*,1997).

Arabidopsis thaliana

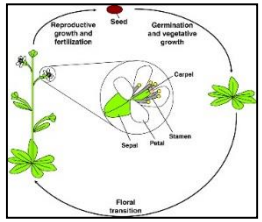
Drosophila of plants

Model plant for host-pathogen interactions

Four weeks old

- *Arabidopsis thaliana*, commonly known as thale-cress or mouse-ear cress, is a member of the Brassicaceae (mustard) family.
- *A. thaliana* has been coined the "Drosophila" of plants, due to the fact that it is a useful model system for plants.
- The *A. thaliana* genome is fairly small in size: 125Mb contained in five haploid chromosomes.
- In 2000, the entire genomic sequence was completed, and all five chromosomes currently have detailed and broad genetic maps (www.arabidopsis.org,2003).
- The genome contains a low amount of non-coding DNA, making *A. thaliana* useful for genetic analysis.

Vaitkunas,2003



Life cycle

Arabidopsis thaliana

Model plant for host-pathogen interactions

- Additionally, *A. thaliana* has a rapid life cycle, going from seed to flower in approximately six weeks.
- Since *Arabidopsis* plants produce many seeds, it is also easy to maintain a stock of seeds.
- These plants are easily cross-pollinated or self-pollinated, and given their small physical size (about a tenth of the size of a rice plant), large crops may be grown at one time for lab experiments.

Vaitkunas,2003

Arabidopsis thaliana
Model plant for host-pathogen interactions


Four weeks old

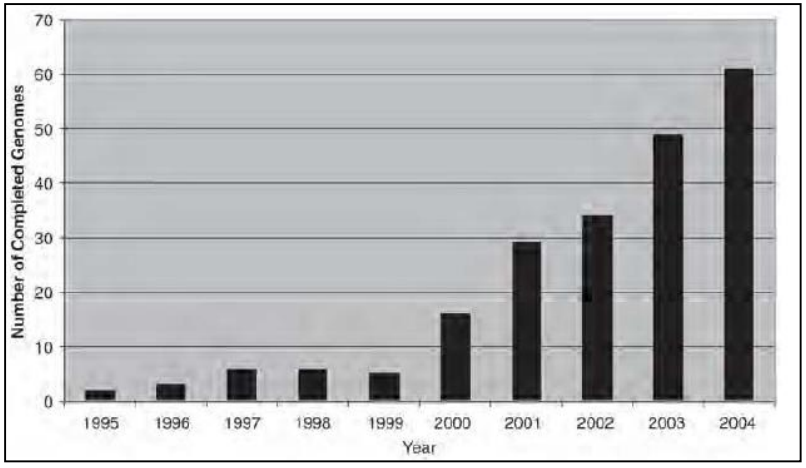
- The varied ecotypes of *A. thaliana*, comprised of over 150 wild isolates, present great opportunities for genetic and pathogenic studies by plant scientists.
- Although there are many varieties of *A. thaliana*, the three significant to this particular study are the:

1. Dijon-17 (Di-17),
2. Dijon-3 (Di-3) and
3. Columbia-0 (Col-0).

Vaitkunas,2003

Genome sequencing in bacteria
From 1995 to 2013

- In Feb 1, 2006:
- 297 prokaryotic genomes have been sequenced, and 500 prokaryotic genomes that are at various stages of completion.
- As of October 2013:
- 6851 bacterial genomes have been completed;
- Sequencing of 18679 are ongoing, and
- 1111 represent targeted genome projects.

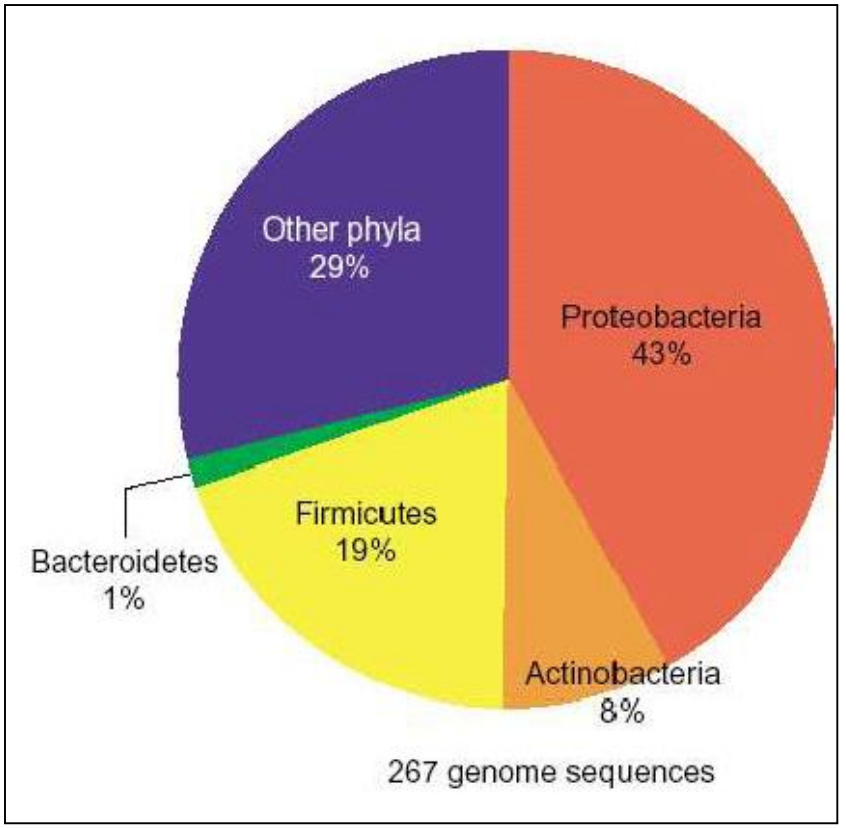


Chan *et al.*,2006;Kishore *et al.*,2015

Biased(undercoverage) sampling of bacterial genomes

For genome sequencings

- A phylum of bacteria comprised of three classes:
  1. Bacteroides,
  2. Flavobacteria, and
  3. Sphingobacteria.
- 43% of genome sequencing analyses are related to the gram-negative bacteria.



Hugenholtz,2004;..

Bacterial phytopathogens and genome science

- Many genomes of bacterial phytopathogens were completed.
- These genomes come from a phylogenetically diverse set of organisms, and range in size from 870 kb to more than 6 Mb.
- The publication of these annotated genomes has significantly helped our understanding of bacterial plant disease.
- These genomes have also provided important information about bacterial evolution.
- Examples of recently completed genomes include:

1. *Pseudomonas syringae* pv. *tomato*, which is notable for its large repertoire of effector proteins;
2. *Leifsonia xyli* subsp. *xyli*, the first Gram-positive bacterial genome to be sequenced; and
3. Phytoplasma asteris, the small genome that lacks important functions previously thought to be essential in a bacterium.

Wood *et al.*,2005

Plant pathogenic bacteria with sequenced genomes

- *Acidivorax avenae*
- *Agrobacterium tumefaciens*
- *Burkhoderia cepacia*
- *Clavibacter michiganensis*
- *Erwinia amylovora*
- *Pantoea*
- *Pectobacterium carotovorum*
- *Pseudomonas syringae* pv. *syringae*
- *Pseudomonas syringae* pv. *tomato*
- *Ralstonia solanacearum*
- *Xanthomonas campestris* pv. *campestris*
- *Xanthomonas oryzae* pv. *oryzae*
- *Xanthomonas axonopodis* pv. *citri*
- Phytoplasmas
- *Spiroplasma citri*
- *Xylella fastidosa*

Top 10 bacterial plant pathogens

The table represents the ranked list of bacteria as voted for by plant bacteriologists associated with the Journal Molecular Plant Pathology

Rank	Bacterial pathogen	Author of bacterial description
1	*Pseudomonas syringae* pathovars	John Mansfield
2	*Ralstonia solanacearum*	Stéphane Genin
3	*Agrobacterium tumefaciens*	Shimpei Magori, Vitaly Citovsky
4	*Xanthomonas oryzae* pv. *oryzae*	Malinee Sriariyanum, P. Ronald
5	*Xanthomonas campestris* pathovars	Max Dow
6	*Xanthomonas axonopodis* pv. *manihotis*	Valérie Verdier
7	*Erwinia amylovora*	Steven V. Beer
8	*Xylella fastidiosa*	Marcos A. Machado
9	*Dickeya* (*dadantii* and *solani*)	Ian Toth
10	*Pectobacterium carotovorum* (and *P. atrosepticum*)	George Salmond

Mansfield *et al.*,2012

Some prokaryotic plant pathogens being sequenced

Common bacteria

PLANT PATHOGENIC BACTERIA AND MOLLICUTES
≤10 - IMMEDIATE PRIORITY SPECIES BACTERIA
AND
≤10 - IMMEDIATE PRIORITY SPECIES PHYTOPLASMAS and SPIROPLASMAS:
(Species are alphabetically, arranged - not ranked within the list)

Organism	Strain	Genome Size	Rationale /Significance
Bacteria			
Burkholderia cepacia	ATCC 25416	7.9 Mb incl 3.5, 3.1, 1.1, and 0.2 Mb replicons	Causes sour skin of onion, cavity disease of mushroom, brown spot of orchids, and rot of caladium bulbs. Strain ATCC 25416 is a plant-pathogen representative of genomovar I strains, which have rarely been isolated as human pathogens. Opportunity for direct genome comparison to genomovar III strains, which are more typically associated with cystic fibrosis patients exhibiting symptoms of Cepacia syndrome.
Clavibacter michiganensis subsp. *insidiosis*	NCPPB 1109	ca. 3.0	Causes an economically important disease on alfalfa. Very little known of its molecular biology due to the difficulty in genetic manipulation of the pathogen. Recent advances in the manipulation of related pathogens and the soon to be available genome sequences of *Clavibacter michiganensis* subsp. *sepedonicus*, *C. michiganensis* subsp. *michiganensis* and *Leifsonia xyli* will enable genomic and functional comparisons across phylogenetically related vascular pathogens..
Pectobacterium carotovorum subsp. *carotovorum*	Ecc71	ca. 5 Mb	Causes soft rot and related diseases of numerous crops worldwide, under temperate as well as tropical conditions and under field conditions, in storage and in transit. Opportunity for genome-scale comparison with enterobacterial pathogens (virulence factors and evolution of pathogenesis). Because this pathogen has world wide economic importance and has been a principal model organism for molecular studies of plant-microbe interactions, the scientific community who would benefit from the genomic sequence is particularly large.
Pseudomonas marginalis		ca 6.5 Mb	Causes soft rot. Sequence would be useful for comparison to other *Pseudomonas* spp. and to *Pectobacterium* spp. This bacterium represents a group of phytopathogenic pseudomonads and its sequence would be useful for exploring the similarities and differences among this important group of plant pathogens.

APS,2008

Some prokaryotic plant pathogens being sequenced

Common bacteria

Pseudomonas viridiflava		ca. 6.0 Mb	Causes leaf spot (soybean and melon, among others) and bacterial blight (kiwi). This bacterium represents a group of phytopathogenic pseudomonads and its sequence would be useful for exploring the similarities and differences among this important group of plant pathogens.
Rhodococcus fascians	D188	5.8 Mb	Causes leafy gall formation on a broad array of monocot and dicot plants. It induces de novo cell division in cortical plant cells, leading to the formation of numerous shoot meristems. It is an adapted endophyte that possibly also enters the plant cells. This strain has been the model strain in molecular studies. As a close relative to *Rhodococcus equi* (virulent on horse) and Mycobacterium species (known human and animal pathogens), it is a good model for understanding general and specific pathogenesis factors from Actinomycetes, from both plant and animal pathogens.
Streptomyces turgidiscabies	Car8	10 Mb	Causes a severe version of potato scab in Japan and possibly in Europe. Not currently found in the United States. Contains a mobilizable pathogenicity island about 660 kb in size that includes the fas operon. This operon is homologous and colinear with the operon in Rhodococcus fascians and represents an introduction to the PAI in Streptomyces. A genome sequence will be highly useful for comparative genomics among pathogenic and nonpathogenic Streptomyces and investigation of the PAI within different genetic backgrounds.
Xanthomonas axonopodis pv. malvacearum		ca. 5.0-5.2 Mb	Causes cotton blight disease, which causes yield losses of up to 50%, with 10-30% losses occurring commonly. Cotton blight disease is a world-wide problem. The pathogen has served as a model bacterial gene-for-gene system with the highest level of race specificity of any xanthomonad (over 20 races described). The African strains are of particular interest because they are quarantined pathogens in the U.S., and they are capable of defeating most of the resistance genes that are known against the disease. The organism is amenable to standard microbial genetic tools, and the histology, biochemistry and physiology of the disease have been very well described, giving enormous leverage to the sequence information..
Phytoplasmas and Spiroplasmas			
Clover Phyllody Phytoplasma		ca. 0.8 Mb	Clover phyllody is widespread in North America and Europe where it is responsible for diseases in fruits and vegetables. Host plants damaged by this phytoplasma include *Trifolium* spp., *Fragaria* (strawberry), *Olea* (olive), *Poa pratensis*, *Anemone*, *Ranunculus*, and *Vitis* (grapevine). This phytoplasma is a

APS,2008

Some prokaryotic plant pathogens being sequenced

Common bacteria

PLANT PATHOGENIC BACTERIA AND MOLLICUTES ≤25 SPECIES - HIGH PRIORITY SPECIES 10 BACTERIA AND ≤25 SPECIES - HIGH PRIORITY SPECIES PHYTOPLASMAS and SPIROPLASMAS: (Species are alphabetically, arranged - not ranked within the list)			
Bacteria			
Acidovorax avenae subsp. *avenae*	CAa4		Infects many important crops including bacterial stripe of rice, bacterial stalk rot and blight of corn, leaf blight of oats, and red stripe of millet and sugarcane. These diseases have become very important in Asia, especially in seedlings. The closely related A. avenae subsp. citrulli causes severe losses in watermelon and other curcurbits.
Brenneria rubrifaciens		ca. 3.0 Mb	Causes deep bark canker of walnut. Former name is *Erwinia rubrifaciens.* Opportunity for comparative genomics with other Enterobacteriacae, including those that cause other plant and animal diseases.
Clavibacter michiganensis subsp. *nebraskensis*	NCPPB 2581	ca. 3.0 Mb	Causes Goss's wilt of corn. Very little is known of its molecular biology. Availability of the *C. m.* subsp. *nebraskensis* genome sequence would enable genomic and functional comparisons across phylogenetically related vascular pathogens, which specifically infect either monocots or dicots.
Pectobacterium betavasculorum	Ecb168		Causes soft rot of sugar beet. Differs from other soft rot pathogens in having very narrow host range, and therefore provides opportunity for discovery of molecular basis of host specificity. Opportunity for genome-scale comparison with enterobacterial pathogens of humans.
Pectobacterium rhapontici			Causes crown rot of rhubarb. Produces pro-ferrosamine (pink iron+2 chelating pigment)
Pseudomonas chicorii		ca. 6.0 Mb	Center rot of "french endive" or wilt of chicory. This bacterium represents a group of phytopathogenic pseudomonads and its sequence would be useful for exploring the similarities and differences among this important group of plant pathogens.
Pseudomonas corrugata			The tomato pith necrosis pathogen, *Pseudomonas corrugata*, is a non-fluorescent pseudomonad that is closely allied to the fluorescent pseudomonads when compared using standard physiological tests. The bacterium is also a severe opportunistic pathogen of tomatoes but has a restricted host range. It can

APS,2008

Some prokaryotic plant pathogens being sequenced

Prokaryotes

PLANT ASSOCIATED BENEFICIAL PROKARYOTES AND EUKARYOTES ≤10 - IMMEDIATE PRIORITY SPECIES: (Alphabetically, arranged - not ranked within the list)			
Organism	**Strain**	**Genome Size Mb**	**Rationale/Significance**
Prokaryotes:			
Bacillus subtilis	GB03	ca. 4.3	One of the most widely distributed bacterial species in agricultural systems. This rhizosphere isolate is used in biocontrol of soilborne root diseases. Well-established commercial applications. Excellent opportunity for genome-scale comparisons with saprophytic and pathogenic species in the genus.
Bacillus subtilis var. *amylo-liquefaciens*	FZB2	ca. 4.3	"Taegro" an EPA-registered microbial biocontrol product.
Bacillus mojavensis	203-7	ca 4.2 Mb	Represents a group of endophytic bacteria of which a patented endophytic biocontrol strain by ARS has been shown to have some effects at reducing the endophytic state of *Fusarium verticillioides*, a serious and common pathogen of corn that produces the fumonisin mycotoxins. Also produces plant growth enhancements for a majority of plants tested. The type is ATCC 51516 (NRRL 14698) and the patented strain is ATCC 55732
Enterobacter cloacae	Ec501		A common, aggressive spermosphere and rhizosphere colonizer of several plant species. Strain Ec501 provides an excellent model system for colonization, biocontrol and metabolism, and would serve as an excellent choice for comparative genomics with other plant and non-plant associated enterics.
Lysobacter enzymogenes	C3	6.5 Mb	Common soil and water inhabitant with antagonistic activity towards various microorganisms, and demonstrated biocontrol activity towards several plant diseases. Producers of multiple forms of chitinases and glucanases and other extracellular, depolymerizing enzymes. Also produces antibiotics and contains a type III secretion system. No *Lysobacter* spp. has been sequenced to date.
Pantoea agglomerans	C9-1	ca 4.5 Mb	Common plant epiphyte. Registration is in progress for commercialization for biological control strain of fireblight. Opportunity for genome-scale comparison with enterobacterial pathogens.

APS,2008

Some prokaryotic plant pathogens being sequenced

Plant associated beneficial prokaryotes

PLANT ASSOCIATED BENEFICIAL PROKARYOTES AND EUKARYOTES ≤25 - HIGH PRIORITY SPECIES: (Alphabetically, arranged - not ranked within the list)			
Prokaryotes:			
Bacillus licheniformis	Type Strain		
Bacillus licheniformis	SB3086		Commericalized biocontrol strain used in Ecoguard (Novozymes). Type strain ATCC14580 already sequenced. Useful for genomic comparisons with biological control strains to identify genes unique to biocontrol.
Bacillus mojavensis	203-7		
Bacillus mycoides	ATCC 6482		Accepted type strain of species. Useful for genomic comparisons with biological control strains to identify genes unique to biocontrol.
Bacillus mycoides	BmJ		
Bacillus pumilis	ATCC 7061		Accepted type strain of species. Useful for genomic comparisons with biological control strains to identify genes unique to biocontrol. Note: B. pumilus strain FO-036b, non biocontrol strain is slated for sequencing in 2006.
Bacillus pumilis	GB34		Commercial biocontrol strain
Bacillus pumilis	QST 2808		Commercial biocontrol strain
Bacillus subtilis	MBI600		Commercial biocontrol strain
Bacillus subtilis	IN937a	ca. 4.3	Well-studied PGPR strain. Known inducer of plant host defenses against multiple diseases on different crops.

APS,2008

Genomes sequencing
Essential to advance our knowledge

- A complete understanding of disease susceptibility and resistance will require understanding the interactions between plant hosts and pathogen, and beneficial microorganisms.
- Sequence data from the genes for such microorganisms is essential to advance our knowledge of:

1. Infection;
2. Interaction of pathogens and host gene products;
3. How these organisms reproduce and spread;
4. Progression of the plant-pathogen co-evolution.

Plant pathogenic bacteria with sequenced genomes

Xanthomonas genome sequences

- With the diversity of complete and ongoing *Xanthomonas* genome sequences, the genus has become a superb(excellent) model for understanding:
- Functional,
- Regulatory,
- Epidemiological, and
- Evolutionary aspects of host- and tissue-specific plant pathogenesis.

Jackson,2009

Genomes sequencing

Helping to develop more efficient and sustainable control

- Effective control will then be achieved through developing host resistance or through means that combat the pathogen directly or indirectly.
- Studies of microorganisms with biocontrol capacities may lead to further development of environmentally safe chemical controls for plant pathogens.

Bacterial genome sequencing
Other advantages

- With these amount of bacterial genome sequencings, we begin to appreciate the enormous diversity of prokaryotic genomes in terms of:

1. Chromosomal structure;
2. Gene content and organization, and
3. The abundance and fluidity(plasticity)of accessory and mobile genetic elements.

Chan *et al.*,2006

Bacterial genome sequencing

Chromosomal structure;Gene content and organization

Core genes, accessory genes and mobile genetic elements

- The genome of a bacterial species is composed of:

1. Conserved core genes(chromosomally-encoded genes), and
2. Variable accessory genes(some other chromosomally-encoded genes are part of the accessory genome (i.e. genes present in some of the strains) along with mobile genetic elements such as plasmids, bacteriophages, transposons and integrons).

- The association between distinct core and accessory genes creates a structure of genetic subgroups within the population.

Genome sequencing

Genomic plasticity

Horizontal gene pool in bacteria

- The horizontal gene pool in bacteria is composed of an array of accessory mobile genetic elements that profoundly influence:

1. Genome plasticity,
2. Organization, and
3. Evolution.

- Genome plasticity results from DNA acquisition by horizontal gene transfer.

Hayes,2004

Genome sequencing

Genomic plasticity

Horizontal gene pool in bacteria

- The genomes of closely related prokaryotes often have different organizations.
- These differences arise from rearrangements (such as inversions) between repeated elements, IS elements, and transposons and from the horizontal transfer of nucleotide sequences between cells.
- The latter phenomenon is mediated most commonly by conjugative plasmids, which are nonessential, autonomous accessory genetic elements that can acquire genes (such as antibiotic resistance genes) and then move them from a donor organism to a recipient.
- The dynamic character of genomic organization in prokaryotes is often designated as genomic plasticity.

Genome sequencing

Accessory genes

Mobile genetic elements

- Mobile genetic elements (part of accessory genes) are:

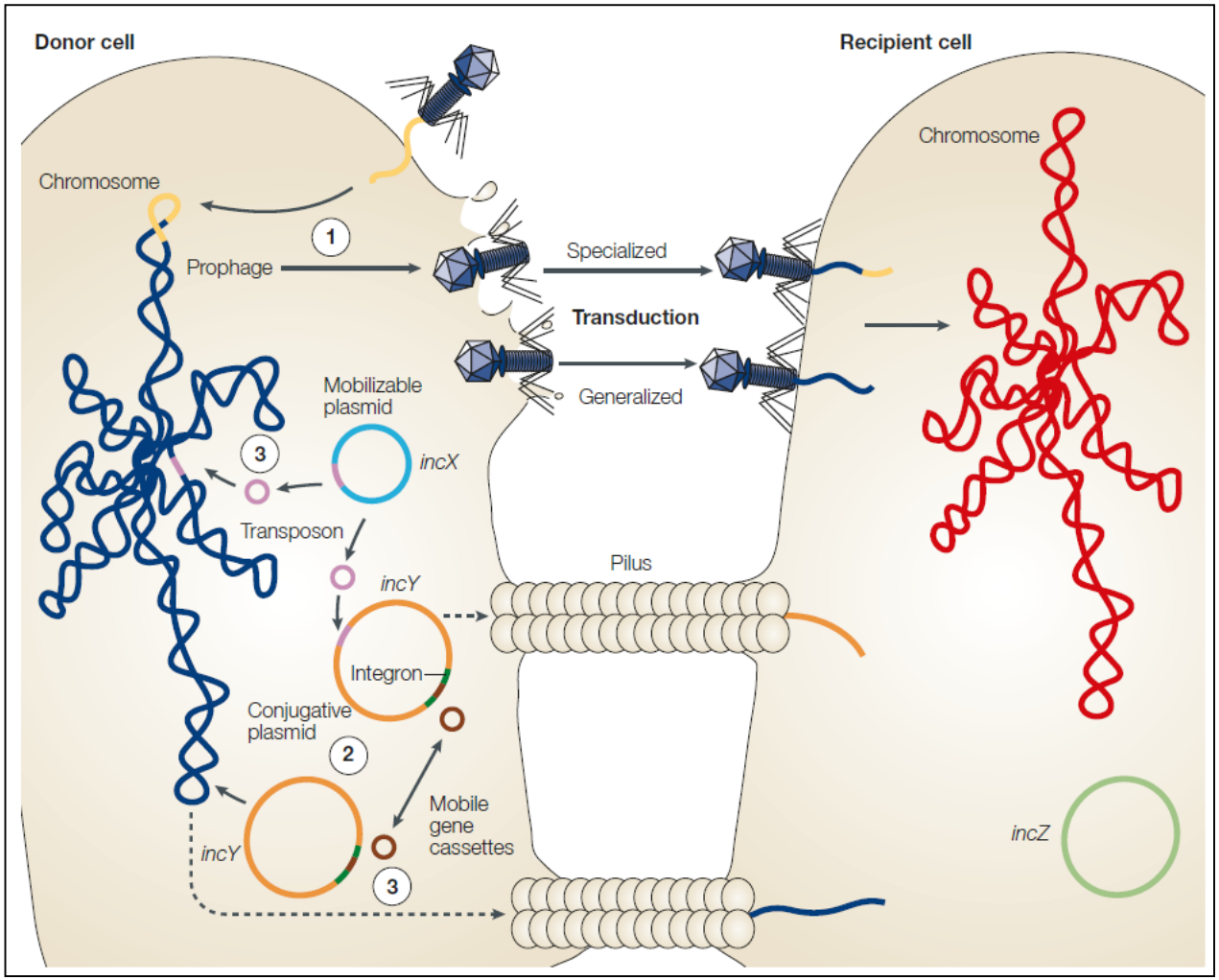
1. Plasmids,
2. Transposons,
3. Insertion sequences,
4. Integrons,
5. Prophages,
6. Genomic islands, and
7. Pathogenicity islands.

- These are part of the accessory genes, which can have a significant influence on the phenotype and biology of the organism.
- These mobile elements facilitate interspecies and intraspecies genetic exchange.
- They play an important role in the pathogenicity of bacteria, and are a major contributor to species diversity.

Chan *et al.*,2006

Genome sequencing

Accessory genes



Frost *et al.*,2005

Accessory genes

Mobile genetic elements involved in HGT in bacteria

HGT, horizontal gene transfer

Mobile element	General features	Examples	Phenotype of specific element
Gene cassette	Mobility of 'simple genes' Immediate constitutive expression Extremely broad host range	IntI1 IntI4	Antibiotic resistance (simple) Gene 'maturation' in *Vibrio*
Transposon	Mobility of genes and operons Very broad host range Loci screening for best performance	Tn21 Tn4651	Antibiotic resistance (complex) Toluene degradation
Plasmid	Mobility of operons and regulons Immediate regulated expression Very broad host rang	pNRG234a pNL1 pCD	Nodulation plasmid of *Rhizobium* Degradation of aromatic compounds Virulence determinants of *Yersinia*
Bacteriophage	Mobility of regulons 'Maturation' in chromosomes (via lysogeny) Narrow host range	CTXϕ	Virulence phage of *Vibrio cholerae*

In cell biology and genetics, a regulon is a collection of genes or operons under regulation by the same regulatory protein.
intI1/intI4 integrase genes

de la Cruz and Davies,2000

Ice nucleation organisms

Ice genes

Might be accessory genes due to horizontal gene transfer

- A phylogenetic analysis of 16S ribosomal RNA gene sequences from a total of 14 ina^+ and ina^- bacterial strains indicated that the ina^+ bacteria are not monophyletic but instead phylogenetically interspersed among ina^- bacteria.
- The relationships of ina^+ bacteria inferred from ina sequence did not coincide with those inferred from the 16S data.
- These results suggest the possibility of horizontal transfer in the evolution of bacterial ina genes.
- Recent evidence supports a role for the horizontal transfer of ice-binding protein genes from bacteria to sea-ice diatoms.

Edward *et al.*,1994; Raymond and Kim, 2012

Integron and prophage

Capture and spread of genes by site-specific recombination

- **Integrons(vectors for antibiotic resistance):**
- Integrons are mobile DNA elements with the ability to capture genes (gene cassettes), notably those encoding antibiotic resistance, by site-specific recombination.

1. Integrons may be found as part of mobile genetic elements such as plasmids and transposons.
2. Integrons can also be found in chromosomes.

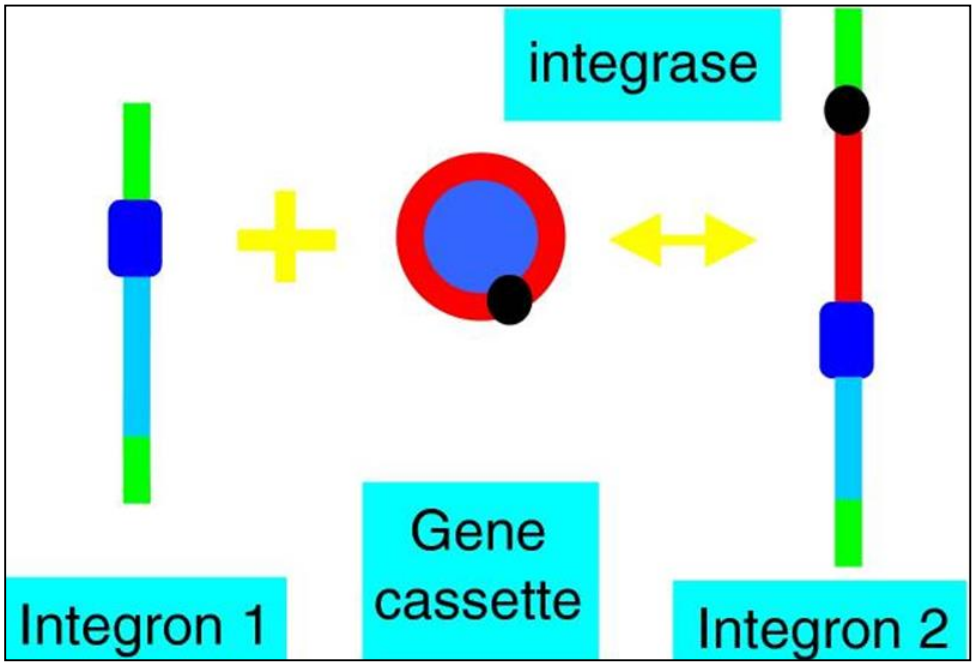
- Integrons are important agents of horizontal gene transfer.

Waites,2000; Wikipedia,2015

Integrons

An acquisition tool for capturing antibiotic resistance genes by pathogenic bacteria

- Cartoon of gene cassette capture by a bacterial integrons (mobile DNA elements).



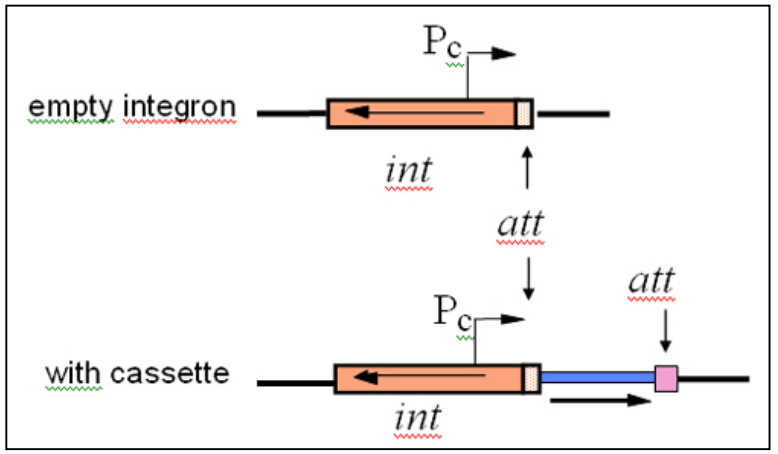
Bennett,2008

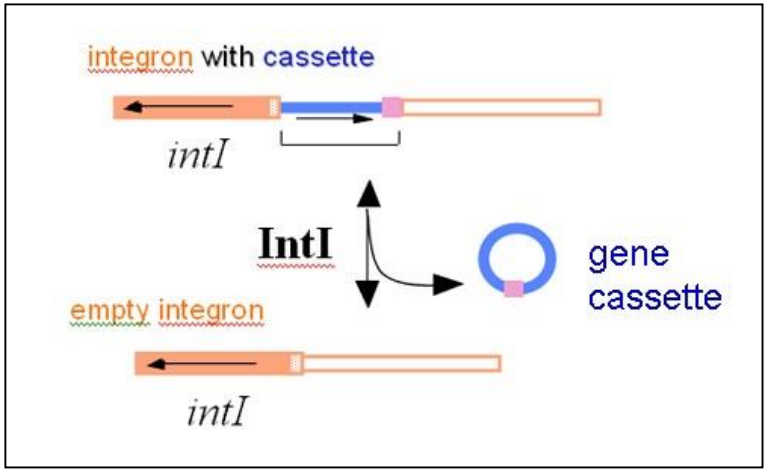
Integrons

- **Integrons have:**

1. an intergase gene (int);
2. a recombination site (attI);
3. a promoter, Pc.

- The *attC* site (pink) in the cassette is recognized by intergase gene (IntI).
- The promoter, Pc, in the integron is needed to transcribe the gene.
- Cassettes usually don't have their own promoter.





Plant pathogens

Types of plant pathogens

Biotrophic & Necrogenic Parasites

Definitions

- **Biotrophs:**
- Obtains nutrients from living cells.
- Exhibit a total lack of specialization.
- Biotrophs are typically obligate parasites.
- Biotrophs tend to cause disease on only one or a few related plant species.
- **Necrotrophs:**
- Lifestyle where the pathogen kills the host tissue to obtain nutrients from the dead cells.
- Necrotrophs use typically toxins and tissue-destroying enzymes as pathogenicity factors.
- Most necrotrophic fungi or bacteria that kill colonized plant tissues.

Necrotrophs
Virulence gene diversity

- Necrotrophs are able to attack many kinds, sometimes hundreds of host plants.
- This is because either:

1. They have many diverse genes for virulence, or
2. Their genes of virulence somehow have much less plant specificity than those of the commonly more specialized pathogens.

Biotrophs

Disease physiology

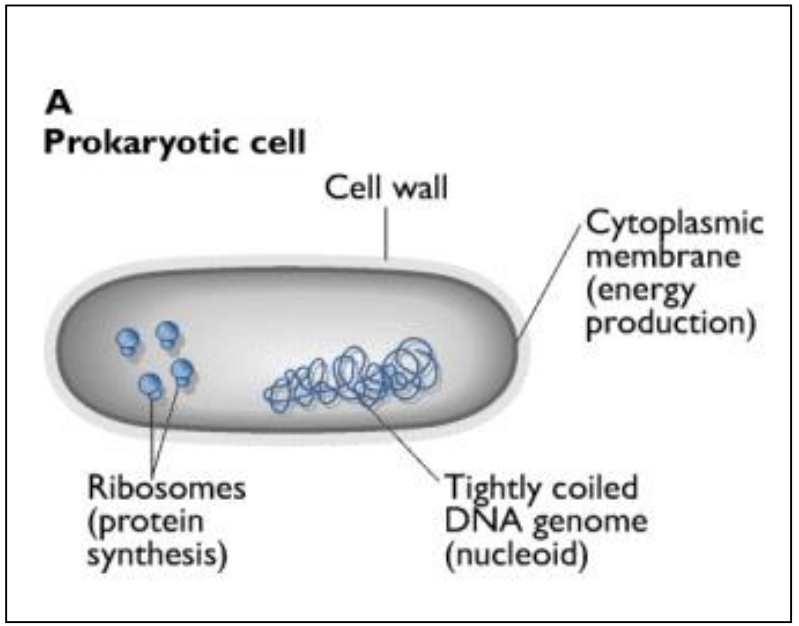
- Necrotrophs have little effect on plant physiology, since they kill host cells before colonizing them.
- Biotrophic pathogens modify various aspects of host physiology, such as:
- Respiration, photosynthesis, translocation, transpiration and growth and development.
- Examples:

1. The respiration rate of plants invariably increases following infection by fungi, bacteria or viruses.
2. The higher rate of glucose catabolism causes a measurable increase in the temperature of infected leaves.

New features of bacteria

- Based on recent findings bacteria are not the simple cells but have:

1. A cytoskeleton
2. Mitotic activity
3. Elaborate control systems
4. Cross-talk.



Singleton,2004;..

New features of bacteria
Cross-talk

- Many of the same signal molecules are perceived by both plants and microbes.
- This is not surprising since angiosperms arose about 3 billion years after bacteria, and evolved in the constant presence of microbial signaling.
- Similar signal molecules are produced by a wide range of bacteria and all bacterial plant pathogens are likely to be exposed to plant signal molecules.

Allen *et al.*,2009

Bacterial new features
Sensing/thinking and mimicking

- This is perhaps misinterpretation to believe that bacteria are headless, heartless, brainless, with a primitive cell for an entire body, one DNA molecule for a chromosome and a life span measured in minutes.
- Sufficient data are now available which show:

1. Bacteria sense (Quorum sensing);
2. Bacteria think (Microminds: the Minds of Microbes);
3. Bacterial creativity.

Pietsch; Holloway,2006

Bacterial sensing
Sensing/mimicking mechanisms

1. Quorum Sensing
2. Sensing/mimicking plant components
3. Sensing the iron-deficient intercellular space.

- *Erwinia* senses both:

1. plant components from wounded plant cells, and
2. the iron-deficient environment of the plant apoplast, that is the intercellular spaces or xylem vessels, causing parenchymatous and vascular or parenchymatous-vascular diseases, respectively.

- But symptom development is only observed after bacterial population reaches a certain size (QS).

Bot452,2001; Buonaurio,2008

Bacterial sensing

Evidence for sensing plant components and increasing the amount of degradation enzymes

- Expression of pectin degrading enzymes in response to plant components.
- Extracellular levels of *PelA* and *PelE* increase if celery extracts (mimicking a wounded plant) are added to *Pectobacterium* cultures.
- Three mutants were isolated that abolish celery inducibility of *PelA* and *E*.

Bacterial sensing

Mimic/inactivate/hijack/abusing plant MAPKinase

- Plants use MAPK cascade for their adaptation and survival and defense.
- MAPK cascade-mediated signaling is an essential step in the establishment of resistance to pathogens.
- Pathogens on the other hand, have evolved strategies to overcome defense responses.
- This can be achieved through:

1. Inactivation, or even
2. Hijack MAPK-mediated defense responses by targeting the MAPK cascade components (Pitzschke *et al.*,2009).

Bacterial sensing

Molecular mimicry of plant pathogens

Mimic/inactivate/hijack/abusing plant MAPKinase

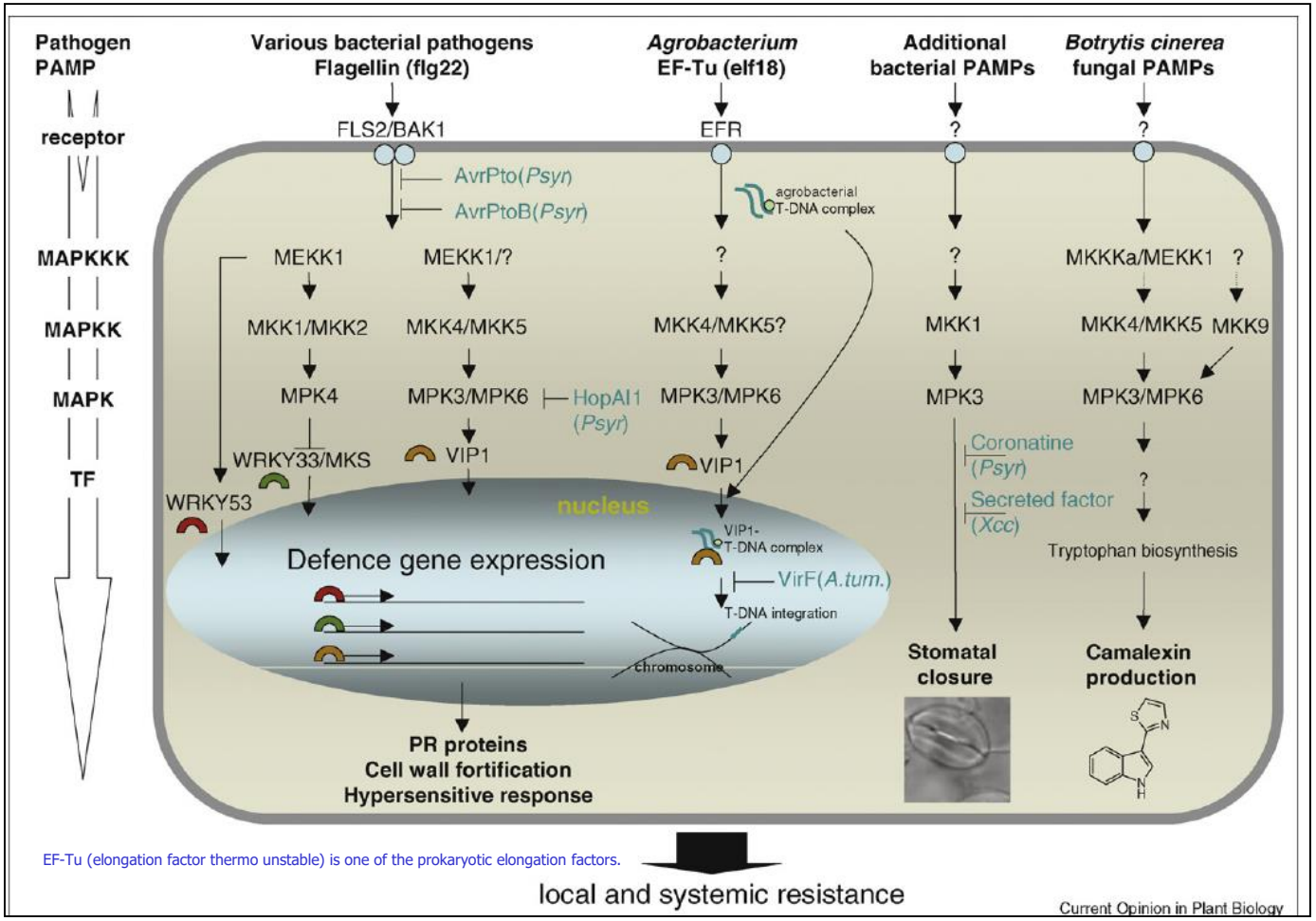
- MPK3/MPK6 in host plants are necessary to induce defence responses.
- Plant nuclear VIP1 protein counteracts with *Agrobacterium* invasion and induce the expression of defence genes.
- The activation of MPK3 in response to flg22 (PAMP) or *Agrobacterium* results in the phosphorylation and subsequent nuclear translocation of the host protein VIP1 (virE2 interacting protein 1).
- *Agrobacterium* has hijacked VIP1 for delivering their T-DNA into the plant nucleus, where it integrates into the host genome.

Pitzschke *et al.*,2009

Bacterial sensing

Inactivation of plant MAPKinase by *A. tumefaceins*

Agrobacterium has hijacked host protein VIP1 through proteolysis and delivers its T-DNA into the plant nucleus



Pitzschke *et al.*,2009

Bacterial sensing

Molecular mimicry of plant proteins by bacterial pathogens

Mimic/inactivate/hijack/abusing plant MAPKinase

- Simultaneously entering of VIP1 and T-DNA of *Agrobacterium* (Friend & Foe) into the nucleus.
- The plant protein VIP1 occurs initially in the cytoplasm of cells and in order to fulfill its role as a regulator/defender- it then needs to migrate into the nucleus.
- During this process the bacterial T-DNA adheres to VIP1, thereby enabling it to infiltrate the nucleus unnoticed.

Djamei *et al.*,2007; Magori and Citovsky,2011

Bacterial sensing

Molecular mimicry of plant pathogens

Mimic/inactivate/hijack/abusing plant MAPKinase

- *Agrobacterium* injects its T-DNA into the nucleus via translocating its F-box effector(VirF) into plant and hijacks the host SCF complex (plant F-box protein) to facilitate bacterial infection.
- Prof. Hirt compares this strategy, which inevitably means that the plants own defences cause its downfall, to the famous Trojan Horse.

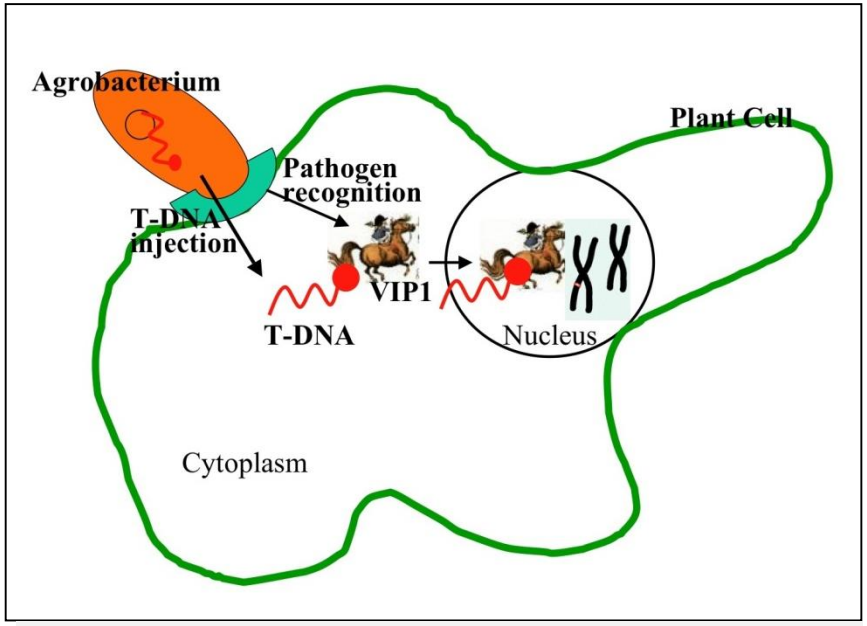
Djamei et al.,2007; Magori and Citovsky,2011; Hughes and Sperandio,2009

Bacterial sensing

Molecular mimicry of plant pathogens

Mimic/inactivate/hijack/abusing plant MAPKinase

- The plant protein VIP1 regulates the defence against pathogen attacks.
- In order to do so it needs to migrate into the nucleus.
- The *Agrobacterium* uses this process to inject its T-DNA into the nucleus.



During this process the bacterial T-DNA adheres to VIP1, thereby enabling it to infiltrate the nucleus unnoticed.

Original publication: Trojan horse strategy in *Agrobacterium* transformation-Abusing MAPK-targeted VIP1 defence signalling. Armin Djamei, Andrea Pitzschke, Hirofumi Nakagami, Iva Rajh, Heribert Hirt, Science 318, 453 (2007).

Bacterial sensing
Molecular mimicry of plant pathogens
Mimic/inactivate/hijack/abusing plant MAPKinase

- *Agrobacterium* hijacks plant nuclear VIP1 through proteolysis of this specific host protein into peptides by a multicatalytic protease complex called the 20S proteasome.
- Proteolysis of specific host protein (VIP1) occurs in early stages of the transformation process by the *Agrobacterium* virulence factor VirF, which encodes an F-box protein.

Proteasomes are very large protein complexes inside all eukaryotes and archeae and in some bacteria. In eukaryotes, they are located in the nucleus and the cytoplasm. The main function of the proteasome is to degrade unneeded or damaged proteins by proteolysis, a chemical reaction that breaks peptide bonds. Enzymes that carry out such reactions are called proteases. The most common form of the proteasome is known as the 26S proteasome.

Plant F-box proteins

Molecular mimicry of plant proteins by bacterial pathogens

- Diverse pathogens have evolved virulence factors that mimic host cell functions.
- This molecular mimicry, presumably acquired through either divergent or convergent evolution.
- A fascinating example of the molecular mimicry is pathogen-encoded F-box proteins (e.g. bacterial F-box proteins).

Divergent evolution: When a species diverges over time into two different species, resulting in a species becoming less like the original one.
Convergent evolution(parallel evolution): The process by which unrelated or distantly related organisms evolve similar body forms, coloration, organs, and adaptations often results from the evolution of similar or identical mutations in independent lineages.

Plant F-box proteins

Molecular mimicry of plant proteins by bacterial pathogens

Mimic/inactivate/hijack/abusing plant MAPKinase

- Viruses and pathogenic bacteria use pathogen-encoded F-box proteins (e.g. bacterial F-box proteins) to mimic/inactivate/hijack plant F-box proteins which are associated with cellular functions (ubiquitination) such as:

1. Signal transduction,
2. Regulation of the cell cycle, and
3. Sensitive to interference by invading bacteria.

Plant F-box proteins

1. Plant F-box proteins

F-box proteins encoded by plants

- The F-box domain is a protein structural motif of about 50 amino acids that mediates protein-protein interactions.
- Numbers of F-box proteins in plants:
- It strikingly, nearly 700 F-box proteins have been predicted in *Arabidopsis*.
- Plant use these vast F-box proteins to control the stability of hundreds of substrates involved in cellular functions such as signal transduction and regulation of the life cycle.

F-box proteins

2. Bacterial F-box proteins

F-box proteins encoded by plant pathogens

- A recent bioinformatic analysis has identified more than 70 F-box proteins (plant pathogen-encoded F-box effectors) in 22 different bacterial species.
- Functions of F-box proteins in bacteria:

1. Use of pathogen-encoded F-box effectors in the host cell may be a wide spread infection strategy.
2. F-box proteins play a key role in virulence.

- Examples of F-box proteins in bacteria:

1. VirF is the first F box protein identified in bacteria(*A. tumefaciens*).
2. GALA proteins are another example of F box proteins were found in *Ralstonia solanacearum*.

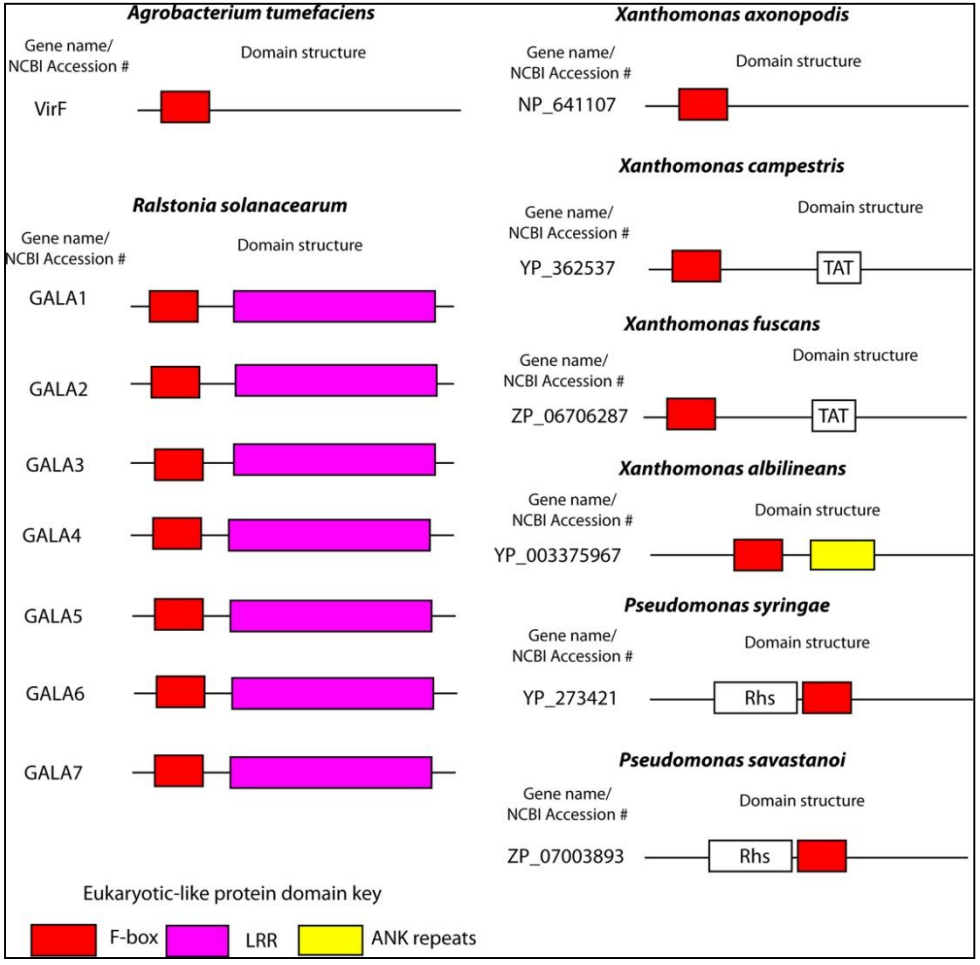
F-box proteins

F-box proteins encoded by plant pathogens

- *Ralstonia solanacearum* encodes several F-box proteins, some of which are translocated into the plant cell through the type III secretion system.
- The *R. solanacearum* F box proteins have been designated "GALA" proteins(GALA 1-7).
- It has been suggested the acquisition of many bacterial F-box proteins from primitive eukaryotic host.

F-box proteins

F-box proteins encoded by plant pathogens



Price and Kwaik,2009

F-box proteins

F-box proteins encoded by plant pathogens

BACTERIAL PATHOGEN			
Agrobacterium tumefaciens	VirF	VIP1	Interacts with the *Arabidopsis* SKP1. Facilitates the T-complex uncoating by destabilizing the host factor VIP1 as well as its associated T-strand coating protein VirE2
Ralstonia solanacearum	GALA family	N/D	At least four (GALA1, 5, 6, 7) out of seven members of this protein family interact with the *Arabidopsis* SKP1. Possess partially overlapping roles in enhancing infection of *Arabidopsis* and tomato. GALA7 is a host range factor required for virulence in *Medicago truncatula*
Pseudomonas syringae	YP_273421	N/D	N/D
Pseudomonas savastanoi	ZP_07003893	N/D	N/D
Xanthomonas axonopodis	NP_641107	N/D	N/D
Xanthomonas campestris	YP_362537	N/D	N/D
Xanthomonas fuscans	ZP_06706287	N/D	N/D
Xanthomonas albilineans	YP_003375967	N/D	N/D

Magori and Citovsky,2011

Bacterial sensing

Mimic/inactivate/hijack/abusing plant MAPKinase

Hijacking autoinducers or AHLs

- Prokaryotes and eukaryotes communicate with each other through various hormone and hormone-like chemical compounds. Hormone-like compounds referred to as autoinducers or AHLs.
- These signals, however, can be 'hijacked' by bacterial pathogens to activate their virulence genes.
- These bacterial signals can modulate eukaryotic cell-signal transduction and that host hormones can cross-signal with QS signals to modulate bacterial gene expression.
- However, plants and algae have evolved multiple mechanisms(e.g. alfalfa plant secretes more AHL mimics after bacterial AHL treatment) to interpret these QS signals and initiate defensive responses.

Hughes and Sperandio,2009

Bacterial sensing

Evidence for sensing low levels of iron

- There is also abundant evidence that many bacteria can sense the low levels of iron in the plant intercellular space and that the ability to acquire iron during the infection process is required for pathogenicity.
- Siderophores in ex. *Erwinia* species:

1. Desferrioxamine by *Erwinia amylovora*;
2. Chrysobactin by *P. carotovorum*.

- Under iron limiting conditions, *P. carotovorum* produces siderophore chrysobactin and three outer membrane transport proteins (for uptake of Chrysobactin-Fe complex)
- Therefore, the mutants defective for chrysobactin or transporter show reduced virulence.

Bacterial thinking

- When bacteria are not in a gradient, they tumble randomly.
- But when they sense graded amounts of attractant, they immediately check the tumbling and swim smoothly on a straight course.
- Thus, bacteria had to make a decision to swim toward or away from substances (Chemotaxis).

Bacterial thinking

- The most famous example of this process is antibiotic resistance.
- One reason that resistant bacteria can spread so quickly in a hospital is that inheritance is not the only way these microbes can get hold of the genes that can fight off a drug.
- Horizontal gene transfer, as it's known, may involve:

1. a single gene, or
2. an entire network of genes.

- And when two networks arrive in an alien genome, they can combine together into a bigger network that can do something entirely new.
- Horizontal gene transfer gives bacteria an extra dimension of creativity.

Horizontal gene transfer

- New biotypes of bacteria seem to arise by means of at least three sexual-like processes.
- It is probable that similar processes occur in mollicutes:

1. Conjugation
2. Transformation
3. Transduction

- Gram-negative bacteria can transmit genetic material readily across species;
- *Agrobacterium* transmits genes across kingdom barriers to plants.
- Such events are called horizontal gene transfers.

Bacterial creativity

Gene swap, slurp and slice

- Bacteria can swap (exchanage) genes.

1. The genes may be carried by viruses that jump from one bacterial host to another.
2. In other cases, bacteria slurp up (extract) DNA from dead microbes and insert it into their own genomes.
3. In still other cases, genes can spontaneously slice themselves out of one genome and get inserted in the DNA of a distantly related species.

Bacterial creativity

The TALE family found in genus *Xanthomonas*

- Transcription activator like effectors (TALEs) are injected via the type III secretion pathway of many plant pathogenic *Xanthomonas* spp. into plant cells where they contribute to disease or trigger resistance by binding to DNA and turning on TALE-specific host genes.
- This new paradigm for protein-DNA interaction has been discovered in bacterial pathogens of plants.

For more information see the pathogenicity section of xanthomonads.

Bogdanove *et al.*,2010; Horvath,2011

Bacterial creativity

The TALE family found in genus *Xanthomonas*

- *Xanthomonas* uses TAL effectors to manipulate gene function in plants in ways that benefit the pathogen.
- Interaction with plants has selected TALEs that activate host genes that facilitate bacterial colonization and spread.

Bogdanove *et al.*,2010; Horvath,2011

Bacterial creativity

The TALE family found in genus *Xanthomonas*

- Xanthomonads utilize proteins that function as eukaryotic transcription activators to effect the host environment.
- Transcription activator like (TAL) effectors use a novel protein motif with an extremely simple DNA recognition code.
- The understanding of this code enables a number of highly useful tools, including:

1. targeted gene activation or repression,
2. engineering of genomes to add, remove, or
3. alter genes, and site-specific gene insertion.

Bogdanove *et al.*,2010; Horvath,2011

Bacterial creativity

Gene silencing/slice

P. aeruginosa

- Humans often worry about being infected by bacteria, but bacteria have to worry about infections of their own.
- As a result, bacteria long ago developed an adaptive immune system to protect themselves by silencing foreign genes introduced by viruses or other bacteria.
- It was observed an enzyme that helps bacteria slice up foreign genetic material.

Doudna,2010

Bacterial pathogenesis on plants
Pathogen derived molecules-Part 1

The path of bacterial plant pathogenesis
Microbial Strategies for Attack

1. Bacterial infection
2. Main virulence factors:
   - Toxins
   - Extracellular polysaccharide
   - Degradative enzymes
   - Hormones
3. Quorum sensing
4. Secretion systems

Bacterial pathogenesis on plants

Important concepts in phytobacteriology

- Bacterial pathogens gain access to plants through wounds or natural openings.
- They do not make their own openings into plants.
- Bacterial pathogens can manipulate plant cells by:

1. Injecting DNA or proteins into plant cells, or
2. With toxins.

- Plants can fight back by evolving ability to recognize these effectors and launch resistance/defense mechanisms.

Bacterial pathogenesis on plants

Important concepts in phytobacteriology

- Some bacterial plant pathogens are insect-transmitted.
- Bacterial pathogens can acquire new virulence genes via horizontal transfer.
- Bacterial pathogens do not rely on just one virulence gene.
- Pathogenesis is due to small and overlapping contributions from many genes.
- Bacteria act as a group.

Pathogenicity/virulence factors

- Pathogenicity is, the ability of a pathogen to cause disease (essential for disease), while, virulence is the degree or measure of pathogenicity of a given pathogen.
- Therefore, a bacterium could be pathogenic yet have varying degrees of virulence.
- Those which contribute to the virulence factors are:

1. Incidence,
2. Rate or severity of wilt symptoms.

- Bacteria use virulence factors to suppress plant defence.

Denny, 2006; Buonaurio,2008;Jakson,2009

Plant pathogens
Biotrophs, necrotrophs and hemibiotrophs

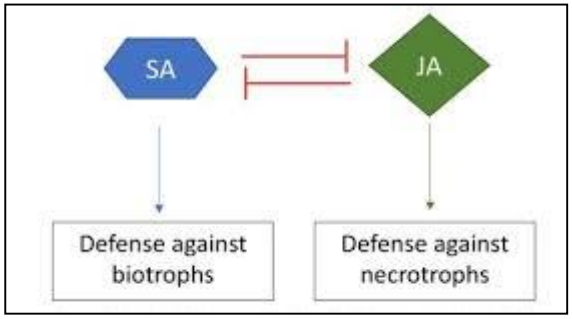
- Plant pathogens are often divided into biotrophs and necrotrophs, (and, more recently, hemibiotrophs) according to their lifestyles.

1. Biotrophs derive nutrients and energy from living cells;
2. Necrotrophs derive their energy from dead or dying cells;
3. Hemibiotrophs An organism that is parasitic in living tissue for some time and then continues to live in dead tissue.

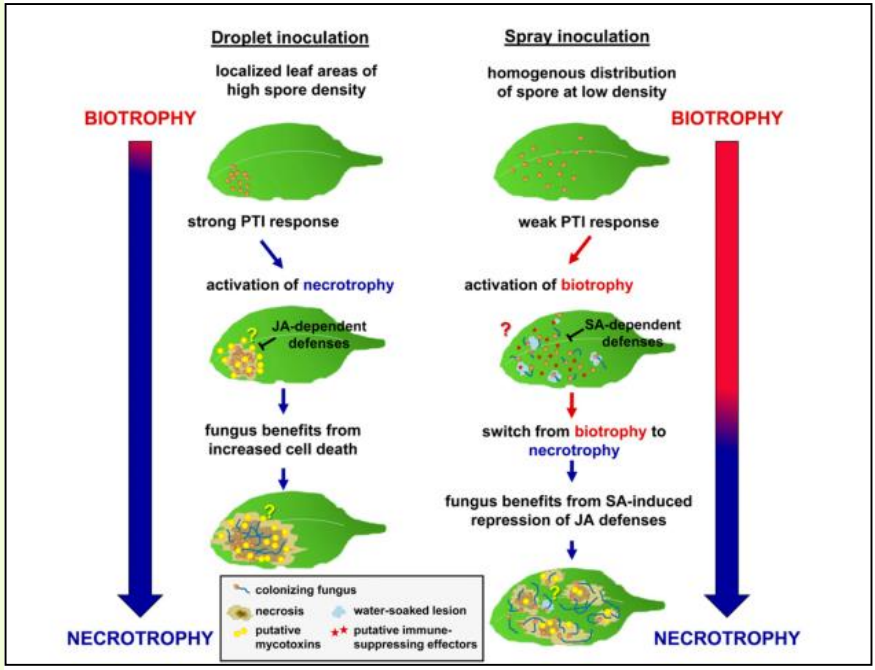
- Hemibiotrophs switch from a biotrophic to a necrotrophic lifestyle in the course of their development.

Häffner *et al.*,2015;..

Colonization
Biotrophs and necrotrophs



- Necrotrophic and biotrophic pathogens are resisted by different plant defenses.
- Necrotrophic pathogens are sensitive to jasmonic acid (JA)-dependent resistance,
- Biotrophic pathogens are resisted by salicylic acid (SA)- and reactive oxygen species (ROS)-dependent resistance.

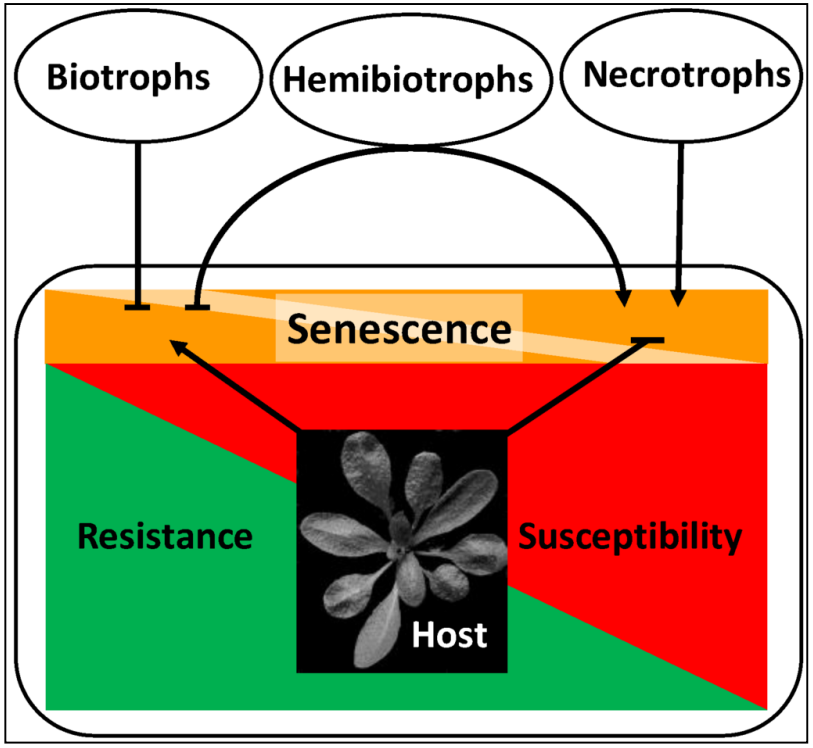


Many pathogens switch from biotrophy to necrotrophy during infection, little is known about the signals triggering this transition. PAMP-triggered immunity (PTI) or basal resistance

Pétriacq *et al.*,2016

Relationship between senescence and resistance/susceptibility in necrotrophic and biotrophic host-pathogen interactions

- Biotrophic pathogens and hemibiotrophs in their biotrophic stage inhibit senescence to increase susceptibility.
- The host can control pathogen growth and promote resistance by activating senescence-like processes.
- Necrotrophic pathogens and hemibiotrophs in their necrotrophic stage induce senescence to increase susceptibility.



Arrows stand for activation/induction, bars for inhibition.

Häffner *et al.*,2015

Model necrogenic Gram-Negative phytopathogens

Examples of necrotrophic/biotrophic bacteria

Pathogen	Host Range; Model Hosts	Typical Diseases	Phenotype of *hrp* (Type III Secretion) Mutants[a]	Phenotype of Type II Secretion Mutants[b]	Other Disease Factors
Necrotrophic					
Erwinia carotovora and *E. chrysanthemi*[c]	Wide; potato, tobacco seedlings, Saintpaulia	Soft rots	HR^{-d}; infectivity reduced but wild-type maceration	No maceration	Pectic enzymes; siderophores; autoinduction
Biotrophic					
E. amylovora	Rosaceae; apple and pear	Fire blight	Hrp^{-d}	Not known	EPS; harpin
E. stewartii	Maize	Stewart's wilt	Wts^{-d}	Not known	EPS; autoinduction
Ralstonia solanacearum[e]	Solanaceae; tomato and tobacco	Wilts	Hrp^{-}	Virulence reduced	EPS; volatile signal and global regulation
Xanthomonas campestris pathovars	Individually narrow; pepper, tomato, brassicas	Foliar spots and blights	Hrp^{-}	Virulence reduced	Avr proteins; global regulation
Pseudomonas syringae pathovars	Individually narrow; tomato, Arabidopsis, legumes	Foliar spots and blights	Hrp^{-}	Not known	Avr proteins; toxins

Erwinia spp. such as *E. amylovora* and *E. chrysanthemi* use type I secretion system to secrete proteases, which have been shown to be required for leaf colonization and act as pathogenicity or virulence factors.

Alfano and Collmer,1996

Virulence/Aggressiveness/phyto pathogenic factors

Small molecules as virulence factors

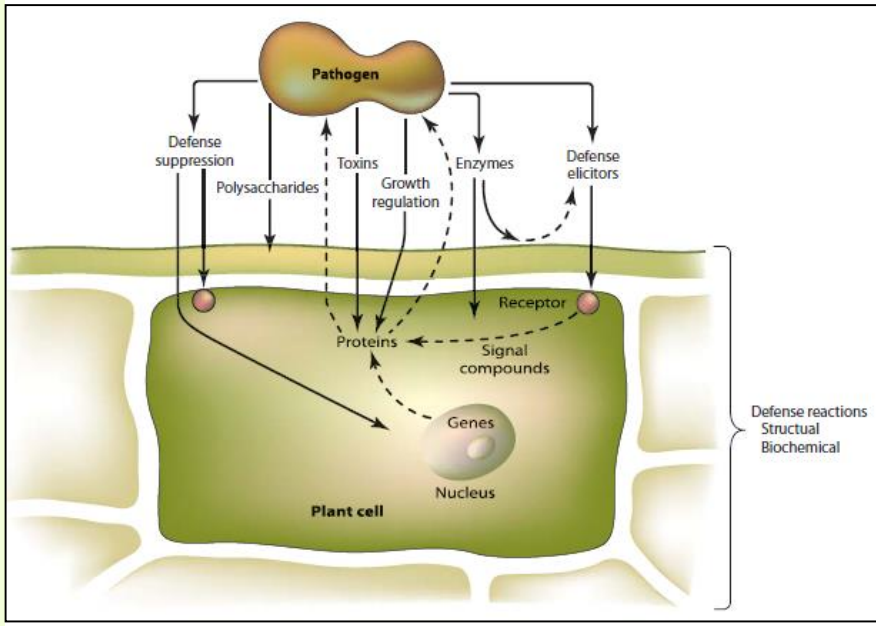
- **Biotrophic bacteria**
- The most important pathogenicity factors to be considered are:
- Extracellular polysaccharides (EPS)
- Extracellular plant cell wall-degrading enzymes
- Toxins
- Hormones
- Other diverse factors such as:
- Autoinducers(hormone-like molecules)
- Siderophores
- Defense elicitors
- These bacteria also use different secretion systems (virulence factors) as well as intercellular signals involved in quorum sensing that activate the expression of numerous genes during infection.

Biotrophic pathogens produce very little extracellular enzymes in comparison to necrotrophs.

Abramovitch *et al.*,2006; Soto *et al.*,2006

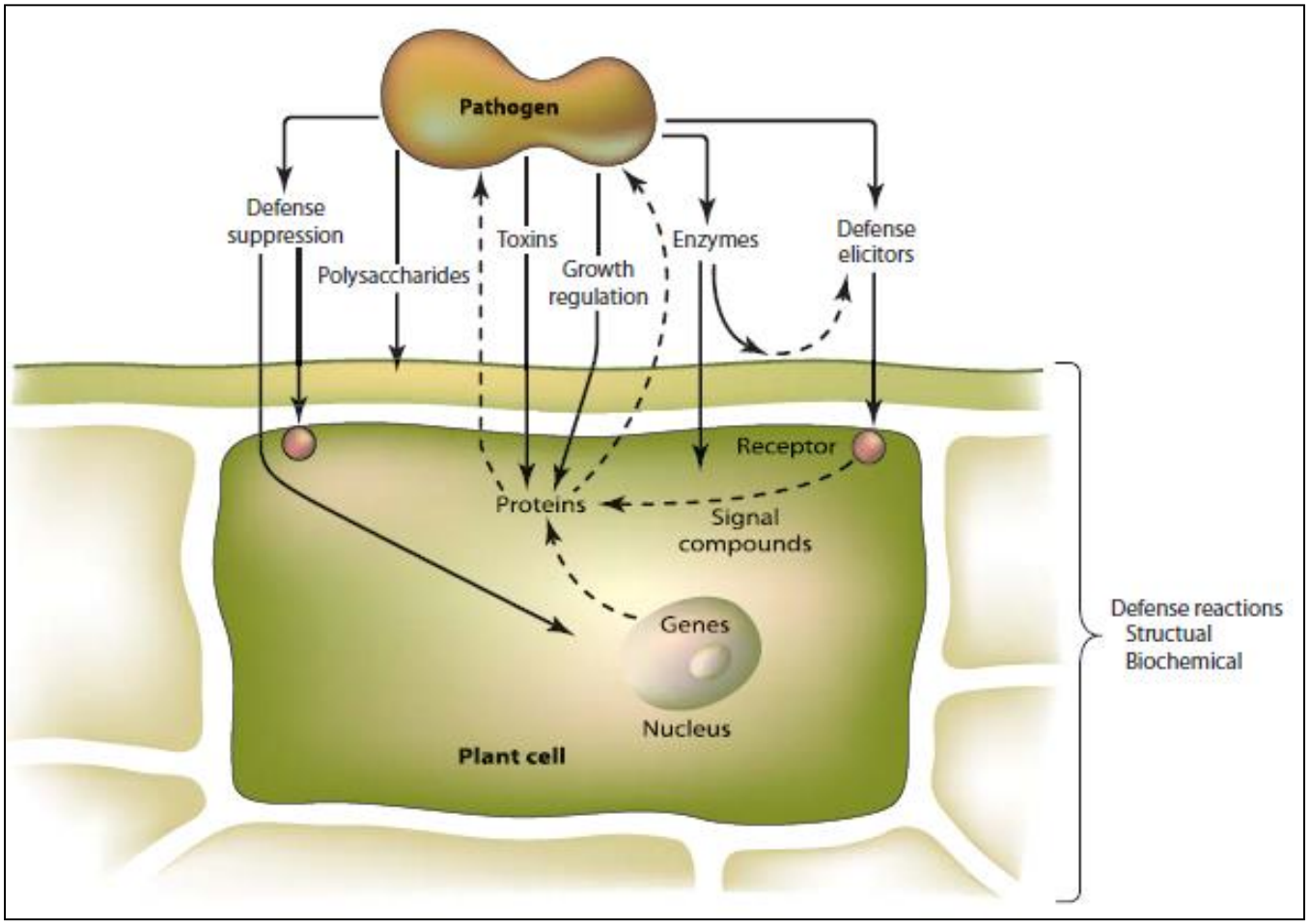
Schematic representation of pathogen interactions with host cells

- Pathogens with a variety of nonspecific elicitors such as PAMPs (defense elicitors) and toxins, enzymes, glycoproteins, etc. stimulate the plant defense.
- *avr* genes implicate the suppression of host defenses.
- Plant cells, react with numerous defenses, which may include cell wall structural defenses (waxes, cutin, suberin, lignin, phenolics callose, etc.) or biochemical wall, membrane, cytoplasm, and nucleus defense reactions.
- The latter may involve bursts of oxidative reactions, production of elicitors, HR, phytoalexins, PR proteins.



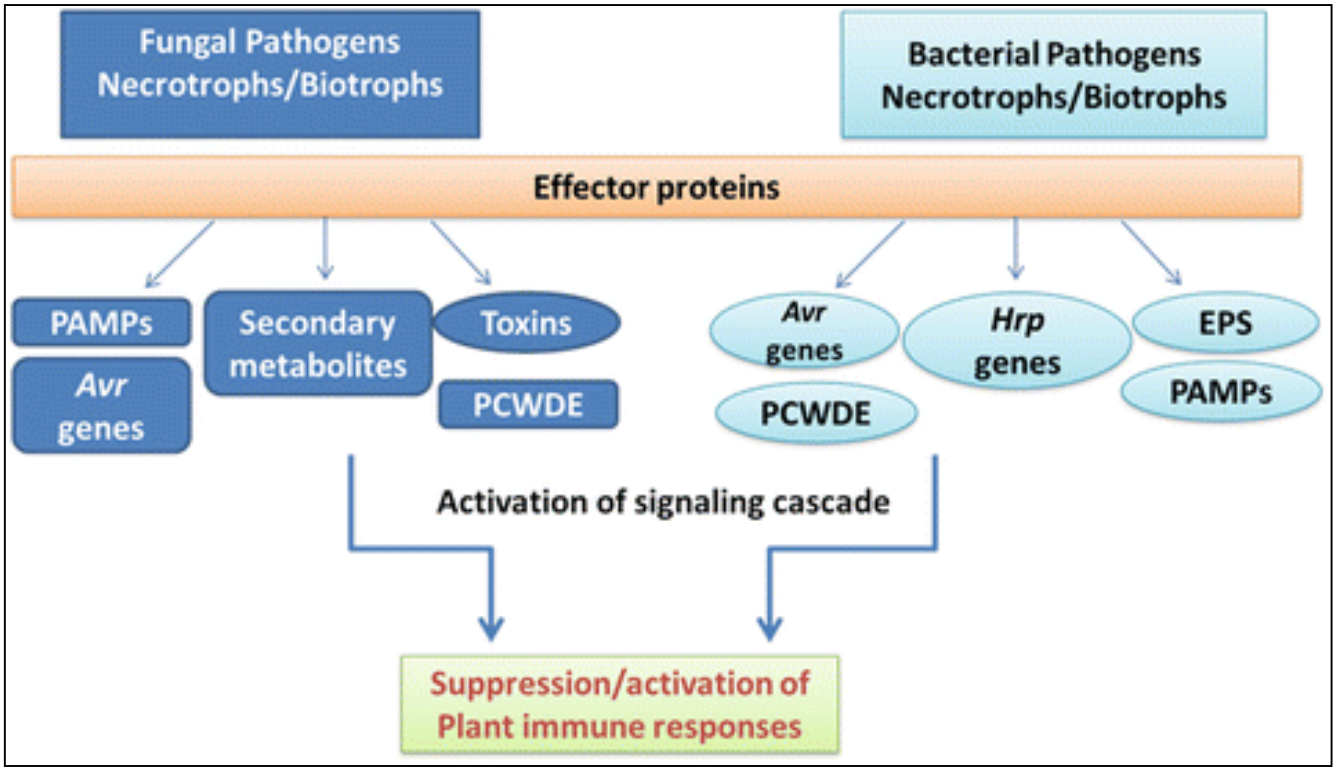
Agrios,2005;..

Schematic representation of pathogen interactions with host cells



Agrios,2005

Fungal and Bacterial Biotrophy and Necrotrophy



Geeta and Mishra,2018

Infection process

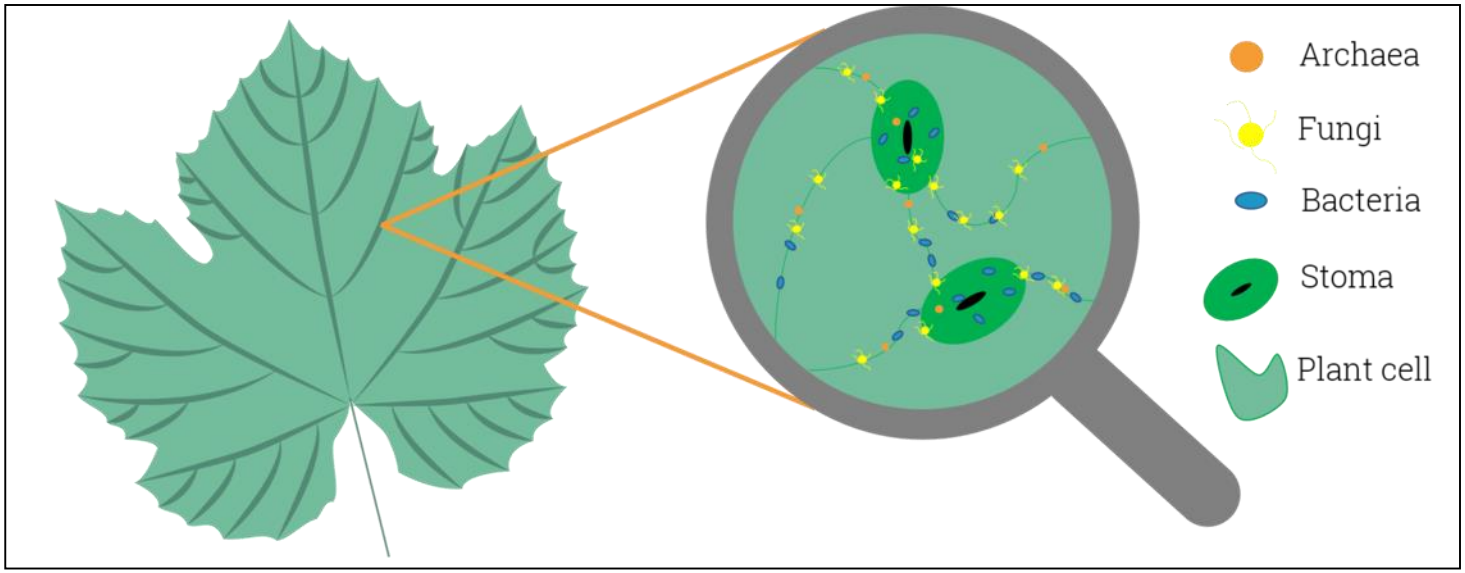
- The "infection process" can be divided into three phases:

1. **Pre-entry:** The motile pathogens, they must find the host and negotiate its surface before entering the host.
2. **Entry:** Pathogenic bacteria and nematodes often enter through stomatal pores when there is a film of moisture on the leaf surface.
3. **Colonization:** A successful infection requires the establishment of a parasitic relationship between the pathogen and the host, once the host has gained entry to the plant.

Plant cell surfaces

Play a key role in plant-microorganism interactions

- The plant aerial surface, mostly occupied by leaves, is inhabited by diverse microorganisms, forming the phyllosphere.



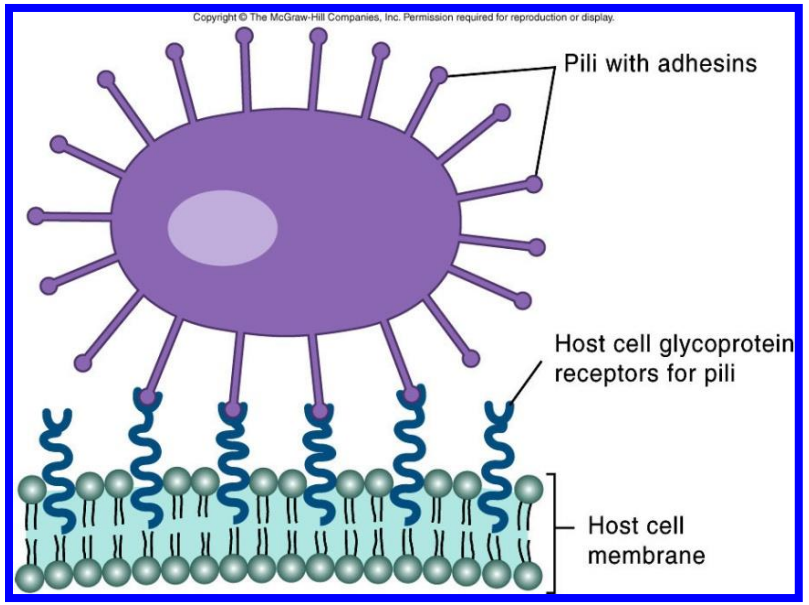
Wikipeda,2021

Adhesion process

Adhesin proteins

Most fimbria of Gram-ve bacteria function as adhesins

- Bacteria typically employ proteins known as adhesins to attach to host tissues, which usually are located on ends of fimbriae.
- Alternatively, adhesins can consist of glycocalyx.



Adhesins are cell-surface components or appendages of bacteria that facilitate bacterial adhesion or adherence to other cells or to inanimate surfaces. Adhesins are a type of virulence factor.

Adherence structures

Terms used to describe adherence factors in microbiology

Adherence factor	Description
Adhesin	A surface structure or macromolecule that mediate the binding of a bacterium to the host cell. E.g. proteins, glycoproteins and EPSs (xanthan, amylovorin).
Receptor	A complementary macromolecular binding site on a (eucaryotic) surface that binds specific adhesins or ligands.
Lectin	Any protein that binds to a carbohydrate.
Ligand	A surface molecule that exhibits specific binding to a receptor molecule on another surface.
Mucous	The mucopolysaccharide layer of glucosaminoglycans covering animal cell mucosal surfaces.
Fimbriae	Filamentous proteins on the surface of bacterial cells that may behave as adhesins for specific adherence.
Common pili	Same as fimbriae
Sex pilus	A specialized pilus that binds mating procaryotes together for the purpose of DNA transfer.
Type 1 fimbriae	Fimbriae in *Enterobacteriaceae* which bind specifically to mannose terminated glycoproteins on eucaryotic cell surfaces.
Type 4 pili	Pili in certain Gram-positive and Gram-negative bacteria. In *Pseudomonas*, thought to play a role in adherence and biofilm formation.

Adherence structures

Terms used to describe adherence factors in microbiology

Adherence factor	Description
Biofilm	Exopolysaccharide or slime produced by bacteria that attaches imbedded cells to a surface.
S-layer	Proteins that form the outermost cell envelope component of a broad spectrum of bacteria, enabling them to adhere to host cell membranes and environmental surfaces in order to colonize.
Glycocalyx	A layer of exopolysaccharide fibers on the surface of bacterial cells which may be involved in adherence to a surface. Sometimes a general term for a bacterial capsules.
Capsule	A detectable layer of polysaccharide (rarely polypeptide) on the surface of a bacterial cell which may mediate specific or nonspecific attachment.
Lipopolysaccharide (LPS)	A distinct cell wall component of the outer membrane of Gram-negative bacteria with the potential structural diversity to mediate specific adherence. Probably functions as an adhesin.
Teichoic acids and lipoteichoic acids (LTA)	Cell wall components of Gram-positive bacteria that may be involved in nonspecific or specific adherence.

Todar,2008

Putative attachment factors described for plant pathogenic bacteria

- EPS, exopolysaccharide;
- CPS, capsular polysaccharide;
- LPS, lipopolysaccharide;
- Hrp, hypersensitive response and pathogenicity;
- HecA, homologous to FHA;
- FHA, filamentous hemagglutinin;
- PA-IIL has high affinity for L-fucose;
- RSL has affinity for L-fucose;
- RSL has affinity for L- fucose>L-galactose>D-mannose/D-arabinose;
- RS-IIL has high affinity for fructose and mannose;
- rhicadhesin a bacterial Ca^{2+}-binding protein(14,000 Da).

Agrobacterium tumefaciens	LPS
Agrobacterium tumefaciens	T-pilus, F-conjugation factor
Agrobacterium tumefaciens	Rhicadhesin
Agrobacterium tumefaciens	att-encoded proteins
Agrobacterium tumefaciens	CPS
Erwinia carotovora	Type 1 fimbriae, Hrp
Erwinia chrysanthemi	Hrp proteins
Erwinia chrysanthemi	HecA (FHA)
Klebsiella aerogenes	Type 3 fimbriae
Pantoea stewartii	Hrp
Pseudomonas aeruginosa[d]	Type IV pili
Pseudomonas aeruginosa	PA-IIL lectin[e]
Pseudomonas aeruginosa	Type II pseudopilus
Pseudomonas fluorescens	Type III?
Pseudomonas fluorescens	Type IVB pili, Hrp
Pseudomonas syringae pathovars	Type IVB pili, Hrp
Ralstonia solanacearum	Type IVB pili, Hrp
Ralstonia solanacearum	FHA homologs[f]
Ralstonia solanacearum	RSL and RS-IIL lectins[g]
Xanthomonas campestris	Type IVB pili, Hrp
Xanthomonas campestris pathovars	FHA homologs
Xylella axonopodis	Flagellum, type IV pili
Xylella fastidiosa	Type IVB pili?
Xylella fastidiosa	FHA homologs

Sapers *et al.*,2006

Virulence factors and their associated functions

Adherence structures

- Bacteria produce numerous virulence factors that contribute to their ability to colonize and infect the host.

1. Pili, flagella, and surface proteins (e.g., adhesins) can facilitate initial interactions and adhesion to host cells.
2. Secretion systems are protein complexes that enable secretion of exotoxins and other virulence factors into the host, causing damage, and enabling the bacteria to more efficiently invade the host cell.
3. The bacterial endotoxin, LPS, triggers an immune response in the host.
4. Bacterial capsules enable immune evasion and adhesion to host cells.

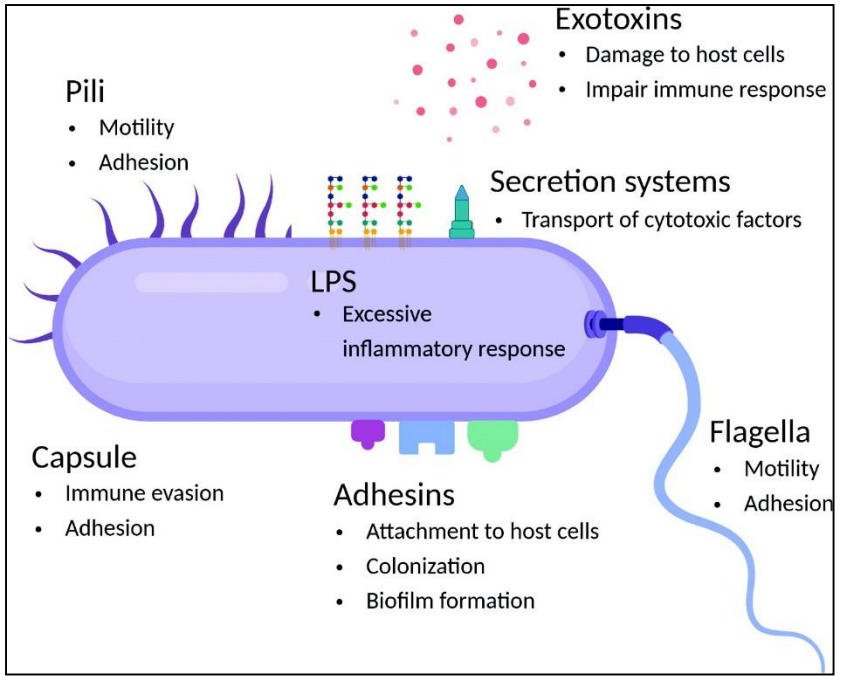
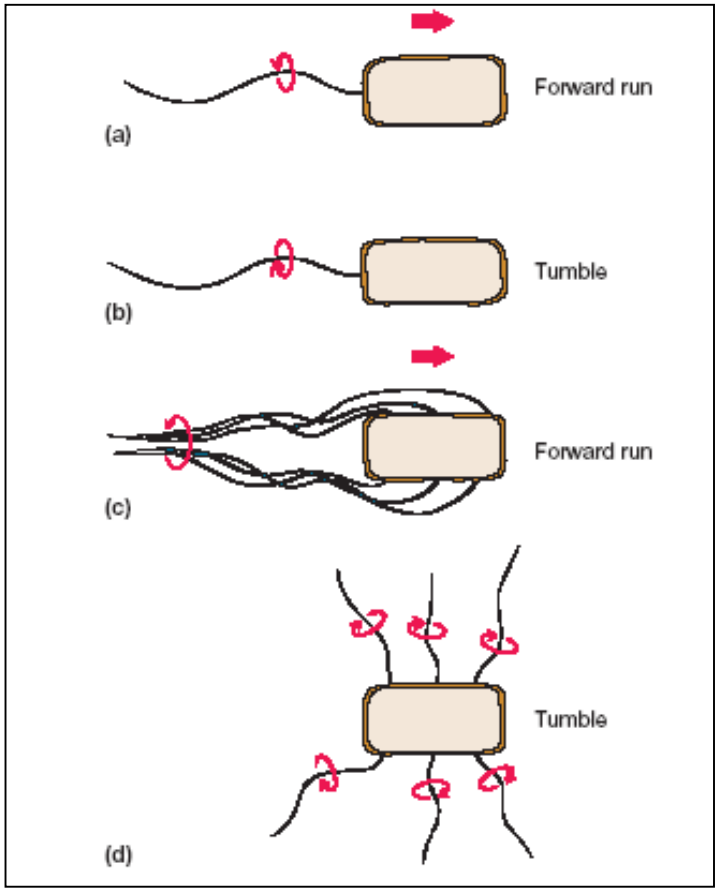


Figure created using BioRender.com.

Stoica and Cox,2021

Pre-entry
Flagella motility and chemotaxis

- Flagella and chemotaxis are required for involves in chemotaxis and penetration through open stomata.
- Parts (a) and (b) describe the motion of monotrichous, polar bacteria.
- Parts (c) and (d) illustrate the movements of peritrichous organisms.



Prescott,2006

Entry

Natural surface openings

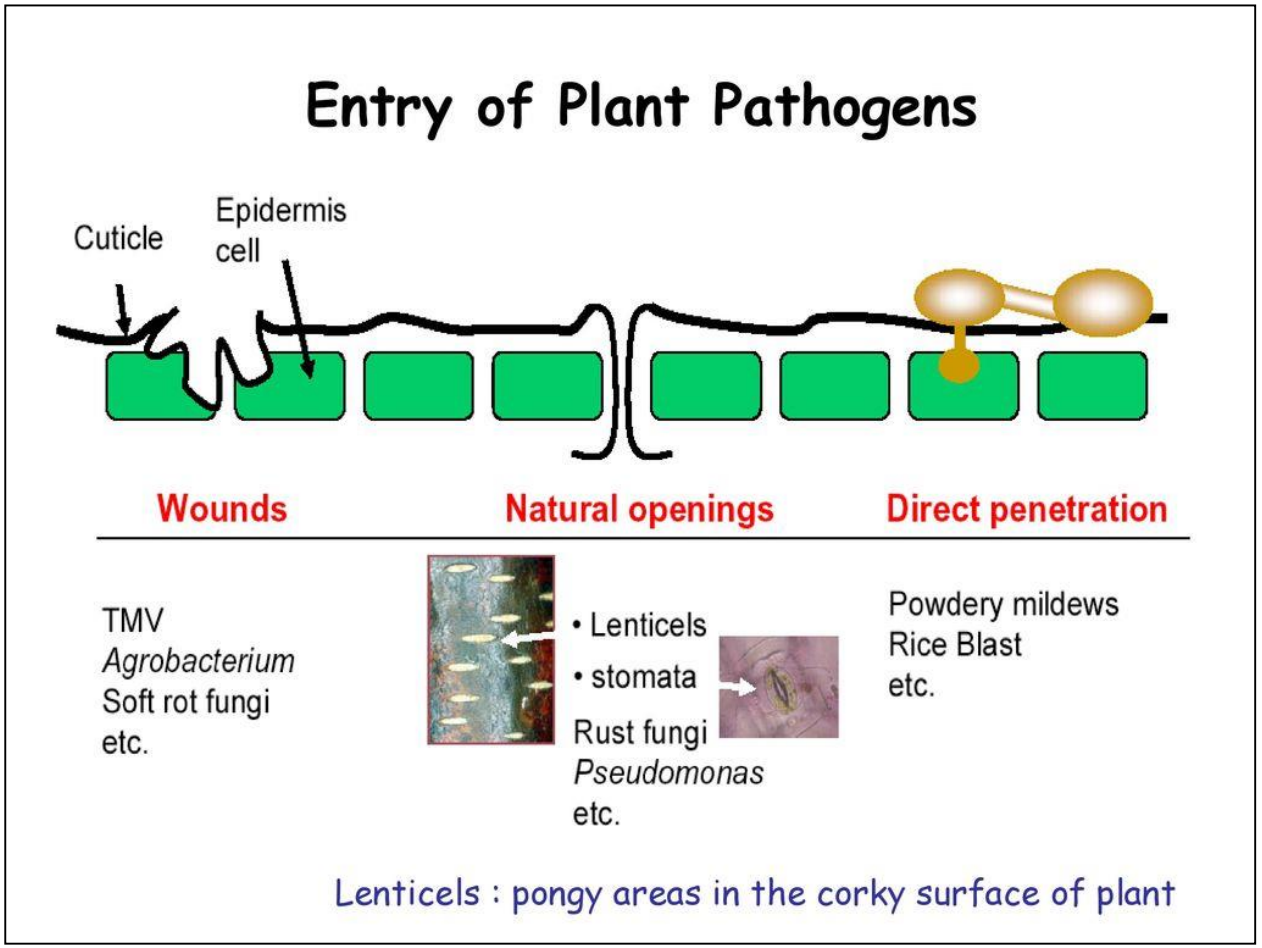
- There are a number of natural surface openings through which bacterial pathogens have been observed to enter the plant:
- These include:

1. Hydathodes(water pores): Water-exuding pores at the edges of leaves (e.g., *Xanthomonas campestris* pv. *campestris*),
2. Stomata: Openings in the aerial part of plants that control gas exchange and water transpiration between the plant interior and the environment (e.g., *Pseudomonas syringae*),
3. Nectarthodes: Nectar-secreting pores at the point of emergence of the styles and stamens (e.g., *Erwinia amylovora*), and
4. Lenticels: Respiration pores in stems and roots (*Pectobacterium atrosepticum*).

Melotto *et al.*,2008;..

Entry

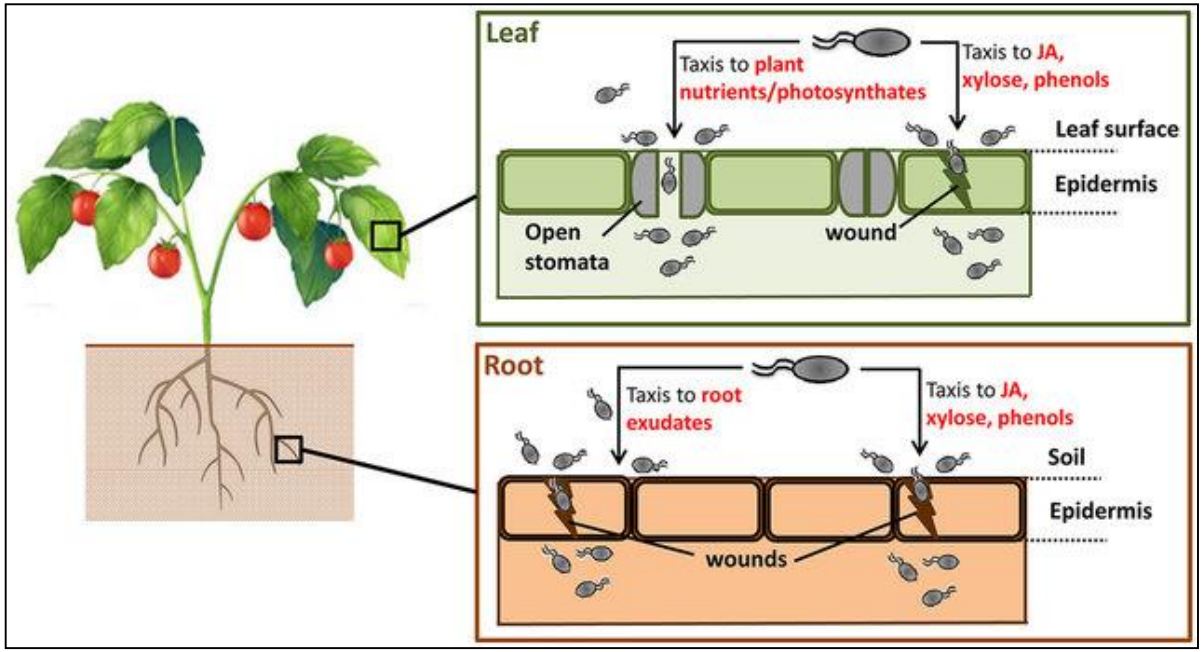
Natural surface openings



Brandon Pierce

Entry
Natural surface openings

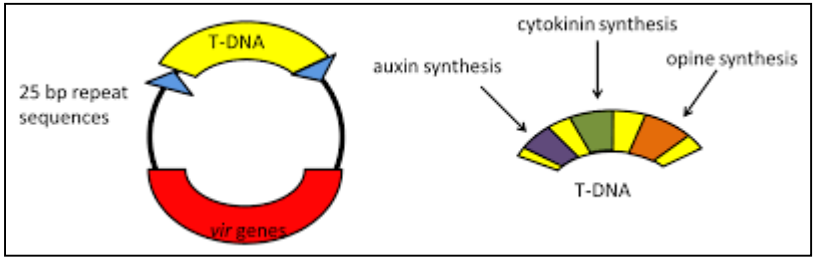
- Chemotaxis towards multiple plant-derived compounds facilitates the bacterial entry through natural openings or wounds. Once inside the plant, bacteria can proliferate at the infection site or disperse via de vascular system.

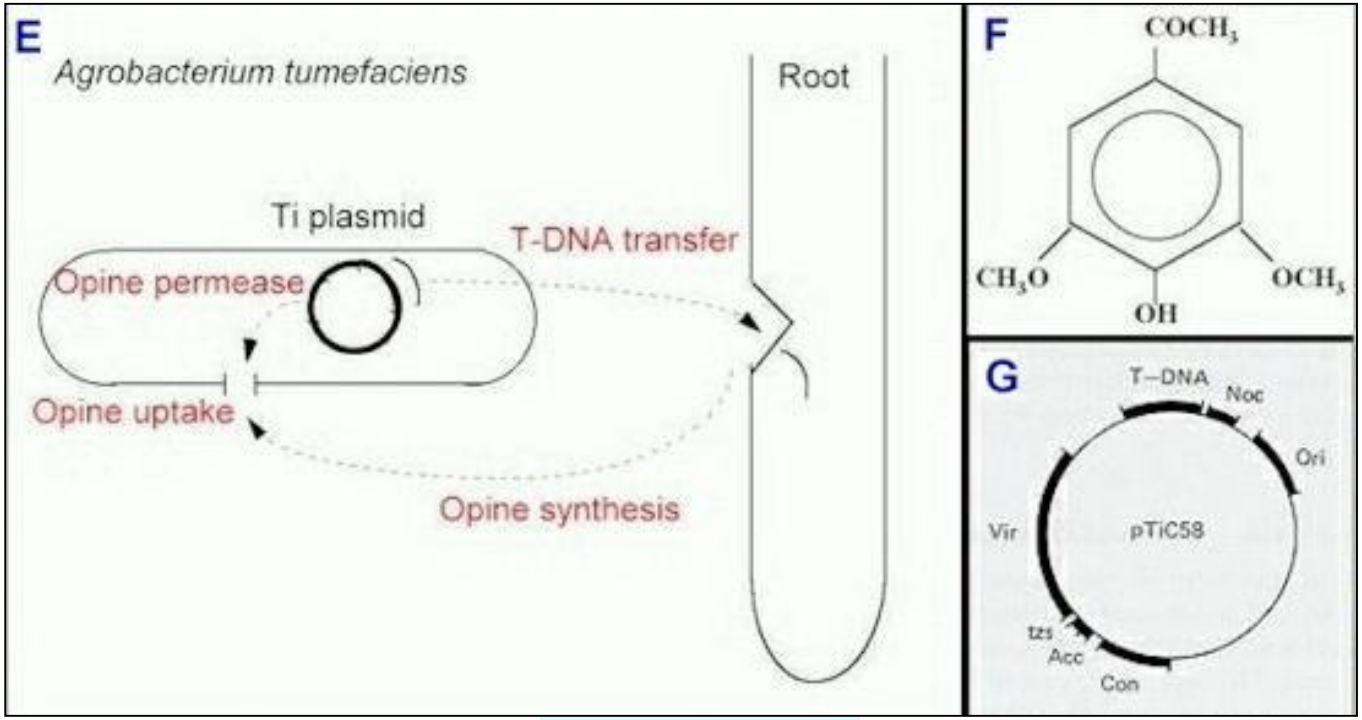


Matilla and Krell,2017

Infection process

Chemotaxies toward acetosyringone, a specific phenolic compound





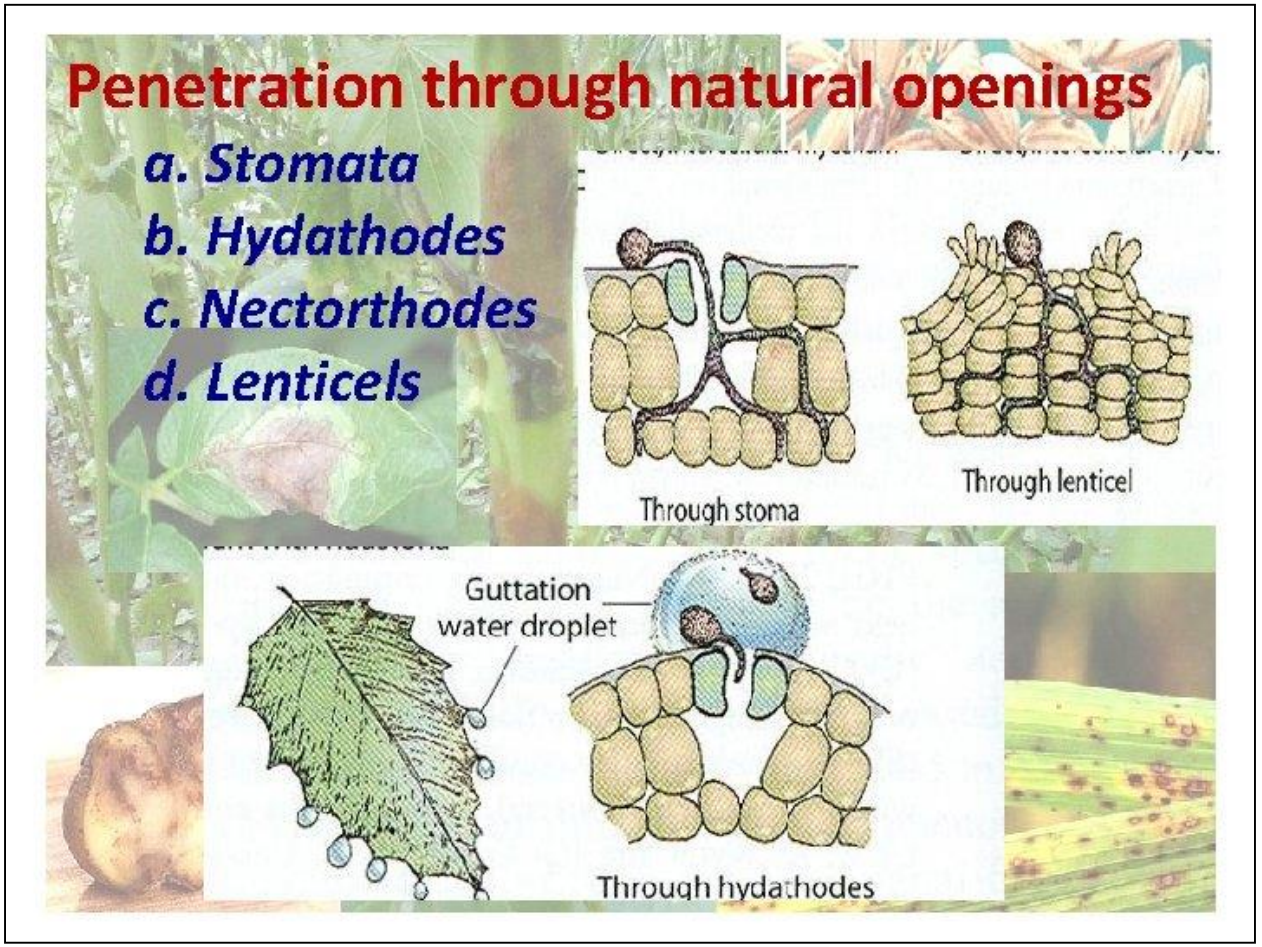
Acetosyringone

Ti plasmid

Jim Deacon;..

Entry

Natural surface openings

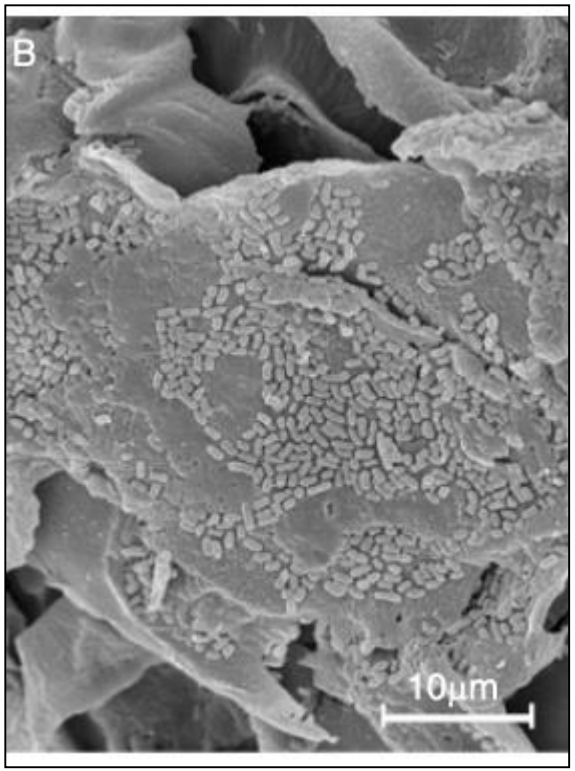


Čia renkasi meistrai

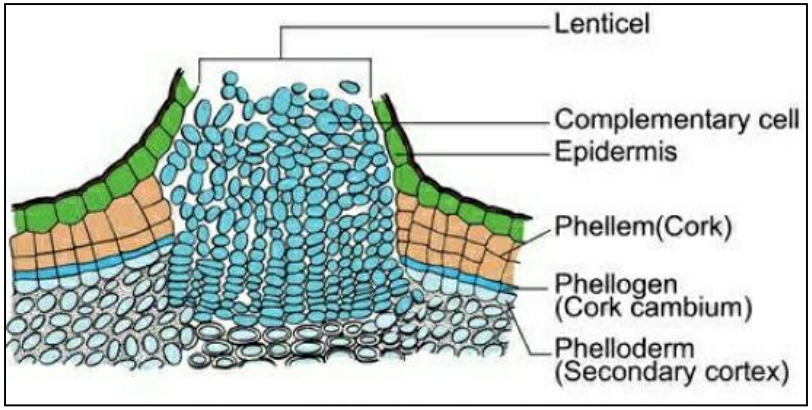
Lenticel

Lenticels are formed beneath the stomata, may be scattered on the stem and also occur on the roots

Colonization



Scanning electron microscopy image showing bacteria on in lenticel (B).



Sapers *et al.*,2006;Smriti,2022

Entry

Hydathodes

Structures containing water pores located at leaf margins that connect to the intracellular spaces and to the xylem vascular system

- Infection of *Xanthomonas campestris* pv. *campestris*, in a cabbage leaf, through the water pores or hydathodes.
- One hydathode infection indicated by red arrow at the leaf margin.
- Symptoms are blackening of the small veins and yellowing due to action of a toxin produced by the bacteria.

Janse,2006;..

Entry
Stomata
Penetration of bacteria through the stomatal pores

- Most previous studies involving inoculation of plant leaves with *P. syringae* have used the 'infiltration' method which bypasses stomata.
- The inoculation method used in the present study allowed Pss to enter through the stomata as it normally would.

SEM of stomata on grapefruit leaf with *Xac* bacteria entering stomatal chamber.

(Courtesy J. Cubero)

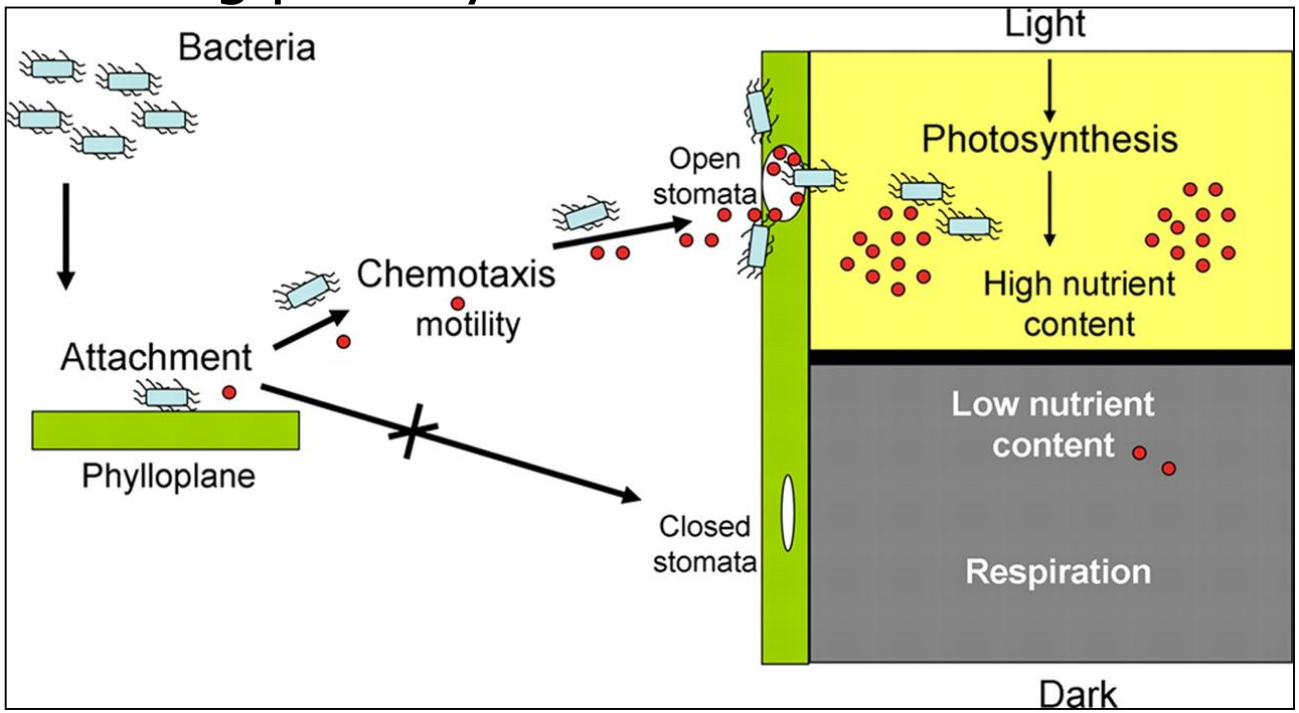
Whitelaw-Weckert *et al.*,2011

Entry

Stomata

Flagella and effect of bacterial chemotaxis on host infection and pathogenicity

- Red circles denote putative chemoattractant nutrients produced by stomatal guard cells and by parenchyma cells during photosynthesis.



Kroupitski *et al.*,2020

Stomata entry

Stomata-based defense against bacterial invasion into host tissue

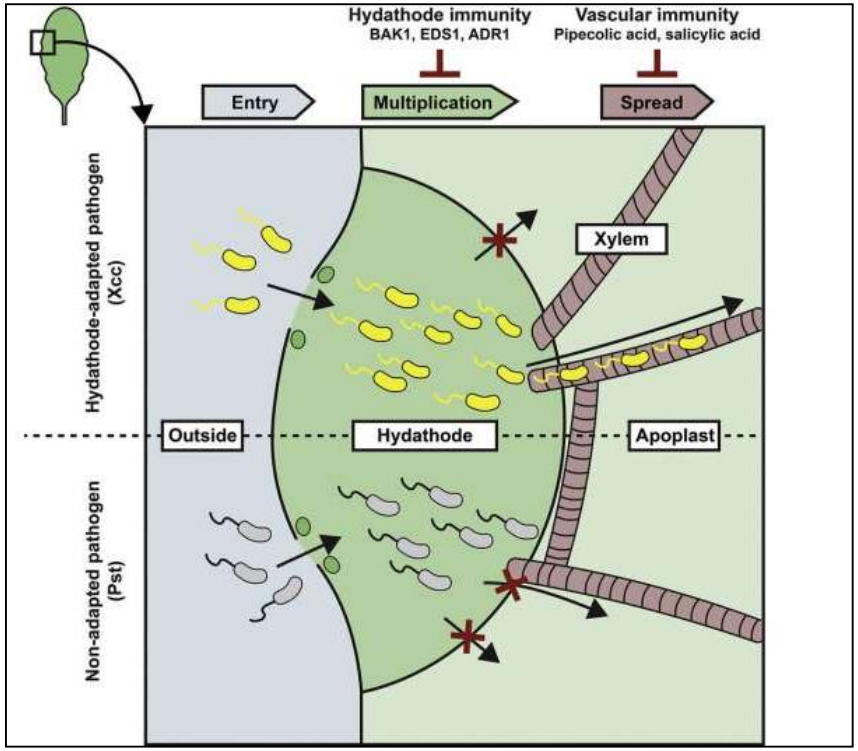
- Historically, surface openings/wounds have been considered as passive portals of entry for plant pathogenic bacteria.
- However, recent studies have shown that plants have evolved effective immune responses to protect wound sites to prevent pathogen invasion.
- e.g. stomata can play an active role in limiting bacterial invasion as part of the plant innate immune system.

Melotto *et al.*,2008; Misas-Villamil *et al.*,2013

Hydrothods entry

Water pores in leaves proven to be part of plant's defense system against pathogens

- We were able to establish that two protein complexes (for those interested: BAK1 and EDS1-PAD4-ADR1) prevent the bacteria from multiplying in the water pores.
- The same immune responses also prevent these bacteria from advancing further into the plant interior.
- In addition, we discovered that when this first line of defense occurs, the water pores produce a signal that causes the plant to produce hormones that suppress further spread of the invading bacteria along the vascular system.



Stomata entry

Stomatal closure is part of a plant immune response to restrict bacterial invasion

Coronatine reopen closed stomata

- Still many pathogens are able to enter leaf tissues and cause disease by producing:

1. A polyketide toxin, coronatine which effectively cause stomates to reopen;
2. Also produce syringolin A (SylA), a small molecule proteasome inhibitor which diffuses from the primary infection site and suppresses acquired resistance in adjacent tissues by blocking signaling by the stress hormone salicylic acid (SA). e.g.

- *Pseudomonas syringae* pv. *syringae*, *P. s.* pv. *atropurpurea*, and *P. s.* pv. *tomato*.

Melotto *et al.*,2008; Misas-Villamil *et al.*,2013

Stomata entry

Stomatal closure is part of a plant immune response to restrict bacterial invasion

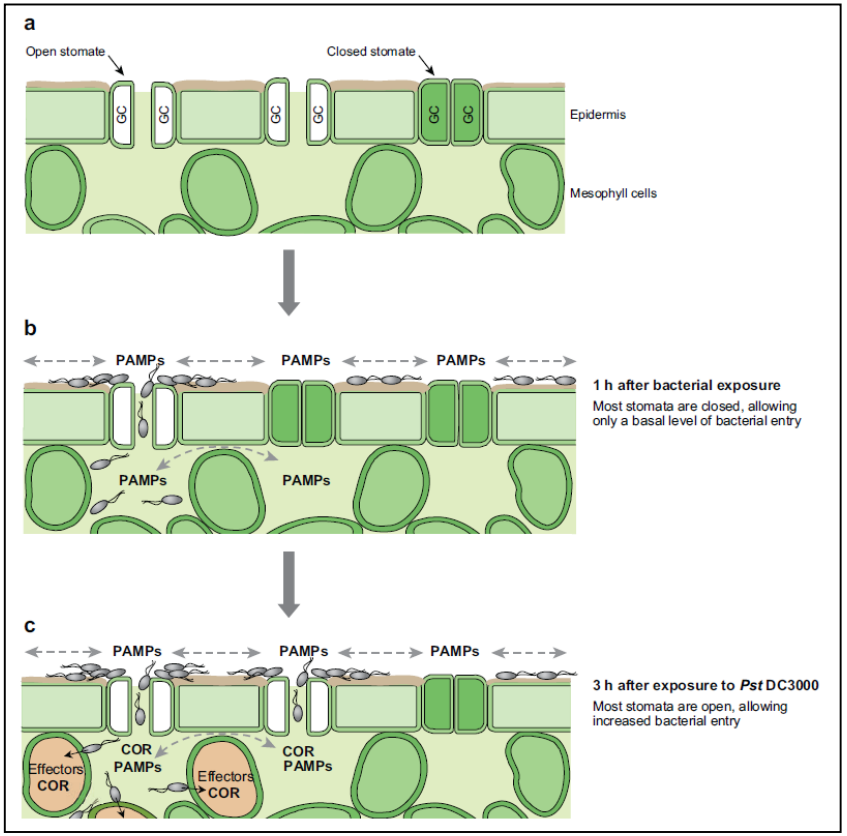
Coronatine reopen closed stomata

- A diagram depicting stomata as entry sites for bacterial invasion.

a) Stomata, formed by pairs of guard cells (GC), in light-adapted *Arabidopsis* leaves are mostly fully open.

b) Upon exposure to bacteria (Pathogen-associated molecular patterns (PAMPs) such as flagella), guard cells perceive PAMPs and many stomata close within 1 h.

c) In the case of the virulent plant pathogen *Pst* DC3000, 3 h after infection bacteria produce diffusible COR in the apoplast and/or on the plant surface to reopen closed stomata (e.g., middle stomata), thereby increasing the number of sites for bacterial invasion.

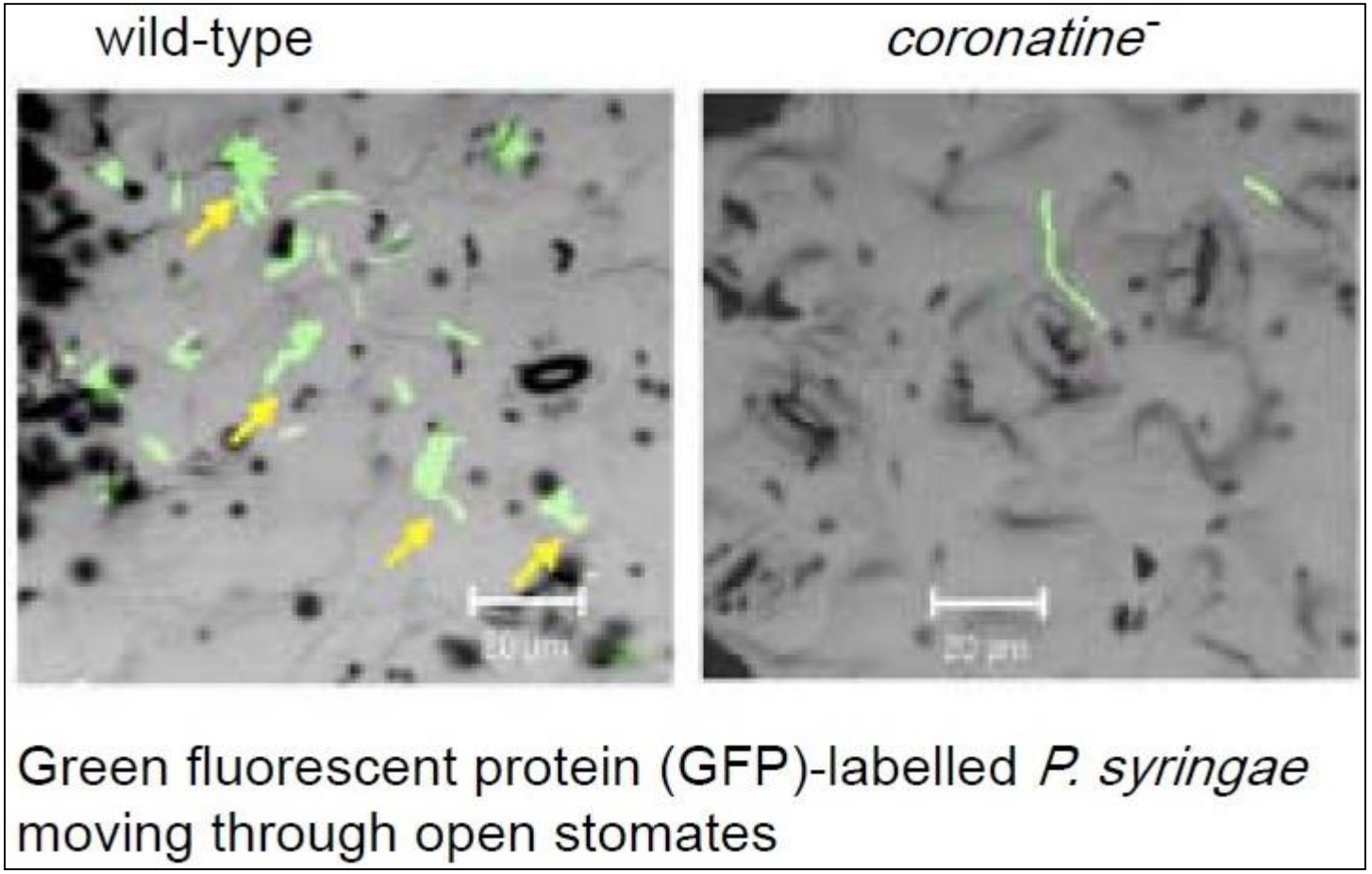


Melotto *et al.*,2008

Stomata entry

Coronatine facilitates pathogen entry into leaf mesophyll by causing stomates to open

Wild type(coronatine^{+}) and mutant (coronatine^{-})

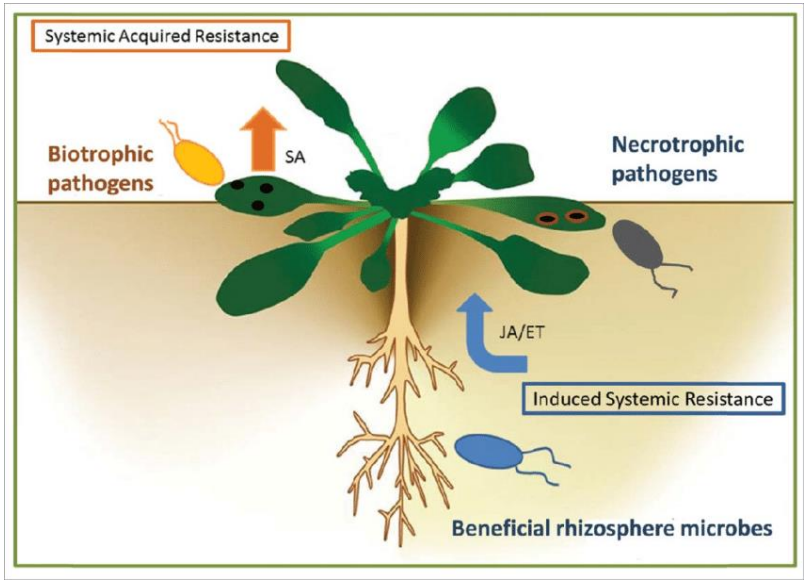


Green fluorescent protein (GFP)-labelled *P. syringae* moving through open stomates

Upon attack by bacterial pathogens
In resistant and susceptible host plant cells

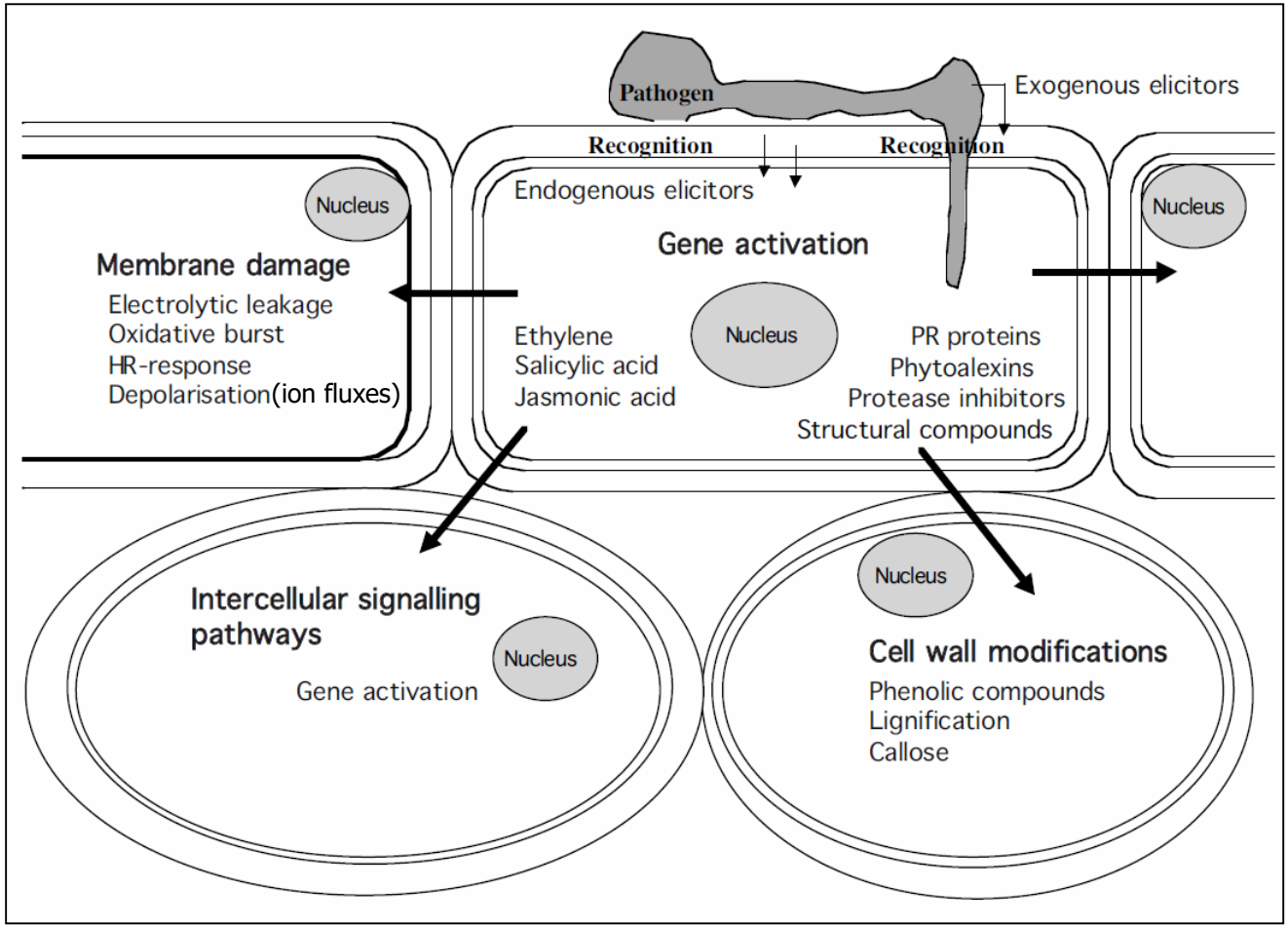
- Plant defense pathways against pathogens are linked to interactions with beneficial rhizosphere microbes.
- Plants use different defense responses to deal with different types of pathogens.
- For successful defense against biotrophic pathogens, plants typically activate:

1. the salicylic acid (SA) pathway, and
2. produce reactive oxygen species.



Chen *et al.*,2014

Schematic representation of plant defense responses in plant-pathogen interactions Modified from Benhamou *et al.*,1996



Santén *et al.*,2009

Schematic representation of pathogen interactions with host cells
Phytoalexin production/degradation

- Phytoalexins are toxic antimicrobial substances produced in appreciable amounts in plants only after stimulation by various types of phytopathogenic microorganisms or by chemical and mechanical injury.
- Phytoalexins are produced by healthy cells adjacent to localized damaged and necrotic cells in response to materials diffusing from the damaged cells.
- Phytoalexins are not produced during compatible biotrophic infections.

Agrios,2005

Schematic representation of pathogen interactions with host cells
Phytoalexin production/degradation

- Fungal pathogens are generally able to rapidly degrade phytoalexins produced by the host plant whereas degradation of phytoalexins from non-host plants is very slow.
- There is little evidence of phytoalexin degradation by bacteria.

Agrios,2005

Specific rhizobial and phytopathogenic factors required for association with plants
Enzymes and toxins as virulence factors

- Rhizobia, and phytopathogenic bacteria have adopted similar strategies and genetic traits to colonize, invade and establish a chronic infection in the plant host (common infection weapons).
- e.g. Deficiencies in QS lead to the reduction or loss of virulence in phytopathogenic bacteria, and to altered nodulation and nitrogen fixation efficiencies in rhizobia.

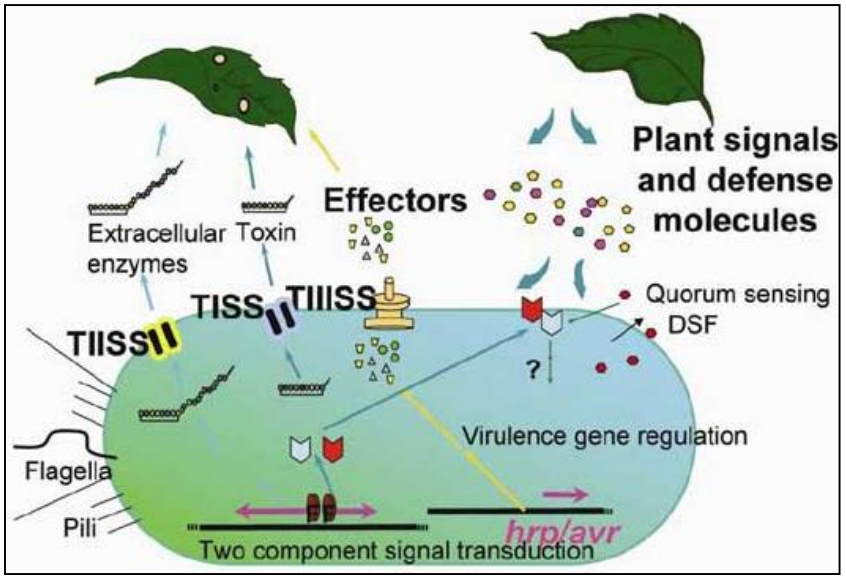
Factor	*Rhizobium* specific	Plant pathogen specific	
	Nod factor	Hydrolytic enzymes	Toxins
Function	Root hair deformation, nodule primordium and preinfection thread formation, nodule-specific gene expression, control of plant defences	Nutrient acquisition and invasion	Interference with plant metabolism, control of plant defences
Phenotype of bacterial mutants	No nodules are formed	Reduction or loss of virulence	Reduction or loss of virulence

Soto *et al.*,2006

Infection process and host-pathogen interactions

Xanthomonas spp., *X. fastidiosa* and *Burkholderia* spp.

- The most common QS signals in G-ve bacteria are N-acyl homoserine lactones (AHLs).
- But some other non-AHLs diffusible signal factor (DSF) have been identified in rhizobia and plant pathogens.
- DSF originally identified from the plant bacterial pathogen *Xanthomonas campestris* pv. *campestris*.
- Later, BDSF was found from *Burkholderia cenocepacia*.



Three secretion systems:
TISS,TIISS and TIIISS

Chatterjee,2009; Deng *et al.*,2010; Wu *et al.*,2011

Infection process and host-pathogen interactions

Xanthomonas spp., *X. fastidiosa* and *Burkholderia* spp.

- DSF-based quorum sensing is a new language in Gram-negative bacteria.
- Diffusible signal factor (DSF) is a fatty acid signal molecule involved in regulation of virulence in several *Xanthomonas* species as well as *Xylella fastidiosa*.
- It also regulates virulence and biofilm dispersal in:

1. *Burkholderia* spp., and
2. *Pseudomonas aeruginosa*.

- BDSF was found from *Burkholderia cenocepacia*.

Chatterjee,2009; Deng *et al.*,2010; Wu *et al.*,2011

Siderophores

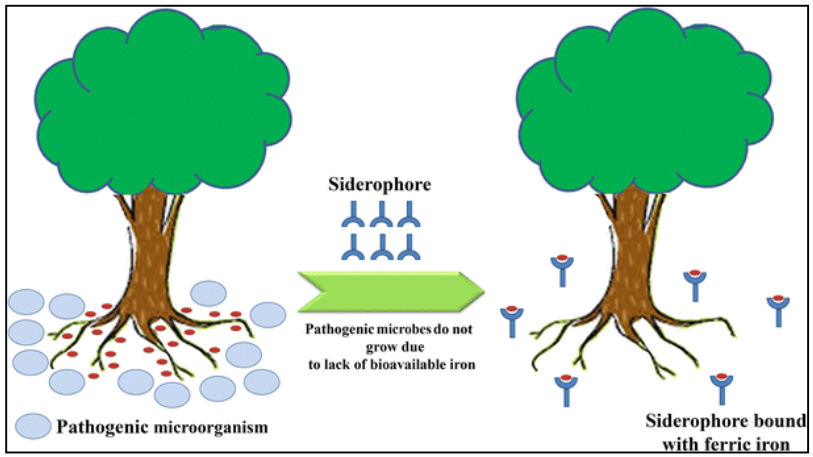
Required for full virulence of some phytopathogenic bacteria

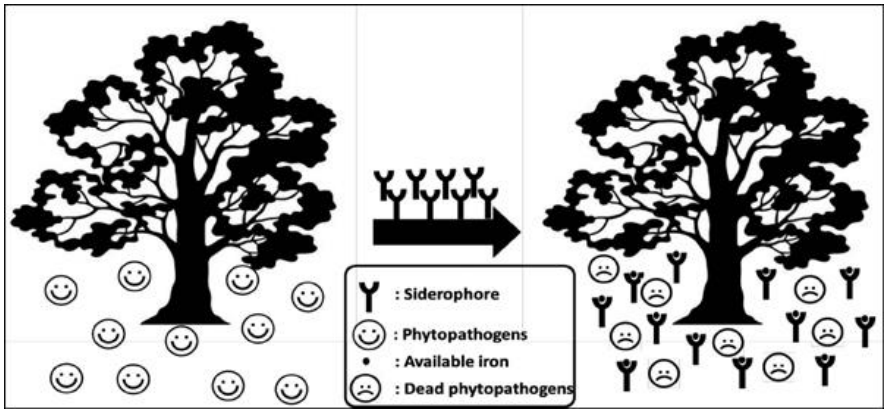
- Many bacteria produce siderophores (low-molecular mass extracellular iron scavenging compounds) to help them acquire essential iron, which is often in forms that are biologically unavailable to aerobes.
- Some phytopathogenic bacteria need their iron acquisition system for full virulence, whereas others do not.
- The production of siderophores by microorganisms is beneficial to plants, because it can inhibit the growth of plant pathogens.

Denny,2006; Sharma & Johri,2003

Siderophores

Required for full virulence of some phytopathogenic bacteria



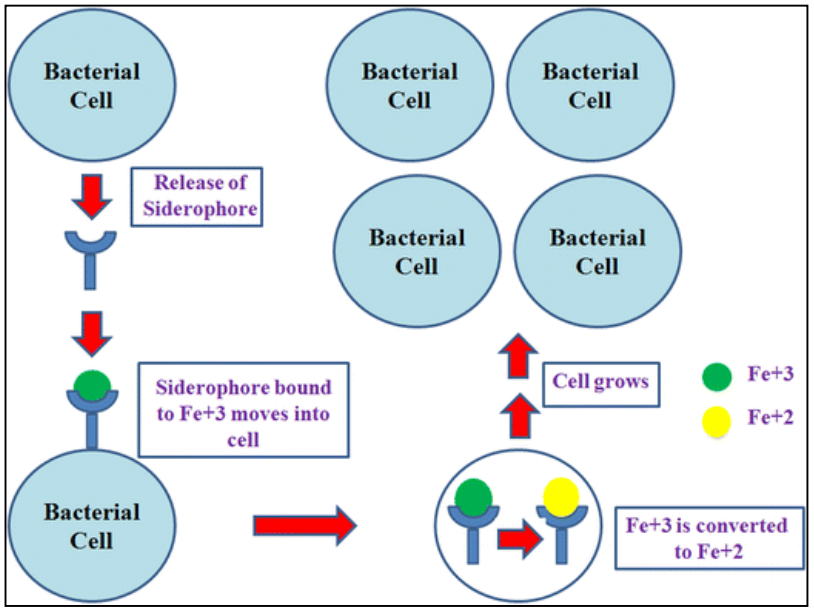


Saha *et al.*,2016; Prabhakar,2020

Siderophores

Required for full virulence of some phytopathogenic bacteria

- Under iron-limited conditions, bacterial cell releases siderophore.
- This siderophore forms complex with the insoluble ferric iron and binds to the surface of the bacterial cell.
- The Fe^{3+}-siderophore complex gets transported inside the cell and the insoluble ferric iron (Fe^{3+}) is converted into the soluble ferrous form (Fe^{2+}).



The siderophore either gets degraded inside the cell or is released in free form outside the cell. Bacterial cell utilizes this ferrous form of iron for their growth and thereby increases in number.

Saha *et al.*,2016

Major (pathogenicity/virulence)Factors

Small molecules as virulence factors used by bacteria to promote disease

These include:

EPS

Enzymes

Toxins

Hormones

and

Translocated effector proteins

Host-pathogen interactions
Major virulence factors

- In the process of host–pathogen interactions, bacterial pathogens always employ some special genes, e.g., virulence factors (VFs) to interact with host and cause damage or diseases to host.
- A number of VFs have been identified in bacterial pathogens that confer upon bacterial pathogens the ability to cause various types of damage or diseases.

Niu *et al.*,2013

Host-pathogen interactions

Major virulence factors

- Virulence genes (encoding virulence factors) are good indicators for bacterial pathogenic potentials.
- Some specific pathogenicity factors enable these bacteria to spread disease in the plants.
- Five major types of factors are well known in this regard.
- These factors are:

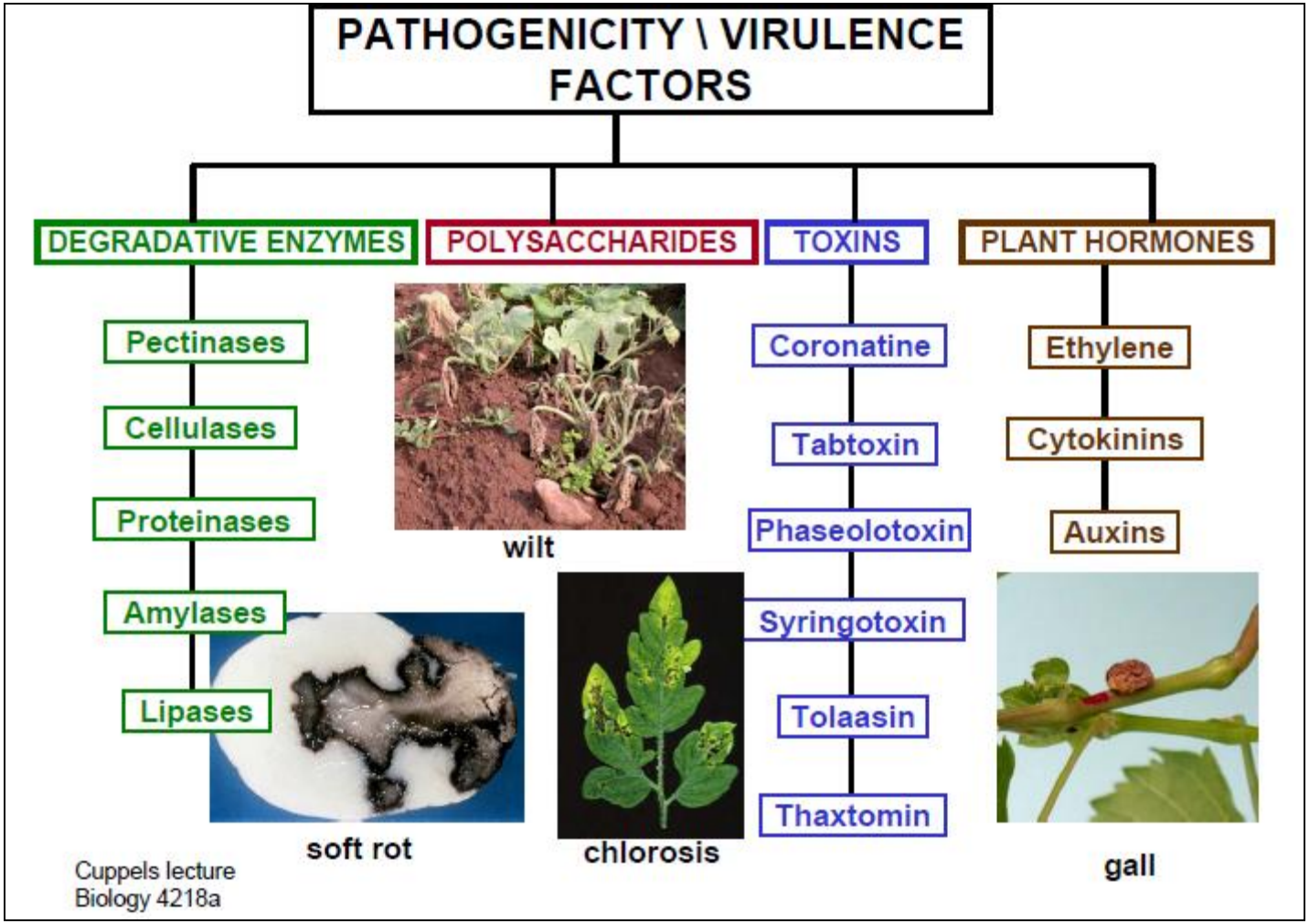
1. Exopolysaccharides,
2. Cell wall-degrading enzymes,
3. Phytohormones,
4. Phytotoxins,
5. Effector proteins.

Major virulence factors
In different bacteria

- *Agrobacterium* species cause the tumor formation through alteration in auxin levels.
- *Ralstonia solanaceraum* generate exopolysaccharides, which may cause blockage in xylem vessels, and often even plant death.
- *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 use the virulence factor coronatine to suppress stomate-based defense.
- *Pectobacterium atrosepticum* produces several degradative enzymes including a protease to cause potato soft rot.

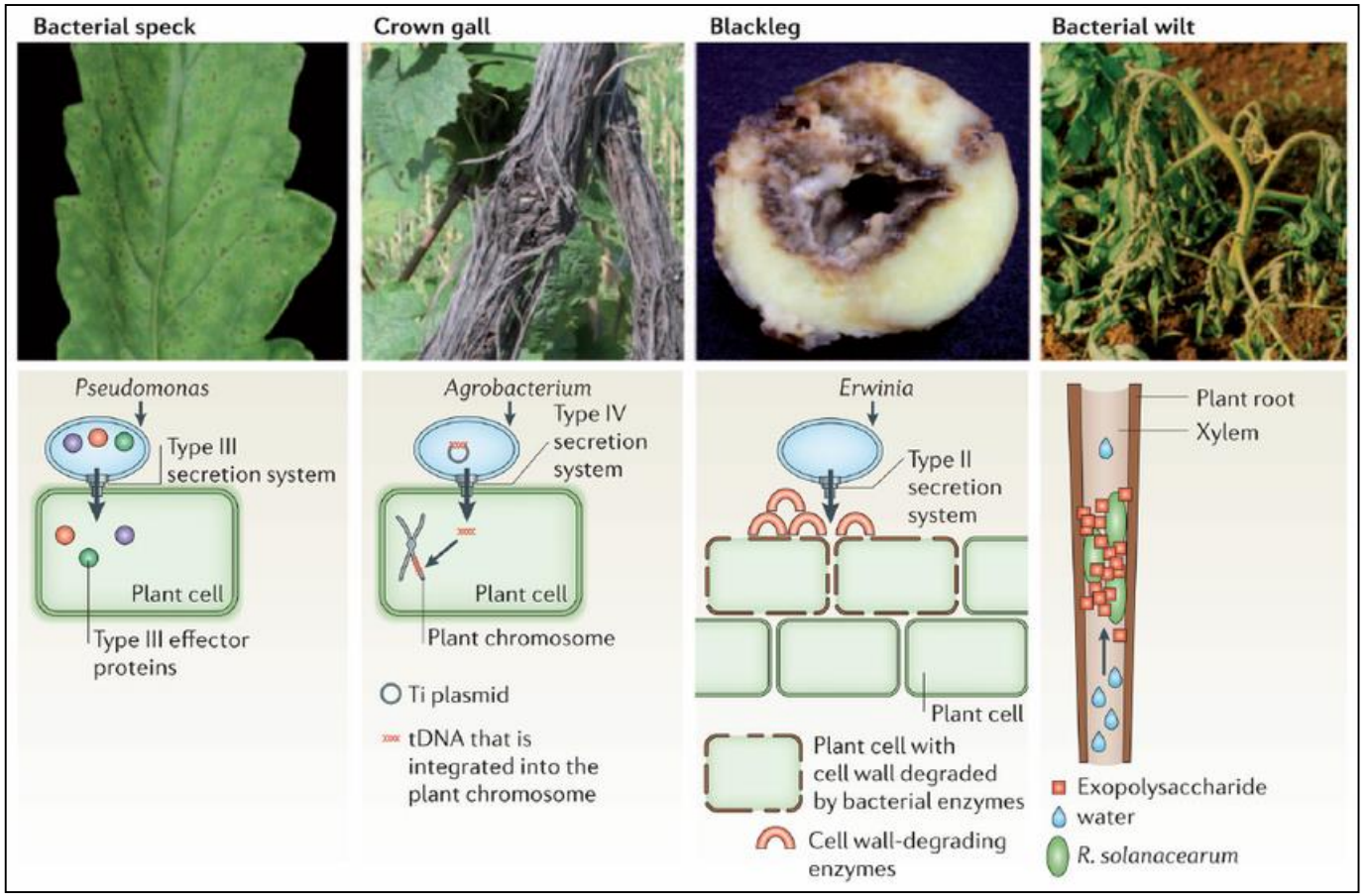
Major disease factors

Pathogenicity/virulence factors



Host-pathogen interactions

Major virulence factors



Host-pathogen interactions

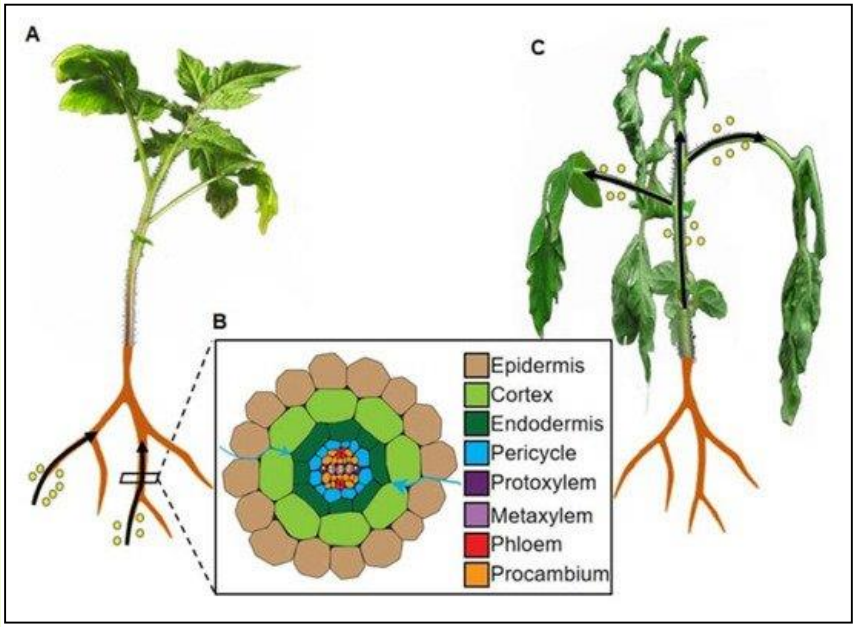
Plant invasion by the pathogenic bacterium *Ralstonia solanacearum*

A. In the first stage of invasion, bacteria (depicted as **yellow circles**) enter roots through wounds, emerging lateral roots, and root tips.

B. In the second stage of invasion, bacteria massively multiply in the intercellular spaces between cortex cells (**blue arrows**), and cause plasmolysis of epidermal cells.

C. In the last stage of invasion, bacteria (depicted as yellow circles) move throughout the plant through xylem vessels, causing clogging of the vascular system and the typical wilting symptoms.

- Bacterial movement is depicted as **black arrows**.



Intercellular spaces (blue arrows) between cortex cells.

Xue *et al.*,2020

Host-pathogen interactions

Severity of disease

Pathogen's virulence factors

- Some pathogens are more virulent than others.
- This is due to the unique virulence factors produced by individual pathogens, which determine the extent and severity of disease they may cause.
- A pathogen's virulence factors are encoded by genes that can be identified using molecular Koch's postulates.
- When genes encoding virulence factors are inactivated, virulence in the pathogen is diminished.

Host-pathogen interactions

Severity of disease

Pathogen's virulence factors

- Furthermore, pathogenic bacteria produce some morepathogenicity and virulence factors such as:

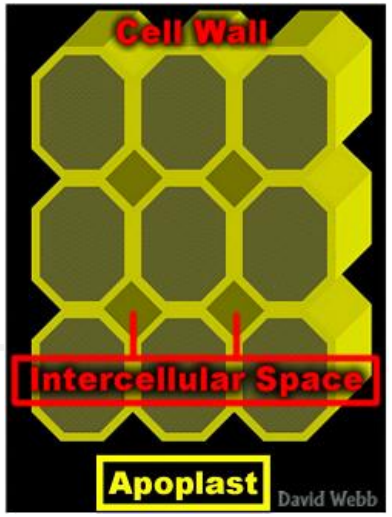
1. ice nuclei proteins,
2. secondary metabolites(e.g. siderophore),
3. quorum sensing system,
4. biofilm formation, and
5. cellular transduction signaling.

Weigele *et al.*,2017:...

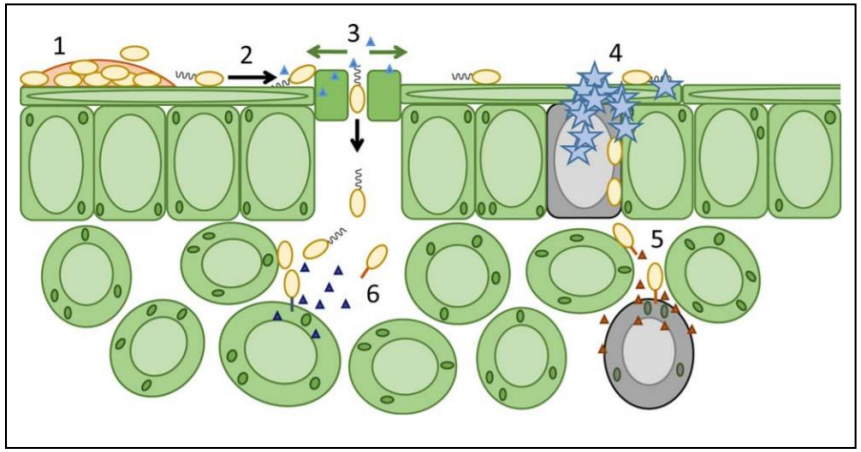
Major disease factors

Pathogenicity/virulence factors

The process of plant infection



1. Surface survival and biofilm formation.
2. Flagella/pili-driven migration across plant surfaces to apoplastic entry sites.
3. Release of phytotoxins to bypass stomatal closure.
4. Ice nucleation to damage plant surfaces.
5. Extracellular enzyme secretion to degrade cell walls and damage plant tissue.
6. Phytotoxin secretion to modify plant physiology, metabolism and immune responses.

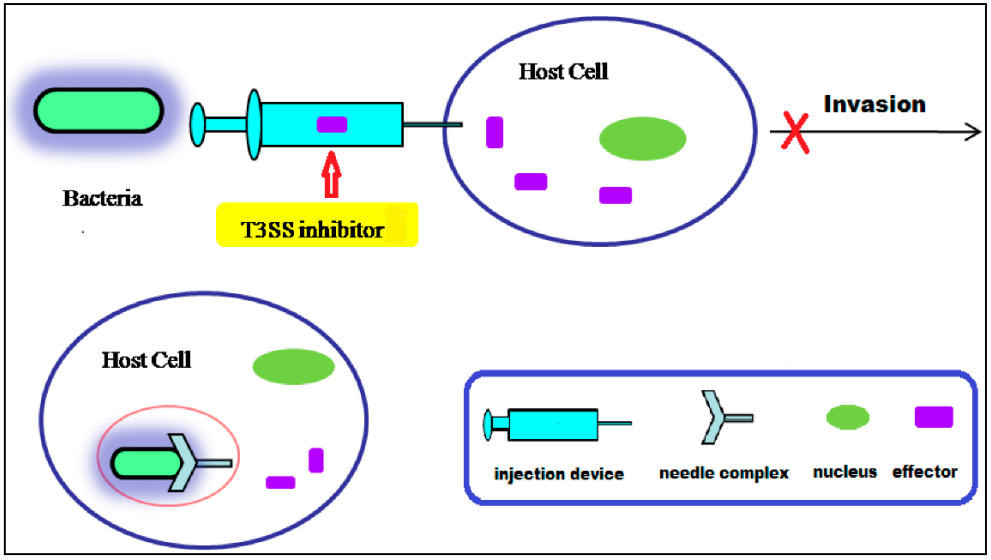


The apoplast is the portion of the plant cell outside the cell membrane. This region includes the cell walls and intercellular space of the plant.

Pfeilmier *et al.*,2016

Host-pathogen interactions
Type III secretion system

- The type III secretion system, for example, is thought to translocate between 20 and 50 effector molecules per second into plant cells. Effector proteins are up to 200 kDa in size.



The atomic weight of hydrogen atom is about one dalton. Alanine is 89 Da.

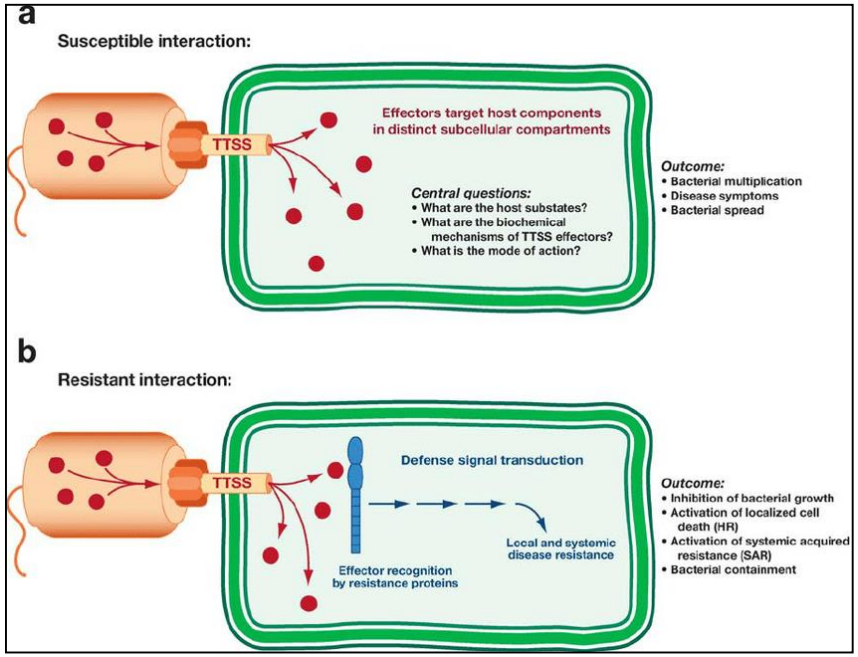
Host-pathogen interactions

Type III secretion system

- Phytopathogenic bacteria inject TTSS effector proteins into plant cells, resulting in:

a. bacterial multiplication and disease symptoms in susceptible plants, and

b. the activation of local and systemic disease resistance in resistant plants.

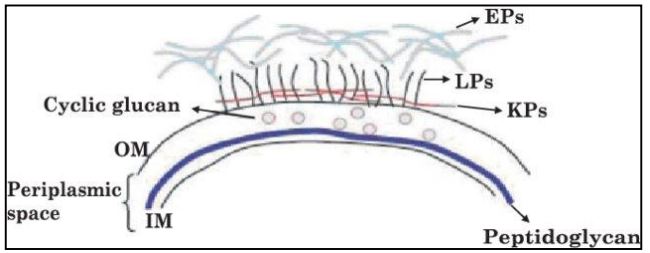


Mudgett,2005

Major virulence factors
1. Extra-cellular polysaccharides (EPS)

- EPS excreted by some prokaryotic (bacteria, archaea) and eukaryotic (algae, fungi) microorganisms.
- EPS molecules are also produced by many of the plant pathogenic bacteria.
- EPS are rich in high molecular weight polysaccharides (10 to 30 kDa) and have heteropolymeric composition.
- EPS role has been well documented in bacteria such as:

1. *Ralstonia solanacearum*,
2. *Pantoea stewartii* subsp. *stewartii*
3. Xanthomonads,
4. *Pseudomonas* spp., and
5. *Erwinia amylovora*.



Schematic representation of bacterial surface polysaccharides. EPS: exopolysaccharides; KPS: exopolysaccharides attached to the bacterial surface; LPS: lipopolysaccharides; IM: cell internal membrane; OM: cell outer.

Major virulence factors

Names of bacterial exopolysaccharides (EPSs)

- Stewartan, the EPS produced by *Pantoea stewartii*,
- The EPS from *Dickeya chrysanthemi*,
- Xanthan from *Xanthomonas campestris*,
- The fastidian gum(EPS) from *Xylella fastidiosa*,
- The alginate from *Pseudomonas aeruginosa*,
- The succinoglycan (EPS I) from *Rhizobium meliloti*, and
- The colanic acid from *Escherichia coli* have specific repeating units and sugar linkages.
- A comparison of the repeating units shows a certain level of diversity.
- Amylovoran and stewartan are highly similar.

Vanneste,2000;..

Extra-cellular polysaccharides (EPS)
Function in soil bacteria

- Soil bacteria such N_2-fixing bacteria that produce EPSs may influence the stability of soil aggregates, which in turn determines the mechanical and physical properties of soil (Burdman *et al.*,1999).
- This has a marked impact on a range of processes influencing crop yield.
- EPSs produced by leguminous nodulating nitrogen-fixing bacteria have also been reported to play an important role in plant-bacteria association and root cells colonization.

Serrato *et al.*,2006

EPSs
General functions

- The involvement of the EPS in the pathogenicity and epiphytic survival of *P. syringae* pv. *syringae* was ascertained.
- Also surface polysaccharides (SPSs) acts as:

1. Virulence factors by increasing the microbial ability of colonizing host tissues;
2. Blocks the xylem causing disease symptoms such as water-soaking or wilting.
3. Avoid or delay the activation of plant defences;
4. Act as shields protecting the bacterium against toxic compounds/enviromental stresses.
5. Acting as signal in the plant-pathogen cross-talk (cross-talk between different signalling pathways).

EPSs

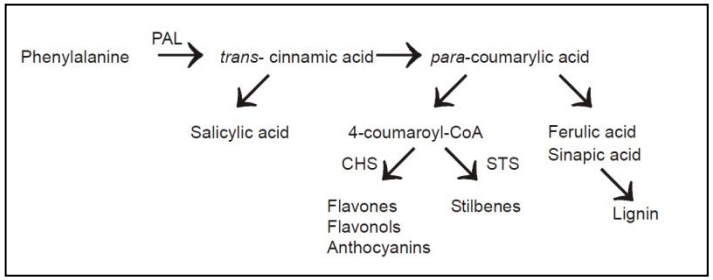
Activation of plant defence responses

Increase in PAL (phenylalanine ammonia-lyase) activity

- EPSs from *P. syringae* pv. *ciccaronei* and *P. savastanoi* pv. *nerii* induced chlorotic symptoms starting from a concentration of 0.01 mg ml^{-1}.
- No effect was induced by the EPS purified from *P. caryophylli*.

EPSs concentrations (mg ml^{-1})	Symptoms observed on the infiltrated area (~1 cm^2)		
	Chlorotic symptoms	Necrotic symptoms	
	24 h	48 h	96 h
P. syringae pv. *ciccaronei*			
	+–	–	–
0.25	+	–	+
0.50	+	–	+
2.50	+	+	++
5.00	+	++	+++
10.00	+	++	+++
P. savastanoi pv. *nerii*			
0.01	–	–	–
0.50	+–	–	–
1.00	+	–	+–
2.50	+	–	+
5.00	+	+–	++
10.00	+	+	+++
P. caryophylli			
2.50	–	–	–
5.00	–	–	–
8.00	–	–	–

de Pinto *et al.*,2003



Activation of plant defence responses

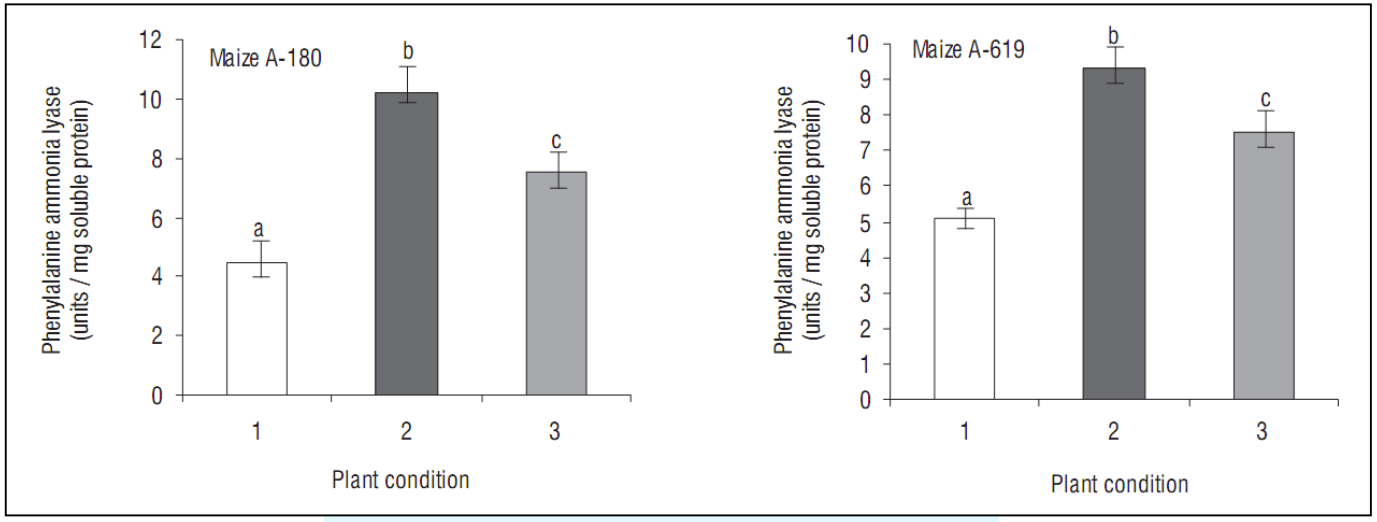
Assessment of the activity of phenylalanine ammonia lyase

- Phenylalanine ammonia lyase activity analysis:
- Leaf samples (200 mg) were homogenized in 2 ml of 25 mM borat buffer, pH 8.8 containing 2 µl β-mercaptoethanol and a pinch of polyvinyl polypyrrolidone (PVP).
- The homogenate was filtered through the cheese cloth, centrifuged at 12,000 × g for 10 min and the supernatant was used for enzyme activity assay according to Sadisivam and Manickam (1992).
- One unit of the enzyme was defined as increase in absorbance of one unit per min.
- The activity of the enzyme was expressed as units per mg of soluble protein.

Gholizadeh and Kohnehrouz,2010

Activation of plant defence responses
Assessment of the activity of phenylalanine ammonia lyase

- Activation of propanoids biosynthetic pathway was assessed in terms of PAL activity analysis in both maize inbreds (A-180 and A-619) as described in materials and methods section. Experiment was carried out at three different plant conditions including 24 h before stress treatment (plant condition 1), 24 h after stress treatment (plant condition 2) and 24 h after recovery (plant condition 3). Data were presented as the means of duplicates analyzed at $P \leq 0.05$.



Gholizadeh and Kohnehrouz,2010

EPSs

Activation of plant defence responses

The EPSs affecting phenylalanine ammonia-lyase also induce an increase in hydrogen peroxide production

- Bacterial pathogens are a potential sources for H_2O_2 accumulation in the plant-bacterial interactions.
- Plants, in response to invasion by microorganism (their metabolites such as EPS), employ diverse defense mechanisms to contest pathogen, and production of active oxygen species (AOS) such as O_2, OH^+ and H_2O_2.
- Oxidative burst is often the first response detected, occurring within 5 min.

1. H_2O_2 may be directly toxic to pathogens.
2. H_2O_2 also induces benzoic acid 2 hydrolase(BA) enzyme activity, which is required for biosynthesis of signal molecule salicylic acid (SA).

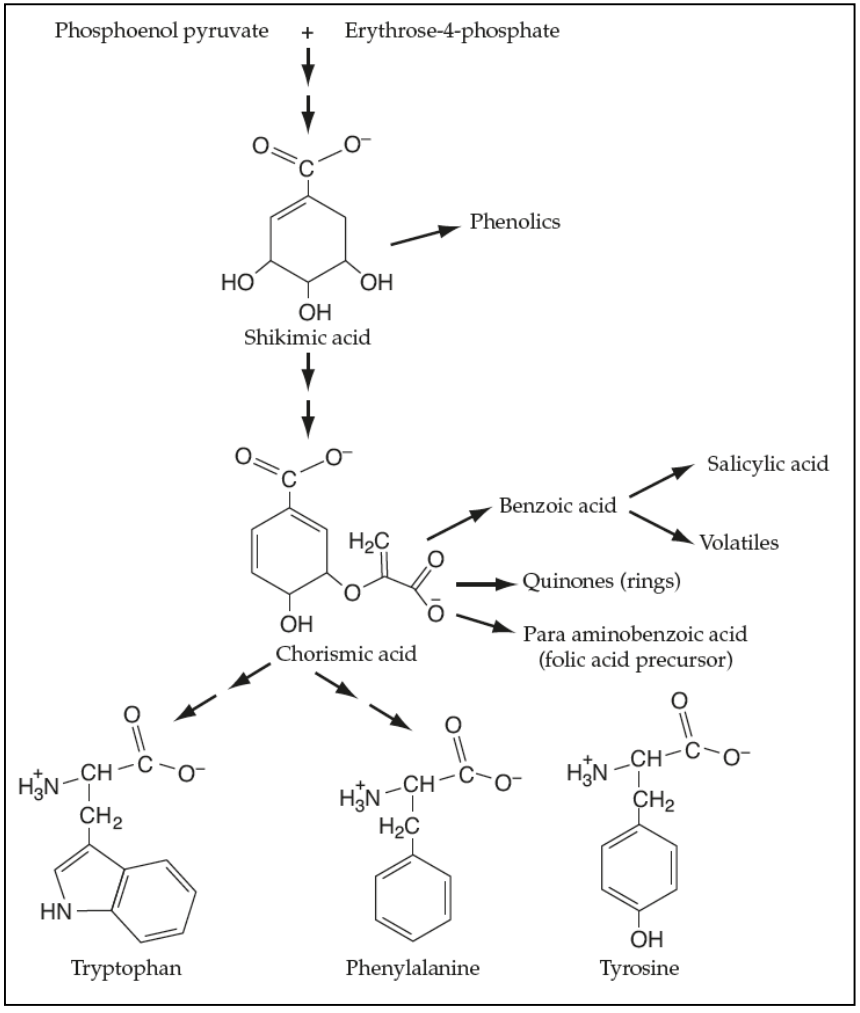
EPSs

Activation of plant defence responses

The EPSs affecting phenylalanine ammonia-lyase also induce an increase in hydrogen peroxide production

- Both salicylic acid (SA) and BA (benzoic acid 2 hydrolase) are derived from the phenylpropanoid pathway and have many roles in plant defence responses.

Salicylic acid is derived from the metabolism of salicin. The salts and esters of salicylic acid are known as salicylates. e.g. ester methyl salicylate or sod. salicylate.
Salicylic acid is used as an ingredient in synthetic salicylates and a variety of other products.



EPSs

Activation of plant defence responses

The EPSs affecting phenylalanine ammonia-lyase also induce an increase in hydrogen peroxide production

- The increase in H_2O_2 production induced by the presence of EPS was tested by both measuring:

1. The H_2O_2 released in the culture medium, and
2. Visualizing the cellular H_2O_2 production on probe labeled with fluorescent dye dihydrorodamine 123.

- All the active EPSs(*P. syringae* pv. *ciccaronei* and *P. savastanoi* pv. *nerii*) but not the *P. caryophylli* EPS induced a slight increase in H_2O_2 release in the external medium/and in TBY-2 cells (cultured cells of *Nicotiana tabacum*) treated with fluorescent probe dihydrorodamine 123.

de Pinto *et al.*,2003

EPSs

Activation of plant defence responses

The EPSs induce an increase in hydrogen peroxide production in culture medium by TBY-2 cells (cultured cells of *Nicotiana tabacum*)

- **1. In the culture medium:**
- The increase in H_2O_2 production induced by the presence of EPS was tested by both measuring the H_2O_2 released in the culture medium.
- All the active EPSs, but not the *P. caryophylli* EPS, induced a slight increase in H_2O_2 release in the external medium.

Treatment	H_2O_2 (nM)
control	702±11
ciccaronei	935±28
cerii	895±33
alginate	883±26
cariophylli	710±21

The analysis was performed after 20 h of treatment with 0.05 mg ml^{-1} EPSs. The values are the means of four independent experiments ± SE.

de Pinto *et al.*,2003

EPSs

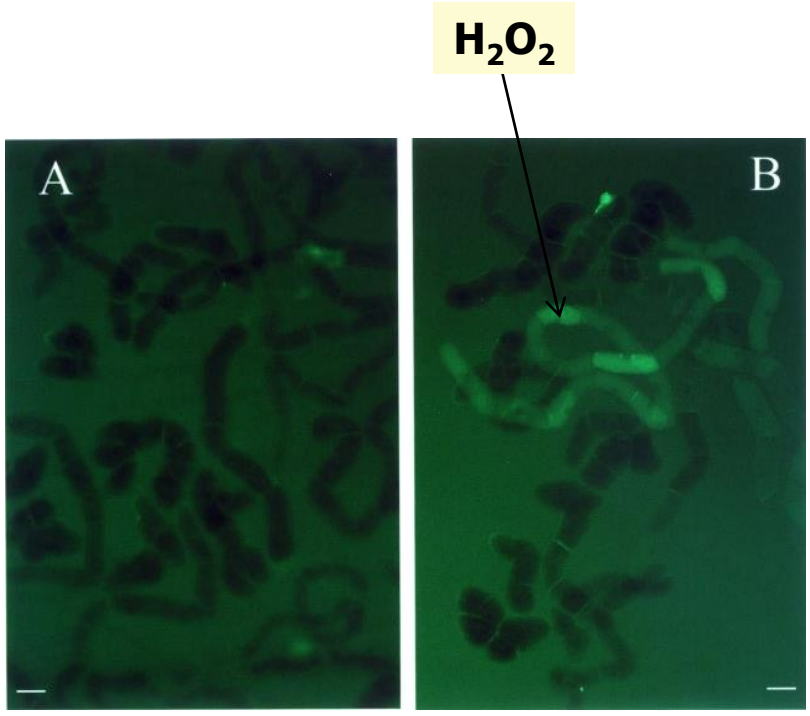
Activation of plant defence responses

Visualising the cellular H_2O_2 production(within TBY-2 cells) with the fluorescent probe dihydrorodamine 123

- **2. With the fluorescent probe:**
- The presence of H_2O_2 within cells was detected by treatment with fluorescent dye dihydrorhodamine 123, a probe giving a green fluorescence when reacts with H_2O_2.

A. Control cells

B. Cells treated with *P. syringae* pv. *ciccaronei* EPS.



de Pinto *et al.*,2003

EPSs

Other functions

Bacteriophage treatments: Enzymes as a tool to understand EPS role in pathogenicity

- An attractive way to understand the role of EPS in more depth and eventually increase disease resistance by reduced EPS production in plant pathogenic bacteria could be through the use of polysaccharide depolymerase enzymes.
- Hartung *et al.*,1986 described the isolation of a polysaccharide depolymerase gene from a bacteriophage of *E. amylovora*.
- The purified recombinant enzyme degraded amylovoran, the acidic component of the EPS:

1. Abolished the virulence of *E. amylovora* in bioassays, and
2. Inhibited bacterial cell growth.

Major virulence factors

Basic structures of EPS molecules

Isolation of EPS

- For isolation of EPS, bacteria were cultured for 2 to 3 days at 20 or 28°C on 5 to 10 culture dishes (100 by 15 mm) containing PAF or PAF (Difco Pseudomonas agar F medium) with 5% (wt/vol) glycerol.
- Increased glycerol content in the medium and/or a low incubation temperature was required to obtain adequate yields of EPS for characterization for some strains.
- It is well established that an increased carbon-to-nitrogen ratio and incubation temperatures lower than those optimal for bacterial growth often favor increased production of EPS.

Fett *et al.*,2004

Major virulence factors

Basic structures of EPS molecules

Extraction of EPS

- Various physical and chemical methods have been reported to extract EPS associated with cells from different sources:

1. Common physical methods include centrifugation, ultrasonication and heating.
2. Chemical methods include extraction of EPS with chemical agents such as Ethylenediamine tetraacetic acid (EDTA), formaldehyde, NaOH and NaOH-formamide.

Xiangliang,2010

Major virulence factors

Basic structures of EPS molecules

Extraction of EPS

- Physical extraction:
- Extraction with high-speed centrifugation: The cell suspension was centrifuged at 20 000 r/min, 4°C for 20 min. The supernatants were filtered through a 0.22 μm membrane. The filtrate was used as the EPS sample.
- Extraction with ultrasonication: The cell suspension was firstly subject to ultrasonication at 40W in an ice bath for 2 min. The sonicated cell suspension was then centrifuged at 20 000 r/min, 4°C for 20 min.

Xiangliang,2010

Major virulence factors

Basic structures of EPS molecules

Extraction of EPS

- Chemical extraction:
- Extraction with EDTA: The cell suspension was extracted with 2% EDTA for 3 h at 4°C.
- Extraction with formaldehyde: The cell suspension was extracted with formaldehyde (36.5%) for 1 h at 4°C.
- All the samples treated with chemical agents were centrifuged at 20000 r/min, 4°C for 20 min and the supernatants were filtered through a 0.22 μm membrane.
- The filtrates were used as EPS samples.

Xiangliang,2010

Major virulence factors

Basic structures of EPS molecules

Determination of EPS components

- The chemical characterization of the different EPSs was carried out using chemical, enzymatic and spectroscopic methods (essentially and 1 and 2D 1H and 13C-NMR and FAB MS techniques). e.g.

1. Sugars and related substances such as uronic acid were determined by colorimetric assay.
2. Sugars were identified by cochromotography.
3. The pyruvate content was determined by an enzymatic assay with lactate dehydrogenase and measured spectrophotometrically.
4. Succinic acid was identified by gas-liquid chromatography (GLC).

de Pinto *et al.*,2003; Fett *et al.*,1995

Major virulence factors
Basic structures of EPS molecules

- EPS may vary in chemical and physical properties but it primarily consists of polysaccharides.
- Backbone of EPS contains 1,3- or 1,4-β linked neutral hexose (e.g. galactose, glucose, mannose) residues.
- Some of the polysaccharides are neutral or polyanionic.
- The presence of uronic acids (e.g. D-galactouronic, mannuronic acid, D- glucuronic acid) confers the anionic properties.
- EPS molecules may associate with metal ions, divalent cations and macromolecules such as proteins, DNA, lipids and even humic substances.

Major virulence factors
Basic structures of EPS molecules

- Thus the EPSs are composed of:

1. Neutral hexoses (e.g. galactose, glucose, mannose)
2. 6-deoxyhexoses (e.g. fucose, rhamnose);
3. With other possible constituents such as uronic acids (e.g. mannuronic acid, glucuronic acid); and
4. Common subsitutent groups (e.g. pyruvate, succinate, and acetate).

- Most EPS molecules are heteropolysaccharides (more than one type of sugar) of high molecular weight, which are composed of repeating units.
- Excess available of carbon source as well as limitation of potassium and phosphate promote the EPS production.

Van den Bulk *et al.*,1991; Vannste,2009;..

Major virulence factors
Basic structures of EPS molecules

- EPS molecules are produced by members of all species of phytopathogenic bacteria.
- These polysaccharides may be present as:

1. A discrete capsule, or
2. More commonly, as extracellular slime, which generally is soluble and diffuses readily in liquid media.

- Amino sugars e.g. glucosamine are less prevalent.
- However, glucosamine is a major component of EPS produced by *R. solanacearum*.
- Also N-acetylglucosamine and N-acetylgalactosamine were reported from *P. savastanoi* pv.*nerii* EPS.

Van den Bulk *et al.*,1991;..

Major virulence factors
Acidic and neutral EPSs
Pseudomonads

- Many plant related Pseudomonads including plant pathogenic bacteria produce:

1. Acidic alginate, a co-polymer composed by D-mannuronic and L-guluronic acids, and
2. Neutral EPS levan (a fructan/a polyfructan), a polymer of fructose.

- Most, if not all, fluorescent pseudomonads are capable of synthesizing alginate.
- The acidic exopolysaccharide (EPS) of the phytopathogens: *P.s.* pv. *glycinea*, *P. aeruginosa*, *P. fluorescens*, *P. putida*, and *P. mendocina* are alginate in structure.
- It is now evident that alginic acid is not the sole acidic EPS produced by fluorescent pseudomonads.

Osman and Fett,1998

Chemical composition of EPSs

Acidic EPSs

The three plant pathogenic Pseudomonads

- The EPS of *P. syringae* pv.*ciccaronei* (Leaf spot of carob tree)is a mannopyranose polymer.
- The EPS of *P. caryophylli* (now *Burkholderia*) is a homopolymer of D-fructose.
- The EPS of P. *savastanoi* pv.*nerii* is more complex and consist of an hetero-polymer of:

1. Fucose,
2. Galactose,
3. Nacetylglucosamine, and
4. N-acetylgalactosamine present in different percentages.

Fett *et al.*,2004; de Pinto *et al.*,2003

Chemical composition of EPSs

Acidic EPSs

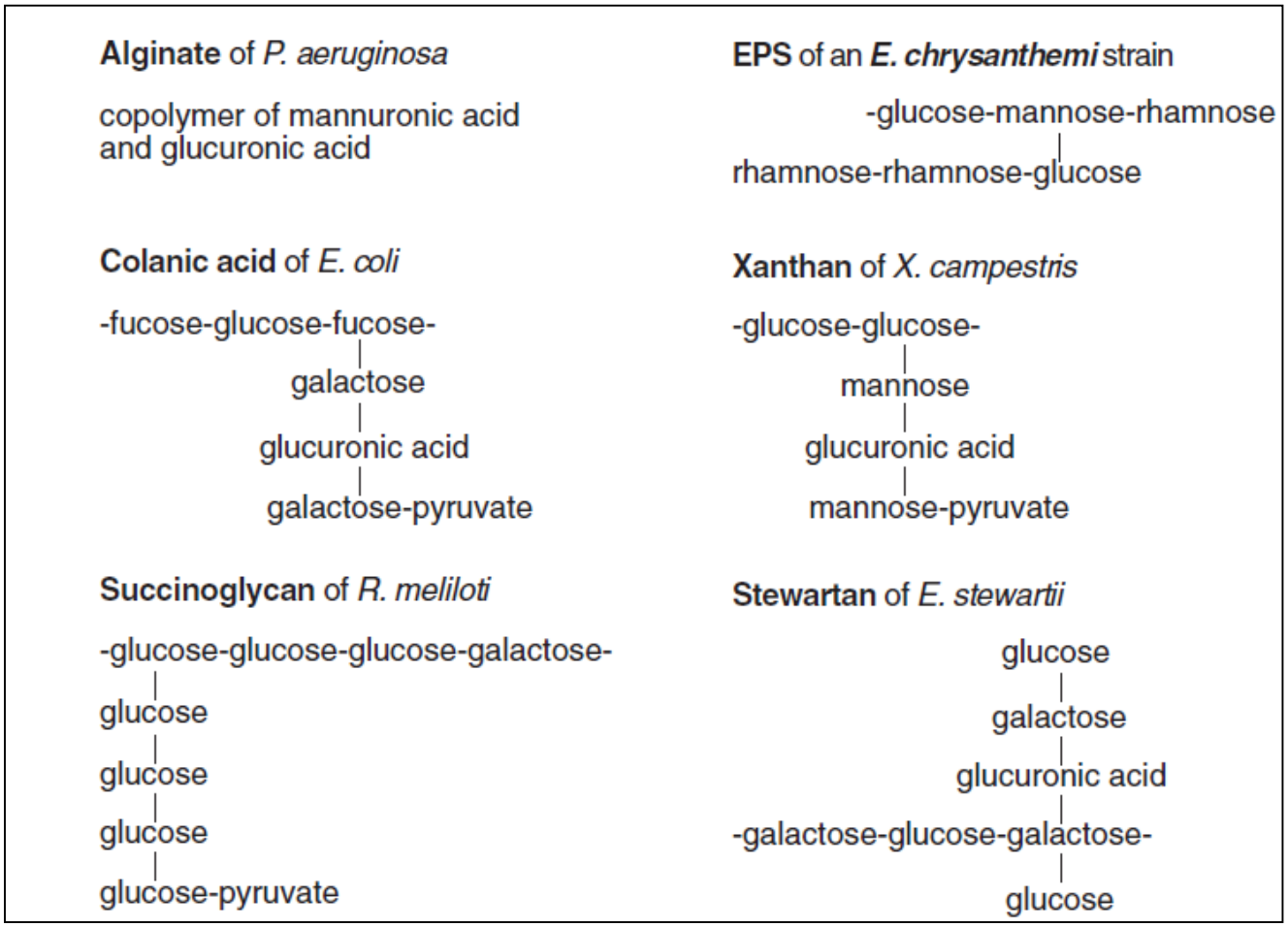
The three plant pathogenic Pseudomonads

EPS sources	Chemical composition
P. syringae pv. *Ciccaronei* Leaf spot of carob tree (*Ceratonia siliqua*)	D-Mannose (100%), D-glucose (in traces)
P. Caryophylli Bacterial wilt or stem crack of carnation (*Dianthus caryophyllus*)	D-Fructose (100%)
P. savastanoi pv. *nerii* Oleander (*Nerium oleander*) knot	L-Fucose (16%), D-galactose (31%), 2-deoxy-acetoammidoglucose (34%), 2-deoxy-2-acetoammidogalactose (19%)

de Pinto *et al.*,2003

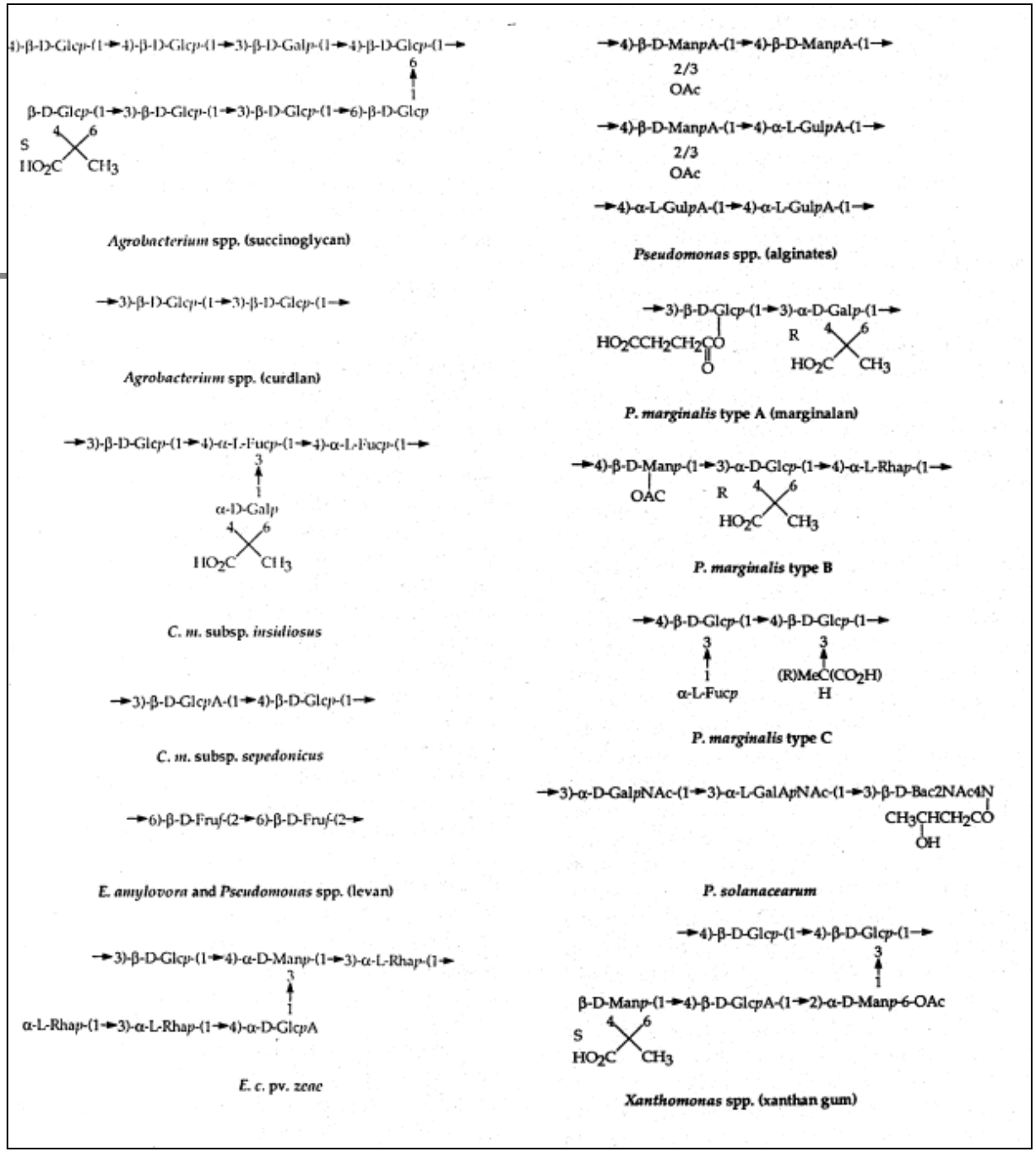
An outline of basic structures for bacterial exopolysaccharides (EPSs)

Acidic EPSs in some bacteria



Vanneste,2000

Exopolysaccharides of plant pathogenic bacteria



Fett,1993

Exopolysaccharides produced by bacteria

- The main examples of bacterial EPS applied in various industrial activities.

EPS	Microorganism	Main use
Alginate	*Azotobacter vinelandii*	Gelling agent
Curdlan	*Alcaligenes faecalis*	Paint thickener
Dextran	*Leuconostoc Mesenteroides, Klebsiella* spp.	Viscosity modifiers, photographic industry, dietary sugar
Gellan	*Sphingomonas paucimobilis (syn.: Pseudomonas elodea)*	Texturizing, stabilizer, thickener, emulsifier and gelling agent
Marinactan	*Flavobacterium uliginosum*	Anticancer and antitumor therapy
Xanthan	*Xanthomonas campestris*	Sauces and syrups, toothpaste, bread, cosmetics, agricultural products, paints
Zanflo	*Erwinia tahitica*	Clay stabilizer for drilling petroleum wells

Bomfeti,2011

Culture conditions for the production of an acidic exopolysaccharide

Plant pathogenic pseudomonads

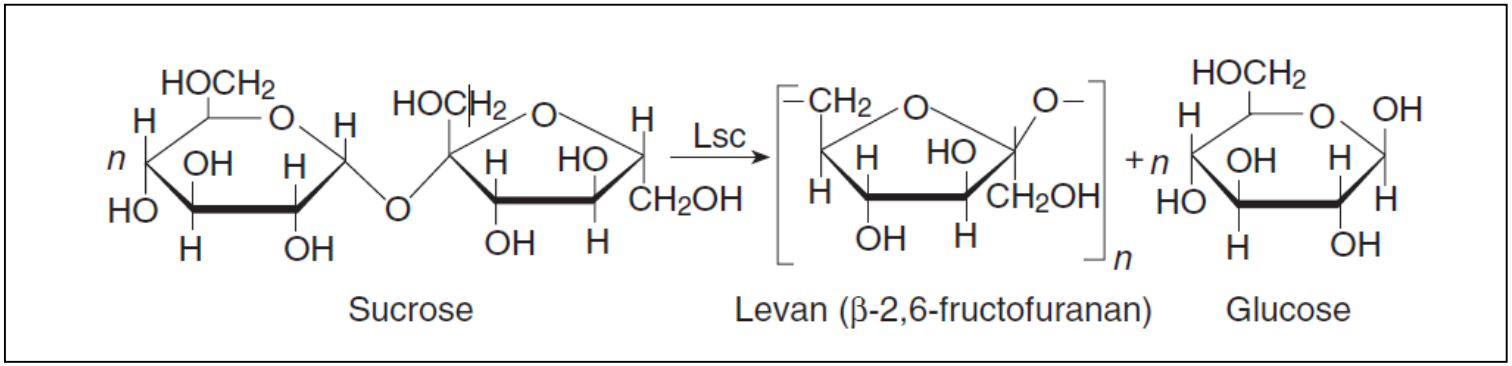
- Alginate production to be common among fluorescent types when glucose or gluconate was used as the carbon source in the culture medium;
- With sucrose as the carbon source, levan, levan and alginate, or alginate alone was produced, depending on the bacterial strain examined.
- *P. marginalis* on glycerol-containing medium produced neither alginate nor levan but marginalan(an acidic galactoglucan).

Osman and Fett,1998

Levan

Basic structure of a neutral EPS

- Synthesis of levan by levansucrase (Lsc) from sucrose in the bacterial environment.
- The constitutively secreted enzyme attaches to the growing chain of the polymer.
- Levan could protect the cells and the released glucose could be used as a carbon source.



Vanneste,2000

Culture conditions for the production of an acidic exopolysaccharide

The endophytic diazotrophic bacterium *Burkholderia tropica*

- The effect of various aeration regimes and carbon source concentrations on EPS production was determined, as well as the effects of temperature and time of growth.

Temperature (°C)	Growth time (h)	EPS yield ($g \cdot L^{-1}$)[a]
26	24	0.350±0.033
	72	0.929±0.061
30	24	0.572±0.042
	72	0.940±0.074

Note: Mean values from triplicates relative to 1 L culture ± SD.
[a]Determined by the PhOH–H_2SO_4 method at 490 nm (Dubois et al. 1956).

Serrato *et al.*,2006

Major virulence factors

Acidic EPSs

Mushroom associated-fluorescent pseudomonads

- The fluorescent pseudomonads are a rich source of novel EPSs.
- All mushroom production-associated fluorescent pseudomonads with mucoid growth on Pseudomonas agar F medium (PAF) were produced acidic exopolysaccharides (EPSs).
- An acidic galactoglucan, previously named marginalan (isolated from *Pseudomonas marginalis*) was produced by mucoid strains of the saprophyte *Pseudomonas putida* and the majority of mucoid strains of saprophytic *P. fluorescens* (biovars III and V).
- The "*P. gingeri*" EPS was unique in containing both neutral sugar and glucuronic acid.
- Mucoid, weakly virulent strains of "*P. reactans*" produced either alginate or marginalan (acidic EPSs).

Fett *et al.*,2004

Major virulence factors

Acidic EPSs

Mushroom-associated fluorescent pseudomonads

- When cultured on PAF containing 1 or 5% glycerol, all mucoid strains of the saprophytic bacterium *P. putida* and most strains of the saprophyte *P. fluorescens* (biovars III and V) isolated from mushroom casing medium produced marginalan as an acidic EPS.

Bacterial strain	Yield of EPS (mg)[a]	EPS production			
		Marginalan	Alginate	Unique	Levan[b]
P. fluorescens					
J1	35–70	−	+	−	+
H13	7–30	−	−	+	−
B4, B14, B15, B20, B22; D12; G2, G7, G8, G13, G19; K17; H6, H8, H9, H11, H16, H21; I15, I19, I22, I23; J12, J19; L16, L23, L24	8–79	+	−	−	−
"*P. gingeri*"					
Pf2, Pf3, Pf9, Pf11, Pf13, Pf31; K20, K23	8–32	−	−	+	−
P. putida					
All strains	25–58	+	−	−	−
"*P. reactans*"					
ATCC 14340; H23; K15, K16; L18	12–55	−	+	−	−
C7, C11; L5	23–43	+	−	−	−

[a] Yields of EPS are expressed per five culture dishes (100 by 15 mm) of PAF containing 1 or 5% glycerol. Cultures were incubated for 2 to 3 days at 20 to 28°C before isolation of EPS.

[b] Data for ability to produce levan are based on results of a previous study (43).

Fett *et al.*,1995

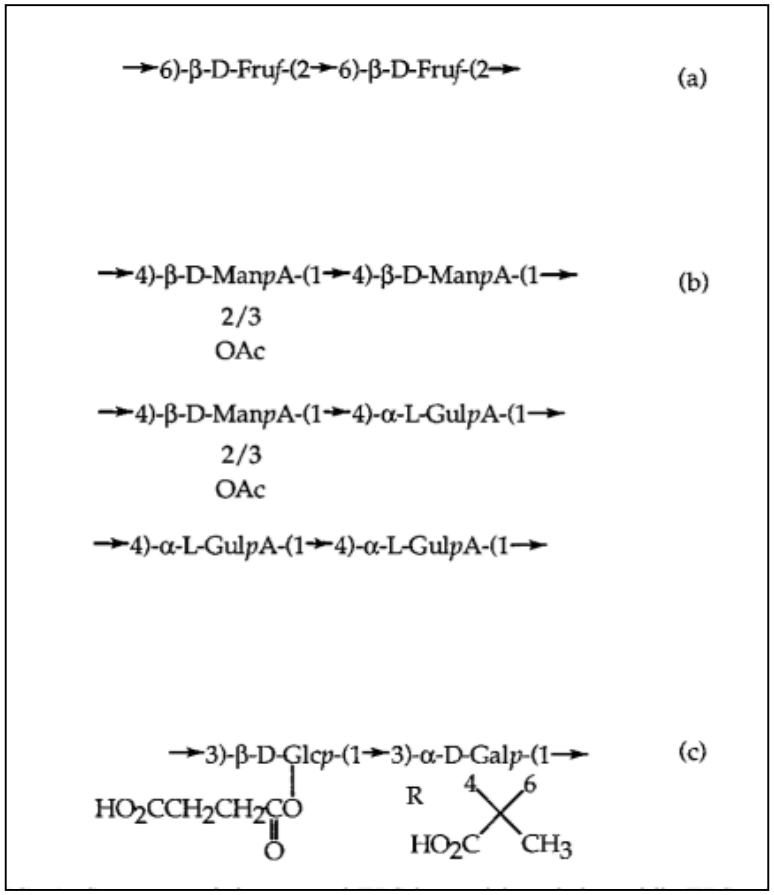
Major virulence factors

Acidic EPSs

Mushroom-associated fluorescent pseudomonads

a) Structure of the neutral EPS levan;
b) The acidic EPSs alginate, and
c) Marginalan (an acidic galactoglucan).

- Fru, fructose;
- ManA, mannuronic acid;
- GulA, guluronic acid;
- OAc, O-acetyl group substituted at the 2 and/or 3 position of mannuronic acid;
- Glc, glucose;
- Gal, galactose;
- *f*, furanose form of the sugar;
- *p*, pyranose form of the sugar.



Fett *et al.*,1995

Major virulence factors

Acidic EPSs

Mushroom-associated fluorescent pseudomonads

- All 10 strains of the pathogen *P. tolaasii*, the causal agent of brown blotch of mushrooms, were nonmucoid on PAF.
- Production of EPS by these 10 strains plus the 2 nonmucoid strains of *P. gingeri* also was negative on several additional solid media as well as in two broth media tested.

Fett *et al.*,2004

EPSs biosynthesis

eps operon

- At least three gene clusters, which are organized more or less similarly in various phytopathogenic bacteria, are important for EPS biosynthesis.
- Regulation of their synthesis is often complex and responds to:
- Numerous environmental, and
- Specific signals.

Synthesis and regulation of EPSs

Acidic EPSs

R. solanacearum

- Production of an acidic, glucosamine-rich EPS is one of the main causes of its pathogenicity.

1. In *R. solanacearum*, an 18-kb operon (eps) with at least nine genes is responsible for the acidic component of EPSs.
2. Another gene cluster (ops) that contains at least seven structural genes seems to be necessary for nucleotide sugar components of both EPSs and lipopolysaccharides.
3. A regulator gene whose overexpression resulted in decreased EPS production and reduced virulence in *R. solanacearum*.

Synthesis and regulation of EPSs

Acidic EPSs

R. solanacearum

- Synthesis of this EPS specifically regulated by:
- Two-component regulatory systems (VsrA/D and VsrB/C) and the activator XpsR.
- Also, EPS synthesis is co-regulated with other virulence factors such as:

1. Endo polygalacturonase A,
2. A pectin methylesterase, and
3. An endoglucanase, through a regulatory cascade that involves the products of the *phcA*, *B*, *R*, and *S* genes.

Synthesis and regulation of EPSs

Acidic and neutral EPSs

Erwinia amylovora

- EPS are the main pathogenicity and virulence factors of *E. amylovora*.
- *E. amylovora* synthesizes two types of EPS:

1. The virulence factor netural levan, and
2. The pathogenicity factor acidic exopolysaccharide (EPS) amylovoran.

- Amylovoran is necessary but not sufficient for the development of fire blight symptoms.
- However, it is quite similar to the units constituting stewartan, the EPS produced by *E. amylovora*, and to those constituting the EPS of *Erwinia stewartii*.

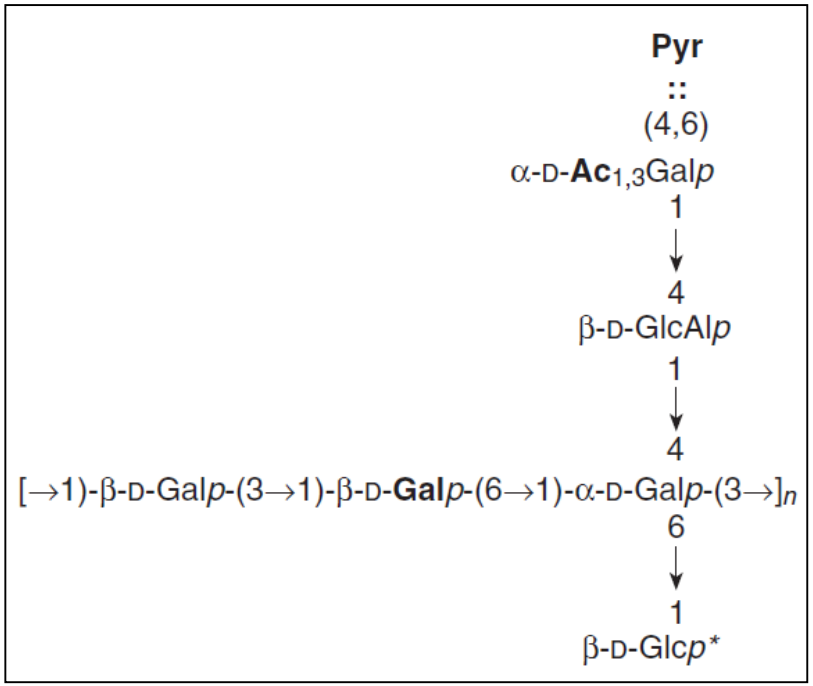
Vanneste,2000

Synthesis and regulation of EPSs

Structure of the repeating unit of amylovoran

Erwinia amylovora

- * About 10% of repeating units carry a glucose as a second side-chain (in the case of stewartan, 90%).
- Gal, galactose;
- GlcA, glucuronic acid;
- Pyr, pyruval residue with keto linkage;
- α,β, sugar linkage at C1;
- D, sugar configuration;
- *P*, pyranoside;
- *n*, degree of polymerization (at least 1000).



Vanneste,2000

Synthesis and regulation of EPSs

Map of the *ams* region encoding amylovoran synthesis

Erwinia amylovora

- Levan is synthesized by levansucrase, encoded by the *lsc* gene.
- Biosynthesis of amylovoran requires the *ams* operon, consisting of 12 genes.
- Most of the structural genes are located in an approximately 17 kb region of the chromosome called *ams* (Bugert and Geider,1995).

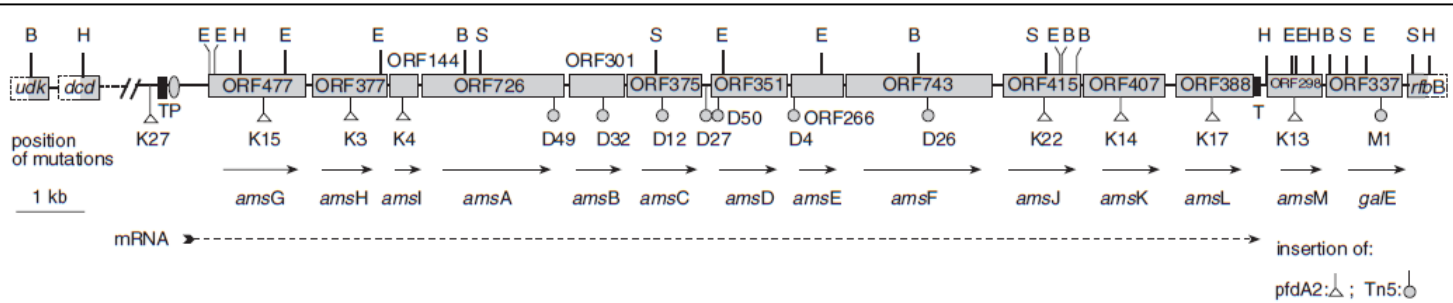


Fig. 7.4. Map of the *ams* region encoding amylovoran synthesis. The 16 kb transcript of the *ams* operon is shown at the bottom. P, promoter; T, terminator; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I. (Mainly based on data from Bugert and Geider, 1995.)

Vanneste,2000

Synthesis and regulation of EPSs

Acidic EPSs

Pantoea stewartii

- EPS is the main virulence factors of *P. stewartii*.
- *P. stewartii* produces stewartan, an acidic exopolysaccharide that contributes to the water-soaking symptoms observed in Stewart's wilt of corn.
- Amylovoran and stewartan are highly similar, differing only in one sugar in the backbone and the terminus of the side-chain.
- Regulation of EPS synthesis in both *P. stewartii* and *E. amylovora* is very similar to that described for the regulation of colanic acid capsule synthesis in *Escherichia coli*.

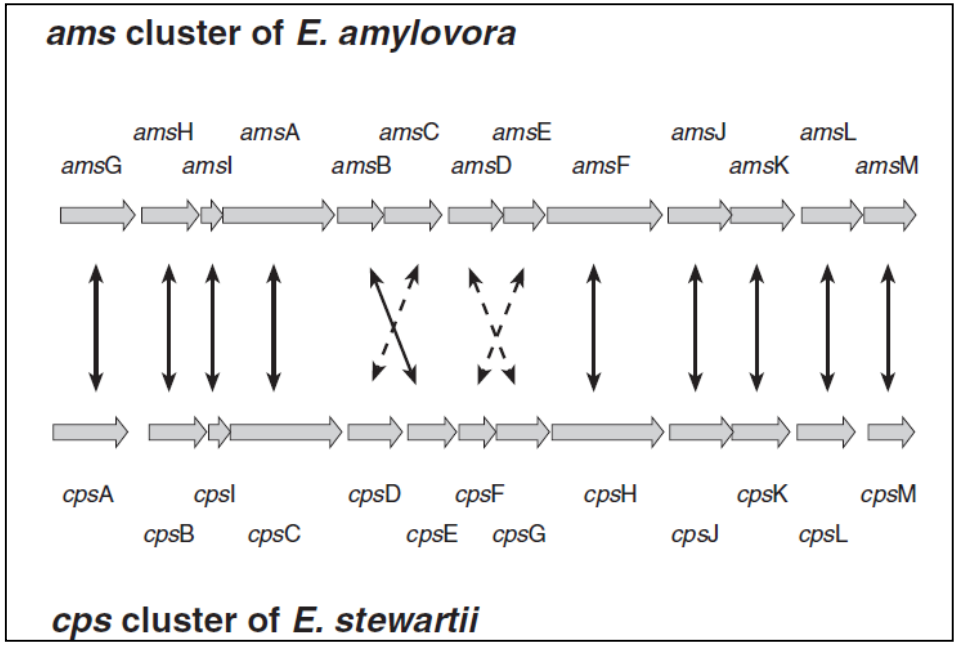
Vanneste,2000

Synthesis and regulation of EPSs

Acidic EPSs

Pantoea stewartii

- The high similarity of most *cps* (capsular polysaccharide) genes in *P. stewartii* to the corresponding *ams* (amylovoran) genes in *E. amylovora* implies similar functions in EPS synthesis.



Vanneste,2000

Synthesis and regulation of EPSs

Acidic EPSs

Dickeya chrysanthemi (formerly grouped in the *Erwinia* genus)

- In case of *D. chrysanthemi* 3937 it was shown that EPS produced by it is composed of L-rhamnose, D-galactose, and galacturonic acid (4:1:1).
- Of course, production of plant cell wall-degrading enzymes, mostly pectinases, is the main cause of *D. chrysanthemi* pathogenicity.

Synthesis and regulation of EPSs

Acidic EPSs

X. campestris pv. *campestris*

- EPS is not the main virulence factors of *X. campestris* pv.*campestris* since EPS-deficient mutants still induced disease symptoms.
- Genes involved in the synthesis of xanthan gum have been cloned recently and found to be clustered in a 13.5-kb region of DNA.
- Acidic EPS assays:
- Acidic EPS (xanthan gum) was quantitatively determined as described previously, using precipitation with cetyltrimethylammonium bromide followed by an anthrone colorimetric assay for total carbohydrates.

Kamoun and Kado,1990

Xanthan gum
Chemical Structure

- Xanthan or xanthan gum is a complex polysaccharide composed of more than one type of sugar (a heteropolymer).
- The backbone of the polysaccharide chain consists of two β-D-glucose units linked through the 1 and 4 positions.
- The side chain consists of two mannose and one glucuronic acid, so the chain consists of repeating modules of five sugar units.
- About half of the terminal mannose units have a pyruvic group.
- The gums help the bacteria stick to the leaves of host plants.

The repeating unit of Xanthan Gum.

Synthesis and regulation of EPSs

Acidic and neutral EPSs

Clavibacter michiganensis subsp. *michiganensis*

- The EPS produced by the strain of in cmm contained:
- The neutral monosaccharides: fucose, galactose, glucose (shown by paper chromatography), and
- Acidic pyruvyl, succinyl, *O*-acetyl groups.
- The approximate molar ratio of L-fuctose, D-galactose, D-glucose, pyruvate, succinate, and acetate were 2:1:1:1:5:1:5, respectively.

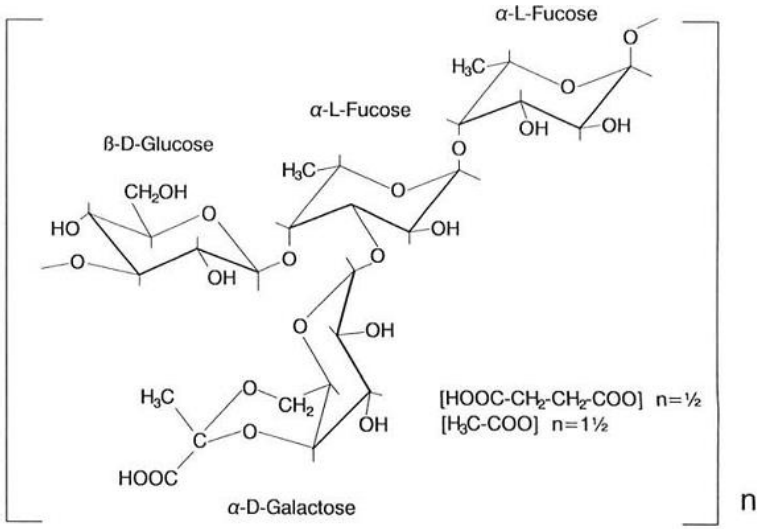


TABLE 1. Composition of the extracellular polysaccharides produced by *Clavibacter michiganensis* subsp. *michiganensis* in vitro

	D-Glucose	D-Galactose	L-Fucose	Pyruvate	Succinate	Acetate
Weight percent	18.1	18.8	34.5	9.2	6.4	7.0
Moles	1.01	1.04	2.10	1.05	0.54	1.61
Molar ratio[a]	0.97	1.00	2.02	1.01	0.52	1.55

[a]Based on the amount of D-galactose (in moles) set to 1.0.

Van den Bulk *et al.*,1991

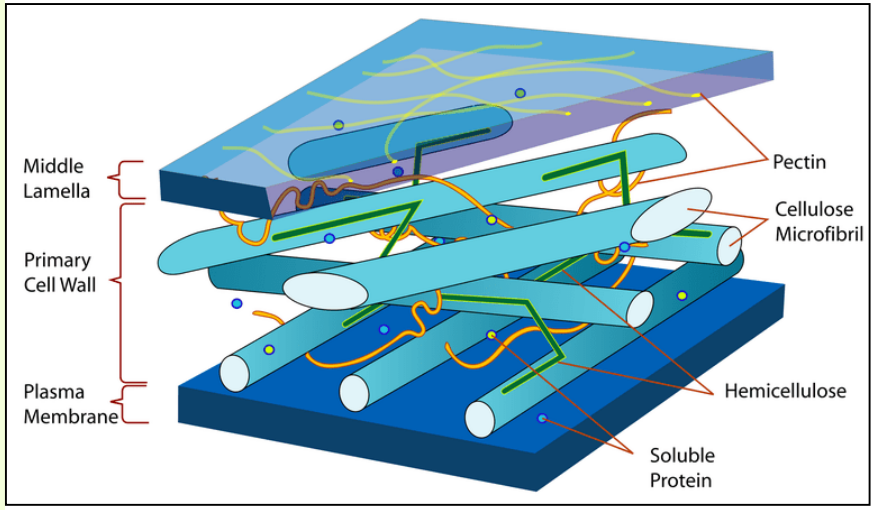
Major virulence factors
2. Bacterial enzymes

- Most fungal and bacterial pathogens of plants produce enzymes that degrade plant materials *in vitro*, but so do a great many saprophytic species.
- Plant pathogenic bacteria do not possess cutinases and can only enter plants through natural openings, e.g. stomata, or wounds associated with resistance.
- The enzyme has a molecular size of 72000 dalton and is composed of two subunits of 36000 dalton.

Plant cell wall components

Schematic diagram of morphology and arrangement of some cell wall components

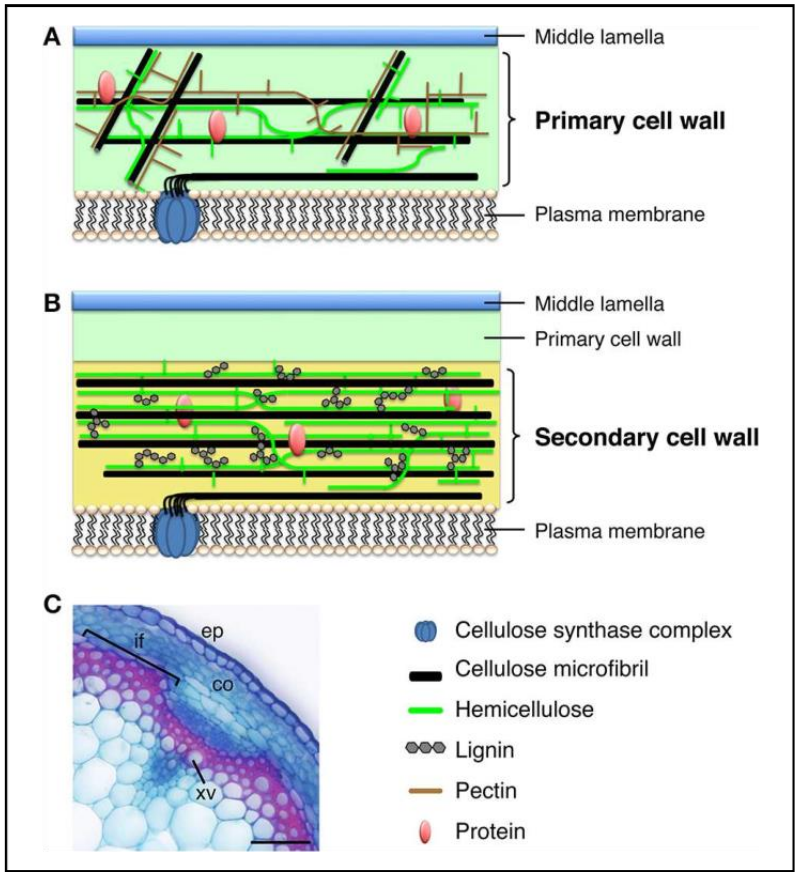
- The cell wall is the rigid, semi-permeable protective layer in some cell types.
- This outer covering is positioned next to the cell membrane (plasma membrane) in most plant cells, fungi, bacteria, algae, and some archaea.
- Animal cells however, do not have a cell wall.
- Cell wall composition varies depending on the organism. In plants, the cell wall is composed mainly of strong fibers of the carbohydrate polymer cellulose.
- Cellulose is the major component of cotton fiber and wood and is used in paper production.



Bailey,2017

Plant cell wall components

- The secondary cell wall mainly contains relatively long and thick cellulose microfibrils, hemicellulosic xylan, and lignin.
- Cellulose is the major component of cotton fiber and wood and is used in paper production.



Nakano *et al.*,2015

Bacterial enzymes
Necrotrophs/biotrophs

- Necrotrophic pathogens produce a wide range of extracellular enzymes enabling them to enter plant cells by degrading plant cell wall polymers.
- Many of these extracellular enzymes exist as isozymes.
- Microbial proteases may degrade plant cell wall proteins.
- Biotrophic pathogens produce very little extracellular enzymes in comparison to necrotrophs.
- Pathogenic fungi possess cutinases enabling them to penetrate cutin on the surface of plants.

Isozymes/Isoenzymes are a group of enzymes that perform the same function, but are different from one another in their structure or how they move.

Bacterial enzymes

Plant surface and cell structures, building blocks serving as nutrients for bacteria, and degradative enzymes of plant pathogenic bacteria degrading these structures

Plant structure	Building block/nutrient	Degradative enzyme
Cutin (cuticle) Suberin (cork layers)	Fatty acid peroxides Fatty acid polyesters	Cutinase Suberin esterase
Cellulose (cell wall) Hemicelluloses (cell wall) Proteins (cell wall)	Glucose monomer β-1,4-linked xylans Polypeptides	Cellulases, C1, C2, Cx, β-glucanase Xylanases Proteases, Proteinases
Pectic substances (cell wall and middle lamellae)	Galacturonans	Pectate lyase, Oligogalacturonase, Pectin methylesterase, Pectin lyase, Polygalacturonase
Proteins (cytoplasmic membrane, CM)	Polypeptides	Proteases, Proteinases
Phospholipids (CM)	Phospholipids	Phospholipidase
Phosphatidyl compounds (CM)	Phospholipids	Phospholipidase
DNA	3-deoxy polynucleotides	Deoxyribonucleases
RNA	Ribopolynucleotides	Ribonucleases

Janse,2006

Bacterial enzymes
Degradative/hydrolytic enzymes

- Different enzymes required to attack different host plants, since pectin structure varies from plant species to species.
- Pectic enzymes and cellulases from the out pathway of ex. *Erwinia* spp.
- Polygalacturonase and others from *Xanthomonas campestris*.
- Production of plant cell wall-degrading enzymes such as proteases and more often pectinases from *Dickeya chrysanthemi* are considered as the main cause of *D. chrysanthemi* pathogenicity.

Bacterial enzymes

Degradative enzyme

Polygalacturonases

- Polygalacturonases (EC 3.2.1.15) are plant cell wall-degrading enzymes that are virulence factors for a number of bacterial and fungal plant pathogens, including:
- *Pectobacterium carotovora*,
- *Agrobacterium vitis*,
- *Xylella fastidiosa*, and
- *Botrytis cinerea*.

Warren *et al.*,2016

Bacterial enzymes

Hydrolytic enzymes

Affect of *Pectobacterium* enzymes on the plant

- Cell walls and middle lamella are degraded;
- Producing individual cells → macerated tissue;
- Cells with broken cell walls can't withstand turgor pressure → BURST → NECROSIS.

Bacterial enzymes

Hydrolytic enzymes

Soft rots

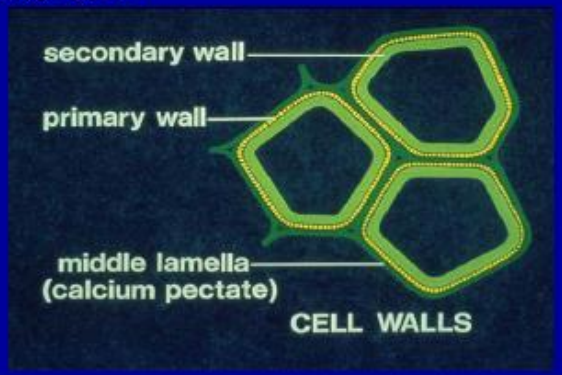
Types of Symptoms

Soft Rots

Bacteria reside in lenticels

Under anaerobic conditions, they hydrolyze the pectin in the middle lamella, which leads to necrosis and collapse of tissue.

Cause disease in stems, fruits, and storage organs (tubers).



Soft rot of potato

Pectin-based
Selective media
for isolation

Degradative/hydrolytic enzymes bacterial enzymes produced by *Pectobacterium* carotovorum subsp. *Carotovorum*.

Enzyme	
Cellulases	CelA
	CelB
	CelS
	CelVI
	CelV
Pectate lyases	PelA
	PelB
	Pel153
	Pel-1
	Pel-3
	PelII
	PelIII
	PelI
	PelX
	PelB
	PelB
	PelC
Pectin lyases	PnlA
	Pnl
Polygalacturonases	Peh
	Peh-1
	PehA
	Peh
Proteases	Prt1
	PrtW

Sjöblom *et al.*,2009

Bacterial enzymes

Pectinases and cellulases

Pectate lyases and pectin methylestrase in *Dickeya* spp. and pectate lyases in *Pectobacterium* spp.

Degradative enzymes - examples for the *Erwinia* species

Erwinia chrysanthemi		*Erwinia caratovora*	
PelA	Pectate lyase	PelA	Pectate lyase
PelB	Pectate lyase	PelB	Pectate lyase
PelC	Pectate lyase	PelC	Pectate lyase
PelD	Pectate lyase		
PelE	Pectate lyase		
ExoPeh	Polygalacturonase	Peh	Polygalacturonase
EGY	Cellulase	CelS	Cellulase
EGZ	Cellulase	CelV	Cellulase
Pem	Pectin methylesterase		
PrtA	Protease	Prt1	Protease
PrtB	Protease		
PrtC	Protease		
PrtG	Protease		
PlcA	Phospholipase		
		PnlA	Pectin Lyase

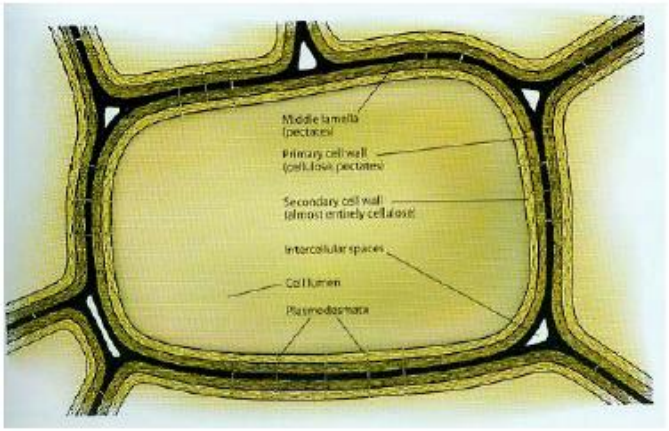
These *Erwinia* species cause soft rot diseases in a wide variety of hosts. The enzymes contribute to a brute force approach to plant cell killing and tissue maceration.

Polygalacturonase, an enzyme that degrades pectin components of the xylem cell wall.

Pectic enzymes
Pectinases

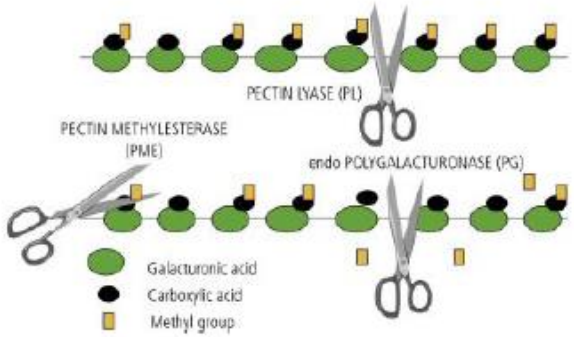
Pectinases

Plant Cell:
from Plant Pathology (Agrios)



- Pectin methyl esterases
- Polygalacturonases
- Pectin lyases

Mode of action of the main pectolytic enzymes



Cuppels lecture
Biology 4218a

www.wynboer.co.za

Pectic enzymes

Secreted by Type II Secretion pathway across inner and outer bacterial membrane

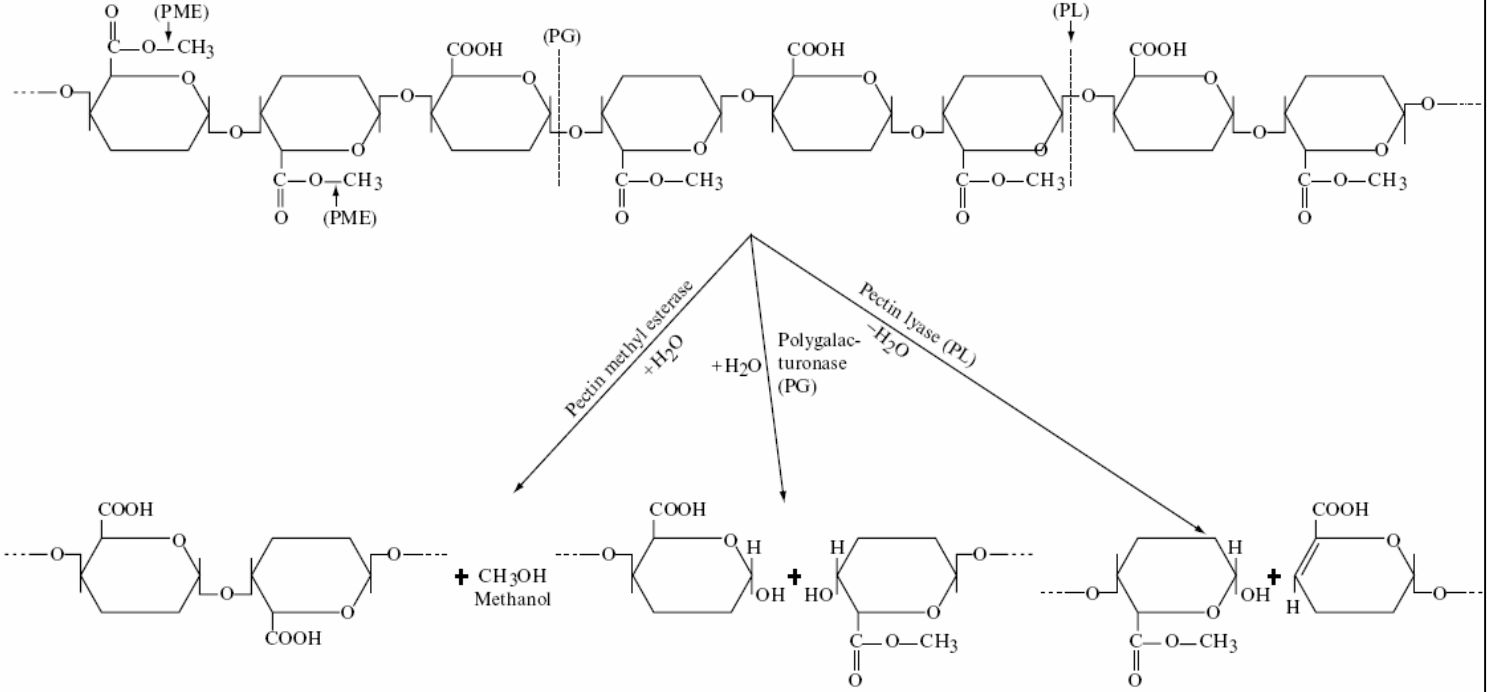


Figure 6.13 Mode of action of pectic enzymes, PME = pectin methyl esterase PG – polygalacturonase, PL = pectate lyase (courtesy of George N Agrios and Academic Press)

Enzymatic activity of *P. syringae* strains

- The cell-wall degradation enzymes:
- *N*-acetyl-β-D-glucosaminidase (NAGase),
- β-glucosidase,
- cellobiohydrolase,
- Cellulase, and
- Protease were detected in culture filtrates of the majority of *P. syringae* strains while only a few strains showed chitinase and glucanase activities.
- Enzymytic activity were detected in cell free culture filtrates.

Cirvilleri *et al.*,2008

Proteases

Proteolytic activity

The thermostable biocatalysts

- The proteases including different families of enzymes:

1. Serine protease (proteolytic enzyme with a serine residue (Ser) in its active site),
2. Cysteine (thiol) protease (EC.3.4.22),
3. Aspartic proteases (EC. 3.4.23), and
4. Metallo-protease (proteolytic enzyme which use a metal for its catalytic mechanism).

- These enzymes constitute one of the most important groups of industrial enzymes accounting for about 60% of the total worldwide enzyme sales.
- Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases.
- And among bacteria, *Bacillus* species are specific producers of extracellular proteases.

Olajuyigbe and Ajele,2005;..

Proteases

Lytic enzymes

Screening for proteolytic activity

- Casein Degradation Test:
- Protease (proteolytic activity) was determined from clearing zones in skim milk agar:
- 50 ml of sterilized skim milk mixed at 55°C with 50 ml of one-fifth volume of tryptic soy agar and 4% agar) after 5 days of incubation at 30°C.

Berg *et al.*,2002; Qureshi *et al.*,2010

Mechanisms of biological control

Lytic enzymes

Protease activity

- Thermostable proteases are advantageous in some applications because higher processing temperatures can be employed, resulting in faster reaction rate,...
- Therefore, depending on the:

1. Zone of clearance and
2. Growth temperature of organism.

- The bacterial isolates such as *Bacillus* species (*B. macerans*, *B. licheniformis* and *B. subtilis*) and soft rot bacteria were selected for further experimental studies.

Olajuyigbe and Ajele,2005;..

Proteases

Proteolytic activity

P. atrosepticum and *X. c.* pv. *campestris*

- Pagel and Heitefuss (1990) showed that several degradative enzymes appeared sequentially in potato tubers infected by *Pectobacterium atrosepticum* including a protease which was detected 19 h after inoculation.
- Dow and co-workers (1990) demonstrated that *Xanthomonas campestris* pv.*campestris* produced two proteases in culture, a serine protease and a zinc- requiring protease and these accounted for almost all the proteolytic activity of the wild-type organism.
- A mutant that lacked both proteases was less virulent than the wild type when introduced into the cut vein endings of turnip leaves.

Lignin-degrading fungi
Ligninases

- Most lignin degradation in nature is by basidiomycetes known as white-rot fungi.
- These fungi produce ligninases that enable the fungi to utilize lignin.
- Most plant pathogenic bacteria have only a limited ability to degrade cell-wall polymers such as lignin.

Lignin-degrading bacteria

- Lignin is degraded and modified by bacteria in the natural world, and bacteria seem to play a leading role in decomposing lignin in aquatic ecosystems.
- Lignin-degrading bacteria approach the polymer by mechanisms such as tunneling, erosion, and cavitation.

Lignin-degrading bacteria
Ligninases

- However, several *Streptomyces* strains are able to degrade lignin during their primary metabolic activity; however, they do not convert it to CO_2 to the same extent as the white rot fungi do.
- Most species of bacteria, other than actinomycetes, do not attack lignified cell walls;
- However, substantial lignin degradation (low-molecular-weight fractions of lignin) was obtained with *Pseudomonas*, *Acinetobacter*, *Xanthomonas* species and several soil (*Bacillus* spp.).
- The question of whether bacteria can depolymerize high molecular-weight lignin remains unresolved.

Seigle-muyandi *et al.*,1996

Poplar lignin decomposition by Gram-negative aerobic bacteria

- Degradation of poplar dioxane lignin(water-soluble milled liginin) and MWL (Milled wood lignin) by bacterial strains in mineral medium (50 ml) after 7 days in agitated cultures (30°C).

Strain	Lignin degradation (%)	
	Dioxane lignin	MWL
Acinetobacter 121	39.5	37.4
Pseudomonas 122	39.0	31.8
Pseudomonas 106	38.0	37.1
Xanthomonas 14	30.2	26.2
Xanthomonas 53	29.9	25.6
Pseudomonas 19	27.9	26.3
Acinetobacter 88	25.0	26.4
Acinetobacter 15	24.6	33.3
Pseudomonas 27	24.6	33.8
Acinetobacter 2	23.2	26.7
Pseudomonas 3	20.9	29.3

Dioxane is classified as an ether. It is mainly used as a stabilizer but occasionally as solvent.

Odier *et al.*,1981

Bacterial enzymes

Global regulation of degradative enzymes

- Several regulatory genes in plant pathogenic bacteria have been discovered by random insertional mutagenesis.
- For example, *aep* genes activate the production of pectate lyase, polygalacturonase, cellulase and protease in *Erwinia*.
- The regulatory sequence was designated rpfA for *r*egulator of *p*athogenicity *f*actors.
- In *Xanthomonas campestris* pv. *campestris*, a pathogen of crucifers, eight such regulatory sequences designated *rpfA-H* was found.

Bacterial vascular diseases

Enzymes and EPSs –The two main pathogenicity factors

- Bacterial vascular diseases are caused by several genera of bacteria, such as *Erwinia*, *Ralstonia*, *Xanthomonas*, and *Clavibacter*.
- In *Pectobacterium carotovorum* subsp. *carotovorum* endoglucanase-defective mutants were less virulent.
- In *Ralstonia solanacearum*, however, in addition to EPS and polygalacturonase, an extracellular endoglucanase is necessary for full virulence but not essential for symptom development, as shown by site directed mutagenesis.
- In the genus *Clavibacter*, production of extracellular enzymes such as endoglucanase, pectin methylesterase, polygalacturonase, and xylanase has been reported.

Major virulence factors
3. Bacterial toxins (phytotoxins)

- Toxins were the first bacterial virulence factors to be identified and were also the first link between bacteria and cell biology.
- **Molecular sizes ranging:**
- Most toxins are:

1. single-chain polypeptides with molecular sizes ranging from about 2-3 kDa for *E. coli*,
2. thermostable enterotoxins up to 300 kDa for *Clostridium difficile* toxins A and B, which are the largest single-chain bacterial protein toxins hitherto identified.

Ladent *et al.*,2005

Kinds of bacterial toxins
Mechanism of action

- The activities of these antimetabolite toxins on nitrogen metabolism usually promote a disorder in amino acid levels, causing:

1. imbalanced levels of intermediates, and
2. depletion of the final product.

- This metabolic imbalance of amino acids leads to chlorosis and even necrosis symptoms in the host plant and probably aids pathogen growth, due to the release of nutrients when cells are affected.

Arrebola *et al.*,2011

Bacterial toxins
Mechanism of action

- Toxin production following colonization.
- Tissue invasion followed by toxin production.
- Most antimetabolite toxins show:

1. Antimicrobial activity and they could
2. Contribute to bacterial competitive ability, and
3. Epiphytic fitness.

- The best-characterized bacterial toxins originate from various pathovars of *P. syringae*.
- Toxigenesis described among different pathovars of *Ps. syringae* appears variable.

Major virulence factors
Mechanism of action

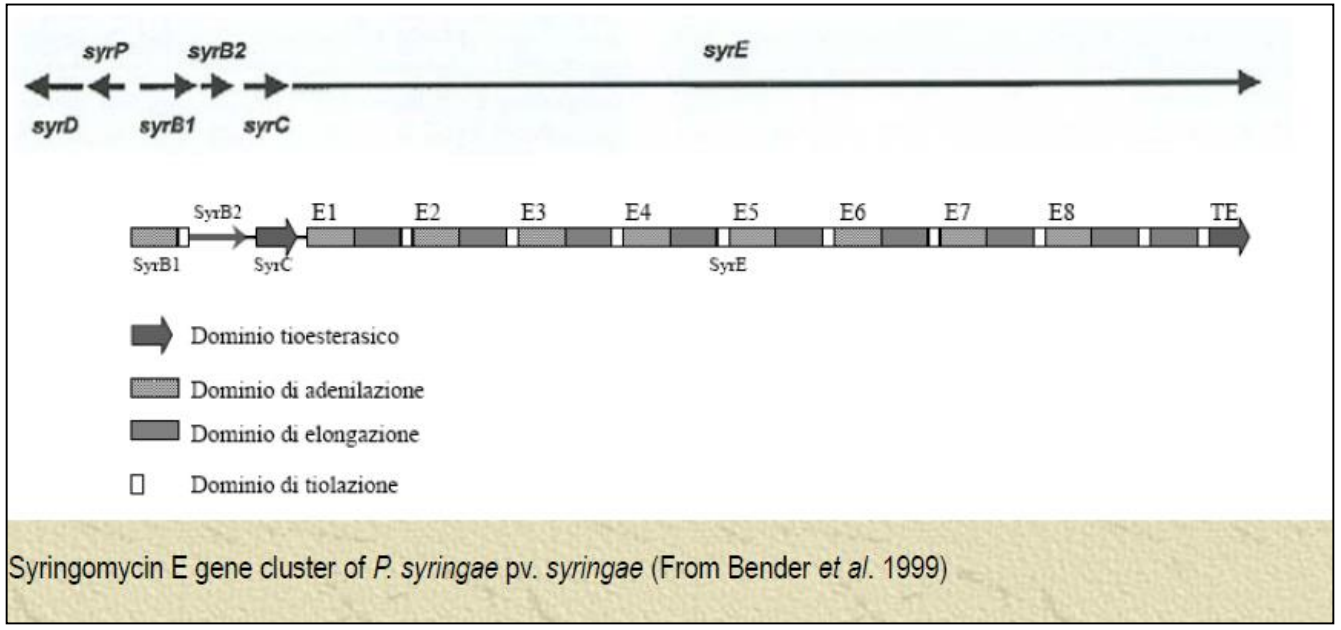
- Although phytotoxins are not required for pathogenicity in *P. syringae*, they generally function as virulence factors for this pathogen, and their production results in increased disease severity.
- For example, *P. syringae* phytotoxins can contribute to:

1. Systemic movement of bacteria *in planta*,
2. Lesion size, and
3. Multiplication of the pathogen in the host.

Bender *et al.*,1999

Toxin production
Genes encoding toxins

- The genes encoding toxins are:
- usually located on the chromosome, but
- sometimes they reside on a plasmid(i.e. coronatine).



Syringomycin E gene cluster of *P. syringae* pv. *syringae* (From Bender *et al.* 1999)

M. Stefani

Non-ribosomal peptides

Cyclic Lipoproteins and lipopeptides (CLP)

- Cyclic Lipoproteins and lipopeptides (CLPs) have extremely broad range of biological activities such as acting as virulence factors.
- They are often:

1. Antibiotics(polymyxins, fusaricidins, and paenibacterin, fengycin,..),
2. Toxins (syringomycin and syringopeptin)
3. Siderophores,
4. Pigments.

Interest in *Pseudomonas* species is due to their diverse lifestyles and complex interactions with multiple hosts, and to their ability to produce an array of metabolites, including enzymes, siderophores, antibiotics, toxins and cyclic lipopeptides (CLPs).

Non-ribosomal peptides
Kinds and functions

- The most potent cell wall-derived inflammatory toxins (pathogenicity factors) of Gram-negative and -positive bacteria are:

1. lipopolysaccharides (LPS) (endotoxins), and
2. Lipoproteins and lipopeptides (LP).

- LPS forms the outer leaflet of the outer membrane for nearly all Gram-negatives.
- Besides LPS, also other amphiphilic molecules, i.e., lipopeptides and lipoproteins (LP) are found in bacterial envelopes, in Gram-negative as well as in Gram-positive bacteria and in mycoplasma.

Wikipedia,2012; Martínez de Tejada *et al.*,2015

Non-ribosomal peptides

Cyclic Lipoproteins and lipopeptides (CLP)

- Nonribosomal peptides often have a cyclic and/or branched structures, can contain non-proteinogenic or unnatural amino acids including D-amino acids.
- The protein moiety of these compounds can be neutral or anionic and the amino acids are often arranged in a cyclic structure.
- Lipoproteins and lipopeptides (LP) are nonribosomal peptides are synthesized by multimodular enzymes known as nonribosomal peptide synthetases (NRPSs), without the aid of ribosomes.

Cyclic lipopeptides (CLPs) are versatile molecules with antimicrobial, cytotoxic, and surfactant properties. Cyclic lipopeptides are composed of a short oligopeptide with a linked fatty acid tail.

Non-ribosomal peptides

Cyclic Lipoproteins and lipopeptides (CLP)

- Hundreds of peptide antibiotics have been described in the past half-century.
- Antimicrobial secondary metabolites (peptides) are classified as either:

1. Ribosomally synthesized peptides (RAMPs), or
2. Non-ribosomally synthesized peptides.

- many siderophores such as iron-siderophore bacillibactinare,
- some toxins such as syringomycin and syringopeptin, and
- cyclic lipopeptides molecules such as surfactin, bacillomycin-D and fengycin are synthesized by nonribosomal peptide synthetases.

Non-ribosomal peptides

Cyclic Lipoproteins and lipopeptides (CLP)

- An NPR synthetase is generally composed of one or more modules and can terminate in a thioesterase domain that releases the newly synthesized peptide from the enzyme.
- Unlike ribosomal peptide synthesis, they do not involve the translation of mRNA in order to begin the synthesis.
- Nonribosomal peptides often have a cyclic and/or branched structures.
- These are not naturally encoded or found in the genetic code of any organisms.

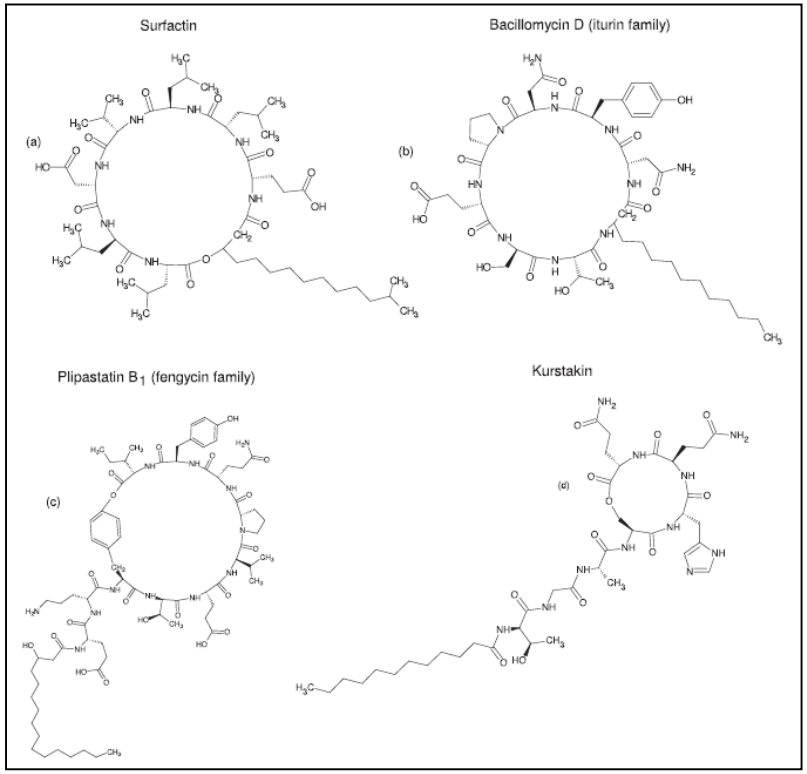
Module: a separable component, frequently one that is interchangeable with others, for assembly into units of differing size, complexity, or function.

Non-ribosomal peptides

Cyclic Lipoproteins and lipopeptides (LP)

- Non-Ribosomal Peptide Synthesis (NRPS) utilizes a large monomer pool including:

1. All the amino acids, and
2. Several unnatural amino acids in total less than 50 amino acids to produce small molecule bioactive peptides.



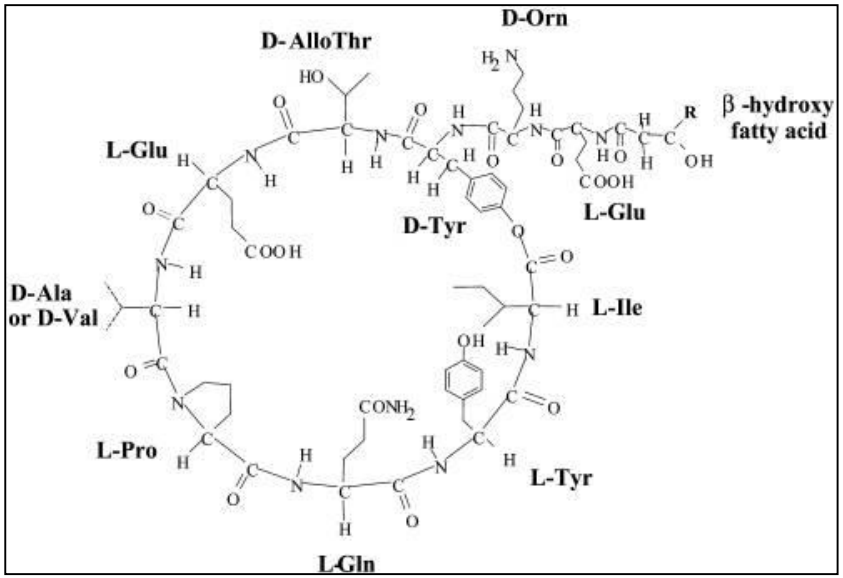
Most prominently, *B. amyloliquefaciens* and *B. subtilis* encompass gene clusters coding for cyclic LPs including surfactin, plipastatin B1(fengycin family), bacillomycin D (iturin family), and kurstakin.

Non-ribosomal peptides

Cyclic Lipoproteins and lipopeptides (CLP)

Fen A and Fen B

- Fengycin is composed of two isoform compounds (isoform A with D-Ala and isoform B with D-Val), each of them containing fatty acid side chains of variable length.



Bacillus fengycin lipopeptides function as quorum-sensing blockers (quorum-quenching).

Deleu *et al.*,2008

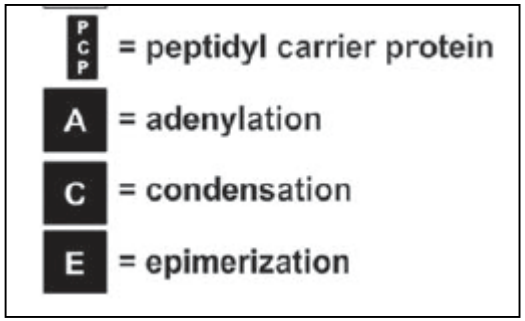
Non ribosomal peptide synthesis

Key of domains used in NRPS

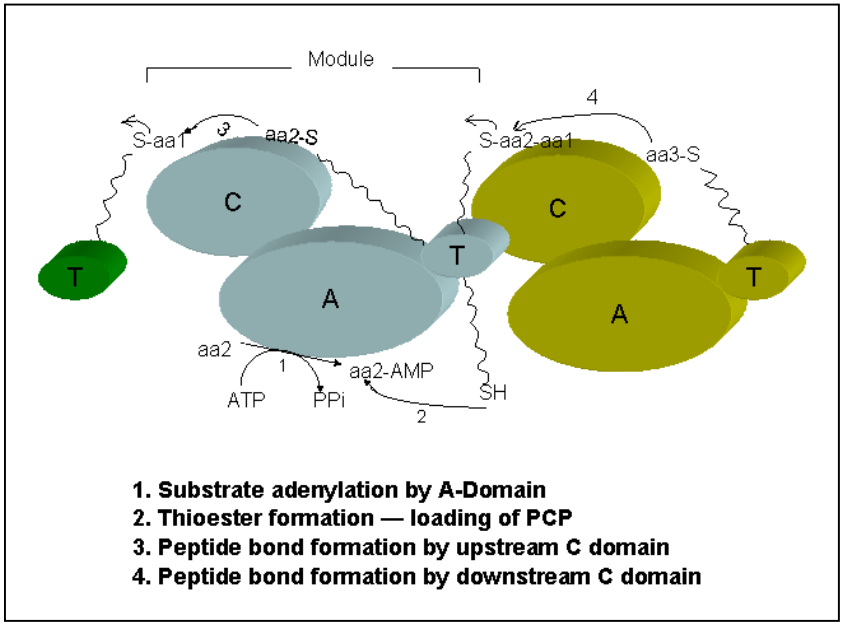
Nonribosomal peptide synthetases (NRPS)

- NRPSs use a set of core domains, known as a module, to accomplish peptide synthesis.
- A minimal module consists of three core domains:

1. An adenylation (A) domain which recognizes and activates the substrate via adenylation with ATP,
2. A thiolation (T) or peptidyl carrier protein (PCP) domain, and
3. Condensation(C) domain.



key of domains in NRPS

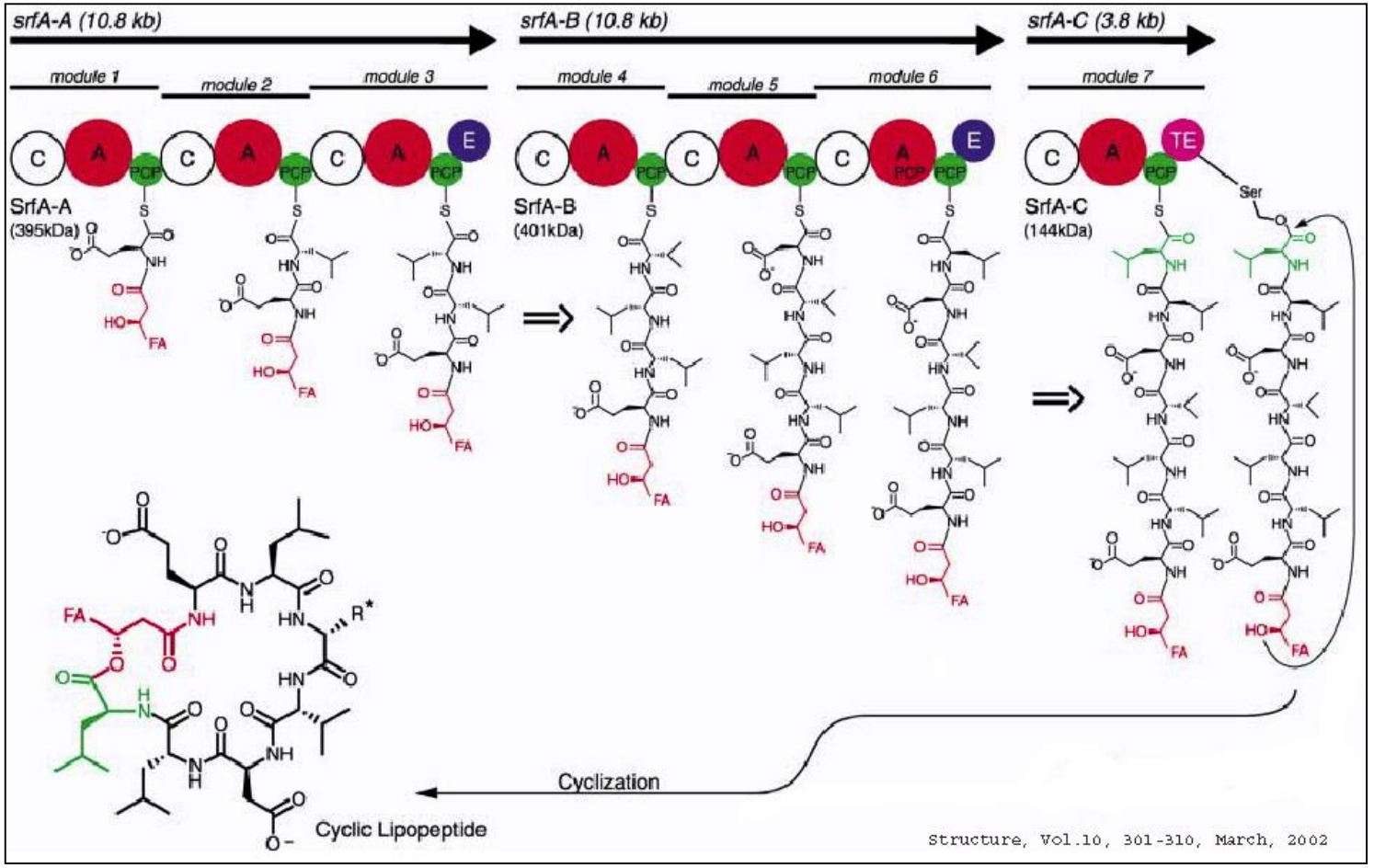


1. Substrate adenylation by A-Domain
2. Thioester formation — loading of PCP
3. Peptide bond formation by upstream C domain
4. Peptide bond formation by downstream C domain

Non ribosomal peptide synthesis

NRPS production of the cyclic lipopeptides

Synthetase c (srfa-c), a non-ribosomal peptide synthetase termination module



Major virulence factors
Kinds of Bacterial toxins (phytotoxins)

- Toxins affecting plants are divided into two classes:

1. **Host specific toxins (HSTs):** Usually essential for pathogenicity, i.e. the ability to cause disease, mutants that have lost their ability to produce the toxin being non-pathogenic.
2. **Non-host specific toxins (NSTs):** Not essential for pathogenicity but may contribute to virulence. These affect a wider range of host and non-host organisms.

- Toxins produced by *P. syringae* are not specific to only one pathovar because the same antimetabolite toxins are produced by several pathovars.

Major virulence factors
Bacterial toxins (phytotoxins)

- The phytotoxins (antimetabolite toxins) produced by *P. syringae* generally induce:

1. Chlorosis (coronatine, phaseolotoxin, tabtoxin,..), or
2. Necrosis (syringomycin, syringopeptin,..)

- *P. tolaasii* also produce necrosis inducing toxin known as tolaasin.

Syringomycin	*syringae*	Cyclic lipodepsipeptide	Forms pores in plasma membrane	Necrosis
Syringopeptin	*syringae*	Cyclic lipodepsipeptide	Forms pores in plasma membrane	Necrosis
Coronatine	*tomato*, *glycinea*, others	Polyketide and cyclized amino acid	Molecular mimic of plant signal molecule methyl jasmonate	Chlorosis
Tagetitoxin	*tagetis*	Hemithioketal	Inhibitor of chloroplast RNA polymerase	Chlorosis
Phaseolotoxin	*phaseolicola*	Sulpho-diamino-phosphinyl tripeptide	Inhibitor of ornithine carbamoyltransferase	Chlorosis
Tabtoxin	*tabaci* and several others	β-lactam–containing dipeptide	Inhibitor of glutamine synthetase	Chlorosis

[a] All references are given in the text or in Gross (1991), except for those for tagetitoxin action; see Mathews and Durbin (1990).

Bender *et al.*,1999; Alfano and Collmer,1996

Kinds of bacterial toxins

Phytotoxins produced by *Pseudomonas* spp.

Toxin	Producing organism	Chemical class or biosynthetic origin
Coronatine	*P. syringae* pv. *atropurpurea*, *glycinea*, *maculicola*, *morsprunorum*, *tomato*	Polyketide
Corpeptin	*P. corrugata*	Lipodepsipeptide
Fuscopeptin	*P. fuscovaginae*	Lipodepsipeptide
Persicomycins	*P. syringae* pv. *persicae*	Fatty acid
Phaseolotoxin	*P. syringae* pv. *actinidiae*, *phaseolicola*	Sulfodiaminophosphinyl peptide
Rhizobitoxine	*P. andropogonis*	Vinylglycine
Syringomycins[a]	*P. syringae* pv. *syringae*, *aptata*, *atrofaciens* *P. fuscovaginae*	Lipodepsinonapeptide
Syringopeptins	*P. syringae* pv. *syringae*	Lipodepsipeptide
Tabtoxin	*P. syringae* pv. *tabaci*, *coronafaciens*, *garcae*	β-Lactam
Tagetitoxin	*P. syringae* pv. *tagetis*	Unknown
Tolaasin	*P. tolaasii*	Lipodepsipeptide
Viscosin	*P. marginalis* (*P. fluorescens*)	Lipodepsipeptide

[a]Includes the related toxins syringotoxin, syringostatin, and pseudomycin.

Bender *et al.*,1999

Kinds of bacterial toxins

Phytotoxins produced by *P. syringae* pathovars and *P. tolaasii*

Pseudomonads	Toxin(s)	Mechanism or site of action	Host plant(s)
1. *P. syringae*			
pv. *atropurpurea*	coronatine		Italian rye grass
pv. *coronafaciens*	tabtoxin-β-lactam	Glutamine synthetase	oat
pv. *garcae*	tabtoxin-β-lactam	Glutamine synthetase	coffee
pv. *glycinea*	coronatine/ polysaccharide		soybean
pv. *lachrymans*	extracellular polysaccharides		cucumber
pv. *maculicola*	coronatine		crucifers
pv. *morsprunorum*	coronatine		sour cherry
pv. *phaseolicola*	phaseolotoxin	Ornithine transcarbamoylase	bean, kudzu
pv. *syringae*	syringomycins syringopeptins syringotoxins	Plasma membrane	peach, maize
pv. *tabaci*	tabtoxin-β-lactam	Glutamine synthetase	tobacco
pv. *tagetis*	tagetitoxin	Chloroplastic RNA polymerase	marigold
pv. *tomato*	coronatine		tomato
2. *P. tolaasii*	tolaasin	Plasma membrane	mushroom

OECD,1997

Kinds of bacterial toxins

Antimetabolite toxins produced by different pathovars of *P. syringae*

Pseudomonads	Toxin(s)	Mechanism or site of action(Target enzyme)
pv. *coronafaciens*	Tabtoxin	Glutamine synthetase(GS)
pv. *garcae*	Tabtoxin	Glutamine synthetase(GS)
pv. *tabaci*	Tabtoxin	Glutamine synthetase(GS)
pv. *phaseolicola*	Phaseolotoxin	Ornithine carbamoyltransferase (OCT)
pv. *actinidae*	Phaseolotoxin	Ornithine carbamoyltransferase (OCT)
pv. *syringae*	Mangotoxin	Glutamate/ornithine N-acetyltransferase (OAT)
pv. *avellanae*	Mangotoxin	Glutamate/ornithine N-acetyltransferase (OAT)

Mangotoxin was detected in the 1990s by *Pseudomonas syringae* pv. *syringae* isolated from mango trees affected by bacterial apical necrosis.

Arrebola *et al.*,2011

Kinds of bacterial toxins

1. Chlorosis-inducing dipeptides

Tabtoxin, coronatine, phaseolotoxin, tagetitoxin

- **Chlorosis:** Yellowing of the leaf tissue typically as a result of chloroplast disruption or inhibition of its formation:
- Tabtoxin, coronatine, phaseolotoxin, tagetitoxin, produced by:
- *Pseudomonas syringae* pv. *tabaci*, *P. s.* pv. *coronofaciens*, *P. s.* pv. *phaseolicola*, *P.s.* pv. *tagetis*, and some other pathovars.
- Mangotoxin on the other hand, seems to increase necrosis symptoms in tomato leaves infected with mangotoxin-producing strains of *P. syringae* pv. *syringae*.

Kinds of bacterial toxins

2. Necrosis-inducing dipeptides (cyclic lipopeptides)

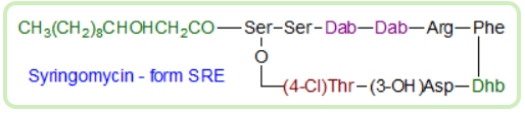
Syringomycins, syringotoxins, syringostatins and tolaasin

- **Necrosis:** The necrosis inducing toxins form ion channels in the cell membrane, causing leakage of cells.
- Cyclic peptides are polypeptide chains taking cyclic ring structure.
- The genus *Pseudomonas* produces many cyclic lipopeptides (lipodepsipeptides) with surfactant, antibacterial and antifungal properties; some have even been reported to have anti-cancer activity.
- Syringomycins, syringotoxins and syringostatins, which are produced by different strains of *P. syringae* pv. *syringae*.
- Tolaasin is another LDP toxin belongs to necrosis group produced by *P. tolaasii*.

Christie,2019;..

Necrosis-inducing dipeptides (cyclic lipopeptides)

Syringomycin, syringostatin, syringotoxin, and pseudomycin

- The four lipodepsinonapeptides differ in the amino acid sequence between positions 2 and 6 (aa_2-aa_6).
- The 3-hydroxy fatty acyl group is a derivative of either:
- decanoic acid (syringomycin) 

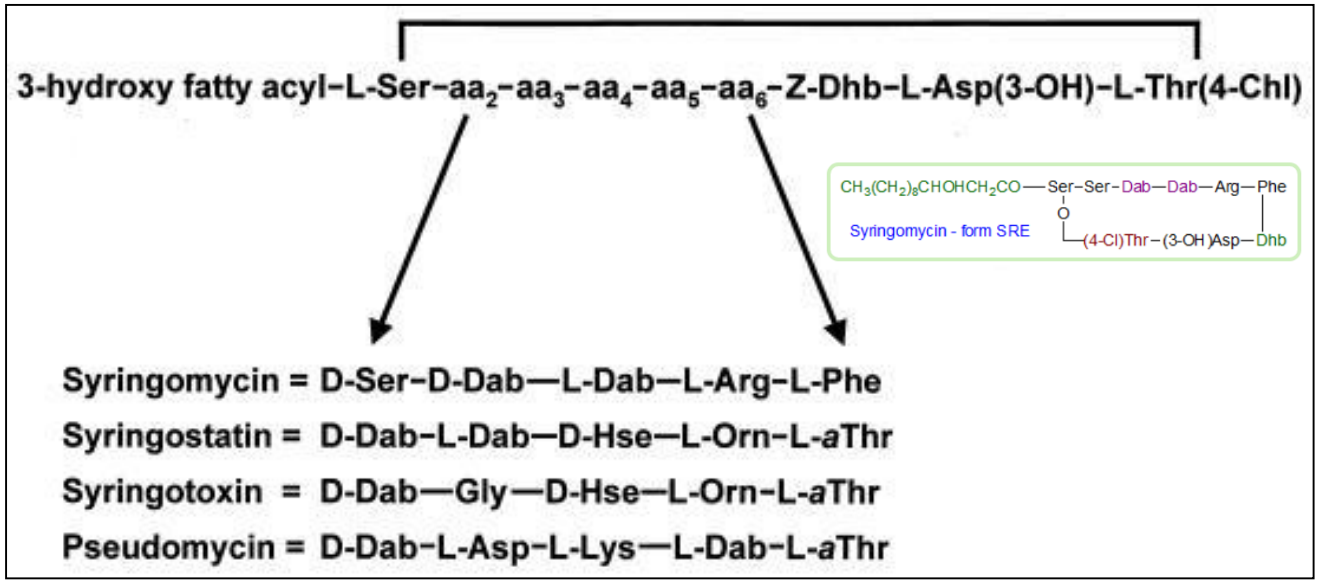
,
- dodecanoic acid (syringomycin and syringostatin),
- tetradecanoic acid (all four lipodepsinonapeptides), or hexadecanoic acid (pseudomycin);
- Some forms of pseudomycin are:
- acylated by 3,4-dihydroxytetradecanoate or
- 3,4-dihydroxyhexadecanoate.

Decanoic acid is a saturated fatty acid. Its formula is $CH_3(CH_2)_8COOH$.

Bender *et al.*,1999

Necrosis-inducing dipeptides (cyclic lipopeptides)

Syringomycin, syringostatin, syringotoxin, and pseudomycin



Syringomycin form SRE. It contains nine amino acids of which three are unusual (Dab = 1,4-diaminobutyric acid; Dhb = 2,3-dehydroamino-butyric acid; 4(Cl)Thr = C-terminal chlorinated threonine residue), while three are of the D-form.

Bender *et al.*,1999; Christie,2019

Necrosis-inducing dipeptides (cyclic lipopeptides)

- Simplified primary structures of cyclic lipopeptides (CLPs) such as syringomycin, syringotoxin, pseudomycin, syringostatin (syringomycin group), tolaasin (tolaasin group) produced by plant-associated *Pseudomonas* spp.

***Syringomycin* Group**

		1	2	3	4	5	6	7	8	9
Syringomycin	3-hydroxy fatty acyl –	L-Ser-	D-Ser-	D-Dab-	L-Dab-	L-Arg-	L-Phe	– z-Dhb-	L-Asp(3-OH)-	L-Thr(4-Cl)
Syringostatin	3-hydroxy fatty acyl –	L-Ser-	D-Dab-	L-Dab-	D-Hse-	L-Orn-	L-aThr-	z-Dhb-	L-Asp(3-OH)-	L-Thr(4-Cl)
Syringotoxin	3-hydroxy fatty acyl –	L-Ser-	D-Dab-	L-Asp-	L-Lys-	L-Dab-	L-aThr-	z-Dhb-	L-Asp(3-OH)-	L-Thr(4-Cl)
Pseudomycin A	3-hydroxy fatty acyl –	L-Ser-	D-Dab-	L-Asp-	L-Lys-	L-Dab-	L-aThr-	z-Dhb-	L-Asp(3-OH)-	L-Thr(4-Cl)
Cormycin A	3-hydroxy fatty acyl –	L-Ser-	D-Orn-	L-Asn-	D-Hse-	L-His-	L-aThr-	z-Dhb-	L-Asp(3-OH)-	L-Thr(4-Cl)

***Tolaasin* Group**

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Tolaasin	C_8 HO acid –	Dhb-	Pro-	Ser-	Leu-	Val-	Ser-	Leu-	Val-	Val-	Gln-	Leu-	--	--	--	--	Val-	Dhb-	aThr-	Ile-	Hse-	Dab-	Lys
FP-B	C_8 HO acid –	Dhb-	Pro-	Leu-	Ala-	Ala-	Ala-	Ala-	Val-	Gly-	Ala-	Val-	Ala-	--	--	--	Val-	Dhb-	aThr-	Ala-	Dab-	Dab-	Phe
Corpeptin A	C_{10}HO acid –	Dhb-	Pro-	Ala-	Ala-	Ala-	Val-	Val-	Dhb-	Hse-	Val-	aIle-	Dhb-	Ala-	Ala-	Ala-	Val-	Dhb-	aThr-	Ala-	Dab-	Ser-	Ile

I. de Bruijn,2009

Necrosis-inducing dipeptides (cyclic lipopeptides)

Genes involved in the biosynthesis and regulation of cyclic lipopeptides (CLPs) produced by plant associated *Pseudomonas* spp.

CLP group	CLP	Species/strain	Gene/protein information	GenBank accession no.	P/C*
Synthesis (including efflux transporters)					
Viscosin	Viscosin	*P. fluorescens* PfA7B	Nonribosomal peptide synthetases	No sequence available	–
	Massetolide A	*P. fluorescens* R1SS101	Nonribosomal peptide synthetases	AY303770; AY303771	P
Amphisin	Amphisin	*Pseudomonas* sp. strain DSS73	*amsY*, peptide synthetase	AJ416154	P
	Arthrofactin	*Pseudomonas* sp. MIS38	*arfA*; *arfB*; *arfC*; nonribosomal peptide synthetases	AB107223	C
	Arthrofactin	*Pseudomonas* sp. MIS38	ORF5; putative outer membrane efflux protein	AB107223	C
	Arthrofactin	*Pseudomonas* sp. MIS38	ORF6; putative ABC transporter protein	AB107223	P
Tolaasin	Tolaasin	*P. tolaasii*	TL1, TL2, TL3; high-molecular weight protein	No sequence available	–
	Tolaasin	*Pseudomonas* NZ17	Homology to syringomycin synthetase	No sequence available	–
	Syringopeptin	*P. syringae* pv. *syringae* B728a	Syringopeptin synthetase genes	CP000075	C
	Syringopeptin	*P. syringae* pv. *syringae* B301D	*sypA*, *sypB*, *sypC*; syringopeptin synthetase	AF286216	C
	Syringopeptin	*P. syringae* pv. *syringae* B301D	*pseABC*; tripartite resistance-nodulation-cell division transporter system	No sequence available	–

C = complete CDS and P = partial sequence

Raaijmakers *et al.*,2006

Necrosis-inducing dipeptides (cyclic lipopeptides)

Genes involved in the biosynthesis and regulation of cyclic lipopeptides (CLPs) produced by plant associated *Pseudomonas* spp.

Syringomycin	Syringomycin	*P. syringae* pv. *syringae* B728a	Syrinogmycin synthetase genes	CP000075	C
	Syringomycin	*P. syringae* pv. *syringae* B301D	*syrE*; nonribosomal peptide synthetase	AF047828	C
	Syringomycin	*P. syringae* pv. *syringae* B301D	*syrB1*; nonribosomal peptide synthetase	U25130	C
	Syringomycin	*P. syringae* pv. *syringae* B301D	*syrC*; thioesterase	U25130	C
	Syringomycin	*P. syringae* pv. *syringae* B301D	*syrB2*; nonheme Fe^{II} halogenase	U25130	C
	Syringomycin	*P. syringae* pv. *syringae* B301D	syrD; putative ABC transporter protein	M97223	C
	Syringomycin	*P. syringae* pv. *syringae* B301D	*pseABC*; tripartite resistance-nodulation-cell division transporter system	No sequence available	–
Other	Putisolvin	*P. putida* PCL1445	*psoA*; putisolvin synthetase	DQ151887	P
	Viscosin-like	*P. fluorescens* Pf-5	Nonribosomal peptide synthetases	CP000076 (PFL2145; PFL2146; PFL2147)	C

C = complete CDS and P = partial sequence.

Raaijmakers *et al.*,2006

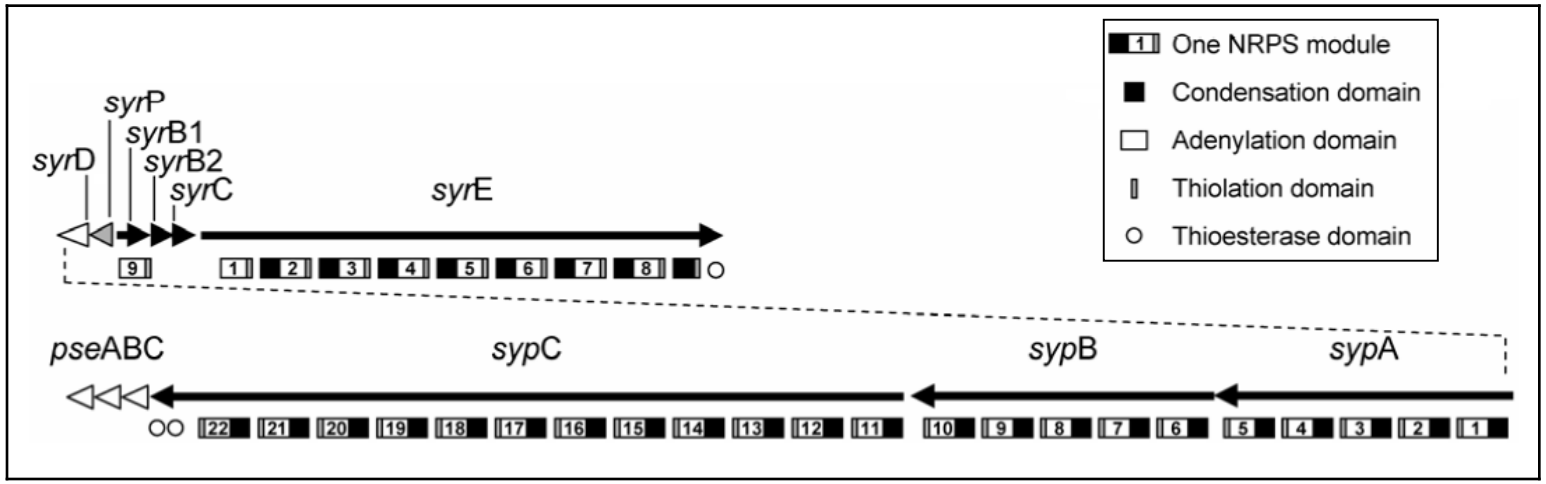
CLP group	CLP	Species/strain	Gene/protein information	GenBank accession no.	P/C*
Regulation					
Viscosin	Viscosin	*P. fluorescens* 5064	AHL biosynthesis	No sequence available	–
Amphisin	Amphisin	*Pseudomonas* sp. strain DSS73	*gacS*; sensor kinase in two-component regulatory system	AJ416155	P
	Arthrofactin	*Pseudomonas* sp. MIS38	ORF1; putative DNA binding protein (luxR type)	AB107223	C
Tolaasin	Tolaasin	*P. tolaasii*	*pheN*; two-component regulatory protein (GacS)	U95300	C
	Syringopeptin	*P. syringae* pv. *syringae* B728a	*gidA*; initiation of chromosome replication	AF302083	C
	Syringopeptin	*P. syringae* pv. *syringae* B301D	*salA*, *syrG*, *syrF*; putative DNA-binding regulatory proteins (luxR type)	AF372703	C
Syringomycin	Syringomycin	*P. syringae* pv. *syringae* B728a	*gidA*; initiation of chromosome replication	AF302083	C
	Syringomycin	*P. syringae* pv. *syringae* B728a	*lemA*; two-component regulatory protein (GacS)	M80477	C
	Syringomycin	*P. syringae* pv. *syringae* B728a	*gacA*; response regulator in two-component regulatory system	CP000075	C
	Syringomycin	*P. syringae* pv. *syringae* B728a	*salA*; putative DNA-binding protein (luxR type)	AF022808	C
	Syringomycin	*P. syringae* pv. *syringae* B301D	*salA*, *syrG*, *syrF*; putative DNA-binding proteins (luxR type)	AF372703	C
	Syringomycin	*P. syringae* pv. *syringae* B301D	*syrA*; *N*-acetylglutamate synthase (arginine biosynthesis)	AY374326	C
	Syringomycin	*P. syringae* pv. *syringae* B301D	*syrP*; histidine kinase in two-component regulatory system (phosphorelay)	U88574	C
Other	Putisolvin	*P. putida* PCL1445	*gacA*; response regulator in two-component regulatory system	No sequence available	–
	Putisolvin	*P. putida* PCL1445	*gacS*; sensor kinase in two-component regulatory system	No sequence available	–
	Putisolvin	*P. putida* PCL1445	*dnaK*; *dnaJ*, *grpE*; heat-shock proteins	AY823737	C

Raaijmakers *et al.*,2006

Necrosis-inducing dipeptides (cyclic lipopeptides)

Genes involved in the biosynthesis and regulation of cyclic lipopeptides (CLPs) produced by plant associated *Pseudomonas* spp.

- In the genome of *P. syringae* pv. *syringae*, the syringomycin and syringopeptin gene clusters are physically linked (indicated by a dotted line).



Raaijmakers *et al.*,2006

Toxins

General methods

Extraction, bioassays and HPLC analyses

- Symptoms of toxic damage to a plant such as wilting, water-soaking, chlorosis and necrosis may be readily observed by the naked eye in many instances but the primary lesion is usually at the biochemical level.
- But sometimes detection and analysis of an unknown toxin requires a suitable bioassay.
- e.g. Phaseolotoxin was assayed initially by its ability to cause chlorotic lesions on bean leaves.
- Once the chemistry of the toxin is known non-biological assays such as:

1. High performance liquid chromatography (HPLC) may be used, or
2. If the toxin is immunogenic or can be rendered immunogenic, immuno-assays.

Toxins

General methods

Preliminary bioassay test

- To demonstrate the production of toxins from fluorescent plant pathogenic *Pseudomonas* spp., plates containing potato dextrose agar were spot-inoculated at four equidistant points with test strains and incubated for up to 7 d at 25 °C.
- Plates were then spray-inoculated either with:

1. Spore suspensions of *Aspergillus niger* , or
2. Faintly turbid cellular suspensions of *Rhodotorula mucilaginosa* prepared from 48 h PDA spread plates.

- After 48 h, the appearance of an inhibitory zone around bacterial colonies was recorded as:

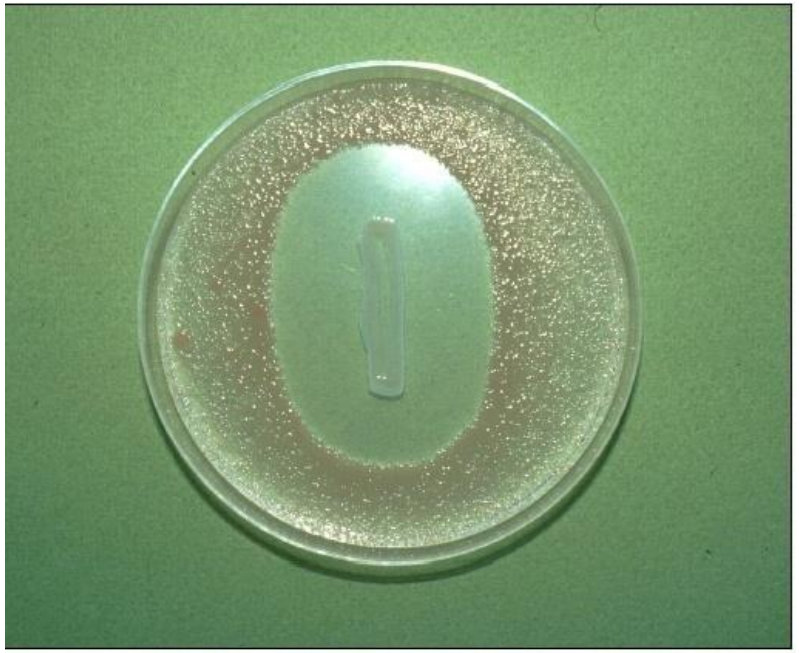
1. Positive (1mm zone around colonies),
2. Weak (1 mm, but detectable around colonies), and
3. Negative (non-detectable zone of inhibition).

Hu*et al.*,1998

Test for production of toxins

Syringomycin/syringopeptin determination using *Rhodotorula pilimanae*

- Detection of toxic lipodepsipeptide production on the improved culture medium peptone-glucose-NaCl (PGNaClA) by using the very toxin-sensitive organism(the yeast) *Rhodotorula pilimanae*.
- More reliable than the PDA test.



Alain Bultreys

Toxin bioassay

General methods

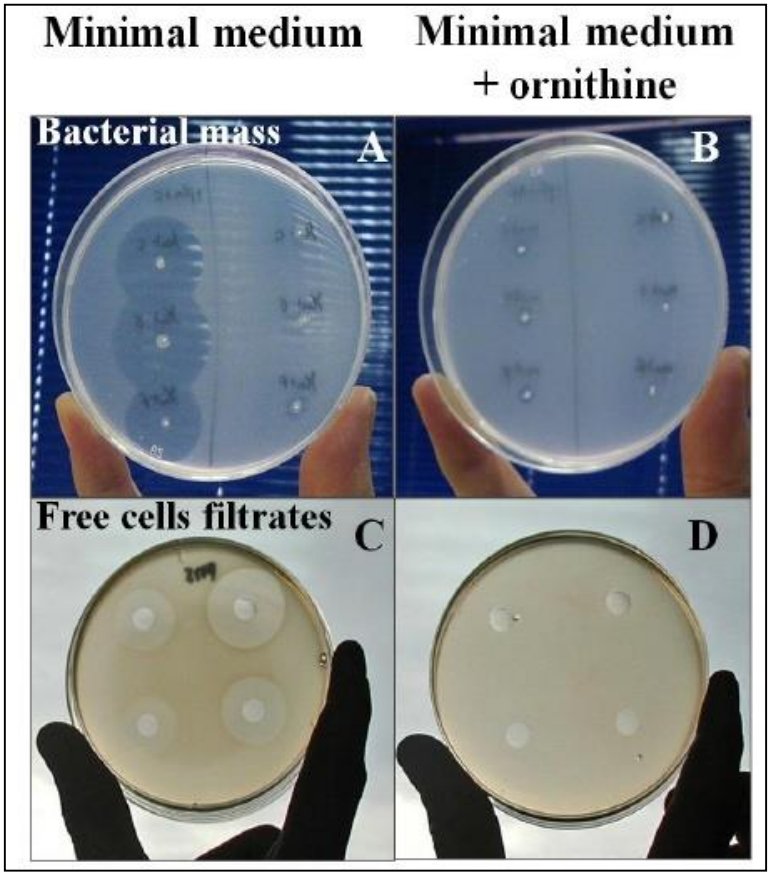
Inactivation of toxin action by adding ornithine

A. The strains of *Pseudomonas syringae* (pv. *phaseolicola*) to be tested are stabbed into the agar and covered with a thin layer of the *Escherichia coli* as an indicator microorganism.

B. The indicator inhibition can be reversed by one or more amino acids.

C. Cell-free filtrates from bacterial cultures can also be used; and

D. The toxic activity can be reversed by one or more amino acids.



Arrebola *et al.*,2011

Toxins

General methods

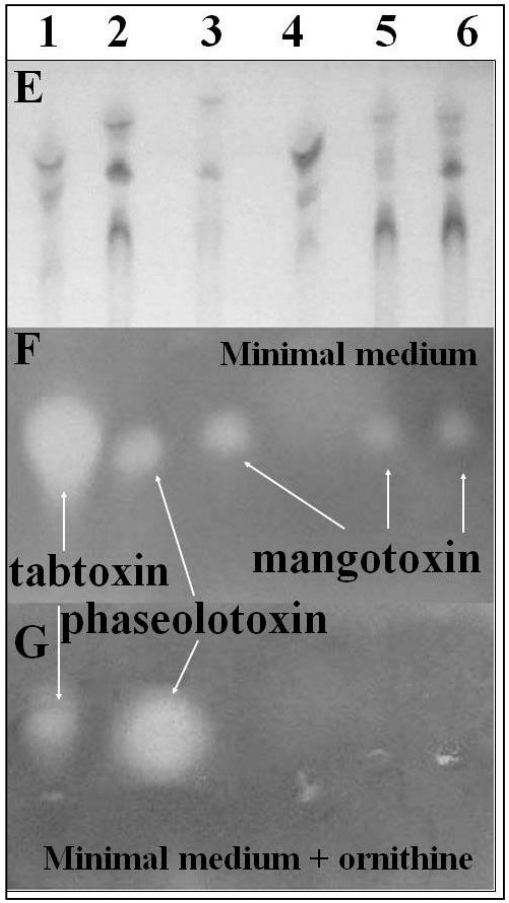
Toxin bioassay and TLC analysis

- TLC analysis of cell-free culture filtrates of:

1. A tabtoxin-producing strain(*P. syringae* pv. *coronafaciens*)
2. A phaseolotoxin-producing strain(*P. syringae* pv. *phaseolicola*)

3,5 & 6. Mangotoxin-producing strains (*P. syringae* pv. *syringae*)

4. A non-mangotoxin-producing strain(mutant strain of*P. syringae* pv. *syringae*).



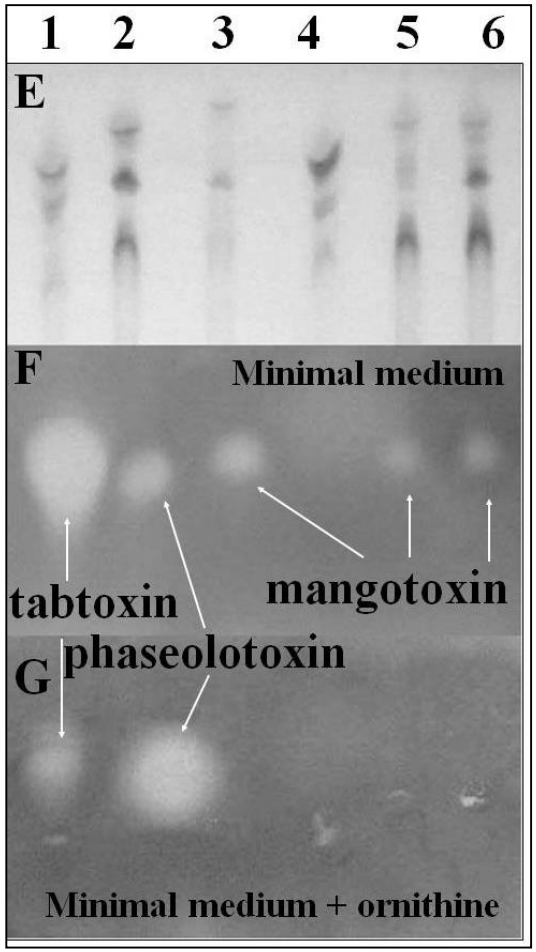
Arrebola *et al.*,2011

Toxins

General methods

Toxin bioassay and TLC analysis

- (E) The fractions were separated by TLC on silica plates, and the chromatograms were visualized under UV light (254 nm);
- (F) The strains' corresponding toxic activities were located on TLC plates by an *E. coli* growth inhibition assay on a thin layer of PMS agar over the TLC plate or
- (G) *Pseudomonas* minimal medium (PMS) supplemented with ornithine.



Arrebola *et al.*,2011

Toxins
General methods
Extraction and semi-purification

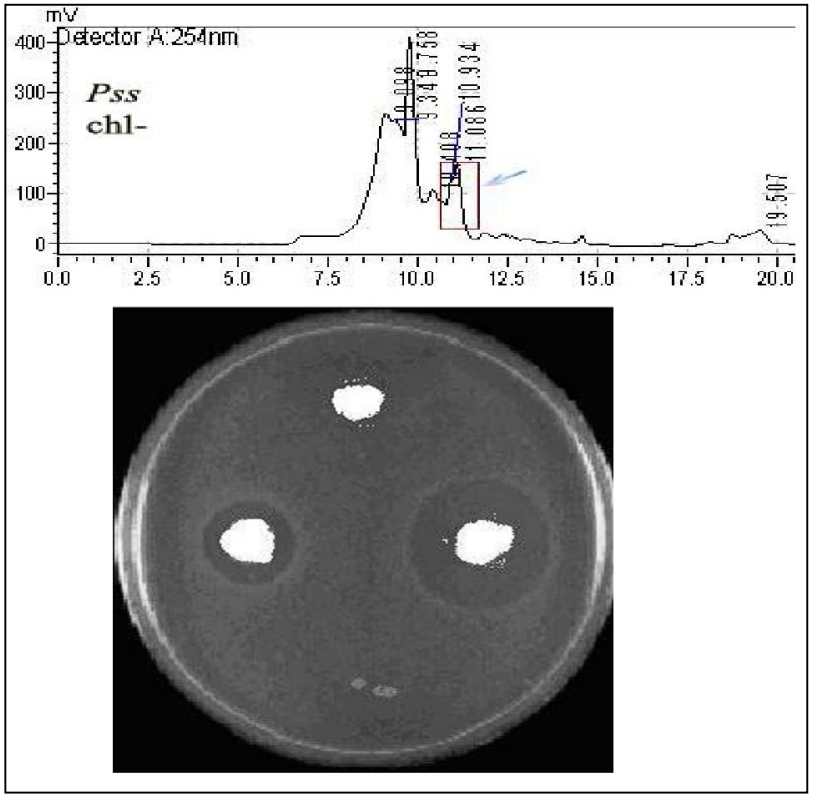
- To extract toxins from fluorescent plant pathogenic *Pseudomonas* spp., such as *Pss*, the strains were grown in 100 ml IMM medium (Surico *et al.*, 1988) as still cultures at 25 °C for 6-8 d.
- Each culture was centrifuged at 10400 *g* for 20 min and the supernatant fluid saved.
- Ammonium sulphate was added to give 80% saturation (57 g 100 ml^{-1}) and the treated culture was allowed to stand at room temperature for 2 h.
- Following centrifugation at 10400 *g* for 20 min, the supernatant fluid was discarded and the centrifugate was resuspended in 5 ml 0.01 mol^{-1} Tris-phosphate buffer (pH 6.5).
- The semi-purified toxin was dialysed at 4 °C for 24 h against Tris-phosphate buffer (2) using Visking tubing (9-17000 Da cut-off) prepared by boiling in Tris-phosphate buffer for 30 min.

Hu*et al.*,1998

Toxin bioassay and HPLC analysis method

Characterization and identification of virulence factors produced by virulent isolates of *P. syringae* pv. *sesami*

- HPLC separations of *Pseudomonas syringae* pv. *sesami*.
- Toxin extractions from acetone of cell free culture filtrate of 7 day old culture.
- All the fractions obtained from different peaks were tested against unknown bacterium.
- The peak enclosed by a box showed toxic activity against unknown bacterium.

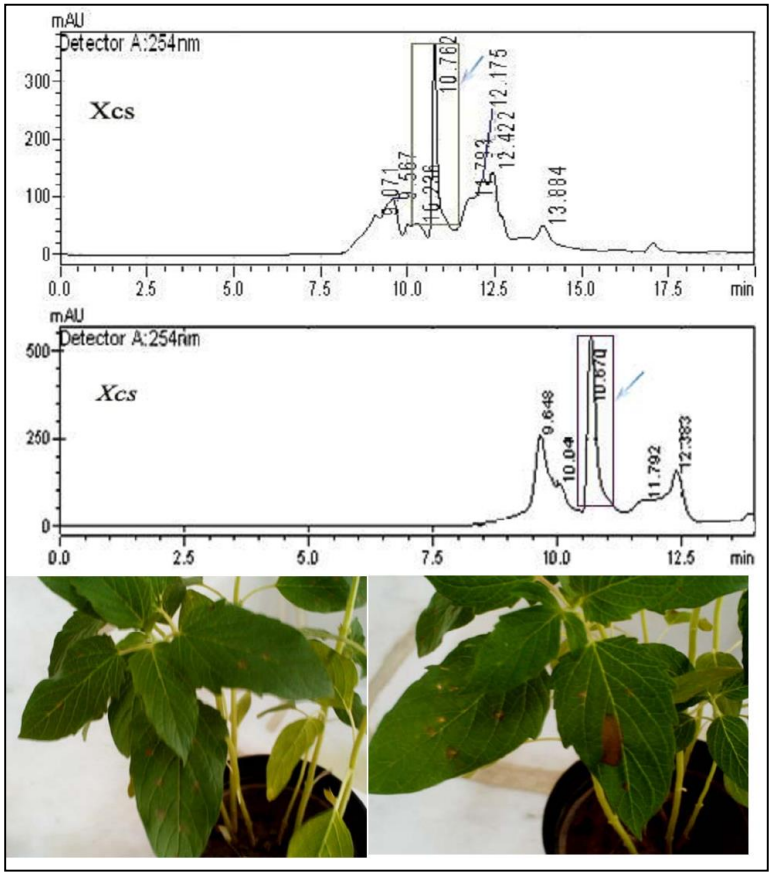


Firdous *et al.*,2010

In vivo toxin assay and HPLC analysis method

Characterization and identification of virulence factors produced by virulent isolates of *X. campestris* pv. *sesami*

- Fractions of peak enclosed in box from *Xanthomonas campestris* pv.*sesami* showed phytotoxic activity on sesame plant.



Firdous *et al.*,2010

In vivo toxin

Role of mangotoxin in bacterial virulence

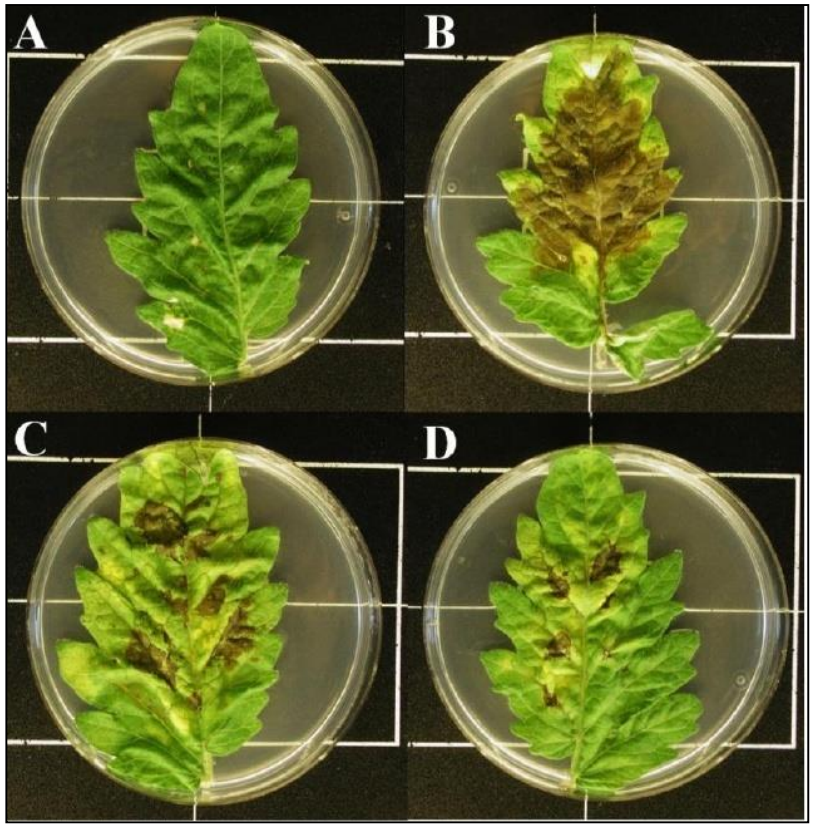
The mangotoxin inhibition on *E. coli* growth was reversed by adding ornithine

- Mangotoxin seems to increase necrosis symptoms in tomato leaves infected with mangotoxin-producing strains of *P. syringae* pv.*syringae*.

(A) Absence of disease symptoms in a control (non-inoculated) leaflet;

(B) Representative symptoms of a mangotoxin-producing strain of *P. syringae*;

(C) and (D) Symptoms produced by its derivative mutant defective in mangotoxin production on tomato leaflets at 7 days after inoculation.

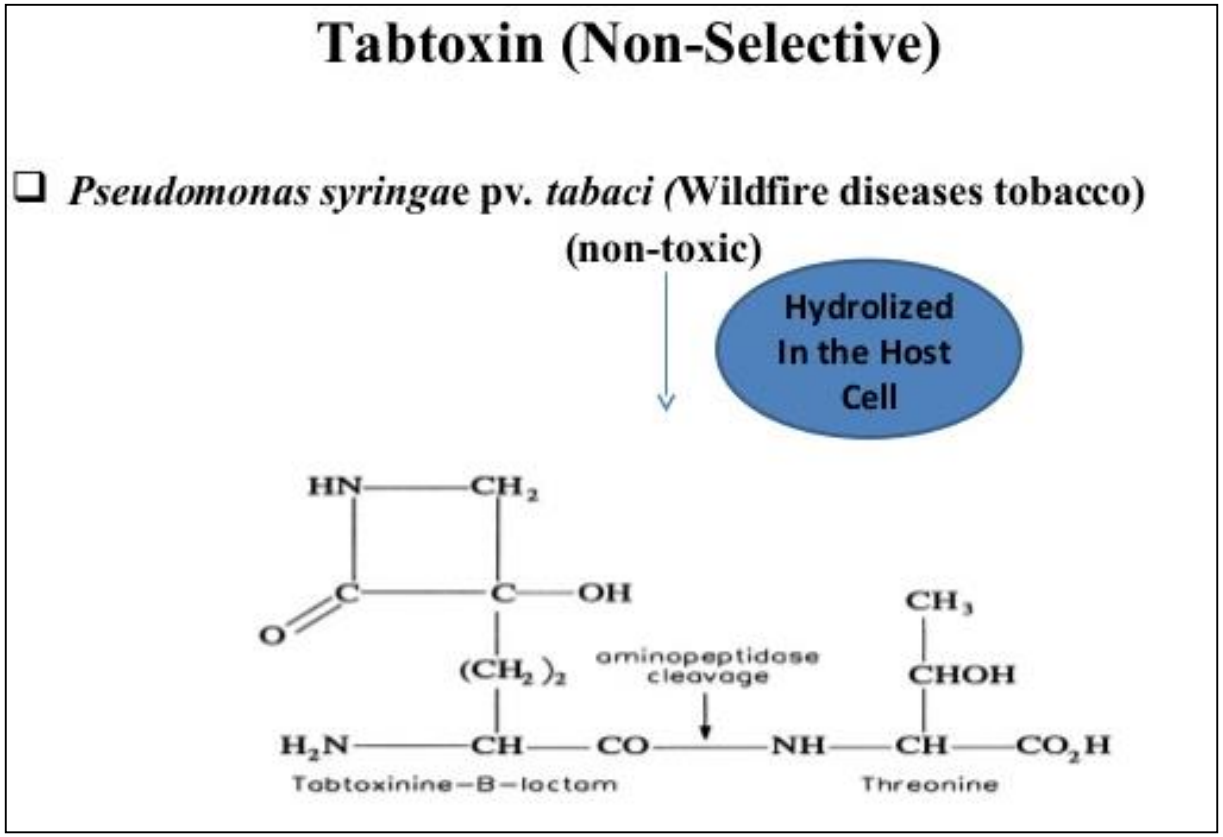


Arrebola *et al.*,2011

Chlorosis-inducing toxins

Tabtoxin

Inhibits glutamine synthease



Tabtoxin is a ß-lactam that inhibits glutamine synthesis.

Chlorosis-inducing toxins

Tabtoxin determination

Reversed by the addition of 10 mM glutamine to the assay medium

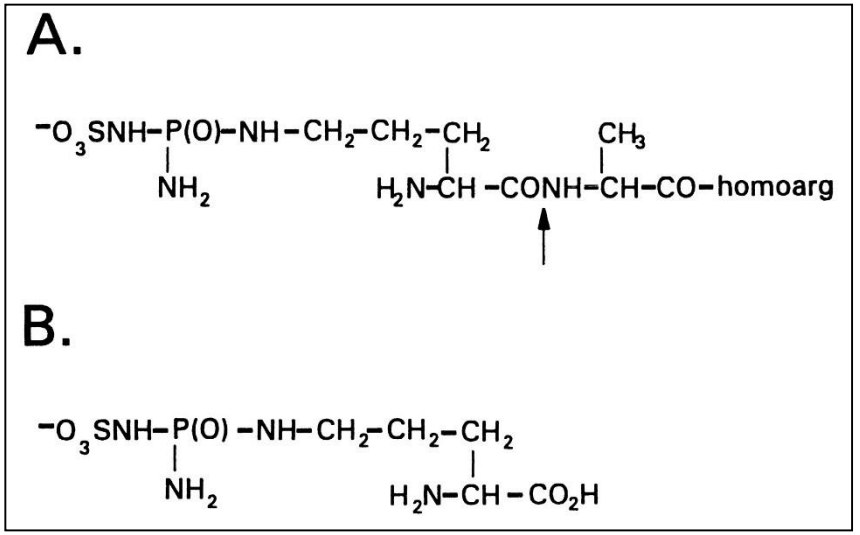
- This toxin inhibits the target enzyme glutamine(glutamine synthetase).
- Glutamine was used to antagonize the toxin by mixing 25 µl of a 0.1% solution of the amino acid with cultures filtrates in the agar hole.
- Production of tabtoxin was determined by an agar plate diffusion test with *E. coli* N99 as the indicator strain.
- Two milliliters of 0.7% molten mineral salts-glucose (MG) agar (maintained at 45°C) was mixed with 2 ml of *E. coli* and poured onto MG agar plates.
- MG-glutamine plates were made by overlaying the *E. coli* MG soft agar mixture with 17 µmol of glutamine.
- Next, 10 µl of an overnight culture of *P. syringae* grown in MG medium was spotted onto the MG-*E. coli* and MG-glutamine-*E. coli* plates, followed by incubation at room temperature for 48 h.
- Strains were scored as positive for tabtoxin when there was a zone of inhibition surrounding the *P. syringae* colonies on the MG plates but not surrounding the corresponding colonies on the MG-glutamine plates (Hwang *et al.*,2005).

Chlorosis-inducing toxins

Strucure of phaseolotoxin & octicidine

A. Structure of phaseolotoxin and
B. Octicidine.

- Plant peptidases cleave phaseolotoxin (arrow) to release the alanine and homoarginine residues, a reaction which results in octicidine formation.



Bender *et al.*,1999

Chlorosis-inducing toxins

Phaseolotoxin production

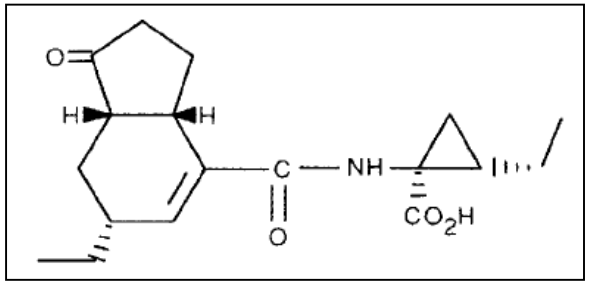
Reversed by L -arginine or L -citrulline, but not by L -glutamine

- Phaseolotoxin production was determined by using a method modified from Staskawicz and Panopoulos,1997.
- *E. coli* N99 was grown in Davis minimal medium for 48 h at 37°C.
- A 2-ml portion of culture was mixed with 2 ml of 2% molten agar in water and overlaid on Davis minimal medium plates.
- *P. syringae* strains were grown in minimal A medium for 48 h at 30°C, and 10 µl of the *P. syringae* culture was spotted onto the *E. coli* test plates.
- The presence of phaseolotoxin was characterized by a zone of inhibition surrounding the *P. syringae* colonies after 24 h.

Hwang *et al.*,2005

Chlorosis-inducing toxins
Coronatine

- Structure of coronatine, a toxin synthesized by several pathovars of *Pseudomonas syringae* e.g. *P. syringae* pv. *atropurpurea*, *P.s.* pv. *tomato*,…which may act by inhibiting active defence in the host.
- Coronatine causes chlorosis in a number of plants and also hypertrophy of potato-tuber tissue but, at the time of writing, no specific receptor has been identified.
- Hypertrophy suggested that the toxin might mimic the action of one of the plant-growth substances such as auxins, cytokinins, gibberellins, abscisic acid or ethylene.



Chlorosis-inducing toxins
Coronatine production

- Production of coronatine was determined by a semi quantitative potato disk bioassay.
- 50 µl of an overnight *P. syringae* culture was added to 1 ml of Hoitink and Sinden medium (HSC) (Hoitink and Sinden,1970), and this was followed by incubation on a 250-rpm rotary shaker at 20°C for 4 days.
- One milliliter of this bacterial suspension was centrifuged at 2,000 x *g* for 10 min at room temperature, and 20 µl of the bacterial supernatant was spotted onto the potato tuber disk prepared as described in Volksch *et al.*,1989.
- The presence of coronatine was characterized by a hypertrophic response (an obvious enlargement of tissue) on the potato disks.

Hwang *et al.*,2005

Chlorosis-inducing toxins

Coronatine

Test for hypertrophy in potato tuber

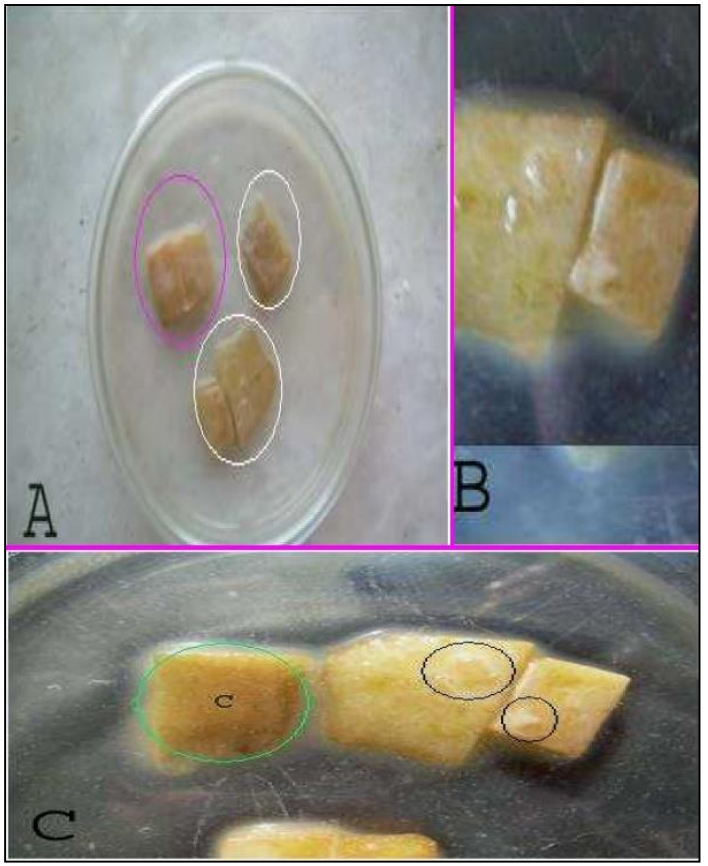
- Production of chlorosis producing toxin was evaluated by a semiquantitative potato disc bioassay (Volkch *et al.*,1989).
- 20 µl cell free culture filtrate as described above was spotted onto the potato discs.
- The presence of chlorosis (chl) producing phytotoxin was characterized by a hypertrophic response (enlargement of tissue) on the potato discs.
- Bacteria have also been assayed directly for hypertrophic outgrowth by placing a loopful of inoculum on the surface of the potato tuber discs.
- Tissue was assessed for hypertrophic outgrowth 5 days after inoculation.

Chlorosis-inducing toxins

Coronatine

Test for hypertrophy in potato tuber

- Cell enlargements due to *P. syringae* pv. *sesami* (chl^+) isolates encircled in white, necrotic (chl^-) isolates did not show any enlargement encircled in pink in A.
- Close images of cell enlargement due to chlorosis in B and C.



prr.hec.gov.pk/Chapters/375S-5.pdf

Chlorosis-inducing toxins

Coronatine

Test for hypertrophy in potato tuber

- Coronatine may be characterized as a substance with high auxin-like activity.
- In potato tuber discs the stimulation of water uptake by coronatine was considerably higher than that of IAA.
- Also our results indicated that the effect of coronatine on the increase in hypertrophy of potato tuber discs was higher than that of IAA.

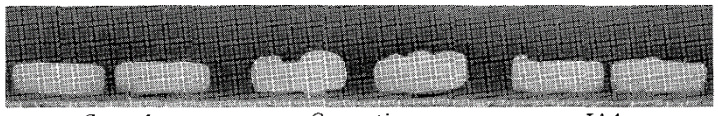


Fig. 2 Effects of coronatine and IAA on hypertrophy response in potato tuber discs. Coronatine (10 mM/ml), IAA (50 mM/ml) and water as control

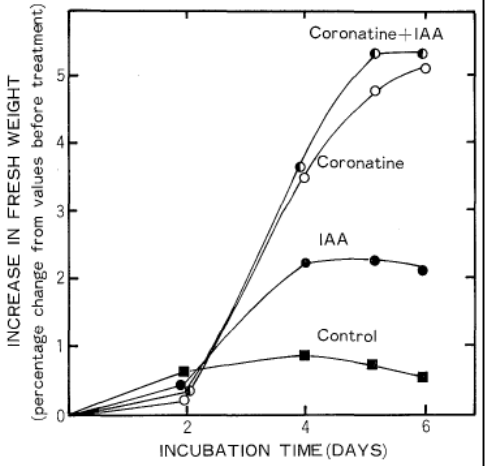


Fig. 1 Time course of water uptake by potato tuber discs treated with coronatine (25 mM/ml), IAA (50 mM/ml) and water as control.

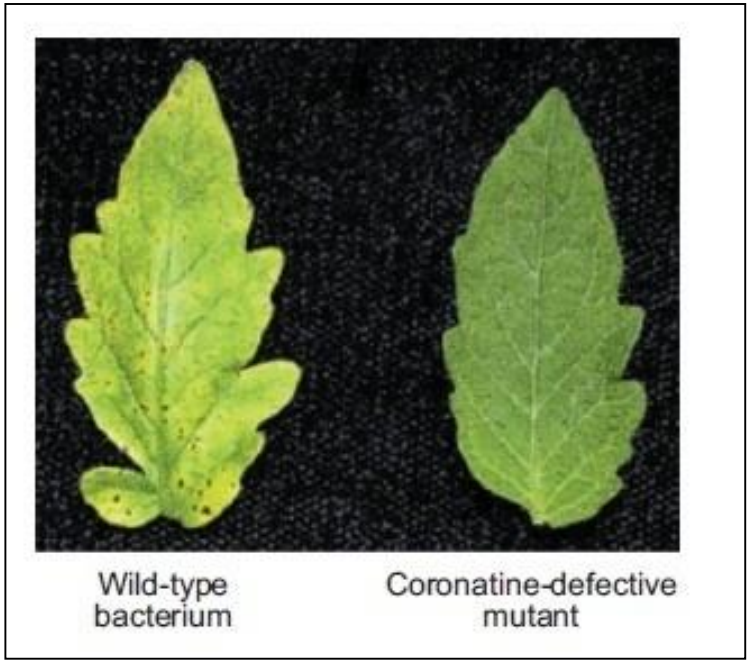
Sakai,1980

Chlorosis-inducing toxins

Coronatine

Test for necrotic lesions of potato leaves

- Tomato leaves 5 days after dip-inoculation with 1×10^7 CFU/ml suspension of the wild-type bacterium *Pst* DC3000 (left) and the coronatine defective mutant *Pst* DC3118 (right).
- Whereas *Pst* DC3000 caused necrotic lesions with diffuse chlorosis, the coronatine-defective mutant bacteria caused only some lesions and no chlorosis.

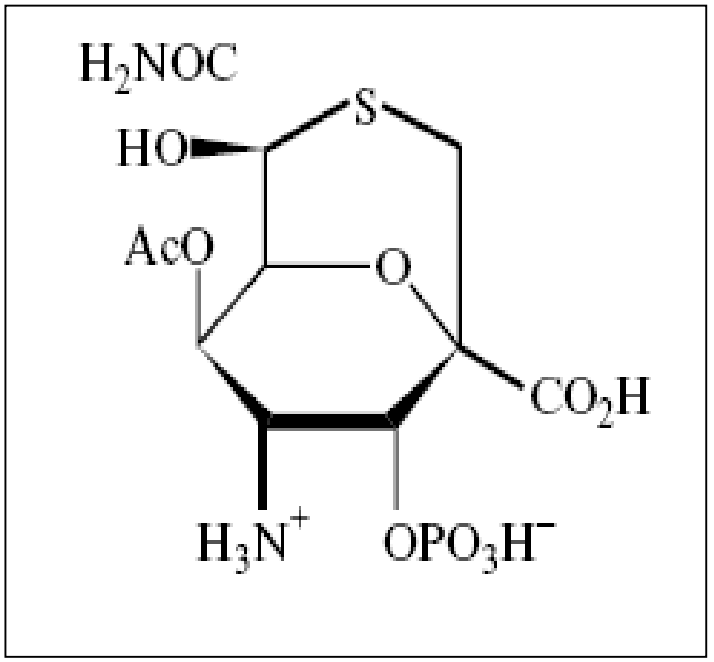


Melotto *et al.*,2008

Chlorosis-inducing toxins

Tagetitoxin

- The structure of tagetitoxin from *Pseudomonas syringae* pv. *tagetis* the toxin is an inhibitor of chloroplast RNA polymerase.



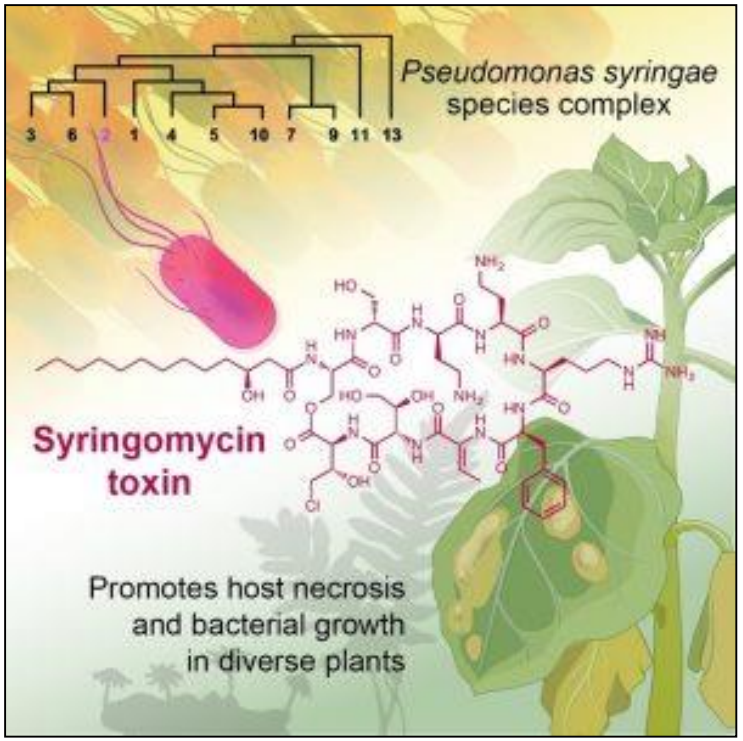
Cyclic lipopeptides (CLPs)
Syringomycin production

- Syringomycin production was assessed using a general method for detecting lipodepsipeptides.
- Twenty microliters of a *P. syringae* overnight culture was spotted onto potato dextrose agar, followed by incubation for 48 h at 30°C.
- Subsequently, the plates were sprayed with an overnight culture of the yeast *Rhodotorula pilimanae* and incubated for 24 h at room temperature.
- The presence of lipodepsipeptide was characterized by the development of a zone of inhibition surrounding the *P. syringae* colonies.

Hwang *et al.*,2005

Characteristics of the species
Syringomycin production

- The toxin syringomycin produced by the most widely infectious *P. syringae* strains, and compared its effect on both non-flowering and flowering plants.
- The toxin syringomycin likely interferes with cell membranes across each of the diverse plants.
- A necrotizing toxin enables *Pseudomonas syringae* infection across evolutionarily divergent plants appears in Cell Host and Microbes.



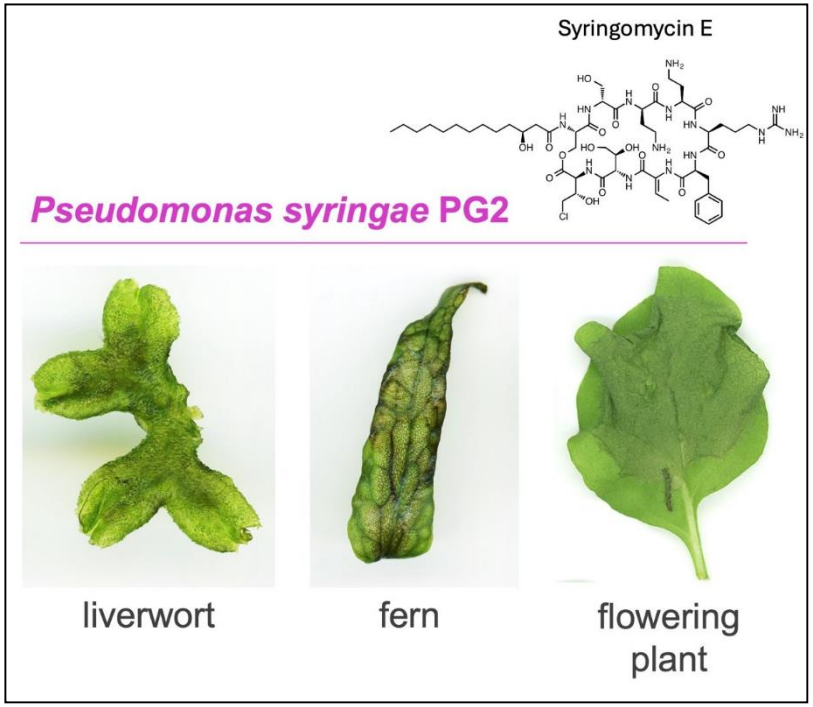
Evolutionary study reveals the toxic reach of disease-causing bacteria across the Plant Kingdom.

The John Innes Centre,,2024

Characteristics of the species

Syringomycin production

- Three model plants showing disease symptoms after infection with *Pseudomonas syringae*.
- The toxin Sringomycin shown is critical to establish disease symptoms.



Cyclic lipopeptides (CLPs)
Tolaasins production

- *Pseudomonas tolaasii*, the causal organism of brown blotch disease of *Agaricus bisporus* and of the yellowing of *Pleurotus ostreatus*, was shown to produce in culture:

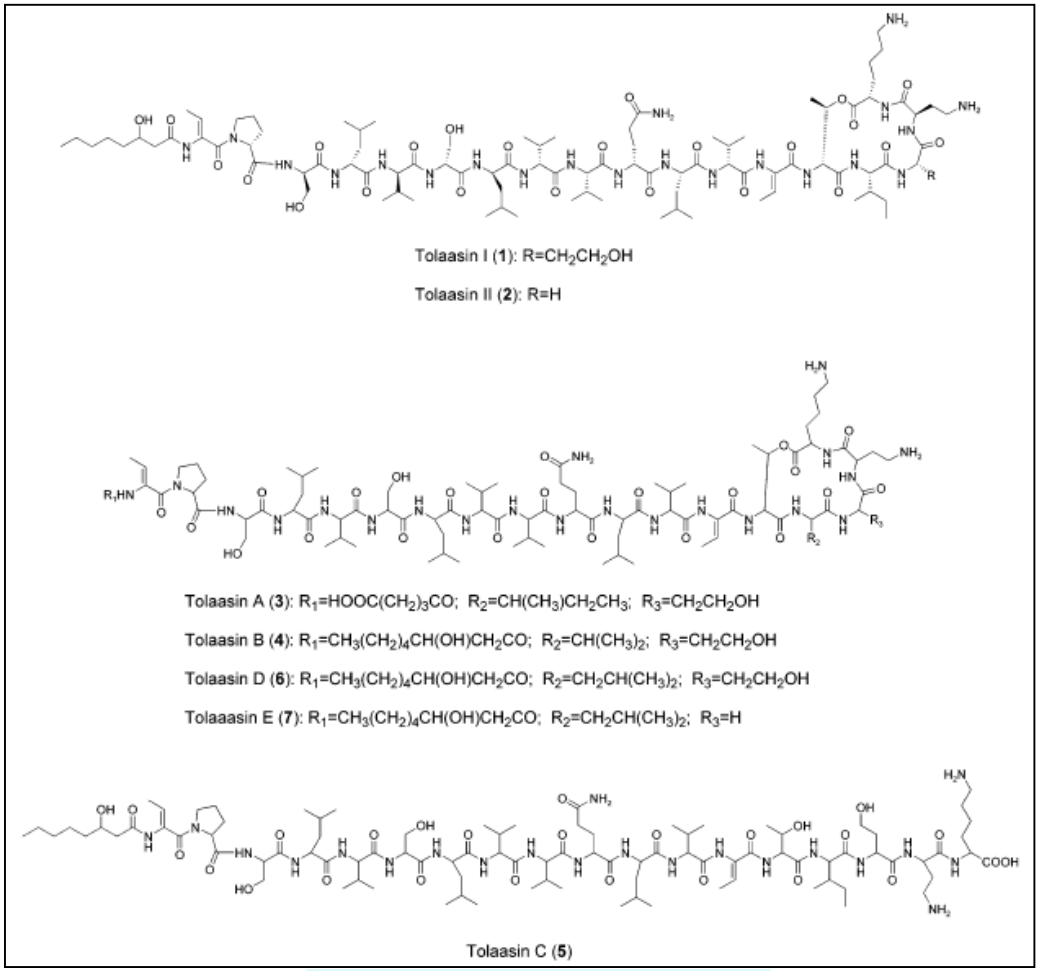
1. Tolaasin I,
2. Tolaasin II, and
3. Five other minor metabolites, tolaasins A, B, C, D, and E.

- These extracellular necrosis inducing toxins were demonstrated to be important in the development of the disease symptoms.

Bassarello *et al.*,2004

Cyclic lipopeptides (CLPs)

Tolaasins production



Tolaasin I (**1**): R=CH_2CH_2OH

Tolaasin II (**2**): R=H

Tolaasin A (**3**): R_1=$HOOC(CH_2)_3CO$; R_2=$CH(CH_3)CH_2CH_3$; R_3=CH_2CH_2OH

Tolaasin B (**4**): R_1=$CH_3(CH_2)_4CH(OH)CH_2CO$; R_2=$CH(CH_3)_2$; R_3=CH_2CH_2OH

Tolaasin D (**6**): R_1=$CH_3(CH_2)_4CH(OH)CH_2CO$; R_2=$CH_2CH(CH_3)_2$; R_3=CH_2CH_2OH

Tolaaasin E (**7**): R_1=$CH_3(CH_2)_4CH(OH)CH_2CO$; R_2=$CH_2CH(CH_3)_2$; R_3=H

Tolaasin C (**5**)

Bassarello *et al.*,2004

Cyclic lipopeptides (CLPs)
Tolaasins production

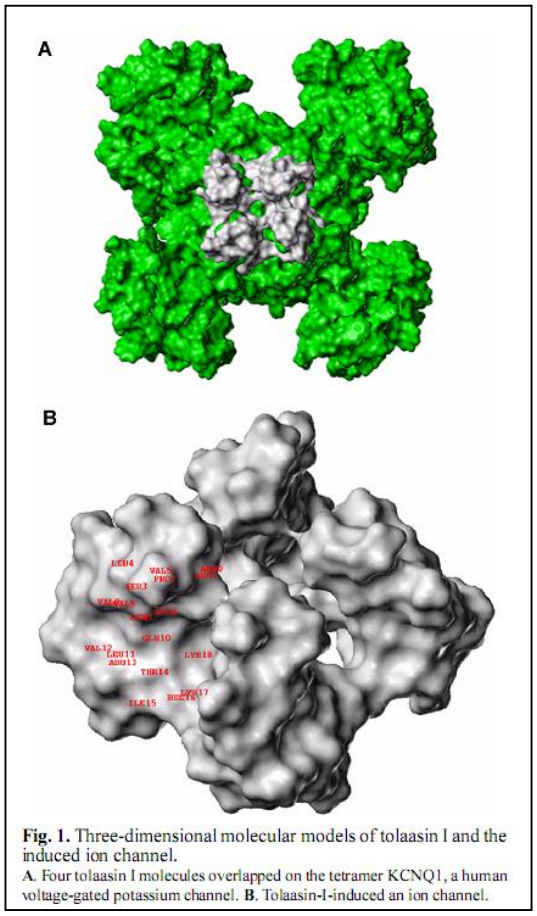
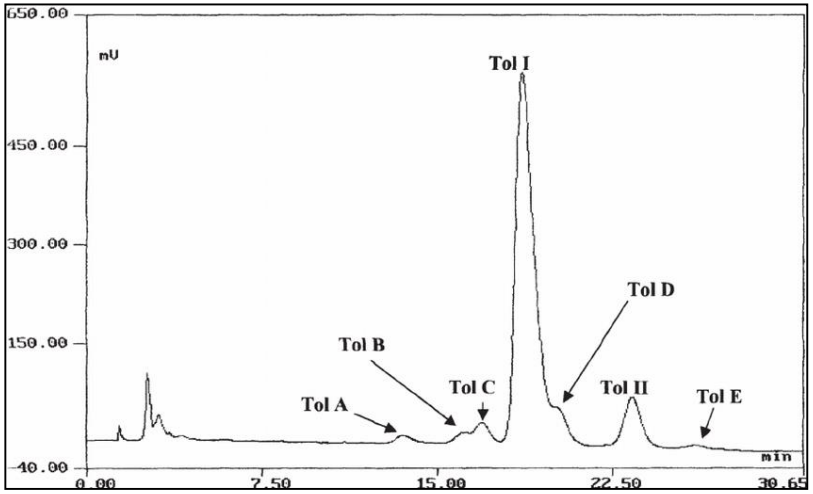


Fig. 1. Three-dimensional molecular models of tolaasin I and the induced ion channel.
A. Four tolaasin I molecules overlapped on the tetramer KCNQ1, a human voltage-gated potassium channel. **B.** Tolaasin-I-induced an ion channel.



Tolaasin-I-8-primary-structure

Bassarello *et al.*,2004; Geunhyeong *et al.*,2011

Cyclic lipopeptides (CLPs)
Tolaasins production

- **Bacterial Growth Conditions:**
- Strain type NCPPB2192 of *P. tolaasii* was grown at 25°C under shaking (180 rpm) in 500 mL Erlenmeyer flasks filled with 150 mL of liquid King's B medium inoculated with 1.5 mL of a bacterial suspension containing 10^8 cfu/mL.
- After 48 h incubation cultures were centrifuged (20000*g* for 15 min), and the resulting supernatants were evaluated for the antimicrobial activity against *Bacillus megaterium* following an already established procedure, lyophilized, and stored at -20°C before further processing.

Bassarello *et al.*,2004

Cyclic lipopeptides (CLPs)
Tolaasins production

- The antimicrobial activity of HPLC grade tolaasins A-E in comparison with tolaasin I and II against:
- The yeast *Rhodotorula pilimanae*,
- The fungus *Rizoctonia solani*,
- The Gram-positive bacteria *Bacillus megaterium* and *Rodococcus fascians*, respectively, and
- The Gram-negative bacteria *Escherichia coli* and *Erwinia carotovora* subsp. *carotovora*.
- *B. megaterium* and *R. fascians* were the most sensitive test microorganisms.

microorganism	tolaasins minimal inhibitory quantity (μg)						
	I	II	A	B	C	D	E
Rizoctonia solani 1583	0.32	0.64	1.28	2.56	5.12	0.16	5.12
Rhodotorula pilimanae ATCC26423	2.56	5.12	5.12	>5.12	>5.12	2.56	>5.12
Bacillus megaterium ITM100	0.32	0.64	1.28	2.56	>5.12	0.16	2.56
Rodococcus fascians NCPPB3067	0.32	0.64	1.28	1.28	>5.12	0.16	2.56
Escherichia coli K12 ITM103	> 5.12	> 5.12	>5.12	> 5.12	>5.12	>5.12	>5.12
Erwinia carotovora subsp. *carotovora* ICMP5702	> 5.12	>5.12	>5.12	> 5.12	>5.12	>5.12	>5.12

Bassarello *et al.*,2004

Tolaasins production
Alternative method

- Bacteria were grown in PSB (potato semi-synthetic broth) or PSA (potato semi-synthetic agar) at 25°C.
- PSB-Tol, a medium that contains tolaasin, was prepared by adding the components of PS-broth to the culture supernatants of *P. tolaasii* strains.
- Culture supernatants of *P. tolaasii* strains were obtained by culturing *P. tolaasii* in PSB at 25°C for 48 h, harvested by centrifugation (4°C, 10,000 × g) and sterilized by placing in boiling water for 10 min.

Tsukamoto *et al.*,1998 and 2002

Tolaasins production
Alternative method

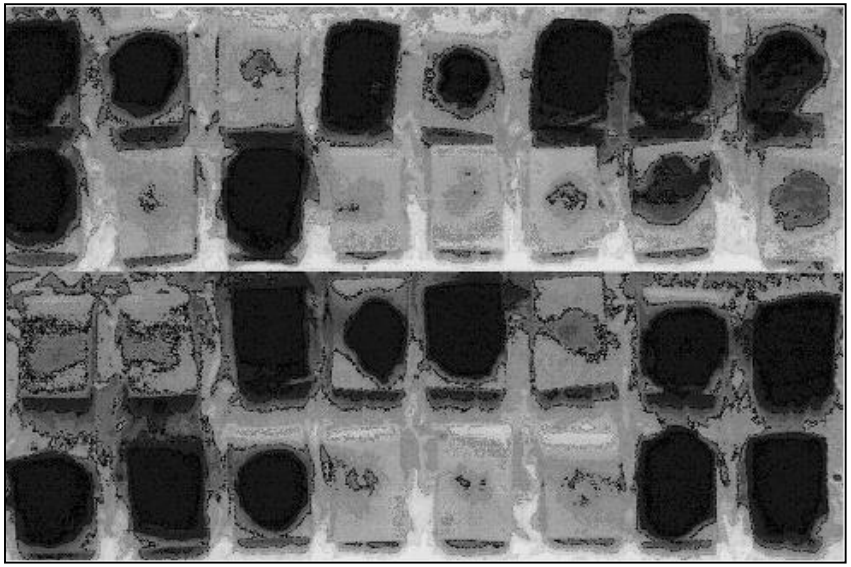
- The 50 µl of samples of the whole cultures were then applied onto potato tuber slices, wherein tuber slices normally become blackened due to the toxicity of tolaasin.

Tsukamoto *et al.*,2002

Tolaasins production

Effects of talaasin(Blackened) and talaasin-detoxifying bacteria(colorless) on potato slices

- Potato slices treated with PSB-Tol (a medium that contains tolaasin) and tolaasin detoxifying bacteria.
- In the presence of tolaasin detoxifying bacteria remained colorless, but those treated together with non-detoxifying bacteria were blackened.



PSB (potato semi-synthetic broth)

Tsukamoto *et al.*,2002

Tolaasins production

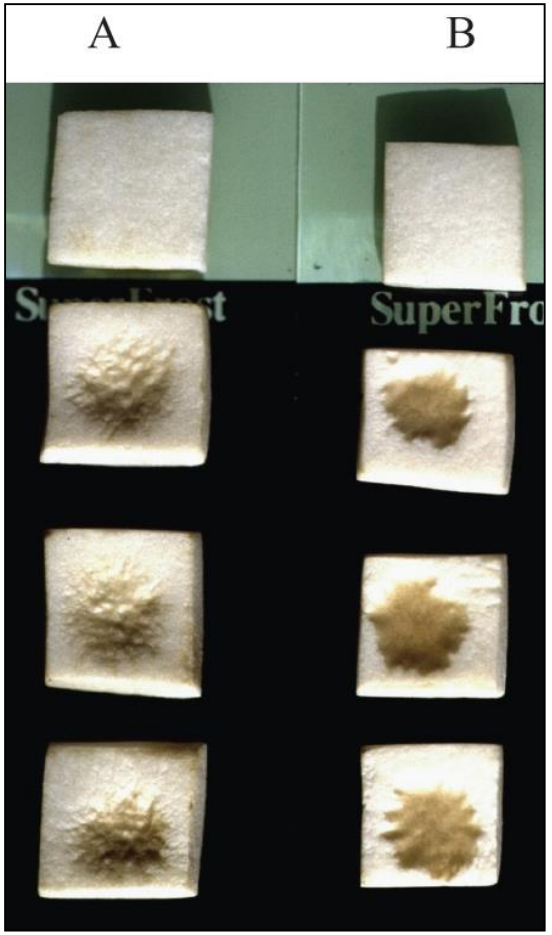
Assay on tissue blocks and whole sporophores of *Agaricus bisporus*

- Brown lesions on tissue blocks of *Agaricus bisporus* (lower three blocks in each treatment), caused by deposition of 5 µl solutions containing:

A. 5.12 µg of WLIP(White Line Inducing Principle), and

B. 0.64 µg of tolaasin I, respectively.

- On upper blocks, 5 µl of sterile water was deposited.
- Brown sunken lesions were observed with both A and B treatments.



Andolfi *et al.*,2009

Toxin production

Persicomycins determination

- *Pseudomonas syringae* pv. *persicae* is a phytopathogenic bacterium responsible for a dieback disease on *Prunus* spp. mainly on *Prunus persica*.
- Several toxic substances called persicomycins are synthesized by *Pseudomonas syringae* pv. *persicae*.
- It is now an accepted fact that *P. s.* pv. *persicae* is heterogeneous in toxigenesis i.e. a variable necrotic capability of strains on the peach tree host/variable biocidal activities(persicomycin production) of each and every individual colonies.
- Three classes of colonies were defined:
- Class 1 with no detectable production,
- Class 2 with a low production and
- Class 3 characterized by a larger production.
- It appears that the level of persicomycin production varied from cell to cell whatever the strain and the assay.

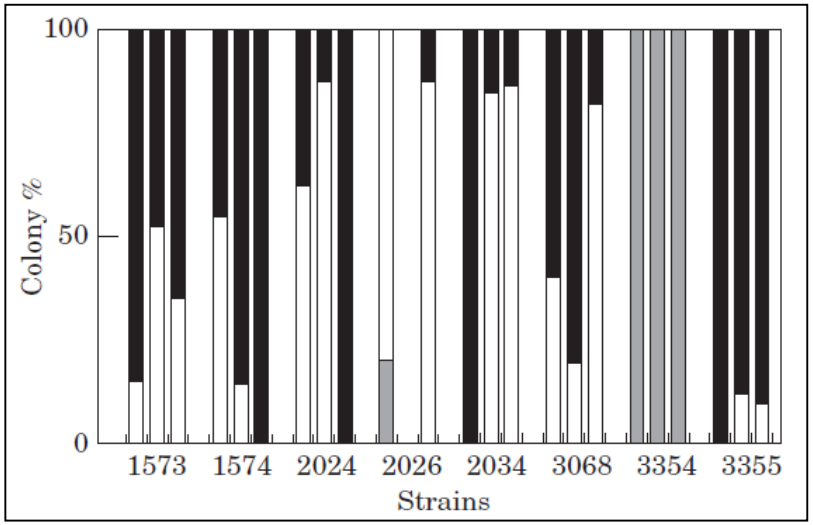
Barzic,1999

Toxin production

Toxin production capability of colonies

Persicomycins determination

- Tests were realized with 10 µl of suspension of each colony.
- Data is from three separate assays with 40 colonies each.
- Colonies were divided into three classes:
- Class 1, colonies with no detectable production;
- Class 2, colonies giving a production scored as the mean growth inhibition ≤5 mm from the edge of the colony;
- Class 3, colonies giving a production scored as the mean growth inhibition >5 mm from the edge of the colony.

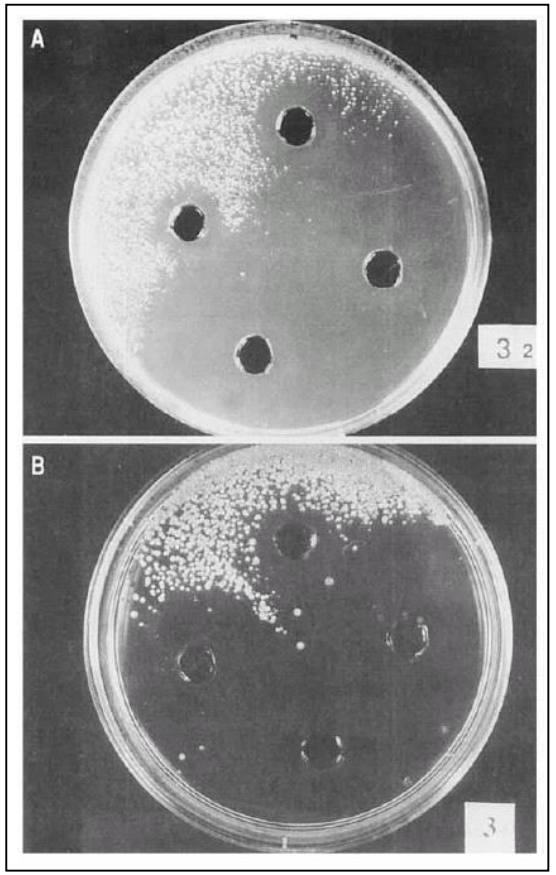


Barzic,1999

Toxin production
Persicomycin determination

- Both plant cells damage (necrosis of peach tree tissues) as well as *in vitro* antibiosis against microorganisms such as *Bacillus thuringiensis* (bacterial indicator) is due to antibiotic property of persicomycins.
- Antibiosis was displayed by persicomycin extracted from:

A. Culture extracts and
B. Necrotic peach tree tissues.

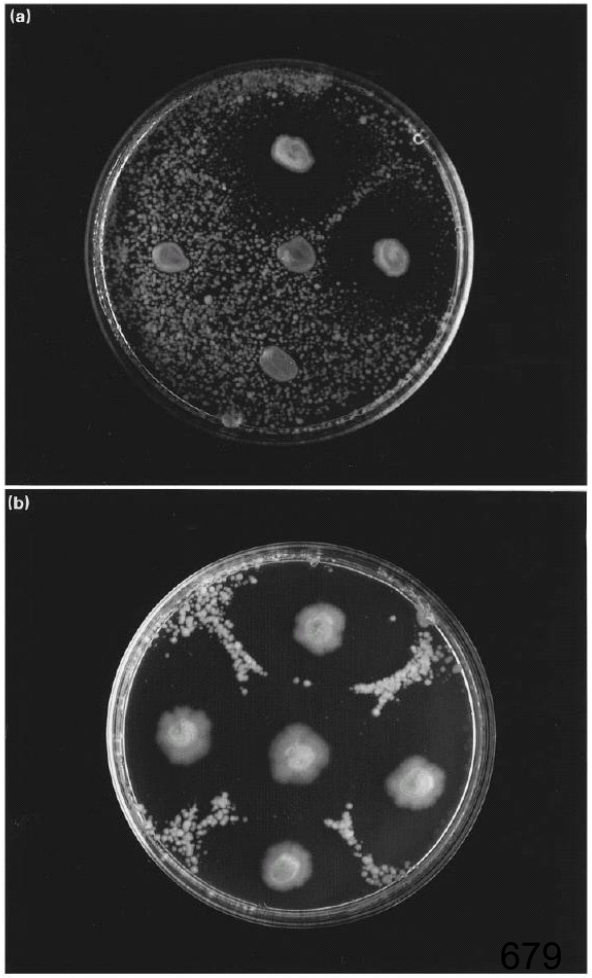


Barzic and Guittet,1996

Toxins

Persicomycin determination

- Toxigenicity among colonies of *Pseudomonas syringae* pv. *persicae* obtained from strain 3354 (a) and strain 3355 (b) shown by antibiosis to *Bacillus thuringiensis* on LPGA medium.
- All *Ps. syr.* pv. *persicae* isolates from a range of geographical origins have been shown to produce persicomycins *in vitro*.
- *Ps. syr.* pv. *persicae* can synthesize its necrosis inducing toxins, and freezing temperatures, e.g. -5°C, that can occur with frosts did not seem to affect bacterial toxigenesis.



Barzic,1999

Toxin production

Scab-inducing toxins

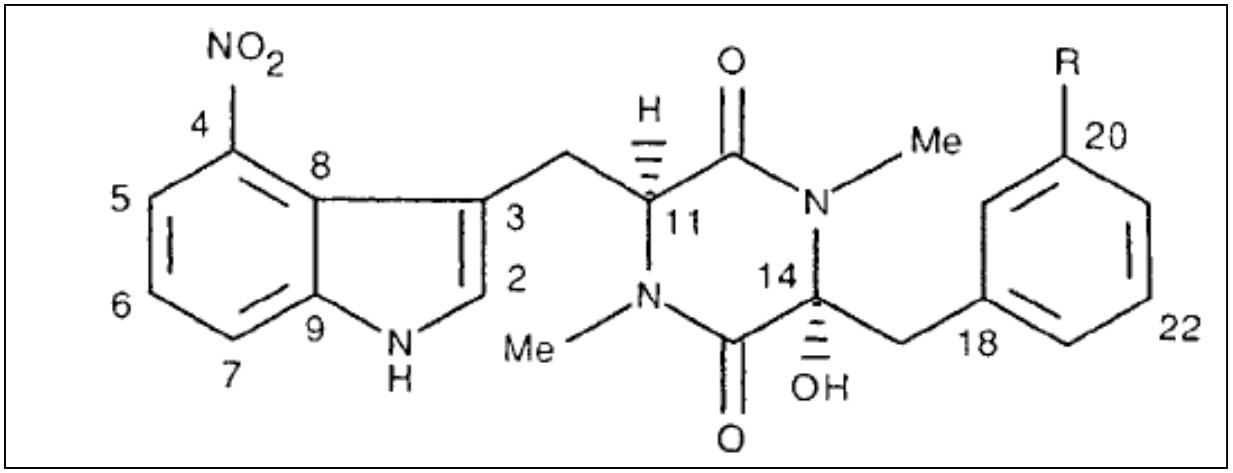
Thaxtomins

- Scab-inducing toxins such as the cyclic dipeptide thaxtomin A and B, produced by *Stretomyces scabiei*, causing common scab of potato.
- Thaxtomins are produced only by plant pathogenic species, cause necrosis and cell hypertrophy on expanding host tissue.
- Production of thaxtomins is perfectly correlated to pathogenicity in all strains tested to date including *S. scabies*, *S. acidiscabies*, and *S. ipomoeae* (King *et al.* 1994), suggesting that thaxtomins may be a common pathogenicity determinant among diverse *Streptomyces* spp. that cause plant disease.

Toxin production

Scab-inducing toxins

Thaxtomins A & B



Structures of thaxtomins A (R = OH) and B (R = H), two piperazine toxins produced by species of *Streptomyces* that are important in the development of scab symptoms on potatoes.

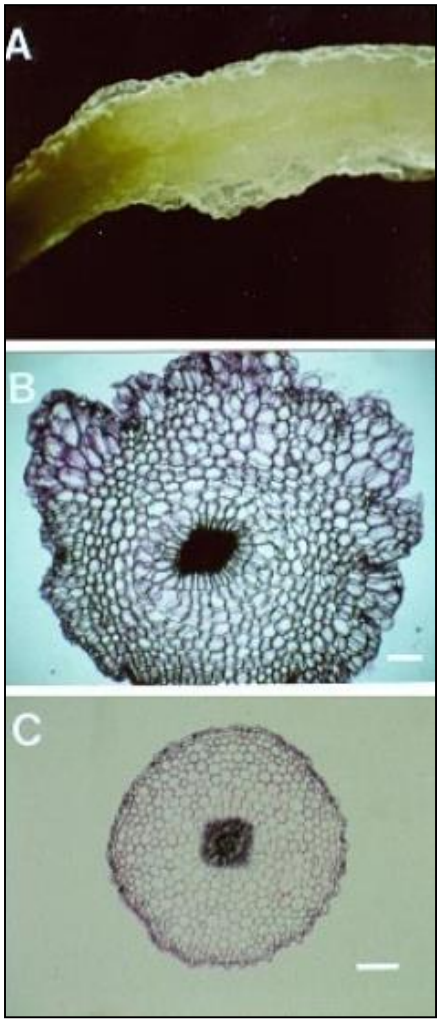
Strange,2003

Scab-inducing toxins

Thaxtomin A

Radial swelling is due to dramatic cell hypertrophy

- Dramatic cell hypertrophy on the hypocotyl of a radish seedling that was treated with thaxtomin A.
- Germinated seeds were incubated on agar containing:
- A and B: thaxtomin A 0.075 µM or
- C: Unamended agar for approximately 7 days before examination.
- Cross sections B and C were approximately 150 µm thick and were stained with toluidine blue.



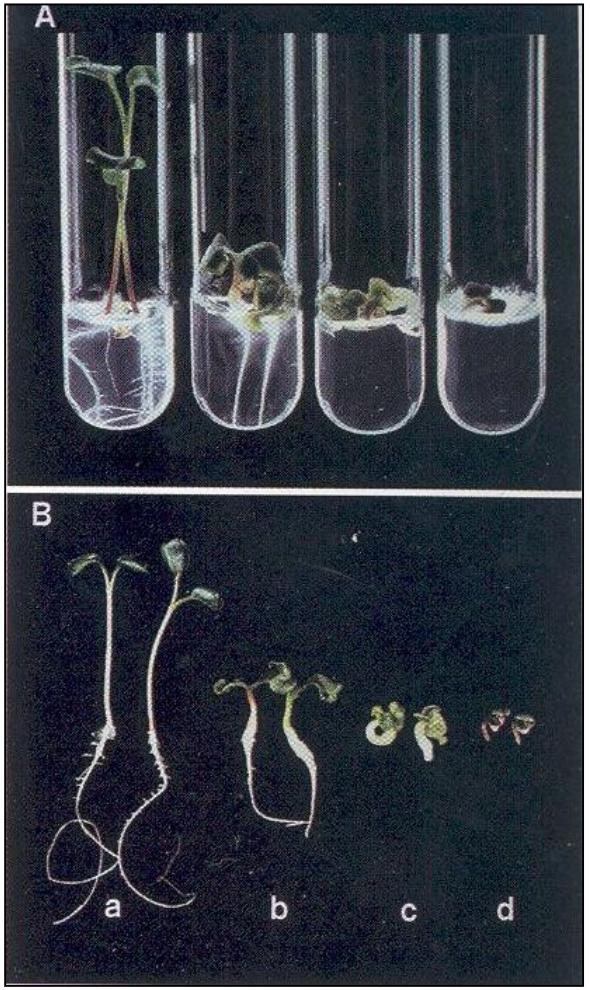
Loria *et al.*,1997

Scab-inducing toxins

Thaxtomin A

Radish seedling assay

- Untreated.
- Treated with pure thaxotamin A.
- Filter-sterilized supernatant of *S. scabies* (stunting and radial swelling).
- *S. scabies* culture (necrosis and seedling collapse).



Schaad *et al.*,2001

Toxin production

Albicidin

- Leaf scald, caused by *Xanthomonas albilineans*, is a serious disease of sugarcane.
- The xylem-invading bacterium produces several toxins of which the major one is albicidin which has only been partially characterized.
- It is a low molecular weight compound with several aromatic rings and is bactericidal to Gram-positive and Gram-negative bacteria at concentrations as low as 1 ng/ml.
- Symptoms of leaf scald include chlorosis, necrosis, wilting and sometimes sudden death of the plant after a long latent period.
- The chlorotic symptoms are caused by inhibition of DNA synthesis in chloroplasts caused by the toxin.
- Tox^- mutants are unable to cause disease symptoms.

Toxin production

Albicidins

Genes that codes for a peptide which detoxifies albicidin

- Albicidins such as Abscisic acid (ABA) are a family of phytotoxins and antibiotics produced by the xylem invading bacterium *Xanthomonas albilineans* and specifically block prokaryotic (plasmid) DNA replication.
- Several genes that confer resistance to albicidins have been cloned from heterologous, biocontrolling bacteria, such as *Klebsiella oxytoca* (albA), *Alcaligenes denitrificans* (albB), *Pantoea dispersa* (albD), and from *X. albilineans* itself.
- These genes may be useful candidates for transfer into the sugarcane genome.
- Indeed, expression of albD in transgenic sugarcane resulted in:

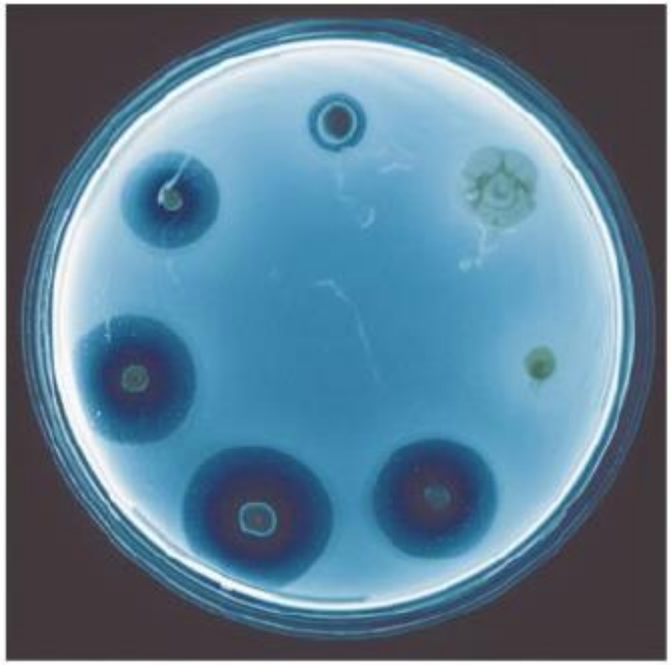
1. Reduced chlorotic disease symptoms, and
2. Conferred resistance to systemic multiplication of the pathogen.

Toxin production

Albicidins bioassay

Xanthomonas spp.

- Genetic and physiological approaches showed that *X. albilineans* produces a family of antibiotics and phytotoxins, which are not only implicated in chlorosis induction but also play a larger role in pathogenesis.
- Inhibition of *E. coli* lawn growth around colonies of *X. albilineans* due to albicidin antibiotics.
- Other tested *Xanthomonas* spp. (top right) are not inhibitory.



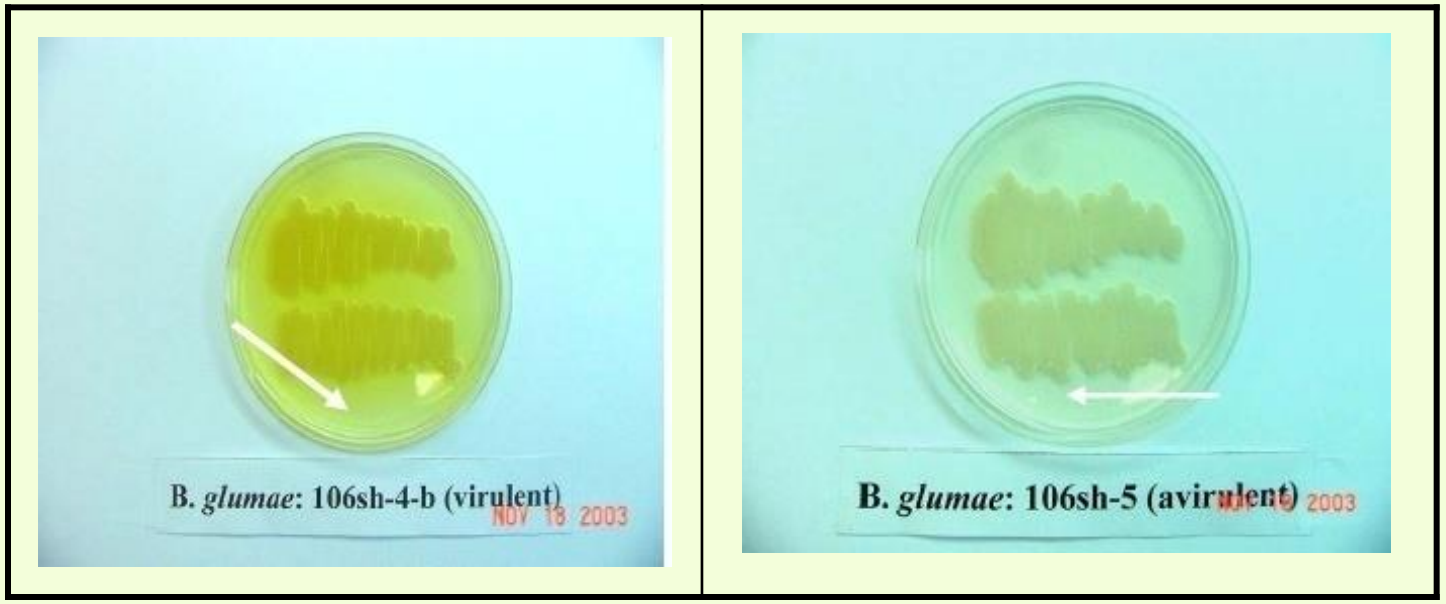
Chlorosis-inducing isolates of *X. albilineans* produce a family of antibiotics called albicidins in culture.

Birch,2001

Pigment (toxin) production

Cultures that produced the yellow or green pigments were pathogenic and virulent

- Cultures of *Burkholderia glumae* with a virulent strain on the left and an avirulent strain on the right.
- Note the lack of toxin in the medium on the right.



Yuan,2004

Control of toxin biosynthesis

- Literature suggest that host factors may be of importance in the production of toxins.
- **e.g.**
- Oat bran was the best medium for production of thaxtomin A.
- Suberin was the only plant polymer that allowed the production of the toxin in minimal medium.
- Arbutin, a phenolic glucoside, and D-fructose were identified as signal molecules which were either necessary for the induction of syringomycin in *Pseudomonas syringae* pv. *syringae* or enhanced its production.
- Leaves of sweet cherry have compounds that activated the *syrB* gene which is required for the synthesis of syringomycin.

Toxin self-protection

- Two major strategies have been adopted by bacteria to protect themselves from their own toxin:

1. Resistance based on the production of target enzymes that are insensitive to the toxin.
2. Resistance via the production of detoxifying enzymes.

Major virulence Factors
4. Classical hormones

- All multicellular organisms produce hormones.
- Plant hormones are also called phytohormones.
- The ability of microorganisms to synthesize or degrade phytohormones is widely known.
- Bacteria, micromycetes, and algae form phytohormones of auxin, cytokinin, or gibberellin nature.

Major virulence Factors
Hormones(meaning set in motion)

- Microorganisms also synthesize phytohormone-like substances including:

1. Ethylene and abscisic acid (ABA), brassinosteroids, oligosaccharines, salicylic acid, and jasmonic acid, and
2. Phytohormone-like substances, such as:
3. Proteins (or peptides) e.g. in Gram+ve bacteria
4. Steroids (a subclass of lipidic hormones) e.g. AHLs in Gram-ve bacteria
5. Amino-acid derivatives (or amines) e.g. AI-3 in Gram-ve bacteria which are resident in the gastrointestinal tract human.

Major virulence Factors

Hormones

- None of the hormones has any enzymatic activity.
- Production of plant hormones alter host physiology.
- Genes for hormone production may be located on:

1. Plasmids, or
2. on the chromosome.

- Each and every plant hormones can be synthesized and/or degraded by bacteria.

Major virulence Factors

Bacterial synthesis and degradation of plant hormones

Hormone	Pathway	Key enzyme(s)	Gene(s)	Representative species
IAA	Trp→IAM→IAA	Trp 2-monooxygenase, IAM hydrolase	*iaaM, iaaH*	*Agrobacterium tumefaciens* *Pseudomonas syringae* pv. *savastanoi* *Bradyrhizobium japonicum* (IAM→IAA) *Pantoea agglomerans* pv. *gypsophilae* *Pseudomonas syringae* pv. *syringae*
	Trp→IPyA→IAAld→IAA	IPyA decarboxylase	*ipdC*	*Enterobacter cloacae* *Azospirillum brasilense* *Pantoea agglomerans* *Pseudomonas putida*
	Trp→TAM→IAAld→IAA	Trp decarboxylase, TAM oxidase	–	*Bacillus cereus* (Trp → TAM) *Azospirillum brasilense* (TAM → IAA)
	Trp→IAAld→IAA	Trp side-chain oxidase	–	*Pseudomonas fluorescens*
	Trp→IAN→IAA	IAN nitrilase	*nitA*	*Alcaligenes faecalis* *Pseudomonas fluorescens*
	IAA→IAA-Lys	IAA-lysine synthase	*iaaL*	*Pseudomonas savastanoi*
	IAA→Cat→	catechol ortho cleavage into β-ketoadipate pathway	*iac* locus, *catABC-pcaD*	*Pseudomonas putida*
	IAA→Ska→Ind→Sal→Cat	–	–	*Pseudomonas* sp.
	IAA→Dio→Isa→IsA→Ant	isatin amidohydrolase	–	*Bradyrhizobium japonicum*
	IAA→2-FABA→Ant	–	–	unidentified
C_2H_4	Met→KMBA→C_2H_4	methionine transaminase	–	*Escherichia coli* *Agrobacterium rhizogenes*
	Glu→2-OG→C_2H_4	ethylene-forming enzyme	*efe*	*Pseudomonas syringae*
	ACC→C_2H_4	–	–	*Bacillus* sp.
	ACC→2-OBA	ACC deaminase	*acdS*	*Agrobacterium rhizogenes* *Enterobacter cloacae* *Achromobacter, Azospirillum, Burkholderia, Pseudomonas, Ralstonia. Rhizobium, Kluyvera* species
	C_2H_4→CO_2	–	–	*Pseudomonas* sp.
ABA	–	–	–	*Bradyrhizobium japonicum* *Azospirillum brasilense*
Z/ZR	AMP→iAMP→Z/ZR	isopentenyl transferase (cytokinin synthase)	*ipt* *ptz* *etz* *fas1*	*Agrobacterium tumefaciens* *Pseudomonas savastanoi* *Rhodococcus fascians* *Erwinia herbicola*

Faure *et al.*,2009

Major virulence Factors
Hormones as signals

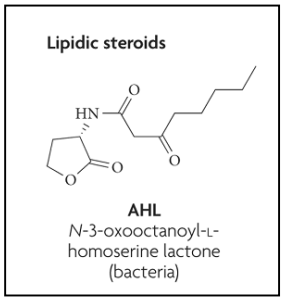
- The role of hormones and hormone-like substances is not limited to intracellular signaling in plants and animals; they also mediate interactions between macro-oganisms (of plant or animal origin) and microorganisms.
- Several plant associated bacteria have evolved ways such as bacterial synthesis or degradation of plant hormones to tap into these hormone signalling pathways and to manipulate plant physiology accordingly and to their own advantage.
- For example, *Pseudomonas syringae* pv. *tomato* DC3000 is able to induce the biosynthesis of the hormones auxin and abscisic acid in *Arabidopsis thaliana*.

Tsavkelova *et al.*,2006;..

Major virulence Factors
Hormones

- Hormones can be classified into following three groups on the basis of their chemical structure:

1. Amino acid derivatives e.g. cytokinins are derivatives of adenine.
2. Peptide hormones e.g. glycoproteins
3. Lipid derivatives e.g. steroid hormones.



- Steroid hormones are derived from cholesterol.
- Most hormones are proteins with specific structure.
- Most hormones have certain biochemical trends in common:

1. Small molecules synthesized from ubiquitous precursors (amino acids, mevalonic acid, nucleotides)
2. Sometimes via multi-step pathways, then deactivated by oxidation or conjugation (linking to other small molecules such as glucose and amino acids).

Plants in action;..

Major virulence Factors

Hormones

1. Classical plant/bacterial hormones

- Plant hormones are signal molecules produced within the plant, and occur in extremely low concentrations.
- Many of plant disease symptoms are mediated by altered concentrations of the five 'classical' plant hormones:

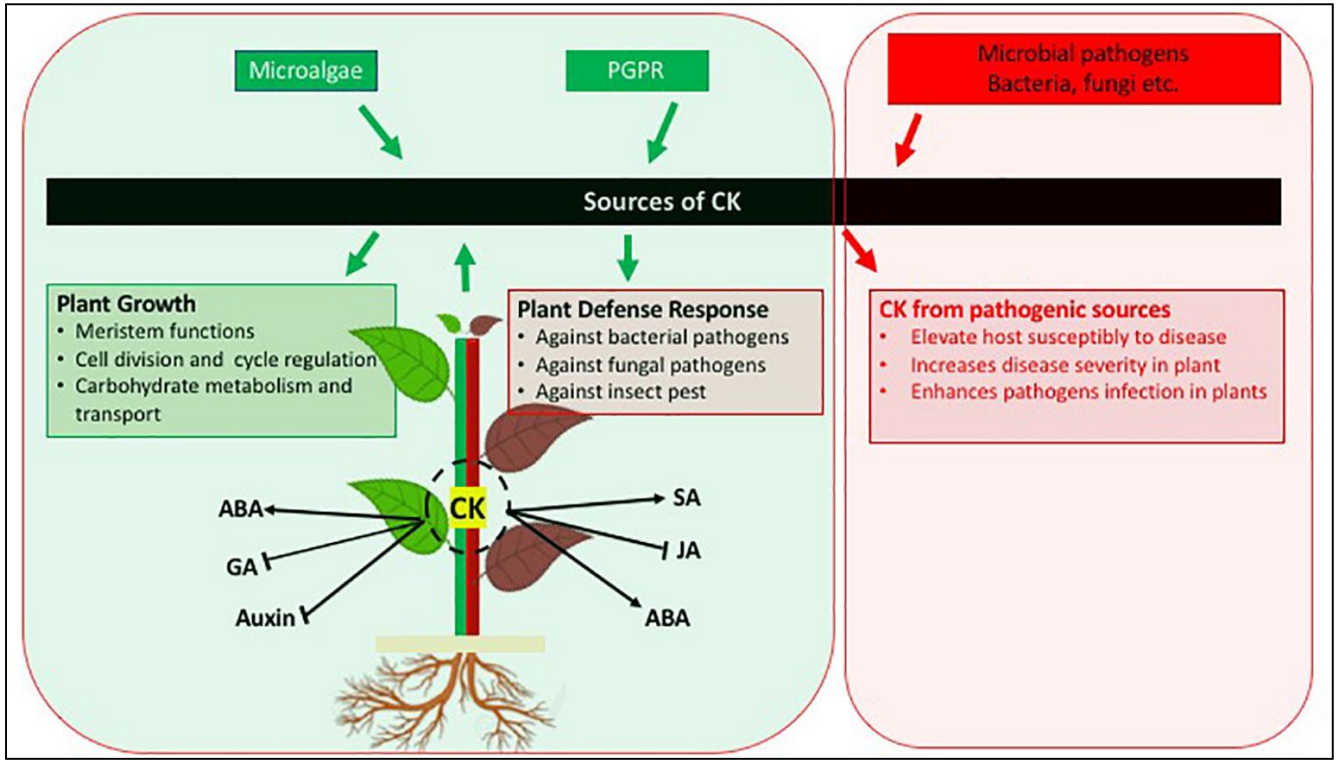
1. Auxins(IAA),
2. Cytokinins(CK),
3. Gibberellins(GA),
4. Ethylene(ETH), and
5. Abscisic acid(ABA).

- Many bacteria are capable of producing more than one type of plant hormone.
- e.g. *A. tumefaciens* induce galls by auxins and cytokinins.

Major virulence Factors

Hormones

1. Classical plant hormones



Schematic diagram indicating the role of cytokinins in plant growth and defence against pathogens. In addition, cross-talk between Cytokinins(CK), and other phytohormones is also presented. Arrows indicate positive interaction; blunt ends indicate negative interaction.

Akhtar *et al.*,2020

Hormones

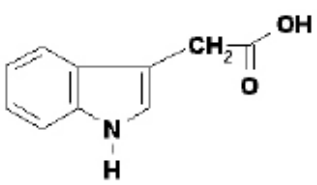
1. Classical bacterial hormones

Bacterial species or pathovar	ß-Indole acetic acid (IAA, auxin)	Cytokinins (CK)	Gibberellin (GA)	Abscisic acid (ABA)	Ethylene (ETH)
A. tumefaciens	+	+	+		+
A. rhizogenes	+	+	+		+
E. carotovora subsp. *carotovora*					+
Pantoea agglomerans (also pvs. *betae, gypsophilae* and *milletiae*)	+				
P. savastanoi pv. *savastanoi*	++	+			
P. savastanoi pv. *fraxini*	- Or W	-			
P. syringae pv. *cannabina*					+
P. syringae pv. *phaseolicola*					+
P. syringae pv. *sesami*					+
Ralstonia solanacearum	+			+	+
Rhizobium spp.	+	+			
Rhodococcus fascians	+	+			
Streptomyces scabiei	+				
Xanthomonas citri	+				
X. vesicatoria					+

Janse,2006

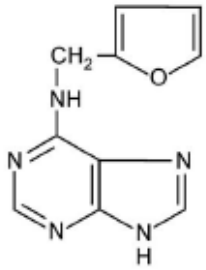
Classical plant hormones

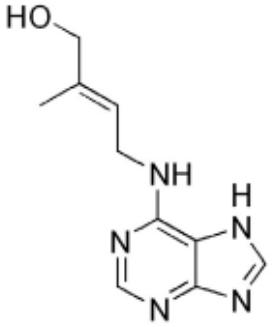
Growth regulators in plant disease



Indole-3-acetic acid (IAA)

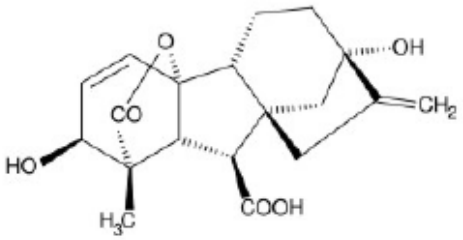
(Auxin)





Kinetin Zeatin

(Cytokinins)



Gibberellic acid

$CH_2 = CH_2$

Ethylene

Auxin production
By plant pathogenic bacteria

- Indole acetic acid (IAA) is the principal auxin of higher plants.
- It is synthesized from tryptophan but three pathways have been proposed via:

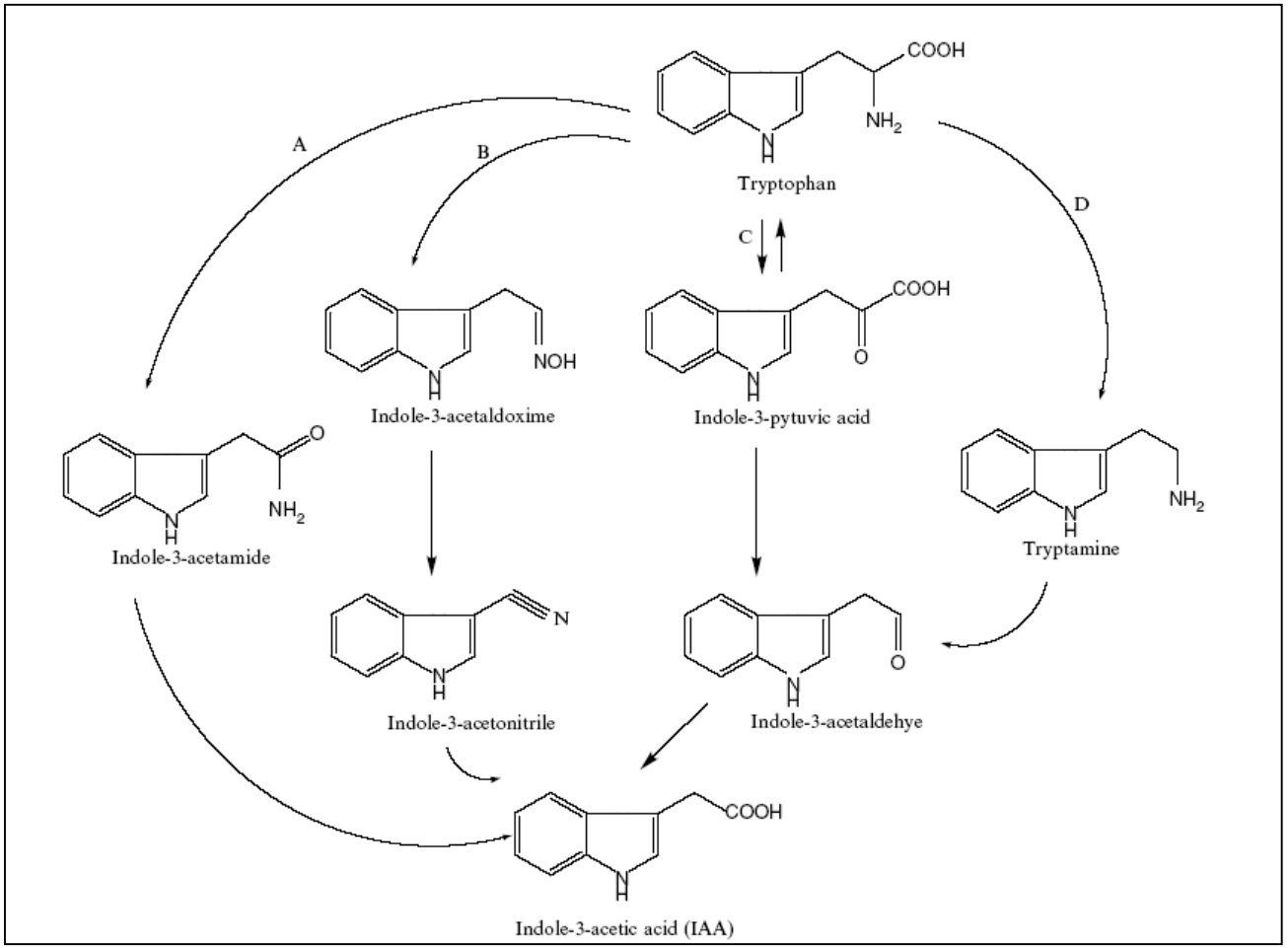
1. Indole-3-pyruvic acid,
2. Tryptamine, or
3. Indole-3-acetonitrile.

- Recently, a tryptophan independent pathway has been described in plants (Taiz and Zeiger,2002).
- Plant pathogenic bacteria such as *Agrobacterium tumefaciens*, however, use a further pathway in which tryptophan is converted to indole-3-acetamide from which IAA is released by the action of indole-3-acetamide hydrolase.

Bacterial pathway vs. plant pathway

Biosynthesis of indole acetic acid

(A) Bacterial pathway; (B), (C) and (D) alternative plant pathways

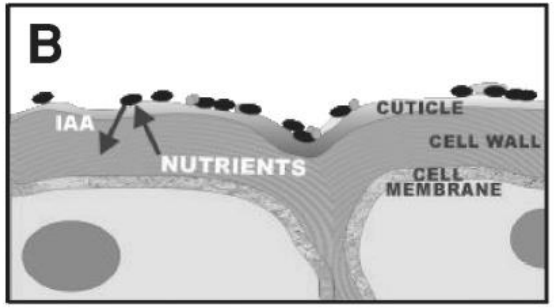


Strange,2003

Biosynthesis of indole acetic acid

By bacterial colonizers of the phyllosphere

- The biosynthesis of the plant growth regulator indole-3-acetic acid (IAA) is widespread among bacterial colonizers of the phyllosphere.
- IAA promotes cell wall loosening at very low concentrations and exogenously applied auxin stimulates the release of saccharides from the plant cell wall.
- Because bacteria on plants are frequently nutrient limited, it was hypothesized that the greater epiphytic fitness of IAA producing strains resulted from enhanced nutrient availability caused by increased leakage of saccharides from plant cells in their vicinity.



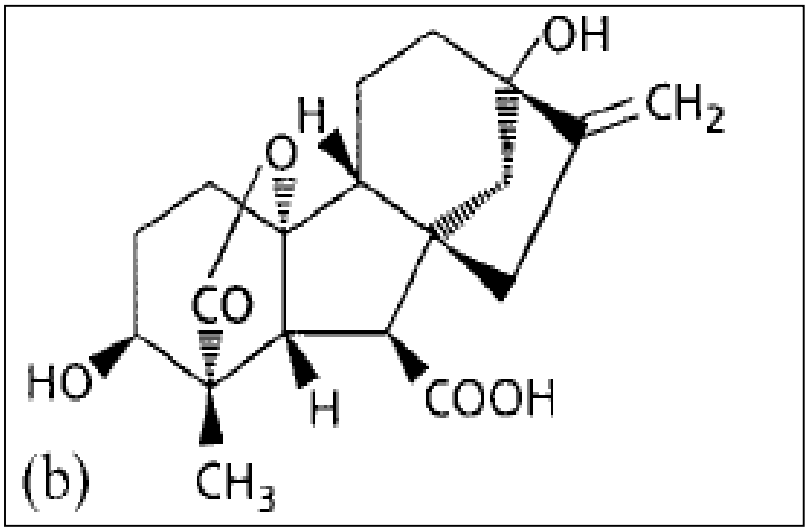
Lindow and Brandl,2003

Cytokinins

- Cytokinins affect many facets of plant metabolism such as:
- Delay of senescence, redirection of nutrients and the proliferation of plant organs.
- They also promote the maturation of chloroplasts and stimulate cell enlargement.
- Their mechanism of action is still unknown although they affect protein synthesis, possibly by stabilizing specific mRNAs.
- Cytokinin-binding proteins have been identified and these are likely to represent the start of one or more signal transduction pathways.

Gibberellins

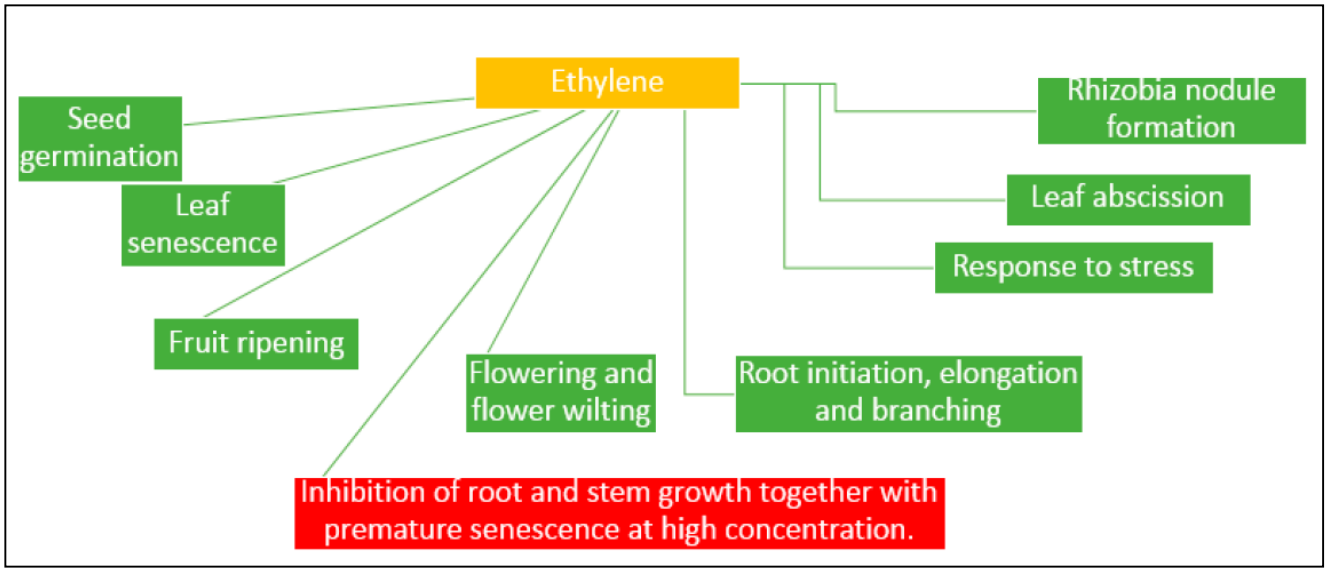
- Gibberellins are a large family of tetracylic diterpenoid growth factors currently numbering well over 100.
- As with other growth regulators, gibberellic acids, have multiple effects on plants.
- However, the one that first attracted attention was the promotion of stem elongation and plant maturity.



Strange,2003

Ethylene
The gaseous hormone

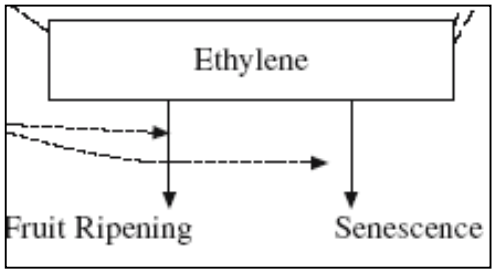
- The phytohormone ethylene affects a large number of different processes in the growth and development of a plant.



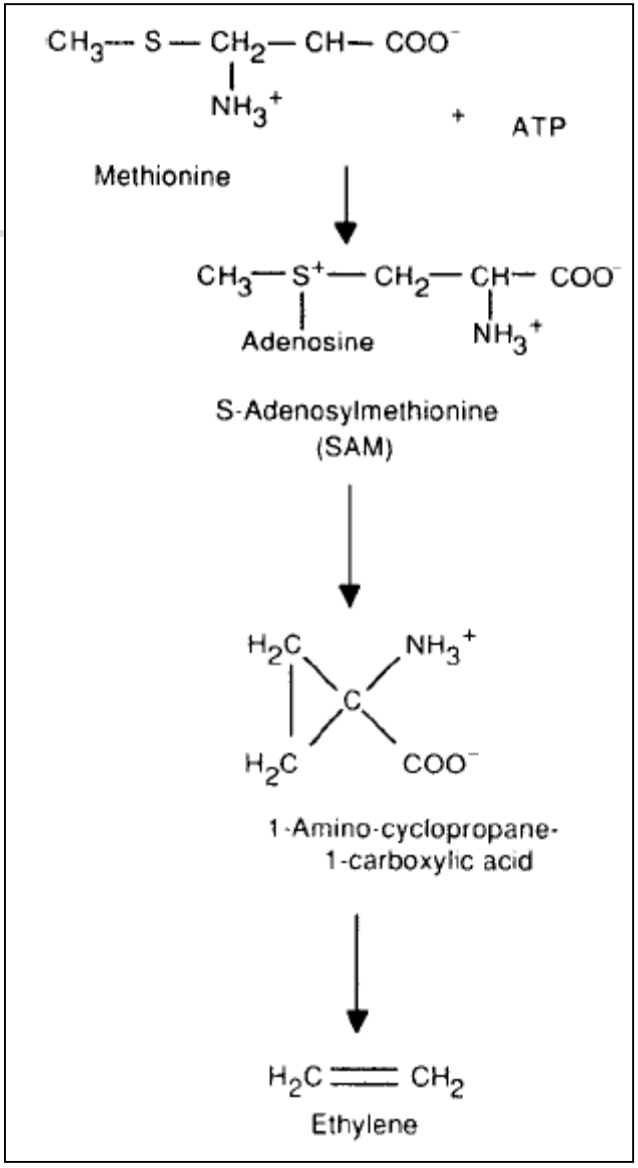
Vejan *et al.*,2016

Ethylene

- Ethylene (= ethene; C_2H_4) is a unique gaseous hormone that diffuses rapidly out of plant tissues.
- Plants synthesize ethylene from methionine via S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid.
- Ethylene treatment cause severe epinasty, enhanced flower senescence, and accelerated fruit ripening.



Strange,2003



Ethylene
The gaseous hormone

- Being a gaseous compound, ethylene may be transported throughout the plant only by diffusion.
- Microorganisms are also capable of synthesizing ethylene.
- The producers include heterotrophic bacteria:
- *Escherichia coli*,
- *Cryptococcus albidus*,
- *Pseudomonas syringae*,
- *Chromobacterium violaceum*, and
- *Ralstonia solanacearum*.

Tsavkelova *et al.*,2006

Ethylene

The gaseous hormone ethylene is another inhibitor of plant growth

- Ethylene slows down growth and cell extension, disrupts geotropism, promotes phylloptosis, and accelerates fruit maturation and ageing.
- Ethylene production is activated in plants under abiotic and biotic stress conditions.

1. Pathogen-derived ethylene acts as a virulence factor of fungal and bacterial pathogens, and
2. Plant-derived ethylene as a signaling compound involves in disease resistance.

- Ethylene can induce certain types of pathogenesis-related proteins or phytoalexins in different types of induced resistance.

Hormones

2. Non-traditional regulators

The hormonal signals

- Phytohormone-like compounds (brassinosteroids, oligosaccharines, salicylic acid, and jasmonic acid) were only discovered at the end of the 20th century.

1. Oligosaccharins, brassinosteroids and jasmonates have been recognized in the healthy plants.
2. Steroid hormones(lipophilic hormones) are very important for physiological and developmental regulation, both in animals and plants.
3. Nitric oxide (NO) serves as signal in hormonal and defense responses.

- All are involved in signal transduction in defence.

Jasmonates

Jasmonate (JA) and its derivatives are lipid-based hormone signals

- JAs are well- recognized lipid-derived stress hormones that regulate plant adaptations to biotic stresses, including herbivore attack and pathogens.
- Jasmonic acid (JA) was first isolated in 1971 as a plant growth inhibitor from culture filtrates of the fungus, *Lasiodiplodia theobromae*, a plant pathogen with a wide host range.
- Jasmonic acid is an organic compound found in several plants including jasmine.
- Jasmonic acid was detected in symbiotic bacteria and cyanobacteria.

Jasmonates

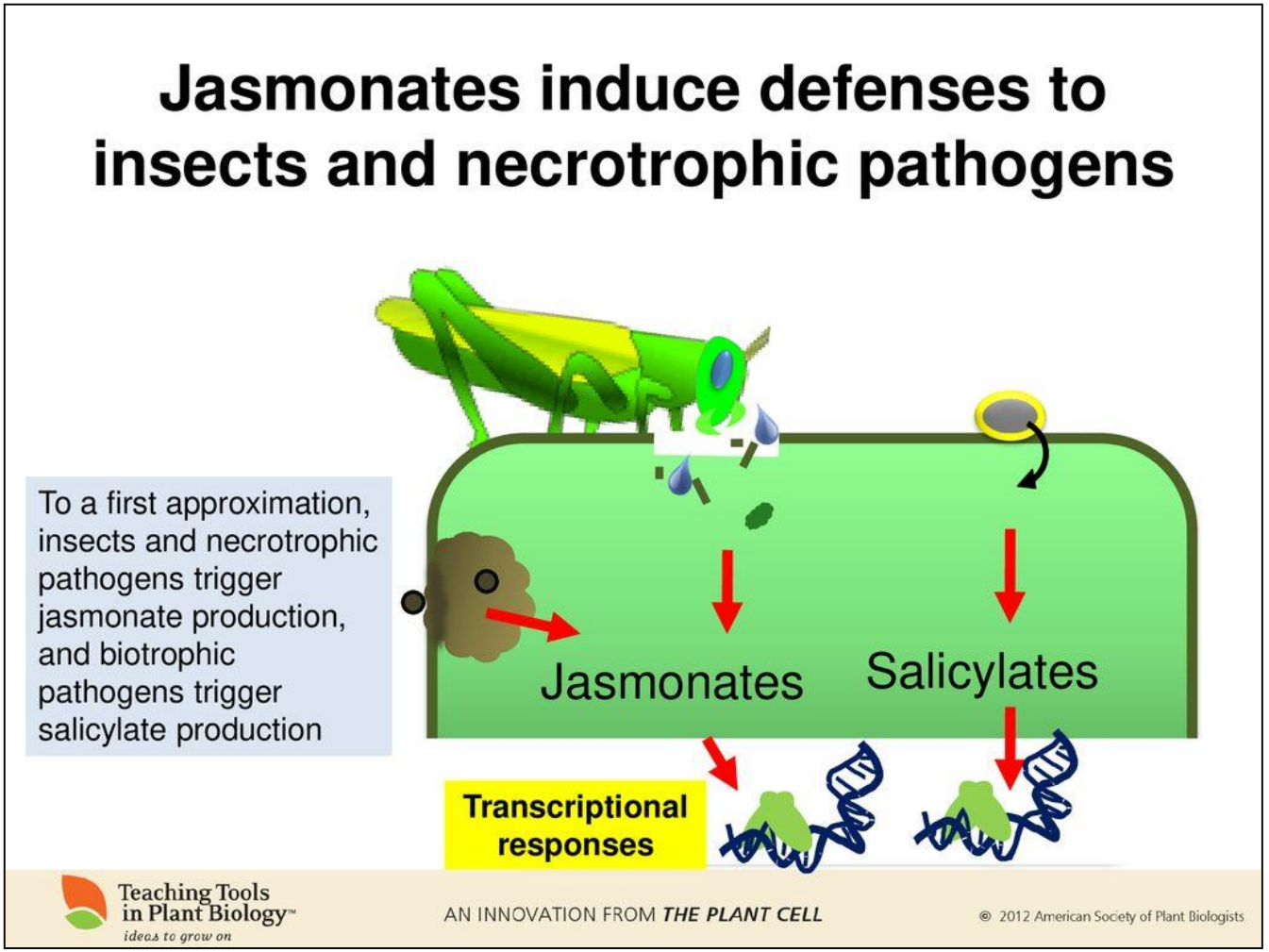
Jasmonate (JA) and its derivatives are lipid-based hormone signals

- Jasmonates affects on:
- Jasmonic acid (a plant immune hormone, fatty acid derivative) and salicylic acid (a type of phenolic acid) play important roles in plant defense systems.
- Jasmonic acid is involved in:

1. Plant cell signaling responses to abiotic and biotic stress.
2. Inhibition of germination of non-dormant seeds and root growth, and
3. The stimulation of the ripening of tomato and apple fruit.

Jasmonates

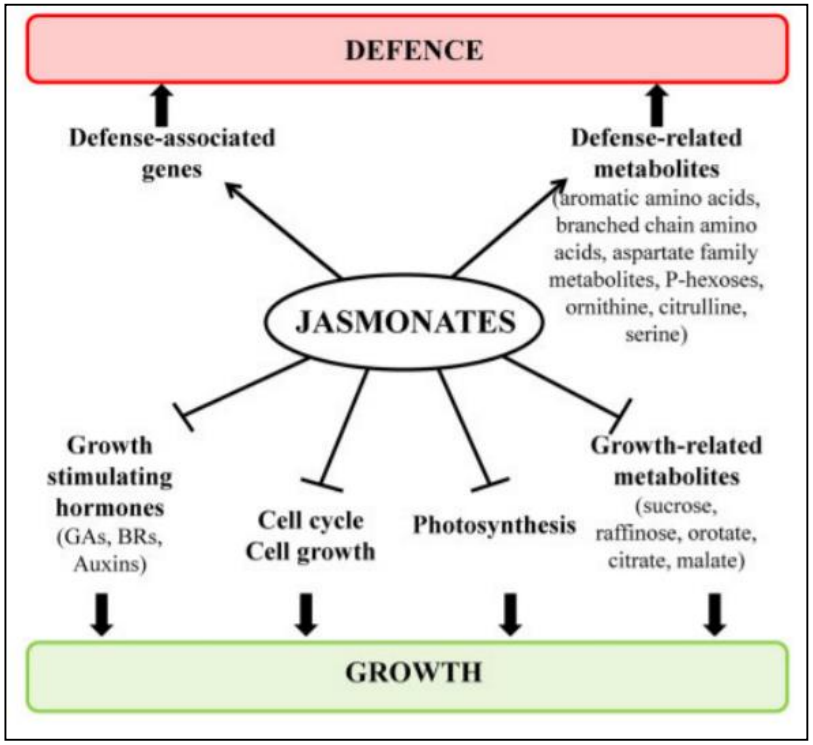
Jasmonate (JA) and its derivatives are lipid-based hormone signals



Jasmonates

Jasmonate (JA) and its derivatives are lipid-based hormone signals

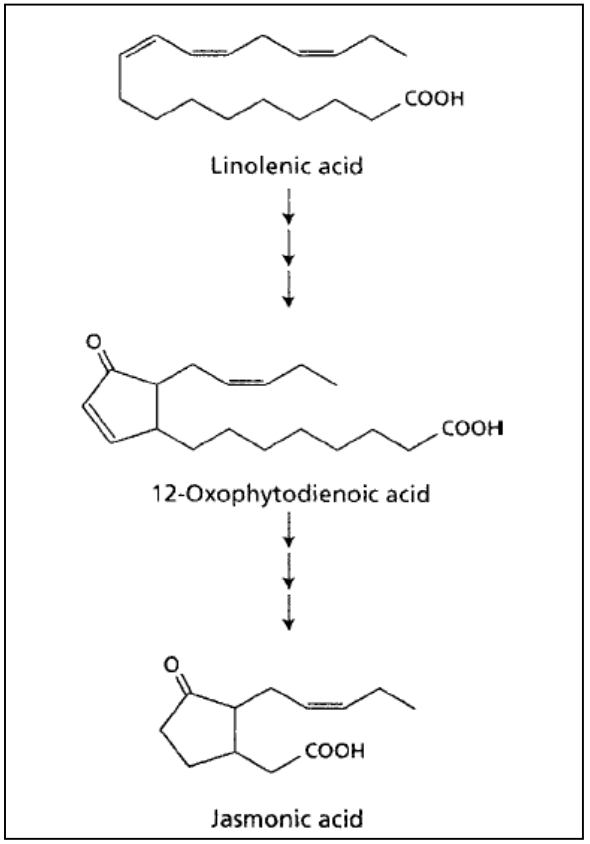
- Simplified model of JAs-mediated shift from 'growth mode' to 'adaptive mode'.



Savchenko *et al.*,2021

Schematic representation of the jasmonate biosynthetic pathway

- Jasmonic acid facilitates the development of symbiotic bacteria.
- Jasmonate activates the expression of *nod* genes in *Bradyrhizobium japonicum* and *Rhizobium leguminosum*;
- Increases in jasmonates may result from the release of linolenic acid(one of two essential fatty acids) from membranes by phospholipases.

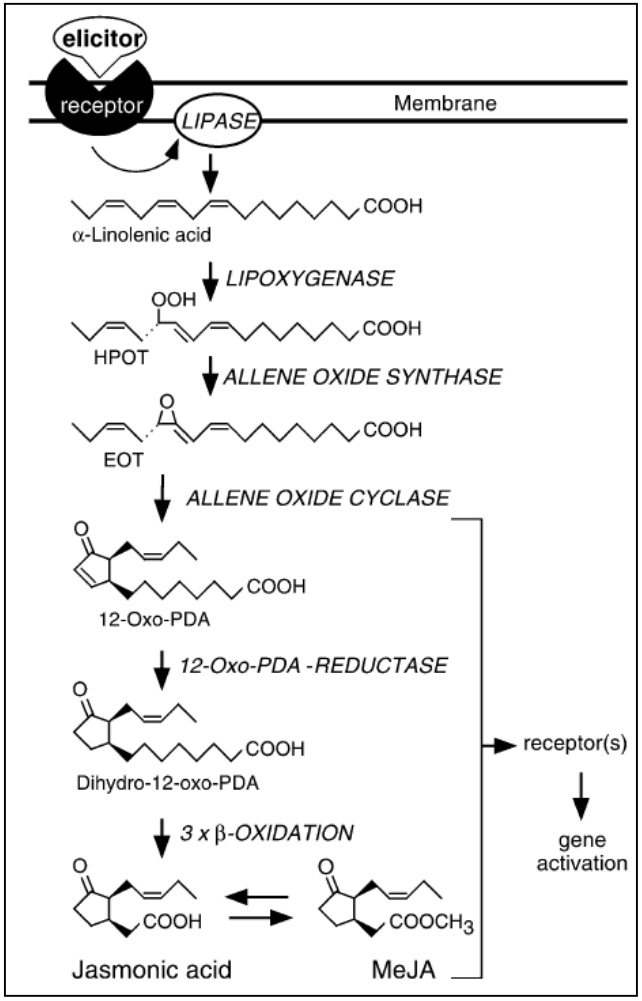


Synthesis of jasmonic acid from linolenic acid.

Strange,2003;..

Schematic representation of the jasmonate biosynthetic pathway

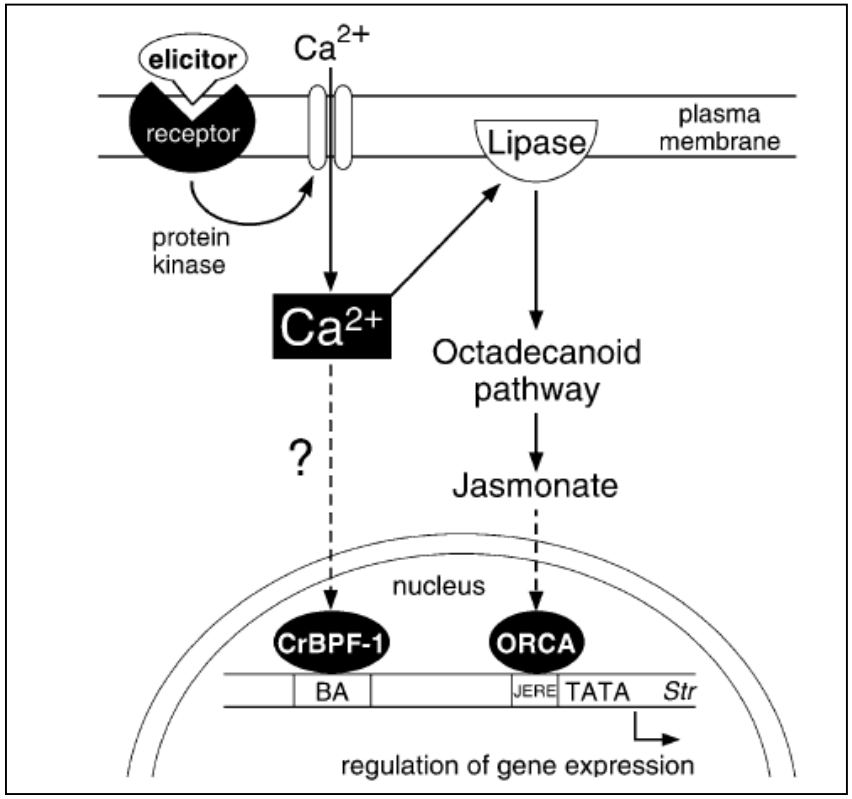
- Details of jasmonate biosynthetic pathway.
- Increase of jasmonates is due to release of linolenic acid from membranes by phospholipases.
- *12-oxo-PDA* 12 Oxophytodienoic acid;
- *HPOT* 13(*S*) hydroperoxyoctadecatrienoic acid;
- *EOT* 12,13(*S*)-epoxyoctadecatrienoic acid;
- *MeJA* methyl jasmonate.



Scheper & Zhong (eds.),2001

Roles of jasmonate in the signaling of plant defense

- Protein phosphorylation and calcium influx are required for elicitor induced jasmonate biosynthesis, as well as for the induction of transcription factors:
- *CrBPF-1*,
- *ORCA2*, and
- *ORCA3*.



Scheper & Zhong (eds.),2001

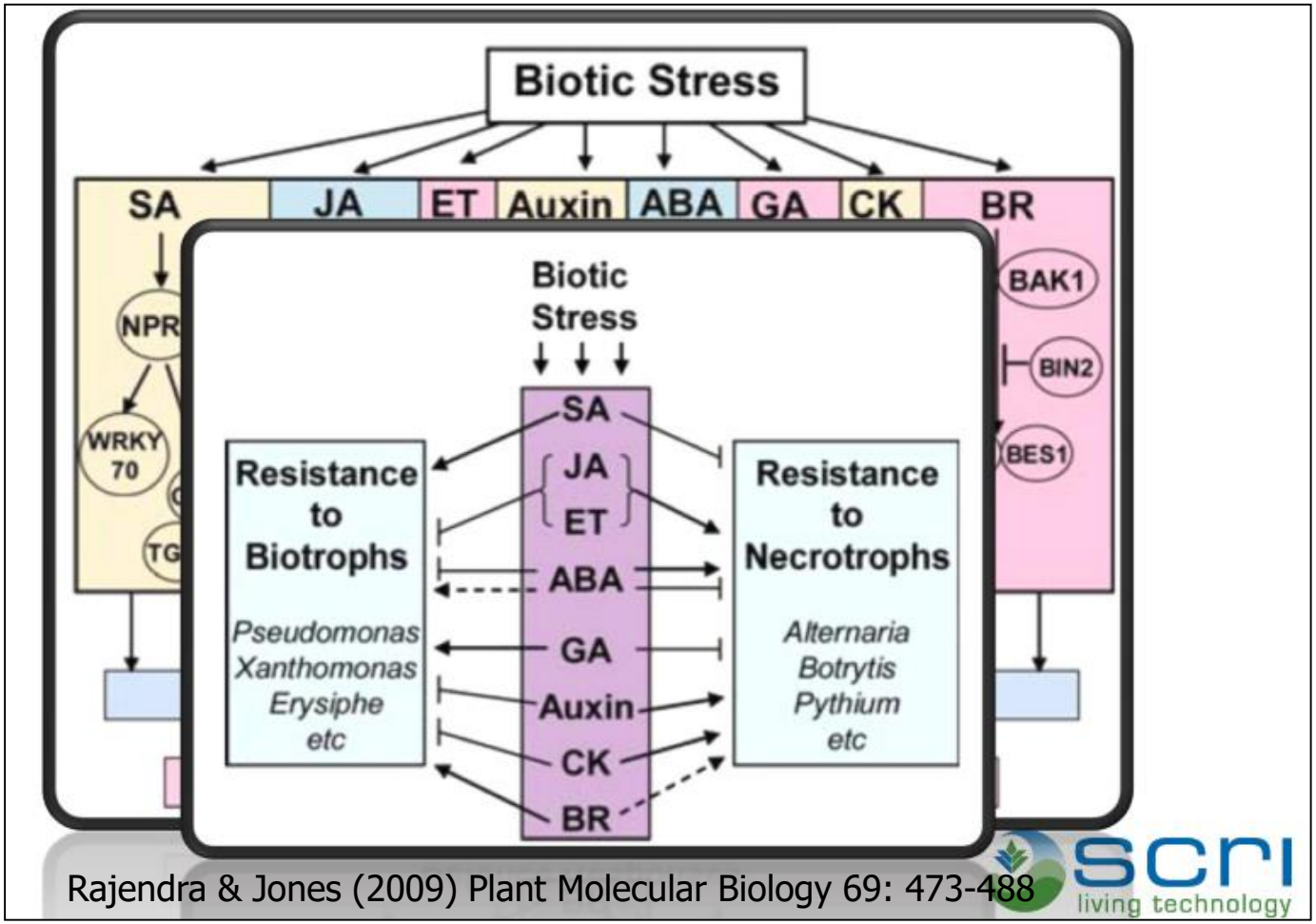
Phosphorylation is a common way of activating (or inactivating) proteins.

Bacterial manipulation of hormone pathways
Pathogen's actions against SAR

- Plant hormones can quickly and potently affect plant physiology; therefore it is not surprising that pathogens manipulate plant hormone signalling to promote disease.
- Recently, it was discovered that plants gain enhanced disease resistance by downregulating auxin levels in response to pathogen challenge.
- Therefore, it is possible that bacterial-derived auxin might function to counter this plant response to suppress plant defences.

Abramovitch *et al.*,2006

Plant Protection
Complex Network of Hormones



Rajendra & Jones (2009) Plant Molecular Biology 69: 473-488

scri living technology

Bacterial manipulation of hormone pathways
Coronatine
Pathogen's actions against SAR

- Coronatine is a molecular mimic of the plant hormone jasmonate.
- Coronatine and jasmonates are believed to stimulate similar responses.
- The SA- and JA-dependent defense pathways can be mutually antagonistic, and some bacterial pathogens take advantage of this to overcome the SAR.
- Coronatine dramatically reprogrammes host gene expression, causing altered expression of hundreds of genes, including the upregulation of genes that are involved in the synthesis of endogenous JA.

Bacterial manipulation of hormone pathways

Coronatine

Pathogen's actions against SAR

- Coronatine-dependent reprogramming of plant gene expression has been shown to induce systemic susceptibility to bacterial pathogens.
- For example, pathogenic strains of *Pseudomonas syringae* produce coronatine, which is similar to JA, to overcome the SA-mediated pathway (He *et al.*,2004).

Quorum sensing (QS)
Autoinduction

Intra and inter-species molecular communications

Species-specific

The GacS/GacA, two-component regulatory system controls regulation of secondary metabolism and many other aspects of bacterial physiology including ecological fitness, QS, pigmentation, motility and tolerance to stress (See also pathogenesis file part II).

Quorum sensing

Controversy

Quorum sensing, diffusion sensing, efficiency sensing

- The release of signal substances is understood to be either a cooperative strategy to determine the cell density (quorum sensing).
- This concept has been challenged.
- The concept of diffusion sensing, is an alternative and complementary model to quorum sensing.
- Diffusion sensing model explains a non-cooperative strategy in which the signal substance is only used to determine the dimensions of the space surrounding the cell (diffusion sensing).
- This third model – called efficiency sensing – combines both theories and first allows an understanding of how bacterial communication works and which purpose it serves.

Quorum sensing

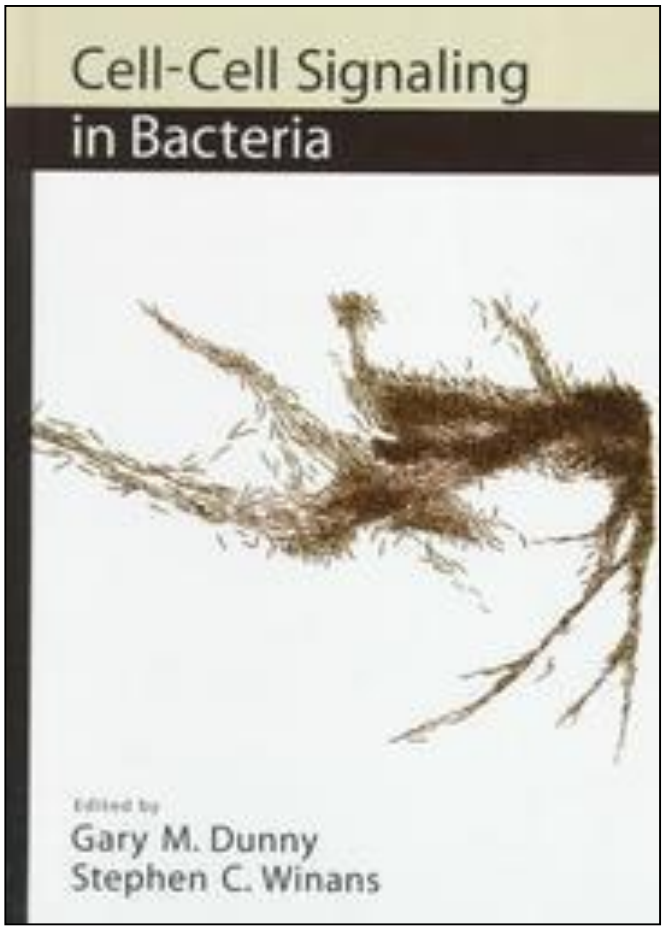
Controversy

Quorum sensing, diffusion sensing, efficiency sensing

- The approach of diffusion sensing is slightly simpler: it is assumed that the bacterium uses the signal substances to measure whether the cell surrounding space is adequate to achieve the concentration of active substances required for efficient action.
- Therefore, the scientists developed a synthesis of the two models, which they named "efficiency sensing":
- Based on efficiency sensing model, the microbes always perceive a mixture of cell density, cell distribution and diffusion limitation due to spatial conditions, because these aspects cannot be strictly separated – it depends on the circumstances and habitat quality which aspect is predominant.

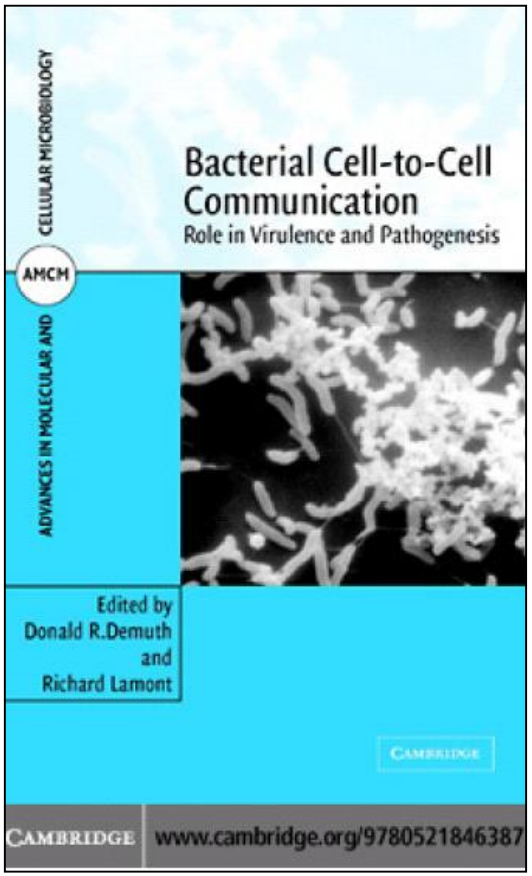
Cell-Cell Signaling in Bacteria

- **Cell-Cell Signaling in Bacteria**
- by Gary M. Dunny and Stephen C. Winans
- Publisher: Amer. Society for Microbiology
- 1999
- 367 pages.



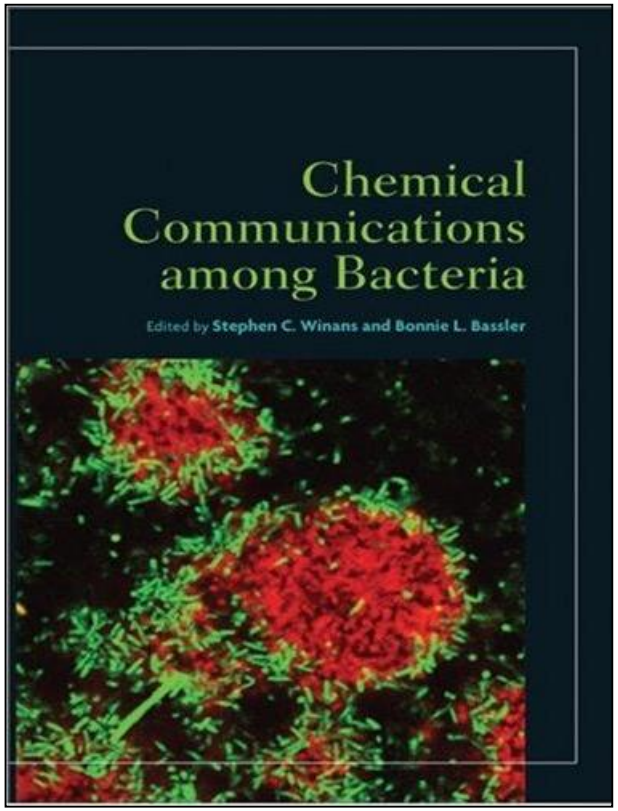
Bacterial Cell-to-Cell Communication
Role in Virulence and Pathogenesis

- **Bacterial Cell-to-Cell Communication-Role in Virulence and Pathogenesis**
- Donald R. Demuth and Richard J. Lamont
- Cambridge University Press
- 2006
- 338 pp.



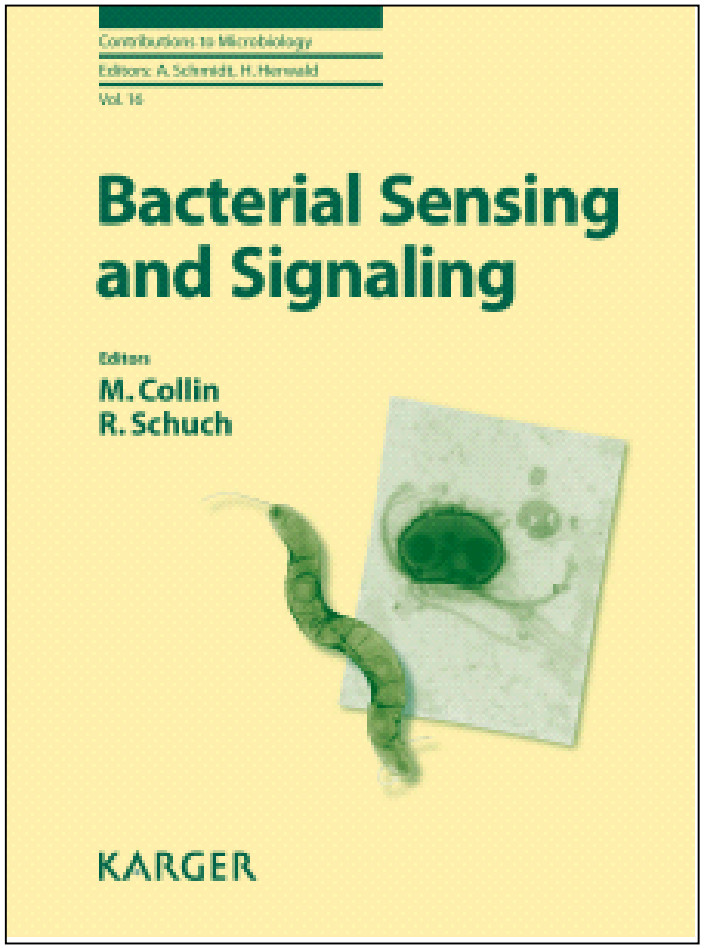
Chemical Communication among Bacteria

- **Chemical Communication among Bacteria**
- by Stephen C. Winans and Bonnie L. Bassler
- Publisher: ASM Press
- 2008
- 483 pages.



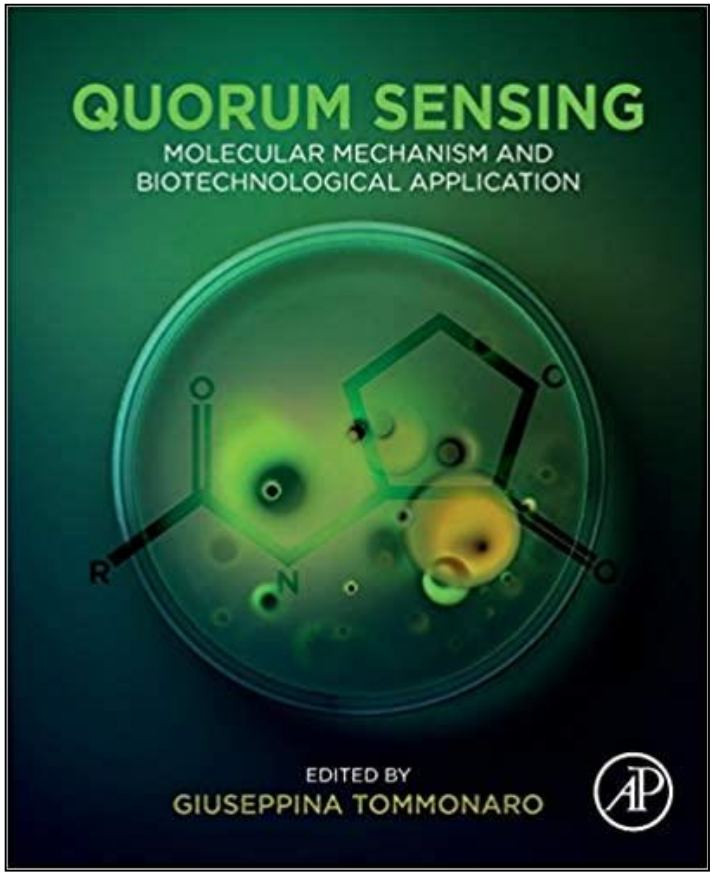
Bacterial Sensing and Signaling

- **Bacterial Sensing and Signaling**
- By Mattias Collin and Raymond Schuch
- by S. Karger AG (Switzerland)
- 2009
- 230 pp.



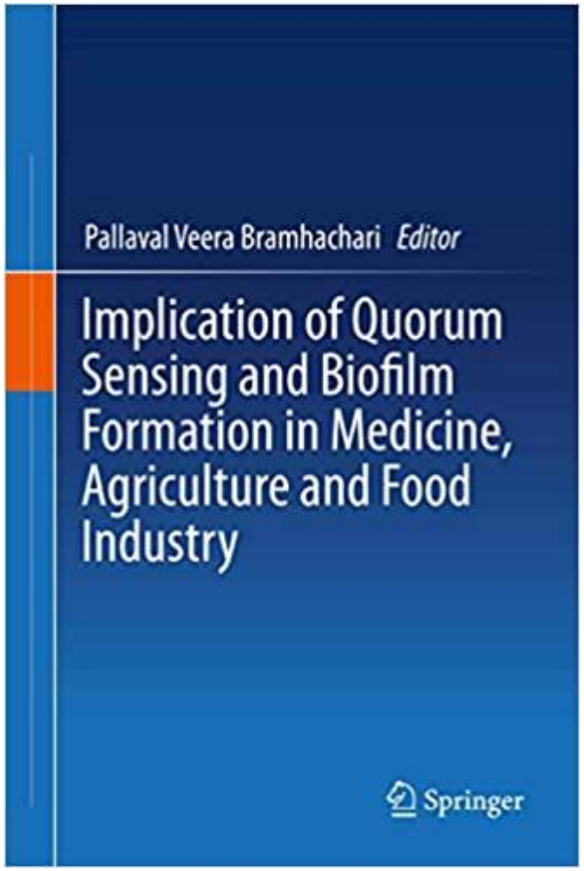
Quorum Sensing:
Molecular Mechanism and Biotechnological Application

- **Quorum Sensing: Molecular Mechanism and Biotechnological Application**
- by by Giuseppina Tommonaro (Editor)
- Academic Press
- 2019
- 309 pages.



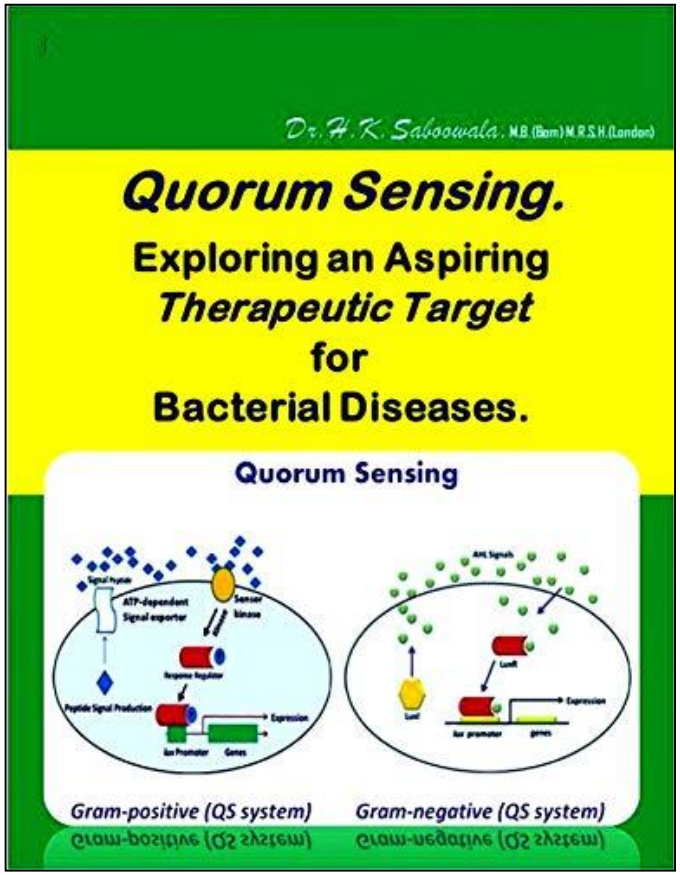
Implication of Quorum Sensing and Biofilm Formation in Medicine, Agriculture and Food Industry

- **Implication of Quorum Sensing and Biofilm Formation in Medicine, Agriculture and Food Industry**
- by Pallaval Veera Bramhachari
- Springer
- 2019
- 346 pp.



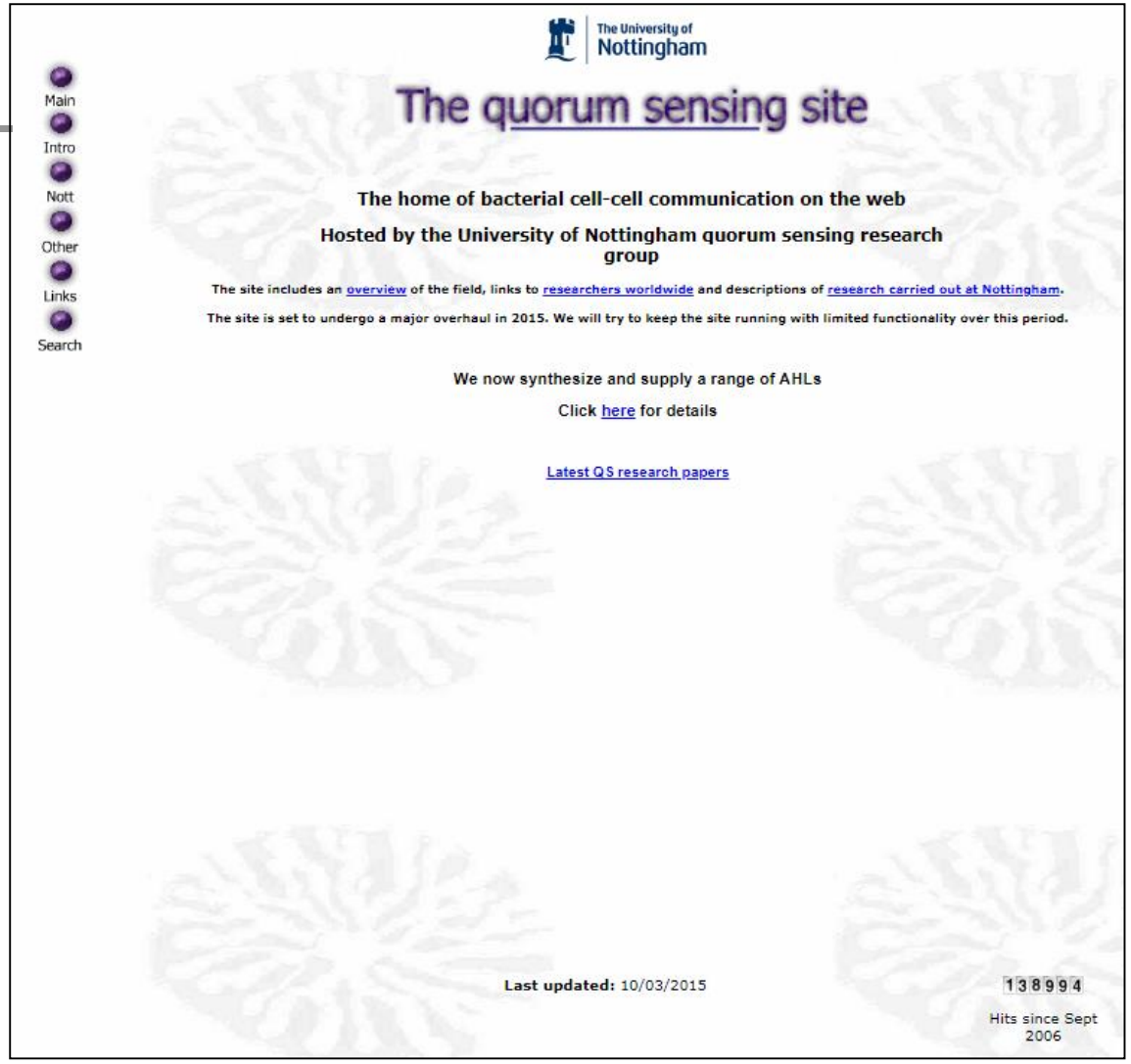
Quorum Sensing: Exploring an Aspiring Therapeutic Target for Bacterial Diseases

- Quorum Sensing: Exploring an Aspiring Therapeutic Target for Bacterial Diseases
- by Hakim. Saboowala
  ASIN: B08D7972WN
- 2020
- 58 pp.



The quorum sensing website

This web site is included in the ISI current web contents.

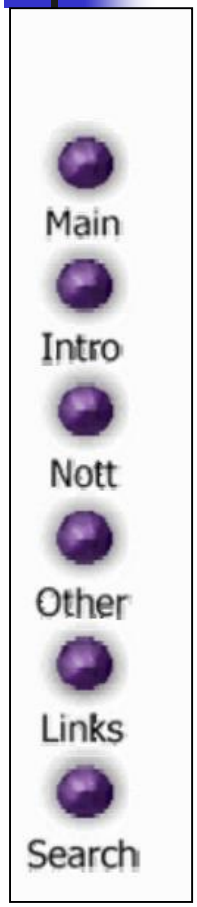


Last updated: 10/03/2015

The quorum sensing site

Links Categories

A list of synthesized AHLs



- **Links Categories**
- University of Nottingham
- Other universities
- Molecular biology
- Genome project
- Microbiology
- Scientific Societies
- Journals
- Search pages

Below is a list of the AHLs which we synthesize and supply:
These chemicals can be purchased from Sigma-Aldrich-Fluka
To place an order please download and complete this form and email it to:
Darren Furniss (darren.furniss@nottingham.ac.uk) to whom you should address all other enquiries.

Another quorum sensing website

Quorumpeps database

A resource of quorum sensing signaling peptides

Quorumpeps®

Quorumpeps® is a resource of quorum sensing signalling peptides. This database is managed by Ghent University. Based upon your input, this search page will give you all information (structure, activity, physicochemical properties and related literature). The database is linked to a manuscript entitled "Quorumpeps database: chemical space, microbial origin and functionality of quorum sensing peptides", in which the origin of the different peptides and their quorum sensing pathways and methods are described.

Reference: BibTeX

Sequence, Trivial name, SMILES, Molecular formula, Receptor, Method, Origin, Literature

Peptide
Sequence, Trivial name,
SMILES, Molecular formula
ERGMT
PhrC
O=C(NC(C(=O)NC(C(=O)O)C(O)C)CCSC)CNC(=O)C(NC(=O)...
C22H41N8O9S1

Receptor
AgrC

Method
Class, Name
Biosensor
Beta-galactosidase

Origin
Species
Staphylococcus aureus

Literature
Author
Title
Year
Journal (full)

Search

Citation

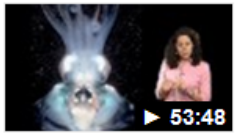
If you are using or downloading data from the database, please cite "Wynendaele E., Bronselaer A., Nielandt J., D'Hondt M., Stalmans S., Bracke N., Verbeke F., Van De Wiele C., De Tré G. and De Spiegeleer B. *Quorumpeps database: chemical space, microbial origin and functionality of quorum sensing peptides*. Nucleic Acids Research 2013, 41 (D1), D655-D659, http://dx.doi.org/10.1093/nar/gks1137"

Bacterial quorum sensing

Videos of Bonnie Bassler talk on how bacteria communicate hosted by TED(Technology, Entertainment, Design)

Bonnie Bassler: How bacteria "talk" | **Video** on TED.com
www.ted.com/.../**bonnie_bassler**_on_how_bacteria_communicate.ht...
TED Talks **Bonnie Bassler** discovered that bacteria "talk" to each other, using a chemical language **...** We have a fancy name for this: we call it **quorum sensing**.

Bonnie Bassler (Princeton) Part 1: Bacterial Communication via **...**



www.youtube.com/watch?v=saWSxLU0ME8
Mar 27, 2010 - Uploaded by ibioseminars
You need Adobe Flash Player to watch this **video**. **...** This process is called **quorum sensing** and it enables **...**

Bonnie Bassler: The secret, social lives of bacteria - YouTube

www.youtube.com/watch?v=TVfmUfr8VPA
Apr 8, 2009 - Uploaded by TEDtalksDirector
http://www.ted.com **Bonnie Bassler** discovered that bacteria "talk" to each other, using **...** You need Adobe **...**

Bonnie Bassler Part 1: Bacterial Communication via **Quorum ...**

www.youtube.com/watch?v=qNkXDlSo4ZI
Sep 10, 2011 - Uploaded by ibioseminars
Alert icon. You need Adobe Flash Player to watch this **video**. **....** **Bonnie Bassler** Part 2: Vibrio Cholerae **...**

Bonnie Bassler Discovers **Quorum Sensing** - YouTube

www.youtube.com/watch?v=1NoxOs-hcRU
Aug 10, 2008 - Uploaded by bigthink
Bonnie Bassler Discovers **Quorum Sensing**. bigthink·9,202 **videos**. Subscribe Subscribed Unsubscribe 301 **...**

Small Talk: Cell-to-Cell Communication in Bacteria

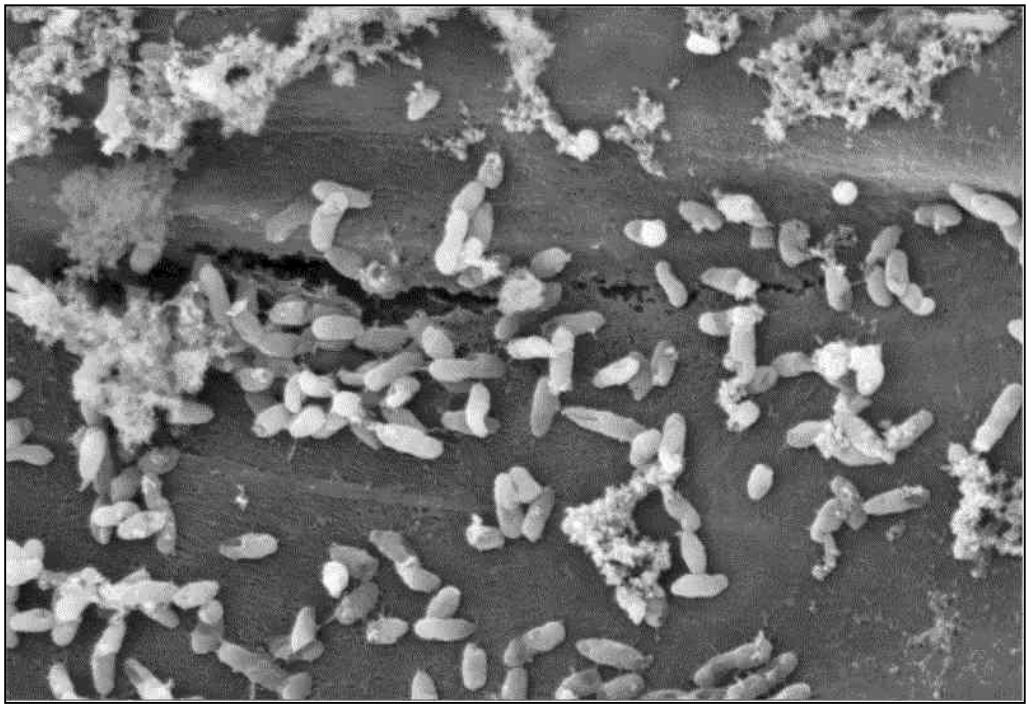
Bacteria make sense

- Bacteria were considered to be lonely 'mutes' for hundreds of years.
- However, recently it was found that bacteria usually coordinate their behaviors at the population level by producing (speaking), sensing (listening), and responding to small signal molecules.
- This so-called quorum sensing (QS) regulation enables bacteria to live in a 'society' with cell-cell communication and controls many important bacterial behaviors.

Yong and Zhong,2013

Small Talk: Cell-to-Cell Communication in Bacteria

Bacteria make sense

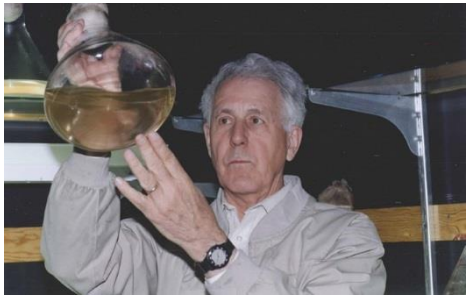


Research into AHL based quorum sensing started in the late 1960s (almost five decades ago).

Microbes seem to talk, listen and collaborate with one another.

Bassler,2002; Hollowaym,2006; Yeo *et al.*,2021

Quorum sensing
The background

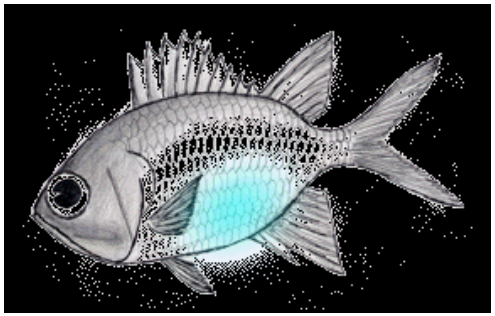

Woody Hastings (1927–2014)

- The study of quorum sensing(bacterial signaling) has its roots in the late 1960s.
- Two scientists - John Woody Hastings and Kenneth H Nealson discovered that a marine bacterium, *Vibrio fischeri*, produced light when its population reached a critical (late- log phase of growth).
- When fewer were present, the bacteria didn't bioluminesce.
- Therefore chemical communication first emerged from investigations into marine bacteria able to grow in the dark.

Quorum sensing
The background

- The two researchers speculated that the bacteria released a signal - something they called an autoinducer - that cried out:

  "We are here! We are here! We are here!"
- When the cacophony became loud enough, the assemblage glowed.
- In 1983, Michael R. Silverman, then at the Agouron Institute in La Jolla, California, and a colleague identified the genes for *V. fischeri*'s autoinducer and its receptor.



Bioluminescent bacteria

Bioluminescent bacteria and their role as light organs in the flashlight fish

- There are bacteria living in the oceans that actually produce light!
- These microbes are easily found in seawater, marine sediments, in the guts of marine animals, and on the surface of decomposing fish.
- Bioluminescent bacteria can be divided into two genres(class):

1. Vibrio, and
2. Photobacteria.

- The most common three are *Vibrio fischeri*, *Vibrio harveyi*, and *Photobacterium phosphoreum*.
- The enzyme responsible for the light generation of these bacteria is the enzyme called luciferase.

Bioluminescent bacteria

Bioluminescent bacteria and their role as light organs in the flashlight fish

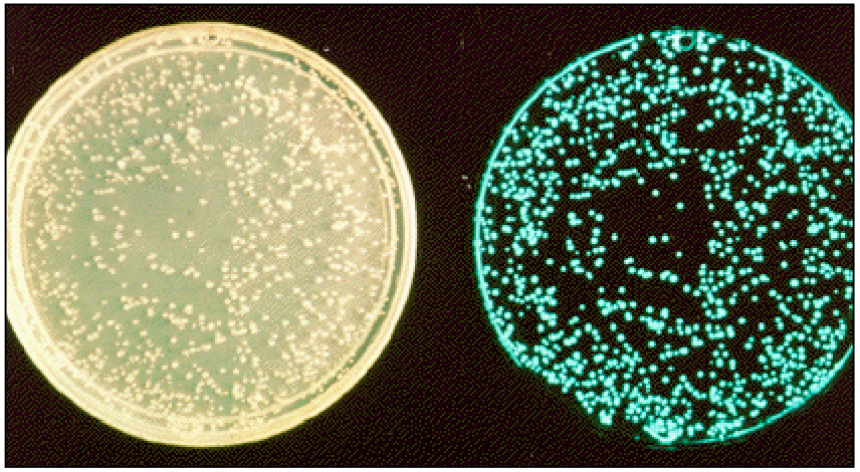
- Pinecone fish utilize luminous bacteria, colonized in the ventral cavity, to:

1. Illuminate the surroundings as well as for
2. Intra-species communication.

Bioluminescent bacteria

Bioluminescent bacteria and their role as light organs in the flashlight fish

- The picture at left shows how bioluminescent bacterial colonies appear on a petri plate under normal light.
- The image on the right is what you will see when you look at the same plate of bacteria in a dark room.



Light is only produced when the organisms are present at high cell densities.

Quorum sensing

The density-dependent cell-cell communication

- Bacteria live in colonies.
- Bacteria do not always act as single cells but have the ability to act as a population analogous to a multicellular organism.
- Quorum sensing (QS) is the phenomenon which allows single bacterial cells to measure the concentration of bacterial signal molecules.
- QS enables bacteria to co-ordinate their behaviour.
- This form of signal dependent communication is present in:

1. Gram-negative
2. Gram-positive bacteria
3. Fungi.

Quorum sensing

Chemical communication signals

The fundamental biological process

- The autoinducer diffusible signal molecules mainly acyl-homoserine lactones(AHLs) are used by a diverse range of microorganisms such as:

1. Opportunistic pathogens such as *Pseudomonas* species;
2. Phytopathogens like *Erwinia* species;
3. Specific symbiotic strains as e.g. *Rhizobium* species;
4. Certain plankton phototrophic bacteria;
5. Signaling molecules can be sensed by fungi (*Candida albicans*; dimorphic; filamentous fungi);
6. AHLs also found in algal blooms.
7. Similarly, social insects like bees and ants use QS to communicate and determine a suitable place to build their nest.

Bacteria sophisticated communication systems

Why do bacteria talk to each other?

- Bacteria utilize a diverse range of signals, including:

1. Their nutritional status, and
2. Population density, to sense and respond to their biotic environment.

- As environmental conditions often change rapidly, bacteria need to respond quickly in order to survive.
- These responses include:

1. Adaptation to availability of nutrients,
2. Defence against other microorganisms which may compete for the same nutrients and the avoidance of toxic compounds potentially dangerous for the bacteria.

Quorum sensing

Bacteria sophisticated communication systems

An important global gene regulatory mechanism

- QS is a beautiful invention because, by talking and listening to each other, the separate bacterial cells in a local population can co-operate with each other.
- In many species QS modulates virulence functions and is important for pathogenesis in several enteric diseases.
- Autoinducers play a critical role in triggering virulence gene expression in QS-dependent pathogens, such as in the production of rotting enzyme.
- QS mutants are defective in virulence and infectivity/stability.

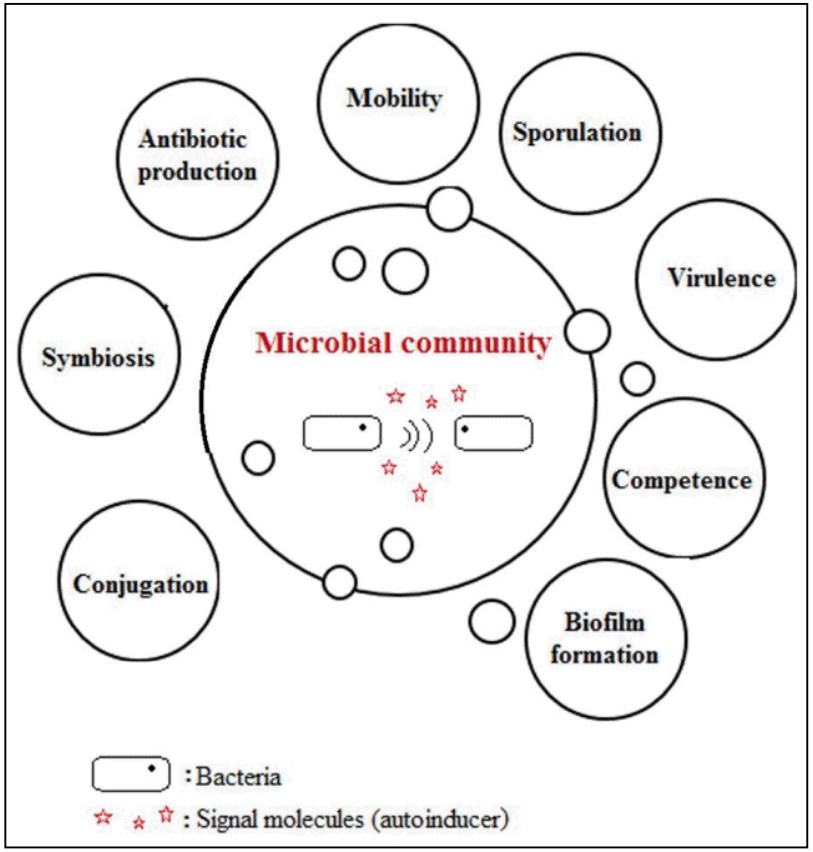
Robinson *et al.*,2003;Chen *et al.*,2013

Quorum sensing

Bacteria sophisticated communication systems

An important global gene regulatory mechanism

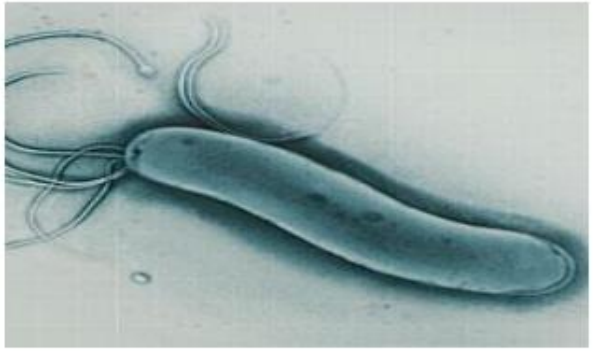
- Quorum sensing through production and diffusion of secreted signal molecules (autoinducers).
- QS coordinates:
- motility, stress responses, mating, nutrient acquisition, antibiotic production, exoenzyme secretion, and biofilm formation.



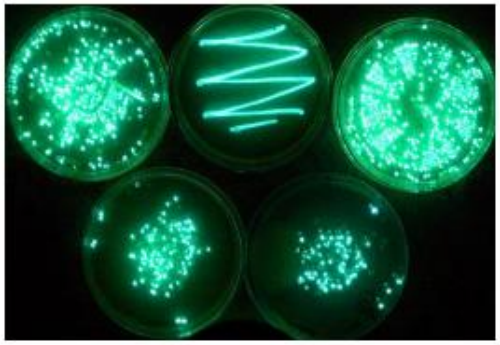
Köse-Mutlu *et al.*,2019; Rosales and Tellez,2021

Quorum sensing

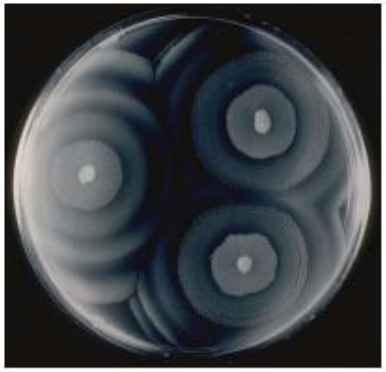
Some other functions



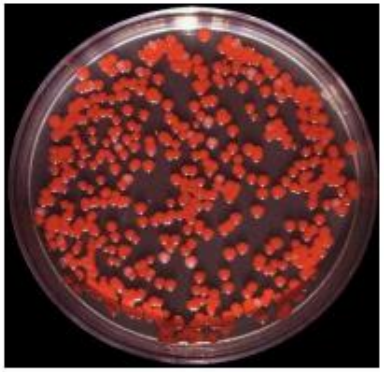
Virulence



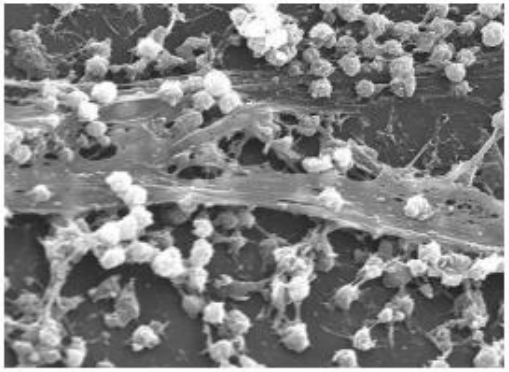
Bioluminiscence



Swarming



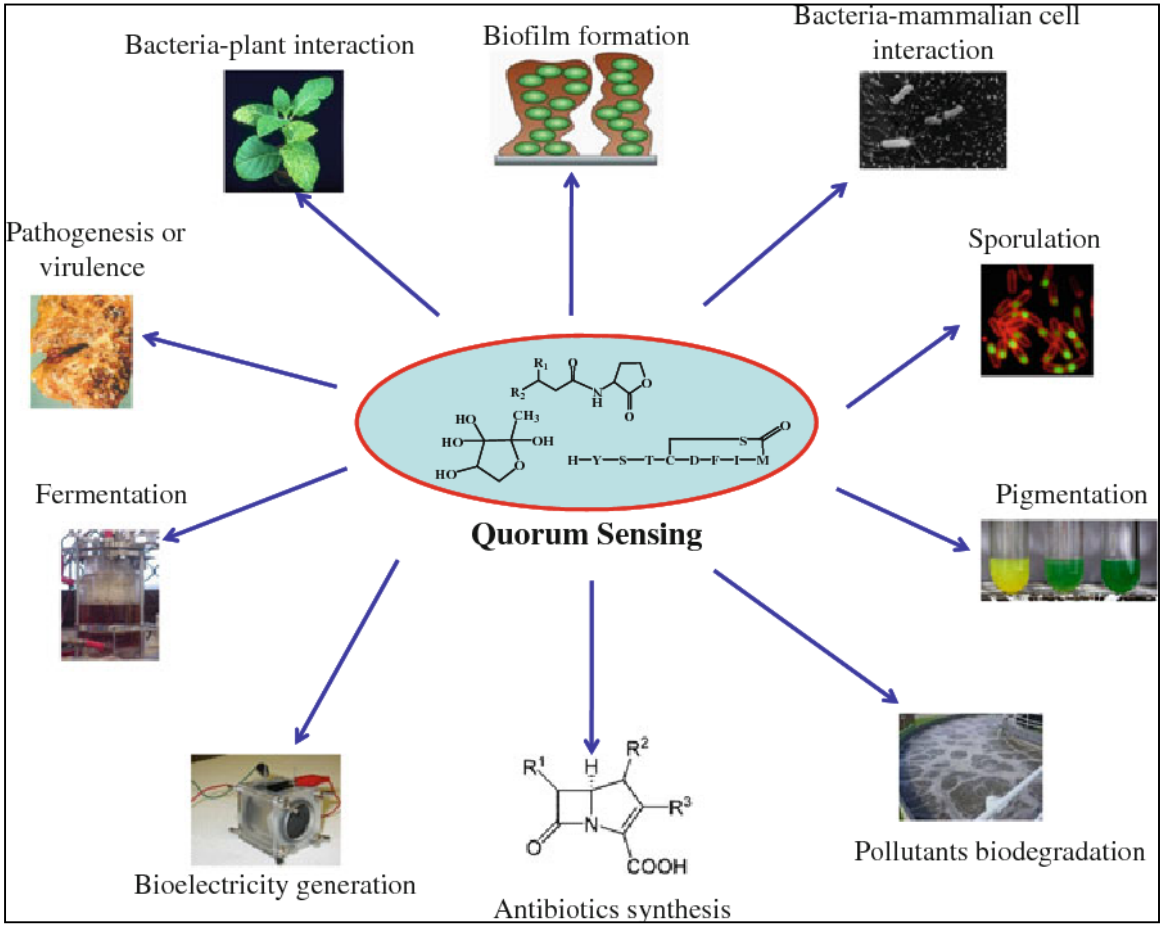
Pigments



Biofilms

Quorum sensing

Impacts of quorum sensing on microbial metabolism and human health

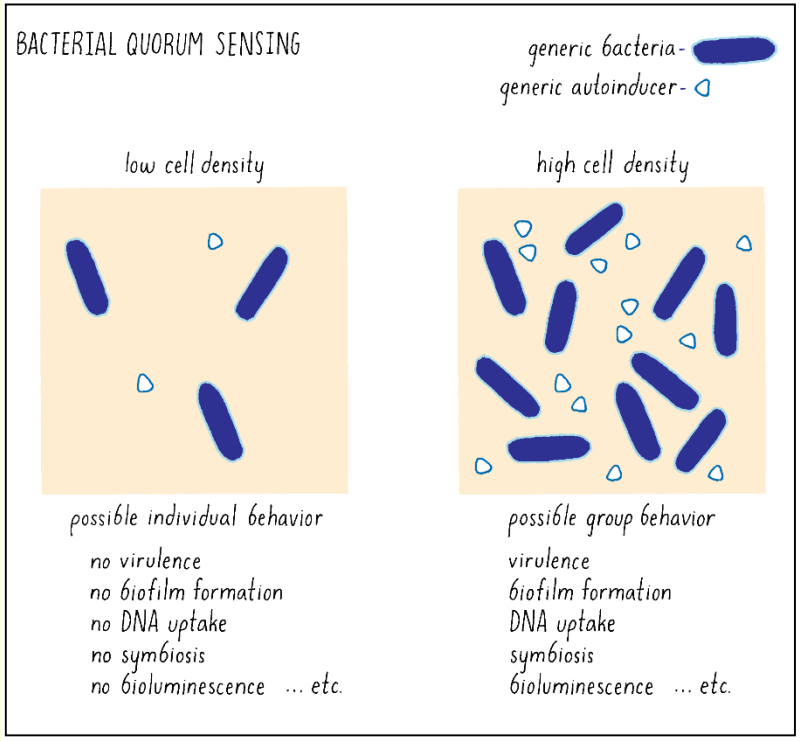


Yong and Zhong,2013

Quorum sensing

Quorum sensing controls many bacterial behaviors at low and high cell density

- Quorum sensing controls the production of toxins that are crucial for bacteria to cause disease.
- Quorum sensing controls biofilm formation.
- Biofilms help many bacteria to act as pathogens and cause disease.
- Quorum sensing controls competence, which is the process that enables bacteria to acquire DNA from other cells.



Quorum sensing
Factors affecting signalling process

- Thus, the signaling process is directly influenced by:

1. Abiotic factors (such as pH, temperature and medium composition), and
2. Biotic factors (such as other members of the bacterial community).

- These can modulate signal genesis, diffusion, interception and degradation and that can produce parasitic signals.

Boyer and Dye',2009

Quorum sensing

Bacterial communication systems

Autoinducer signals

- Bacterial communication systems include:

1. Long- and short-range chemical signaling channels;
2. One-way, two-way, and multi-way communication;
3. Contact mediated and contact-inhibited signaling; and
4. The use and spread of misinformation, or
5. More dramatically, even deadly information.

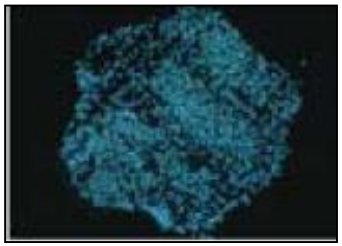
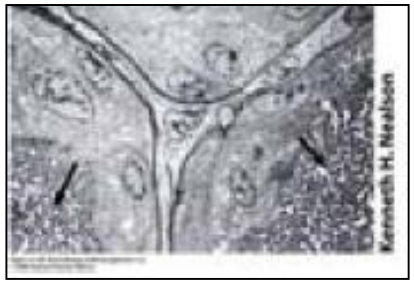
Even without physical contact, bacteria living at the same community likely secrete small extra-cellular molecules to interact with each other.

Bassler and Losick,2006

Bioluminescent bacteria
Making Seawater Complete (SWC) media

- Materials: natural or artificial seawater (you can get instant ocean salt mix from any pet store that sells marine fish), tap water, agar, yeast extract, peptone, glycerol, 20 sterile petri plates, container suitable to boil 1 liter of liquid, heat source to boil water SWC agar recipe:
- 1 L water
- 24 g sea salt
- 5 g peptone
- 3 g yeast extract
- 3 ml glycerol
- 15 g agar (if making solid media)

Bioluminescent bacteria and their role as light organs in the flashlight fish.

	
(b) Colonies of *Photobacterium phosphoreum* photographed by their own light.	The flashlight fish *Photoblepharon palpebratus*; the bright area is the light organ containing bioluminescent bacteria.
	
Underwater photograph taken at night of *Photobacterium palpebratus* in coral reefs in the Gulf of Eilat.	Electron micrograph of a thin section through the light-emitting organ of *Photobacterium palpebratus*, showing the dense array of bioluminescent bacteria (arrows).

Diversity_1.pdf

Autoinducing signals

The languages by which bacteria communicate take the form of chemical signals

- In the natural environment, there are many different bacteria living together which use various classes of signalling molecules.
- As they employ different languages they cannot necessarily talk to all other bacteria.

1. Phylogenetically unrelated bacteria produce the same AHL;
2. Other bacteria form more than one AHL.

- How these compounds inter-play in natural habitats is still completely unknown.

Quorum sensing
Autoinducing signals

- QS has not only been described:

1. Between cells of the same species (intraspecies),
2. Between species (interspecies), and
3. Between bacteria and higher organisms (inter-kingdom).

Diggle *et al.*,2009

Quorum sensing
Bacteria and eukaryotes communications
Interkingdom signaling

- It is also evident that QS is not limited to the bacterial kingdom.
- They are capable of listening in and broadcasting to unrelated species, either for the good of the population or for the benefit of one species over another.
- The study of two-way intercellular signalling networks between bacteria and both uni- and multicellular eukaryotes as well as between eukaryotes is just beginning to unveil a rich diversity of communication pathways.

Atkinson and Williams,2009

Quorum sensing
Bacteria-nematode communications
Interkingdom signaling

- *Caenorhabditis elegans* is a free-living terrestrial nematode that feeds on bacteria in its environment.
- The nematode *Caenorhabditis elegans* is also capable of adapting its behaviour in the presence of AHLs.
- Recent data show that *C. elegans* uses odors produced by bacteria to identify food sources.
- *C. elegans* could sense the acylated homoserine lactone (AHSL) autoinducers produced by many gram-negative bacteria possessing quorum-sensing (QS) systems.

Beale *et al.*,2006; Niu *et al.*,2010

Quorum sensing
Bacteria-nematode communications
Interkingdom signaling

- The nematode *Caenorhabditis elegans*, can be infected by microbes, including certain human-pathogenic bacteria.
- While *Pseudomonas* species can serve as food for *C. elegans*, some species are also pathogens.
- The nematoxic bacterium *P. aeruginosa* can produce acylated homoserine lactones, the signal molecules in quorum sensing, as attractants for nematodes.

Beale *et al.*,2006; Niu *et al.*,2010

Quorum sensing
Bacteria-nematode communications
Interkingdom signaling

- Some species of *P. aeruginosa* are natural pathogens of *C. elegans*, whereas others can serve as a food source for the nematodes.
- The common human pathogenic bacterium *Pseudomonas aeruginosa* kills *C. elegans* with quorum-sensing controlled-virulence factors.
- In an experiment when synthetic AHLs were used in the absence of bacteria, the worms were significantly more likely to migrate towards the AHL than the controls.

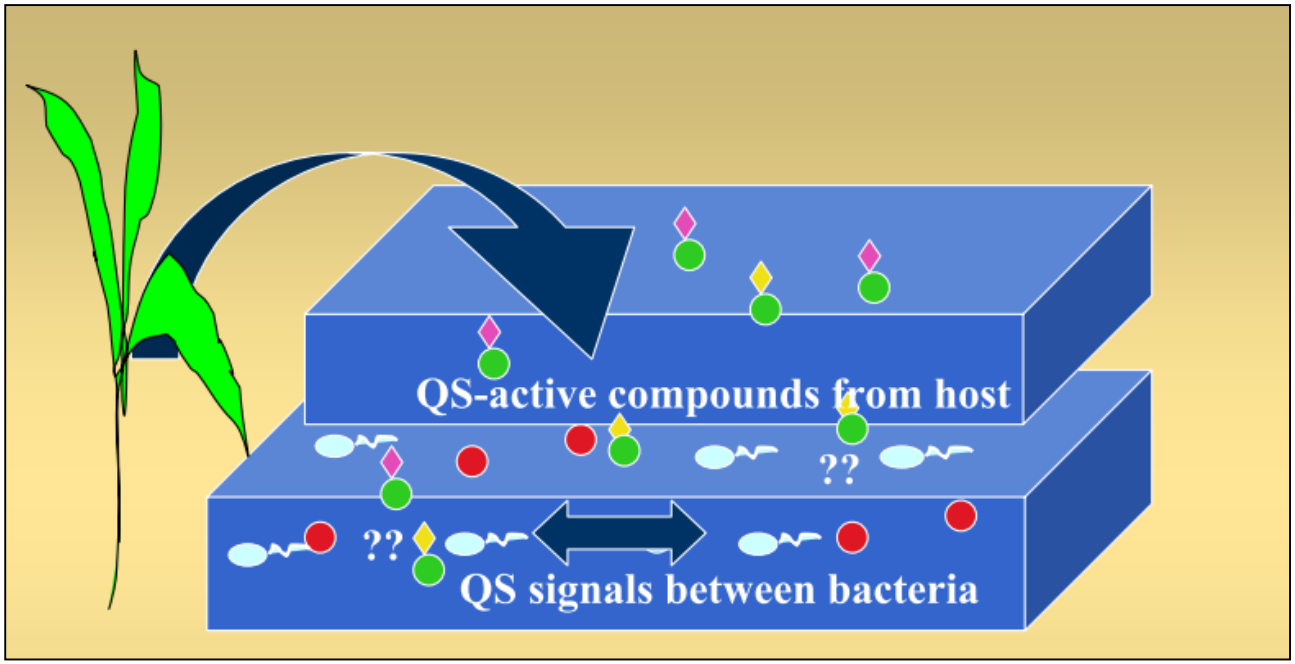
Beale *et al.*,2006; Atkinson and Williams,2009

Quorum sensing

Bacteria-Plant communications

Interkingdom signaling

- QS is crucial to many host-bacterial interactions.



Robinson *et al.*,2003

Quorum sensing
Bacteria-Mammal communications
Interkingdom signaling

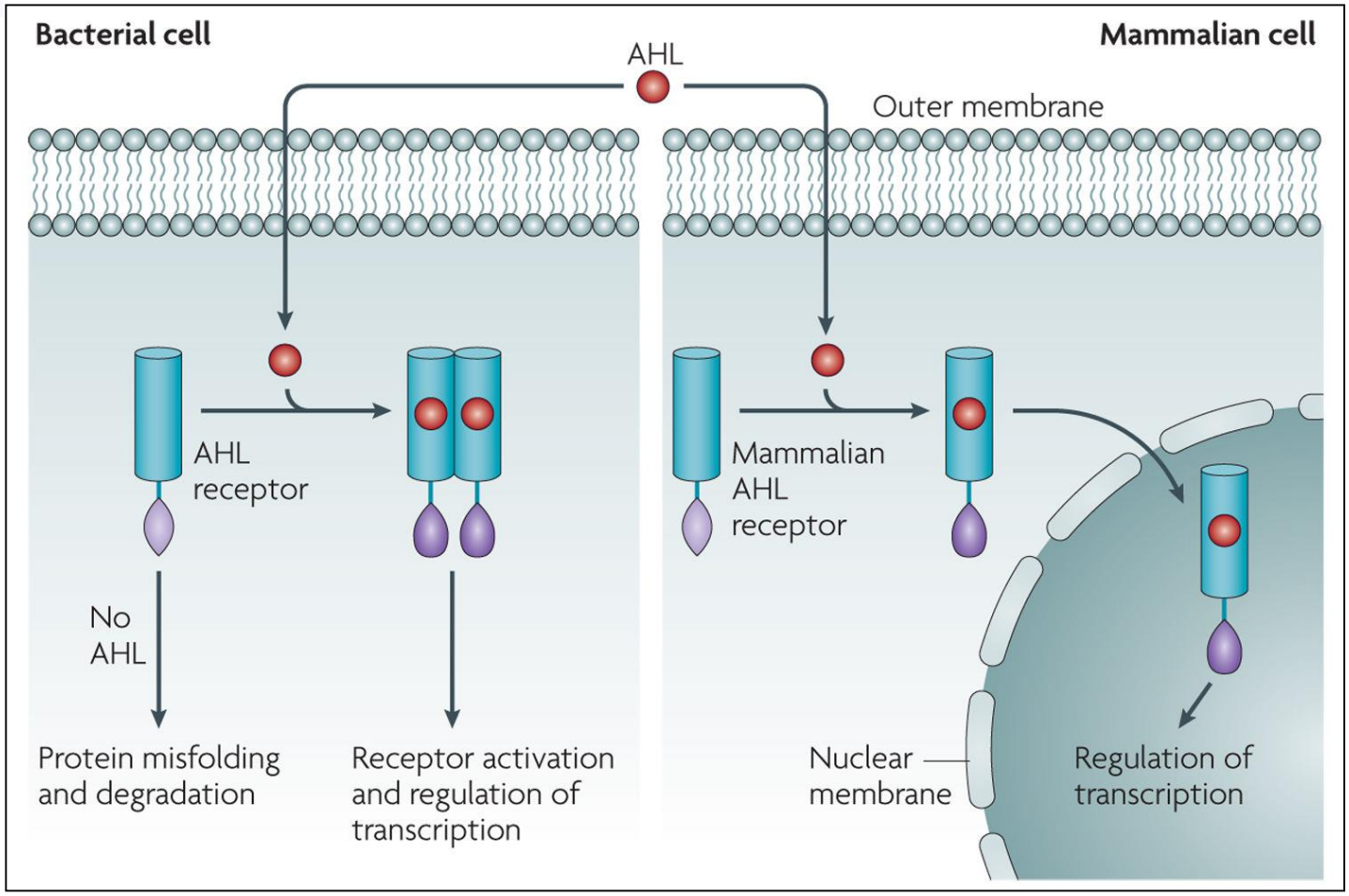
- In mammalian cells, AHLs also gain access to the cytoplasm by crossing the plasma membrane.
- The identity of the mammalian receptor (or receptors) for AHLs in mammalian cells is unknown.
- However, if there are intracellular receptors, it is proposed that the interaction with the AHL ligand activates these receptors and thereby allows their transportation into the nucleus, where they could control gene expression.

Hughes and Sperandio,2009

Quorum sensing

Bacteria-Mammal communications

Interkingdom signaling



Hughes and Sperandio,2009

Quorum sensing
Human pathogens

- **Quorum sensing** occurs in more than 70 types of microorganisms, including bioluminescent bacteria, which together produce light from a chemical reaction but alone must remain dark.
- Among human pathogens that use quorum sensing to estimate their population densities are:

1. *Pseudomonas aeruginosa*, which can cause serious infections, particularly in burn victims;
2. *Yersinia pestis*, the bubonic plague bacterium that killed millions during the Middle Ages.

Bacterial Quorum Sensing

Gram-positive vs. Gram-negative bacteria

Typical Gram-negative quorum sensing mechanism

- Acyl homoserine lactone molecules, synthesized by LuxI, Due to the small size and lipophilicity of AHL autoinducers, they readily pass the cell membrane by means of passive diffusion.
- If the concentration of AHL is sufficiently high, the AHL autoinducer binds to the intracellular LuxR protein and provokes the LuxR DNA binding domain to reveal.
- Subsequently, the LuxR protein binds to DNA, causing activation of target gene transcription.

Verbeke *et al.*,2017

Bacterial Quorum Sensing

Gram-positive vs. Gram-negative bacteria

Typical Gram-negative quorum sensing mechanism

- Quorum sensing peptides are synthesized by the bacterial ribosomes as pro-peptidic proteins and undergo posttranslational modifications during excretion by active transport.
- As the population density increases, the AIPs accumulate in the environment.
- When a certain threshold level is reached, binding of an AIP to a receptor initiates activation of the receptor kinase by phosphorylation on a conserved histidine residue.
- Subsequently, the activated receptor kinase transfers the phosphoryl group to a conserved aspartate residue of the intracellular response regulator, which in turn will be activated.
- The activated response regulator influences the transcription of target genes, including the AIP genes, genes for the receptor kinase and response regulator and genes for the ABC transporter.
- These phosphorylated response regulators induce increased target gene expression.

Verbeke *et al.*,2017

Quorum sensing
The QS system

- The QS system can be seen as being based on the following crucial elements:

1. The autoinducers;
2. The signal synthase;
3. The signal receptor;
4. The signal response regulator;
5. The regulated genes (which form the so-called QS regulon).

A regulon is a collection of genes or operons under regulation by the same regulatory protein.

Nazzaro *et al.*,2013

AHL production
Gram negative bacteria

- *N*-acyl homoserine lactones (AHLs) are the best-studied QS signals among Gram-negative bacteria.
- Quorum sensing systems in Gram negative bacteria consist of two basic protein components:

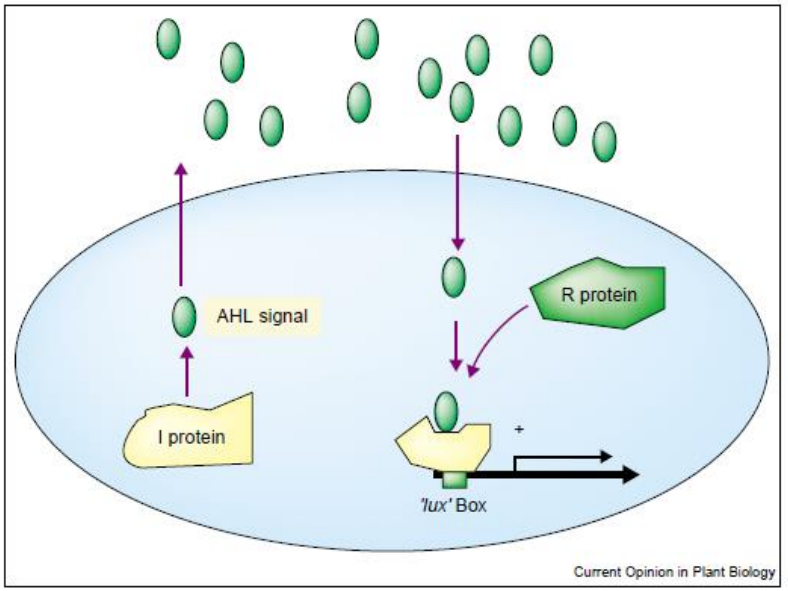
1. I protein (synthesized by AHL synthases), and
2. R protein (AHL receptor).

- The concentration of the autoinducers (I proteins) are sensed and monitored by the response regulator family of transcription factors known as R proteins.
- As the bacterial population grows, the level of autoinducer in the environment increases.

There is a low likelihood of a bacterium detecting its own secreted inducer.

Quorum sensing
LuxI/LuxR system

- Bacterial cells (shown in blue) contain an I protein that is responsible for the synthesis of freely diffusible signals (green ovals).
- At high cell density, the signal accumulates intracellularly and interacts with the R protein.
- This interaction induces a conformational change in the R protein, which alters the affinity of the R protein for specific DNA sequences, known as '*lux*' boxes, that are located within the promoters of the AHL-regulated genes.



LuxI/LuxR is an integrated part of the lux operon, which is involved in bioluminescence through activation of the structural *lux* genes (lux*CDABE*).

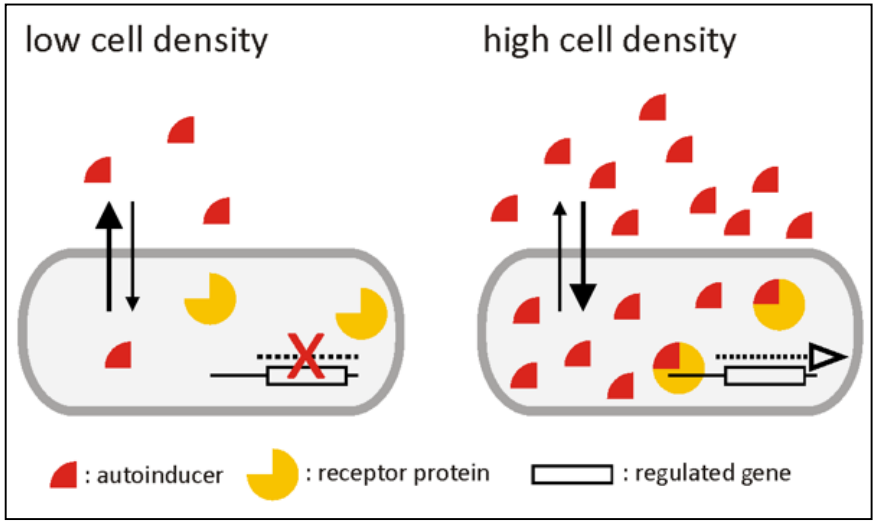
Loh *et al.*,2002;..

Quorum sensing
LuxI/LuxR system

- QS in Gram-negative organisms involves two regulatory components:

1. the transcriptional activator protein (receptor protein), and
2. the autoinducer (AI) molecule produced by the AI-synthase.

- Accumulation of AI occurs in a cell-density-dependent manner until a threshold level is reached.
- At this time the AI binds to and activates the receptor protein, which in turn induces gene expression.



Signaling System
Detailed of gene circuit.

Weigel,2016; Kievit and Iglewski,2020

AHL receptors
LuxR

- The specific receptors for acyl-HSL signals are members of the LuxR family of transcriptional regulators.
- LuxR family members have been proposed to consist of two domains:

1. N-terminal acyl-HSL-binding domain,
2. C-terminal DNA-binding domain.

- AHL receptors (R proteins) are typically transcriptional activators that form dimers or multimers and bind to "receptor box" promoter sequences in the bacterial DNA, thus enhancing expression of sets of genes with these promoter sequences.

The light emission is due to transfer of electrons from a substrate, in presence of an enzyme called *Luciferase*. The electrons are transferred to a lower energetic level, with an output of energy in the form of light radiation.

Quorum sensing

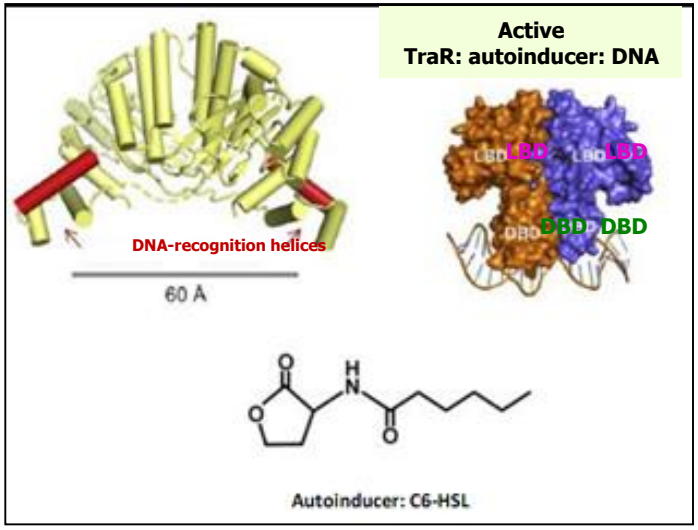
Bioreporter (indicator) strains

lux-like genes expression in *C. violaceum* CV026

- All receptors share a common modular structure. E.g. Ers.

1. Centrally located is a highly conserved DNA binding domain (DBD);
2. C-terminal to the DBD is the ligand-binding domain (LBD).
3. Activation functions are located within the LBD and the *N*-terminal regions.

-DNA binding domain (DBD)
-ligand-binding domain (LBD)



AHL production
Mechanism

- There is a low likelihood of a bacterium detecting its own secreted inducer.
- Thus, in order for gene transcription to be activated, the cell must encounter signaling molecules secreted by other cells in its environment.
- When only a few other bacteria of the same kind are in the vicinity, diffusion reduces the concentration of the inducer in the surrounding medium to almost zero, so the bacteria produce little inducer.
- However, as the population grows, the concentration of the inducer passes a threshold, causing more inducer to be synthesized.

Wikipedia,2017

AHL production
Mechanism

- This forms a positive feedback loop, and the receptor becomes fully activated. Activation of the receptor induces the up-regulation of other specific genes, causing all of the cells to begin transcription at approximately the same time.
- This coordinated behavior of bacterial cells can be useful in a variety of situations. For instance, the bioluminescent luciferase produced by *Vibrio fischeri* would not be visible if it were produced by a single cell.
- By using quorum sensing to limit the production of luciferase to situations when cell populations are large, *V. fischeri* cells are able to avoid wasting energy on the production of useless product.

Wikipedia,2017

AHL production
Mechanism

- By detecting population density, bacteria can also sense when a host site has become more or less saturated with bacteria and activate quorum sensing genes, such as those involved in motility, in order to move to and colonize a new site.

A simplified model for AHL-mediated gene regulation in bacteria

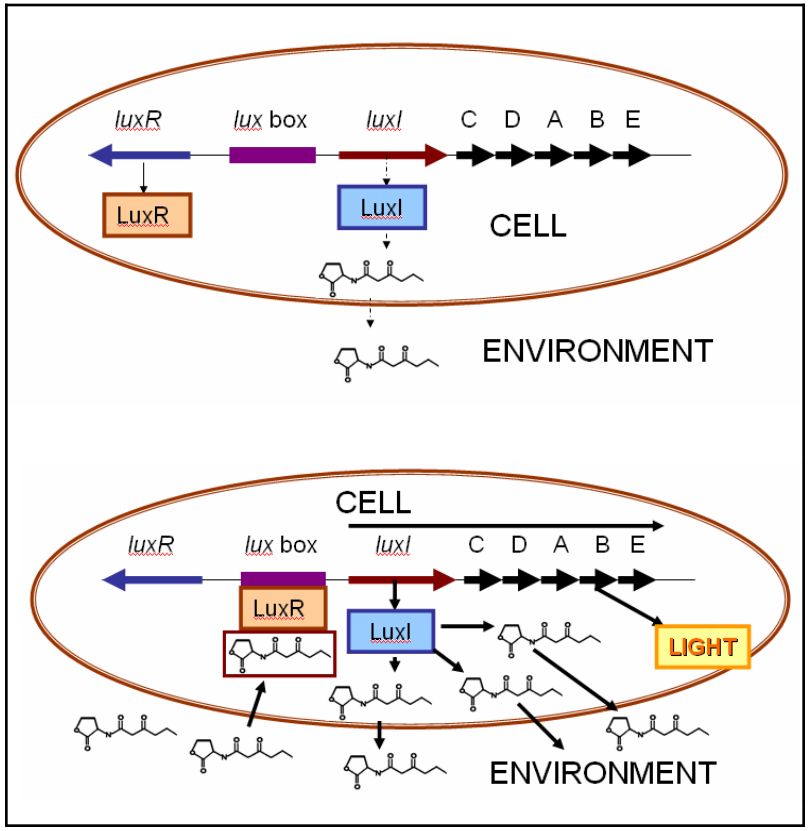
In Gram negative bacteria

Vibrio fischeri

a) When there are few bacteria nearby, the cell produces very little AHL.

b) As the concentration of cells in the surroundings increases, the signal accumulates.

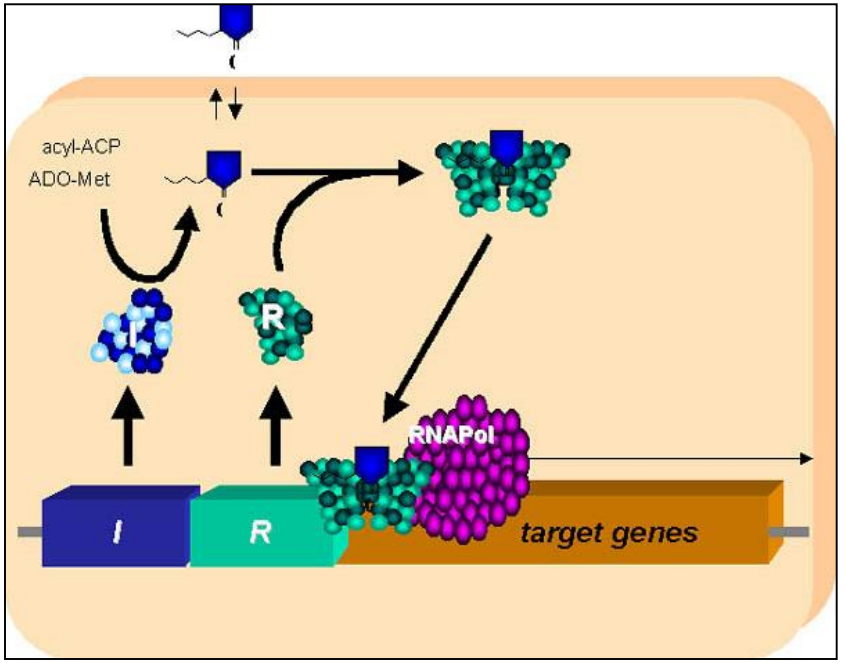
- The AHL binds to LuxR and the LuxR/AHL complex binds to a region of DNA called the lux box.
- This activates the transcription of structural genes whose products give rise to luminescence.
- LuxI production also increases, leading to increased synthesis of the AHL.
- Homologues of the *V. fischeri* LuxR and LuxI proteins have been identified in over 25 species of gram-negative bacteria.



Lal,2009

A simplified model for AHL-mediated gene regulation in bacteria
In Gram negative bacteria

- The genes *I* and *R* represent the genes encoding the AHL synthase and regulatory protein, respectively.
- In the presence of sufficient AHL signal, the R regulatory protein is activated, possibly by dimerization.
- The activated R regulatory protein binds to a specific binding site and stimulates (or represses) transcription initiation by RNA Polymerase holoenzyme (RNAP).



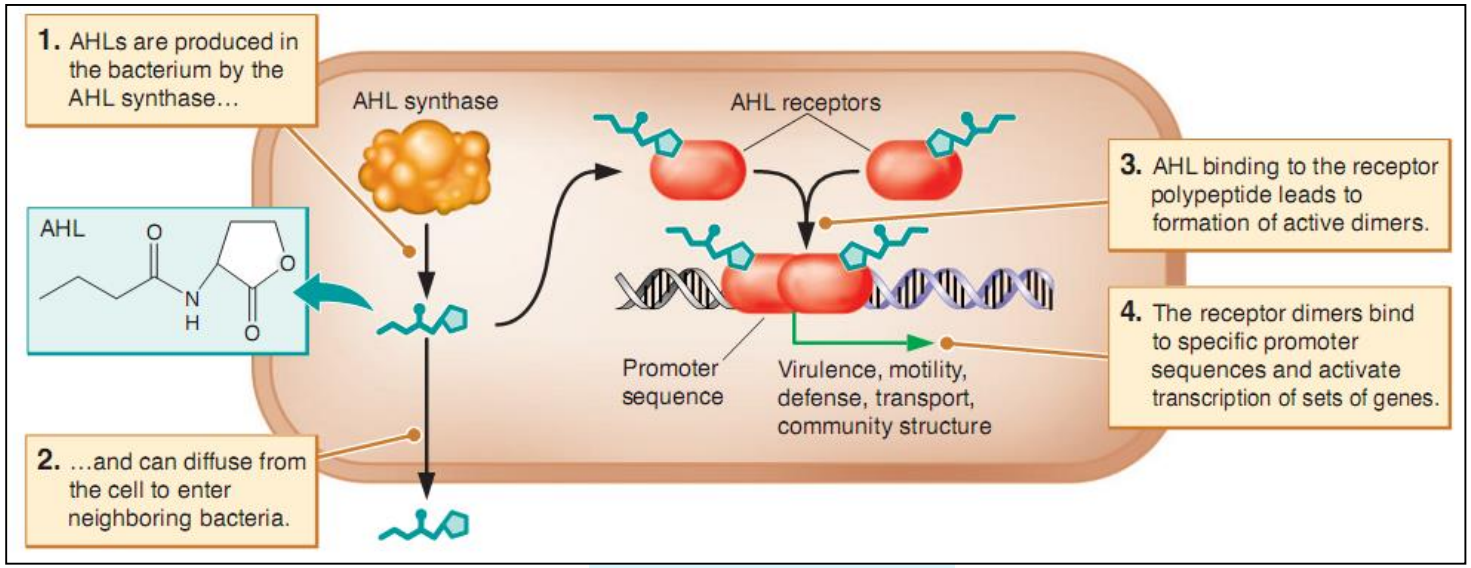
RNA polymerase (RNAP) is the enzymatic machinery responsible for transcription, a key regulatory step in gene expression. The prokaryotic RNAP is a highly conserved, "crab claw" shaped enzyme with a molecular mass of ~400kD.

Pierson III,2005;..

Quorum sensing

Molecular mechanics of AHL-mediated QS

- AHLs are produced in the bacterium can diffuse from the cell to enter neighboring bacteria.
- AHL binding to the receptor polypeptide leads to formation of active dimers.
- The receptor dimers bind to specific promoter sequences in the bacterial genome and activate transcription of sets of genes.



Bauer *et al.*,2005

Quorum sensing
The QS system

- In bacteria, acyl homoserine lactones (AHLs) cross the cell membrane and interact with cytoplasmic receptors of the LuxR family.

1. Binding the AHL to the LuxR-type receptor allows proper folding of this protein, which allows the receptor to dimerize and bind to its target sequence on DNA to regulate gene expression.
2. In the absence of signal, LuxR-type proteins misfold and are targeted for degradation.

Hughes and Sperandio,2009

AHL production
Gram negative bacteria

1. At a low bacterial cell density, the low level of transcription of the lux operon is insufficient for the activation of *LuxR*.
2. When the cell density increases and signal levels reach a specified threshold level, LuxR activation can take place.

- The LuxR/3-oxo-C6-HSL complex thereby activates transcription via the lux operon promoter, giving rise to the expression of other genes, including (in *Vibrio fischeri*) lux AB genes encoding luciferase and lux CDE, which encodes the enzymes that produce the substrate for luciferase and, hence, bioluminescence.

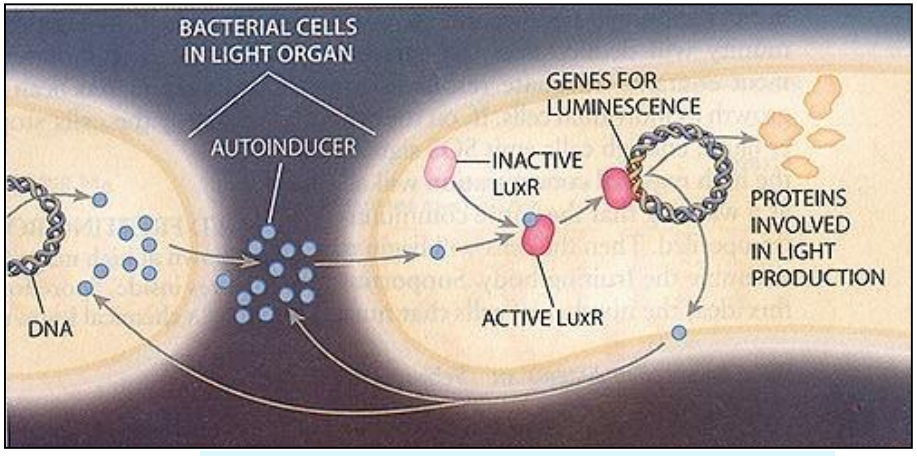
Nazzaro *et al.*,2013

Quorum Sensing

Light production

Three kinds of single celled marine organisms produce light: bacteria, dinoflagellates, radiolarians, all with different luciferins

Gene	Gene Product	Function
LuxI	autoinducer	always expressed, this is the signal.
LuxR	receptor	always expressed, the receptor detects the signal(autoinducer) and in response turns on expression of luciferase genes.
LuxAB	luciferase	makes light, only expressed when the receptor is active.



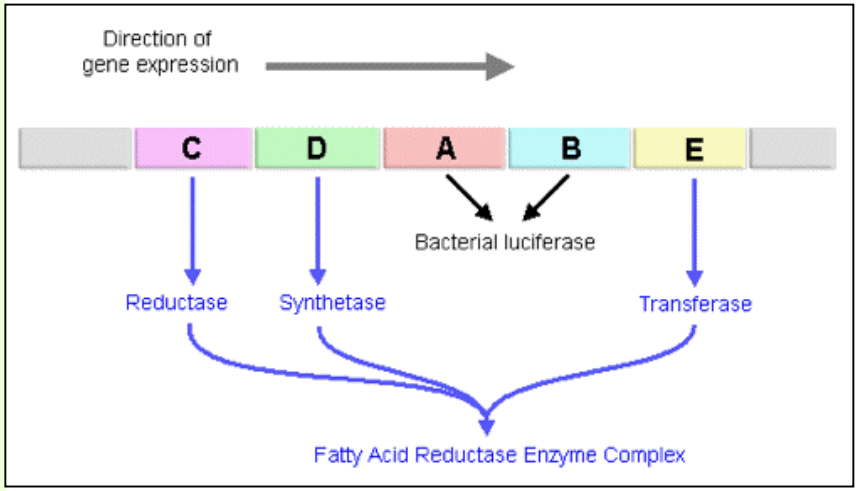
cibt.bio.cornell.edu/workshops

Quorum sensing

LuxR-type receptor

lux genes expression in mutant *E. coli*

- The constant light emission in luminous bacteria depends on several different enzymes which continuously generating the substrates for the bioluminescence reaction.
- In particular, the fatty acid reductase, a multienzyme complex, whose lux genes (*luxC*, *luxD*, and *luxE*) immediately flank the *lux*A and *lux*B genes of luciferase.



The arrangement of the *lux*CDABE open reading frames.

The light emission is due to transfer of electrons from a substrate, in presence of an enzyme called Luciferase. The electrons are transferred to a lower energetic level, with an output of energy in the form of light radiation.

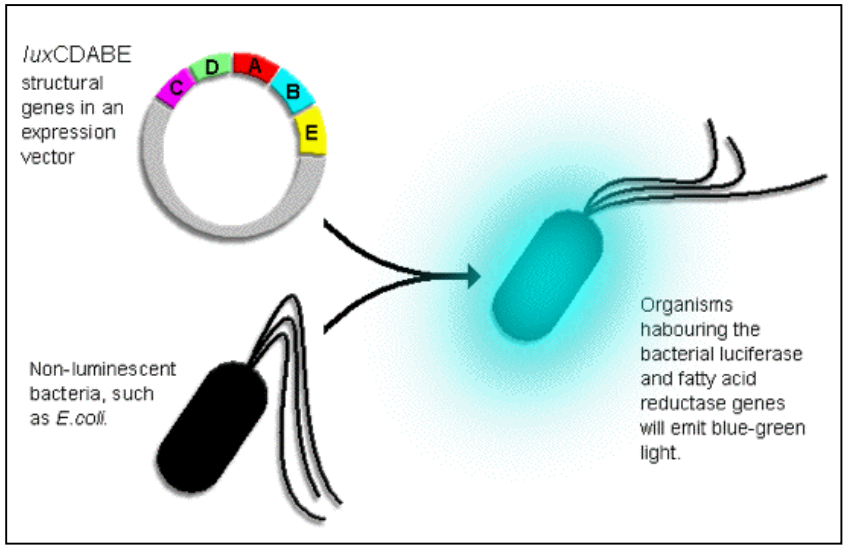
Lin and Meighen,2009

Quorum sensing

LuxR-type receptor

LuxCDABEG genes expression in mutant *E. coli*

- The fact that the conversion of a nonluminous bacterium, such as *Escherichia coli*, e.g. *E. coli* MM294 cells to a light-emitter requires only the insertion of the *lux*CDABE genes, encoding the bacterial luciferase and the fatty acid reductase complex, into the cell.



Chatterjee *et al.*,2002; Lin and Meighen,2009

Methods

Biomonitor/Bioreporter/Biosensor or reporter strains

Biosensor systems using reporter bacteria

Overview of plasmid-based AHL reporter bacteria

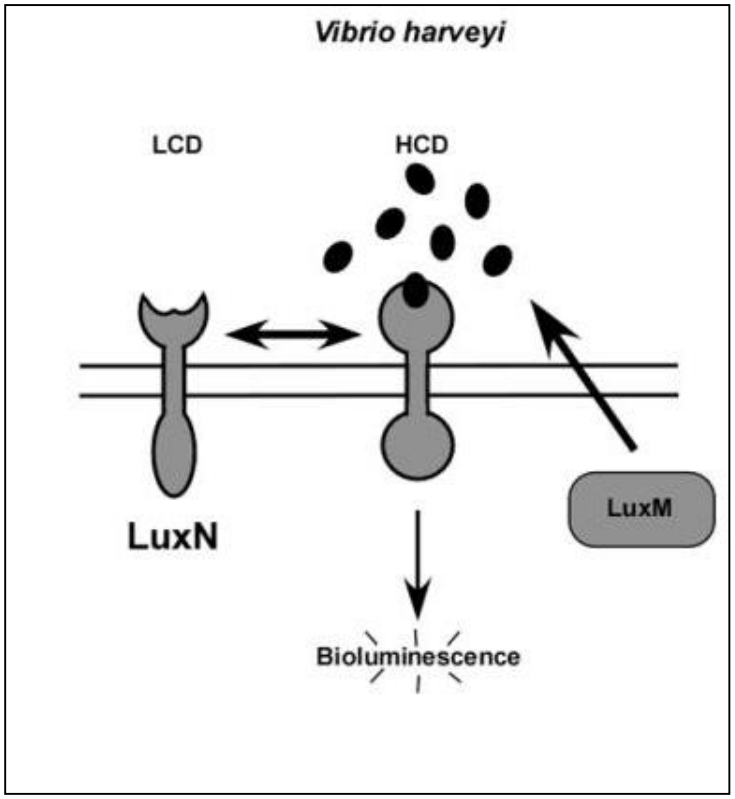
Plasmid	Bacterial strain	Analyte	Promoter	Reporter
pSB401	*E. coli*	3-oxo-C4- to 3-oxo-C14-HSL C4- to C12-HSL	*luxI*	*luxCDABE*
pSB1075	*E. coli*	3-oxo-C12- to 3-oxo-C16-HSL C12- to C16-HSL	*lasI*	*luxCDABE*
pECP61.5	*P. aeruginosa*	C4-HSL	*rhlA*	*lacZ*
pKDT17	*E. coli*	3-oxo-C10- to 3-oxo-C12-HSL C10- to C12-HSL	*lasB*	*lacZ*
pCF218, pMV26	*A.tumefaciens*	3-oxo-C6- to 3-oxo-C12-HSL C4- to C12-HSL	*traI*	*luxCDABE*
pCF218, pCF372	*A.tumefaciens*	3-oxo-C4- to 3-oxo-C12-HSL C5- to C10-HSL	*traI*	*lacZ*
pSB406	*E. coli*	3-oxo-C4- to 3-oxo-C14-HSL C4- to C12-HSL	*rhlI*	*luxCDABE*
	C. violaceum	3-oxo-C6- to 3-oxo-C8-HSL C4- to C8-HSL	*cviI*	Violacein
pAL105	*E. coli*	3-oxo-C12-HSL	*lasI*	*luxCDABE*
pAL101	*E. coli*	C4-HSL	*rhlI*	*luxCDABE*
pSB536	*E. coli*	C4-HSL	*ahyI*	*luxCDABE*
pSB403	*Broad host range*	3-oxo-C4- to 3-oxo-C14-HSL C4- to C12-HSL	*luxI*	*luxCDABE*
pHV2001	*E. coli*	3-oxo-C6- to 3-oxo-C8-HSL C6- to C8-HSL	*luxI*	*luxCDABE*
pZLR4	*A.tumefaciens*	All 3-oxo-HSLs C6- to C14-HSL 3-OH-C6- to 3-OH-C10-HSL	*traI*	*lacZ*
pJZ384, pJZ410, pJZ372	*A.tumefaciens*	3-oxo-C4- to 3-oxo-C18-HSL C4- to C18-HSL	*traI*	*lacZ*
pSF105, pSF107	*P. fluorescens*	3-OH-C6-HSL C6-HSL 3-OH-C8-HSL	*phzI*	*lacZ*
pUCP18	*P. aeruginosa*	3-oxo-C12-HSL	*rsaL*	*luxCDABE*
pMS402	*P. aeruginosa*	3-oxo-C12-HSL	*rsaL*	*luxCDABE*
pUCGMAT1-4	*E. coli*	3-oxo-C6-HSL	*ahlI*	*mcherry*
pREC-FF	*E. coli*	3-oxo-C6-HSL	*luxI*	*cfp*
M71LZ	*P. aeruginosa lasI* $^-$	3-oxo-C10 to 3-oxo-C12-HSL	*lasI*	*lacZ*
pAS-C8	*Broad host range*	C8- to C10-HSL	*cepI*	*gfp*
pKR-C12	*Broad host range*	3-oxo-C10- to 3-oxo-C12-HSL	*lasI*	*gfp*
pJBA-132	*Broad host range*	3-oxo-C6-HSL C6- to C10-HSL	*luxI*	*gfp*

Verbeke *et al.*,2017

Quorum sensing

Vibrio harveyi Quorum-Sensing Circuits

- The membrane-bound quorum-sensing receptor, LuxN from *Vibrio harveyi* binds to the AHL autoinducer (black ovals) at high cell density (HCD) resulting in a phosphorylation cascade that activates expression of the *lux* genes required for bioluminescence.
- LuxM is the 3OH-C4-HSL autoinducer synthase.



Swem *et al.*,2010

Biochemistry and Quorum Sensing
How do they make light?

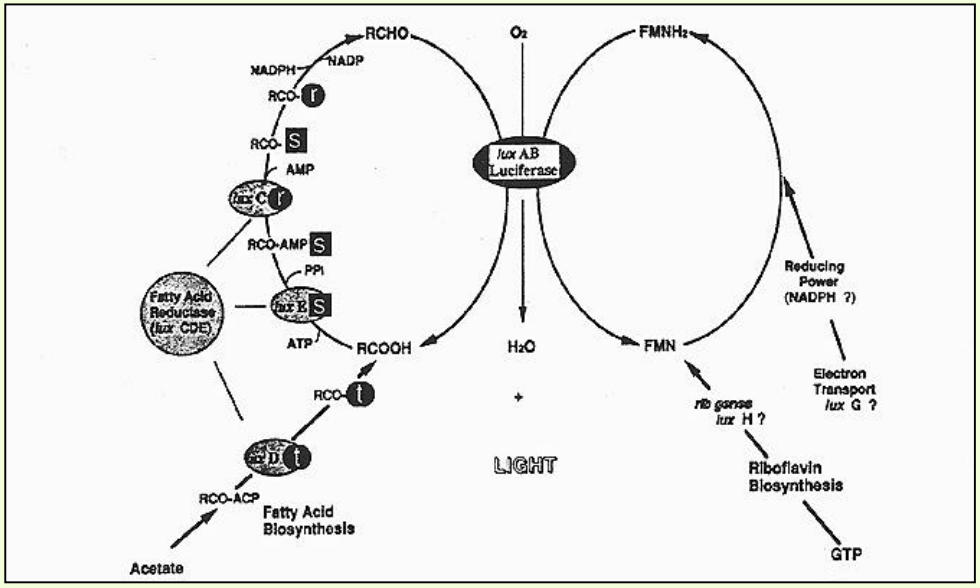
- The bacterial luminescence reaction, which is catalyzed by luciferase, involves the oxidation of a long-chain aliphatic aldehyde and reduced flavin mononucleotide ($FMNH_2$) with the liberation of excess free energy in the form of a blue-green light at 490nm:
  ($FMNH_2 + O_2 + RCHO \rightarrow FMN + RCOOH + H_2O + Light$)
- LuxA and LuxB catalyze the luciferase reaction, using oxygen and a reduced flavin mononucleotide (FMNH2) to oxidize a long chain aldehyde RCHO.
- It results in the production of light and and inactive oxyluciferin, ATP used as energy to produce more luciferin.
- Sometimes luciferin and luciferase are bound together in a single molecule called "photoprotein", which can be triggered by calcium ions to produce light.
- Most of the energy produced is emitted as light rather than heat, and the creation of light occurs only when organisms are present in high cell densities.

MicrobeWiki; Bluth and co-workers

Biochemistry and Quorum Sensing

How do they make light?

Relationship between the bacterial bioluminescence reaction and the genes and enzymes linked to the *lux* operon



- Sometimes luciferin and luciferase are bound together in a single molecule called "photoprotein", which can be triggered by calcium ions to produce light.
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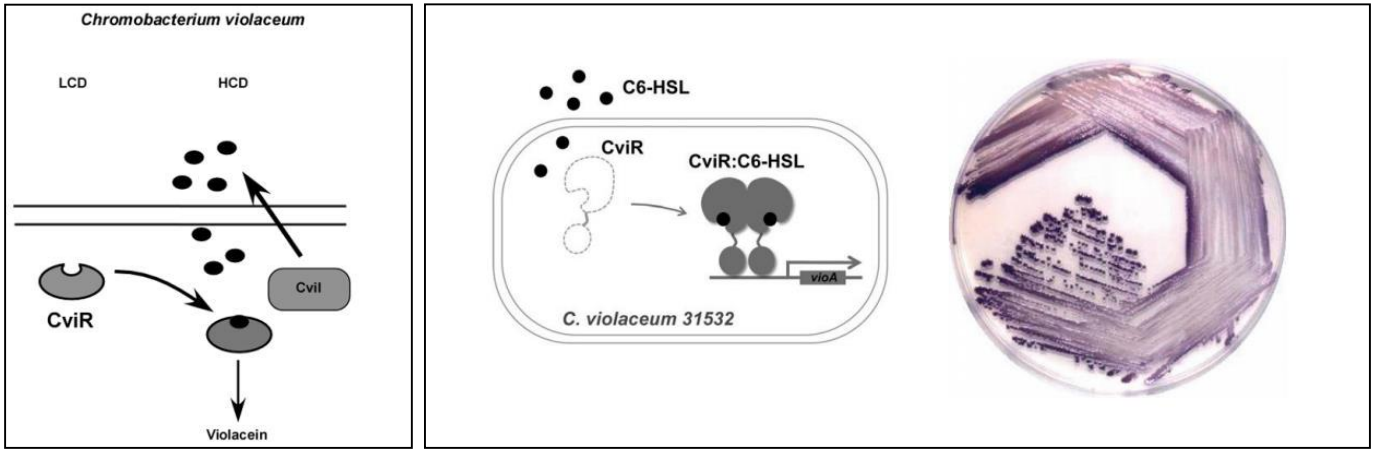
MicrobeWiki; Bluth and co-workers

Bioreporter (indicator) strains

lux-like genes expression in *C.violaceum* CV026

CvirI and CvirR

- ***Chromobacterium violaceum* CV026** contains a LuxI and LuxR homlog, termed CvirI and CvirR, respectively.
- Violacein production requires CviR(quorum-sensing receptor, CviR) binding to C6-HSL, which accumulates at high cell density, and subsequent activation of transcription of the *vioABCDE* biosynthetic gene cluster.



HCD, high cell density

Camilo Gómez;..

Quorum sensing
C. violaceum Quorum-Sensing Circuits

- *Chromobacterium violaceum* CV026 synthesizes and responds to C6-HSL.
- The AHLs induce the production of a purple compound violacein which is visualized on agar plate.
- It is more sensitive to C4 to C8 un-substituted homoserine lactones.
- Consistent with the inability of long chain AHL molecules to induce CviR transcriptional activation, C10-HSL, C12-HSL, and C14-HSL have been reported to antagonize the CviR protein.

Swem *et al.*,2010;…

Quorum sensing
Other pathogenic relationships

- QS is now known to regulate a wide range of biological functions including:

1. Production of EPSs (Biofilm formation);
2. Production of degradative enzymes, siderophores, antibiotics and pigments;
3. Swarming motility;
4. Conjugative transfer of Ti plasmid;
5. Expression of type III secretion apparatus (*hrp* genes).

- Since many of these functions are directly or indirectly related to pathogenesis or to controlling competitors, most phytopathogenic bacteria also possess a QS system, which probably functions as a preventive means to avoid early activation of local and systemic plant defense responses.

Quorum sensing
Production of degradative enzymes

- In Gram negative bacteria such as *P. carotovorum* mutants were found that were deficient in the production of enzymes (pectinase, cellulase and protease) required for pathogenicity.
- One class of mutants was found to be deficient in production of the autoinducer N-3-(oxohexanoyl)-L-homoserine lactone (Jones *et al.*,1993).
- Addition of HSL to the mutants of *P. carotovorum* restored enzyme production and virulence.

Quorum sensing

QS activity of bacterial isolates from diseased *Salix* plants

INA activity

- Quorum densing is a system known to be active in several regulatory mechanisms in many bacteria, e.g. pathogenicity, but its possible role in INA regulation has yet to be elucidated.
- Here provide evidence that pathogenic ice-nucleation bacteria with inducible INA produce QS signals that in other bacteria have been shown to be in the control of genes of importance for pathogenicity.

Genera	Strain	QS-activity	INA[a]	Temp. inducible[b]
Bacillus spp.	12	+	+	+
	294	−	+	−
	118	−	+	nt
Erwinia rhapontici	262	+	+	+
Erwinia sp.	113	−	+	−
Frigoribacterium faeni	275	−	−	nt
Pseudomonas fluorescens	69	+	+	+
P. fluorescens	198	−	+	−
P. fluorescens	103	−	+	−
P. syringae	269	+	+	nt
P. syringae	217	+	+	+
P. syringae	226	+	+	nt
P. syringae	229	+	+	−
P. syringae	9	−	+	−
P. graminis	148	−	+	−
P. veronii	254	+	+	nt
P. brenneri	109	−	+	−
Sphingomonas yanoikuyae	200	+	+	+
Pedobacter sp.	253	−	+	nt
Xanthomonas campestris	117	−	+	−

[a]Ice-nucleation-active, initiate freezing above −9°C

[b]Ice-nucleation-active only after cultivation at certain temperatures

Nejad and Ramstedt,2006

Quorum sensing
Synthesis of Acyl-HSLs

- Gram-negative LuxIR systems:
- This system exhibits great specificity.
- The autoinducer (AI/AHL)molecule produced by one species of bacteria can rarely, if ever, interact with the LuxR-type regulator of another species.

Kaper and Sperandio,2005

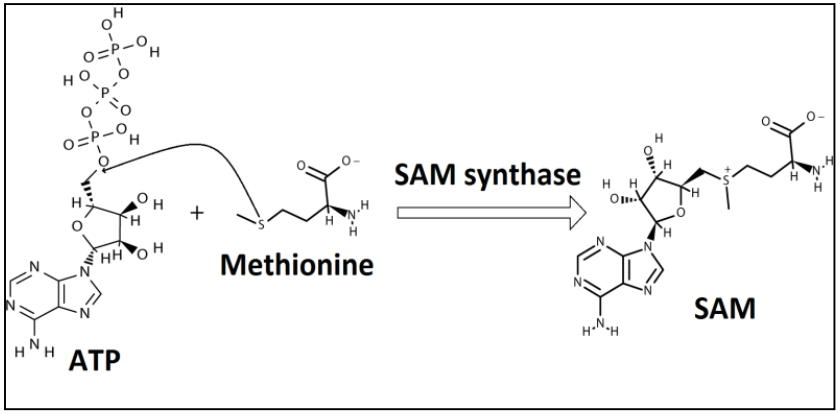
Quorum sensing

Synthesis of Acyl-HSLs

Biosynthesis of *S*-adenosylmethionine (SAM)

- The *S*-adenosylmethionine (SAM or AdoMet) is the main substrate for the acyl-HSL synthesis.
- Biosynthesis of SAM starting from:

1. ATP, and
2. methionine.



Camilo Gómez; Fontecave *et al.*,2004

Quorum sensing

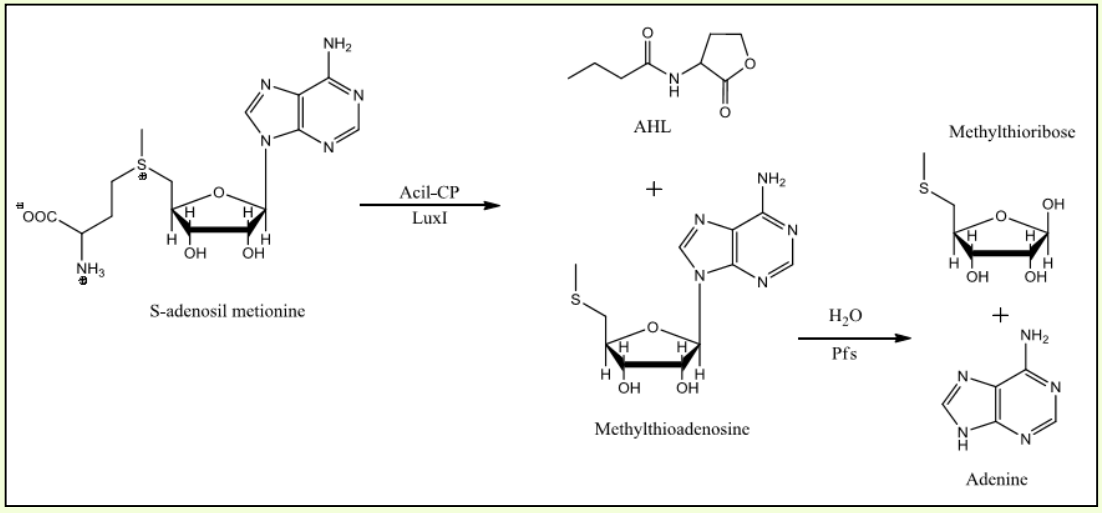
Synthesis of Acyl-HSLs

Two substrates, acyl-ACP and SAM were combined

- AHLs are synthesized from substrates:

1. S-adenosylmethionine (SAM) and
2. acyl-acyl carrier proteins (acyl-ACP) by the autoinducer synthetase coded by the *luxI* gene.

- The result is the formation of an acylated-homoserine lactone.

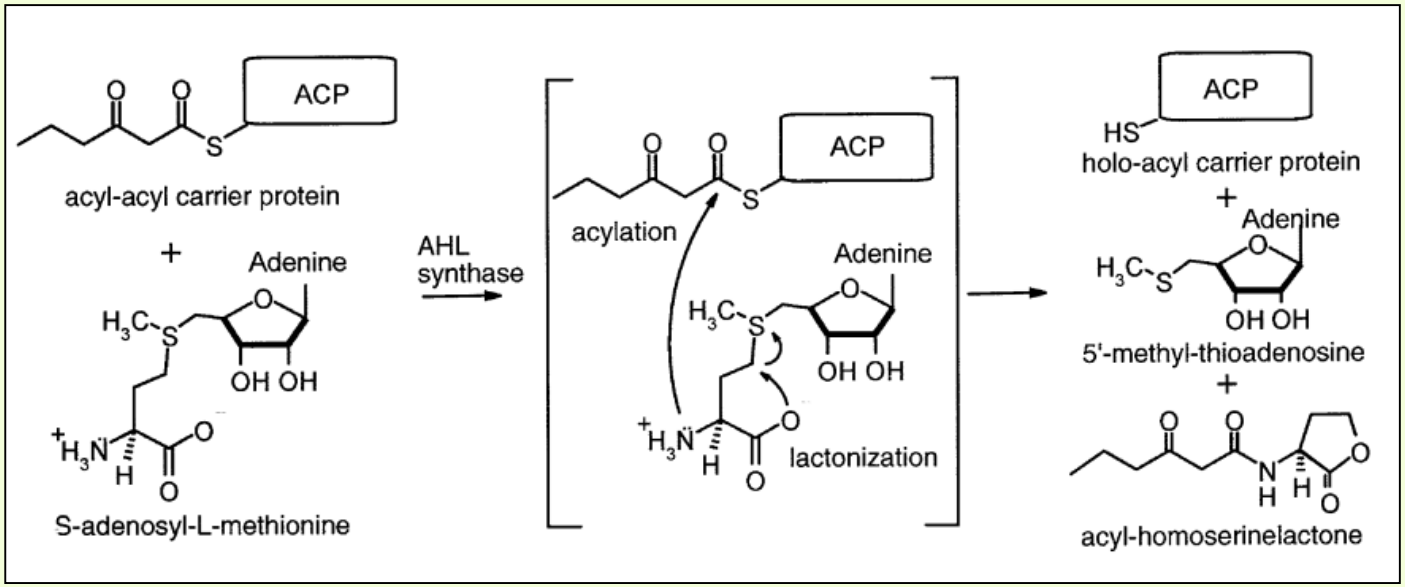


Camilo Gómez; Bassler and Miller,2006;Sufrin *et al.*,2009

Quorum sensing

The biosynthetic pathway for acylated homoserine lactone (AHL) autoinducers

- Two substrates, acyl-ACP and SAM, bind to the AHSL synthase.
- After the acylation and lactonization reactions, the product AHSL and holo-ACP and 5'-methylthioadenosine are released.



Adapted from Watson *et al.*,2002.

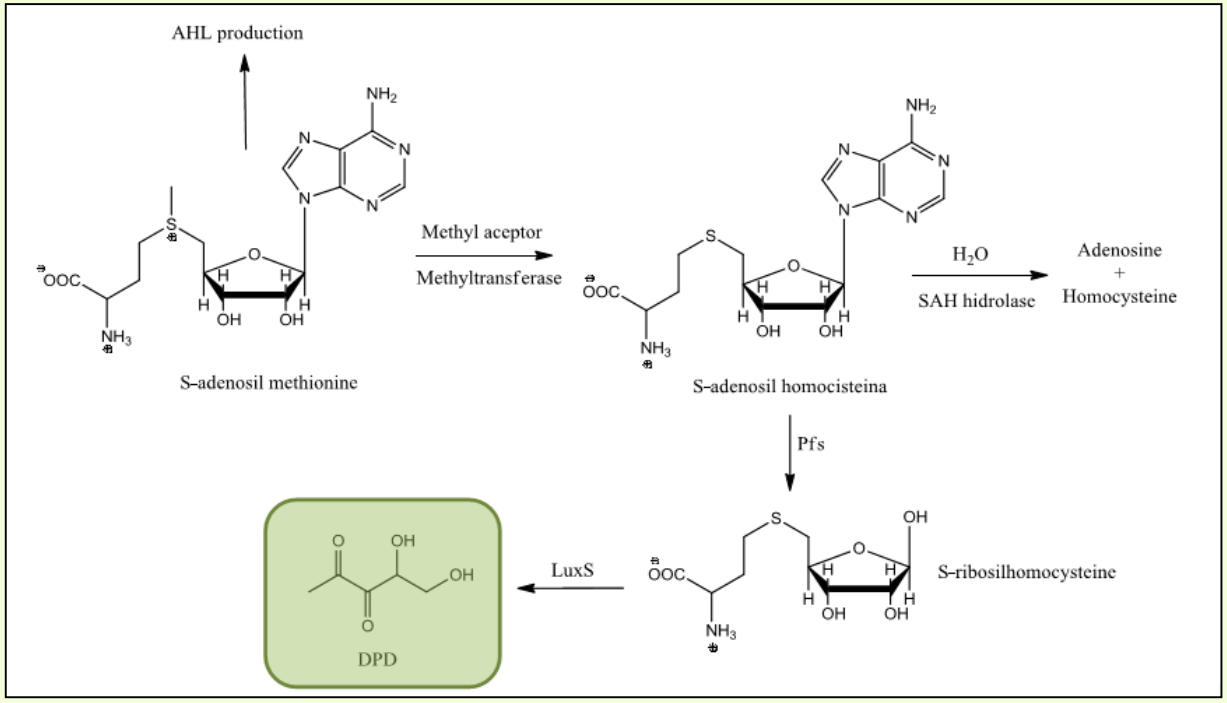
Sjöblom *et al.*,2009

Quorum sensing

Synthesis of other universal signal

AI-2 (LuxS)

- The *S*-adenosylmethionine (SAM or AdoMet) is the main substrate for the acyl-HSL synthesis.



Quorum sensing

Autoinducing peptide (AIP) QS in Gram-positive bacteria

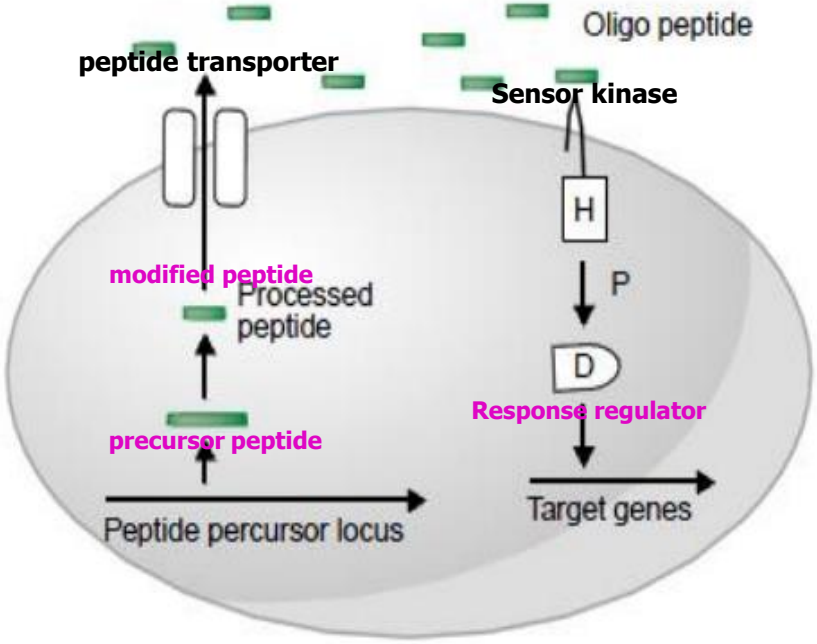
- QS systems in Gram-positive bacteria are much more diverse than the Gram-negative AHL QS system and there are many variations in the nature of the QS signal.
- Gram-positive bacteria speak with oligopeptides.
- The QS system of Gram-positive bacteria generally consists of:

1. A signal peptide, and
2. A two-component regulatory system made up of a membrane-bound sensor, and
3. An intracellular response regulator.

Note: The receptors for AHLs in G-ve bacteria exist in the cytoplasm, whereas in the case of G+ve bacteria these are located on membrane (membrane receptor).

Autoinducer peptides (AIP) or quorum sensing peptides

Gram-positive bacteria use mainly peptides



Oligopeptide Autoinducers

B. subtilis/ComX	ADPITRQWGD
B. subtilis/CSF	ERGMT
S. aureus/subgroup 1	YSTCDFIM
S. aureus/subgroup 2	GVNACSSLF
S. aureus/subgroup 3	YINCDFLL
S. aureus/subgroup 4	YSTCYFIM

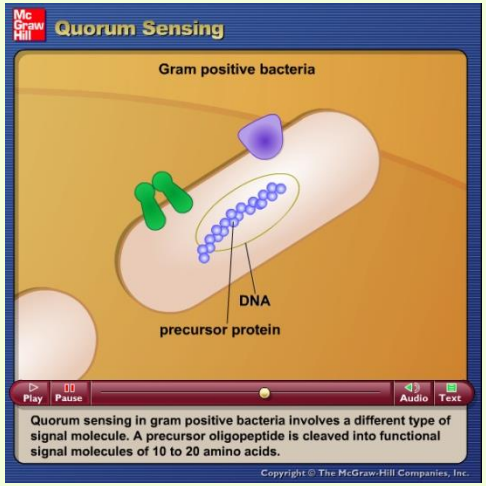
Oligopeptide signalling molecules have only been found in Gram positive bacteria.
The precursor peptide autoinducers are modified and transported.

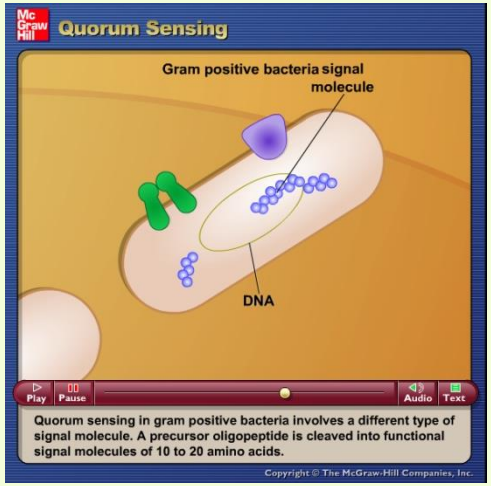
Camilo Gómez

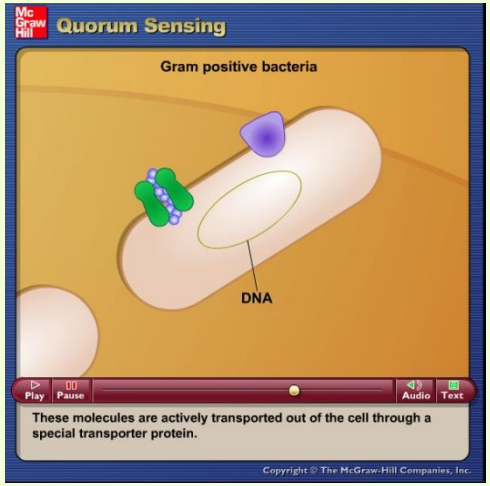
Autoinducing peptide (AIP) QS in Gram-positive bacteria

Autoinducing peptides (AIPs)

- The precursor peptide autoinducers are modified and transported out of the cell by ATP-binding cassette exporter complex.





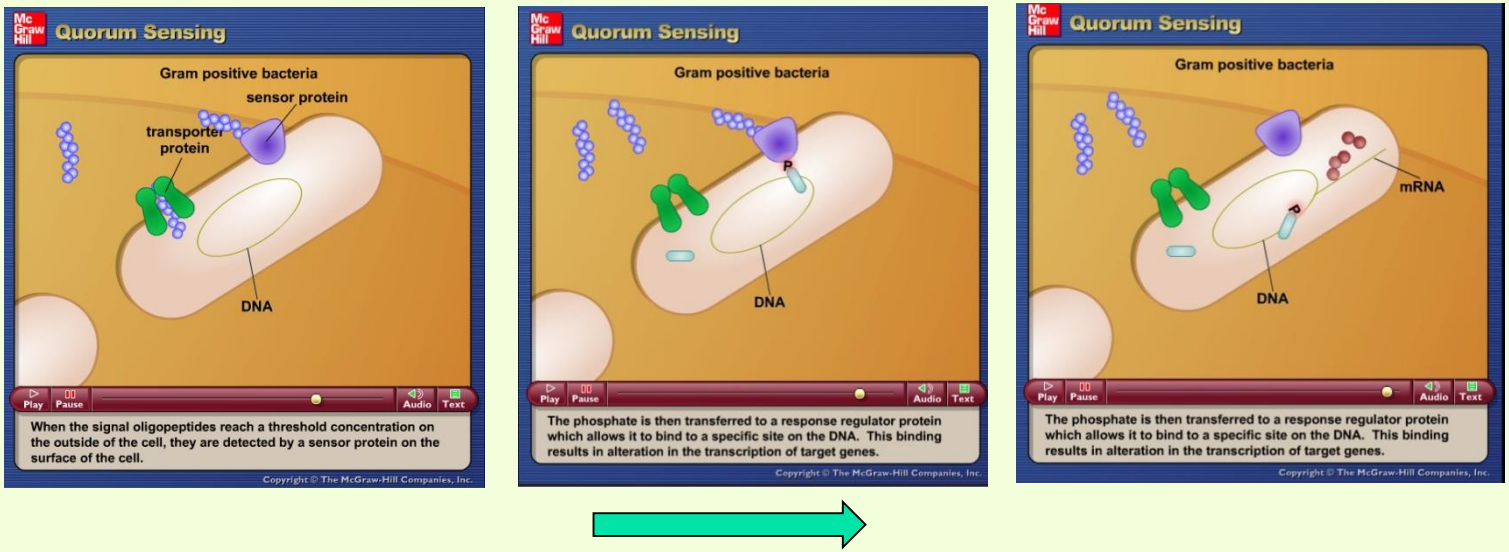


Rutherford and Bassler,2012;Koh *et al.*,2013

Autoinducing peptide (AIP) QS in Gram-positive bacteria

Autoinducing peptides (AIPs)

- The oligopeptide binds to a cognate membrane-bound two-component histidine kinase receptor.
- The phosphorylated response regulator activates transcription of the genes in the QS regulon.

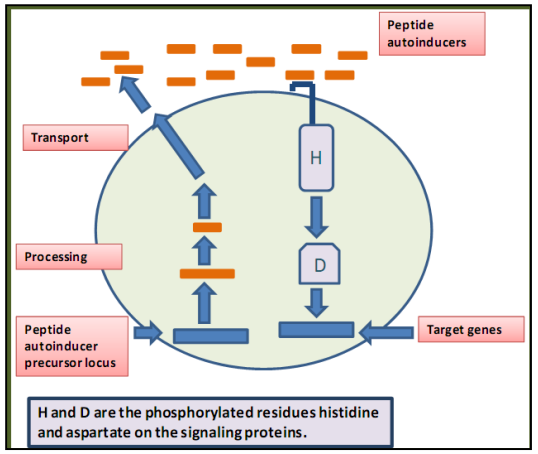


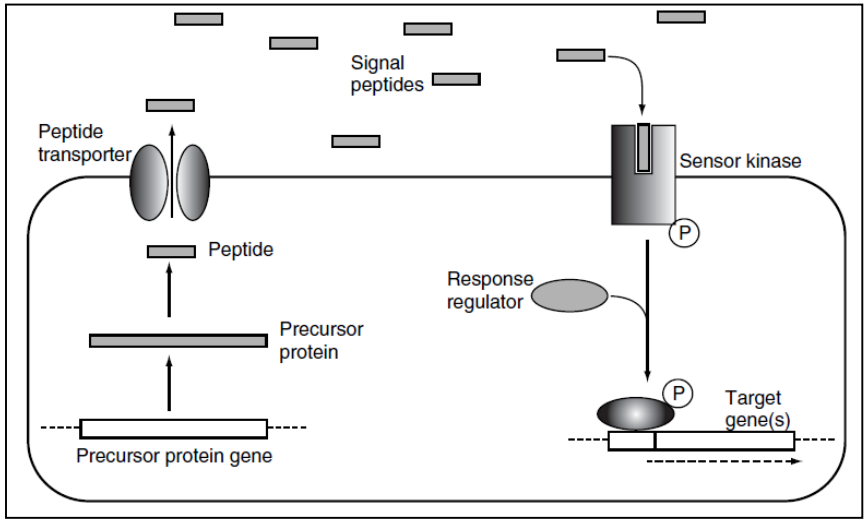
Rutherford and Bassler,2012;Koh *et al.*,2013

Autoinducing peptide (AIP) QS in Gram-positive bacteria

Autoinducing peptides (AIPs)

- Quorum sensing in Gram-positive bacteria is typically mediated by small peptides(oligopeptides) which are transported across the membrane by a specific peptide transporter.
- These peptides are not taken up by the target cells, but are recognized by a membrane receptor which transmits the signal to the interior of the cell.





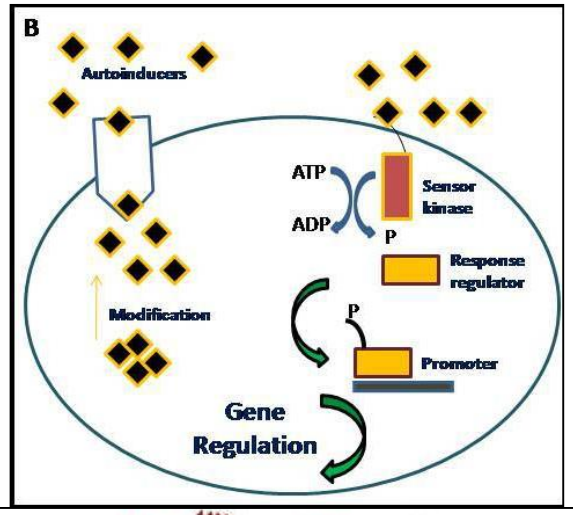
Dale and Park,2004; Winans and Bassler,2012

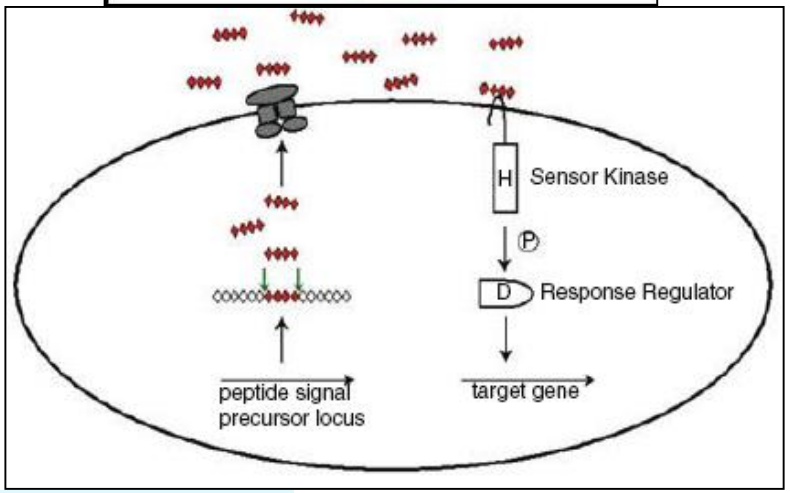
Gram-Positive Quorum Sensing

Peptide signals and two- component signal transduction

Autoinducing peptides (AIPs)

- In Gram-positive bacteria, the autoinducer is a peptide signal (red diamonds).
- As the bacterial population grows, the peptide signal accumulates extracellularly, where the signal can then be detected by a two-component system.
- Following interaction with the peptide signal, the sensor kinase protein of the two-component system autophosphorylates on a conserved histidine residue (H).
- This autophosphorylation event initiates a phospho-relay cascade that results in phosphorylation of the cognate response regulator protein on a conserved aspartic acid residue (D).
- The phosphorylated response regulator activates the transcription of the target gene(s).
- The P in the circle represents the phosphorylation cascade.

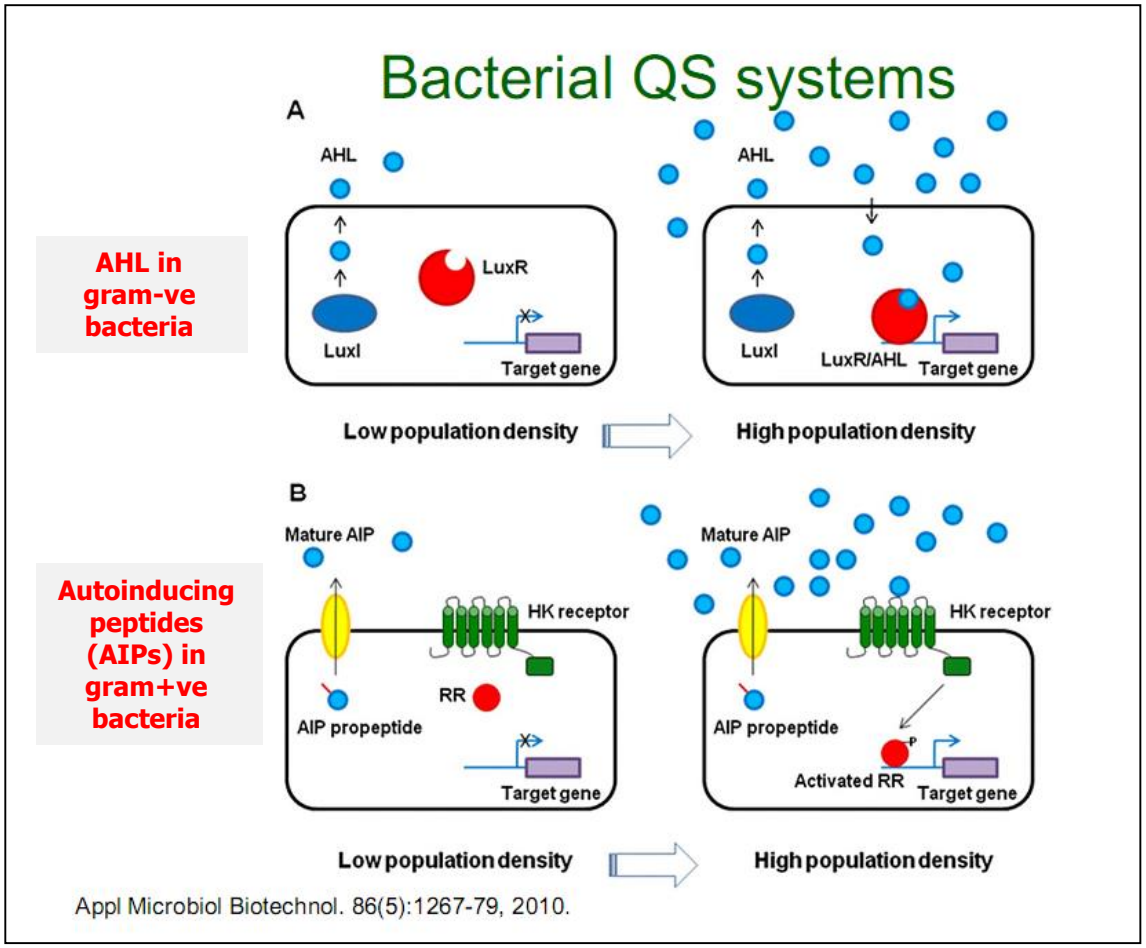




The Prokaryotes (chapter 1.12); Koh *et al.*,2013

Bacterial Quorum Sensing

Gram-Positive vs. Gram-negative bacteria



AHL in gram-ve bacteria

Autoinducing peptides (AIPs) in gram+ve bacteria

Haung,2011

Bacterial Quorum Sensing
Gram-positive vs. Gram-negative bacteria

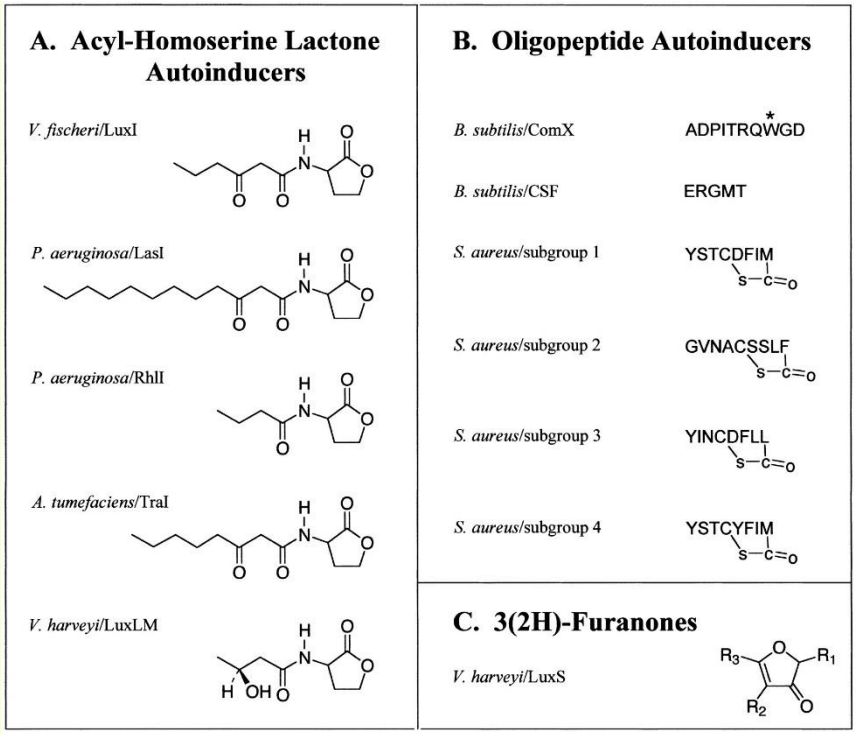
- Three classes of molecules used as autoinducers:

A. Representative AHLs used by Gram-negative bacteria.

B. Representative oligopeptide autoinducers used by Gram-positive bacteria.

C. The general structure of the 3(2H) class of furanones. A 3(2H)-furanone is suspected to be the LuxS-dependent autoinducer called AI-2.

- The latter found both in Gram-ve and Gram+ve bacteria.



Schauder and Bassler 2001;..

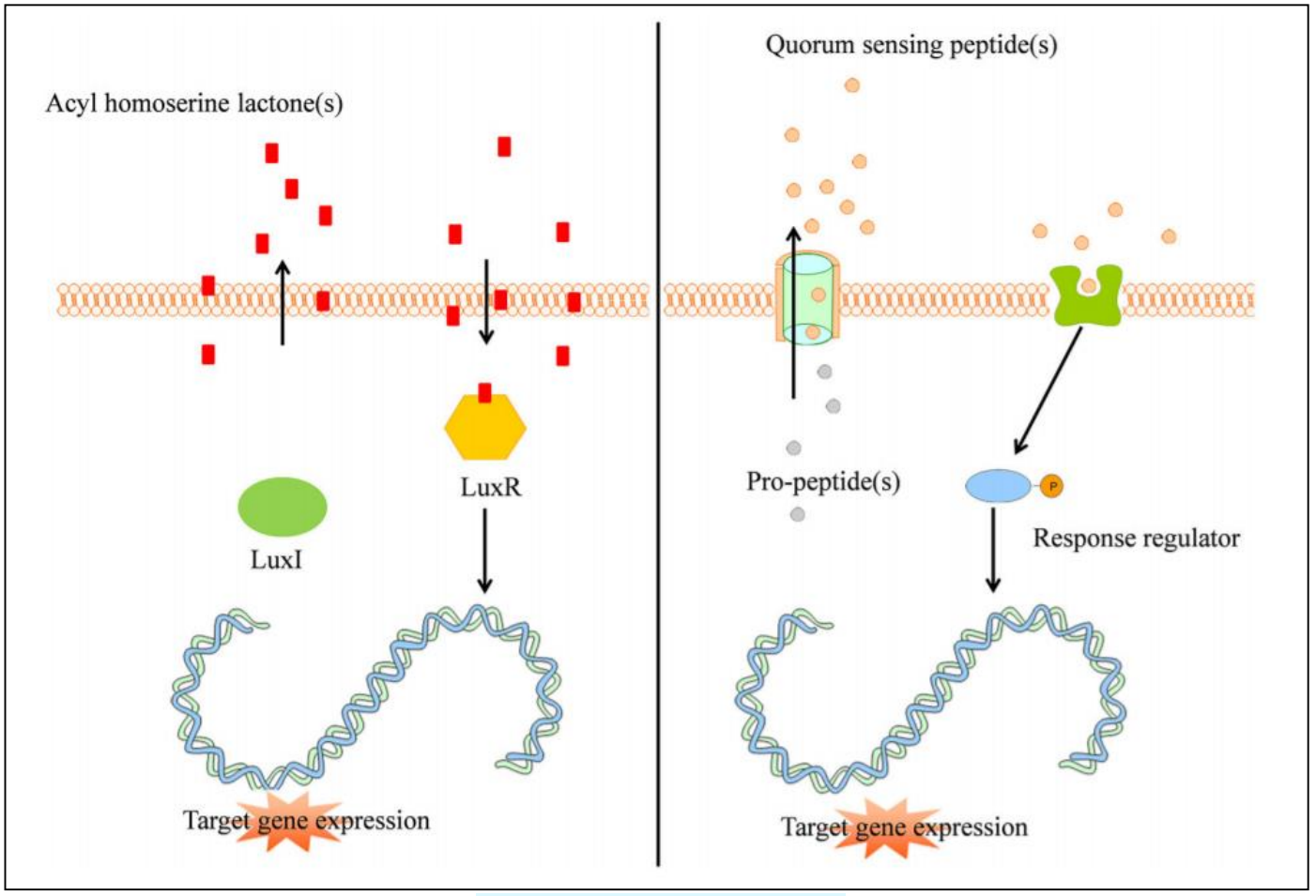
Bacterial Quorum Sensing
Gram-positive vs. Gram-negative bacteria

- Typical Gram-negative quorum sensing mechanism:
- Acyl homoserine lactone molecules, synthesized by LuxI, passively pass the bacterial cell membrane and when a sufficient concentration is reached (threshold level) activate the intracellular LuxR which subsequently activates target gene expression in a coordinated way.
- Typical Gram-positive quorum sensing mechanism:
- Quorum sensing peptides are synthesized by the bacterial ribosomes as pro-peptidic proteins and undergo posttranslational modifications during excretion by active transport. The quorum sensing peptides bind membrane associated receptors which get autophosphorylated and activate intracellular response regulators via phosphor-transfer. These phosphorylated response regulators induce increased target gene expression.

Verbeke *et al.*,2017

Bacterial Quorum Sensing

Gram-positive vs. Gram-negative bacteria



Verbeke *et al.*,2017

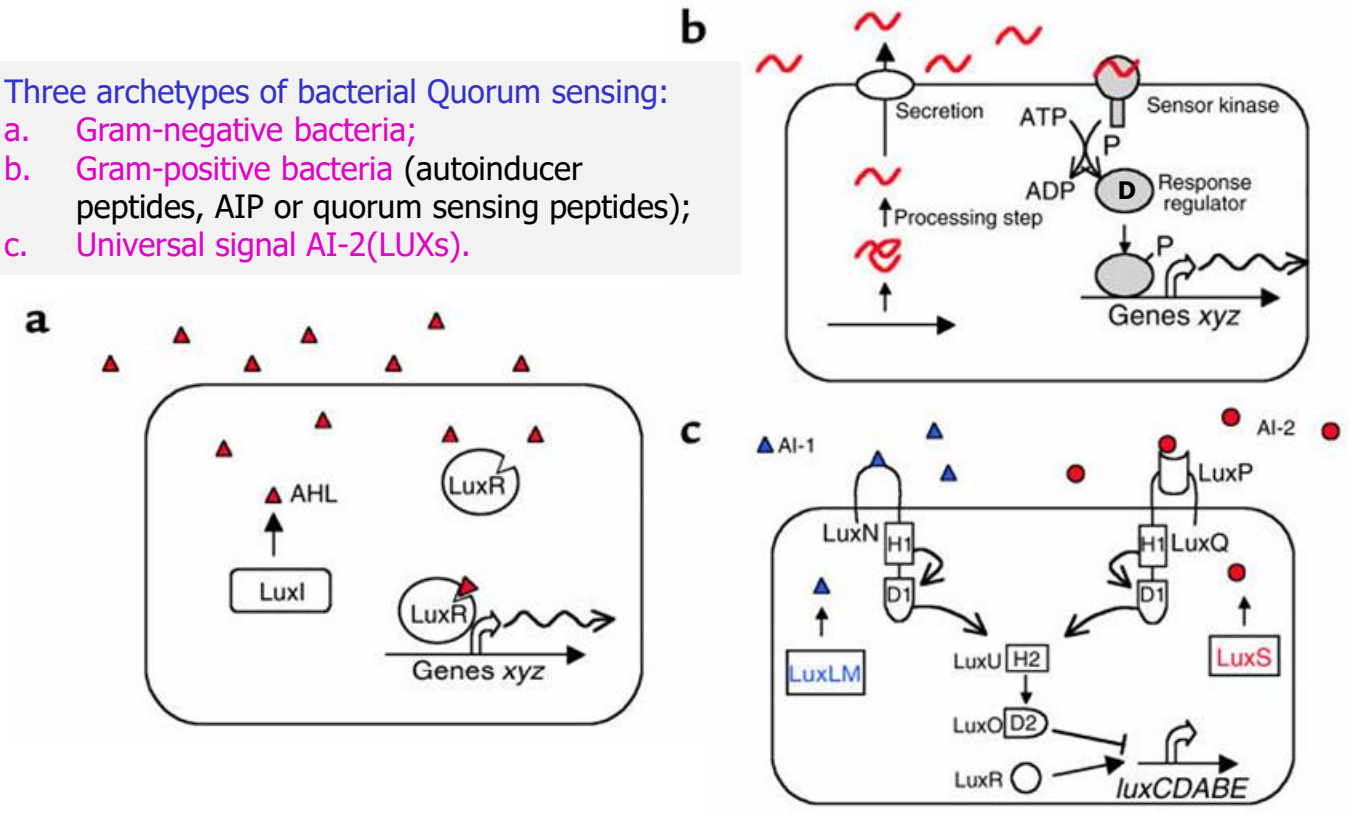
Autoinducers

Three typical quorum sensing circuits in G-ve and G+ve bacteria

a) AHL, b) Autoinducer peptide (AIP), C) Autoinducer 2(AI-2) or LuxS

Three archetypes of bacterial Quorum sensing:

a. Gram-negative bacteria;
b. Gram-positive bacteria (autoinducer peptides, AIP or quorum sensing peptides);
c. Universal signal AI-2(LUXs).

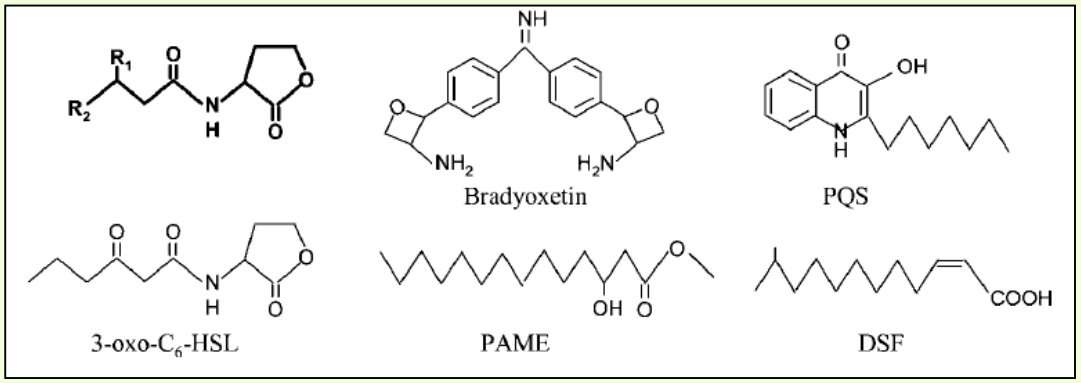


Haung,2011

Structures of AHL signals and non-AHL autoinducers

Gram negative bacteria

- **AHL signals:**
- N-Acylhomoserine lactones (AHLs).
- 3-oxohexanoylhomoserine lactone (3-oxo-C6-HSL) produced by several plant pathogens.
- **Non-AHL signals:**
- Bradyoxetin identified in *Bradyrhizobium japonicum*.
- 3-OH PAME (3-hydroxypalmitic acid methyl ester) produced by *Ralstonia solanacearum*.
- DSF (diffusible signal factor, cis-11-methyl-2-dodecenoic acid) of *Xanthomonas campestris*.



Soto *et al.*,2006

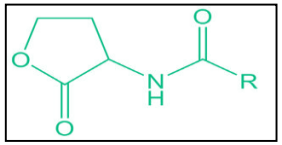
Autoinducers

Gram-negative bacteria

Six types of autoinducers

- Two main types of AIs have been described in Gram-negative bacteria (AI-1 and AI-2).

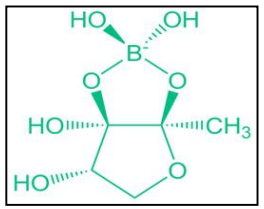
1. **AI-1 molecules or Acyl homoserine lactones (AHLs). Also called autoinducer 1(AI-1):**



- Used for intraspecies communication.
- Example: in *Vibrio fischeri* and many other Gram-negative bacteria such as *Erwinia* spp., and *Escherichia* spp.

2. **AI-2 is a unique furanosyl borate diester. Also called Autoinducer 2(AI-2) or LuxS:**

- It is considered to be a universal system used for interspecies communication (between species).
- Example: in *Vibrio fischeri* and *Salmonella enterica*.
- Exception: it is also found in Gram+ve bacteria.



Autoinducers

Gram-negative bacteria

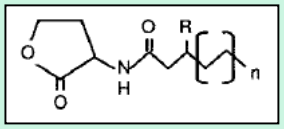
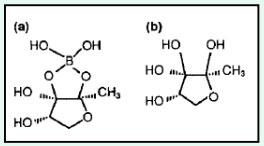
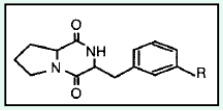
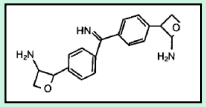
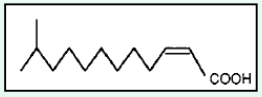
Six types of autoinducers

3. **AI-3. Also termed as Autoinducer 3(AI-3)**
   - A new bacterial signal was decoded in some species of Gram-negative bacteria which are resident in the human gastrointestinal tract and might also be involved in inter-kingdom signaling.
4. **Cyclic dipeptides:**
   - A new class of autoinducers was recently identified in strains of *Pseudomonas*.
5. **Bradyoxetin:**
   - A cell density factor was proposed to be involved in population density-dependent regulation of the *nod* genes in *Bradyrhizobium japonicum*.
6. **Diffusible signal factor (DSF):**
   - In *Xanthomonas* spp. and few more Gram-ve bacteria.

Autoinducers

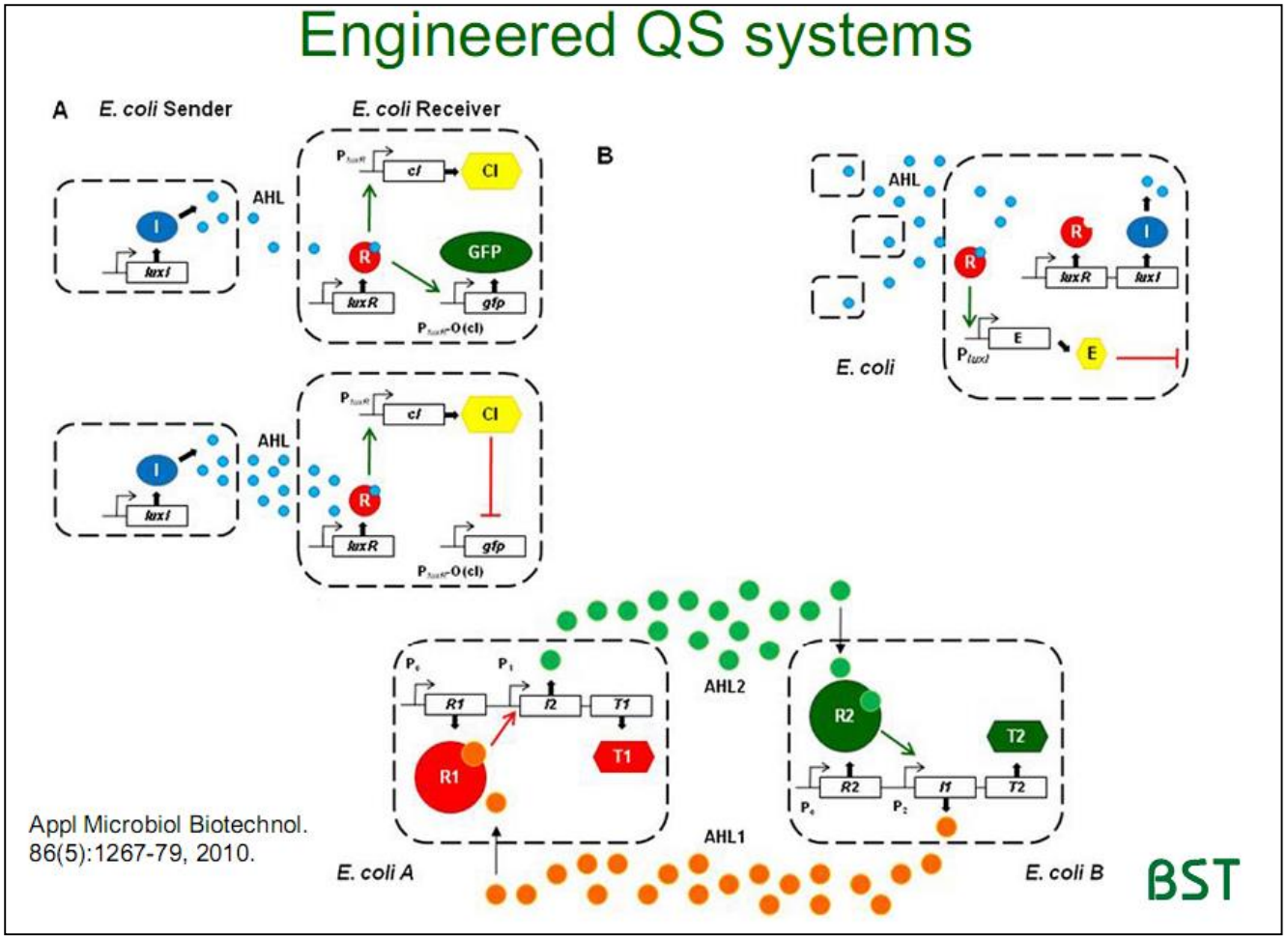
Gram-negative bacteria

Six types of autoinducers

Autoinducer(s)	General structure	Producing species	Phenotype(s) regulated
AHL(AI-1)		Many Gram-ve bacteria	Motility, exopolysaccharides, biofilms, others
AI-2 (LuxS)		Both Gram-ve and Gram+ve bacteria	Bioluminescence, ABC transporters
AI-3		Some species of Gram-negative bacteria	responsible for activating gene expression in *Salmonella*
Cyclic dipeptides		*P. fluorescens*, *P. alkaligenes*	Cross activate quorum sensing indicator strains
Bradyoxetin		*B. japonicum*	Nodulation
DSF		*X. campestris*	Endoglucanase production

González and Keshavan,2006;..

Engineered quorum sensing circuits(systems)



Haung,2011

Autoinducers

Common signal for both G-ve and G+ve bacteria

The universal system: Autoinducer 2 (AI-2) or LuxS

- AI-2 was first recognized as a quorum-sensing signal in marine bacterium *Vibrio harveyi*.
- Since then, this type of signaling has been discovered in many gram-negative bacteria, such as *Salmonella* spp., *Erwinia* spp., and *Escherichia* spp.
- The AI-2-type signaling is involved in the:

1. Regulation of bioluminescence in *V. harveyi*,
2. Type III secretion in *Escherichia coli* O157:H7,
3. The virulence factor VirB in *Shigella flexneri*.

González and Keshavan,2006

Autoinducers

Common signal for both G-ve and G+ve bacteria

The autoinducer-2 (AI-2) or LuxS QS system

- *E. pyrifoliae*: Black stem blight (necrotic disease) of Asian pear trees.
- Both *E. billingiae* and *E. tasmaniensis* (nonpathogenic species) are considered part of the apple and pear microbiota. These epiphytic bacteria may represent antagonists for biocontrol of fire blight.
- *Erwinia piriflorinigrans* : causal agent of pear blossom necrosis.

Species	Capsular EPS	Levan formation from sucrose	AHL synthesis	AI-2 Production	Virulence on apple/pear	HR on tobacco
E. amylovora	+	+	−	+	+/+	+
E. pyrifoliae	+	−	−	+	−/+	+
E. piriflorinigrans	ND	+	ND	ND	−/+	+
E. billingiae	+	−	+	+	−/−	−
E. tasmaniensis	−	+	−	+	−/−	+

- AHL acyl-homoserine lactones, AI-2 autoinducer 2, HR hypersensitivity response, ND Not determined.
- *E. billingiae* possess both autoinducers of AHL and A1-2.

Palacio-Bielsa *et al.*,2012

AHL signals in Gram-ve bacteria

Non-acyl HSL-based systems

Diffusible signal factor (DSF and BDSF)

- Some bacteria such as *Xanthomonas* spp., *Burkholderia* spp., *Ralstonia solanacearum*, rhizobia, etc. utilize non-acyl HSL QS signal molecule known as DSFs.
- DSF-based quorum sensing is a new language in Gram-negative bacteria.
- Diffusible signal factor (DSF) regulates virulence and biofilm dispersal in *Xanthomonas* spp. and *Burkholderia* spp.
- BDSF was found from *Burkholderia cenocepacia*.

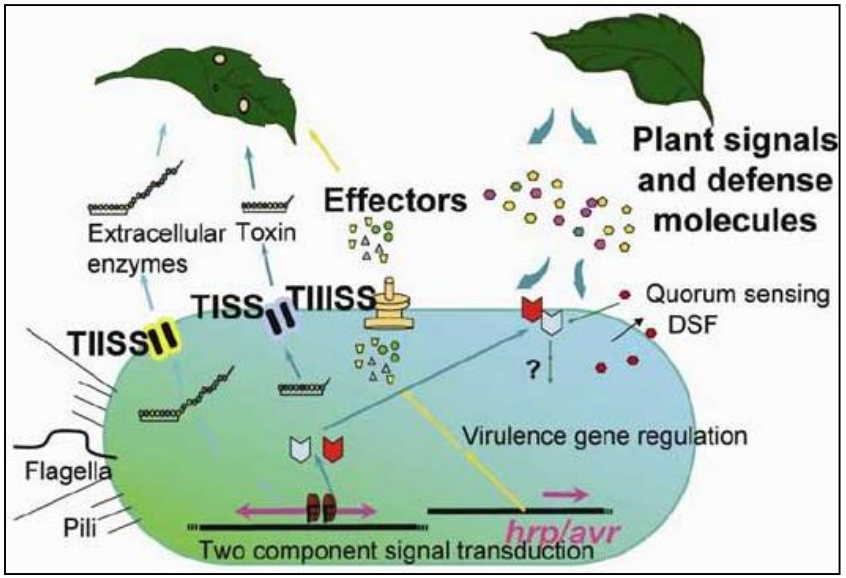
Chatterjee,2009; Deng *et al.*,2010; Wu *et al.*,2011

AHL signals in Gram-ve bacteria

Non-acyl HSL-based systems

Diffusible signal factor (DSF and BDSF)

- DSF originally identified from the plant bacterial pathogen *Xanthomonas campestris* pv. *campestris*.
- BDSF was found from *Burkholderia cenocepacia*.
- In these cases, two QS systems appear to act in conjunction in the regulation of *B. cenocepacia* virulence (fingertip rot disease of banana).



Three secretion systems:
TISS,TIISS and TIIISS

Chatterjee,2009; Deng *et al.*,2010; Wu *et al.*,2011

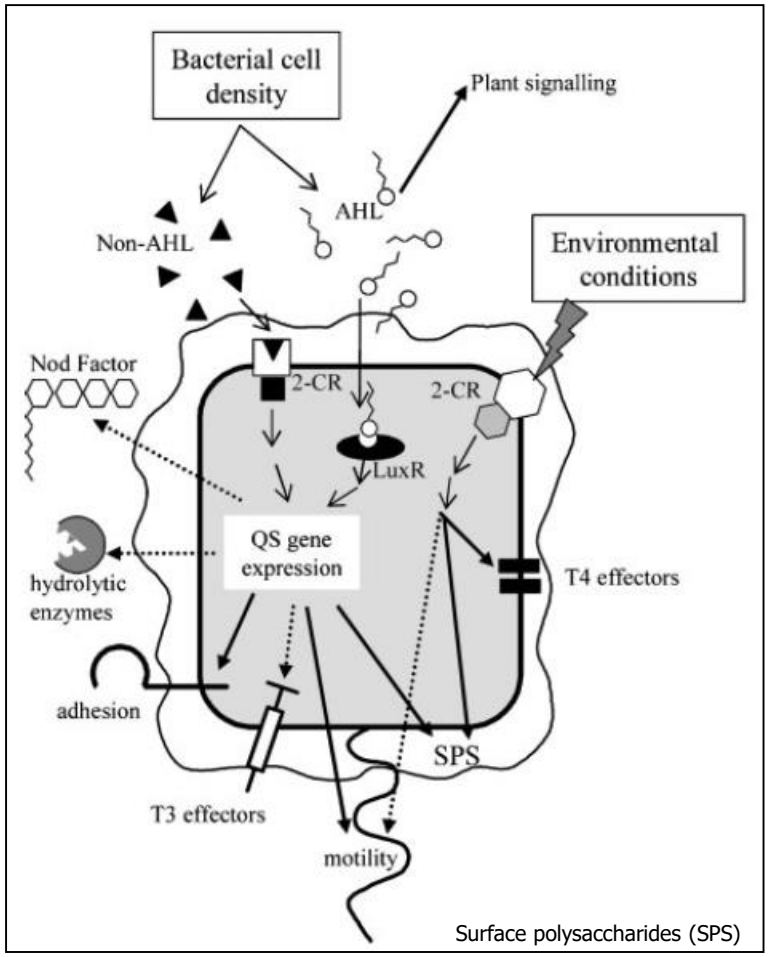
DSFs: non-AHL signals

DSF-based quorum sensing

Xanthomonas spp.

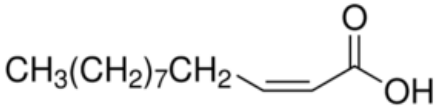
- Host colonization and invasion mediated by quorum sensing (QS) signals and two component regulatory (2-CR) systems.

1. Detection of N-acylhomoserine lactones (AHL, loop and tail) by cytoplasmic LuxR-type transcriptional activators (black oval), and
2. Non-AHL (black triangles) by 2-CR systems (white and black squares), allow plant interacting bacteria to coordinate the expression of important genes for host colonization and invasion in response to cell density.

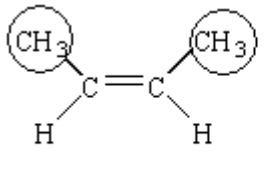


Soto *et al.*,2006

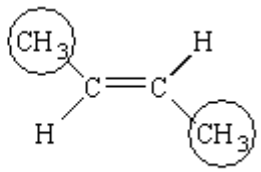
DSFs



(*cis* -2-dodecenoic acid)



cis-2-Butene



trans-2-Butene

Structure of Non-AHL signals

- Diffusible signal factor (DSF) of *Xanthomonas campestris* pv. *campestris* is reported as *cis*-11-methyl-2-dodecenoic acid.
- *Burkholderia cenocepacia* produce a DSF QS signal molecule known as BDSF(*cis* -2-dodecenoic acid) analogue of *cis*-11-methyl-2-dodecenoic acid.
- A DSF, 12-methyl-tetradecanoic acid was shown to be produced by *Xylella fastidiosa*.
- In these cases, the signals seem to be detected through two-component regulatory systems.

Dodecanoic acid($C_{12:0}$). 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."

Soto *et al.*,2006; Deng *et al.*,2010

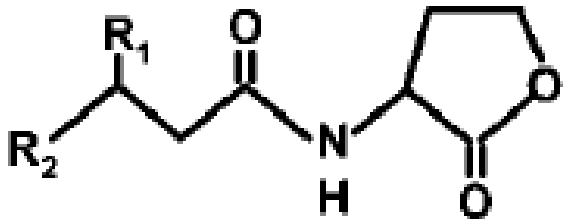
AHL signals in Gram-ve bacteria
Structure of acyl-HSLs

- AHL was the very first QS signal to be detectedin Gram-negative bacteria (Eberhard *et al.*,1981).
- It consists of a lactonized homoserine moiety attached by an amide bond(-CONH2) to an acyl side chain.
- Homoserine lactone ring is conserved and connected to a variable acyl chain.
- The acyl side chain, however, can vary in:

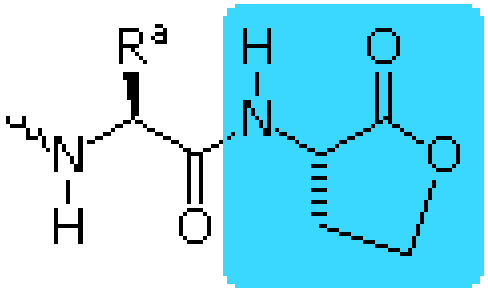
1. Length, and
2. Degree of substitution.

AHL autoinducers all share a common homoserine lactone moiety and differ only in their acyl group.

Fine structure of N-Acylhomoserine lactones (AHLs)



Acyl homoserine lactone (AHL)

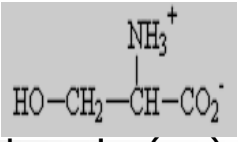


Lacton moiety of AHL

Acyl homoserine lactones are lactone rings with a carbonyl tail (C_4-C_{16}) with varying functional lengths (Nagy,2010).

Lactones are cyclic esters, a ring of two or more carbon atoms and a single oxygen atom with a ketone group at one of the carbons adjacent to the other oxygen.

Fine structure of N-Acylhomoserine lactones (AHLs)



L- serine(ser)

- L-Homoserine is not one of the common amino acids encoded by DNA.
- Homoserine (also called isothreonine) is an α-amino acid with the chemical forumula: $HO_2CCH(NH_2)CH_2CH_2OH$.
- L-Homoserine differs from the amino acid serine by insertion of an additional -CH2-unit into the backbone.
- Homoserine, or its lactone form, is the product of a cyanogen bromide cleavage of a peptide by degradation of methionine.

Wikipedia,2013

AHL signals in Gram-ve bacteria
Structure of acyl-HSLs

- The family of N-Acyl homoserine lactones (AHL/ASL) seem to be an almost universal signal factor in Gram negatives.
- Because in the most well-studied systems the autoinducers(AIs) were N-acylated derivatives of L-homoserine lactone (acyl-HSL).
- Signal specificity of the acyl side-chain is conferred by:

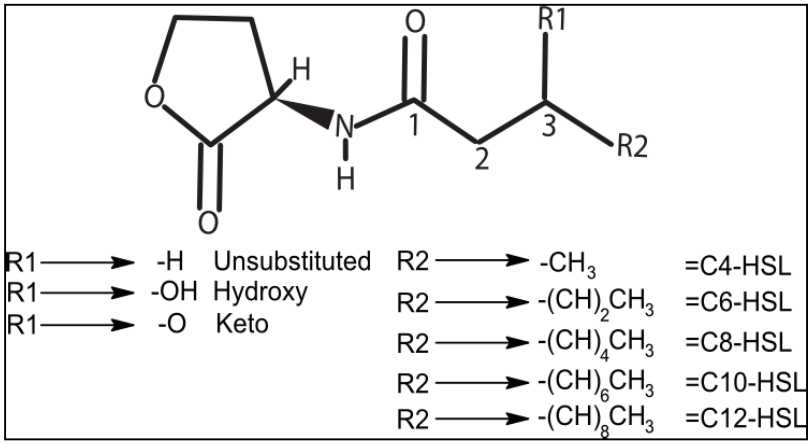
1. An acyl chain C-3 substituent (oxo or hydroxy), or
2. The length of the N-acyl side chain.

AHL signals in Gram-ve bacteria

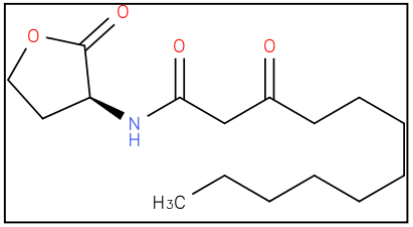
Structure of Acyl homoserine lactones (AHLs)

1. R1 group is defined as substitutions on the third carbon, and
2. R2 group is defined as acyl chain length.

- The acyl chains can vary with even carbon numbers starting from C4, C6, C8 to up to 12 carbons or even more.
- Odd number of carbons in the side chains has also been reported.



Usual substitutions are on the third position (R1 in Figure 3) with hydrogen (un-substituted), a keto (3-oxo), or a hydroxy (3-OH).



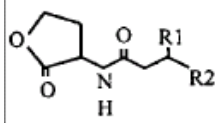
N-(3-oxododecanoyl)-L-homoserine lactone.

Annous *et al.*,2009

AHL signals in Gram-ve bacteria

Structure and functions of AHLs

- Acyl side-chain length and the substitutions on the side chain provide signal specificity.
- Acyl side chains of the signals can be fully saturated, they can have hydroxyls(OH) or carbonyls(C=O) on the third carbon, and they can have lengths of 4 to 16 carbons.
- R_1= –H, –OH or =O;
- R_2= $–CH_3$, $–(CH_2)_{2–14}CH_3$ or $–(CH_2)_5CH=CH(CH_2)_5CH_3$.

Structure	R		AHL molecule	Bacteria	Behaviour
	R1	R2			
 The general structure of AHLs (acyl-HSLs)	H	H	C4-HSL	*S. liquefaciens*	Cell motility/swarming
	OH	H	3-hydroxy-C4-HSL	*V. harveyi*	Bioluminescence
	H		C6-HSL	*C. violaceum*	Pigments/antibiotics/chitinase
	O		3-oxo-C6-HSL	*E. carotovora* *P. aureofaciens*	Pathogenicity/antibiotics Biocontrol activity/antibiotics
	H		C7-HSL	*R. leguminosarum*	?
	O		3-oxo-C8-HSL	*A. tumefaciens*	Conjugation
	OH		3-hydroxy-C14:1-HSL	*R. leguminosarum*	Growth inhibition/rhizosphere genes

Fray,2001;..

AHL signals in Gram-ve bacteria
Diffusibility of the acyl-HSLs

- The diffusion speed is correlated with the nature of the acyl chain and long acyl chain AHLs, if they can diffuse at all, would diffuse more slowly than short acyl chain AHLs.

1. **Short-chain signals** such as C4-C6 HSLs (detected by biosensor *C. violaceum* CV026)diffuse freely through the cell membrane.
2. **Long chain signals** such as 3-oxoC12-HSL(detected by biosensor *A. tumefaciens* NTL4 (pZLR4) partitions into cells, presumably in the membrane.
3. **Signals like 3-oxoC12-HSL** can diffuse into the surrounding environment but export is enhanced by the mexAB-oprM (a multi-component channel) and perhaps other, efflux pumps.

Parsek and Greenberg,2000;Nagy,2010

Efflux pumps

Single or a multi-component channels

- Structurally, efflux pumps occur either as:

1. Single component, or
2. Multi-component systems.

- **TetA or CmlA as single-component efflux:**
- In gram-negative bacteria, single-component efflux pumps, such as TetA or CmlA, transfers substrates from the cytoplasm to the periplasm space.
- **MexAB-oprM as multi-component efflux:**
- In the multi-component pump, such as mexAB-oprM, substrates were transferred into external medium.
- MexAB-OprM protein is expressed by *MexAB-OprM* gene.

Lomovskaya and Watkins,2001;..

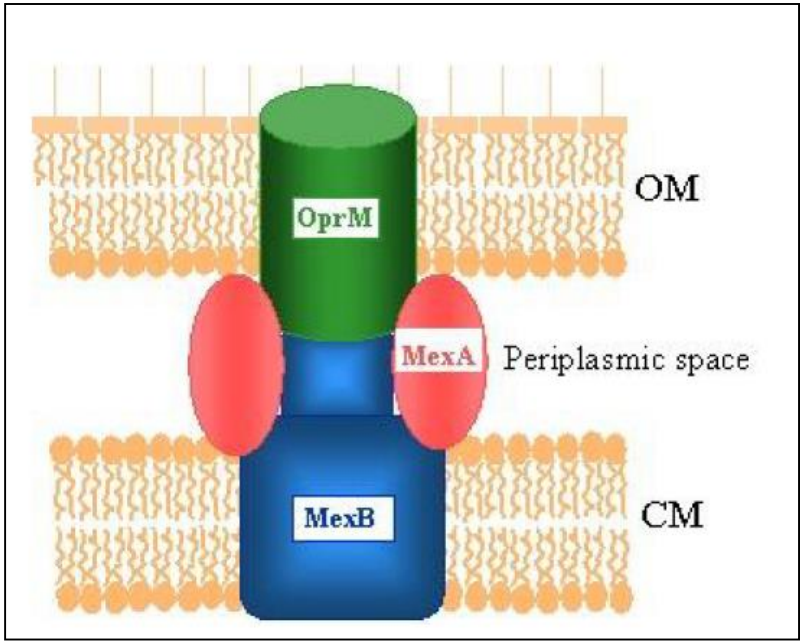
Efflux pumps

MexAB-OprM multidrug efflux pump

- Efflux-pumps are present in all organisms.
- These efflux pumps can remove (expel) antibiotics, dyes, detergents, solvents and other compounds from the cell e.g. bacterial cell before they can cause harm.
- There are two types of efflux pumps that are seen in bacteria:

1. Those which are encoded in the chromosome and
2. Those which are plasmid borne.

- MexAB-OprM is under quorum sensing control.



OM: outer membrane, CM: cytoplasmic or inner membrane.

Nagy,2010

Efflux pumps

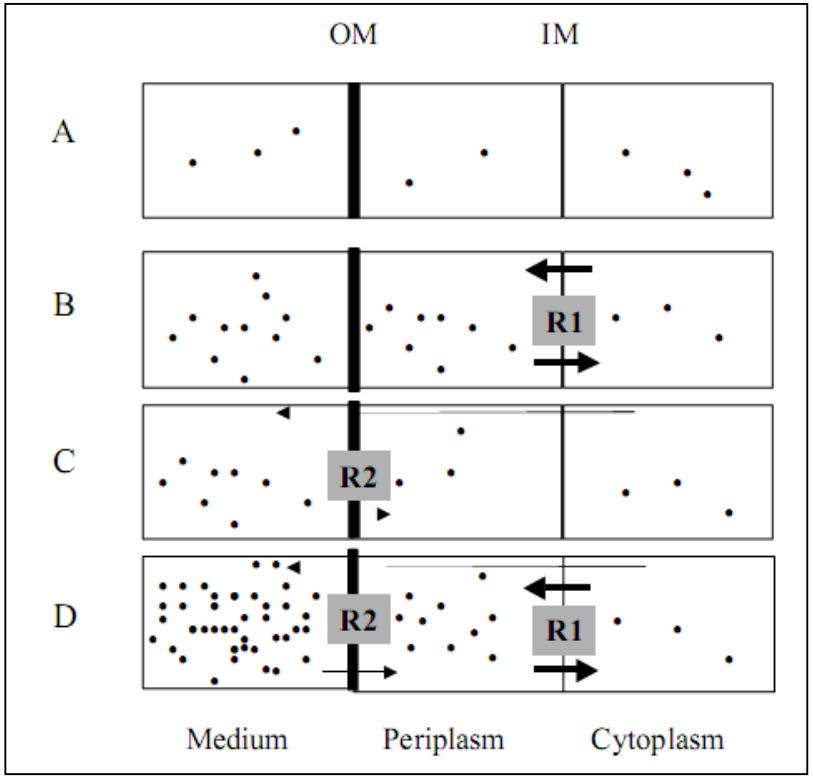
Single or a multi-component channels

A. In cells without efflux pumps all compartments are essentially in equilibrium.

B. In cells expressing a single-component pump, substrates were transferred from the cytoplasm to the periplasm space.

C. In the multi-component pump, such as mexAB-oprM, substrates were transferred into external medium.

D. In the case of simultaneous expression of both efflux pumps, concentration gradients are maintained at both the inner and outer membranes.



R1: A concentration gradient exists at the inner membrane; R2: A concentration gradient exists across the outer membrane.

Lomovskaya and Watkins,2001

The names and abbreviations of some signaling molecules (compounds) in bacteria

- C4-HSL, N-butanoyl-L-homoserine lactone;
- C6-HSL, N-hexanoyl-L-homoserine lactone;
- C7-HSL, N-heptanoyl-L-homoserine lactone;
- C8-HSL, N-octanoyl-L-homoserine lactone;
- C10-HSL, N-decanoyl-L-homoserine lactone;
- C12-HSL, N-dodecanoyl-L-homoserine lactone;
- C14-HSL, N-tetradecanoyl-L-homoserine lactone;
- 3-oxo-C6-HSL, N-(3-oxohexanoyl)-L-homoserine lactone;
- 3-oxo-C8-HSL, N-(3-oxooctanoyl)-L-homoserine lactone;
- 3-oxo-C10-HSL, N-(3-oxodecanoyl)-L-homoserine lactone;
- 3-oxo-C12-HSL, N-(3-oxododecanoyl)-L-homoserine lactone;
- 3-oxo-C14-HSL, N-(3-oxotetradecanoyl)-L-homoserine lactone.

Numerical multiplier (or multiplying affix

Number	Multiplier	Number	Multiplier
1	mono-	32	dotriaconta-
2	di-	40	tetraconta-
3	tri-	50	pentaconta-
4	tetra-	60	hexaconta-
5	penta-	70	heptaconta-
6	hexa-	80	octaconta-
7	hepta-	90	nonaconta-
8	octa-	100	hecta-
9	nona-	200	dicta-
10	deca-	300	tricta-
11	undeca-	400	tetracta-
12	dodeca-	500	pentacta-
13	trideca-	600	hexacta-
14	tetradeca-	700	heptacta-
15	pentadeca-	800	octacta-
16	hexadeca-	900	nonacta-
17	heptadeca-	1000	kilia-
18	octadeca-	2000	dilia-
19	nonadeca-	3000	trilia-
20	icosa-	4000	tetralia-
21	henicosa-	5000	pentalia-
22	docosa-	6000	hexalia-
23	tricosa-	7000	heptalia-
30	triconta-	8000	octalia-
31	hentriconta-	9000	nonalia-

Definition of a QS signal

Criteria for characterizing a molecule as a cell-to-cell signal molecule cell-to-cell signal molecule (CCSM)

- With increasing number of signals being detected (both in number and type) over the last couple of decades, there has arisen a need for defining

1. what is a signal, and
2. what is not a signal.

- Keeping this in mind some researchers have set up certain criteria for characterizing a molecule as a cell-to-cell signal molecule (CCSM), for review on this topic see Winzer *et al*., and Monds and O'Toole *et al*., (Winzer *et al*.,2002, Monds and O'toole,2008).

Definition of a QS signal

Criteria for characterizing a cell-to-cell signal molecule (CCSM)

- These criteria are as follows:

1. CCSM should be produced at a certain phase of growth (example lag, log or stationary), and it should be produced at certain conditions or as a response to a change in condition of the environment.
2. CCSM should be present in good amounts outside the cell and it should also bind to a receptor.
3. There should be a response once a critical threshold concentration of CCSM has been reached.
4. There should also be a cellular response to neutralize or destroy the CCSM.
5. The concentration of CCSM should not be at a toxic level.
6. The last and the most difficult to prove is that the CCSM is adaptive to the level of the community.

- This is hard to show as it involves evolutionary experiments, for example assessing whether a functional signaling network provides a fitness advantage in a challenging environment among competitors.

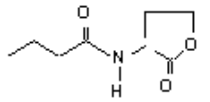
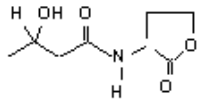
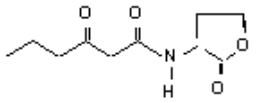
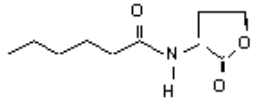
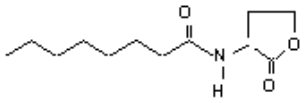
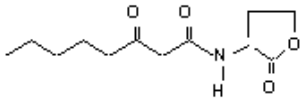
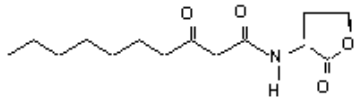
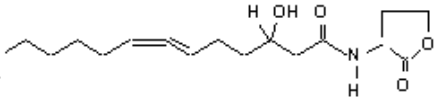
Synthesis of AHLs
Under laboratory conditions

- Could we synthetize some AHLs in the lab?
- The best you can do to get those standards is to grow bacteria which produce them and extract them.
- For example you can grow *B. cepacia* to get C8, and C6, and *P. aeruginosa* or *P. putida* to get OC10 and OC12.
- That would allow you to have them.
- You may then load them on a TLC and once developed, you may re-extract each spot to have a cleaner standard.

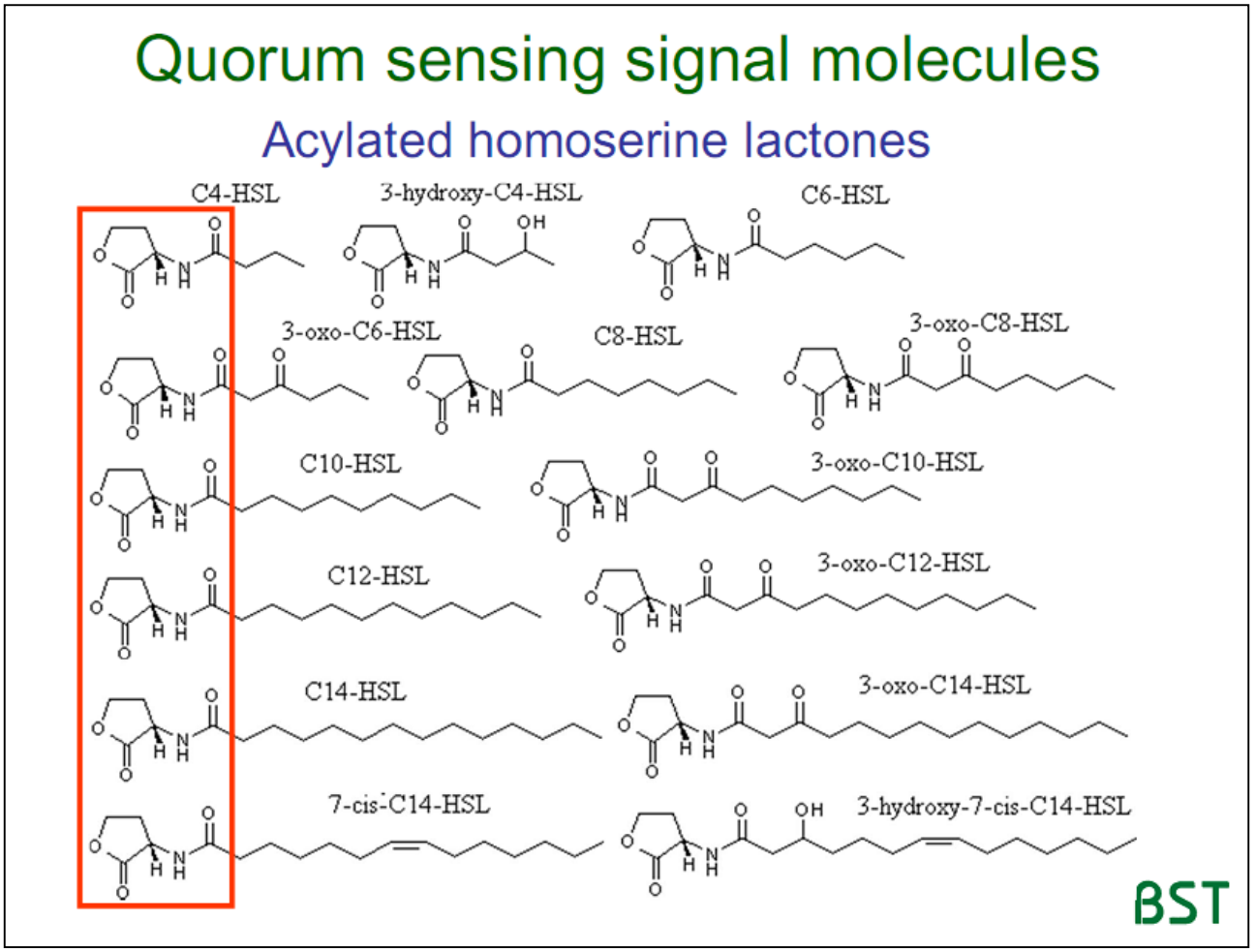
Structure of AHL signals produced by G-ve bacteria

The standard AHL/luxI/luxR signaling systems

- **Note that the homoserine lactone moiety is conserved.**
- **Variation occurs primarily in the fatty acyl side chain.**

Pseudomonas aeruginosa	Butyryl AHL	
Vibrio harveyi	Hydroxybutyryl AHL	
Erwinia caratovora *Pantoea stewartii*	3-oxohexanoyl AHL	
Pseudomonas aureofaciens	Hexanoyl AHL	
Ralstonia solanacearum	Octanoyl AHL	
Agrobacterium tumefaciens	3-oxooctanoyl AHL	
Pseudomonas aeruginosa	3-oxododecanoyl AHL	
Rhizobium leguminosarum	3R-hydroxy-7-*cis*-tetradecenoyl AHL	

AHL signals in Gram-ve bacteria
Abbreviations



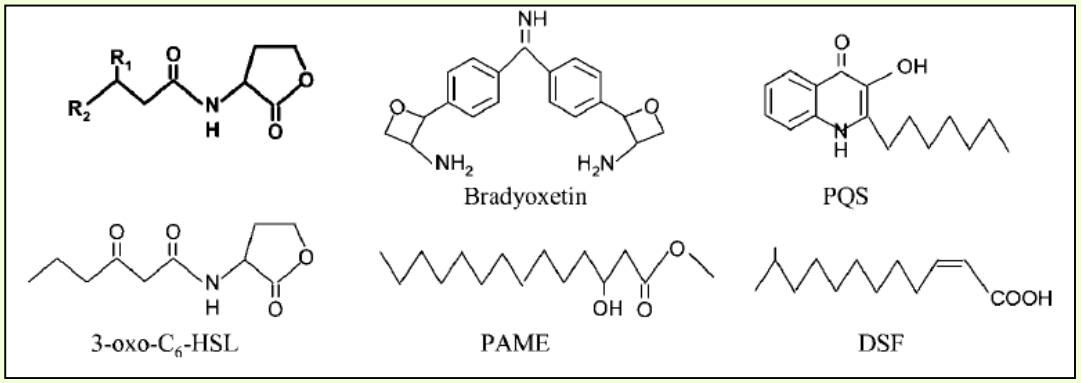
AHL signals in Gram-ve bacteria
Abbreviations

- **AHL:** N-acyl homoserine lactone
- **OHL:** N-octanoylhomoserine lactone
- **OHHL:** N-3 oxohexanoyl- L-homoserine lactone
- **HHL:** N-hexanoyl-L-homoserine lactone
- **3O,C12-HSL:** 3-Oxo,dodecanoyl-homoserine lactone
- **C4-HSL:** Butyroyl-homoserine lactone
- **C6-HSL:** Hexanoyl-homoserine lactone
- **C12-HSL:** Dodecanoyl-homoserine lactone
- **C14-HSL:** Tetradecanoyl-homoserine lactone

Structures of AHL signals and non-AHL autoinducers

Gram negative bacteria

- **AHL signals:**
- N-Acylhomoserine lactones (AHLs).
- 3-oxohexanoylhomoserine lactone (3-oxo-C6-HSL) produced by several plant pathogens.
- **Non-AHL signals:**
- Bradyoxetin identified in *Bradyrhizobium japonicum*.
- 3-OH PAME (3-hydroxypalmitic acid methyl ester) produced by *Ralstonia solanacearum*.
- DSF (diffusible signal factor, cis-11-methyl-2-dodecenoic acid) of *Xanthomonas campestris*.

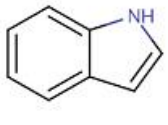


Soto *et al.*,2006

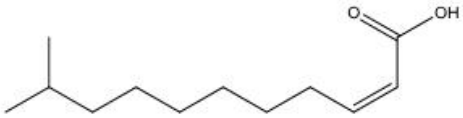
Structures of AHL signals and non-AHL autoinducers

Gram negative bacteria

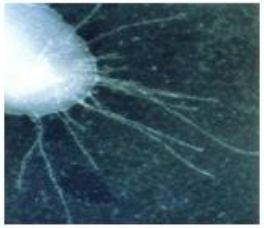
Escherichia coli: Indol

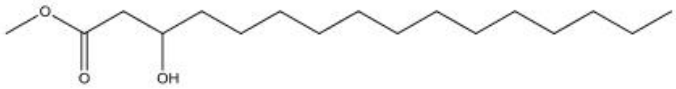


Xanthomonas campestris: DSF

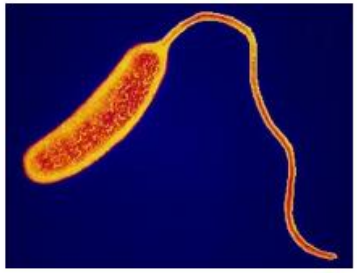


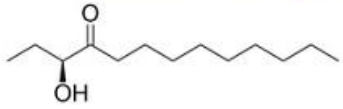
Ralstonia solanacearum: 3OH-PAME





Vibrio cholerae: CAI-1





Microbiol Rev 2008, 32(5):842-57.

Structure & Functions of AHLs

Some diffusible signals utilized by Gram +ve and-ve bacteria

Signal	Mediates
N-acyl-homoserine lactones (AHLs)	Antibiotic synthesis, virulence gene expression, etc.
Butyrylactones	Antibiotic synthesis in *Streptomyces* spp.
Amino acids	Swarming in *Proteus* spp.
Peptides	• competence in *Bacillus* spp. • fruiting body formation in *Myxococcus* spp. • conjugal plasmid transfer in *Enterococcus* spp.

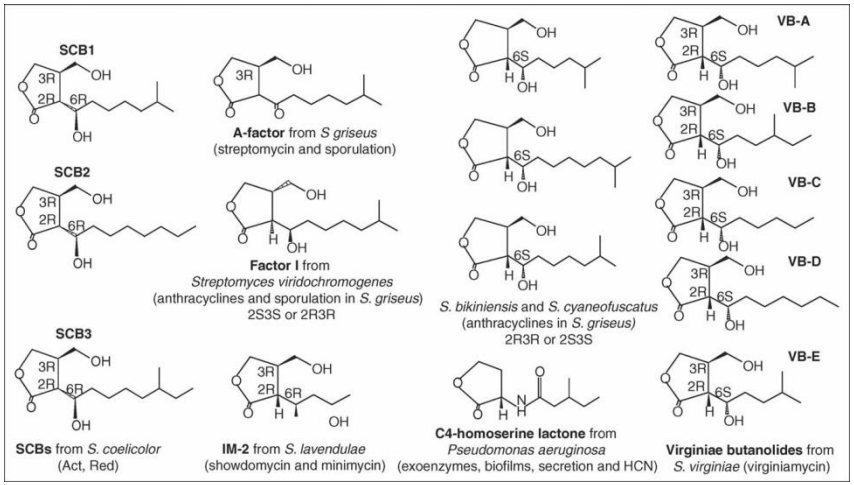
In Gram-positive bacteria, such as *B. subtilis* oligopeptides are the signalling molecules (Lazdunski *et al.*,2004)

Pierson III,2005

Structure & Functions of AHLs

Some diffusible signals utilized by Gram+ve streptomyces and-ve bacteria

- Small signaling molecules called gamma-butyrolactones (colorless oily liquid with a weak characteristic odor and is soluble in water) are mainly produced by *Streptomyces* species in which they regulate antibiotic production and morphological differentiation.



The chemical structure of gamma-butyrolactones is similar to that of AHLs except for the carbon side-chain.

Takano,2006

Structure & Functions of AHLs

Examples of N-acyl homoserine lactone based regulatory systems

Bacterial species	Signal molecules	Regulatory Proteins	Target function(s)
Agroabcterium tumefaciens	N-3-(oxooctanoyl)-homoserine lactone (AAI)	TraI/TraR-TraM	tra genes, traR, Ti plasmid conjugal transfer
Erwinia carotovora **subsp. *carotovora*** **SCRI193**	VAI-1	ExpI/ExpR	pel, pec, pep, exoenzyme synthesis
Erwinia carotovora **subsp. *carotovora*** **SCC3I93**	VAI-1	CarI/CarR	cap, carbapenem antibiotic synthesis
Erwinia stewartii	VAI-1	EsaI/EsaR	wts genes, exopolysaccharide synthesis, virulence factors
Pseudomonas aeureofaciens	(PRAI)	PhzI/PhzR	phz, phenazine biosynthesis

Whiteley *et al.*,2005

Structure & Functions of AHLs

Examples of N-acyl homoserine lactone based regulatory systems

Bacterial species	Activities modulated by quorum sensing
Agrobacterium tumefaciens	conjugal transfer of the Ti plasmid
Erwinia caratovora	production of protease, cellulase, pectinase, exopolysaccharide, and antibiotics
Escherichia coli	cell division, but inducer molecule is not HSL
Photobacterium fischeri	bioluminescence
Pseudomonas aeruginosa	alkaline protease, elastase, cyanide (HCN) production, hemolysin, exotoxin A, neuraminidase, pyocyanin and rhamnolipid production
Rhizobium leguminosarum	expression of rhizosphere genes
Serratia liquefaciens	expression of rhizosphere genes

Szenthe and Page,2003

Multilingual bacteria
Multiple QS signals

- The presence of multiple QS signals in culture supernatants of gram-negative bacteria is not a rare finding. E.g.

1. *Pseudomonas aeruginosa*
2. *Pseudomonas aureofaciens*
3. *Pseudomonas putida*

González *et al.*,2009

Multilingual bacteria

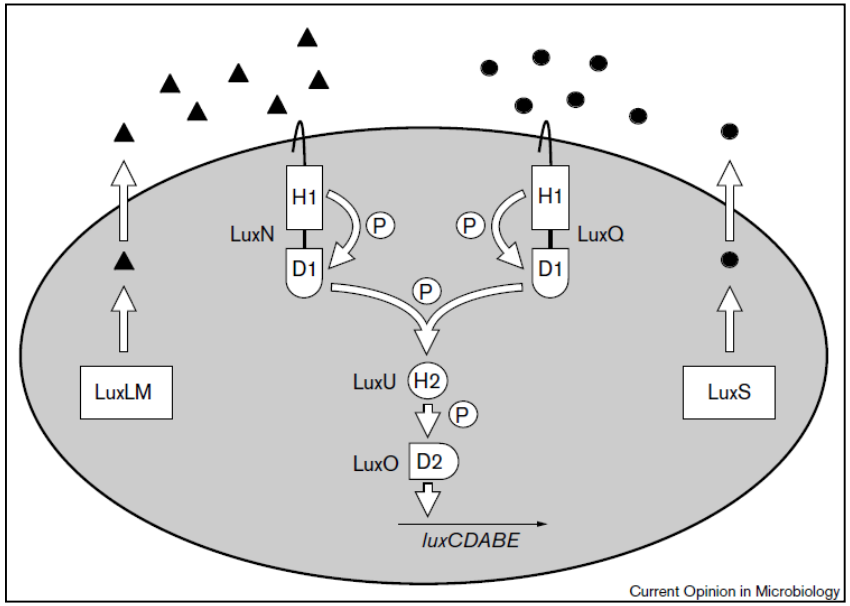
Two QS systems

Vibrio harveyi

- The two quorum sensing circuits of *V. harveyi* are shown.

1. **Autoinducer 1(AI-1):** A HSL autoinducer (triangles).
2. **Autoinducer 2(AI-2):**

- Synthesis of AI-1 and AI-2 is dependent on LuxLM and LuxS, respectively.



Bassler,1999

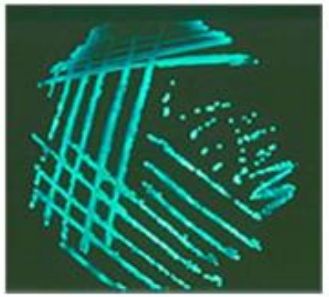
Different AHLs synthases have been described in different quorum sensing signalling pathways. For instance, in *V. harveyi* a 3-hydroxy-C4-HSL is synthesized by the LuxM synthase and received by luxN protein. The genes coding for these proteins show no homology to the previously described LuxR/I quorum sensing system.

Multilingual bacteria

Two QS systems

Vibrio harveyi

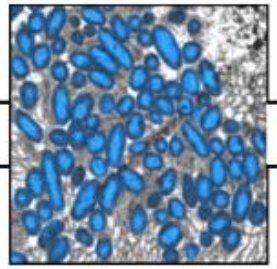
Multilingual bacteria



Vibrio harveyi

- A bioluminescence Gram (-) marine bacterium ; free living in the ocean

Communication system



AI-1 molecule

AI-2 molecule

Speaks language unique to V. harveyi

Speaks universal language used by many quorum-sensing bacteria

Haung,2011

Multilingual bacteria
Multiple QS signals

- *Pseudomonas aeruginosa*, in which quorum sensing systems have been extensively characterized, two main AHSLs are produced:

1. C4-HSL, and
2. 3oxo-C12-HSL.

- While the same signaling systems can synthesize other AHSLs in smaller amounts:

3. 3-oxo-C6-HSL, and
4. C6-HSL.

González *et al.*,2009

Multilingual bacteria
Multiple QS signals

- *In Pseudomonas putida*
- Main QS signals are:

1. 3-oxo-C10-HSL,and
2. 3-oxo- C12-HSL.

- Minor QS signals which are secreted in minor amounts are:

3. 3-oxo-C8-HSL, and
4. 3-oxo-C6-HSL.

González *et al.*,2009

Quorum sensing

Functions of different AHLs

Various gene systems regulated by quorum-sensing

1. *Pseudomonas aeruginosa*

- Many Gram-negative bacteria have been shown to possess one or more(multiple)quorum sensing systems.
- These systems regulate a variety of physiological processes, including the activation of virulence genes and the formation of biofilms.
- In case of ***Pseudomonas aeruginosa***, several genes including those required for the production of rhamnolipids and extracellular enzymes and toxins were regulated by two hierarchical quorum-sensing systems, each with its own transcriptional regulator and diffusible signal molecules.

Rhamnolipids are a quorum-sensing-controlled biosurfactant which increases the solubility of PQS in aqueous solutions.

Quorum sensing

LasI-R and RhlI-R systems

Pseudomonas aeruginosa

- Different LuxI homologs generate different acyl-HSLs.
- *Pseudomonas aeruginosa* has two quorum-sensing systems(cascading regulatory systems or Q-S circuits):

1. LasI/R
2. RhlI/R

- Both systems regulate the:

1. Production of virulence factors(e.g. elastase); protease, and
2. Biofilm formation.

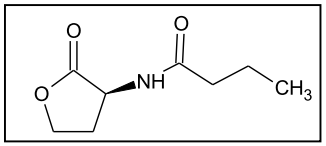
Elastase is an enzyme from the class of proteases (peptidases) that break down proteins.

Quorum sensing

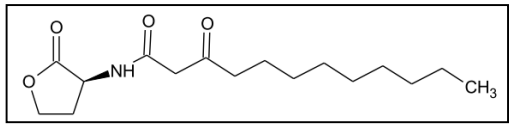
Types and functions of different AHLs

Pseudomonas aeruginosa

1. RhlI synthetase manufacture the autoinducer signaling molecule *N*-(butanoyl)-L-homoserine lactone (C_4-HSL) or BHL (C_4-HSL).



2. LasI synthetase directs the synthesis of autoinducer signaling molecule *N*-(3-oxododecanoyl)-HSL or 3-oxo-C_{12}-HSL or OdDHL.



This opportunistic pathogen controls many of its virulence factors and cellular functions through the activity of three cell-to-cell signals, *N*-butyryl-L-homoserine lactone, *N*-(3-oxododecanoyl)-L-homoserine lactone, and the *Pseudomonas* quinolone signal (PQS). The later is relatively insoluble in aqueous solutions (Calfee *et al.*,2005).

Quorum sensing

LasI-R and RhlI-R systems

Pseudomonas aeruginosa

- Both C4-HSL and 3-oxo-C_{12}-HSL signaling molecules diffuse out into the environment and, upon reaching a threshold concentration, activate receptors *lasR* and *rhlR*.

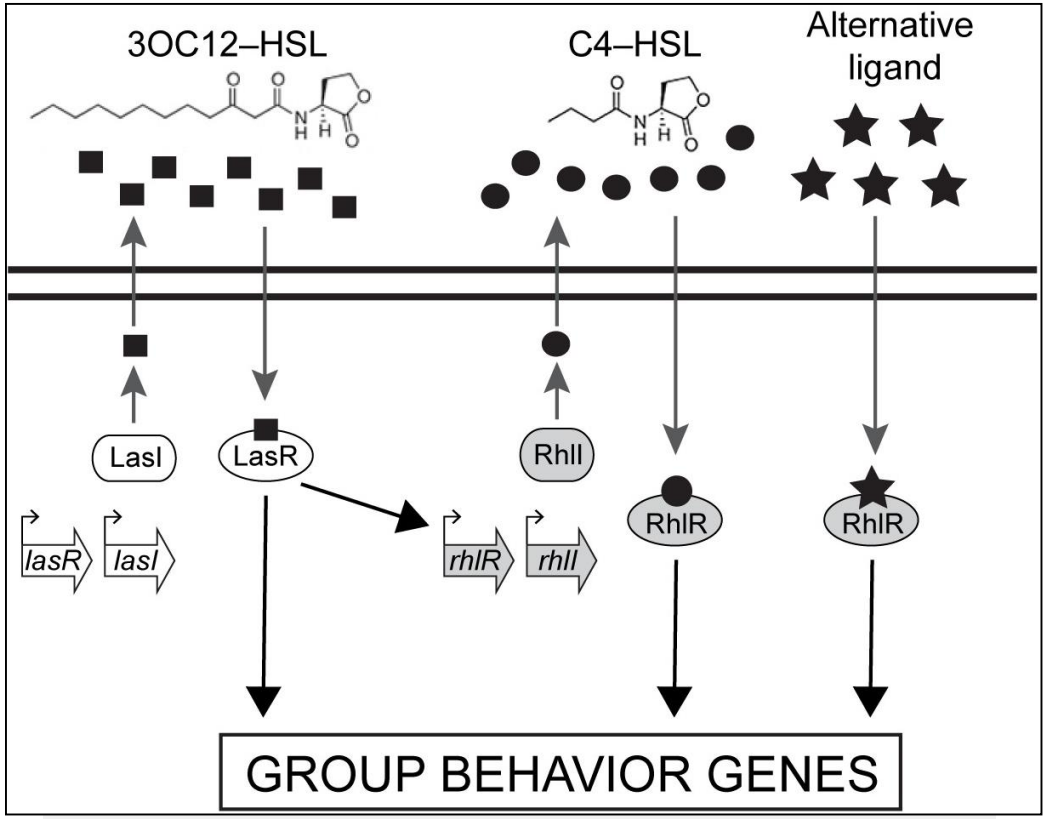
1. The virulence factors LasA (staphylolytic protease) and LasB (elastase) are thought to be under control of the lasI/R system, whereas
2. Pyoverdin is believed to be under rhlI/R control.

Adonizio,2008

Quorum sensing

LasI-R and RhlI-R systems

Pseudomonas aeruginosa



Either LasR or RhlR can bind with varying efficiency to any *lux*-box-like element.

Adonizio,2008; Mukherjee *et al.*,2017

Quorum sensing

LasI-R and RhlI-R systems

Pseudomonas aeruginosa

- When the 3-oxo-C_{12}-HSL signal molecule binds to the Las R regulator protein, two functions are carried out.

1. It triggers modulation of QS-regulated genes including genes associated with exoenzymes- elastase, alkaline and acid proteases, exotoxin A, secretion apparatus (Xcp) and biofilm development.
2. It also triggers the cascading regulatory expression of Rhl I/R genes.

- Rhl I/R gene products control the expression of a number of secondary genes including regions that code for: elastase, lectins, hydrogen cyanide, rhamnolipids and siderophores.

Nagy,2010

Alternative sigma factors and their roles in bacterial virulence

The stationary phase sigma factor RpoS

- Among the pathogenic bacteria, *Pseudomonas aeruginosa* is perhaps the best understood in terms of the virulence factors regulated and the role the Quorum sensing plays in pathogenicity.
- Regulation of Quorum sensing by RpoS in *Pseudomonas aeruginosa*.
- RsmA, RpoS, QocR all negatively regulate the Rhl or Las Quorum sensing systems, thus preventing early activation of these systems.

Whiteley *et al.*,2000; Deep *et al.*,2000

Quorum sensing

LuxI/LuxR type QS systems

Vibrio spp.

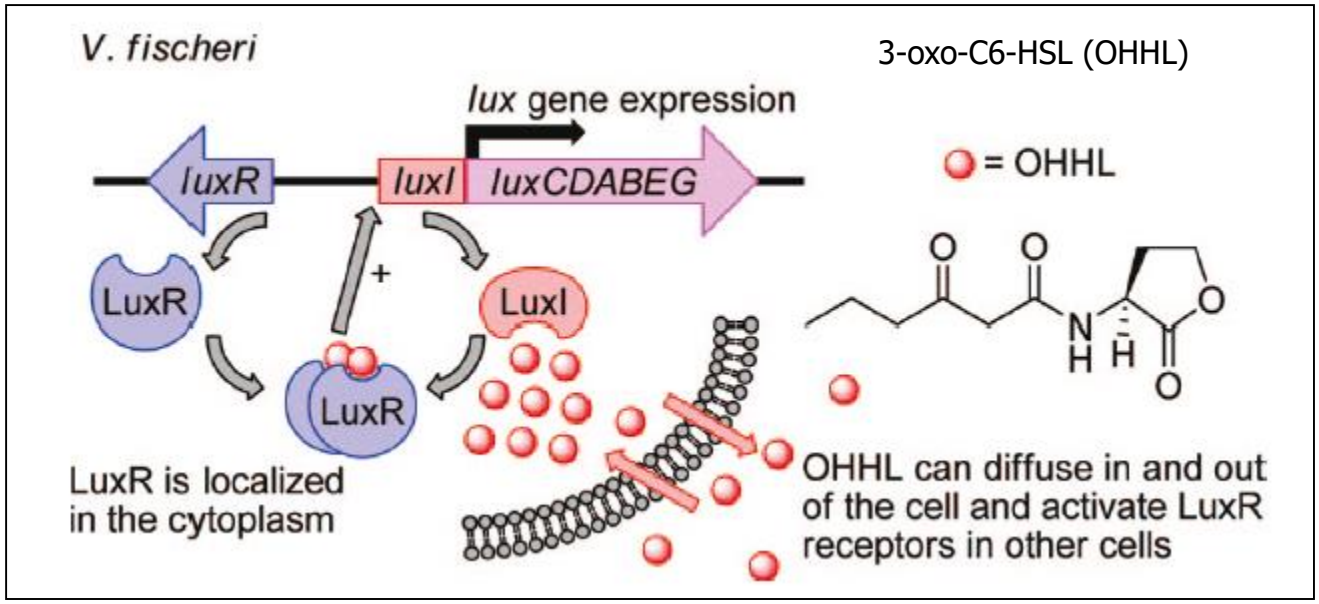
2. *Vibrio fischeri* (Photo-bacterium)

- The main machinery for the light production luxAB (luciferase) and luxCDE (aliphatic-aldehyde synthesis) have the same organization.
- The main difference is in the arrangement of luxR genes.
- The lux operon of *A. salmonicida* strain NCMB 2262 has been reported as novel with respect to arrangement of genes, as compared to that of *A. fischeri* and *V. harveyi* lux operons.

Quorum sensing

LuxI/LuxR quorum sensing system

V. fischeri



Galloway *et al.*,2011

Quorum sensing

Functions of different AHLs

Various gene systems regulated by quorum-sensing

3. *P. aureofaciens* 30-84

- Regulates production of phenazine antibiotics by quorum-sensing.
- These antibiotics have been implicated in the ability of this bacterium to control take-all disease of wheat in disease-suppressive soils.

Functions of different AHLs

Various gene systems regulated by quorum-sensing

Examples of LuxI-LuxR-type QS systems in plant bacterial pathogens

4. Plant Bacterial pathogens

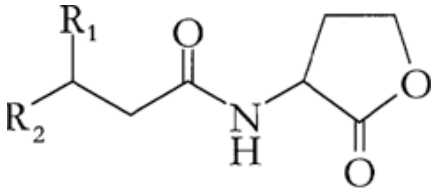
- *Agrobacterium tumefaciens*
- *Pectobacterium carotovoraum*
- *Pantoea stewartii*
- *Pantoea agglomerans* pv. *gypsophilae*
- *Ralstonia solanacearum*
- *Xanthomonas campestris* pv. *campestris.*
- Regulate the expression of traits involved in pathogenesis by quorum sensing systems.

Functions of different AHLs

Various gene systems regulated by quorum-sensing

Examples of LuxI-LuxR-type QS systems in plant bacterial pathogens

Bacteria	Regulators	Chain length (R2)	β R-group (R1)	Target function
Agrobacterium tumefaciens	TraI-TraR	C8 (4)	=O	Conjugal transfer
Erwinia carotovorum subsp. *carotovorum*	ExpI-ExpR	C6 (2) C8 (4)	=O =O	Extracellular enzymes and antibiotics
Pantoea stewartii	EsaI-EsaR	C6 (2)	=O	Exopolysaccharide



Sjöblom *et al.*,2009

Quorum sensing

TraI/TraR QS system

Agrobacterium tumefaciens

- In *A. tumefaciens*, the key QS regulators are encoded by the Ti plasmid, which includes TraI and TraR.
- TraI synthesizes the AHL signal *N*-3-oxooctanoyl-l-homoserine lactone (3OC8HSL), which binds to and activates TraR.
- TraR then binds to the palindromic *tra* box and thereby activates a number of operons that encode proteins necessary for Ti plasmid replication and conjugation.

tra genes for transfer of the Ti plasmid
between strains of *A. tumefaciens*, and genes for opine catabolism.

Wang *et al.*,2014

Quorum sensing

TraI/TraR QS system

Agrobacterium tumefaciens

- We further identified additional homologs (TraI2 and TraR2) in *A. tumefaciens*.
- *A. tumefaciens* A6 carries a second QS system(QS2) that may play a redundant role in the regulation of the replication and conjugation of the Ti plasmid.
- TraR2 is a functional homolog of TraR that recognizes 3OC8HSL and activates the QS-responsive genes.
- A *traI* mutant still produces an AHL signal that is likely to be 3OC8HSL, suggesting the presence of one or more additional AHL synthetases in this bacterial strain.
- The QS2 genes are located on a transmissible genetic element in *A. tumefaciens* A6.

Wang *et al.*,2014

Quorum sensing

TraI/TraR QS system

Agrobacterium tumefaciens

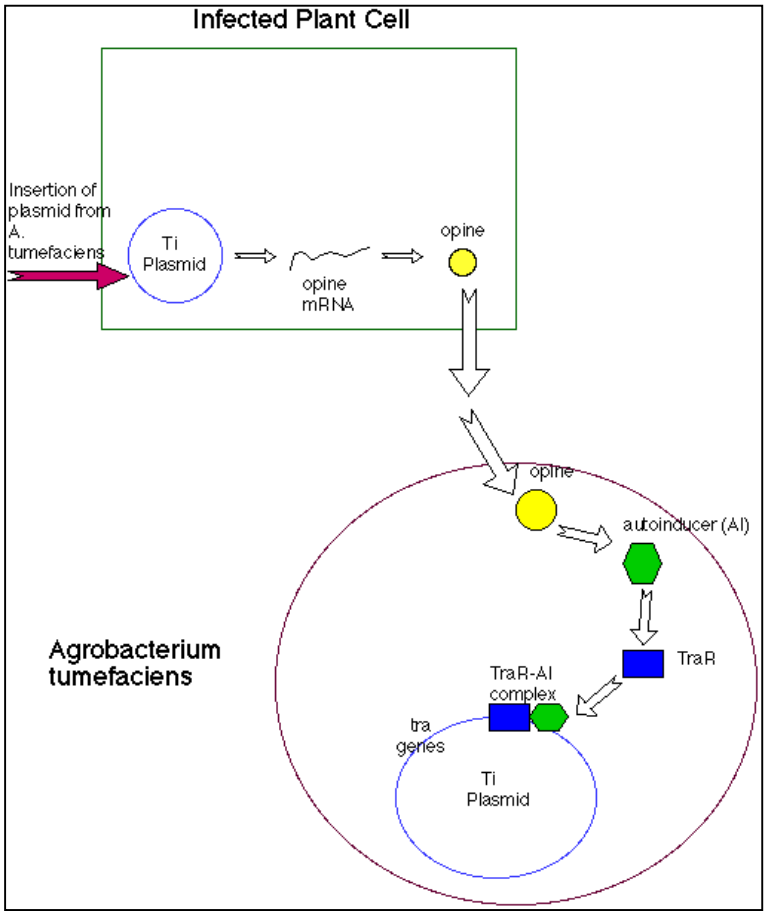
- During infection, a copy of the T-DNA is transfered into the host cell and is stably incorporated into the plant genome.
- The integrated DNA (T-DNA) contains genes for auxin and cytokinin synthesis and their presence explains the ability of explants of tumours to grow in tissue culture without auxin or cytokinin supplements.
- T-DNA also contains genes for the synthesis of arginine derivatives, known as opines, which may be catabolized by the bacterium but are unavailable to the plant.

Quorum sensing

TraI/TraR QS system

Agrobacterium tumefaciens

- Mechanism of conjugal transfer within *A. tumefaciens* by the process of quorum sensing.
- AHL signals produced by the TraI synthase.
- Opines also induce synthesis of the TraR response regulator.
- TraR activates the *tra* regulon.
- Tra M titrates the activity of TraR at higher AHL concentrations.



Coval *et al.*,1999; Bodman *et al.*,2003

Quorum sensing

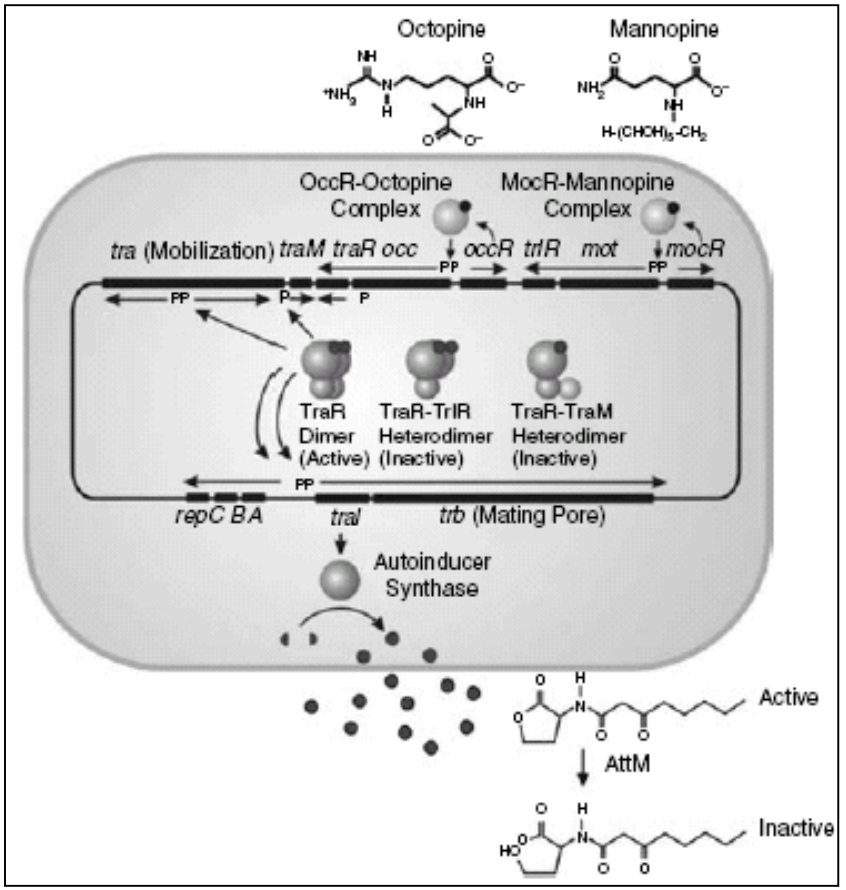
TraI/TraR QS system

Agrobacterium tumefaciens

- The TraR–TraI quorum sensing system of *Agrobacterium tumefaciens*.
- TraI synthesizes OOHL, which diffuses across the cellular envelope.
- At high population density, OOHL accumulates in the cell and binds to TraR.
- TraR activates expression of the two *tra* promoters:
- The *trb* operon which includes :

1. *traI*, *traM*, and
2. *repABC*.

- OccR activates transcription of *traR* in the presence of octopine, and MocR activates *trlR* in the presence of mannopine.
- TraM and TrlR are anti-activators of TraR.
- Finally, at the stationary phase of growth, *attM* is transcribed, resulting in inactivation of OOHL.



Demuth and Lamont,2006

Quorum sensing

AHL(AI-1) and AI-2(LuxS)-based QS systems

Pectobacterium carotovorum

- In *Pectobacterium carotovorum*, at least two QS systems exist being specified by the nature of chemical signals involved.

1. N-acylhomoserine lactone (AHL)
2. Autoinducer-2 (AI-2)

- Pectobacteria may use QS to make sure that gene products (e.g., PCWDE) are produced.

Virulence factors	Gene-associated	Major metabolic precursors	Abbreviation in iPC1209
Lipopolysaccharide			
Lipid A	LpxA, LpxB, LpxC, LpxD, LpxH, LpxK, LpxL, LpxM, kdsA, KdsB, KdsC,WaaA	UDP-*N*-acetyl-D-glucosamine	uacgam
		(*R*)-3-Hydroxytetradecanoyl-ACP	3hmrsACP
		Myristoyl-ACP	myrsACP
		D-Arabinose 5-phosphate	ara5p
		Phosphoenolpyruvate	pep
Core oligosaccharide	GmhA, GmhB, GmhC, GmhD, WaaC, WaaF, WaaQ	Sedoheptulose 7-phosphate	s7p
		UDP-glucose	udpg
		UDP-*N*-acetyl-D-glucosamine	uacgam
		dTDP-4-dehydro-6-deoxy-L-mannose	dtdp4d6dm
		L-Alanine	ala_L
Ara4N modification	Ugd, ArnA, ArnB, ArnC, ArnT	UDP-glucose	udpg
		L-Glutamate	glu_L
		Undecaprenyl phosphate	udcpp
Quorum sensing			
AHL	CarI, CarR	*S*-Adenosyl-L-methionine	amet
		Octanoyl-ACP	ocACP
		Hexanoyl-ACP	hexACP
AI-2	LuxS, LuxR	*S*-Ribosyl-L-homocysteine	rhcys

Põllumaa *et al.*,2012; Wang *et al.*,2015

Quorum sensing

AHL(AI-1) and AI-2(LuxS)-based QS systems

Pectobacterium carotovorum

- *Pcc* and *Pba* (*Pectobacterium atrosepticum*) strains have been divided into two classes on the basis of produced AHL.

1. Class I strains, such as *Pcc* EC153 and SCC3193 synthesize predominantly N-3-oxooctanoyl-L-homoserine lactone (3-oxo-C8-AHL), along with lower amount of 3-oxohexanoyl-L-homoserine lactone (3-oxo-C6-AHL).
2. In contrast, class II strains which include *Pcc* ATCC39048 (and its derivatives, e.g., GS101, ATTn10, and MS1), *Pcc* 71, *Pcc* SCC1 and *Pba* SCRI1043 produce predominantly 3-oxo-C6-AHL, whereas little or none of 3-oxo-C8-AHL.

Põllumaa *et al.*,2012

Quorum sensing

AHL(AI-1) and AI-2(LuxS)-based QS systems

P. carotovorum

Strain	Major AHL	LuxI/R homologue(s)	QS regulated phenotype(s)
Class I strains			
Pcc SCC3193	3-oxo-C8-AHL 3-oxo-C6-AHL	ExpI/ExpR1/ExpR2	production of PCWDE; virulence
Pcc EC153	3-oxo-C8-AHL	AhlI/ExpR	production of PCWDE; virulence
Class II strains			
Pcc 71	3-oxo-C6-AHL 3-oxo-C8-AHL	AhlI/ExpR1/ExpR2	production of PCWDE; virulence
Pcc SCRI193	3-oxo-C6-AHL	ExpI/ExpR1/ExpR2	production of PCWDE; virulence
Pcc ATCC390048	3-oxo-C6-AHL	CarI/CarR ExpR1/VirR	production of carbapenem and PCWDE; virulence
Pba SCRI1043	3-oxo-C6-AHL	ExpI/ExpR/VirR	production of PCWDE, Nip

Põllumaa *et al.*,2012

Quorum sensing
D. chrysanthemi

- The pathogens produce a range of pectinases as key virulence factors which degrade various components of pectins as well as other degradative enzymes such as cellulase isozymes, protease isozymes, xylanase, and phospholipase.
- In addition, the pigment indigoidine and the siderophores chrysobactin and achromobactin have been implicated in the bacterial systemic infections.
- The AHL-type QS signal plays an essential role in modulation of *E. chrysanthemi* pv.*zeae* cell motility and the ability to form multicell aggregates and is involved in regulation of bacterial virulence.

Mumtaz *et al.*,2008

Quorum sensing
Two QS systems and IAA
Dickeya chrysanthemi

- *Dickeya* spp. produce:
1. N-3 oxohexanoyl-L-homoserine lactone (OHHL) or
2. N-3-oxo-octanoyl-L-homoserine lactone (3-oxo-C8-HSL), and
3. A molecule of the autoinducer-2 family (AI-2).
- *Dickeya* spp. also produced the indole-3-acetic acid (IAA) in tryptophan-rich conditions.
- Production of N-acyl-homoserine lactones (NAHSLs) is lower in *Dickeya* than in *Pectobacterium*.
- NAHSL-dependent QS appears less important in virulence of other soft-rot bacteria such as *Dickeya dadantii* 3937 and *Dickeya chrysanthemi*.

Crépin *et al.*,2012

Quorum sensing
Dickeya vs. *Pectobacterium*

- OHHL is the most abundant and was thus postulated to be the most physiologically important QS signal in *E. chrysanthemi*.
- But mutation of the AHL synthase gene, *expI*, results in a decrease of some pectinase gene expression but does not seem to significantly change the total pectinase activity of the pathogen.
- This indicates that the two genera of soft-rot bacteria(*Dickeya* and *Pectobacterium*) have similarities but also differences in the mechanisms of communication via the diffusible molecules.

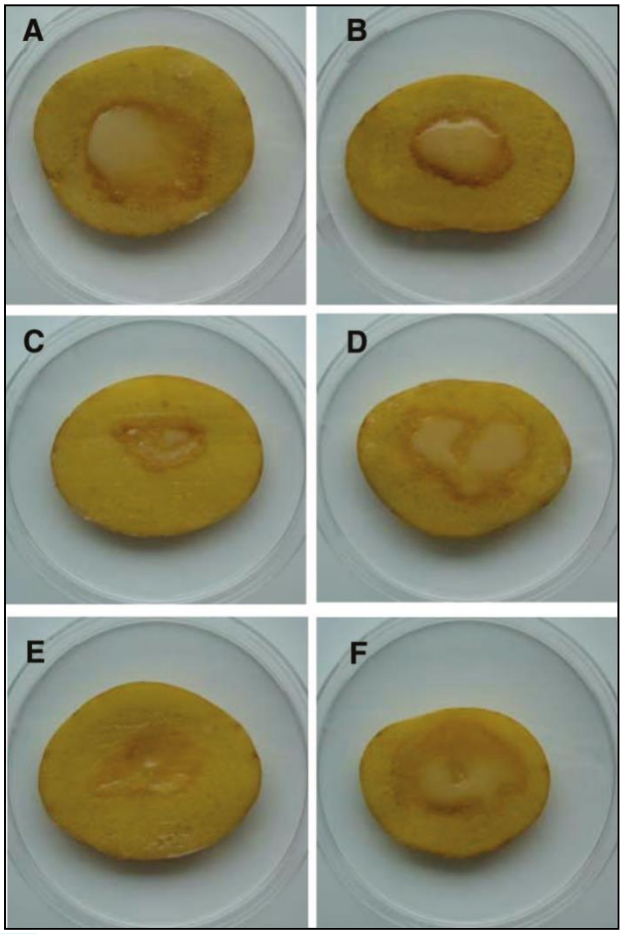
Mumtaz *et al.*,2008; Crépin *et al.*,2012

Quorum sensing

ExpI/ExpR Quorum Sensing

Dickya chrysanthemi

- AHL-deficient mutants showed attenuated soft rot symptoms on potato tubers.
- Each cut tuber was inoculated with 2µl of fresh bacterial cells at an OD_{600} of 1.2.
- The bacterial strains inoculated were EC1 (A), EC3937 (B), WM3 (C), WM3expI (D), WM6 (E), and WM6expI (F).
- Photographs were taken 24 h after incubation at 28°C.



Mumtaz *et al.*,2008

Quorum sensing

EsaI/EsaR quorum-sensing (QS) system

Pantoea stewartii

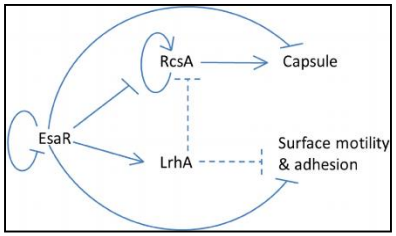
- The quorum-sensing regulation in *P. stewartii*, is different from most other described systems.
- For *Pantoea stewartii* subsp. *stewartii*, the extracellular polysaccharide (EPS) stewartan is a major virulence factor.
- EPS does not produced until the cell density reaches 10^8 cfu/ml.
- Two genes, *esaI* and *esaR*, encode essential regulatory proteins for quorum sensing.
- EsaR unlike the other Gram-ve bacteria repress EPS synthesis at high cell density.

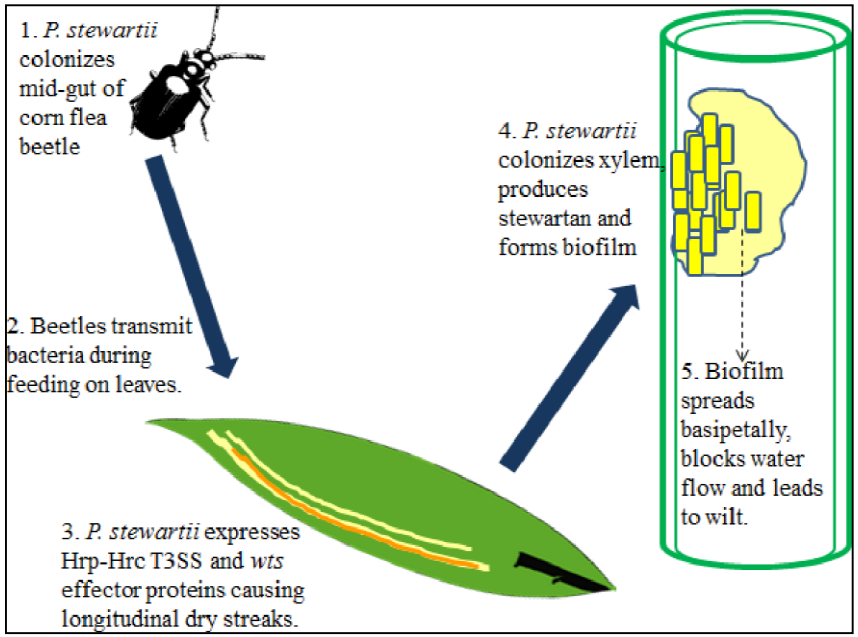
Regulation is achieved through a quorum-sensing (QS) system consisting of the acyl-homoserine lactone (AHL) synthase, EsaI, and the transcription regulator EsaR.

Bacterial wilt (Stewart's disease) of corn
Pantoea stewartii subsp. *stewartii*

- Regulation is achieved through a quorum-sensing (QS) system consisting of the acyl-homoserine lactone (AHL) synthase, EsaI, and the transcription regulator EsaR.
- The QS master regulator EsaR was shown to regulate two major virulence factors of *P. stewartii*:

1. capsule production, and
2. surface motility.





Ramachandran,2014; Burke,2015; Duong,2018

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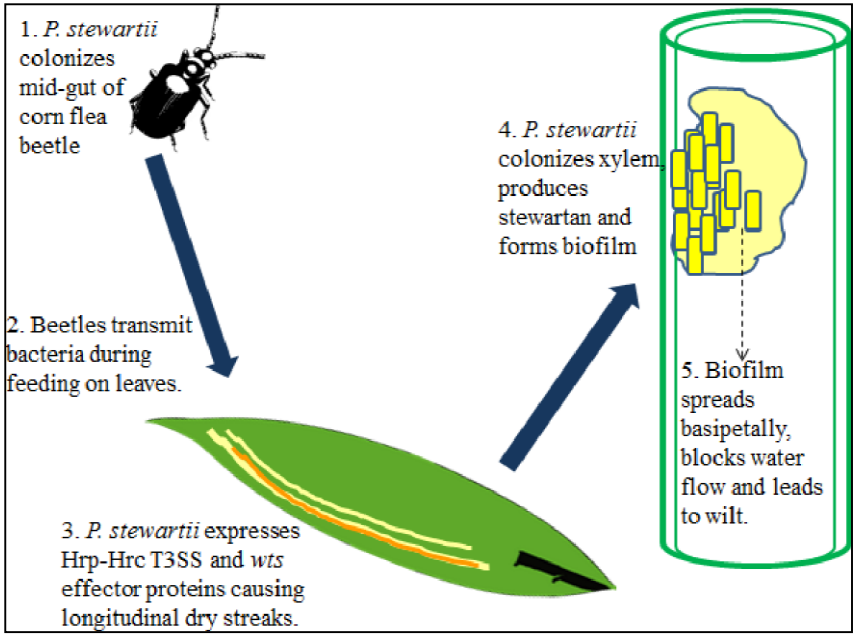


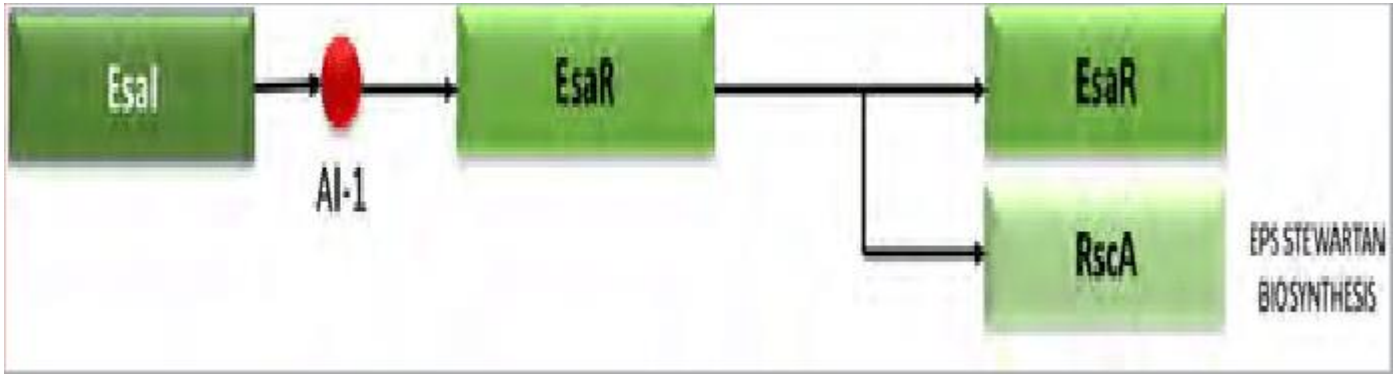
Ramachandran,2014; Duong,2018

Quorum sensing

EsaI/EsaR quorum-sensing (QS) system

Pantoea stewartii

- EPS synthesis in *P. stewartii* is governed by the Rcs phosphorelay signal transduction system.
- EsaR does not regulate the primary promoter of the *cps* gene system, which encodes the functions required for stewartan EPS synthesis.
- Instead, EsaR controls the expression of the *rcsA* gene, which is an essential co-activator necessary for *cps* gene activation.



Neupane *et al.*,2020;..

Quorum sensing

EsaI/EsaR quorum-sensing (QS) system

Pantoea stewartii

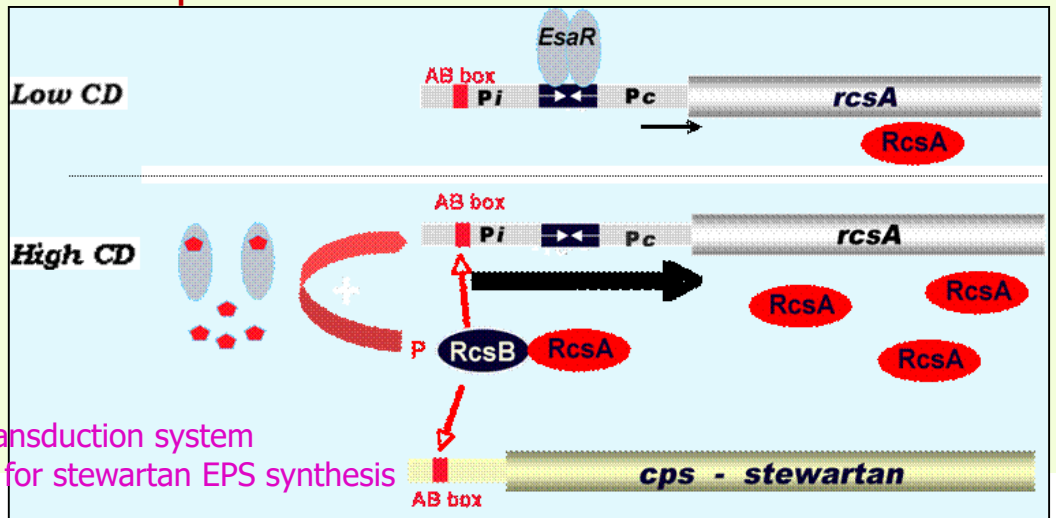
- Mutations were constructed to establish the regulatory role of EsaR.
- *esaI*, *esaR*, and *esaI-esaR* mutations were constructed to establish the regulatory role of EsaR.
- Strains containing an *esaR* mutation produce high levels of EPS independently of cell density and in the absence of the AHL signal.

Quorum sensing

Alternative model for QS regulation in bacteria

Pantoea stewartii subsp. *stewartii*

- EPS (stewartan) synthesis in *P. stewartii* is Rcs-dependent.
- Stewartan in *P. stewartii* ssp.*stewartii* is a function of EsaR-mediated repression of the *rcsA* gene.
- EsaR when represses the transcription of *rcsA*, the levels of RcsA protein will remain less and thus was subject to enzyme degradation.
- But when EsaR repression of *rcsA* is relieved, RcsA protein levels will exceed the degradation capacity of Lon enzyme and thus *cps* gene activated for stewartan production.

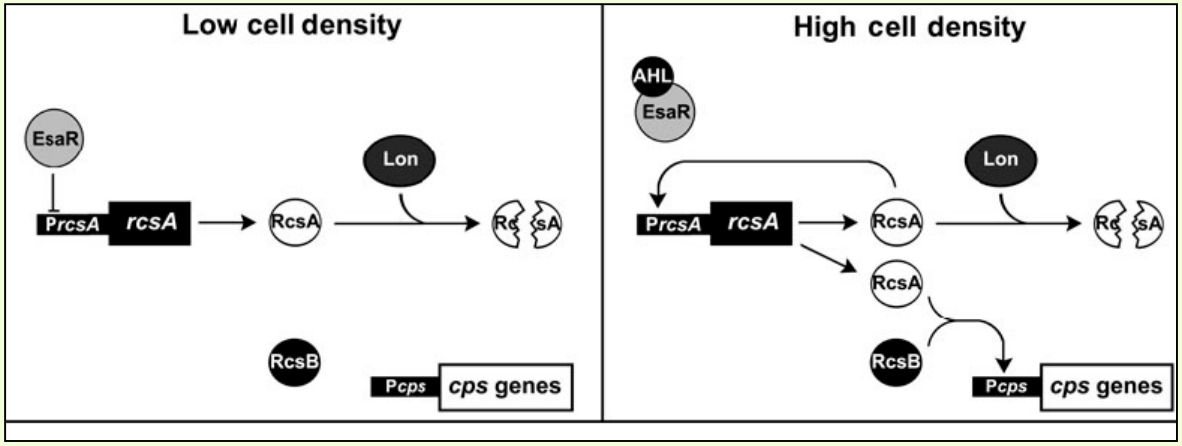


Rcs phosphorelay signal transduction system
cps gene system, required for stewartan EPS synthesis

A model depicting the hierarchical EsaR QS regulatory pathway *Pantoea stewartii*

1. At low cell density, EsaR represses the transcription of *rcsA*, yielding basal levels of RcsA protein that is subject to degradation by Lon protease preventing significant RcsA/RcsB activation complex formation.
2. At high cell density, EsaR repression of *rcsA* is relieved resulting in RcsA levels exceeding the degradation capacity of Lon.

- RcsA recruits(inforce) RcsB to form an activation complex for the positive feedback regulation of *rcsA* and activation of the *cps* gene cluster.



Rcs phosphorelay signal transduction system
cps gene system, required for stewartan EPS synthesis

Minogue *et al.*,2006

Quorum sensing
Pantoea agglomerans pv. *gypsophilae*

- Expression of pathogenicity factors in a variety of plant pathogenic bacteria might be critically dependent on QS.
- The gall forming *Pantoea agglomerans* pv. *gypsophilae* produced:

1. N-butanoyl-L-homoserine lactone (C4-HSL) as a major, and
2. N-hexanoyl-L-homoserine lactone (C6-HSL) as a minor QS signal.

- Homologs of *luxI* and *luxR* regulatory genes, *pagI* and *pagR*, were characterized in wild strain of *P. agglomerans* pv. *gypsophilae* Pag824-1.

Chalupowicz *et al.*,2006

Quorum sensing

Gall induction bioassays

Pantoea agglomerans pv. *gypsophilae*

- Effect of gene disruption on gall size.
- Gypsophila cuttings were inoculated with 10^6 cells/ml of:

1. lane 1, *Pag*824-1 (wild type);
2. lane 2, *Pag*MxI (mutant on *pagI*);
3. lane 3, *Pag*MxR (mutant on *pagR*);
4. lane 4, *Pag*MxΔIR(double deletion in *pagI* and *pagR*); and
5. lane 5, water control.

- Symptoms were observed at 18 days after inoculation and severity of disease was expressed as gall size.

Chalupowicz *et al.*,2008

Quorum sensing

Gall induction bioassays

Pantoea agglomerans pv. *gypsophilae*

- The population of *Pag*824-1 (wild type) was $3 \pm 1.44 \times 10^9$ CFU/g compared with a range of $7 \pm 1.14 \times 10^7$ to $9 \pm 1.29 \times 10^7$ CFU/g with the three mutants.
- Thus, disruption of *pagI* or *pagR* was associated with a lower growth rate *in planta*.
- It should be pointed out that the adverse effect of these mutants on gall size could be overcome by applying an excess inoculum concentration (e.g., 10^9 cells/ml).

Chalupowicz *et al.*,2008

Quorum sensing

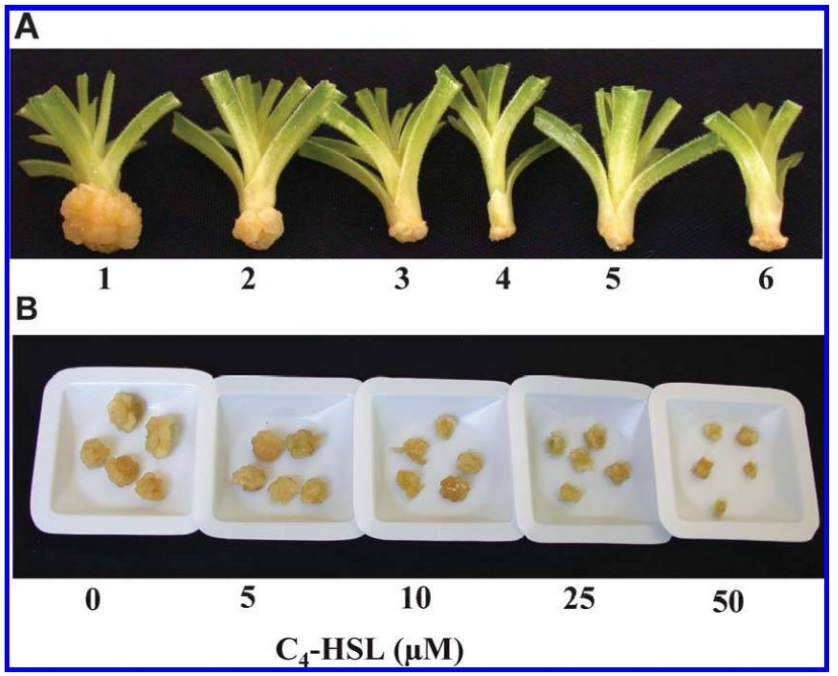
Gall induction bioassays

Pantoea agglomerans pv. *gypsophilae*

- Overexpression of *pagI* or additions of N-butanoyl-L-homoserine lactone (C4-HSL) reduce gall development.

A. Gypsophila cuttings were inoculated with: lane 1, *Pag*824-1; lane 2, *Pag*MxI; lane 3, *Pag*824-1/pVS1; and lane 4, *Pag*MxI/pVS1 or addition of synthetic C4-HSL (50 µM) during inoculation with lane 5, *Pag*824-1 and lane 6, *Pag*MxI.

B. Galls were reduced by *Pag*824-1 following inoculation of gypsophila cuttings in the presence of various C4-HSL concentrations (0, 5, 10, 25, and 50 µM).



Chalupowicz *et al.*,2008

Quorum sensing

AHL-based quorum sensing

Xanthomonas translucens

Genus, species	Strain	Reporter strain	
		A.t.[b]	*C.v.*[c]
P. cichorii	9a	20	++++
	293a	8	+
P. fluorescens	2-79	16	+
P. fluorescens	315	10	++
(biotype 1)	334	14	++
P. fluorescens	38	22	–
(other biotypes)	101b	18	+++++
	142	18	+++++
	260	13	++
	447	10	+++++
P. geniculata	29	11	+
P. lemonnieri	110	18	+
P. putida	153	10	++
	146	11	+++++
	170	7	+++++
	262a	5	+++
	262b	13	++
P. saccharophila	488	5	nd
Pseudomonas spp.	36	13	–
	88	4	–
	149	12	+++++
	279	20	+++++
	320	17	+++++
	K19	3	–
Xanthomonas ampelina	11	–	+++
X. campestris pv. *malvacearum*	20	8	+++
X. campestris pv. *translucens*	6	7	++
X. campestris pv. *vesicatoria*	7	10	++
	340	15	++

[a]Strains *A. tumefaciens* C58 and 1D1, *C. violaceum* CVWT, *P. aureofaciens* 30-84 and *P. fluorescens* 2-79 were used as positive controls.

Genus, species	Number of strains tested	Reporters[a]	
		NT1/pZLR4	CV026
Erwinia herbicola	13	7	13
E. herbicola pv. *gypsophilae*	1	0	1
Pantoea stewartii	1	1	1
Pseudomonas aeruginosa	6	4	3
P. chlororaphis (= *P. aureofaciens*)	39	13	11
P. cichorii	6	2	2
P. geniculata	20	2	1
P. fluorescens (biotype 1)	25	2	2
P. fluorescens (other biotypes)	51	5	4
P. lemonnieri	10	1	1
P. marginata	1	0	0
P. mendocina	1	0	0
P. putida	30	5	5
P. saccharophila	1	1	nd
Pseudomonas spp.	49	6	3
Xanthomonas ampelina	1	0	1
X. campestris pv. *malvacearum*	1	1	1
X. campestris pv. *vesicatoria*	2	2	2
X. campestris pv. *translucens*	1	1	1
X. maltophilia	41		0
X. maltophilia	(26)	0	

[a]Number of strains giving a positive response with one or both reporters. The bacteria tested as controls are not included; nd – not determined.

Veselova *et al.*,2003

Quorum sensing

DSF-based quorum sensing

Xanthomonas citri and *Xylella fastidiosa*

- In *Xanthomonas citri* and *Xylella fastidiosa*, as in other bacteria, cell-to-cell signalling plays an important role in:
- Colonization, pathogenesis and biofilm formation.
- Both bacteria have a cell-to-cell signalling system mediated by a a new language (non-AHL signals) known as DSF (diffusible signal molecule).
- Genes within the *rpf* cluster (for regulation of pathogenicity factors) encode the components of the DSF signalling system.

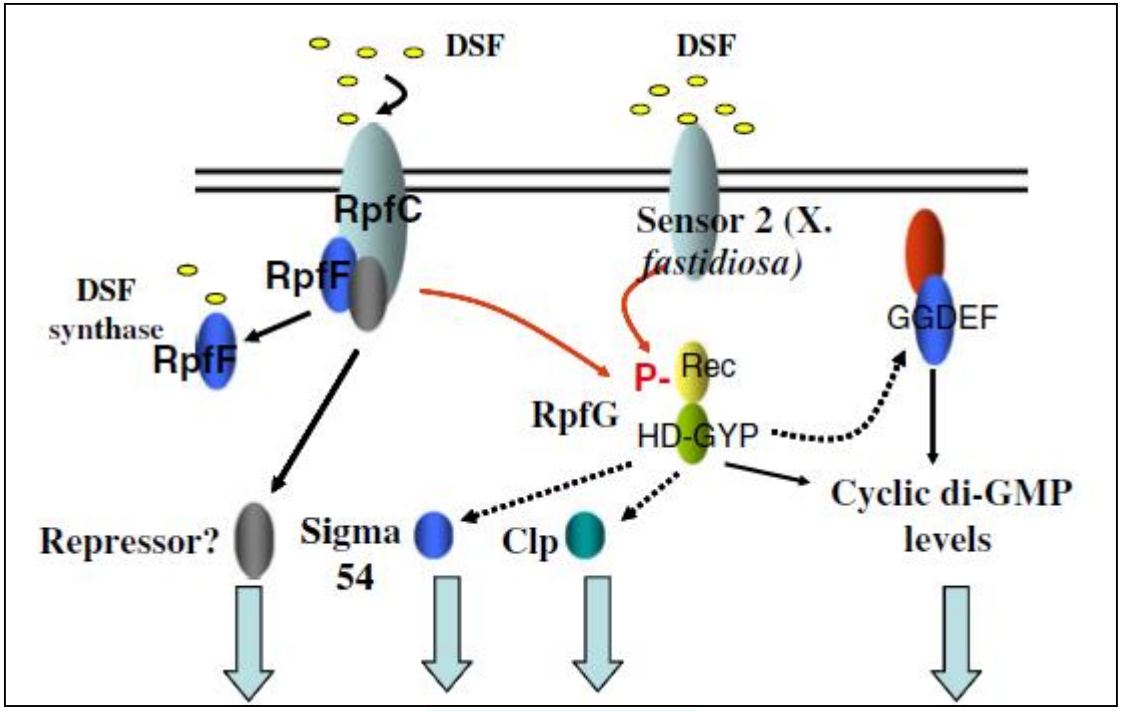
Vojnov *et al.*,2010

Quorum sensing

DSF-based quorum sensing

X. citri and *X. fastidiosa*

- RpfF is responsible for the synthesis of DSF whereas RpfC and RpfG comprise a two-component system implicated in DSF perception and signal transduction.



Vojnov *et al.*,2010

Quorum sensing

DSF-based quorum sensing

Xanthomonas campestris

- Pathogenicity of *X. campestris* pv. *campestris* (Xcc) is dependent on:

1. Various extracellular enzymes, viz. proteases, pectinases and exoglucanases, and
2. EPS.

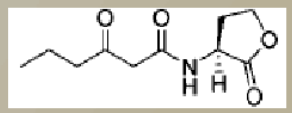
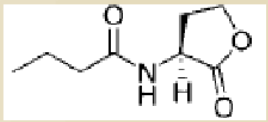
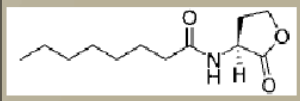
- Production of exocellular enzymes and EPS formation in Xcc like *X. citri* is regulated by rpf (regulation of pathogenicity factors) cluster, which comprises of 9 genes (rpfA-I).
- Two of these genes, rpfB and rpfF, are implicated in the regulation mediated by a small diffusible molecule called DSF (diffusion signal factor).

Diffusible signaling molecule

DSF-based quorum sensing

Xanthomonads and *Burkholderia cenocepacia* produce DSF signals in regulation of virulence factor production

- DSF was identified as methyl dodecenoic acid, which represents a new class of QS signals that seems to be conserved in a range of bacterial species.
- *Xanthomonas* makes an extracellular fatty acid like signaling molecule called as Diffusible Signaling Molecule (DSF), regulates virulence and biofilm dispersal in *Xanthomonas* spp.

Organism	AHL Structure	Chemical Name	Synthase	Receptor
Vibrio fischeri		3-Oxo-C6-HSL (OHHL)	Lux I	Lux R
Pseudomonas aeruginosa		C4-HSL (BHL)	Rhl I	Rhl R
Burkholderia cenocepacia		C8-HSL (OHL)	Cep I	Cep R

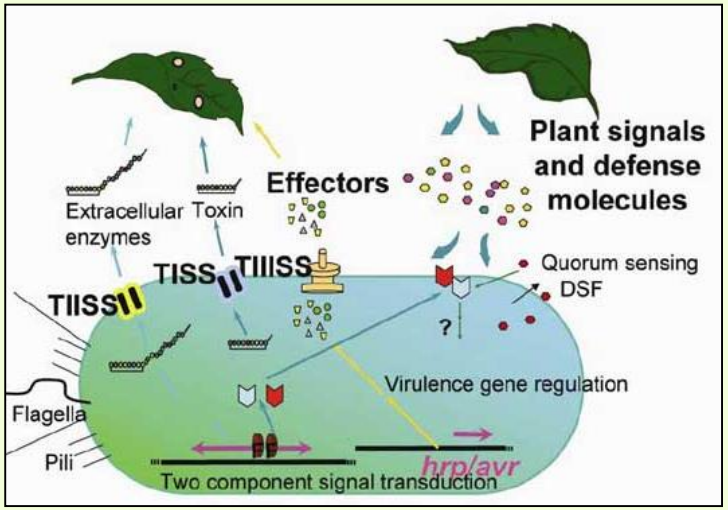
Chatterjee,2009; Wu *et al.*,2011;Ambri et al

Diffusible signaling molecule

DSF-based quorum sensing

Xanthomonads and *Burkholderia cenocepacia* produce DSF signals in regulation of virulence factor production

- More recently, a DSF has been identified from *Burkholderia cenocepacia* (fingertip rot disease of banana), structurally determined as dodecenoic acid and designated as BDSF.
- The DSF signalling system comprises several key regulatory proteins and a second messenger cyclic-di-GMP (c-di-GMP).



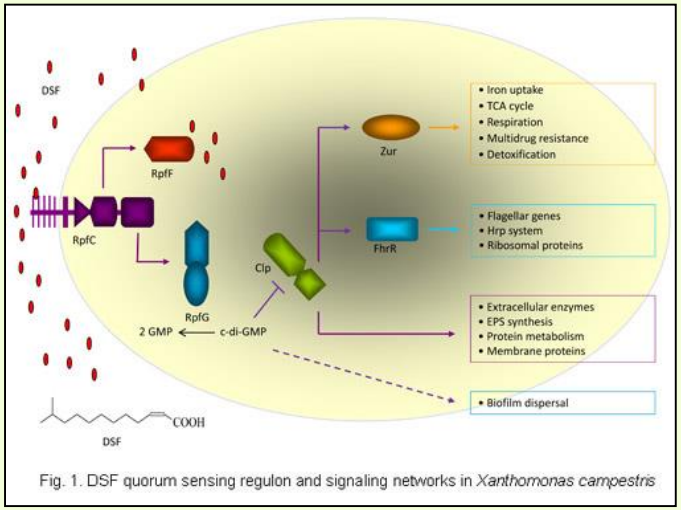


Fig. 1. DSF quorum sensing regulon and signaling networks in *Xanthomonas campestris*

Wu *et al.*,2011;Chatterjee,2009; Wu *et al.*,2011

Multiple DSF-family signals

DSF-based quorum sensing

Xanthomonas oryzae

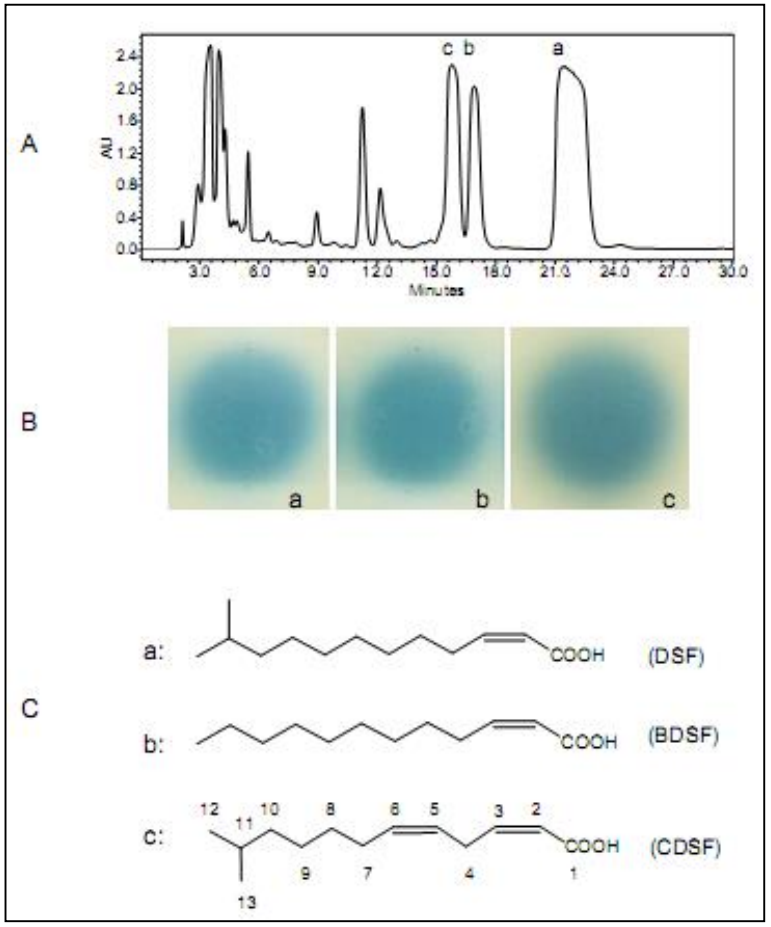
- Xoo produces multiple DSF-family signals.

A. HPLC analysis of the active fractions after flash column chromatography.

B. The compounds in fractions a, b, and c showed strong DSF-like activity.

C. Chemical structures of the compounds in fractions a, b, and c as confirmed by ESI-MS and NMR analysis.

- Molecular weight of CDSF is 209.1555 dalton.



He *et al.*,2010

Multiple DSF-family signals

DSF-based quorum sensing

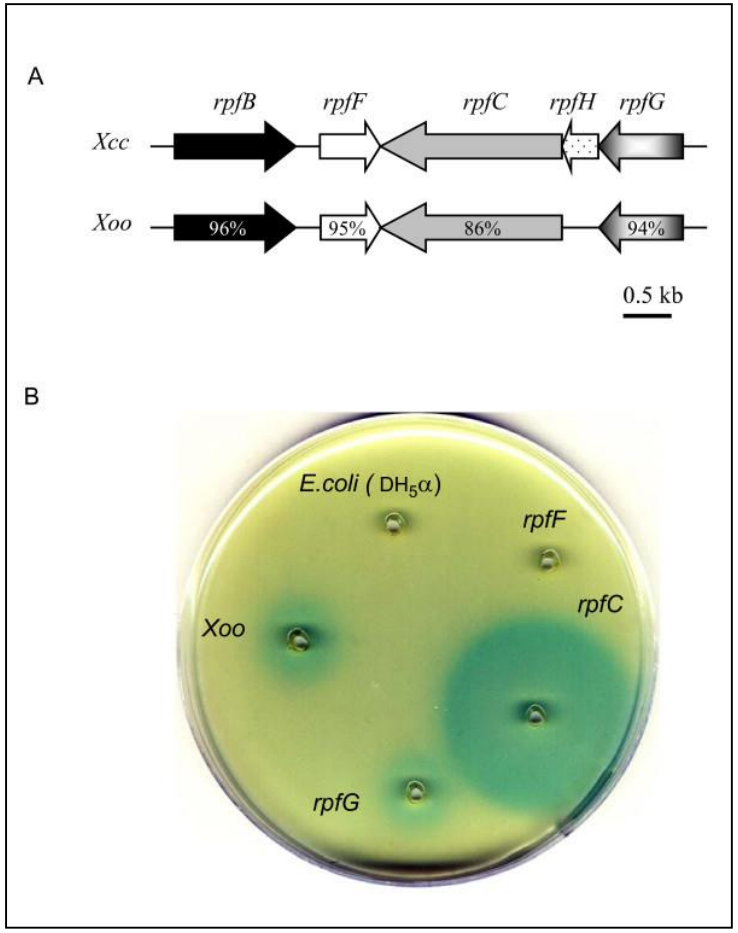
Xanthomonas oryzae

- *Xoo* produces multiple DSF-family signals (i.e. DSF, BDSF and CDSF) in regulation of virulence factor in rich media.

(A) Physical map of the part of the *rpf* gene cluster from *rpfB* to *rpfG* in *Xoo* strain KACC10331 and *Xcc* strain ATCC33913.

- The organization of ORFs predicted by sequence analysis together with predicted directions of transcription are indicated by the broad arrows.

(B) DSF production of *Xoo* strain KACC10331 and derivatives.



He *et al.*,2010

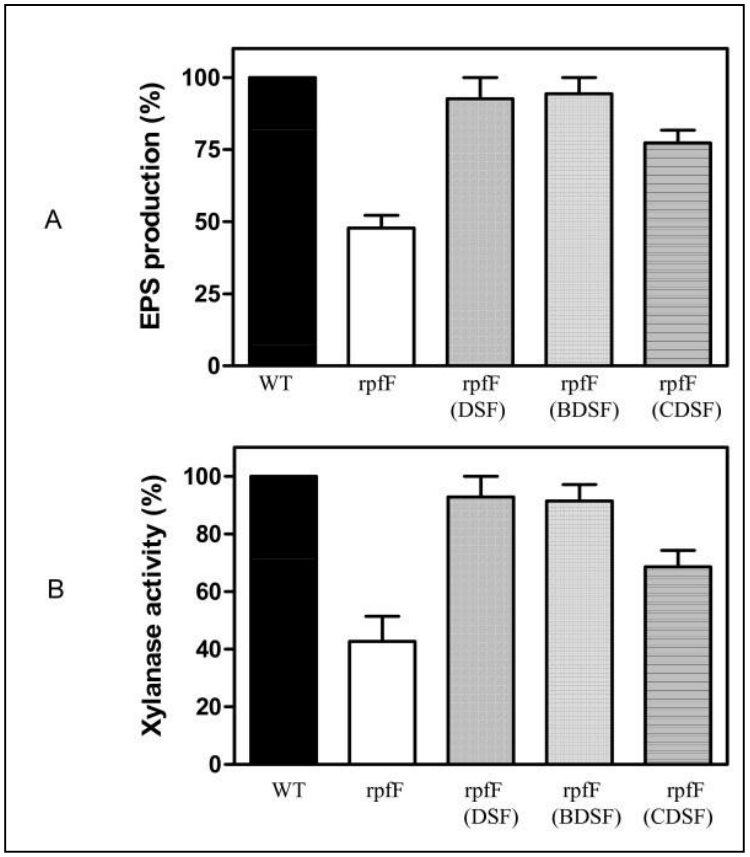
Effects of DSF, BDSF and CDSF on EPS production and extracellular xylanase activity *Xanthomonas oryzae*

- Effects of DSF, BDSF and CDSF on EPS production and extracellular xylanase activity of *rpfF* mutant of *Xoo* strain KACC10331.

A. EPS production at OD_{600} = 2.5.

B. The xylanase activity in the supernatant of cell culture at OD_{600} = 2.5.

- DSF, BDSF and CDSF were separately added to *rpfF* mutant at early growth stage at a final concentration of 3 μM.



He *et al.*,2010

Quorum sensing

An array of AHLs

Burkolderia gladioli pv. *agariciola*, *P. agarici* and *P. gingeri*

- Strains of *Burkolderia gladioli* pv.*agariciola*, *Pseudomonas agarici* and *Pseudomonas gingeri*, but not those of *Pseudomonas tolaasii* and *Pseudomonas reactans*, produced an array of AHLs depending on the strain.
- This is the first report of AHL production by mushroom bacterial pathogens.
- In the culture filtrates of a certain number of isolates/strains the AHL-hydrolyzed forms were also present.

Shanmugam *et al.*,2011

Quorum sensing

Unusual Multiple Production of *N*-Acylhomoserine Lactones a by *Burkholderia* sp. Strain C10B Isolated from Dentine Caries

C6-HSL, C8-HSL, C10-HSL and C12-HSL

- Four unusual multiple production of AHLs by *Burkholderia* sp. strain C10B were isolated from dentine caries.
- To the best of our knowledge, this is the first example where *Burkholderia* sp. produced four types of AHL (C6-HSL, C8-HSL, C10-HSL and C12-HSL).
- *Chromobacterium violaceum* CV026 was used as short chain AHLs biosensor in this study.
- For all four AHLs, mass spectrometry (MS) analyses were made.

Goh *et al.*,2014

Quorum sensing

Unusual Multiple Production of *N*-Acylhomoserine Lactones a by *Burkholderia* sp. Strain C10B Isolated from Dentine Caries

C6-HSL, C8-HSL, C10-HSL and C12-HSL

- High resolution MS was performed as previously described by Ortori and coworkers,2011, using an Agilent 1290 Infinity LC system coupled together with an Agilent ZORBAX Rapid Resolution High Definition SB-C18 column (50 mm × 2.1 mm, 1.8 µm particle size), with its temperature, flow rate and injection volume as described previously.
- The mobile phases A and B used in this study were water and acetonitrile (both mobile phases added with 0.1%v/v formic acid), respectively and set to a ratio of 80:20.
- The parameter for high resolution electron spray ionization mass spectrometry (ESI-MS) set for the run was as described previously.
- The precursor ion scan mode targeting the *m/z* 102 product ion that indicates that [M + H]+ ion of the lactone ring.
- The *m/z* value range to detect the precursor ions was set at 150–400 and the MS data analysis was done using Agilent MassHunter software.

Goh *et al.*,2014

Quorum sensing

Burkholderia sp. strain C10B. AHL Profiling by High Resolution Tandem Liquid Chromatography Mass Spectrometry

C6-HSL, C8-HSL, C10-HSL and C12-HSL

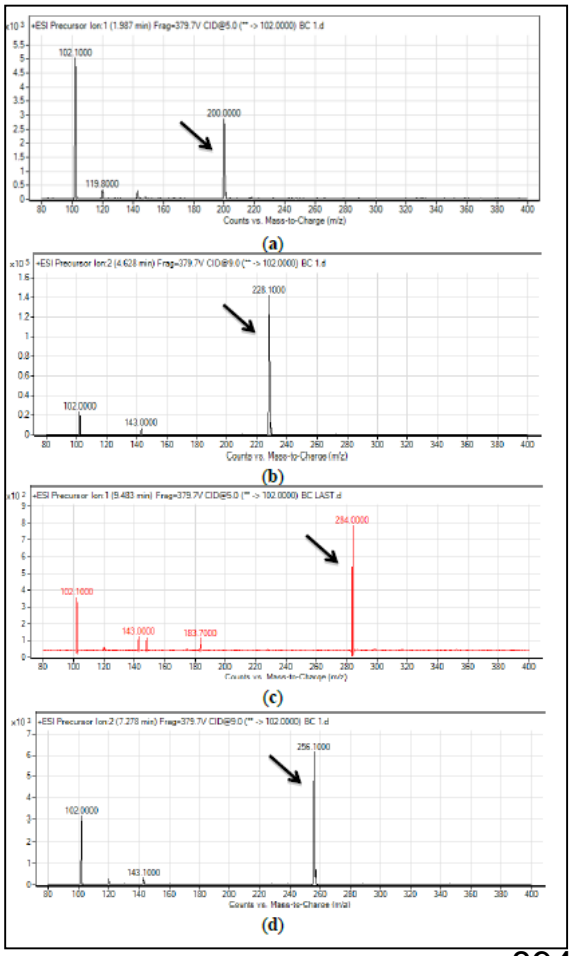
- Mass spectrometry analysis of spent supernatant extract of *Burkholderia* sp. strain C10B.
- All corresponding *m/z* for respective AHLs are marked by arrows;

a) mass spectrum of C6-HSL (*m/z* 200.0000),

b) mass spectrum of C8-HSL (*m/z* 228.1000),

c) mass spectrum of C10-HSL (*m/z* 256.1000),

d) mass spectrum of C12-HSL (*m/z* 284.1000).



Goh *et al.*,2014

Quorum sensing
Erwinia amylovora

- We report for the first time the production of acyl homoserine lactones (AHLs) by *Erwina amylovora*, the causal of fire blight in plants.
- *E. amylovora* produces:

1. One N-acyl homoserine lactone [a N-(3-oxo-hexanoyl)-homoserine lactone, or
2. A N-(3-hydroxy-hexanoyl)-homoserine lactone] quorum sensing signal molecule both *in vitro* and *in planta* (pear plant).

- Given the involvement of AHLs in plant pathogenesis, we speculate that AHL-dependent quorum sensing could play an important role in the regulation of *E. amylovora* virulence.

Venturi *et al.*,2004

Quorum sensing
Erwinia amylovora

- Here we present the first evidence for autoinduction in *E. amylovora* and a role for an *N*-acyl-homoserine lactone (AHL)-type signal.
- Two major plant virulence traits:

1. Production of extracellular polysaccharides (amylovoran and levan), and
2. Tolerance to free oxygen radicals, were controlled in a bacterial-cell-density-dependent manner.

Molina *et al.*,2005

Quorum sensing

Two standard autoinducer biosensors, *Agrobacterium tumefaciens* NTL4 and *Vibrio harveyi* BB886 were used *Erwinia amylovora*

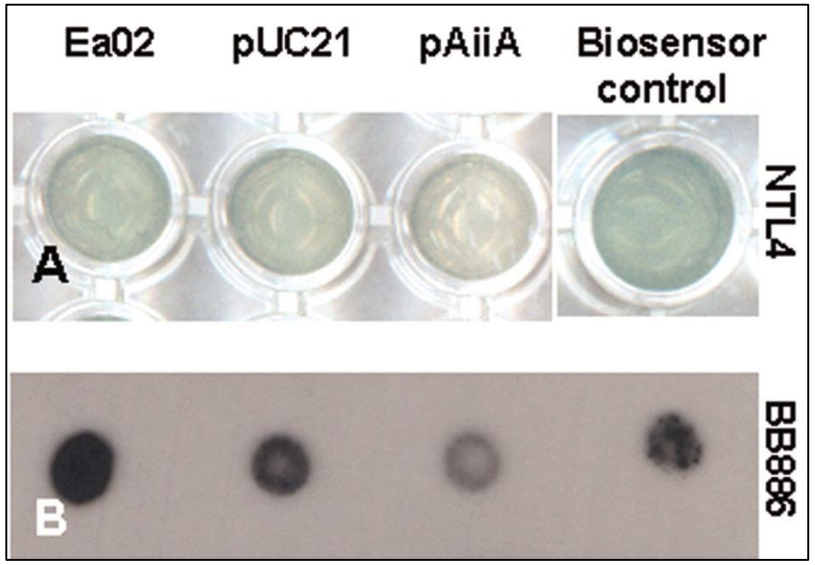
- Culture supernatants of wild-type *E. amylovora* induced the β-galactosidase activity (blue color) of the 3-oxo and 3-hydroxy AHL derivative biosensor *A. tumefaciens* NTL4/pZLR4 when they were added to solid or liquid LB medium (Fig.1A).
- Also, stimulation of light production by *Vibrio harveyi* BB886 (Fig. 1B), the specific *N*-(3-hydroxybutanoyl)-homoserine lactone (autoinducer type I) reporter, was observed when this strain was cultured in AB medium supplemented with 10% (vol/vol) *E. amylovora* culture supernatant (Fig. 1B).
- However, no induction of AHL-mediated violacein production was observed when *E. amylovora* was coinoculated with *C. violaceum* CV026, a biosensor sensitive to short-chain AHLs.

Note: CV026 does not respond to long chain AHLs and AHL production (N-acyl side chains C4-C8) was judged by its ability to induce violacein production.

Quorum sensing
Erwinia amylovora

- Autoinducer production by *E. amylovora* and autoinducer degradation in a derivative strain carrying the *aiiA* acyl-homoserine lactonase-encoding gene.
- Heterologous expression of the *Bacillus* sp. strain A24 acyl-homoserine lactonase gene *aiiA* in *E. amylovora:*

1. Abolished induction of AHL biosensors,
2. Impaired extracellular polysaccharide production and tolerance to hydrogen peroxide, and
3. Reduced virulence on apple leaves.



Autoinducer production was revealed by the appearance of a blue color resulting from β-galactosidase activity in the NTL4/pZLR4 AHL biosensor or by an increase in the intensity of black spots visualized after autoradiography to detect light emission by the *Vibrio* autoinducer biosensor strain.

Molina *et al.*,2005

Quorum sensing

Lux-box-like sequences in *E. amylovora*

Autoinduction in *Erwinia amylovora*: evidence of an Acyl-homoserine lactone signal in the fire blight pathogen

Lux box sequence[a]	AHL-regulated gene	Phenotype	Accession no.
ACCTGGCAGCCTGA**GCTG**CCAGG	*E. amylovora srlMR*	Regulators of sorbitol uptake	Y14603
T**CCTGGCAA**CAA**GTT**GCCAGA	*E. amylovora hrpL*	Regulator of secretion system type III	AF083877
GG**CCTG**ATTAA**T**C**GAG**GCC**G**	*E. amylovora foxR*	Desferrioxamine receptor	AJ223062
CCTGGCTTATA**G**CTGC**C**AAT	*E. amylovora ams*	Amylovoran synthesis genes	X77921
ACCTGCACTA**T**A**GGT**ACA**GG**C	*P. stewartii esaI*	AHL synthase	L32183
ACCTCCC**TGTT**CT**GGGAGGT**	*P. putida ppuA*	Possible chain fatty acid coenzyme A ligase	A4115588
ACCTGCCAGTTCT**GGT**A**GGA**	*P. putida ppuI*	AHL synthase	A4115588
ACCTGCCAGTTCT**GGCAGGT**	*P. aeruginosa lasB*	Protease (pseudolysin) precursor gene	AB029328
CCCTACCAGATCT**GGCAGGT**	*P. aeruginosa rhlI*	Autoinducer synthase	U40458
ACCTA**CCAG**AATT**GGCAGG**G	*P. aeruginosa hcnA*	Cyanide synthase	AE0046446
ACCTGTACT**T**A**GGTGCAGGT**	*P. fluorescens afmI*	AHL synthase	AF232768
ACCTGACCT**TTC**G**GTCAGGT**	*Serratia marcescens spnI*	AHL synthase	AF389912
T**ACCTG**TTCC**T**A**GGTACAGTA**	*P. syringae psmI*	AHL synthase	AF2344628

[a] Boldface type indicates bases with more than 50% homology among the various sequences.

Molina *et al.*,2005

Quorum sensing
Pseudomonas syringae

- Quorum sensing in *P. syringae* regulates traits that contribute to:
- Epiphytic fitness,
- Distinct stages of disease development during plant infection including:

1. Motility,
2. EPS production, and
3. Virulence.

Quorum sensing

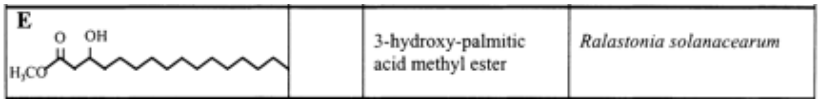
3-hydroxypalmitic acid methyl ester(3-OH)PAME

Ralstonia solanacearum

- The most common quormone group in Gram-negative bacteria consists of acyl homoserine lactones (AHLs).
- In *R. solanacearum* another type of quormone (3-hydroxypalmitic acid methyl ester) is also known (Flavier *et al.*,1997).
- The phytopathogenicity of *R. solanacearum* is dependent on the:

1. Production of an acidic extracellular polysaccharide (EPS), and
2. Plant cell-well degrading extracellular enzymes.

- The PhcA regulatory system(network) is responsible for regulation and control of EPS and extracellular enzymes (virulence factors) in *R. solanacearum*.



Sági,2003; Chen *et al.*, 2009

Quorum sensing

PhcA (Phc, phenotype conversion)

Ralstonia solanacearum

- The main transcriptional regulator is PhcA (Phc, phenotype conversion), which simultaneously

1. Activates diverse virulence genes such as those of EPS biosynthesis and production of Pme and Egl exoproteins, and
2. Represses others such as *hrp* genes and those related to production of polygalacturonases, and motility.

Alvarez *et al.*,2010

Quorum sensing

PhcA and *solI*/*solR* quorum-sensing system

R. solanacearum

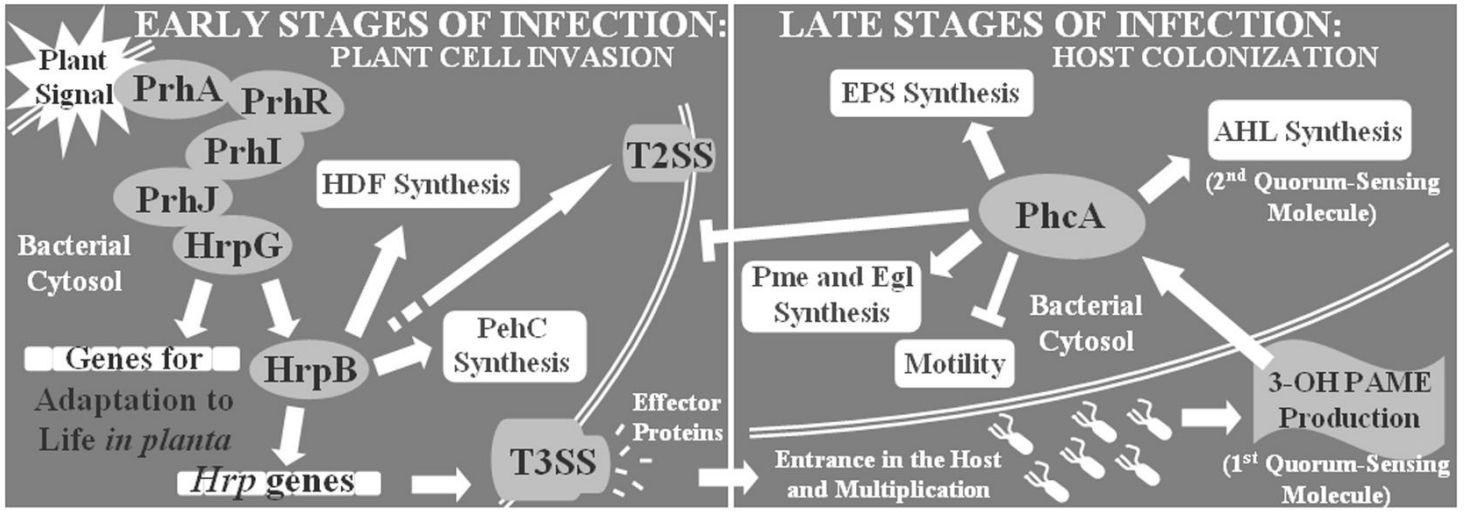
1. Therefore, PhcA as the central transcriptional regulator is modulated by an endogenous signal molecule, the 3-hydroxypalmitic acid methyl ester (3-OH PAME).
2. *R. solanacearum* also possesses a *solI*/*solR* quorum-sensing system that is a *luxI*/*luxR* homologue.

- Inactivation of *solIR* eliminates the synthesis of C6- and C8- HSLs, but does not affect disease or virulence factor production.
- *R. solanacearum* senses the presence of a plant cell by an outer membrane receptor, PrhA, which transduces the signal through a complex regulatory cascade progressively integrated by PrhR, PrhI, PrhJ, HrpG and HrpB regulators.

Chen *et al.*,2009

Quorum sensing
R. solanacearum

- Control of the main pathogenicity genes in early and late stages of host infection by *R. solanacearum*.
- PhcA has a central role in regulating virulence, but there are additional regulatory proteins in the supporting cast.
- Mutation in *phcA* results in the phenotypic conversion of *R. solanacearum* colonies into the afluidal type.



Alvarez *et al.*,2010

Quorum sensing

Acidovorax avenae subsp. *citrulli*

- *Acidovorax avenae* subsp.*citrulli* the causal agent of bacterial fruit blotch (BFB) in cucurbits use the acyl-homoserine lactone (AHL)-type quorum sensing (QS) system in growth, swimming motility and virulence.
- **Wild-type strain:**
- The AHL synthase gene of the QS system from strain XJL12, defined as *aacI*.
- **Mutant strain:**
- An *aacI* disruption mutant was also generated.
- The *aacI* mutant XJL13 abolished the ability to produce AHL molecules.
- **In minimal medium:**
- The *aacI* mutant exhibited a significant decrease in growth rate relative to the wild type in minimal medium, and was partially impaired in swimming motility.

Fan *et al.*,2011

Quorum sensing

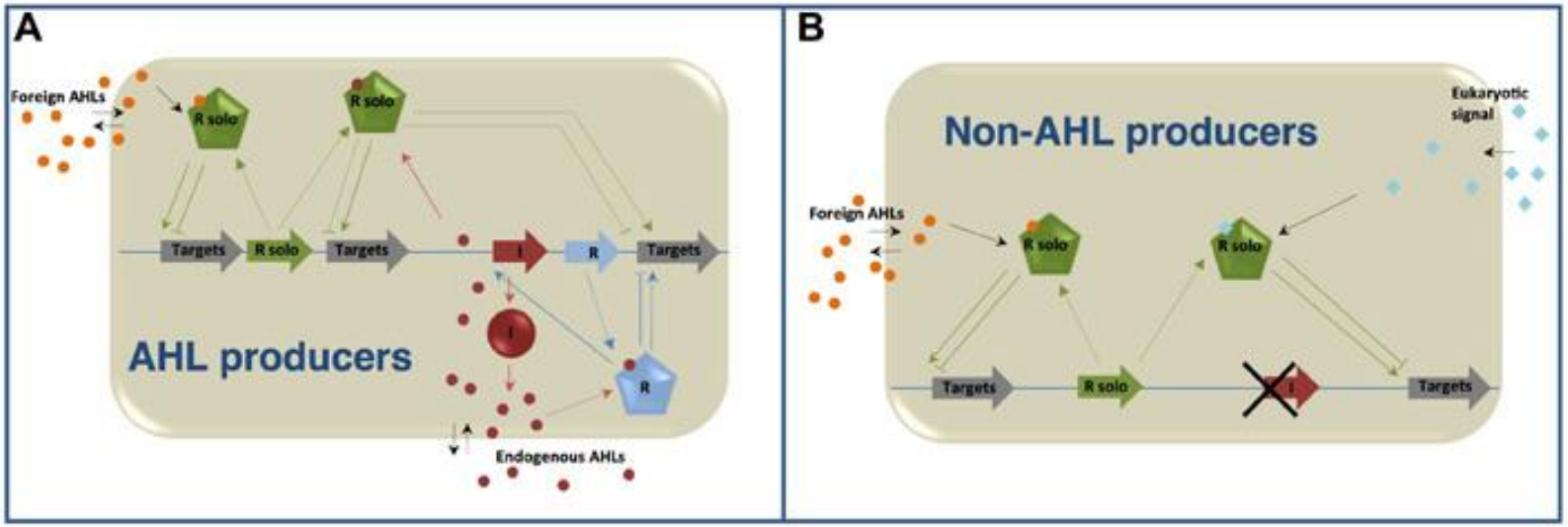
Acidovorax avenae subsp. *citrulli*

- **In plants:**
- In plants, the *aacI* mutant showed a significant reduction of virulence in watermelon fruits and melon seedlings when compared to the wild-type strain.
- However, the *aacI* mutation in strain XJL12 had no effects on biofilm formation, exopolysaccharide production, or induction of hypersentitive response in *Nicotiana tabacum*.
- Our data suggest that the AHL-type QS may play a key role in pathogen virulence and this may provide an opportunity to explore novel approaches for managing BFB in cucurbits by QS interference.

Fan *et al.*,2011

Bacterial inter-cellular communication (QS) in rice associated bacteria

(i) rice-rhizosphere beneficial *Pseudomonas* spp., (ii) in beneficial rice endophytic *Burkholderia* spp., and (ii) in rice pathogenic *Xanthomonas oryzae*, *Burkholderia glumae* and *Pseudomonas fuscovaginae*



LuxR-family 'solos'

Venturi *et al.*,2010

Isolation of *N*-acyl-homoserine lactone-producing bacteria from plant surfaces

Materials

- Selection of recently collected plant roots and leaves. These should be stored in plastic bags at 4ºC until use.
- Vortex mixer
- Sonicating water bath (optional);
- 10-ml tube of sterile phosphate buffered saline (PBS) ;
- Sterile 1.5-ml snap-top microfuge tubes (have containers full of open sterile microfuge tubes);
- Pipetor (P-20 & P-100) and a box of sterile yellow tips;
- 6 LB + Cycloheximide (100 mg/ml) agar plates (Cycloheximide is an antibiotic that prevents the growth of fungi);
- 3 LB agar plates;
- 20-ml LB broth (bottles);
- Jar of ethanol plus glass spreader.

Isolation of *N*-acyl-homoserine lactone-producing bacteria

Media

Luria Broth and Agar Plates

- For 1,000-ml D.H_2O:
- 10 g of Tryptone
- 5 g of Yeast Extract
- 5 g of NaCl
- For agar plates add 15 g of agar

Pierson III, 2005

Alternative composition for Luria-Bertani (LB) agar:
1% w/v peptone, 0.5% w/v yeast extract, 0.5% w/v NaCl,
1.5% agar in 100 mL distilled water.

Isolation of *N*-acyl-homoserine lactone-producing bacteria

Media

Phosphate Buffered Saline (PBS)

- Per 1000-ml:
- 1.6 g of NaCl
- 0.2 g of KH_2PO_4
- 2.9 g of Na_2HPO_4-$12H_2O$
- 800 ml of dionized H_2O
- Adjust pH to 7.4
- Adjust final volume to 1000 ml.

Pierson III, 2005

Isolation of *N*-acyl-homoserine lactone-producing bacteria

Materials

Glass Spreaders

- Take a glass rod approximately 6 inches long.
- Heat an area approximately 2 inches from one end over a flame until it can be bent to a 90-degree angle.
- Let cool.

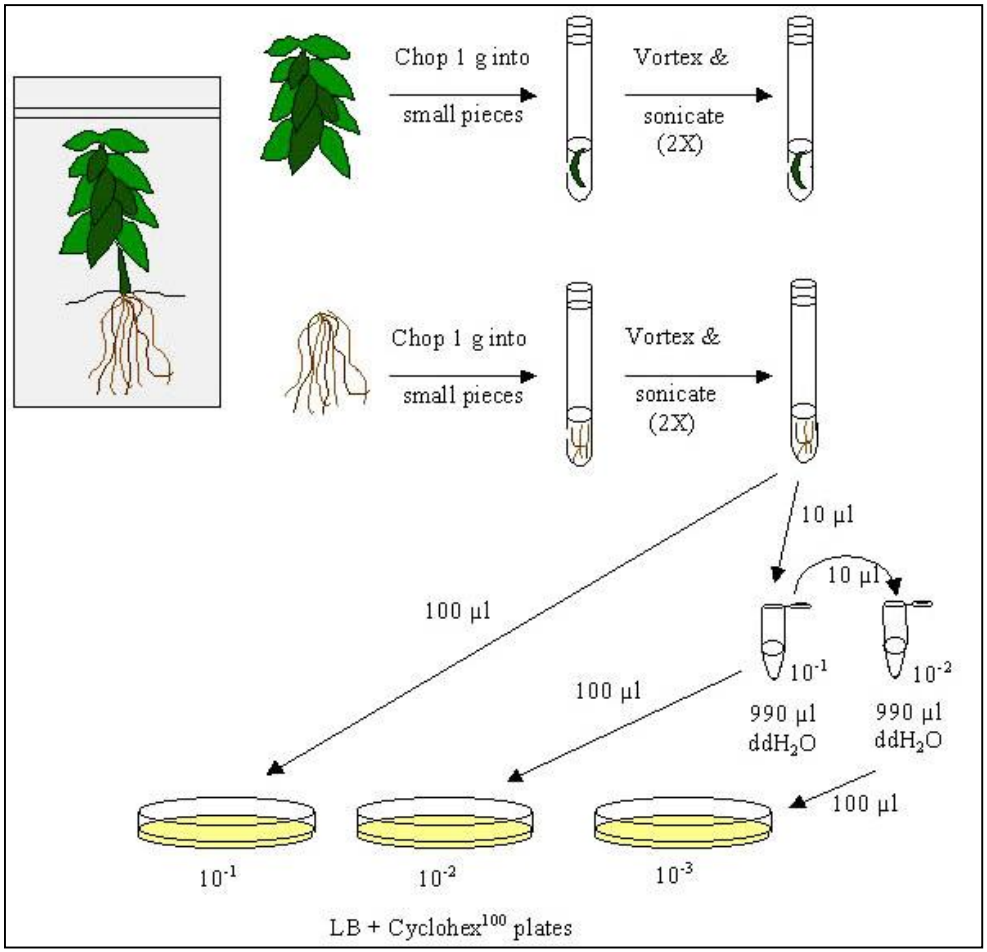
Pierson III, 2005

Isolate and plate bacteria from plant surfaces

- Collect several samples of different plants, both roots and leaves. Store in labeled plastic bags at 4ºC.
- Take about 1 g each of your different plant materials, and cut both roots and leaves separately into small pieces with a sterile razor blade.
- Place the pieces of each sample into separate, sterile glass test tubes.
- Add 2 ml of sterile PBS buffer to each test tube.
- Vortex each tube for 5 sec, sonicate 30 sec. Repeat (the sonication step can be omitted but some of the more tightly adhering bacteria may not be recovered).
- Serially dilute each to 10^{-2} in sterile microfuge tubes (each 1/10 dilution is achieved by adding 100-µl sample to 900-µl sterile PBS) and plate on LB + Cycloheximide plates at 10^{-1}, 10^{-2}, and 10^{-3}.
- Incubate at 28ºC up to 3 days.

Pierson III, 2005

Isolate and plate bacteria from plant surfaces



Pierson III, 2005

Inoculating cultures

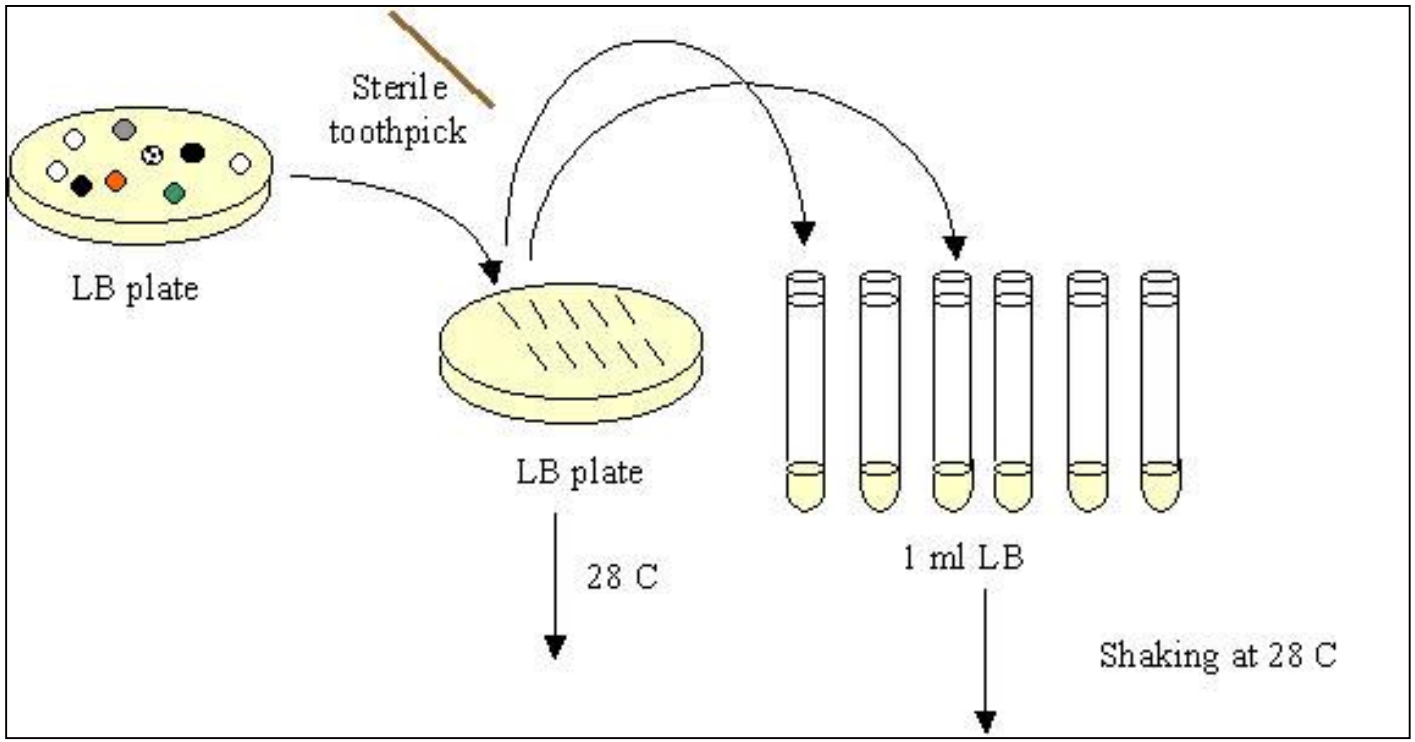
Bacterial strains

Pseudomonas aureofaciens

- After sufficient numbers of colonies have appeared, pick 12 (or more) isolated colonies that appear distinctly different in shape, color, etc. (this is your test strain collection).
- At the same time, transfer by picking a colony and touching it onto an LB agar plate and then using the same toothpick to inoculate 1-ml LB cultures of each test (unknown) strain in sterile glass test tubes.
- Shake at 28ºC overnight.
- Also inoculate 1-ml LB cultures of bioreporter (indicator) strains with and without phz operon strains:
- 30-84 (wild strain: I^+ and R^+)
- 30-84I (mutant strain: I^- and R^+)
- 30-84Ice (mutant positive control strain: I^+ and R^-)
- 30-84Gac (mutant negative control strain: I^- and R^-).
- Shake at 28ºC overnight.

Pierson III, 2005

Inoculating cultures
Bacterial strains



Pierson III,2005

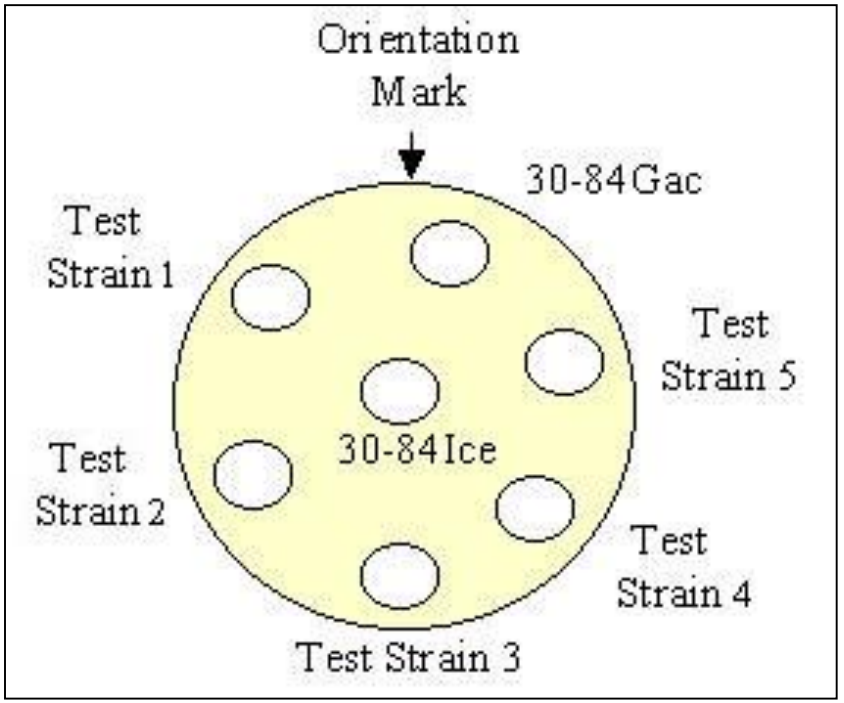
Pseudomonas aureofaciens

Bioreporter(indicator) strains

1. *Pseudomonas aureofaciens* strain 30-84 (A wild-type isolate that produces AHL signal (I^+/R^+) and therefore the orange phenazine antibiotics.
2. *Pseudomonas aureofaciens* strain 30-84I (A derivative of strain 30-84 that does not produce AHL signal (I^-/R^+). It will produce phenazines if certain exogenous AHL signals are provided.
3. *Pseudomonas aureofaciens* strain 30-84Ice (A derivative of strain 30-84 that does produce AHL signal but not phenazines (I^+/R^-).
4. *Pseudomonas aureofaciens* strain 30-84Gac (A derivative of strain 30-84 that does not produce any AHL signal (I^-/R^-).

Preparation of AHL screen plates

- Arrangement of spot tests on LB agar plates seeded with either:
- wild strain 30-84 (I^+/R^+) or
- mutant strain 30-84I (I^-/R^+).
- There should be two LB agar plates:
- one seeded with strain 30-84 and
- a second plate seeded with strain 30-84I.



Pierson III, 2005

Preparation of AHL screen plates

- Aseptically microfuge (max. speed) for 1 min both cultures of strains 30-84 and 30-84I.
- Resuspend each pellet in 500 µl of LB.
- Repeat again.
- Make an orientation mark on the outside of each plate as shown in next Fig.
- Spread 100 µl of each culture (30-84 and strain 30-84I) onto a separate LB agar plate.
- Let the remaining liquid soak into the plate.
- Spot 5 µl of the overnight culture of each test isolate onto the lawn.
- Include strain 30-84Ice as a positive control (produces an AHL signal recognized by strain 30-84I) and strain 30-84Gac as a negative control (does not produce any AHL signal).
- Let spots soak into the plate.
- Incubate plates at 28ºC for 2-3 days.

Pierson III, 2005

Identification of *N*-Acyl-Homoserine Lactone-Producing Bacteria

Scoring screening plates for cross-communication

- Pate A: Positive cross-communication
- **Seeded with strain 30-84I(I^-/R^+):**
- The presence of orange halos surrounding the test spots indicates restoration of phenazine production by a signal produced by the test spots (**test bacteria**).
- Pate B: Negative cross-communication
- **Seeded with strain 30-84(I^+/R^+):**
- The presence of white halos surrounding the test spots indicates inhibition of phenazine production by **strain 30-84** by a signal produced by the test spots.

Pierson III, 2005

Data collection chart

Score the effect of each test strain on 30-84I or 30-84

Isolates/source	30-841	30-84
P1		
P2		
P3		
P4		
P5		
etc.		

Pierson III, 2005

Similarity of I and R proteins of *Enterobacteriaceae* strain A2JM to I and R proteins of other strains

Protein type	Strain	Protein	Similarity (%)	GenBank accession no.
I	*E. carotovora*	HslI	97	AAA62483
	E. carotovora	CarI	97	P33880
	E. carotovorum subsp. *betavasculorum*	EcbI	96	AAB69645
	E. carotovora	ExpI	88	P33882
	E. chrysanthemi	EchI	78	Q46968
	S. liquefaciens	SwrI	78	P52989
	E. chrysanthemi	AhlI	77	AAM46699
	Serratia sp. strain ATCC 39006	SmaI	76	CAB92553
	Yersinia pestis	YpeI	73	AAF21290
	S. marcescens	SpnI	67	AAN52498
	Serratia proteomaculans	SprI	65	AAK76733
R	*E. carotovorum* subsp. *betavasculorum*	EcbR	99	AAB69464
	E. carotovora	ExpR	81	CAA56646
	E. chrysanthemi	EchR	79	Q46967
	E. chrysanthemi	ExpR	78	Q47188
	Serratia sp. strain ATCC39006	SmaR	75	CAB92554
	Hafnia alvei	HalR	73	AAP30848
	Yersinia pestis	YspR	72	NP_404602
	S. proteomaculans	SprR	71	AAK76734
	S. marcescens	SpnR	69	AAN52499
	E. carotovora	CarI	61	Q46751

Bioassay (Biosensing on solid medium)

A. tumefaciens strain NT1was used as acyl-HSL bioreporter

- Bioassay is performed on agar plates with bacteria containing different reporter genes.
- *A. tumefaciens* strain NT1(pZLR4) was used as indicator strain.
- This bacterium is sensitive to 3-oxo-AHLs and upon receiving the molecules it expresses β-galactocidase, which in turn breaks down Xgal present on the agar plate to give blue color.
- In this strain the traI (AHL synthase) gene has been deleted and when AHL binds TraR it starts expressing traG::lacZ fusion reporter gene present on a plasmid.

Cha *et al.*,1998;…

Surveying plant-associated bacteria for the production of compounds with acyl-HSL activity

A. tumefaciens strain NT1was used as acyl-HSL bioreporter

- Using petri dish assay, we surveyed 106 isolates representing seven genera of plant-associated, gram-negative bacteria: *Agrobacterium*, *Erwinia*, *Pantoea*, *Pseudomonas*, *Ralstonia*, *Rhizobium*, and *Xanthomonas* for the production of compounds with acyl-HSL activity.
- Petri dish assays were performed in ABM agar medium (AB minimal medium supplemented with 0.2% mannitol) supplemented with 40 µg of 5-bromo-3-indolyl-β-D-galactopyranoside (X-Gal) per ml.
- *A. tumefaciens* strain NT1(pZLR4) was used as indicator strain.
- Plates were incubated at 28°C for 12 to 16 h and examined for X-Gal hydrolysis (blue color).
- The strain NT1 produces a blue color in the presence of X-Gal in response to a wide range of AHLs.

Cha *et al.*,1998;…

Surveying plant-associated bacteria for the production of compounds with acyl-HSL activity

A. tumefaciens strain NT1was used as acyl-HSL bioreporter

- AB medium is a minimal growth medium used for bacterial cultures. It has some changes compared to M9 medium and is supposed to cause less problems with precipitation.
- Recipe:

5xA
for 1 liter AB medium
2 g $(NH_4)_2SO_4$
6 g Na_2HPO_4
3 g KH_2PO_4
3 g NaCl
200 ml H_2O

1xB
for 1 liter AB medium
1 ml 0.1 M $CaCl_2$ (sterile)
1 ml 1.0 M $MgCl_2$ (sterile)
1 ml 0.003 M $FeCl_3$ (sterile)
797 ml H_2O (sterile)

Mix 200ml 5xA and 800ml B to get 1 liter AB. Do never add B salts to 5xA!
If you want agar-plates add 16g agar to 1xB, autoclave and than add 5xA. Prepare and autoclave.
Add 0.2% sterile mannitol as sole carbon source.

MediaWiki,2012

Surveying plant-associated bacteria for the production of compounds with acyl-HSL activity

- Most of the *Agrobacterium*, *Rhizobium*, and *Pantoea* isolates and about half of the *Erwinia* and *Pseudomonas* isolates gave positive reactions.
- Only a few isolates of *Xanthomonas* produced a detectable signal.

- Reaction (R): Assessed by Petri dish assay.
- +++, wide (3 cm dia.); ++, somewhat narrower (1.5–3 cm dia.); +, very narrow (1.5 cm dia.); –, no detectable zones of diffusing blue color in the overlay surrounding colony or culture supernatant being tested.
- In most cases the colony or spot was approximately 0.5 to 1 cm in diameter.

Cha *et al.*,1998

Production of compounds with AHL activity by plant-associated and soil-borne bacteria.

Genus, species	Strain	Reporter strain	
		A.t.[b]	*C.v.*[c]
Agrobacterium tumefaciens	C58	5	–
	1D1	3	–
Chromobacterium violaceum	CVWT	10	++++
Erwinia herbicola	194	4	+++
	204	3	+++
	206	3	+++
	207	3	+++
	216	3	+++
	217	5	+++
	221	–	++
	227	–	++++
	229	–	+++
	230	–	+++
	234	–	+++
	241	3	+++
	243	–	+++
E. herbicola pv. *gypsophilae*	824-1	–	++++
Pantoea stewartii	183	20	++++
Pseudomonas aeruginosa	303	10	++++
	42	24	+++
	74a	1	–
	75	8	++++
P. chlororaphis	30-84	5	++++
(= *P. aureofaciens*)	21	2	–
	34a	16	+++++
	35a	3	–
	62	7	+++++
	64	10	++++
	65	10	+++++
	66	17	+++++
	67	8	++++
	205	7	+++++
	445	9	+++++
	449	9	+++++
	454	11	+
	464	10	+++++

Genus, species	Strain	Reporter strain	
		A.t.[b]	*C.v.*[c]
P. cichorii	9a	20	++++
	293a	8	+
P. fluorescens	2-79	16	+
P. fluorescens	315	10	++
(biotype 1)	334	14	++
P. fluorescens	38	22	–
(other biotypes)	101b	18	+++++
	142	18	+++++
	260	13	++
	447	10	+++++
P. geniculata	29	11	+
P. lemonnieri	110	18	+
P. putida	153	10	++
	146	11	+++++
	170	7	+++++
	262a	5	+++
	262b	13	++
P. saccharophila	488	5	nd
Pseudomonas spp.	36	13	–
	88	4	–
	149	12	+++++
	279	20	+++++
	320	17	+++++
	K19	3	–
Xanthomonas ampelina	11	–	+++
X. campestris pv. *malvacearum*	20	8	+++
X. campestris pv. *translucens*	6	7	++
X. campestris pv. *vesicatoria*	7	10	++
	340	15	++

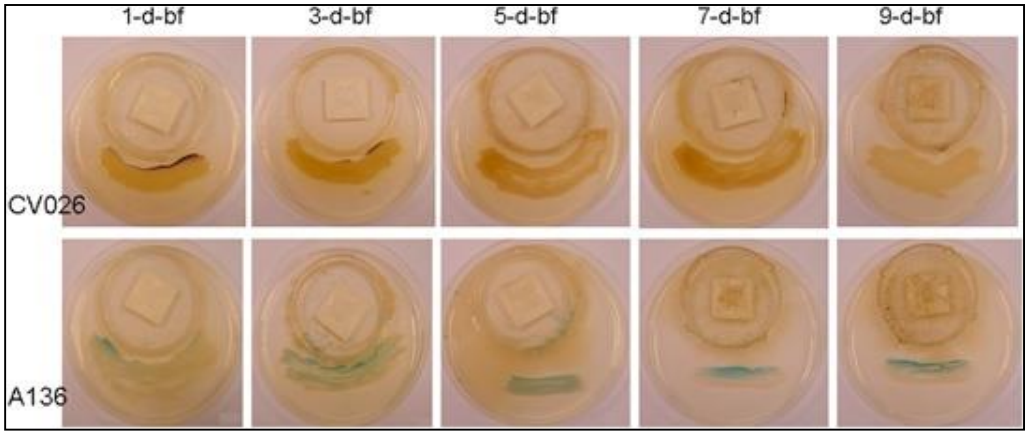
Strains *A. tumefaciens* C58 and 1D1, *C. violaceum* CVWT, *P. aureofaciens* 30-84 and *P. fluorescens* 2-79 were used as positive controls.

Vesolova *et al.*,1998

Detection of AHLs in subtidal biofilms by AHL reporter strains

***Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* A136**

- Detection of acylated homoserine lactones (AHLs) in subtidal biofilms of different ages (1-9-day-old biofilms, 1-9-d-bf) using the reporter strains *Chromobacterium violaceum* CV026 (fist raw panels) and *Agrobacterium tumefaciens* A136 (second raw panels).
- The strain A136 produces a blue color in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and due to X-Gal hydrolysis.
- whereas the strain CV026 produces a purple pigment when induced by short- to medium-chain AHLs, such as C4-, C6-, C8-HSL and 3-oxo-C6-, C8-HSL.



Huang *et al.*,2009

Commercially available AHL products

Catalog No.	Product Name
09945	N-Butyryl-DL-homoserine lactone BioChemika 25 mg
09926	N-Hexanoyl-DL-homoserine lactone BioChemika 25 mg
10939	N-Heptanoyl-DL-homoserine lactone BioChemika 25 mg
10940	N-Octanoyl-DL-homoserine lactone BioChemika 25 mg
10937	N-Tetradecanoyl-DL-homoserine lactone BioChemika 25 mg
17248	N-Decanoyl-DL-homoserine lactone BioChemika 25 mg
17247	N-Dodecanoyl-DL-homoserine BioChemika 25 mg
10942	N-Butyryl-DL-homocysteine thiolactone BioChemika 25 mg
K3255	N-(beta-Ketocaproyl)-DL-homoserine lactone (3-Oxo-hexanoyl-DL-homoserine lactone) 25 mg, 50mg

Sigma-Aldrich Co.

AHL screen assay
Pectobacterium sp.

- AHL-producing transgenic tobacco plants restore pathogenicity to an avirulent AHL-deficient *Erwinia carotovora* subsp. *carotovora* mutant (PNP22).
- The photograph shows the leaves 4 d after infection:

1. Wild-type tobacco inoculated with wild-type *Erwinia carotovora*;
2. Wild-type tobacco inoculated with AHL-negative *Erwinia carotovora* mutant PNP22;
3. AHL-producing tobacco line inoculated with *Erwinia carotovora* PNP22.

Fray,2001

AHL screen assay
Pectobacterium sp.

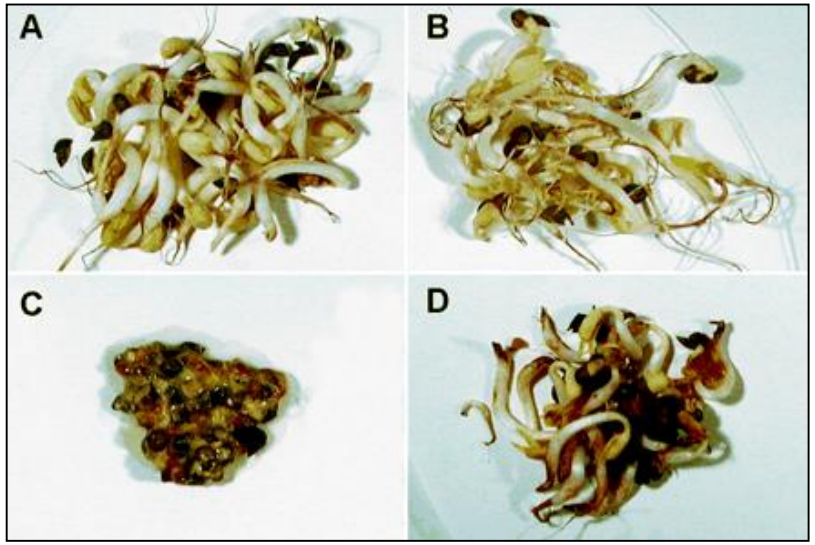
- Appearance of bean sprouts, where the soaking water has been inoculated with different bacterial strains to determine their spoilage potentials.

A. Control (uninoculated);

B. Bean sprouts inoculated with the nonspoiling strain C1JM;

C. Bean sprouts inoculated with the spoiling strain *Pectobacterium* sp. strain A2JM;

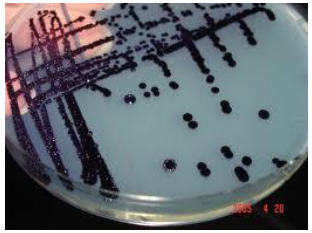
D. Bean sprouts inoculated with the AHL-deficient A2JM *luxI* mutant.



Thin layer chromatogram showing the range of AHLs produced by various *Erwinia carotovora* species

- After chromatography, *Erwinia carotovora* AHLs were located by overlaying the chromatogram with agar seeded with the *Chromobacterium violaceum* (CV) reporter strain mutant for *cviI* (the gene required for AHL synthesis).
- The presence of AHLs is indicated by complementation of the mutation and restoration of the production of the purple pigment, violacein.
- A, 3-oxo-C6-HSL standard
- B, C6-HSL standard
- C– F, spent bacterial culture supernatants of *Erwinia carotovora* subsp. *carotovora* SCRI 193 (C); *Erwinia carotovora* subsp. *atroseptica* SCRI 1043 (D); *Erwinia carotovora* subsp. *atroseptica* SCRI 1039 (E); and *Erwinia chrysanthemi* SCRI 1043 (F).
- Most strains produce both 3-oxo-C6-HSL and C6-HSL as well as additional AHLs in some cases.

Chromobacterium violaceum
Colony morphology



- It is a Gram-negative, facultative anaerobic, non-sporing coccobacillus.
- It is part of the normal flora of water and soil of tropical and sub-tropical regions of the world.
- It produces a natural antibiotic called violacein.
- It grows readily on nutrient agar, producing distinctive smooth, low convex, circular, with an entire margin colonies with a dark violet metallic sheen (due to violacein production).

Reverse phase thin-layer chromatography (TLC)

Thin layer chromatogram showing the range of AHLs produced by various bacteria

- TLC is a powerful technique both for analysis and separation of different AHLs, that can be analysed either by biological methods or analytical techniques.
- Extracts are spotted on TLC plates and allowed to run in a mixture of organic solvents.
- Plates are usually C18 silica matrix plates.
- Separated AHLs are either visualized with UV or by chromic agents like potassium dichromate in sulphuric acids.

If you are working with highly polar compounds, reversed-phase mode is more suitable. Reversed-phase chromatography employs a polar (aqueous) mobile phase.

Thin layer chromatography (TLC)

Thin layer chromatogram showing the range of AHLs produced by various bacteria

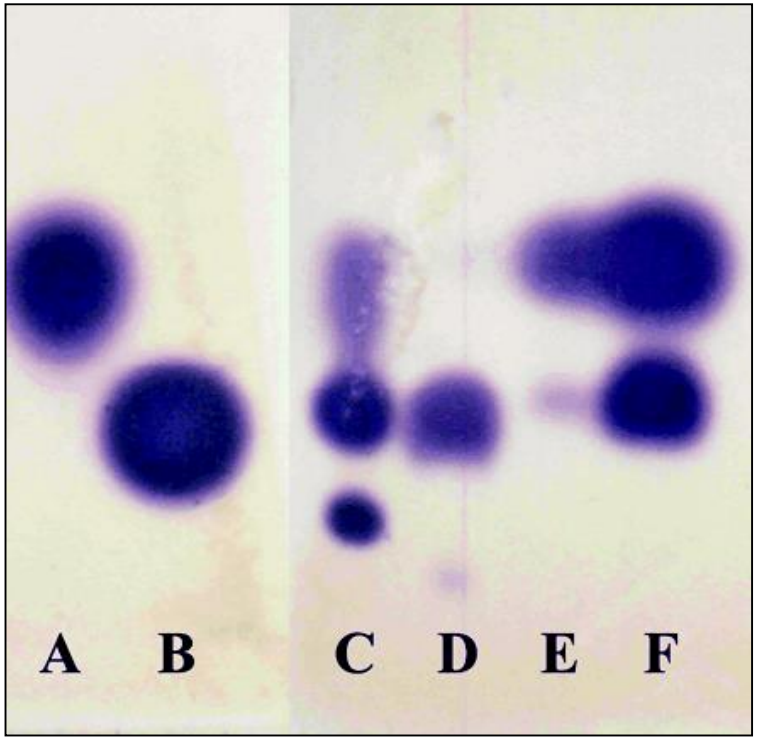
- Alternatively, spots could be scraped off and the material obtained extracted with either dichloromethane or ethyl acetate for further analysis.
- One disadvantage is that:

1. No structural information can be obtained, and
2. Interference of metabolites is hard to avoid.

- For example two unknown spots found in a report which could not be verified.

Thin layer chromatogram showing the range of AHLs produced by various *Erwinia carotovora* species

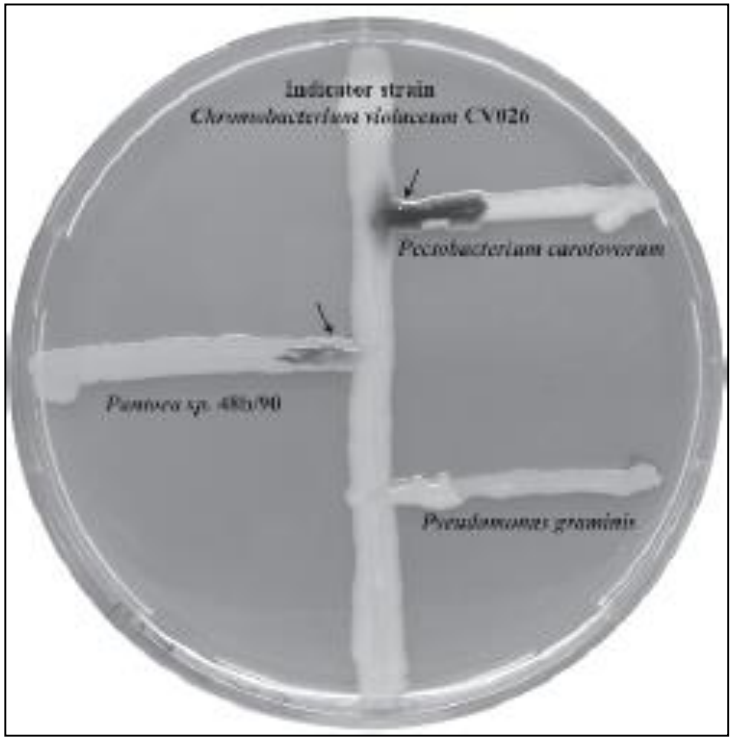
- The sensitivity of the *Chromobacterium violaceum* bioreporter(indicator) strain/a biosensor sensitive to short-chain AHLs) varies according to the AHL being detected.
- Thus the intensity of pigment is not a direct indicator of the relative abundance of each AHL.



Fray,2001;..

Quorum Sensing
Pantoea sp. strain 48b/90

- The antagonist *Pantoea* sp. strain 48b/90 isolated from soybean leaf induced weakly the production of violacein in the *Chromobacterium violaceum* CV026.
- Probably, it produced an acyl-homoserine lactone (AHL) with a short acyl side chain or another class of quorum sensing signal molecules.
- In this test, different strains were screened on solid medium in a streak plate assay using AHL biosensor strain *Chromobacterium violaceum* CV026.
- AHL production (N-acyl side chains C4-C8) was judged by its ability to induce violacein production (purple pigment: arrows).

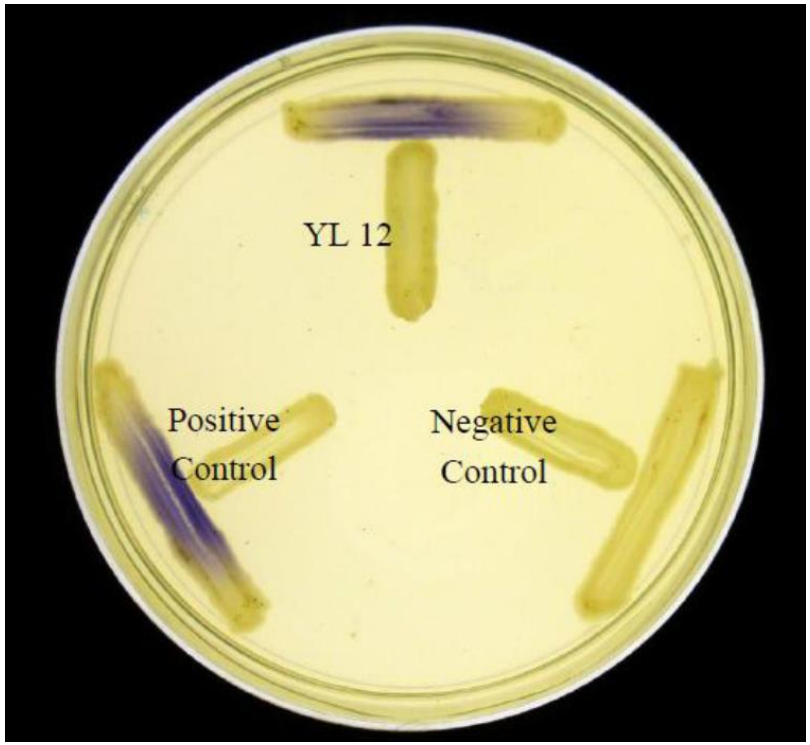


Völksch and Sammer,2008

Quorum Sensing

Cross-streaking of *Aeromonas caviae* strain YL12 with *C. violaceum* CV026

- Cross-streaking of strain YL12 with *C. violaceum* CV026 was observed to trigger violacein production in the biosensor.
- *E. carotovora* GS101 and *E. carotovora* PNP22 were used as positive and negative controls, respectively.
- In the cross-streaking experiment conducted, strain YL12 triggered CV026 violacein production, which suggests that YL12 produces diffusible short chain AHLs.



Lim *et al.*,2014

Phylogenetic analysis of LuxI and LuxR homologues present in diverse members of the Gram-negative *Proteobacteria*

Sequence analysis

- The majority of quorum sensing systems that fall under the two-gene (*I* and *R*):

1. An autoinducer synthase coupled with
2. A receptor molecule.

- A comparison between the Proteobacteria phylogeny as generated by 16S rRNA sequences and phylogenies of LuxI-, LuxR-, or LuxS-homologs shows a notably high level of global similarity.
- Overall, the quorum sensing genes seem to have diverged along with the Protecobacteria phylum as a whole.
- This indicates that these quorum sensing systems are quite ancient, and arose very early in the Proteobacteria lineage.

Wikipedia,2011

Phylogenetic analysis of LuxI and LuxR homologues present in diverse members of the Gram-negative *Proteobacteria*

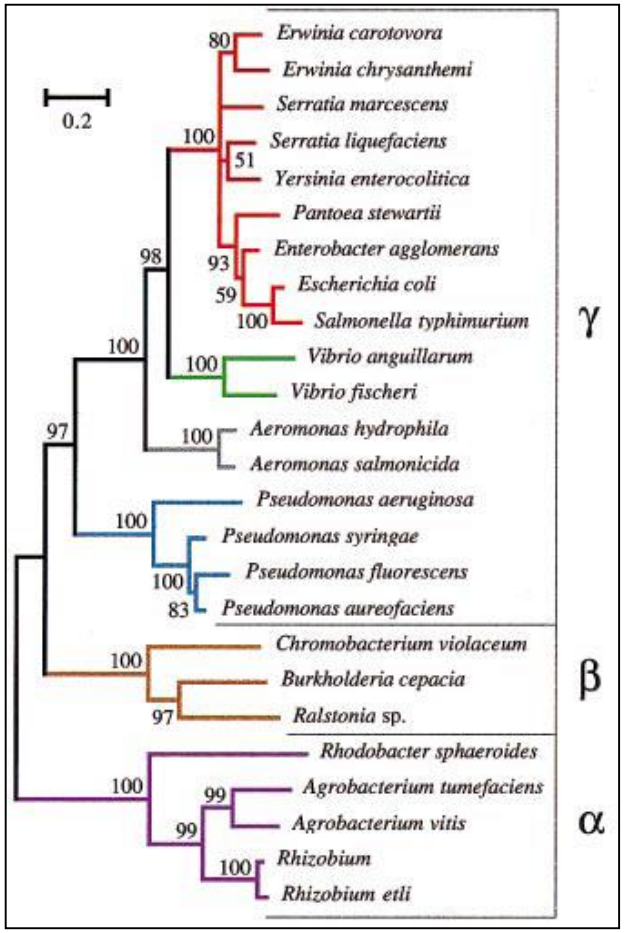
Single or multiple LuxI/R homologues

- Homologues of *luxI* and *luxR* genes have been identified both on bacterial chromosomes and on extrachromosomal elements such as the symbiosis (Sym) and tumour-inducing(Ti) plasmids of *Rhizobium* and *Agrobacterium* spp., respectively.
- Many bacterial species even contain multiple LuxI and/or LuxR homologues for the production or detection of multiple, distinct signals.
- Multiple LuxR homologues can also permit the independent activation of different gene functions(such as exoenzyme synthesis, conjugation, antibiotic production, luminescence and biofilm formation) in response to a single autoinducer signal.

Gray and Garey,2001

These 16S rRNA groupings were used as the basis for comparison with phylogenetic trees derived from our analysis of LuxI and LuxR family members

- Neighbour-joining tree of 16S rRNA sequences from bacteria that contain LuxI and/or LuxR family members.
- Species belonging to the α, β and γ-*Proteobacteria* are grouped.
- Our results suggest that these quorum sensing regulators arose early in the evolution of the *Proteobacteria* and subsequently diverged within each group of organisms, although instances of horizontal gene transfer have also occurred.
- Surprisingly, most bacteria that contain multiple LuxI and/or LuxR family members appear to have acquired each of their different homologues from separate sources.



Gray and Garey,2001

Microbial biofilms

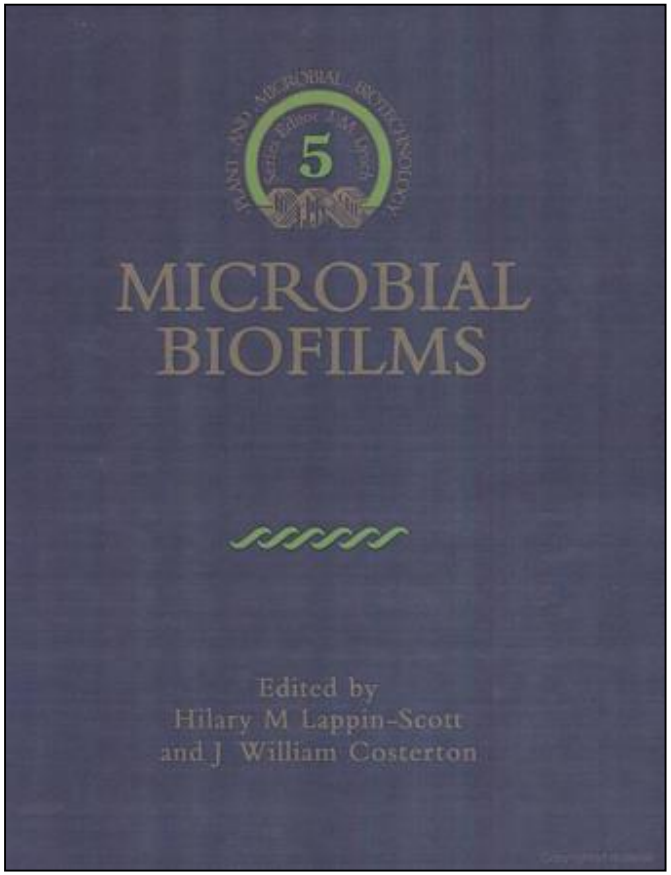
Thick, sugary coating coatings that help microbes stick to plants

City of Microbes

Key to understanding and controlling bacterial growth

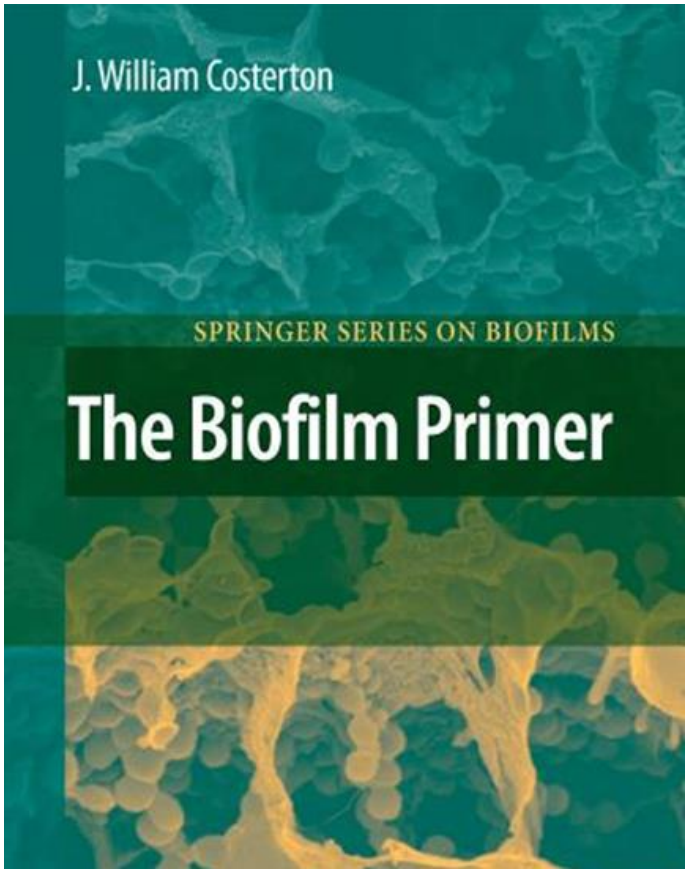
Microbial Biofilms

- **Microbial Biofilms**
- Editors: Hilary M. Lappin-Scott and J. William Costerton
- Cambridge University Press
- 2003
- 328 pages.



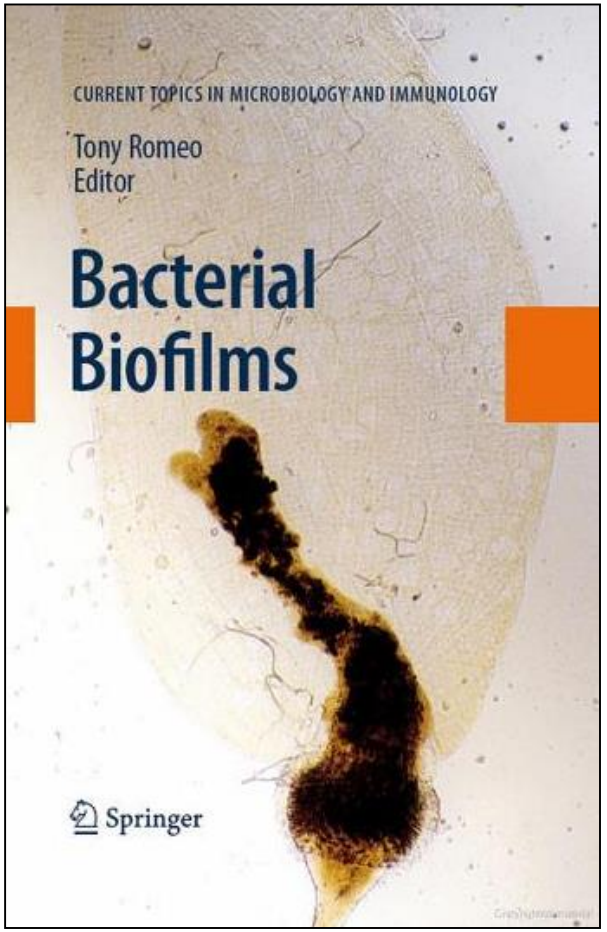
The Biofilm Primer

- **The Biofilm Primer**
- Author: J. William Costerton
- Springer
- 1 edition
- April 19, 2007
- 207 pages.



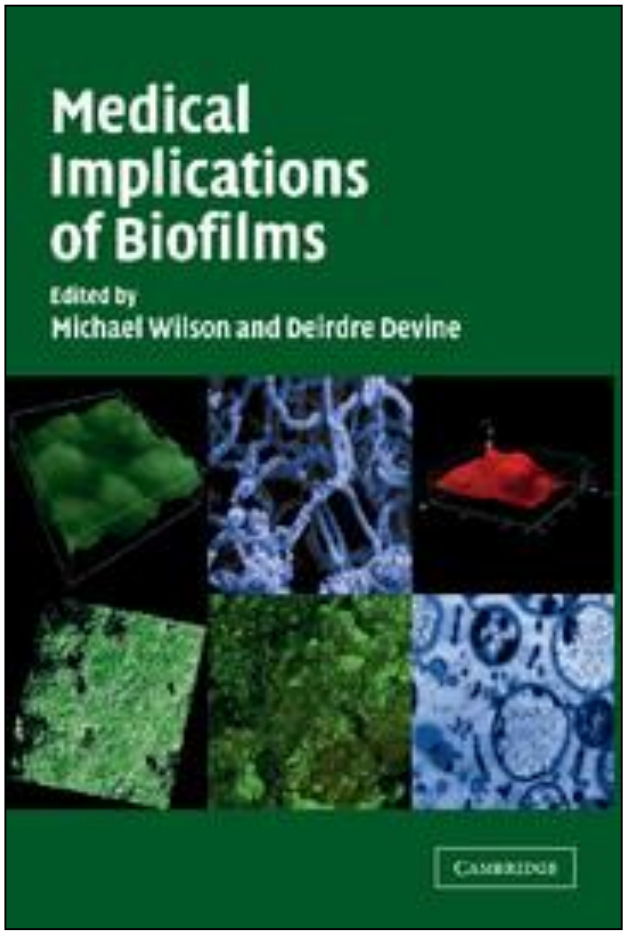
Bacterial Biofilms

- **Bacterial Biofilms**
- Current Topics in Microbiology and Immunology, Vol. 322.
- Editor: Tony Romeo
- Springer, 2008.
- 308 pages.
- 3.2 Mb.



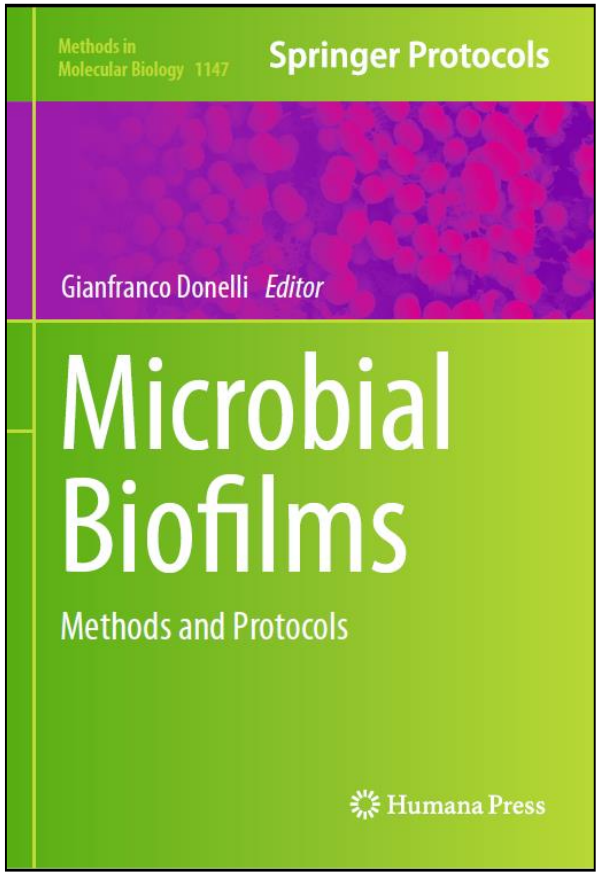
Medical Implications of Biofilms

- **Medical Implications of Biofilms**
- Michael Wilson and Deirdre Devine
- Publisher: Cambridge University Press.
- Cambridge books online
- Date Published: 2011
- 314 pages.



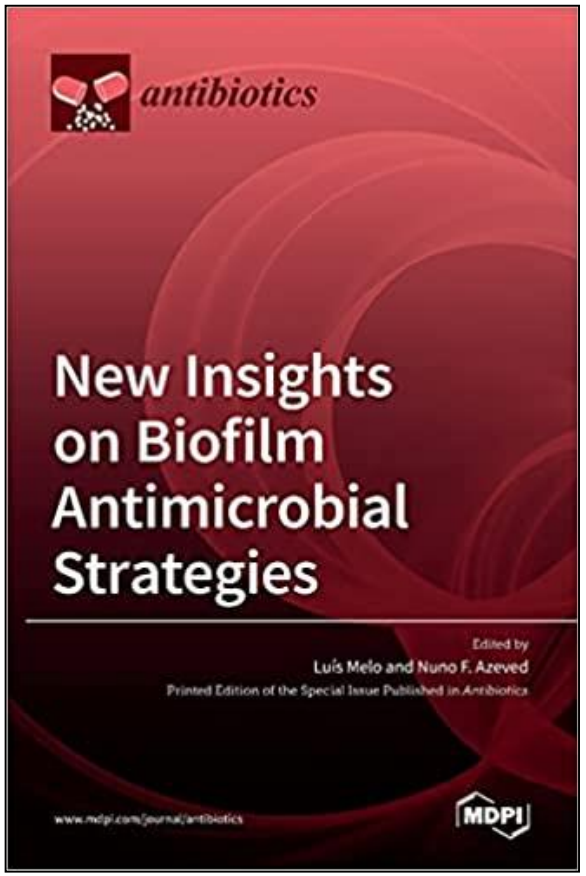
Microbial Biofilms
Methods and Protocols

- **Microbial Biofilms-Methods and Protocols**
- Editor: Gianfranco Donelli
- Publisher: Humana Press.
- Date Published: 2014
- 380 pp.



New Insights on Biofilm Antimicrobial Strategies

- **New Insights on Biofilm Antimicrobial Strategies**
- Editors: Luís Melo and Nuno F Azevedo
- Publisher: Mdpi AG
- Date Published: 2021
- 264 pp.



Quorum sensing and biofilm

1. Quorum sensing control gene expression in groups of bacteria, and
2. Biofilms organized groups of bacteria.

- Bacteria often tend to attach to surfaces and form communities enmeshed in a self-produced polymeric matrix.
- These communities are called a biofilm.

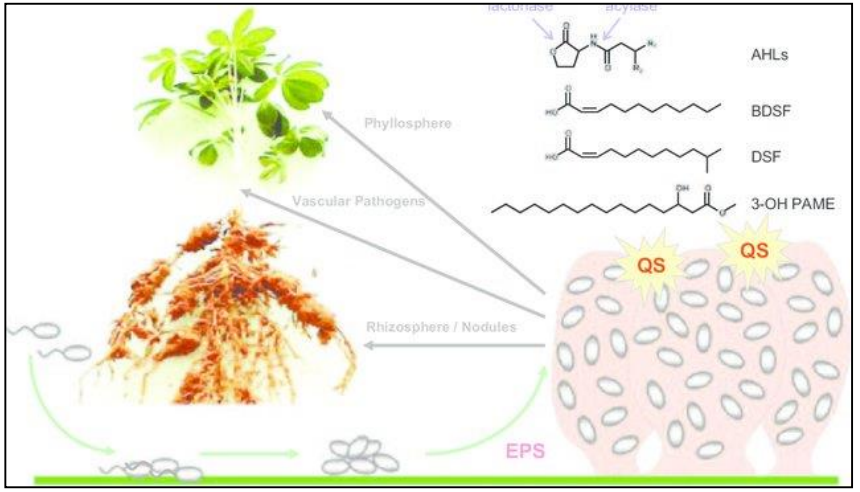
Wikipedia,2010;…

Quorum sensing and biofilm

- In general, bacterial pathogens use quorum sensing to ensure that virulence genes are only expressed after their population has reached a critical size.
- This unified attack strategy makes it more difficult for the host to mount an effective defense.

Microbial biofilms on and within plants

- In many plant-associated bacteria biofilm formation is controlled by QS.
- These bacterial communication systems utilize chemically diverse signal molecules.
- The site of action of two classes of enzymes that inactivate AHL signal molecules is indicated by blue arrows.



Carlier *et al.*,2015

Plant and soil-associated biofilm-forming bacteria: Their role in green agriculture

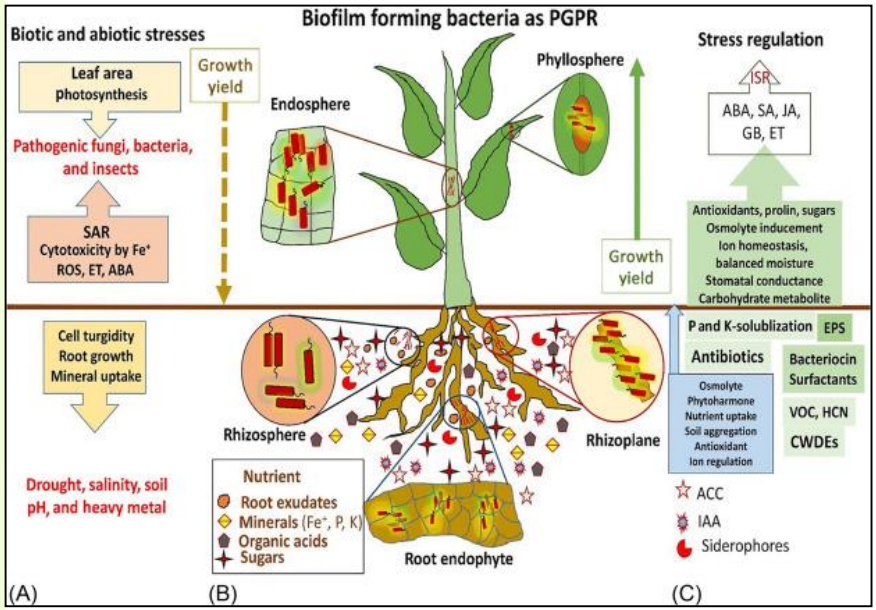
Sites of microbial colonization on terrestrial plants

- Soil and plant-associated bacteria play a significant role as:

1. plant growth regulators,
2. plant defense activators, and

- can exist in:

1. planktonic (free-swimming) form, or
2. sessile form (biofilm; attached to surfaces).



Solanki *et al.*,2020

Quorum sensing and biofilm

- Quorum sensing has been shown to directly control:

1. Biofilm formation (A highly structured polymer matrices produced by sessile bacteria (bacteria living within a biofilm).
2. The expression of virulence genes,
3. Swarming,
4. Conjugation, and
5. Production of secondary metabolites.

Bacterial autoaggregation and biofilm development, and their relationship with plant colonization

- Cell aggregation and biofilm formation in plant-bacterial associations are regulated by:

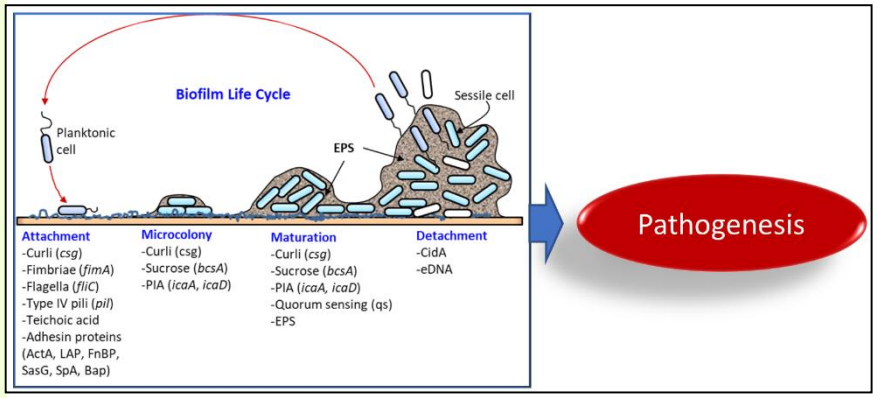
1. Environmental signals,
2. Nutrient limitation of growth,
3. Quorum sensing,
4. EPSs,
5. Flagella,
6. LPSs, and
7. Other factors.

Bogino *et al.*,2013

Schematic showing the different stages of biofilm formation

Staphylococcus aureus

- (i) attachment, (ii) microcolony formation, (iii) maturation with cellular differentiation, and (iv) detachment or dispersion, and participation of bacterial virulence factors in each step.



Abbreviations:

ActA, actin polymerization protein; Bap, biofilm-associated protein; bcsA, bacterial cellulose synthesis; CidA, cell death effector protein; csg, curli synthesis gene; EPS, extracellular polymeric substance; eDNA, extracellular DNA; FnBP, fibronectin-binding proteins; icaA, intercellular adhesion; LAP, Listeria adhesion protein; PIA, polysaccharide intercellular adhesin; SasG, *S. aureus* surface protein G; SpA, *S. aureus* protein A. Figure adapted with permission from Ray and Bhunia 2014.

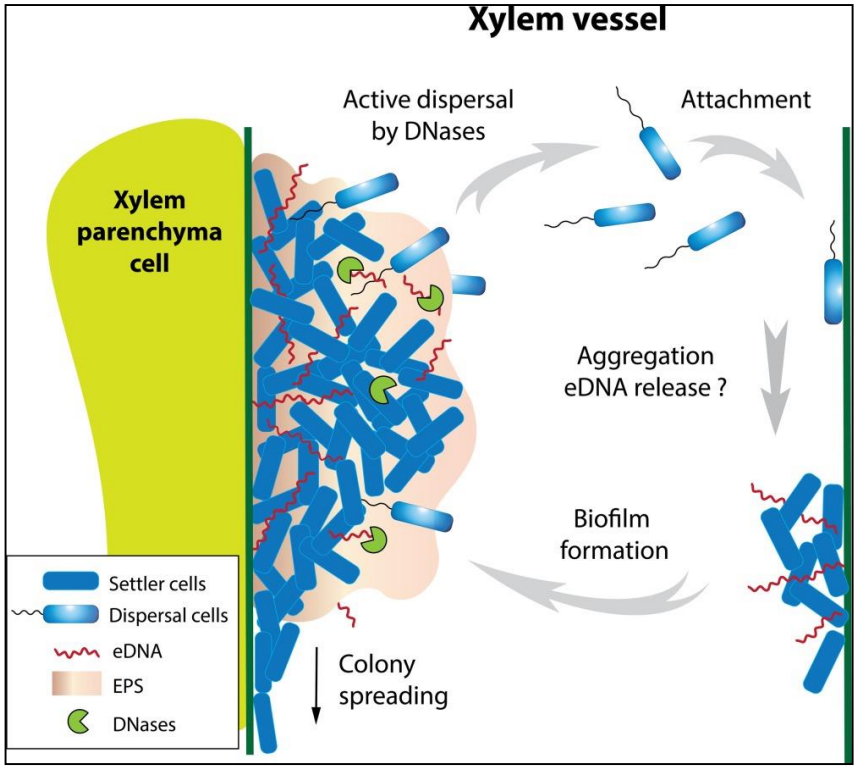
Bai *et al.*,2021

Biofilm formation by plant bacterial pathogens

- *Xanthomonas campestris* pv.*campestris*
- *Pseudomonas syringae* pv.*syringae*
- *Dickeya chrysanthemi*
- *Pantoea stewartii* subsp.*stewartii*
- *Ralstonia solanacearum*
- *Clavibacter michiganensis* subsp.*sepedonicus*
- *Xylella fastidiosa*
- '*Candidatus Liberibacter asiaticus*'

Extracellular DNases of *Ralstonia solanacearum* modulate biofilms and facilitate bacterial wilt virulence

- *Ralstonia solanacearum* is a soil-borne vascular pathogen that colonizes plant xylem vessels, a flowing, low-nutrient habitat where biofilms could be adaptive.
- During tomato infection, *R. solanacearum* forms biofilm-like masses in xylem vessels.
- These aggregates contain bacteria embedded in a matrix including chromatin-like fibres commonly observed in other bacterial biofilms.

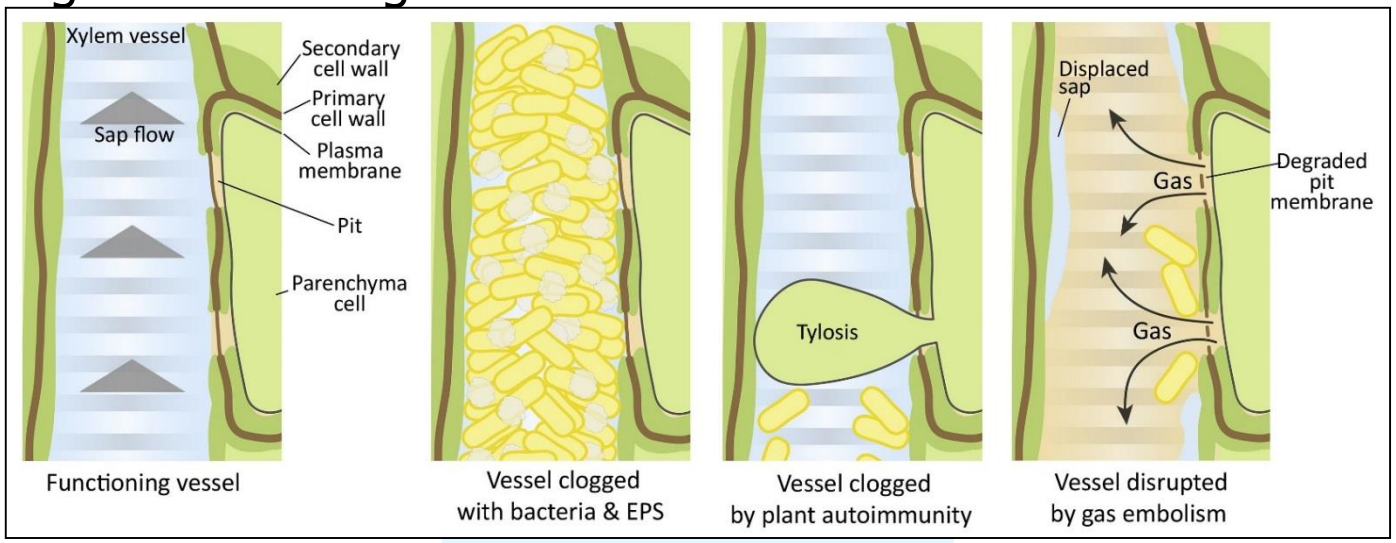


Tran *et al.*,2016

Tylosis form in xylem vessels

Ralstonia solanacearum

- Tyloses are ingrowths of parenchyma cells into the lumen of embolized xylem vessels, thereby protecting the remaining xylem from pathogens. Gas embolisms are a less-discussed mechanism of plant vascular dysfunction during wilt disease.
- Air (gas)embolism is thought to accelerate pathogen progression during Pierce's disease.



Lowe-Power*et al.*,2018;.

Biofilm

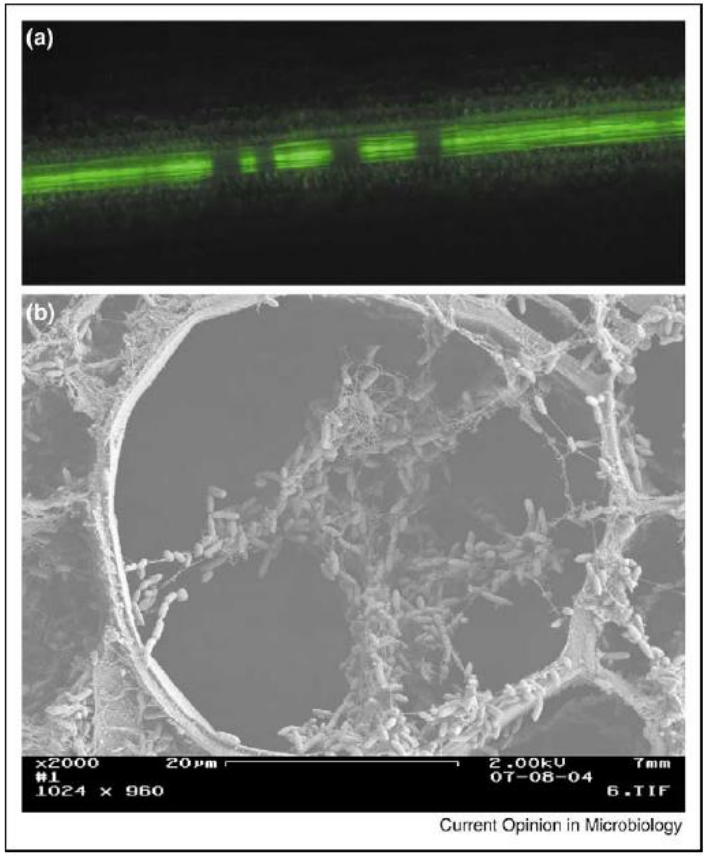
Plant pathogenic bacteria

Pantoea stewartii subsp. *stewartii*

- Colonization of the vasculature by *Pantoea stewartii* subsp. *stewartii*.

(a) GFP-tagged wild-type strain DC283 colonizing leaf xylem vessels of a susceptible maize cultivar. Obtained using an Olympus IX70 inverted epifluorescence microscope, 40Xmagnification.

(b) Scanning electron micrographs depicting colonization of sweet corn xylem vessels.

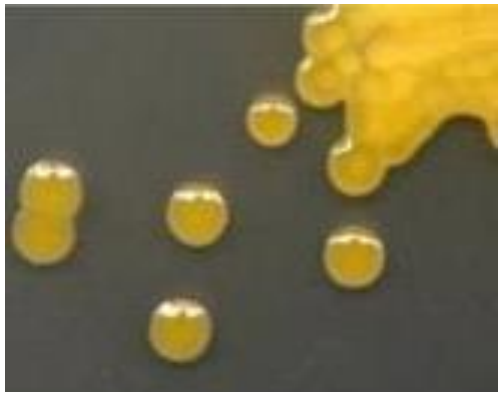


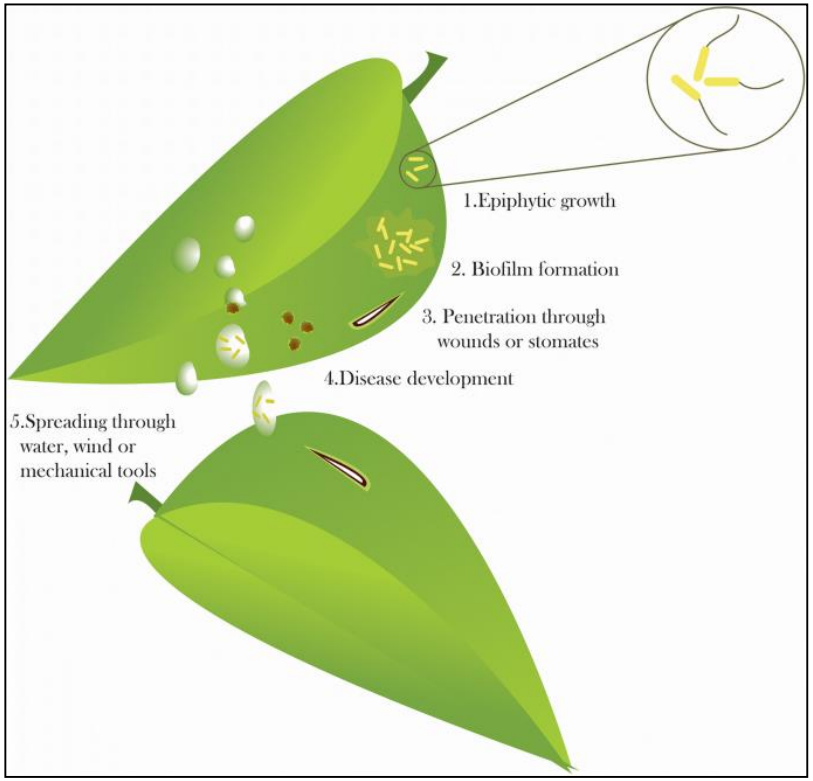
Ramey *et al.*,2008

Biofilm

Biofilm formation in *Xanthomonas* genus

Xanthomonas citri subsp. *citri*



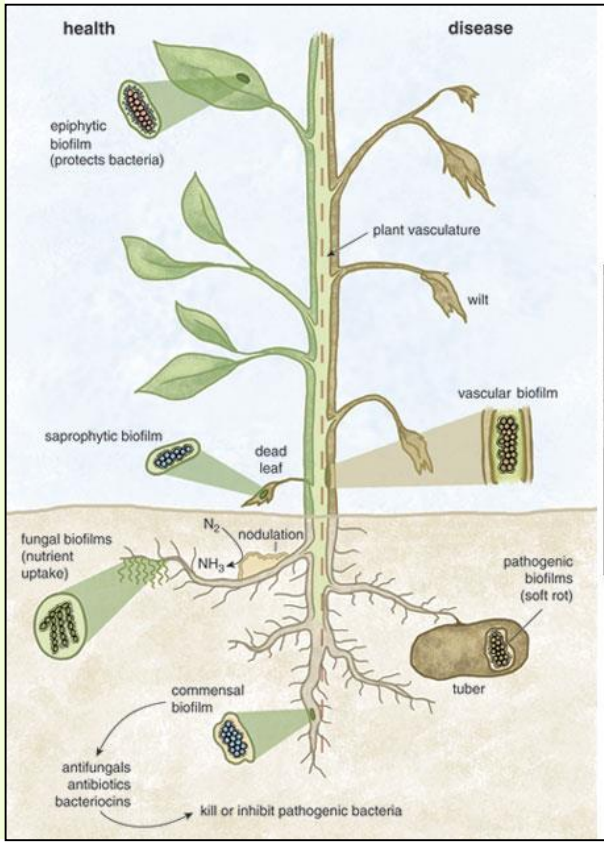


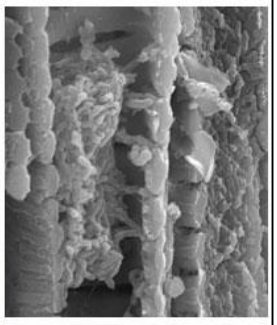
Cirad,2019; Moreira Martins *et al.*,2020

Biofilm formation by plant pathogenic bacteria

Xylella fastidiosa

- Certain epiphytic populations with the genetic potential to initiate a pathogenic interaction with the host grow large enough to overwhelm the host's defense mechanisms.
- An example *Xylella fastidiosa*. This pathogen causes Pierce's disease in grapevines and citrus variegated chlorosis in sweet oranges-diseases with economic losses exceeding $14 billion in the past decade.

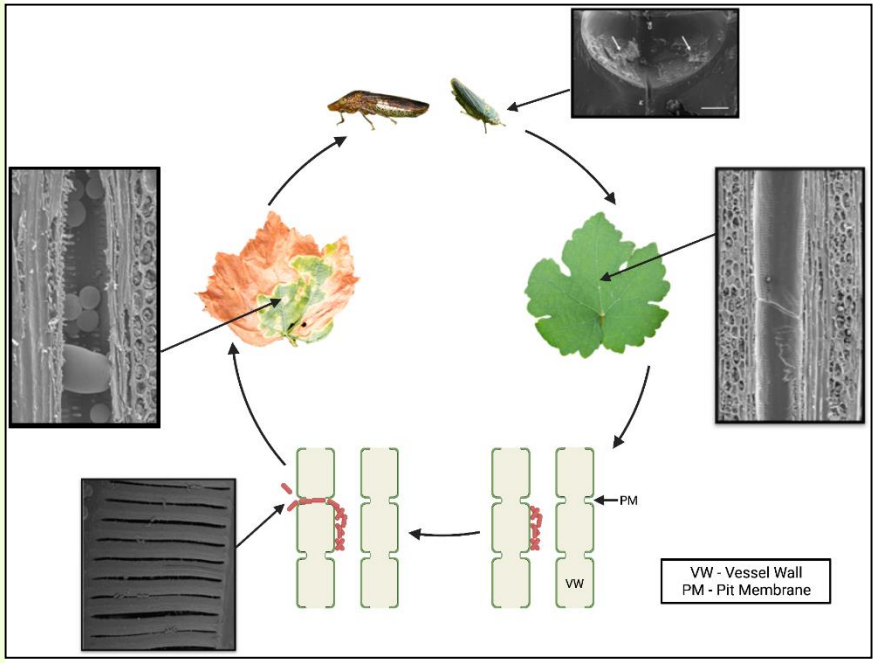




Harrison *et al.*,2021

PD of grapevine cycle
Biofilm formation by *Xylella fastidiosa*

- *Xylella fastidiosa* is transmitted to a new host plant when the insect vector feeds on a new plant and deposits *X. fastidiosa* cells directly into the plant xylem.
- Systemic colonization and xylem vessel occlusion by bacterial biofilms and excess tylose production lead to Pierce's disease (PD) of grapevine.



Castro *et al.*,2021

Biofilm

Production, composition, and functional roles of exopolysaccharides

EPSs in beneficial and pathogenic plant-associated bacteria

Bacteria/plant association	Exopolysaccharide	Chemical composition	Function
B. japonicum symbiosis with *Glycine max*	EPS [100,101]	Pentasaccharide units (mannose:galacturonic acid:glucose:galactose 1:1:2:1)	Biofilm formation on both inert and biotic surfaces. Roles during the early stages of interaction with the host plant (initial attachment of rhizobia to root epidermal cells) [102]
M. tianshanense symbiosis with *Glycyrrhiza uralensis*	EPS	ND	Involved in biofilm formation and successful establishment of symbiosis [103]
A. tumefaciens ubiquitous plant pathogen	Succinoglycan [104]	See above	Increased production of succinoglycan results in reduced attachment and biofilm formation [105]
X. fastidiosa plant pathogen	Putative Fastidian gum [106]	Putative tetrasaccharide units (glucose-1-phosphate, glucose, mannose, and glucuronic acid)	Possibly involved in bacterial pathogenicity [106] Cell attachment and overall biofilm formation [107]
X. campestris *X. axonopodis* plant pathogens	Xanthan gum [108]	Pentasaccharide units (glucose:mannose:glucuronic acid 2:2:1 derivatized with acetyl and pyruvyl moieties)	Essential for microcolony formation [74] Formation of structured biofilms on abiotic surfaces and in infected

Bogino *et al.*,2013

Biofilms

Common examples

- Biofilms are a collection of microorganisms surrounded by the slime they secrete, attached to either an inert or living surface.
- You are already familiar with some biofilms:

1. The slippery slime on river stones and in a water pipe,
2. The plaque on your teeth,
3. The gel-like film on the inside of a vase which held flowers for a week.

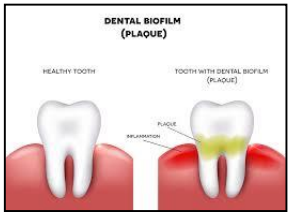
- Thus, among infectious bacteria, talk is not cheap.
- Biofilm exists wherever surfaces contact water.

Biofilms

Biofilm in a water pipe

- The interiors of almost all water distribution systems eventually develop biofilms that may harbor pathogenic microbes and promote metal pipe corrosion, scaling and sediment buildup.
- Biofilms can discolor water or cause it to take on disagreeable tastes or odors.

Reiff,2013

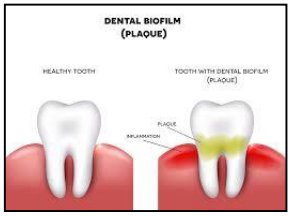


Biofilms

Bacterial species involved with dental biofilm accumulation and tooth decay. All employ quorum sensing

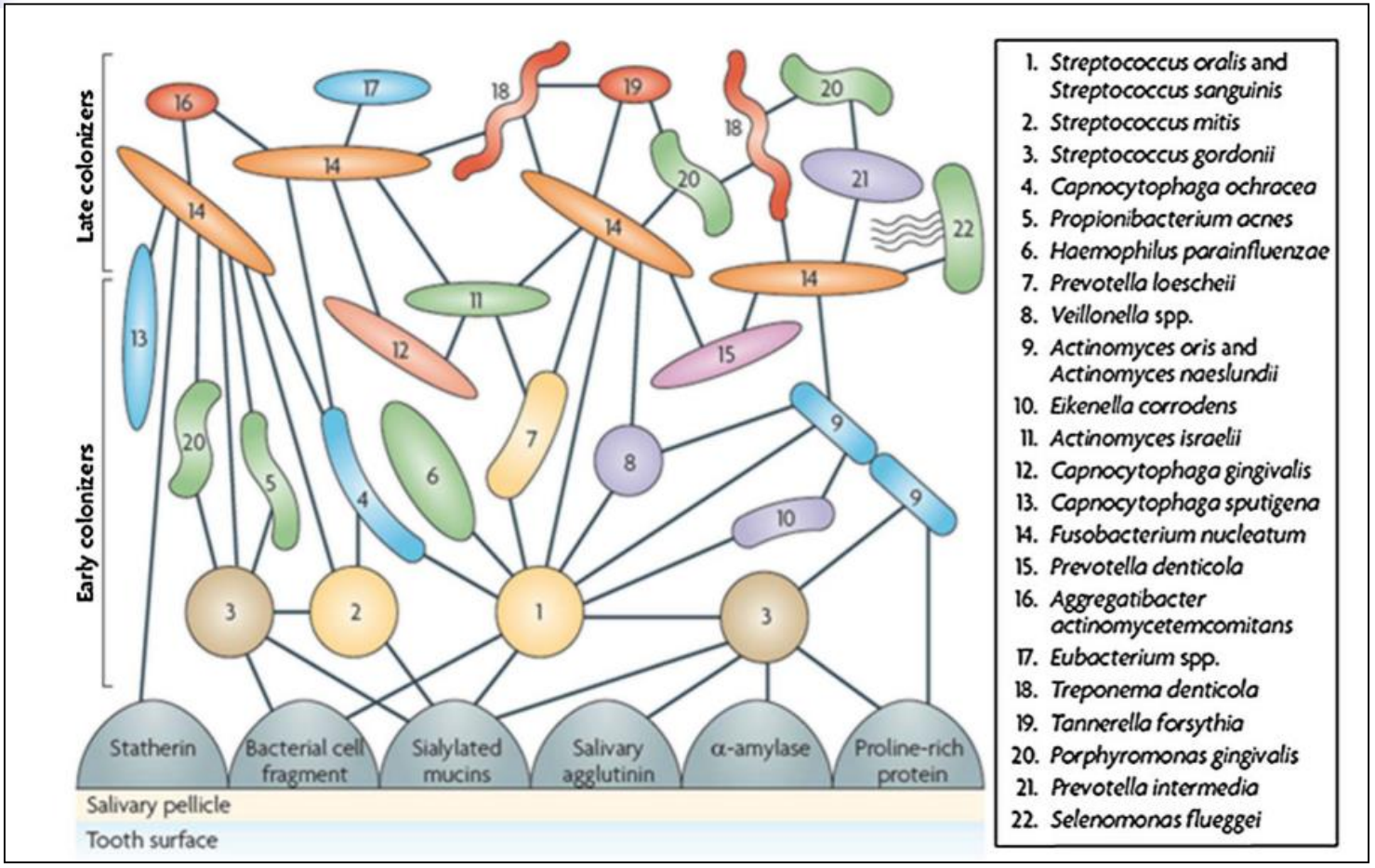
- That "fuzzy" feeling that can be felt on unbrushed teeth is an accumulation of a biofilm of bacteria.
- There are several bacteria within a person's mouth which make up this biofilm and are cariogenic, meaning they have a tendency to cause dental caries.
- These include:
- *Lactobacillus acidophilus*,
- *Streptococcus mutans* (and *S. gordonii*, *S. salivarius*),
- *Actinomyces odontolyticus*.

Cain *et al.*,2017

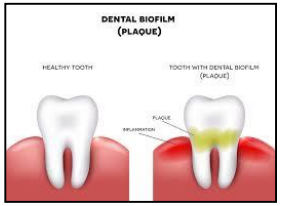


Biofilms

Bacterial species involved with dental biofilm accumulation and tooth decay. All employ quorum sensing



Parashar *et al.*,2015



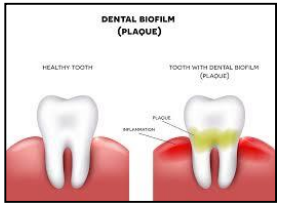
Biofilms

Bacterial species involved with dental biofilm

The Snyder Test

- The Snyder Test is used to determine a person's susceptibility to dental caries based on acid production that is assumed to be a result of the growth of lactobacilli on the teeth or in other areas of the mouth.
- Note that the Snyder Test does not test for cariogenic streptococci or actinomycetes.
- The Snyder Test agar contains 2% glucose and has a pH of about 4.8.
- This low pH inhibits the growth of most organisms, but it is ideal for lactobacilli.

Cain *et al.*,2011



Biofilms

Bacterial species involved with dental biofilm

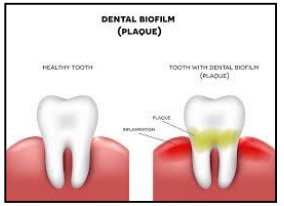
The Snyder Test

- MEDIA NEEDED: (per student)

1. 1 Snyder deep agar tube
2. 1 Sterile empty tube or beaker

- CULTURES NEEDED: Saliva from student's oral cavity

Cain *et al.*,2017



Biofilms

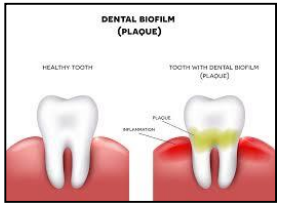
Bacterial species involved with dental biofilm

The Snyder Test

- Procedure:

1. Obtain a sterile test tube or small beaker. Collect approximately ¼ to ½ inch of saliva in the test tube or beaker.
2. Obtain a melted Snyder Agar deep tube that has been tempered in a 45°C water bath.
3. While the Snyder Agar deep tube is still liquefied, aseptically pipette 0.2 ml of saliva into the tube.
4. Place the cap loosely on the tube and roll the tube gently between the palms of your hands. Let solidify.
5. Incubate the Snyder tube at 37°C for 48 hours.

Cain *et al.*,2017



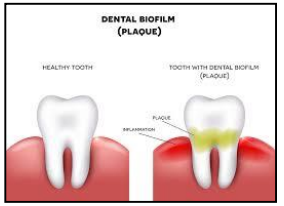
Biofilms

Bacterial species involved with dental biofilm

The Snyder Test

- If growth occurs, and lactic acid is produced by the lactobacilli, the pH will drop below 4.8 to about 4.4.
- The indicator used to show this change in pH is bromcresol green, which will change from green to yellow upon acid production.
- The rate of change is another factor to be considered. The faster the rate of change, the more organisms present.

Cain *et al.*,2017



Biofilms

Bacterial species involved with dental biofilm

The Snyder Test

Color of Agar	Green	Light Green	Yellow-Green	Yellow
Susceptibility	Slight	Mild	Moderate	High

SNYDER TEST

- This test measures the ability of salivary microorganisms to form organic acid from a carbohydrate medium.
- The classical formula of Snyder's agar per litre of purified water is

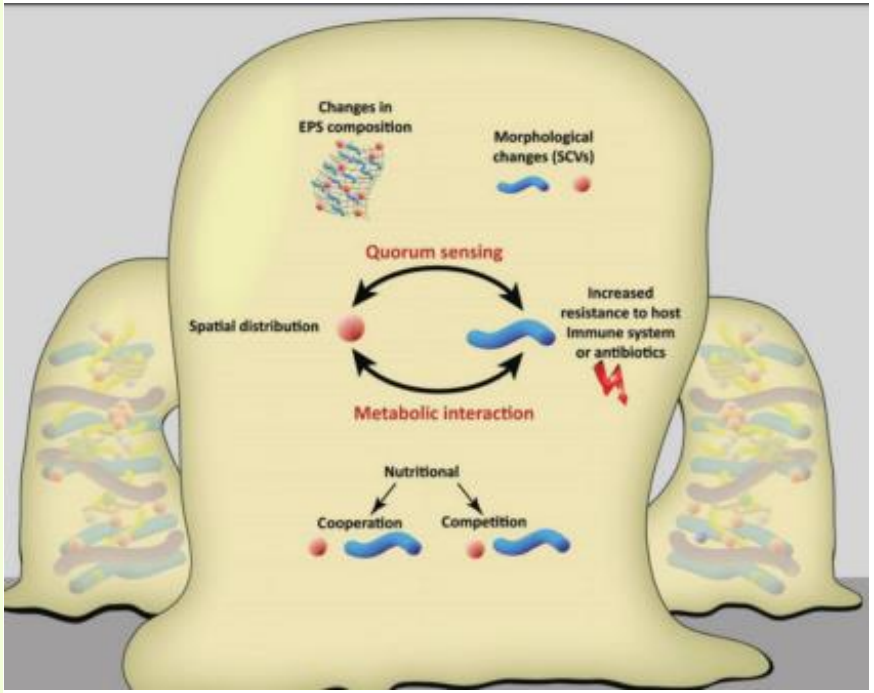
pancreatic digest/ casein	-13.5 gm
yeast extract	-6.5 gm
dextrose	-20 gm
sodium chloride	-5 gm
agar	-16 gm
Bromocresol green	-0.029 gm

Cain *et al.*,2017; SlideToDoc.com

Biofilm

Multispecies/monospecies microbial community

- Multiple bacterial species as opposed to the mono-species biofilms.
- Interactions among different species within a biofilm can be antagonistic, such as competition over nutrients and growth inhibition, or synergistic.



Schaechter,2004; Elias and Banin,2012

Biofilm

Biofouling or biological fouling

Microbiological fouling/microfouling

- Biofouling or biological fouling: The damage caused to a surface by microorganisms attached to a surface.
- Biofouling can be either:

1. Micro (by bacteria, slimes, algae, and the like);
2. Macro (by clams, mussels,…) in nature.

Daniels and Selby,2007

Biofilm development

Five steps in biofilm development

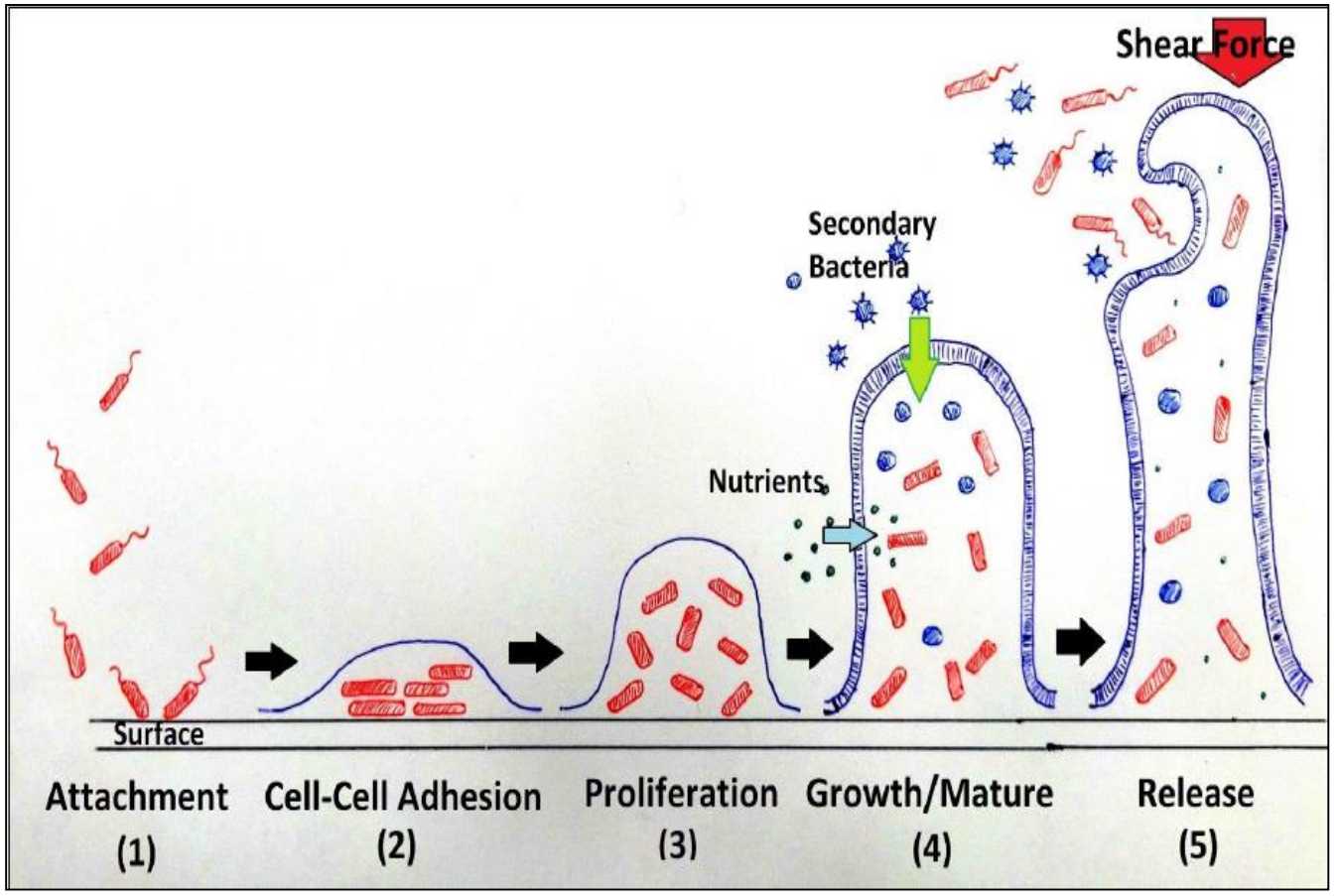
- Biofilm development can be divided into several key steps including:

1. attachment,
2. Cell to cell adhersion
3. Proliferation (micro colony formation),
4. biofilm maturation, and
5. Dispersion.

- In each step bacteria may recruit different components and molecules including flagella, type IV pili, DNA and exo polysaccharides.

Biofilm development

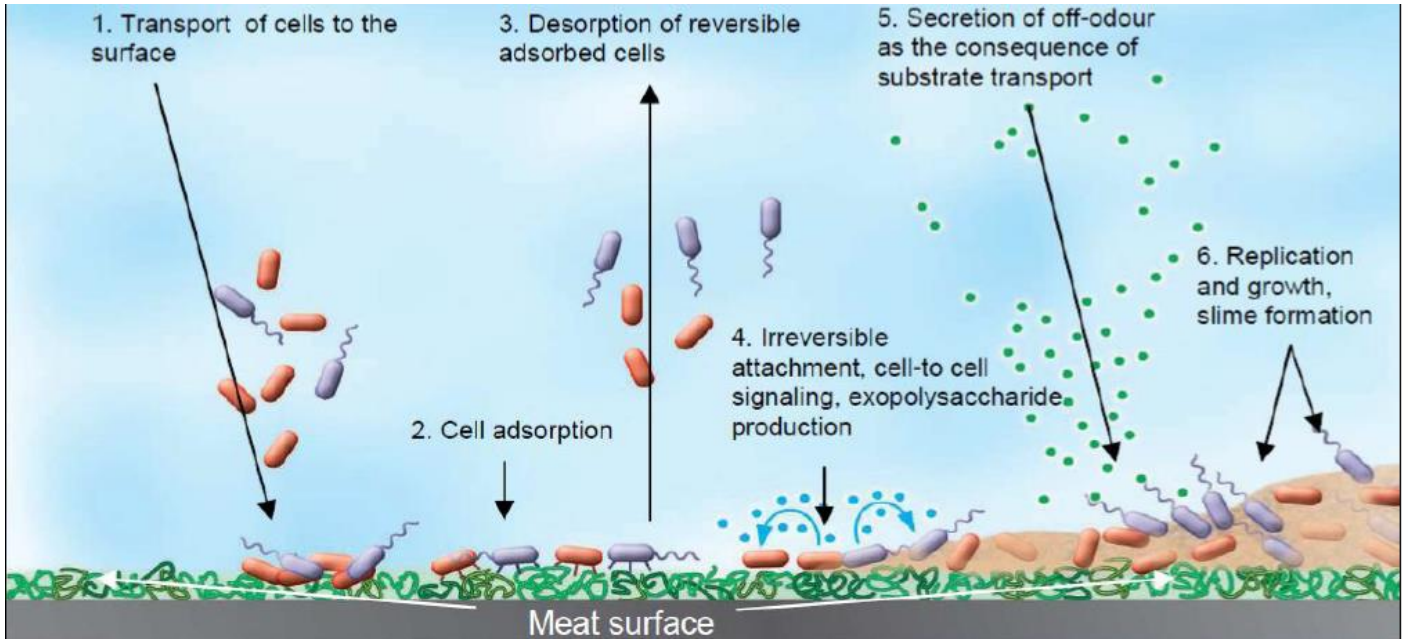
Five steps in biofilm development



Aguinaldo,2015

Biofilm development

On meat surface



Desorption is a phenomenon whereby a substance is released from or through a surface. The process is the opposite of sorption/adsorption (that is, either adsorption or absorption).

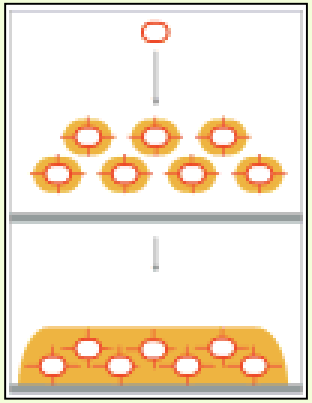
Marta *et al.*,2011

Biofilm development

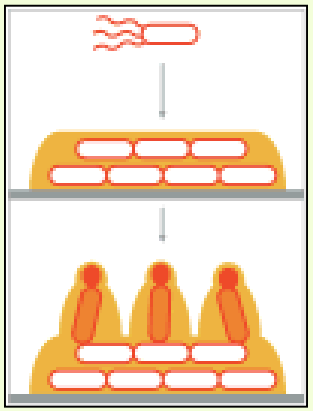
Two general models for biofilm development

Nonmotile and motile bacteria

- In nonmotile bacteria, changes in cell surface proteins, along with the production of EPS, play a critical role in the initiation of biofilm formation.



- For a number of motile organisms, the dominant role for flagella in initiation of biofilm formation is to provide motility as flagella-minus and paralyzed flagella mutants are comparably defective in biofilm formation.



Lemon *et al.*,2008

Biofilm development

Twitching motility and Biofilm formation

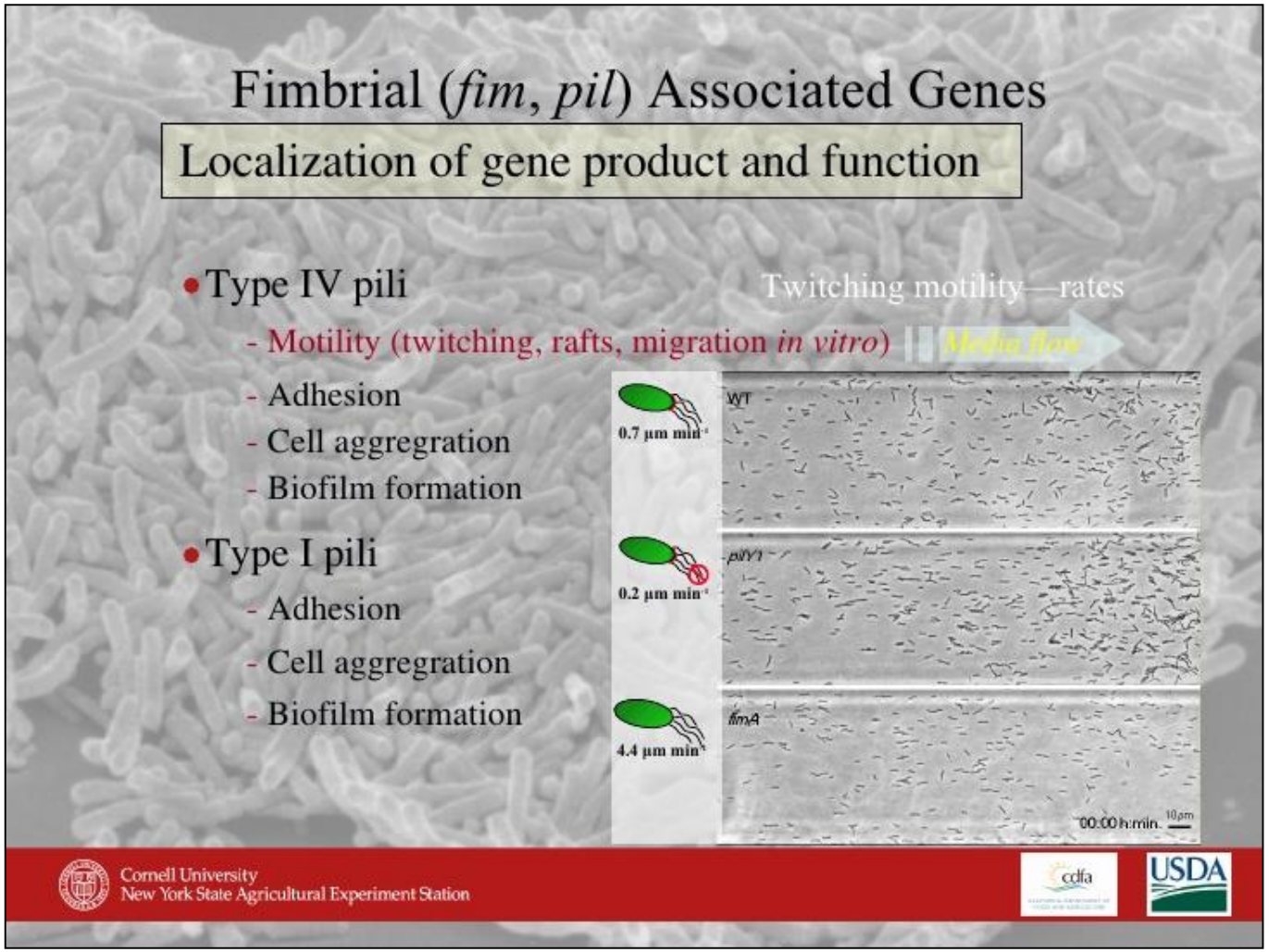
Type IV pili/fimbriae ("tfp")

- The Type IV pili are architectural marvels of biology.
- Type IV pili are remarkable multifunctional organelles expressed by diverse pathogenic bacteria.
- Twitching motility (surface motility) via type IV pili has been observed in a number of gram-negative bacteria.
- Type IV pili are essential for host colonization and virulence for many Gram negative bacteria, and may also play a role in pathogenesis for some Gram positive bacteria.

Ramey *et al.*,2008; Wikipedia,2010

Attachment pili

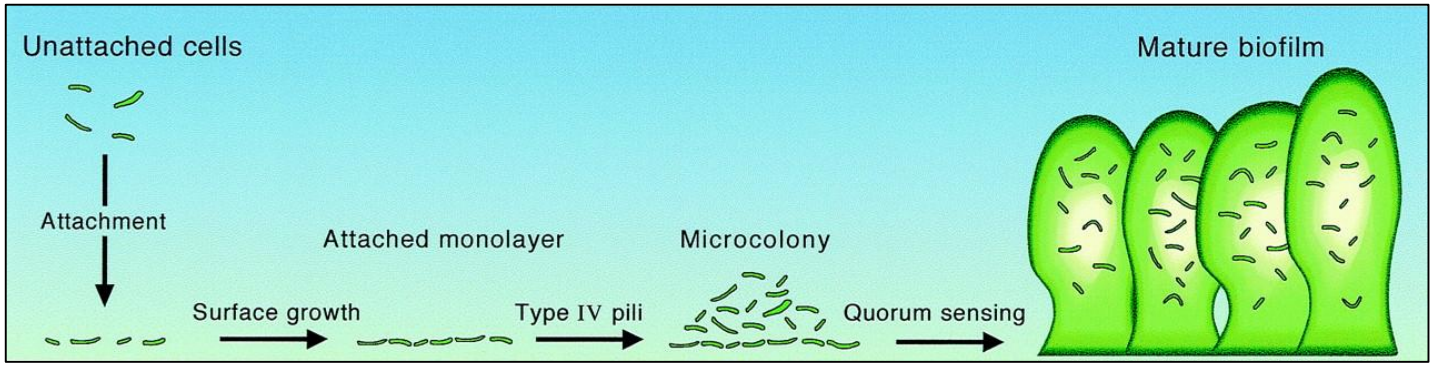
Can be an important virulence factor a feature of the organism that enhances its ability to cause disease



Biofilm development

Diagram of the *P. aeruginosa* biofilm-maturation pathway

- Unattached cells that approach a surface may attach.
- Attachment involves specific functions.
- Attached cells will proliferate on a surface and use specific functions to actively move into microcolonies.
- The high-density microcolonies differentiate into mature biofilms by a 3OC12-HSL-dependent mechanism.

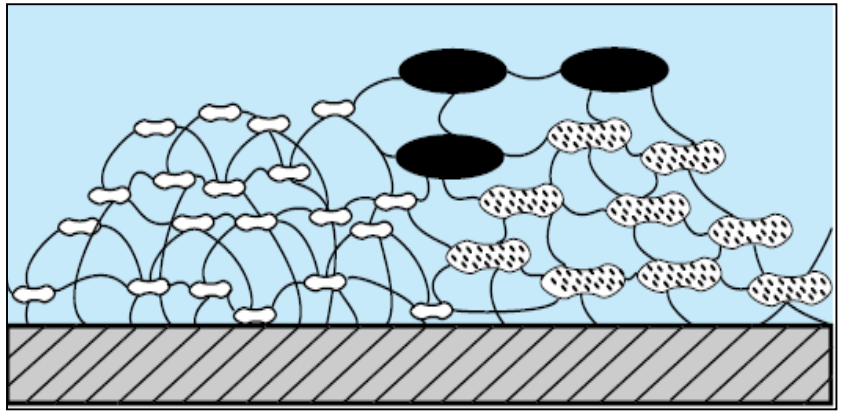


Parsek and Greenberg, 2000

Biofilm development

Pioneer cells

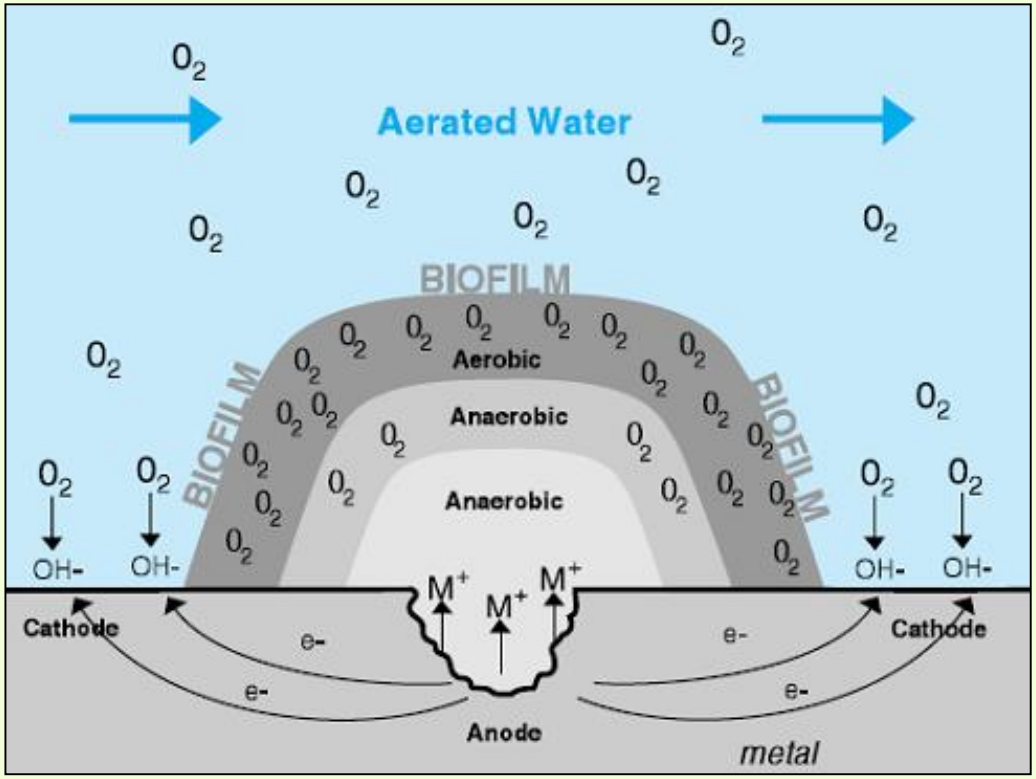
- A biofilm starts when a few pioneer cells use specialized chemical hooks to adhere to a surface.
- Bacteria in biofilms bind together in a sticky web of tangled polysaccharide fibers which anchor them to surfaces and to each other.



Steps in biofilm development

Oxygen is present at measurable concentrations mainly at the periphery of the biofilm

- Nonuniform colonization by bacteria results in differential aeration cells. This schematic shows pit initiation due to oxygen depletion under a biofilm (Borenstein,1994).



Biofilm

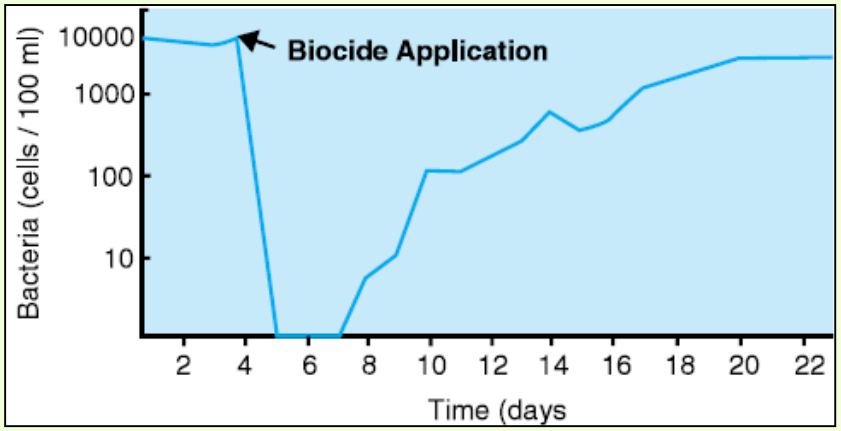
Range of composition of biofilm matrices

Component	% of matrix
Water	Up to 97%
Microbial cells	2-5% (Many species)
Polysaccharides (homo-and heteropolysaccharides)	1-2% (Neutral and polyanionic)
Proteins (extracellular and resulting from lysis)	<1-2% (Many, including enzymes)
DNA and RNA	<1-2% (From lysed cells)
Ions	? (Bound and free)

Sutherland,2001

Biocide application
Removal and recovery of biofilms

- Reducing levels of nutrients in water can help reduce biofilm growth.
- It is common to observe a rapid regrowth of biofilm immediately biocide application.
- Incomplete removal of the biofilm will allow it to quickly return to its equilibrium state.
- Example of sanitization followed by biofilm recovery.
- Bacteria count samples were taken on a daily basis.



Biofilm formation

Methods to detect biofilm production

- There are various methods to detect biofilm production. These include:

1. Congo Red Agar method (CRA),
2. The Tissue Culture Plate (TCP),
3. Tube method (TM),
4. Bioluminescent assay,
5. Piezoelectric sensors, and
6. Fluorescent microscopic examination.

Hassan *et al.*,2011

Methods to detect biofilm production
Biofilm congo red agar medium

- Congo red agar medium consists of (per litre): brain heart infusion 37g, sucrose 50 g, agar 10 g, and congo red stain 0.8g.
- Congo red stain was prepared as a concentrated aqueous solution and autoclaved at 121°C for 15 min separately and added to the growth medium cooled to 55°C.
- The test isolates were inoculated on to the CRA plates and incubated aerobically at 37°C for 24 hrs.

The production of black colonies with a dry crystalline consistency by the organisms was taken to indicate biofilm production(positive result). Non-biofilm-producing strains develop red colonies. Weak slime producers were indicated by pink colonies with occasional darkening at the centre of colonies.

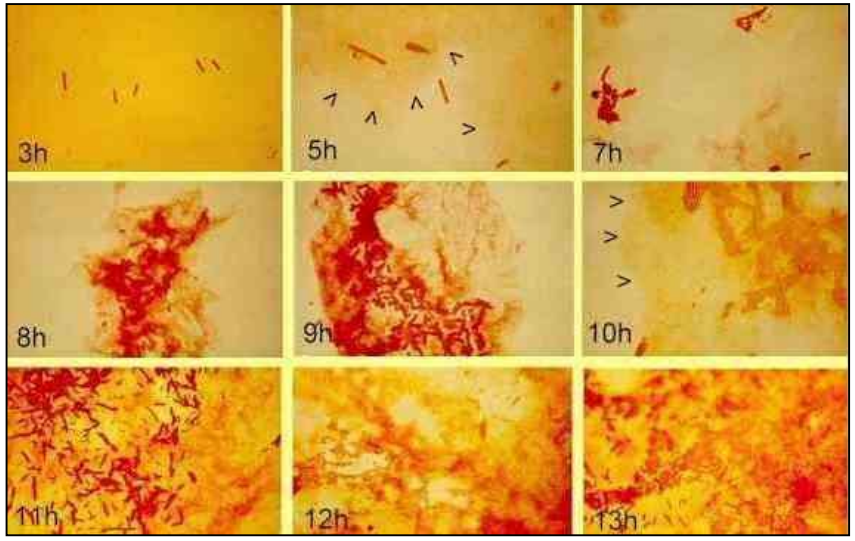
Niveditha *et al.*,2013;..

Biofilm formation

Microscopic examination

Biofilm congo red staining method

- *Pseudomonas* strain S61 was allowed to develop as a biofilm on glass slides immersed in nutrient medium(broth) containing 1% glucose.
- The formation of biofilm was studied at 6 different time intervals of growth at 3, 5, 7, 8, 9,10 and 11-13 hours. Development of the biofilm was followed by a specific staining technique (congo red) in which:
  - The bacterial cells stain dark red and
  - The exopolysaccharide stains orange-pink.
- Attached cells can be seen at 3 hours.
- They then divide and form microcolonies.
- After 5 hours the development of exopolysaccharide is clearly seen (arrowheads) and it increases as the microcolonies increase in size.



Allison and Sutherland

Biofilm formation

Microscopic examination

Biofilm Gram staining method

- The bacterial strains were first tested for biofilm formation on 18mm glass cover slips being immersed in 15mL nutrient media (broth) with 1% glucose in 50mL sterile falcon tubes.
- The organism was inoculated and incubated at 30°C for 4 days.
- The cover slips were recovered from the culture tubes, washed thoroughly in 1% saline solution aseptically, air-dried and Gram-stained.
- Formation of biofilm was viewed under 100X oil immersion objective using Nikon's DN100 microscope.
- The formation of biofilm on thin glass cover slips was also studied for hourly development of film by the strain KPW.1-S1 and stained at 6 different time intervals of growth at 6th, 12th, 18th, 24th, 36th and 48th hours.

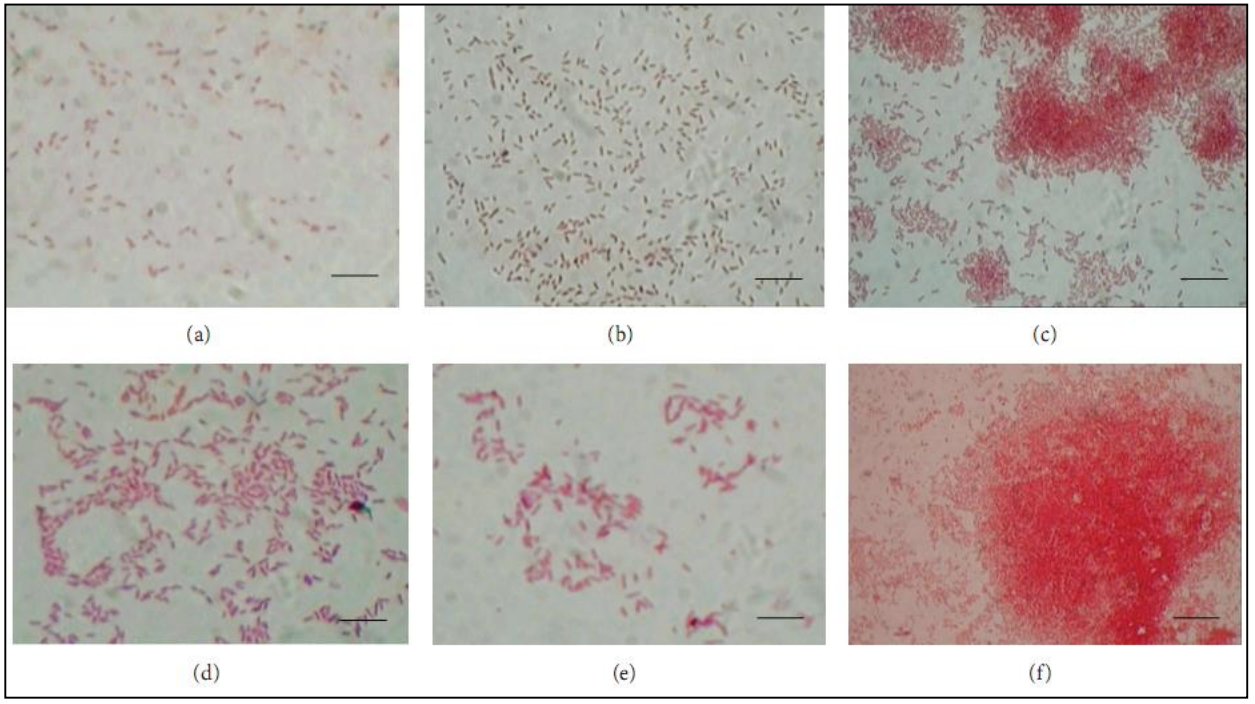
Dasgupta *et al.*,2013

Biofilm formation

Microscopic examination

Biofilm Gram staining method

- Hourly development of biofilm on thin glass cover slip at different time interval by KPW.1-S1 at (a) 6th, (b) 12th, (c) 18th, (d) 24th, (e) 36th, and (f) 48th hours. Bars, 23 μm.



Dasgupta *et al.*,2013

Biofilm formation

Tube method (TM)

Staining with crystal violet

- Tube method Described by Christensen *et al.*,1982 is a qualitative method for biofilm detection.
- A loopful of test organisms was inoculated in 5 mL of trypticase soy broth+1% glucose or fresh LB with 1% glucose in test tubes. The tubes were incubated at 37°C for 24 h. After incubation, tubes were decanted(free unbound cells were removed) and biofilm layer was washed 3-4 times with water and dried.
- The formed biofilm in tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position and observed for biofilm formation.

1. Biofilm Production was considered positive when a visible film lined the wall and bottom of the tube.
2. Ring formation at the liquid interface was not indicative of biofilm formation.

Mohamed *et al.*,2013; Sabur et al.,?

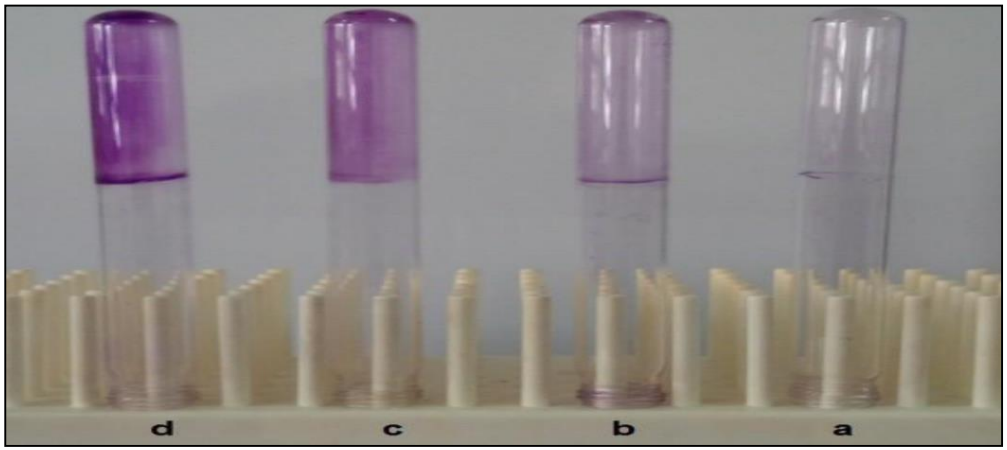
Biofilm formation

Tube method (TM)

Staining with crystal violet

- Detection of biofilm producers by Tube method:

a. Non biofilm producer;
b. Weak biofilm producer;
c. Moderate biofilm producer, and
d. Strong biofilm producer.

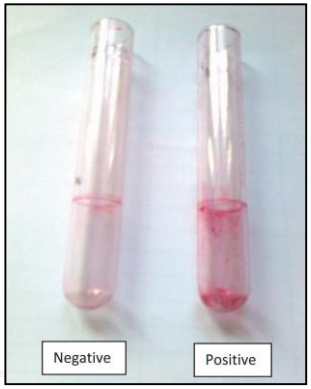


Mohamed *et al.*,2013;

Biofilm formation

Tube method (TM)

Staining with safranin



- The suspensions of the tested strains were incubated in glass tubes which contained Brain Heart Infusion Broth (broth) aerobically at a temperature of 35°C for a period of 2 days.
- Then, the supernatants were discarded and the glass tubes were stained with a 0.1% safranin solution, washed with distilled water 3 times and dried.

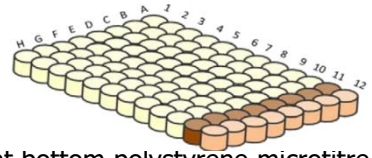
1. A positive result was defined as the presence of a layer of the stained material which adhered to the inner wall of the tubes.
2. The exclusive observation of a stained ring at the liquid-air interface was considered as negative.

Niveditha *et al.*,2013

Biofilm formation

Tissue culture plate method (TCP)

Staining with crystal violet



Flat bottom polystyrene microtitre plates

- Biofilm formation on TCP was carried out by pipetting 200 µl aliquot of 10^8 CFU/ml suspension of the *E. coli* into 9 wells of sterile 96-well TC plates (flat bottom polystyrene microtitre plates) while control wells were only filled with Tryptic Soy Broth (TSB).
- The plates were later incubated at 10, 27 and 37°C for 24, 48, 72 and 168 hours in order for the bacteria to attach to the pirates of the wells.
- Following the attachment step, the planktonic bacteria were removed using a multichannel pipette and each well was washed twice with 200 ml of PBS (pH 7.3) to remove the loosely attached cells.
- After the removal of loosely attached cells, the wells were renewed with 200 µl of TSB every 24 h up to the 7^{th} day (168 hours) to allow for bacterial growth and biofilm development.

Muazu *et al.*,2013

Biofilm formation

Tissue culture plate method (TCP)

Staining with crystal violet

- Biofilms formed by adherent 'sessile' organisms in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v).
- Excess stain was rinsed off by thorough washing with deionized water and plates were kept for drying.
- Adherent cells usually form biofilm and wells were uniformly stained with crystal violet.
- Optical density (OD) of stained adherent bacteria were determined with a micro ELISA auto reader (model PR 601, Qualigens) at wavelength of 570 nm (OD_{570} nm).
- These OD values were considered as an index of bacteria adhering to surface and forming biofilms.

Al-Mulla,2013; Muazu *et al.*,2013

Biofilm formation

Tissue culture plate method (TCP)

Staining with crystal violet

- This method is quantitative detection of slime production which was carried out according to Christensen *et al.*,1985.
- The adherence of the biofilm to the polystyrene,96-well, flat-bottomed tissue culture plates was observed by reading the optical density of the stained wells bottoms with microELISA auto reader (Beckman) at 570 nm wavelength.
- Isolates with OD values above 0.24 were considered strong producers.
- These OD values were taken as index of bacteria adhering the surface and formed biofilm.

Al-Mulla,2013

Biofilm formation

Tissue culture plate method (TCP)

Interpretation of Results

- OD readings from sterile medium, fixative and dye were averaged and subtracted from all test values.

Mean OD values	Biofilm formation
<0.120	Non / weak
0.120-0.240	Moderate
>0.240	High

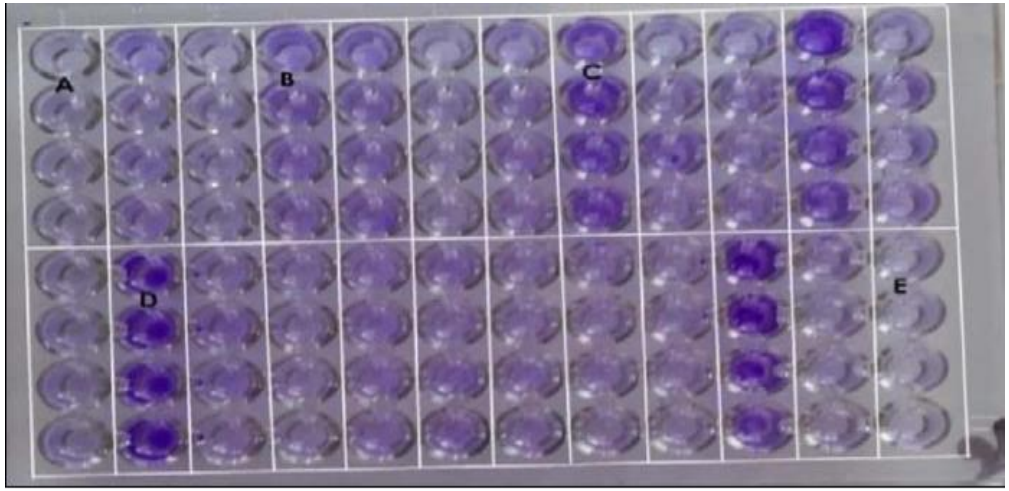
Vasanthi *et al.*,2014

Biofilm formation

Tissue culture plate method (TCP)

- Detection of biofilm production by tissue culture plate method (TCP):

A. non biofilm producer,
B. week producer biofilm,
C. moderate producer biofilm,
D. strong producer biofilm, and
E. negative control.



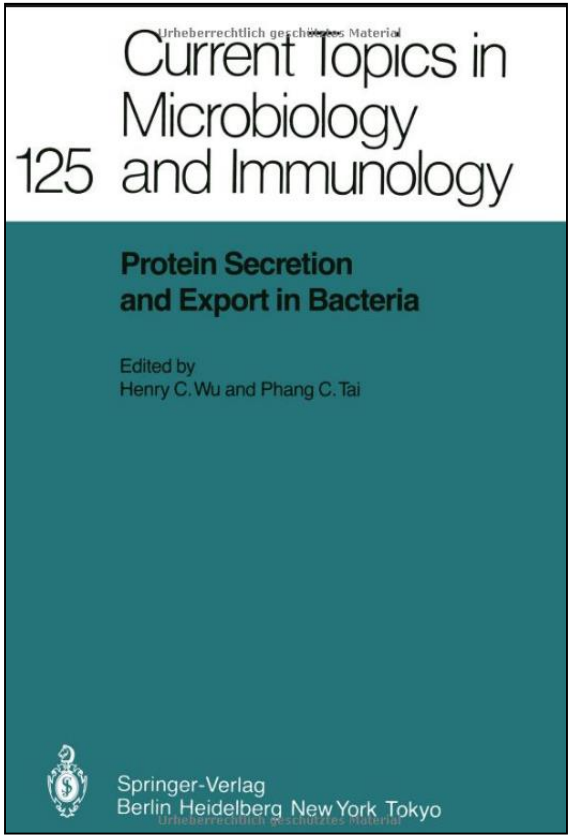
Mohamed *et al.*,2013

Bacterial Secretion Systems (SSs)
Protein-secretion pathways

Export bacterial virulence factors(secreted proteins)

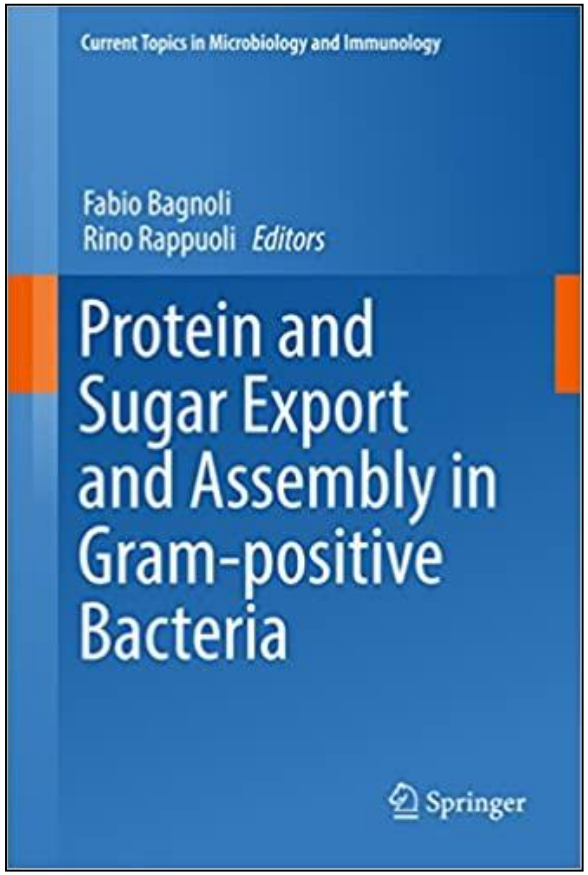
Protein Secretion and Export in Bacteria

- Protein Secretion and Export in Bacteria
- Author: Henry C. Wu and Phang C. Tai
- Springer
- 2011
- 221 pages.



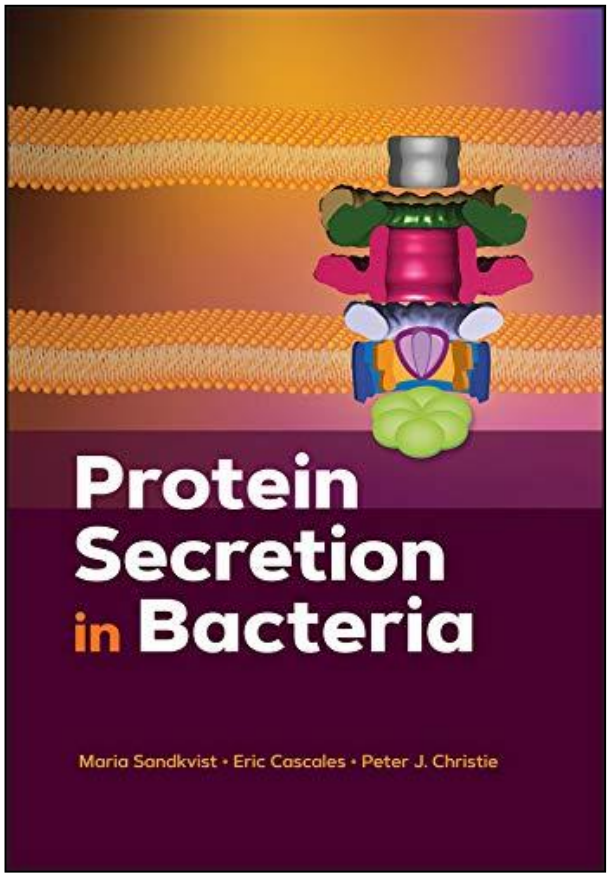
Protein and Sugar Export and Assembly in Gram-positive Bacteria

- Protein and Sugar Export and Assembly in Gram-positive Bacteria
- Editors: Fabio Bagnoli and Rino Rappuoli
- Springer
- 2017
- 347 pages.



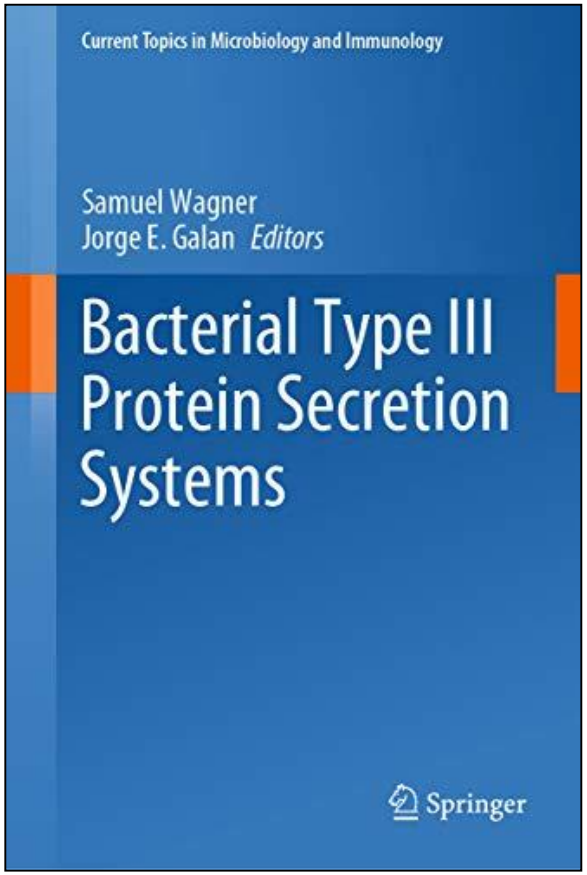
Protein Secretion in Bacteria

- Protein Secretion in Bacteria
- Editors: Maria Sandkvist, Eric Cascales and Peter J. Christie
- ASM Press
- 2020
- 410 pages.



Bacterial Type III Protein Secretion Systems

- Bacterial Type III Protein Secretion Systems
- Editors: Samuel Wagner and Jorge E. Galan (Editors)
- Springer
- 2021
- 230 pages.



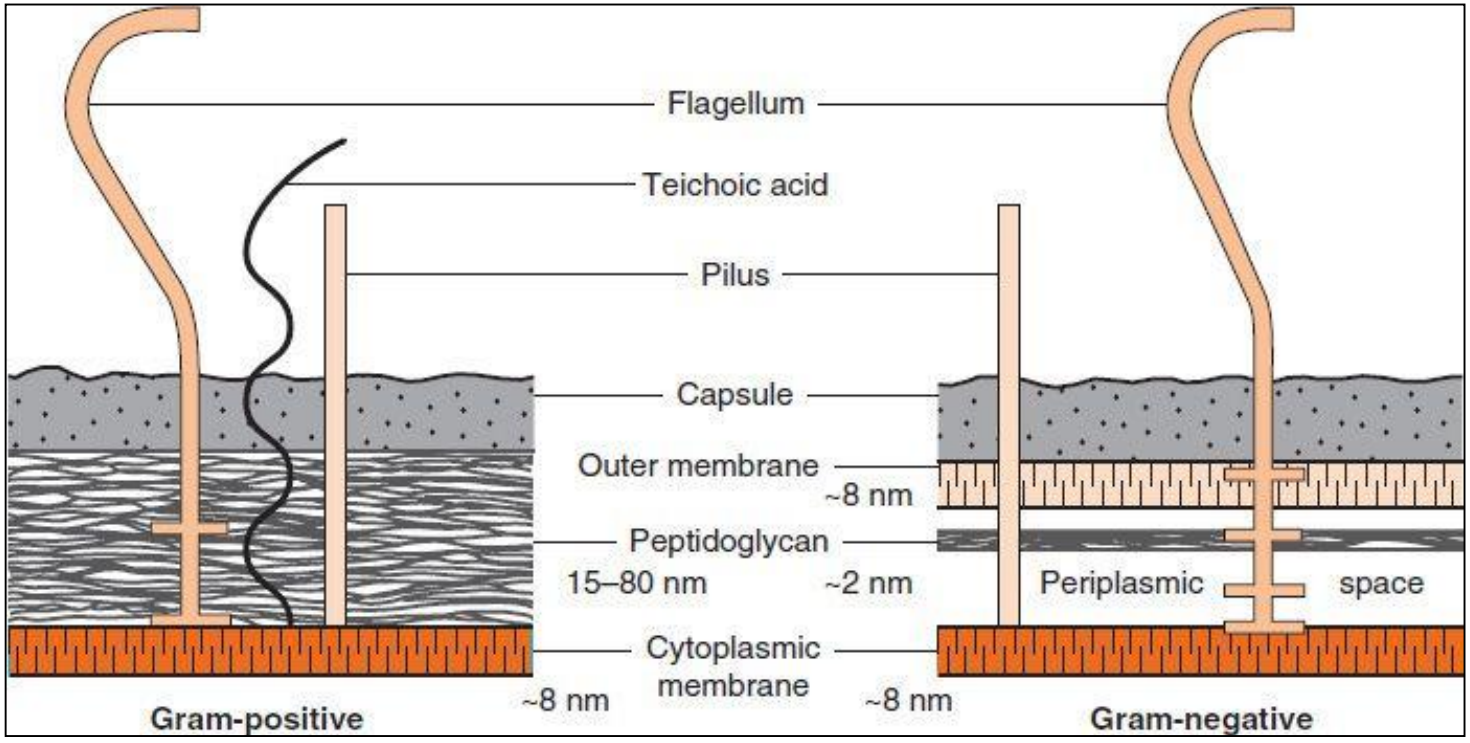
Protein translocation pathways
Bacterial cell wall structure
Gram-ve vs Gram+ve

- **Gram-ve bacteria:**
- Protein secretion in Gram-negative bacteria is particularly complex because these bacteria are surrounded by two membranes that the secreted proteins must pass through to enter the extracellular environment or a host cell.
- **Gram+ve bacteria:**
- Protein secretion in Gram-positive bacteria is simple in structure because they lack a second membrane; consequently, secretory proteins of Gram-positive bacteria only need to traverse both cytoplasmic membrane and peptidoglycan layer to enter the extracellular environment.

Abdallah *et al.*,2007

Protein translocation pathways

Gram-ve and G+ve negative bacterial cell wall



Gram-negative - translocation past the cytoplasmic/periplasm/outer membrane

Gram-positive - translocation cytoplasmic membrane/cell wall

Ingraham *et al.*,1983

Bacterial secretion systems

Secretion vs Translocation

- Secretion: delivery of effectors outside the bacterial cell. Secreted proteins were transport into the extracellular milieu.
- Translocation: delivery of effectors into host cells (transport into the eukaryotic cell cytosol(liquid portion of cytoplasm).

Oh *et al.*,2010; Economou *et al.*,2006

Protein translocation pathways
In all bacteria

- All bacteria including non-pathogenic bacteria secrete proteins and transport these proteins through some secretion systems.
- The number of proteins and type secretion systems in Gram negative bacteria are more variable and complex than Gram positive bacteria.
- This is because of outer membrane in Gram-negative bacteria cell wall.

Protein translocation pathways
11 different secretion pathways

- In addition, bacteria have evolved a vast repertoire of secretion pathways to transport effectors across the cell envelope.
- Up to date, 11 different secretion pathways (T1SS–T11SS), involved in:

1. substrate transport,
2. protein exposition at the cell surface,
3. pili assembly, and
4. motility.

Arechaga and Cascales,2022

Protein translocation pathways
11 different secretion pathways

- These 11 protein secretion pathways transport the proteins in two ways:

1. Directly or one-step secretion systems: some Gram-negative bacteria are recognizing their substrates in the cytoplasm and transporting them simultaneously across both bacterial membranes,
2. Indirectly or two-step secretion systems: whereas other Gram-negative bacteria rely on the Sec and/or Tat pathways to first translocate substrates to the periplasm.

Note: T11 secretion system was isolated from sheep erythrocyte membrane and not from bacteria.

Palmer *et al.*,2020

Protein translocation pathways
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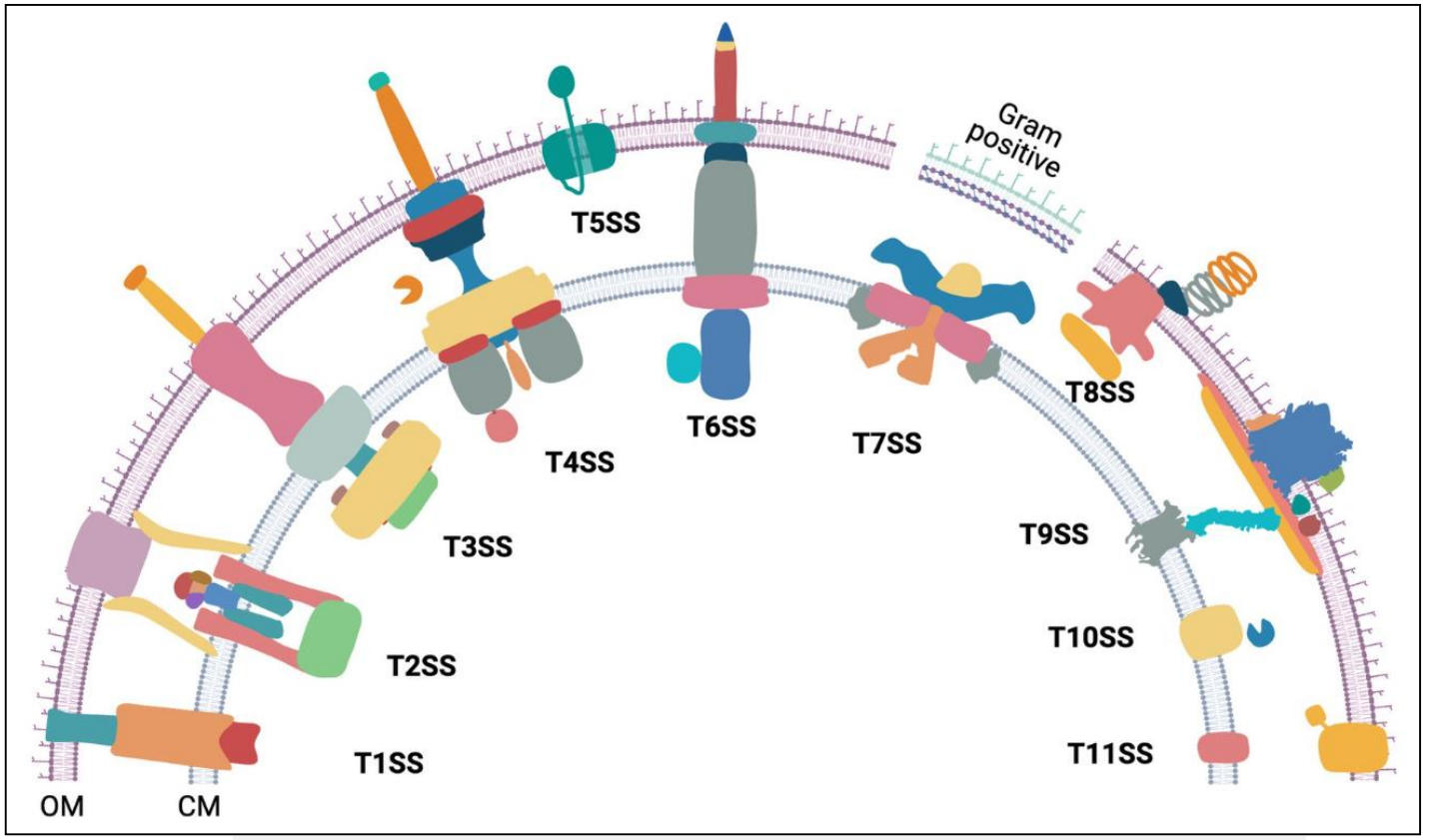
Palmer *et al.*,2020

Protein translocation pathways

11 different secretion pathways

Mostly in Gram-ve bacteria

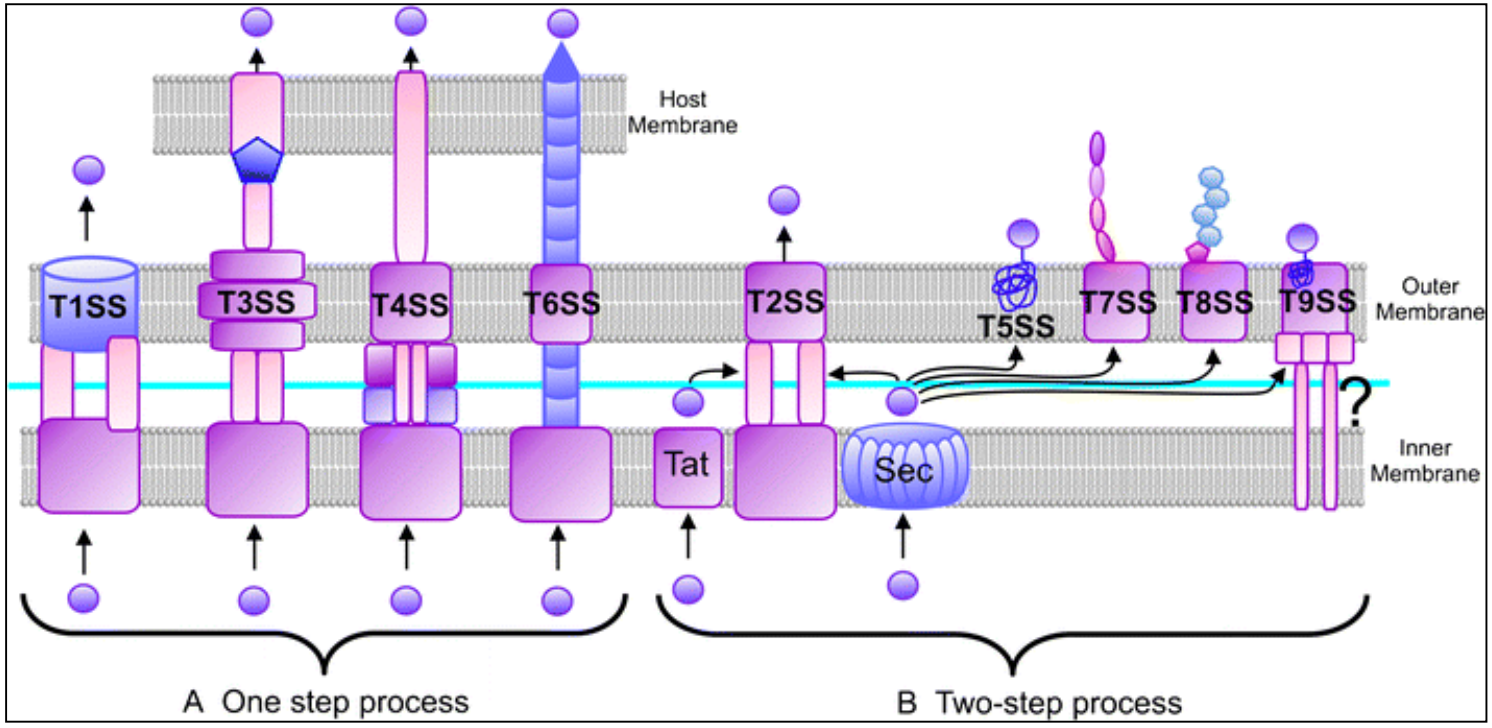


Note: T11 secretion system was isolated from sheep erythrocyte membrane and not from bacteria.

Wikipedia,2023;..

Bacterial secretion systems

Nine different secretion pathways. Type 10 Secretion System (TXSS) was also described



Bocian-Ostrzycka *et al.*,2017; Palmer *et al.*,2010

Bacterial secretion systems
In symbiotic bacteria

- Type III protein secretion systems (TTSSs) have recently been reported in non-pathogenic bacteria.
- TTSSs were described in the symbiotic bacteria such as:
- *Rhizobium parasponia* NGR234,
- *Sinorhizobium fredii*,
- *Mesorhizobium loti*, and
- *Bradyrhizobium japonicum*.
- TTSSs in these bacteria are responsible for biological nitrogen fixation in the roots of legumes.

Mazurier *et al.*,2004

Bacterial secretion systems

In saprophytic and nonpathogenic bacteria

- Many nonpathogenic organisms secrete proteins which are adaptive to their life-styles.
- e.g.
- Saprophytic bacteria may also secrete:

1. cellulases, or
2. other degradative enzymes.

- Secretion of these enzymes is governed in large part by the structure of the bacterial cell envelope.

Protein translocation pathways

Type III protein secretion systems (TTSSs)

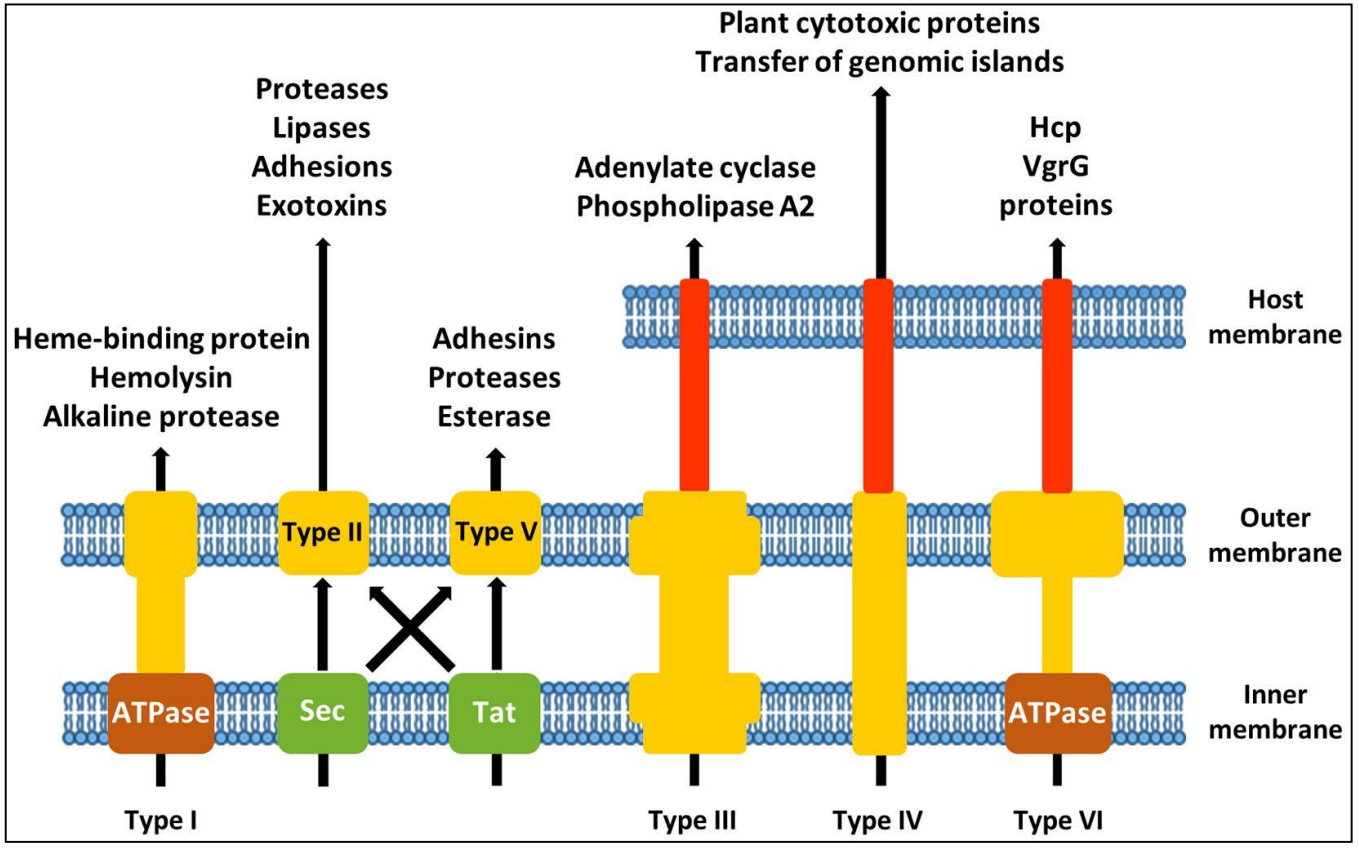
In some non-pathogenic bacteria

- T3SSs are essential for the pathogenicity (the ability to infect) of many pathogenic bacteria.
- Defects in the T3SS may render a bacterium non-pathogenic.
- It has been suggested that some non-invasive strains of gram-negative bacteria have lost the T3SS because the energetically costly system is no longer of use.

Tseng *et al.*,2008;Yuan *et al.*,2020; Wikipedia,2023

Bacterial secretion systems

Different secretion pathways



Depluverez *et al.*,2016

Protein translocation pathways
In pathogenic bacteria

- A few hundred bacterial species, belonging to the Proteobacteria, Mollecutes and Actinomycetes cause a large number of different plant diseases, some of which are devastating for agricultural crops.
- Symptoms of bacterial plant diseases are diverse and include necrosis, tissue maceration, wilting, and hyperplasia.
- For successful infection to occur, the pathogen must overcome plant defense mechanisms, which it often does by injecting effector molecules directly into plant cells to suppress a host response.

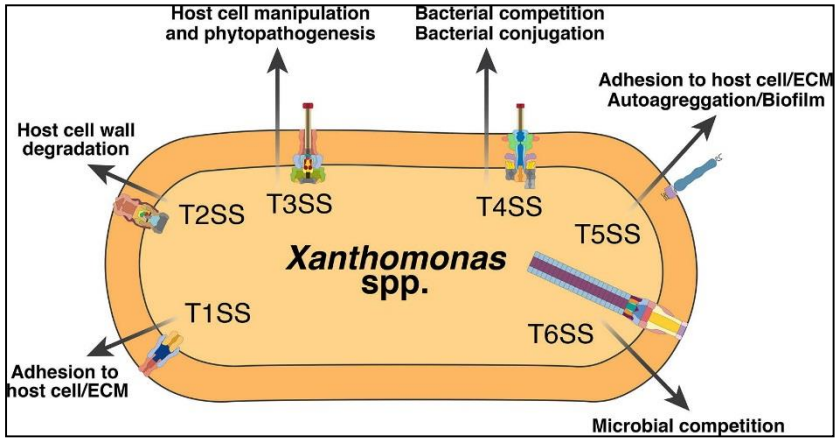
Wolf and De Boer,2014

Protein translocation pathways

In pathogenic bacteria

Multiple secretion systems among different *Xanthomonas* species and pathovar

- The architecture and mode of action of bacterial type I to type VI secretion systems and the distribution and functions associated with these important nanoweapons within the *Xanthomonas* genus.



Alvarez-Martinez *et al.*,2020

Protein translocation pathways
Coordination between secretion systems

- Secretion systems can translocate hundreds of cargos(proteins) that function together as a well-orchestrated virulence system.
- There is also a higher-level coordination between the different secretion systems.
- In *X. vesicatoria*, evidence suggests that the T2SS and T3SS reciprocally regulate the expression and translocation of cargo by each secretion system.
- This cross talk is conceptually appealing, as it implicates a collaborative attack between two systems that secrete cargo with primary functions in countering different components of plant defense.

Chang *et al.*,2014

Protein translocation pathways
Coordination between secretion systems

- Enteropathogenic *E. coli* provides an even more astonishing (extremely surprising) example of coordination between two secretion systems.
- The pathogen uses a T3SS to inject Tir, which is a receptor for a virulence factor intimin (adhensin), a T5eSS surface localized adhesion.
- Together, the two mediate attachment of the pathogen to the host cell.
- Secretion systems must also be coordinately regulated to limit conflict.

Chang *et al.*,2014

Protein translocation pathways

Bacterial secretion systems

Signal peptides (SPs) or translocation signals

- Bacteria often secrete suites of proteins that contribute to:

1. environmental functioning, and
2. bacterial virulence.

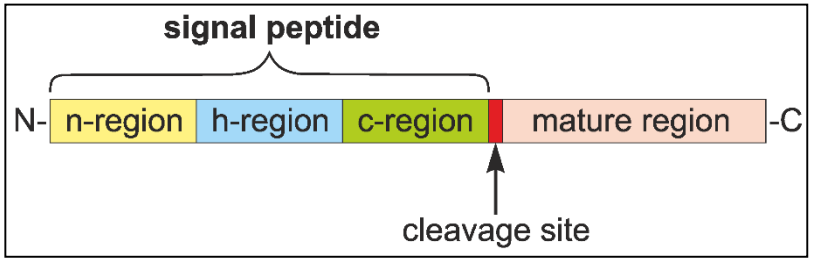
- The secreted proteins generally possess appropriate signal peptides (SPs) or translocation signals that enable **targeting** of a variety of conventional secretion pathways, including type I to VII secretion systems (T1SS to T7SS), in addition to Sec- and Tat-dependent translocation pathways.

Protein translocation pathways

Bacterial secretion systems

Signal peptides (SPs) or translocation signals

- Signal peptides (SPs) consist of short peptide sequences present at the N-terminal of newly synthesizing proteins and act as a zip code for the translocation of the proteins to the endoplasmic reticulum (ER).



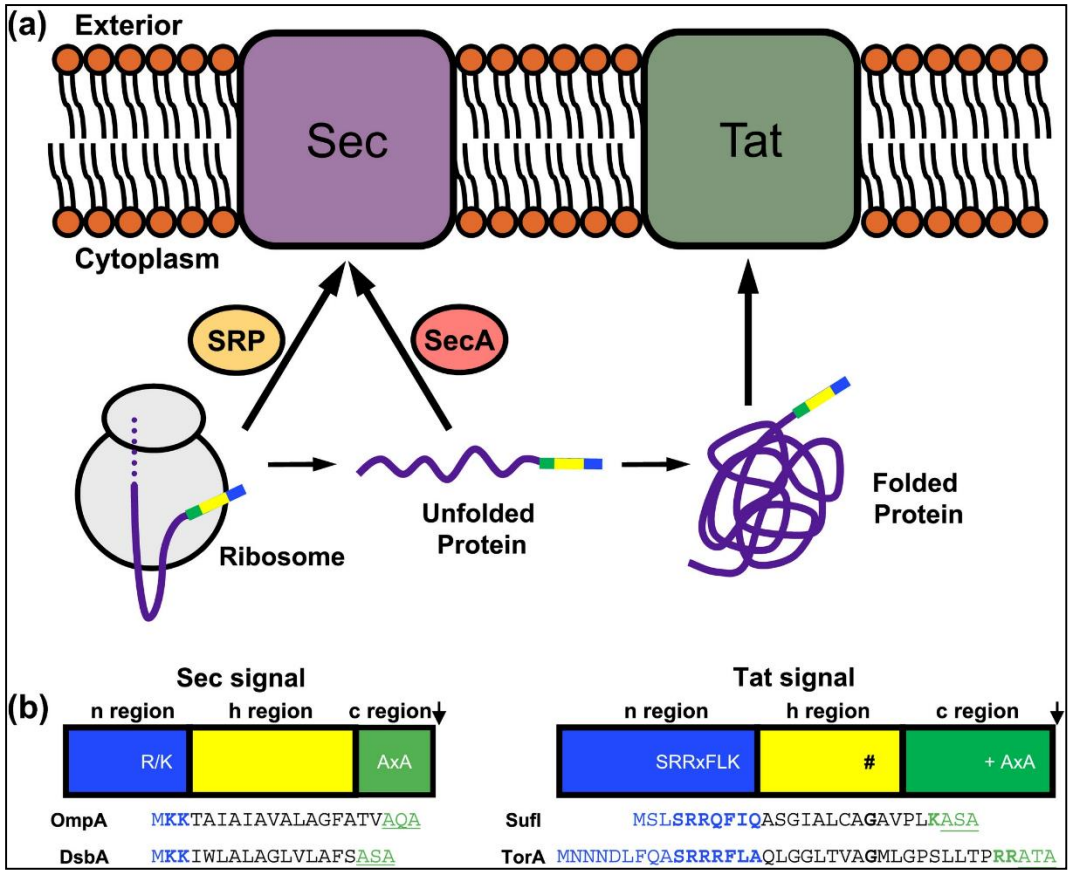
A general structure of the SP was proposed and it includes three main parts: n-region, h-region and c-region.

Burdukiewicz *et al.*,2018;..

Protein translocation pathways

Targeting of proteins to the twin-arginine translocation pathway(TAT)

Signal peptides (SPs) or translocation signals



Palmer and Stansfeld,2020

Protein translocation pathways
Specific and common secretion systems

- There are at least:

1. eight types specific pathways to Gram-negative bacteria,
2. four to Gram-positive bacteria,
3. while three pathways are common to both.

Wikipedia,2023;..

Protein translocation pathways
Common secretion systems

- Three common secretion systems are responsible for secretion of proteins across the bacterial inner (cytoplasmic) memebrane.
- Proteins are transported through one of three mechanisms:

1. General secretory pathway (Sec, GSP),
2. Twin-arginine translocation (TAT) pathway,
3. ATP-binding cassette (ABC) pathway.

Common secretion systems

1. Sec system(SecA)

Transport unfolded proteins into periplasm

- Transport unfolded proteins into periplasm.
- The Sec protein translocation system is best characterized in *E. coli*, comprising at least:
- 11 proteins, and
- one RNA species (Economou,1999).
- Among these proteins, SecY, SecE, SecG and SecA constitute a translocase complex that acts as export machinery at the cytoplasmic membrane.
- Among different secretion systems, only the Sec system is essential for cell viability.

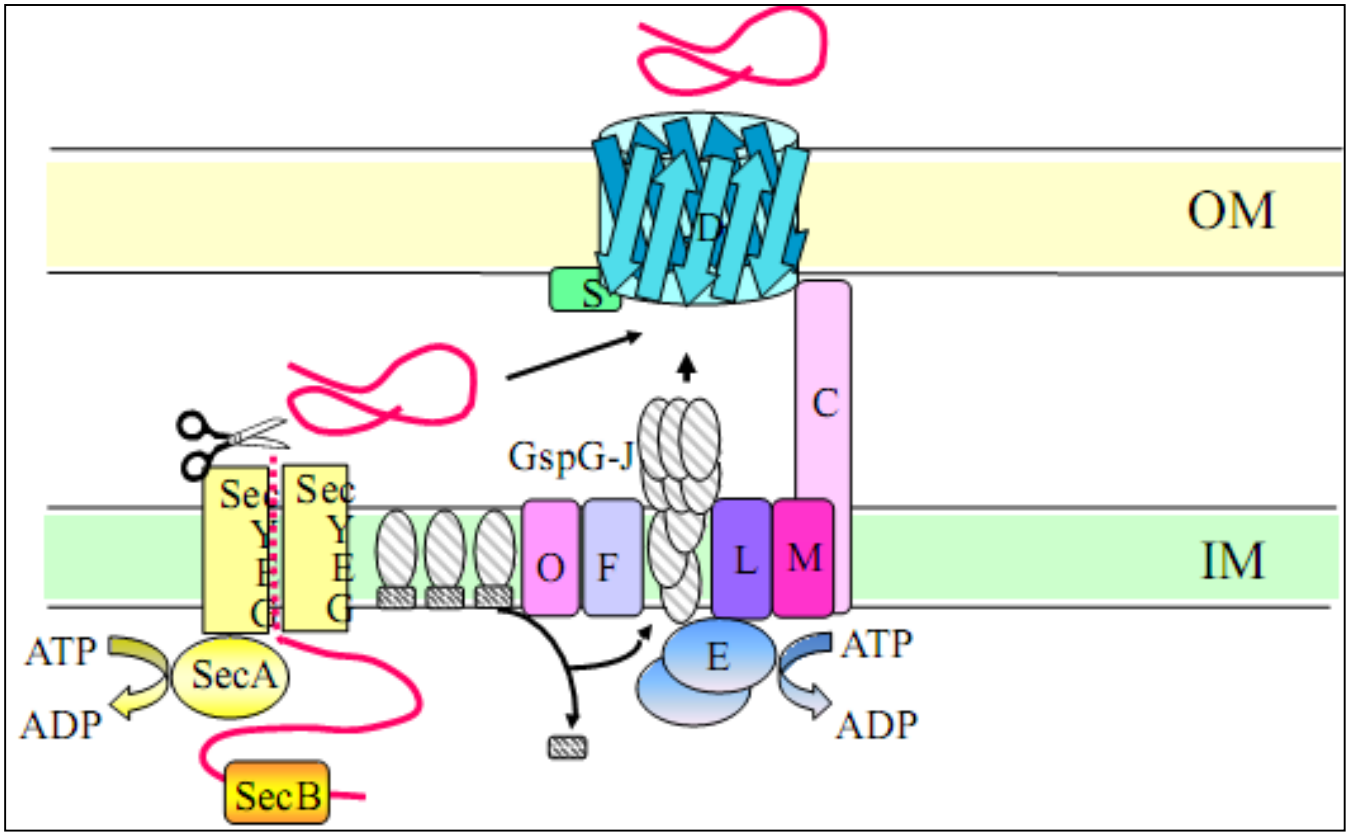
Weintraub and Jones,2010; De La Fuente,2009

Common secretion systems

Sec system(SecA)

Structure

- Transport unfolded proteins into periplasm.



Common secretion systems

2. Twin-arginine translocation(TAT)

Transports fully folded proteins across the membrane

- Bacteria as well as mitochondria and chloroplasts also use Twin-arginine translocation (TAT) pathway too.
- In contrast to Sec system, this system transports fully folded proteins across the membrane.
- The name of the system comes from the requirement for two consecutive arginines in the signal sequence required for targeting to this system.

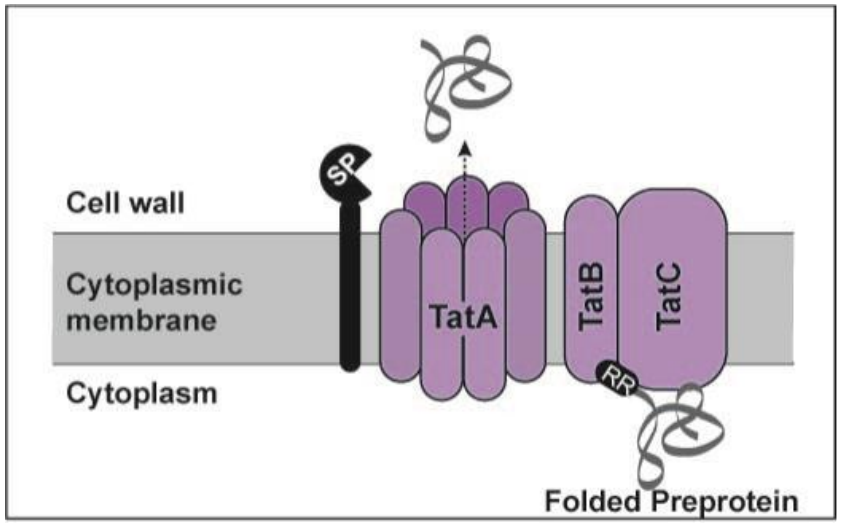
De La Fuente,2009; Braunstein,2016

Common secretion systems

2. Twin-arginine translocation(TAT)

Transports fully folded proteins across the membrane

- Prior to export, Tat exported proteins are folded and they contain an N-terminal signal sequence/SP (black oval) with a twin arginine (RR) motif.
- This folded preproprotein is recognized by the TatB and TatC complex and then delivered to TatA.



Common secretion systems

3. ATP-binding cassette (ABC) pathway

Utilize ATP hydrolysis to transport various substrates

- ATP-binding cassette (ABC) transporter complex is a common pathway to all the three domains of life.
- This is one of the largest, and most ancient families with representatives in all three domains of life from prokaryotes to humans.
- ABC-transporters utilize the energy of ATP hydrolysis to transport various substrates across cellular membranes.

ATP-binding cassette transporters
Functions

- These are transmembrane proteins that function in the transport of a wide variety of substrates across extra- and intracellular membranes, including:
- Lipids and sterols,
- Ions and small molecules,
- Drugs such as antibiotics
- Large polypeptides.
- Within bacteria, ABC-transporters mainly pump essential compounds such as sugars, vitamins, and metal ions into the cell.
- ATP-binding cassette (ABC) superfamily is also considered as one of the fifth multidrug-resistance efflux pumps.

Wikipedia,2008

ATP-binding cassette transporters
Functions

ABC transporters

- ATP-binding cassette transporters
- observed in bacteria, archaea, and eucaryotes

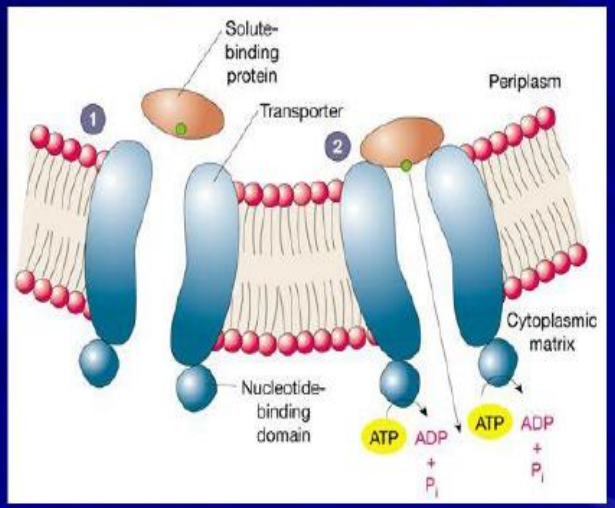


Figure 5.3

Classification of ABC transporters

- Proteins are classified as ABC transporters based on the:

1. Sequence, and
2. Organization of their ATP-binding domain(s), also known as nucleotide-binding folds (NBFs).

ATP-binding casssette transporters
Subfamilies

- **There are many known ABC transporters present in prokaryotes:**
- **Prokaryotic subfamilies**
- **Importers** (e.g.):
- Carbohydrate Uptake Transporter-1 (CUT1)
- Polar Amino Acid Uptake Transporter (PAAT)
- Peptide/Opine/Nickel Uptake Transporter (PepT)
- Thiamin Uptake Transporter (ThiT)
- Siderophore-Fe^{3+} Uptake Transporter (SIUT)
- Lipid Exporter (LipidE)
- Molybdate Uptake Transporter (MolT)
- **Exporters** (e.g.):
- Capsular Polysaccharide Exporter (CPSE)
- Teichoic Acid Exporter (TAE): An enzyme found in Gram-positive bacteria that exports teichoic acid.
- β-Glucan Exporter (GlucanE)
- Protein-1 Exporter (Prot1E)
- Protein-2 Exporter (Prot2E)
- Peptide-1 Exporter (Pep1E)
- Peptide-2 Exporter (Pep2E)
- Peptide-3 Exporter (Pep3E)
- Drug/Siderophore Exporter-3 (DrugE3)

Wikipedia,2008

Protein translocation pathways
In Gram-negative bacteria
Multiple secretion systems

1. **Protein transport across the inner membrane in Gram-negative/positive bacteria:**
   - 1.1. General secretory pathway (GSP, Sec)
   - 1.2. Twin-arginine translocation (TAT) pathway
   - 1.3. ATP-binding cassette (ABC) pathway
2. **Protein translocation across the outer membrane in Gram-negative bacteria:**
   - 2.1. Type I pathway (ABC-dependent pathway)
   - 2.2. Type II pathway (Sec-dependent)
   - 2.3. Type III pathway
   - Chaperone/usher(CU) pathway (used for pilus assembly)
   - 2.4. Type IV pathway (Sec-like dependent)
   - 2.5. Type V pathway (Sec-dependent)
   - 2.6. Type VI pathway
   - 2.8. Type VIII pathway (used for fimbriae assembly).
   - 2.9. Type IX pathway
   - 2.10. Type X pathway

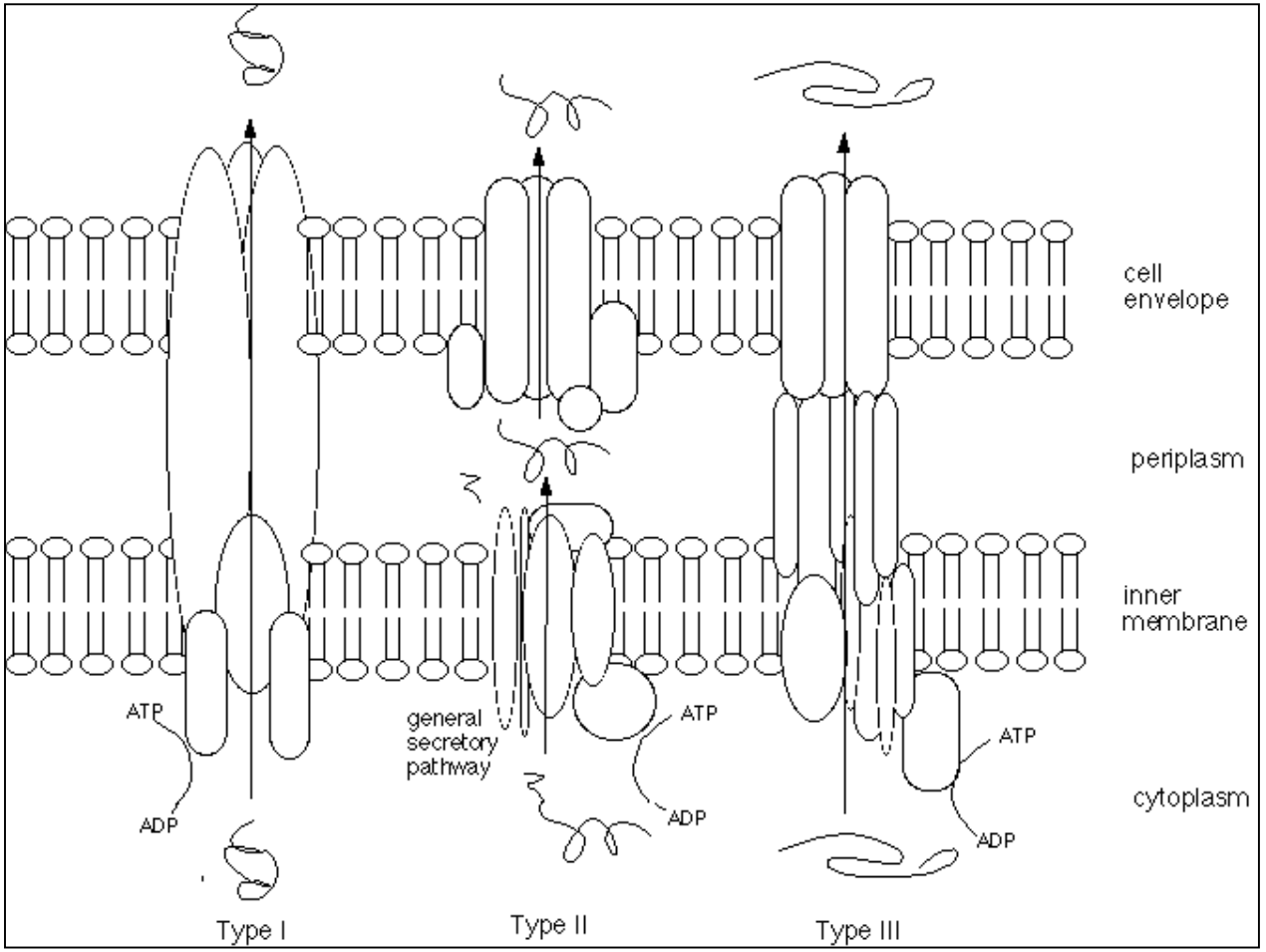
Protein translocation pathways

In Gram-positive bacteria

Multiple secretion systems

1. **Protein transport across the inner membrane in Gram-positive and Gram-negative bacteria:**
   - 1.1. General secretory pathway (GSP, Sec)
   - 1.2. Twin-arginine translocation (TAT) pathway
   - 1.3. ATP-binding cassette (ABC) pathway
2. **Protein translocation across the outer membrane in Gram-positive bacteria:**
   - 2.1. Type IV pathway
   - 2.2. Type VII pathway or ESX1-5 systems. *Mycobacterium tuberculosis* use five of these secretion systems, named ESX-1 to ESX-5.

Three main bacterial secretion systems
Gram-negative bacteria
Signal peptide cleavage in Type II



Mecsas and Strauss,1996

Type I-V secretion systems in Gram-negative bacteria

One/two step translocation

- **One step translocation:**
- T1SSs, T3SSs, T4SSs, T6SSs and T7SSs transport proteins from the cytoplasm across both membranes of the cell and are believed to transport proteins in one step from the bacterial cytosol to the bacterial cell surface and external medium.
- **Two steps translocation:**
- T2SSs, T5SSs, T8SS, and the chaperone-usher pathway only facilitate secretion across the outer membrane. These systems transport proteins in two steps.
- In the latter, proteins are first transported to the periplasm via the Sec or Tat system before reaching the cell surface.

Filloux *et al.*,2008;..

Common and specialized secretion systems
Gram-negative bacteria
Types III and IV are bacterial injection machines

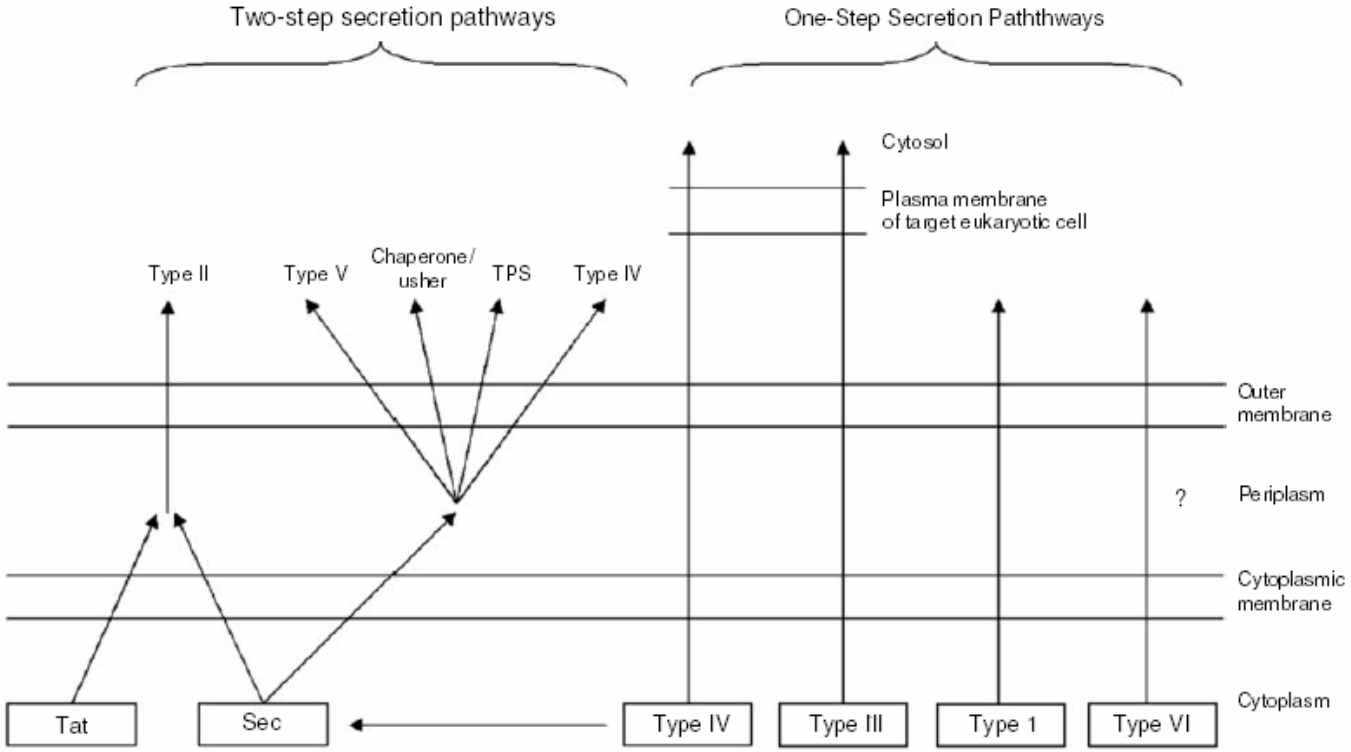


Fig. 5.1 Overview of the protein secretion systems of Gram-negative bacteria. Two-step secretion pathways require Sec or Tat translocases for secretion across the bacterial inner (cytoplasmic) membrane. One-step secretion pathways are Sec (Tat) independent. Type IV can be either a two-step (Sec-dependent) or a one-step mechanism. Type III and two-step Type IV pathways secrete effector molecules across three membranes and directly into the cytosol of the target eukaryotic cell. The newly discovered Type VI is probably a one-step secretion pathway; however, its secretion apparatus and mechanism of secretion remain currently unknown

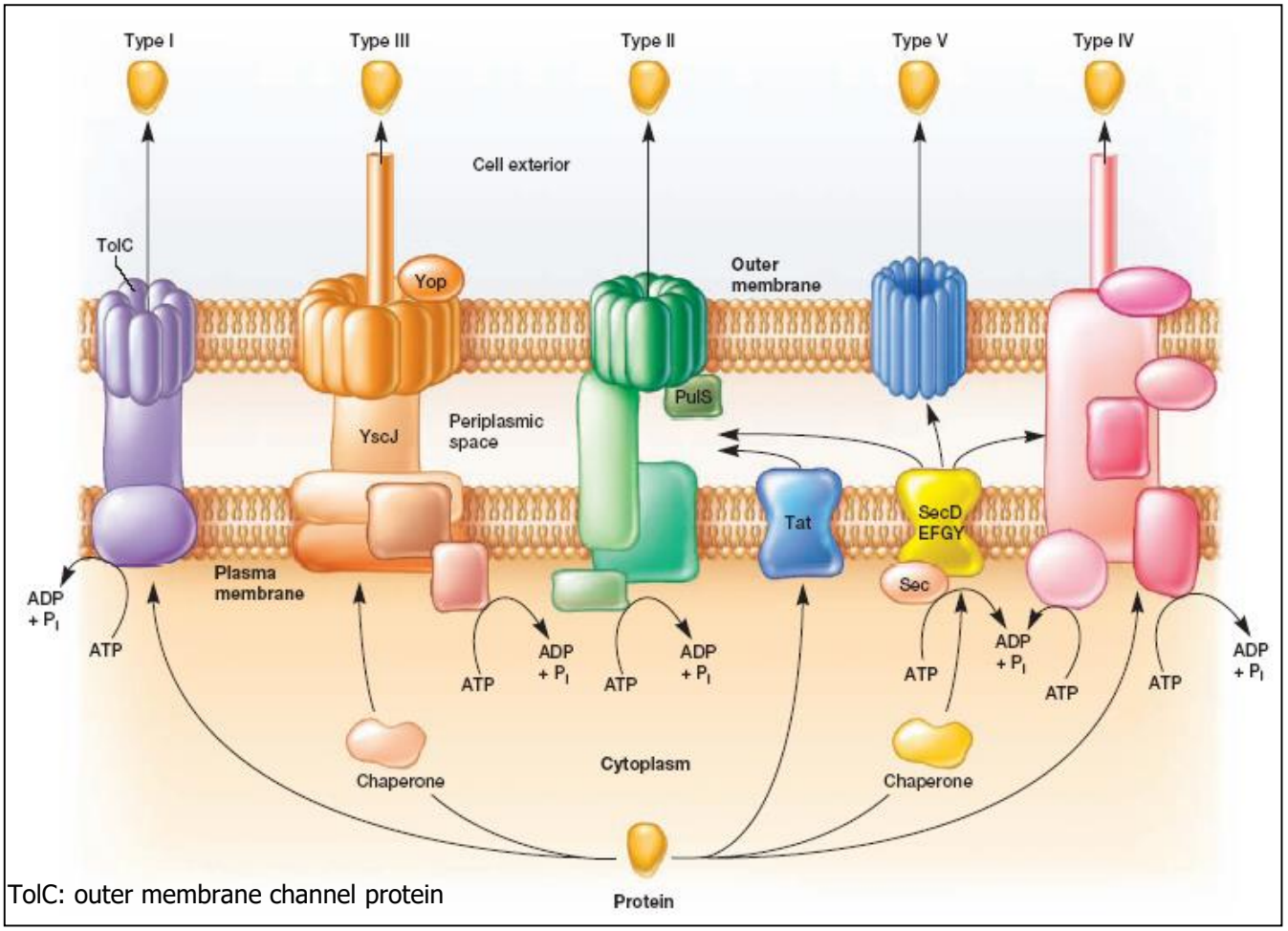
TPS: two-partner secretion systems

Type 1: Depends on ATP-binding cassette (ABC) pathway.

El-Sharoud,2008

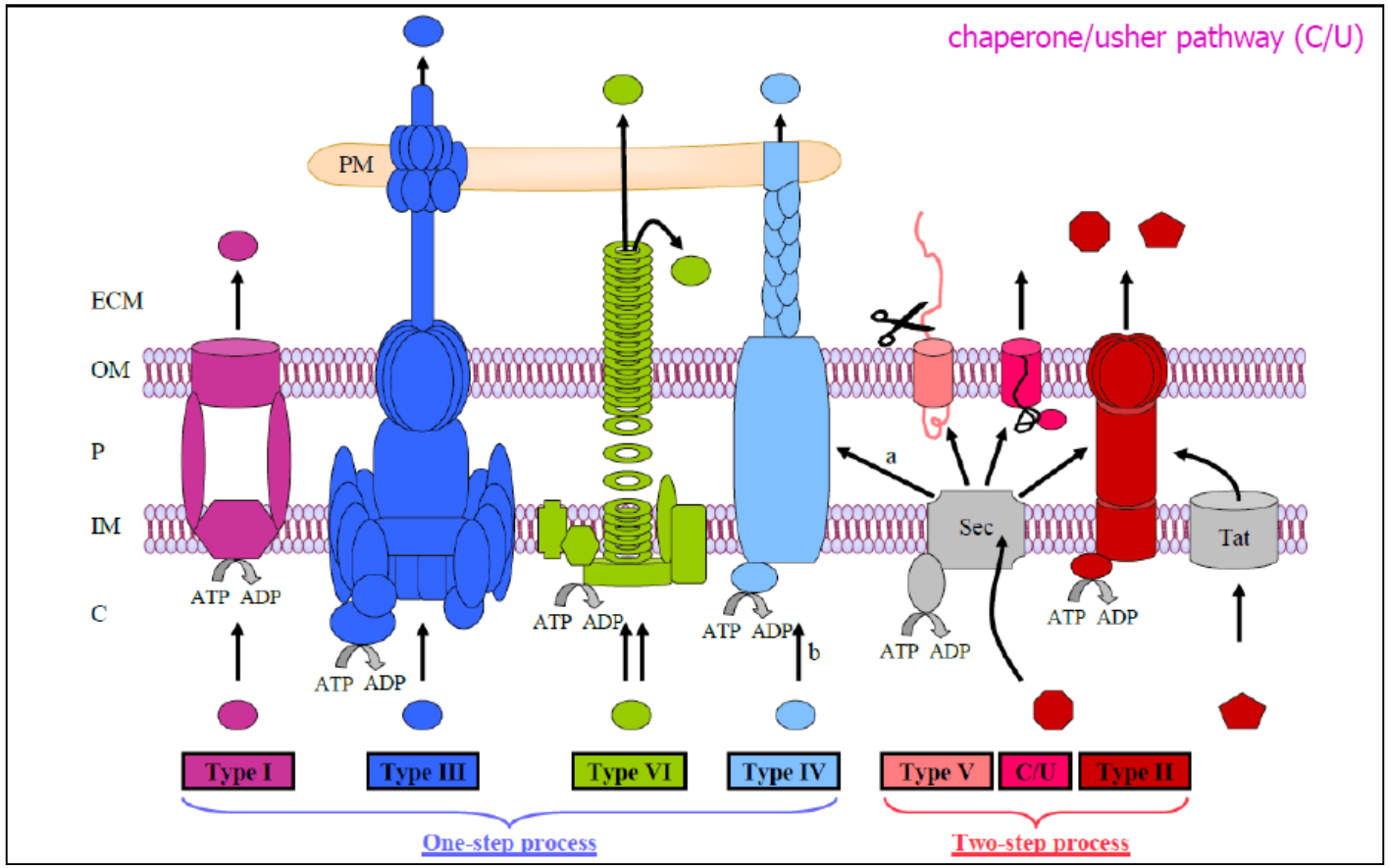
General features of secretion pathways

The protein secretion systems of Gram-negative bacteria



Prescott,2006

Type I-VI secretion systems in Gram-negative bacteria



Beeckman and Vanrompay,2010

Protein translocation pathways

In Gram-negative plant pathogenic bacteria

1. Attachment to the tissue surface is the first step for pathogenic bacteria in establishing infection.
2. The next step is using secretion systems (SSs) to deliver extracellular enzymes and effectors to host cells.
3. Three classes of secretion systems i.e. type I, III and IV have been implicated in virulence, and are widely distributed and highly conserved among bacterial pathogens of plants and humans.

Effectors: A term for virulence proteins injected into host cells by a TTSS, which is broadly applicable to various plant and animal pathogens.

Protein translocation pathways

In Gram-negative plant pathogenic bacteria

Organism	Sec (GSP)	Tat secretion	Type I secretion	Type II secretion	Type III secretion	Type IV secretion	Type V secretion
Agrobacterium tumefaciens C58	Yes	Yes	Yes	No	No	Yes	Yes
Erwinia carotovora subsp. *atroseptica* SCRI1043	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Ralstonia solanacearum GMI1000	Yes	Yes	Yes	Yes	Yes	Partial	Yes
Pseudomonas aeruginosa PAO1	Yes	Yes	Yes	Yes	Yes	No	Yes
Pseudomonas syringae pv. *tomato* DC3000	Yes	Yes	Yes	Yes	Yes	No	Yes
Xanthomonas axonopodis pv. *citri* 306	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Xanthomonas campestris pv. *campestris* ATCC33913	Yes	Yes	No	Yes	Yes	Yes	Yes
Xylella fastidiosa 9a5c	Yes	Yes	Yes	Yes	No	Yes	Yes
Xylella fastidiosa Temecula1	Yes	Yes	Yes	Yes	No	Yes	Yes

We queried the KEGG Pathway database for the occurrence of Sec, Tat, Type I, Type II, Type III, Type IV and Type V secretion systems. Our diagnostic for the occurrence of Type V (autotransporter) secretion was the presence of proteins with a significant match to Pfam model PF03797.

Preston *et al.*,2005

Protein translocation pathways

In Gram-negative plant pathogenic bacteria *Candidatus* Liberibacter asiaticus

- "*Candidatus* Liberibacter asiaticus" (CLas), as a Gram-negative bacterium, lacks secretion systems T3SS, T4SS and T6SS, but it has the Sec secretion system.
- Several Sec-dependent secretory proteins have been identified.
- CLas does not secrete classical proteins (also termed effectors) but secretes nonclassically secreted proteins (ncSecPs).

Zhang,2019; Du *et al.*,2021

Bacterial secretion systems

Multiple mechanisms of protein secretion

In Gram-negative plant pathogenic bacteria

- It is important to note that many pathogens rely on multiple mechanisms of protein secretion.
- For example:

1. Many *Erwinia* species require both a T2SS and a T3SS to cause disease, and
2. Several strains of *Xanthomonas* have T2SS, T3SS and T4SS.

Abramovitch *et al.*,2006

Bacterial secretion systems

Multiple protein secretion systems

Pectobacterium species

- ***P. carotovorum***
- ***P. atrosepticum***
- ***P. brasiliensis***
- ***P. wasabiae***

System	Function	Role in virulence	Studied species
T1SS	Export of PrtW, an adhesin and maybe other proteases and toxins	Required for full virulence	*P. carotovorum* (+) *P. carotovorum* subsp. *brasiliensis* (+) *P. atrosepticum* (+)
T2SS	Export of PCWDE, Svx protein and maybe other virulence determinants	Necessary for pathogenicity	All species (+)
T3SS	Export of plant physiology modifying T3SS effectors. Only one known effector in *Pectobacterium* spp. (DspE/F)	Required for full virulence but T3SS is not present in all of the strains	*P. atrosepticum* (+) *P. carotovorum* (+) *P. carotovorum* subsp. *brasiliensis* (+/-) *P. wasabiae* (-)
T4SS	Export of unknown molecules	Required for full virulence but T4SS is not widely present in the genus	*P. atrosepticum* (+) *P. carotovorum* subsp. *brasiliensis* (+) *P. carotovorum* (-)
T5SS	Possibly secretion of large proteins such as serine protease, hemolysin and hemagglutinin. Bacterium-bacterium interaction (Cdi/Rhs)	Not investigated	*P. carotovorum* (+) *P. carotovorum* subsp. *brasiliensis* (+) *P. atrosepticum* (+) *P. wasabiae* (+)
T6SS	Export of unknown T6SS effectors	Required for full virulence and may be related to microbe-microbe interaction	*P. atrosepticum* (+)

Nykyri *et al.*,2013

Bacterial secretion systems

Multiple protein secretion systems

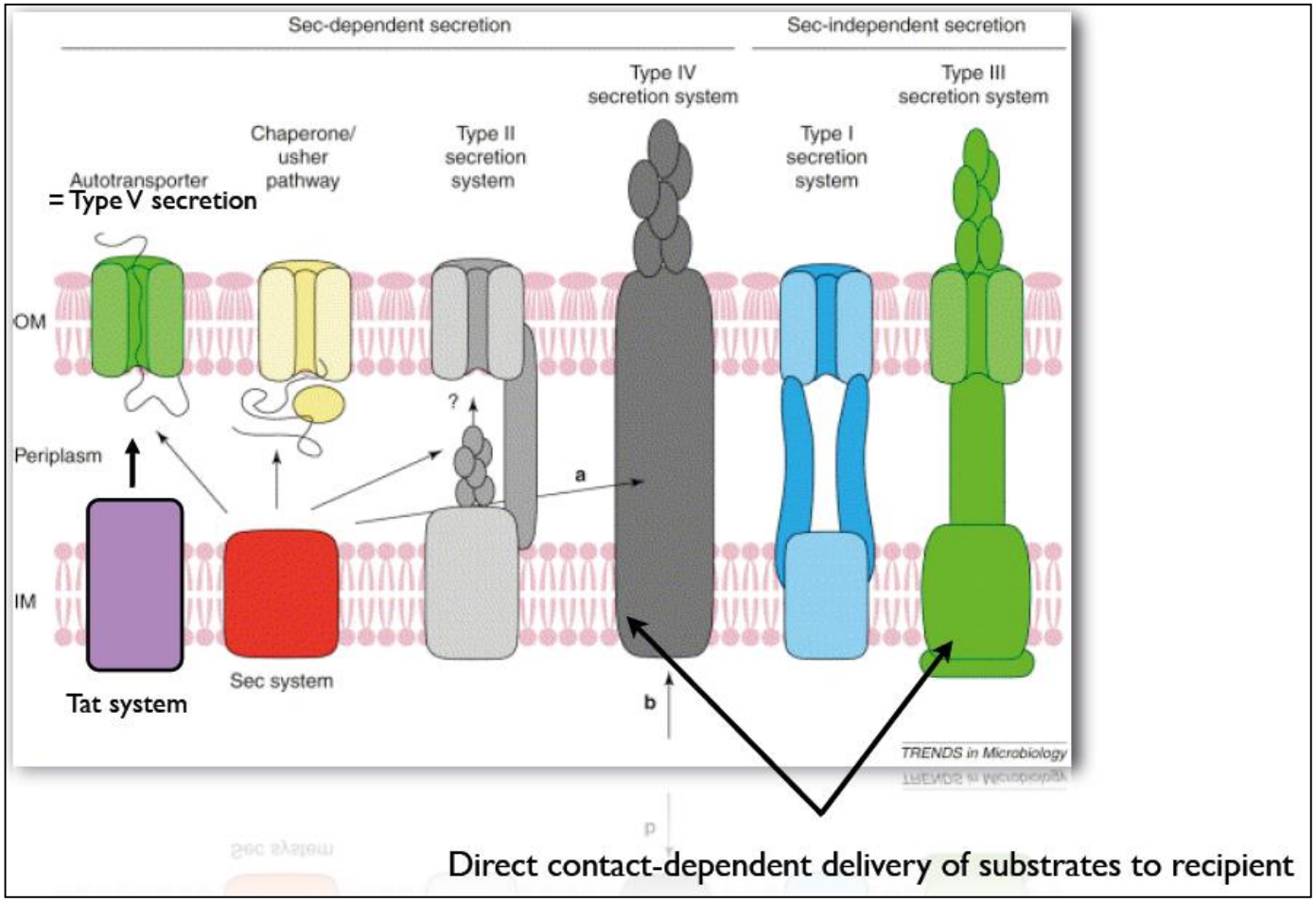
Xanthomonas spp.

- *Xanthomonas* spp. also contain genes for all known protein transport systems in Gram-negative bacteria, i.e.
- The Sec,
- Signal recognition particle, and
- TAT pathways;
- Type I, type II, type III, and type IV secretion systems of different types,
- Type V autotransporters,
- Two-partner secretion systems, and
- A type VI secretion system.
- However, for most *Xanthomonas* secretion systems the substrates and their importance for bacterial virulence are unknown.

Kay and Bonas,2009

Xanthomonas spp.

Most type of secretion systems including type V



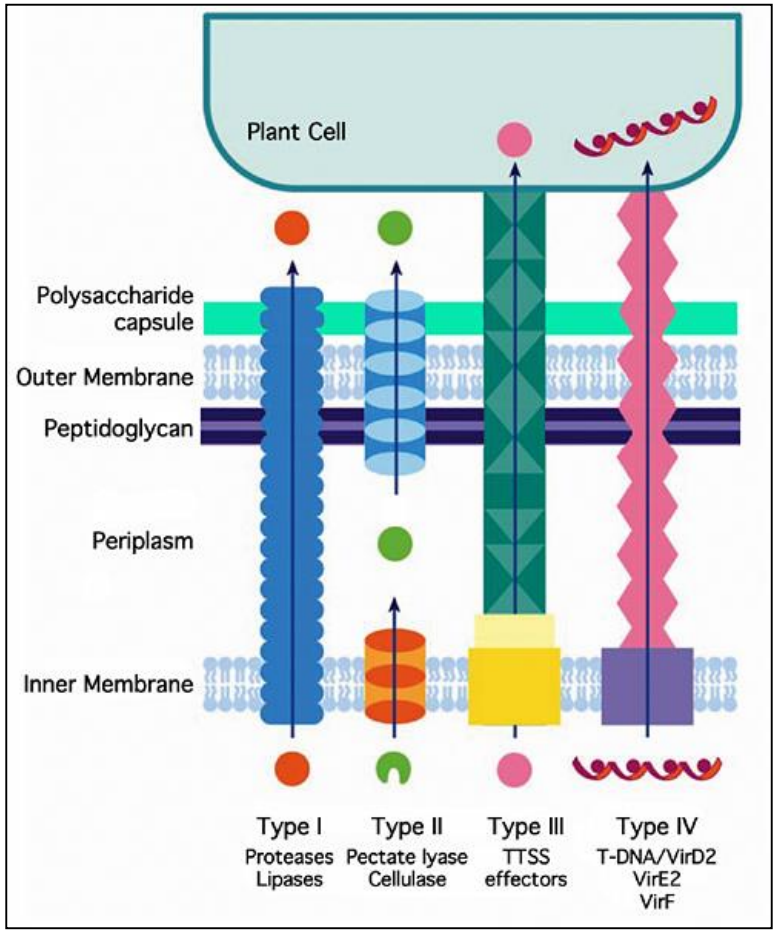
Büttner and Bonas,2002

Bacterial secretion systems

In Gram-negative plant pathogenic bacteria

Types I-IV

- Gram-negative phytopathogenic bacteria use four major pathways to secrete effectors (type I and II) to the intercellular spaces, or directly translocate effectors (type III and IV) into the plant cell.
- The effectors are pathogen-produced proteins that alter the plant cell or its environment to cause disease or to trigger defense.
- Specific examples of effectors transported by each pathway are indicated.

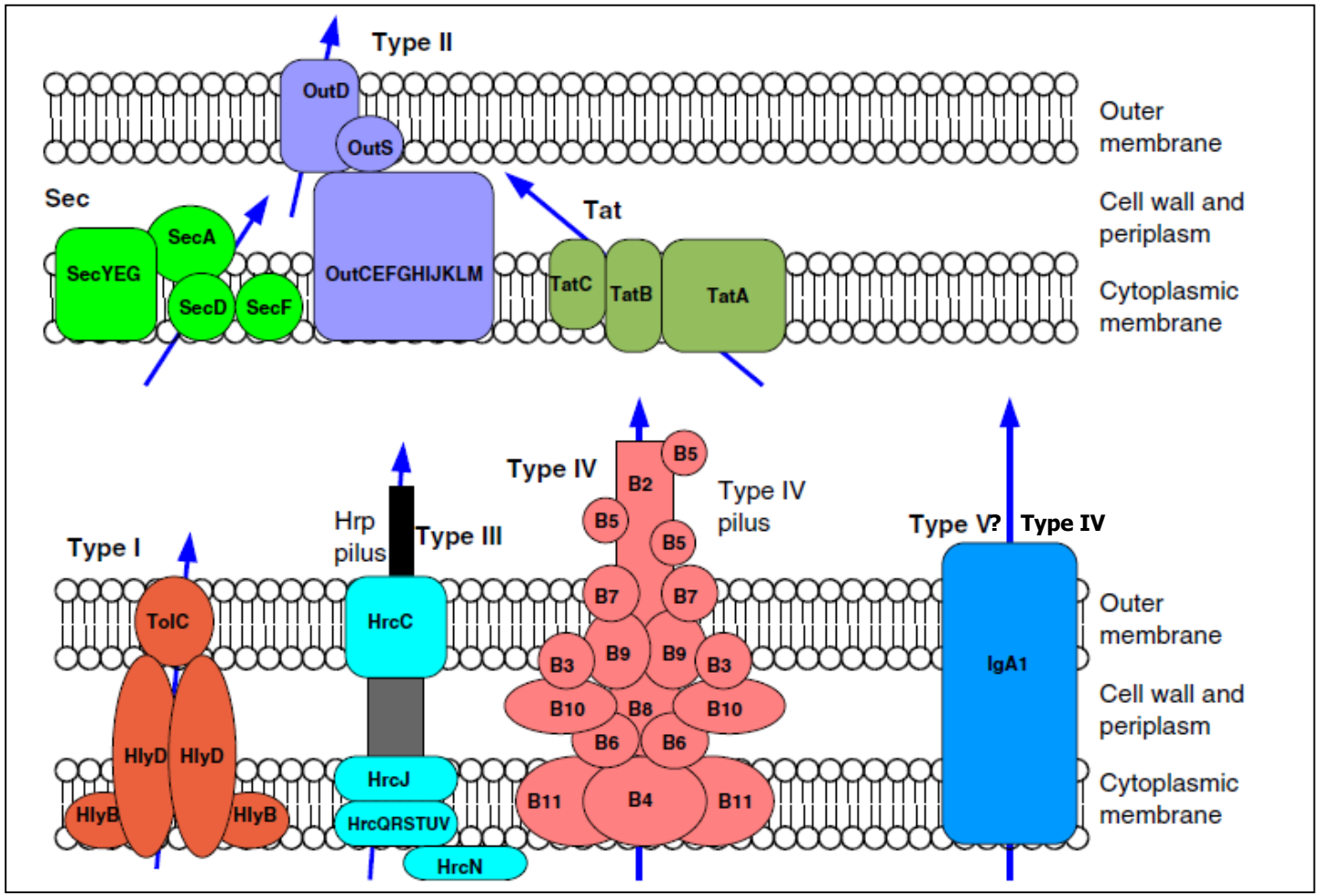


Vidaver and Lambrecht,2004

Specialized secretion systems

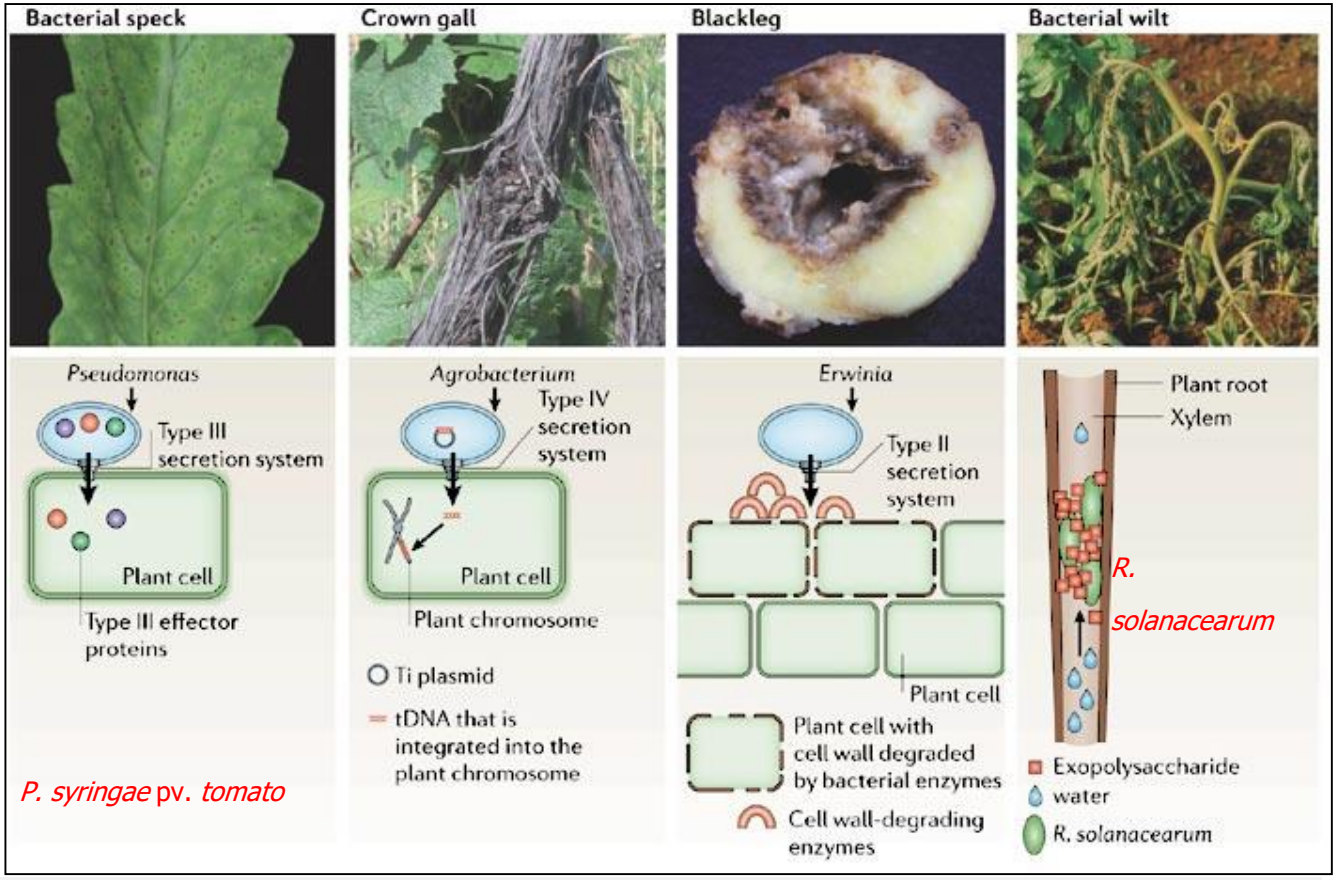
Gram-negative bacteria

Secretion systems encoded in completely sequenced genomes of plant pathogenic bacteria



Preston *et al.*,2005

Disease symptoms caused by some bacterial pathogens of plants and representative virulence mechanisms used by these pathogens



After *R. solanacearum* enters the vascular system of plants, it will secrete a large number of EPS to block the vascular bundle and eventually cause the wilting of plants.

Abramovitch *et al.*,2006

Type I secretion system

Depends on ATP Binding Cassette (ABC) Transporters

Structure

- The type I secretion system is the simplest secretion system found in Gram negative bacteria.
- Type I secretion system is similar to the ABC transporter, however it has additional proteins that, together with the ABC protein, form a contiguous channel traversing the inner and outer membranes of Gram-negative bacteria.
- ATP is needed for this type of translocation.

Type I secretion system
Examples of plant bacterial pathogens

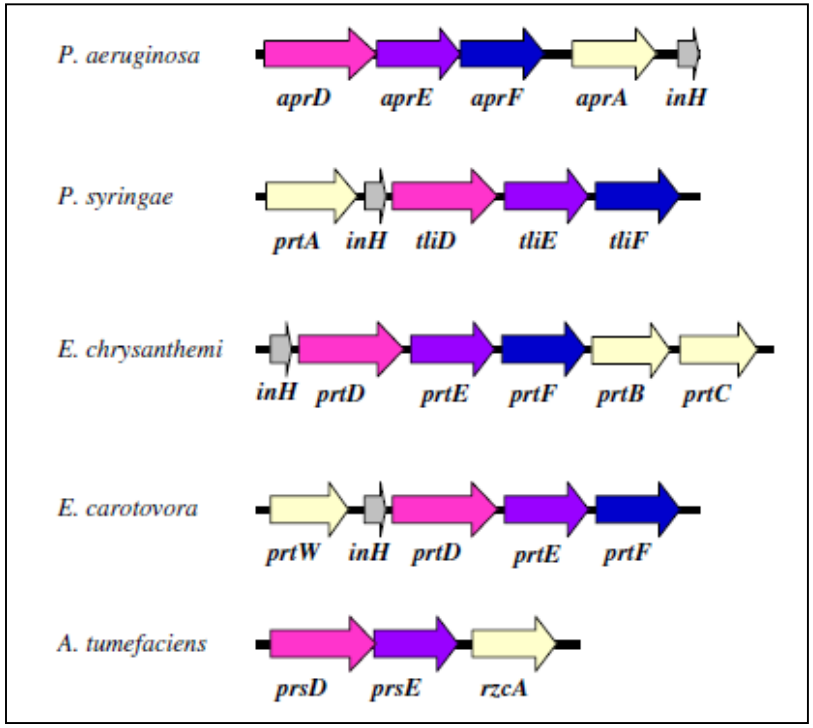
1. *Agrobacterium tumefaciens*
2. *Pseudomonas syringae* pv. *tomato*
3. *Ralstonia solanacearum*
4. *Xanthomonas axonopodis* pv. *citri*
5. *Xylella fastidiosa*
6. *Pectobacterium* spp. and *Dickeya chrysanthemi* uses TISS to secrete proteases.
7. *Erwinia amylovora*
8. *Serratia marcescens*
9. *Pseudomonas syringae* pv.*syringae* and *P. phaseolicola* use this system to secrete anti-freeze protein, and
10. *P. syringae* pv.*syringae* and *P. phaseolicola* use this system to secrete toxin phaseolotoxin.

Tseng *et al.*,2009

Type I secretion system

Gene organization in type I secretion system

- Gene organization in type I secretion/protease clusters from
- *P. aeruginosa*,
- *P. syringae*,
- *D. chrysanthemi*, *P. carotovoraum*,
- *A. tumefaciens.*
- *inH* codes for a protease inhibitor.
- Genes encoding the type I system and type I-secreted proteins are typically located in close proximity.
- For example, the genes coding for protease PrtW in *E. carotovora*, or PrtB and PrtC in *E. chrysanthemi* are clustered with genes encoding PrtD, PrtE, and PrtF which form the ABC exporter.



The direction of arrows indicate the transcription orientation.

Preston *et al.*,2005

Type I secretion system

Functions

- Type I secretion system transports various molecules, from ions, drugs, to proteins of various sizes (20-900 kDa).
- Many secreted proteins are particularly important in bacterial pathogenesis.
- It secretes:

1. Extracellular proteins such as proteases and lipases. e.g. the virulence factors metalloproteases (a family of enzymes from the group of proteases);
2. Toxins (e.g. phaseolotoxin);
3. Bacteriocins;
4. Non-proteinaceous substrates like cyclic β-glucans and polysaccharides.

Type II secretion system

Out system

Structure

- This pathway represents a third branch of the GSP system.
- Thus it is called the General Secretion(Sec system).
- More than 12 different proteins are involved in this pathway.
- Proteins secreted through the type II system initially transport into the periplasm.
- Then they pass through the outer membrane via a multimeric complex of secretin proteins.
- In addition to the secretin proteins, 10-15 other inner and outer membrane proteins compose the full secretion apparatus, many with as yet unknown function.
- ATP is needed for the translocation.

Type II secretion system
Structure

- Several important pathogens of humans and plants use the system to deliver different virulence factors.
- The major outer membrane T2SS protein is the 'secretin' GspD which forms one of the largest multimeric assemblies in the outer membrane of bacteria.
- The GspD secretin, indicated here as a complex with the GspS lipoprotein, serves as a gated channel for secretion of substrates to the cell surface.
- Signal peptide cleaved in periplasmic space.
- Secreted proteins (secretomes) have an N-terminal signal sequence that is cleaved during transport.

Type II secretion system
Out system

- The T2SS or the Out system, is essential for G-ve bacteria such as

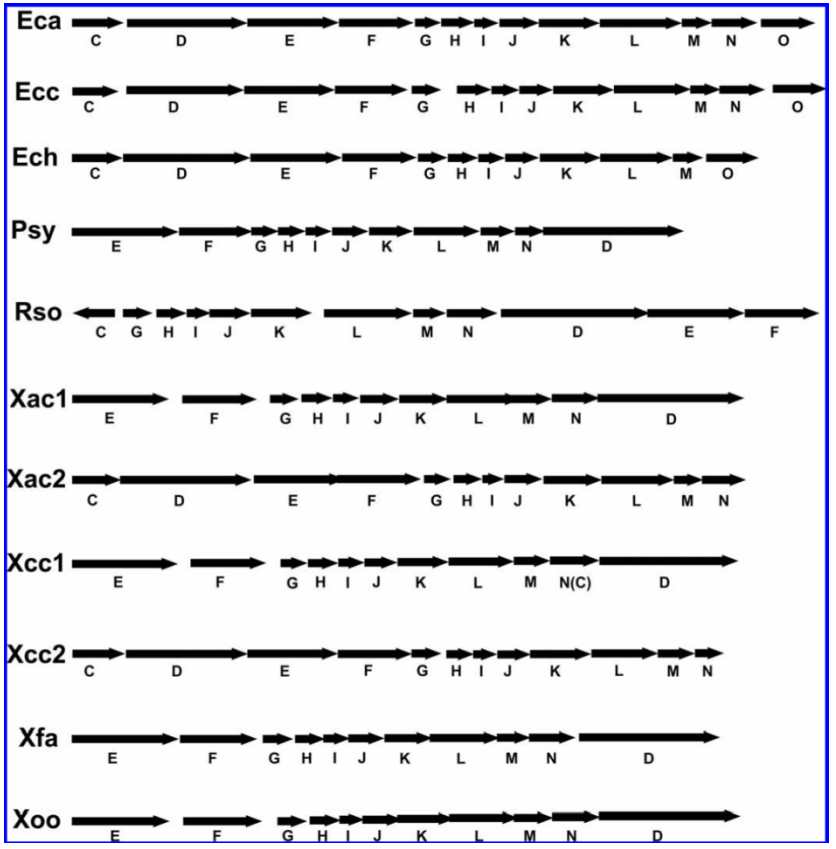
1. soft-rotting bacteria (e.g. *Pectobacterium* spp.),
2. *Pseudomonas fluorescence*,
3. *R. solanacearum*,
4. *X. oryzae* pv. *oryzae*,
5. *X. campestris* pv. *campestris*,
6. Some more G-ve bacteria.

The pathogenicity of *R. solanacearum* is the result of the cooperation and coordination of various pathogenic factors, mainly including 1) extracellular polysaccharide (EPS), 2) type II secretion system (T2SS), and 3) type III secretion system (T3SS).

Type II secretion system

Schematic of the organization of type two secretion system (T2S) gene clusters from various phytopathogenic bacteria

- Eca: *Erwinia c.* subsp. *atroseptica*
- Ecc: *E. carotovora* subsp. *carotovora*
- Ech: *E. chrysanthemi*
- Psy: *Pseudomonas syringae* pv. *tomato*
- Rso: *Ralstonia solanacearum*
- Xac2: *xps* and *xcs* in *Xanthomonas axonopodis* pv. *citri*
- Xcc1 and Xcc2: *xps* and *xcs* in *Xanthomonas campestris* pv. c*ampestris*
- Xfa: *Xylella fastidiosa*
- *Xoo: Xanthomonas oryzae* pv. *oryzae*
- C, D, E, F, G, H, I, J, K, L, M, N, and O represent genes encoding various components of T2S.
- The direction of arrows indicate the transcription orientation.



Jha *et al.*,2005

A notable exception is *Agrobacterium tumefaciens*, which does not appear to encode a T2S.

Type II secretion system

Out system

Functions

- The plant pathogenic bacteria uses this system to export hydrolytic enzymes (cell wall degrading enzymes) including:

1. Pectinases,
2. Proteases,
3. Amylase,
4. Xylanses,
5. Endoglucanases,
6. Cellulases.

- These and other exoenzymes are associated with pathogenicity of soft rot bacteria.
- +Toxins (enterotoxin).

An enterotoxin (not to be confused with endotoxin) is a protein toxin released by a microorganism in the intestine. e.g. Heat-labile enterotoxin (LT) from *Escherichia coli* (ETEC) and cholera toxin (CT) from *Vibrio cholera*.

Type II secretion system

Schematic of the organization of type two secretion system (T2S) gene clusters from various phytopathogenic bacteria

- Pectinases: *Pectobacterium* spp.
- Endoglucanases: *Ralstonia solanacearum*; *D. chrysanthemi*; *Pectobacterium* spp.
- Cellulase: *X. oryzae* pv. *oryzae*; *Pectobacterium* spp.
- Lipase: *X. campestris* pv. *campestris*
- Amylase: *X. campestris* pv. *campestris*
- Protases: *X. campestris* pv. *campestris*

Name of the bacterium	Known effectors
Erwinia carotovora pv. *carotovora*	Pectate lyases Cellulase/endoglucanase Polygalacturonase
E. carotovora pv. *atroseptica*	Pectate lyase Endoglucanase/cellulase Putative exported plant-proteoglycan hydrolase Putative cellulase Svx (homologous to AvrXca)
E. chrysanthemi	Pectate lyases Polygalacturonase Pectin methylesterases Cellulase/endoglucanase Pectin acetylesterases Rhamnogalacturonate lyase Avr-like protein (AvrL, homologous to AvrXca) FaeD esterase
Ralstonia solanacearum	Polygalacturonases Cellulase/endoglucanase Pectin methyl esterase Exo-cellobiohydrolase
Xanthomonas campestris pv. *campestris*	Polygalacturonate lyases Cellulase/endoglucanase Proteases α-Amylase
X. oryzae pv. *oryzae*	Xylanase Cellulase/endoglucanase Putative cysteine protease Putative cellobiosidase Lipase/esterase

Jha *et al.*,2005

Type III secretion system
Also called injectisome

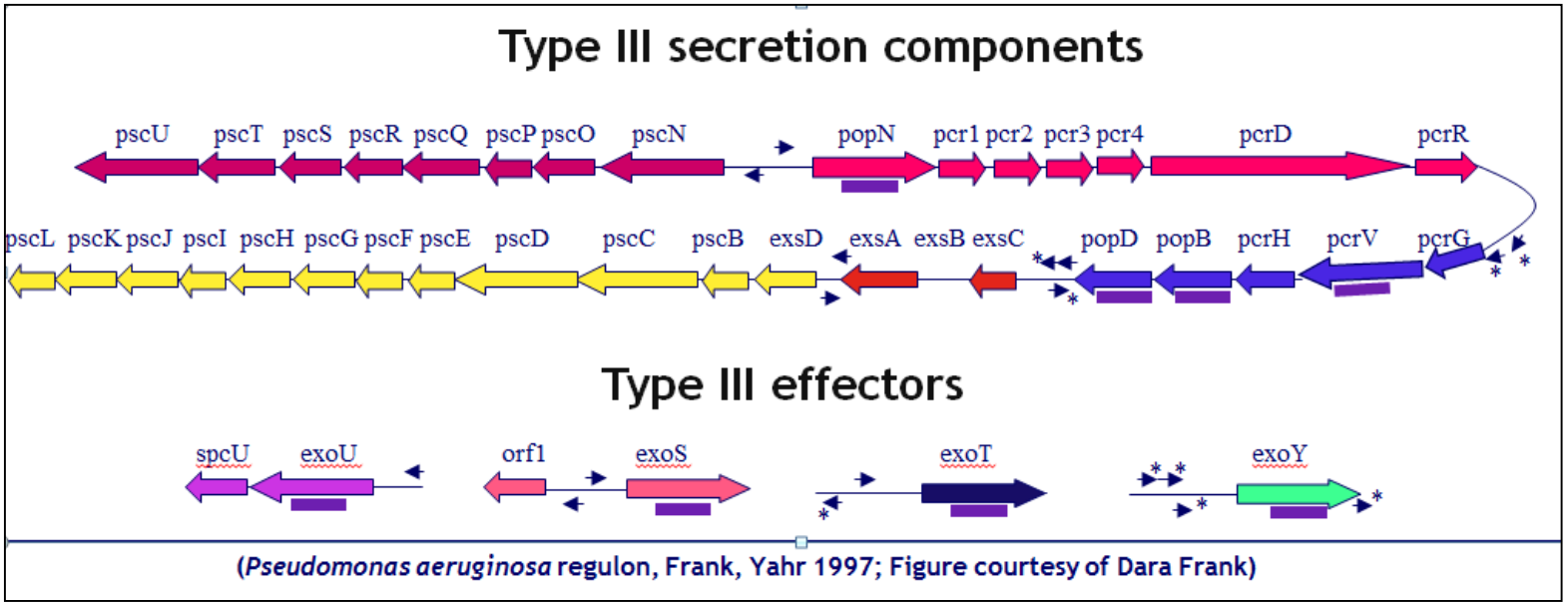
- Discovered in 1994.
- Induced by contact with host cell.
- Genes encoding type III secretion systems are predominantly located on pathogenicity islands (PAIs).
- The type III secretion system (TTSS) was first identified in pathogenic *Yersinia* spp. for the secretion of Yop proteins.

Arnold and Jackson,2011

Protein translocation pathways

Studying bacterial secretion processes

Type III secretion components



Olson,2006

Type III secretion system(T3SS)

TTS systems exhibit the most complex architecture

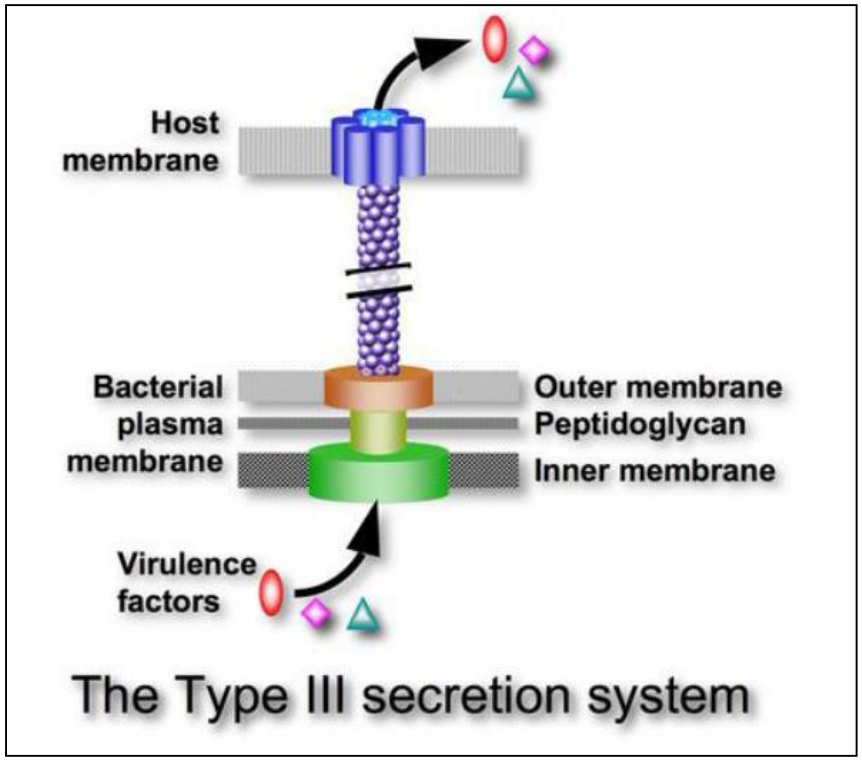
Structure

- Multiple-component Type III secretion systems (T3SSs) widely distributed among proteobacterial pathogens of plants, animals, and humans.
- Many Gram-negative plant and animal pathogenic bacteria utilize a specialized type III secretion system as a molecular syringe to inject effector proteins directly into host cells.
- The low Ca^{2+} concentration in the cytosol opens the gate that regulates T3SS.
- Here also ATP is needed for the translocation.
- No signal peptide cleavage was occurred in this system.

The T3SS machinery is absent in both *X. fastidiosa* and *Ca.* Liberibacter asiaticus.

Type three secretion system

- Injection apparatus, together with effector proteins, for parasitising euckaryotic cells.
- Required for by proteobacteria to produce infection of plants and animals.
- Required for colonization and plant defence activation in growth promoting strains.



Type III secretion system

Hrp pilus or Hrp TTSS

In plant pathogenic bacteria

- Pathogenicity most other Gram-negative phytopathogenic bacteria depends on a conserved type III secretion (T3S) system which injects more than 25 different effector proteins (T3SE) into the plant cell.
- Since this discovery, TTSS have been identified in several mammalian and plant pathogens including:

1. *Ralstonia solancearum*
2. *Pseudomonas syringae*.

- Type III secretion pathway, also referred to as the Hrp pathway in *P. syringae* pathovars.
- It is directly responsible for many of the most devastating diseases known to plants and humans.

Type III secretion system

Hrp pilus or Hrp TTSS

Endogenous versus exogenous bacteria

- While most phytopathogenic bacteria are exogenous and colonize the apoplast, the phloem-restricted and the xylem-restricted bacteria are endogenous, and they are introduced directly into the phloem or the xylem by their insect vectors.
- The type III secretion system is present in all the major groups of exogenous Gram-negative, plant pathogenic bacteria.
- The T3SS substrate effector proteins (T3SE) are secreted via a pilus from the bacterial cell to inside a plant cell and are subject to considerable change as the pathogen and host co-evolve.

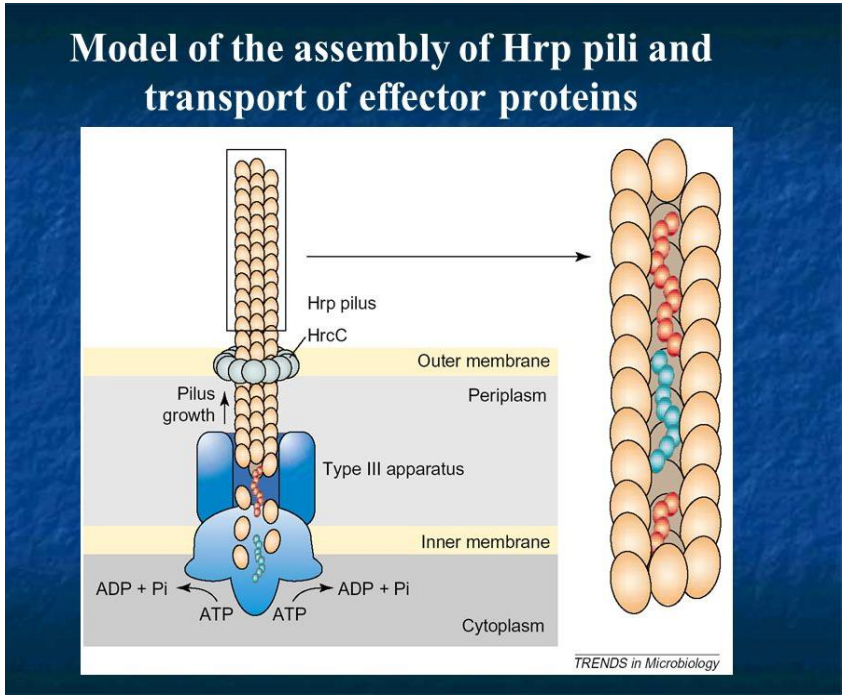
Exceptions: *Agrobacterium* and *X. fastidiosa* do not contain the genes of a type III system.

Type III secretion system

Hrp pilus or Hrp TTSS

A surface filamentous appendage

- Primary, or "classical," Hrp-T3SS Hrp described for:
  1. *P. syringae*
  2. *R. solanacearum*
  3. *Erwinia amylovora*
  4. *X. vesicatoria*.
- Hrp pili elongate distally with the addition of their major component, Hrp pilin subunits, likewise T3SS effectors are secreted from the pilus tip.
- Each *hrp* gene clusters contains more than 20 genes and many of them encode components of the 'type-III' protein-secretion pathway.



These are tubular structures that constitute the delivery system by which effector molecules are secreted into the plant cell.

Type III secretion pathway
The Hrp pathway

- A type III secretion pathway, broadly conserved among gram-negative pathogens of plants and animals.
- Macromolecular structure, Hrp pilus, acts as conduit for traffic (called needle complex in animal pathogens).

1. Encoded by clustered *hrp* genes.
2. Required for hypersensitive reaction and pathogenicity.
3. Expression induced *in planta* and in defined minimal media.
4. Capable of delivering proteins into host cells.
5. Secretes and delivers effector proteins:

a) virulence factors

b) avirulence factors

Bogdanove

Type III secretion system

Hrp pilus or Hrp TTSS

A surface filamentous appendage

- The injectisome is composed of two parts:

1. An envelope-embedded multiring base, and
2. A long protruding surface appendage, called the hrp pilus, 2 µm or longer, with an outer diameter 6-10 nm and an inner one probably up to 2 nm.

- Considering the dimensions of the pilus, one has to assume that the effector proteins, which are up to 200 kDa in size, move within the channel in an at least partially unfolded state.
- Many of the T3SS effector proteins have been shown to be dependent on chaperones, which keep the effector in a partially unfolded form in the bacterial cytoplasm.

Buonaurio,2008

Type III secretion system

Hrp pilus or Hrp TTSS

Name and common features of pilus proteins in some bacteria

- Although the pilus proteins,

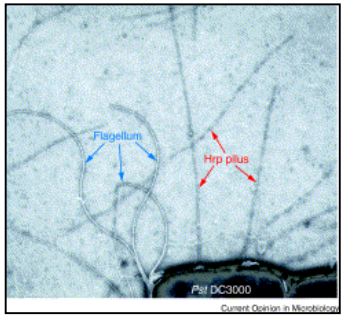
1. HrpA (*P. syringae* and *E. amylovora*),
2. HrpY (*R. solanacearum*), and
3. HrpE (*X. vesicatoria*) do not share any significant sequence homology, they have a number of physicochemical features in common:
4. They are small (8.7-11.3 kDa),
5. Predicted to consist almost exclusively of α-helices,
6. Show very similar hydrophobicity profiles, and
7. Resemble each other in their instability and aliphatic indices.

Buonaurio,2008

Aliphatic: Acyclic or cyclic, not aromatic carbon compounds.

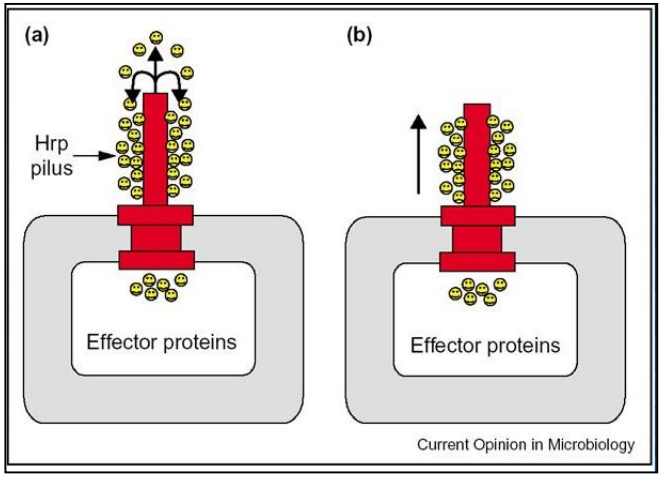
The Hrp pilus

Pseudomonas syringae



- There are two alternative models to explain the localization of secreted proteins along the Hrp pilus during active type III secretion.
- The newly synthesized AvrPto or HrpZ is

1. extruded from the tip (as in the conduit model) or
2. the base (as in the guiding filament/conveyor model) of the Hrp pilus.



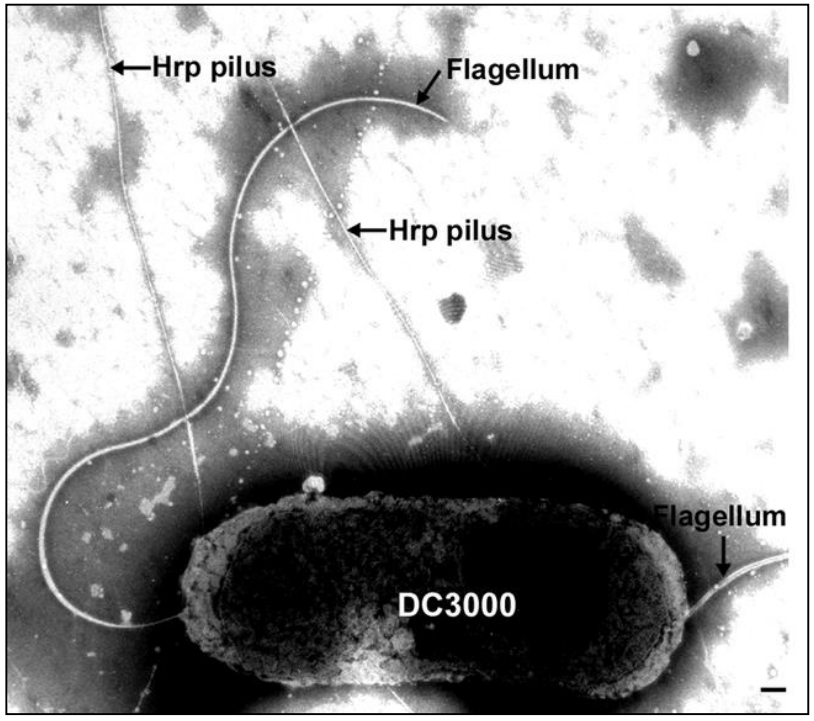
In *Pseudomonas syringae* Hrp pilus elongates from its tip and acts as a conduit for translocation of the effector protein HrpZ.

Li *et al.*,2003; He,2004

Transmission electron microscope image of *Pseudomonas syringae* pv. *tomato* DC3000

The Hrp pilus

1. Flagella enable bacteria to swim toward or away from specific chemical stimuli.
2. Hrp pili are involved in type III secretion of avirulence and virulence proteins.



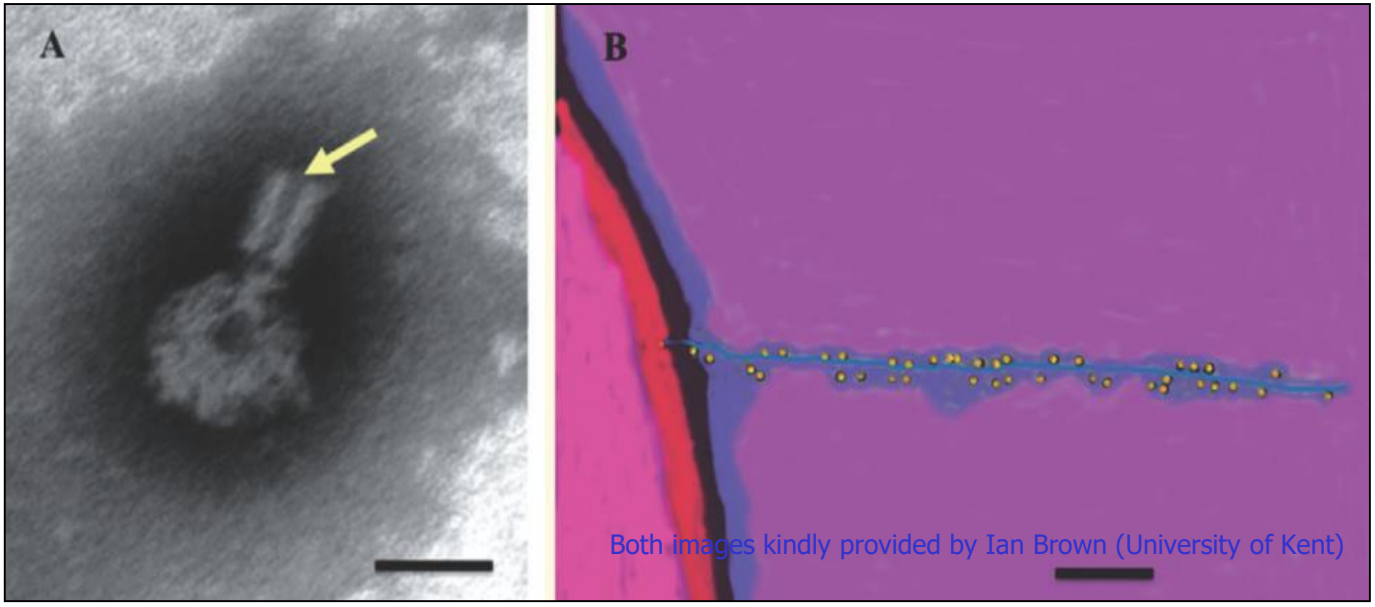
Note that DC3000 produces polar flagella (15 nm in diameter) and a few Hrp pili (8 nm in diameter).

Katagiri *et al.*,2002;BIOL 350 Spring 08

The type III secretion system (T3SS)
P. syringae pv. *tomato*

A. Putative basal body of the T3SS released from membrane preparations after growth in hrp inducing medium. The arrow marks the attachment point of the Hrp pilus. Bar, 25 nm.

B. False colour image of the Hrp pilus gold labelled with antibodies to the subunit protein HrpA, emerging from the bacterial surface. Bar, 50 nm.



Mansfield *et al.*,2012

Type III secretion system

Differences between plant (A) and animal (B) pathogenic bacteria TTSSs

- **In mammalian bacterial pathogens:**
- The translocation of T3SS effectors into host cells requires T3SS-secreted accessory proteins called translocons, which forms pores in the host plasma membrane.
- **In plant bacterial pathogens:**
- Although little is known about T3SS translocons in phytopathogenic bacteria, recent evidence suggests that:
- HrpF in *X. vesicatoria*, and
- HopB1 and HrpK in *P. syringae* pv. *tomato* have this function.

Buonaurio,2008

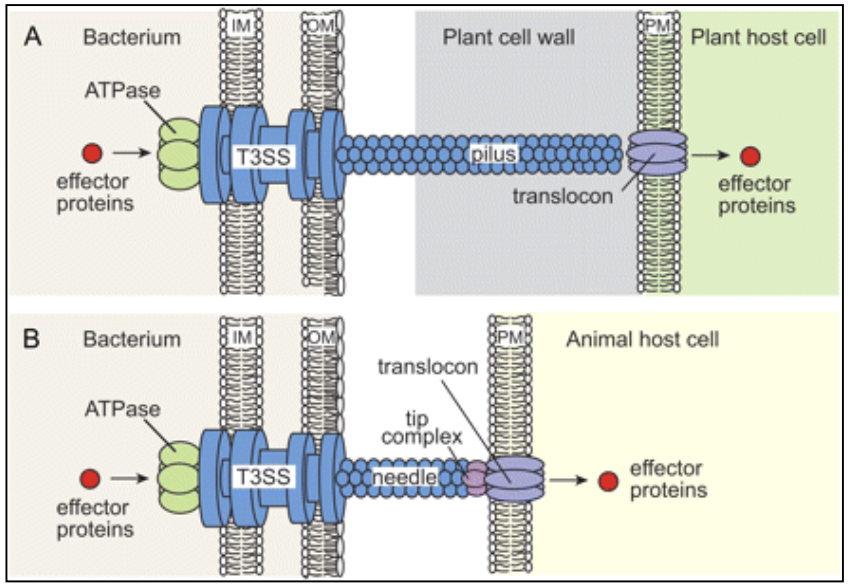
Type III secretion system

Differences between plant (A) and animal (B) pathogenic bacteria TTSSs

A. The T3SS from plant pathogenic bacteria is connected to an extracellular pilus that presumably spans the plant cell wall.

B. The T3SS system from animal pathogenic bacteria is associated with a short extracellular needle, which serves as a transport channel for secreted proteins.

- The needle is linked via the so-called tip complex to the translocon, which forms a proteinaceous channel in the host plasma membrane and allows transport of effector proteins into the host cell cytosol.
- Evidence for the presence of a tip complex in plant pathogenic bacteria is still missing.



IM, Inner membrane; OM, outer membrane; PM, plasma membrane.

Büttner and He,2009

Genes encoding for
Type III secretion apparatus

- In phytopathogenic bacteria, the TTSS is encoded by hypersensitive reaction/pathogenicity (*hrp*) genes.
- As mentioned earlier, 20 or more genes (around 20 proteins) are involved in the formation of a membrane-spanning secretion apparatus, which is associated with an extracellular filamentous structure.
- These genes are highly conserved and generally located on a single:

1. Plasmid, or
2. Chromosomal locus.

Type III secretion system (T3SS)
Similarity of TTSS and syringe

- The analogy between the TTSS and the hypodermic syringe, coupled with the ability of the systems to inject proteins directly into the host cytosol, has allowed the phrase "injectisome" to be coined for this protein secretion system.

Type III secretion system
Similarity of TTSS and flagella

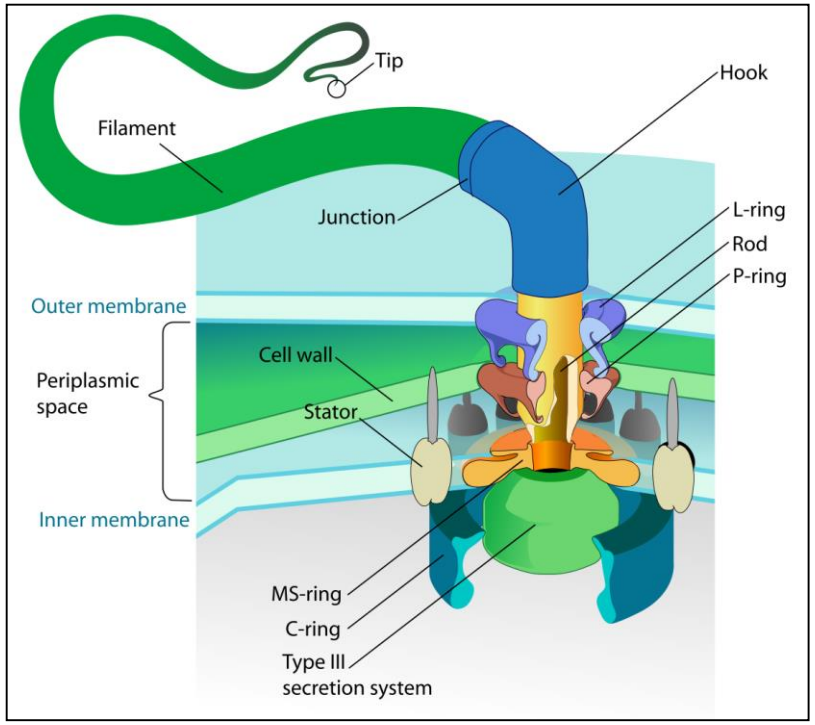
- The T3SS and flagella are evolutionarily related and they share:

1. A remarkably similar basal structure. e.g. a number of the inner membrane components of the TTSS show similarity to proteins of the flagellar basal body.
2. Sequence similarity confirm these high degree similarities between TTSS proteins and flagellar proteins.

- It has often been suggested that TTSS genes evolved from genes encoding flagellar proteins (flagellar assembly apparatus probably represents an evolutionary ancestor of the TTS system).

Flagellum of Gram-negative Bacteria
Distinction of TTSS and flagella

- An important distinction between T3SSs and flagellar systems is that the former are typically located in pathogenicity islands (PAIs) and found to be transferred horizontally among bacterial species.

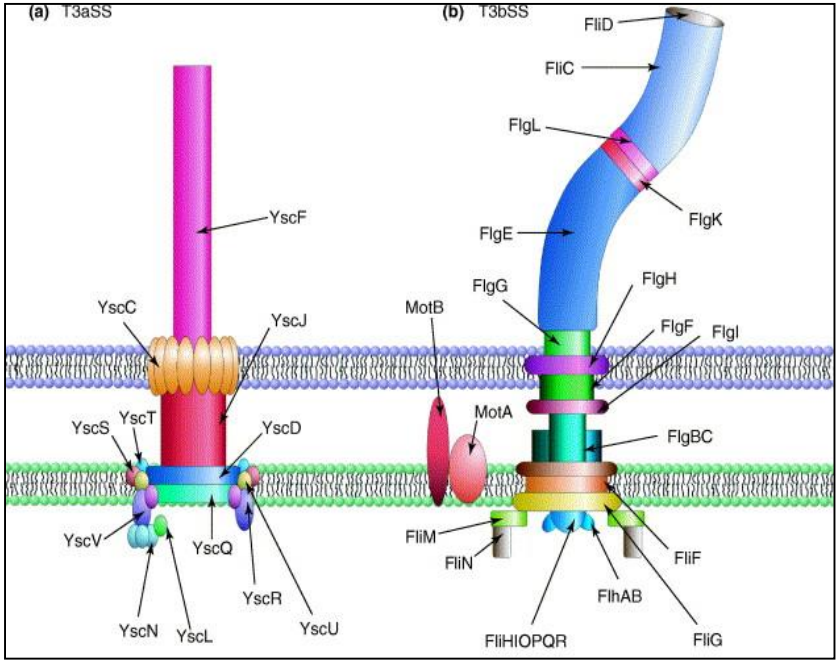


Type III secretion system
Similarity of TTSS and flagella

- The evolution of the T3SS and flagellar systems from a common ancestor.
- Type III apparatus resembles the basal body of flagella, with an injection needle at the tip replacing the hook and flagellar filament.

a) The Ysc–Yop system (type III secretion system (T3aSS) found in *Yersinia* species.

b) The bacterial flagellum (T3bSS).



Note homology between T3aSS and T3bSS components.

Type III secretion system

Pathogenic T3SS substrates

Effector proteins

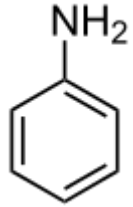
- The pathogenic T3SS substrates are multi-domain proteins that are typically subdivided into two distinct regions:

1. An N-terminal domain that contains secretion and translocation signals that function within the bacterium.
2. One or more C-terminal domains that harbor the host cell effector activities.

Type III secretion system

Pathogenic T3SS substrates

Effector proteins



Alanine an aromatic carbon compound.

- All effectors have particular profile in first 50 amino acids (AA):

1. > 10% serines.
2. 1 aliphatic AA (isoleucine, leucine, valine, or proline) in 3rd or 4th position.
3. No acidic AA (aspartic or glutamic acid) in the first 12AA.

Aliphatic: Acyclic or cyclic, not aromatic carbon compounds. Aromatic hydrocarbon or arene (or sometimes aryl hydrocarbon) is a hydrocarbon characterized by general alternating double and single bonds between carbons forming rings.

De La Fuente,2008

Some phytopathogen type III effectors
T3E activities and plant targets

T3E	Species	Activity	Target
AvrB	*P. syringae* pv. *glycinea* race 0	Induces phosphorylation	RIN4/RAR1
AvrBs3	*X. vesicatoria* race 1	Transcription activator-like	Upa20(transcription factor)/Bs3
AvrPphB	*P. syringae* pv. *phaseolicola* race 3	Cysteine protease	cleaves the Arabidopsis protein kinase PBS1
AvrPto	*P. syringae* pv. *tomato* JL1065	Kinase inhibitor	Pto/EFR/FLS2
AvrPtoB	*P. syringae* pv. *tomato* DC3000	E3 ubiquitin ligase	Fen(tomato kinase protein)
AvrRpm1	*P. syringae* pv. *glycinea* race 0	Induces phosphorylation	RIN4 protein(regulated basal defense in *Arabidopsis*)
AvrRpt2	*P. syringae* pv. *tomato* T1	Cysteine protease	RIN4 protein(regulated basal defense in *Arabidopsis*)
AvrXa27	*X. oryzae* pv. *oryzae* PXO99^{A}	Transcription activator-like	Rice R gene *Xa27*
AvrXv4	*X. vesicatoria* T3	DeSUMOylating cysteine protease	Unknown
GALA	*R. solanacearum* GMI1000	F-box and LRR domains	*A. thaliana* Skp1-like proteins
HopAI1	*P. syringae* pv. *tomato* DC3000	Phosphothreonine lyase	MPK3/MPK6

Some phytopathogen type III effectors
T3E activities and plant targets

T3E	Species	Activity	Target
12	*P. syringae* pv. *tomato* DC3000	Protein tyrosine phosphatase	Unknown
HopI1	*P. syringae* pv. *maculicola* ES4326	J-domain protein (possible Hsp70 cochaperone)	Unknown
HopM1	*P. syringae* pv. *tomato* DC3000	Unknown	AtMIN7 and other immunity associated *Arabidopsis* protein
HopU1	*P. syringae* pv. *tomato* DC3000	Mono-ADP-ribosyltransferase	GRP7 and other RNA-binding proteins
HsvB	*Pantoea agglomerans* pv. *betae* 4188	Transcriptional activator-like	Unknown
HsvG	*Pantoea agglomerans* pv. *gypsophilae* 824-1	Transcriptional activator-like	Unknown
PthXo1	*X. oryzae* pv. *oryzae* PXO99^{A}	Transcriptional activator-like	host gene *Os8N3*
PthXo6/7	*X. oryzae* pv. *oryzae* PXO99^{A}	Transcriptional activator-like	*OsTFX1 OsTFIIAg1*
XopD	*X. vesicatoria* 85-10	DeSUMOylating cysteine protease	Unknown

Block *et al.*,2008

Bioinformatics

Identification of effector proteins using bioinformatics

- Bioinformatics pipeline used for the identification of pathogen effectors such as '*Ca.* Phytoplasma asteris' (aster yellows) effectors.
- The computer software, SignalP was successfully used to identify candidate effector proteins in the genome of '*Ca*. Phytoplasma asteris' (aster yellows).
- Recognition of the N-terminal signal peptide (SP) and cleavage sites of SPs in proteins was done by SignalP.
- The SP is cleaved during the protein export process across the bacterial cell wall, leading to the presence of a mature protein without SP in the extracellular environment.

Bioinformatics

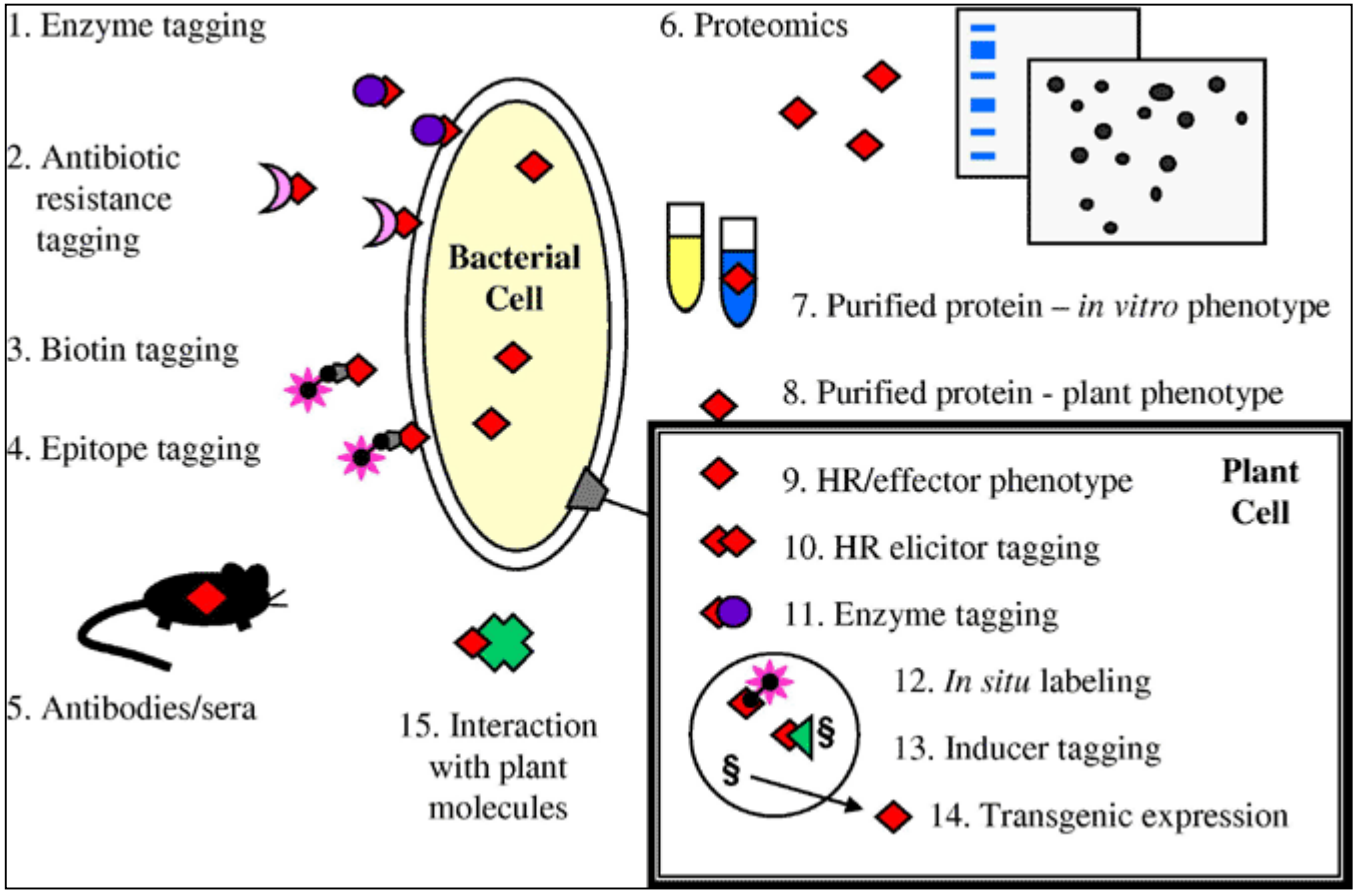
Assays used to identify secreted proteins

- Knowledge of host-microbe interactions has been used to develop a range of techniques to identify proteins delivered into the cytoplasm of plant cells by type III and type IV systems.
- The next figure gives a schematic overview of some of the tools and techniques currently in use, including a few techniques that have yet to be applied to plant pathogenic bacteria.

Preston *et al.*,2005

Bioinformatics

Assays used to identify secreted proteins



Preston *et al.*,2005

Bioinformatics

Bioinformatics resources for prediction of N-terminal secretion signals in bacterial protein sequences

Name of application	Type of algorithm used	Availability
CELLO	Support vector machine	Webserver: http://cello.life.nctu.edu.tw/
NNPSL	Neural network	Webserver: http://www.doe-mbi.ucla.edu/astrid/astrid.html
Phobius	Hidden Markov model	Webserver: http://phobius.binf.ku.dk/
PSORT-B	Hidden Markov model and support vector machine	Webserver: http://psort.org
SigCleave	Scoring matrix	Part of the EMBOSS suite **[230]**: http://www.hgmp.mrc.ac.uk/Software/EMBOSS/
SignalP3.0	Neural networks and hidden Markov model	Webserver: http://www.cbs.dtu.dk/services/SignalP/
SPEPlip	Neural network	Webserver (requires authentication): http://gpcr.biocomp.unibo.it/predictors/
SPScan	Scoring matrix	Part of the GCG suite: http://www.accelrys.com/about/gcg.html
SubLoc	Support Vector Machine	Webserver: http://www.bioinfo.tsinghua.edu.cn/SubLoc/

Preston *et al.*,2005

Type III secretion system

Biochemically diverse functions of TSS effectors

- TTSS effectors have biochemically diverse functions.
- These are interacted with host proteins both inside and outside the host cell, manipulating host cell biological systems such as:

1. Cytoskeletal structure,
2. Signal transduction,
3. Cell cycle progression, and
4. Programmed cell death.

- These allow bacteria to precisely modulate host tissues and systems for the benefit of the pathogen.

Type III secretion system

Direct /indirect connection of TSS effectors

- Direct connections:
- Usually TTSS effectors (harpin and avirulence proteins) are directly injected into the host cytosol (cytoplasm) via the TTSS pilus.
- Indirect connections:
- Nevertheless, investigators should be reminded that not all TTSS are contact dependent and some effector molecules secreted by TTSS are released into the external environment.

Type III secretion system

Functions of TSS effectors

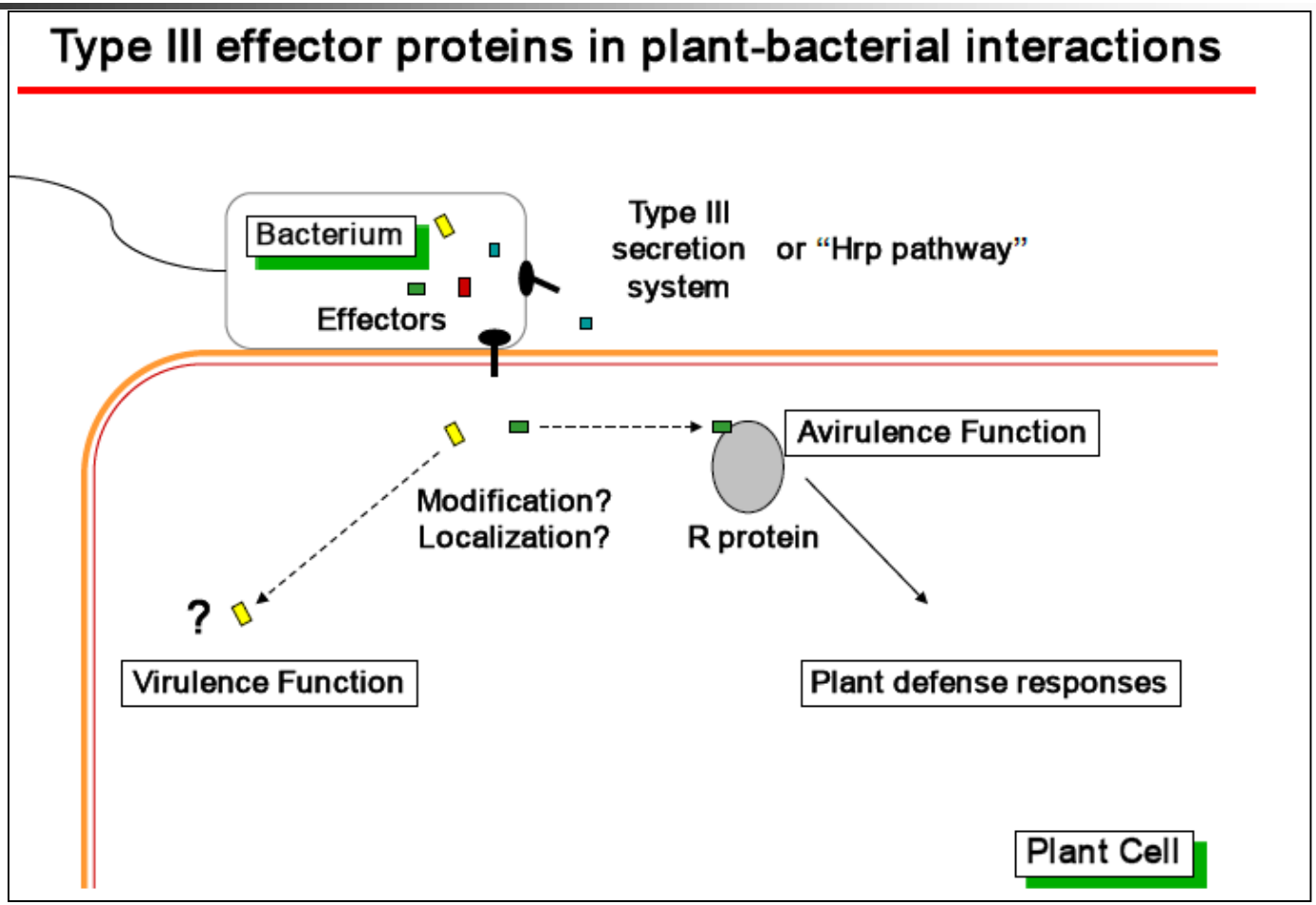
Host specificity

- In many cases, effectors are necessary for pathogenesis and are directly responsible for determining host specificity.
- Pathogens that are deficient in type III secretion are unable to grow *in planta* or to cause disease, suggesting that the proteins secreted by the TTSS are essential virulence factors.
- These proteins:

1. Suppress resistance, but can also
2. Trigger plant immune system.

Type III secretion pathway

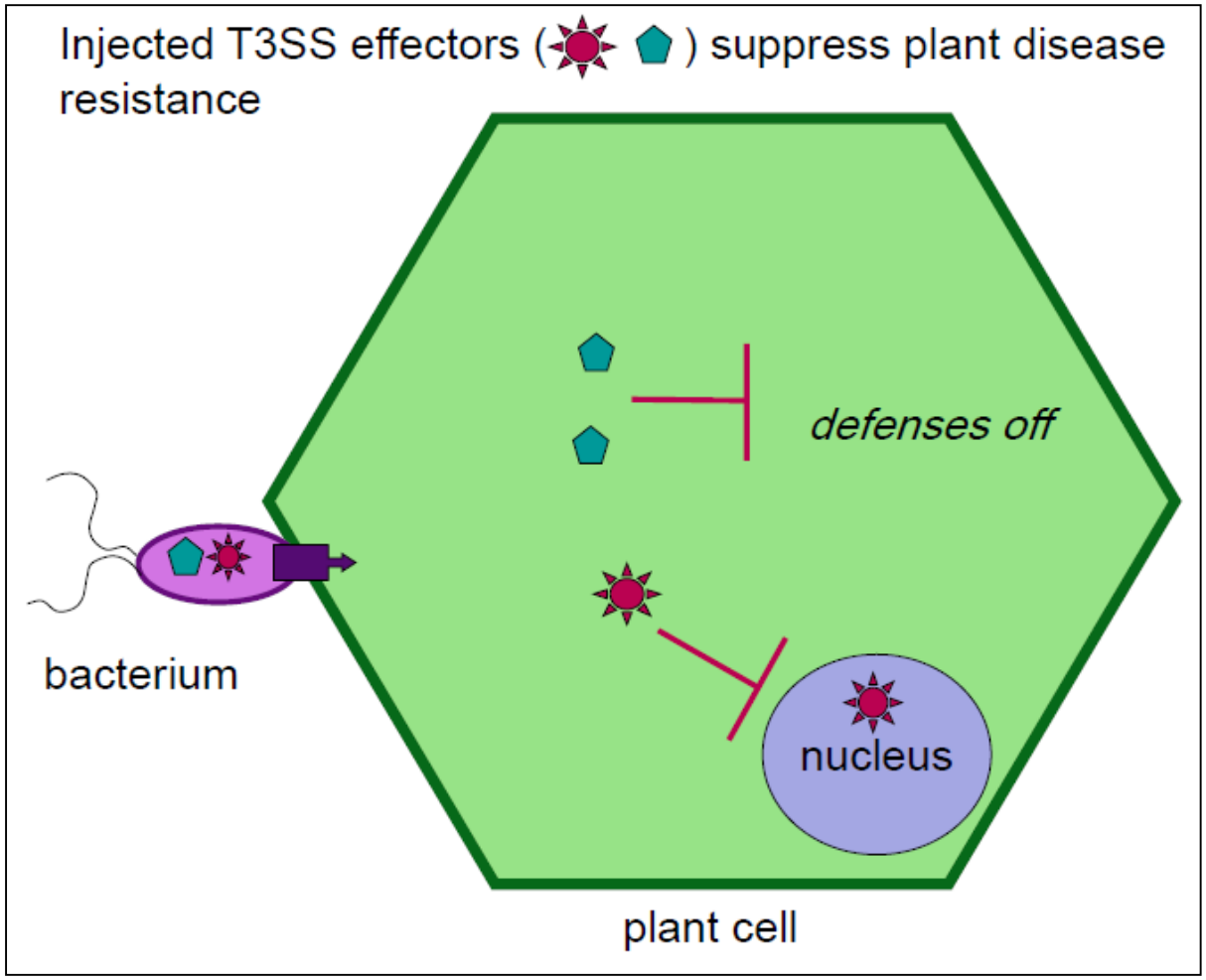
Functions of TSS effectors



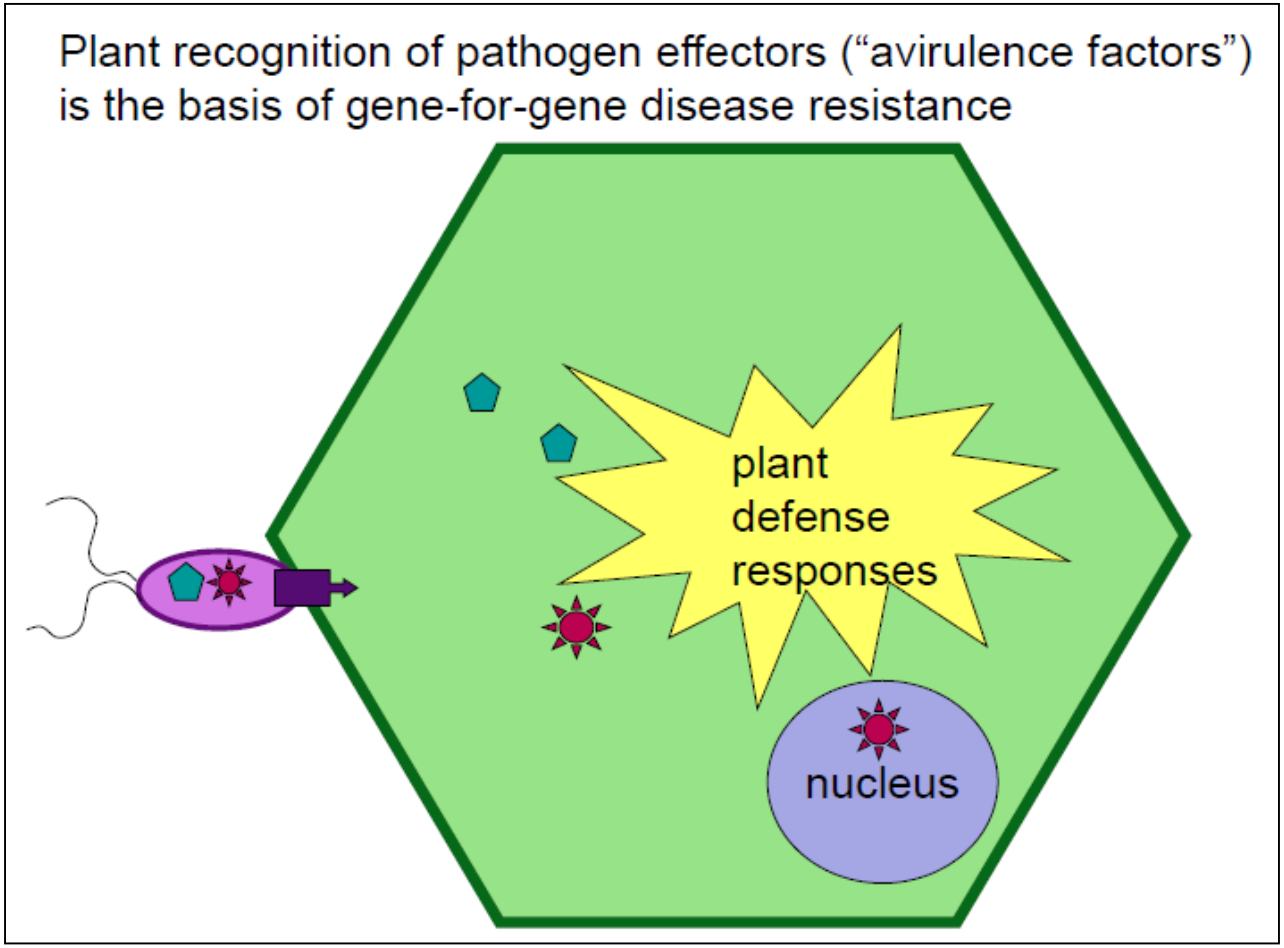
Bogdanove

Type III secretion system

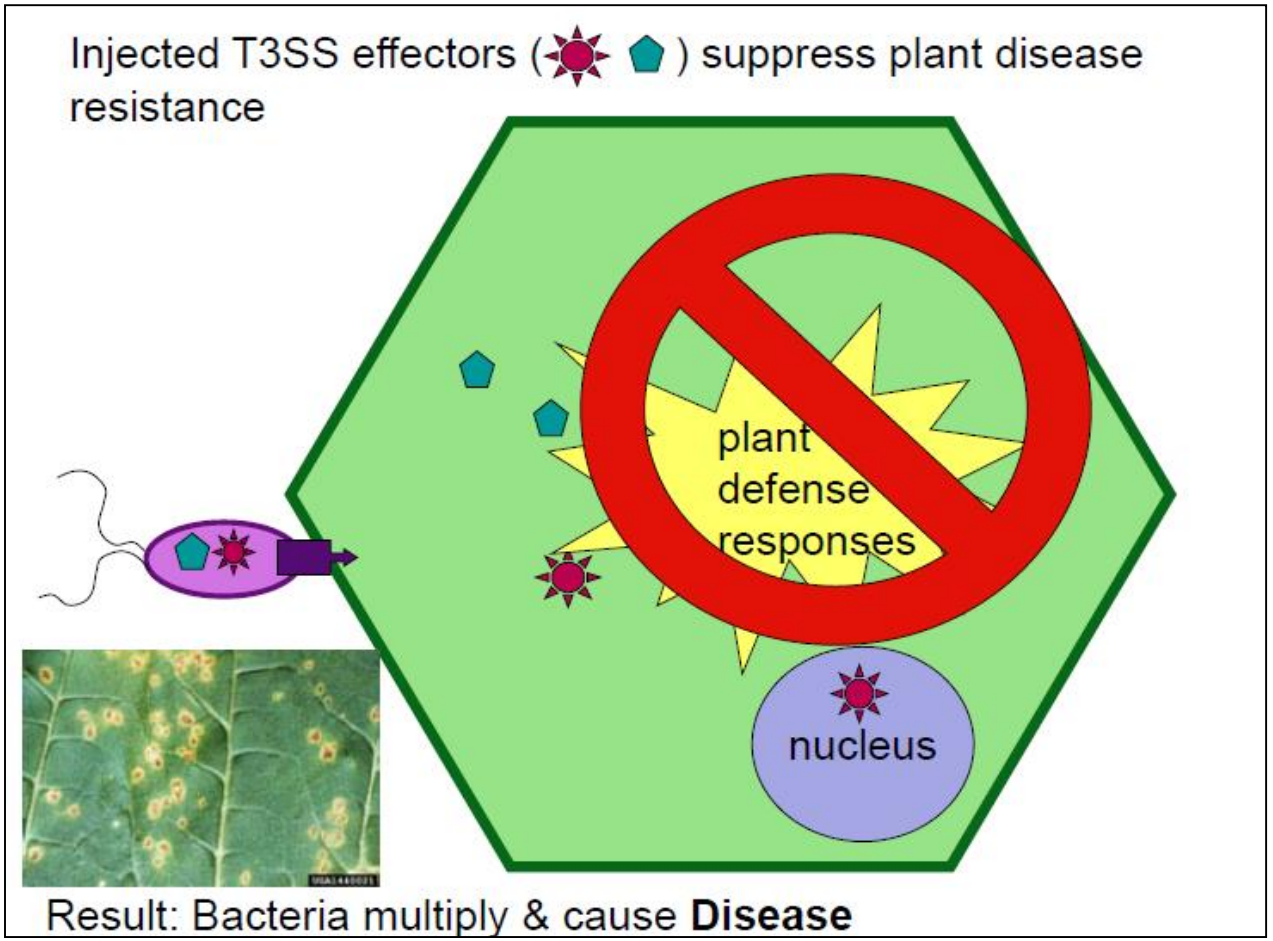
A syringe-like apparatus that injects proteins ("effectors") directly into host plant cells



T3SS
Gene for gene reaction

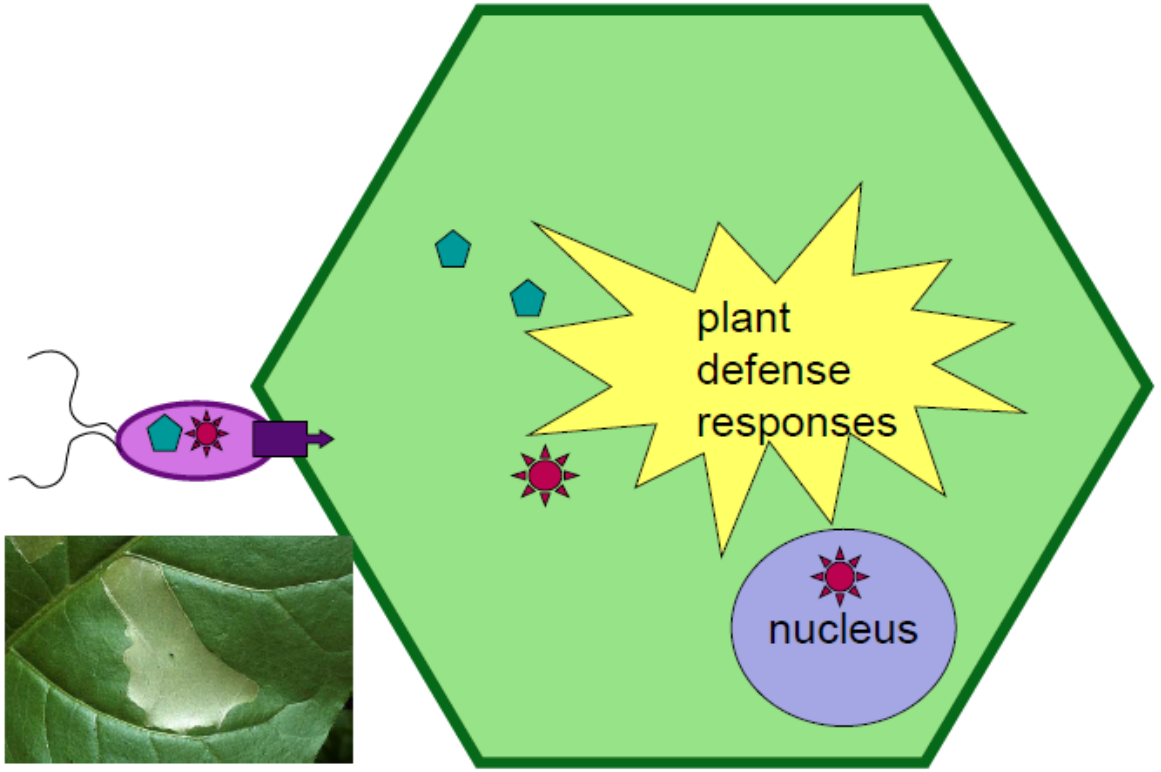


T3SS
Disease development



T3SS
HR reaction and disease resistance

BUT…plants can evolve to recognize injected T3SS effectors and launch plant disease resistance (often HR)



Result: Rapid plant cell death, bacteria can't multiply: **Resistance**

The Type III Secretion System

Approaches to the study of plant-phytopathogen interactions: *in vivo* and *in vitro* assay systems of phytobacterial pathogenesis

Rice-*Xanthomonas oryzae pv. oryzae* (*Xoo*) interactions

- Many *in vivo* and *in vitro* systems have been developed to study the plant-phytopathogen interactions to cause disease or resistance in plant and each system has its own merits and limits.

1. *In vivo* system is easy to monitor the effector translocation from phytopathogen to plant and has been used to study the resistance mechanism of plant like Hypersensitivity Response (HR).
2. *In vitro* system is useful to study the pathogenic mechanism of phytopathogen such as pathogenic gene expression.
3. Recently, new *in vitro* system was developed, which enables us to monitor the time-dependent gene expression of phytopathogen upon the interaction with host plant.

Kim *et al.*,2017

The Type III Secretion System

Approaches to the study of plant-phytopathogen interactions: *in vivo* and *in vitro* assay systems of phytobacterial pathogenesis

Rice-*Xanthomonas oryzae pv. oryzae* (*Xoo*) interactions

- *In vivo* and *in vitro* systems have been successfully developed to study plant-phytopathogen (rice-*Xoo*) interactions and each system has its own merits and limits.

1. The resistance mechanisms of host rice could be studied better with the *in vivo* systems,
2. Whereas for the pathogenic mechanism of phytopathogen the *in vitro* systems are generally more suitable.
3. Recently, a new *in vitro* system, mimicking both the *in vivo* and *in vitro* systems, was developed, which initiates and activates the *Xoo* pathogenicity by adding fresh rice leaf extracts into *Xoo* culture medium.

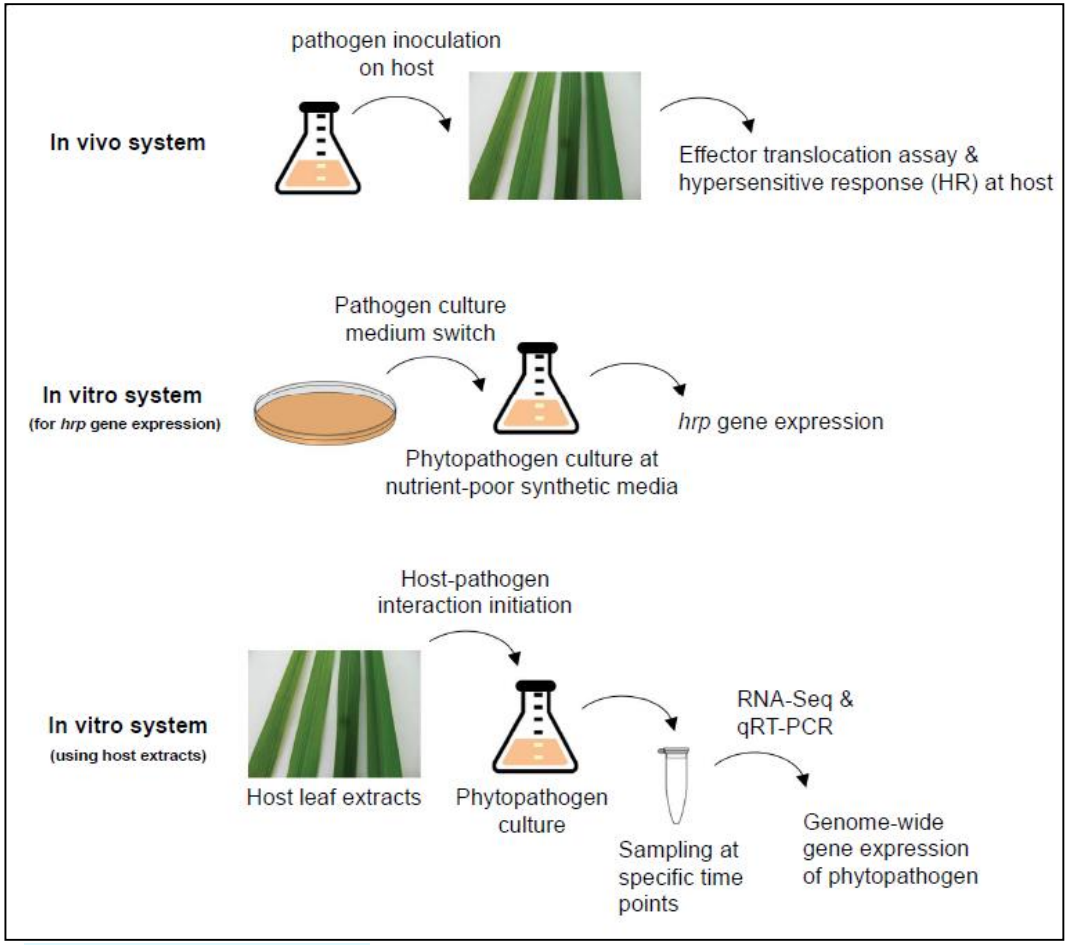
- The *in vitro* system was also combined with RNA-Seq to study the genome-wide gene expression of the phytopathogen *Xoo*.

Kim *et al.*,2017

The Type III Secretion System

Approaches to the study of plant-phytopathogen interactions: *in vivo* and *in vitro* assay systems of phytobacterial pathogenesis

Rice-*Xanthomonas oryzae* pv. *oryzae* (*Xoo*) interactions



The *in vitro* system was also combined with RNA-Seq to study the genome-wide gene expression of the phytopathogen *Xoo*.

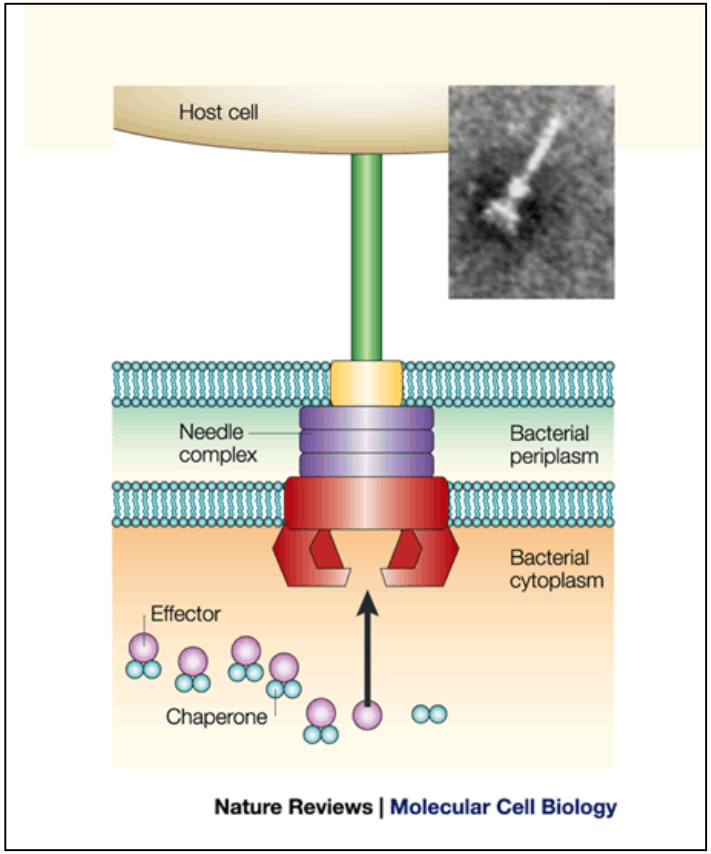
Kim *et al.*,2017

Type III secretion systems (T3SS)
Chaperones

- Chaperones as proteins and protein complexes bind to these straight (misfolded or unfolded) polypeptide chains and affect the folding processes of these chains.
- Chaperones are found in all types of cells and cellular compartments, and have a wide range of binding specificities and functional roles.

Type III secretion systems (T3SS)
Chaperones

- Schematic representation of the type III secretion needle complex used by Gram-negative plant and animal pathogenic micro-organisms.
- The chaperone effector molecules secreted into the host cells.

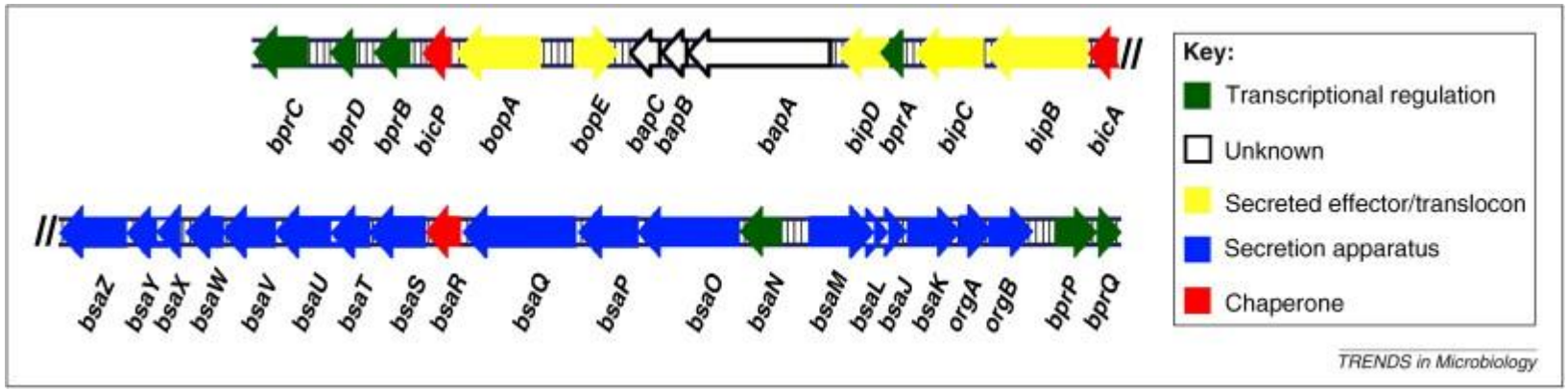


Naum *et al.*,2009;..

Type III secretion systems (T3SS)

Chaperones

Burkholderia pseudomallei



Sun and Gan,2010

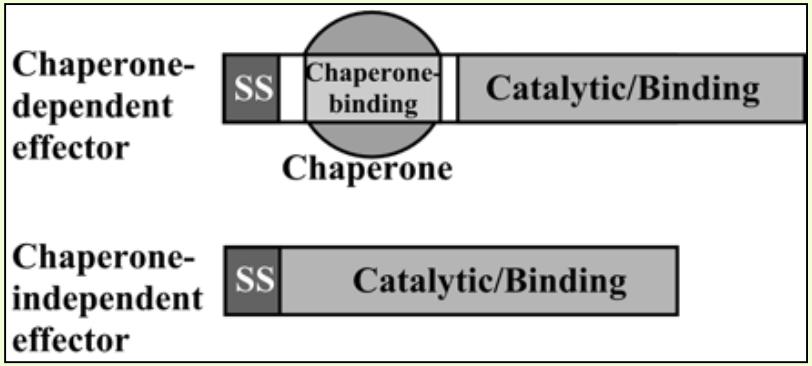
Chaperones

Proteins and protein complexes effects on protein folding

- All proteins emerges from the cell's ribosome are straight chains, but during assembly must be folded into a precise, three-dimensional shape (conformation) in order to perform their specific functions.
- Although only 20 to 30 percent of polypeptide chains require the assistance of a chaperone for correct folding under normal growth conditions, molecular chaperones are absolutely required for cell viability.

TTSS chaperones
Specificities and functional roles

- Physiologically significant translocation of many effectors depends on the action of TTSS chaperones, which generally bind to a chaperone-binding region that follows the secretion signal; the chaperone is shown as a circle.
- Effector activities, either catalytic or host cell target binding, are encoded by domains that usually follow the chaperone-binding region.
- Some effectors apparently have no cognate chaperones and are translocated independently of chaperone action.



TTSS chaperones

The role of specialized secretion in type III delivery

- It seems chaperones are as prevalent in bacterial plant pathogen type III systems.
- Recently, TTS chaperones have also been identified in other plant pathogenic bacteria.
- Chaperones help effectors compete for access to TTSSs.
- The identification of phytopathogen chaperones may reveal which effectors are translocated first into the plant cell and thus lead to a better understanding of the early stages of plant pathogenesis.

TTSS chaperones

Functions

1. To assist in the proper folding of the proteins during assembly;
2. To stabilize folded proteins;
3. To protect the effector protein from coming in contact with other proteins;
4. To facilitate the export of an effector protein secreted through a type III secretion system of bacteria;
5. Unfold them for translocation across membranes or for degradation.
6. In some cases to regulate the TTSS.

Chaperones
Structure

- Biochemically, the T3SS chaperones are:

1. Small acidic proteins,
2. Show no ATP binding or hydrolytic activity,
3. Possess no easily detectable sequence similarity to other proteins or other T3SS chaperones, and
4. Form highly stable dimers in solution.

Chaperones
Common classes

- TTSS chaperones have been separated into classes based on whether they interact with:

1. One effector, or
2. Multiple effectors.

Chaperones
Heat shock proteins (HSPs)

- Many chaperones are heat shock proteins, that is, proteins expressed in response to elevated temperatures or other cellular stresses.
- The reason for this behavior is that protein folding is severely affected by heat and, therefore, some chaperones act to repair the potential damage caused by misfolding.

Common chaperone systems
Hsp60, Hsp70 and Hsp90

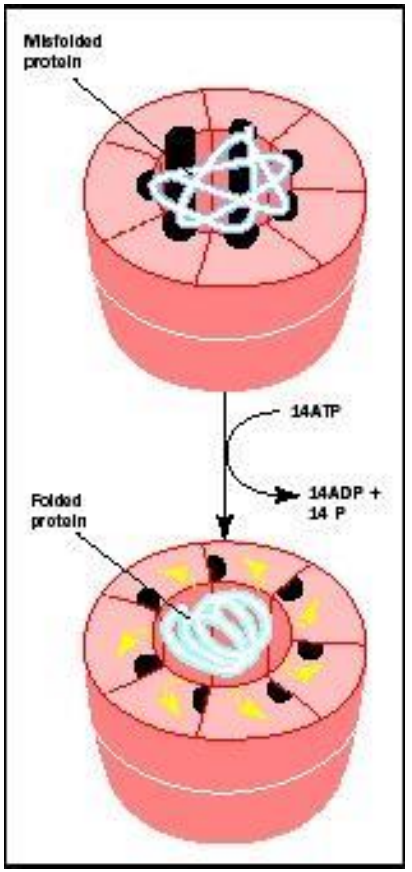
- Hsp70 chaperones (so called because their size is approximately 70,000 daltons, or atomic mass units) are a very large family of proteins whose amino acid sequences are very similar.
- Hsp60 chaperones (also called "chaperonins") are barrel-shaped structures composed of 14 to 16 subunits of proteins that are approximately 60,000 daltons in size.
- A single cell or cellular compartment may contain multiple Hsp70 chaperones, each with a specific function.
- Some of the well-studied Hsp70 chaperones include DnaK from the bacterium *Escherichia coli*.

All molecular chaperones perform the same function, but structurally they are divided into groups of chaperones and chaperonins.

Chaperones

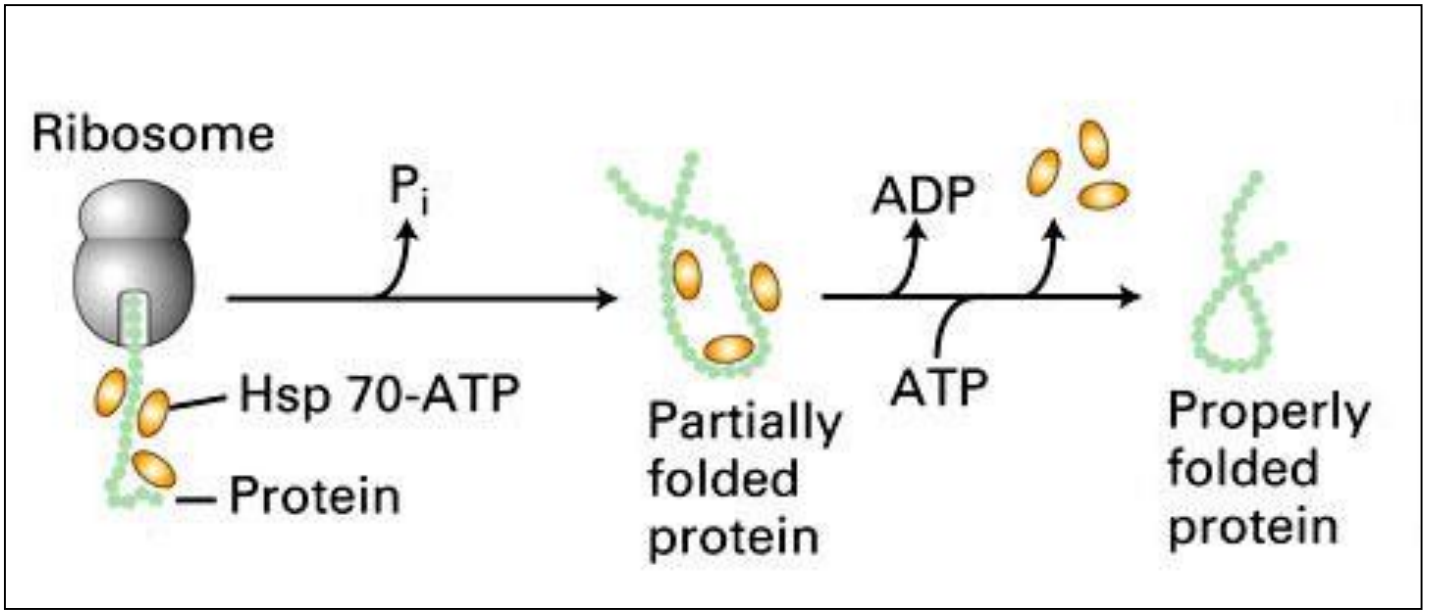
Help misfolded proteins refold properly

- **Molecular Chaperones,** are families of proteins to help "properly fold" a new protein under temperature elevation (25° --> 32° C) cells make heat shock proteins (HSPs).
- To refold properly, chaperones use energy from ATP.



Chaperones

To refold properly, chaperones use energy from ATP

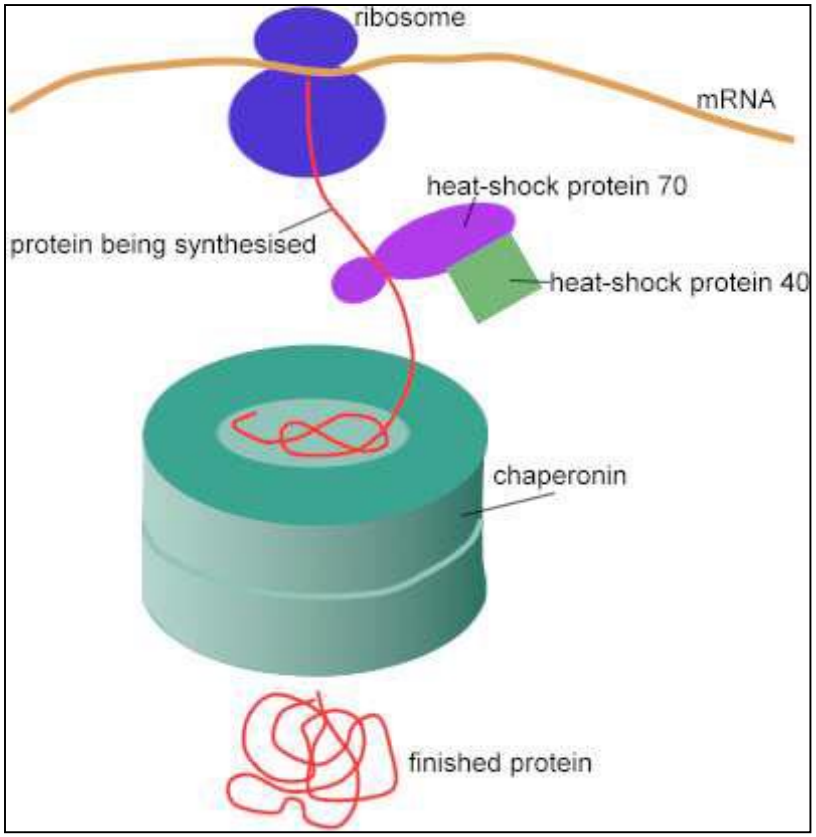


Chaperones are proteins whose function is to assist other proteins in achieving proper folding.

Chaperones

Heat-shock proteins and chaperonins cooperate to fold newly synthesized proteins

- The correct three-dimensional conformation is essential for protein function.
- Hsp60 chaperones are also called chaperonins.



TTSS chaperones

Most common TTSS chaperones in phytobacteria *Erwinia amylovora* and *Pseudomonas syringae*

- TTS chaperones have been identified in plant pathogenic bacteria:

1. *Erwinia amylovora* (DspA/E-DspB/F):

- DspA/E is a type III effector of *Erwinia amylovora*.
- DspB/F is the chaperone of DspA/E.
- *dspB/F* and *dspA/E* together constitute the *dspEF* locus.

2. *Pseudomonas syringae* (HopPsyA-ShcA):

- ShcA acting as a chaperone for HopPsyA.
- The ShcA protein is a molecular chaperone that assists in the secretion of the HopPsyA effector from TTSS of *Pseudomonas syringae*.

TTSS chaperones

Most common TTSS chaperones in phytobacteria *P. syringae, R. solanacearum, X. campestris*

- Besides chaperone ShcA, several other candidate chaperones have been identified in *P. syringae*.
- Three of these candidate chaperones:
- ShcM, ShcV, and ShcF, act as chaperones for the effectors HopPtoM, HopPtoV, and HopPtoF, respectively.
- TTSS chaperones have yet to be demonstrated in:

1. *R. solanacearum*
2. *X. campestris*
3. *X. axonopodis*

Hop (Hrp outer protein): The term "Hop" applies generically to expressed proteins that are secreted or translocated by the TTSS of *P. syringae* and related plant pathogens (e.g. *Erwinia* and *Pantoea* spp.).

TTSS chaperones

Chaperone binding motif in *E. amylovora*

Binding of virulence factors to bacterial secretion chaperones

- Chaperones generally bind to the N-terminal regions of effectors.
- In *Erwinia amylovora* the chaperones bind at the C terminus of DspA/E.
- We identified two DspB/F-binding motifs that are present in the first 210 amino acids of the N terminus of DspA/E.
- We also found that the first 210 amino acids of the N terminus of DspA/E is important for its secretion.

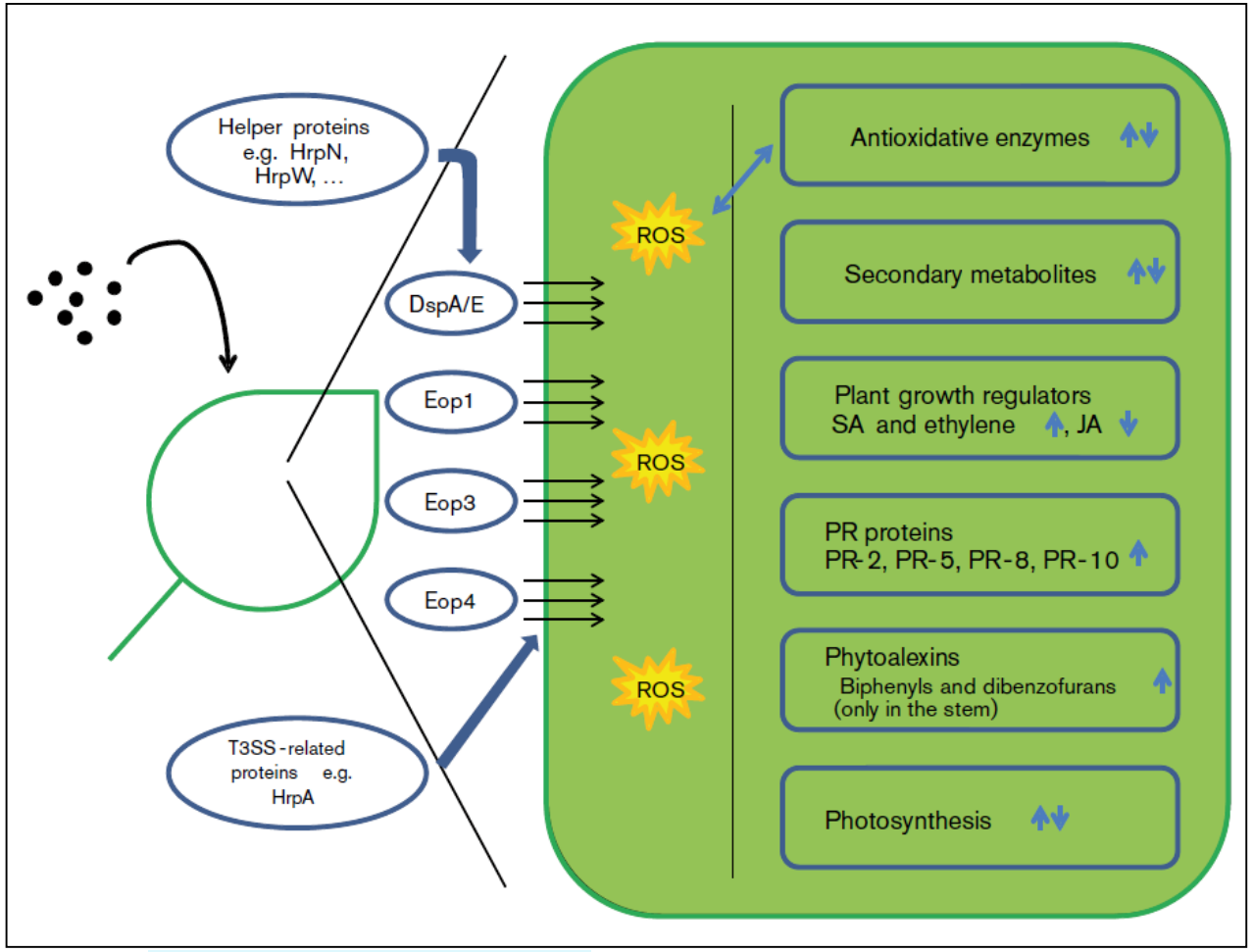
Oh *et al.*,2010

TTSS chaperones

Chaperone binding motif in *E. amylovora*

Binding of virulence factors to bacterial secretion chaperones

Infection by *E. amylovora* causes the effector-related proteins DspA/E, Eop1, Eop3 and Eop4 to be secreted in the plant tissue, together with helper proteins and T3SS-related proteins. The result is an increase in ROS and different mechanisms to be triggered.



Vrancken *et al.*,2013

TTSS chaperones

Chaperone binding motif in *E. amylovora*

Binding of virulence factors to bacterial secretion chaperones

- Translocation, secretion, and interaction with DspB/F of the N-terminal region of DspA/E.
- Translocation was performed by infiltrating *N. tabacum* with *E. coli* strain MC4100 containing pCPP431.
- Secretion was determined by the method of Ham et al. (1998) and immunoblotting using an anti-AvrRpt2 antibody (+, secretion).
- Interaction between N-terminal DspA/E and DspB/F in yeast was determined by blue colour on X-Gal medium and growth on SD quadruple-dropout medium (++, strong interaction; +, weak interaction; 2, no interaction).

N-terminal length of DspA/E (aa)	Translocation*		Secretion†		DspB/F interaction in yeast‡
	+DspB/F	−DspB/F	+DspB/F	−DspB/F	
35	−	−	+	+	−
50	−	−	+	+	−
70	−	−	+	+	+
109	++	+/−	+	+	++
152	+	ND	+	ND	ND
210	+	ND	+	ND	++

Oh *et al.*,2010

Type IV secretion system

Three types of T4SS have been described

Versatility and diversity in function

- It is capable of transporting both DNA and proteins.
- It was discovered in *Agrobacterium tumefaciens*.
- Type IV secretion (T4S) systems are widely distributed among:

1. Many species of Gram-negative and Gram-positive bacteria,
2. Wall-less bacteria, and
3. The Archaea.

Alvarez-Martinez and Christie,2009

Type IV secretion system
Examples

- Type IV secretion system(T4SSs) have been described for many Gram-negative plant bacterial pathogens:

1. *P. syringae*
2. *Xylella fastisiosa*
3. *Xanthomonas* spp. (e.g. *X. vesicatoria*).

- *P. syringae* pathovars use this system to mediate attachment of various pathovars to the leaves of both susceptible and non-susceptible plants.
- Type IV mutant strains exhibit reduced aggregation in the laboratory, but still retain wild type virulence toward their tomato host.

Seubert *et al.*,2004;..

Type IV secretion system
Versatility and diversity in function

- T4SSs permits the movement of big molecules such as:

1. DNA (horizontal gene transfer), or
2. Protein-DNA complexes,
3. Proteins e.g. toxin proteins

- Peptide secreted through this pathway is exported through the cytoplasmic membrane by the General secretory pathway, GSP(Sec).
- Gram-negative bacterial pathogens also use this system to spread plasmids harboring antibiotic resistance genes.

One exception for type IV: Gram-negative *Bordetella pertussis*, causes whooping cough secretes the pertussis toxin via sec-system(two steps) and through the type IV system.

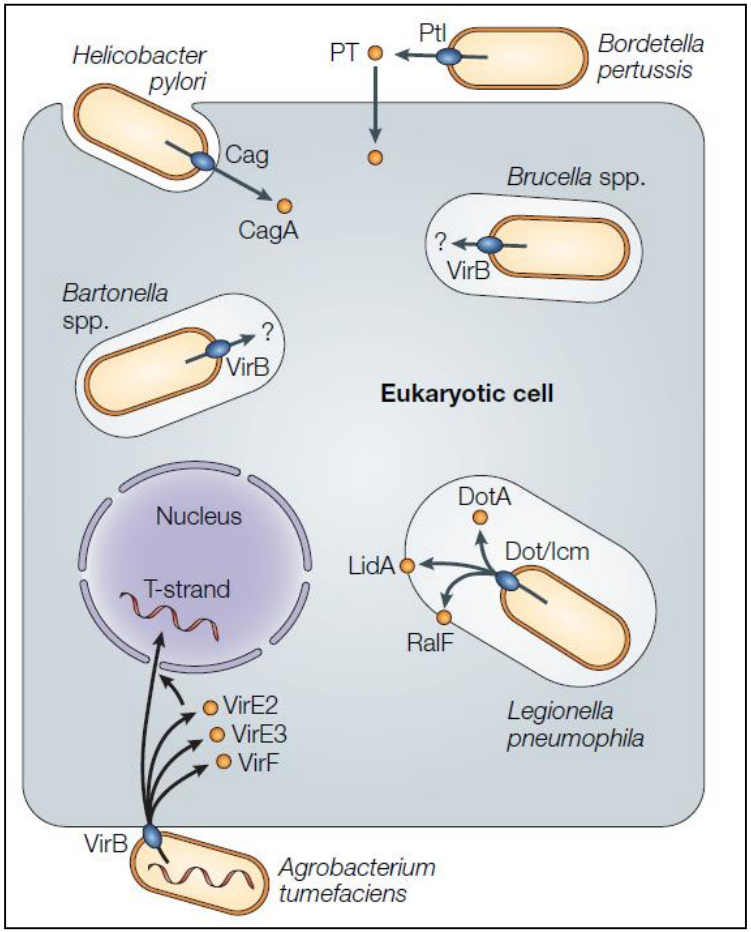
Type IV secretion system
Versatility and diversity in function

Type IV secretion system	Examples
1. Contact-dependent delivery of DNA to eukaryotic or bacterial cells (Type IVA)	*A. tumefaciens* - spread of conjugative plasmids.
2. Contact-dependent delivery of effector proteins into eukaryotic cells (Type IVB)	injection of virulence factors into the host cytoplasm.
3. Export DNA to external environment	Gram-ve *Neisseria gonorrhoeae* T4SS.
4. Import DNA from external environment	Gram-ve *Helicobacter pylori* competence system.

Helicobacter pylori causes chronic inflammation in the stomach and is a common cause of ulcers. *Neisseria gonorrhoeae* causes the sexually transmitted disease (STD) gonorrhea and *Legionella pneumophila* causes lung inflammation.

Type IV secretion system
Versatility and diversity in function

1. *A. tumefaciens* - spread of conjugative plasmids.
2. *Bordetella pertussis*, secretes the pertussis toxin via sec-system and through the type IV system.
3. *Helicobacter pylori* import DNA from external environment.



Cascales and Christie,2003; Mark Pallen

Type IV systems

Agrobacterium tumefaciens vir-encoded type IV secretion system

Infection process

- Some of these factors mediate the generation of a single-stranded copy of T-DNA (T-strand) and its transport into the host cell through a type IV secretion system.
- In addition to the T-strand, several Vir proteins are also translocated into plant cells.
- These exported effectors, together with multiple host factors, facilitate the nuclear import of the T-strand and its subsequent integration into the host genome.
- Finally, genes involved in auxin and cytokinin biosynthesis are expressed from the integrated T-DNA, leading to abnormal cell proliferation in the infected tissues and the formation of tumours, i.e. crown galls.

Mansfield *et al.*,2012

Type IV secretion system

Agrobacterium tumefaciens vir-encoded type IV secretion system

Structure

- The *A. tumefaciens* VirB/D4 system is composed of 12 proteins.

1. 11 proteins named VirB1-11 and encoded by the virB operon; and
2. At least 1 protein, VirD4, encoded by the virD operon.

- Vir D4: DNA binding-may function in DNA transfer.

Seubert *et al.*,2004;..

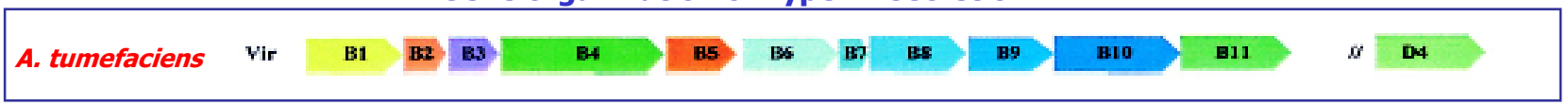
Type IV secretion system

Agrobacterium tumefaciens vir-encoded type IV secretion system

Structure

- Used in export of protein complexes/DNA.
- Can translocate directly into host cell.
- Show homology to pilus-mediated conjugal transfer systems.
- Sec-like dependent translocation into periplasm.
- Vir B11: related to ATP-ases of type II system.
- Vir D4: DNA binding - may function in DNA transfer.
- Virs B6, B7, B8 B9, B10: are core periplasmic components.
- VirB2 and VirB5 are pilus components and built up T-pilus.

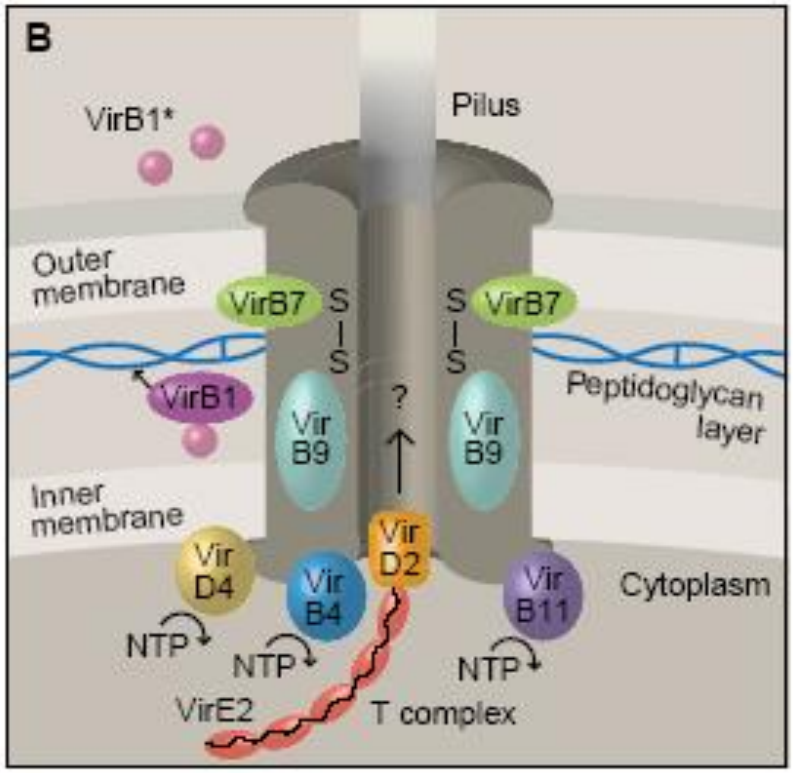
Gene organization of Type IV secretion

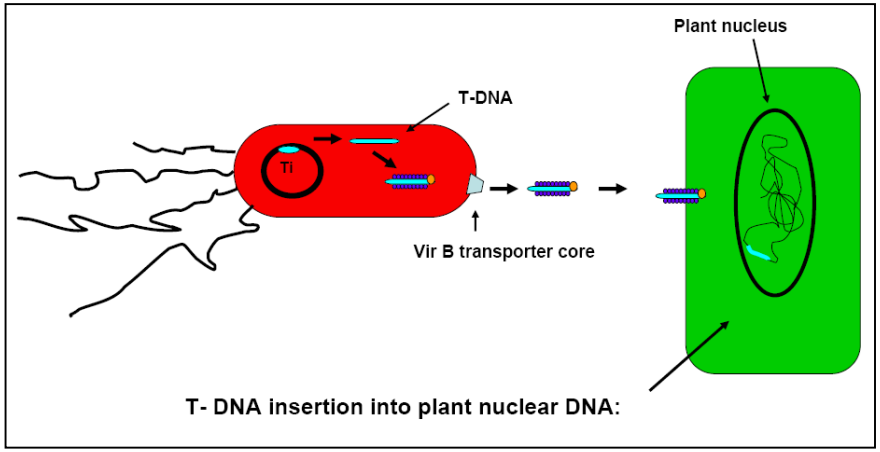


The Type IV systems

Agrobacterium tumefaciens vir-encoded type IV secretion system

Vir D4, DNA binding may function in DNA transfer



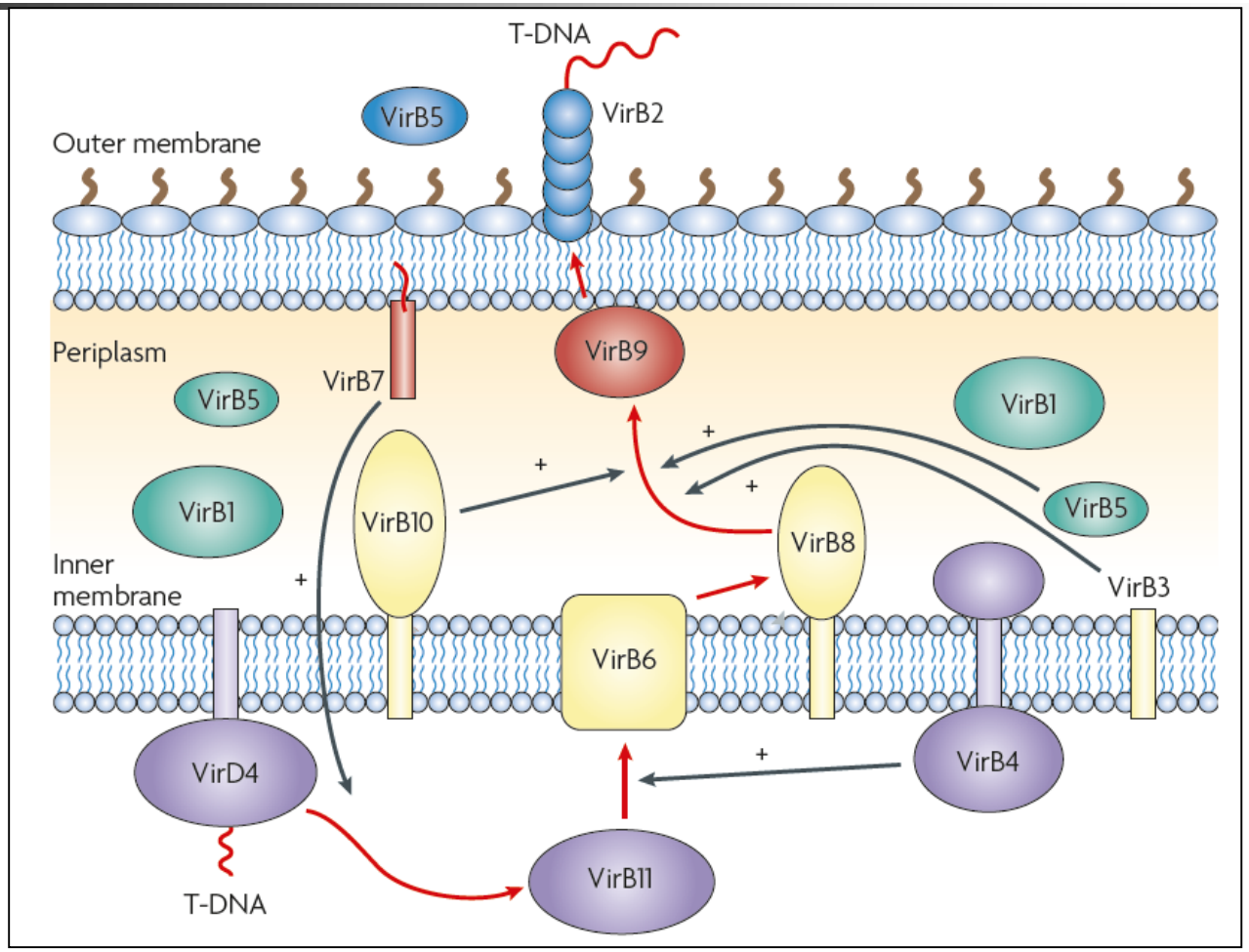


Vir D4, DNA binding may function in DNA transfer. *Agrobacterium* also translocates its F-box effector(VirF) into plant to hijack the host SCF complex (plant F-box protein) in order to facilitate bacterial infection. virF, is the host-range-determining virulence gene of *A. tumefaciens* (See also F-box section).

Type IV systems

Agrobacterium tumefaciens vir-encoded type IV secretion system

Vir D4, DNA binding may function in DNA transfer



Fronzes *et al.*,2009

Type V Protein secretion system

Simplest protein secretion system

In plant pathogenic bacteria

- Most of the T5SS secreted proteins characterized to date contribute to the virulence of animal or human pathogens.
- But also found in:

1. *Pectobacterium* spp.
2. *Xanthomonas axonopodis* pv. *citri*
3. *X. campestris* pv. *campestris*
4. *Dickeya* (*dadantii* and *chrysanthemi*)
5. *Xylella fastidiosa*
6. *R. solanacearum*.

- *P. syringae* contains the highest number of autotransporter-like proteins, with 9 candidate proteins.

Preston *et al.*,2005;..

Type V secretion system

Autotransporter system(AT)

Structure

- Autotransporter pathway is probably the simplest protein secretion system since all the necessary secretion components are included in one polypeptide.
- The autotransporter pathway is produced as a single polypeptide with two domains:

1. The C terminal (β-domain or helper domain) of the protein forms a pore in the outer membrane (a β-barrel structure).
2. The passenger or N-terminal (α-domain or extracellular domain) is released to the extracellular environment.

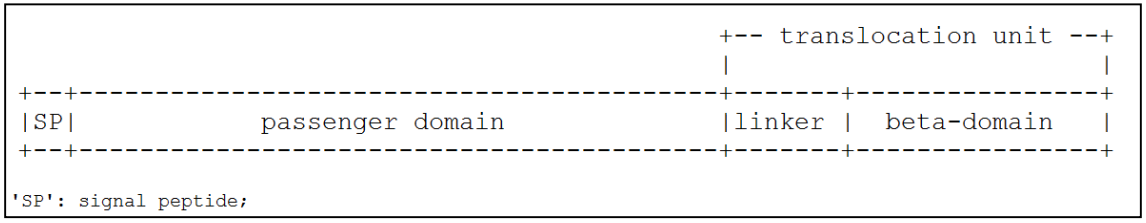
Type V secretion system
Autotransporter system (AT)
Structure

- It is one of the simplest secretion mechanisms, and is widely distributed, especially in pathogenic bacteria.
- Autotransporter (AT) proteins consisting of

1. an N-terminal signal peptide (SP)
2. a passenger domain
3. a C-terminal module called the "translocation unit".

- Translocation unit consists of:

1. a short linker region with an α-helical secondary structure,
2. a β-domain of 250 to 300 amino acid residues.



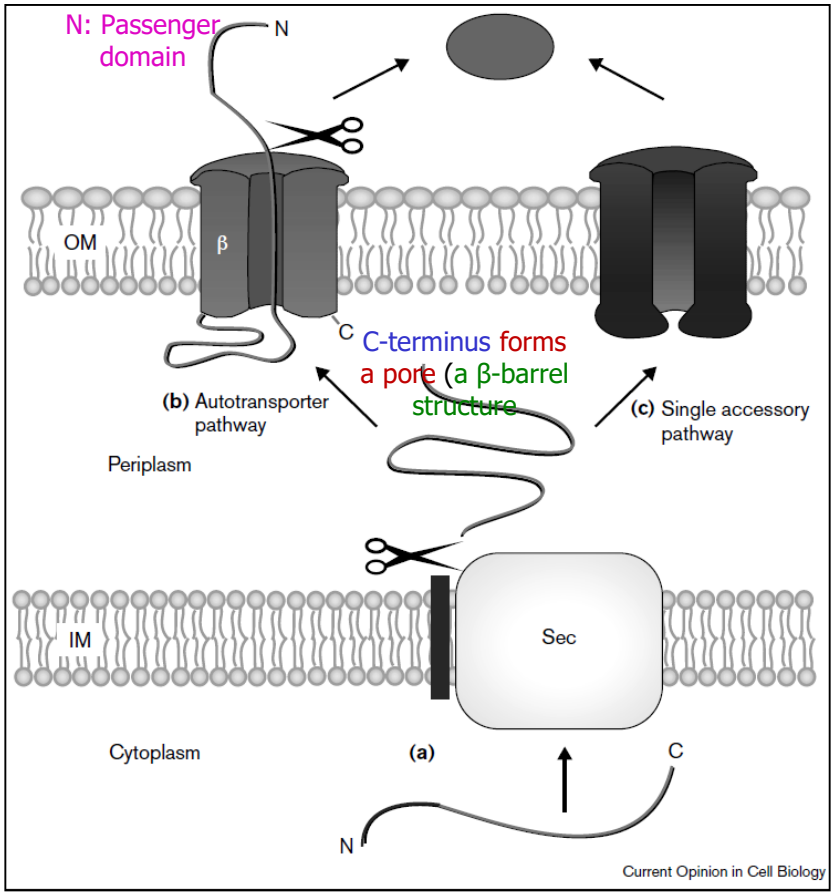
PROSITE documentation PDOC51208

Type V protein secretion system

Models for the autotransporter and single accessory pathways

- Many autotransporter proteins undergo a proteolytic cleavage in the course of secretion, which results in their separation into two polypeptides:

1. the β-domain in the outer membrane (forms a pore in OM), and
2. the passenger protein exposed at the surface or released into the milieu.



Thanassi and Hultgren,2000;..

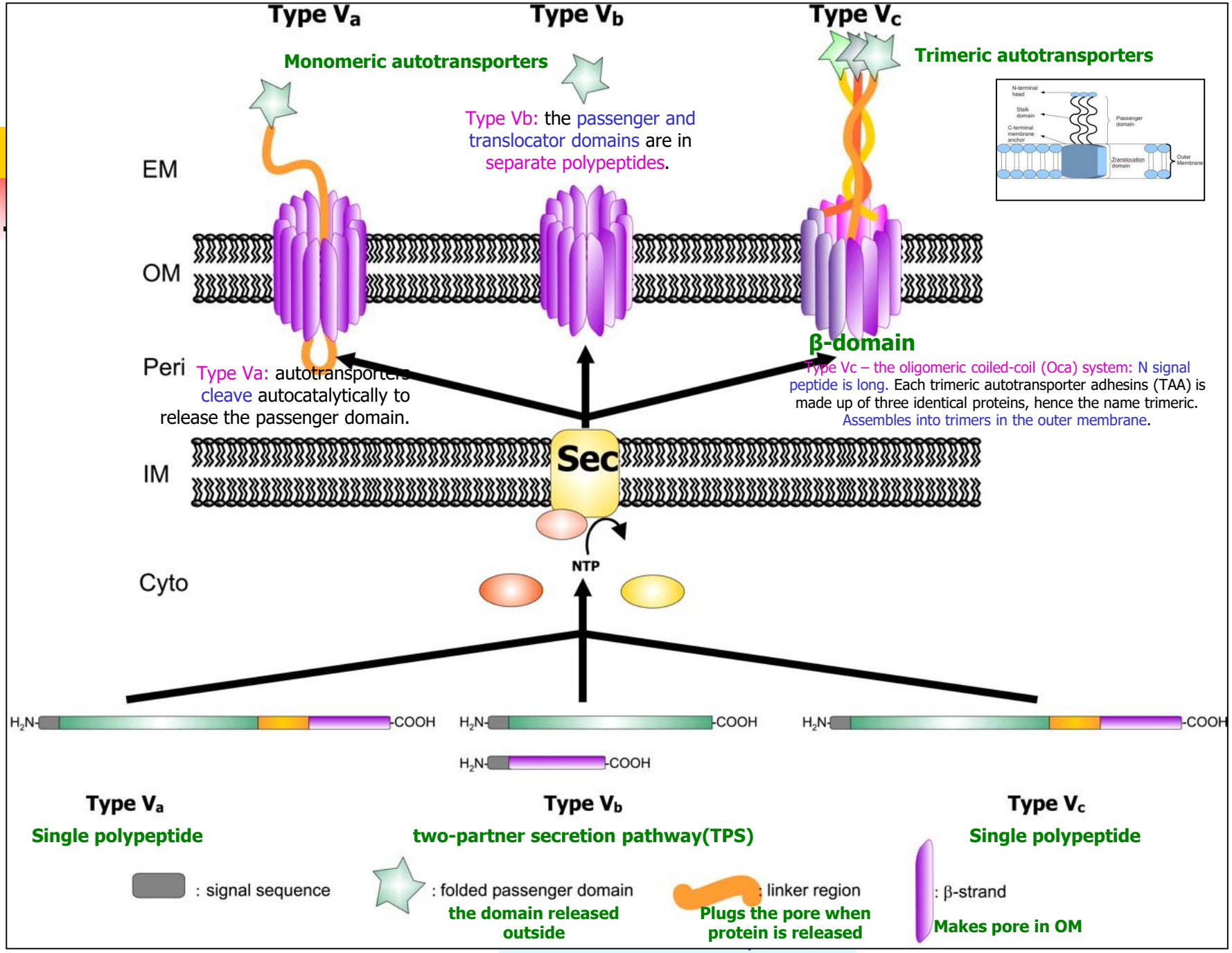
Type V protein secretion system
Five sub-classes of type V secretion

- There are five (sub-) classes in type V secretion pathways:

1. Type Va or AT-1: the classical monomeric autotransporters;
2. Type Vb: two-partner secretion systems (TPS);
3. Type Vc or AT-2: trimeric autotransporters;
4. Type Vd: passenger domains can be fused to their transport (β) domains in type Vd autotransporters;
5. Type Ve: Autotransport proceeds from the N- to the C-terminus in type Ve autotransporters.

Most autotransporters export numerous domains to the cell surface, leading to multi-domain passenger 'domains'.

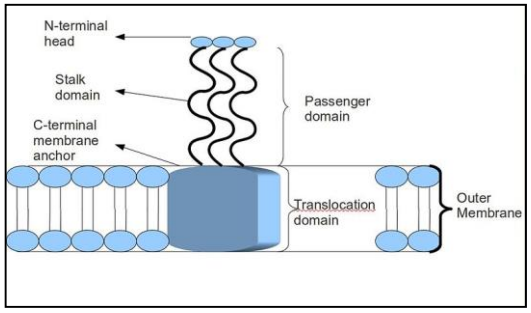
Leo *et al.*,2011; van ulsen *et al.*,2014


Type Va
Type Vb
Type Vc
Monomeric autotransporters
Trimeric autotransporters
Type Vb: the passenger and translocator domains are in separate polypeptides.
N-terminal head
Stalk domain
C-terminal membrane anchor
Passenger domain
Translocation domain
Outer Membrane
EM
OM
Peri
IM
Cyto
Type Va: autotransporters cleave autocatalytically to release the passenger domain.
β-domain
Type Vc – the oligomeric coiled-coil (Oca) system: N signal peptide is long. Each trimeric autotransporter adhesins (TAA) is made up of three identical proteins, hence the name trimeric. Assembles into trimers in the outer membrane.
Sec
NTP
H2N-
-COOH
Type Va
Single polypeptide
Type Vb
two-partner secretion pathway(TPS)
Type Vc
Single polypeptide
: signal sequence
: folded passenger domain
the domain released outside
: linker region
Plugs the pore when protein is released
: β-strand
Makes pore in OM

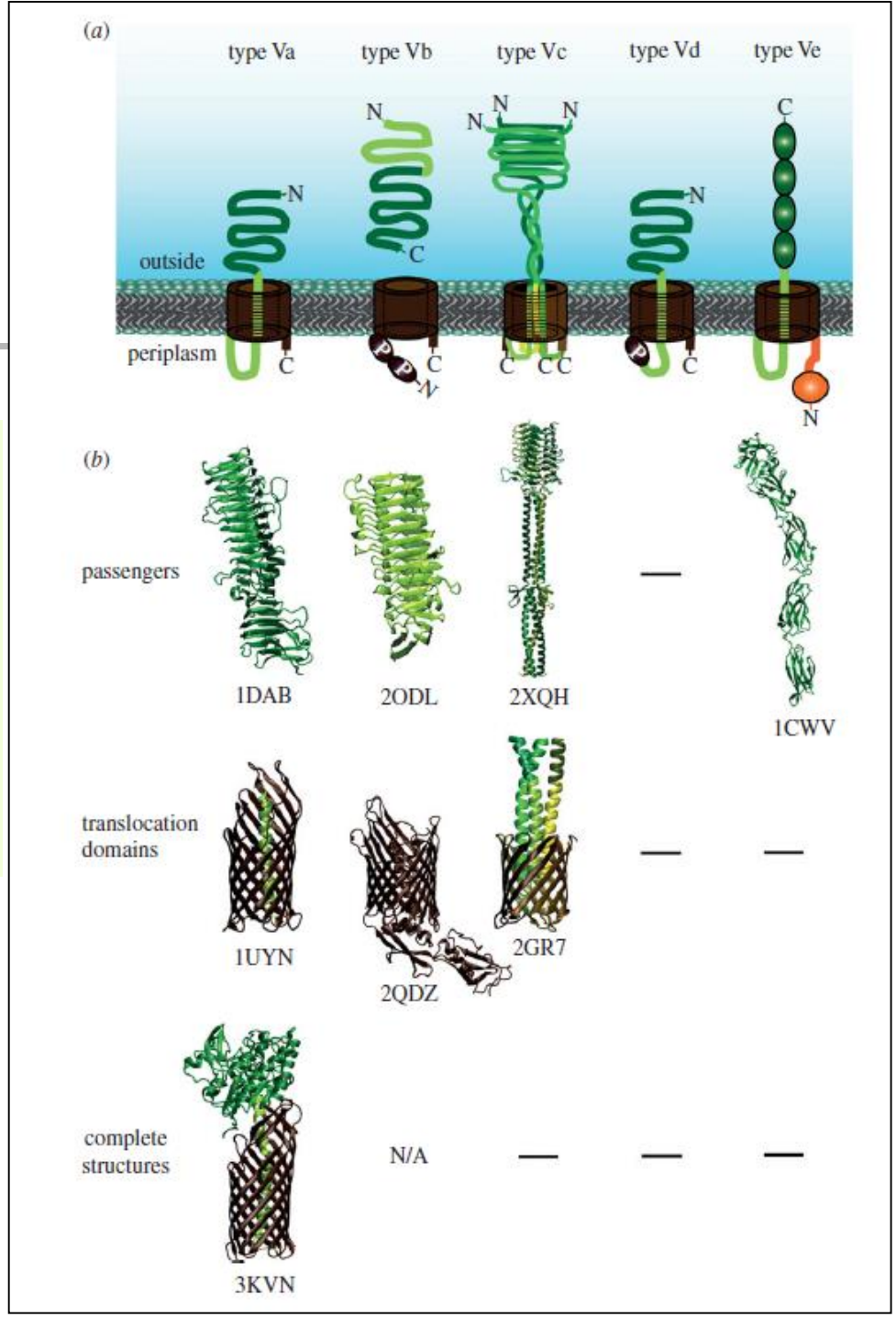
Schematic overview of the type V secretion systems

1. The translocation domain (β-domain) is displayed in brown (type Va),
2. linker/Tps regions in light green (AT-2),
3. passenger domains in dark green.

TPS: two-partner secretion systems



Leo *et al.*,2011



Type V protein secretion system
Functions

- Several of type V proteins play essential roles in the pathogenesis of bacterial infections.
- Most of the effector proteins released are involved in:

1. Adherence,
2. Invasion, and
3. Degradation.

- Also:

4. To condense host cell actin, and
5. To modulate apoptosis.

- Autotransporter proteins can function as proteases, adhesins, toxins and mucinases that aid Gram-negative bacteria in pathogenesis.

Preston *et al.*,2005; Rodou *et al.*,2010;..

Type V Protein secretion system
Autotransporter system(AT)
Functions

- In Gram-ve bacteria, the virulence factors associated with T5SS passenger are numerous including:

1. Enzymatic activity (protease, peptidase, lipase and esterase);
2. mediating actin promoted bacterial motility;
3. acting as adhesins,
4. immunomodulatory proteins,
5. Toxins
6. cytotoxins,
7. Biofilm formation,
8. permitting the maturation of other virulence proteins.

PROSITE documentation PDOC51208

Type V Protein secretion system

Antifreeze bacteria

Pseudomonas putida

- *Pseudomonas putida*
- It was found that the secreted antifreeze protein from *P. putida* GR12-2 lacks a conserved canonical N-terminal signal peptide.
- However, they speculate secretion may occur using either:

1. a hemolysin-like secretion, or
2. type V autotransportation system.

Hemolysins or haemolysins are lipids and proteins that cause lysis of red blood cells by destroying their cell membrane.

Lorv *et al.*,2014

Type VI secretion system

Fluorescent *Pseudomonas* and *P. atrosepticum*

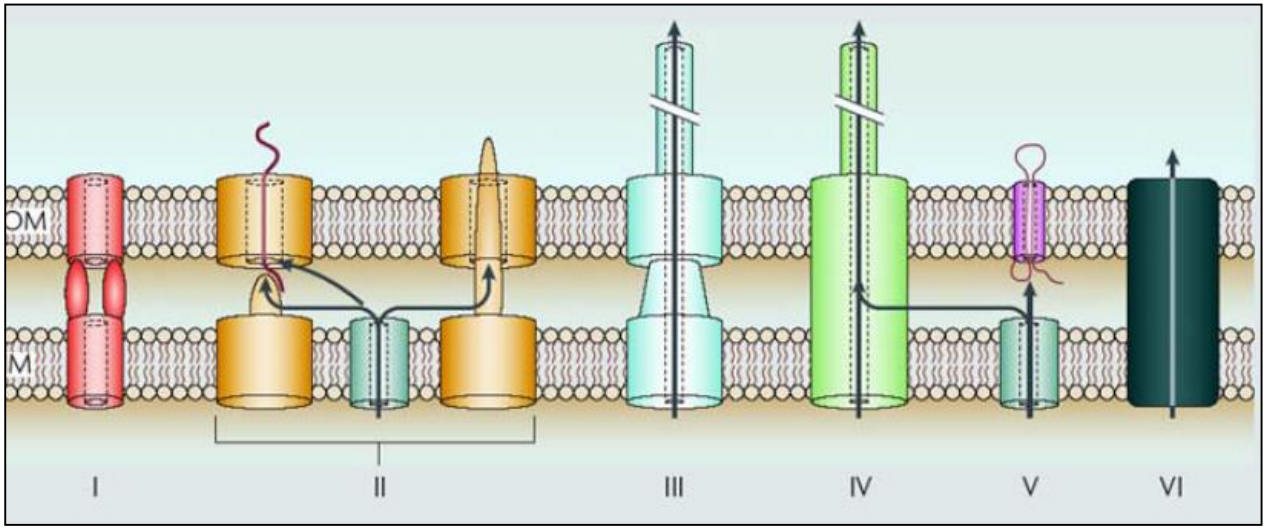
- Type VI secretion systems have been found in most genomes of Gram-negative bacteria, including:

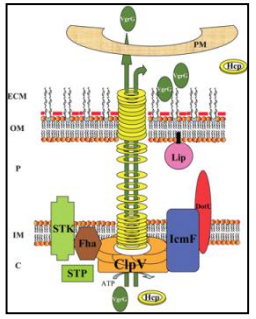
1. Plant pathogens (*Pseudomonas syringae*; *P. atrosepticum*; *Agrobacterium tumefaciens* and *X. oryzae*),
2. Animal, human pathogens;
3. Soil, environmental or marine bacteria.

- Like the type 4 secretion system (T4SS), T6SS can injects effector molecules directly into host cells through a channel.

Schematic representation of a T6SS
Hcp and VgrG proteins

- T6SS protein secretion system found in both pathogenic and non-pathogenic Gram-negative bacteria.
- Key virulence factor in some important pathogenic bacteria.
- Injects effector molecules directly into host cells through a channel.





T6SS

Abdallah *et al.*,2008; Bryndis Bjornsdottir

Type VI secretion system
Structure

- The Type VI secretion system gene clusters contain from 15 to more than 20 genes.
- Two of which, have been shown to be nearly universally secreted substrates of the system.

1. Hcp (haemolysin coregulated protein), and
2. VgrG (valine-glycine repeat G).

Haemolysin: any substance that causes lysis.

Type VI secretion system

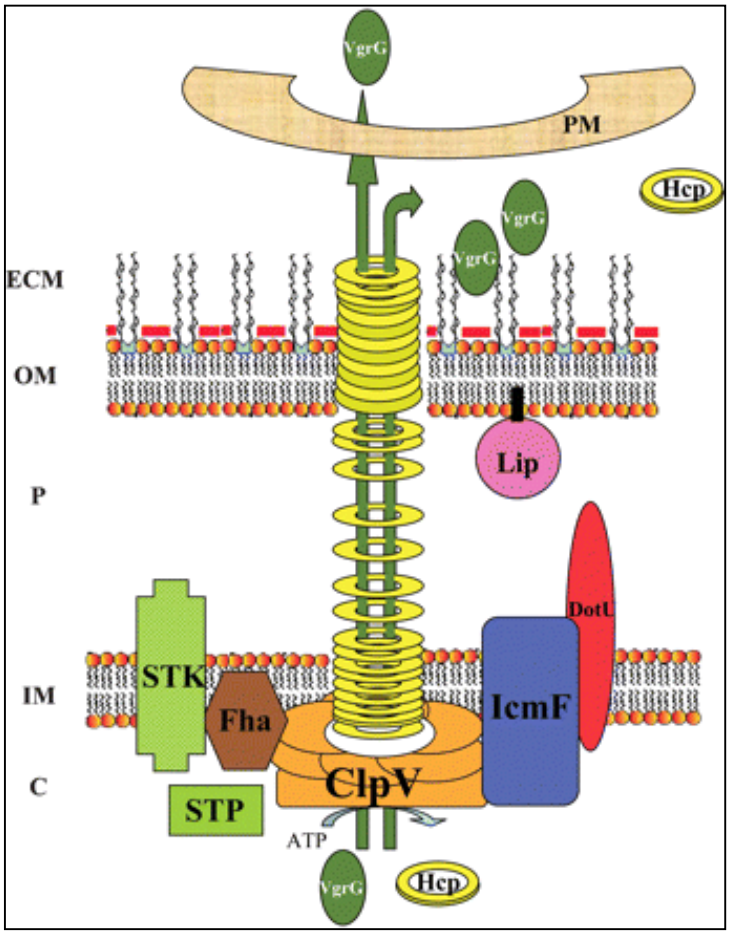
Schematic representation of a T6SS

The two effectors: Hcp and VgrG proteins

- Hcp and VgrG proteins secreted through T6SS.

1. Hcp (haemolysin co-regulated protein);
2. VgrG (valine-glycine repeat protein G)

- Many bacteria contain more than one T6SS.
- Several T6SS subgroups exist.



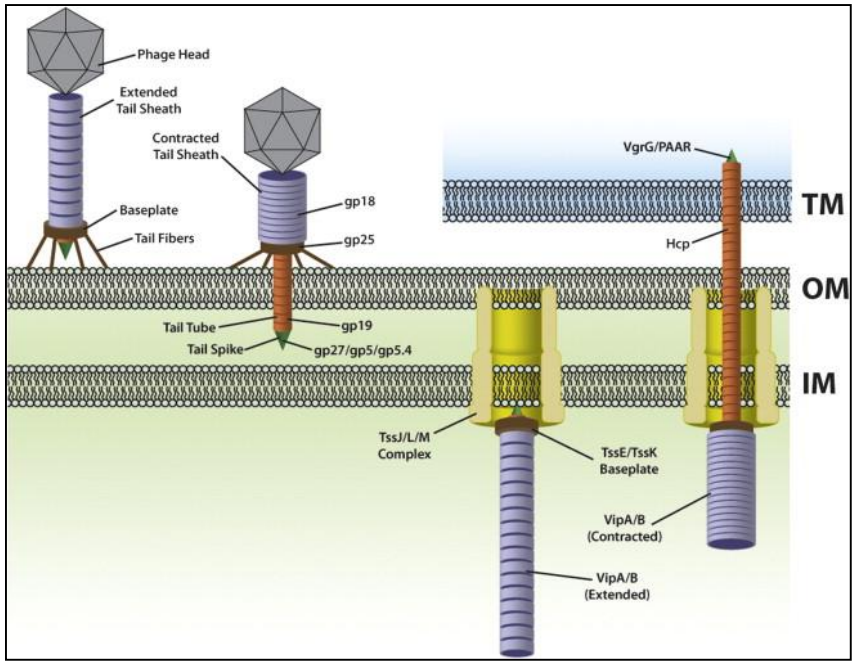
Haemolysin: any substance that causes lysis.

Type VI secretion system

Similarity with the bacteriophage tails

Hcp and VgrG proteins

- The structure of the type VI secretion systems presents a significant similarity with the bacteriolphage tails which inject their effector proteins.
- VgrG proteins show significant structural homology to a complex called $(gp27)_3$-$(gp5)_3$, which corresponds to the tail spike or needle of the T4 phage.

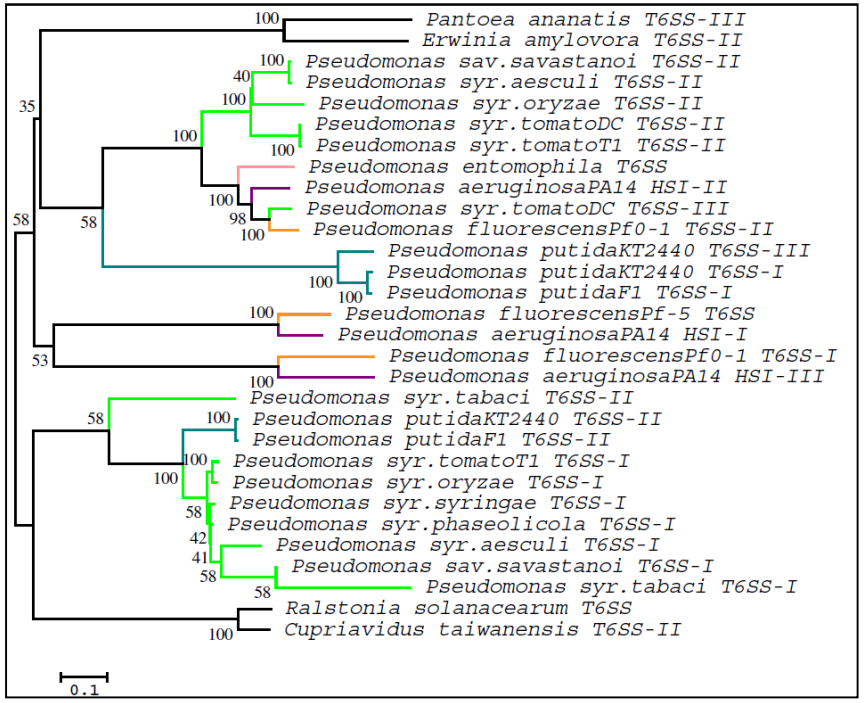


Ho *et al.*,2013

Type VI secretion system

T6SS evolutionary relationships of 30 fluorescent *Pseudomonas* T6SSs

- The evolutionary history was inferred using four T6SS core proteins (ImpC, ImpG, ImpH, ImpL), by the Neighbor-Joining method (Saitou & Nei,1987).
- Difference in tree branch colors indicates the different *Pseudomonas* species, while the outgroup species are presented with black.

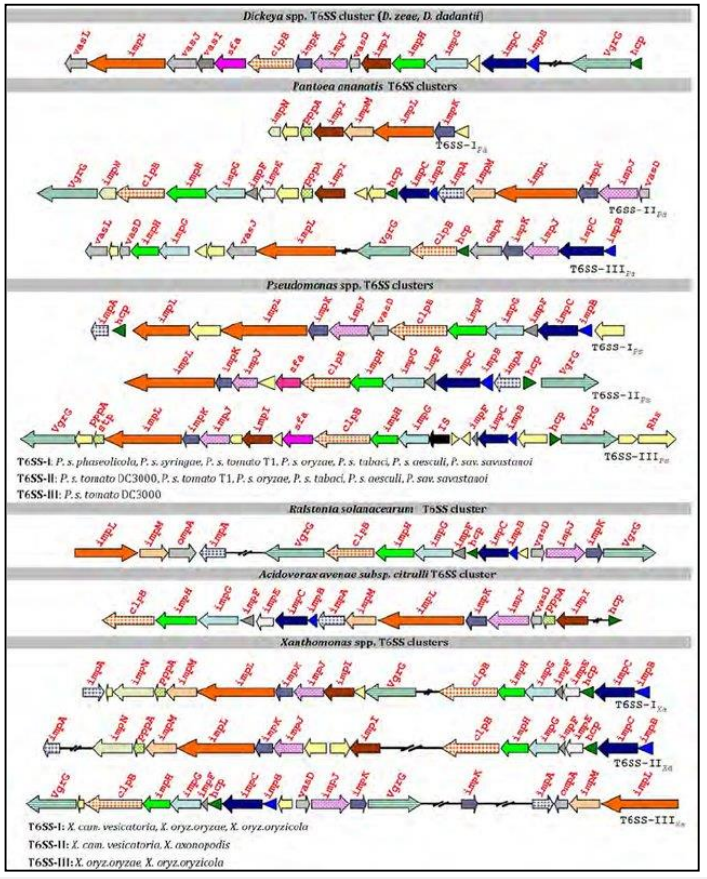


Sarris *et al.*,2012

Type VI secretion system

Maps of T6SS clusters of plant-associated bacteria

- Orthologs are indicated by the same color.
- The genes adjacent to or encoded by the T6SS gene clusters but not recognized as orthologs are indicated by light beige arrows.
- Arrows indicate the transcriptional direction.
- The gene locus numbers are referred in the text and the published or annotated gene designations are indicated above the genes of each cluster.



Sarris *et al.*,2012

Orthologues: Genes in different species that are homologous (similar) because they are derived from a common ancestral gene.

Type VI secretion system
Functions

- It involves in translocation of numerous pathogenicity determinants including:

1. Host colonization;
2. Biofilm formation;
3. Quorum sensing;
4. Antibacterial toxins.

Sarris *et al.*,2012; Benali *et al.*,2014

The chaperone/usher pathway
Involved in pilus assembly

- Fimbriae are cell-surface protein polymers that mediate interactions important for host and environmental persistence, development of biofilms, motility, colonization and invasion of cells, and conjugation.
- Four general assembly pathways for different fimbriae have been proposed:

1. chaperone–usher,
2. alternate chaperone,
3. general secretion and
4. Type VIII.

Gibson *et al.*,2007;..

The chaperone/usher pathway
Involved in pilus assembly

- The chaperone/usher pathway (C/U) is an additional system consists of:

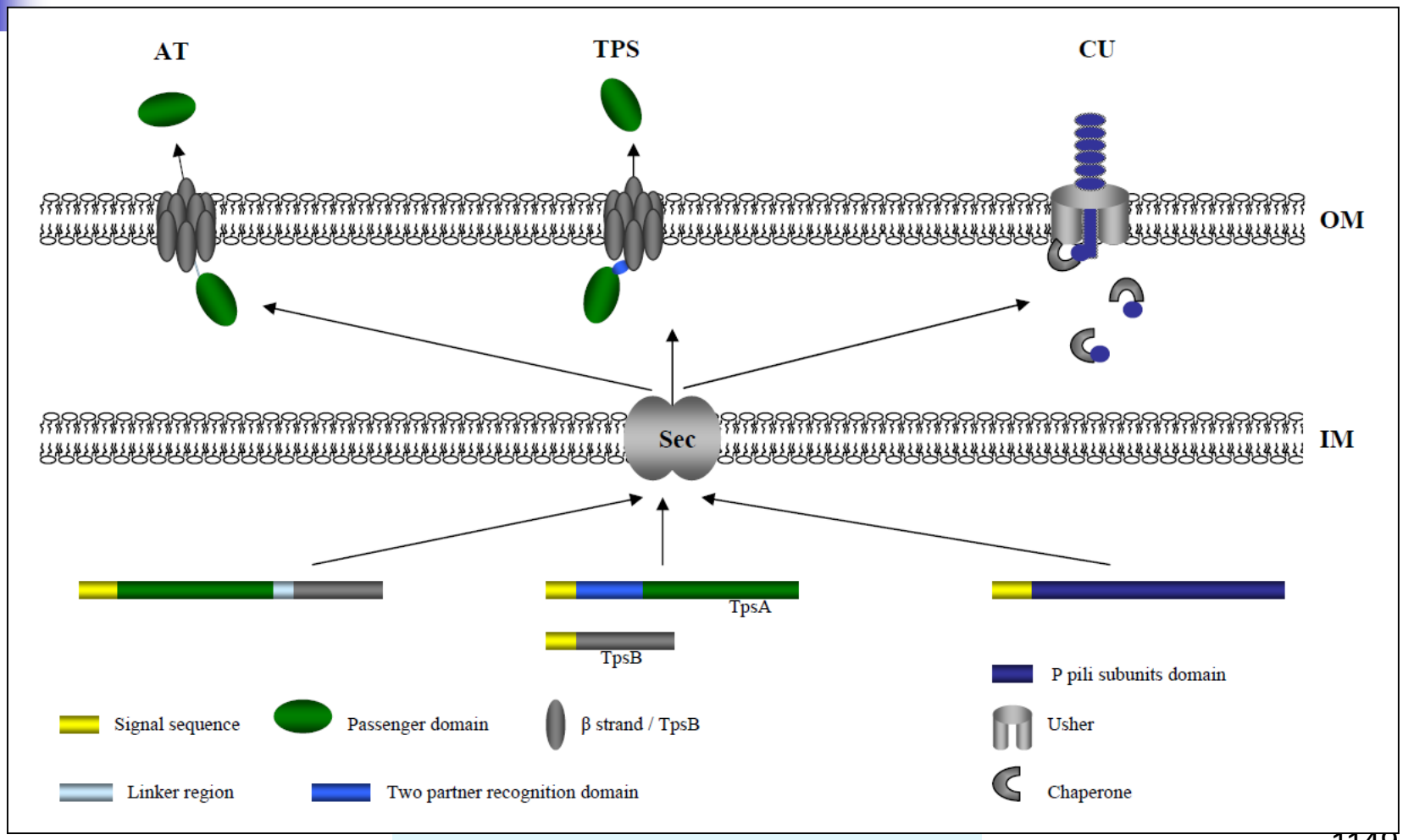
1. An OM protein, termed as usher β-barrel protein, and
2. A periplasmic chaperone.

- The chaperone-usher (CU) system that is used for pilus assembly on the Gram-negative bacterial cell surface.
- During transportation, the substrates are exported across the inner membrane via the Sec system, followed by the immediate binding of the chaperone; then the amino-terminal signal sequences of the substrates are cleaved off and the substrates are released into the periplasm.

An usher is a person who shows people where to go or sit.

Li *et al.*,2007;..

The chaperone/usher pathway
Involved in pilus assembly



Henderson *et al.*,2004; Li, 2007;..

Type VIII secretion system

Responsible for the secretion and assembly of the curli fimbriae or aggregative fimbriae (curli or Tafi)

- The Type VIII secretion system (T8SS) is responsible for the secretion and assembly of the fimbriae called as curli fimbriae or thin aggregative fimbriae (Tafi).
- It is also known as the extracellular nucleation-precipitation (ENP) pathway.
- T8SS is found in Gram negative bacteria.
- Curli or Tafi or aggregative fimbriae were first discovered in the late 1980s on enteric bacteria *Escherichia coli* strains and *Salmonella* spp.
- Tafi or curli are important in pathogenesis and biofilm formation in *Salmonella* spp.

Leo *et al.*,2012;..

Type VIII secretion system

Responsible for the secretion and assembly of the fimbriae called as curli fimbriae or Tafi

- Curli (Tufi or aggregative fimbriae) are morphologically thin (d=4-6 nm) and curled.
- These are the major proteinaceous component of a complex extracellular matrix produced by many *Enterobacteriaceae*.
- Function:

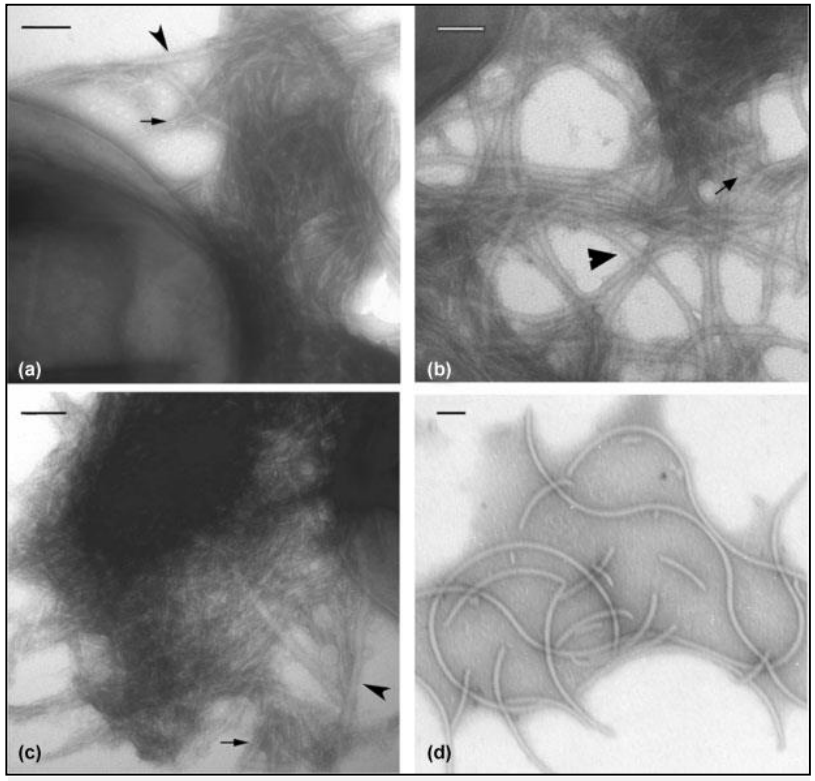
1. Curli fibers are involved in adhesion to surfaces, cell aggregation, colonization and biofilm formation.
2. Curli also mediate host cell adhesion and invasion, and they are potent inducers of the host inflammatory response.

Leo *et al.*,2012; Barnhart and Chapman,2006

Type VIII secretion system

TEM micrographs of *Salmonella sereotype Enteritidis* cell-surface curli fibers

- In the *Salmonella* Enteritidis the operon was termed agf (*agfBAC* Tafi operon).
- *agfA* and *agfB* encode the major and minor fimbrial subunits AgfC and AgfE, respectively.
- AgfC and AgfE in turn, facilitate extracellular thin aggregative fimbriae synthesis.
- Fibres 20 nm in diameter were abundant on the cell surface of the ΔagfC mutant (Fig. 3b),
- Fibres 20 nm in diameter were fewer in wild-type (wt) (Fig. 3a).



Large arrowheads indicate thick (~20 nm diameter) fibres in mutants and small arrows indicate thin normal Tafi (5-7 nm) in wt.

Gibson *et al.*,2007;..

Type IX secretion system
A Deadly Weapon or A Peaceful Tool?

- Type IX secretion system (T9SS), a complex translocon found only in some species of the Bacteroidetes phylum.
- T9SS translocates proteins, especially virulence factors, across the outer membrane (OM).
- T9SS plays two roles, depending on the lifestyle of the bacteria.
- It provides either a means of:

1. movement (called gliding motility) for peace-loving environmental bacteria, or
2. a weapon for pathogens.

Lasica *et al.*,2017

Type IX secretion system

A Deadly Weapon or A Peaceful Tool?

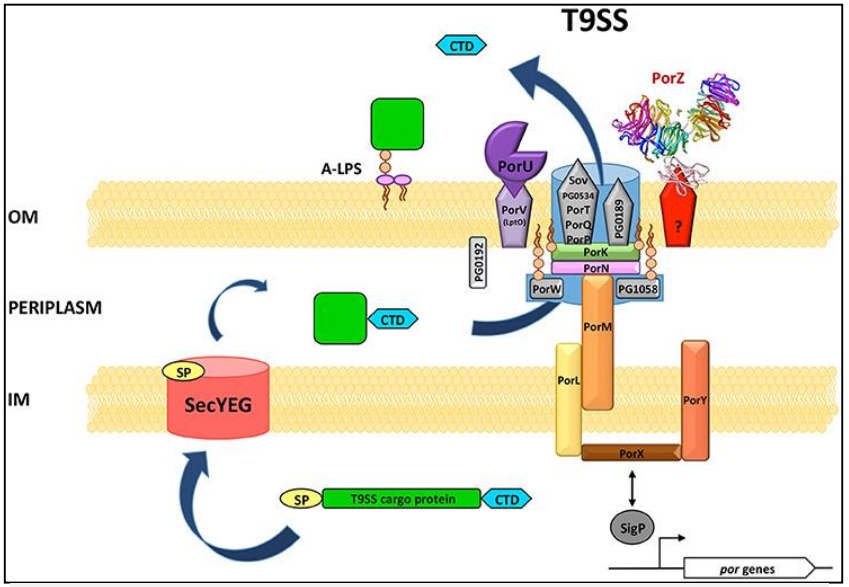
- The phylum "Bacteroidetes" is composed of three large classes of Gram-negative, nonsporeforming, anaerobic or aerobic, and rod-shaped bacteria that are widely distributed in the environment, including in soil, sediments, and sea water, as well as in the guts and on the skin of animals.

Lasica *et al.*,2017;..

Type IX secretion system

A Deadly Weapon or A Peaceful Tool?

- Hypothetical model of the structure and function of *P. gingivalis* T9SS.
- The overall translocon structure and the protein(s) forming a pore in the OM (outer membrane).
- M β-barrel proteins are depicted as pentagons.
- PorZ is presently the only T9SS protein with the known atomic structure.



The gram-negative anaerobe *Porphyromonas gingivalis*, a human oral pathogen belongs to the family Porphyromonadaceae, order Bacteroidales in the phylum Bacteroidetes.

Lasica *et al.*,2017;..

Type X secretion system

A holin/peptidoglycan hydrolase-dependent protein secretion system

- Eight Gram-negative protein secretion pathways (named Type I–VI, Type VIII, and Type IX) have previously been described.
- Here we describe the Type X (10) Secretion System, a two-step pathway that relies on a holing (a holin membrane protein in tandem) and peptidoglycan hydrolase pair for secretion across the outer membrane.
- Chitinase secretion by *Serratia marcescens* DB10 as a paradigm pathway dependent on a holin and a peptidoglycan hydrolase.

Palmer *et al.*,2020

Type X secretion system

A holin/peptidoglycan hydrolase-dependent protein secretion system

- *Serratia* are a genus of ubiquitous Gram-negative bacteria found in water and soil, and some species have been identified as opportunistic pathogens of humans and insects.
- *Serratia* produce a vast number of extracellular enzymes and as a result several different protein secretion systems.
- Most strains encode at least four chitinases (ChiA, ChiB, ChiC, and Cbp21), although some contain more that collectively degrade chitin to chitobiose, which is taken up into the periplasm assisted by the outer membrane chitoporin, ChiP.

Palmer *et al.*,2020

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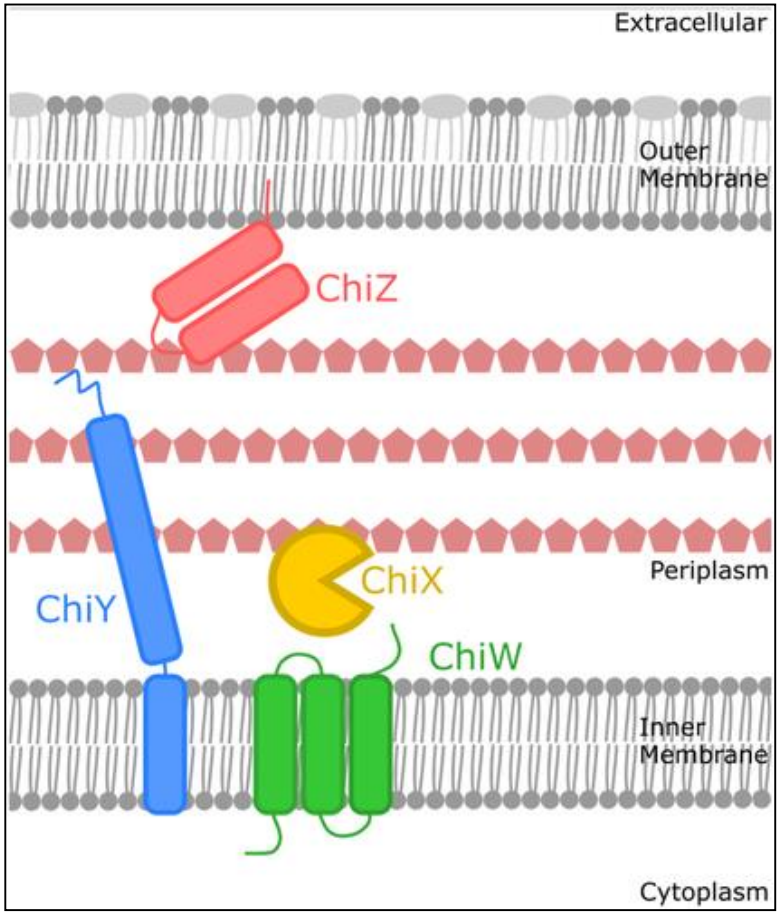
Palmer *et al.*,2020

Type X secretion system

A holin/peptidoglycan hydrolase-dependent protein secretion system

- Subcellular location and predicted topology of components of the *S. marcescens* chitinase secretion system.
- Secretion system component topology and simplified structure based on transmembrane and secondary structure predictions of ChiW, ChiY, and ChiZ using SignalP, LipoP, TMHMM, and Phyre, and the known 3-D crystal structure of ChiX.



Palmer *et al.*,2020

Type X secretion system

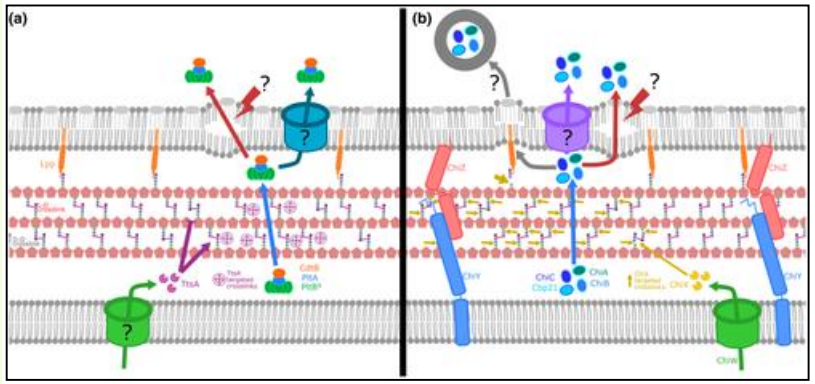
A holin/peptidoglycan hydrolase-dependent protein secretion system

- Models for holin/peptidoglycan hydrolase-mediated protein secretion.

a. Typhoid toxin secretion.

b. Chitinase secretion.

1. ChiX is exported to the *S. marcescens* periplasm *via* Chi W, a holin-like protein, where it specifically cleaves between L-Ala and D-Glu on the stem peptide cross-links of the peptidoglycan layer (gold arrows).



Palmer *et al.*,2020

Protein translocation pathways

In Gram-positive bacteria

Multiple secretion systems

1. **Protein transport across the inner membrane in Gram-positive and Gram-negative bacteria:**
   - 1.1. General secretory pathway (GSP, Sec)
   - 1.2. Twin-arginine translocation (TAT) pathway
   - 1.3. ATP-binding cassette (ABC) pathway
2. **Protein translocation across the outer membrane in Gram-positive bacteria:**
   - 2.1. Type IV pathway
   - 2.2. Type VII pathway or ESX1-5 systems. *Mycobacterium tuberculosis* use five of these secretion systems, named ESX-1 to ESX-5.

Bacterial secretion systems

Gram-positive bacteria

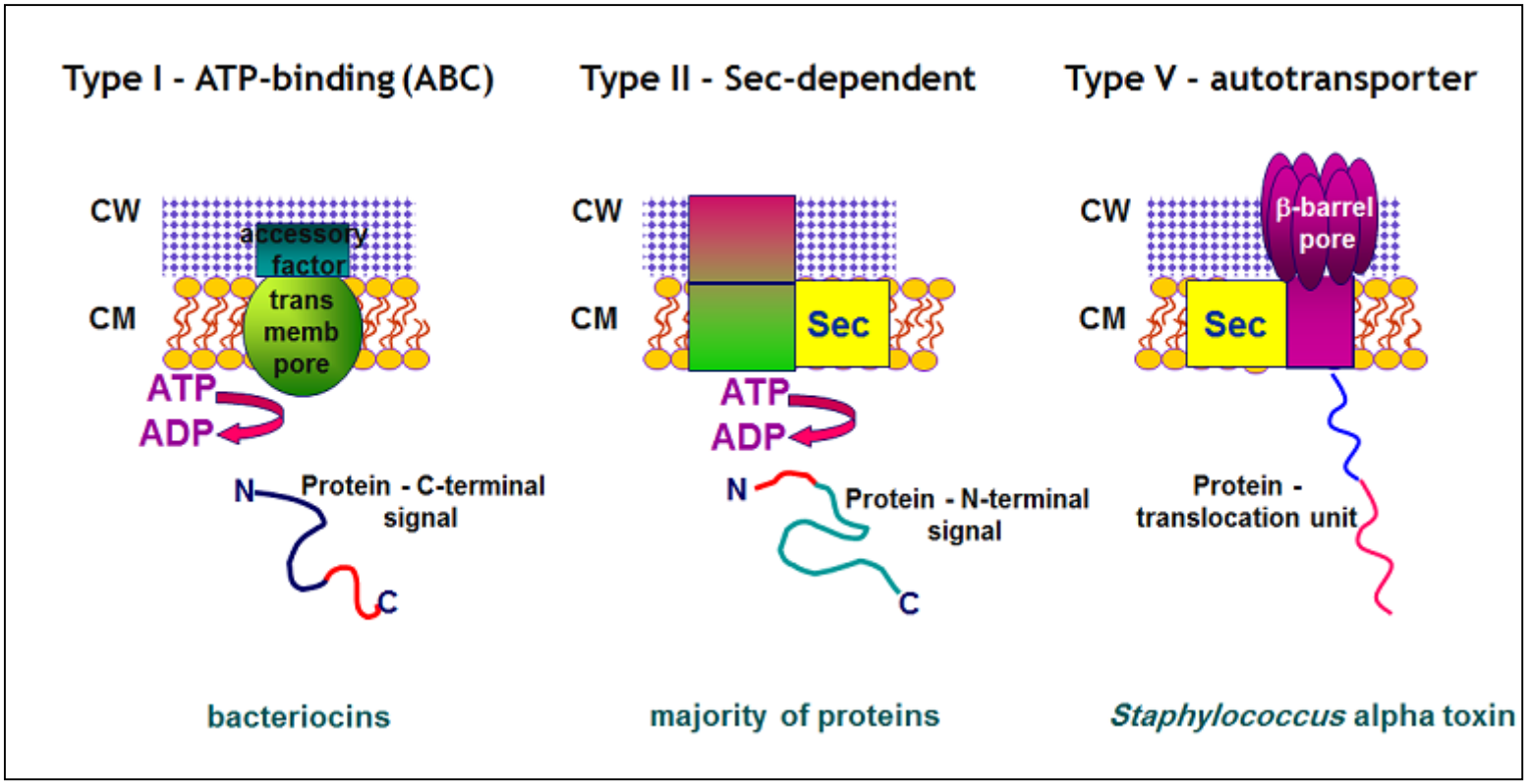
Common SecA, ABC and TAT pathways

1. The majority of exported proteins (extracellular and surface proteins) are exported from the cytoplasm via the general Secretory (Sec) pathway.
   - Phytoplasmas mostly depend on the SecA-dependent system for secretion of proteins (see also phytoplasma pathogencity section).
2. Specific ATP-binding cassette (ABC) transporters direct the export and processing of small antibacterial peptides called bacteriocins.
3. Some Gram-positive bacteria also contain the Tat-system that is able to translocate folded proteins across the membrane.

Bacterial secretion systems

Gram-positive bacteria

SecA, ABC and Type V(?) pathways



Bacterial secretion systems

Gram-positive bacteria

T4SS

- The Gram+ve pathogenic bacteria transport of only a few proteins.
- Type IV secretion (T4S) systems are widely distributed among many species of:
- Gram-negative and Gram-positive bacteria,
- Wall-less bacteria, and
- The Archaea.
- Phytoplasmas phylogenetically belong to Gram-positive bacteria.

Bacterial secretion systems

Gram-positive bacteria

T7SS in some pathogenic bacteria

- T7SS gene clusters are also found in partially acid-fast (mycolic) bacteria *Corynebacterium diphtheriae* and *Nocardia*.
- Also in the genomes of:

1. *Streptomyces* species(*Streptomyces scabies*),
2. *Bacillus* and *Clostridium* spp.,
3. *Staphylococcus aureus*,
4. *Streptococcus agalactiae*,
5. *Listeria monocytogenes.*

The mycolic layer or mycomembrane contains various free lipids.

Tseng *et al.*,2009

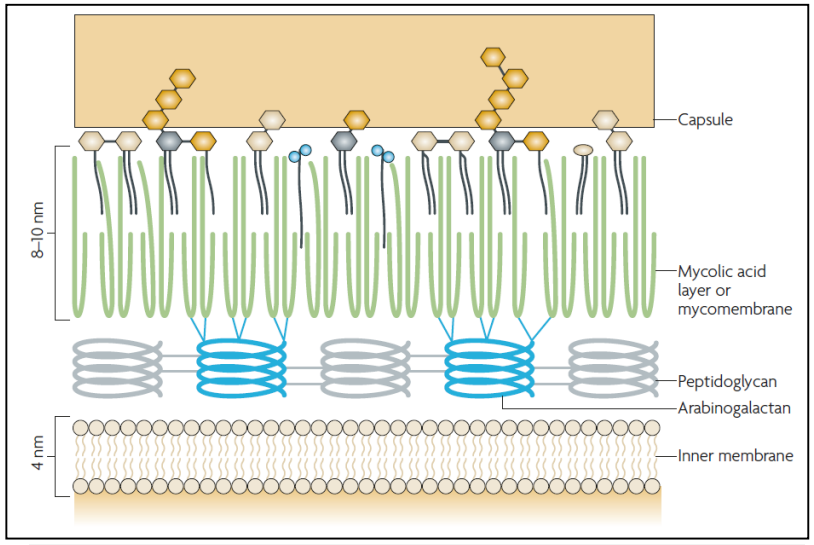
Bacterial secretion systems

Gram-positive bacteria

Cell envelope of *Mycobacterium tuberculosis*

- The cell wall is mainly composed three different covalently linked structures:
  1. peptidoglycan (grey),
  2. arabinogalactan (blue) and
  3. mycolic acids (green).
- The mycolic layer or mycomembrane contains various free lipids.
- The outer layer, which is generally called the capsule, mainly contains polysaccharides (glucan and arabinomannan).

Schematic representation of the cell envelope of *Mycobacterium tuberculosis*



Arabinogalactan is a biopolymer consisting of arabinose and galactose ... The microbial arabinogalactan is a major structural component of the mycobacterial cell wall.

Abdallah *et al.*,2007

Bacterial secretion systems

Five ESX secretion systems have been identified (Esx1-5)

M. tuberculosis

- The genome of *M. tuberculosis* encodes 5 of such secretion systems:
- ESX-1
- ESX-2
- ESX-3
- ESX-4
- ESX-5
- At least three of these secretion systems are essential for mycobacterial virulence and/or viability.

Mycobacterium tuberculosis (acid fast positive and causal of tuberculosis in humans).

Houben *et al.*,2014

Bacterial secretion systems

Gram-positive bacteria

Type VII system or five systems of ESX-1 to ESX-5

- According to sequential secretion-system nomenclature system, it has been called type VII secretion.
- ESX systems can also be categorized on the basis of their function.

1. Thus far only ESX-1, ESX-3 and ESX-5 have been shown to be involved in the secretion of proteins.
2. Active secretion of substrates via ESX-2 or ESX-4 has not been proven.

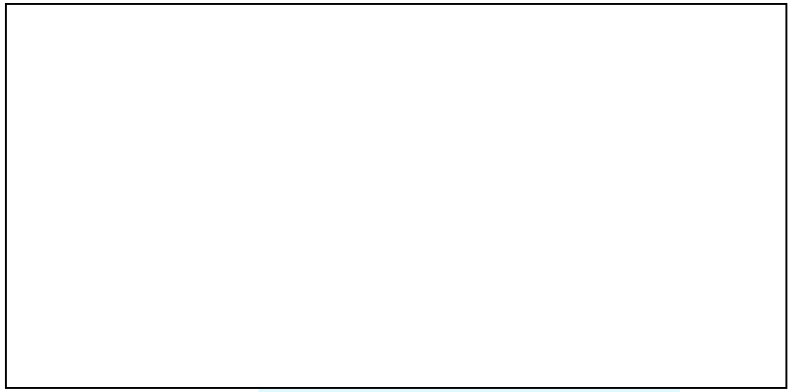
Abdallah *et al.*,2007; Simeone *et al.*,2009;Houben *et al.*,2014

T7SS secretion systems

Five ESX secretion systems have been identified (Esx1-5)

M. tuberculosis

1. ESX1 system: responsible for the secretion two proteins (effectors) ESAT6 and CFP10.
2. ESX3 system: involved in Fe^{3+} and Zn^{2+} uptake and is a central virulence factor in *M. tuberculosis*.
3. ESX5 system: large numbers of PE/PPE proteins were exported via this pathway. Disruption of ESX-5 in *M. tuberculosis* resulted in the loss of PPE protein secretion, changes in cell wall integrity and strong attenuation.

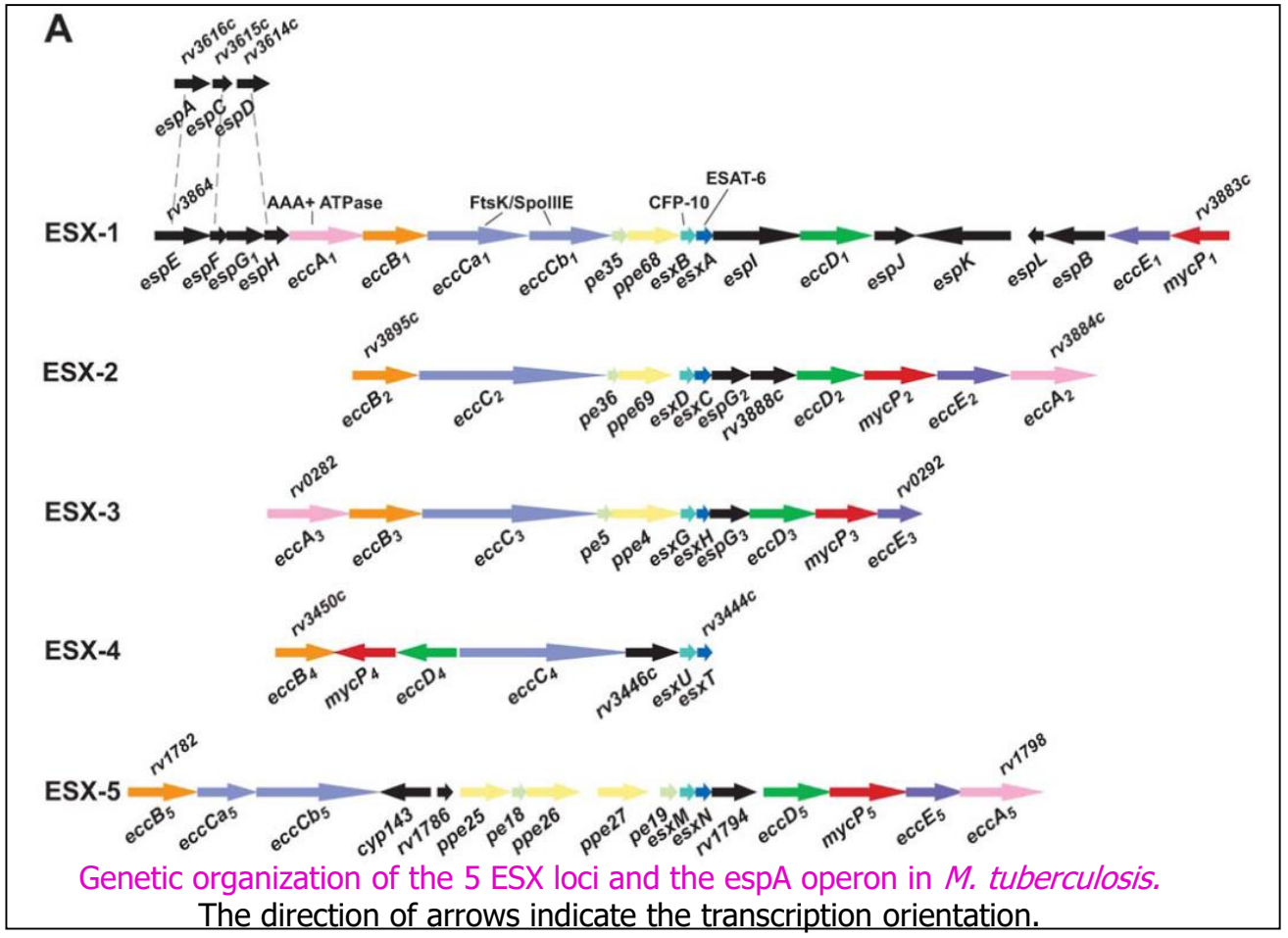


Conserved Pro-Glu (PE) and Pro-Pro-Glu (PPE) Protein Domains.

Morandi *et al.*,2013

T7SS secretion systems

The genome of *M. tuberculosis* encodes 5 of such systems (ESX-1 to-5)

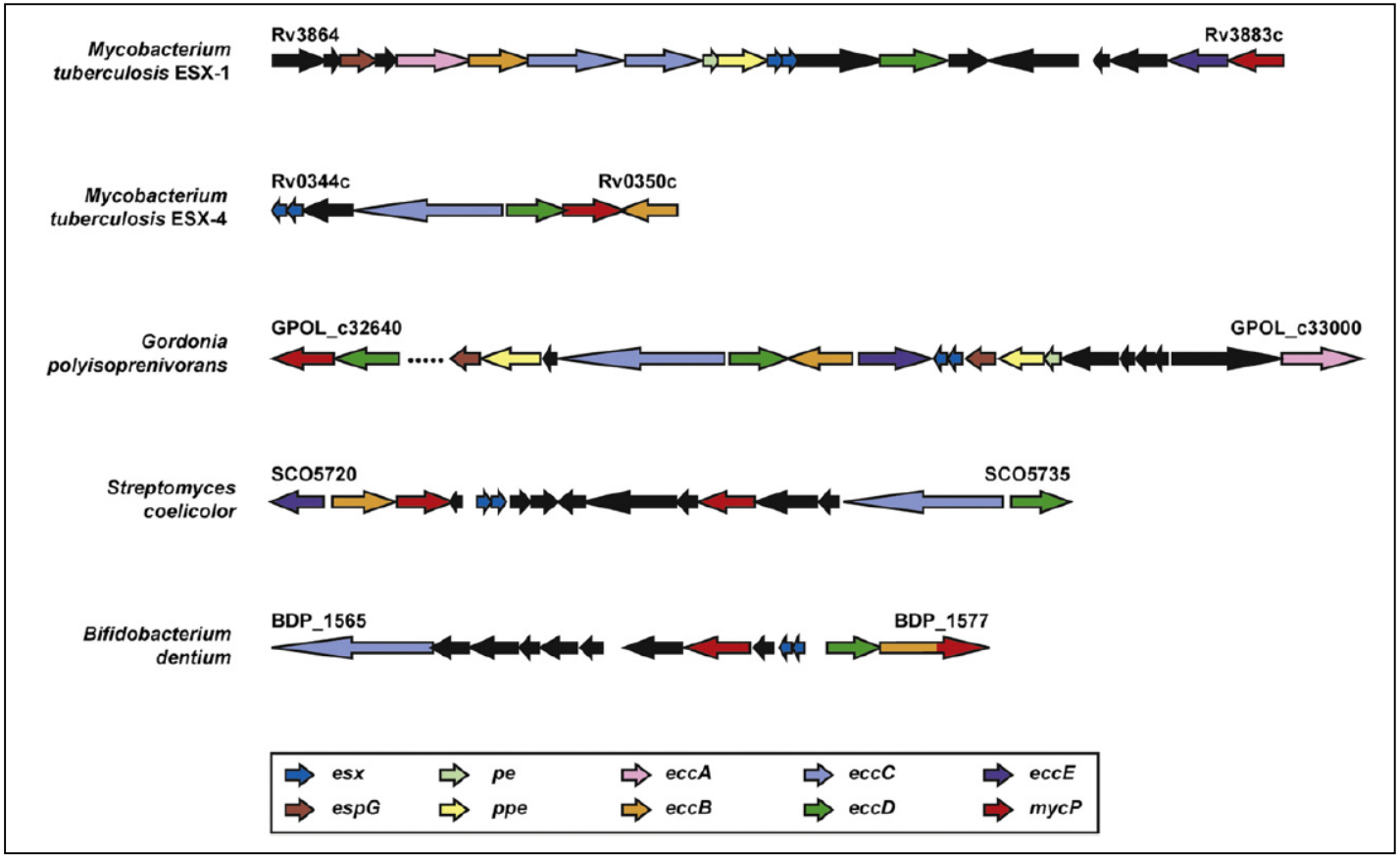


Genetic organization of the 5 ESX loci and the espA operon in *M. tuberculosis.*
The direction of arrows indicate the transcription orientation.

Bitter *et al.*,2009

T7SS secretion systems

The genome of *M. tuberculosis* encodes 5 of such systems (ESX-1 to-5)

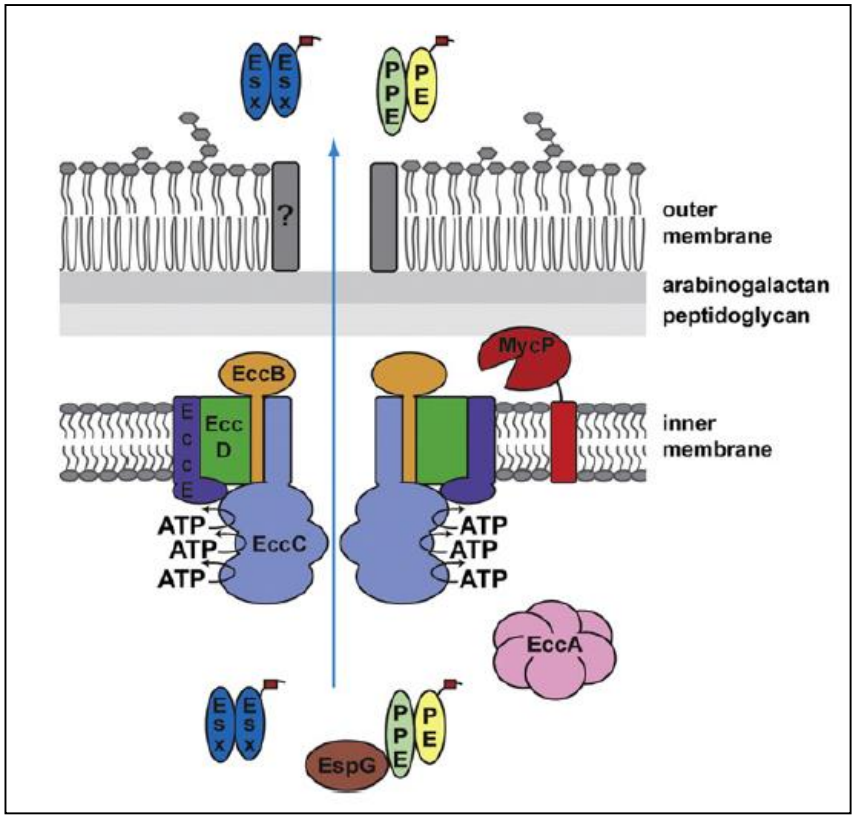


Comparison of different gene clusters that encode type VII secretion systems. The color coding for the figure is presented in the key. The black arrows indicate region-specific genes. The dotted line represents a considerable distance between the two adjacent genes and is not in scale.

Bacterial secretion systems

Both Esx and PE/PPE proteins are exported as dimers by the T7S secretion machinery

- **Mechanism of transport: a one-step process:** Since T7S substrates do not contain classical signal sequences and therefore do not depend on Sec or Tat for secretion, one might therefore assume that T7S is a one-step process.
- Indeed, the size of the T7S membrane complex allows it to span both the inner and the outer membrane.



J.M. Chen, M. Zhang, J. Rybniker, L. Basterra, N. Dhar, A.D. Tischler, F. Pojer, S.T. Cole.2013. Phenotypic profiling of Mycobacterium tuberculosis EspA point-mutants reveals blockage of ESAT-6 and CFP-10 secretion in vitro does not always correlate with attenuation of virulence. J. Bacteriol. 195 (24): 5421–5430.

Houben *et al.*,2014

Bacterial secretion systems

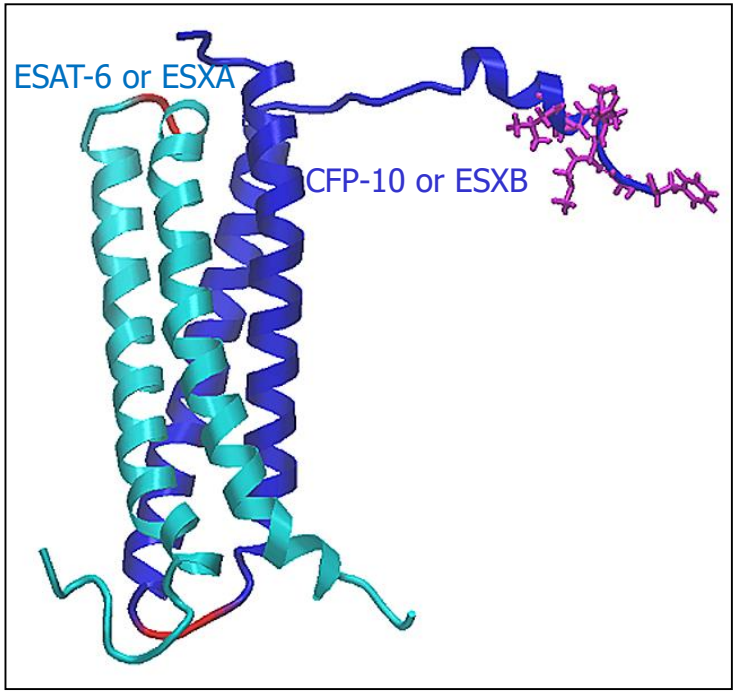
The first T7S system: ESX-1

The two effectors: ESAT-6(EsxA) and CFP-10(EsxB)

- ESAT-6 (light blue) and CFP-10 (dark blue) form a tight 1:1 complex.

1. ESAT-6 (EsxA), a 6 kDa protein;
2. CFP-10 (EsxB), a protein of 10 kDa).

- CFP-10 (EsxB) is a putative chaperone of ESAT-6 (EsxA).
- WxG motifs are shown in red;
- Secretion signal motifs are shown in pink.



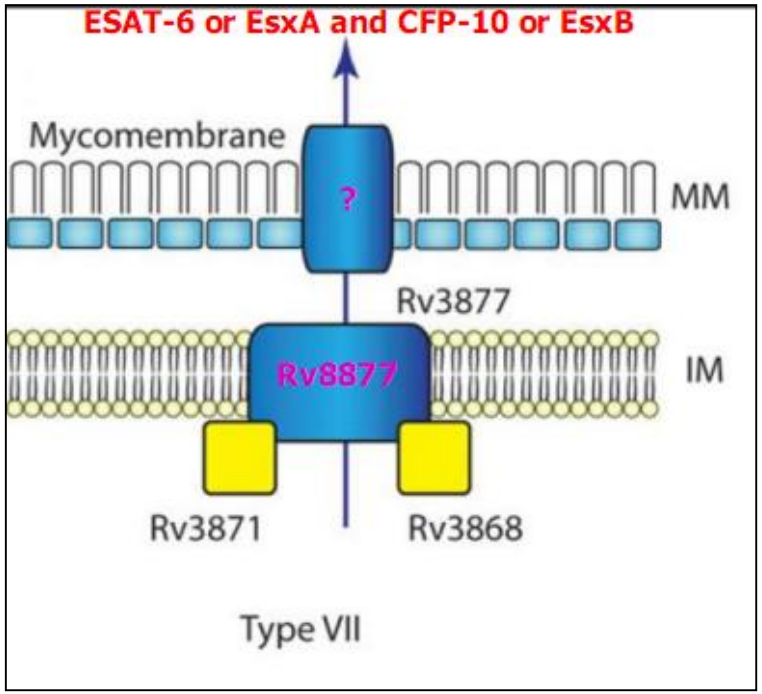
Abdallah *et al.*,2008;..

Bacterial secretion systems

The first T7S system: ESX-1

The two effectors: ESAT-6(EsxA) and CFP-10(EsxB)

- The structure and operation of the T7SS are still being pieced together.
- Current models suggest involving of two protein(s) for secretion of ESAT-6--CFP-10 complex:
- The translocation channel in the IM is probably formed by the integral membrane protein Rv3877.
- But it is unknown which protein (or proteins) forms the channel in the mycomembrane (MM).



Tseng *et al.*,2009;..