Milestone report

COMPRO approaches recommended to participating universities for incorporation in their degree and short-course curricula

Project

Institutionalization of quality assurance mechanism and dissemination of top quality commercial products to increase crop yields and improve food security of smallholder farmers in sub-Saharan Africa – COMPRO-II

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Project Website: www.compro2.org

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Foreword

The COMPRO-II project has an important component on capacity building intended to various stakeholders including farmer organizations, extension agents, policy makers, regulatory officers, graduate students etc. A training manual has been developed to that effect (i.e. *Training manual for product screening and inspection*). However, for long-term impact and sustainability there is a need of continuous training particularly for the youth.

In addition to training graduate students (MSc and PhD candidates), there was a need to broaden the capacity building to other categories of stakeholders such as undergraduate students (university curricula) and in-service professionals (short courses). In fact, using the sample of the graduate students supported by COMPRO-II (five for PhD and 10 for MSc programs) and following interaction with the participating universities, the project noted a need to work closely with partners for course maintenance in areas related to soil microbiology.

The project has therefore recommended this content to create a solid foundation in soil microbiology for students, particularly for undergraduate candidates. The content is also relevant to in-service professionals who are interested to develop their career in the field of soil microbiology. This content, or part of it, has been considered by participating universities for course maintenance.

Table of Content

I. Introduction to Microbiology

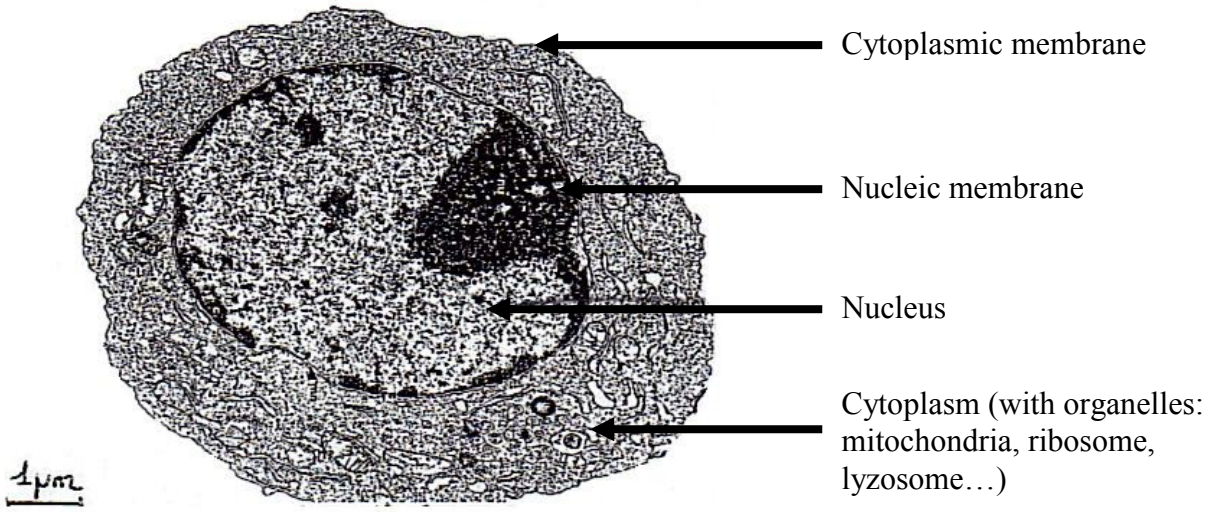
Microbiology has been created as a science in 1676 by Van Leeuwenhock when he discovered the third kingdom (after the animal and plants ones). This third kingdom is called Protists kingdom.

A. Definition of Microbiology

Micro = small
Bio = life
Logy = science.
Microbiology is the science which studies alive microorganisms, which are not visible with naked eyes because they are few micrometers big (rough estimate).

B. Eukaryotic Protists or Superior Protists

1. Structure:



2. Observations:

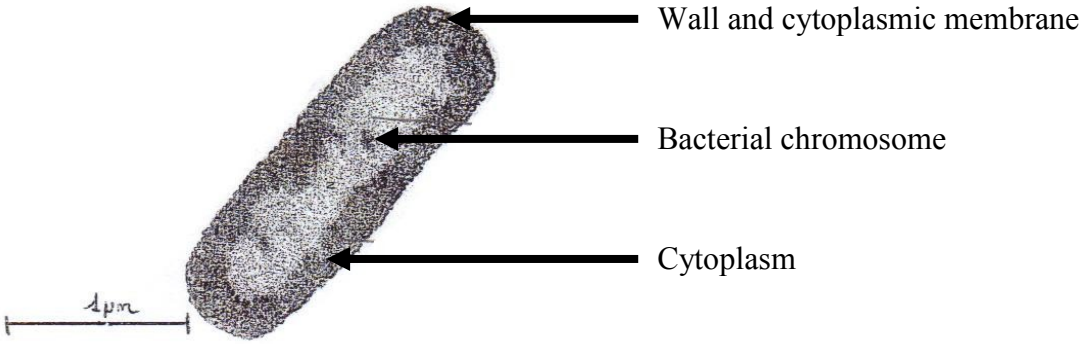
These are unicellular organisms, or if they are multicellular, all the cells are similar and there is no cellular differentiation.
Every cell is formed by:

- a nucleus which includes the DNA (several chromosomes) and which is enclosed by a membrane called nuclear membrane,
- the cytoplasm, where all the organelles are (for example mitochondria),
- a cytoplasmic membrane, which delimits the cell.

A cell is about ten micrometers (up to one hundred for the biggest).
Eukaryotic protists include fungi, algae or protozoa.

C. Prokaryotic Protists or Inferior Protists

1. Structure:

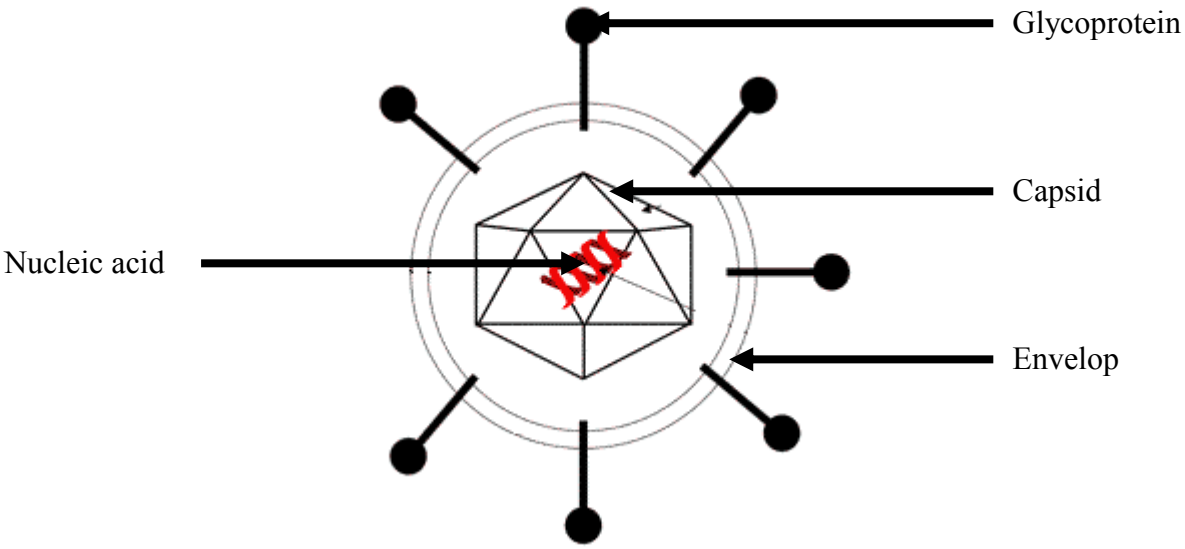


2. Observations:

Prokaryotic protists are unicellular organisms; their structure is really basic and simple. They don't have a real nucleus: the single chromosome is diffused in the cytoplasm because they do not have a nuclear membrane. Few organelles are present in the cytoplasm (basically ribosomes). The cytoplasmic membrane delimits the cell, and a cell wall protects every cell.
The size of these cells is about one micrometer (up to one hundred times smaller than eukaryotic protists), and this group mainly includes bacteria and cyanophytes.

D. A Special case: Viruses

1. Structure:



2. Observations:

They form a new kingdom because their cell organisation doesn't look like any other cell. They just have a nucleic acid (DNA or RNA, never both at the same time) included in a protective proteinic capsid. Some viruses also have an envelope, and therefore are called enveloped viruses. They are 10 to 100 times smaller than bacteria, and they can't reproduce

on their own as bacteria do. They need another cell, named host. They use proteins or organelles from the host cell to develop themselves, and kill the cell. That's why they are called obligatory intracellular parasites.

E. Fields of microbiology

1. Pathogenic micro organisms:

They are able to penetrate a host cell and to cause disorders (and make the host die) by too many multiplications or by secretion of toxins.

2. Environmental saprophytic micro organisms:

These micro organisms live in water, soils, air, but they are not related with others micro organisms. That means that they don't need any host cell to grow and to reproduce. They feed on organic matter and are often important part of biological cycles (nitrogen, carbon, sulphur).

3. Human commensal micro organisms:

They are associated to human mucous membranes, but they don't affect the health of the organisms they live with. They also play major roles in different physiological functions as pH regulation, vitamin secretion, or protection against several infections…

4. Micro organisms used for technological processes

In food industries, micro organisms are used because of their metabolism to transform raw materials into new products (interesting because of their taste or nutritional effect). Some of them can also be used to produce interesting molecules (ethanol, hormones, vitamins,, enzymes…) at a very low cost.
Microorganisms are also used in different sectors, such as medicine (antibiotics production), or environmental sciences (degradation agents, fertilizer…).

F. Conclusion

Microbiology is a wide speciality and can be divided into different fields: bacteriology, mycology, parasitology, virology…
Microbiology is closely linked with molecular biology, and immunology, so it is really helpful to connect all the information to get a better knowledge of the bacteria.

Bacteria are widely present in the environment. For example, we assume that the cell concentration in soil is around 10^{10}cells/g.

II. Bacterial Classification

A. Gender, Specie, Strain

Gender: group of many species which are morphologically very close or identical, and physiologically relative.

Specie: group of bacteria which have many characteristics in common, about morphology and physiology.

Variety: within the specie, a variation could occur, which makes the bacteria stable and different from the "mother bacteria" for few minor criteria (most of the time, presence or lack of few enzymes).

Strain: culture which is the conclusion of the growth of a single cell. This cell is isolated from a pure colony, from a pure culture. Every cell in a strain has exactly the same characteristics because they all come from the same cell. Most of the time, a strain is called by the name of the person who has isolated it.

Bacteria are classified using different criteria depending on the fields of studies.

B. Classification depending on the nutrition of the bacteria

The criteria are:

- Energy source: phototrophic bacteria use light energy whereas chemotrophic bacteria use chemical energy through oxido-reduction reactions.
- Carbon source: autotrophic bacteria use Carbon from CO_2 whereas heterotrophic bacteria use Carbon from organic molecules (protids, lipids or glucids).
- Electron donor: if the donor is an organic compound, bacteria are called organotrophic, whereas bacteria which use inorganic compound are lithotrophic.

C. Classification using physiologic groups

Bacteria which are involved in the same cycle (Nitrogen, Carbon, Sulphur, Iron…) can be pool together in a group.
Example: *Nitrobacter* and *Nitrosomonas* are involved in the organic matter mineralization:

$$NH_3 \rightarrow NO_2^- \rightarrow NO_3^-$$

D. Taxonomic classification

Taxonomy is a science. It is used to classify all micro organisms depending on each other, using several criteria.

These criteria are:

- Morphology (phenotype): shape, antigen of the surface, reaction to Gram staining, sporulation ability, mobility…
- Biochemical characteristics: nutrients which can be used, enzymes, metabolic pathways, products of this metabolism…

- DNA structure: Chargaff coefficient (which is the percentage of G-C bases), DNA hybridization, or melting temperature (Tm). For example, if the difference of G-C bases percentage between the both bacteria is bigger than 5%, they are not part of the same specie. DNA extracted from two cells from same specie hybridizes at least at 70%. Melting temperature has also to be less different than 5°C.
- RNAr 16S sequence: 97% homology is required to classify two bacteria in the same specie.

Using these criteria, different classifications have been created, the major one is still the Bergey's one. The *Bergey's manual* is the book where you can find all the references for this classification.

E. Nomenclature

Bacteria are named by their gender with a capital letter, then by their specie and the strain if there is one. The entire name is underlined, or written in italics if typed, but not the name of the strain:

Example: *Corynebacterium diphteriae*, strain Park-William
Corynebacterium diphteriae, strain Park-William

III. Bacterial cell morphology

A. General points

The observation of the cells is done with a microscope, with or without fixation and staining.

1. Size

A cell is about ten micrometers but this size varies with species.

2. Shape

The discussion of bacterial morphology has been dominated by questions about how a cell manages to create different shapes and what advantages that may give to the organisms (Young, 2007). What has not been as well explored is why bacteria find it advantageous to exhibit such a prodigious number of different shapes. Two evolutionary arguments also support the utility of bacterial shape. First, shape has a vector through evolutionary time – rod-like organisms having arisen first and coccoid forms being derivatives at the ends of evolutionary lines (Stackebbrandt and Woese, 1979; Woese et al. 1982; Siefert and Fox, 1998; Gupta, 2000; Tamames et al. 2001). Progressive development of a trait implies that selective forces are operating. Secondly, prokaryotes with different genealogies may converge morphologically, indicating that a similar shape may confer advantages in certain Bacterial cell shape differs depending on groups. It is important to note that even in a group, the morphology of a cell can be different, depending on the media they have been grown on, or if they are observed in their environment. The simplest conclusion is that morphological adaptation serves an important biological function.

Shape contributes a measure of survival value in the face of three "Primary" selective pressures: 1) nutrient acquisition, 2) cell division, and 3) predators; and in optimizing five "Secondary" mechanisms: 4) attachment to surfaces, 5) passive dispersal, 6) active motility, and 7) internal or 8) external differentiation (Young, 2006) The first three are Primary in that they represent fundamental conditions that determine whether cells live or die, because cells must grow and multiply and keep from being killed. The last five are Secondary in that they represent a suite of morphologically associated mechanisms that bacteria use to deal with the Primary forces.

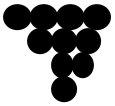
Shape isn't everything. The point, though, is that morphology is a significant selectable trait in many circumstances and that the subject can be approached experimentally like any other. As we understand more about the mechanisms that regulate cell shape we may soon be able to manipulate bacterial morphology with enough confidence to ask how morphological changes affect survival in different conditions. And as evidence accumulates for the utility of cell shape, we can hope that investigators will be motivated to ask these types of questions more directly.

Examples of common bacterial shapes

They may be round or oval, and are called **Cocci** (Coccus if singular):

The way the cells are clustered can be a tool for identification:

- Isolated cells: ● ●

- Cluster of two : Diplococci  Example : *Pneumococci*
- Chaplet or small chain:  Example: *Streptococci*
- Cluster of 4:  Example: *Micrococcus luteus*
- Cubic pile:  Example: *Sarcina maxima*
- Irregular pile:  Example: *Micrococci*
- Grape cluster:  Example: *Staphylococci*

If the cells are elongated, with an erect stick shape, they are called **<u>Bacillus</u>**:
The stick shapes can have:

- Round ends:  Example: *Escherichia coli*
- Square ends:  Example: *Bacilllus anthrasis*
- Bulging ends:  Example: *Corynebacterium*
- Spindle shaped:  Example: *Fusobacterium*

The cocci can form clusters:

- Isolated: 
- Cluster of two: Diplobacillus 
- Chain, pretty much long: Streptobacillus 
- Palisade: 
- V: 

The cells can also have a shape between the cocci and the bacillus: **Coccobacillus** :

For example, *Pasteurella*: 

Some cells are curved and stick shaped: Spirilallacea

- Comma shape: *Vibrio* 
- Curl shape : *Spirillum* 

3. Structure

Bacterial cells include two types of organs:

- *The "permanent organs"*

They are included in every cell, and are integrated in the genetic information of the bacteria (they are a part of the genotype) and the genes encoding for them are always expressed (they are a part of the phenotype). They are composed of:

- The wall: it protects and delimits the bacteria.
- The cytoplasmic membrane: it limits the cytoplasm, allow the ATP synthesis, and the regulation of exchanges between outer and inner environment.
- The cytoplasm: it provides metabolism because it includes every organelle.
- Genetic material: There is no real nucleus in prokaryotic cells. The bacteria have a single circular chromosome, which is in charge of development of everything in the cell (regulation of cellular life)

- *The "fickle organs"*

They are not present in all cells, depending on species but also on the environment and the media where bacteria are growing. If a bacterium possesses a fickle organ, then it is part of the genotype of the bacterium, and a part of the phenotype if the bacterium is growing under favourable conditions to express the genes encoding for it.

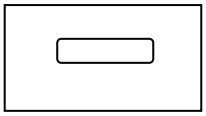
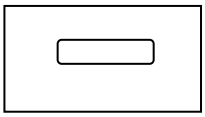
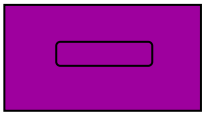
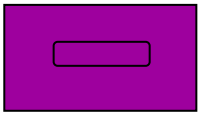
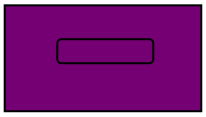
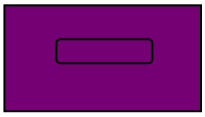
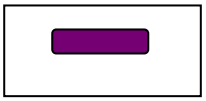
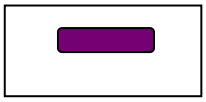
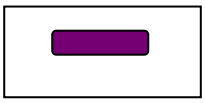
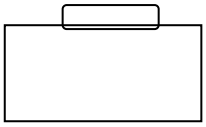
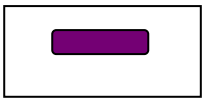
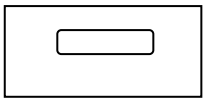
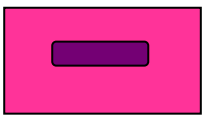
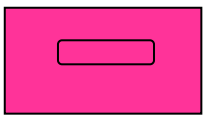
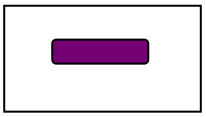
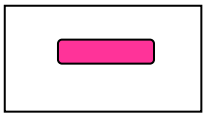
B. The wall

Every cell has a wall and it represents 20 to 30% of the bacteria's dry weight.
It is composed by different chains of molecules like peptidoglycane, amino acids, teichoic acid, polysaccharides, and lipids (phospholipids).

Two groups of bacteria can be formed depending on the composition of the wall.
Because of this difference of composition, the cells react differently to coloration: Gram coloration. Consequently, the two groups are called Gram Positive and Gram Negative.

Gram positive wall is composed by only one stratum and is full of peptidoglycane, and not really in lipids.

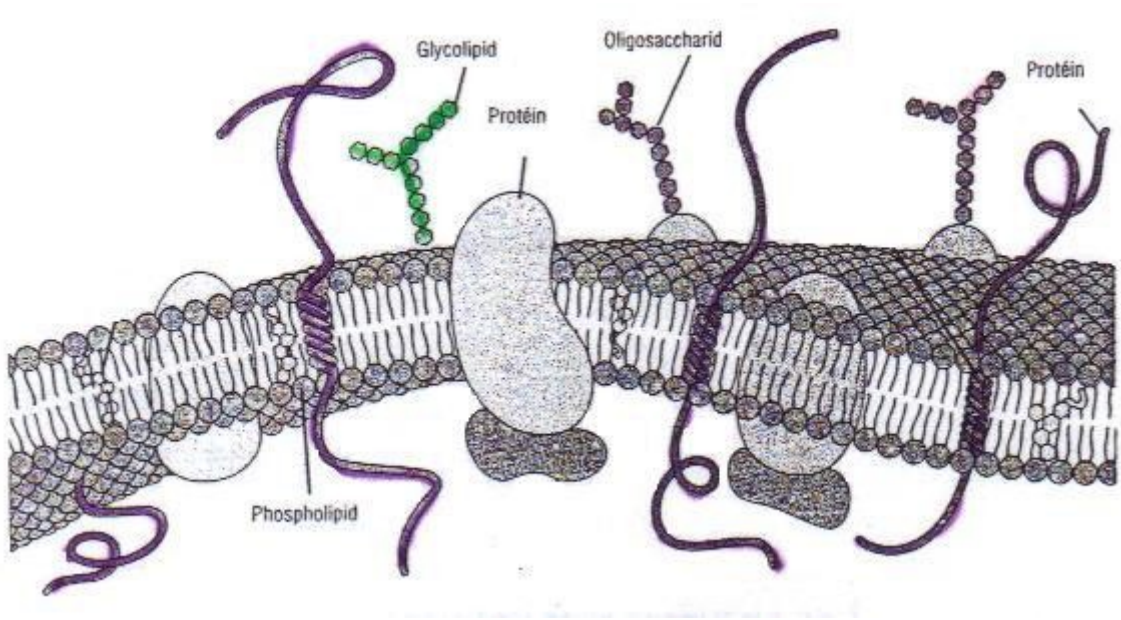
Gram negative wall is composed by three layers, and the upper one is full of polysaccharides and lipids.
The coloration of Gram can be determined in five steps:
First of all, the cells are fixed on a slide with the flame of the burner.
Then, a purple colorant called Crystal Violet is added. It is able to go through every wall (positive or negative) and makes every cell purple.
The Lugol (a mordant) is added to strengthen the purple coloration.
Then, alcohol is added. Alcohol makes lipids soluble so, the walls with a lot of lipids are made permeable, and the cells become colourless. Peptidoglycanes make Gram positive cells impermeable.

Steps	Reactions of cells	Gram positive	Gram negative
1st step: A smear of bacteria is made on a slide, and is fixed by heating	Cells stick to the slide and the walls are weakened, making cells permeable to colorants.		
2nd step: The slide is cover with Crystal Violet	The colorant goes through the wall and makes the cells purple coloured.		
3rd step: Mordant Lugol is added	Lugol goes into the cell and bound to the Crystal Violet, it makes the coloration stronger.		
The slide is gently rinsed with water	Surplus stain is eliminated		
4th step: Alcohol is added on the slide	Alcohol dissolves lipids and eliminates the purple coloration: In the Gram positive, alcohol does not go into the cell because of the low lipid concentration of the wall.		
The slide is gently rinsed with water	Alcohol is eliminated, so are the colorants of Gram negative cells.		
5th step: The slide is covered with fuchsine (pink colorant)	Fuchsine is less strong than purple. So it has no effect on Gram positive cells (still purple). The Gram negative cells are coloured in pink.		
Surplus of colorant is eliminated	The cells are ready to be watched with a microscope.		

Finally, a pink colorant is added mainly used is here safranin (fuchsine): the cells with Gram positive wall (without lipids) are still purple, while Gram negative cells are coloured in pink with fuchsine.
The wall has many functions: it helps to support the cell, it gives the shape of the cell, prevents the lysis of the cell (because it regulates osmotic pressure in the cell) and protects the bacteria from outer physical aggressions.

C. The cytoplasmic membrane

It envelops the bacterial cell, just below the wall. It represents about 20% of the cell dry weigh.
It is composed by two stratums of phospholipids, with proteins, steroids and glycoproteins.

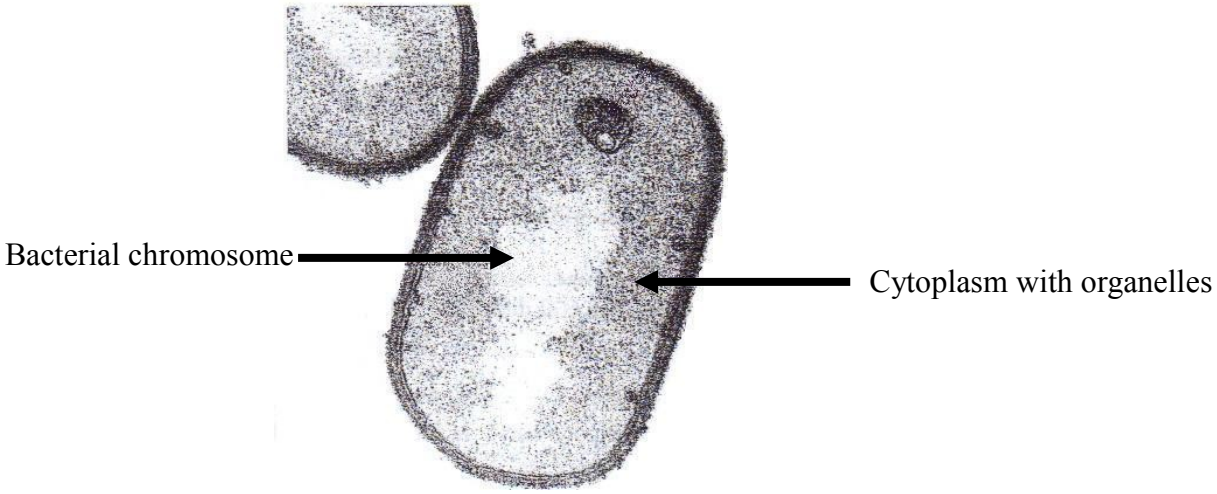


The membrane has two major functions:

- Selective permeability: it acts as a gate to regulate nutrients entries and waste exit. It also helps to stabilize osmotic pressure of the cell.
- Enzymes: enzymes are proteins which have a catalytic capacity. They make the chemical reaction much faster. The cytoplasmic membrane includes three types of enzymes: exo enzymes, which cut the big nutrient molecules into smaller portions to allow them to enter into the cell; permeases, which are able to carry the molecules from the outer environment to the cell; respiratory enzymes which produce ATP.

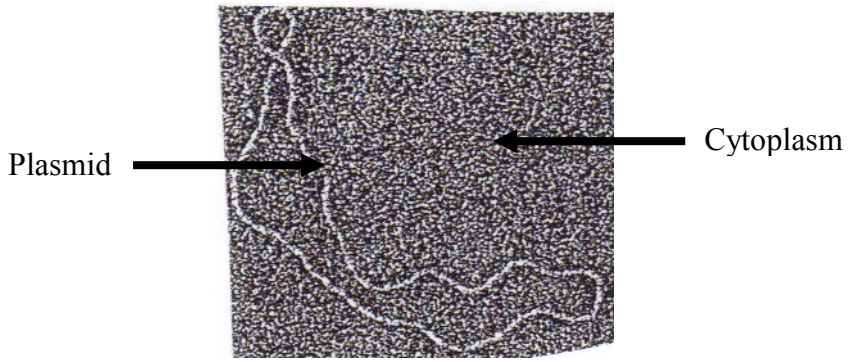
D. Cytoplasm

Cytoplasm = cytosol + organelles.



It includes very few organelles which are mainly enzymes and ribosomes.
A cell has about 50000 ribosomes, more than in a eukaryotic cell, whereas it is smaller.
They are formed by proteins and ribosomal RNAs and act as a part of protein synthesis because they translate the messenger RNAs and catalyse the formation of peptidic bounds between amino acids.

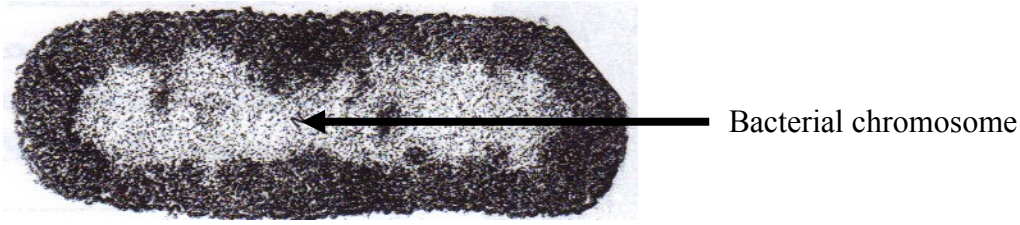
Many bacteria also have one or several plasmids in their cytoplasm. A plasmid is a piece of DNA (double strand), which is able to replicate on its own. It looks like a chromosome (double strand and circular DNA), but it is really smaller.



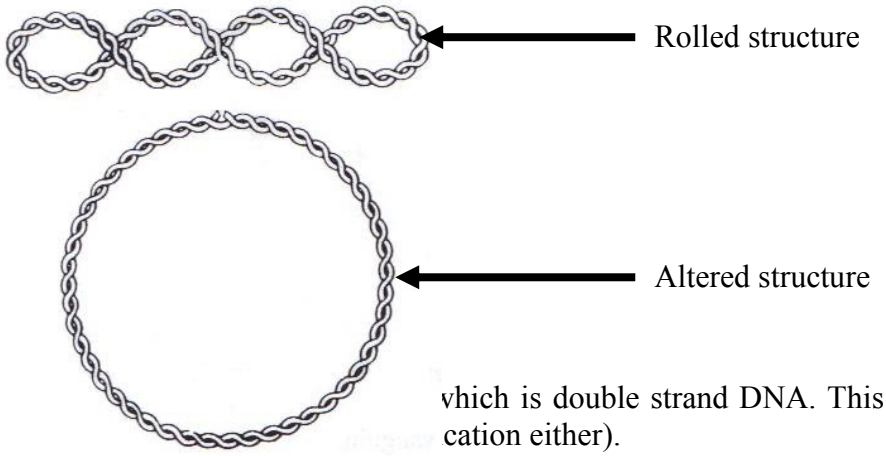
Different types of plasmids exist, they can make the cell resistant to different types of molecules as antibiotics (Plasmid R for Resistance), or they can encode for the sexual pili, which are used for mating (Pili F for Fertility).

E. Nucleus or bacterial chromosome

It is not a real nucleus since there is no membrane or nucleolus. There is no mitosis either. So we can't call it nucleus, but "nucleoid" or bacterial chromosome.



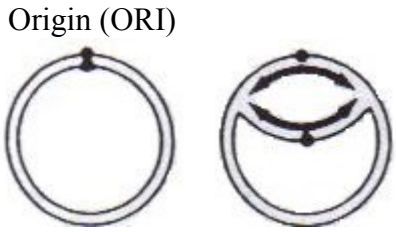
1. **Structure:**



The chromosome is forı vhich is double strand DNA. This filament is entangled, cii cation either).

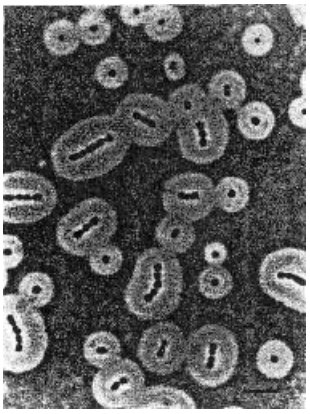
2. **Replication:**

The replication is half conservative. The two strands separate from each other. Every single strand is used as a template to synthesize the new strands. The replication is done following the both directions from the replication origin (ORI).



F. Capsule

The capsule is a fickle organ, which is outer form the wall. Few bacteria have it and they are called encapsulated bacteria.
To be able to see it with the microscope, a staining is necessary. Black ink is basically used and then, the bacteria appear black and the capsules are white.



Capsules can also be stained by fuchsine (Ziehl staining) or they can be seen with an electronic transmission microscope.
The capsule is slimy or jelly-like and can border several cells. It is not necessary for the bacterium to live, and even without it the bacterium is able to reproduce. The capsule is a virulence factor, because it hides the fixation sites which are recognized by the immune cells. Like this, immune cells can't kill the encapsulated bacteria. It is also helpful to avoid predators which are not able to recognize the cell since the fixation sites are hidden.

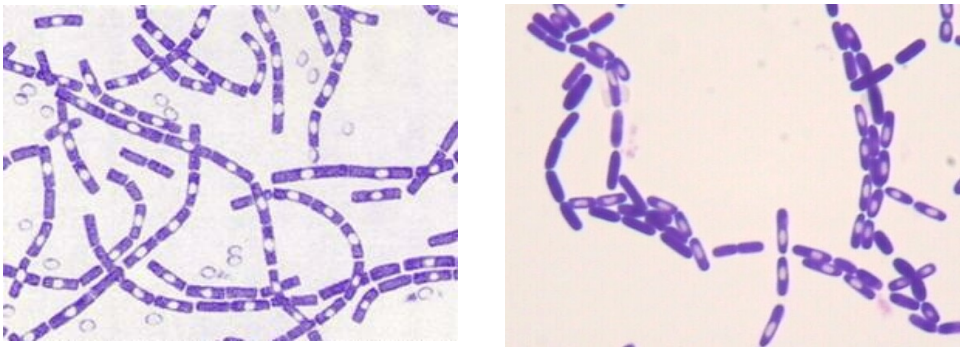
G. Spore

The spore is an inconstant organ for the bacterial resistance. Bacteria will produce it responding to unfavourable environmental conditions. A spore is in a slow life, a kind of "sleepy statement", and it is also a way of dissemination. Metabolism is almost stopped and a very low quantity of energy is produced (metabolism requires water and spores are very dry).
Many types of bacteria are able to make spore, but they are found mainly in Gram positive bacillus, like *Bacillus* and *Clostridium*.
In bacteria, spores are intracellular, whereas fungal spores are out of the cell (extracellular spores).

1. Observation

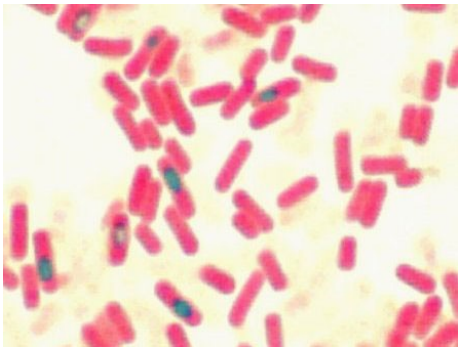
Spores are visible with microscope without staining. They appear as bright, shinning and refringent spaces.

Spores have a strong wall, so they are impermeable to colorants if they are used at room temperature. So, with Gram staining for example, the spore will be colourless while the rest of the cell will be pink or purple, depending on the type of bacteria.



Bacillus anthrasis and *Bacillus cereus*, Gram staining (x40)

Spore can be coloured with warm colorants: Benito staining uses warm Malachite green stain and fuchsine (not warm). The green warm colorant is able to go through the cell's wall and the spore's wall. At this point, the entire cell is green. Then, the fuchsine colours the cytoplasm but not the spore because of the wall of the spore. So, the cells will appear pink, with green spores.

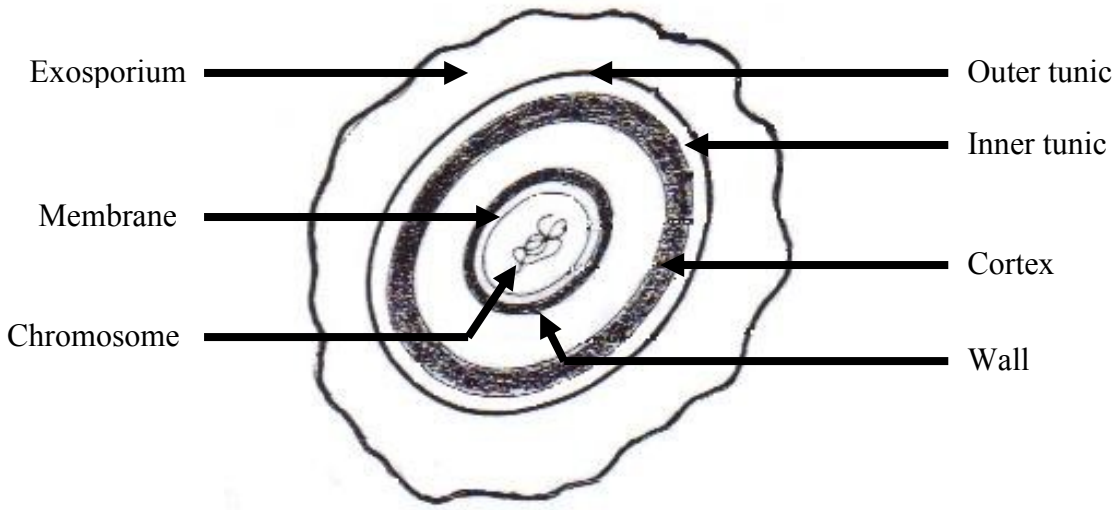


Bacillus subtilis, Benito staining (x40)

The Moeller staining can also be used, but it is more complicated because it uses chromic acid, warm fuchsine, absolute alcohol and methylene blue stain. At the end of the process, the cells are blue, and the spores are pink.

The spores are a tool to identify the species. The shape (round, oval), the location in the cell (central, terminal, subterminal) or the size (if the spore is bigger than the cell, it is called distorting spore) are more or less specific of a specie.

2. Structure



The spore has a lot of protection.

3. Properties

The spore is resistant to a lot of unfavourable conditions:

- High temperature: Thermo resistance: a spore resists up to 100°C temperatures, some of them even up to 120°C for several minutes (the most resistant resist to a 135°C temperature). The only way to kill every spore in a media is to autoclave them at 121°C for 20 minutes. Therefore they resist to pasteurization (around 80°C), drying and freeze drying processes, cooking (even when it is boiling) and warming.

- Cold temperature: Spores have been found in ice lands, and they can be freeze dried as said above.
- Physical and chemical agents: The spores are not killed by pH or pressure variations, they are resistant to UV and dehydration.
- Antiseptic and antibiotics: only sporicides kill them.
- Time: They live much longer than vegetative bacterial cell. The oldest spores were found in pyramids and are 4000 year old.

The resistance of a spore depends on several factors: species, age (when the spore gets old, it is less resistant), environmental conditions when it was formed (the richer the media the more the resistance)
The spore is resistant because of the number of envelops (tunics) and because it is very dry (there is no water inside, so there is no denaturation).

4. Spore formation: Sporulation:

Spores are formed when the environmental conditions become unfavourable: lack of nutrient, dehydration, accumulation of wastes which are toxic. It is a compound process, which is regulated by the genetic control.

Step 1: new organization of the cell with doubling of the chromosome (but no mitosis)

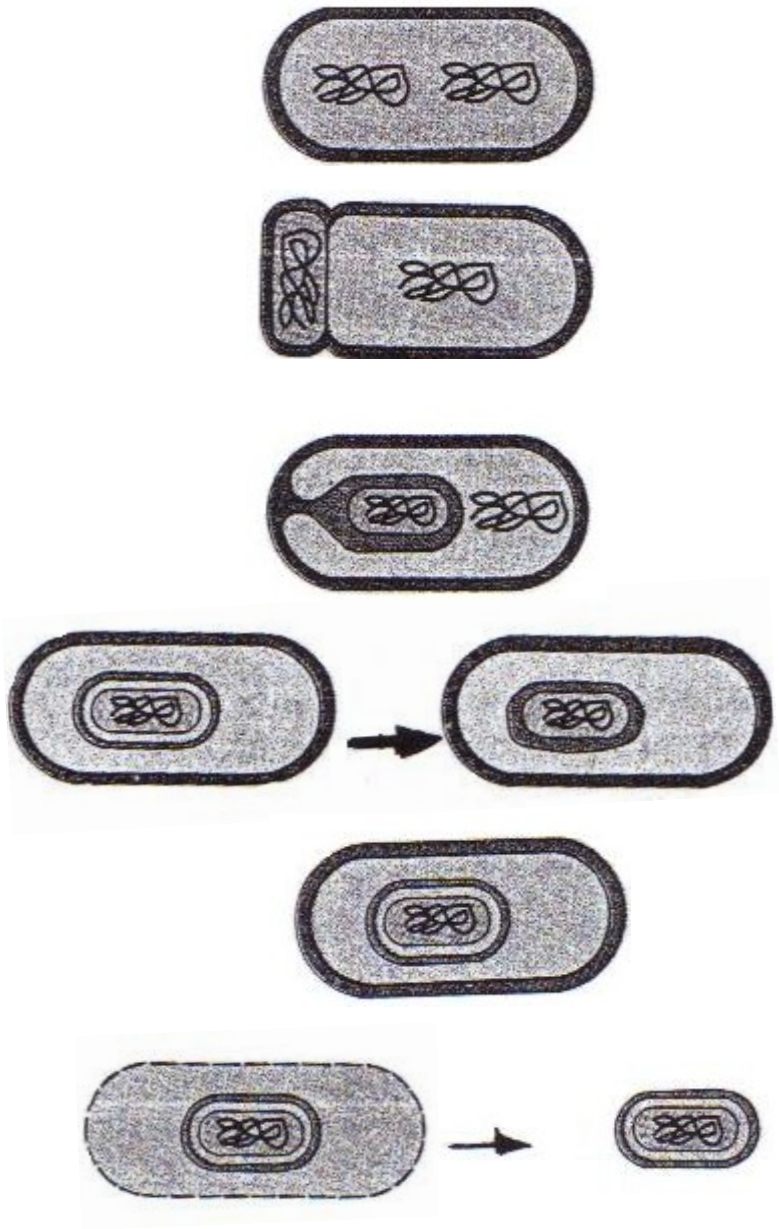
Step 2: scission of the second chromosome and formation of a septum (division) which is transversal and divides the bacteria in two unequal parts.

Step 3: formation of the "pre spore" with a distinct intracellular wall which is completely independent from the cell.

Step 4 and 5: formation of the others envelops: cortex, tunics (1 and 2), expsporium.

Step 6: maturation of the spore. It becomes highly refringent and dry.

Step 7: the cell dies and releases the mature spore, using enzymes able to lyses the cell from inside.

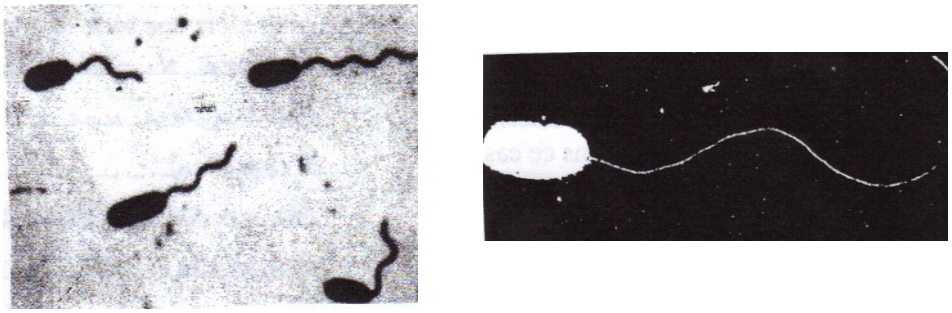


5. Germination of the spore

It happens when the conditions become favourable again: temperature, pH, nutrients…
The process lasts 40 to 60 minutes:
First, the spore gets wet, and swells. It gets back respiratory function, and metabolism (big enzymes synthesis). The envelop break and a new vegetative cell is born. This cell is exactly the same than the cell before the sporulation. It is able to divide itself, and the bacteria reproduce until a nutrients lack occurs. Then, the cell starts the sporulation again. This is a cycle process (from spore to spore).

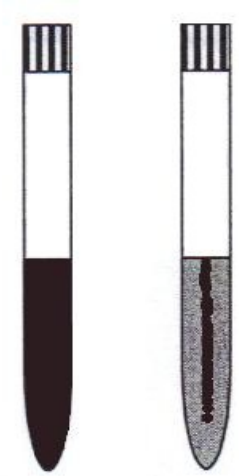
H. Flagella

They are inconstant and make the cell able to move. They can not be seen with a microscope without staining, but it is possible to see their effect: the mobility of the bacteria. To see them, it is necessary to make them bigger, with Silver, for example. They also can be seen with an electronic microscope.



Flagellum seen after Silver staining (left) and with a electronic microscope (right)

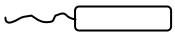
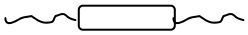
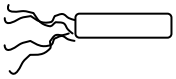
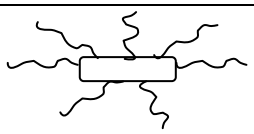
To assess if a strain is mobile or not, it is also possible to use a media, with a low concentration of agar, in a tube. The media is sowed by a straight sting in the middle of the tube. If the bacteria grow just near to the sting, they are not mobile. If they are, the growth is visible in all the tube.



Culture in all the tube: Bacteria mobile, with flagellum

Culture just near to the sting: Bacteria without flagellum

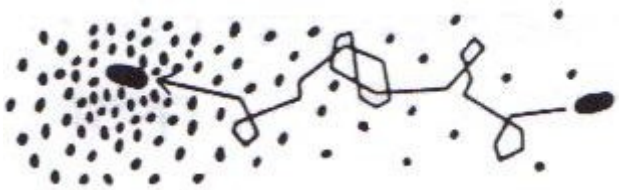
The flagellum is a sinuous and thin filament. It has a variable length, often longer than the cell itself, and it s diameter is the same all the long of the flagellum.
The flagellum turns as a propeller to allow the movement of the bacteria.
The way the flagellum is inserted in the cell is a tool for the identification:

Disposition		Way of moving	Example
One flagellum at one end		Straight	*Pseudomonas*
One flagellum at each end		Sinusoidal	*Bacillus megaterium*
Many flagella at one end		Sinusoidal	*Spirillum*
Many flagella all over the cell		Sinusoidal	*Bacillus*, Entérobacteria

The way the bacteria move is also depending on the composition of the media. If there is not attractive substances (nutrients for example), the bacteria move without specific direction. They turn around at random. If attractive substances are present in the media, the bacteria don't turn around (or less) and progress in direction of the signal.

No attractive substances

Attractive substances

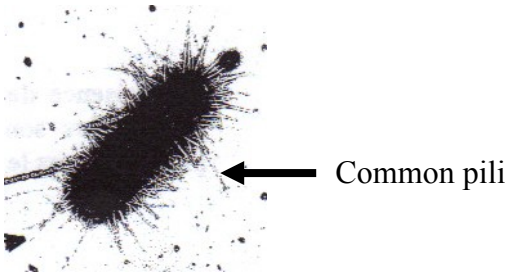


I. Pili

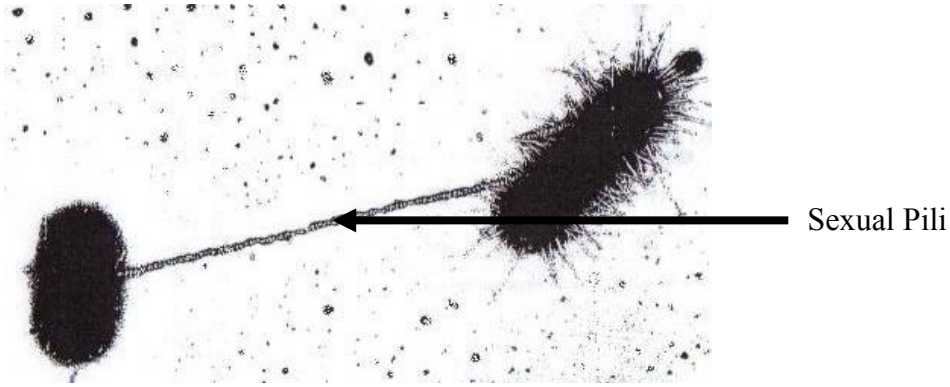
They are also inconstant organs and they are distinct from the flagella because they don't have any mobility function. They also are smaller and straight whereas flagella are sinuous.
There are two types of pili:

1. Fimbriae or Common Pili (CP)

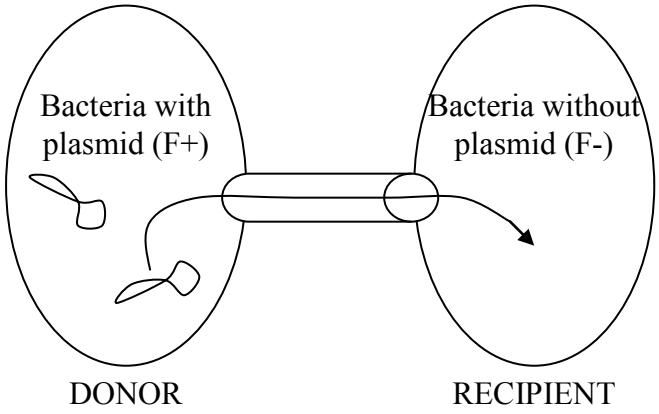
They are short and many, all over the cell surface. They take part of the adherence of the bacteria on a support (tissue of the host for example).



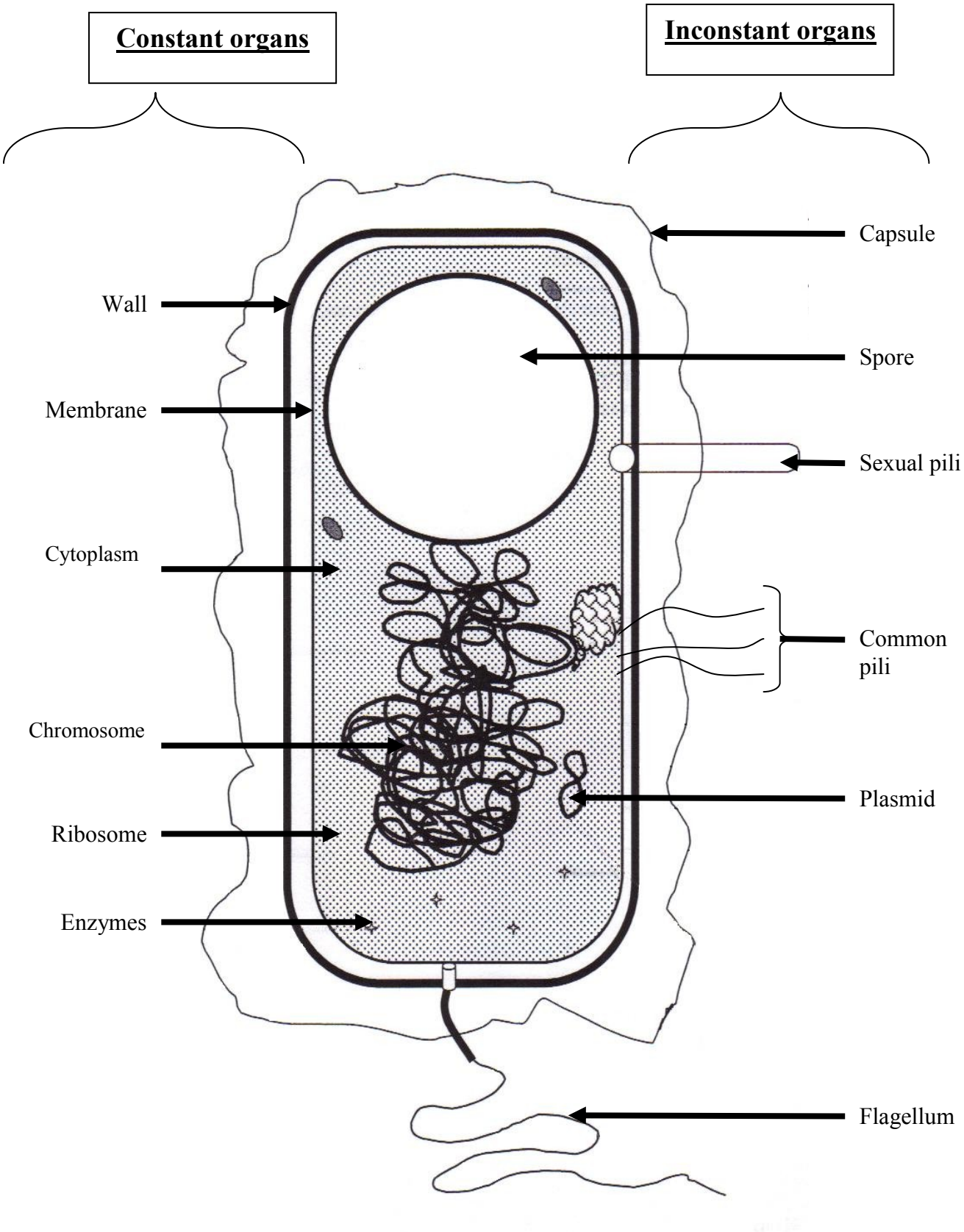
2. Sexual Pili (SP)



They are bigger and thicker and they have a tubulous structure (empty). A cell doesn't have many pili, from 1 to 4 approximately. They take part in bacterial mating which is a transfer of DNA (a piece of chromosome or a plasmid) between two bacteria.
A plasmid can easily be given from a bacterium to another which doesn't have it yet. The "donor" bacterium makes a copy of the plasmid which goes to the "recipient" one through the pili which is a kind of tunnel. At the end, both have the plasmid. This is the most common way for a bacterium to acquire new properties, as antibiotic resistance for example.



J. Conclusion



IV. Bacterial reproduction

A. Mechanism

Bacteria reproduce by binary division or scissiparity. The different steps are:

"Mother cell"

The cell becomes bigger because of wall and membrane development (from the mesosome which is a fold of the cytoplasmic membrane)

Replication of the chromosome

Separation of the chromosomes because of the mesosome. This is not a mitosis phenomenon.

The cytoplasmic membrane starts to separate the both chromosome

The wall covers progressively the membrane, forming a sort of partition called "septum".

Both cells are finished, and could (or not) separate from each other, if they cut the septum. This step, if present, is generally very slow.

Every cell formed is able to be a "mother cell" to give two new cells. This is a cycle process which can be more or less fast.
For *E.coli* for example, it takes only around twenty minutes.

B. Classification

If the bacteria don't separate at the end of the replication, they form some groups, which can be characteristic of the specie:

- If they separate: bacteria will be isolated
- If they don't separate and reproduce always in one direction: they will form chain
- If they don't separate and reproduce in two directions: they will form squares clusters
- If they don't separate and reproduce in three directions: they will form cubes
- If they don't separate and reproduce in every direction without order: they will form grape shaped cluster.

(See chapter on Morphology for more details)

A single cell reproduces until there are no more enough nutrients or until it is too old to grow. On solid media, a cell will reproduce to give a colony, which contains about 10^7 exactly identical cells. Then, the growth slowdown, and the bacteria die.
On liquid media, the growth is seen when the media becomes unclear.

V. Bacterial nutrition

A. Introduction

The nutrition of the bacteria includes the nutrients and the source of energy which help the growth and multiplication of the bacteria in a favourable environment.

A nutrient is a chemical molecule which allows the bacterial growth.
There are two types of nutrients:

- Elementary nutrients: water, carbon, nitrogen, sulphur, phosphorus, minerals.
- Necessary nutrients: growing factors which are not produced by the bacteria. Bacteria have to find them in their media.

To be used, nutrients have to enter into the cell, so they have to be able to go through the wall and the membranes.

Bacteria can use the light or a chemical source (oxido-reduction reactions) to produce its energy.

A favourable environment is defined by different factors such as pH, temperature, osmotic pressure, moisture, oxygen concentration etc.

Studying nutrition is useful because it helps in preparation of the best culture media for the bacteria, specific to their needs. It is also used to classify micro organisms.

B. Chemicals factors for nutrition

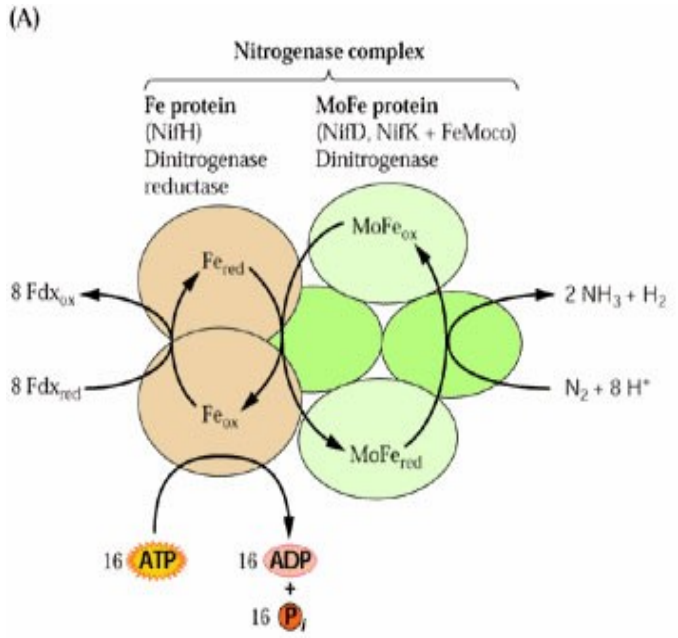
1. Elementary nutrients

- Water: It represents about 75% of the bacterial mass, and it is necessary to the bacterial multiplication process. So, bacteria can be conserved without water (freeze-drying) but rehydration is needed to allow growth again.

- Carbon: autotrophic bacteria use CO_2 as Carbon source whereas heterotrophic bacteria use Carbon from organic molecules (protids, lipids or glucids). Heterotrophs can use one or several molecules as Carbon source, depending on the species.

- Nitrogen: It is needed for the protein synthesis. Bacteria can find 3 Nitrogen sources.
    - Molecular Nitrogen N_2: atmospheric gas, it is used mainly by soil bacteria, like *Azotobacter* or *Rhizobium* for example, because of a nitrogenase.

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP$$



- Inorganic compounds : ammonium NH_4^+, nitrates NO_3^-, nitrites NO_2^- are used with two enzymes: nitrate reductase: NO_3^- → NO_2^- and nitrite reductase: NO_2^- → NH_4^+
- Organic molecules: peptone, urea…: different enzymes are necessary according to the molecules. For example, urease cuts the urea molecule: urea → $2NH_3 + CO_2$.

- Sulphur: it is necessary to produce few amino acids such as cysteine or methionine. Bacteria get sulphur from sulphates or organic compounds which contain sulphur.

- Phosphorus: it is a major part of nucleic acid and ATP/ADP (Adenosine Tri/Di Phosphate) and is mainly taken from inorganic phosphorus HPO_4^{2-}, H_2PO^{4-} or PO_4^{3-}. It is also needed to synthesize phospholipids and few coenzymes. Some phosphorus compounds can act as electrons donor, such as SH_2, or as an electron receptor in anaerobic respiration.

- Others minerals elements: the trace-elements are necessary in a very low concentration. They include sodium, magnesium, potassium, chlorine, which participate in the chemical balance of the bacteria, and iron, nickel, selenium, copper, zinc manganese, cobalt, molybdenum which are parts of many enzymes, pigments, vitamins or antibiotics.

2. Growth factors

Growth factors are organic substances which bacteria need to reproduce whereas they are not able to synthesize from elementary nutrients. So they have to find them in their environment.

They could be amino acids, vitamins, or bases for DNA and RNA…
These molecules are active in very low concentrations, and increase the growth correspondingly with their concentration, up to a limit.

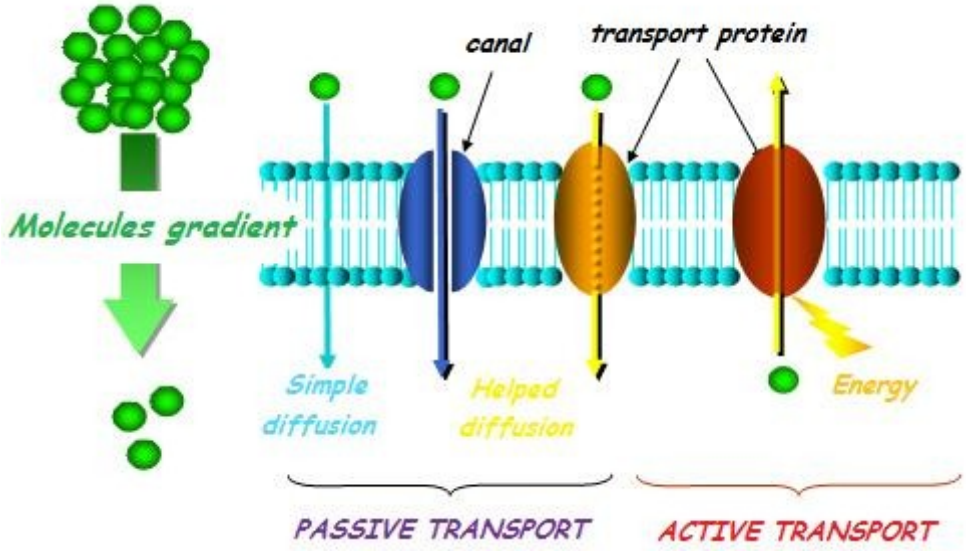
Bacteria which need these factors are called auxotrophs but they don't need the same compounds depending on their specie.
Some bacteria don't need any of these molecules and are called prototrophs.

3. Transport of the nutrients

To be used by the bacteria, the nutrients have to go into the cell, so they have to go across the membranes.
Two sorts of transport exist:

- Simple and helped diffusion: the molecule enters in the cell because of the difference of its concentration between the outside and the inside environments. The flow of the nutrients follows the gradient from the highest (out of the cell) to the lowest (in the cell) molecule concentration. This way concerns small molecules mainly (O_2 for example) but some bigger the molecules may use a canal (like porins) to enter into the cell. Then the diffusion is called helped diffusion. If molecules do not use these canals, it is a simple diffusion.

- Active transport: the molecules flow is opposed to the concentration gradient. This kind of transport needs an energy source (ATP, protons gradient…) and uses permeases or any other membrane transport proteins. The most known system is ABC transporters (ATP Binding Cassette Transporter) which is included in the membrane of many bacteria, as *Enterobacteriaceae*.



C. Physical factors for nutrition

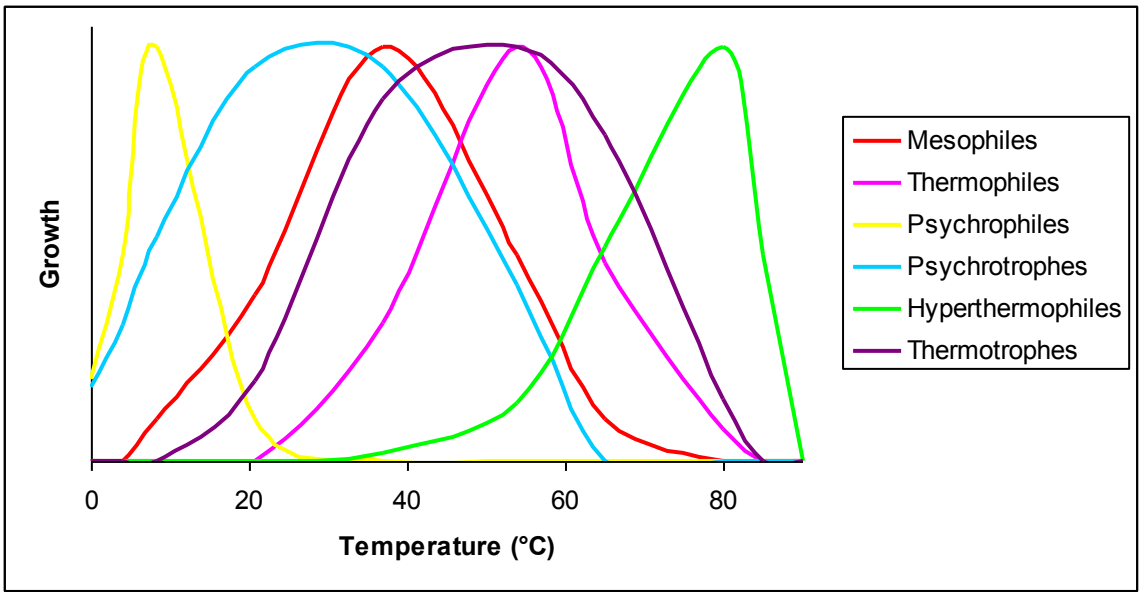
1. Temperature

Bacteria can be divided in three major groups depending on their optimal temperature to grow:

- Mesophiles: their optimum is between 20 and 45°C (Example : *Escherichia coli*),
- Psychrophiles: their optimum is around 10°C but they can grow down to 0°C (example : *Pseudomonas aeruginosa*),

- Thermophiles: their optimum is between 45 and 65°C (Example: *Clostridium perfringens).*

However, overlaps between these groups exist: psychrothrophes and thermotrophes have the same optimum with mesophiles, but psychrotrophes can grow down to 0°C; thermotrophes up to 80°C. Some bacteria are also able to grow in extreme conditions. For example, hyperthermophiles are able to grow up to 85°C.



2. pH

Most of the bacteria need a neutral pH around 7, but some tolerate a pH lower than 7 and are called acidophiles, or to a pH higher than 7 which are basophiles or alcalophiles. For example, *Vibrio* is able to grow at pH = 9. If the pH increases up to 11 and more, or decreases down to 3.2 and less, no bacteria are able to grow.

3. Activity of water

Activity of water (aw) corresponds to the quantity of water which is available for the bacteria growth.
It is always between 0 and 1.
Most of the microorganisms are unable to grow if aw < 0.86, and sometimes down to 0.6. But some species support a lower water activity: they are halophiles, such as *Pseudomonas* or *Staphylococcus*

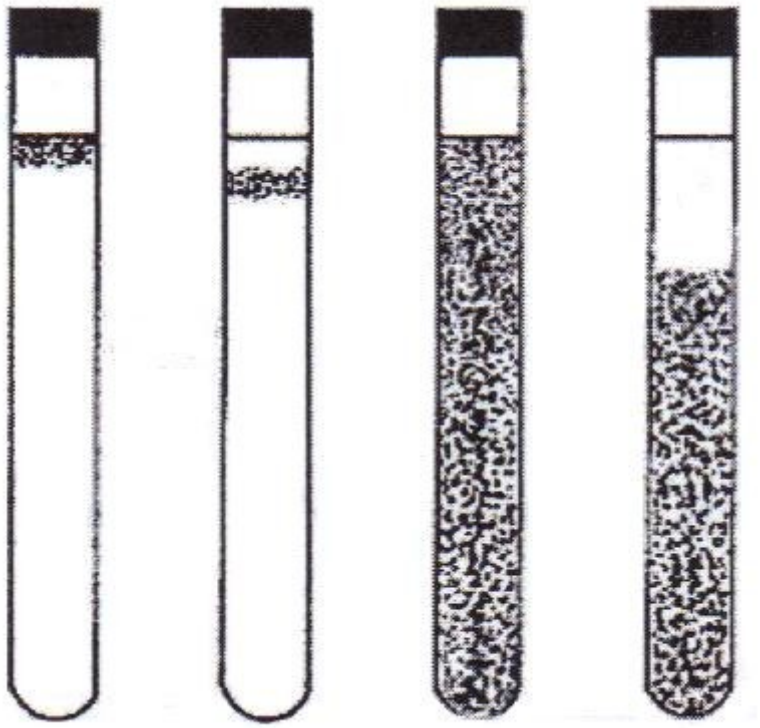
4. Oxygen

Bacteria have different needs in oxygen:

- Obligate Aerobic bacteria: they can't survive without O_2

- Microaerophile: they support a low concentration of O_2 only (around 2 to 10%). They can't grow without any O_2, neither with a too high concentration.
- Obligate Anaerobic bacteria: They only grow in environments where O_2 is completely absent.
- Facultative Anaerobic bacteria: these bacteria can grow with or without O_2. Some of them are just O_2 tolerant: they support the O_2 in their environment, but they don't use it to their growth.

To determine the respiratory type, (so the O_2 tolerance), a media is made with glucose, liver and meat and some agar, and pour into long and thin tubes.
The media is kept for a while in a water bath (about 20 min) to make it liquid and to eliminate the O_2 in the bottom of the tube. An O_2 gradient is formed from the top where the O_2 concentration is the highest to the bottom where there is almost no O_2. Before sowing, the media has to cool down a little bit (or the bacteria will die) but has to stay liquid.
With the loop, the bacteria are sown by a straight movement down to the bottom of the tube, and then the loop is removed by a curl movement to inoculate all the tube without adding O_2 in the lower part. Then, the media becomes solid and is incubated for few days (depending on the bacteria you are using). The way the bacteria grow indicates the way they use (or don't use) O_2:



A: growth at the top of the tube only:
Obligate Aerobic bacteria

B: growth in the upper part of the tube, but not at the extreme top:
Microaerophile

C: growth in all the tube:
Facultatively Anaerobic bacteria

D: growth in the lower part of the tube only:
Obligate Anaerobic bacteria

- Why is O_2 toxic for bacteria?

O_2 is essential for the life of many organisms, but it is first a very toxic compound because it is very easily reduced, producing toxic molecules as hydrogen peroxide, superoxide or hydroxide.

These molecules can oxidize and destroy the components of a cell. To survive in an environment where O_2 is present, the organisms have developed several protection mechanisms: the major one is the use of enzymes which are able to transform these toxics: superoxide dismutase, catalase or peroxidise.

Anaerobic bacteria don't have these protection mechanisms, that's why they can't grow or survive in an aerobic environment.

D. Energy source

There are two major energy sources: light and chemical source, used to classify the bacteria:

- Chemotrophic bacteria: use chemical energy, oxidizing mineral or organic compounds which act as electrons donor. If the bacteria use organic source, they are called chemo organotrophic bacteria. Many molecules used in medicine as antibiotics are produced by this kind of bacteria. If the compound is mineral, the bacteria are chemo lithotrophic. They are involved in the transformation of the matter in soils and water.
- Phototrophic bacteria: they use the light to produce energy by the photosynthesis. If the electrons donor is an organic compound, the cell will be called photolithotrophic, and photoorganotrophic if it is an inorganic compound.

E. Conclusion

Group of bacteria	Considered element	Meaning
Autotrophic	Carbon source	CO_2 as Carbon source
Heterotrophic		Organic compounds as Carbon source
Auxotrophic	Growth factor	Needed
Prototrophic		Not needed
Phototrophic	Energy	Light as source
Chemotrophic		Chemical molecules as source
Lithotrophic	Nature of electrons donor	Mineral
Organotrophic		Organic
Obligate Aerobic	Respiratory type	O_2 needed in high concentration (about 20%)
Microaerophile		O_2 needed in low concentrations (2 and 10%)
Facultatively anaerobic		With or without O_2
Obligate Anaerobic		No O_2
Mesophile	Temperature	Optimum between 20 and 45°C
Thermophile		Optimum between 45 and 60°C
Thermotrophe		Optimum between 20 and 45°C but growth up to 65°C
Hyperthermophile		Optimum between 65 and 85°C
Psychrophile		Optimum around 10°C
Psychrotrophe		Optimum between 20 and 45°C but growth up to °C
Acidophile	pH	pH lower than 7
Alcalophile (or Basophile)		pH higher than 7

VI. Bacterial Metabolism

A. Definitions

We will just study the metabolism of chemo organotrophic and heterotrophic bacteria which use organic molecules as electrons donor to produce chemical energy (oxido-reduction reactions) and Carbon sources.

- Metabolism: it includes every chemical reaction which occurs in the bacterial cell, such as:
    - Catabolism: degradation of nutrients, producing chemical energy,
    - Anabolism: synthesis of the different bacterial organs and constituents, using energy produced by catabolism.

Catabolism is used as a tool to identify bacteria. That's why this chapter will just focus catabolism and not anabolism.

- Catabolism: for heterotrophic bacteria, it represents every enzymatic reaction, divided into three steps:
    - Exogenous organic substances digestion: glucids, lipids, proteins which are too big to enter into the cell are cut by enzymes produced by the bacteria, and which are secreted out of the cell, in the media. They are hydrolysed in smaller and simpler elements by enzymes called hydrolases.
    - Degraded products go into the cell: it is possible by two ways, helped or simple diffusion, and active transports (see nutrition chapter for details). For the helped diffusion, some proteins are needed as permeases. They are expressed only if the substrates are present in the media, and only if the bacteria need it.
    - Carbonated substrates degradation: the aim is to produce enough energy which is necessary to synthesize every cellular component.

B. Energetic metabolism

Nutrition produces chemical energy which is needed for life and growth of the bacteria. Chemical energy is contained in atomic or molecules bonds. It is released when these bonds are broken.

1. Oxido-reduction reaction, transfer of electrons

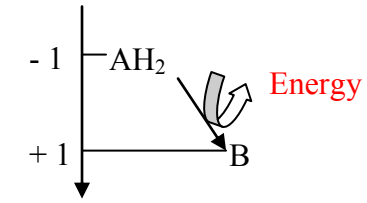
Microorganisms produce energy using oxidation of different kinds of organic or inorganic molecules. Those reactions need an electrons donor, which is the oxidized molecule, and a receptor, which is reduced and receives the electrons. Acceptors are also called coenzymes. Their nature depends on the reaction, but the most known are NAD, NADP, FAD or CoA.

If AH_2 is a molecule (donor) and B a coenzyme (receptor) the reaction occurs as following:

$$AH_2 \rightarrow A + 2H^+ + 2e^-$$
$$B + 2H^+ + 2e^- \rightarrow BH_2$$
$$\text{So } AH_2 + B \rightarrow BH_2 + A + \text{energy}$$

$\Delta G = - nF \Delta E$
nF = constants
ΔE = oxido-reduction potential difference

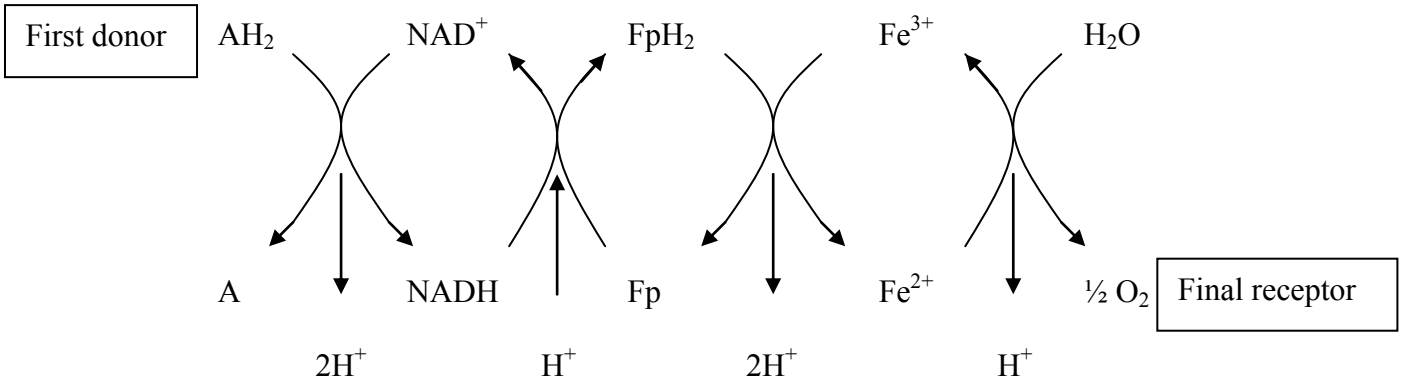
if $\Delta G < 0$ => energy is produced, if $\Delta G > 0$ => energy is consumed.



Oxido–reduction potential (E)

$\Delta E = E_B - E_{AH2} = 1 - (-1) = 2$
$\Delta E > 0$ so $\Delta G < 0$
→ Reaction releases energy

Then, the receptor can act as a donor, and makes a reaction in chain, such as the respiratory chain:



2. Oxido-reduction and chemical groups transfer

Transfer of electrons is not the only way to produce energy. It is also possible to transfer some chemical groups which are very energy giving. The mainly one which has a lot of electrons is phosphate, principally in ATP (Adenosine Tri Phosphate) which becomes ADP (Adenosine Di Phosphate), giving one Phosphate: ATP → ADP + Pi

3. ATP genesis

ATP can be formed by two ways in heterotrophic bacteria:

- Substrate phosphorylation: While a substrate is oxidized, an inorganic Phosphate is attached to an electrons receptor because of an enzyme. This receptor transfers this Phosphate to an ADP to make an ATP: ADP + Pi → A-P~P~P. The P bonds are very energetic.

- Oxidative phosphorylation: ATP can be synthesized during an oxido-reduction reaction during the respiratory chain for example, because of an enzyme, the ATP synthase.

4. Respiration and fermentation

Molecules degradation process produces reduced coenzymes, such as NADH. These coenzymes are re oxidized by a transporter chain, such as respiratory chain. For aerobic bacteria, the process takes place in plasmic membrane, and the final receptor is oxygen. This is an aerobic respiration.
Respiratory chain (or oxidative phosphorylation) is composed of several proteic complexes, four or five depending on the bacteria. During the process, protons gradient is created, which is an energy source to produce ATP at the end of the chain, because of the enzyme, the ATP synthase.
Bacteria which have the entire respiratory chain are called positive oxidase (cytochrome oxidase is the enzyme used in complex IV). Negative oxidase bacteria have a smaller chain, without the complex IV.
In few bacteria, the final receptor can be another molecule than oxygen, such as nitrate (NO^{3-}) or sulphate (SO_4^{2-}). It is an anaerobic respiration and this kind of process produces less energy than aerobic respiration.

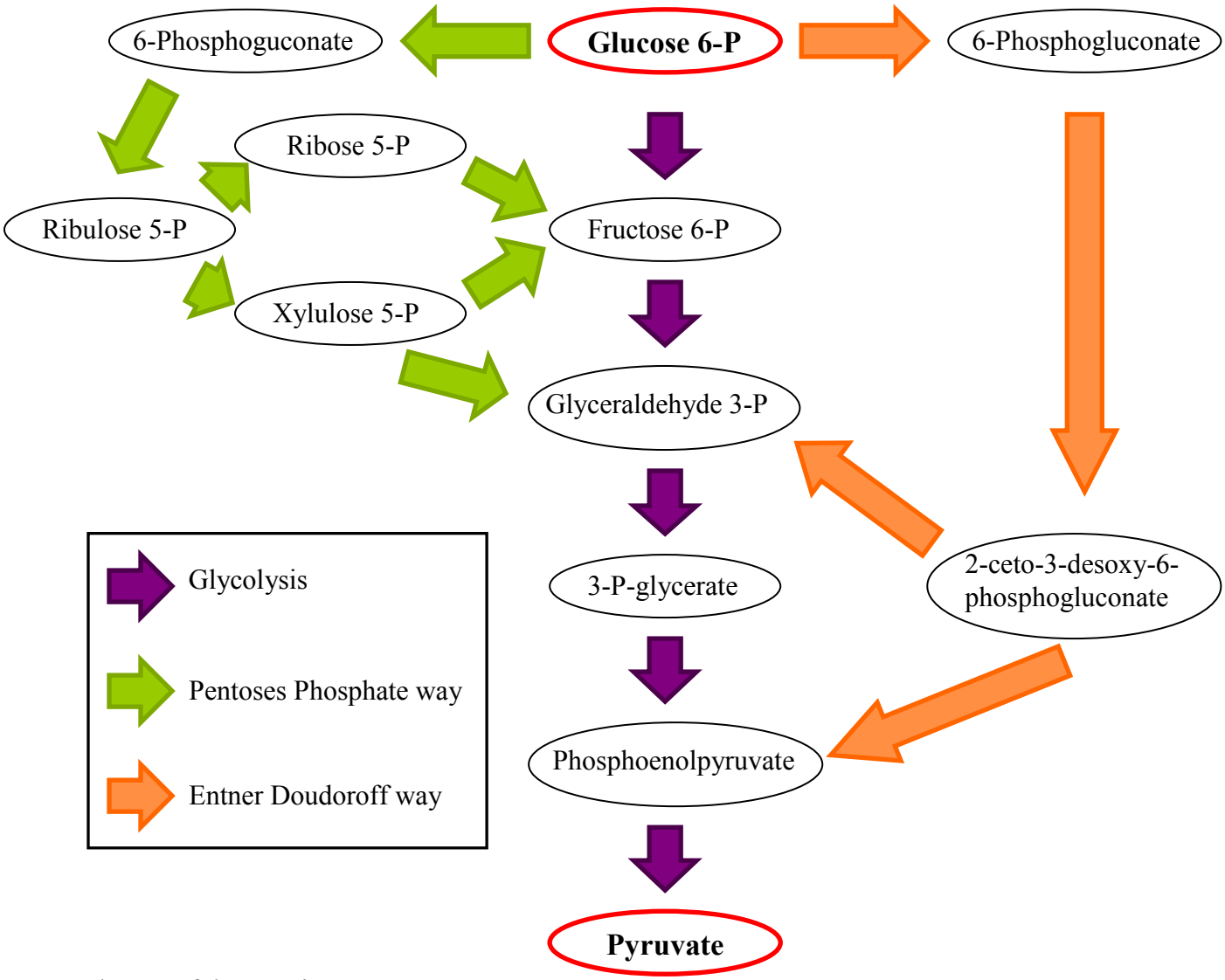
If O_2 is completely absent in the environment, some bacteria are able to produce energy and ATP from a different process, using carbon source, such as glucose most commonly: fermentation. This is an oxido-reduction and the final receptor is the final product (most of the time). The carbon source is partially oxidized and energy amount produced is low.
Different types of fermentation exist, and they will be seen later.

C. Glucidic metabolism

Glucids are big molecules which need to be cut in smaller and smaller molecules to be used by the cell by many ways.
An enzyme family called glucidase is active on holosides, heterosides (glucosidases), starch (amylase), cellulose (cellulases), pectins (pectinases), or glycogen (glycogenase).

1. Glucose degradation

Glucids are broken in simple sugars, mainly in glucose, which is the leading glucidic metabolite for the catabolism of the bacteria. When the glucose ($C_6H_{12}O_6$) is into the cell, it has to be phosphorylated to be used by the different pathways: glycolysis, pentoses Phosphate way, Entner Doudoroff way.



Balances of the reactions:

Glycolysis:

1 Glucose-6-P + 2 ADP + 2 Pi + 2 NAD^+ → 2 Pyruvate + 2 NADH, H^+ + 2 ATP

Pentoses Phosphates pathway:

3 Glucose-6-P + 6 $NADP^+$ + 3H_2O → 2 Fructose-6-P + Glyceraldehyde-3-P + 3CO_2 + 6 NADPH, H^+

Entner Doudoroff way:

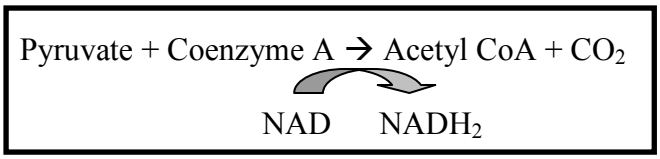
1 Glucose-6-P + 1 NADP + 2 ADP + 1 NAD^+ → 2 Pyruvate + 2 ATP + 1 NADPH, H^+ + 1NADH, H^+

Glycolysis is the main way, but it can be passed around if a molecule can not be produced (such as fructose for example).
Entner Doudoroff way is specific to the bacteria.

2. **Pyruvate oxidation**

- Krebs cycle

At the end of the glycolysis, Pyruvates (CH_3-CO-COOH) are made and are used to transform a coenzyme (CoenzymeA) into AcetylCoA which is the molecule used to start the Krebs cycle:



There are 10 steps in the Krebs cycle, producing at every cycle many reduced coenzymes ($NADH_2$, $FADH_2$), which are very energy giving (Thauer, 1988). It ends the degradation of the glucose, using the pyruvate to produce CO_2, ATP, H_2O and coenzymes. This cycle takes place in aerobic conditions only, and is closely linked to the respiratory chain, which reduces the coenzymes produced during the Krebs cycle to produce even more ATP.

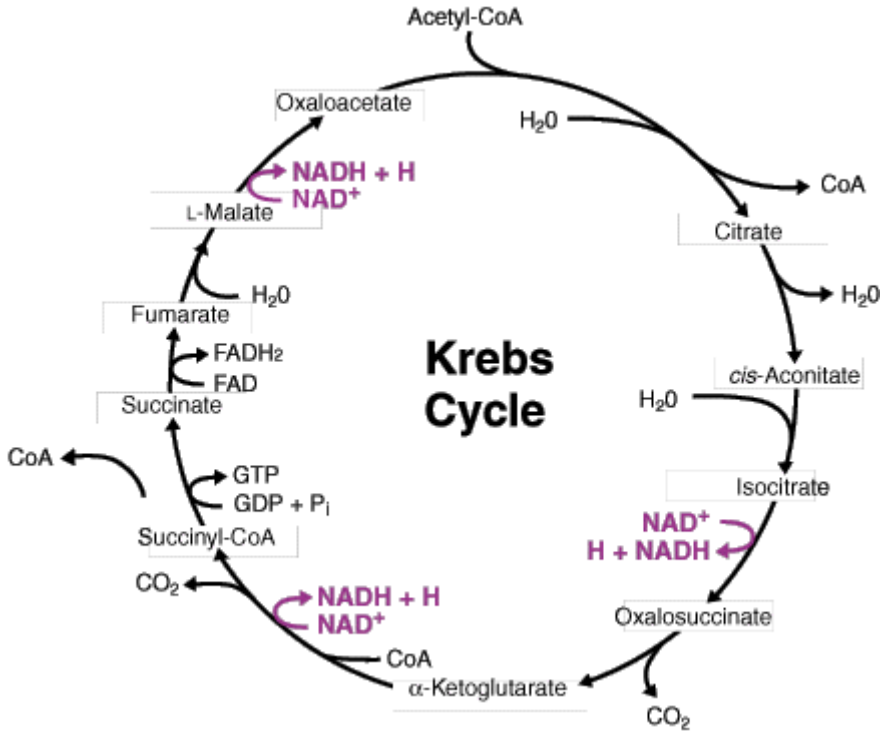


Fig. The Krebs cycle is a series of enzyme-catalyzed reactions, three of them involving NAD, that transform chemical energy stored in the foods we eat into energy in a form that cells can use. The ultimate product, although it does not appear in the Krebs cycle itself, is the energy-rich molecule ATP

Source: Block, 2000

In aerobic conditions, electrons and protons produced during the Krebs cycle are used for the respiratory chain. This one is the most energy giving reaction. Reduced coenzymes $NADH_2$, $FADH_2$ are reoxidized, producing respectively 3 and 2 ATP. So, at the end of one cycle in the Krebs cycle, for each Acetyl CoA, 12 ATP are produced.

The reaction from the Glucose to the end of the respiratory chain is:

$$6\ C_6H_{12}O_6\ (Glucose) + 6\ O_2 \rightarrow 6\ H_2O + 6\ CO_2 + 38\ ATP$$

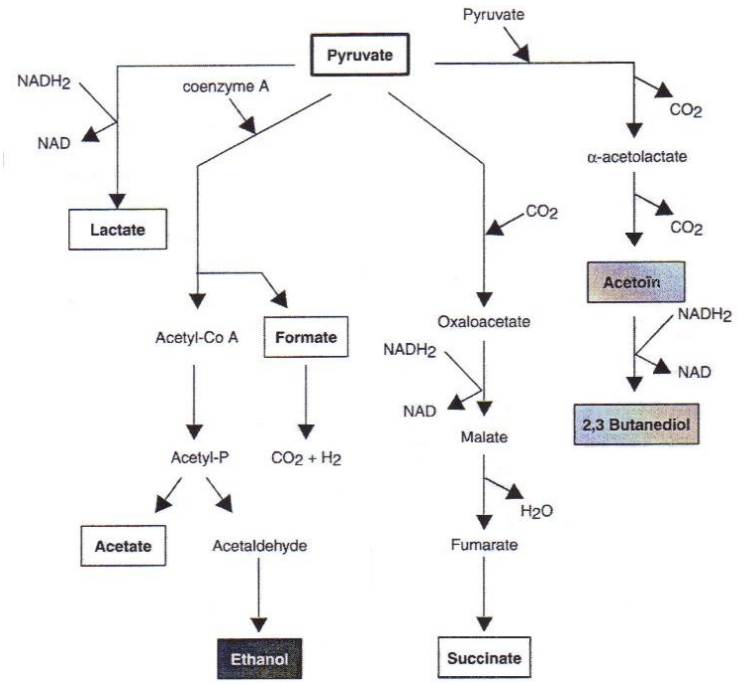
- Why 38 ATP?

Glycolysis produces 2 ATP and 2 NADH, H^+. These NADH, H^+ will be reoxidized, producing 6 ATP. So glycolysis produces 8 ATP.
The reaction 2 pyruvates → 2 Acetyl CoA produce 6 ATP (reoxidation of 2 NADH,H^+)
The degradation of the 2 molecules of Acetyl CoA through the Krebs cycle produces 24 ATP.
So, from Glucose to the end of the reaction: 8 + 6 + 24 = 38 ATP.

- Fermentations

They all start with the pyruvate. There are different types of fermentations: mixed: If there is a single product at the end of the process, the fermentation is called homofermentation. If there are several products, this is a heterofermentation. Sometimes, fermentations also produce gas (CO_2, H_2 or CH_4), which can be a tool for the identification.

Lactic fermentation for example: Lactose is hydrolysed to form glucose and glucose is destroyed to form pyruvate. Degradation of pyruvate produces lactic acid only if the pathway is homofermentation. Others products such as ethanol, CO_2, aromatic molecules, acetate… can be formed by different pathways: tagatose pathway, pentose pathway…

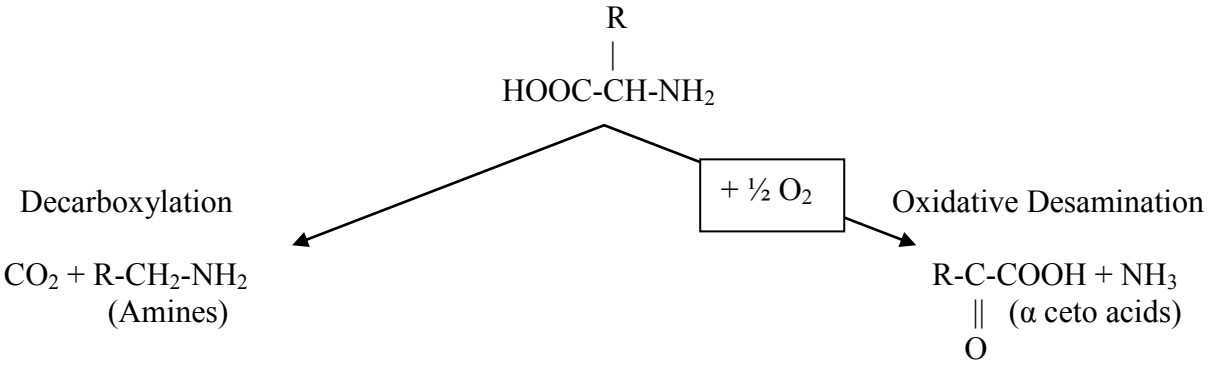


D. Proteic metabolism: Protein degradation and amino acids catabolism

Proteins are broken in small peptides then in amino acids because of enzymes, proteases and peptidases.

These enzymes can be used as a tool to identify micro organism. For example, *Bacillus* is able to hydrolyse casein and gelatine because of caseinase and gelatinase respectively.

Amino acids produced because of the proteins degradation are metabolised by oxidative desamination or decarboxilation.



E. Lipidic metabolism: Lipids degradation and fatty acids catabolism

Lipids are hydrolysed to produce glycerol and fatty acids because of extracellular lipases.

CH_2O-CO-R1		CH_2-OH		R1-COOH
CHO-CO-R2	→	CHOH	+	R2-COOH
CH_2O-CO-R3		CH_2OH		R3-COOH
(Triglyceride)		(Glycerol)		(3 fatty acids)

Then, glycerol is phosphorylated and becomes integrated into the glycolyse reaction. Fatty acids are transformed by coenzyme A and oxidized, producing acetyl CoA which is the start molecule of Krebs cycle.

F. Metabolism as a tool for bacteria identification

To identify the bacteria, metabolism can be very helpful, and more specifically the enzymes involved in metabolism.

1. Bacterial enzymes

Bacterial enzymes are biological catalysts, which are able to increase the kinetic of chemical reactions. An enzyme is specific for a reaction and a substrate, and is active in very low concentration, even if it sometimes needs cofactors.
The enzyme allows the reaction: Substrate (S) → Product (P)
This reaction is reversible but can be irreversible if the product of the first reaction is used as substrate for a second reaction: S → P_1 → P_2

The speed of the reaction depends on environmental pH, temperature and concentrations in enzyme and substrate.

Enzymes are classified depending on:

- The kind of reactions they catalyse: permeases (transport), synthetases and ligases (formation of bonds), hydrolases (break of bonds)…
- Their localisation in the cell: exoenzymes (extracellular), endoenzymes (intracellular)
- Genetic expression: constitutive enzymes (genes encoding for them are always expressed, so they are always produced by the cell), induced enzymes (are produced when the cell needs them, if the substrate is present in the environment or if it needs the product of the reaction)

2. Revelation of an enzyme presence

A product "A" becomes "B" because of the enzyme "E". It is possible to reveal the disappearance of A, or the appearance of B. That means that A and B have to be easily differentiated:

- They can have a different colour: A $\xrightarrow[\text{(enzyme)}]{}$ B (coloured)

  A $\xrightarrow[\text{(enzyme)}]{}$ B $\xrightarrow[\text{(+ reagent)}]{}$ C (coloured)

  A $\xrightarrow[\text{(enzyme)}]{}$ B ; rest of A + reagent $\rightarrow$ C (coloured)

- They can have a different pH: some molecules have a different colour depending on the pH: they are called pH indicator. For example, the BBT (Blue of Bromothymol) has a blue colour in basic or neutral pH, and is yellow if the pH is acidic. So, if A is basic and disappears to produce B which is acidic, the colour of the media will change. Some of the most commonly used indicators are given in the following table:

Indicator	Colour if pH< pH_c	pH of change (pH_c)	Colour if pH> pH_c
Blue of Thymol	Yellow	1.2 - 2.8	Blue
Blue of Bromophenol	Yellow	3.0 – 4.6	Blue
Red of Methyle	Red	4.4 – 6.2	Yellow
Blue of Bromophenol	Yellow	6.2 – 7.6	Blue/Green
Red of Phenol	Yellow	6.8 – 8.2	Red
Red of Cresol	Yellow	7.0 – 8.8	Red
Phenolphtaleine	Colourless	8.0 – 10.0	Red/Pink

- Another substrate is recognized by the same enzyme and produces a coloured product: A second substrate is added in the media, and the both reactions will occur during the growth of the bacteria. The first reaction will not be seen, but it is assumed that if the enzyme is produced, it catalyses the both reactions.

$$A \xrightarrow[\text{(enzyme)}]{} B \quad \text{and} \quad C \xrightarrow[\text{(enzyme)}]{} D \text{ (coloured)}$$

- Antibody – Antigen reaction: Some enzymes are able to make organisms produce antibodies (pathogenic potential). These antibodies are used to see the enzymes by coloured or agglutination reactions.

VII. Growth of microorganisms

The life of a bacterial cell is feast or famine depending on availability of growth factors. To survive the bacterium must rapidly adapt to changing environmental conditions. Nutrient-rich conditions lead to a decrease in mass doubling time and an increase in cell size, whereas nutrient poor conditions curtail growth and reduce cell size (Fantes et al. 1977; Schaechter et al. 1958). Changes in growth rate must be accompanied by changes in the cell cycle to ensure that cell division stays coordinated
with mass doubling, chromosome replication and chromosome segregation.

A. Definitions and general information

- Growth: orderly increasing of every component of an organism. As every cell, a microorganism increases its weight and size. As they are really small, to assess their growth, it is easier to measure the result of the growth and not the growth itself.

So, growth study in microbiology is the study of the evolution, down the time, of a homogeneous population composed of strictly identical organisms (pure strain).

The study of the growth helps to understand and assess the needs and the physiological and biochemical characteristics of a microorganism in order to identify it. It also allows the massive production of many organisms, or substances produced by them (proteins, antibiotics for example). That's why it is necessary to define exactly the best growth conditions to get the best yield, growth rate and generation time.

- Differential equations of the growth law: in a liquid media, the total number of cells per mL increases of dX during a time dt. The speed of the growth is proportional to the population:

$$\boxed{\frac{dX}{dt} = \mu X} \quad \text{or} \quad \ln(X) = \mu.t + \text{constante (C)}$$

For $t = 0$: $\ln(X_0) = 0 + C = C$

So $\ln(X) = \mu.t + \ln(X_0)$ or $\boxed{X = X_0.e^{-\mu t}}$

- The director factor, μ, is called napierian growth rate: $\boxed{\mu = \frac{\ln(X/X_0)}{t}}$

- Generation time Tg or G: it is the interval between two successive divisions of a cell. So it is the time needed to multiply the population by two:
  so $X/X_0 = 2$ and $\mu = \ln(2)/Tg$

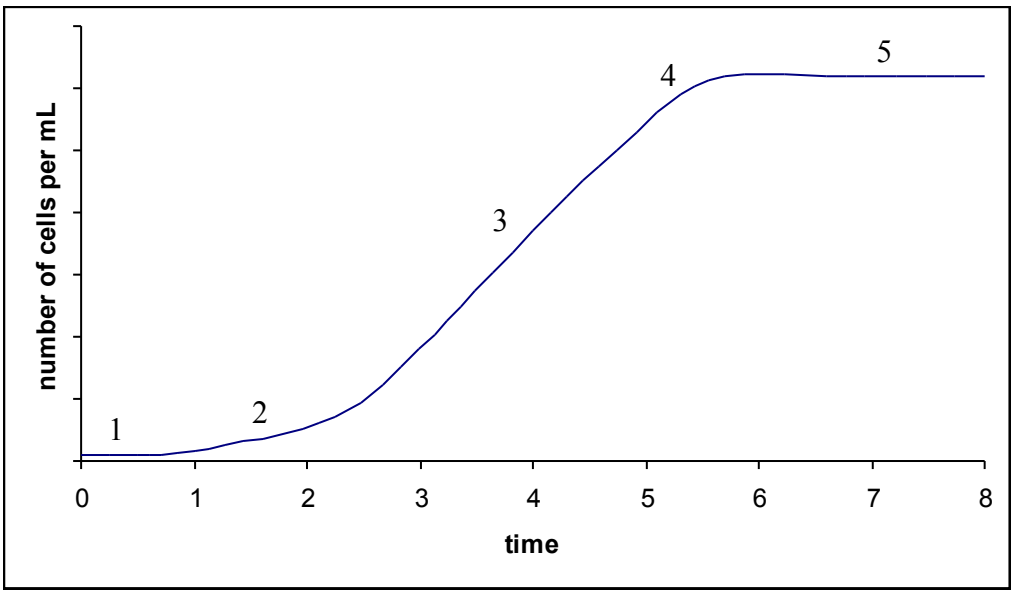
$$\boxed{Tg = \frac{\ln(2)}{\mu}}$$

- Growth rate r: number of generation by unit of time (hour for example).

$$r = \frac{1}{Tg} \quad \text{or} \quad \mu = r \times \ln(2)$$

B. Physical and chemical factors influence

Many parameters have a significant impact on the culture, such as temperature, pH, osmotic pressure, or oxygen concentration.
If the conditions are optimized, the growth curve has the aspect of the following curve:



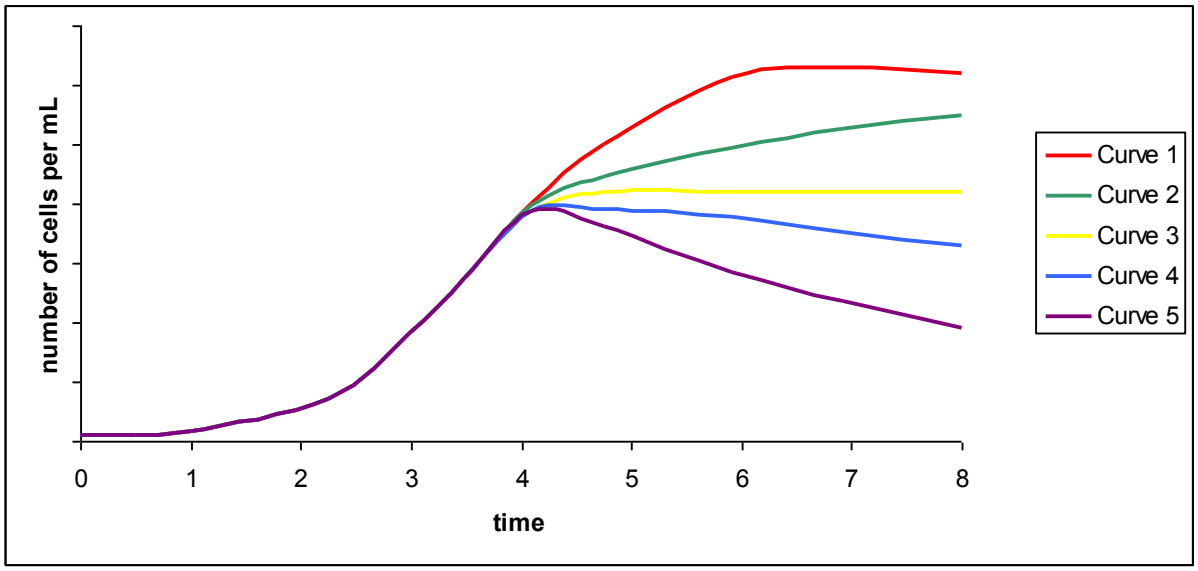
The growth can be broken down into 5 phases:

- Latency: cells have to adapt to the culture conditions and to the media composition which represent a stress for them. There is no multiplication so the cellular concentration stay stable (1)
- Acceleration: cells start to grow. Growth rate increases from 0 to a maximal value μ_{max} and Tg decreases down to its minimal value Tg_{min} (2)
- Exponential growth: cells are growing the fastest they can: $\mu = \mu_{max}$ constantly and $Tg = Tg_{min}$. This is the part used to do the different calculations (3)
- Slowing down phase: bacteria have used almost all the nutritive compounds in the media. Nutritive elements decrease and the molecules produced by the bacteria increase, such as toxins. Growth rate decreases progressively down to 0, so Tg is inclined to get very high value, even up to everlasting (4)
- Stopped growth: Cells don't develop anymore but there is no cellular lyses and the concentration stay stable (5)

If a factor is modified during the growth, the curve will be affected. This change can have different effects, as seen on the following curves:

- Curve 1: optimized conditions

- Curve 2: slowing down action: director factor μ slows down, but the bacteria are still able to develop. It might happen when a slight change occurs, or if some toxic molecules are in the media (but just traces).
- Curve 3: bacteriostatic action: the growth is stopped, so μ=0, but the bacteria don't die. Examples: low temperature conservation, food conservatives…
- Curve 4: bactericidal action: The growth is stopped, and the cells start to die. It could be because of antiseptics or antibiotics (at low concentration), or conservatives in high concentration.
- Curve 5: bacteriolytic action: microorganisms are destroyed: high temperature increasing, antiseptic and antibiotics at use concentration.



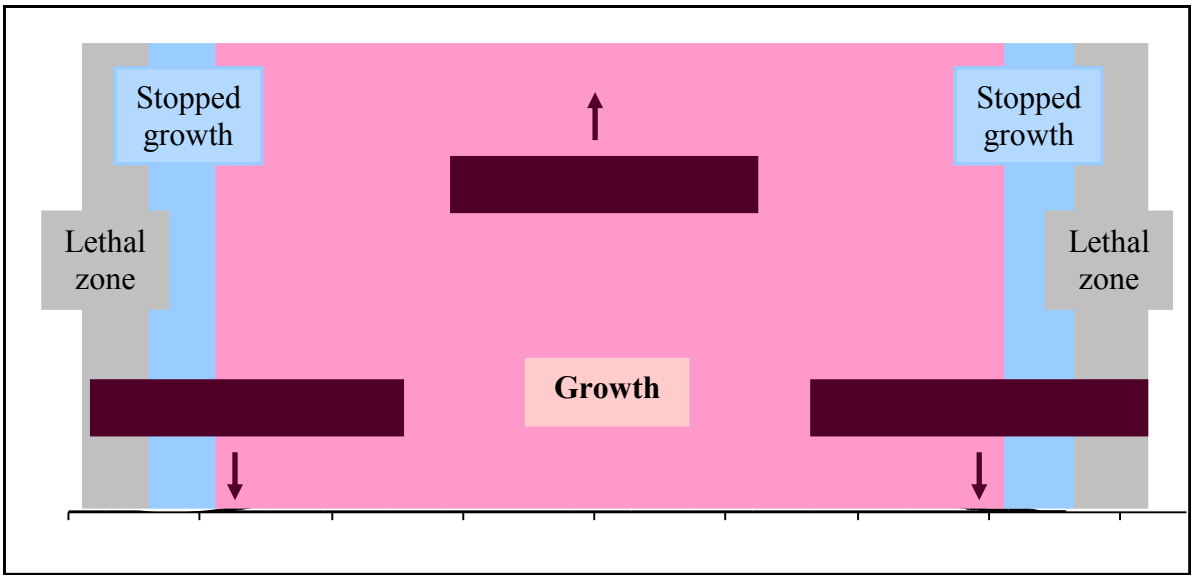
To assess the growth rate and generation time values, the curve log (X) = f (t) has to be built. The director factor of the linear curve is calculated with two points, A(xa;ya) and B(xb;yb) and represents the growth rate r :

$$r = \frac{|y_b - y_a|}{|x_b - x_a|}$$

Tg is calculated with the relation Tg = 1/r.

1. Temperature (see Nutrition Chapter also)

Temperature has an effect on metabolic reactions speed. If the change is moderate, the growth will just slow down. But if it is too important, the growth can stop.
Every microorganism behaves differently regarding to the temperature. It is necessary to define the "optimal growth temperature", specific to one specie. This temperature is got when the director factor of the growth curve is the highest: $\mu = \mu_{max}$. Near to this temperature, the bacteria will develop but at a slow rate. Minimal and maximal temperatures can also be defined. Under and above these temperatures respectively, the growth is stopped. The microorganisms are able to survive but can not develop down or up to the lethal temperature, where the bacteria are lysed.

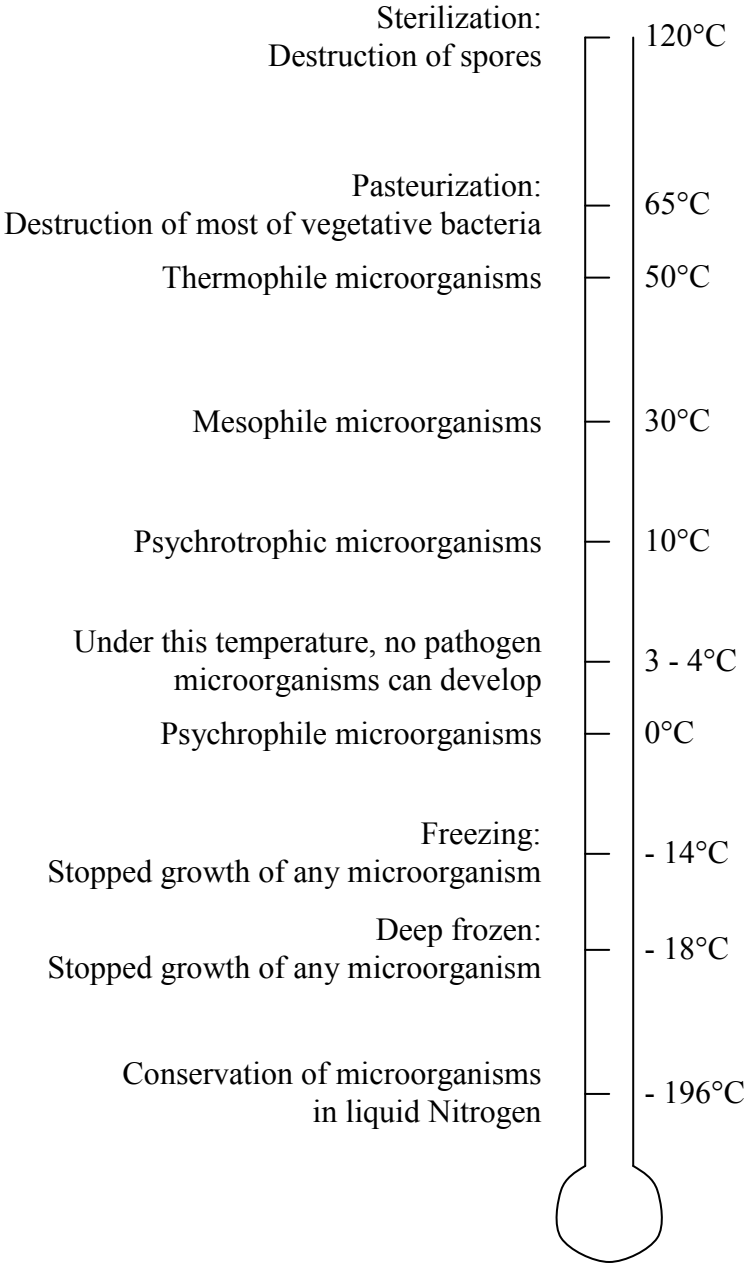


Depending on the value of the optimal temperature, microorganisms are classified into three major groups:

- Mesophile bacteria: They grow between 20 and 40°C approximately.
- Psychorphile bacteria: They develop under 10°C down to 0°C.
- Thermophile bacteria: They prefer higher temperature, from 45 up to 60°C.

These groups can overly and some groups can be created with bacteria which are more tolerant: psychrotrophic bacteria for example, are able to grow between 4°C up to 45°C, or the thermotrophics, which support temperature between 20 and 70°C.

It is interesting to know how bacteria behave according to the temperature to be able to make them grow with the best yield, or to the contrary, to avoid their development, for example in the food industry.



2. pH (see Nutrition Chapter also)

Most of bacteria need a pH between 6.5 and 7.5 but as for the temperature, the limits are not strict and many bacteria can develop when the pH is acid or alkaline. It is necessary to define the optimal, minimum and maximum pH. It is admitted that if the pH is lower than 5, no pathogen is able to grow, and many toxins productions are inhibited.

Some species have an optimum around 3.5 up to 5.5 and are called acidophiles (most of them are yeasts, fungi, *Lactobacillus*, *Acetobacter*). *Thiobacillus thiooxydans* is even able to grow at pH = 0, that means in a sulphuric acid solution. To cultivate these bacteria, a tartric acid solution is usually used to decrease the pH down to the good value.
Others bacteria prefer pH from 8.5 up to 9.5: they are called basophiles or alkalophiles.

During their growth, bacteria can release different molecules which might have an effect on the media pH. That can stop the growth if the change is significant. For example, bacteria can produce lactic acid with lactose. To confine this effect, a buffer solution can be added in the media. This way, even if molecules are produced, the media stay at the optimal value to allow the growth of the bacteria until the end of the process. Phosphates buffers (K_2HPO_4, KH_2PO_4) are the most commonly used because they cover a wide zone of pH around neutrality.

3. Osmotic pressure

There are three ways to modify the osmotic pressure in a media:

- To vary salts concentration (mainly NaCl)
- To add high molecular weigh compounds, sometimes even not used by the microorganisms. The most used is saccharose.
- To remove a part of the water. That concentrates every compound of the media. The water activity (aw) decreases.

Depending on their behaviour according to the NaCl concentration, microorganisms are classified:

- Halosensitive: they can't develop if the media contains a too high salt concentration.
- Halotolerant microorganisms: they can't grow on a salty media but they don't die.
- Halophile microorganisms: they need a high (up to 20%) concentration in salt to grow.
- Extreme halophile microorganisms: They can grow at very high concentration, even in a saturated solution (31%).

If the sugars (saccharose) concentration is taken into consideration, microorganisms are divided into three groups:

- Osmosensitives: they don't survive if the sugars concentration is too high.
- Osmophiles: they can grow with a high sugars concentration (between 200 and 400 g of saccharose per litre of media), as many yeasts and fungi.
- Osmotolerants: their growth is stopped if the sugars concentration is high, but the cells stay alive.

When a part of the water is removed, the water activity is changed. This activity (aw) represents the "free water" in the media, and not the bounded water contained in a product or in big molecule.

$$aw = \frac{\text{partial pressure of water}}{\text{partial pressure of distilled water at the same temperature}}$$

So, aw < 1. The more the media contains free water the more the aw is close to 1. The free water is the only water which can be used by the microorganisms.

Most of microorganisms actively grow when aw is around 1 (0.98 down to 0.9) but, as for the others factors, a minimum value exists, where the bacteria don't grow, but don't die. It is admitted that under 0.5, no microorganism are able to grow. Some fungi and yeasts grow with aw as low as 0.6.

4. Oxido-reduction potential

The oxido-reduction potential (rH_2) of a media reflects the oxidative or the reducer activity of this media, jus as the pH reflects the ionic activity.

$rH_2 = - \log (H_2)$. H_2 is the hydrogen pressure, expressed in atmospheric unit.

Every microorganism living in a specific media has a maximal activity for a specific rH_2 value. This behaviour depends on the respiratory type of the specie:

- Obligate Aerobic bacteria: $rH_2 = 20$ down to 14
- Microaerophile bacteria: $rH_2 = 14$ down to 12.5
- Obligate Anaerobic bacteria: $rH_2 = 7.5$
- Facultaive Anaerobic bacteria can support a wide range of rH_2.

When a solid media is poured into a tube and autoclaved, a rH_2 gradient is formed, from 20 at the top of the tube (in contact with the air) down to 7 in the bottom of the tube (no oxygen). So, if the bacteria are sown into the tube by straight injection, the location of the growth will allow assessing approximately the value of the potential.

5. Dissolved oxygen concentration (see Nutrition Chapter also)

Depending on the respiratory type of a microorganism, the Oxygen needs are different. As for the others factors, an optimal can be defined and critical limits where the growth is stopped:

- Obligate aerobics need O_2 to grow in high concentration (around 20%)
- Microaerophiles need O_2 in slow concentration
- Obligate anaerobics can't grow in aerobic conditions. O_2 is a toxic for them.
- Facultative anaerobics are able to develop with or without O_2.

6. Chemical products

They include antiseptics and additives such as acids, sulphites, or some antibiotics.
The most commonly used antiseptics are sodium hypochlorite, ethanol, phenols…
A low concentration of these products just makes the growth slow down, but the aim of their use is to quickly kill every microorganism. Nature, concentration and contact time are very important but it is also necessary to take the toxicity for the humans and for the environment in consideration and the targeted microorganisms as well.

The additives are classically used in the food industry to preserve food during a long time, especially for sensitive products. They don't kill the bacteria (the quantity in the product is too low) but they stop the growth: bacteriostatic action.

7. Light

If the microorganisms are photosynthetic, light is a very important factor because it is used as energy source. Intensity but also the alternation light/obscurity has to be considered. For these organisms, a specific CO_2 concentration is also needed.

C. Factors affecting the different phases of the growth curve

Factors will affect one or many phases of the population growth and have different nature.

The latency will be affected by the genetic factors because the cells have to adapt themselves to their new environment and it could be more or less fast depending on the cells species.
The latency length of time will also be related with the quantity and the age of bacteria which are inoculated.

The exponential phase will be affected by the media composition. When a microorganism grows, it uses nutrients for its metabolism. When one of these nutrients is missing, it could stop the growth because the cells obligatory need it to develop. The nutrient is called restrictive factor. The nature of the different sources (C, energy, ions…) is affecting the growth because a bacterium uses preferentially some molecules before the others. If the favourite nutrient for the C source for example is not present in the media, the bacteria will still grow, but not as fast as they could.
The genetic factors are also involved because they control the ability of a cell to use a nutrient or another.

It is possible to make the exponential phase longer, by the choice of the best media for the bacteria (concentration and nature of each component) but others factors can also help to improve the culture.
Most of the time, the growth slows down because bacteria don't have access to all the molecules they need, and because an Oxygen gradient is formed from the top to the bottom of the culture container (tubes, flasks…).
Shaking the media during the incubation allows the bacteria to have access to every molecule they need, and to make the O_2 concentration homogeneous on all the media.

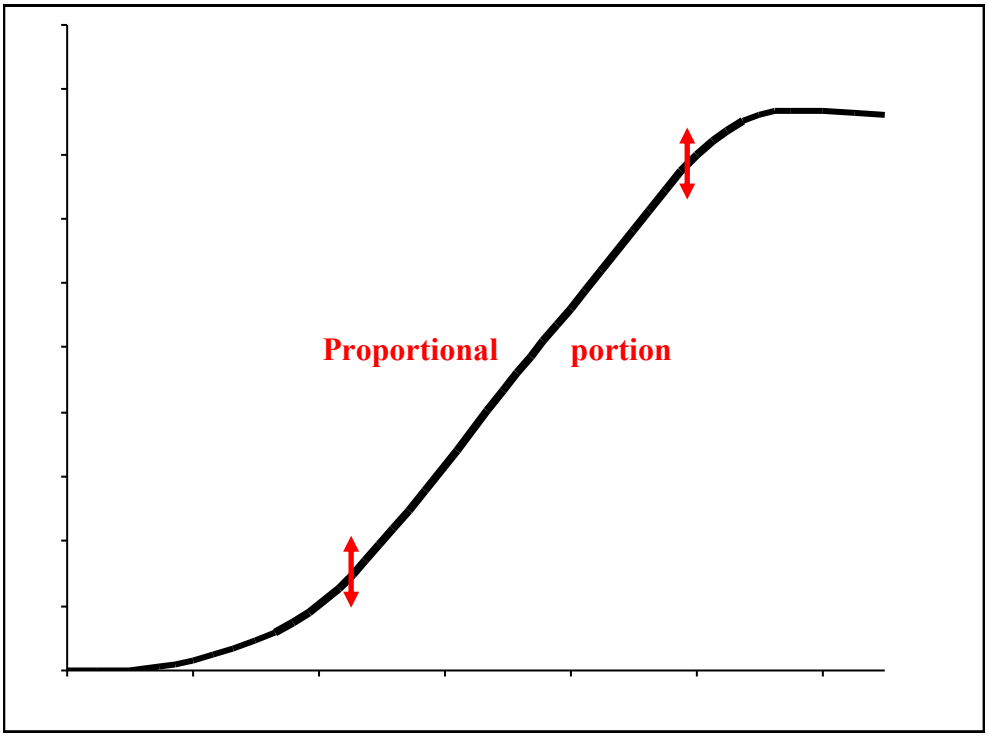
D. Growth study methods

Growth study is the study of the evolution of a population from a pure strain during time. Every method has advantages and limits. So, it is useful to use several methods to compare the results.

1. Optic Density measurement (OD)

Also called opacity or cloudiness measurements, OD measurement is the fastest and simplest method, classically used to assess the microbial weigh and its evolution with time.

OD is proportional with the cellular concentration in the media, between limits as seen on the graph.



Into the proportional portion, the following relation can be written:

$$OD = k \times l \times C$$

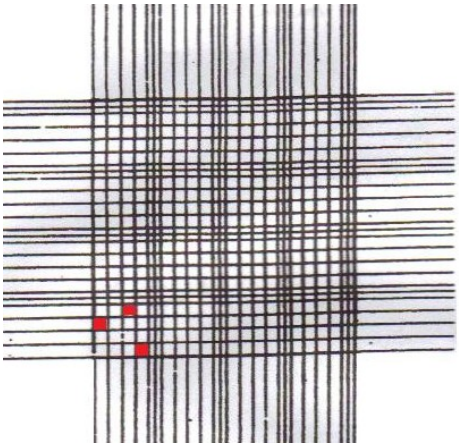
k is a proportional coefficient called absorption factor, and noted ε.
l is the length of the container used in the spectrophotometer (which is crossed by the light rays)
C is the cellular concentration (or any other molecule concentration depending on the wavelength λ).

Limits:

- With high concentrations of cells in the solution, the formula can not be used because there is no proportionality between the OD and the cells concentration. A dilution can be made, and then, the concentration obtained is multiplied by the dilution factor to get the final result.
- The culture has to be rich enough to be measurable, depending on the sensitivity of the used spectrophotometer (the limit is regularly around 10^6 cells per mL)
- This method cannot be used if the media has too strong colours, or precipitates. The negative control is done with sterile media, but the errors are increased.
- Dead and alive cells are counted without any distinction.
- For every studied microorganism, a previous calibration curve has to be established, to estimate the absorption factor.

2. Cells counting

Many methods exist to count cells in a suspension, and are called direct methods. The simplest are the Thoma and the Malassez cells.
Thoma cell is a slide with an engraved lines network. This network is formed by 400 small squares, 1/20 mm long each. A drop of the suspension is put on the network, and is covered with a specific lamella which is really flat. The sides of the slide are slightly upswept, so the lamella supports on them, and between the slide and the lamella, there is exactly 1/10 mm. So the volume of a small square is $1/(4x10^6)$ cm^3 or mL.

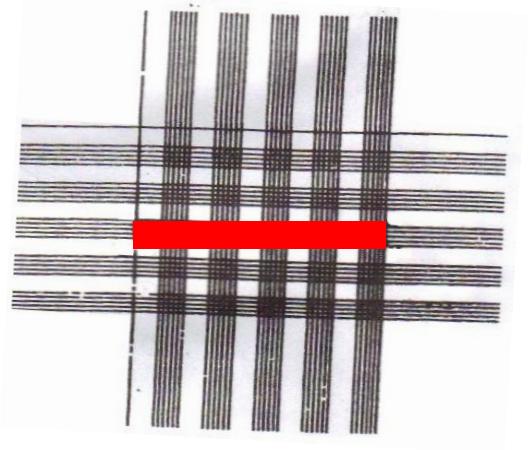


The cells are counted in several small squares (squares in red), and the cellular concentration of the suspension is calculated as following:

$$N = \frac{n_1 + n_2 + n_3 + \ldots + n_p}{p} \times 4 \times 10^6 \times C$$

N: cellular concentration (number of cells per mL)
n_1, n_2, n_3, n_p: number of counted cells in every square.
p: number of counted squares
C: dilution factor if the suspension is diluted

The Malassez cell is based on the same principle. The differences are the size and the disposition of the lines in the network. That means that the volume is different. The network is composed of 100 rectangles, into 10 lines. Every rectangle is composed by 20 squares and the total volume of the network is 1 mm^3. The counting is usually done on a line, as seen on the schema, and it is better to repeat the counting on two or three lines.



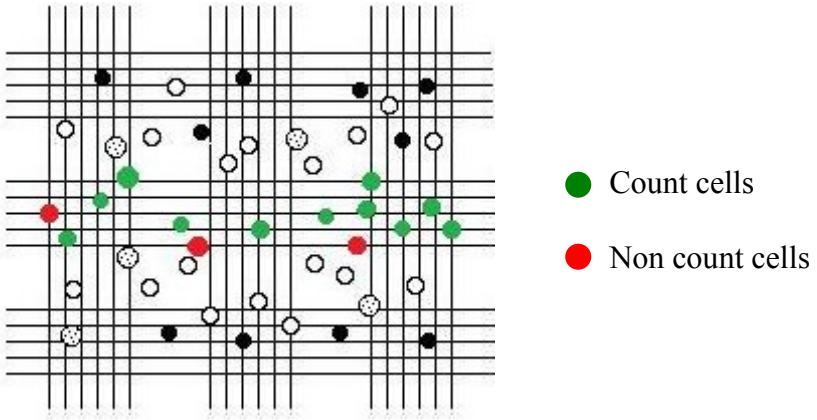
The lines represent a 0.1 mm^3 volume each so the formula is:

$$N = \frac{n \times 10^3 \times C}{v}$$

N: cellular concentration (number of cells per mL)
n: total number of cells counted
v: studied volume in mm^3 (number of lines where the cells are counted x 0.1)
C: dilution factor if the suspension is diluted

For the both counting methods, to be sure not to count twice the same cell when they are on the marks, are counted the cells on the marks at the top and on the right side of the square/line.

For example, on a Malassez cell, if the counting is done on the middle line, the green cell will be counted, and the red ones will not.



Limits:

- If the cellular concentration is too low, the counting is not efficient. It is possible to repeat many counting to confirm the results.
- A fixative agent has to be added if the cells are moving to fast because of their mobility. The dilution created by the agent has to be taken into consideration.
- If the cells are too small, the zoom lens x40 is not sufficient, and it is not easy too count with the x100 lens.
- Dead and alive cells are counted without any distinction.
- Suspensions have to be diluted if more than 5 cells are counted in a small square.

3. Determination of the dry weight

It is an indirect method because the cells are not directly counted.
First, the suspension is concentrated to make the method more accurate, and easier. It could be done by centrifugation or by membrane filtration:

- Before the filtration, note the weight of the membrane in a container. Filter the suspension and replace the membrane in the container. Dry at 100°C and weigh frequently until the values are stable. The difference between the two weighs represents the dry weigh of the cells of the suspension. This method is quick and there is no loss during the process but the weights are usually very small so errors can occur. As well, the membrane can block up, and then, filtration is not correct.
- Centrifuge the suspension and remove gently the supernatant. Rinse the pellet with distilled water or buffer, and centrifuge again. Repeat twice. Re-suspend the pellet in a small quantity of water and transfer it into a tube after have weighed it. Add a small quantity of Fontainebleau sand (to avoid projections during the drying), and dry at 100°C at least for 24 hours. Let the sand cool down and weigh. The difference between the two weighs represents the dry weight of the cells of the suspension. This method can be used whatever the initial concentration and the volume of the suspension, but a loss may occur at every centrifugation when the supernatant is removed. The method is quite long, and has to be done very carefully to give good results.

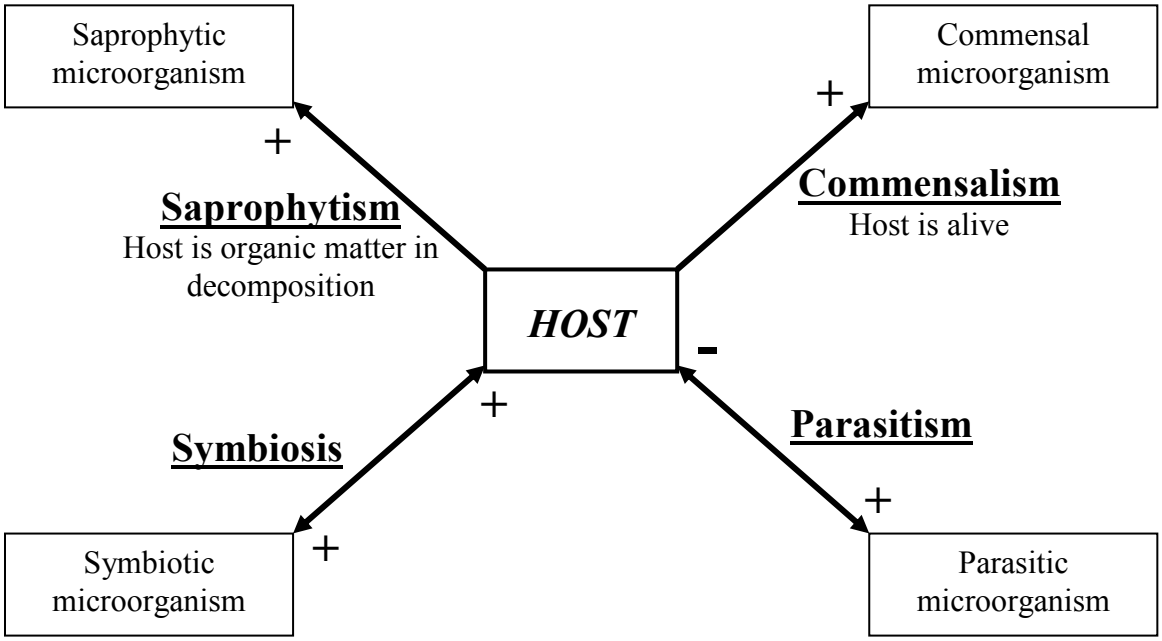
4. ATP measurement

This is also an indirect method, sensitive and fast. ATP is the best biotic marker. It is never stored and is quickly destroyed when the cell dies.
The reaction of bioluminescence (transformation of the luciferine by the luciferase) is related with the quantity of ATP.
First, the luciferine is activated by the ATP. Then, it is oxidized because of O_2, and decarboxylated, producing light. The intensity of the light is proportional to the ATP quantity.

VIII. Bacteria – Hosts relations

A. Introduction

Chemo-organotrophic bacteria need an organic matter source to grow. To satisfy this need, they develop many relations with their environment. Four groups can be distinguished depending on the nature of these relations between the microorganism and its host.



B. Saprophytism

Saprophytes are commonly plants which use organic substances in decomposition as nutrients. Some bacteria are also able to use these substances and are called saprophytes.
Saprophytic bacteria are found in soils (*Clostridium* for example), water (*Pseudomonas*), or air (*Micrococcus luteus*, *Bacillus*). They ferment animal, plant and human wastes, and are part of the biological cycles and food chain.

C. Commensalism

"Commensal" means "eating at the same table". Commensal bacteria take the food they need from their host, but don't cause any damage to it. It can be compared to cohabitation.
Human have an important commensal flora, also called normal flora. They are present on skin (*Micrococcus*, *Corynebacterium*…), respiratory mucous membrane (superior part only, *Neisseria*, *Lactobacillus*…), vaginal mucous membrane (*Lactobacillus acidophilus* also called Döderlein bacillus), or digestive system (coliformes, enterococci…).

D. Symbiosis

Symbiosis is a durable and necessary association which offers benefits to both distinct organisms.

For example, the lichens are composed by the association of a cyanophyte (bacterium) and a fungal. The cyanophyte produces organic matter through its photosynthesis, and the fungal takes this organic matter, which it exchanges with mineral salts taken from the rocks.

Mycorrhizaes also belong to microsymbionts: in roots, a fungal exchanges water and mineral salts from the soils with organic matter produced by the tree through the photosynthesis process.

E. Endophytes

An endophyte is an endosymbiont, bacterium or fungus, which lives within a plant for at least part of its life without causing apparent disease. Endophytes are ubiquitous and have been found in many species of plants. However, most of these endophyte/plant relationships are not well understood. Endophytic species are very diverse; and most of them are known to colonize multiple species of plants (there is no host specificity). A single plant can also harbor many different species of endophytes, both bacterial and fungal.

Endophytes may benefit host plants by preventing pathogenic organisms from colonizing them. Colonization of the plant tissue by endophytes creates a "barrier effect", so there is a competition which prevents pathogenic organisms from taking hold. Endophytes may also produce chemical molecules which inhibit the growth of competitors, including pathogenic organisms.
This kind of relations may improve the ability of the hosts to tolerate abiotic stresses such as drought, as well as improve their resistance to insect and mammalian herbivores.

F. Parasitism

Parasitism is an unbalanced association: one of the partners takes all the benefits and the other one takes damages. Parasites live at the expense of the host. If the parasite engenders a disease, it is called pathogenic organism.
There are two types of pathogenic bacteria:

- Specific pathogenic bacteria: they always cause a disease when then enter into a host. Then can be killed with an antibiotic use for example if the host is a human. Sometimes, the pathogen stays inside the body even if the disease is over. The patient is called sane carrier.
- Opportunist pathogenic bacteria: In human bodies (hosts), they are part of the normal flora. They become pathogenic if the immune defences of the hosts are weaker than usually. For example, *Escherichia coli* is part of part of normal flora, but if a dysfunction occurs, it can cause diseases.

1. Pathogenicity

Pathogenicity is the ability of a microorganism to induce a disease in another organism, the host.
It is expressed by two properties: virulence and toxin synthesis.

2. Virulence

It is the ability of the pathogen to enter into the host and to develop in order to colonize it. It is the invasive ability (Casadevall and Pirofski, 2001).

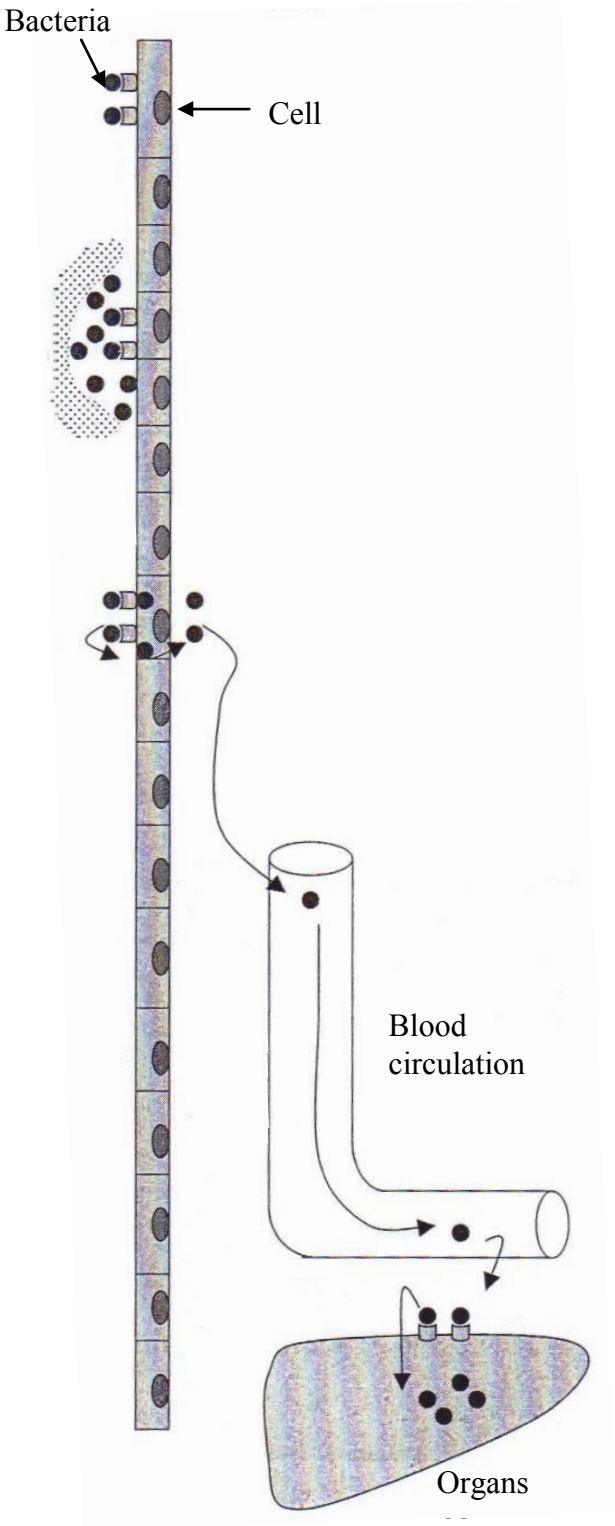
Step 1: Adherence of the bacterial cell to the mucous membrane cells, if specific receptors are present on this one.

Step 2: colonization of the mucous membrane. The bacteria develop and form a biofilm on the external side.

Step 3: invasion of the mucous membrane. Bacteria go through the membrane of the cells because of phagocytosis process. They develop and make the host cell explode. So, the bacteria are inside the host.

Step 4: spreading in the all host organism. Bacteria are carried with the biological fluids.

Step 5: colonization of organs. Bacteria are carried to organs. They stick to it, go into it, and develop. The organ is not able to operate as usual and the host will present symptoms of a disease.



Many factors can have an effect on virulence:

- **Host factors**: To be sensitive to a pathogen, different host characteristics are needed: The cells of the mucous membrane have to present fixation receptors for the pathogen, the macrophages cannot recognize the pathogen, so they can't kill it, specific antibodies against the pathogen are not present (that means that the host and the pathogen have never been in contact before) and the physiological conditions (especially temperature) of the host are propitious to its growth.
  Virulence also depends on the host specie, age, physiological stress, and the entry site of the pathogen (every mucous membrane doesn't have the same receptors).

- **Environmental factors**: for example, cold decreases the strength of the host barriers, and inhibits the immune system.

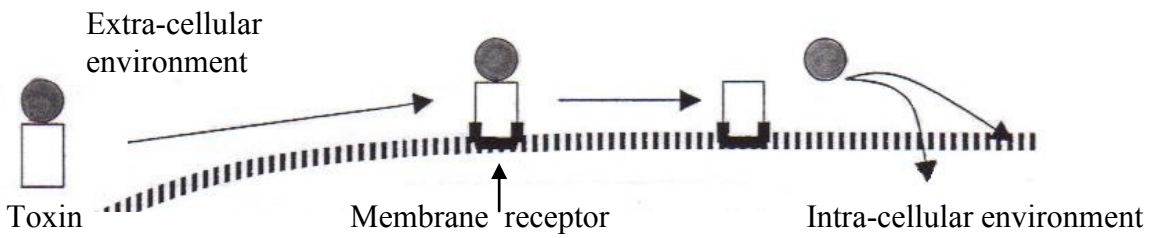
- **Pathogen factors**: many surface components as pili, capsule or flagella can have a positive effect on the virulence because they make the adherence phase easier, or they inhibit the macrophages activity. The quantity of pathogens is also a factor, because the more they are many, the more efficient the infection is. Some pathogens are able to produce exo-enzymes, which help their diffusion into the host.

3. Toxigenesis

The bacteria can produce toxic substances (toxins) to cause disorders in the host cell. Many bacterial toxins are proteins, encoded by the bacterial chromosomal genes, plasmids or phages (Lubran, 1988). The toxin can infect the entire organism (human body for example) even if the bacteria cannot enter into it (Masignani *et al.*, 2006)).

A toxin is a poison. It is a microbial product which cause damages or destroy the healthy cells of the host, even at very low concentration. It is macromolecules which are immunogenic. That means that they are able to activate the immune system of the host, inducing antibodies synthesis by the host. Many toxins combine with specific receptors on the surface membrane, frequently glycoproteins or gangliosides, and penetrate the cell to reach their intracellular target. A common mechanism of entry is absorptive endocytosis. Many protein toxins have an A-B structure, B being a polypeptide which binds to the receptor and A being an enzyme. Many toxins are activated, either when produced by the bacterium or when bound to the membrane receptor, by proteases (nicking) (Lubran, 1988).
The diversity of the toxins is really vast, and there is no classification. Usually, they are divided into two groups: proteic toxins, or exo-toxins, released by the pathogen, and the lipo-polysaccharidic toxins, or endo-toxins, compounds of the pathogen membrane.

- Exo-toxins: they have a proteic structure, with two subunits. They are able to stick to the membrane receptors of the target cells. Then, the both subunits are divided:



They are able to induce an immune response, an antibodies production, which are specific of the toxin, and which neutralize it when it is not bound to a cell receptor (free toxin). Toxins are around 1000 times stronger than chemical and animal poison. For example, 1mg of botulic toxin kills 15000 human bodies or 10 million mice (1000 tons of "living matter").
Some of the exo-toxins can stick to one type of tissue only (target tissue), or don't have any specificity (and are therefore called cytotoxin).
Symptoms generally appear few hours after the toxin fixation on its receptor.
They are mainly secreted by gram positive bacillus and cocci, sometimes by gram negative bacillus. They are destroyed at high temperature (thermolabile) and by H_2O_2, sodium hypochlorite, UV…

- Endo-toxins: They are a component of the negative Gram bacterial membrane and are released when the cell is destroyed. They have a lipo-poly-saccharidic structure, and the toxicity is due to the lipidic part (Lipid A). They are less toxic than the exo-toxins and the toxicity is expressed only if the pathogenic cell is destroyed. They don't have any targeting activity, and attack every organ. The symptoms appear immediately after the cell lyses. The significance of the disease depends on the toxins dose, not on the nature of the pathogen. They are thermo-resistant up to 100°C during two hours and a half, but are destroyed by formol.

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