Chapter IV: Bacterial growth

Introduction

Growth is the orderly increase of all components of an organism. In unicellular organisms (as bacteria and yeasts), it results in an increase in the number of individuals.

A bacterial population thus grows at the rate of cell divisions, which occur through **binary fission**: a transverse septum forms, gradually separating the two future daughter cells, each of which receives a copy of the mother cell's chromosome.

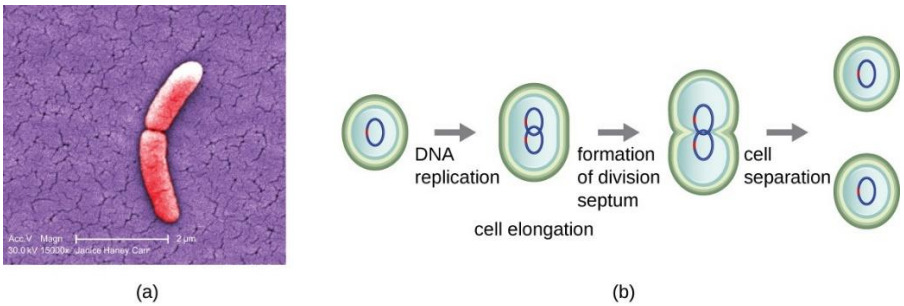


Figure 1. Binary fission in bacteria

Cell separation is not always complete, resulting in chains, clusters, and other arrangements.

Bacterial growth is highly dependent on environmental physicochemical conditions, including nutrient availability, antibacterial agents, temperature, pH, oxygen levels, and more.

1. Bacterial growth parameters

Bacterial growth can be studied by measuring either the number of cells over time or the total biomass of the bacterial population.

A cell division cycle (the formation of two daughter cells from a mother cell) is called a **generation**. The key growth parameters are:

- ✓ Number of generations (**n**)
- ✓ Generation time (**g**)
- ✓ Division rate (**k**)

1.1. Number of generations (n)

Bacteria replicate by binary fission, a process by which one bacterium splits into two. Therefore, bacteria population doubles every generation time following a **base-2 geometric progression**: $2^0 \rightarrow 2^1 \rightarrow 2^2 \rightarrow 2^3 \rightarrow \ldots \rightarrow 2^n$ yielding cell counts of **1, 2, 4, 8, 16**, respectively

At time **t**, the population size is:

$$\mathbf{N = N_0 \times 2^n}$$

Where:

- **N_0**: Initial cell counts (cells/unit volume) at time t_0
- **N**: Final cell counts (cells/unit volume) at time t

Taking logarithms:

$\log N = \log N_0 + n \log 2$

$\log N - \log N_0 = n \log 2$

Thus, the number of cell divisions (generations, **n**) is calculated as:

$$\boxed{n = (\log N - \log N_0) / \log 2}$$

1.2. Generation time (g)

It is the time it takes for a population of bacteria to **double in number**. For any given bacterial species, the generation time under specific growth conditions (nutrients, temperature, pH, and so forth) is genetically determined. For many common bacteria, the generation time is quite short, 20-60 minutes under optimum conditions.

Generation time **(g) = t/n**

where:

- **t** represents the duration of exponential growth
- **n** represents the number of generations.

1.3. Division rate (k)

It represents the number of cellular divisions per unit time. It is the inverse of the generation time, and it is expressed as: **k= 1 /g** [generations/hour]

where g = generation time (hours).

$$k = \frac{n}{t} = \frac{\text{Log N - Log } N_0}{\text{Log 2 } (t_n - t_0)}$$

Example species:

- *Escherichia coli*: k=3 h^{-1}
- *Lactobacillus acidophilus*: k=0.75 h^{-1}
- *Mycobacterium tuberculosis*: k=0.075 h^{-1}

2. The Growth curve

Microorganisms grown in closed culture (also known as a batch culture), in which no nutrients are added and most waste is not removed, follow a reproducible growth pattern referred to as the **growth curve.**

The bacterial growth curve graphically represents the number of living cells in a bacterial population over time, showcasing a predictable pattern of growth when bacteria are grown in culture.

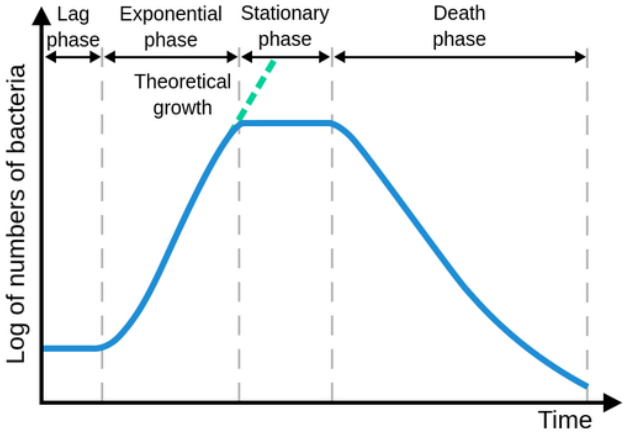


Figure 2. The growth curve of a bacterial culture

2.1. Growth curve phases

Bacterial growth cycles in a growth curve consist of **four** phases:

- **Lag Phase**: This is the adaptation phase during which bacteria acclimate to their culture medium. The **growth rate** is zero (no cell divisions occur). Bacteria do not divide but adapt by activating enzymatic

systems tailored to the nutrients in the new substrate. The duration of this phase varies depending on the bacterial species and factors such as medium composition, temperature, and inoculum size, etc.

- **Exponential phase (log phase)**: This phase begins with an **acceleration period** (end of the lag phase) where bacteria metabolize nutrients and initiate cell division. The **generation time** (g) shortens to reach the species-specific minimum, and the **growth rate** reaches its maximum value (μmax). During the log phase, the relationship between time and number of cells is not linear but exponential. This is where the bacteria are at their peak metabolic activity, and the ideal moment to extract samples for inoculating other fresh cultures.
- **Stationary phase:** In a closed system, such as a laboratory flask or petri dish, growth cannot be sustained indefinitely. The bacterial population will run out of resources, space or produce an overwhelming amount of toxic end products from high metabolic activity. Growth slows down to a negligible rate, but this phase is inadequate for a population count as it is difficult to differentiate between viable and dying cells. Bacteria will also start producing antibiotics and spores, which could affect the estimation of viable cells.
- **Decline phase (Death phase):** As the culture medium accumulates toxic byproducts and nutrients become depleted, bacterial cells suffer severe damage, irreversibly losing their ability to reproduce and entering a state of accelerated death. The generation time progressively lengthens, creating a growing imbalance between new cells (increasingly scarce) and aging cells dying in greater numbers. Soon, the number of dying cells exceeds the number of dividing cells, leading to an exponential population decline.

2.2. Growth curve modifications

a) Continuous growth

This refers to an open system used to maintain microbial populations in continuous exponential growth. It requires a constant supply of fresh nutrients and simultaneous removal of culture volume to maintain a renewed growth medium. Equipment such as **chemostats** is typically employed. This process is commonly used to produce:

- Age-synchronized bacterial cells for bacterial vaccine preparation
- Bacterial metabolites: vitamins and bacterial toxins (anatoxins).

Figure 3. A continuous culture system: The chemostat

b) Diauxie

In synthetic media, when bacteria are supplied with **two carbon substrates**, a biphasic growth curve can be observed. This curve is characterized by:

1. A **first exponential growth phase**
2. An **intermediate lag phase**
3. A **second exponential growth phase**

This phenomenon occurs because one substrate is preferentially metabolized until depletion before the second substrate is assimilated.

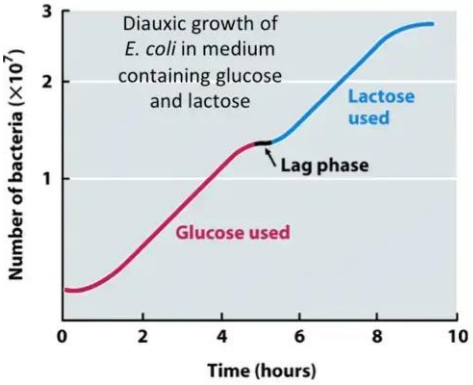


Figure 4. Diauxic growth of bacteria

3. Measurement of bacterial growth

Bacterial growth estimation can be performed using either direct methods (bacterial cell counting) or indirect methods (biomass measurement).

a) Direct Methods: Bacterial Enumeration (Cell Counting)

a-1 Total Count:

This method utilizes:

• Optical microscope examination using a gridded counting chamber (hemocytometer). All bacteria present in liquid suspensions are counted using specialized counting chambers such as:

- Malassez cell (or Thoma cell): Used for enumerating larger cells
- Petroff-Hausser chamber: Specifically designed for counting small cells (e.g., bacteria)

Limitation: This technique cannot differentiate between viable and dead bacteria.

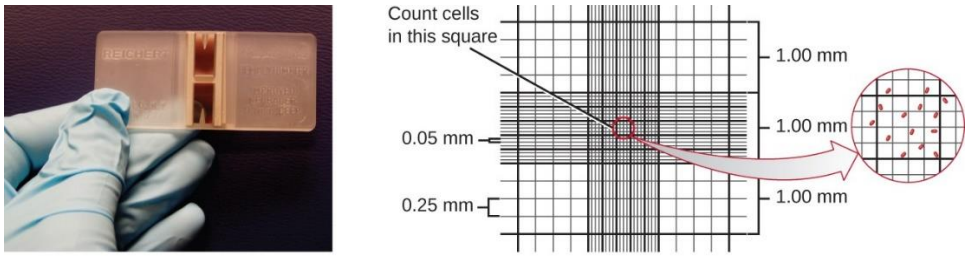


Figure 5. Counting chambers

• Automated particle counting using a particle counter for bacteria suspended in an electrolyte solution.

Limitation: Cannot discriminate between dead and live cells.

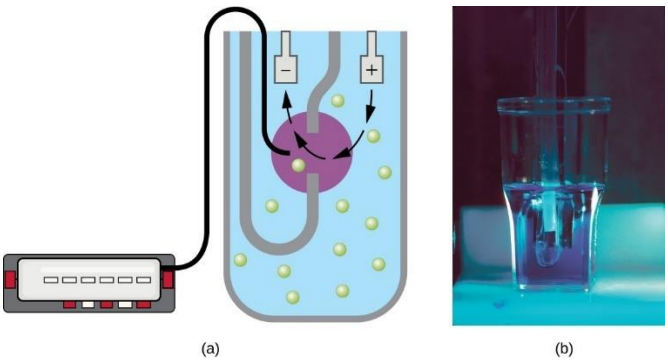


Figure 6. Automatic particle counter

- Epifluorescence microscopy method: Bacteria are stained with a fluorochrome (e.g., acridine orange) and observed under fluorescence microscopy.

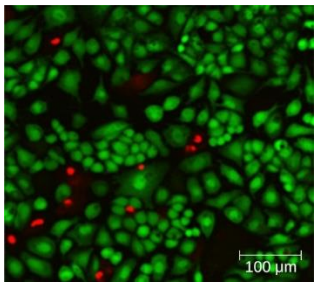


Figure 7. Bacteria counting with epifluorescence microscopy method

a-2 Viable cell counting

Viable cell enumeration is performed after culturing on either solid or liquid media. Three methods are distinguished (two using solid media and one using liquid media):

- **Solid Media Enumeration**

This method counts culturable (viable) bacteria that form colonies or CFUs (Colony-Forming Units) on appropriate culture media in Petri dishes.

Limitations :

- Cell aggregates may produce only a single colony
- Many isolated cells may fail to form visible colonies

✓ **Counting (enumeration) after dilution: Dilution Plating**

A diluted bacterial sample is spread on solid media or incorporated (mixed) with agar before solidification. Each microorganism or cell cluster grows into a distinct colony during incubation at an appropriate temperature. Since colonies may originate from cell clusters rather than single cells, results are expressed as **Colony-Forming Units (CFU)** rather than absolute microorganism numbers. For reliable results, plates should contain 15-300 colonies.

$$N\ (UFC/ml) = \frac{\Sigma C}{V\ (n_1 + 0.1\ n_2)\ d}$$

N: Number of microorganisms per mL of suspension

- **Σc**: The sum of the colonies counted on all selected plates from two successive dilutions.
- **V**: The volume of the inoculum plated on each dish in mL (1 mL or 0.1 mL).
- **n_1**: Number of selected plates from the first dilution.
- **n_2**: Number of selected plates from the second dilution.
- **d**: The dilution corresponding to the first selected dilution.

The calculated result should be rounded to **two significant figures**:

- If the last digit is **less than 5**, the preceding digit remains unchanged.
- If the last digit is **5 or greater**, the preceding digit is increased by one.

The final result should be expressed as a number between **1.0 and 9.9**, multiplied by the appropriate power of **10**.

Example**:**

$$N = \frac{\sum C}{V(n_1 + 0{,}1n_2)d} = \frac{168 + 215 + 14 + 25}{1(2 + 0{,}1\times 2)\times 10^{-2}} = \frac{422}{0{,}022} = 19\ 182$$

N = 19,182, after rounding as prescribed, the result is **19,000** or **1.9×10^4 CFU/mL**.

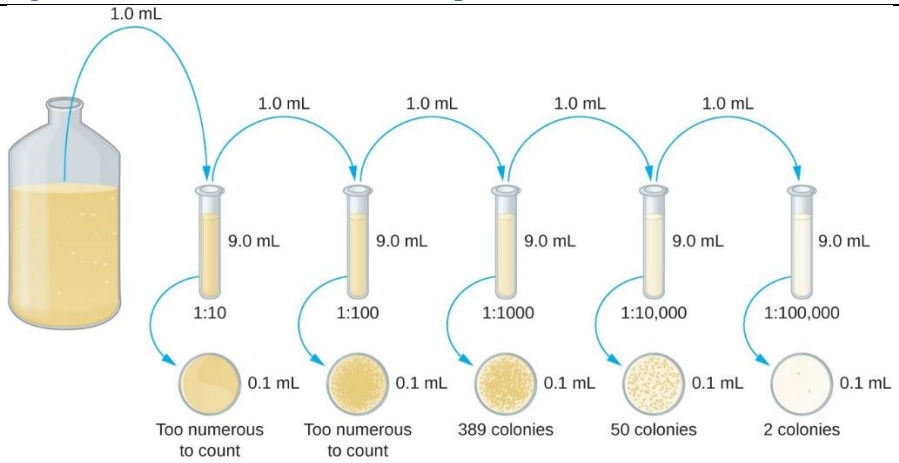


Figure 8. Standard Plate count Method

✓ **Membrane filtration**

This technique filters liquid through a membrane that retains cells larger than the filter pore size. It is commonly used for bacteriological water analysis.

The number of colonies present on the membrane allows for the calculation of the bacterial concentration N, expressed in **Colony-Forming Units** per **millilitre (CFU/mL)**, using the following formula:

N = n/V

Where:

- n: Number of CFUs on the membrane
- V: Volume of the filtered product in mL

Limitations: Not suitable for large liquid volumes

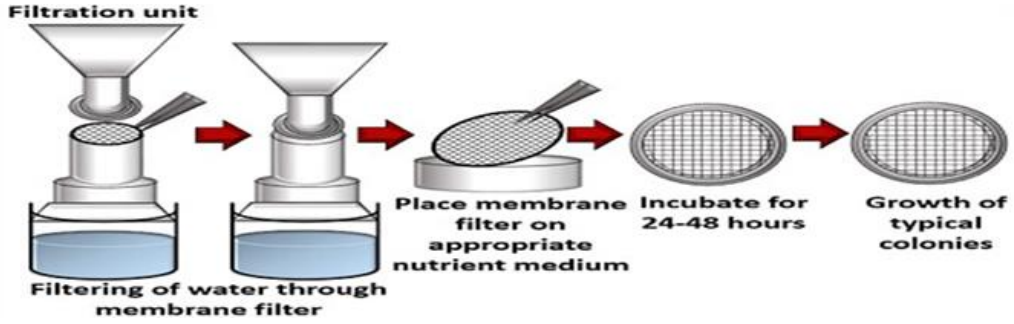


Figure 9. Method of membrane filtration

Enumeration in liquid medium: Most Probable Number (MPN) method

It is a **statistical technique** used to **estimate** the concentration of viable microorganisms. This technique is especially useful for analysing **microorganisms that do not grow well on solid media** and provides a reliable approximation when direct plate counts are impractical.

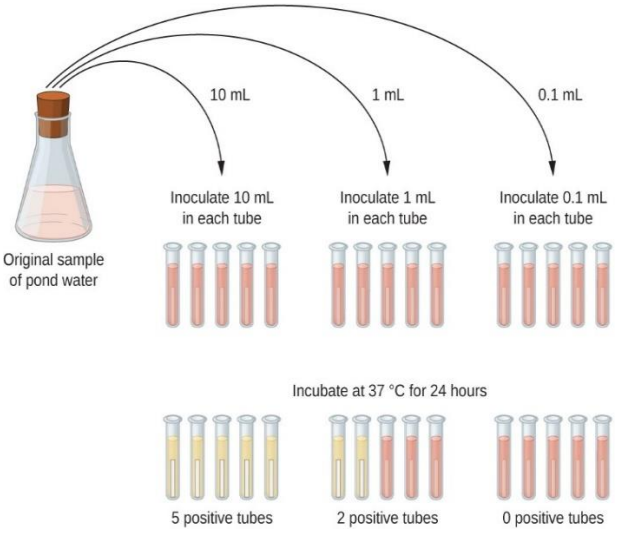
The **MPN method** involves inoculating multiple tubes (generally three or five tubes) with serial dilutions of the sample and then observing **growth indicators** such as turbidity, gas production, or colour changes.

The results follow a **binary system**:

- **Negative result**: No turbidity and/or no change in the medium.
- **Positive result**: Presence of turbidity and/or alteration of the medium.

To estimate the bacterial concentration, the number of **positive tubes** is expressed as a **three-digit code** (e.g., 3-2-1), where each digit represents the **number of positive tubes** at a given dilution.

This code is then compared to a standardized MPN index table, which provide the **Most Probable Number (MPN)**.



MPN index (when five tubes are used per dilution)

Number of tubes giving positive reaction out of				Number of tubes giving positive reaction out of			
5 undiluted samples (dilution factor-1)	5 dilutions of 10 (dilution factor-10)	5 dilutions of 100 (dilution factor-100)	MPN Index per 100 mL	5 undiluted samples (dilution factor-1)	5 dilutions of 10 (dilution factor-10)	5 dilutions of 100 (dilution factor-100)	MPN Index per 100 mL
0	0	0	< 2	4	2	1	26
0	0	1	2	4	3	0	27
0	1	0	2	4	3	1	33
0	2	0	4	4	4	0	34
1	0	0	2	5	0	0	23
1	0	1	4	5	0	1	30
1	1	0	4	5	0	2	40
1	1	1	6	5	1	0	30
1	2	0	6	5	1	1	50
2	0	0	4	5	1	2	60
2	0	1	7	5	2	0	50
2	1	0	7	5	2	1	70
2	1	1	9	5	2	2	90
2	2	0	9	5	3	0	80
2	3	0	12	5	3	1	110
3	0	0	8	5	3	2	140
3	0	1	11	5	3	3	170
3	1	0	11	5	4	0	130
3	1	1	14	5	4	1	170
3	2	0	14	5	4	2	220
3	2	1	17	5	4	3	280
4	0	0	13	5	4	4	350
4	0	1	17	5	5	0	240
4	1	0	17	5	5	1	300
4	1	1	21	5	5	2	500
4	1	1	26	5	5	3	900
4	2	0	22	5	5	4	1600
				5	5	5	≥ 1600

Figure 10. MPN test

B- Indirect methods: Biomass measurement

B-1- Dry weight determination

Microbial cells growing in a liquid medium are centrifuged, washed, oven-dried, and weighed. This technique is particularly useful for measuring the growth of filamentous fungi.

-Limitation: The entire cellular mass is measured, and the technique is time-consuming and delicate.

B-2- Optical Density (OD) measurement

The OD of the growth medium is measured over time at a specific wavelength (typically between 450 and 550 nm) using the Beer-Lambert Law.

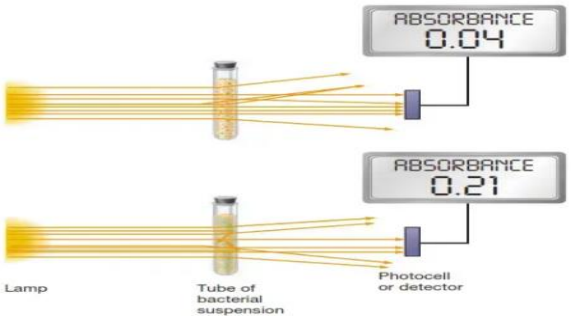


Figure 11. An illustration of turbidity measurement using a spectrophotometer.

3. Antimicrobial Agents

4.1. Definition

An antimicrobial agent refers to any substance or process that inhibits or kills microorganisms through various mechanisms.

4.2. Classification

4.2.1. Physical agents

a) Temperature

- Moist heat: Autoclave, pasteurisation, tyndallisation
- Dry heat: Hot-air oven, Bunsen burner, etc.

b) Radiation : UV rays, X-rays, etc.

c) Filtration

4.2.2. Chemical agents

Oxidizing agents, alcohols, heavy metals and their salts, soaps and detergents, phenolic compounds, aldehydes, etc.

4.2.3. Antimicrobial chemotherapeutic agents (Antibiotics)

4.2.3.1. Definition of Antibiotics

They are chemical substances of natural or synthetic origin that can inhibit bacterial growth (bacteriostatic antibiotics) or destroy bacteria (bactericidal antibiotics).

4.2.3.2. Classification of antibiotics

<u>According to antimicrobial action:</u>

- **Microbiostatic antibiotics**: Agents that inhibit growth (e.g., bacteriostatics, fungistatics).
- **Microbicidal antibiotics**: Agents that completely kill pathogenic microbes (but not necessarily spores), such as bactericides, fungicides, and algicides.

<u>According to spectrum of activity:</u>

- **Broad-spectrum antibiotics:** they are a type of antibiotics that are effective against a wide range of bacteria.
- **Narrow-spectrum antibiotics:** they are active against a selected group of bacterial types.

<u>According to chemical composition:</u> The most commonly used classification.

Example:

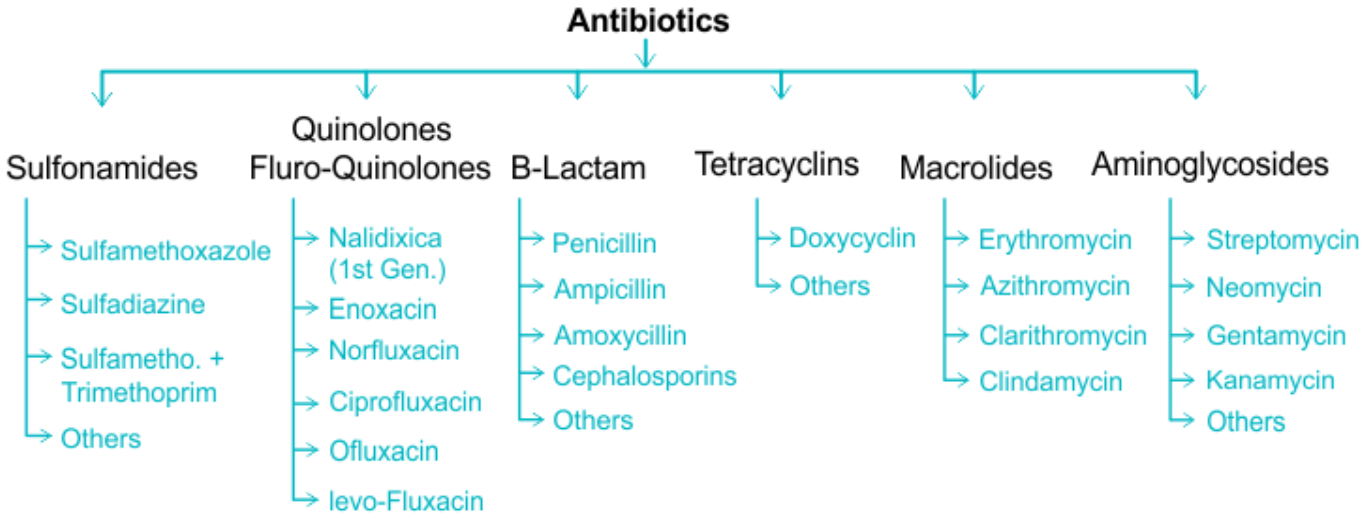


Figure 12. Common antibiotic families

4.2.3.3. Mechanism of action of antibiotics

The targets typically affected by antibiotic action are those that represent an essential and constant element for bacterial cell survival. The bacterial cell wall, membrane, DNA, and ribosomes are antibiotic target sites.

5.2.3.4. Antibiotic (antimicrobial) Resistance

Bacteria have a high adaptive capacity, allowing them to develop resistance to antibiotics. Two main types of resistance exist:

- **Natural resistance**: Affects all strains of a bacterial species. This resistance is chromosomal, permanent, and transmitted to daughter cells during replication.
- **Acquired resistance**: affects only some strains of a normally susceptible bacterial species and emerges following antibiotic use. The acquisition of a new resistance mechanism results from:
  ✓ either a mutation occurring in the bacterial chromosome,
  ✓ or the acquisition of plasmids from an already resistant bacterium.

5.2.3.4.1. Mechanisms of antibiotic resistance

Three main resistance mechanisms are currently known:

1. **Antibiotic inactivation by bacterial enzymes**
2. **Reduction of antibiotic concentration reaching the target**: The antibiotic remains unmodified but cannot access its target within the bacterium:

- *Either* due to decreased membrane permeability preventing entry
- *Or* because it is actively expelled to the outside of the bacterium by proteins acting as pumps (efflux systems)

3. **Target modification** :

- Quantitative modifications: For example, the absence of a cell wall in bacteria of the genus *Mycoplasma* is responsible for their natural resistance to β-lactams.
- Qualitative modifications: Modification of the target's structure may decrease its affinity for the antibiotic. This is a frequent mechanism of acquired resistance.
- Target protection: This is a reversible protection of the target (by proteins preventing the binding of quinolones, for example).

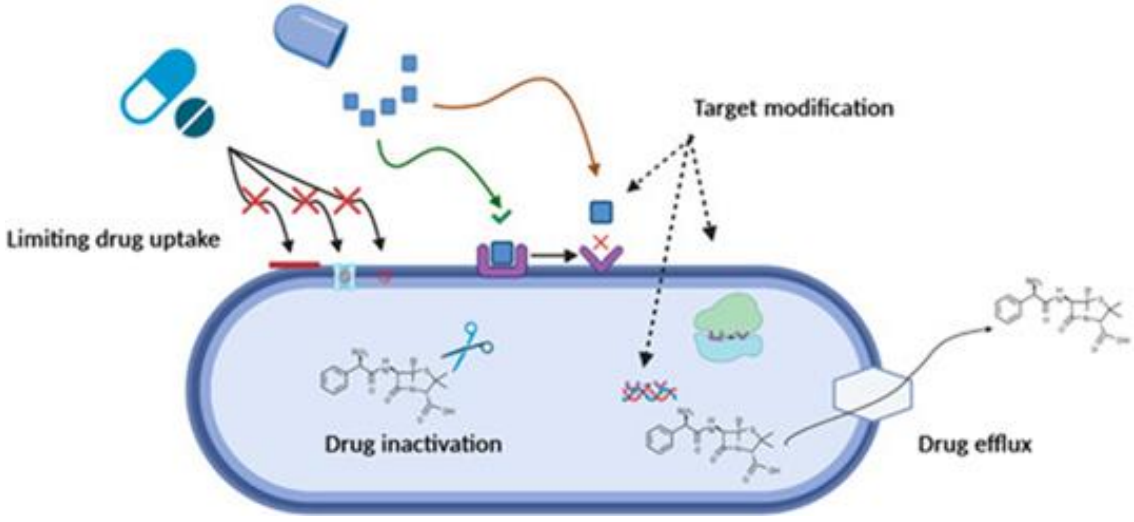


Figure 13. Summary of AMR mechanisms in bacteria

5.2.3.4.2. Antibiotic Susceptibility Testing (AST)

Also known as ***antibiotic sensitivity testing***, it is "**in vitro"** assay which determines bacterial susceptibility or resistance to specific antibiotics.

It is performed by exposing microorganisms to various antibiotics in a controlled laboratory environment to evaluate their effectiveness in inhibiting or killing bacteria.

AST results guide clinicians in selecting the most effective antibiotic(s) for treating bacterial infections.

AST Methods

Several standardized methods exist to assess bacterial antibiotic susceptibility. The most widely used include:

1. Disk diffusion method (Kirby-Bauer)

A standardized bacterial inoculum (typically **0.5 McFarland**) is swabbed onto a **Mueller-Hinton agar** (MH) plate. Antimicrobial-impregnated filter paper disks are placed on the agar surface.

After 16–18 hours of incubation at 35±2°C, the **inhibition zone diameter** around each disk is measured and interpreted using **clinical breakpoints** (e.g., CLSI/EUCAST criteria).

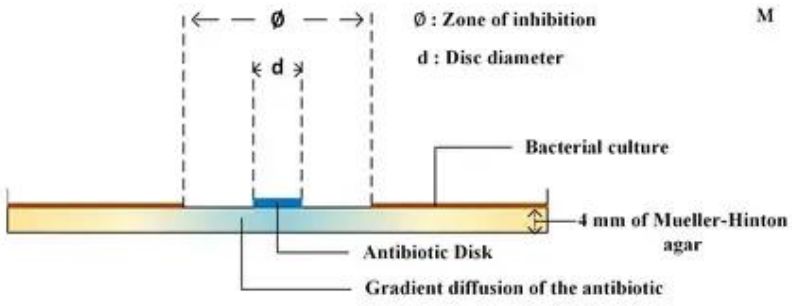


Figure 14. Kirby Bauer disk disc diffusion method

2. Broth dilution

It quantifies Minimum Inhibitory Concentration (MIC) using serial antibiotic dilutions which are prepared in tubes (macro-dilution) or microtitration plates (micro-dilution). A bacterial suspension is added and the MIC is determined as the lowest concentration inhibiting visible growth.

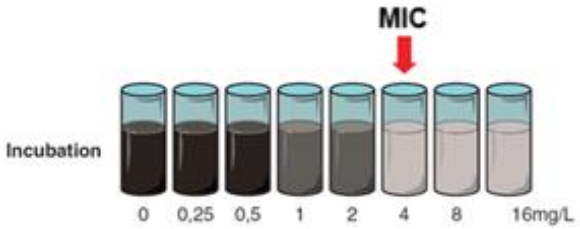


Figure 15. Determination of MIC using broth dilution

3. Gradient diffusion (E-test)

Combines disk diffusion and dilution principles via plastic strips with predefined antibiotic gradients.

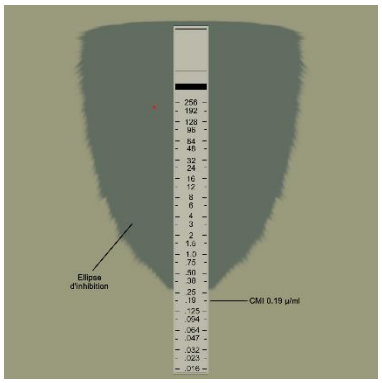


Figure 16. E Test gradient diffusion method