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Pedagogical Handout – Directed works- General Microbiology

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PREAMBLE

Microbiology is taught in the second year of the Bachelor's degree in Food Sciences and Agricultural Sciences, during the third semester of the curriculum. It is the discipline that studies microorganisms—such as bacteria, archaea, viruses, fungi, algae, and protozoa—that are usually too small to be seen with the naked eye. Microbiology encompasses research on the structure, physiology, genetics, ecology, and evolution of these microorganisms, as well as their roles in disease and their interactions with other organisms and with the environment. It has major applications in numerous fields, including human and animal health, agrifood systems, environmental management, and various industrial sectors.

Microbiology includes several main subdisciplines:

- **Bacteriology**: the study of bacteria;
- **Virology**: the study of viruses;
- **Mycology**: the study of unicellular microscopic fungi;
- **Parasitology**: the study of parasites (including protozoa and certain helminths);
- **Clinical microbiology**: the application of microbiological methods to the diagnosis and management of infectious diseases;
- **Food microbiology**: the study of microorganisms associated with food, including those responsible for spoilage, foodborne disease, and beneficial fermentations;
- **Industrial microbiology**: the use of microorganisms and their metabolites for the production of substances such as antibiotics, vitamins, organic acids, enzymes, and other biotechnological products.

The applications of microbiology are diverse, notably in:

- **Health**: identification of pathogens, development of antimicrobial agents and vaccines, and laboratory diagnosis of infectious diseases;
- **Food**: production of fermented foods (e.g., yogurt, cheese, bread, wine), microbiological quality control, and hygiene of food products;

- **Agriculture**: use of biopesticides, biofertilizers, and microbial inoculants to enhance plant growth and soil fertility;
- **Environment**: involvement of microorganisms in biogeochemical cycles (carbon, nitrogen, sulfur, etc.) and their use in the bioremediation of pollutants.

Intended Audience

This tutorial handout is designed for second-year students enrolled in the Bachelor's program in Food Sciences and Agricultural Sciences.

Learning Objectives

The Microbiology module enables students to acquire fundamental concepts related to the microbial world, including the diversity of microorganisms, the methods used to observe and study them, and the principles of bacterial growth and classification. It provides students with a solid understanding of microbial structure and physiology, basic microbial genetics, and interactions between microorganisms and their environment. These foundations are essential for understanding biological processes at the microscopic scale and for their applications in food science and agriculture.

Prerequisites

Students are expected to have a solid background in General Biology, Biochemistry, Genetics, and Molecular Biology. They should also possess a general understanding of pathogenic microorganisms and the basic principles of infectious diseases.

Course Content

This handout covers six (06) core courses, each accompanied by illustrations, detailed explanations, and corrected exercises:

1 **Course I**: General Information on the Microbial World
2 **Course II**: The Bacterial Cell
3 **Course III**: Bacterial Nutrition
4 **Course IV**: Bacterial Growth
5 **Course V**: Antimicrobial Agents

6 **Course VI**: Virulence Factors

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Introduction

Microbiology is both fundamental and applied biological science concerned with the study of microorganisms and their interactions with the environment. More specifically, microbiology is dedicated to the identification and characterization of microorganisms; the investigation of their origin and evolution; the description of their structural and physiological characteristics, metabolic activities, and nutritional requirements; and the analysis of the relationships they maintain with one another and with their natural or artificial environments.

The historical foundations of microbiology are closely linked to the development of microscopy. In 1665, Robert Hooke provided the first description of "cells" in cork, laying the groundwork for what would later become the cell theory, which postulates that the cell is the basic structural and functional unit of living organisms.

Antonie van Leeuwenhoek (1632–1723), a Dutch merchant and skilled lens maker, was the first to observe and describe the microbial world in detail. In a series of letters addressed to the Royal Society of London between 1674 and 1687, he reported microscopic observations of what he called "animalcules" in rainwater, dental plaque, and his own fecal material, thereby revealing the existence and diversity of microorganisms.

Historically, unicellular organisms with a simple cellular organization—lacking a true nucleus, nuclear membrane, mitotic apparatus, and organelles such as mitochondria—were grouped among the so-called "lower protists". Today these organisms are recognized as prokaryotes, which include bacteria and archaea. Bacteria, in particular, are characterized by a typically single, circular chromosome located in the nucleoid region and by a relatively simple cellular architecture.

In 1878, Charles-Emmanuel Sédillot introduced the term microbes to designate microscopic living agents, a category within which bacteria and, later, viruses were distinguished. The term virus originally referred to any infectious agent but is now reserved for obligate intracellular parasites that possess only one type of nucleic acid (DNA or RNA) and lack the cellular machinery required to synthesize their own macromolecular constituents independently.

The “golden age” of microbiology and its recognition as a distinct scientific discipline were made possible by technological and methodological advances in the 19th century. The development of more powerful microscopes, the introduction of culture media solidified with agar, the use of Petri dishes, the establishment of pure culture techniques, and the refinement of staining methods (e.g., Gram staining) were decisive. Louis Pasteur, Robert Koch and their students made major contributions, particularly in medical bacteriology and the study of diseases such as tuberculosis and cholera.

The direct relationship between a specific bacterium and a particular disease was rigorously demonstrated by the German physician Robert Koch (1843–1910), notably through his work on tuberculosis and its etiological agent, *Mycobacterium tuberculosis*. To demonstrate a causal link between a microorganism and an infectious disease, Koch formulated a series of criteria known as Koch’s postulates, which have played a central role in the development of medical microbiology and infectious disease epidemiology.

This textbook is a collection of microbiology tutorials that complements the lectures delivered in the Microbiology module. It is intended primarily for second-year (L2) students enrolled in Biological Sciences and Food Sciences programs, but it may also serve as a useful didactic resource for students in Medical Sciences and related disciplines.

1 General Information on the Microbial World

1.1 Introduction

Microbiology is one of the most applied branches of the biological sciences. It did not emerge as an autonomous discipline until the late nineteenth century.

Microbiology is concerned with the study of microorganisms, i.e. living entities of microscopic size that are too small to be clearly perceived with the naked eye. In practice, any organism with a diameter less than or equal to 1 mm is considered a microorganism and falls within the domain of microbiology. Since most microorganisms measure only a few micrometres in length, they must be observed using a microscope and investigated with specialized techniques. Because of this invisibility and the methodological challenges involved in their study, microbiology was the last of the three major classical branches of biology (the others being botany and zoology) to develop.

There is now a general consensus that the microbial world comprises five major groups of microorganisms:

1. **Bacteria**
2. **Archaea**
3. **Fungi** (including yeasts and molds)
4. **Protists** (including microscopic algae and protozoa)
5. **Viruses**

Microorganisms occupy virtually all environments, both aquatic and terrestrial, where they play a central role in the decomposition of dead organic matter and in biogeochemical cycles. Among them, some are pathogenic (disease-causing), others are beneficial (e.g. members of the intestinal microbiota), and many are commensal or non-pathogenic under normal conditions.

Microbiology studies microorganisms in all their aspects: their morphology and physiology, ecology and habitats, genetic and evolutionary relationships, their interactions with one another and with animals and plants, and their importance for human and animal health, the environment, agriculture, food systems, and industry.

1.2 Historical Overview

1.2.1 The Age of Discovery

This period corresponds to the first visual evidence of the microbial world and is dominated by the work of Antonie van Leeuwenhoek.

Antonie van Leeuwenhoek (1632–1723), a native of Delft in the Netherlands, was the first to observe and accurately describe microorganisms—bacteria and protozoa—using simple microscopes that he constructed himself. In 1676, he reported these observations to the Royal Society of London, referring to the organisms he saw as "animalcules" ("little animals").

Van Leeuwenhoek's meticulous observations and detailed descriptions of bacteria and protozoa were unique for his time. For this extraordinary contribution, he is regarded as the "father of bacteriology and protozoology."

1.2.2 The Transitional Period

Although substantial progress had been made since van Leeuwenhoek's discoveries, many questions remained, particularly concerning the origin of microorganisms and their relationship to disease. Two main issues dominated this transitional period:

- The controversy over spontaneous generation (the idea that living organisms could arise from non-living matter).
- The mechanisms involved in the transmission of disease.

Key contributions include:

1.2.2.1 Francesco Redi (1626–1697)

Redi, an Italian physician, was the first to experimentally challenge the traditional belief in spontaneous generation. He tested the hypothesis that maggots arose spontaneously from rotting meat. In his classic experiment, he placed meat in three jars: one left uncovered, one covered with fine gauze, and one sealed with paper. Flies entered only the uncovered jar, laid eggs on the meat, and maggots developed there. In the gauze-covered jar, flies laid eggs on the gauze, and maggots appeared on the gauze but not on the meat. No maggots appeared in the sealed jar. Redi concluded that maggots originated from fly eggs and not from a "vital force" in the meat.

1.2.2.2 John Needham (1713–1781)

Needham, a strong defender of spontaneous generation, conducted experiments with mutton broth. He briefly boiled the broth, poured it into flasks, and sealed them with cork. After a period of incubation, he consistently observed microbial growth and concluded that "animalcules" could arise spontaneously from the broth.

1.2.2.3 Lazzaro Spallanzani (1729–1799)

Spallanzani, an Italian naturalist, sought to refute Needham's conclusions. He boiled nutrient broths (such as meat broths) for longer periods to ensure more complete heating, then sealed the flasks hermetically. After incubation, no microbial growth was observed. When the sealed flasks were later opened and exposed to air, microorganisms appeared. Spallanzani demonstrated that properly heated and sealed nutrient solutions remained sterile, and that contamination occurred when air carrying microorganisms was allowed to enter, thereby arguing against spontaneous generation.

1.2.2.4 Nicolas Appert (1749–1841)

Building on these ideas, Nicolas Appert, a French food preservation pioneer, showed that soups and other liquids could be preserved by heating them for prolonged periods in sealed glass containers (originally thick glass bottles similar to champagne bottles). His work laid the foundations for modern appertization and industrial food canning.

1.2.2.5 Ignaz Semmelweis (1818–1865) and John Snow (1813–1858)

Semmelweis and Snow contributed to the growing awareness of how infectious diseases are transmitted. Semmelweis demonstrated that handwashing with chlorinated lime drastically reduced puerperal fever in maternity clinics. Snow, through epidemiological investigations of cholera outbreaks in London, provided evidence that the disease was waterborne.

1.2.2.6 Theodor Schwann (1810–1882) and Theodor Schulze (1815–1873)

These German scientists proposed that microbes present in the air were responsible for contaminating sterile infusions. They passed air through red-hot tubes or strong chemical solutions before allowing it into flasks containing boiled infusions. Under these conditions, the

infusions remained free of microbial growth, supporting the idea that microbes in untreated air were the source of contamination.

1.2.2.7 Georg Schroeder and Theodor von Dusch (1854)

Schroeder and von Dusch introduced the use of cotton plugs to filter air entering flasks containing sterile media. These plugs retained airborne microorganisms and prevented contamination, further supporting the concept that microbes are carried by dust particles in the air.

1.2.2.8 Charles Darwin (1809–1882)

In On the Origin of Species (1859), Darwin proposed a naturalistic explanation for the origin and evolution of species. His theory reinforced the idea that humans and other organisms, including their diseases, are subject to biological laws rather than supernatural forces, thus contributing indirectly to the conceptual framework in which infectious diseases came to be understood as biological phenomena.

1.2.3 The Golden Age of Microbiology

The Golden Age of Microbiology began in the second half of the nineteenth century with the work of Louis Pasteur and Robert Koch, who founded their own research institutes and established microbiology as a rigorous experimental science. Their findings were rapidly accepted by the international scientific community and stimulated an intense period of discovery and innovation.

1.2.3.1 Louis Pasteur

Louis Pasteur (1822–1895), a French chemist and microbiologist, performed a series of decisive experiments demonstrating that microorganisms are present in the air and do not arise spontaneously. He prepared nutrient broths in flasks with long, curved “swan-neck” tubes. The flasks were boiled to sterilize the contents, and the open ends of the curved necks allowed air to enter while causing airborne dust particles to settle in the bends by gravity.

After incubation, no microbial growth appeared in the broths as long as the flasks remained upright and the dust trapped in the neck did not reach the liquid. When the necks were broken or the flasks tilted so that the broth came into contact with the trapped dust, microbial growth

rapidly occurred. These experiments clearly demonstrated that microorganisms present on dust and in the air were responsible for the contamination of sterile solutions.

In 1861, Pasteur definitively resolved the controversy between spontaneous generation and biogenesis, showing that microorganisms originate from pre-existing microorganisms.

As a professor of chemistry at the University of Lille, Pasteur also made fundamental contributions to the fermentation industry. He showed that alcoholic fermentation of sugars to produce wine and beer is carried out by specific microorganisms (yeasts) and that undesirable bacteria were responsible for spoilage. He demonstrated that moderate heating of beverages (around 62–63 °C for 30 minutes) could destroy spoilage organisms without significantly altering the organoleptic properties of the product. This heat treatment, later termed pasteurization, was introduced commercially in the United States in the 1890s.

Pasteur's work on fermentation, spoilage, and sterilization provided crucial evidence for the germ theory of disease, which holds that specific microorganisms are the causative agents of specific infectious diseases.

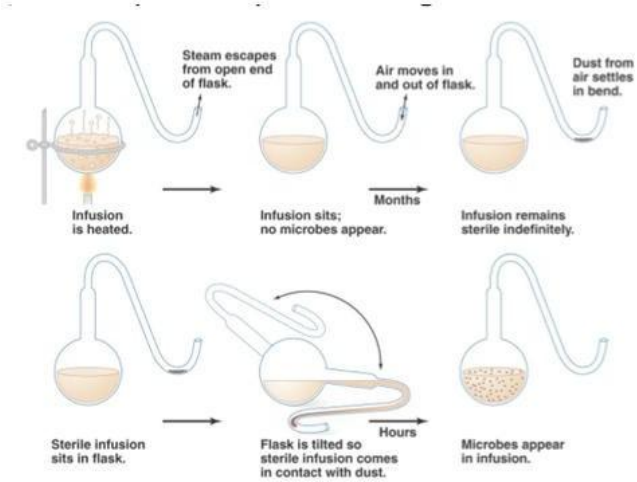


Figure 1. Pasteur's swan-neck flask experiment refuting the theory of spontaneous generation

1.2.3.2 John Tyndall (1820–1893)

John Tyndall, an English physicist, delivered the final experimental blow to the doctrine of spontaneous generation in 1877. Working with specially designed aseptic chambers, he

demonstrated that airborne dust particles carry microorganisms. He showed that, in the absence of dust, sterilized broth remained indefinitely free of microbial growth, even when it was in direct contact with air.

While studying hay infusions, Tyndall also discovered a highly resistant bacterial form, later identified as the endospore. He observed that ordinary boiling was insufficient to destroy these structures. To achieve complete sterilization, he developed a method of intermittent heating—alternating periods of boiling and cooling over several days—allowing spores to germinate and then be killed in the vegetative state. This process, known as tyndallization, ensured the elimination of both vegetative cells and spores.

1.2.3.3 Joseph Lister (1827–1912)

Lord Joseph Lister, a British surgeon, is renowned for his pioneering work in the development of antiseptic surgery. Applying the principles of the germ theory of disease, Lister concluded that wound infections were caused by microorganisms introduced during surgical procedures.

In 1867, he introduced an antiseptic technique based on the use of phenol (carbolic acid). He applied phenol to surgical dressings, instruments, and sometimes sprayed it in the operating area to reduce the microbial load. These procedures markedly decreased postoperative infections and mortality. Lister's systematic use of chemical agents to control microorganisms in the surgical environment laid the foundations of modern aseptic and antiseptic techniques, and he is regarded as the father of antiseptic surgery.

1.2.3.4 Robert Koch (1843–1910)

Robert Koch, a German physician, was the first to demonstrate directly the causal role of bacteria in specific diseases. In 1876, he isolated and identified the etiologic agent of anthrax, *Bacillus anthracis*, and showed its ability to cause disease in experimental animals.

Koch greatly improved bacteriological methods by:

- Perfecting techniques for obtaining pure cultures of bacteria;
- Introducing solid culture media in 1881, initially using gelatin as a solidifying agent;
- Describing in 1882 the etiological agent of tuberculosis, *Mycobacterium tuberculosis*.

To demonstrate a causal relationship between a microorganism and a disease, Koch proposed a set of criteria, published in 1884, known as Koch's postulates, which became a cornerstone of the germ theory of disease:

1. The suspected microorganism must be present in all cases of the disease and absent from healthy individuals.
2. The microorganism must be isolated from the diseased host and grown in pure culture on an appropriate nutrient medium.
3. The cultured microorganism must reproduce the same disease when inoculated into a healthy, susceptible host.
4. The same microorganism must be reisolated from the experimentally infected host and shown to be identical to the original isolate.

Although certain pathogens (e.g. many viruses and some obligate intracellular bacteria) cannot be grown on standard artificial media and thus do not strictly fulfil all postulates, Koch's framework remains a fundamental reference in medical microbiology.

Koch's initial use of gelatin as a solidifying agent presented two major drawbacks: (i) gelatin, being a protein, is degraded by many bacteria that produce the proteolytic enzyme gelatinase, and (ii) it melts at temperatures above approximately 25 °C, which is unsuitable for the cultivation of many human pathogens. Nevertheless, Koch's methodological innovations and his discovery of major pathogens profoundly shaped the development of bacteriology.

1.2.3.5 Fannie Hesse (1850–1934) and Richard Petri (1852–1921)

Fannie Eilshemius Hesse, an associate in Koch's laboratory, suggested the use of agar-agar as a solidifying agent in culture media. Agar proved superior to gelatin because it is not readily degraded by most bacteria and has a high melting point (around 90–96 °C) and a relatively low solidification temperature (about 40–45 °C), making it ideal for microbial culture.

Richard Julius Petri, another of Koch's assistants, designed the Petri dish in 1887, a shallow, lidded glass (now often plastic) vessel used to contain solidified agar media. The combined contributions of Koch, Hesse, and Petri made it possible to routinely obtain pure cultures of microorganisms and greatly accelerated progress in all areas of microbiology.

1.2.3.6 Edward Jenner (1749–1823)

Edward Jenner, an English physician, was the first to develop a practical method for preventing smallpox. He observed that milkmaids who had contracted cowpox (a benign disease caused by a virus related to the smallpox virus) were subsequently protected against smallpox.

On 14 May 1796, Jenner inoculated material (pus) from cowpox lesions into a young boy. The boy developed a mild illness but became resistant to subsequent exposure to smallpox. In 1798, Jenner published his results based on a series of successful inoculations. This procedure became known as vaccination, from the Latin vacca ("cow"), in reference to cowpox. The use of cowpox virus to protect humans against smallpox progressively replaced the riskier practice of variolation (inoculation with smallpox material).

Jenner's work was later recognized by Louis Pasteur, who extended the principle of vaccination to other diseases. Pasteur developed attenuated vaccines against anthrax and, subsequently, rabies, demonstrating that weakened forms of pathogens could stimulate protective immunity. These successes laid the conceptual and practical foundations for modern vaccination programs against diseases such as diphtheria, tetanus, pertussis, poliomyelitis, and measles.

1.2.3.7 Élie Metchnikoff (1845–1916)

Élie (Ilya) Metchnikoff proposed the phagocytic theory of immunity in 1883. While studying starfish larvae and later mammalian systems, he discovered that certain leukocytes (white blood cells) could engulf and destroy invading microorganisms. These cells were termed phagocytes, and the process phagocytosis.

Metchnikoff's work demonstrated that host cells play an active role in defence against infection, establishing the concept of cell-mediated immunity and contributing significantly to the foundation of modern immunology.

1.2.3.8 Émile Roux (1853–1933), Alexandre Yersin (1863–1943), Emil von Behring (1854–1917) and Shibasaburo Kitasato (1852–1931)

Émile Roux and Alexandre Yersin, French bacteriologists working in the Pasteurian tradition, showed that cultures of Corynebacterium diphtheriae produce a soluble toxin that can be detected in cell-free filtrates of culture broth.

Emil von Behring and Shibasaburo Kitasato, collaborators of Koch, discovered that animals immunized with inactivated toxins of Clostridium tetani or Corynebacterium diphtheriae produced circulating antitoxins capable of neutralizing these toxins. In 1890, von Behring reported successful serum therapy against diphtheria using antitoxin-containing serum.

The elucidation of the toxin–antitoxin relationship and the development of antitoxin therapies represented major milestones in humoral immunology and opened the way to the production of toxoid vaccines.

1.2.3.9 Paul Ehrlich (1854–1915)

Paul Ehrlich made fundamental contributions to chemotherapy, the use of chemical substances to selectively inhibit or kill pathogens with minimal harm to the host. In 1904, he discovered that the synthetic dye trypan red was active against Trypanosoma spp., the protozoan parasites that cause African sleeping sickness. He coined the idea of a “magic bullet”—a compound that would specifically target pathogens.

In 1910, in collaboration with Sahachiro Hata, Ehrlich introduced Salvarsan (arsphenamine), an arsenic-based compound effective against Treponema pallidum, the causative agent of syphilis. Salvarsan was one of the first successful antimicrobial drugs and is considered the beginning of modern systemic chemotherapy.

1.2.3.10 Gerhard Domagk (1895–1964)

In 1935, the German researcher Gerhard Domagk tested numerous synthetic dyes and discovered that Prontosil, a red azo dye used in the leather industry, was effective in protecting mice against infections caused by streptococci and staphylococci, although it showed little direct activity in vitro.

Later that year, the French researchers Jacques and Thérèse Tréfouël and their colleagues demonstrated that Prontosil was metabolized in vivo to sulfanilamide, the true active antimicrobial agent. Sulfonamides became the first widely used systemic antibacterial drugs. Domagk was awarded the Nobel Prize in Physiology or Medicine in 1939 for his discovery of the first sulfonamide.

1.2.3.11 Alexander Fleming (1881–1955), Howard Florey (1898–1968) and Ernst Chain (1906–1979)

The first antibiotic in the modern sense—penicillin—was discovered by Sir Alexander Fleming, a Scottish physician and bacteriologist. In 1928, after returning from a short holiday, Fleming noticed that a culture plate of *Staphylococcus aureus* had been contaminated by a greenish mold, later identified as *Penicillium notatum* (now *Penicillium rubens*). Around the mold colonies, there was a clear zone where staphylococcal growth had been inhibited.

Rather than discarding the contaminated plate, Fleming hypothesized that the mold produced a diffusible antibacterial substance. He isolated the mold and extracted the active compound, which he named penicillin after the producing genus *Penicillium*. In 1929, he published his findings, showing that penicillin could kill or inhibit several pathogenic bacteria.

Large-scale production of penicillin became feasible only during the Second World War, through the work of Howard Florey, Ernst Chain, and their team, who purified and stabilized penicillin and optimized its industrial production. Commercial manufacture began in the United States around 1941. Fleming, Florey, and Chain jointly received the Nobel Prize in Physiology or Medicine in 1945 for their contributions to the discovery and development of penicillin.

For industrial production, *P. notatum* was later replaced by high-yielding strains of *Penicillium chrysogenum*, selected and improved by mutation, which produce vastly greater quantities of penicillin than the original strain.

1.2.3.12 Selman A. Waksman (1888–1973) and Other Antibiotics

In 1944, Selman Abraham Waksman of Rutgers University in the United States discovered streptomycin, an antibiotic produced by the actinomycete Streptomyces griseus. Streptomycin was the first effective antibiotic against tuberculosis caused by *Mycobacterium tuberculosis* (identified by Koch in 1882). Waksman received the Nobel Prize in Physiology or Medicine in 1952 for this discovery.

In the late 1940s and early 1950s, several other important antibiotics produced by Streptomyces species were identified, including:

- **Chloramphenicol** (chloromycetin), from *Streptomyces venezuelae* (Paul R. Burkholder, 1947);
- **Aureomycin** (chlortetracycline), from *Streptomyces aureofaciens* (B. M. Duggar, 1948);
- **Terramycin** (oxytetracycline), from *Streptomyces rimosus* (Finlay, Hobby and collaborators, 1950).

The death of Robert Koch in 1910 and the outbreak of the First World War marked the end of the classical golden age of microbiology. The Pasteur Institute reduced its research activities, and many German laboratories were redirected toward war-related medical needs. Although microbiological research continued, this period is often seen as the conclusion of the first great era of microbiology.

1.2.4 The Twentieth Century: The Era of Molecular Biology

By the early twentieth century, microbiology had become firmly established as a major branch of biology. Microorganisms, because of their relative simplicity, rapid reproduction, and genetic uniformity, soon emerged as ideal model systems for investigating fundamental biological processes. This led to the development of a closely related discipline: molecular biology.

The use of microorganisms as tools for exploring the mechanisms of life became particularly attractive for several reasons:

- **Rapid growth and reproduction**: Many bacteria and fungi have short generation times, enabling multiple generations to be studied over brief periods.
- **Ease of cultivation**: Microorganisms can be grown in defined media under controlled conditions, in both small and large volumes.
- **Experimental manipulability**: Their growth can be readily influenced by physical and chemical factors, and their cells can be disrupted to separate cellular components into fractions of different sizes and densities.

1.3 The Place of Microorganisms in the Living World

Before the discovery of microorganisms, living beings were traditionally classified into two kingdoms: Animalia (animals) and Plantae (plants). The recognition of microscopic life forms posed a major challenge to this dichotomy, because many microorganisms did not fit clearly into either category.

In 1866, Ernst Haeckel proposed a third kingdom, Protista, to accommodate unicellular organisms such as algae and protozoa, which differed from both plants and animals. With time, fungi and bacteria were also distinguished as separate groups, and modern systems now commonly recognize several kingdoms and, above all, three domains: Bacteria, Archaea, and Eukarya.

Microorganisms represent the dominant form of life on Earth, both in terms of numbers and ecological importance. They have inhabited the planet for more than 3 billion years and constitute the evolutionary foundation from which more complex life forms emerged. It is estimated that microbial biomass accounts for a substantial proportion of all organic matter on Earth.

Microorganisms are distinguished from other living beings by several key features:

- **Cellular organization:** Microorganisms generally lack tissue and organ differentiation. Each cell is **autonomous**: it is capable of taking up nutrients, metabolizing them, excreting waste products, reproducing, responding to environmental changes, and undergoing genetic mutation and adaptation.
- **Metabolic potential:** Many bacteria and archaea exhibit an exceptional metabolic versatility compared with multicellular organisms. Their high surface-to-volume ratio facilitates rapid exchange of nutrients and waste products with the environment, supporting fast growth and allowing them to exploit a wide range of energy and carbon sources (phototrophy, chemolithotrophy, chemoorganotrophy, etc.).
- **Abundance and ubiquity:** Microorganisms are extremely abundant and ubiquitous. They are found in virtually all environments: air, fresh and marine waters, soils, sediments, extreme environments (hot springs, deep-sea vents, hypersaline lakes), and on or within the bodies of animals and plants.
- **Unicellularity:** Most microorganisms are unicellular, consisting of a single cell that performs all vital functions. Some, however, form multicellular filaments, mycelia, or colonies with varying degrees of organization.
- **Small size:** Microorganisms are typically microscopic, and thus invisible to the naked eye. Prokaryotic cells (bacteria and archaea) are generally much smaller than eukaryotic cells, which has major implications for their physiology and ecology.

Microbiology, as a discipline, investigates microorganisms in all these aspects: their morphology, physiology, genetics, ecology, and evolution; their interactions with one another and with plants, animals, and humans; and their roles in health, agriculture, the environment, and industry.

1.4 General Characteristics of the Prokaryotic Cell

Bacteria and archaea have long been grouped under the term prokaryotes. The term, derived from the Greek pro (“before”) and karyon (“nucleus”), reflects the fact that these organisms lack a membrane-bound nucleus. The concept of the prokaryote–eukaryote dichotomy was clearly formulated by R. Y. Stanier and C. B. van Niel in 1962, based not on what prokaryotes possess, but on what they lack in comparison with eukaryotes.

Prokaryotic cells are typically 0.2–10 μm in size and may occur as isolated cells or form simple multicellular arrangements (chains, clusters, filaments). Their main characteristics are as follows:

- **Genetic material**

Prokaryotic cells do not possess a nuclear membrane. Their genetic material is located in the nucleoid, a region of the cytoplasm containing a single, usually circular, double-stranded DNA molecule. This chromosome carries the essential genetic information required for cell growth, metabolism, and reproduction. Chromosomal replication is coupled to cell division and occurs without mitosis.

In addition, most prokaryotes harbour small, circular, extrachromosomal DNA molecules called plasmids. Plasmids often carry non-essential but advantageous genes, such as those conferring antibiotic resistance, virulence factors, or specific metabolic capabilities, and they can be transferred between cells.

- **Cytoplasmic membrane and internal organization**

The cytoplasmic (plasma) membrane is a selectively permeable phospholipid bilayer containing proteins involved in transport, energy generation, and signal transduction. In many prokaryotes, the membrane can form invaginations or internal vesicles and lamellae, increasing the surface area for metabolic activities.

Energy-transducing processes such as respiration and, in phototrophic prokaryotes, photosynthesis, are associated with the cytoplasmic membrane and its internal derivatives, since prokaryotes lack mitochondria and chloroplasts.

The cytoplasm contains numerous 70S ribosomes, which are the sites of protein synthesis, and various enzymes and macromolecules required for metabolism. It is generally homogeneous and lacks the complex compartmentalization seen in eukaryotic cells. Nutrients are assimilated in molecular form, after being transported across the membrane.

- **Cell wall and cell envelope**

Most prokaryotes possess a rigid cell wall external to the cytoplasmic membrane, which maintains cell shape, protects against osmotic lysis, and contributes to pathogenicity. In bacteria, the major structural component of the cell wall is peptidoglycan (murein), a carbohydrate–peptide heteropolymer. Some prokaryotes (e.g. *Mycoplasma* species) lack a cell wall and are instead surrounded only by a plasma membrane.

- **Motility**

Prokaryotic cells may be non-motile or motile. Motility is often mediated by flagella, which in bacteria are thin, helical filaments composed of flagellin, driven by a rotary motor embedded in the cell envelope. Other types of movement include gliding and twitching motility, which may involve surface structures such as pili or secretion-based mechanisms.

Although prokaryotes are frequently unicellular, they can form visible colonies, filamentous structures, or mycelium-like networks, especially among actinomycetes and certain cyanobacteria. They are widespread in all environments and play crucial roles in biogeochemical cycles and symbiotic associations.

Table 1. Main Distinguishing Characteristics of Prokaryotes and Eukaryotes

Characteristic	Prokaryotes	Eukaryotes
Usual cell size	~0.5–5 μm	≥5 μm (many 10–100 μm)
Nuclear membrane	Absent; DNA in nucleoid	Present; DNA in membrane-bound nucleus
Number and form of	Usually one circular	Multiple linear chromosomes

chromosomes	chromosome (± plasmids)	
Mode of genetic division	Binary fission; no mitosis or meiosis	Mitosis and meiosis present
Location of DNA	Nucleoid and plasmids	Nucleus; additional DNA in mitochondria and plastids (if any)
Membrane-bound organelles	Absent	Present (mitochondria, Golgi apparatus, ER, etc.)
Membrane sterols	Generally absent (except some groups)	Present in most (e.g. cholesterol in animal cells)
Cell wall	Peptidoglycan (murein) in most bacteria	Cellulose in plants, chitin in fungi, absent in animals
Flagella and cilia	Simple flagella; no true cilia	Complex 9+2 microtubule-based flagella and cilia

From an evolutionary standpoint, prokaryotes are among the earliest forms of life, and modern systematics recognizes two distinct prokaryotic domains:

1. **Eubacteria (Bacteria)**: the "true" bacteria, which include most familiar bacterial groups found in soil, water, and in association with plants, animals, and humans.
2. **Archaea (formerly Archaebacteria)**: a distinct lineage of prokaryotes. Many archaea are extremophiles, thriving in environments with high temperature, extreme salinity, or anoxia (e.g. hydrothermal vents, hypersaline lakes, anaerobic sediments), although numerous archaeal species also inhabit more moderate environments.

These two domains differ markedly in the structure of their cell envelopes, membrane lipids, and genetic machinery, reflecting deep evolutionary divergence within the prokaryotic world.

2 The Bacterial Cell

2.1 Introduction

The term bacterium (from the Greek *bakterion*, meaning "little rod") was originally used by early microscopists to describe rod-shaped organisms considered to belong to the "lowest plant order" and defined as "microscopic, unicellular, non-chlorophyllous organisms reproducing by fission."

Today, bacteria are recognized as unicellular prokaryotic organisms of small size and diverse morphology, characterized by a simple cellular organization lacking a true nucleus and membrane-bound organelles. The mass of a typical bacterial cell is on the order of 10^{-12} g and it contains approximately 70% water. On a dry-weight basis, a bacterial cell is mainly composed of proteins, nucleic acids, lipids and polysaccharides. Proteins account for roughly half of the dry mass, RNA is more abundant than DNA, and structural polymers such as peptidoglycan and lipopolysaccharides contribute significantly to the cell envelope.

2.2 Structure of Bacterial Cells

Bacteria show considerable variation in size and shape, but all are sufficiently small that they cannot be resolved by the naked eye and must be observed with a microscope (Figure 2). Most medically and agriculturally important bacteria are between about 0.5 and 2 μm in width and 1–5 μm in length.

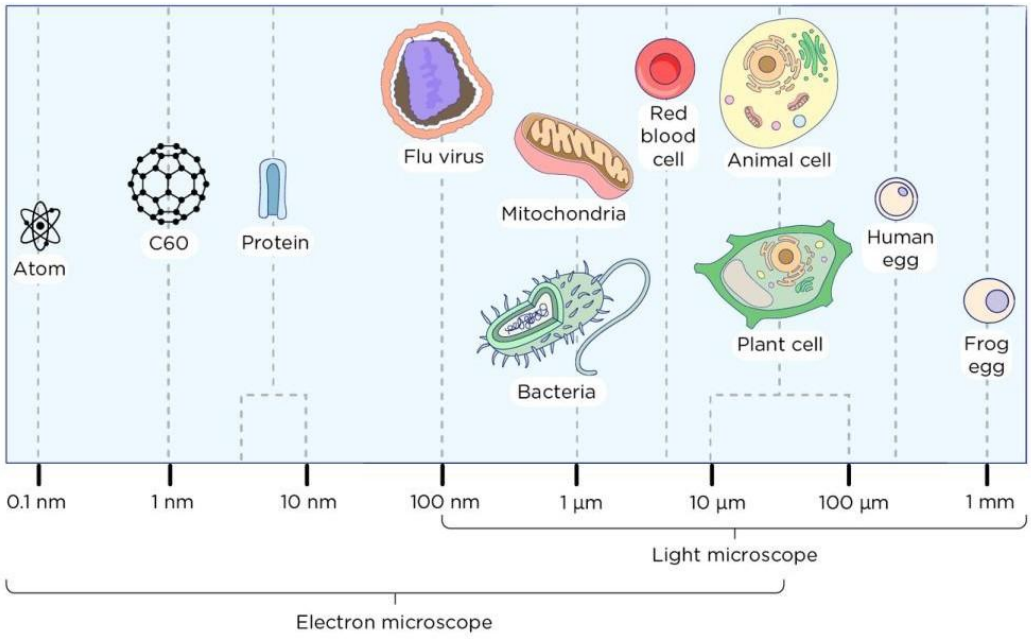


Figure 2. Schematic representation of the size range of bacterial cells

2.3 General Characteristics of Bacteria

Bacteriology is the branch of microbiology devoted to the study of the morphology, physiology, genetics, ecology and reproduction of bacteria. The main general characteristics of bacteria can be summarized as follows:

1. **Ubiquity**: Bacteria are ubiquitous: they are present in soil, air, water, and on or within plants, animals and humans.
2. **Prokaryotic, unicellular organization**: Bacteria are unicellular prokaryotes. Each cell is an independent unit capable of carrying out all essential life functions.
3. **Cell wall**: The bacterial cell is generally surrounded by a rigid cell wall external to the plasma membrane. This structure maintains cell shape, protects against osmotic lysis and contributes to pathogenicity.
4. **Nutritional diversity**: Bacteria display wide metabolic and nutritional diversity. They may be:
    a) **Autotrophic** (e.g. photoautotrophs, chemoautotrophs),
    b) **Heterotrophic**, which includes:
        - **Parasitic** forms (living at the expense of a host),

- **Saprophytic** forms (living on dead organic matter),
- **Symbiotic** forms (engaged in mutualistic relationships).

5. **Photosynthetic pigments**: Most bacteria lack true chlorophyll a as found in higher plants, but some photosynthetic bacteria possess specialized pigments such as bacteriochlorophylls and carotenoids.
6. **Absence of a true nucleus**: Because of their prokaryotic nature, bacteria lack a membrane-bound nucleus. Their genetic material is localized in the cytoplasm in a region termed the nucleoid, without a nuclear envelope or nucleolus.
7. **Cell wall composition**: The cell wall of most bacteria contains peptidoglycan (murein), a mucopeptide polymer distinct from cellulose (in plants) or chitin (in fungi).
8. **Absence of membrane-bound organelles**: Bacteria lack mitochondria, Golgi apparatus, plastids, and endoplasmic reticulum. Functions analogous to those of mitochondria and chloroplasts are performed by the cytoplasmic membrane and its internal infoldings.
9. **Nature of DNA**: Bacterial chromosomal DNA is typically a single, circular, double-stranded molecule that is not associated with canonical histone proteins (although histone-like proteins are present).
10. **Ribosomes**: Bacteria possess 70S ribosomes (composed of 50S and 30S subunits), which are smaller than the 80S ribosomes found in eukaryotic cytoplasm.
11. **Membrane invaginations (mesosomes)**: In older descriptions, invaginations of the plasma membrane termed mesosomes were reported and thought to be involved in respiration or cell division. It is now recognized that many mesosome-like structures are artifacts of chemical fixation; nevertheless, the cytoplasmic membrane remains the central site for energy-transducing processes in bacteria.

2.4 Techniques for Observing the Bacterial Cell

Because bacterial cells are typically of the order of 1 μm, they must be examined using a light (optical) microscope (Figure 3), which provides sufficient magnification (M) and resolution to visualize them.

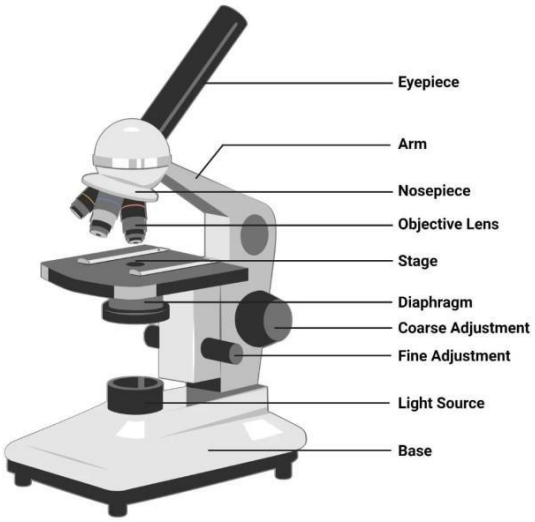


Figure 3. Schematic diagram of a light (photon) microscope

In a bright-field light microscope, light (a stream of photons) passes through a condenser, which focuses the beam onto the specimen. The objective lens forms the first magnified image of the specimen (e.g. 4×, 10×, 40×, 100×), and the ocular (eyepiece) lens produces a second magnification (commonly 10×). The total magnification is the product of the objective and ocular magnifications (e.g. 1000× for a 100× objective with a 10× ocular).

The quality of the image depends on:

- The quality of the lenses,
- The wavelength of the light used,
- The resolving power of the microscope, i.e. its ability to distinguish two closely spaced points as separate.

A standard bright-field optical microscope with oil immersion objectives can achieve a final magnification of about 1000–1500×, which is sufficient to detect and roughly characterize bacterial cells. Bacteria can be observed:

- **Unstained**, in a wet mount or hanging drop, to study their general shape and motility;
- **After simple staining**, for example with methylene blue or basic fuchsin, to increase contrast and better visualize cell morphology.

More advanced techniques (phase-contrast microscopy, fluorescence microscopy, electron microscopy) provide additional information but are beyond the scope of this introductory section.

2.5 Morphology of the Bacterial Cell

Bacteria exhibit a wide variety of shapes (Figure 4). Historically, bacterial classification relied heavily on cell morphology, although modern taxonomy is now based primarily on genetic and molecular criteria. For descriptive purposes, however, several basic morphological types are still recognized.

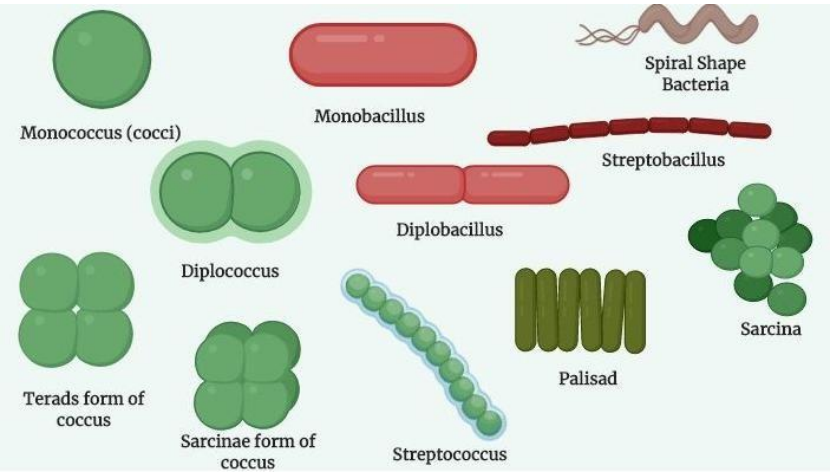


Figure 4. Schematic representation of the main bacterial cell shapes

2.5.1 Cocci (Spherical or Ovoid Forms)

The term coccus (plural: cocci) comes from the Greek kokkos, meaning "grain" or "berry." Cocci are approximately spherical or slightly ovoid cells, usually 0.5–1.5 μm in diameter, and many lack flagella. Depending on the way they divide and remain attached after division, they form characteristic arrangements:

- **Micrococci:** Cocci that occur singly, e.g. *Micrococcus* spp.
- **Diplococci:** Cocci that occur in pairs, resulting from division in one plane with cells remaining attached, e.g. *Streptococcus pneumoniae* (historically *Diplococcus pneumoniae*).
- **Streptococci:** Cocci arranged in chains as a result of repeated division in one plane, e.g. *Streptococcus lactis*, *Streptococcus pyogenes*.

- **Staphylococci:** Cocci arranged in irregular, grape-like clusters due to division in multiple planes, e.g. *Staphylococcus aureus*.
- **Tetrads:** Groups of four cocci arranged in a square, formed by division in two perpendicular planes, e.g. some *Micrococcus* spp.
- **Sarcinae:** Cocci arranged in cubic packets of eight, sixteen or more cells, formed by division in three perpendicular planes, e.g. *Sarcina* spp.

2.5.2 Rod-Shaped Bacteria (Bacilli)

The term bacillus (plural: bacilli) refers to rod-shaped bacteria. Their ends may be rounded, square, or tapered. Typical sizes range from about 0.5–1.2 μm in width and 1–7 μm in length. Bacilli may be motile (with flagella) or non-motile. Many plant and animal pathogens are rod-shaped. They may appear as:

- **Single rods (monobacilli)**: isolated cells.
- **Diplobacilli**: pairs of rods, remaining attached after division.
- **Streptobacilli**: chains of rods, e.g. *Streptobacillus moniliformis*.
- **Palisades**: rods aligned side by side in parallel or in a picket-fence arrangement, as sometimes observed with *Corynebacterium* spp.

A medically important rod-shaped species is *Mycobacterium tuberculosis*, the causative agent of tuberculosis.

2.5.3 Spiral and Helical Forms

The term spirillum (plural: spirilla) comes from the Greek speira, meaning “coil.” These bacteria are rigid, spiral-shaped cells resembling a corkscrew, with one or more helical turns. They usually occur as free-living single cells and are often motile by means of external polar flagella. Their length is typically 10–50 μm and their diameter 0.5–3 μm, e.g. Spirillum minus, Spirillum volutans.

2.5.4 Vibrios (Comma-Shaped Rods)

Vibrios are curved, comma-shaped rods with a single, gentle curve, often possessing one or more polar flagella. They typically measure about 1.5–3 μm in length and 0.5–0.8 μm in width. A classical example is Vibrio cholerae, the causative agent of cholera.

2.5.5 **Spirochetes**

Spirochetes are long, thin, flexible, helical bacteria with multiple tight coils. Unlike spirilla, their motility is mediated by internal axial filaments (endoflagella) located between the cell wall and outer membrane. Their length greatly exceeds their diameter. Important genera include Treponema, Borrelia and Leptospira.

2.5.6 **Filamentous Bacteria**

Some bacteria form filamentous structures, consisting of long chains of cells often surrounded by a sheath. These forms are frequently found in wastewater, nutrient-rich aquatic environments and iron-rich waters. Examples include Sphaerotilus natans, Leptothrix, Cladothrix, Nocardia and Beggiatoa. Filamentous bacteria may resemble fungal hyphae and can form complex mycelium-like networks.

2.5.7 **Stalked (Prosthecate) Bacteria**

Stalked or prosthecate bacteria possess one or more cellular extensions (prosthecae) that increase surface area and facilitate attachment to surfaces. The prostheca is a tubular or stalk-like appendage enveloped by the cell wall and membrane. Two main types are distinguished:

1. Bacteria whose prosthecae do not participate directly in reproduction, e.g. *Caulobacter* spp., where a stalked mother cell produces a motile swarmer cell.
2. Bacteria whose prosthecae participate in budding reproduction, e.g. *Hyphomicrobium* spp., where a new cell buds from the tip of the prostheca.

The stalk may reach lengths of several micrometres and is often formed in nutrient conditions rich in certain ions (e.g. phosphate). The basal end of the stalk frequently bears adhesive material allowing attachment to surfaces.

2.5.8 **Pleomorphic Bacteria**

Pleomorphic bacteria exhibit variability in shape and size depending on growth conditions, age of the culture or environmental factors. Instead of having a single, stable morphology, they can appear as coccoid, rod-shaped or filamentous forms within the same species. Examples include certain species of *Acetobacter* and *Corynebacterium*.

2.5.9 **Budding Bacteria**

Some bacteria reproduce by budding, in which a new cell develops as a small protrusion from a larger mother cell. These organisms often show asymmetrical, club-shaped or "pear-shaped" cells with a thin tubular extension. The bud enlarges at the distal end of the tube, eventually forming a new globular cell and, in some species, generating a branched cellular network. An example is *Rhodomicrobium* spp., a photosynthetic budding bacterium.

2.6 The structure of a bacterial cell

In bacterial cells, a distinction is made between constant (obligate) structures, present in almost all bacteria, and non-constant (facultative) structures that characterize only certain bacterial groups (Table II and Figure 5).

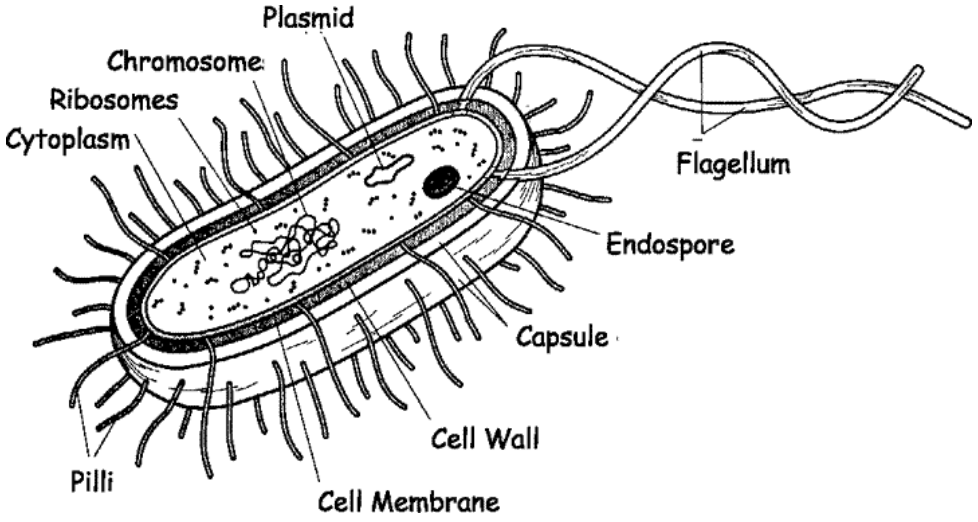


Figure 5. Structure of a typical bacterial cell

Table 2. Constant and non-constant structures of a bacterial cell

Constant structures	Non-constant (facultative) structures
Cell wall	Endospore
Cytoplasmic (plasma) membrane	Capsule
Cytoplasm	Flagella
Ribosomes	Conjugative (sex) pili
Bacterial chromosome	Fimbriae

2.6.1 Constant structures

2.6.1.1 The cell all

In bacteria, the cell wall is located beneath the external structures (such as capsules and flagella) and external to the cytoplasmic membrane. It is a rigid structure that determines and maintains the shape of the cell. In most species it is composed of multiple layers, whose overall thickness varies between Gram-positive and Gram-negative bacteria (Figure 6). The walls of Gram-negative species are generally thinner (approximately 10–15 nm) than those of Gram-positive species (approximately 20–25 nm or more).

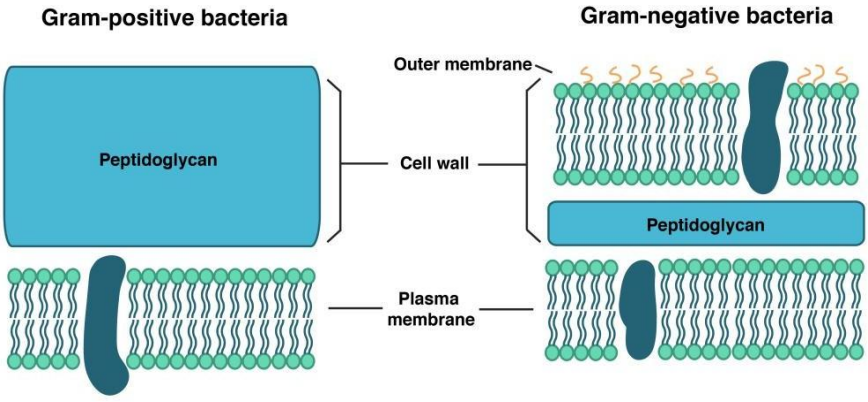


Figure 6. Structure of the bacterial cell wall

The cell wall of most bacteria contains peptidoglycan (also called murein), an insoluble, porous, highly cross-linked polymer that is extremely strong and rigid. Peptidoglycan is characteristic of bacterial cell walls and is absent from eukaryotic cells. It consists essentially of a polymer of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), to which short peptide chains of

four amino acids (classically L-alanine, D-alanine, D-glutamate and a diamino acid such as meso-diaminopimelic acid or L-lysine) are attached (Figure 7).

Tetrapeptide side chains on neighboring glycan strands are cross-linked, forming a three-dimensional network that surrounds the cell and provides high mechanical strength and rigidity. Several antibiotics, such as β-lactams (e.g. penicillin), inhibit key steps in peptidoglycan synthesis and thereby block cell wall formation, ultimately leading to cell lysis.

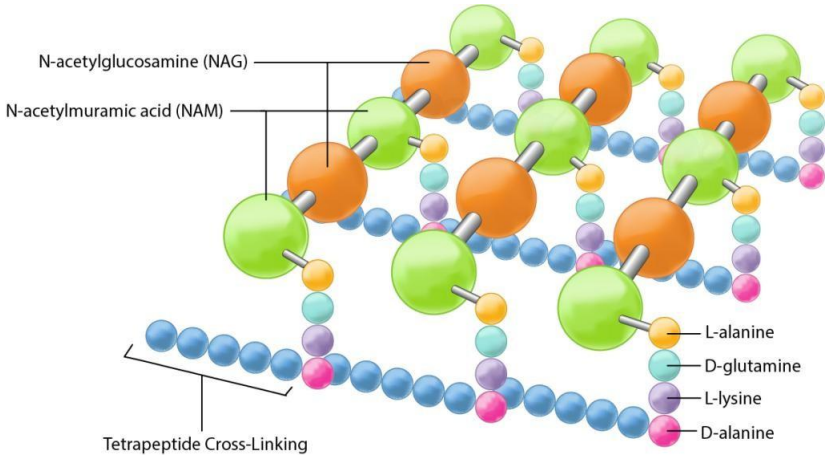


Figure 7. Structure of peptidoglycan (murein)

2.6.1.2 The cytoplasmic membrane

The cytoplasmic (plasma) membrane lies immediately beneath the cell wall and is similar in overall organization in both Gram-positive and Gram-negative bacteria. It is a phospholipid bilayer approximately 7–8 nm thick, composed mainly of phospholipids (about 20–30%) and proteins (about 60–70%). The phospholipids form a bilayer into which most proteins are embedded as integral membrane proteins, while others are peripherally associated.

The cytoplasmic membrane acts as a selectively permeable, hydrophobic barrier that prevents the free passage of most water-soluble molecules. Transport of solutes is mediated by specific membrane proteins that facilitate or actively drive the passage of small molecules (nutrients, metabolic intermediates and waste products). The membrane also houses many enzymes and protein complexes involved in energy metabolism (e.g. components of the respiratory chain), as well as enzymes required for the synthesis of cell wall polymers and, in some species, capsule components.

2.6.1.3 The mesosome

Bacterial cells lack membrane-bound organelles such as mitochondria, chloroplasts and Golgi apparatus. Historically, electron microscopy of especially Gram-positive bacteria revealed complex invaginations of the cytoplasmic membrane, appearing as convoluted tubules and vesicles termed mesosomes. They were often prominent in bacilli and could be present in several copies (2–4 or more) per cell, with higher numbers reported in bacteria with high respiratory activity, such as *Azotobacter* species.

These structures were once thought to increase the membrane surface area and to participate in processes such as respiration, cell division and chromosome segregation. However, it is now widely accepted that classical mesosomes are largely artifacts produced by chemical fixation during sample preparation for electron microscopy. *In vivo*, bacteria may form functional membrane invaginations, but these are not generally referred to as mesosomes in modern microbiology.

2.6.1.4 The cytoplasm

The cytoplasm is the region of the bacterial cell enclosed by the cytoplasmic membrane. Approximately 80% of the cytoplasm is water; the remaining fraction consists of nucleic acids, proteins, lipids, carbohydrates, inorganic ions and a variety of low–molecular weight metabolites.

In contrast to eukaryotic cells, the cytoplasm of bacteria does not exhibit cytoplasmic streaming and lacks membrane-bound organelles. Nevertheless, bacteria possess a protein-based cytoskeleton (e.g. FtsZ, MreB, CreS) that plays an important role in cell shape determination, cell division and intracellular organization.

2.6.1.5 The ribosomes

Ribosomes are abundant in the bacterial cytoplasm, dispersed throughout as free particles or organized in clusters. They account for a substantial fraction of the cell mass (often around 25–30% of the dry weight), with approximately 10,000–15,000 ribosomes or more in a rapidly growing bacterial cell. During protein synthesis, multiple ribosomes simultaneously translate the same mRNA molecule, forming polyribosomes (polysomes). The number of ribosomes correlates closely with the rate of protein synthesis and the growth rate of the cell.

Prokaryotic ribosomes are of the 70S type (Svedberg units) and have a molecular mass of about 2.7 MDa. Each 70S ribosome consists of two subunits: a large 50S subunit and a small 30S subunit. At low concentrations of Mg^{2+} ions, 70S ribosomes dissociate into their respective subunits.

2.6.1.6 Cytoplasmic inclusions (reserve materials)

Concentrated deposits of various reserve materials are often detectable in the cytoplasm of certain bacteria. These inclusions are typically of high molecular weight and are usually osmotically inert. The main types include:

2.6.1.6.1 Polyphosphate granules (volutin or metachromatic granules)

These are composed of linear polymers of orthophosphate residues, often represented as (PO_3^-)□. They stain reddish-purple with basic dyes such as methylene blue (metachromatic staining). Polyphosphate granules are commonly observed in *Spirillum volutans*, *Corynebacterium diphtheriae* and various mycobacteria. Polyphosphate serves as a reserve of phosphate and energy and is required for the synthesis of nucleic acids and other phosphorylated compounds.

2.6.1.6.2 Poly-β-hydroxybutyrate (PHB) granules

Poly-β-hydroxybutyrate is a hydrophobic polyester that functions as a carbon and energy storage material. PHB granules can be visualized with lipid stains such as Sudan black. They are prominent in several species, for example *Bacillus megaterium,* in which PHB may constitute a large proportion (up to ~60%) of the dry cell weight under certain growth conditions.

2.6.1.6.3 Polyglucan (glycogen or starch-like) granules

These inclusions consist of branched or unbranched polymers of glucose, such as glycogen or starch-like polyglucans. When stained with iodine, they appear blue, reddish-blue or brown, depending on the structure of the polymer. They serve primarily as carbon and energy reserves.

2.6.1.6.4 Sulfur granules

Elemental sulfur inclusions occur in bacteria that oxidize reduced sulfur compounds. They are typically found in phototrophic purple sulfur bacteria, which use hydrogen sulfide (H_2S) as an electron donor during photosynthesis, and in certain non-photosynthetic chemolithotrophic

bacteria such as *Beggiatoa* and *Thiothrix*. In these organisms, sulfur granules represent intermediate or reserve forms of oxidized sulfur.

2.6.1.7 Genetic material

Like all prokaryotes, bacteria lack a membrane-bound nucleus. Their genetic material occupies an irregularly shaped region near the center of the cell, termed the nucleoid, also referred to in older literature as the chromatin body or bacterial chromosome. The nucleoid consists of a single, covalently closed circular double-stranded DNA molecule in which essentially all essential genes are organized.

The nucleoid can be visualized with DNA-specific stains such as the Feulgen reaction under the light microscope. In electron micrographs, it appears as a relatively electron-lucent region with a fine fibrillar structure. If fully extended, the DNA molecule of a typical bacterial chromosome would be on the order of 1,000 μm in length and approximately 2 nm in diameter, with an estimated molecular mass of about 5×10^9 Da.

A typical bacterial chromosome encodes on the order of ~4,000 genes (open reading frames), which are replicated by a semi-conservative mechanism. In contrast to eukaryotic chromosomes, the bacterial chromosome is not organized into nucleosomes with canonical histone proteins. Instead, it is associated with various histone-like DNA-binding proteins (e.g. HU, H-NS, Fis) that compact and organize the DNA into a highly condensed nucleoid structure.

2.6.1.8 Plasmids and episomes

In many bacteria, in addition to the chromosomal nucleoid, there are small, circular, double-stranded DNA molecules known as plasmids. These extrachromosomal genetic elements replicate autonomously and are stably inherited during cell division. Plasmids were recognized as distinct hereditary elements in the mid-20th century, notably through the work of Joshua Lederberg (1952).

Plasmids typically range in size from about 20 to 100 kilobase pairs (kbp), whereas a bacterial chromosome often comprises several thousand kilobase pairs (e.g. ~4,000 kbp). Plasmids frequently carry genes that confer selective advantages under particular environmental

conditions, such as genes encoding antibiotic resistance, virulence factors, metabolic pathways or conjugation functions.

The term episome is used for plasmids that are capable of integrating into the bacterial chromosome and existing either in an autonomous (plasmid) state or integrated within the chromosome (e.g. the F factor in Escherichia coli).

2.6.2 Non-constant (facultative) structures

2.6.2.1 Pili (fimbriae)

Pili, or fimbriae, are hair-like appendages present on the surface of many Gram-negative bacteria (e.g. members of the *Enterobacteriaceae*, Pseudomonadaceae and Caulobacter spp.) and in some Gram-positive species. They are thinner and generally shorter than flagella and do not participate in motility. Individual pili are typically a few micrometres long (approximately 0.2–2 μm) and about 5–8 nm in diameter. A bacterial cell may carry from tens to several hundred pili distributed over its surface.

Pili arise from the bacterial cell envelope and extend through the peptidoglycan layer (and, in Gram-negative bacteria, also through the outer membrane). Chemically, they are composed almost entirely of a protein subunit called pilin (older term: fimbrillin), with a molecular mass of approximately 15–20 kDa and a length of about 150–170 amino acids.

Two main functional types of pili are commonly distinguished:

1. **Somatic (common) pili (fimbriae)**: Each bacterial cell may carry around 100 or more common pili. Their primary function is to mediate adhesion of the bacterium to biotic or abiotic surfaces, including host cells, tissues, and inert substrates. This adhesion is often a critical determinant of colonization and pathogenicity.
2. **Sex pili (conjugative pili, F pili)**: Sex pili are longer and fewer in number (typically 1–10 per cell). They are encoded by conjugative plasmids (e.g. the F plasmid of *Escherichia coli*). During conjugation, the sex pilus of the donor (F^+ or male) cell recognizes specific receptor structures on the surface of a recipient (F^- or female) cell and establishes initial contact. Subsequent retraction of the pilus brings the cells into close proximity, allowing the formation of a conjugation bridge through which DNA (usually plasmid DNA) is transferred.

In several pathogenic bacteria, adhesive pili or fimbriae are important virulence factors because they facilitate attachment to host cells and tissues.

2.6.2.2 Gas vacuoles (gas vesicles)

Some aquatic bacteria form intracellular gas-filled structures known as gas vacuoles, which confer buoyancy and enable cells to position themselves optimally in the water column with respect to light and nutrients. Under the light microscope, gas vacuoles appear as highly refractile bodies that can collapse under increased external pressure, resulting in loss of refractility.

The vacuoles are composed of many small, spindle-shaped gas vesicles with proteinaceous walls that are permeable to gases but impermeable to water. Gas vesicles occur particularly in planktonic, phototrophic and some heterotrophic microorganisms, such as certain cyanobacteria and purple or green phototrophic bacteria.

2.6.2.3 Intracytoplasmic photosynthetic membranes (lamellae, chromatophores)

Many photosynthetic bacteria possess specialized intracytoplasmic membrane systems in the form of lamellae or vesicles, often referred to as chromatophores in purple bacteria. These membranes arise as invaginations or internal extensions of the cytoplasmic membrane and are distributed throughout the cytoplasm.

Chromatophores are typically spherical or vesicular structures, with diameters on the order of a few tens of nanometres (historically described as ~300 Å). They contain photosynthetic pigments (bacteriochlorophylls and carotenoids), together with the protein complexes and electron transport chain components required for the light-dependent reactions of photosynthesis and photophosphorylation.

In contrast, the light-independent ("dark") reactions of carbon fixation occur in the cytoplasm, using enzymes that are not restricted to these membranes. Thus, intracytoplasmic photosynthetic membranes primarily house the light-harvesting and energy-transducing machinery of bacterial photosynthesis.

2.6.2.4 The capsule

Some bacterial cells are surrounded by a viscous, gelatinous layer outside the cell wall, termed a capsule when it is well organized and closely associated with the cell surface. Electron microscopy has revealed that capsules may consist of a network of thin fibrils or lamellae. Only a subset of bacterial species produce capsules, but in those species capsules are often important virulence determinants.

Capsules can be classified as:

a. **Macrocapsules:** These are sufficiently thick (on the order of ≥0.2–1 μm) to be visible by light microscopy after appropriate staining (e.g. negative staining).
b. **Microcapsules:** These are thinner, not clearly visible by light microscopy, and are typically demonstrated by immunological methods or electron microscopy.

Chemically, capsules are usually composed of polysaccharides, although polypeptide capsules also exist (e.g. the poly-D-glutamic acid capsule of *Bacillus anthracis*). Capsule polysaccharides may be:

a) **Homopolysaccharides** (composed of a single type of monosaccharide), e.g. certain glucan polymers produced by *Streptococcus mutans*.
b) **Heteropolysaccharides** (composed of several different monosaccharides), e.g. the capsular polysaccharides of *Klebsiella pneumoniae*.

Major functions of capsules include:

- **Protection against desiccation**, by binding and retaining water.
- **Protection against host defenses**, notably by inhibiting phagocytosis and complement-mediated killing, thereby enhancing invasive capacity and virulence in pathogenic species.
- **Facilitation of adhesion** to surfaces and the formation of biofilms.

2.6.2.5 Sheaths

Some freshwater and marine bacteria form chains or trichomes enclosed within a hollow, tube-like extracellular structure known as a sheath. The sheath is typically composed of polysaccharide and/or protein and may provide mechanical protection and structural support.

In certain sheathed bacteria, the sheath can become impregnated with iron or manganese oxides (e.g. ferric hydroxides or manganese oxides), which strengthens the structure and contributes to its characteristic appearance. Sheathed bacteria include genera such as *Sphaerotilus* and *Leptothrix*.

2.6.2.6 Flagella

Bacteria may be motile or non-motile. Motile bacteria swim by means of long, thin, flexible appendages called flagella. Prokaryotic flagella are structurally and compositionally distinct from the cilia and flagella of eukaryotic cells.

A typical bacterial flagellum has a diameter of about 12–18 nm (120–180 Å) and a length that is often several times the length of the cell (commonly 4–10 μm). Flagella are composed mainly of subunits of the protein flagellin, which is synthesized in the cytoplasm and transported through the hollow core of the growing filament to be added at the distal (apical) end. Thus, the flagellar filament elongates from its tip rather than from the base.

The bacterial flagellum is structurally differentiated into three main parts (Figure 8):

1. **Basal body** – embedded in the cell envelope and functioning as a rotary motor;
2. **Hook** – a curved, flexible connector that links the basal body to the filament;
3. **Filament** – the long, helical propeller that extends into the external environment.

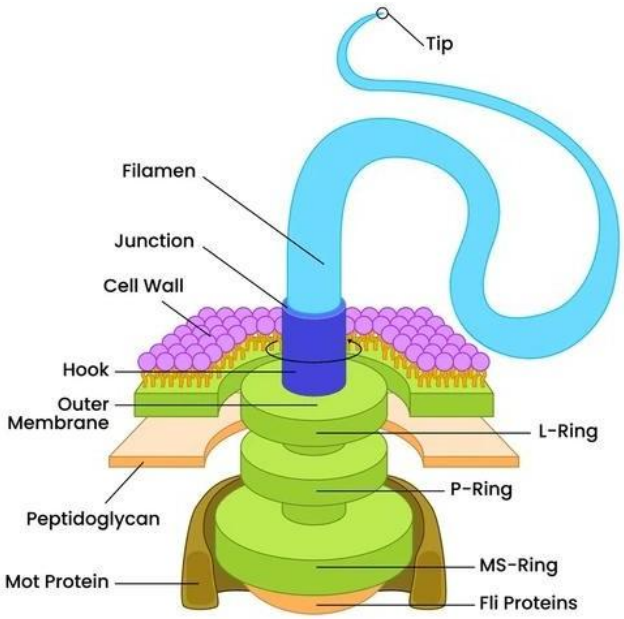


Figure 8. Structure of a bacterial flagellum

The presence, number and arrangement of flagella vary among species. Bacteria that lack flagella are described as atrichous (e.g. many cocci, some Lactobacillus and Pasteurella species). The principal patterns of flagellar arrangement (Figure 9) are:

- **Monotrichous** – a single flagellum at one pole, e.g. *Vibrio cholerae*, *Pseudomonas aeruginosa*.
- **Lophotrichous** – a tuft of two or more flagella at one or both poles, e.g. *Spirillum volutans.*
- **Amphitrichous** – a single flagellum or tuft of flagella at both poles, e.g. some *Spirillum* and *Nitrosomonas* species.
- **Peritrichous** – numerous flagella distributed over the entire cell surface, e.g. *Salmonella* spp., *Escherichia coli* and *Clostridium* spp.

The primary function of flagella is to confer motility, allowing bacteria to move toward favorable environments or away from harmful conditions in response to chemical, light, or other gradients (taxis).

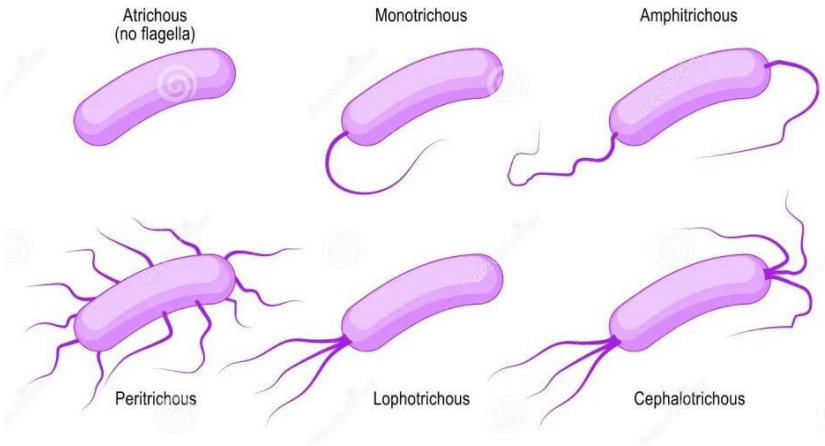


Figure 9. Types of bacterial flagellar arrangements

2.6.2.7 Glycocalyx, prosthecae and stalks

The glycocalyx is a layer of extracellular polymeric material, typically composed of polysaccharides (and sometimes proteins), present on the surface of many bacteria. It includes both capsules (well-organized layers) and slime layers (diffuse, loosely associated material). The glycocalyx promotes adhesion of bacteria to foreign materials, host tissues and abiotic surfaces (e.g. medical implants and prostheses). For example, the glucan-containing glycocalyx produced by *Streptococcus mutans* contributes to the formation of dental plaque and dental caries.

In addition, some bacteria, particularly aerobic freshwater and marine species, possess prosthecae and stalks, which are semi-rigid extensions of the cell envelope that contain cytoplasm and are continuous with the cytoplasmic membrane and cell wall. These structures are characteristic of genera such as *Caulobacter* and *Ancalomicrobium*. Prosthecae may be single or multiple, and their main function is to increase the cell surface-to-volume ratio, thereby enhancing nutrient uptake in dilute environments and facilitating attachment to surfaces.

Stalks (also called hyphae in some organisms) are non-motile, often tubular or ribbon-like extensions produced by certain bacteria, such as *Planctomyces* (now *Planctomycetes*). Stalks assist in surface attachment and can play a role in the formation of complex biofilm communities.

2.6.2.8 Photosynthetic organelles of cyanobacteria

Cyanobacteria (also known historically as *Cyanophyceae* or "blue-green algae") perform oxygenic photosynthesis using specialized internal membrane systems called thylakoids. Unlike eukaryotic algae and plants, cyanobacteria do not possess chloroplasts; instead, their thylakoids are free within the cytoplasm.

Cyanobacterial thylakoids contain chlorophyll a and a variety of accessory pigments, including phycobiliproteins (phycocyanin, phycoerythrin), which collectively impart the characteristic blue-green coloration. These pigments capture light energy, which is converted into chemical energy through the light-dependent reactions of photosynthesis. Water serves as the electron donor, and molecular oxygen (O_2) is released as a by-product.

Through the combined light-dependent and light-independent reactions, cyanobacteria fix CO_2 into organic matter while simultaneously generating ATP and reducing power, thereby sustaining cellular metabolism and growth.

2.6.2.9 The spore

Under adverse environmental conditions, such as nutrient depletion, extreme desiccation or lack of oxygen, certain Gram-positive rod-shaped bacteria (notably species of *Bacillus* and *Clostridium*) differentiate into highly resistant dormant forms known as endospores. Each vegetative cell forms a single endospore within its cytoplasm, which is eventually released upon lysis of the mother cell.

Some other bacteria, such as *Methylosinus*, produce exospores by budding from the poles of vegetative cells. These exospores (often termed cysts in such organisms) generally exhibit increased resistance compared with vegetative cells, but are usually less resistant than the true endospores of Bacillus and Clostridium.

2.6.2.9.1 Types of spores

Bacterial spores vary in shape (spherical, cylindrical or oval). A spore is described as swollen (deforming) when its diameter is greater than that of the vegetative cell, and non-swollen (non-deforming) when it does not visibly distend the cell. Depending on its position within the cell, the spore may be:

a) Central
b) Subterminal
c) Terminal

These variations are illustrated in Figure 10.

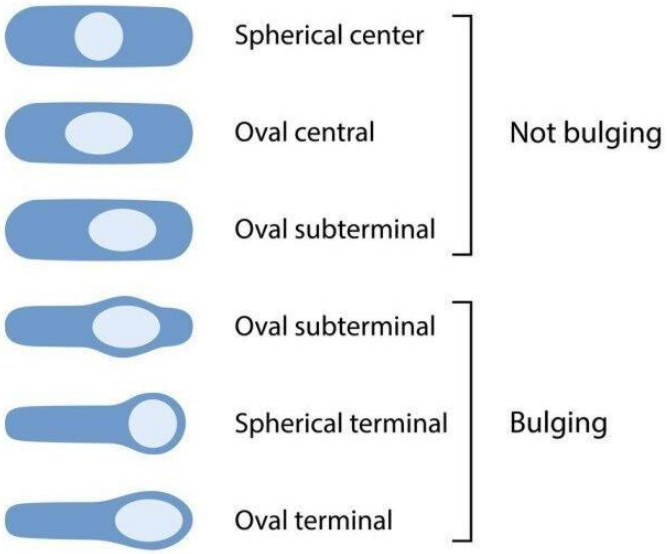


Figure 10. Shape and position of bacterial endospores

2.6.2.9.2 Structural characteristics of the endospore

Compared with the vegetative cell, the endospore contains relatively low amounts of RNA, enzymes and water (while the DNA content remains similar). The spore cytoplasm has a dense, homogeneous appearance.

From inside to outside, the endospore is organized into several distinct layers (Figure 11):

- **Core** – containing the chromosome, ribosomes and essential enzymes; highly dehydrated and rich in dipicolinic acid and calcium ions (calcium dipicolinate).
- **Inner membrane** – a permeability barrier surrounding the core.
- **Germ cell wall (spore wall)** – which becomes the cell wall of the emerging vegetative cell.
- **Cortex** – a thick layer of modified peptidoglycan containing high levels of calcium dipicolinate; critical for dehydration and heat resistance.

- **Spore coats** – multiple protein layers (inner and outer coats) that provide chemical and enzymatic resistance.
- **Exosporium** (present in some species) – a thin, outermost proteinaceous or glycoprotein layer.

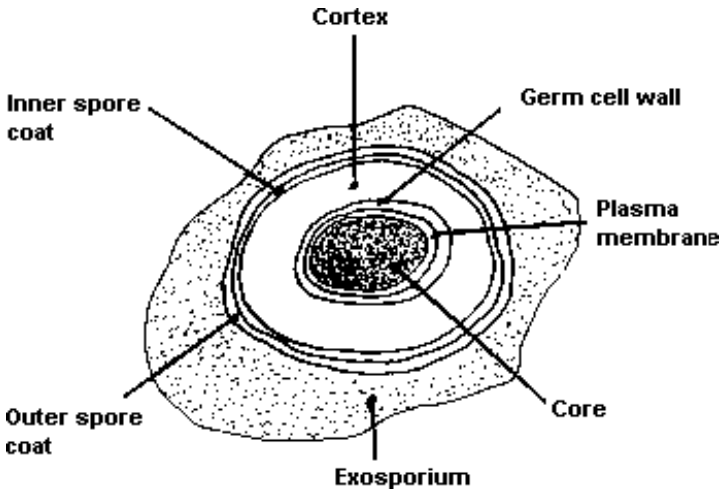


Figure 11. Structure of a bacterial endospore

Endospores differ from vegetative cells in morphology, structure, enzyme composition and, above all, in their extraordinary resistance to heat, ultraviolet and ionizing radiation, many chemical disinfectants and severe desiccation. This resistance results from several factors, including the low water content of the core, the presence of calcium dipicolinate and small acid-soluble spore proteins (SASPs), and the protective nature of the spore coats.

2.6.2.9.3 Formation of the endospore (sporulation)

Sporulation typically begins as the bacterial culture enters the stationary phase, when nutrients become limiting. In *Bacillus subtilis* at 37 °C, the complete process of sporulation may require approximately 6–8 h. The main stages of sporulation (Figure 12) are:

- **Axial filament formation and DNA replication:** The chromosome is replicated and the DNA is arranged along the cell's longitudinal axis.
- **Asymmetric septum formation:** An asymmetric septum (the sporulation septum) forms by invagination of the cytoplasmic membrane, dividing the cell into a larger mother-cell compartment and a smaller forespore (prespore) compartment.
- **Engulfment of the forespore:** The mother-cell membrane engulfs the forespore, surrounding it with a second membrane and forming a cell-within-a-cell structure.
- **Cortex synthesis:** Peptidoglycan is deposited between the two forespore membranes to form the cortex.
- **Coat synthesis:** Protein layers (spore coats) are assembled around the cortex.
- **Maturation:** The spore dehydrates, accumulates calcium dipicolinate and SASPs, and acquires full resistance properties.
- **Release:** The mother cell undergoes autolysis, releasing the mature endospore into the environment.

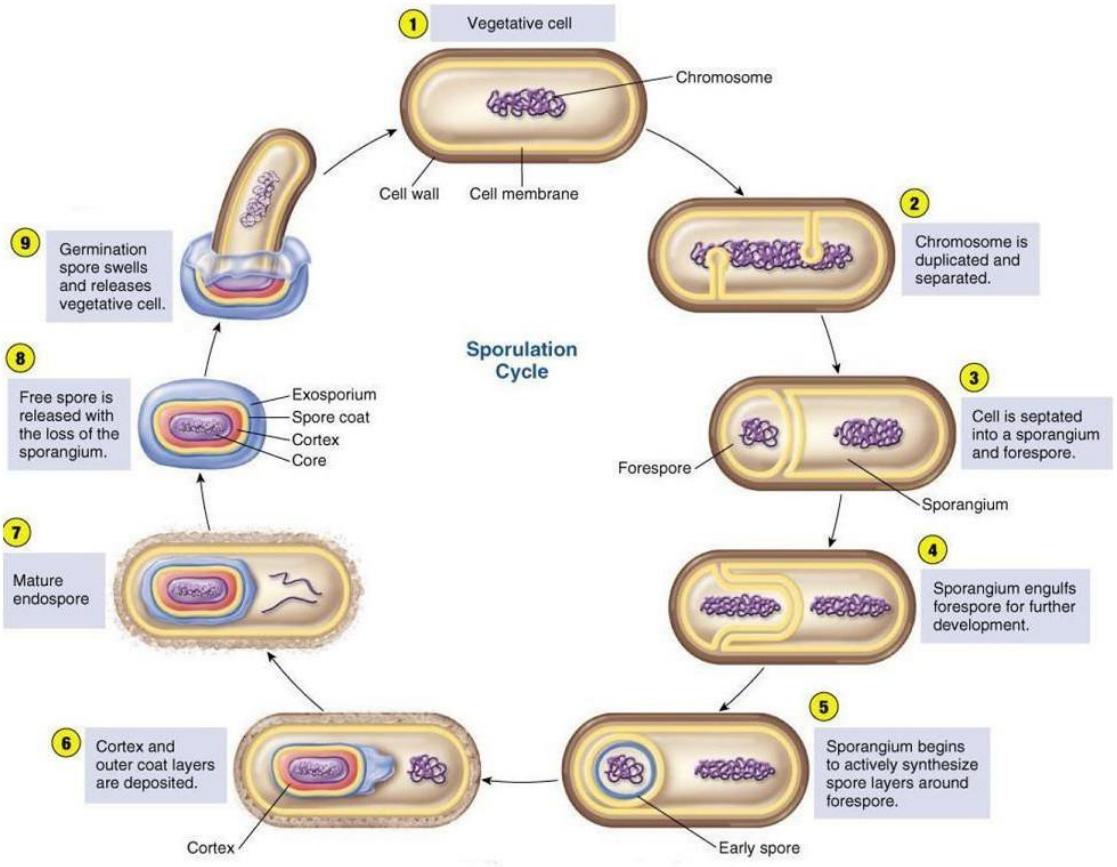


Figure 12. Sporulation and germination of a bacterial endospore

The resistance of the mature endospore is due to:

- **Heat resistance** – survival at temperatures of 70–80 °C for 10 min or longer, largely attributable to low water content, calcium dipicolinate and protective proteins.
- **Resistance to physical and chemical agents** – including UV and X-rays, many disinfectants, antiseptics and antibiotics.
- **Resistance to desiccation and aging** – due to the highly dehydrated core and greatly reduced metabolic activity (dormant state).

2.6.2.9.4 Germination of the spore

When environmental conditions become favorable (e.g. availability of nutrients, appropriate temperature and moisture), an endospore can germinate and give rise to a vegetative cell genetically identical to the original. Germination is the process by which a dormant spore is

converted into a metabolically active bacterium. It is initiated by specific environmental signals, particularly the presence of germinants such as certain amino acids, sugars or inorganic ions.

The germination process can be divided into three phases:

1. **Activation:** A reversible preparatory step during which the spore becomes capable of germinating. Activation may be triggered by sublethal heat treatment or other stimuli and is associated with early physiological changes.
2. **Germination:** An irreversible phase characterized by rapid rehydration of the core, release of calcium dipicolinate, degradation of SASPs and early resumption of metabolism. Macroscopically, the spore swells, the spore coats and cortex are partially degraded, the spore loses its refractility and resistance, and the internal pH rises.
3. **Outgrowth (post-germinative growth):** The germinated spore transforms into an actively growing vegetative cell. Macromolecular synthesis resumes, the cell elongates and eventually divides, giving rise to a new bacterial population (colony) under suitable conditions.

2.7 Questions (Student Versions)

2.7.1 Part I – Fill in the blanks

1. Microbiology is a _____ of biology that deals with the study of _____ and their _____ with the environment (from Greek: _____= _____, _____= _____).
2. Microorganisms include the following major groups: _____, _____, _____, _____ and _____.
3. According to their cellular organization, protists are subdivided into _____and _____.
4. A bacterium is a , “” microorganism of _____ morphology that reproduces by _____. Some bacteria are _____ to humans, whereas others are _____.

2.7.2 **Part II – Multiple choice**

Circle all correct answers. Any wrong answer cancels a correct answer.

1. Flagella:
    - Allow bacteria to move
    - Are constant structures of bacteria
    - Consist mainly of a protein called pilin
    - Exist only in non-motile bacterial species
    - Are involved in bacterial chemotaxis
    - No answer is correct
2. The spore:
    - Is synthesized in all bacterial species
    - Is a form of bacterial resistance in adverse environmental conditions
    - Occurs in different morphological forms
    - Prevents the attachment of bacteriophages
    - No answer is correct
3. Bacterial genetic material consists of:
    - A single chromosome made up of several double-stranded DNA molecules
    - A single chromosome formed from a single double-stranded DNA molecule
    - Several chromosomes made up of several double-stranded DNA molecules
    - Several chromosomes made up of several single-stranded DNA molecules
    - No answer is correct
4. The plasmid is:
    - Extrachromosomal genetic material
    - Composed of a circular, double-stranded DNA molecule
    - Capable of autonomous replication
    - Carrying accessory genes (e.g. antibiotic resistance factors)
    - No answer is correct
5. Peptidoglycan:
    - Is also called mucopeptide (murein)
    - Is a simple polymer formed from three different components

- Is a complex polymer formed from three different components
- Is composed of an alternation of N-acetylglucosamine and N-acetylmuramic acid molecules
- No answer is correct

6. The mesosome:
    - Was described as a structure formed by invagination of the cytoplasmic membrane
    - Was described as being closely associated with nuclear material
    - Was described as giving rise to the septum separating the two daughter cells
    - Has nothing to do with cell wall synthesis
    - No answer is correct
7. The gas vacuole is:
    - Found in all bacteria
    - Found in cyanobacteria
    - Found in some archaea
    - Found in photosynthetic bacteria
    - No answer is correct

2.7.3 Part III – Short answer questions

1. State the different functions of the cytoplasmic membrane.
2. In a comparison table, list the constant and non-constant (facultative) elements of a bacterial cell.
3. Bacteria can be observed in two main ways. Which ones? Explain.
4. Depending on the energy source used, bacteria can be classified into phototrophic and chemotrophic bacteria. Explain.
5. Caption the figure below and provide an appropriate title.

    Title: _____

2.8 Answers (Answer Keys)

2.8.1 Part I – Fill in the blanks

1. Microbiology is a sub-discipline of biology that deals with the study of microorganisms and their interactions with the environment (from Greek: mikros = small, bios = life).
2. Microorganisms include the following major groups: algae, protozoa, fungi, bacteria and archaea.
3. According to their cellular organization, protists are subdivided into unicellular protists and multicellular (higher) protists.
4. A bacterium is a single-celled, "prokaryotic" microorganism of diverse morphology that reproduces by binary fission. Some bacteria are pathogenic to humans, whereas others are beneficial.

2.8.2 Part II – Multiple choice

1. Flagella:
    - ✓ Allow bacteria to move
    - ✓ Are involved in bacterial chemotaxis
    - ✗ Are constant structures of bacteria
    - ✗ Consist mainly of a protein called pilin (they consist mainly of flagellin)
    - ✗ Exist only in non-motile species
    - ✗ No answer is correct
2. The spore:
    - ✓ Is a form of bacterial resistance in adverse environmental conditions
    - ✓ Occurs in different morphological forms (spherical, oval, cylindrical; central, subterminal, terminal; deforming or non-deforming)
    - ✗ Is synthesized in all bacterial species
    - ✗ Prevents the attachment of bacteriophages
    - ✗ No answer is correct
3. Bacterial genetic material consists of:
    - ✓ A single chromosome formed from a single double-stranded DNA molecule

- ✗ A single chromosome made up of several double-stranded DNA molecules
- ✗ Several chromosomes made up of several double-stranded DNA molecules
- ✗ Several chromosomes made up of several single-stranded DNA molecules
- ✗ No answer is correct

4. The plasmid is:
    - ✓ Extrachromosomal genetic material
    - ✓ Composed of a circular, double-stranded DNA molecule
    - ✓ Capable of autonomous replication
    - ✓ Carrying accessory genes (e.g. antibiotic resistance factors, virulence factors, metabolic pathways)
    - ✗ No answer is correct
5. Peptidoglycan:
    - ✓ Is also called mucopeptide (murein)
    - ✗ Is a simple polymer formed from three different components
    - ✓ Is a complex polymer formed from three major components (N-acetylglucosamine, N-acetylmuramic acid and short peptide chains)
    - ✓ Is composed of an alternation of N-acetylglucosamine and N-acetylmuramic acid molecules
    - ✗ No answer is correct
6. The mesosome (historical view):
    - ✓ Was described as a structure formed by invagination of the cytoplasmic membrane
    - ✓ Was described as being closely associated with nuclear material
    - ✓ Was described as giving rise to the septum separating the two daughter cells
    - ✗ Has nothing to do with cell wall synthesis (it was also historically implicated in wall synthesis)
    - ✗ No answer is correct

Modern note: Mesosomes are now considered mainly artifacts of chemical fixation in electron microscopy and are not regarded as true organelles in living bacteria.

7. The gas vacuole is:
    - ✗ Found in all bacteria
    - ✓ Found in cyanobacteria
    - ✓ Found in some archaea (e.g. Halobacterium species)
    - ✓ Found in photosynthetic bacteria (e.g. certain purple and green phototrophic bacteria)
    - ✗ No answer is correct

2.8.3 Part III – Short answer questions

1. **State the different functions of the cytoplasmic membrane.**
    - Selective transport of substances: controlled entry and exit of solutes (nutrients, ions, waste products).
    - Biosynthesis of macromolecules: localization of enzymes involved in cell wall and membrane synthesis, and sometimes capsule synthesis.
    - Energy metabolism (respiration, sometimes photosynthesis): housing components of the electron transport chain and ATP synthase.
    - Signal reception and transduction: hosting receptors and sensor systems that detect and respond to environmental signals (chemotaxis, regulatory systems).
2. **In a comparison table, list the constant and non-constant (facultative) elements of a bacterial cell.**

Constant elements (present in nearly all bacteria)	Non-constant (facultative) elements (present only in some bacteria)
Cell wall	Capsule
Cytoplasmic (plasma) membrane	Plasmids
Cytoplasm	Flagella
Ribosomes	Pili / fimbriae
Nucleoid (bacterial chromosome)	Endospores
	Gas vacuoles
	Cytoplasmic inclusions (reserve granules)
	Intracytoplasmic photosynthetic membranes (chromatophores, thylakoids)
	Sheaths, prosthecae, stalks, glycocalyx (when treated

	separately)

3. **Bacteria can be observed in two main ways. Which ones? Explain.**
    - In the fresh state (wet mount): A drop of biological fluid (e.g. pus, sputum, urine, culture in liquid medium) is placed between slide and coverslip and observed directly. This allows examination of motility, general morphology and cell arrangement in living cells.
    - After fixation and staining (fixed smear): A thin film of the sample is spread on a slide, air-dried, heat-fixed and then stained (e.g. simple stain, Gram stain). This improves contrast and allows better visualization of cell morphology, Gram reaction, spore formation, capsules, etc., but the cells are no longer alive.
4. **Depending on the energy source used, bacteria can be classified into phototrophic and chemotrophic bacteria. Explain.**
    - Phototrophic bacteria: Use light as their primary energy source. Light energy is converted into chemical energy via photosynthetic or photoheterotrophic processes (e.g. cyanobacteria, purple and green phototrophic bacteria).
    - Chemotrophic bacteria: Use chemical compounds as their energy source.
        - Chemolithotrophic bacteria oxidize inorganic compounds (e.g. NH_3 , H_2 S, Fe^{2+}) to obtain energy.
        - Chemoorganotrophic bacteria oxidize organic compounds (e.g. sugars, amino acids, organic acids) as their energy source.
5. **Caption the figure below and provide an appropriate title.**

Suggested title: “Structure of a Typical Prokaryotic (Bacterial) Cell”

Suggested labels (depending on the figure provided):

- Capsule (or glycocalyx)
- Cell wall
- Cytoplasmic (plasma) membrane
- Cytoplasm
- Nucleoid (bacterial chromosome)
- Ribosomes
- Flagellum

- Pili / fimbriae
- Cytoplasmic inclusion (reserve granule)
- Endospore (if present)
- Intracytoplasmic membrane system / mesosome (if represented, with a note that it is an artifact in classic electron microscopy images)

The list of labels should be adapted to the specific structures represented in the figure used in the exercise.

3 Bacterial Nutrition

3.1 Introduction

For growth and survival, a bacterium must obtain from its environment all nutrients required for the synthesis and maintenance of its cellular components. These include both energy-yielding substrates and precursor molecules that constitute macromolecules such as proteins, nucleic acids, lipids, and polysaccharides. In culture, bacterial multiplication depends on the availability of suitable nutrients in the growth medium.

All bacteria share a number of basic requirements: water, a source of energy, a source of carbon, a source of nitrogen, and mineral salts. Under appropriate conditions, many species can grow and divide when only these basic nutrients are provided. Other species, however, are unable to grow because they lack specific organic compounds that they cannot synthesize de novo; these compounds are termed growth factors.

Microorganisms that can grow on a simple mineral medium supplemented with a single carbon and energy source, without the addition of growth factors, are referred to as prototrophs. In contrast, auxotrophs are mutant or naturally fastidious strains that, in addition to the basic nutrients, require one or more growth factors (e.g., specific amino acids, vitamins, or nucleotides).

3.2 Nutrient Requirements

The nutrient requirements of bacteria can be broadly divided into two categories:

1. Basic requirements, which are common to most microorganisms.
2. Specific requirements, corresponding largely to growth factors.

3.2.1 Basic Requirements

All microorganisms require three fundamental resources: a carbon source, an energy source, and a source of electrons (reducing power). Specific terms are used to classify organisms according to the origin of each of these resources.

3.2.1.1 Carbon Source

All organisms are composed of carbon-containing macromolecules (proteins, carbohydrates, lipids, and nucleic acids), in which carbon forms the structural backbone. Carbon is the most abundant element in bacterial cells and is central to cellular metabolism.

On the basis of their carbon source, microorganisms are divided into two major groups:

3.2.1.1.1 Heterotrophs

Heterotrophic microorganisms use reduced, preformed organic compounds as their principal carbon source. In other words, they require organic molecules—such as sugars and their derivatives, organic acids, peptides, or amino acids—for growth. Some heterotrophs can utilize a wide variety of organic substrates, whereas others are metabolically specialized and can grow only on a limited number of substrates, sometimes on a single compound.

3.2.1.1.2 Autotrophs

Autotrophic microorganisms use carbon dioxide (CO_2) as their principal or sole carbon source. They reduce or "fix" this inorganic form of carbon into organic cell material and are therefore described as self-feeding. These organisms can grow in an entirely inorganic (mineral) medium, using CO_2 or hydrogen carbonate ions (HCO_3^-) as the only carbon source for the synthesis of cellular constituents.

3.2.1.2 Energy Source

The energy required for biosynthesis and other cellular processes is provided mainly in the form of adenosine triphosphate (ATP), which bacteria synthesize through various catabolic pathways. From the energetic point of view, there are two principal sources:

1. Light energy, originating from solar radiation.
2. Chemical energy, derived from the oxidation of organic or inorganic compounds.

According to their energy source, bacteria are classified as:

3.2.1.2.1 Chemotrophs

Chemotrophic bacteria obtain energy from the oxidation of chemical compounds. Depending on the nature of the electron donor, they are subdivided into:

1. **Chemolithotrophs**, when the electron donor is an inorganic compound (e.g., H_2 , NH_3 , reduced sulfur compounds, Fe^{2+}).
2. **Chemoorganotrophs**, when the electron donor is an organic compound (e.g., glucose, acetate, amino acids).

3.2.1.2.2 Phototrophs

Phototrophic bacteria use light as their primary energy source. They convert light energy into ATP through photosynthetic or phototrophic electron transport systems mediated by pigments such as chlorophylls, bacteriochlorophylls, and carotenoids.

Irrespective of whether the ultimate energy source is light or chemical compounds, organisms can also be classified according to the nature of their electron (hydrogen) source:

- An organism that uses inorganic electron donors is called a lithotroph.
- An organism that uses organic electron donors is called an organotroph.

When combined with the energy source, these terms yield designations such as photolithotrophs (light as energy source, inorganic electron donor) and photoorganotrophs (light as energy source, organic electron donor). The same logic applies to chemotrophs (e.g., chemoorganoheterotrophs, chemolithoautotrophs).

These prefixes can be combined to describe in a single term the sources of energy, electrons, and carbon used by an organism (Table 3).

Table 3. Major nutritional types of microorganisms

Energy source	Electron source	Carbon source	Nutritional type (example)
Light (photo-)	Organic (organo-)	Organic (heterotrophic)	**Photoorganoheterotroph** – many purple non-sulfur bacteria
Light (photo-)	Organic (organo-)	CO_2 (autotrophic)	**Photoorganoautotroph** – rare, few well-characterized examples
Light (photo-)	Inorganic (litho-)	Organic (heterotrophic)	**Photolithoheterotroph** – rare in nature
Light (photo-)	Inorganic (litho-)	CO_2 (autotrophic)	**Photolithoautotroph** – cyanobacteria, many algae-like prokaryotes
Chemical (chemo-)	Organic (organo-)	Organic (heterotrophic)	**Chemoorganoheterotroph** – most pathogenic and commensal bacteria
Chemical (chemo-)	Organic (organo-)	CO_2 (autotrophic)	**Chemoorganoautotroph** – uncommon, specialized bacteria
Chemical (chemo-)	Inorganic (litho-)	Organic (heterotrophic)	**Chemolithoheterotroph** – some sulfur-oxidizing bacteria under certain conditions
Chemical (chemo-)	Inorganic (litho-)	CO_2 (autotrophic)	**Chemolithoautotroph** – nitrifying, sulfur-oxidizing, and hydrogen-oxidizing bacteria

3.2.1.3 Macronutrients

In addition to carbon, hydrogen, and oxygen, bacterial cells require several other elements in relatively large amounts, known collectively as macronutrients. Among these, nitrogen, phosphorus, sulfur, potassium, magnesium, calcium, sodium, and iron are particularly important.

3.2.1.3.1 Nitrogen Source

Nitrogen is essential for the synthesis of proteins, nucleic acids, and several other cellular constituents. Bacteria can obtain nitrogen from a variety of sources:

1. **Organic nitrogen**, such as amino acids and nitrogenous bases.
2. **Inorganic nitrogen**, including:
    - Ammonium ions (NH_4^+), which can be directly assimilated.
    - Nitrate (NO_3^-) and nitrite (NO_2^-), which many bacteria reduce to ammonium via nitrate and nitrite reductases.
    - Molecular nitrogen (N_2), which can be fixed by certain diazotrophic microorganisms.

Nitrogen fixation is carried out by a limited number of bacteria and archaea, some of which live in symbiosis with plants. For example, species of *Rhizobium* form nodules on the roots of leguminous plants and reduce atmospheric N_2 to ammonia via the enzyme nitrogenase, thereby contributing significantly to soil fertility. Other diazotrophs, such as *Azotobacter* and some species of *Clostridium*, are free-living in soil and likewise participate in biological nitrogen fixation.

3.2.1.3.2 Sulfur Source

Sulfur is particularly important for microorganisms because it is a constituent of the sulfur-containing amino acids cysteine and methionine, which contribute to the tertiary and quaternary structure of many proteins through disulfide bonds. Sulfur is also required for the synthesis of several vitamins and coenzymes, including biotin and coenzyme A.

In many culture media, sulfur is supplied as sulfate ions (SO_4^{2-}). Sulfate is taken up by the cell and reduced sequentially to sulfite (SO_3^{2-}) and then to sulfide (H_2S). The sulfide is

subsequently incorporated into serine to form cysteine, which then serves as the primary sulfur donor in anabolic reactions.

3.2.1.3.3 Phosphorus Source

Phosphorus is an essential component of ATP, nucleic acids, phospholipids, and various coenzymes (e.g., NAD^+ , $NADP^+$, and flavin nucleotides). Bacteria generally take up phosphorus in the form of inorganic phosphate (Pi), usually as orthophosphate (PO_4^{3-}), which is then incorporated into organic molecules by kinases and other phosphotransfer enzymes.

3.2.1.3.4 Other Mineral Elements

- **Potassium (K^+)**: functions primarily as an enzymatic cofactor and contributes to osmotic balance and maintenance of intracellular pH.
- **Magnesium (Mg^{2+})**: acts as a cofactor for numerous enzymes, including those involved in DNA replication and transcription. It also stabilizes ribosomes, membranes, and nucleic acids.
- **Calcium (Ca^{2+})**: contributes to the heat resistance of endospores, particularly in genera such as Bacillus and *Clostridium* (often as calcium dipicolinate), and participates in the stabilization of the cell envelope.
- **Sodium (Na^+)**: is essential for the growth of halophilic bacteria, in which it plays roles in osmotic balance and, in some cases, in sodium-dependent bioenergetic processes.
- **Iron (Fe)**: is a critical trace metal involved in multiple redox reactions. It forms part of cytochromes, iron–sulfur proteins, and other components of the respiratory chain, particularly in aerobic and facultatively anaerobic bacteria. Because ferric iron (Fe^{3+}) is poorly soluble at neutral pH and in the presence of oxygen, many bacteria synthesize siderophores, high-affinity iron-chelating compounds that solubilize environmental iron and transport it into the cell via specific receptor systems.

3.2.2 Need for Growth Factors

Some microorganisms can synthesize all the organic molecules they require for growth, provided that they are supplied with an appropriate carbon source and inorganic salts. Others, however, are unable to synthesize certain essential organic compounds de novo and must obtain them from their environment. These exogenously required organic compounds are termed growth factors. A

growth factor may therefore be defined as an organic molecule that a microorganism cannot synthesize and must acquire from its surroundings in order to grow.

A growth factor should not be confused with an essential metabolite. Both growth factors and essential metabolites are organic compounds that are indispensable for cellular metabolism. However, an essential metabolite can, in principle, be synthesized by the bacterium from simpler precursors, whereas a growth factor cannot and must be present in the external medium. Thus, every growth factor is an essential metabolite, but not every essential metabolite is a growth factor.

For example, in a minimal medium containing glucose, a nitrogen source, and mineral salts, a bacterium such as *Escherichia coli* can proliferate, whereas *Proteus vulgaris* cannot. The growth of *P. vulgaris* in such a medium requires the addition of nicotinamide (or nicotinic acid, vitamin B_3). Nicotinamide is indispensable for the growth of both *E. coli* and *P. vulgaris*, but *E. coli* can synthesize it, whereas *P. vulgaris* cannot. Consequently, nicotinamide is an essential metabolite for both species but constitutes a growth factor only for *P. vulgaris*. The concept of a growth factor is therefore relative and must always be considered with respect to a particular genus, species, or even strain.

Growth factors can be grouped into three main classes:

1. **Amino acids**, required for protein synthesis.
2. **Nitrogenous bases** (purines and pyrimidines), required for the synthesis of nucleic acids.
3. **Vitamins and coenzymes (or their precursors)**, which serve as cofactors in numerous metabolic reactions.

Some illustrative examples include:

- *Escherichia coli*, a Gram-negative member of the family *Enterobacteriaceae*, is able to grow in a purely mineral medium supplemented with a single carbon source such as glucose. It can synthesize all of its carbon-containing cellular constituents from this single substrate. Under these conditions, *E. coli* is described as prototrophic, because it does not require any exogenous growth factor.
- *Proteus vulgaris*, a Gram-negative bacterium of the family *Morganellaceae*, can grow in the same type of minimal medium only if small amounts of nicotinic acid (vitamin B_3)

or its amide are supplied. *P. vulgaris* is therefore said to be auxotrophic for nicotinic acid, which, for this species, functions as a specific growth factor.

3.3 Nutrient Uptake

To sustain growth, survival, and metabolic activity, bacterial cells must acquire essential nutrients from the external environment (the culture medium). These solutes cross the cytoplasmic membrane via several distinct membrane transport systems. In bacteria and archaea, the major transport mechanisms are summarized in Figure 13.

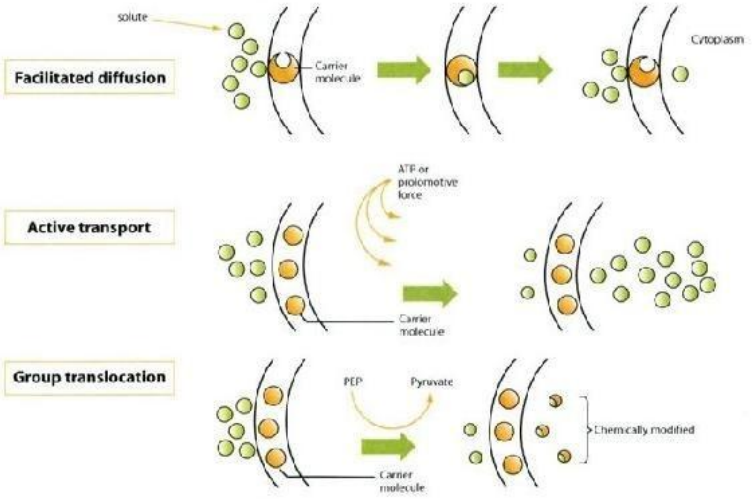


Figure 13. Schematic representation of the main transport mechanisms in bacteria

3.3.1 Passive Diffusion

Passive (simple) diffusion allows the direct passage of small nonpolar molecules and gases, such as CO_2 , O_2 , and, to a lesser extent, H_2 O, across the cytoplasmic membrane. Transport occurs down the concentration gradient, from a region of higher solute concentration to one of lower concentration, without expenditure of cellular energy. As the solute accumulates inside the cell, the gradient progressively decreases and the net rate of diffusion declines.

3.3.2 Facilitated Diffusion

Facilitated diffusion also operates down a concentration gradient, with the solute concentration higher outside the cell than inside, but it requires specific membrane proteins, often termed carrier proteins or permeases. These integral membrane proteins form transient pores or channels that enable the passage of larger or more polar molecules that cannot diffuse freely through the

lipid bilayer. When the concentration gradient is dissipated, net transport ceases. Each carrier protein typically exhibits high substrate specificity, recognizing and transporting only a particular molecule or a small group of closely related molecules.

3.3.3 Active Transport

Many nutrients are present in the environment at very low concentrations, such that efficient uptake requires transport against a concentration gradient (i.e., from a lower external concentration to a higher internal concentration). In these cases, the cell uses metabolic energy to drive the movement of solutes via carrier proteins embedded in the cytoplasmic membrane; this process is termed active transport. All active transport systems are carrier mediated.

Three major categories of active transport are recognized: primary active transport, secondary active transport, and group translocation (Figure 13).

3.3.3.1 Primary Active Transport

Primary active transport uses chemical energy, most commonly derived from ATP hydrolysis, to power solute movement. A prominent example is the ATP-binding cassette (ABC) transporter family. Each ABC transporter typically consists of three functional components (Figure 14):

1. **Transmembrane domains (TMDs)** – integral membrane proteins that form the translocation pathway (transport channel) across the cytoplasmic membrane;
2. **Nucleotide-binding domains (NBDs)** – cytoplasmic domains that bind and hydrolyze ATP, providing the energy required for substrate translocation;
3. **Substrate-binding proteins (SBPs)** – peripheral proteins that bind the specific solute and deliver it to the transmembrane domains. In Gram-negative bacteria, SBPs are located in the periplasm, whereas in Gram-positive bacteria they are usually anchored to the external face of the cytoplasmic membrane or associated with the cell wall.

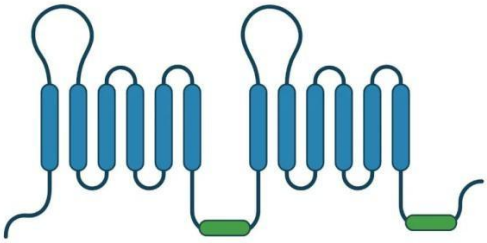


Figure 14. Basic organization of an ATP-binding cassette (ABC) transporter

3.3.3.2 Secondary Active Transport

Secondary active transport systems exploit the energy stored in an ion gradient, most commonly the proton motive force (PMF). The PMF is an electrochemical gradient generated when electrons are transferred along the electron transport chain during energy-conserving processes. As a result, protons (H^+) accumulate outside the negatively charged cytoplasm, establishing both a charge difference ($\Delta\psi$) and a proton concentration gradient (ΔpH) across the membrane.

Carrier proteins involved in secondary active transport can be classified into three basic transport modes (Figure 15):

1. **Uniporters** transport a single type of solute across the membrane, either into or out of the cell. Uniport is often associated with facilitated diffusion.
2. **Symporters** simultaneously transport two different substances in the same direction across the membrane, typically coupling the inward movement of a solute to the inward flow of protons down their electrochemical gradient.
3. **Antiporters** transport two different substances in opposite directions; the inward movement of one solute is coupled to the outward movement of another.

In symporters and antiporters, the energy released by ions moving down their electrochemical gradient drives the uphill transport of the coupled solute.

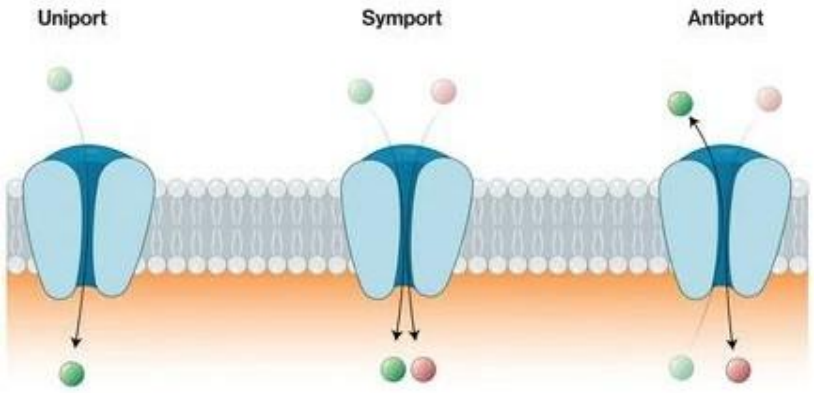


Figure 15. Major carrier transport modes: uniport, symport, and antiport

3.3.4 **Group Translocation**

Group translocation is a specialized form of active transport in which the transported solute is chemically modified as it crosses the cytoplasmic membrane. This process is driven by the energy released from a high-energy organic compound other than ATP. In contrast to simple transport and ABC transporters, group translocation couples substrate uptake directly to its covalent modification, usually phosphorylation.

The best-characterized example of group translocation is the phosphoenolpyruvate (PEP)–dependent sugar phosphotransferase system (PTS). In this system, the energy contained in the high-energy phosphate bond of PEP is used to drive the uptake of specific sugars into the cell. During transport, a phosphate group is transferred stepwise from PEP through a series of cytoplasmic proteins to the membrane-associated components of the PTS, and finally to the incoming sugar. As a result, the sugar enters the cytoplasm in a phosphorylated form (Figure 16).

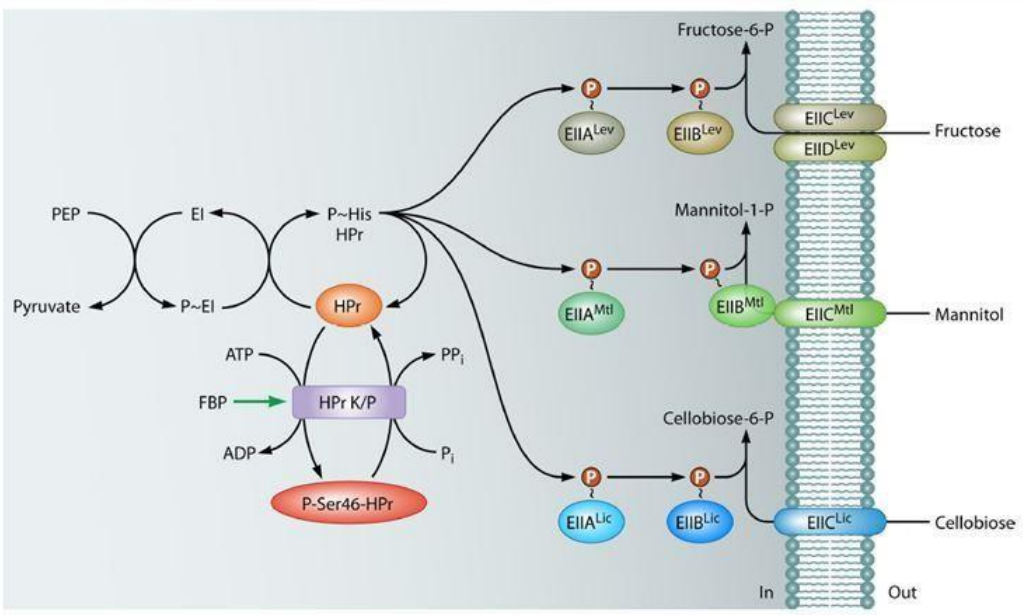


Figure 16. Schematic representation of group translocation via the phosphoenolpyruvate-dependent phosphotransferase system

3.3.5 **Iron Uptake**

Iron is an essential micronutrient for microorganisms because it serves as a critical cofactor in cytochromes and many enzymes involved in respiration, DNA synthesis, and other metabolic

processes. Under physiological conditions, however, free ferric iron (Fe^{3+}) is poorly soluble and therefore scarcely available in most natural environments. Consequently, iron acquisition often becomes a growth-limiting factor for bacteria.

To overcome iron limitation, many bacteria synthesize and secrete siderophores, low-molecular-mass organic chelators that bind ferric iron with very high affinity. Siderophores are released by the cell into the surrounding environment, where they scavenge available Fe^{3+} and form stable iron–siderophore complexes. These complexes are then specifically recognized by high-affinity receptors located on the cell surface. Binding of the iron–siderophore complex to its cognate receptor initiates a series of transport steps that mediate the passage of iron across the cell envelope and into the cytoplasm (Figure 17). Once inside the cell, iron is released from the siderophore and incorporated into cellular proteins and cofactors.

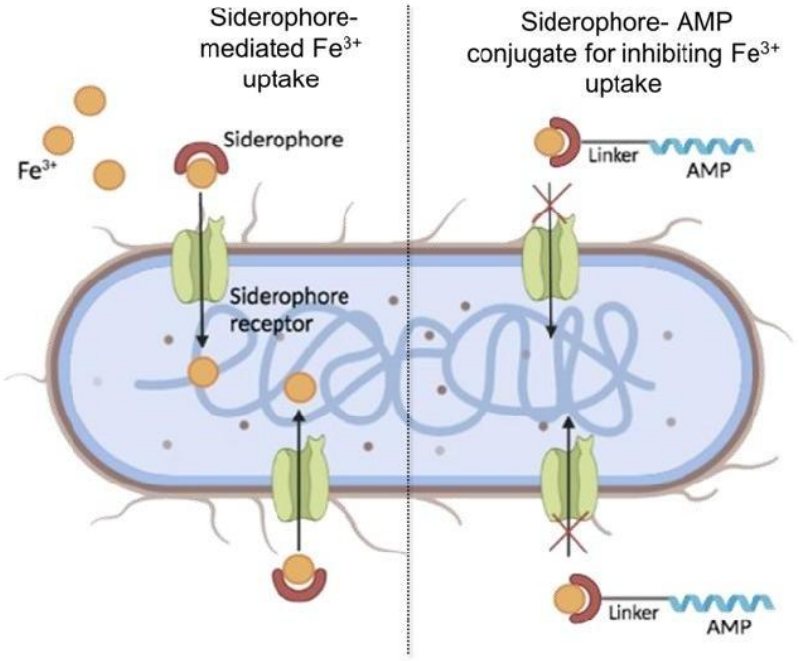


Figure 17. Iron uptake mediated by siderophores and their specific cell-surface receptors

3.4 Physicochemical Parameters

Physicochemical parameters are environmental factors—such as water availability, temperature, pH, osmotic pressure, and oxygen concentration—that strongly influence microbial growth and metabolism. Depending on their values, these parameters can promote, limit, or completely inhibit microbial proliferation.

3.4.1 Water Availability

Water typically accounts for 80–90% of a bacterial cell's mass. It plays a fundamental role in solubilizing nutrients, enabling their transport, and participating in hydrolytic and other biochemical reactions.

The availability of water to microorganisms is quantified by the parameter water activity (a_w). In a given substrate, part of the water is bound to solutes (e.g., salts, proteins) and is therefore not accessible to microorganisms, which require free (unbound) water for growth. Water activity is defined as the ratio of the equilibrium vapor pressure of the medium to the saturation vapor pressure of pure water at the same temperature. This dimensionless ratio, which is ≤ 1, can be approximated by the relative humidity of the medium (expressed as a fraction).

Most bacteria require relatively high water activity; when aw is low, their growth is slowed or completely inhibited. Bacterial endospores, however, can survive in environments that lack free water. The moisture content and water activity of foods strongly influence their shelf life: drying and related preservation processes are based in large part on reducing water activity to prevent microbial growth.

3.4.2 Temperature

Each bacterial species can grow only within a characteristic temperature range, defined by a minimum temperature below which growth ceases, a maximum temperature above which growth is no longer possible, and an optimum temperature at which the growth rate is maximal. On the basis of their optimum growth temperature, microorganisms are commonly classified as follows:

1. **Psychrotrophs (facultative psychrophiles)**: microorganisms that can grow at 0 °C but have an optimum temperature for growth between approximately 20 and 25 °C. They are important in the spoilage of refrigerated foods and biological products.

2. **Psychrophiles**: microorganisms with a maximum growth temperature near 20 °C and an optimum below 15 °C. They are adapted to permanently cold environments.
3. **Cryophiles (extreme psychrophiles)**: microorganisms that can grow at very low, often subzero, temperatures; for example, *Trichococcus patagoniensis*, isolated in Patagonia from penguin feces, can grow at −5 °C.
4. **Mesophiles**: microorganisms that grow best at intermediate temperatures, generally between 25 and 40 °C, with an optimum around 37 °C for many species. Most bacteria associated with humans and other mammals, including the majority of pathogenic bacteria, are mesophilic.
5. **Thermophiles**: microorganisms that grow optimally at relatively high temperatures, typically between 50 and 60 °C (e.g., species of *Bacillus* and *Clostridium*).
6. **Hyperthermophiles**: microorganisms capable of growth at extremely high temperatures, usually with optimum temperatures ≥ 80 °C and in some cases up to 110–113 °C. Examples include *Methanothermus sociabilis* (optimal growth around 97 °C), *Pyrobaculum islandicum* (100 °C), *Pyrococcus furiosus* (100 °C), *Pyrodictium occultum* (105 °C), *Methanopyrus kandleri* (up to 110 °C), and *Pyrolobus fumarii*, which can multiply at 113 °C.

Note. The commensal and pathogenic bacteria comprising the normal flora of mammals, as well as most pathogens of mammals and birds, are mesophilic. Psychrotrophic and psychrophilic bacteria are of particular practical importance because they can contaminate and spoil blood, blood products, and foods stored at low temperatures.

3.4.3 **pH**

The pH of a medium reflects its hydrogen ion activity and has a major impact on the ionic balance of the environment, cell membrane permeability, and nutrient availability. Most bacteria grow optimally at near-neutral pH values (approximately 6.5–7.5), although many species can tolerate a broader pH range; for instance, *Escherichia coli* can multiply between pH 4.4 and 9.0.

Based on their optimal pH for growth, bacteria can be classified into:

1. **Acidophiles**: microorganisms that grow best at low pH values, typically between pH 1.0 and 5.5 (e.g., *Lactobacillus* spp.).

2. **Neutrophiles**: microorganisms with an optimal growth pH between about 5.5 and 8.5, usually around pH 7; the majority of bacteria fall into this category.
3. **Alkaliphiles (basophiles)**: microorganisms that grow optimally at high pH values, generally between pH 8.5 and 11.5. For example, *Vibrio cholerae* has an optimal pH around 9.0; *Exiguobacterium aurantiacum* grows between pH 8.5 and 9.5; *Alkaliphilus transvaalensis* can grow at pH values up to 12.5.

During cultivation, bacterial metabolism often generates acidic or basic end products that can significantly alter the pH of the medium and thereby inhibit growth. To minimize such pH shifts, culture media are commonly buffered, most often with phosphate buffers.

3.4.4 Oxygen Requirements

Oxygen (O_2) is a colorless, odorless gas that constitutes about 21% of the Earth's atmosphere. It serves as the terminal electron acceptor in aerobic respiration and is therefore crucial for the energy metabolism of many organisms. However, oxygen can also give rise to reactive oxygen species (ROS), which are toxic unless they are detoxified by specific enzymes.

Bacteria can be classified according to their requirements and tolerance for molecular oxygen:

1. **Obligate (strict) aerobes**: microorganisms that require free oxygen for growth and use O_2 as the terminal electron acceptor in their respiratory chain.
2. **Obligate (strict) anaerobes**: microorganisms that can grow and survive only in the absence of oxygen. They generally lack enzymes such as catalase, peroxidase, and superoxide dismutase, and are therefore unable to detoxify ROS (e.g., hydrogen peroxide and superoxide anion). Oxygen is toxic to these organisms.
3. **Facultative anaerobes (facultative aero-anaerobes)**: microorganisms that can grow in the presence or absence of oxygen. They typically use aerobic respiration when oxygen is available but can shift to anaerobic respiration or fermentation under anoxic conditions.
4. **Microaerophiles**: microorganisms that require oxygen for growth but at lower concentrations than that of air; high oxygen levels are inhibitory or lethal.
5. **Aerotolerant anaerobes**: microorganisms that do not use oxygen for their energy metabolism but can grow in its presence because they possess mechanisms to detoxify ROS.

3.4.5 **Osmotic Pressure**

Osmotic pressure is the force that drives the movement of water across a semipermeable membrane separating two solutions of different solute concentrations. It results from dissolved solutes (such as salts and sugars) that cannot freely cross the membrane.

Most bacteria, with the notable exception of members of the order *Mycoplasmatales* (which lack a rigid cell wall), are relatively resistant to moderate changes in osmotic pressure because their peptidoglycan cell wall helps prevent osmotic lysis. Some marine microorganisms are adapted to environments containing approximately 35 g of NaCl per liter (about 0.6 M NaCl).

According to their tolerance to sodium chloride, microorganisms (including many bacteria and archaea) can be divided into three main categories:

1. **Non-halophiles**: organisms that grow only at low NaCl concentrations, generally below 0.2 M.
2. **Halophiles**: organisms that grow optimally at elevated NaCl concentrations, from just above 0.2 M in slightly halophilic species (e.g., *Cobetia marina*) to about 5.2 M in extreme halophiles such as *Halobacterium salinarum*, *Halococcus morrhuae*, and *Halorubrum sodomense*.
3. **Halotolerant organisms**: microorganisms such as *Staphylococcus*, *Listeria*, and *Lactobacillus* spp. that do not require high salt concentrations for growth but can tolerate NaCl concentrations of 7.5–15%.

Food preservation methods such as salting (e.g., cured meats) and sugaring (e.g., jams) increase osmotic pressure by adding salt or sugar, thereby limiting the growth of many microorganisms. Only osmophilic microorganisms (often yeasts) can multiply in the presence of very high sugar concentrations, and only true halophiles can grow in media with very high salt concentrations.

3.4.6 **Pressure**

Barophilic or piezophilic bacteria (from the Greek *baros*, weight; *piezein*, to press) are characterized by the fact that their growth is enhanced when they are incubated at pressures higher than atmospheric pressure. These organisms are typically isolated from deep-sea environments and other high-pressure habitats.

Most barophilic bacteria described to date belong to the class Gammaproteobacteria, particularly the genera *Colwellia* (e.g., *Colwellia hadaliensis*), *Moritella* (e.g., *Moritella abyssi*, *Moritella japonica*, *Moritella profunda*, *Moritella yayanosii*), *Photobacterium* (e.g., *Photobacterium profundum*), *Psychromonas* (e.g., *Psychromonas kaikoae*, *Psychromonas profunda*), and *Shewanella* (e.g., *Shewanella benthica*, *Shewanella violacea*). Among these species, *Colwellia hadaliensis*, *Moritella yayanosii*, and *Psychromonas kaikoae* are strictly barophilic and can be cultured only under pressures higher than atmospheric pressure.

Barophilic microorganisms not belonging to the Proteobacteria have also been characterized. Examples include the hyperthermophilic archaeon *Thermococcus barophilus* and the anaerobic bacterium *Marinitoga piezophila*.

3.5 Questions

3.5.1 Part I. Choose the correct answer

1. For many bacteria, a common primary energy source in standard laboratory media is:
    - Vitamins
    - Glucose
    - Nucleic acids
    - Proteins
    - Lipids
2. Chemotrophic organisms:
    - use chemical compounds (organic or inorganic) as a source of energy.
    - use organic matter exclusively as an electron donor.
    - use water as an electron donor.
    - use light as a source of energy.
3. Lithotrophic organisms:
    - use organic matter as a source of energy.
    - use organic matter as an electron donor.
    - use reduced inorganic compounds as electron donors.
    - use light as a source of energy.
4. Phototrophic organisms:

- Use light as a source of energy.
- Use organic matter as a source of energy.
- Use water as an electron donor.
- Use organic matter as an electron donor.

5. Animals, many bacteria, and fungi are:
    - Chemolithotrophs
    - Photo-organotrophs
    - Photo-lithotrophs
    - Chemo-organotrophs
6. Chlorophyllous plants, algae, and cyanobacteria are:
    - Chemolithotrophs
    - Photo-organotrophs
    - Photo-lithotrophs (photolithoautotrophs)
    - Chemo-organotrophs

3.5.2 **Part II**

1. Indicate whether the following statements are true or false, and justify your answer.
    a) *The term auxotroph refers to microorganisms that are able to grow with CO_2 as their only source of carbon.*
    b) *The term growth factor refers to a substance that must be used in a culture medium designed to study the growth of microorganisms.*
    c) *Microorganisms are used to produce proteins for human use because they have no constraints for human consumption.*

2. Define the following terms:
    a) Halophile
    b) Heterotroph

3. Complete the following sentences:
    a) Bacteria that grow at temperatures between 20 and 25 °C are called: …….

b) Bacteria that grow at very low temperatures (around −5 °C) are called: …….

3.5.3 **Part III**

Complete the legend of Figure 18.

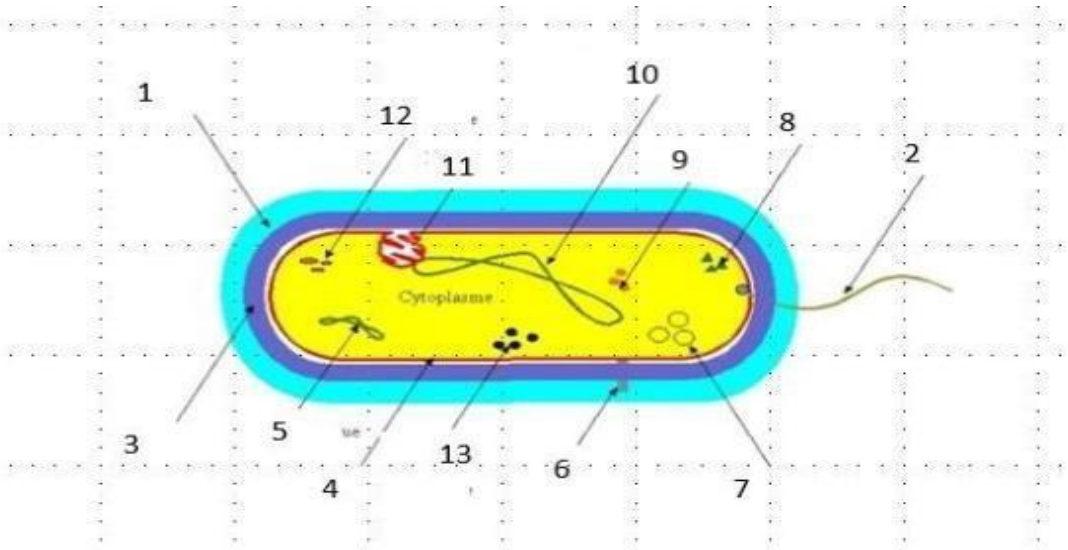


Figure 18. The bacterial cell

a) What are the functions of element 4?

b) Element 3 is a selective barrier.

Complete the legend for Figure 19.

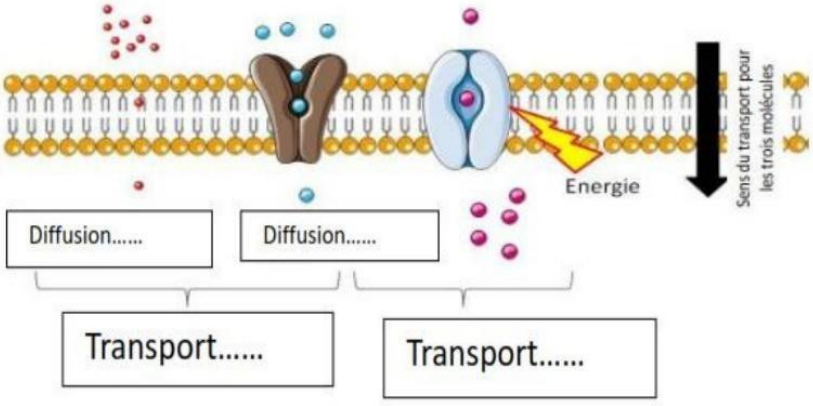


Figure 19. The different types of membrane transport

a) What are the differences between active and passive transport?

b) What are the differences between facilitated diffusion and simple diffusion?

3.5.4 **Part IV**

A continuous culture of a photo-litho-autotrophic bacterium shows that the bacterium grows optimally at 37 °C, at a pH of about 3, and at a high NaCl concentration (1.5 M). The bacterium is able to grow both in the absence and in the presence of oxygen.

a. Complete the following tables.

Table 4. Trophic type

Parameter	Answer
Energy source	……..
Carbon source	……..
Electron donor	……..

Table 5. Growth parameters

Parameter	Answer
Temperature	……..
pH	……..
Oxygen	……..
NaCl	……..

3.6 **Corrected (Answer Key)**

3.6.1 **Part I**

1. The main primary energy source used for many bacteria in standard laboratory media is **glucose**.
2. Chemotrophic organisms: **Correct answer:** use chemical compounds (organic or inorganic) as a source of energy.
3. Lithotrophic organisms: **Correct answer:** use reduced inorganic compounds as electron donors.
4. Phototrophic organisms: **Correct answer:** use light as a source of energy.

5. Animals, many bacteria, and fungi are: **Correct answer:** Chemo-organotrophs.
6. Chlorophyllous plants, algae, and cyanobacteria are: **Correct answer:** Photo-lithotrophs (photolithoautotrophs).

3.6.2 Part II

1. True/false with justifications

a. The term auxotroph refers to microorganisms that are able to grow with CO_2 as their only source of carbon. **False**. Auxotrophy is the inability of a microorganism to synthesize one or more specific organic compounds (such as certain amino acids, vitamins, or nucleotides) required for its growth. An auxotrophic strain must obtain these compounds preformed from the environment. Microorganisms that grow with CO_2 as their sole carbon source are termed autotrophs, not auxotrophs.

b. The term growth factor refers to a substance that must be used in a culture medium designed to study the growth of microorganisms. **False**. A growth factor is an organic compound that a given microorganism cannot synthesize and must acquire from its environment in small amounts (e.g., certain vitamins, amino acids, purines, pyrimidines). Growth factors therefore need to be added only to media used for microorganisms that specifically require them (often auxotrophic strains), not to all culture media.

c. Microorganisms are used to produce proteins for human use because they have no constraints for human consumption. False. Microorganism-derived proteins may present several constraints for human consumption. For example:

- They may have a high nucleic acid content, which can be undesirable.
- They may be contaminated with toxins, endotoxins, or other harmful metabolites.
- The proteins produced may exhibit physiological or immunological properties that are not suitable for direct human consumption and may require purification or modification.

2. Definitions

a) Halophile: Halophiles are microorganisms that require elevated concentrations of salt (typically NaCl) for growth. Many halophiles grow optimally at NaCl concentrations significantly higher than those tolerated by non-halophilic organisms; extreme halophiles may require very high salt concentrations (e.g., ≥ 2–5 M NaCl).

b) Heterotroph: A heterotroph is an organism that uses organic compounds as its principal carbon source. Heterotrophs cannot synthesize all cellular carbon skeletons from CO_2 alone; instead, they rely on preformed organic molecules to build biomass and synthesize their metabolites.

3. Completion

a) Bacteria that grow at temperatures between 20 and 25 °C are called psychrotrophs.

b) Bacteria that grow at very low temperatures (around −5 °C) are called cryophiles (extreme psychrophiles).

3.6.3 **Part III**

a. Suggested legend (to be adapted to the specific figure):

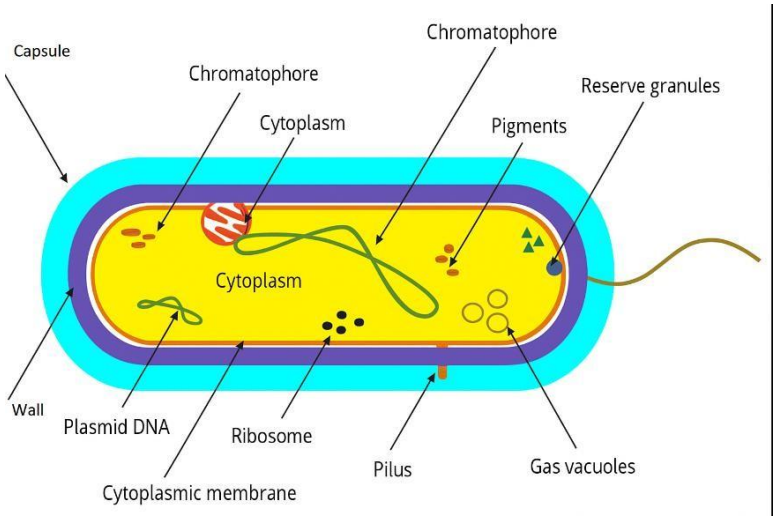


Figure 18: The bacterial cell.

Schematic representation of the main structural components of a typical bacterial cell (cell wall, cytoplasmic membrane, cytoplasm, nucleoid, ribosomes, flagella, pili, etc.).

b. Functions of element 4 (bacterial cell wall, if element 4 corresponds to the wall):

- Confers mechanical strength and maintains the characteristic shape of the cell.
- Protects the cell against osmotic lysis by resisting internal turgor pressure.
- Carries surface structures and receptors, including those that can serve as binding sites for bacteriophages.
- Bears important antigenic determinants (e.g., O-antigen, teichoic acids) involved in serotyping and immune recognition.

c. Legend for Figure 19 (membrane transport):

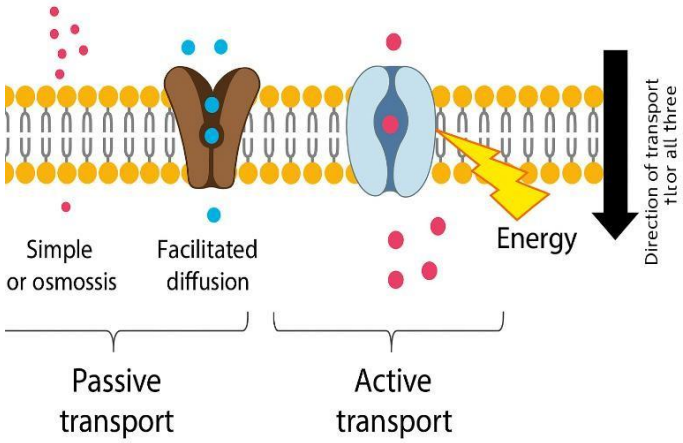


Figure 19: The different types of membrane transport.

Diagram illustrating passive diffusion, facilitated diffusion, primary active transport, secondary active transport (uniport, symport, antiport), and group translocation across the cytoplasmic membrane.

d. Differences between active and passive transport

1. Passive transport (including simple and facilitated diffusion):
    - Occurs down a concentration gradient (from high to low solute concentration).
    - Does not require metabolic energy (no direct consumption of ATP or other energy sources).
    - Simple diffusion does not involve specific transport proteins; facilitated diffusion is mediated by carrier or channel proteins.

2. Active transport:
    - Allows transport against a concentration gradient (from low to high solute concentration) or against an electrochemical gradient.
    - Requires metabolic energy, usually from ATP hydrolysis (primary active transport) or from an ion gradient such as the proton motive force (secondary active transport).
    - Always involves specific carrier proteins embedded in the membrane.

e. Differences between facilitated diffusion and simple diffusion

1. Simple diffusion:
    - Direct movement of small, nonpolar or weakly polar molecules (e.g., O_2 , CO_2 , some lipophilic substances) through the lipid bilayer.
    - Driven solely by the concentration gradient, without involvement of specific transport proteins.
    - Rate of transport increases linearly with the concentration gradient (no saturation).
2. Facilitated diffusion:
    - Transport of molecules via specific membrane proteins (carriers or channels).
    - Occurs down the concentration gradient, without energy expenditure, but the solute does not cross the lipid bilayer directly.
    - Shows saturation kinetics: the rate of transport reaches a maximum when all carriers are occupied.

3.6.4 **Part IV**

Table 4. Trophic type

The bacterium is described as **photo-litho-autotrophic**.

Parameter	Correct answer
Energy source	Light
Carbon source	CO_2 (autotrophic)

Electron donor	Inorganic compounds (e.g., H_2 O, H_2 , H_2 S)

Table 5. Growth parameters

From the data: optimal temperature 37 °C, pH ≈ 3, NaCl 1.5 M, growth in the presence and absence of O_2 .

Parameter	**Correct classification**
Temperature	Mesophilic
pH	Acidophilic
Oxygen	Facultative anaerobe (facultative aero-anaerobic)
NaCl	Moderately halophilic (1.5 M NaCl)

4 Bacterial Growth

4.1 Introduction

Bacteria grow when appropriate nutritional and physicochemical conditions are available in their environment. In microbiology, growth is defined as an increase in cellular mass and cellular constituents, which in most bacteria results in an increase in cell number through binary fission (also called scissiparity) (Figure 20) or, less frequently, budding. During binary fission, a bacterial cell elongates, replicates its chromosome, and divides to produce two daughter cells of approximately equal size. Growth may also be expressed as an increase in individual cell size or length without cell division.

In coenocytic (multinucleate) microorganisms, nuclear division may occur in the absence of cytokinesis. In such cases, growth leads primarily to enlargement of a single multinucleate cell rather than an increase in cell number.

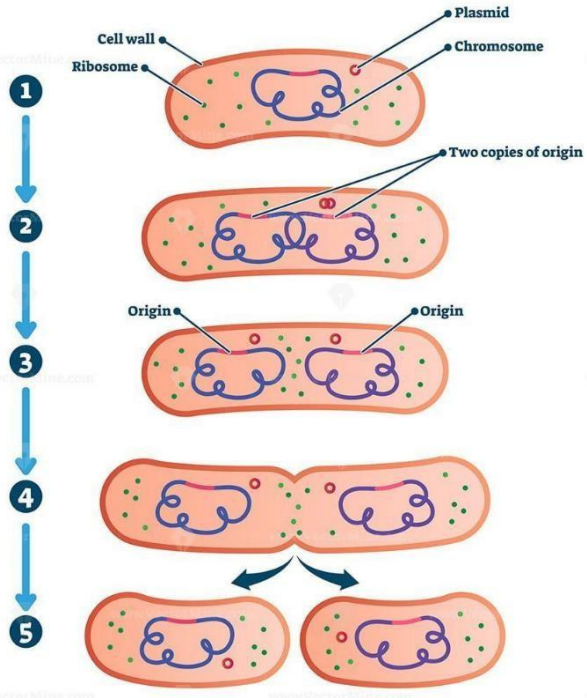


Figure 20. Binary fission (scissiparity) in bacteria

In laboratory culture, bacterial growth is evidenced by the appearance of discrete colonies on solid media and by the development of turbidity in liquid media (Figure 21). Each visible colony typically originates from a single colony-forming unit (CFU), that is, a single cell or a small cluster of cells capable of forming a colony.

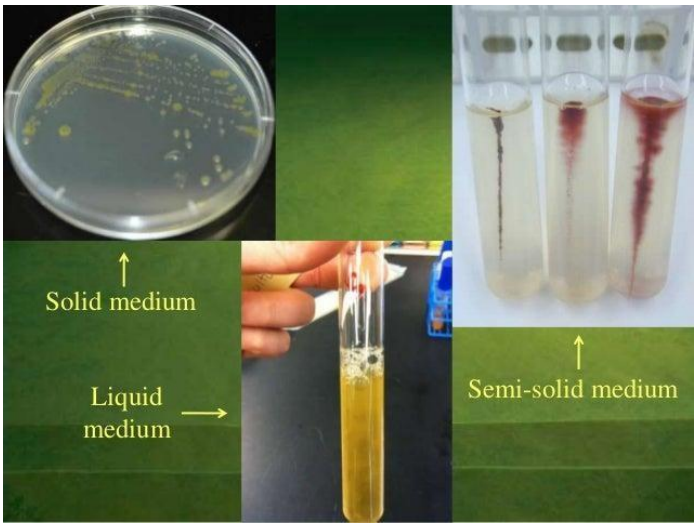


Figure 21. Macroscopic appearance of bacterial growth

4.2 Measurement of Bacterial Growth

Bacterial growth can be quantified using direct methods, which are based on cell counts, or indirect methods, which estimate biomass or metabolic activity.

4.2.1 Direct Methods

4.2.1.1 Direct Microscopic Enumeration

From a known volume of a bacterial suspension, a total cell count can be obtained microscopically using a counting chamber. This method is simple, inexpensive, and relatively rapid. It also provides information on the size and morphology of the microorganisms. A hemocytometer (e.g. Malassez, Thoma, or Helber counting chambers) is commonly used to count medium-sized bacteria or yeasts (Figure 22).

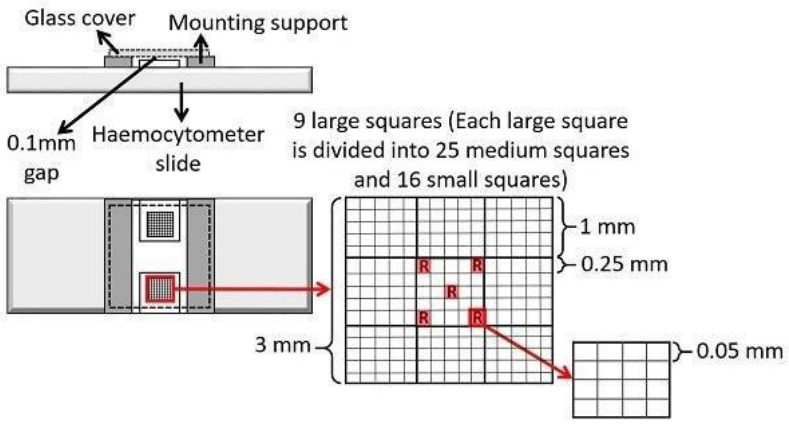


Figure 22. Example of a hemocytometer (counting chamber)

For small bacteria, a specialized slide known as the Petroff–Hausser counting chamber is often used. These chambers have a defined depth and a finely ruled grid etched on the surface (Figure 23), allowing calculation of the number of cells per unit volume.

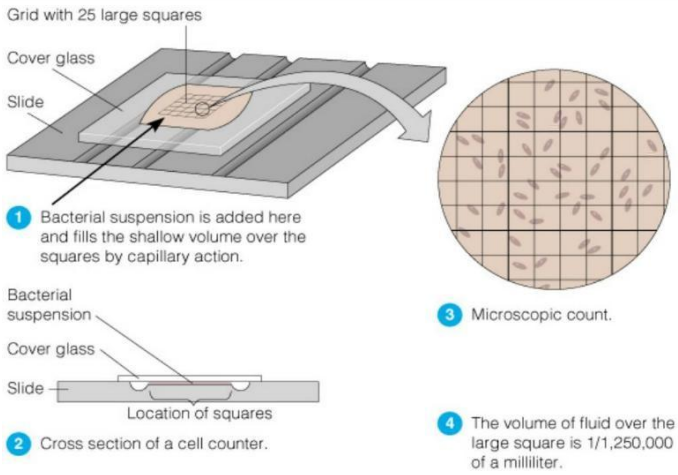


Figure 23. Use of the Petroff–Hausser counting chamber for direct microscopic counts

To calculate the number of microorganisms in the original sample, both the volume of the chamber and any dilution factors must be taken into account. This technique has several limitations: the microbial population density must be relatively high because only a small volume is examined, and the method generally does not distinguish between living and dead cells.

4.2.1.2 Particle Counter (Electrical Resistance Method)

Larger microbial cells, such as yeasts, can be counted automatically using an electrical particle counter (e.g. a Coulter counter). In this method, the microbial suspension passes through a small aperture within the instrument. An electric current is applied across the aperture, and electrodes on either side measure electrical resistance. Each time a microbial cell passes through the aperture, it displaces electrolyte, transiently increasing electrical resistance (or decreasing conductivity); this change is recorded as a single particle (Figure 24).

A major limitation of this method is that it counts all particles of the appropriate size, including non-biological or inert particles, which may lead to overestimation of microbial numbers.

Figure 24. Particle counter for microbial enumeration

4.2.1.3 Epifluorescence Technique

Epifluorescence microscopy can, in principle, be used to differentiate between viable and non-viable cells in a microbial population. This technique uses acridine orange or other DNA-binding fluorochromes. When samples are examined under an epifluorescence microscope with ultraviolet illumination, acridine orange bound to double-stranded DNA typically emits green fluorescence, whereas binding to single-stranded nucleic acids results in red/orange fluorescence.

Under idealized conditions, cells with predominantly green fluorescence are interpreted as viable, whereas cells with red fluorescence are interpreted as non-viable.

However, this method has several important limitations:

- During active growth, opening of the double-stranded DNA during replication generates single-stranded regions that may emit red fluorescence, leading to misclassification of viable cells as dead.
- Populations below approximately 10^5 cells/mL for yeasts and 10^6 cells/mL for bacteria cannot be reliably quantified because of the detection limits of the method.
- In organisms that form chains, clusters, or mycelial structures, individual cells are difficult to distinguish, making enumeration inaccurate.

4.2.1.4 Bacterial Colony Counts after Culture

Enumeration of viable cells by plate count after culture on solid media is a reference method and is widely used in microbiology. These culture-based methods selectively detect cells that are viable and capable of forming colonies under the specific culture conditions employed.

The two most commonly used techniques are:

1 the spread plate method (surface plating), and
2 the pour plate method (depth plating).

In both methods, a series of decimal (or other) dilutions of the sample is prepared prior to inoculation of the agar medium. After incubation, microbial growth appears as discrete colonies. Because it cannot be guaranteed that each colony originates from a single cell, results are expressed as colony-forming units per millilitre (CFU/mL) rather than as an absolute number of cells. For optimal statistical reliability, only plates containing between 30 and 300 colonies are used for calculations (Figure 25).

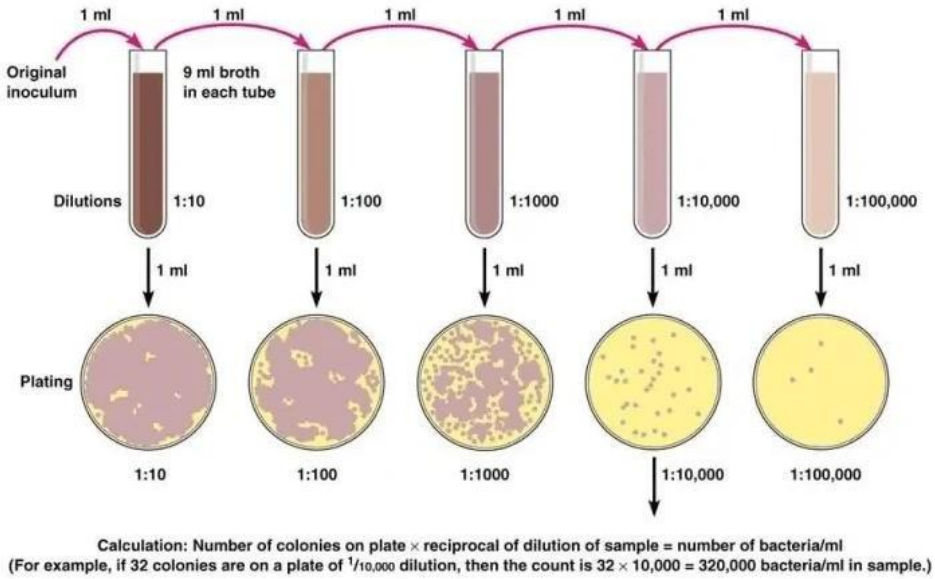


Figure 25. Enumeration of bacteria by serial dilution and plate count

4.2.1.5 Membrane Filtration Method

In certain liquid products, such as water, oral solutions, or injectable pharmaceutical preparations, microorganisms may be present at very low concentrations. In these cases, enumeration is performed using membrane filtration. A relatively large volume of the sample (typically at least 100 mL) is filtered through a membrane that retains microorganisms.

The membrane filter usually has pores with a nominal diameter of 0.45 μm, arranged in a three-dimensional network that efficiently retains bacteria on its surface. After filtration, the membrane is aseptically transferred onto the surface of an appropriate agar medium in a Petri dish (Figure 26) and incubated. Viable bacteria retained on the membrane multiply and form colonies that can be counted. This is possible because the pore diameter of the filter is smaller than the size of most bacterial cells commonly encountered.

For very small bacteria, slender bacilli (such as *Pseudomonas aeruginosa*), or bacterial spores that could potentially pass through 0.45 μm pores, membranes with a smaller pore size, such as 0.22 μm, are used to ensure effective retention.

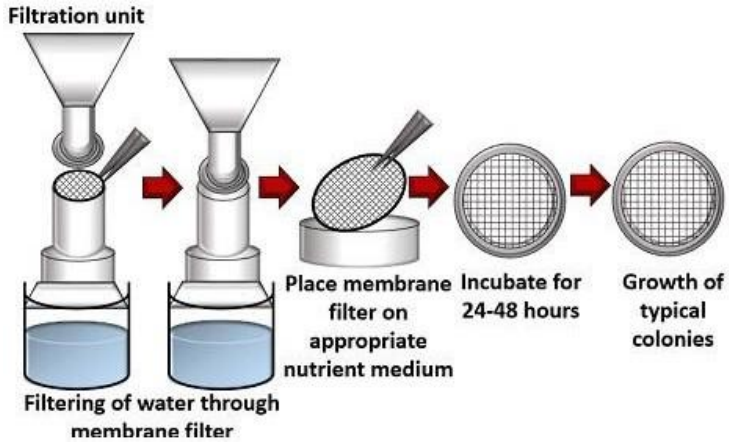


Figure 26. The membrane filtration process for enumeration of microorganisms in liquids

4.2.2 Indirect Methods

4.2.2.1 Biomass Measurement

Biomass refers to the total mass of living organisms present in a defined environment or sample, whether they are plants, animals, fungi, or microorganisms. In microbiology, biomass generally denotes the total mass of microbial cells in a culture or population.

Several techniques can be used to estimate bacterial biomass, including: determination of dry weight, measurement of optical density (OD), quantification of one or more cellular constituents, monitoring of substrate consumption, measurement of excreted metabolic products, and assessment of physicochemical changes induced by growth.

4.2.2.2 Optical Density Measurement

Optical density is commonly measured using a spectrophotometer. This general optical method is based on the property of a suspension to absorb and/or scatter part of the intensity of a light beam that passes through it in a straight line. Depending on the configuration, one may measure:

- the apparent absorbance or optical density (opacimetry),
- the decrease in transmitted light (turbidimetry), or
- the intensity of light scattered at an angle (nephelometry).

Each of these parameters can be correlated with the bacterial concentration in the medium. For routine bacterial growth measurements, the wavelength commonly used is 600–650 nm

($OD_{600} - OD_{650}$). As the number of microorganisms increases, more light is scattered, less light reaches the detector, and the recorded optical density (absorbance) increases.

This technique is widely used because it is simple, rapid, and inexpensive. Its major limitation is its relatively low sensitivity: bacterial concentrations of at least about 10^7 cells/mL are generally required to obtain reliably measurable optical density values. In addition, OD measurements do not distinguish between living and dead cells.

4.2.2.3 Indirect Measurement by Determination of Dry Biomass

Determination of dry biomass provides one of the most satisfactory measures of growth for filamentous microorganisms such as molds, and it can also be applied to bacteria.

For filamentous fungi, the mycelium is separated from the culture medium, usually by filtration, washed to remove medium components, transferred to a pre-weighed container, dried in an oven or desiccator to constant weight, and then weighed. The increase in dry weight reflects the increase in fungal biomass.

A similar approach can be used for bacteria. In this case, bacterial cells are usually harvested by centrifugation, washed to remove residual medium, dried, and weighed. The bacterial population can then be estimated on the assumption that 1 mg of bacterial dry weight corresponds to approximately 10^9 cells (the exact value depends on species and growth conditions). Over a wide range, the total dry biomass of a population is directly proportional to the number of cells.

4.2.2.4 Indirect Measurement by Metabolic Activity

The number of bacteria in a population can also be estimated indirectly by measuring their metabolic activity. This approach relies on the assumption that the rate or extent of a given metabolic process is proportional to the number of metabolically active cells.

Commonly used indicators include:

- changes in pH, for example acidification of the medium due to organic acid production during fermentation;
- production of carbon dioxide (CO_2), measured volumetrically or by gas analysis;
- consumption of oxygen (O_2), measured by respirometry;

- quantification of adenosine triphosphate (ATP), often by luminescence or fluorescence-based assays.

These methods are particularly useful when direct counting is difficult, but they require careful calibration and standardization, since metabolic activity per cell can vary with environmental conditions and growth phase.

4.2.2.5 I.2.4. Measurement of Cellular Constituents

If the amount of a particular cellular component is relatively constant per cell under defined conditions, the total quantity of that component in a sample will be directly related to the total microbial biomass.

For example, it is possible to determine the total protein or nitrogen content of a suspension of washed cells obtained from a known volume of culture. An increase in cell number will result in a proportional increase in total protein or nitrogen content.

Similarly:

- the chlorophyll content of a sample is commonly used to estimate the biomass of algae and cyanobacteria,
- the total ATP content serves as an indicator of the amount of living microbial biomass.

These biochemical measurements, when calibrated against cell counts or dry weight, provide reliable indirect estimates of microbial population size and growth.

4.3 Growth Kinetics

4.3.1 Growth Parameters

Bacterial growth in a homogeneous population can be characterized by two essential kinetic parameters: generation time (G) and specific growth rate (μ).

4.3.1.1 Generation time (G)

Generation time is the time required for a bacterial population to double in number, or equivalently for a single cell to complete one division cycle. It depends on the microbial species and on environmental conditions (e.g. nutrient availability, temperature, pH, oxygen).

Under optimal culture conditions, the generation time is very short for many bacteria. For example, the generation time is approximately 13 minutes for *Vibrio parahaemolyticus* and about 20 minutes for *Escherichia coli*.

If a population undergoes *n* generations over a period *t*, the generation time *G* is:

$$G = \frac{t}{n}$$

Where *t* = time (e.g. in minutes) and *n* = number of generations (cell divisions).

4.3.1.2 Specific growth rate (μ)

The specific growth rate μ expresses the number of generations per unit time. It is often given in reciprocal hours (h^{-1}). If a population undergoes *n* generations in a time *t*:

$$\mu = \frac{n}{t}$$

Thus,

$$n = \mu t \quad \text{and} \quad G = \frac{1}{\mu}$$

For example, if *E. coli* divides three times in one hour under given conditions, then $\mu = 3\ h^{-1}$ and the generation time is approximately 20 minutes.

4.3.2 Growth Curve (Batch Culture)

The growth of a microbial population is commonly studied by analyzing the growth curve obtained in a batch culture. In a batch culture, microorganisms are inoculated into a fixed volume of liquid medium contained in a closed vessel (e.g. a flask or tube). No fresh medium is added and no culture is removed during incubation. Consequently, nutrient concentrations progressively decrease, while metabolic waste products accumulate.

The growth curve is obtained by plotting the change in cell concentration or biomass (X) as a function of time (t), often on a semi-logarithmic scale:

$$X=f(t)$$

In a typical batch culture, where growth is asynchronous and nutrients are gradually depleted, the population passes through several characteristic phases. Classically, four major phases are recognized (lag, exponential, stationary, and death), but in a more detailed analysis, intermediate acceleration and deceleration phases can be distinguished, giving a 5–6 phase curve (Figure 27).

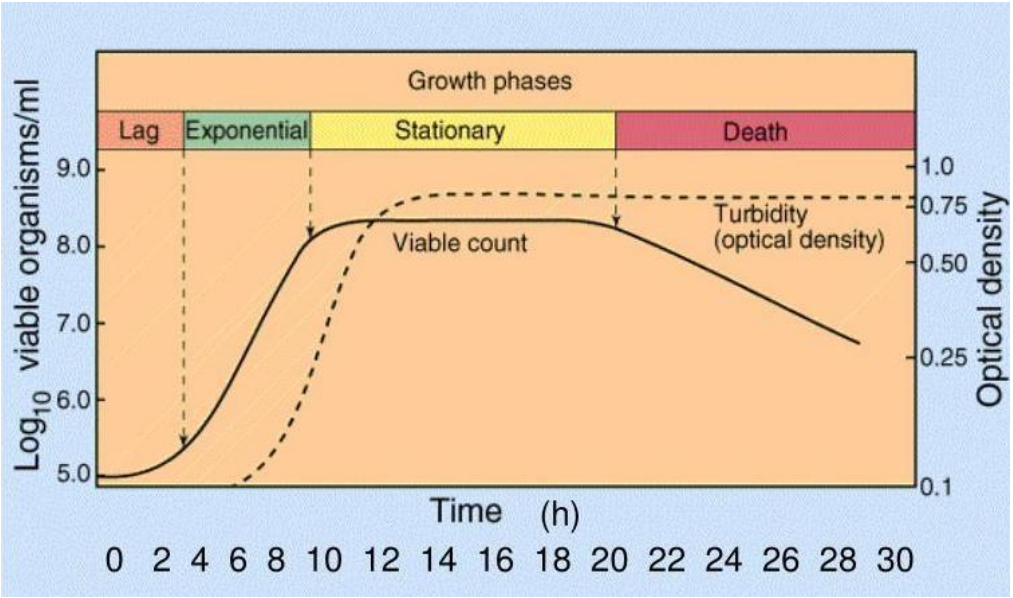


Figure 27. General bacterial growth curve in batch culture

The phases of the growth curve are:

1 **Lag phase**: The net growth rate is approximately zero ($\mu \approx 0$). The duration of this phase depends on the physiological state (age) of the inoculum and on the composition of the new medium. During lag phase, cells adapt to the new environment, repair damage, synthesize RNA, proteins, and enzymes required to metabolize the available substrates. If an actively growing culture is transferred into fresh medium of identical composition, the lag phase may be very short or even absent.

2 **Acceleration (transition) phase**: During this phase, the specific growth rate increases from $\mu = 0$ to $\mu = \mu_{max}$. Cells progressively complete their adaptation; the proportion of cells actively dividing increases, and the culture approaches exponential growth.

3 **Exponential (logarithmic) phase**: The specific growth rate reaches a maximum and remains constant ($\mu = \mu_{max}$). Cell number and biomass increase exponentially, and the generation time is minimal and constant. Mortality is negligible, and the population is

physiologically relatively homogeneous. This is the phase most often used for physiological, biochemical, and genetic studies, as well as for kinetic measurements.

4 **Deceleration phase**: The growth rate progressively decreases as nutrients become limiting and inhibitory metabolites accumulate. Cell division slows, and the balance between anabolic and catabolic processes shifts. Autolytic processes may begin in a fraction of the population.

5 **Stationary phase**: The net growth rate becomes zero ($\mu \approx 0$) because the rate of cell division roughly equals the rate of cell death. Nutrient depletion, accumulation of toxic metabolites, and changes in pH contribute to growth arrest. The total number of cells remains approximately constant. This phase is sometimes described as involving cryptic growth, in which some cells continue to grow by utilizing nutrients released from lysed cells. Many bacteria in this phase express stress-resistance mechanisms, and some species may form spores.

6 **Death (decline) phase**: The apparent growth rate becomes negative ($\mu < 0$) as the death rate exceeds the division rate. Nutrient resources are exhausted, toxic metabolites accumulate, and cell lysis increases, often under the action of endogenous (autolytic) enzymes and external stressors. The number of viable cells decreases markedly. Cryptic growth may still occur in a minority of cells that are able to use materials released from lysed cells, but overall the viable population declines.

4.3.3 Other Growth Patterns

4.3.3.1 Diauxic Growth

The term diauxie (from the Greek *dia* = twice, *auxein* = to grow) was introduced by Jacques Monod to describe a two-phase growth pattern observed when microorganisms are grown in a batch culture containing a mixture of two carbon sources. In English, this phenomenon is commonly referred to as diauxic growth.

Diauxic growth is typically observed in a defined (synthetic) medium containing two different sugars as carbon and energy sources. For example, in a medium containing both glucose and lactose, bacteria preferentially utilize glucose first. Glucose is metabolized by constitutive enzymes, and its presence represses the synthesis of the inducible enzymes required for lactose catabolism (e.g. β-galactosidase). This regulatory mechanism is known as catabolite repression.

As long as glucose is available, lactose is not significantly metabolized. Once glucose is depleted, repression is lifted, the enzymes necessary for lactose metabolism are synthesized, and after an adaptation (lag) period, a second exponential growth phase begins, now based on lactose utilization. This results in a characteristic biphasic (two-step) growth curve (Figure 28).

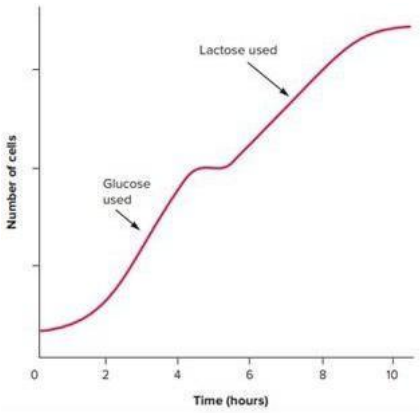


Figure 28. Diauxic growth of bacteria cultured in the presence of glucose and lactose as mixed carbon sources

4.3.3.2 Continuous Culture

Continuous growth of microorganisms can be achieved in an open system in which culture conditions are kept essentially constant over time by continuous or semicontinuous input of fresh medium and simultaneous removal of culture fluid containing cells and waste products. Such systems, known as continuous culture systems, allow a microbial population to be maintained for extended periods in a defined physiological state, often close to exponential growth and at an approximately constant biomass concentration.

The two principal types of continuous culture systems are the chemostat and the turbidostat.

4.3.3.2.1 Chemostat

A chemostat is a continuous culture device in which sterile fresh medium is continuously introduced into the culture vessel at the same rate as culture broth (containing microorganisms and metabolic products) is removed (Figure 29). The culture volume therefore remains constant.

The medium in a chemostat contains one essential nutrient in limiting concentration (e.g. a specific carbon source, nitrogen source, or an amino acid). Under steady-state conditions, the

specific growth rate of the microorganism is determined by the dilution rate (flow rate/volume) and limited by the concentration of this growth-limiting nutrient. The final cell density (biomass concentration) depends on the concentration of the limiting nutrient in the inflowing medium.

By adjusting the dilution rate and nutrient concentration, the chemostat makes it possible to control and study microbial growth under precisely defined and reproducible conditions.

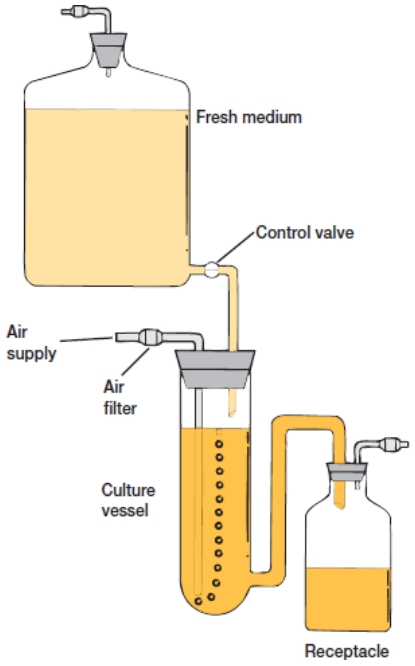


Figure 29. Example of a continuous culture system: the chemostat

4.3.3.2.2 Turbidostat

Continuous culture can also be carried out in a turbidostat. In this system, the culture vessel is equipped with a photocell (or other optical sensor) that continuously measures the turbidity (optical density) of the culture (Figure 30).

The flow rate of fresh medium into the vessel, and the corresponding outflow of culture, are automatically adjusted to maintain a predetermined turbidity or cell density. When the culture becomes too turbid (cell concentration too high), the flow rate increases; when the turbidity decreases, the flow rate decreases.

In contrast to the chemostat, where the growth rate is set by the dilution rate and limited by a specific nutrient, the turbidostat operates by keeping cell density constant and allows

microorganisms to grow close to their maximal specific growth rate under the given conditions. Turbidostats are particularly useful for studying organisms at or near μ_{max}.

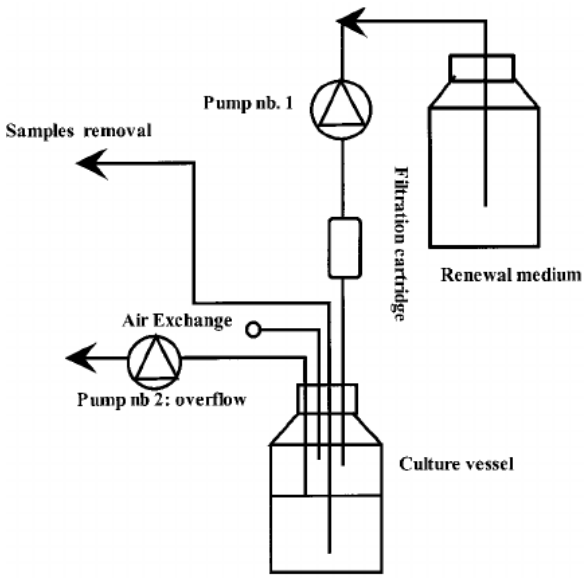


Figure 30. Schematic representation of a turbidostat

4.4 Bacterial Culture

The growth and maintenance of microorganisms in the laboratory are fundamental operations on which microbiology depends. These are made possible by the availability of suitable culture media.

4.4.1 Culture Media

Microorganisms require nutrients for growth. In the laboratory, these are provided by culture media (or nutrient media). For a medium to support microbial development, it must:

- contain all essential nutrients in sufficient quantities and in appropriate relative proportions (i.e. it must be nutritionally adequate and balanced);
- exhibit physicochemical characteristics compatible with microbial life, including appropriate pH, osmotic pressure, viscosity, and water activity.

Because microorganisms differ greatly in their nutritional requirements and growth conditions, there is no universal medium that supports the growth of all species. However, some media are suitable for the cultivation of a wide range of microorganisms. The choice of medium is guided

by knowledge of the natural habitat and nutritional physiology of the microorganisms to be cultivated.

4.4.1.1 Definition

A culture medium is a prepared substrate in which microorganisms (bacteria, yeasts, and moulds) can multiply, thereby allowing their isolation, enumeration, and study. In principle, cells find in this environment the essential components for rapid multiplication in large numbers and, in some cases, additional elements that selectively favour a particular genus, species, or group.

A suitable culture medium must therefore meet the nutritional requirements (source of energy, carbon, nitrogen, minerals and essential ions such as calcium, phosphate, sulphate, etc.) and physical requirements (appropriate pH, temperature, water availability, and osmotic balance) of the microorganism under study.

4.4.1.2 General Classification of Culture Media

Different types of media exist. Some are basic (or simple) media that support the growth of non-fastidious species. Others are enriched by the addition of substances such as serum, egg, blood, or vitamins to promote the growth of fastidious bacteria. Media can also be made selective by adding antibiotics, antiseptics, salts, or dyes that inhibit certain microorganisms while permitting the growth of others.

Culture media may be classified according to several criteria, including:

- physical consistency (liquid, solid, semi-solid),
- chemical composition (complex vs defined),
- intended use (basic, selective, differential, enrichment, chromogenic, etc.).

4.4.1.3 Classification of Media According to Consistency

Microorganisms can be grown on:

1 **Liquid media (broths)**: Microbial growth appears as turbidity or a surface pellicle.
2 **Solid media**: These are obtained by adding agar (a polysaccharide extracted from red algae) to a liquid base. Depending on the agar concentration, one distinguishes:
    - solid agar (≈ 1.5%),

- semi-solid or soft agar (≈ 0.3–0.75%), often used for motility tests or microaerophilic cultivation.

It is generally unnecessary to exceed about 1.5% agar, as higher concentrations increase gel firmness and may affect diffusion properties without improving growth.

Solid media can be prepared in Petri dishes or in tubes (as slants or deeps). Agar melts when heated to around 100 °C and solidifies upon cooling at approximately 40–45 °C. Petri dishes are typically poured with agar cooled to about 50 °C to avoid thermal damage to microorganisms.

On solid media, bacterial development appears as colonies. The morphology of colonies (shape, size, surface, edge, elevation), as well as their colour and odour, depends on both the species and the medium used.

Examples of commonly used media: nutrient agar, trypticase soy agar (TSA), MacConkey agar, Mueller–Hinton agar.

4.4.1.4 Classification of Media According to Composition

4.4.1.4.1 Complex (empirical) media

Complex media have an imprecisely defined composition. They contain complex organic materials in which the exact concentration of individual components (e.g. amino acids, peptides, vitamins) is not known. They are widely used for the cultivation of chemoheterotrophic bacteria.

Complex media may be:

- of animal origin: milk, serum, nutrient broth and nutrient agar, gelatin, blood, etc.;
- of plant origin: soya peptone, potato infusion, etc.

Examples of complex media: Gassner agar, Trypticase soy agar, chocolate agar, blood agar.

4.4.1.4.2 Synthetic (defined) media

In defined media, the qualitative and quantitative composition is precisely known. All components are chemically identified and present at known concentrations. These media are particularly useful for studying autotrophic or non-fastidious bacteria, and for analysing the specific nutrient requirements or metabolic pathways of a microorganism.

They are less frequently used for routine diagnostics, but some defined media are used for specific tests, such as Simmon's citrate medium, Christensen's citrate medium, and certain urea–tryptophan (urea–indole) media.

4.4.1.5 Classification of Media According to Use

4.4.1.5.1 Basic (general-purpose) media

Also called usual or non-selective media, basic media do not contain inhibitory molecules. They are generally simple and relatively inexpensive. They contain a nutrient base composed of nitrogenous substances (peptones, amino acids, organic nitrogen compounds) and other organic molecules derived from the hydrolysis of products of animal, plant, or fungal origin (e.g. peptones, meat extract, yeast extract).

Examples: nutrient agar and nutrient broth, Mueller–Hinton agar and Mueller–Hinton broth.

4.4.1.5.2 Isolation media

Isolation media are solid media used to obtain isolated colonies, perform identification tests, or study the antibiotic susceptibility of bacteria. Depending on their formulation and purpose, they may be:

4.4.1.5.2.1 Selective media

Selective media favour the growth of one or a limited number of bacterial species while inhibiting others. This is achieved by incorporating substances such as bile salts, high concentrations of sodium chloride, certain dyes (e.g. crystal violet), or specific antibiotics. These agents are chosen according to the properties of the target microorganism. Selective media are particularly useful for the analysis of polymicrobial samples.

Example: *Salmonella–Shigella* (SS) agar, which favours the growth of *Salmonella* species; Shigella can also grow, but more slowly (typically 48–72 hours before a usable culture is obtained).

4.4.1.5.2.2 Differential (or indicator) media

Differential media allow distinct types of microorganisms to be distinguished on the same medium. They contain specific substrates and indicators that reveal particular biochemical properties (most often the ability to degrade or ferment a substrate) through visible changes.

Indicators may be pH indicators or redox indicators, such as neutral red, phenol red, eosin, or methylene blue.

Example: Chapman agar (mannitol salt agar, MSA) is both selective for staphylococci (high NaCl concentration) and differential for mannitol fermentation, which produces a colour change and facilitates the presumptive identification of *Staphylococcus aureus*.

4.4.1.5.2.3 Enrichment media

In addition to basic components, enrichment media contain growth factors or nutrients that certain bacteria cannot synthesize (e.g. vitamins, amino acids, blood, serum). They are generally rich liquid media designed to promote the multiplication of fastidious microorganisms or to increase the relative proportion of a target organism in a mixed population. Some enrichment or enriched media are formulated to support strict anaerobes.

Common examples include nutrient broth, Schaedler broth or agar, and brain–heart infusion (BHI) medium, sometimes supplemented with reducing agents for anaerobic culture.

4.4.1.5.2.4 Chromogenic (discriminating) media

Chromogenic media may be selective and/or differential. Their principle is based on the inclusion of one or more substrates conjugated to a chromogenic molecule. When a specific bacterial enzyme hydrolyses or modifies the substrate, the associated chromogen is released or transformed into a coloured compound (chromophore) visible directly in the colony.

If the enzyme is characteristic of a particular species or group, colonies of that organism appear with a distinctive colour, allowing rapid presumptive identification.

Example: MRSA-ID agar, which permits the direct detection of methicillin-resistant *Staphylococcus aureus* (MRSA) by producing characteristic coloured colonies.

4.4.2 Inoculation (Sowing)

Microorganisms are ubiquitous in natural, clinical, and built environments and often occur in highly diverse polymicrobial communities. For example, the human gastrointestinal tract can be colonized by hundreds of different bacterial species, and one gram of soil commonly contains 10^8–10^9 bacteria belonging to numerous bacterial and fungal species.

Because of this complexity, direct study of such mixed populations is difficult. Microbiologists therefore rely on separation, isolation, and purification techniques, all of which require mastery of basic inoculation (sowing) methods.

4.4.2.1 Key Terms

- **Inoculation**: Introduction of a microbial sample (inoculum) into a sterile culture medium (broth or agar) using a suitable instrument (e.g. Pasteur pipette, platinum loop, disposable plastic loop).
- **Inoculum**: A sample or quantity of microorganisms taken from a mother culture or clinical/environmental specimen, intended to be introduced into a culture medium favourable to their multiplication.
- **Isolation**: Inoculation onto culture media with the aim of separating individual cells or small groups of cells so as to obtain distinct, well-isolated colonies derived from single cells (or CFUs).
- **Subculture (replating, "transplanting")**: Transfer of microorganisms from a primary (or older) culture to a fresh culture medium, often to maintain a pure culture, increase biomass, or perform additional tests.
- **Species vs strain**: A species is a fundamental taxonomic unit that encompasses a group of related bacteria sharing key phenotypic and genotypic characteristics. A strain is a clonal lineage or isolate within a species, differing from other members of the same species by minor but identifiable genetic and/or phenotypic characteristics (e.g. biotype, serotype, antimicrobial resistance profile).

4.4.2.2 Inoculation Techniques

4.4.2.2.1 Inoculation of broth cultures

A loopful of a bacterial colony (or a measured volume of liquid sample) is introduced into a sterile liquid medium (broth) and dispersed by gentle agitation to obtain a uniform suspension.

4.4.2.2.2 Streak inoculation of agar media (streak plate)

An inoculum is deposited on the surface of a solid agar medium (in Petri dishes or on agar slants). A platinum/plastic loop or Pasteur pipette tip is then drawn repeatedly over the agar

surface in a controlled pattern to dilute and separate cells. This is the standard method for obtaining isolated colonies from a mixed or dense culture.

4.4.2.2.3 Flood (lawn) inoculation

The surface of an agar plate is covered (flooded) with a microbial suspension, which is then evenly distributed with a sterile spreader or rake to create a continuous bacterial lawn. Excess inoculum is removed, and the plates are allowed to dry (e.g. at 37 °C for a few minutes). This technique is commonly used for antimicrobial susceptibility testing (e.g. antibiogram) and for certain phage or bacteriocin assays.

4.4.2.2.4 Inoculation in the mass (pour plate)

The inoculum is mixed with molten agar (cooled to about 45–50 °C) before the medium solidifies, and the mixture is poured into a Petri dish. After solidification, colonies develop both on the surface and within the depth of the agar. This method is often used for bacterial enumeration by plate count.

4.4.2.2.5 Spot inoculation (spot seeding)

Small volumes of a concentrated inoculum are deposited as discrete spots (a few millimetres in diameter) on the surface of an agar plate. This technique allows multiple strains or conditions to be tested on the same plate (e.g. screening several strains for resistance or enzyme production).

4.4.2.2.6 Central stab inoculation

Performed in tubes containing solid or semi-solid agar. A Pasteur pipette, needle, or loop loaded with inoculum is inserted vertically into the medium to the bottom of the tube and withdrawn along the same track. This is used, for example, in motility–indole–urea or mannitol–motility media and for testing oxygen requirements or motility in semi-solid media.

4.4.2.2.7 Swab inoculation

A sterile swab is soaked in a liquid specimen or bacterial suspension and then rolled or streaked over the surface of the agar to obtain a uniform inoculum. This method is widely used for antimicrobial susceptibility testing and for culturing from mucosal or skin surfaces.

4.4.2.3 Isolation Techniques

Isolation techniques in microbiology aim to separate microorganisms present in a mixture, to purify a contaminated strain, or to verify the purity of a culture. Isolation of bacteria is mainly carried out on solid media, using different streaking strategies depending on the objective.

4.4.2.3.1 Four-Quadrant Streak (Exhaustion Technique in Four Sectors)

This technique separates the different microorganisms present in the initial sample by progressively diluting the inoculum over the surface of the agar (Figure 31).

1. On the underside of the Petri dish, two perpendicular lines are drawn to divide the plate into four sectors (quadrants A, B, C, and D).
2. The inoculum is first applied to the surface near one edge of the plate in the area corresponding to quadrants A and B.
3. Using a sterile loop, tight streaks are made over the first half of the plate (quadrants A and B), distributing the inoculum.
4. The loop is sterilized and cooled, and the plate is rotated approximately 90°.
5. The loop is then drawn from quadrant B into quadrant C and streaked over quadrants B and C, diluting the number of cells.
6. After re-sterilizing and cooling the loop, the plate is again rotated about 90°, and streaking is continued from quadrant C into quadrant D, producing well-separated streaks.

By successive dilution of the inoculum over the four sectors, isolated colonies can be obtained, particularly in the last quadrant.

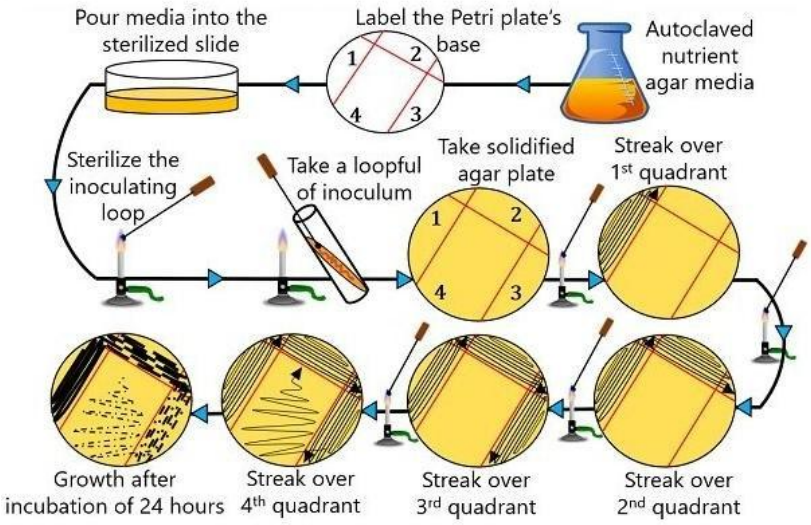


Figure 31. Four-quadrant streak plate

4.4.2.3.2 Three-Quadrant Streak (Exhaustion Technique in Three Sectors)

In this variant (Figure 32), the plate is divided into three sectors instead of four and streaking is performed in three stages:

1 The first third of the plate is inoculated by sweeping the agar surface with non-overlapping lines using a loop loaded with inoculum.

2 The loop is then sterilized and cooled. The second third is streaked by lightly crossing into the first sector to pick up a reduced number of cells, which are then spread over the second sector.

3 After a new sterilization and cooling, the third sector is streaked by slightly crossing into the second one, drawing well-spaced lines to obtain isolated colonies.

This method also aims at progressive dilution of the inoculum and the appearance of isolated colonies, typically in the last sector.

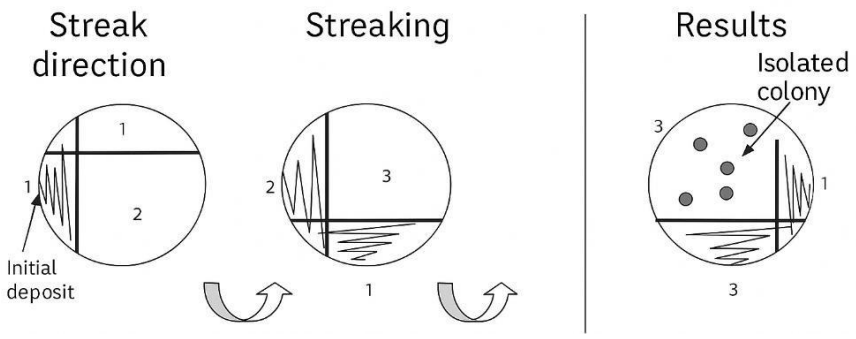


Figure 32. Three-quadrant streak plate

4.4.2.4 Serial Dilution Technique

In some cases, the bacterial load in a culture (liquid or plate) is so high that the surface of the Petri dish becomes covered with confluent growth (a bacterial mat or film), preventing the formation of distinct, countable colonies and making subculture and enumeration difficult.

To overcome this problem, a serial (successive) dilution of the sample is performed.

1 A stock suspension of the product to be analysed is prepared.
2 A series of tubes containing 9 mL of sterile diluent (usually physiological saline or buffered diluent) is prepared.
3 One millilitre of the stock suspension is transferred into the first tube containing 9 mL diluent, yielding a 10^{-1} (1:10) dilution.
4 From this tube, 1 mL is transferred into the next tube containing 9 mL diluent to obtain a 10^{-2} dilution.
5 The procedure is repeated to obtain a dilution series (e.g. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} , etc.), as illustrated in Figure 33.

Aliquots from appropriate dilutions are then plated (e.g. using the pour plate or spread plate method). After incubation, plates with 30–300 colonies are selected for counting, and the original bacterial concentration is calculated by taking into account the dilution factor.

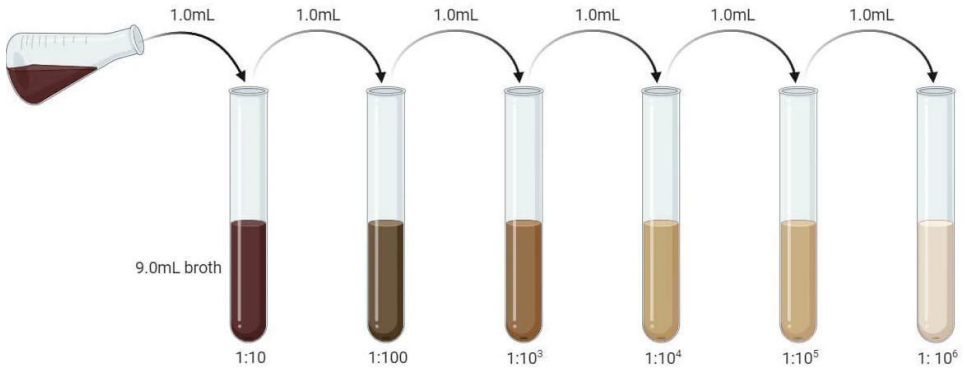


Figure 33. Schematic representation of decimal (10-fold) serial dilutions

4.5 Questions

4.5.1 Part I

Specify an appropriate method of sterilization for each of the following items:

- 100 mL of 0.9% NaCl
- A glass test tube
- Plastic Petri dishes
- An antibiotic solution
- A culture medium containing agar
- A glass Pasteur pipette

4.5.2 Part II

Answer the following statements with True or False and justify your answer:

1 Culture media can be sterilized in either a Pasteur oven or an autoclave.
2 An antibiotic solution can be added to the culture medium before sterilization.
3 A medium sterilized for 20 minutes at 120 °C in the autoclave no longer contains viable cells.
4 60° alcohol destroys germs more easily than 95° alcohol.
5 A vial of autoclaved culture medium can remain open on the bench. A culture will only develop there if it is inoculated.
6 Nutrient agar broth is suitable for all bacteria that exist in nature.

7 Can a pure culture contain only one bacterial species? A single bacterial strain?

8 Subculturing (“transplanting”) makes it possible to obtain a “fresh” culture, i.e. containing metabolically active cells.

9 In an autoclave, the maximum temperature reached depends on the power of the heating elements that heat the water.

10 You must let a sterilized medium cool down to a temperature of about 60 °C before pouring the medium into a plate.

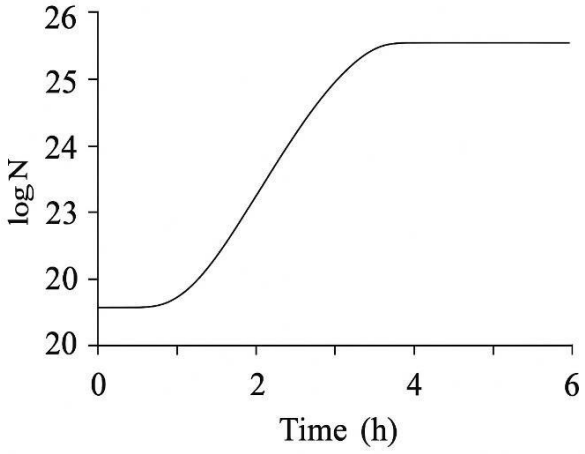
4.5.3 Part III: Enforcement Exercise

4.5.3.1 Growth of a *Pseudomonas* Strain in a Defined Medium

A strain of *Pseudomonas* isolated from soil is able to grow on the following medium:

- Glucose: 16 g/L
- $(NH_4)_2 SO_4$: 1 g/L
- $K_2 HPO_4$: 7 g/L
- $KH_2 PO_4$: 3 g/L
- $MgSO_4 \cdot 7H_2 O$: 0.1 g/L

To study the growth of this strain, this medium was inoculated from a 24-hour culture of the same strain grown on nutrient agar, and then incubated under optimal temperature and pH conditions. The evolution of the bacterial population over time is represented in the growth curve provided (Figure).

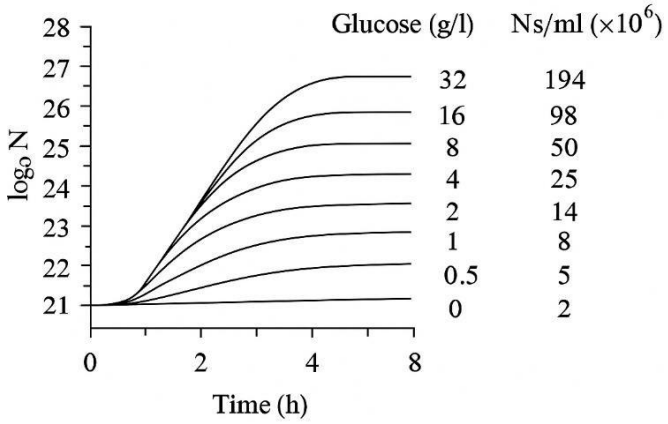


1 Delimit the different phases of growth on the graph and name and interpret each of them.

2 Determine the numerical values of the parameters that are necessary and sufficient to characterize this growth.

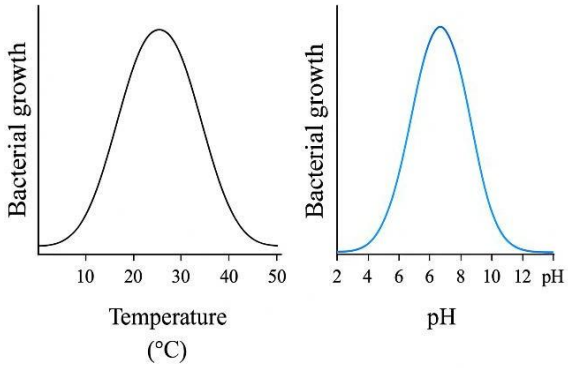
3 Under the experimental conditions described, on which factors does the first phase of the curve depend?

4 Explain the third phase of the curve based on the correlation between bacterial growth and glucose consumption (as shown in the following figure).



4.5.3.2 Effect of Glucose Concentration on Growth

The same growth experiment is repeated with the same strain and the same culture medium, but using different initial glucose concentrations. The different growth curves are represented on the same graph (Figure).



- Compare the total growth (Ns – N_0) and the maximum specific growth rate μmax for glucose concentrations of 32, 4, and 0.5 mg/mL (i.e. 32, 4, and 0.5 g/L).

4.5.3.3 Effect of Temperature and pH on Growth

An optimization of bacterial growth was carried out by varying the temperature and the pH of the growth medium. The results are presented in the corresponding figures.

- Interpret these results and indicate the ecological/physiological type of this bacterial strain (with respect to temperature and pH).

4.6 Corrected Answers

4.6.1 Part I – Answer Key

Specify an appropriate method of sterilization for each item.

- **100 mL of 0.9% NaCl**: Heat-stable aqueous solution → Autoclave sterilization (e.g. 121 °C, 15–20 min, saturated steam under pressure). Sterile membrane filtration is possible but more costly and less practical for routine use.
- **Glass test tube**: Heat-resistant glassware → Autoclave or dry-heat sterilization in a hot-air (Pasteur) oven (e.g. 160–180 °C for 2–1.5 h, respectively).
- **Plastic Petri dishes**: Heat-sensitive plastic (e.g. polystyrene) → sterilized industrially by ionizing radiation (gamma rays) or gas sterilization (ethylene oxide). In the laboratory they are typically purchased pre-sterilized.
- **Antibiotic solution**: Generally heat-labile → must be sterilized by membrane filtration (e.g. 0.22 µm). The filter-sterilized antibiotic is then added aseptically to an autoclaved medium cooled to about 45–55 °C, not before autoclaving.
- **Culture medium containing agar**: Heat-stable aqueous medium → Autoclave sterilization (e.g. 121 °C, 15–20 min).
- **Glass Pasteur pipette**: Heat-resistant glass instrument → Dry heat (Pasteur oven) or autoclave after appropriate packaging.

4.6.2 Part II – Answer Key

1. Culture media can be sterilized in either a Pasteur oven or an autoclave. **False**. Aqueous culture media must be sterilized by moist heat in an autoclave (saturated steam under pressure). Dry-heat sterilization in a Pasteur oven is not appropriate for liquids, as it leads

to excessive evaporation and does not provide the same level of microbial inactivation in water-containing media.

2. An antibiotic solution can be added to the culture medium before sterilization. **False**. Most antibiotics are heat-labile and are degraded by autoclaving. The correct procedure is to autoclave the basal medium alone, cool it to about 45–55 °C, and then add a previously filter-sterilized antibiotic solution under aseptic conditions.
3. A medium sterilized for 20 minutes at 120 °C in the autoclave no longer contains viable cells. **True**. Under standard autoclave conditions (approx. 121 °C, 15–20 min, saturated steam under pressure), vegetative cells and bacterial endospores are inactivated, provided the cycle is properly validated and the load is appropriate. The medium can therefore be considered sterile.
4. 60° alcohol destroys germs more easily than 95° alcohol. **True**. For ethanol or isopropanol, concentrations around 60–80% (v/v) are more effective disinfectants than 95%. The presence of water enhances cell penetration and protein denaturation, which are essential for microbial killing. Very high concentrations may rapidly dehydrate cell surfaces and limit deeper penetration.
5. A vial of autoclaved culture medium can remain open on the bench. A culture will only develop there if it is inoculated. **False**. An open sterile vial is rapidly contaminated by airborne microorganisms (bacteria, fungal spores, dust). These can grow without deliberate inoculation. Sterile media must be kept closed or manipulated only under aseptic conditions (e.g. near a flame or in a laminar-flow hood).
6. Nutrient agar broth is suitable for all bacteria that exist in nature. **False**. The majority of environmental microorganisms are not culturable under standard laboratory conditions, and many culturable species require specific nutrients or environmental conditions not provided by simple nutrient agar/broth. Thus, nutrient agar supports only a subset of bacterial diversity.
7. Can a pure culture contain only one bacterial species? A single bacterial strain? **True / True**. A pure culture is obtained by inoculating a medium from a single isolated colony. Such a colony arises from the multiplication of one cell or a group of genetically identical cells. By definition, a pure culture contains cells of one species, and ideally of one strain (a genetically uniform population).

8. Subculturing (“transplanting”) makes it possible to obtain a “fresh” culture, i.e. containing metabolically active cells. **True**. In aging cultures, nutrients become depleted and inhibitory metabolites accumulate, leading to stationary and death phases, where many cells are metabolically inactive or dead. Subculturing a small inoculum into fresh medium provides new nutrients and conditions that favor metabolically active, “fresh” cells.
9. In an autoclave, the maximum temperature reached depends on the power of the heating elements that heat the water. **False**. The maximum sterilization temperature is determined mainly by the pressure of saturated steam inside the autoclave chamber (e.g. about 121 °C at ≈1 bar overpressure). The heating elements supply the energy to reach this pressure, but the equilibrium temperature is a function of pressure, not of heater power. Power mainly affects how quickly the set temperature is reached.
10. You must let a sterilized medium cool down to a temperature of about 60 °C before pouring the medium into a plate. **True** (with refinement). After autoclaving agar media, they should be cooled to a temperature at which:
    - the container can be handled safely;
    - condensation in Petri dishes is minimized;
    - heat-labile additives (e.g. antibiotics) are not degraded.

In practice, agar is usually poured at ~45–55 °C. Above this range, condensation is excessive; below it, agar begins to solidify and cannot be poured homogeneously.

4.6.3 Part III

4.6.3.1 I. Growth of a *Pseudomonas* Strain on a Defined Medium

1. Delimitation and interpretation of the growth phases (Figure 1)

A typical batch culture growth curve (semi-logarithmic plot) shows:

- **Lag phase (adaptation phase)**
    - Cell number remains almost constant.
    - Cells adapt to the defined medium, synthesize the necessary enzymes and transport systems, and repair any damage from transfer.
- **Exponential (log) phase**

    - Population increases at a **constant maximum specific growth rate (μmax)**.
    - The generation time (*G*) is constant.
    - Reflects the intrinsic growth capacity of the strain under the given conditions.
- **Stationary phase**
    - Net cell count becomes constant; cell division is balanced by cell death.
    - Usually due to nutrient depletion, accumulation of inhibitory metabolites, and/or unfavorable environmental changes (pH, oxygen, etc.).
- **Death (decline) phase** (if shown)
    - Viable cell number decreases because the death rate exceeds the division rate.

2. Parameters necessary and sufficient to characterize this growth

Key quantitative parameters are:

- **Maximum specific growth rate, μmax (h^{-1}):** From the slope of the exponential phase on a semi-log plot:

$$\mu_{\max} = \frac{\log_2 N_2 - \log_2 N_1}{t_2 - t_1}$$

where N_1 and N_2 are cell concentrations (CFU/mL) at times t_1 and t_2, respectively. From the provided graph, $\mu_{max} \approx 2\ h^{-1}$.

- **Generation time, G (doubling time)**

$$G = \frac{1}{\mu_{\max}}$$

For $\mu_{max} \approx 2\ h^{-1}$, $G \approx 0.5$ h=30 min.

- **Total growth (biomass increase), $N_s - N_0$**

Total growth = $N_s - N_0$

Using the example values (in $\log_2$):

- From $\log_2 N_0 = 21$:

$$\log_2 N_0 = 21 \ \Rightarrow \ N_0 = 2^{21} \approx 2.1 \times 10^6\ \mathrm{CFU/mL}$$

- From $\log_2$ N5=25.5:

$$\log_2 N_s = 25.5 \;\Rightarrow\; N_s = 2^{25.5} \approx 4.7 \times 10^7 \text{ CFU/mL}$$

Thus: Ns−N0 ≈ $4.7\times10^7 - 2.1\times10^6 \approx 4.5\times10^7$ CFU/mL

These three parameters (μmax, G, and Ns − N_0) are sufficient to characterize growth under the given conditions.

3. Factors influencing the first phase (lag phase)

Under the experimental conditions, the duration of the lag phase depends on:

- **Nature and richness of the medium**: The culture is transferred from a rich, complex medium (nutrient agar) to a poorer, chemically defined medium. This requires metabolic adaptation, leading to a longer lag phase.
- **Physiological state of the inoculum**: Cells from a 24-hour culture (often stationary phase) require time to resume active metabolism and division.
- **Metabolic adaptation and enzyme synthesis**: The bacterium must synthesize the enzymes and transporters needed to utilize glucose as carbon source and ammonium as nitrogen source, as well as to adjust central metabolism. This gene expression takes time and contributes to the lag phase.
- **Inoculum size and stress**: Small inocula or stressed cells may further increase lag duration.

4. Explanation of the stationary phase in relation to glucose consumption

The third phase of the curve (stationary phase) corresponds to:

- **Depletion of glucose (limiting carbon/energy source)**: As glucose is consumed, its concentration falls below the level needed to sustain exponential growth. Energy production and biosynthesis slow, halting net growth.
- **Accumulation of inhibitory metabolic products**: Metabolites (organic acids, other by-products) may reach toxic concentrations, further restricting growth even if some glucose remains.

- **Unfavorable physico-chemical changes**: Metabolism can modify pH, redox potential, and dissolved oxygen. For an aerobic genus such as *Pseudomonas*, oxygen limitation in a closed, poorly aerated system also contributes to growth arrest.
- **Space limitation**: At high biomass densities, physical space and nutrient diffusion become limiting.

The observed stationary phase thus results from the combined effects of glucose limitation, accumulation of inhibitory metabolites, and altered environmental conditions, as shown by the correlation between the growth curve and the glucose consumption curve.

4.6.3.2 Effect of Glucose Concentration on Growth

Comparison of total growth ($Ns - N_0$) and μ_{max} for 0.5, 4, and 32 g/L glucose

From the given data:

[Glucose] (g/L)	μ_{max} (h^{-1}) (approx.)	($Ns - N_0$) (CFU/mL)
0.5	≈ 0.25	$\approx (5-2)\times 10^6 = 3\times 10^6$
4	≈ 2	$\approx (25-2)\times 10^6 = 2.3\times 10^7$
32	≈ 2	$\approx (194-2)\times 10^6 \approx 1.92\times 10^8$

Interpretation

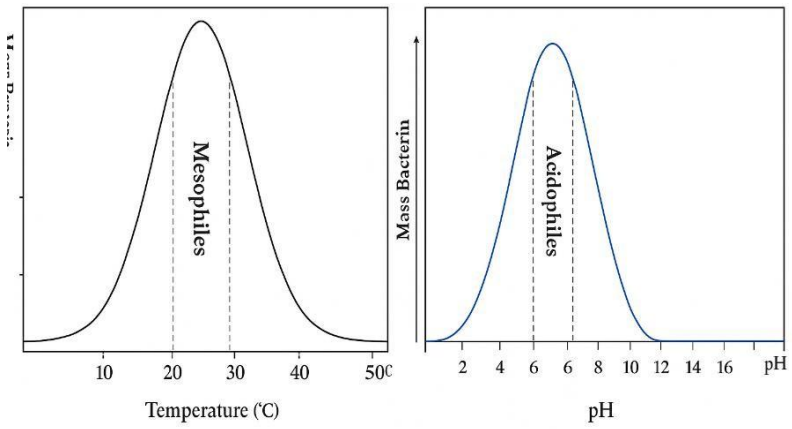
1. **Total growth ($Ns - N_0$)**: Total biomass produced increases with glucose concentration, being lowest at 0.5 g/L and highest at 32 g/L. Within this range, glucose acts as a yield-limiting substrate: more glucose allows more biomass to be formed.
2. **Maximum specific growth rate, μmax**: The relationship between μmax and glucose concentration shows two distinct regions:
    - Phase I (0 < [Glucose] < ~4 g/L): μmax increases and generation time G decreases as glucose concentration rises. In this region, glucose is growth-rate-limiting.
    - Phase II ([Glucose] ≥ ~4 g/L): μmax reaches a constant maximum and G remains minimal and constant, even when glucose concentration is further increased. Here, glucose is no longer limiting for the rate of growth, although it still affects

the yield (final biomass). Other factors (e.g. oxygen transfer, other nutrients, or inhibitory products) become rate-limiting.

The threshold (limiting) concentration is therefore around 4 g/L glucose, above which μmax no longer increases.

4.6.3.3 Effect of Temperature and pH: Type of Bacterial Strain

- **Temperature dependence**: The growth curve as a function of temperature shows that the strain grows well between 25 and 35 °C, with an optimum around 30 °C. This pattern is characteristic of a mesophilic bacterium (optimal growth typically between ~20 and 45 °C).
- **pH dependence**: The strain shows good growth in an acidic range, with significant growth between pH 4 and 6 and an optimum around pH 5. This indicates that the organism is acid-tolerant and can be described as moderately acidophilic, exhibiting very good growth at relatively low pH, where its enzymes and transport systems function optimally.



Conclusion: The soil *Pseudomonas* strain studied is a mesophilic bacterium with optimal growth at mildly acidic pH (moderately acidophilic) under the tested conditions.

5 Antimicrobial Agents

5.1 Introduction

Antimicrobial agents play a crucial role in the control of infectious diseases by inhibiting or eliminating the microorganisms responsible for them, including bacteria, viruses, fungi, and parasites.

The discovery and use of antimicrobial agents have revolutionized medicine, saving millions of lives and enabling numerous therapeutic and surgical advances. In the agri-food sector, antimicrobial agents are widely used at all stages of the food chain, from primary production (livestock, crops) to food processing, packaging, and distribution. Their main role is to control hygienic quality, limit the proliferation of pathogenic microorganisms, and extend the shelf life of food products.

Their use is strictly regulated by national and international authorities in order to guarantee food safety while limiting the emergence and spread of antimicrobial resistance. However, massive and sometimes inappropriate use in animal and plant production constitutes a major risk factor for the selection and dissemination of resistant microorganisms, thereby necessitating prudent use practices and strengthened surveillance systems.

5.2 Definition and General Concepts

An antimicrobial is a class of substances that either kill microorganisms (microbicidal effect) or inhibit their growth (microbiostatic effect). These agents act on microbes such as:

- bacteria (antibacterial activity),
- fungi (antifungal activity),
- viruses (antiviral activity), and
- parasites (antiparasitic activity).

Their use makes it possible to control microbial growth and thereby to prevent, treat, or modulate infections, as well as to direct microbial activity for experimental or industrial purposes.

5.3 Some Terminological Distinctions

5.3.1 Sterilization

Sterilization (from the Latin sterilis, sterile, infertile) is a process that ensures the destruction or removal of all forms of microbial life, including vegetative cells, spores, and viruses. A product is said to be sterile when no viable microorganisms are capable of growing on or in it under defined conditions. Sterilization may be achieved by physical (e.g. moist heat, dry heat, filtration, radiation) or chemical agents.

5.3.2 Disinfection

Disinfection is an operation that produces a temporary result and aims at the destruction, inhibition, or elimination of potentially pathogenic microorganisms and/or the inactivation of viruses on an inert surface or object. Disinfection reduces the microbial load to a level that is not harmful for health, but does not necessarily achieve sterility.

5.3.3 Decontamination

Decontamination is an operation that, with a temporary result, reduces or eliminates undesirable microorganisms on either inert or living supports, depending on the objectives set (e.g. safety for handling, reduction of environmental contamination). It may involve cleaning, disinfection, and/or sterilization.

5.3.4 Disinfectants

Disinfectants are chemical agents used on inanimate (non-living) objects and surfaces to destroy or inactivate microorganisms. Disinfectants do not necessarily achieve sterility, because viable spores may remain after treatment.

5.3.5 Antisepsis

Antisepsis (from the Greek anti, against, and sepsis, putrefaction) is an operation that, with a temporary result, reduces or eliminates microorganisms and/or inactivates viruses on living tissues (e.g. skin, mucous membranes), within the limits of their tolerance. The effect is restricted to the microorganisms and viruses present at the time of application and does not prevent subsequent contamination.

5.3.6 Antiseptic agents

Antiseptic agents are chemical substances used on living tissues. Their spectrum of activity is limited to the microorganisms against which they are effective. They must not cause unacceptable damage to host tissues and are therefore generally less toxic than disinfectants. Examples include preparations based on iodine (e.g. povidone-iodine), chlorhexidine, hydrogen peroxide, and potassium permanganate. Their action is usually rapid, but often not long-lasting over time, which justifies repeated applications in some situations.

5.3.7 Aseptic

Aseptic describes a medium or environment that is free of microorganisms.

5.3.8 Septic

Septic describes an environment or product that contains microorganisms. A product is classified as non-sterile or septic if viable microorganisms are present.

5.3.9 Asepsis

Asepsis refers to the set of measures implemented to prevent any exogenous introduction of microorganisms into a site, product, or process (e.g. aseptic surgical technique, aseptic handling in microbiology). This term is mainly used for procedures and manipulations.

5.4 Factors Influencing Antimicrobial Action

The destruction or elimination of microorganisms and the inhibition of their growth are complex processes. The effectiveness of an antimicrobial agent depends on several interrelated factors.

5.4.1 Type and characteristics of the microorganism

The efficacy of an antimicrobial agent is closely related to the nature of the target microorganism, as microorganisms differ markedly in their intrinsic susceptibility. For example:

- Bacterial endospores are more resistant to most antimicrobial agents than vegetative cells.
- Young, actively growing cells are generally more sensitive than older, stationary-phase cells.
- The size of the microbial population influences efficacy: a small initial inoculum is destroyed more rapidly than a very large population, under identical conditions.

5.4.2 Concentration of the antimicrobial agent

In many, but not all, situations, the antimicrobial effect increases with the concentration of the active substance. Within certain limits, higher concentrations often reduce the time required for microbial destruction. However, this relationship is not always linear, and excessively high concentrations may be impractical, toxic, or may not significantly improve activity.

5.4.3 Duration of exposure

The contact time between the antimicrobial agent and the microorganisms is a critical factor. Generally, the longer a microbial population is exposed to an effective germicidal agent, the greater the proportion of organisms that are killed or inactivated, provided the agent remains active over time.

5.4.4 Temperature

An increase in temperature usually enhances the activity of chemical antimicrobial agents and accelerates physical inactivation processes, because reaction rates and diffusion increase with temperature. However, temperature must remain compatible with the stability of the antimicrobial agent and, for antisepsis, with the tolerance of living tissues.

5.4.5 Local environment

The surrounding environment can either protect microorganisms or favor their destruction. Important factors include:

- Organic matter (e.g. blood, serum, pus, food residues), which can protect microorganisms by binding or inactivating disinfectants or by shielding microbes from heat;
- Biofilms, which provide a structured matrix that limits the penetration and efficacy of many antimicrobial agents;
- pH, which can modulate the activity of both microorganisms and antimicrobial agents. For many agents, acidic pH enhances their activity, and microorganisms may be more readily killed at moderately acidic pH combined with elevated temperature;
- Presence of salts, hardness of water, and other chemical components, which may inhibit or enhance antimicrobial activity depending on the agent.

5.5 The Action of Antimicrobial Agents

The main role of antimicrobial agents is to eliminate microorganisms, inhibit their growth, or destroy them. A specific suffix is used to describe the type of action exerted by an antimicrobial agent.

5.5.1 –cide (from the Latin *caedere*, to kill)

This suffix is reserved for substances that kill microorganisms (lethal treatment). A germicide destroys microorganisms but does not necessarily inactivate all bacterial endospores.

1. **Bactericidal**: kills bacteria, with the possible exception of endospores.
2. **Sporicidal**: kills bacterial spores (endospores).
3. **Fungicidal**: kills fungi, including their spores, which, although often more resistant than vegetative fungal cells, do not have the same resistance level as bacterial endospores.
4. **Virucidal (viricidal)**: inactivates or destroys viruses.

5.5.2 –static (from the Greek statikos, causing to stand, stopping)

This suffix is reserved for substances that do not kill microorganisms but inhibit their growth or multiplication (stabilizing treatment). Their effect is generally reversible once the agent is removed and favorable conditions are restored.

- **Bacteriostatic**: temporarily inhibits bacterial growth and division.
- **Fungistatic**: temporarily inhibits fungal (including mycelial) growth.
- **Virustatic**: temporarily inhibits viral replication.

5.6 Classification of Antimicrobial Agents

The growth and survival of microorganisms are strongly influenced by environmental (physicochemical) conditions such as pH (acidity or alkalinity), temperature, the presence or absence of gases such as O_2 and CO_2, and water availability, commonly expressed as water activity (a_v or $a\square$, a_w: activity of water).

To achieve the reduction or destruction of microorganisms, several categories of antimicrobial means can be applied:

1. **Physical agents:**
    - Heat (e.g. moist heat, dry heat),
    - Radiation (e.g. ultraviolet, ionizing radiation).
2. **Mechanical methods:**
    - *Filtration* (retention of microorganisms by membrane filters),
    - *Centrifugation* (concentration or separation of cells, often as a preparatory step).
3. **Chemical agents:**
    - Disinfectants, antiseptics, preservatives, and other biocidal or biostatic compounds used on inert surfaces, in products, or on living tissues.
4. **Chemotherapeutic agents:**
    - Antimicrobial drugs (e.g. antibiotics, antifungals, antivirals, antiparasitics) used in vivo to treat infections in humans and animals.

The appropriate choice and rational use of these different agents depend on the type of microorganism, the treated material or matrix, the intended purpose (sterilization, disinfection, preservation, therapy), and safety and regulatory constraints.

5.6.1 Physical Agents

5.6.1.1 Heat

Temperature is a major physical factor used in food preservation and in the control of microorganisms. Low temperatures (refrigeration and freezing) inhibit microbial growth and metabolic activity, thereby slowing spoilage and the proliferation of pathogens. Elevated temperatures, by contrast, can inactivate microorganisms and are used for pasteurization and sterilization.

The efficacy of heat treatment depends on the characteristics of the medium (e.g., composition, pH, water activity), the physiological state of the cells, and the size of the microbial population. Vegetative bacterial cells are generally highly susceptible to heat; many are inactivated by exposure to 50–60 °C for approximately 30 min. In contrast, bacterial endospores are extremely resistant and require more severe conditions for inactivation.

In practice, heat sterilization is achieved either by moist heat or by dry heat.

5.6.1.2 Moist Heat

Moist heat sterilization is typically performed at temperatures above 100 °C to ensure the destruction of bacterial endospores. This generally requires the use of saturated steam under pressure.

5.6.1.2.1 Appertization (Canning)

Appertization is a reliable and effective method developed by Nicolas Appert in the early nineteenth century and later given a microbiological explanation by Pasteur. It consists of heating foods that have been hermetically sealed in containers (e.g., glass bottles, metal cans) to temperatures sufficient to destroy vegetative cells and most spores of pathogenic and spoilage microorganisms. This process achieves commercial sterilization, meaning that microorganisms capable of growing under normal storage conditions are eliminated. Appertization is widely used in the canning industry.

5.6.1.2.2 Autoclaving

Steam sterilization in an autoclave is the standard method for achieving true sterilization in laboratory and clinical settings. The autoclave is a robust, hermetically sealed metal chamber in which saturated steam under pressure is generated. A typical cycle uses saturated steam at 121 °C under approximately 103 kPa overpressure (about 2 atm absolute) for 15–20 min. Under these conditions, saturated steam efficiently transfers heat and denatures proteins, leading to the destruction of all vegetative cells and endospores, provided the load is properly prepared and air is effectively removed.

However, some heat-sensitive substances cannot withstand these conditions without degradation. In such cases, alternative methods are used, including fractional sterilization.

5.6.1.2.3 Tyndallization (Fractional Sterilization)

Tyndallization is a method designed for materials that are sensitive to prolonged exposure to high temperatures. The process typically involves heating the material at 100 °C (usually in flowing steam) for about 20–30 min on three successive days, with incubation at a suitable growth temperature between heating cycles. During the incubation periods, surviving endospores germinate into vegetative cells, which are then killed by the subsequent heating step. Although

less commonly used today, tyndallization allows for the sterilization of certain heat-labile media and solutions that cannot tolerate autoclaving.

5.6.1.2.4 Pasteurization

Pasteurization, developed by Louis Pasteur in the nineteenth century, is a heat treatment conducted at temperatures below the boiling point of water. It is primarily designed to reduce the microbial load, particularly pathogenic and spoilage microorganisms, without causing major alterations in the organoleptic properties (colour, odour, flavour) or the nutritional value of foods. Pasteurization targets non–spore-forming pathogens such as Salmonella spp., Listeria monocytogenes, and pathogenic Escherichia coli, as well as many spoilage bacteria.

Pasteurization does not achieve sterilization, because bacterial endospores generally survive the treatment. It is therefore used when the goal is to extend shelf-life and improve safety for a limited period under appropriate storage conditions (e.g., refrigeration).

Common pasteurization regimens include:

- **Low-Temperature, Long-Time (LTLT) pasteurization**: The product is typically heated to about 63 °C for 30 min and then cooled rapidly.
- **High-Temperature, Short-Time (HTST) pasteurization**: The product (e.g., milk) is heated to approximately 72 °C for at least 15 s and then rapidly cooled, often to ≤ 10 °C. This regimen is widely used in the dairy industry.
- **Ultra-High-Temperature (UHT) treatment**: In UHT processing, products such as milk or certain fruit juices are heated to about 135–150 °C for a few seconds and then rapidly cooled and aseptically packaged. UHT treatment can achieve commercial sterility, permitting ambient storage for extended periods. Although sometimes referred to as "UHT pasteurization," it is more accurately considered a form of ultra-high-temperature processing rather than classical pasteurization.

5.6.1.3 Dry Heat

Dry heat sterilization is achieved using hot-air ovens (e.g., Pasteur ovens) heated by electricity or gas, usually with forced air circulation to ensure uniform temperature distribution. Sterilization is typically obtained by maintaining:

- 160 °C for 2 h, or
- 170–180 °C for 1 h,

after the set temperature has been reached throughout the load.

Dry heat sterilizes primarily by oxidative processes and by denaturing and dehydrating cellular components. It is used to sterilize glassware, metal instruments, and other heat-resistant, non-aqueous materials (e.g., powders, oils) that cannot be safely or effectively sterilized by moist heat. Steam sterilization is generally preferred for most surgical equipment, but dry heat remains important for items that are moisture-sensitive or that could be corroded by steam.

5.6.1.4 Radiation

Radiation can be used to reduce or eliminate microorganisms through ionizing or non-ionizing mechanisms, depending on the wavelength and energy of the radiation.

5.6.1.4.1 Ionizing Radiation

Ionizing radiation includes gamma rays, X-rays, and high-energy electron beams. These radiations have short wavelengths and high energy, providing significant penetrating power. Ionizing radiation acts directly on nucleic acids and other cellular components and indirectly by radiolysis of water, generating highly reactive species such as hydroxyl radicals. These radicals induce single- and double-strand breaks in DNA and cause extensive oxidative damage to proteins and lipids, resulting in cell death or loss of reproductive capacity.

Gamma radiation from a cobalt-60 source is widely used for the terminal sterilization of heat-sensitive medical and laboratory materials, including certain antibiotics, surgical sutures, and single-use plastic items such as Petri dishes and syringes. In the food industry, ionizing radiation is employed at carefully controlled doses to reduce microbial loads, inactivate pathogens, and extend shelf-life in products such as meat, poultry, spices, and other foods; this process is often referred to as food irradiation or “cold pasteurization.”

5.6.1.4.2 Non-Ionizing Radiation

Non-ionizing radiation used for microbial control is primarily ultraviolet (UV) radiation, particularly in the UV-C range with wavelengths around 254–260 nm, which corresponds closely to the absorption maximum of DNA. UV radiation induces the formation of abnormal covalent

bonds between adjacent pyrimidine bases (especially thymine) in DNA, resulting in thymine dimers. These lesions disturb the normal base-pairing and interfere with DNA replication and transcription, ultimately leading to mutations or cell death if not repaired.

Solar UV radiation is partially filtered by the atmosphere and is less effective than artificial germicidal UV in microbial control. Some microorganisms synthesize protective pigments or possess efficient DNA repair systems (e.g., photoreactivation, nucleotide excision repair) that mitigate UV-induced damage.

Due to its poor penetrating power and the shadowing effect of particles and surfaces, UV radiation is best suited for the disinfection of air and exposed surfaces rather than for bulk sterilization of materials. Germicidal UV lamps, often installed on ceilings, within air-handling systems, or inside biological safety cabinets, can significantly reduce airborne and surface contamination in laboratories, hospital rooms, and other controlled environments. However, this method is more accurately described as disinfection rather than true sterilization, because complete destruction of all microbial forms, including protected or shielded spores, cannot be guaranteed.

5.6.2 Mechanical Agents

Mechanical processes used to remove microorganisms from a liquid medium in which they are suspended include filtration and centrifugation.

5.6.2.1 Filtration

Filtration, often referred to as cold sterilization, is a highly effective method for reducing the microbial load in solutions, particularly those containing heat-labile substances (e.g., certain vitamins, antibiotics, or enzyme preparations). Its principle is based on the physical removal of microorganisms rather than their inactivation by heat or chemicals.

Two major types of filters are used:

1 **Depth (thick) filters:** These filters are composed of fibrous or granular materials (e.g., diatomaceous earth, unglazed porcelain, or glass fibers) consolidated into a thick matrix containing a network of tortuous channels of small diameter. Microorganisms are retained by a combination of mechanical entrapment, adsorption to the filter material,

and, to a lesser extent, sieving. Depth filters are particularly suitable for clarifying turbid solutions and retaining larger particles and microbial aggregates.

2 **Membrane (surface) filters:** Membrane filters are thin (usually < 0.1 mm) discs made of cellulose acetate, cellulose nitrate, or various synthetic polymers. They contain precisely defined pores that act primarily by sieving. The most commonly used membranes for microbiological sterilization have pores of approximately 0.2 µm in diameter, which retain bacteria and many larger microorganisms while allowing the passage of most solutes. Membrane filtration is widely used for the sterilization of heat-sensitive aqueous solutions and for microbiological quality control (e.g., enumeration of bacteria in water).

5.6.2.2 Centrifugation

Centrifugation is used to separate suspended particles based on their size, shape, and density by subjecting them to high centrifugal forces. Microbial cells can be concentrated into a pellet at the bottom of the tube, while the supernatant becomes depleted in microorganisms. Although centrifugation can substantially reduce the microbial load or concentrate organisms for further analysis, it does not result in complete elimination of microorganisms and therefore cannot be considered a sterilization method.

5.6.3 Chemical Agents

Chemical antimicrobial agents are widely used as disinfectants and antiseptics. However, not all chemical antimicrobials are suitable for direct application to living tissues. Some substances, although highly active against microorganisms, are too toxic or irritating for use on skin or mucous membranes and are thus restricted to inanimate objects or surfaces. Others may have excellent penetrating power or solubility but are unstable or rapidly inactivated by organic matter (e.g., blood, pus, or feces).

In contrast to antibiotics, which typically have specific targets and selective toxicity, many disinfectants and antiseptics act rapidly and non-specifically on multiple cellular components.

5.6.3.1 Modes of Action

Chemical agents act on microorganisms through a variety of mechanisms, often affecting several targets simultaneously:

5.6.3.1.1 Oxidation and Denaturation of Proteins

- Oxidizing agents such as hydrogen peroxide ($H_2 O_2$) generate reactive oxygen species that oxidize free sulfhydryl (–SH) groups in enzymes and structural proteins, irreversibly altering their function.
- Heavy metal salts can bind to sulfhydryl groups and other functional groups in proteins, leading to their inactivation or precipitation.
- Alcohols (e.g., ethanol, isopropanol) cause protein denaturation and coagulation, in a manner analogous to heat, and also disrupt lipid-containing structures.

5.6.3.1.2 Disruption of the Cytoplasmic Membrane

Lipid-soluble agents such as phenolic compounds, soaps, and especially cationic detergents (quaternary ammonium compounds) interact with membrane lipids and proteins, leading to increased permeability or complete disruption of the plasma membrane. Cellular contents may leak out, resulting in cell lysis and death.

5.6.3.1.3 Interference with Metabolism and Genetic Material

- Metabolic inhibitors such as cyanides and fluorides interfere with key enzymes of the respiratory chain, inhibiting energy production.
- Certain basic dyes (e.g., methylene blue, gentian violet) bind to nucleic acids and may interfere with replication or transcription.
- Some agents are mutagenic (e.g., acridine derivatives) or act as chelators (e.g., some quinoline derivatives), disrupting essential metal-dependent enzymes or DNA function.

5.6.3.2 Classes of Antimicrobial Chemical Agents

5.6.3.2.1 Phenols and Phenolic Compounds

Phenol and its derivatives are classic disinfectants. They act mainly by denaturing proteins and disrupting cell membranes, leading to leakage of intracellular constituents. Phenolic disinfectants retain activity in the presence of organic matter and are used for environmental and surface disinfection. In laboratories and health-care settings, cresols, xylenols, and orthophenylphenol are commonly employed phenolic compounds.

5.6.3.2.2 V.3.2.2. Alcohols

Alcohols are widely used as both disinfectants and antiseptics. They are bactericidal and fungicidal but generally not sporicidal. Ethanol and isopropanol are the most commonly used alcohols in clinical and laboratory practice, typically at concentrations around 60–90%. Their antimicrobial activity is due primarily to protein denaturation and disruption of membrane lipids. Alcohols act rapidly and evaporate quickly, which is advantageous for skin antisepsis but limits residual activity.

5.6.3.2.3 Heavy Metals

Heavy metal ions such as mercury, silver, arsenic, zinc, and copper have long been used as germicides. They bind to functional groups of proteins, particularly sulfhydryl groups, leading to enzyme inactivation and protein precipitation.

Because of their toxicity and the potential for environmental accumulation, the use of many heavy metal compounds is now restricted. In addition, the presence of plasmid-encoded resistance to heavy metals in some bacteria reduces their effectiveness. Silver compounds (e.g., silver nitrate, silver sulfadiazine) are still used in certain topical applications (e.g., prevention of infection in burns).

5.6.3.2.4 Oxidizing Agents

Oxidizing agents act mainly by oxidizing essential cellular components, including sulfhydryl groups of enzymes, nucleic acids, and membrane lipids, causing irreversible damage.

- **Hydrogen peroxide (H_2O_2):** Hydrogen peroxide is used as a disinfectant and antiseptic. It decomposes to water and oxygen, and its antimicrobial activity is due to the formation of reactive oxygen species. Its rapid breakdown, especially in the presence of catalase-producing organisms or organic matter, limits its persistence. Stabilized or higher-concentration formulations are employed for more intensive disinfection and sterilization procedures.
- **Chlorine and Chlorine Derivatives:** Chlorine, applied as gas or in chemical combinations (e.g., hypochlorites and chloramines), is the disinfectant of choice for potable water and swimming pools and is widely used in the food industry and health-care facilities. In aqueous solution, chlorine forms hypochlorous acid (HClO), a strong

oxidizing agent that reacts with cellular constituents, including proteins, nucleic acids, and lipids. Sodium hypochlorite (bleach, NaOCl) is an inexpensive, effective, and easy-to-use disinfectant that rapidly kills vegetative bacteria and fungi on floors, equipment, and utensils. Spore-forming bacteria, however, require higher concentrations and longer contact times for inactivation.

- **Iodine and Iodophors:** Iodine is a potent oxidizing agent with broad-spectrum antimicrobial activity, including activity against many spores at sufficient concentrations and exposure times. Poorly soluble in water, iodine is traditionally used in alcoholic solution (tincture of iodine) or in combination with iodide salts. It is applied for skin disinfection (e.g., preoperative antisepsis) and the treatment of superficial wounds, but concentrated solutions can cause irritation or burns. Iodophors (e.g., povidone-iodine, known by trade names such as Betadine) are complexes of iodine with organic carriers that slowly release free iodine in aqueous solution. They are less irritating, non-staining, and widely used as antiseptics and disinfectants in medical and laboratory settings.

5.6.3.2.5 Detergents and Soaps

Detergents are organic molecules that act as wetting agents and emulsifiers. They reduce surface tension, facilitate the removal of dirt and organic material, and help solubilize lipids.

- **Soaps** are sodium or potassium salts of fatty acids. Their antimicrobial activity is modest and varies with microbial species, but they are highly effective as cleansing agents because they emulsify oils and suspend particulate matter. Microorganisms become trapped in the foam and are removed during rinsing.
- **Synthetic detergents**, especially cationic detergents (quaternary ammonium compounds), possess significant antimicrobial activity by disrupting cell membranes. They are widely used for disinfection of non-critical surfaces and equipment.

5.6.3.2.6 Sterilizing Gases

Sterilizing gases are used to treat products that are heat- or moisture-sensitive, as well as to decontaminate enclosed spaces. They are particularly useful for sterilizing plastics and other materials that might be damaged by high temperatures or radiation.

Ethylene oxide (EtO): Ethylene oxide is a highly effective gaseous sterilant that is both germicidal and sporicidal. It acts by alkylating functional groups in proteins, nucleic acids, and other cellular macromolecules, thereby preventing normal cellular function and replication. Ethylene oxide readily penetrates packaging materials, including many plastics, making it suitable for sterilizing disposable medical and laboratory items such as Petri dishes, syringes, catheters, and tubing. Because of its flammability, toxicity, and potential carcinogenicity, its use requires strict control of exposure and careful aeration of sterilized materials.

Remarks

- Hydrogen peroxide has also been used in vapor form (vaporized or hydrogen peroxide gas plasma systems) to decontaminate biological safety cabinets, rooms, and sensitive equipment.
- Essential oils of plant origin can exhibit antiseptic and disinfectant properties, largely attributable to their phenolic, terpenoid, and related components (including alcohols and aldehydes). Commonly used examples include eucalyptus oil and clove oil. These essential oils may also be employed as natural food preservatives, contributing to the preservation of products such as fruit and meat.

5.6.3.2.7 Dyes

Certain dyes possess antimicrobial activity and are used as topical antiseptics or in specific therapeutic or diagnostic applications. Examples include malachite green and brilliant green for superficial infections, methyl violet as a urinary antiseptic, and gentian violet as a skin and mucosal disinfectant.

In microbiology, dyes are added to culture media for their selective properties; some inhibit particular groups of microorganisms while allowing others to grow. Many are more active against Gram-positive bacteria, thereby relatively favoring the growth of Gram-negative organisms in mixed cultures.

5.6.3.2.8 Food Preservatives

An effective antimicrobial food additive should inhibit bacteria and fungi while preserving the organoleptic properties (taste, smell, texture, appearance) and nutritional quality of the product.

Since antiquity, various physical and chemical methods have been used for food preservation, including salting, smoking, drying, and the use of organic acids such as lactic acid and acetic acid. Modern food preservation also employs a variety of chemical preservatives, for example:

- sulphurous acid and sulphites (e.g., sodium bisulphite),
- benzoic acid and its salts,
- salicylic acid and related compounds,
- selected essential oils and their components.

These agents act by lowering pH, interfering with microbial metabolism, or disrupting cell membranes, thereby inhibiting spoilage organisms and, in some cases, foodborne pathogens.

5.6.4 Antimicrobial Chemotherapeutic Agents

Chemotherapy, in the context of infectious diseases, refers to the use of chemical agents to treat infections. This approach truly emerged in 1909 when Paul Ehrlich formulated the principle of selective toxicity: to be useful in the treatment of infectious diseases, a substance must be deleterious to the parasitic microorganism while being relatively harmless to the host's cells.

5.6.4.1 Historical Background

The discovery and development of antibiotics is one of the major achievements of twentieth-century medicine. Until the early twentieth century, infectious diseases were the leading cause of human mortality.

The modern antibiotic era began in 1929, when Alexander Fleming, an English physician, made a key observation: on a Petri dish seeded with *Staphylococcus aureus*, contamination by a mold of the genus Penicillium resulted in a clear zone of inhibition around the fungal colonies. He deduced that the mold secreted a substance capable of inhibiting bacterial growth. Fleming cultivated Penicillium at scale and showed that the filtrates contained an active principle with bactericidal activity and low toxicity for animal cells. He named this substance penicillin.

Fleming's discovery might have remained a laboratory curiosity had it not been for the work of Howard Florey and Ernst Chain, who in 1939 succeeded in extracting and purifying penicillin in sufficient quantities for therapeutic trials. The clinical results were spectacular, particularly in the

treatment of severe bacterial infections. The Second World War accelerated and industrialized the production of penicillin.

From 1939 and over the following two decades, hundreds of antibiotics were isolated, especially from actinomycetes of the genus Streptomyces. During this period, many of the major antibiotic classes still used today were discovered, including β-lactams, aminoglycosides (e.g., streptomycin), tetracyclines, and chloramphenicol. From the mid-1950s onwards, semi-synthetic derivatives were introduced, further expanding the therapeutic arsenal.

5.6.4.2 Definition

Classically, antibiotics are defined as molecules produced by microorganisms (bacteria or fungi) that, at low concentrations, can inhibit the growth (bacteriostatic effect) or kill (bactericidal effect) other bacteria, with minimal toxicity to the host's eukaryotic cells.

By extension, the term now also encompasses:

- **Semi-synthetic antibiotics**, which are chemical derivatives of natural antibiotic molecules (e.g., methicillin derived from penicillin), and
- **Synthetic agents**, which are chemically synthesized compounds with antibiotic activity (e.g., sulfonamides, fluoroquinolones).

Since their discovery, antibiotics have radically changed the prognosis of bacterial infections and have made it possible to cure diseases that were previously almost invariably fatal, such as bacterial endocarditis, syphilis, and tuberculous meningitis.

5.6.4.3 Classification

Because of their diversity, antibiotics can be classified according to several criteria.

5.6.4.3.1 According to Their Antimicrobial Effect

- **Static agents:** These inhibit microbial growth and multiplication without immediately killing the organisms. Examples include bacteriostatic and fungistatic agents.
- **Cidal agents:** These kill microorganisms. They include bactericidal, fungicidal, algicidal, and virucidal agents. (Strictly speaking, the term *antibiotic* is reserved for agents active against bacteria, while agents active against fungi or viruses are more precisely termed antifungals and antivirals.)

5.6.4.3.2 According to Their Origin

1 **Natural antibiotics:** Produced by microorganisms such as:
   - Fungi: penicillins, cephalosporins;
   - Bacteria: aminoglycosides (e.g., streptomycin), polypeptide antibiotics, chloramphenicol.

2 **Synthetic antibiotics:** Entirely obtained by chemical synthesis, e.g. sulfonamides, nalidixic acid and related quinolones.

3 **Semi-synthetic antibiotics:** Derived from a natural core structure to which chemical substituents are added, modifying the spectrum, stability, or pharmacokinetic properties (e.g., methicillin derived from penicillin).

5.6.4.3.3 According to Their Spectrum of Activity

The spectrum of activity of an antibiotic is the range of bacterial species that can be inhibited or killed at clinically achievable concentrations.

- **Broad-spectrum antibiotics:** Active against a wide range of Gram-positive and Gram-negative bacteria.
- **Intermediate or limited-spectrum antibiotics:** Active mainly against Gram-positive bacteria and a subset of Gram-negative organisms.
- **Narrow-spectrum antibiotics:** Active only against a restricted group of bacteria, for example mainly Gram-positive cocci or specific Gram-negative bacilli.

5.6.4.3.4 According to Their Site of Action

Antibiotics act on well-defined bacterial targets (Figure 34). Some interfere with cell wall synthesis, others with nucleic acid or protein synthesis, and still others with essential metabolic pathways.

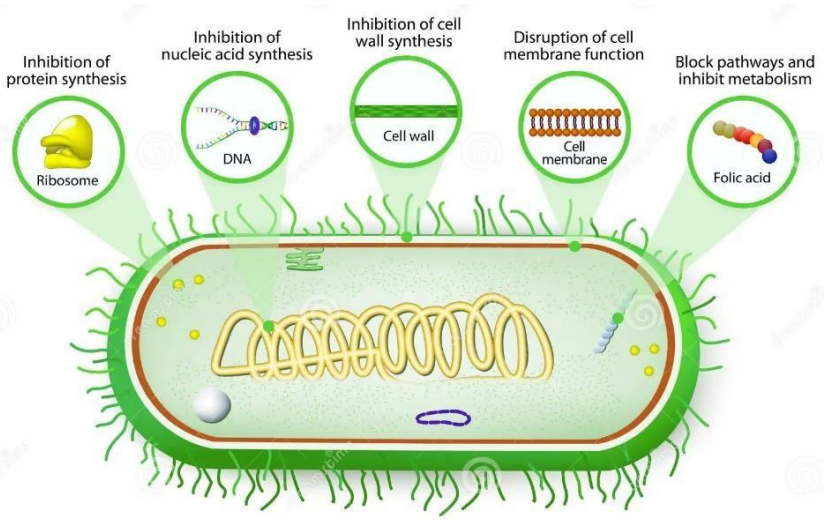


Figure 34. Mechanisms of action of antibiotics

1 **Antibiotics acting on the bacterial cell wall:** These inhibit peptidoglycan synthesis, which is essential for cell wall integrity:

- β-lactams: penicillins, cephalosporins, carbapenems, monobactams;
- Glycopeptides: e.g., vancomycin, teicoplanin;
- Fosfomycin and other agents interfering with peptidoglycan precursors.

2 **Antibiotics acting on protein synthesis:** These bind to bacterial ribosomal subunits (30S or 50S) and inhibit the translation of mRNA into protein, often blocking elongation of the polypeptide chain:

- Aminoglycosides (gentamicin, amikacin, tobramycin, neomycin, etc.);
- Macrolides (erythromycin, spiramycin, clarithromycin, azithromycin), lincosamides (clindamycin), and related agents;
- Tetracyclines (tetracycline, doxycycline, minocycline);
- Phenicols (chloramphenicol, thiamphenicol).

3 **Antibiotics acting on nucleic acid synthesis:** These inhibit the synthesis of DNA or RNA or interfere with their structure:

- Fluoroquinolones (e.g., ciprofloxacin, levofloxacin), which inhibit DNA gyrase and topoisomerase IV;
- Rifamycins (e.g., rifampicin), which inhibit DNA-dependent RNA polymerase;

- Nitroimidazoles (e.g., metronidazole), which cause DNA strand breakage under anaerobic conditions.

4 **Antibiotics acting on essential metabolic pathways:** These act as antimetabolites that compete with natural substrates, particularly in the folate synthesis pathway, which is crucial for nucleic acid synthesis:

- Sulfonamides, which inhibit dihydropteroate synthase;
- Trimethoprim, which inhibits dihydrofolate reductase;
- Combined use (e.g., cotrimoxazole) exerts a sequential blockade of folate synthesis.

5.6.4.3.5 According to Their Chemical Structure

Antibiotics that share a common basic chemical structure are grouped into the same family. Antibiotics within the same family generally have similar mechanisms of action and overlapping spectra of activity.

Chemical classification is therefore widely used and is based on the core chemical structure of each antibiotic. Table 6 summarizes the main families, their principal mode of action, major adverse effects, and predominant activity against Gram-positive and/or Gram-negative bacteria.

Table 6. Main families of antibiotics, principal modes of action, key adverse effects, and main spectrum of activity

Family	Predominant mode of action	Major adverse effects (examples)	Main activity on bacteria*
β-Lactams	Bactericidal; inhibit cell wall (peptidoglycan) synthesis	Allergic reactions, gastrointestinal disturbances, occasional nephrotoxicity	Many Gram+ and Gram− (spectrum depends on subclass)
Aminoglycosides	Bactericidal; inhibit protein synthesis (30S)	Ototoxicity, nephrotoxicity	Mainly aerobic Gram−; some Gram+ with synergy
Macrolides	Mainly bacteriostatic; inhibit protein synthesis (50S)	Gastrointestinal intolerance, cholestatic hepatitis, rare allergy	Mainly Gram+ cocci, some Gram− and atypical bacteria
Lincosamides	Mainly bacteriostatic; inhibit protein synthesis (50S)	Gastrointestinal disturbances, risk of	Mainly Gram+ cocci and

		Clostridioides difficile colitis, hepatotoxicity	anaerobes
Streptogramins (Synergistins)	Mainly bacteriostatic/bactericidal (combination); inhibit protein synthesis (50S)	Arthralgia, myalgia, hepatic enzyme elevation, infusion-related reactions	Gram+ cocci (including some resistant strains)
Tetracyclines	Mainly bacteriostatic; inhibit protein synthesis (30S)	Gastrointestinal disturbances, photosensitivity, dental and bone effects, hepatotoxicity	Broad spectrum: Gram+ and Gram−, atypical bacteria
Fluoroquinolones	Bactericidal; inhibit DNA gyrase and topoisomerase IV	Gastrointestinal symptoms, tendinopathy, QT prolongation, CNS effects	Many Gram− and some Gram+
Sulfonamides	Bacteriostatic; inhibit folate synthesis (antimetabolites)	Hypersensitivity reactions, hematologic toxicity, crystalluria	Broad, but variable; often used in combination (cotrimoxazole)
Glycopeptides	Bactericidal; inhibit cell wall synthesis	Infusion-related reactions, nephrotoxicity, ototoxicity	Mainly Gram+ (e.g., MRSA, enterococci)
Phenicols (Chloramphenicol)	Mainly bacteriostatic; inhibit protein synthesis (50S)	Bone marrow suppression, rare aplastic anemia, gray baby syndrome	Broad spectrum (Gram+ and Gram−), limited by toxicity
Imidazoles	Bactericidal; cause DNA strand breakage in anaerobes	Gastrointestinal disturbances, metallic taste, disulfiram-like reaction with alcohol	Anaerobic bacteria and certain protozoa
Polymyxins	Bactericidal; disrupt cytoplasmic membranes	Nephrotoxicity, neurotoxicity	Mainly Gram− (e.g., *Pseudomonas*, *Acinetobacter*)
Others (e.g., oxazolidinones, lipopeptides)	Mechanism depends on family (protein synthesis, membrane depolarization, etc.)	Various, depending on the agent	Often Gram+ pathogens (including resistant strains)

* Actual spectrum depends on individual agents within each family.

5.6.4.4 Mode of Action

The usual targets of antibiotics are structures or functions that are essential and conserved in bacteria but absent or sufficiently different in the host. The bacterial cell wall, cytoplasmic membrane, DNA, and ribosomes are major sites of action (Figure 35).

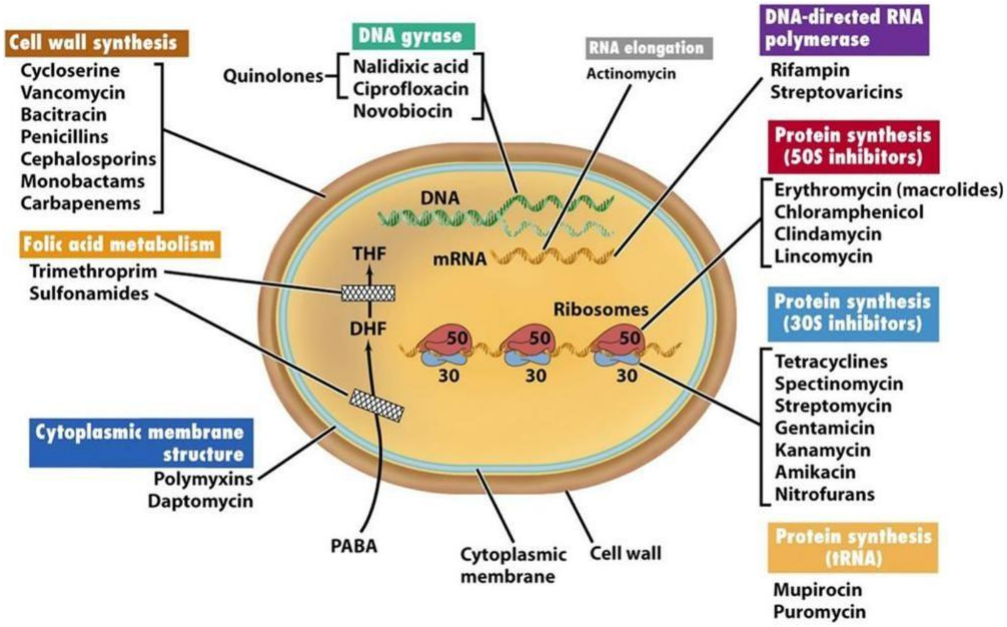


Figure 35. Schematic representation of antibiotic sites of action

5.6.4.4.1 Action of Antibiotics on the Bacterial Cell Wall

- **Binding to enzymes involved in peptidoglycan synthesis:** β-lactams (e.g., penicillins, cephalosporins) bind to penicillin-binding proteins (PBPs), inhibiting the transpeptidation and transglycosylation reactions required for cell wall assembly, leading to cell lysis.
- **Blocking peptidoglycan synthesis by binding to precursors:** Glycopeptides (e.g., vancomycin) bind to the D-Ala–D-Ala terminus of peptidoglycan precursors, preventing their incorporation into the cell wall.
- **Inhibition of intracytoplasmic synthesis of peptidoglycan precursors:** Fosfomycin inhibits an early enzymatic step in the cytoplasmic synthesis of peptidoglycan precursors, thereby impairing cell wall formation.

5.6.4.4.2 Damage to the Cytoplasmic Membrane

Some antibiotics act primarily on the bacterial cytoplasmic membrane, altering its permeability and leading to leakage of cellular contents and cell death.

Example: Polymyxins, which interact with the lipopolysaccharides and phospholipids of the outer and cytoplasmic membranes of Gram-negative bacteria, are active almost exclusively against Gram-negative bacilli.

5.6.4.4.3 Action on Nucleic Acids and Folate Metabolism

1. **Inhibition of DNA topoisomerases:** Quinolones and fluoroquinolones inhibit DNA gyrase and/or topoisomerase IV, enzymes essential for DNA replication, transcription, and repair.
2. **Inhibition of folate-dependent nucleotide synthesis:** Sulfonamides and trimethoprim interfere with the synthesis of tetrahydrofolate, a cofactor required for purine and thymidine synthesis:
    - Sulfonamides inhibit dihydropteroate synthase;
    - Trimethoprim inhibits dihydrofolate reductase.
3. **Direct damage to DNA:** Nitroimidazoles (commonly referred to here as imidazoles, e.g., metronidazole) undergo reduction in anaerobic conditions to generate reactive intermediates that cause fragmentation of the DNA double helix.

5.6.4.5 Resistance Issues

Antimicrobial resistance (AMR) arises when microorganisms develop mechanisms that reduce or abolish the efficacy of drugs used to treat infections. A bacterium is considered resistant to an antibiotic when it can grow at concentrations of the antibiotic that exceed the levels usually achievable and safe in human therapy.

The inappropriate and excessive use of antibiotics in human and veterinary medicine, as well as in agriculture, has contributed to the emergence and spread of resistant strains. Two major forms of resistance are distinguished:

1. Intrinsic (natural) resistance
2. Acquired resistance.

5.6.4.5.1 Natural (Intrinsic) Resistance

Intrinsic resistance is an inherent property of a bacterial species and is encoded by chromosomal genes. It is shared by all or nearly all strains of that species. Following cell division, the resistance determinants are transmitted vertically to daughter cells.

Examples include:

- Many Gram-positive bacteria are intrinsically resistant to colistin and to certain quinolones such as nalidixic acid.
- Anaerobic bacteria are intrinsically resistant to vancomycin's mechanism of action on the outer membrane, but more classically, most Gram-negative bacilli and anaerobes are intrinsically resistant to vancomycin because the drug cannot cross the outer membrane or is ineffective in that environment.
- *Enterobacteriaceae* of the genus *Klebsiella* are intrinsically resistant to aminopenicillins such as amoxicillin and to certain carboxy- and ureidopenicillins (e.g., ticarcillin, piperacillin) because of the production of chromosomal β-lactamases.
- *Enterococcus faecalis* is intrinsically resistant to cephalosporins and to lincosamides (e.g., lincomycin, clindamycin).

Table 7. Examples of intrinsic (natural) resistance

Bacterial group / genus	**Examples of intrinsic resistance**
Gram-positive bacteria	Colistin, nalidixic acid
Gram-negative anaerobes and many Gram-negative bacilli	Vancomycin
Klebsiella spp.	Aminopenicillins (e.g., amoxicillin), ticarcillin, piperacillin
Enterococcus faecalis	Cephalosporins, lincosamides (lincomycin, clindamycin)

Intrinsic resistance must be taken into account when choosing empirical antimicrobial therapy.

5.6.4.5.2 Acquired Resistance

In acquired resistance, bacteria that were originally susceptible become resistant as a result of genetic change. The relevant genetic information may reside on the bacterial chromosome and/or on mobile genetic elements such as plasmids, transposons, or integrons. Transmission of acquired resistance can occur:

1. **Vertically**, from a resistant cell to its progeny, or
2. **Horizontally**, between bacteria of the same or different species.

The three main mechanisms of horizontal gene transfer are:

1. **Transformation** (uptake of free exogenous DNA),
2. **Transduction** (transfer mediated by bacteriophages), and
3. **Conjugation** (direct transfer of DNA, often plasmid-borne, between donor and recipient cells).

5.6.4.5.2.1 Genetic Basis

Acquired resistance may arise in two principal ways:

1. **Chromosomal mutation:** Spontaneous mutations in structural or regulatory genes can alter the target of the antibiotic or its expression. These mutations are transmitted vertically to daughter cells. Although they account for a smaller proportion of clinical resistance (approximately 10%), they can cause serious therapeutic problems, as in resistance of *Mycobacterium tuberculosis* to first-line antituberculous drugs.
2. **Acquisition of foreign genetic material:** Resistance genes located on mobile genetic elements (plasmids, transposons, integrons) can be acquired from other bacteria through horizontal gene transfer. This mechanism plays a major role in the rapid dissemination of resistance within and between species.

5.6.4.5.2.2 Biochemical Mechanisms

Acquired resistance can be expressed through several biochemical strategies, whose common purpose is to prevent effective interaction between the antibiotic and its target. Major mechanisms include:

1. **Modification of the target:** Structural changes in the antibiotic's target reduce or abolish its affinity for the drug. This may result from:
    - Mutations in chromosomal genes encoding the target;
    - Production of enzymes that modify the target (e.g., methylation of ribosomal RNA conferring macrolide resistance).

2 **Production of antibiotic-inactivating enzymes:** This is one of the most widespread and clinically important mechanisms. Bacteria produce enzymes that chemically modify or hydrolyse the antibiotic, thereby preventing its action. Examples include:
    - β-lactamases that hydrolyse β-lactam antibiotics;
    - Aminoglycoside-modifying enzymes (acetyltransferases, phosphotransferases, nucleotidyltransferases).
3 **Reduced permeability (impermeability):** Particularly in Gram-negative bacilli, resistance may result from decreased penetration of the antibiotic through the outer membrane. This is often due to alterations in porin proteins, reducing pore size or number. With the exception of polymyxins and aminoglycosides, most antibiotics active against Gram-negative bacteria rely on passive diffusion through porins to cross the outer membrane.
4 **Active efflux of antibiotics:** Efflux pumps are energy-dependent membrane transport systems that expel antibiotics from the bacterial cell, reducing their intracellular concentration below inhibitory levels. Some efflux systems are intrinsic, while others are encoded by mobile genetic elements (plasmids, transposons, integrons). Efflux can confer resistance to one antibiotic or to multiple structurally unrelated drugs (multidrug efflux pumps).

5.6.4.6 Multidrug-Resistant Bacteria

Multidrug-resistant (MDR) bacteria are defined as bacteria that have acquired resistance to at least one agent in three or more different antimicrobial classes, leaving only a limited number of effective therapeutic options. Infections caused by these organisms are often difficult to treat and are associated with poorer clinical outcomes.

MDR bacteria represent a major public health challenge, particularly in hospitals and other health-care facilities, for several reasons:

- **Difficulty of treatment:** Infections due to MDR organisms may not respond to standard empirical regimens and may require the use of last-resort antibiotics, sometimes with suboptimal efficacy or higher toxicity.

- **Increased morbidity and mortality:** These infections are associated with higher rates of complications, prolonged hospital stays, and increased mortality.
- **Nosocomial spread:** MDR bacteria can spread readily in health-care environments, particularly among vulnerable patients (e.g., the elderly, immunocompromised individuals, and those with chronic diseases or invasive devices).
- **Increased health-care costs:** Management of MDR infections often necessitates more expensive drugs, additional diagnostic and monitoring procedures, and longer hospitalisation, thereby increasing overall costs.

Given their frequency, the severity of the infections they cause, and their capacity for rapid dissemination, MDR bacteria must be subject to rigorous surveillance and prevention programmes, including antimicrobial stewardship, infection control measures, and epidemiological monitoring.

Examples of clinically important MDR bacteria include:

- Third-generation cephalosporin-resistant *Enterobacteriaceae* producing extended-spectrum β-lactamases (ESBLs);
- Carbapenemase-producing Enterobacterales (CPE);
- Methicillin-resistant *Staphylococcus aureus* (MRSA);
- Carbapenem-resistant *Pseudomonas aeruginosa*;
- Vancomycin-resistant enterococci (VRE), including *Enterococcus faecalis* and *Enterococcus faecium*.

These pathogens exemplify the critical need for prudent antibiotic use, strict infection prevention and control practices, and ongoing development of new antimicrobial agents and alternative therapeutic strategies.

6 Virulence Factors

6.1 Introduction

Virulence factors constitute the repertoire of molecular and structural tools that enable pathogenic microorganisms to invade a host, evade immune defenses, and cause disease. Understanding these factors is essential for elucidating the pathogenicity of infectious agents and for developing novel strategies for prevention and treatment. The study of virulence mechanisms therefore sheds light on the dynamics of infection as well as on major public health challenges.

Virulence, or pathogenicity, is the capacity of a microorganism to induce an infectious disease, whereas virulence factors are the microbial products that contribute to this pathogenicity. In recent years, research on the virulence of enteric pathogens has progressed considerably, leading to a more detailed description of the virulence factors of intestinal bacteria and their relationship to distinct stages of the infectious process.

Work on enteropathogenic bacteria of the genera Salmonella, Shigella, and Yersinia has highlighted at least two recurrent features:

- The clustering of virulence genes at specific loci on the bacterial chromosome, known as pathogenicity islands.
- The presence of specialized secretion systems that enable the targeted delivery of virulence factors into host cells (e.g. type III secretion systems).

Virulence factors can be viewed as “weapons” carried by pathogens. They are crucial for the ability to cause disease but are generally not required for basic microbial survival in non-host environments. Different classes of virulence factors act at various stages of infection: some mediate attachment to and/or entry into host tissues; others enable escape from or resistance to intracellular defenses; still others facilitate tissue invasion and dissemination within the host. Finally, certain factors, such as toxins, directly damage or intoxicate host cells. These factors may be simple molecules or complex structures, are encoded by specific genes, and are often characteristic of particular pathogens.

6.2 I. Definition

A virulence factor is any microbial component—such as a protein, glycoprotein, lipid, or other molecule—expressed or secreted by bacteria, viruses, fungi, or protozoa that contributes to:

- Invasion of and passage across host barriers.
- The induction of disease.
- Evasion or modulation of the host immune response.

More specifically, virulence factors help to:

- Colonize a niche within the host (including adherence to host cells).
- Achieve immunoevasion, i.e. escape from recognition or elimination by the immune system.
- Induce immunosuppression, i.e. dampening or inhibition of immune responses.
- Mediate entry into and exit from host cells (for intracellular pathogens).
- Acquire nutrients from the host (e.g. iron, amino acids, carbohydrates).

6.3 Virulence Genes

Virulence genes are those genes that encode proteins or other factors that enable a microorganism to cause disease in a susceptible host. They contribute to the establishment of infection, multiplication of the pathogen, and the damage caused to host tissues.

Key aspects include:

- When a bacterium infects a host, it encounters substantial changes in environmental conditions (e.g. pH, osmolarity, nutrient availability, oxygen tension) to which it must adapt.
- These adaptive responses are mediated by pre-existing genetic systems that include virulence genes. Such genes are often tightly regulated and expressed only under specific conditions encountered during infection.
- The expression of virulence genes is influenced by external environmental signals (e.g. temperature, osmolarity, nutrient levels, host-derived signals). Bacteria therefore possess sensor and regulatory systems (e.g. two-component systems, alternative sigma factors,

quorum-sensing systems) that detect environmental cues and transmit signals leading to the activation or repression of virulence genes.

- For example, expression of flagella in motile species is often regulated according to environmental conditions that favor or disfavor motility.

6.4 Koch's Postulates

Robert Koch (1843–1910), a German physician and microbiologist, formulated a series of postulates intended to establish a causal relationship between a specific microorganism and a particular disease. He applied these postulates to determine the etiologies of tuberculosis and anthrax. The original Koch's postulates are as follows:

1 The microorganism must be found in all cases of the disease and be associated with the characteristic lesions.
2 The microorganism must be isolated from the diseased host and grown in pure culture.
3 The cultured microorganism should cause the same disease when introduced into a healthy, susceptible host.
4 The same microorganism must be re-isolated from the experimentally infected host and shown to be identical to the original isolate.

Subsequent advances in bacteriology and the advent of molecular biology led to the adaptation of Koch's postulates to the genetic level, resulting in the formulation of the molecular Koch's postulates. These do not contradict the original postulates but rather complement them:

1 The candidate virulence gene should be present in all pathogenic strains of the species and absent (or nonfunctional) in non-pathogenic strains.
2 Inactivation (mutation or deletion) of the virulence gene should result in a measurable reduction in virulence or pathogenicity.
3 Restoration of the gene (complementation) in the mutant strain should restore virulence.
4 The virulence gene should be expressed during infection in the host.

6.5 Types of Virulence Factors

Virulence factors (Figure 36) can be classified according to their mode of action, including:

- Surface adhesion factors.
- Invasion factors (cellular and whole-host invasion).
- Factors involved in immune evasion and modulation.
- Toxins (exotoxins and endotoxins).
- Factors involved in nutrient acquisition (e.g. siderophores).

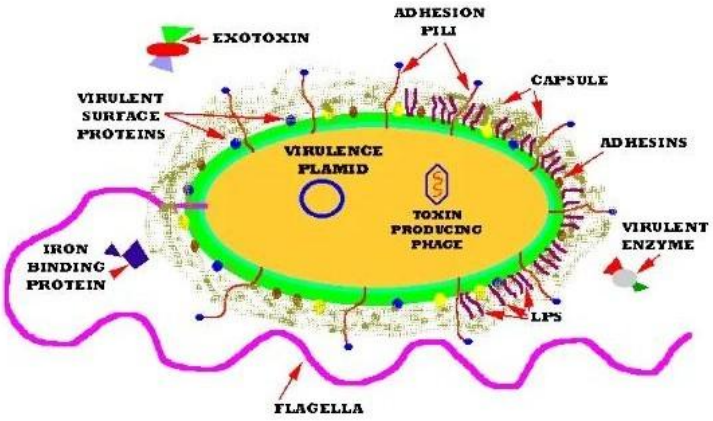


Figure 36. Types of virulence factors

6.5.1 Surface Adhesion Factors

Adhesion factors are key determinants of bacterial pathogenesis. They enable bacteria to attach to host cells or extracellular matrix components and often differ between Gram-negative and Gram-positive bacteria.

6.5.1.1 In Gram-Negative Bacteria

Major adhesion factors in Gram-negative bacteria include:

- **Pili (fimbriae)**: Filamentous protein structures, several micrometers long, regularly distributed over the bacterial surface. They are composed mainly of polymerized pilin subunits and may include minor subunits with adhesin functions. The adhesins of common pili interact with specific carbohydrate-containing receptors (glycolipids or glycoproteins) on the surface of eukaryotic cells.

- **Hemagglutinins**: Adhesive structures or proteins that can mediate attachment to erythrocytes and other host cells.
- **Lipopolysaccharides (LPS)**: In addition to their structural and endotoxin roles, certain LPS components can contribute to adherence and biofilm formation.

6.5.1.2 In Gram-Positive Bacteria

Representative adhesion factors in Gram-positive bacteria include:

- **M protein**: A fibrillar surface protein of *Streptococcus pyogenes* that mediates adhesion to epithelial cells and confers antiphagocytic properties.
- **Protein F and other adhesins**: Fibronectin-binding proteins and related surface adhesins promote binding to extracellular matrix components and host cells.
- **Biofilm formation and glycocalyx (slime)**: Many bacteria form biofilms—complex, often multispecies communities of microorganisms (bacteria, microfungi, microalgae, protozoa) attached to each other and to a surface, embedded in a self-produced extracellular polymeric matrix. This matrix (capsule or slime layer/glycocalyx) enhances adherence, protects against environmental stresses, and contributes to persistence and resistance to antimicrobial agents.

6.5.2 Invasion Factors

6.5.2.1 Cellular Invasion Factors

Cellular invasion factors facilitate bacterial penetration into host cells, movement through tissues, and intracellular survival. They may act by:

1 **Degradation of extracellular matrix and basement membranes**

- Clostridium collagenase: Degrades collagen networks that support tissues.
- *Pseudomonas* aeruginosa elastase: Degrades elastin and other matrix proteins, aiding tissue invasion.

2 **Depolymerization of cellular glycoprotein complexes**

- Hyaluronidases produced by Streptococcus spp.: Degrade hyaluronic acid, a major component of the extracellular matrix, thereby facilitating spread through tissues. In

Streptococcus pyogenes, the hyaluronic acid capsule mimics host tissue components, rendering it poorly immunogenic.

3 **Disruption of cell membranes**

- Lecithinase (phospholipase) of Clostridium spp.: Hydrolyses phospholipids in host cell membranes, leading to cell lysis and tissue necrosis.

4 **Modulation of the host cytoskeleton**

- Certain bacteria (e.g. *Listeria monocytogenes*) induce polymerization or depolymerization of host cell actin to promote their entry, intracellular movement, and cell-to-cell spread.

6.5.2.2 Whole-Host Invasion Factors

Some virulence factors promote dissemination throughout the host:

- **Coagulase (e.g. *Staphylococcus aureus*)**: A fibrin-coagulating enzyme that converts fibrinogen to fibrin. The fibrin clot can wall off bacteria at a local site (e.g. in abscesses), protecting them from phagocytosis and immune attack.
- **Hemolysins (e.g. from *Streptococcus*, *Bacillus*, and others)**: Toxins that lyse erythrocytes (e.g. β-hemolysis), releasing hemoglobin and iron, and may contribute to anemia and tissue damage.
- **Streptokinase (e.g. *Streptococcus* spp.)**: A fibrinolytic enzyme that activates plasminogen to plasmin, thereby dissolving fibrin clots. This promotes the spread of bacteria by disrupting clots that would otherwise localize the infection.
- **Chemotaxis and motility**: Flagella-mediated motility enables bacteria to move toward favorable environments (e.g. nutrients) and away from hostile conditions (e.g. high concentrations of immune effectors), facilitating colonization and escape from local defenses.
- **Siderophores**: High-affinity iron-chelating molecules secreted by bacteria to sequester iron from host proteins. By outcompeting host iron-binding systems, siderophores ensure bacterial iron acquisition, which is essential for growth and virulence.
- **Leukocidins (e.g. streptolysins)**: Pore-forming exotoxins that damage or lyse leukocytes. Lysis of neutrophils and macrophages leads to release of lysosomal enzymes and a reduction in the effectiveness of host immune defenses.

The pathogenicity of a bacterium rarely depends on a single virulence factor. Rather, highly virulent bacteria typically express multiple factors sequentially or simultaneously during different stages of infection.

6.6 Mechanisms of Bacterial Entry into Host Cells

Bacterial penetration into host cells can occur via several mechanisms. Entry generally involves initial contact with host surfaces and exploitation of natural portals of entry such as skin breaches and mucosal surfaces (oral, respiratory, genital, gastrointestinal, etc.). These surfaces are also colonized by commensal microbiota that compete with pathogens for space and nutrients (e.g. sugars, iron, ions).

Adhesion to epithelial surfaces is usually a prerequisite for colonization. Bacterial surface proteins called adhesins recognize and bind to specific receptors on host cells, thereby anchoring the bacteria to host tissues. For example, certain enteropathogenic *Escherichia coli* strains (causing infantile diarrhea) possess surface adhesins that facilitate attachment to the intestinal epithelium. This initial adhesion step is essential for subsequent pathogenic events.

Two major mechanisms of bacterial entry into non-phagocytic host cells have been described (Figure 37): the “zipper” mechanism and the “trigger” mechanism.

6.6.1 Zipper Mechanism

In the zipper mechanism, close contact between bacterial adhesins and host cell receptors induces progressive “zippering” of the host plasma membrane around the bacterium. The bacterium is internalized via receptor-mediated endocytosis. This mechanism is characteristic of pathogens such as *Listeria monocytogenes*, where bacterial surface proteins (e.g. internalins) bind host receptors, leading to actin rearrangement and uptake into the cell. After crossing epithelial barriers, bacteria can disseminate to normally sterile tissues.

6.6.2 Trigger Mechanism

In the trigger mechanism, bacteria such as *Salmonella* and *Shigella* inject effector proteins into host cells via specialized secretion systems (typically type III secretion systems). These effector proteins profoundly remodel the host cytoskeleton and induce membrane ruffling, thereby

triggering bacterial internalization. Here, the host cell actively engulfs the bacterium as a consequence of effector-mediated signaling rather than simple receptor engagement.

Once mucocutaneous barriers are breached, the pathogen encounters the host immune system and must evade or modulate immune responses to replicate and spread.

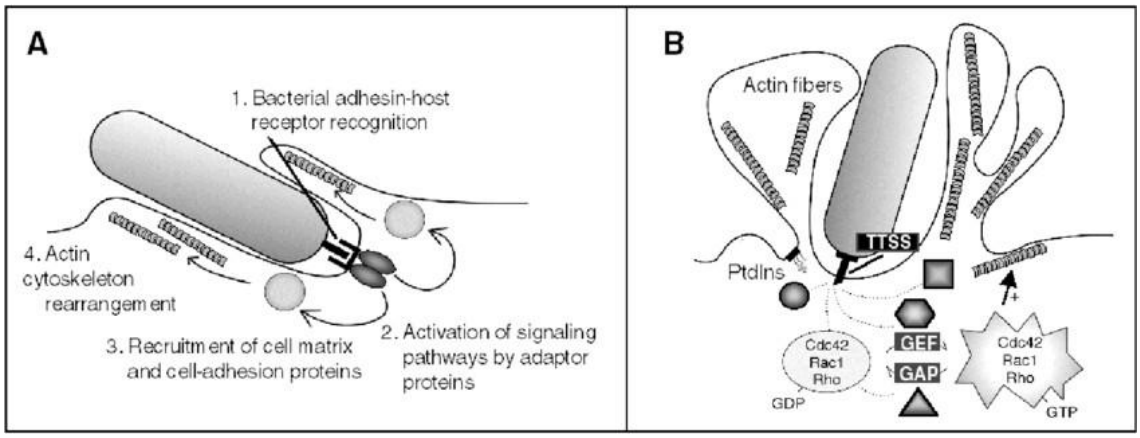


Figure 37. Mechanisms of bacterial penetration into host cells

A "zipper" and B"trigger" mechanisms

6.7 Origin and Transmission of Virulence Factors

Virulence factors arise from and are transmitted through various genetic sources:

- **Chromosomal genes**: Many virulence factors are encoded by genes located on the bacterial chromosome as part of the organism's core or accessory genome.
- **Mobile genetic elements**: Other virulence factors are encoded by mobile elements such as transposons, plasmids, and bacteriophages, or by chromosomal regions known as **pathogenicity islands**. These elements can be transferred horizontally between bacteria, contributing to the rapid dissemination of virulence traits.

Key mobile genetic elements include:

- **Transposons**: Mobile DNA segments capable of moving within and between DNA molecules. Their insertion can modulate expression of virulence genes or introduce new virulence determinants.

- **Plasmids**: Small, circular, extrachromosomal DNA molecules that replicate independently and can be transferred by conjugation. Many plasmids carry genes for antibiotic resistance and virulence factors (e.g. toxins, adhesins).
- **Bacteriophages (phages)**: Viruses that infect bacteria. Some phages carry virulence genes (e.g. toxin genes) in their genomes and can introduce these genes into new bacterial hosts by transduction.
- **Pathogenicity islands (PAIs)**: Large chromosomal regions present in pathogenic strains but absent from non-pathogenic relatives. They often have a distinct G+C content, are associated with mobile genetic elements, and encode multiple virulence-related proteins, including secretion systems and effector proteins.

6.8 Evasion of the Host Immune Response

Evasion of host response refers to the various strategies employed by pathogens (parasites, bacteria, viruses) to avoid, resist, or actively modulate the host immune system, thereby promoting survival, replication, and persistence.

6.8.1 Capsule

Some bacteria (e.g. encapsulated strains of Streptococcus pneumoniae, Neisseria meningitidis, Haemophilus influenzae type b) possess a polysaccharide capsule that interferes with phagocytosis. Encapsulation generally increases virulence compared with non-encapsulated strains. However, capsule-specific opsonizing antibodies can bind the capsule and restore efficient phagocytosis.

6.8.2 Enzymes

Certain bacterial proteins with enzymatic activity contribute to immune evasion and tissue invasion. Examples include proteases, hyaluronidases, neuraminidases, elastases, and collagenases, which enhance local spread through tissues.

Some invasive bacteria (e.g. Shigella flexneri, Yersinia enterocolitica) can invade intact epithelial cells, thereby breaching mucosal barriers.

A number of bacteria produce IgA-specific proteases (e.g. Neisseria gonorrhoeae, Neisseria meningitidis, Haemophilus influenzae, Proteus mirabilis, various Clostridium spp.,

Streptococcus pneumoniae). These enzymes cleave and inactivate secretory IgA at mucosal surfaces, diminishing the effectiveness of this first-line immune defense.

6.8.3 Toxins

Microorganisms may secrete exotoxins, which are protein toxins that can initiate or aggravate disease (e.g. in diphtheria, cholera, tetanus, botulism, and clostridial enterocolitis). Most exotoxins bind specific receptors on target cells and exert enzymatic or pore-forming activities. With the exception of preformed toxins associated with certain foodborne diseases (e.g. botulism, staphylococcal food poisoning, *Bacillus cereus* intoxication), exotoxins are usually produced in situ during infection.

Endotoxin refers to lipopolysaccharide (LPS) found in the outer membrane of Gram-negative bacteria. The lipid A component is responsible for potent biological activities, including activation of complement, coagulation, fibrinolysis, and kinin systems. Excessive endotoxin release can lead to septic shock and is a major cause of morbidity and mortality in Gram-negative sepsis. In Gram-positive bacteria, components such as teichoic acids and peptidoglycan fragments can also trigger strong inflammatory responses with endotoxin-like effects.

6.8.4 Other Immune Evasion Mechanisms

Additional mechanisms that enhance virulence include:

- **Interference with antibody production**: Some bacteria induce regulatory (suppressor) cells, interfere with antigen processing and presentation, or inhibit lymphocyte activation and proliferation, thereby altering or dampening humoral responses.
- **Resistance to oxidative killing**: Certain intracellular pathogens (e.g. *Legionella*, *Listeria*) avoid or inhibit the oxidative burst in phagocytes. Others produce enzymes such as catalase, superoxide dismutase, and glutathione reductase that detoxify reactive oxygen species, thereby attenuating oxidative killing.
- **Resistance to complement-mediated lysis**: Some pathogens evade destruction by the complement system through surface structures that prevent complement deposition or by expressing proteins that bind and inactivate complement components.
- **Production of superantigens**: Superantigens cause non-specific, polyclonal activation of large numbers of T cells by bridging the T-cell receptor and MHC class II molecules

outside the conventional antigen-binding site. This results in massive release of pro-inflammatory cytokines, leading to systemic inflammation and immune dysregulation, as seen in toxic shock syndromes.

6.9 Involvement of Virulence Factors in Therapeutic Strategies

Knowledge of virulence factors is crucial for the development of innovative therapeutic approaches and for improving clinical outcomes. Instead of solely targeting bacterial viability, many modern strategies aim to disarm pathogens by interfering with specific virulence mechanisms. This can reduce tissue damage and limit selection for antibiotic resistance.

6.9.1 Targeting Virulence Factors in Therapy

Some therapeutic agents are designed to neutralize toxins or block bacterial adhesion rather than to kill the bacteria directly. By inhibiting toxin activity or preventing adherence, these strategies reduce pathogenicity and limit host damage while exerting less selective pressure for resistance than conventional bactericidal or bacteriostatic drugs.

6.9.2 Inhibition of Quorum Sensing

Quorum sensing is a cell–cell communication system in bacteria based on the production and detection of small signaling molecules. It regulates the expression of many virulence factors, including toxins and biofilm formation. Inhibitors of quorum sensing (e.g. signal analogs, degrading enzymes) can disrupt this communication, thereby reducing virulence factor production and impairing the formation of antibiotic-resistant biofilms.

6.9.3 Immunotherapy

Immunotherapy aims to enhance the host immune system’s ability to clear infection. Approaches include the use of monoclonal or polyclonal antibodies that specifically neutralize key virulence factors (e.g. toxins, adhesins) or block host–pathogen interactions. Such strategies can be used alone or in combination with antimicrobial agents.

6.9.4 Vaccine Development

Virulence factors often serve as attractive vaccine antigens. Vaccines based on inactivated toxins (toxoids), surface proteins, or polysaccharide–protein conjugates can elicit protective immune

responses and prevent infection or disease. By targeting critical virulence determinants, vaccines can provide long-lasting, pathogen-specific immunity.

6.9.5 Early Diagnosis

Identification of virulence factors in clinical samples underpins the development of sensitive and specific diagnostic tests (e.g. PCR assays for toxin genes, detection of particular adhesins or capsules). Early diagnosis facilitates prompt, targeted therapy and improves prognosis.

6.9.6 Prevention of Nosocomial Infections

Understanding the virulence factors involved in transmission, persistence, and biofilm formation in healthcare environments supports the design of more effective infection prevention and control measures. This includes strategies to limit environmental contamination, prevent device-associated biofilms, and reduce the spread of multidrug-resistant organisms in hospitals and other healthcare settings.