CHAPTER 1

Introduction

Microbiology is the study of living organisms of microscopic size. This term was introduced by French chemist Louis Pasteur, who demonstrated that fermentation was caused by the growth of bacteria and yeasts. **Medical microbiology** is the study of microorganisms (bacteria, viruses, prions, fungi and parasites) capable of infecting and causing disease in humans, and the prevention, diagnosis and treatment of infectious diseases. It also deals with the response of the human host to microbial infection. Although the primary interest is in the diseases caused by the microorganisms, it must also be appreciated that microorganisms play a critical role in human survival.

The normal commensal population of microbes participates in the metabolism of food products, provides essential growth factors against infections with highly virulent microorganisms, and stimulates the immune response. In the absence of these organisms, life as we know would be impossible.

The term **microbe** was first used by Sedillot in 1878, but it has now been replaced by **microorganism**. Microbes were probably the first living things to appear on the earth, and the study of fossil remains indicates that microbial infections and epidemic diseases existed thousands of years ago.

Infectious diseases have been the bane of mankind for centuries and continue to cause high morbidity and sufferings worldwide. Disease and death have always attracted the attention of the human mind. The emergence of acquired immunodeficiency syndrome (AIDS) as a major modern day scourge with tremendous public health importance has brought into limelight even those diseases which were considered rare in the past.

The construction and use of the compound microscope (*micro*, small; and *skop*, to see) was an essential prerequisite to study the microbial forms. To **Antonie van Leeuwenhoek** (1632–1723) must be ascribed the credit of placing the science of microbiology on the firm basis of direct observation. This Dutch maker of lenses from Holland devised an apparatus and technique which enabled him to observe and describe various microbial forms with accuracy and care.

He observed, drew and measured a large number of minute living organisms including bacteria and protozoa, and communicated them to Royal Society of London in 1683. The significance of these observations was not realized then and to Leeuwenhoek the world of '*little animalcules*' represented only a curiosity of nature. Their importance in medicine and in other areas of biology came to be recognized two centuries later. Antonie van Leeuwenhoek first accurately described different shapes of bacteria (coccal, bacillary and spiral) and pictured their arrangement in infected material in 1683.

Antonie van Leeuwenhoek (1632–1723)

ORIGIN OF MICROBIAL LIFE

In 1856, **Louis Pasteur** (1822–1895) was commissioned by an industrialist of Lille to investigate the problem which had arisen in manufacture of alcohol. The beet juice, from which alcohol was derived, was contaminated with a grey material which interfered with alcohol production. During the course of investigation, his attention was abruptly focused on the role of microorganisms in alcohol fermentation and spoilage. Undesirable forms of life could be destroyed at temperatures of 50–60°C in a short period of time. Subsequently, this modified process of heating came to be known as **pasteurization**. Pasteur established that different types of fermentations were due to the activity of different kinds of microbes.

Louis Pasteur (1822–1895)

John Tyndall (1820–1893), an English physicist, was able to explain satisfactorily the need for prolonged heating to eliminate microbial life from infusions. He concluded, by exposing infusions to heat for varying times, that bacteria existed in two forms—a heat-stable form and a heat-sensitive

form. It required either prolonged or intermittent heating to destroy heat-stable forms. Intermittent heating, now called **tyndallization**, killed both forms since between periods of heat treatment, the heat-stable forms changed to heat-sensitive forms.

Ferdinand Cohn (1828–1898) described heat-stable forms as **spores**. Spores as well as vegetative forms were responsible for the appearance of microbial life in inadequately heated infusions.

Joseph Lister (1827–1912), an English surgeon and contemporary of Pasteur, was among the first to appreciate the ramifications of the emerging **germ theory** of disease. He attributed the frequent disastrous consequences following repair of compound fractures to invasions by airborne micro-organisms. Lister introduced antiseptics in surgery. By spraying carbolic acid on surgical instruments, wounds and dressings, he reduced surgical mortality due to bacterial infection considerably. He established the guiding principle of antisepsis for good surgical practice upon which the present day specialists depend. For this work he is known as '**father of antiseptic surgery**'.

THE DEVELOPING SCIENCE OF MICROBIOLOGY

In the course of studies, Pasteur introduced the techniques of sterilization and developed steam sterilizer, hot air oven and autoclave. **Robert Koch** (1843–1910), a German physician, perfected the bacteriological techniques, staining procedures and methods of obtaining bacteria in pure culture using solid media during his studies on the culture and characters of anthrax bacillus.

Robert Koch (1843–1910)

The causative agents of various diseases were reported rapidly by different investigators. Robert Koch discovered bacillus of tuberculosis (1882) and Vibrio cholera (1883); Hansen described the leprosy bacillus in 1874; Neisser discovered the gonococcus in the pus discharge from urethra in 1879; Alexander Ogston in 1880 described the staphylococci in abscesses and suppurative lesions; Eberth observed the typhoid bacillus in 1880; Klebs (1883) and Loeffler (1884) observed and described the diphtheria bacillus; Rosenbach (1886) demonstrated the tetanus bacillus with round terminal spore; Weichselbaum (1887) described and isolated the meningo-coccus from the cerebrospinal fluid of a patient; Bruce (1887) identified the causative agent of malta fever in 1905 and Schaudinn and Hoffmann described the spirochaete of syphilis.

As the agents were being reported in such profusion, it became necessary to introduce criteria for proving the claims that a microorganism isolated from a disease was indeed causally related to it. **Henle** indicated such criteria but were enunciated by Koch which consisted of guidelines for the association of particular microorganisms with specific infectious diseases.

Koch observed thread-like organisms in the blood of animals that had died of anthrax, a disease that was serious threat to farmers killing their sheep and cattle herds periodically. He cultivated anthrax bacteria in pure culture in clear sterile vitreous humor of an ox's eye. He then injected pure cultures of the bacilli into mice and showed that the bacilli invariably caused anthrax. On autopsy, the blood was swarming with the thread-like bacteria and reisolated them in the vitreous humor. The cycle was now complete.

KOCH'S POSTULATES

A microorganism can be accepted as the causative agent of an infectious disease only if following postulates, known as Koch's postulates, are satisfied (Fig. 1.1).

1. The microorganism must be present in the lesions in every case of the infectious disease.
2. It should be possible to isolate the microorganism in pure culture from the lesions.
3. Inoculation of the pure culture, by a suitable route, into a suitable laboratory animal should produce a similar disease.
4. It should again be possible to re-isolate the microorganism in pure culture from the lesions produced in the experimental animals.

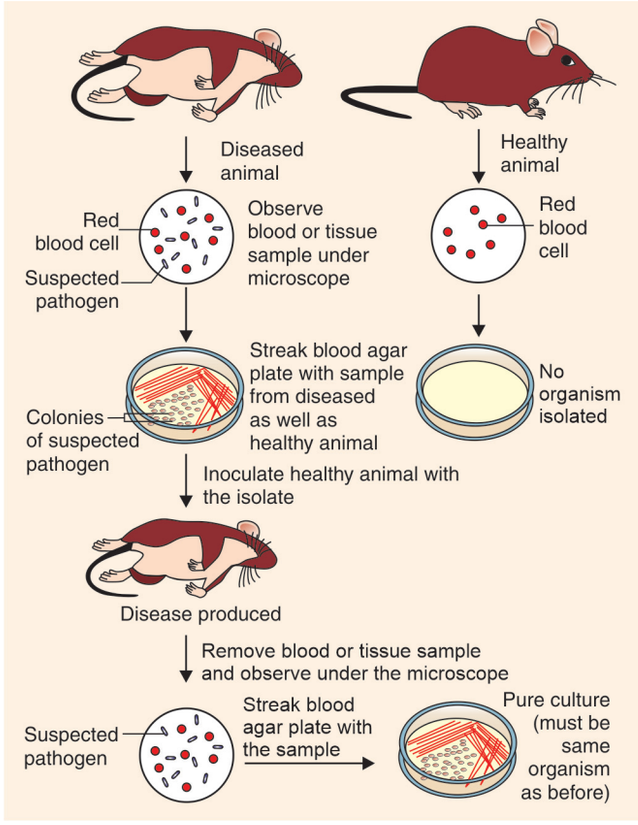


Fig. 1.1: Koch's postulates.

A fifth criterion introduced subsequently states that specific antibodies to the organism should be demonstrable

in the serum of the patient suffering from the disease. These postulates have proved extremely useful in confirming the authenticity of doubtful claims made regarding the causative agents of infectious diseases.

Exceptions to Koch's postulates

Koch's postulates have remained a mainstay of microbiology; however, many microorganisms that do not meet the criteria of Koch's postulates have been shown to cause disease. For example:

- *Treponema pallidum* and *Mycobacterium leprae*, causative agents of syphilis and leprosy, respectively, cannot be grown *in vitro*; however, there are animal models of infection with these agents.
- *Neisseria gonorrhoeae*, which causes gonorrhoea, there is no animal model even though the bacteria can readily be cultured *in vitro*.

THE BEGINNING OF VIROLOGY

For many years the term virus was used to describe any poison or microbial agent capable of causing an infection. In a large number of diseases such as smallpox, chickenpox, measles, influenza, poliomyelitis and the common cold, no bacterial cause could be established. Pasteur had suspected that rabies in dogs could be caused by a microbe too small to be seen under the microscope.

The first man to describe a filtered extract capable of causing disease in plants was Dmitri Iwanowski (1864–1920), a Russian scientist, who started his studies on diseases of tobacco while he was still a student. He reproduced mosaic disease in tobacco plant by applying juice from diseased plants to healthy leaves from which all bacteria had been removed by passage through fine filters (1892). In 1898, Martinus Beijerinck, unaware of Iwanowski's work, attributed the cause of tobacco-mosaic disease to *Contagium vivum fluidum*, a living liquid virus.

In 1898, Loeffler and Paul Frosch from Germany reported that the causative agent of foot-and-mouth disease in cattle would pass through a bacterial filter. Walter Reed (1902) in Cuba proved that the causative agent of yellow fever was not only a filterable virus but also transmitted through the bite of infected mosquitoes. The term 'filterable' was dropped in time and the tiny infectious agents were merely called **viruses**. Larger viruses could be seen under light microscope after appropriate staining but their detailed morphology could only be studied by electron microscope introduced by Ruska (1934). The technique of growing them on chick embryos developed by Goodpasture in 1930s and the application of cell culture in virology expanded the scope of virological techniques considerably.

Ellerman and Bang (1908) suggested the possibility that virus infection could lead to malignancy. Peyton Rous (1911) isolated a virus causing sarcoma in fowls. Several viruses have been blamed to cause natural and experimental tumours in birds and animals. Experimentally, viruses can cause malignant transformation of infected cells in tissue cultures. The discovery of viral and cellular oncogenes have put forth the possible mechanisms of viral oncogenesis.

CONTRIBUTIONS OF VARIOUS SCIENTISTS IN THE FIELD OF MICROBIOLOGY

A large number of scientists has contributed in the field of microbiology. Principal contributions of some of them are given below.

Louis Pasteur

Louis Pasteur (1822–1895) was born in the village of Dole, France on December 27, 1822, the son of humble parents. His father was a tanner. He was originally trained as a chemist, but his studies on fermentation led him to take interest in microorganisms. His discoveries revolutionized medical practice, although he never studied medicine.

1. The term **microbiology**, as the study of living organisms of microscopic size, was coined by Pasteur.
2. He also coined the term **vaccine**.
3. He used various forms of nutrient fluid to grow micro-organisms.
4. He showed that some organisms were not destroyed by boiling. For the sterilization of fluids he advocated heating to 120°C under pressure and for glassware the use of dry heat at 170°C. He showed that cotton plugs (a primitive air-filtration device) could prevent microbes from reaching otherwise air-exposed sterile broths.
5. In 1860–61, he disapproved the **theory of spontaneous generation**. In a series of classic experiments, Pasteur proved conclusively that all forms of life, even microbes, arose only from their alike and not *de novo*.
6. In 1860–64, he gave experimental evidence that fermentation and putrefaction are effects of microbial growth.
7. In 1863–65, he devised the process of destroying bacteria, known as **pasteurization**.
8. He introduced the techniques of sterilization and developed stream sterilizer, hot air oven and autoclave.
9. In 1877, Koch and Pasteur demonstrated that anthrax is caused by bacteria. Pasteur grew the organisms in sterilized yeast water and kept them in the laboratory for several months, transferring them frequently to new culture fluid, in which they multiplied readily, and showed that these cultures would always cause anthrax when inoculated into healthy animals.
10. In 1880, he prevented chicken cholera by injection of live attenuated culture. He found that pure cultures of the germ of this disease which had been kept in the laboratory for some time would not kill his animals as fresh cultures did, but would merely cause a passing illness from which the chickens recovered. Then he discovered that the animals that had recovered from a previous inoculation of weakened germs were immune, and did not succumb to the disease. Pasteur immediately perceived that it might be possible to make individuals

resistant by inoculating them with the weakened (and therefore harmless) germs of a particular disease.
11. In 1880, he first cultured staphylococci in liquid medium and produced abscesses by inoculating them into rabbits.
12. In 1881, he developed **live attenuated anthrax vaccine**.
13. In 1881, pneumococci were first noticed by Pasteur and Sternberg independently.
14. The crowning achievement of Pasteur was the successful application of the principle of vaccination to the prevention of rabies, or hydrophobia, in human beings and developed **Pasteur rabies vaccine** in 1885. He obtained fixed rabies virus by serial intracerebral passage in rabbits. The rabies vaccine was prepared by drying pieces of spinal cord from rabbits infected with fixed virus. Rabies vaccine prevented the development of this fatal disease if the inoculations are given soon after the bite of the rabid animal. He gave the first treatment for rabies in 1885 to a young boy bitten by a rabid dog.
15. In 1887, Pasteur and Joubert first described *Clostridium septicum* and called it *Vibrion septique*.

In 1888, in recognition of his incomparable achievements, the Pasteur Institute of Paris was built by public contribution during his lifetime for investigations of infectious diseases and preparation of vaccines. Acclaimed the world over for his epoch making discoveries, Pasteur died in Paris on September 28, 1895. His body lies in Pasteur Institute of Paris. Today the Pasteur Institute is a thriving research centre—an appropriate memorial to its founder.

Robert Koch

Contributions of Robert Koch, to microbiology, are variegated and enormous.

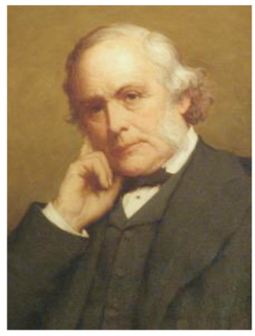
1. In 1876, Robert Koch reported the isolation of anthrax bacillus in pure culture, formation and germination of its spores and the proof of its infectiousness. This agent as the sole cause of anthrax was confirmed by Pasteur.
2. In 1877, he introduced the method of making smears of bacteria on glass slides, and of staining them with the aniline dyes.
3. In 1881, he described means of cultivating bacteria on solid media, thus making it possible to obtain pure cultures by transferring material from a single colony.
4. The **hanging-drop method** of studying bacteria as used today is a product of his genius.
5. In 1882, Koch startled the world by announcing his discovery of **tubercle bacillus** (*Mycobacterium tuberculosis*), the causative agent of tuberculosis. He described a special staining method for detection of this organism and grew it in pure cultures in the laboratory.
6. In 1883, he discovered the causative agents of cholera (*Vibrio cholerae*), Egyptian ophthalmia (pink eye) and Koch Weeks bacillus.
7. In 1884, Koch expounded the postulates or laws by which an organism may be proved to be the cause of a particular disease. These are known as **Koch's postulates**.
8. Koch continued his work on tuberculosis and in 1890–91 he showed how a normal guinea pig and an already infected guinea pig behaved differently to an infection with tubercle bacillus. This is known as **Koch's phenomenon** (*see* Chapter 28).

In 1905, he was awarded the **Nobel Prize in Medicine** for his work on tuberculosis. Together with Louis Pasteur, he laid the foundations of modern bacteriology.

Antonie van Leeuwenhoek

Antonie van Leeuwenhoek was expert in the grinding of simple magnifying lenses. He made these lenses of small bits of glass, polished them very carefully, and mounted each separately between two brass, copper, silver, or gold plates, to which he fastened an adjustable holder for the object to be examined. He constructed many of these 'microscopes' each containing a single lens ground by himself. The best of lenses magnified about 200 times. He observed, drew and measured a large number of living organisms including bacteria and protozoa in materials such as rain water, pond and well water, and saliva and the intestinal contents of healthy subjects and communicated them to the Royal Society of London in 1683. He was the first to accurately describe different shapes of bacteria (coccal, bacillary and spiral) and picture their arrangement in infected material.

Leeuwenhoek observed that very large numbers of bacteria appeared in watery infusions of animals or vegetable matter which were left to stand for a week or two at room temperature. He believed that these huge populations were the progeny of a few parental organisms, or seeds, that were originally present in the materials of the infusion or had entered it from the air. The significance of these observations was not realized then and to Leeuwenhoek the world of '*little animalcules*' represented only a curiosity of nature. Their importance in medicine and other areas of biology came to be recognized two centuries later.



Edward Jenner (1749–1823)

Edward Jenner

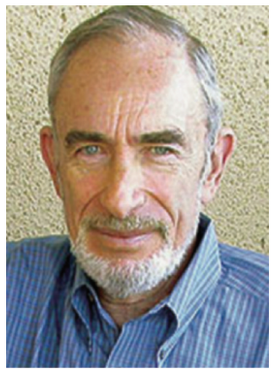
Edward Jenner *introduced the modern method of vaccination to prevent smallpox*. He observed that milkmaids who contracted cowpox or vaccinia while milking were subsequently immune to smallpox. On May 14,1796, he devised a brave experiment. He performed a vaccination against smallpox by transferring material from a cowpox pustule on the hand of a milkmaid, Sarah Nelmes, to the arm of a small boy named James Phipps, his gardener's son. Six weeks later the boy was inoculated with smallpox. He failed to develop the disease. *The terms vaccine and vaccination were first used by Pasteur out of deference to Jenner*.

In 1967, the World Health Organization masterminded a final global plan to eradicate smallpox. Success was announced in 1980 with the declaration: Smallpox is dead.

Thanks to Jenner. Edward Jenner's discovery has now been developed into one of the most important parts of modern medicine—**immunology**.

Paul Ehrlich

1. In 1882, he reported the *acid-fastness* of tubercle bacillus.
2. From 1890 to 1900, he did important research in immunology. He soon found that the specific effect of immune serum could be demonstrated *in vivo* and *in vitro* and introduced methods of standardizing toxin and antitoxin. To him goes the credit of *minimum lethal dose*.
3. In 1898, he proposed *side chain theory of antibody production* (*see* Chapter 15).
4. In 1909, he introduced salvarsan, an arsenical compound, sometimes called the '*magic bullet*'. It was capable of destroying the spirochaete of syphilis with only moderate toxic effects. He continued his experimentation until 1912 when he announced the discovery of neosalvarsan. Thus he created a new branch of medicine known as *chemotherapy*.



Paul Ehrlich
(1854–1915)

KEY POINTS

- Microbiology is the biology of ***microscopic organisms***, its subjects being microorganisms.
- Microorganism is an organism that ***cannot be seen without the use of a microscope***.
- ***Medical microbiology*** deals with those ***organisms which are responsible for infectious diseases of humans***.
- A microorganism is generally accepted as the causative agent of an infectious disease if it satisfies ***Koch's postulates***.
- *Treponema pallidum*, *Mycobacterium leprae* and *Neisseria gonorrhoeae* do not fulfil all the criteria of Koch's postulates; the first two ***cannot be grown in vitro*** and for the third there is ***no animal model***.

Important Questions

Write short notes on:
(a) Koch's postulates.
(b) Contributions of Louis Pasteur in microbiology.
(c) Contributions of Robert Koch in microbiology.
(d) Contributions of Antonie van Leeuwenhoek in microbiology.
(e) Contributions of Edward Jenner in microbiology.
(f) Contributions of Paul Ehrlich in microbiology.

Multiple Choice Questions

1. Which of the following organisms **does not** meet all the criteria of Koch's postulates?
   (a) *Streptococcus pneumoniae*.
   (b) *Treponema pallidum*.
   (c) *Leptospira interrogans*.
   (d) *Mycobacterium scrofulaceum*.
2. The construction and use of the compound microscope is attributed to:
   (a) Antonie van Leeuwenhoek.
   (b) Louis Pasteur.
   (c) Robert Koch.
   (d) Ferdinand Cohn.
3. Salvarsan was discovered by:
   (a) Karl Landsteiner.
   (b) Paul Ehrlich.
   (c) Gerhardt Domagk.
   (d) Howard Florey.
4. Bacillus of tuberculosis was discovered by:
   (a) Hansen.
   (b) Loeffler.
   (c) Robert Koch.
   (d) Bruce.
5. The term microbiology, as the study of living organisms of microscopic size, was coined by:
   (a) Antonie van Leeuwenhoek.
   (b) Robert Koch.
   (c) Louis Pasteur.
   (d) Edward Jenner.
6. The term vaccine was coined by:
   (a) Edward Jenner.
   (b) Kitasato.
   (c) Ehrlich.
   (d) Louis Pasteur.

7. Rabies vaccine was developed for the first time in 1885 by:
   (a) Louis Pasteur.
   (b) Semple.
   (c) Edward Jenner.
   (d) Paul Ehrlich.
8. Who discovered *Mycobacterium tuberculosis* and *Vibrio cholerae*?
   (a) Loeffler. (b) Welch.
   (c) Pfeiffer. (d) Robert Koch.
9. Who introduced the method of vaccination to prevent smallpox?
   (a) Louis Pasteur.
   (b) Edward Jenner.
   (c) Paul Ehrlich.
   (d) John Hunter.
10. Who of the following scientists proposed side chain theory of antibody production in 1898?
   (a) Paul Ehrlich.
   (b) Elie Metchnikoff.
   (c) John Hunter.
   (d) Edward Jenner.
11. Living organisms including bacteria and protozoa were first observed by:
   (a) Louis Pasteur.
   (b) Robert Koch.
   (c) Antonie van Leeuwenhoek.
   (d) Christian Gram.
12. Who is known as father of antiseptic surgery?
   (a) Alexander Fleming.
   (b) Joseph Lister.
   (c) Charles Nicolle.
   (d) Christian Gram.
13. Attenuated vaccine was first developed by:
   (a) Louis Pasteur.
   (b) Robert Koch.
   (c) Landsteiner.
   (d) Ehrlich.
14. Which of the following pioneers of microbiology is credited with the discovery of microorganisms using high-quality magnifying lenses (early microscopes)?
   (a) Robert Koch.
   (b) Louis Pasteur.
   (c) Antonie van Leeuwenhoek.
   (d) Hooke.
15. Which of the following is **not** a condition of Koch's postulates?
   (a) Isolate the causative agent of a disease.
   (b) Cultivate the microbe in the laboratory.
   (c) Inoculate a test animal to observe the disease.
   (d) Produce a vaccine.

Answers

1. b **2.** a **3.** b **4.** c **5.** c **6.** d **7.** a **8.** d **9.** b **10.** a **11.** c **12.** b
13. a **14.** c **15.** d

CHAPTER

2

Morphology of Bacteria

Bacteria are free-living, microscopic, unicellular organisms capable of performing all the essential functions of life, e.g. growth, metabolism and reproduction. They possess both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and lack chlorophyll. Bacteria have been placed in a kingdom separate from the animal and plant kingdoms, **Monera**.

Cells that have a well-defined nucleus are called **eukaryotes** (*eu*, true; and *karyon*, nucleus), whereas cells that lack a well-defined nucleus are called **prokaryotes** (*pro*, primitive; and *karyon*, nucleus). Bacteria are prokaryotes, while fungi, algae, protozoa, plants and animals are eukaryotes. In general, the interior organization of eukaryotic cells is more complex than that of prokaryotic cells. The comparison of prokaryotes and eukaryotes is given in Table 2.1.

SIZE OF BACTERIA

Bacteria are very small in size. The unit of measurement of bacteria is called micrometre (μm). One μm is a millionth part of a metre or a thousandth part of a millimetre (mm). One nanometre (nm) is a thousandth part of a μm, and one Angstrom unit (Å) is one-tenth of a nanometre. The diameter of the smallest body that can be resolved and seen clearly with naked eye is about 200 μm.

Table 2.1: Comparison of prokaryotes and eukaryotes

Characteristics	Prokaryotes	Eukaryotes
Major groups	Bacteria	Algae, fungi, protozoa, plants and animals
Genetic material		
Location	Free in the cytoplasm attached to a structure called mesosome located in the cell membrane	Contained within a membrane-bound nucleus inside the cell
Form	A single circular piece of DNA	Multiple chromosomes, which are surrounded by basic proteins called histones
Nucleolus	Absent	Present
Replication	By binary fission	By mitosis and meiosis
Extrachromosomal DNA	Plasmids—small circular pieces of DNA containing accessory information, present in the cytoplasm	In mitochondria
Protein production site	No endoplasmic reticulum; ribosomes—free in the cytoplasm or attached to the cell membrane	• Rough endoplasmic reticulum, a membrane covered with ribosomes, where protein is made • Smooth endoplasmic reticulum or Golgi complex, where secreted proteins are packaged and transported to the cell surface
Ribosomes	70S in size, consisting of 50S and 30S subunits	80S in size, consisting of 60S and 40S subunits
Energy production site	Electron transport chain located in the cell membrane; no mitochondria present	Within membrane-bound mitochondria
Intracellular organelles (lysosomes)	Absent	Contain hydrolytic enzymes
Cytoplasmic membrane	With the exception of *Mycoplasma*, bacterial cytoplasmic membrane lacks sterols	Does contain sterols
Cell wall	Present; is a complex structure containing peptidoglycans, protein, and lipids	Usually absent except in fungi, which contain chitin in the cell wall

Medically important bacteria, generally, measure 0.2–1.5 μm in diameter and 3–5 μm in length. Therefore, to visualize most bacteria one must use the higher powers of magnification of a good light microscope and enlarge them about 1000 times. To visualize their surfaces distinctly, it is usually necessary to stain them. Electron microscopy is essential for clear visualization of internal structures of the bacteria.

Microscopy

The study of the morphology of bacteria requires the use of microscopes. Following types of microscopes are used for examination of bacteria.

Light microscope

Bacteria may be examined under light microscope, either in the living state or after fixation and staining. The arrangement, motility and approximate size of the organisms can be observed by the examination of wet films or 'hanging drops'. But, due to lack of contrast, details cannot be appreciated.

Phase-contrast microscope

The phase-contrast microscope takes advantage of the fact that light waves passing through transparent objects such as cells, emerge in different phases depending on the properties of the materials through which they pass. A special optical system converts difference in phase into difference in intensity, so that some structures appear darker than others. It can be used to reveal some details of the internal structures in living cells.

Dark-ground (dark-field) microscope

This microscope renders visible delicate organisms such as *Treponema pallidum*, a spirochaete which is less than 0.2 μm in diameter and therefore cannot be observed with direct light. Dark-field microscopy is frequently performed on the same microscope on which bright field microscopy is performed. By means of a special condenser with a circular central stop, the specimen is illuminated by oblique light only. The rays do not enter the tube of the microscope, and in consequence, do not reach the eye of the observer unless they are scattered by objects (e.g. bacteria) of different refractive index from the medium in which they are suspended. As a result, the organisms appear brightly illuminated against a dark background.

The advantage of this method is that the resolving power of the dark-field microscopy is significantly improved compared with that of bright-field microscopy. The disadvantage of this method is that the light passes around rather than through the organisms, making it difficult to study their internal structure.

Fluorescence microscope

When ultraviolet or short-wavelength or invisible light falls on a fluorescent substance, the wavelength of the invisible light increases, so that it becomes luminous and is said to fluoresce. If tissues, cells or bacteria are stained with a fluorescent dye and are examined under the microscope with ultraviolet light instead of ordinary visible light, they become luminous and are seen as bright objects against a dark background. Moreover, fluorescent dyes have a selective action for various microorganisms and cells and for their constituents which thus become readily recognized and identified. The fluorochrome dyes auramine and rhodamine can be used to demonstrate acid-fast bacilli. Viewed by fluorescence microscopy, the bacterial cells appear yellow against a dark background when potassium permanganate is used as a counterstain. For immunofluorescence *see* Chapter 13.

Electron microscope

The greatly increased resolving power of the electron microscope (EM) has enabled scientists to observe the detailed structures of prokaryotic and eukaryotic cells. The superior resolution of the EM is due to the fact that electrons have a much shorter wavelength than the photons of white light. In EM, a beam of electrons is employed instead of the beam of light used in the optical microscope. The electron beam is focused by circular electromagnets, which are analogous to the lenses in the light microscope. The object which is held in the path of the beam scatters the electrons and produces an image which is focused on a fluorescent viewing screen.

The wavelength of electrons used in an EM is 0.005 nm, as compared to 500 nm with visible light, i.e. about 100,000 times shorter than that of ordinary light. Theoretically, if conditions were identical in the optical and electron microscopes, the resolving power of the EM should be 100,000 times (resolution down to 0.0025 nm). However, the numerical aperture of an EM lens is very small (the diameter of the aperture is only a few micrometers) and does not approach the width of that of an optical microscope objective. In practice, the best resolution that can be obtained is 0.3–0.5 nm, a hundred times better than that of the light microscope.

An important technique in electron microscopy is the use of 'shadowing'. This involves depositing a thin layer of heavy metal (such as platinum) on the specimen by placing it in the path of a beam of metal ions in a vacuum. The beam is directed at a low angle to the specimen, so that it acquires a 'shadow' in the form of an uncoated area on the other side. When an electron beam is then passed through the coated preparation in the EM and a positive print is made from the 'negative' image, a three-dimensional effect is achieved.

Another technique in electron microscopy includes the use of ultrathin sections of embedded material—a method of freeze-drying specimen, which prevents the distortion caused by conventional drying procedures; and the use of negative staining with an electron-dense material such as phosphotungstic acid or uranyl salts. This technique enables the study of cellular ultrastructure as it appears in the living state.

SHAPE OF BACTERIA

Bacteria exist in different shapes as under (Fig. 2.1):

1. **Cocci** (from *kokkos* meaning berry) are round or oval cells.

2. **Bacilli** (from *baculus* meaning rod) are rod or stick-shaped. In some of the bacilli the length of the cells may be equal to width. Such bacillary forms are known as **coccobacilli**. The latter have to be carefully differentiated from cocci.
3. **Vibrios** are curved or comma-shaped rods.
4. **Spirilla** are non-flexuous spiral forms with one to three fixed curves in their rigid bodies.
5. **Spirochaetes** (from *spira* meaning coil and *chaite* meaning hair) are slender and flexuous spiral forms.
6. **Mycoplasmas** are cell wall deficient organisms. Therefore, they do not possess stable morphology. They occur as round or oval bodies or as interlacing filaments.

GROUP PATTERNS

The most frequent method of reproduction among bacteria is asexual binary fission, that is each cell splits in half, forming two new cells. As they increase in number they form distinct groups. Cocci that split along one plane only tend to arrange themselves in pairs (**diplococci**) or in chains (**streptococci**). When the division occurs alternatively in each of two planes, groups of four (**tetrads**) or eight (**octads**) are formed. Haphazard splitting in several planes results in the formation of clusters of cocci (Fig. 2.1).

Bacilli split only across their short axes, therefore, the patterns formed by them are limited. They may appear as end-to-end pairs (**diplobacilli**), or chains (**streptobacilli**) (Fig. 2.1). In some instances, there occurs incomplete separation of the daughter cells after binary fission. The bacilli remain attached to each other at various angles, resembling the letters V or L. This is called **Chinese letter arrangement** and is characteristic of *Corynebacterium diphtheriae*.

ANATOMY OF A BACTERIAL CELL

The principal structure of a bacterial cell is shown in Fig. 2.2. The interior of the cell, the protoplast, is differentiated into cytoplasm and nuclear material. Cytoplasm is bounded by a thin, elastic and semipermeable cytoplasmic membrane. Outside this lies cell wall, which gives the bacterium its shape

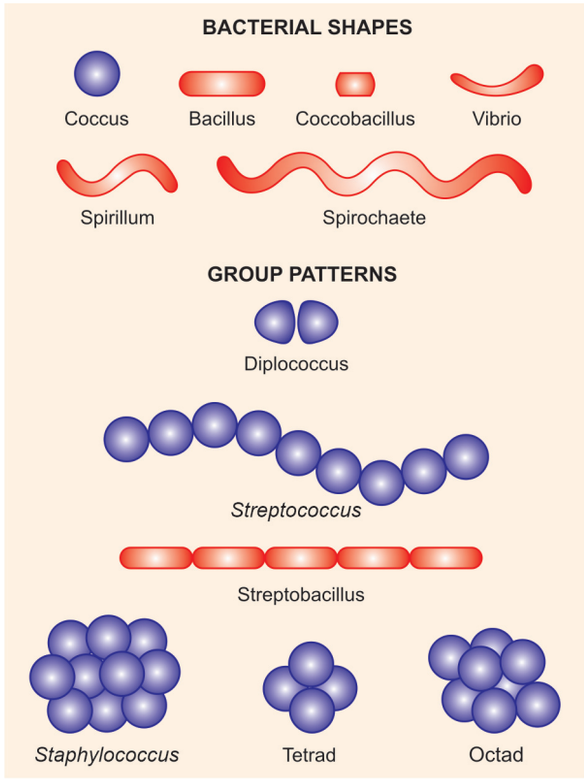


Fig. 2.1: Shapes and group patterns of bacteria.

and rigidity. Cell wall, in many bacteria, is enclosed by a protective gelatinous covering layer called capsule. Many bacteria also possess flagella which are the organelles of motility and some species have fimbriae (pili) too.

Bacterial cell wall

- It is a complex rigid structure which gives bacteria their definite shape.
- It is permeable to passage of liquid nutrient material into the cell, and to outward passage of substances produced within the cell.
- It is about 10–20 nm in thickness and constitutes 20–30% of dry weight of the cell.

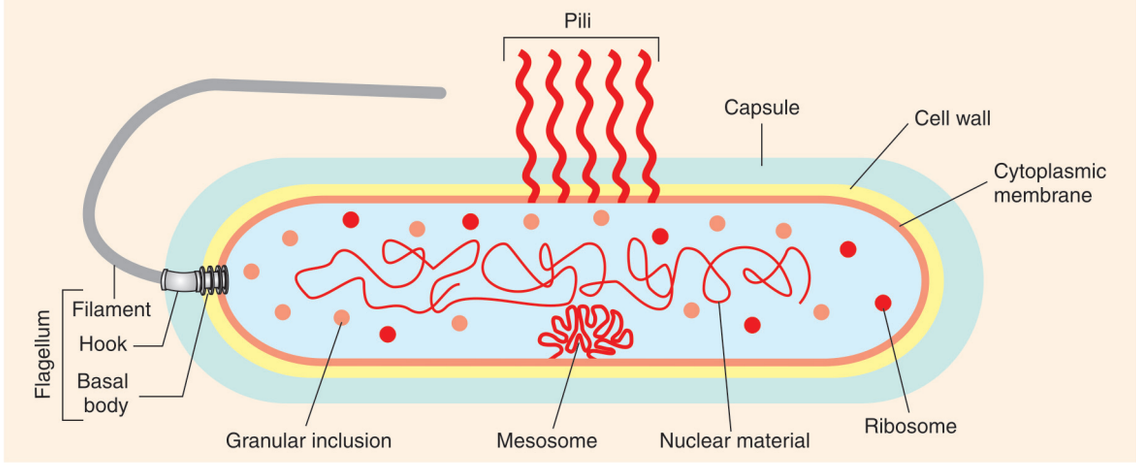


Fig. 2.2: Anatomy of a bacterial cell.

- The cell walls of Gram-positive bacteria are generally thicker than those of Gram-negative bacteria.
- The strength of the bacterial cell wall is due to the presence in it of a substance referred to as **peptidoglycan**, mucopeptide or murein.

Peptidoglycan consists of three parts—a backbone, composed of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid; a set of identical tetrapeptide side chains attached to *N*-acetylmuramic acid; and a set of identical pentapeptide cross-bridges (Fig. 2.3). In all bacterial species the backbone is the same, however, tetrapeptide side chains and pentapeptide cross-bridges vary from species to species.

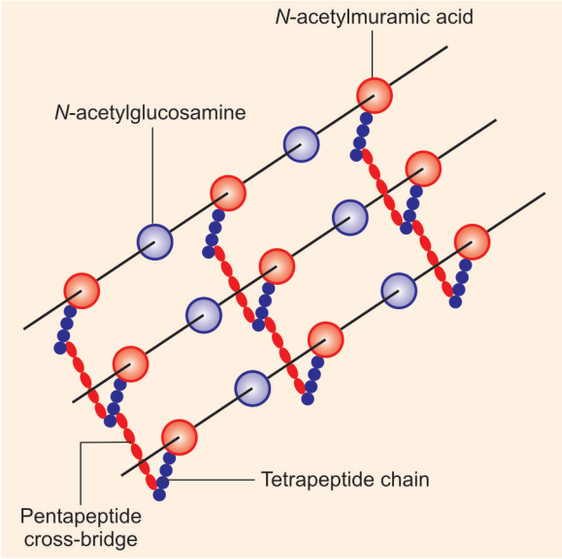


Fig. 2.3: Chemical structure of peptidoglycan.

Gram-positive bacterial cell wall

- The Gram-positive bacterial cell wall (Fig. 2.4) is 16–80 nm thick and is composed mostly of several layers of **peptidoglycan**.
- It constitutes **50–90%** of the dry weight of the wall.
- In addition to peptidoglycan, Gram-positive cell wall also contains teichoic acids and polysaccharides. There are two types of teichoic acids—cell wall teichoic acid, covalently linked to peptidoglycan; and membrane **lipoteichoic acid**, covalently linked to cytoplasmic membrane.

Gram-negative bacterial cell wall

- The cell wall of Gram-negative bacteria (Fig. 2.5) is thinner (2 nm) than that of Gram-positive bacteria but is structurally more complex. It consists of peptidoglycan, lipoprotein, outer membrane, and lipopolysaccharide.
- Peptidoglycan layer is a single-unit thick. It constitutes 5–10% of the dry weight of the wall of Gram-negative bacteria.
- Outside the thin peptidoglycan layer, is the outer membrane. In this membrane are embedded various large molecules like outer membrane protein and porin protein. Large antibiotic molecules penetrate outer membrane relatively slowly, which accounts for the relatively high antibiotic resistance of Gram-negative bacteria. Outer membrane is anchored to the peptidoglycan layer by strongly lipophilic lipoprotein.
- A structural component that is unique to the Gram-negative outer membrane is **lipopolysaccharide (LPS)**. It consists of a complex lipid, called lipid A, to which is attached a core polysaccharide. From core polysaccharide extends outward O polysaccharide. It has several peculiar sugars and varies in composition between bacterial strains, conferring species-specific antigen specificity. It represents a major surface antigen of the bacterial cell. It is known as **O antigen**. Bacteria carrying LPS containing O antigen form smooth colonies in bacteriological media in contrast to those lacking O antigen, which form rough colonies. LPS is firmly bound to the cell surface and is released only when the cells are lysed. It is, therefore, known as **endotoxin** and is extremely toxic to animals. All the toxicity of the endotoxin is due to lipid A.
- The space between the inner membrane (cytoplasmic membrane) and outer membrane is known as **periplasmic space**. It contains the peptidoglycan layer and a gel-like solution of proteins.

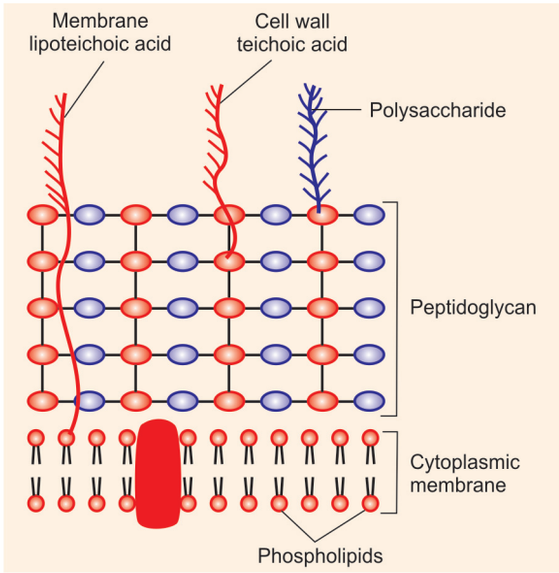


Fig. 2.4: Gram-positive cell wall.

The differences between cell walls of Gram-positive and Gram-negative bacteria are given in Table 2.2.

Acid-fast cell wall

Certain genera (*Mycobacterium* and *Nocardia*) have a Gram-positive cell wall structure but, in addition, contain a waxy layer of glycolipids and fatty acids (**mycolic acid**) bound to the exterior of the cell wall. This makes *Mycobacterium* species difficult to stain with the Gram stain. The mycobacteria and nocardiae can be stained with an acid-fast stain, in which the bacteria are stained with carbol fuchsin, followed by treatment with sulphuric acid or acid alcohol as decolourizer. Other bacteria are decolourized, whereas mycobacteria and nocardiae retain the stain. They are, therefore, known as **acid-fast bacteria**.

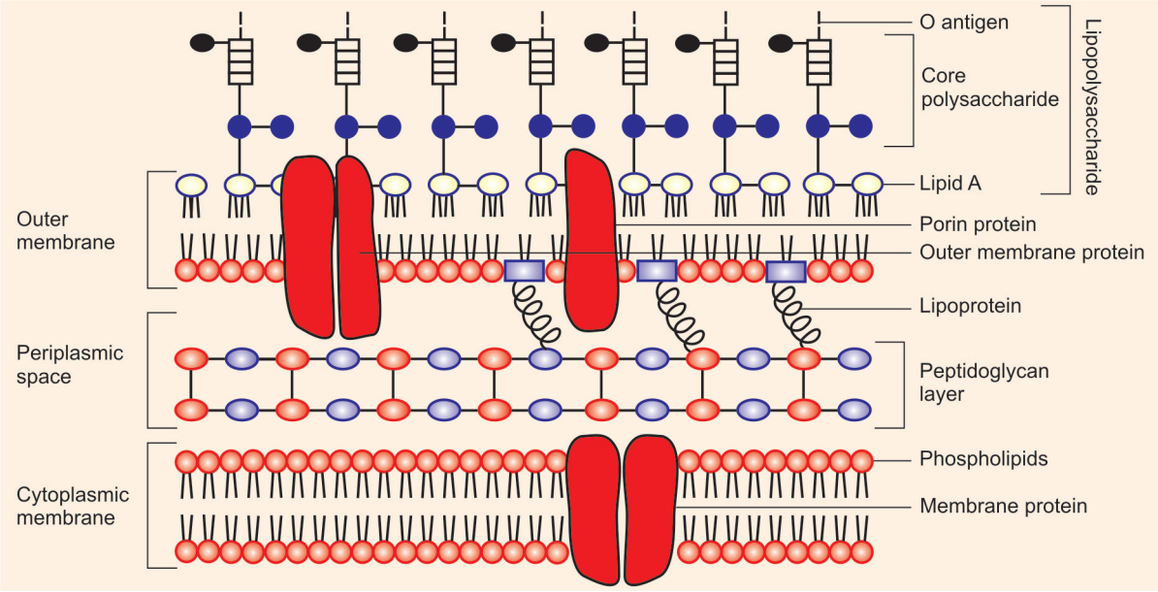


Fig. 2.5: Gram-negative cell wall.

Table 2.2: Differences between cell walls of Gram-positive and Gram-negative bacteria

Character	Gram-positive cell wall	Gram-negative cell wall
1. Thickness	Thicker	Thinner
2. Peptidoglycan	Thick layer (16–80 nm)	Thin layer (2 nm)
3. Lipid content	2–5%	15–20%
4. Teichoic acid	Absent	Present
5. Porin proteins	Absent	Present
6. Variety of amino acids	Few	Several
7. Periplasmic space	Absent	Present
8. Endotoxic activity	Absent	Present

Demonstration of cell wall

The cell wall cannot be seen by light microscopy and does not stain with simple dyes. It can be demonstrated by:

- Plasmolysis
- Microdissection
- Exposure to specific antibody
- Mechanical rupture of the cell
- Differential staining procedure
- Electron microscopy.

Protoplasts and spheroplasts

The cell wall may be removed by treating the bacteria with lysozyme, which is normally present in animal secretions (tears, saliva, nasal secretions) and in egg white. It acts by hydrolyzing linkages of the peptidoglycan backbone.

Protoplast

When a Gram-positive bacterium is treated with lysozyme in a hypotonic solution it lyses. If the osmotic strength of the solution is raised to balance the internal osmotic pressure of the cell, a free protoplast is liberated consisting of cytoplasmic membrane and its contents.

Spheroplast

When Gram-negative bacteria are treated with lysozyme, the outer membrane of the cell wall prevents access of lysozyme unless disrupted by an agent such as ethylenediamine-tetraacetic acid (EDTA). Lysozyme-EDTA treated bacteria result in the formation of spheroplasts which still possess remnants of the complex Gram-negative cell wall.

Removal of the bacterial cell wall may also be accomplished by growing the organisms in the presence of a substance such as penicillin, bacitracin or cycloserine that blocks peptidoglycan biosynthesis. A similar result may be obtained by growing the organisms on a medium lacking nutrients like diaminopimelic acid, lysine or hexosamine which are essential for cell wall synthesis.

If maintained on osmotically protective medium, protoplasts metabolize and grow in size but they do not multiply. Spheroplasts, on the other hand, when kept on osmotically protective agar medium containing cell wall inhibitor such as penicillin, may multiply by fission or budding and reproduce through many serial subcultures.

Because spheroplasts retain a residual cell wall, therefore, they are osmotically less sensitive than protoplasts and are often capable of growing on an ordinary agar medium. Spheroplasts are capable of reverting to parent bacterial form when cell wall inhibitor is removed from the culture medium. Protoplasts perhaps cannot do so. Because of their resemblance with L-forms of bacteria, spheroplasts may be called **unstable L-forms**.

Cytoplasmic membrane

Bacterial cytoplasmic membrane, also called cell membrane, limits the bacterial protoplast externally. It is thin (5–10 nm), elastic and consists of a phospholipid bilayer in which various

constituent proteins are embedded (Figs 2.4 and 2.5). With the exception of *Mycoplasma*, bacterial cytoplasmic membrane lacks sterols. **It acts as a semipermeable membrane controlling the inflow and outflow of metabolites to and from the protoplasm.** It permits the passive diffusion of water and other small molecular substances inward and outward, but it actively effects the selective transport of specific nutrients into the cell and that of waste products out of it. This is mediated through specific enzymes (permeases) present in the cytoplasmic membrane.

Cytoplasm

Cytoplasm of the bacterial cell is a viscous watery solution of soft gel, containing a variety of organic and inorganic solutes. It contains all biosynthetic components required by the bacterium for the growth and cell division, together with genetic material. The cytoplasm of bacteria differs from that of higher eukaryotic organisms in not containing endoplasmic reticulum, Golgi apparatus, mitochondria, lysosomes and in not showing signs of internal mobility, e.g. cytoplasmic streaming, the formation, migration and disappearance of vacuoles and amoeboid movement. Cytoplasm contains ribosomes, mesosomes and intracytoplasmic inclusion bodies (Fig. 2.2).

Ribosomes

Ribosomes are composed of ribosomal RNA (rRNA) and ribosomal proteins and are designated by their sedimentation coefficient (S or Svedberg unit). They are slightly smaller than those of eukaryotic cells. They measure 10–20 nm in diameter and have a sedimentation coefficient of 70S. Each 70S particle is composed of a 30S and a 50S subparticle. Each cell contains thousands of ribosomes strung together on strands of messenger RNA (mRNA) to form polysomes and it is at this site that code of mRNA is translated into peptide sequences. There are certain considerable differences between bacterial and host cell ribosomes. This allows us to use antibacterial agents such as streptomycin which interfere with bacterial metabolism at the ribosomal level without unduly upsetting human ribosomal function.

Mesosomes

These are convoluted or multilaminated membranous bodies which develop by invagination of cytoplasmic membrane into the cytoplasm (Fig. 2.2). They provide increased membrane surface and are principal sites of respiratory enzymes in bacteria. They are analogous to mitochondria of eukaryotes and are more prominent in Gram-positive bacteria. There are two types of mesosomes—septal and lateral. The septal mesosome is attached to bacterial chromosome and is involved in DNA segregation and in the formation of cross walls during binary fission.

Intracytoplasmic inclusions

Many species of bacteria produce cytoplasmic inclusion bodies which appear as round granules. They are not permanent or essential structures and may be absent under certain conditions of growth. They are large polymeric complexes consisting of volutin (polyphosphates), lipid, glycogen, starch or sulphur. Generally, they are present in larger number when bacteria have access to an abundance of energy-yielding nutrients and diminish or disappear under conditions of energy source starvation.

Bacterial nucleus

The genetic information of a bacterial cell is contained in a single, circular, double-stranded molecule of DNA. It is often accompanied by a smaller extrachromosomal DNA known as **plasmid**. It is 1000 μm or more in length, about 1000 times the length of the cell. Therefore, it occurs tightly coiled like a skein of woollen thread. Since it is not bound to proteins, therefore, it does not stain like a eukaryotic chromosome. **Bacterial nucleus does not possess nuclear membrane, nucleolus, deoxyribonucleoprotein and does not divide by mitosis.**

Capsule and slime layer

Cell wall in many bacteria is enclosed by a protective gelatinous covering layer. If it is easily washed off and does not appear to be associated with the cell in any definite fashion it is referred to as a **slime layer**, on the other hand, if it appears as discrete, thickened gel around each cell, it is called a **capsule** (Figs 2.2 and 2.6). If capsule is too thin to be seen with light microscope, it is called **microcapsule**. In most species, it is made up of a complex polysaccharide (e.g. pneumococcus), though in some species its main constituent is polypeptide (e.g. anthrax bacillus). When slime forming bacteria are grown on a solid culture medium, the slime remains around the bacteria as a matrix in which they are embedded and its presence confers on growth a **mucoid** character.

Demonstration of capsule

- Capsule cannot be stained with ordinary stains like Gram staining.
- It can be visualized by suspending the organisms in India ink and observing microscopically the exclusion of the colloidal ink particles from the area around the cell that is occupied by the capsule.
- It may also be visualized by reaction with specific antibody, which causes a characteristic swelling of the capsule. It is known as **Quellung reaction**. This phenomenon is seen in and allows rapid identification of capsular serotypes of *Streptococcus pneumoniae*, *Neisseria meningitidis*, several groups of streptococci, *Klebsiella* and *Haemophilus influenzae*.

For demonstration of polypeptide capsule of *Bacillus anthracis* (McFadyean reaction) refer to Chapter 26.

Functions

- Capsules protect the bacteria from antibacterial agents such as lytic enzymes found in nature.
- They inhibit phagocytosis, thus contributing to the virulence of the bacteria.
- The capsular antigen is specific for bacteria, therefore, it can be used for identification and typing of bacteria.

Loss of capsule by mutation may render the bacterium avirulent. Bacteria tend to lose capsules on repeated sub-cultures *in vitro*.

Flagella

Flagella are long, hollow, helical filaments usually several times the length of the cell. They are 10–20 nm in diameter, 3–20 µm in length and are found on both Gram-positive and Gram-negative bacteria. Their number varies from 1 to 20 flagella per bacterial cell. They are organelles of locomotion.

Arrangement

There are four types of arrangement of flagella (Fig. 2.6):

1. **Monotrichous:** These organisms have a single polar flagellum.
2. **Lophotrichous:** They have a tuft of flagella at one pole.
3. **Amphitrichous:** They have single polar flagellum or tuft of flagella at both poles.
4. **Peritrichous:** Flagella are distributed all around the cell.

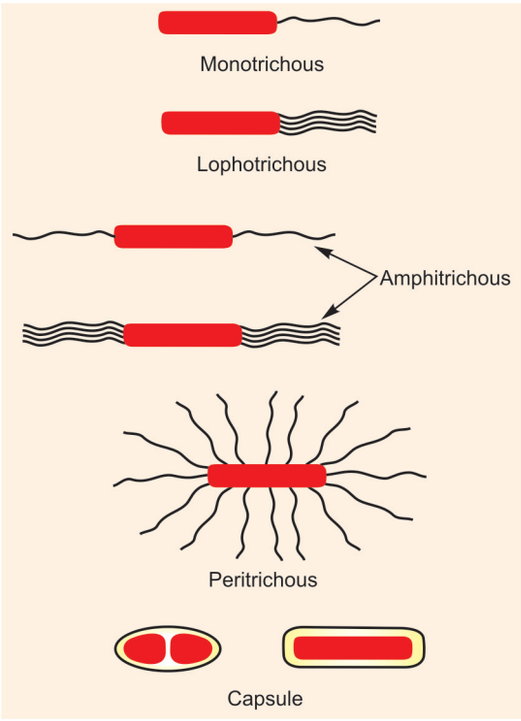


Fig. 2.6: Arrangement of flagella and capsule.

Flagella consist mainly of a protein called flagellin which belongs to the same chemical group as myosin—the contractile protein of muscle. Though, flagella of different genera of bacteria have the same chemical composition, they are antigenically different. Flagellar antigens induce specific antibodies in high titres. These antibodies are non-protective but are useful in serodiagnosis.

Demonstration of flagella

Flagella can be demonstrated by:

- Ordinary light microscope by special staining techniques in which their thickness is increased by mordanting.
- Dark-ground microscopy.
- Electron microscopy.

Indirect methods by which motility of bacteria can be demonstrated

- Occurrence of spreading growth in semisolid agar medium.
- On microscopic examination of wet films, motile bacteria are seen swimming in different directions across the field with darting (*V. cholerae*), very active (*Proteus* spp.), active (*Escherichia coli*), sluggish and tumbling (*Listeria monocytogenes*) motility. True motility should be differentiated from Brownian movement which is a rapid oscillation of the bacterium within a very limited area due to bombardment by the water molecules.

Structure of flagellum

The flagellum consists of three parts—the **filament**, the **hook** and the **basal body**. The basal body, anchored in the cytoplasmic membrane, comprises a rod and two or more sets of encircling rings. In Gram-negative bacteria four types of rings (M, S, P and L) are seen. Through ring M it attaches to the cytoplasmic membrane, ring S is located just above cytoplasmic membrane and through rings P and L it is attached to peptidoglycan and outer lipopolysaccharide membrane, respectively (Fig. 2.7). Rings P and L are absent in Gram-positive bacteria.

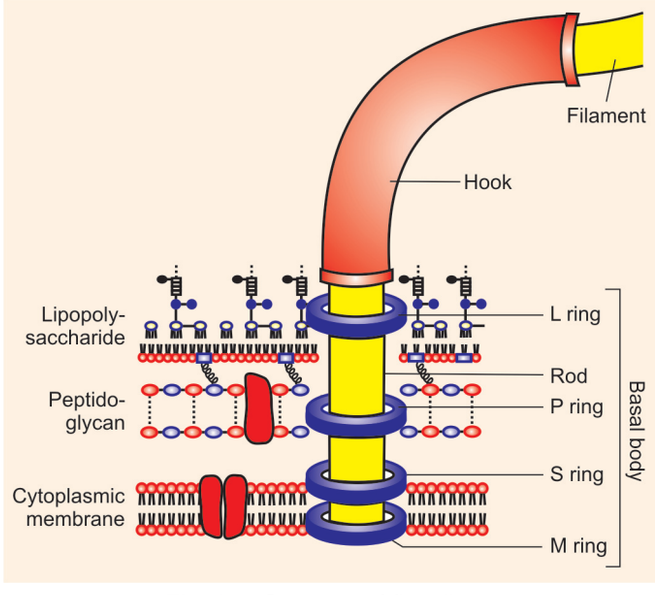


Fig. 2.7: Structure of flagellum.

Fimbriae or pili

They are hair-like surface appendages 1–1.5 µm in length and 4–8 nm in diameter. They are straighter, thinner and shorter than flagella (Fig. 2.2). They are present on many Gram-negative cells and provide a means for adherence to other cells, either bacterial or animal. They are an example of a class of surface structures termed **adhesins** that allow attachment of bacterial cell-to-cell surfaces (organelles of

adhesion). Therefore, they are very important for bacterial survival in an animal host. They occur in both flagellated and non-flagellated bacteria and are far more numerous than flagella. Each bacterium possesses 100–500 peritrichously-borne fimbriae. They can be seen by electron microscopy. They originate in the cytoplasmic membrane and are composed of self-aggregating protein monomers.

In stagnant liquid medium, the fimbriate bacteria grow attached together in the form of a pellicle that floats on the surface of the medium where the growth is greatly enhanced by the free supply of oxygen.

Certain bacteria possess specialized fimbriae or pili which are longer and thinner than the common type. These appear to be hollow and constitute conjugation tubes through which DNA is transferred from one organism to another during conjugation. They are determined by sex factors and are referred to as **sex pili** or conjugation pili.

Detection of fimbriae

- Electron microscopy.
- Haemagglutination: Most of the fimbriae can adhere to the red blood cells (RBCs) of many animal species. They bind very strongly to guinea pig, fowl, horse and pig; moderately strongly to human; and scarcely to ox RBCs. Therefore, a simple haemagglutination test can be used to determine whether a culture contains fimbriate bacilli or not.

Bacterial spore

Some species of bacteria (Gram-positive only), particularly those of the genera *Bacillus* and *Clostridium*, are capable of forming spores inside original cell. These spores can be released from original cell as free spores. Each bacterium forms one spore which on germination forms a single vegetative cell. Sporulation in bacteria, therefore, is a method of preservation and not of reproduction.

Sporulation

It develops from a portion of protoplasm near one end of the cell. This part of the bacterial cell is known as **forespore** and the remaining part as **sporangium** (Fig. 2.8). Bacterial DNA replicates and partitions into two halves and one of them, which is equivalent to one genome of the cell, is incorporated into forespore. A transverse septum derived from the cytoplasmic membrane is then formed by a process of invagination which divides forespore and sporangium. The forespore is, subsequently, completely encircled by dividing septum as a double layered membrane.

The two spore membranes now engage in active synthesis of various layers of the spore. The inner layer becomes the **inner membrane**. Between the two layers is laid **spore cortex** and outer layer is transformed into **spore coat** which consists of several layers. In some species from outer layer

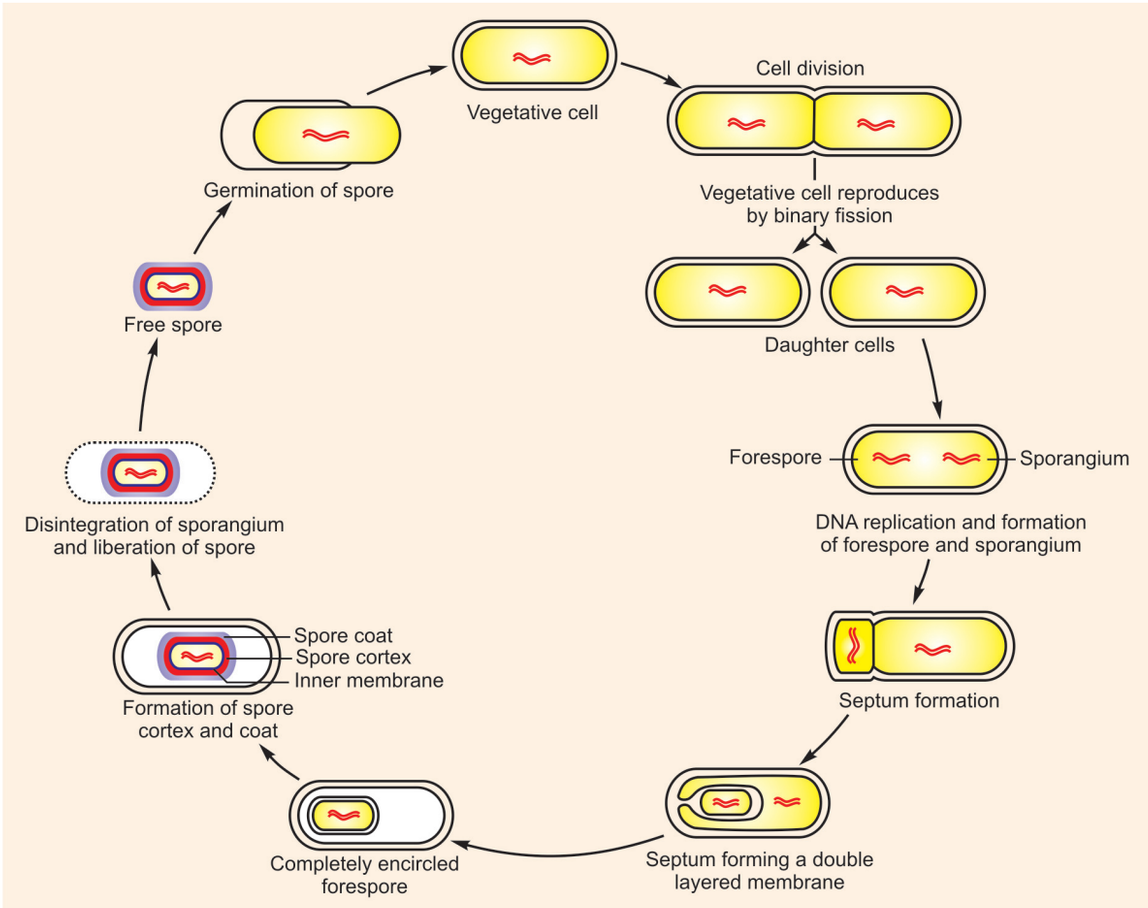


Fig. 2.8: Morphological events in sporulation.

also develops **exosporium**, which bears ridges and folds (Fig. 2.9). Finally, sporangium disintegrates and the spore is freed. Mesosomes appear to play a role in the development of spores and may be involved in the compartmentation of the spore's share of the nuclear material.

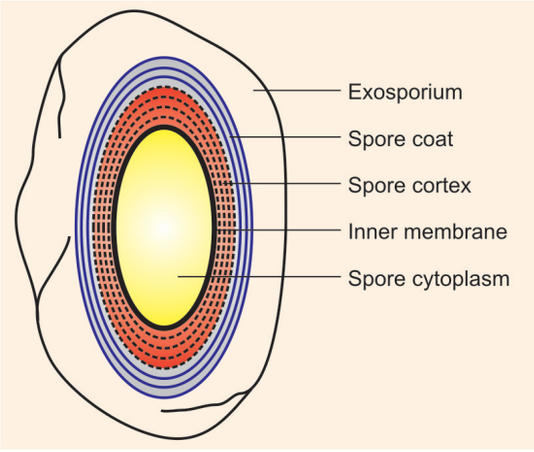


Fig. 2.9: Bacterial spore.

Shape and position

The spores may be round, oval or elongated occupying a terminal, subterminal or central position. They may be narrower than the width of the bacilli or broader and bulging (Fig. 2.10).

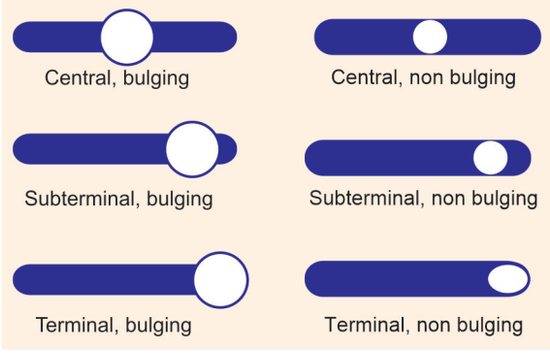


Fig. 2.10: Types of spores.

Resistance

Spores can remain dormant for many years. They are extremely resistant to chemical and physical agents. Their killing requires moist heat at 100–120°C for 10 minutes while vegetative cells can be killed by heating at 60°C for 10 minutes. Marked resistance of the spores is due to:

- The impermeability of their cortex and outer coat,
- Their high content of calcium and dipicolinic acid,
- Their low content of water, and
- Their very low metabolic and enzymatic activity.

Germination

Spores are able to **germinate** when the external conditions become favourable to growth by access to moisture and nutrients. It swells after which the spore coat ruptures, and a new vegetative cell grows out.

Demonstration

- In unstained preparations, the spore is recognized within the parent cell by its greater refractility. In simple stains like Gram it remains unstained and appears as a clear space within the stained cell protoplasm (Fig. 2.11).
- They are slightly acid-fast and may be demonstrated by modified Ziehl-Neelsen staining.

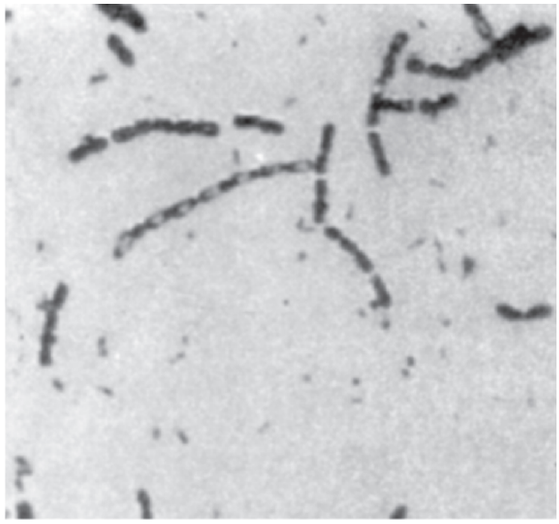


Fig. 2.11: Gram-stained smear of *Bacillus subtilis* showing Gram-positive bacilli in chains with spores which appear as unstained areas within the bacilli (×1,000).

L-forms of bacteria

L-forms (after Lister Institute, London) of bacteria are cell wall deficient bacteria derived by variation, usually in the laboratory, from bacteria of normal morphology. They are stable in the sense that special conditions of culture, such as presence of penicillin, are not required to prevent their reversion to the parental bacterial forms. They lack regular size and shape. They may be spherical or disc-like and measure 0.1–20 μm in diameter.

Cultural characteristics

L-forms are difficult to grow and usually require a medium that is solidified with agar as well as having the right osmotic strength. L-forms are produced more readily with penicillin than with lysozyme.

Colonies of L-forms of bacteria on agar medium show a characteristic **'fried-egg'** appearance with a dark thick centre, where many of the organisms embed themselves and grow within the agar, and a lighter periphery consisting of organisms lying on the surface of the agar. In liquid medium they grow in the form of clumps. Some L-forms are capable of reverting to normal bacillary forms upon removal of the inducing stimulus. Other L-forms are, however, stable and never revert. Presence of residual peptidoglycan is essential for reversion. It acts as a primer in its own biosynthesis.

KEY POINTS

- Prokaryotes such as ***bacteria are simple cells*** with no internal membranes or organelles.
- ***Eukaryotes have a nucleus and organelles*** such as mitochondria, and complex ***internal membranes*** (e.g. fungi and human cells).
- ***Structures external*** to cell wall of bacteria are ***flagella, pili*** or ***fimbriae, capsule*** and ***slime layer***.
- Flagella are used for movement, pili for adhesion, and capsules protect the bacteria from antibacterial agents such as lytic enzymes and inhibit phagocytosis thus contributing to the virulence of bacteria.
- ***Peptidoglycan is present in the cell wall of both Gram-positive and Gram-negative bacteria***, but is thicker in the former and gives rigidity and shape to the organism.
- Peptidoglycan comprises long chains of *N*-acetylglucosamine and *N*-acetylmuramic acid; a set of identical tetrapeptide side chains attached to *N*-acetylmuramic acid, and a set of identical pentapeptide cross-bridges.
- In the outer membrane of Gram-negative bacteria are embedded various large molecules like outer membrane protein and porin protein. Large antibiotic molecules penetrate outer membrane relatively slowly, which accounts for the ***relatively high antibiotic resistance of Gram-negative bacteria***.
- Lipopolysaccharide (LPS) is the outermost layer of outer membrane of Gram-negative (but not Gram-positive) bacteria; ***LPS is endotoxin*** and, therefore, Gram-positive bacteria do not produce endotoxin.
- ***Bacterial cytoplasm contains*** chromosomal nuclear material, ribosomes, mesosomes and inclusions/storage granules.
- ***Sporulation*** is a response to starvation in ***Bacillus*** spp. and ***Clostridium*** spp. They are a means of survival, not reproduction.

Important Questions

1. Differentiate between prokaryotes and eukaryotes in a tabulated form.
2. Draw a labelled diagram of a bacterial cell.
3. Describe the cell wall of bacteria.
4. Differentiate between flagella and fimbria in a tabulated form.
5. Write short notes on:
   (a) Capsule
   (b) Intracytoplasmic inclusions in bacteria
   (c) Bacterial spore
   (d) Mesosomes

Multiple Choice Questions

1. Which of the following bacteria is cell wall deficient?
   (a) *Mycoplasma*.
   (b) *Treponema*.
   (c) *Staphylococcus*.
   (d) *Klebsiella*.
2. Chinese letter arrangement is characteristic of:
   (a) *Mycobacterium tuberculosis*.
   (b) *Bacillus anthracis*.
   (c) *Corynebacterium diphtheriae*.
   (d) *Clostridium tetani*.
3. Peptidoglycan is major constituent of cell wall of:
   (a) Gram-positive bacteria.
   (b) Gram-negative bacteria.
   (c) fungi.
   (d) protozoa.
4. Lipopolysaccharide is a constituent of cell wall in:
   (a) Gram-positive bacteria.
   (b) Gram-negative bacteria.
   (c) fungi.
   (d) protozoa.
5. Sterols are present in the cytoplasmic membrane of:
   (a) *Mycoplasma*. (b) *Bacillus*.
   (c) *Clostridium*. (d) *Proteus*.
6. Mesosomes of bacteria are analogous to:
   (a) mitochondria of eukaryotes.
   (b) lysosomes of eukaryotes.
   (c) Golgi apparatus of eukaryotes.
   (d) nucleolus of eukaryotes.
7. When flagella are distributed all around the bacterial cell, the arrangement is known as:
   (a) monotrichous.
   (b) lophotrichous.
   (c) amphitrichous.
   (d) peritrichous.
8. Ultraviolet light is used in:
   (a) fluorescence microscope.
   (b) dark-ground microscope.
   (c) phase-contrast microscope.
   (d) interference microscope.
9. Polymers *N*-acetylglucosamine and *N*-acetylmuramic acid are found in:
   (a) cell membrane.
   (b) cell wall.
   (c) outer membrane.
   (d) capsule.

10. Teichoic acid is present in:
    (a) Gram-negative organisms.
    (b) Gram-positive organisms.
    (c) *Mycoplasma*.
    (d) *Rickettsia*.

11. Cell wall deficient forms of Gram-positive bacteria are called:
    (a) spheroplasts.
    (b) trophozoites.
    (c) cysts.
    (d) protoplasts.

12. Sedimentation coefficient of bacterial ribosomes is:
    (a) 50S. (b) 60S.
    (c) 70S. (d) 80S.

13. Bacterial structure concerned with respiration is:
    (a) mesosomes.
    (b) ribosomes.
    (c) mitochondria.
    (d) Golgi apparatus.

14. Which of the following bacteria has peritrichous flagella?
    (a) *Vibrio cholerae*.
    (b) *Proteus*.
    (c) *Pseudomonas*.
    (d) *Shigella*.

15. Which of the following bacteria has a single polar flagellum?
    (a) *Vibrio*.
    (b) *Salmonella*.
    (c) *Proteus*.
    (d) *Citrobacter*.

16. Which of the following bacterial structures is involved in attachment to cell surface?
    (a) Flagella. (b) Fimbria.
    (c) Capsule. (d) Mesosomes.

17. Which of the following are eukaryotes?
    (a) Fungi.
    (b) Bacteria.
    (c) Chlamydiae.
    (d) Mycoplasmas.

18. Which of the following structures is found in Gram-negative and not in Gram-positive bacteria?
    (a) Capsule.
    (b) Cytoplasmic membrane.
    (c) Ribosomes.
    (d) Outer membrane.

19. Which of the following structures is found in Gram-positive and not in Gram-negative bacteria?
    (a) Capsule.
    (b) Cytoplasmic membrane.
    (c) Spores.
    (d) Outer membrane.

20. Which of the following is a distinctive component of the cell wall of Gram-negative bacteria?
    (a) Capsule.
    (b) Lipopolysaccharide.
    (c) Peptidoglycan.
    (d) Cytoplasmic membrane.

21. When determining distances and sizes, the smallest unit of measure is:
    (a) centimetre.
    (b) millimetre.
    (c) micrometre.
    (d) nanometre.

22. What function does a condenser serve in light microscope?
    (a) focuses the light onto our eyes.
    (b) focuses the light rays on the sample.
    (c) increases light intensity.
    (d) reduces glare.

23. Bacterial capsules are generally viewed by:
    (a) Albert staining.
    (b) Ziehl-Neelsen staining.
    (c) Negative staining.
    (d) All of the above.

24. Fimbriae present on the outer surface of bacteria are used for:
    (a) inhibition of phagocytosis.
    (b) bacterial motility.
    (c) adherence to surfaces.
    (d) none of the above.

Answers

1. a	**2.** c	**3.** a	**4.** b	**5.** a	**6.** a	**7.** d	**8.** a	**9.** b	**10.** b	**11.** d	**12.** c
13. a	**14.** b	**15.** a	**16.** b	**17.** a	**18.** d	**19.** c	**20.** b	**21.** d	**22.** b	**23.** c	**24.** c

CHAPTER 3

Growth and Nutrition of Bacteria

BACTERIAL GROWTH

Bacteria reproduce by a process called binary fission, in which a parent cell divides to form a progeny of two cells. This results in a logarithmic growth rate—one bacterium will produce 16 bacteria after four generations.

Generation time

The time required for a bacterium to give rise to two daughter cells is known as **generation time**. Under constant conditions, the generation time for any organism is quite reproducible, but differs greatly among different bacteria. The fastest growing bacteria have generation time of 15–20 minutes under optimum growth conditions. Many bacteria, however, have generation times of hours or even days. In *Escherichia coli* it is 20 minutes, in tubercle bacilli it is 14–15 hours and in lepra bacilli it is 12–13 days.

Batch culture

When bacteria are grown in liquid medium, multiplication is arrested after a few cell divisions due to depletion of nutrients and/or accumulation of toxic products. This is known as batch culture.

Continuous culture

By use of special devices like chemostat or turbidostat in which nutrients are replaced and bacteria are removed continuously it is possible to maintain continuous culture of bacteria for industrial and research purposes.

When pathogenic bacteria multiply in the host tissues the situation is intermediate between batch culture and continuous culture because they get inexhaustible source of nutrients but they have to face host defence mechanisms.

In liquid media, growth of bacteria is diffuse and on solid media they form colonies. Each colony consists of a clone of cells derived from a single parent cell. Bacteria in a culture medium or clinical specimen can be counted by two methods:

1. Total count

This is total number of bacteria present in a specimen irrespective of whether they are living or dead. This is done by counting the bacteria under microscope using counting chamber and by comparing the growth with standard opacity tubes.

2. Viable count

This measures only viable (living) cells which are capable of growing and producing a colony on a suitable medium.

BACTERIAL GROWTH CURVE

When a bacterium is inoculated into a suitable culture medium and incubated, its growth follows a characteristic course. If both total and viable counts are made at different intervals and plotted in relation to time, then a characteristic growth curve is obtained. A typical growth curve contains four major phases (Fig. 3.1).

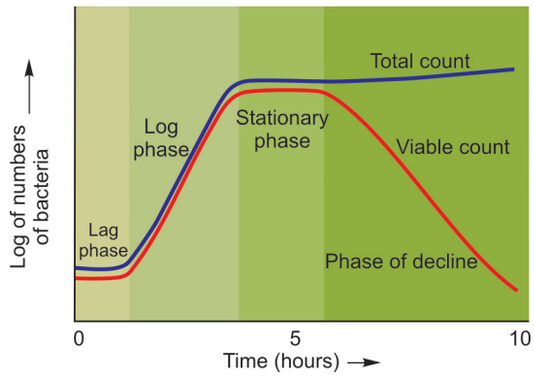


Fig. 3.1: Bacterial growth curve.

1. Lag phase

When bacteria are seeded into fresh medium, multiplication usually does not begin immediately. The period between inoculation and beginning of multiplication is known as lag phase. **During this period the organisms adapt themselves to growth in fresh medium and increase in size and metabolic activity. Therefore, lag phase is regarded as a period not of rest but of intense metabolic activity**.

The duration of lag phase varies with the species, nature of culture medium, temperature of incubation, etc. Depending

on the growth medium, the lag phase may be short or very long. For example, if a culture in a rich growth medium that supplies most of the cells' requirements is inoculated into a poor medium that requires the cells to make most of their own amino acids and vitamins, the lag phase will be very long. The cells must activate the metabolic pathways for amino acid and vitamin synthesis and must make enough of these nutrients to begin active growth. In contrast, the cells that are inoculated from one medium to a fresh tube of the same medium may show virtually no lag phase, since they need not change their metabolism.

2. Log or exponential growth phase

During this phase the bacteria are multiplying at their maximum rate and their number increases exponentially or by geometric progression with time. If logarithm of bacterial count is plotted against time a straight line is obtained. **In the log phase, the bacterial cells are smaller and stain uniformly.** Exponential phase is of limited duration because of:

- Exhaustion of nutrients,
- Accumulation of toxic metabolic end products,
- Rise in cell density,
- Change in pH, and
- Decrease in oxygen tension (in case of aerobic organisms).

3. Stationary phase

Due to above reasons exponential growth slows down and the bacterial population enters the stationary phase in which the number of viable cells remains constant. There is almost a balance between the bacterial reproduction and bacterial death. **During this phase, bacteria become Gram-variable, show irregular staining and spores start forming in spore-forming bacteria.**

4. Phase of decline

Stationary phase is followed eventually by the phase of decline because rate of death exceeds the rate of reproduction and the number of viable cells declines. **Finally, after a variable period, all the cells die and culture becomes sterile.**

Bacterial nutrition

The growth of microorganisms depends upon an adequate supply of suitable nutrients, pH, oxygen and temperature. They require the elements present in their chemical composition. Nutrients must provide these elements in a metabolically accessible form. All bacteria have three major nutritional needs for growth:

1. A source of carbon for making cellular constituents.
2. A source of nitrogen for making proteins.
3. A source of energy (ATP) in order to synthesize macromolecules and maintain essential chemical gradients across their membranes.

Nutritional requirements for growth

Bacteria are divided into two basic groups according to how they meet their nutritional needs:

1. Autotrophs

Some organisms possess considerable synthetic power, therefore, they can utilize very simple inorganic compounds, such as carbon dioxide as carbon source and ammonium salts as nitrogen source. These are known as autotrophs or lithotrophs. Autotrophs obtain energy either photosynthetically (phototrophs) or by oxidation of inorganic compounds (chemotrophs).

2. Heterotrophs

Bacteria that are unable to synthesize their own metabolites and depend on preformed organic compounds are known as heterotrophs. They require an organic source of carbon, such as glucose, and obtain energy by oxidizing or fermenting organic substances. Often, the same substance (for example, glucose) is used as both the carbon source and energy source. Some bacteria require certain organic compounds in minute quantities. These are known as growth factors or bacterial vitamins.

All bacteria that inhabit the human body fall into the heterotrophic group. Within this group, however, nutritional needs vary greatly. Some bacteria like *E. coli* can grow under a wide range of conditions while others like *Neisseria gonorrhoeae* and *Haemophilus influenzae* are exacting and restrictive in their requirements.

CULTURE MEDIA

Numerous culture media have been devised. The original media used by Louis Pasteur were liquids such as urine or meat broth. Liquid media have many disadvantages. Bacteria growing in these media may not exhibit specific characteristics for their identification. With liquid media it is difficult to isolate different types of bacteria from mixed populations. However, liquid media are used for obtaining bacterial growth from blood or water when large volumes have to be used as inoculum, for preparing bulk cultures for antigens and vaccines, and for preparation of inoculum for biochemical reactions and antibiotic susceptibility testing.

In 1881, Robert Koch described means of cultivating bacteria on solid media. First he used as his growth medium pieces of potato, then 2.5–5.0% gelatin to prepare solid media fortifying them with 1% meat extract as an essential ingredient. But gelatin is not satisfactory as it liquefies at 24°C (incubation temperature for most pathogenic bacteria is 37°C) and many proteolytic bacteria liquefy gelatin. At the suggestion of Anglina Hesse, the American wife of his assistant, he substituted agar-agar in place of gelatin as solidifying agent for the media. She had used it to solidify broths in her kitchen.

Agar-agar or '**agar**' for short is prepared from a variety of seaweeds; the product is clarified, dried and supplied as a powder. It does not add to the nutritive properties of medium and is not affected by the growth of bacteria. The exact concentration to be used may require some adjustment according to the batch of agar. A concentration of 1–2% usually yields a suitable gel. In preparing agar media, the

appropriate amount of agar powder is added to the liquid medium and dissolved by placing the mixture in a steamer at 100°C for 1 hour or longer. Most agars dissolve to give a clear solution but sometimes it is necessary to filter off particulate impurities.

The melting and solidifying points of agar solutions are not the same. At the concentrations normally used, most bacteriological agars melt at about 95°C and solidify only when cooled to about 42°C. Agar can be added to any nutrient liquid medium if the advantages of a solid medium are desired. Most of the culture media are sterilized by autoclaving at 121°C for 15 minutes. Nutrients that are damaged by autoclaving are sterilized separately by filtration, etc. The sterilized agar base is then melted in the steamer and cooled to about 45–50°C followed by addition of heat-labile ingredients, but once these are added the medium must at once be poured into Petri dishes because it cannot be remelted without damaging the heat-sensitive ingredients.

Another important ingredient of common media is **peptone**. It consists of water-soluble products obtained from lean meat or other protein material such as heart muscle, casein, fibrin or soya flour, usually by digestion with the proteolytic enzymes pepsin, trypsin or papain. Its constituents are peptones, proteoses, amino acids, a variety of inorganic salts including phosphates, potassium and magnesium, and certain accessory growth factors such as nicotinic acid and riboflavin. Special brands of peptone such as neopeptone and proteose peptone are available for special use.

Other common ingredients of the culture media include casein hydrolysate, meat extract, yeast extract, malt extract, blood and serum.

While bacteria grow diffusely in liquid media, they produce discrete visible growth on solid media in Petri dishes. If a mixed culture is inoculated in suitable dilution on solid medium, different bacteria form well-separated colonies, which are clones of cells originating from a single bacterial cell. On solid media, bacteria have distinct colony morphology and exhibit many other characteristic features such as pigment production or haemolysis.

Types of culture media

Culture media have been classified in many ways:

1. Solid, semisolid and liquid.
2. Simple (basal), complex, synthetic, defined, semidefined and special media. Special media are further divided into enriched, selective, enrichment, indicator or differential, sugar media and transport media.
3. Aerobic media and anaerobic media.

Basal media

These include peptone water, and nutrient broths which form the basis of most media used in the study of the common pathogenic bacteria.

Meat extract broth is most commonly used nutrient broth. It consists of peptone, meat extract, sodium chloride and water. It can be made solid by addition of 1–2% agar. If the concentration of agar is reduced to 0.2–0.5%, it is called **semisolid agar**. If its concentration is raised to 6%, it is called **hard agar**. In semisolid agar the motile organisms show growth in entire medium, and on surface of hard agar swarming of *Proteus* is inhibited.

Enriched media

These are prepared to meet the nutritional requirements of fastidious organisms by addition of substances such as blood, serum and egg to a basal medium. Important examples of enriched media are blood agar for isolation of *Streptococcus*, chocolate agar for isolation of *Neisseria* and *Haemophilus*, and Loeffler's serum slope for the isolation of *Corynebacterium diphtheriae*.

Selective media

When a substance is added to a solid medium which inhibits the growth of unwanted bacteria but permits the growth of wanted bacteria in the form of colonies, it is known as selective medium. Important examples of this type of media are MacConkey agar for *E. coli*, deoxycholate citrate agar (DCA) for *Salmonella* and *Shigella*, Wilson and Blair's medium for *Salmonella*, Lowenstein-Jensen medium for *Mycobacterium tuberculosis*, and blood tellurite agar medium for isolation of *Corynebacterium diphtheriae*.

Enrichment media

When a substance is added to a liquid medium which inhibits the growth of unwanted bacteria and favours the growth of wanted bacteria it is known as enrichment medium. Important examples of this type of media are tetrathionate broth and selenite F broth for *Salmonella* and *Shigella*, and alkaline peptone water for *Vibrio cholerae*.

Indicator media or differential media

When a substance is added into a medium which would produce a visible change in the medium following the growth of a particular organism, it is designated as indicator or differential medium. For example, MacConkey medium contains lactose and neutral red. Lactose fermenting organisms, after growth on this medium, produce acid and in acidic pH neutral red becomes red in colour. Thus, *E. coli* which is lactose fermenter produces red or pink colonies on this medium.

Christensen's medium contains urea and phenol red. When urease producing organisms like *Proteus* and *Klebsiella* grow on this medium, urea is split up into ammonia and carbon dioxide. Ammonia makes the medium alkaline and in alkaline pH the medium becomes pink in colour (in alkaline pH phenol red is pink in colour). Enriched media can also be differential on the basis of certain growth characteristics evident on the medium. Blood agar is considered both an enriched and differential medium because it differentiates organisms based on whether they are α-haemolytic, β-haemolytic or non-haemolytic.

Transport or holding media

When a clinical sample is being transported from the hospital to the laboratory, delicate organisms like *Neisseria*

gonorrhoeae may not survive or the normal flora (*E. coli*) may overgrow pathogenic flora (*Salmonella*, *Shigella* and *V. cholerae*). Transport media maintain the viability of microorganisms present in a specimen without supporting the growth of any organism. These maintain the organisms in a state of suspended animation so that no organism overgrows another or dies out. These media typically contain only buffers and salts. They lack carbon, nitrogen and organic growth factors, hence do not facilitate microbial multiplication. Stuart's transport medium and Amies transport medium are examples of transport media.

Storage media

Bacteria are best preserved and stored by lyophilization. But for preservation and storage for a few months or so, they can be stab inoculated on semisolid agar or on Dorset egg medium followed by incubation. When growth appears they can be stored in refrigerator.

Defined synthetic media

These media are prepared from pure chemical substances, therefore, their exact composition is known. These are used for research purposes.

Sugar media

For the identification of most of the organisms, sugar fermentation reactions are carried out. Glucose, lactose, sucrose and mannitol are widely used sugars. For the preparation of sugar media, 1% of the concerned sugar is added to peptone water with a suitable indicator. Durham's tube (a small tube) is kept inverted in the tube containing this medium to detect gas production. For fastidious organisms like *C. diphtheriae* and pneumococci, Hiss's serum sugar media are used.

Anaerobic media

For the growth of anaerobes, the media used contain reducing substances. These include thioglycollate broth and cooked meat broth. The sterile muscle tissue, in cooked meat broth, contains reducing substances, particularly glutathione, which permit the growth of many strict anaerobes. In addition to its reducing effect, the meat provides a variety of nutritional substances for bacterial growth. In this medium, saccharolytic clostridia rapidly produce acid and gas but do not digest the meat. The cultures may have slight sour smell and the meat is often reddened. The proteolytic clostridia produce blackening of the meat, decomposing it and reducing it in volume with the formation of foul-smelling products.

ENVIRONMENTAL FACTORS INFLUENCING GROWTH

Oxygen

On the basis of the influence of oxygen on growth and viability, the bacteria are divided into two categories—aerobes and anaerobes:

- **Aerobes** require oxygen for their growth. They may be obligate aerobes like *Pseudomonas aeruginosa* which can grow only in the presence of oxygen, and facultative anaerobes. The latter are ordinarily aerobes, but they can also grow in the absence of oxygen though less abundantly.
- **Anaerobes**, on the other hand, are organisms that do not require oxygen for life and reproduction. In addition, oxygen's direct toxic effect may prohibit the growth of these organisms in environments in which oxygen is present. They may be obligate anaerobes such as *Clostridium tetani* which cannot grow even in the presence of traces of oxygen, and **microaerophilic** such as *C. perfringens* which can grow under microaerophilic conditions.

Carbon dioxide

Some organisms such as *Brucella abortus* require extra CO_2 in the air in which they are grown and others such as pneumococci and gonococci grow better in air supplemented with 5–10% CO_2 (**capnophilic**).

Temperature

Each bacterium multiplies best within a restricted temperature range. For most of the pathogenic bacteria optimum temperature for growth is 37°C (our body temperature) with upper and lower temperature limits of 40–50°C and 15–20°C, respectively. The organisms with optimum temperatures of 37°C, less than 20°C and 55–80°C are known as mesophiles, psychrophiles and thermophiles, respectively.

Moisture and desiccation

Moisture is very essential for the growth of bacteria because 80% of their body weight is made up of water. However, the effect of drying varies in different organisms. For example *Treponema pallidum*, gonococci and human immunodeficiency virus die quickly after drying while tubercle bacilli and staphylococci may survive drying for several weeks. However, bacterial spores can survive for several years, and drying in cold and vacuum (**lyophilization**) is a method for preservation of bacteria and viruses.

pH

Like other living organisms, microorganisms are very susceptible to changes in the acidity or alkalinity of the surrounding medium. Most of the medically important bacteria can grow at neutral or slightly alkaline pH (7.2–7.6). Some bacteria like lactobacilli and cholera vibrios grow at acidic and alkaline pH, respectively.

Light and other radiations

Darkness provides a favourable condition for growth and viability of bacteria. Ultraviolet rays from direct sunlight or a mercury lamp are bactericidal. Bacteria are also killed by ionizing radiations. Photochromogenic mycobacteria form pigment only on exposure to light.

Osmotic effect

Because of the mechanical strength of the cell wall, bacteria are more tolerant to osmotic variation, therefore, they can

grow in media with widely varying contents of salt, sugar and other solutes. Sudden exposure of bacteria to solutions of high salt concentration may cause **plasmolysis**. This is due to osmotic withdrawal of water leading to shrinkage of protoplast and its retraction from the cell wall. This occurs more readily in Gram-negative than Gram-positive bacteria. On the other hand, sudden transfer of bacteria from concentrated solution to distilled water may cause **plasmoptysis** due to excessive osmotic imbibition of water leading to swelling and bursting of cell.

Mechanical and sonic stresses

In spite of the mechanical strength of the cell wall, bacteria can be ruptured and killed by vigorous shaking with glass beads and ultrasonic vibrations.

CULTURE METHODS

The methods of bacterial culture used in the clinical laboratory include streak culture, lawn culture, stroke culture, stab culture, pour-plate culture, shake culture and liquid culture.

Streak culture

This method is routinely employed for the isolation of bacteria in pure culture from clinical specimens. A platinum or nichrome wire loop of 2–4 mm diameter is used. The loop is first sterilized in the bunsen flame by making it red hot and cooled by touching on uninoculated part of the medium. Then a loopful of the specimen is smeared thoroughly over area *A* (Fig. 3.2), on the surface of a well dried plate, to give a well-inoculum or 'well'. The loop is re-sterilized and drawn from the well in 2 or 3 parallel lines onto the fresh surface of the medium (*B*). This process is repeated as shown (*C*, *D* and *E*), care being taken to sterilize the loop, and cool it on unseeded medium, between each sequence. On incubation, growth may be confluent at the site 'well', but becomes progressively thinner, and well separated colonies are obtained over the final series of streaks.

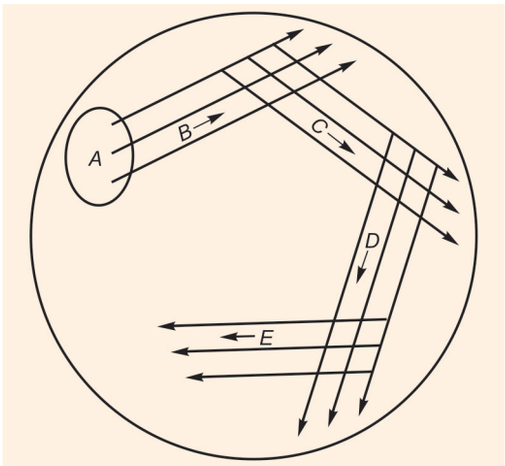


Fig. 3.2: Streak culture.

Lawn culture

Lawn cultures are prepared by flooding the surface of the plate with a liquid culture or suspension of bacteria and pipetting off the excess inoculum or by applying a swab soaked in the bacterial culture or suspension. After incubation, lawn culture provides a uniform surface growth. It is useful for antibiotic susceptibility testing by disc diffusion method and bacteriophage typing.

Stroke culture

Stroke culture is made in tubes containing agar slope or slant. Slopes are seeded by lightly smearing the surface of agar with loop in a zig-zag pattern taking care not to cut the agar. It is used for obtaining pure growth for slide agglutination and other diagnostic tests.

Stab culture

Stab cultures in solid media (nutrient gelatin or glucose agar) are inoculated by plunging the charged wire into the centre of the medium and withdrawing it in the same line to avoid splitting the medium. These are employed mainly for demonstration of gelatin liquefaction and for the maintenance of stock cultures.

Pour-plate culture

This method is used for counting the number of living bacteria or groups of bacteria in a liquid culture or suspension. Prepare serial 10-fold dilutions of the bacterial suspension over a range (6–9 tubes) ensuring that one dilution will contain between 50–500 viable bacteria/ml (number which can be accurately counted). Starting with the greatest dilution, pipette 1 ml amounts of each dilution into each of three 9 cm Petri dishes. Then pour into each dish about 10 ml of clear nutrient agar, melted, and cooled at 45–50°C. Mix well by rapidly moving the plate for about 10 seconds. Allow the agar to set and incubate at 37°C for 48 hours. After incubation, colonies will be seen well distributed throughout the depth of the medium and can be enumerated using colony counters. Count the colonies in three plates containing 50–500 colonies/plate. Multiply the average number/plate by the dilution factor to obtain the viable count/ml in the original suspension.

Shake culture

It is made by melting nutrient agar in a test tube, cooling it to 45°C and inoculating it while molten from a liquid medium with a drop from a capillary pipette. Withdraw the pipette, replace the cap or plug and discard the pipette into disinfectant. Mix the contents of the tube by rotation between the palms of the hands before the agar solidifies and incubate it at 37°C for 24 hours and look for the growth of the organisms.

Liquid culture

Liquid cultures in tubes, bottles or flasks may be inoculated by touching with a charged loop or by adding the inoculum with pipettes or syringes. Large inocula can be employed in

liquid cultures and hence this method is adopted for blood culture and for sterility tests, where the concentration of bacteria in inocula are expected to be small. Liquid cultures are also preferred when large yields are desired.

AEROBIC CULTURE

For cultivation of aerobes the incubation is done in an incubator under normal atmospheric condition. The temperature of incubation for most of the human pathogenic bacteria is 37°C. To prevent drying of the medium when prolonged incubation is necessary, as in the cultivation of the tubercle bacilli, screw-capped bottles should be used instead of test tubes or plates.

CULTURE IN AN ATMOSPHERE WITH ADDED CARBON DIOXIDE

Some organisms such as *Brucella abortus* and capnophilic streptococci, require extra CO_2 in the air in which they are grown and others, such as the pneumococcus and gonococcus grow better in air supplemented with 5–10% CO_2. For this, CO_2 jars are used. The required amount of air is withdrawn with a vacuum pump and replaced with CO_2 from a cylinder. CO_2 incubators which provide a predetermined and regulated amount of CO_2 in a suitably humid atmosphere are commercially available. Screw caps on containers of liquid media must not be tight and should preferably be replaced by a closure that allows entry of CO_2.

CULTURE IN MICROAEROPHILIC ATMOSPHERE

Microorganisms like *Campylobacter*, *Helicobacter pylori* and *Actinomyces israelii* are microaerophilic. Culture of such organisms is done by an evacuation replacement method with 5% O_2, 10% CO_2 and 85% N_2.

ANAEROBIC CULTURE

Anaerobic culture methods

A variety of methods are available for the culture of anaerobic organisms in the clinical laboratory. Exclusion of oxygen from the medium is the simplest method, and is effected by growing the organisms within the culture medium such as freshly steamed liquid media containing reducing agents such as glucose, ascorbic acid, cysteine, sodium thioglycollate and cooked meat pieces.

Cooked meat broth (**CMB**, an original medium known as 'Robertson's bullock-heart medium') has a special place in anaerobic bacteriology; and **thioglycollate broth** and its modifications are also very useful. CMB is suitable for growing anaerobes in air and also for the preservation of stock cultures of aerobic organisms. The inoculum is introduced deep in the medium in contact with the meat. Cooked meat pieces are placed in 30 ml bottles to a depth of about 2.5 cm and covered with about 15 ml broth.

Anaerobic Jars

When an oxygen-free or anaerobic atmosphere is required for obtaining surface growths of anaerobes, anaerobic jars provide the method of choice. The most reliable and widely used anaerobic jar is the **McIntosh-Fildes anaerobic jar**. It is a cylindrical vessel made of glass or metal with a metal lid which is held firmly in place by a clamp (Fig. 3.3). The lid has two tubes with taps, one acting as gas inlet and the other as the outlet. On its undersurface it carries a gauze sachet carrying alumina pellets coated with palladium. It acts as a room temperature catalyst for the conversion of hydrogen and oxygen into water. It acts as a catalyst, as long as the sachet is kept dry.

Inoculated culture plates are placed inside the jar and the lid clamped tight. The outlet tube is connected to a vacuum pump and the air inside is evacuated. The outlet tap is then closed and the inlet tube connected to a hydrogen supply. Hydrogen is drawn in rapidly. As soon as this inrush of gas has ceased, the inlet tap is also closed. After about 5 minutes inlet tap is again opened. There occurs again an immediate inrush of hydrogen since the catalyst creates a reduced pressure within the jar due to the conversion of hydrogen and left over oxygen into water. If there is no inrush of hydrogen, it means the catalyst is inactive and must be replaced. The jar is left connected to the hydrogen supply for about 5 minutes, then the inlet tap is closed and the jar is placed in the incubator, catalysis will continue until all the oxygen in the jar has been used up.

The **GasPak** is now the method of choice for preparing anaerobic jar. The GasPak is commercially available as a disposable envelope containing chemicals which generate hydrogen and carbon dioxide on the addition of water. After the inoculated plates are kept in the jar, the GasPak envelope with water added, is placed inside and the lid screwed tight. Hydrogen and carbon dioxide are liberated and the presence of a cold catalyst in the envelope permits the combination of hydrogen and oxygen to produce an anaerobic environment. The outstanding feature of the GasPak system is the

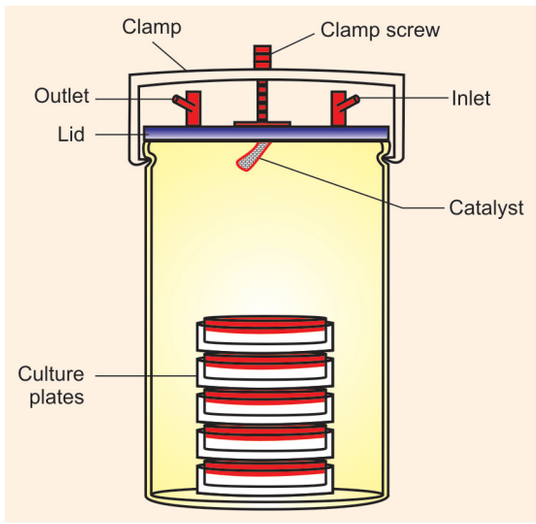


Fig. 3.3: Anaerobic jar.

disposable gas generator envelope, which does away with the need for a vacuum pump and cylinders of compressed gas; the operation of the jar is consequently very quick and simple. As the standard GasPak jar is not evacuated before use a relatively large volume of water is formed during catalysis.

An **indicator** should be used for verifying the anaerobic condition in the jar. Methylene blue is generally used for this purpose. When it is placed in an anaerobic environment, it is reduced from its coloured oxidized form to a colourless reduced leuco compound.

In addition to, or instead of, using a chemical indicator, some workers include in the jar a plate inoculated with a known strict anaerobe such as *Clostridium tetani* or *Bacteroides fragilis*, and a strict aerobe, such as *Pseudomonas aeruginosa*. This method is quite reliable if the indicator anaerobe grows and the aerobe does not.

The major disadvantage of any anaerobic jar system is that the plates have to be removed from the jar to be examined. This, of course, exposes the colonies to oxygen, which is especially hazardous to the anaerobes during their first 48 hours of growth. For this reason, a suitable holding system should always be used in conjunction with anaerobic jars, placed in an oxygen-free holding system, removed one by one for rapid microscopic examination of colonies, and then quickly returned to the holding system. Plates should never remain in room air on the open bench.

Anaerobic chamber

This is an ideal anaerobic incubation system, which provides oxygen-free environment for inoculating media and incubating cultures. Identification and susceptibility tests can also be performed in anaerobic chambers.

Anaerobic chambers may be fitted with airtight rubber gloves to insert hands and manipulate specimens, plates, tubes or they may be gloveless where airtight rubber sleeves fit tightly against user's bare forearms. All anaerobic chambers contain a catalyst, dessicant, H_2 gas (5–10%), CO_2 gas (5–10%), N_2 gas (80–90%) and an indicator.

Anaerobic bags or pouches

These bags are available commercially and one or two inoculated plates are placed into a bag and an oxygen removal system is activated and the bag is sealed and incubated. Plates can be examined for growth without removing the plates from bag, thus without exposing the colonies to oxygen. But as with anaerobic jar, plates must be removed from the bags in order to work with the colonies at the bench. These bags are also useful in transport of biopsy specimen for anaerobic cultures.

Incubation

Inoculated plates should be incubated at 37°C for at least 48 hours, and reincubated for another 2–4 days to allow slow-growing organisms (certain species of *Actinomyces* and *Eubacterium*) to form colonies.

KEY POINTS

- Bacteria ***reproduce*** by ***binary fission*** leading to logarithmic growth of cell numbers; generation time of bacteria varies from minutes to hours or days.
- Bacterial ***growth*** in laboratory media can be divided into a ***lag*** phase, ***log*** phase, ***stationary*** phase and phase of ***decline***.
- Depending on their oxygen requirements, bacteria can be divided into ***obligate aerobes***, ***facultative anaerobes***, ***obligate anaerobes*** and ***microaerophiles***.
- For *cultivation* and *identification* of bacteria, basal media, enriched media, selective media, enrichment media, differential media, etc. are used.
- Depending upon the expected organisms, the inoculated media are incubated in ***aerobic culture, culture in an atmosphere with added carbon dioxide, culture in microaerophilic atmosphere and anaerobic culture***.

Important Questions

1. Describe bacterial growth curve.
2. Define generation time of bacteria. Discuss briefly batch culture and continuous culture.
3. What are culture media? Classify and discuss them in brief.
4. Write short notes on:
   (a) Basal media
   (b) Enriched media
   (c) Selective media
   (d) Enrichment media
   (e) Transport media
   (f) Differential media
   (g) Sugar media
   (h) Synthetic media
5. Discuss in detail anaerobic culture methods.

Multiple Choice Questions

1. Generation time of *Escherichia coli* is:
   (a) 20 seconds. (b) 20 minutes.
   (c) 20 hours. (d) 20 days.

2. The period between inoculation of bacteria in a culture medium and beginning of multiplication is known as:
   (a) lag phase. (b) log phase.
   (c) stationary phase. (d) decline phase.

3. Peptone water and nutrient broth are:
   (a) basal media.
   (b) enriched media.
   (c) selective media.
   (d) enrichment media.

4. When a substance is added to a solid medium which inhibits the growth of unwanted bacteria but permits the growth of wanted bacteria, it is known as:
   (a) selective medium.
   (b) enrichment medium.
   (c) enriched medium.
   (d) differential medium.

5. When a substance is added to a liquid medium which inhibits the growth of unwanted bacteria and favours the growth of wanted bacteria, it is known as:
   (a) selective medium.
   (b) enrichment medium.
   (c) enriched medium.
   (d) differential medium.

6. MacConkey medium is an example of:
   (a) transport medium. (b) differential medium.
   (c) enrichment medium. (d) enriched medium.

7. Stuart's transport medium is used for transport of specimen containing:
   (a) *Salmonella*.
   (b) *Vibrio cholerae*.
   (c) *Neisseria gonorrhoeae*.
   (d) *Shigella*.

8. Bacteria, whose optimum temperature for growth is 37°C, are known as:
   (a) mesophiles. (b) psychrophiles.
   (c) thermophiles. (d) None of the above.

9. Which of the following bacteria dies quickly after drying?
   (a) *Mycobacterium tuberculosis*.
   (b) *Staphylococcus aureus*.
   (c) *Pseudomonas aeruginosa*.
   (d) *Treponema pallidum*.

10. Which of the following bacteria can grow in alkaline pH?
    (a) Lactobacilli.
    (b) *Vibrio cholerae*.
    (c) *Salmonella*.
    (d) *Shigella*.

11. Which of the following bacteria can grow in acidic pH?
    (a) Lactobacilli.
    (b) *Vibrio cholerae*.
    (c) *Salmonella*.
    (d) *Shigella*.

12. All bacteria that inhabit the human body are:
    (a) autotrophs.
    (b) heterotrophs.
    (c) phototrophs.
    (d) chemolithotrophs.

13. Which of the following is **not** a differential medium?
    (a) Christensen's medium.
    (b) MacConkey medium.
    (c) Blood tellurite agar.
    (d) Mueller-Hinton agar.

14. Chocolate agar is an example of which of the following media?
    (a) Non-selective media.
    (b) Selective media.
    (c) Differential media.
    (d) Enriched media.

Answers

1. b **2.** a **3.** a **4.** a **5.** b **6.** b **7.** c **8.** a **9.** d **10.** b **11.** a **12.** b **13.** d **14.** d