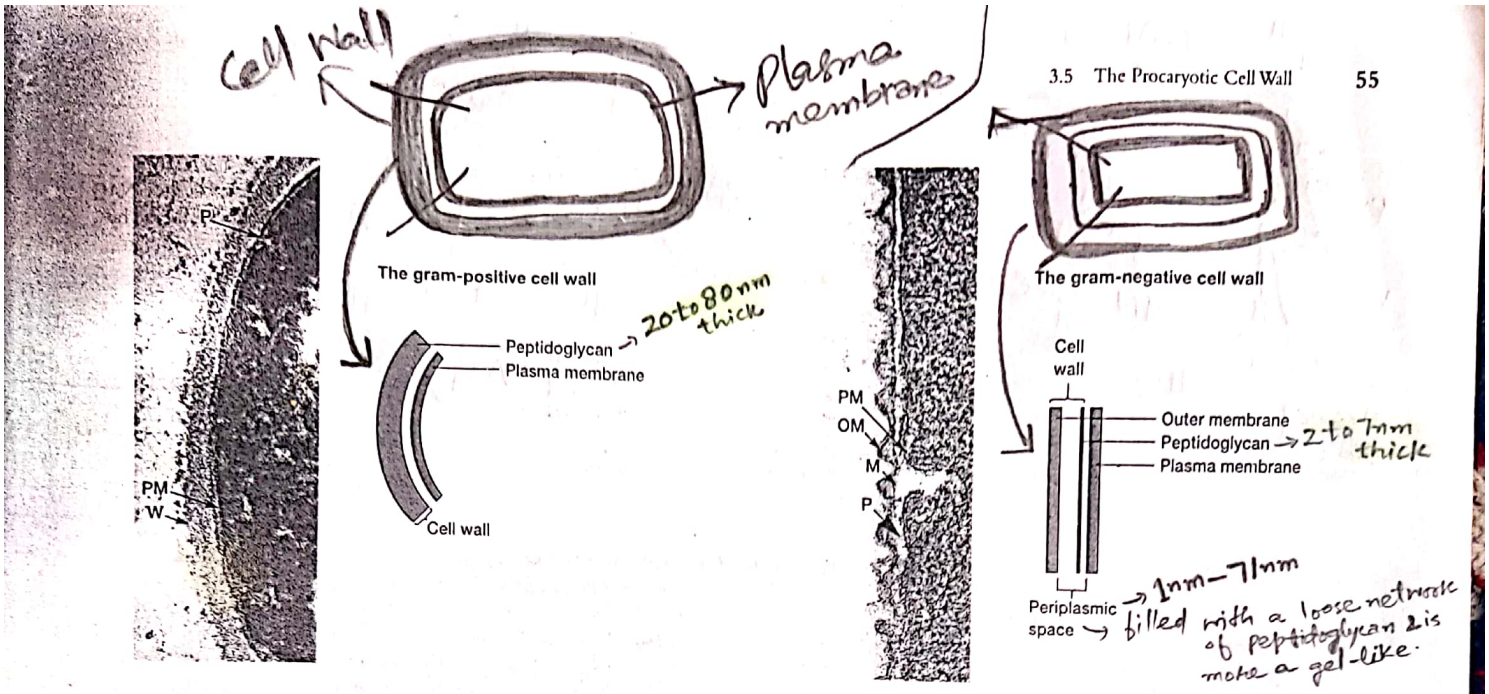


Figure 3.15 Gram-Positive and Gram-Negative Cell Walls. The gram-positive envelope is from *Bacillus licheniformis* (left), and the gram-negative micrograph is of *Aquaspirillum serpens* (right). M; peptidoglycan or murein layer; OM, outer membrane; PM, plasma membrane; P, periplasmic space; W, gram-positive peptidoglycan wall.

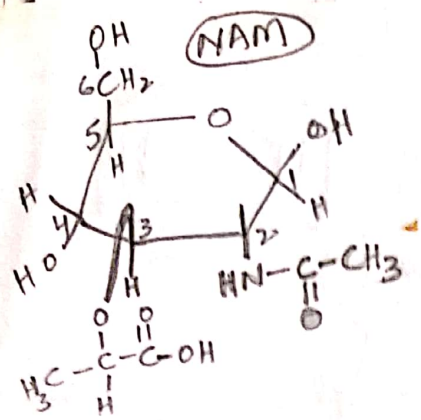
3.5 The Procaryotic Cell Wall

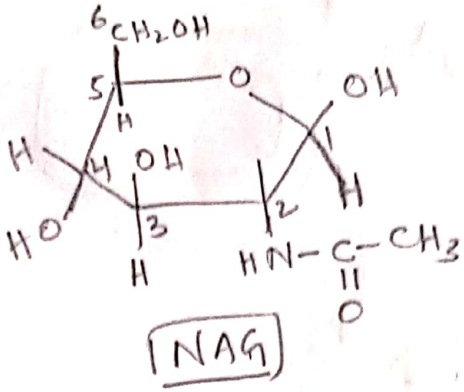
The cell wall is the layer, usually fairly rigid, that lies just outside the plasma membrane. It is one of the most important parts of a procaryotic cell for several reasons. Except for the mycoplasmas (*see Section 23.1*) and some Archaea (*see chapter 20*), most bacteria have strong walls that give them shape and protect them from osmotic lysis (p. 61); wall shape and strength is primarily due to peptidoglycan, as we will see shortly. The cell walls of many pathogens have components that contribute to their pathogenicity. The wall can protect a cell from toxic substances and is the site of action of several antibiotics.

After Christian Gram developed the Gram stain in 1884, it soon became evident that bacteria could be divided into two major groups based on their response to the Gram-stain procedure (*see table 19.9*). Gram-positive bacteria stained purple, whereas gram-negative bacteria were colored pink or red by the technique. The true structural difference between these two groups became clear with the advent of the transmission electron microscope. The gram-positive cell wall consists of a single 20 to 80 nm thick homogeneous **peptidoglycan** or **murein** layer lying outside the plasma membrane (**figure 3.15**). In contrast, the gram-negative cell wall is quite complex. It has a 2 to 7 nm peptidoglycan layer surrounded by a 7 to 8 nm thick **outer membrane.** Because of the thicker peptidoglycan layer, the walls of gram-positive cells are stronger than those of gram-negative bacteria. Microbiologists often call all the structures from the plasma membrane outward the **envelope** or cell envelope. This includes the wall and structures like capsules (p. 61) when present. Gram-stain procedure (p. 28)

Frequently a space is seen between the plasma membrane and the outer membrane in electron micrographs of gram-negative bacteria, and sometimes a similar but smaller gap may be observed between the plasma membrane and wall in gram-positive bacteria. This space is called the **periplasmic space.** Recent evidence indicates that the periplasmic space may be filled with a loose network of peptidoglycan. Possibly it is more a gel than a fluid-filled space. The substance that occupies the periplasmic space is the **periplasm.** Gram-positive cells may have periplasm even if they lack a discrete, obvious periplasmic space. Size estimates of the periplasmic space in gram-negative bacteria range from 1 nm to as great as 71 nm. Some recent studies indicate that it may constitute about 20 to 40% of the total cell volume (around 30 to 70 nm), but more research is required to establish an accurate value. When cell walls are disrupted carefully or removed without disturbing the underlying plasma membrane, periplasmic enzymes and other proteins are released and may be easily studied. The periplasmic space of gram-negative bacteria contains many proteins that participate in nutrient acquisition—for example, hydrolytic enzymes attacking nucleic acids and phosphorylated molecules, and binding proteins involved in transport of materials into the cell. Denitrifying and chemolithoautotrophic bacteria (*see sections 9.6 and 9.10*) often have electron transport proteins in their periplasm. The periplasmic space also contains enzymes involved in peptidoglycan synthesis and the modification of toxic compounds that could harm the cell. Gram-positive bacteria may not have a visible periplasmic space and do not appear to have as many periplasmic proteins; rather, they secrete several enzymes that ordinarily would be periplasmic in gram-negative bacteria. Such secreted enzymes are often called **exoenzymes.** Some enzymes remain in the periplasm and are attached to the plasma membrane.

The Archaea differ from other procaryotes in many respects (*see chapter 20*). Although they may be either gram positive or gram negative, their cell walls are distinctive in structure and





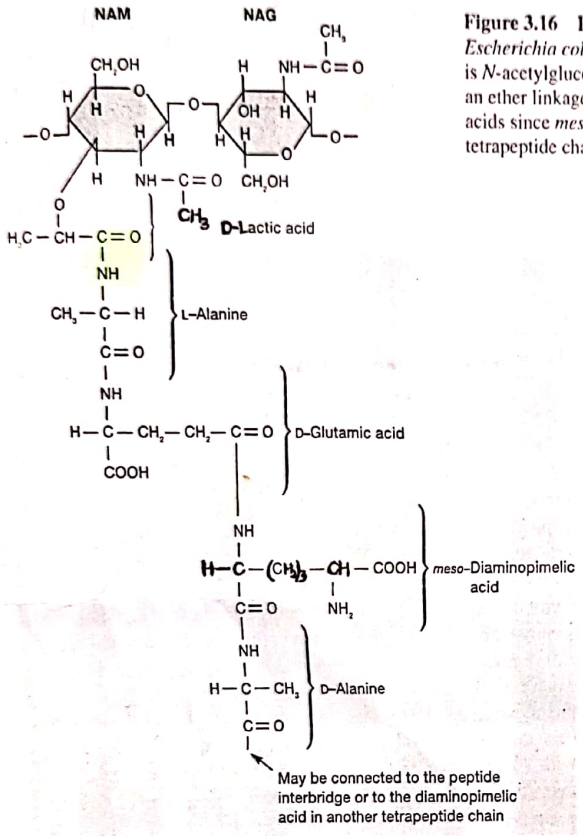


Figure 3.16 Peptidoglycan Subunit Composition. The peptidoglycan subunit of *Escherichia coli*, most other gram-negative bacteria, and many gram-positive bacteria. NAG is *N*-acetylglucosamine. NAM is *N*-acetylmuramic acid (NAG with lactic acid attached by an ether linkage). The tetrapeptide side chain is composed of alternating D- and L-amino acids since *meso*-diaminopimelic acid is connected through its L-carbon. NAM and the tetrapeptide chain attached to it are shown in different shades of color for clarity.

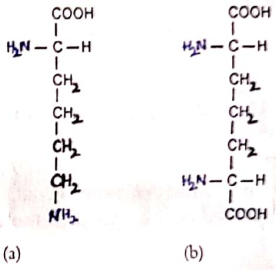


Figure 3.17 Diaminoacids Present in Peptidoglycan. (a) L-Lysine. (b) *meso*-Diaminopimelic acid.

chemical composition. The walls lack peptidoglycan and are composed of proteins, glycoproteins, or polysaccharides.

Following this overview of the envelope, peptidoglycan structure and the organization of gram-positive and gram-negative cell walls are discussed in more detail.

Peptidoglycan Structure

Peptidoglycan or murein is an enormous polymer composed of many identical subunits. The polymer contains two sugar derivatives, *N*-acetylglucosamine and *N*-acetylmuramic acid (the lactyl ether of *N*-acetylglucosamine), and several different amino acids, three of which—D-glutamic acid, D-alanine, and *meso*-diaminopimelic acid—are not found in proteins. The presence of D-amino acids protects against attack by most peptidases. The peptidoglycan subunit present in most gram-negative bacteria and many gram-positive ones is shown in **figure 3.16.** The backbone of this polymer is composed of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues. A peptide chain of four alternating D- and L-amino acids is connected to the carboxyl group of *N*-acetylmuramic acid. Many bacteria substitute another diaminoacid, usually L-lysine, in the third position for *meso*-diaminopimelic acid (**figure 3.17**). A review of the chemistry of biological molecules (appendix D: Peptidoglycan structural variations (pp. 521–22)

Chains of linked peptidoglycan subunits are joined by cross-links between the peptides. Often the carboxyl group of the terminal D-alanine is connected directly to the amino group of diaminopimelic acid, but a **peptide interbridge** may be used instead (**figure 3.18**). Most gram-negative cell wall peptidoglycan lacks the peptide interbridge. This cross-linking results in an enormous peptidoglycan sac that is actually one dense, interconnected network (**figure 3.19**). These sacs have been isolated from gram-positive bacteria and are strong enough to retain their shape and integrity (**figure 3.20**), yet they are elastic and somewhat stretchable, unlike cellulose. They also must be porous, as molecules can penetrate them.

Gram-Positive Cell Walls

Normally the thick, homogeneous cell wall of gram-positive bacteria is composed primarily of peptidoglycan, which often contains a peptide interbridge (figure 3.20 and **figure 3.21**). However gram-positive cell walls usually also contain large amounts of **teichoic acids,** polymers of glycerol or ribitol joined by phosphate

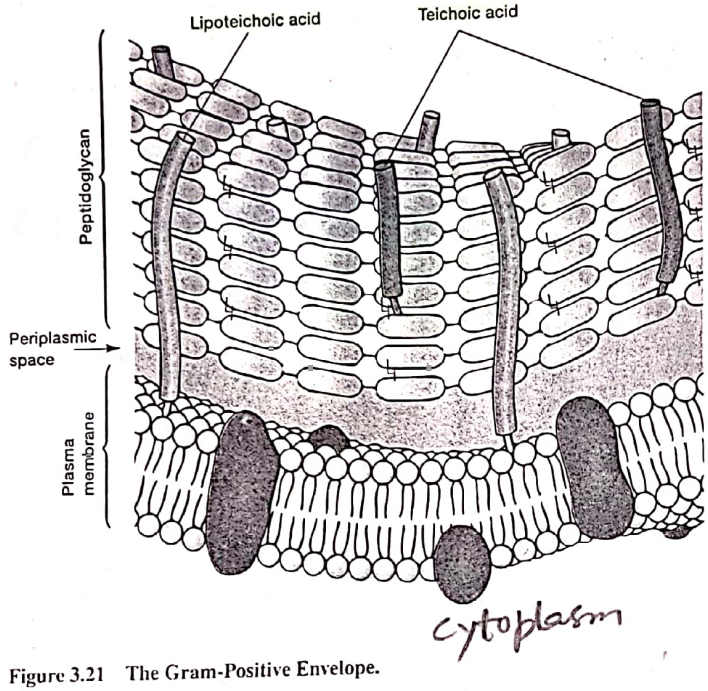


Figure 3.21 The Gram-Positive Envelope.

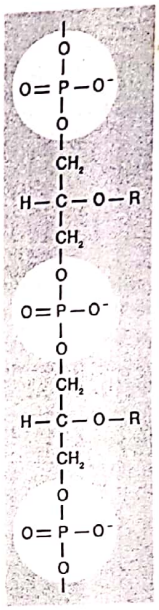


Figure 3.22 Teichoic Acid Structure. The segment of a teichoic acid made of phosphate, glycerol, and a side chain, R. R may represent D-alanine, glucose, or other molecules.

groups (figures 3.21 and 3.22). Amino acids such as D-alanine or sugars like glucose are attached to the glycerol and ribitol groups. The teichoic acids are connected to either the peptidoglycan itself by a covalent bond with the six hydroxyl of *N*-acetylmuramic acid or to plasma membrane lipids; in the latter case they are called lipoteichoic acids. Teichoic acids appear to extend to the surface of the peptidoglycan, and, because they are negatively charged, help give the gram-positive cell wall its negative charge. The functions of these molecules are still unclear, but they may be important in maintaining the structure of the wall. Teichoic acids are not present in gram-negative bacteria.

Gram-Negative Cell Walls

Even a brief inspection of figure 3.15 shows that gram-negative cell walls are much more complex than gram-positive walls. The thin peptidoglycan layer next to the plasma membrane may constitute not more than 5 to 10% of the wall weight. In *E. coli* it is about 2 nm thick and contains only one or two layers or sheets of peptidoglycan.

The outer membrane lies outside the thin peptidoglycan layer (**figures 3.23** and **3.24**). The most abundant membrane protein is Braun's lipoprotein, a small lipoprotein covalently joined to the underlying peptidoglycan and embedded in the outer membrane by its hydrophobic end. The outer membrane and peptidoglycan are so firmly linked by this lipoprotein that they can be isolated as one unit.

Another structure that may strengthen the gram-negative wall and hold the outer membrane in place is the adhesion site. The outer membrane and plasma membrane appear to be in direct contact at many locations in the gram-negative wall. In *E. coli* 20 to 100 nm areas of contact between the two membranes are seen in plasmolyzed cells. Adhesion sites may be regions of direct contact or possibly true membrane fusions. It has been proposed that substances can move into the cell through these adhesion sites rather than traveling through the periplasm.

Possibly the most unusual constituents of the outer membrane are its **lipopolysaccharides** (**LPSs**). These large, complex molecules contain both lipid and carbohydrate, and consist of three parts: (1) lipid A, (2) the core polysaccharide, and (3) the O side chain. The LPS from *Salmonella typhimurium* has been studied most, and its general structure is described here (**figure 3.25**). The **lipid A** region contains two glucosamine sugar derivatives, each with three fatty acids and phosphate or pyrophosphate attached. It is buried in the outer membrane and the remainder of the LPS molecule projects from the surface. The **core polysaccharide** is joined to lipid A. In *Salmonella* it is constructed of 10 sugars, many of them unusual in structure. The **O side chain** or **O antigen** is a polysaccharide chain extending outward from the core. It has several peculiar sugars and varies in composition between bacterial strains. Although O side chains are readily recognized by host antibodies, gram-negative bacteria may thwart host defenses by rapidly changing the nature of their O side chains to avoid detection. Antibody interaction with the LPS before reaching the outer membrane proper may also protect the cell wall from direct attack. Antibodies and antigens (chapters 32 and 33)

In staphylococci, these surface proteins are covalently joined to the pentaglycine bridge of the cell wall peptidoglycan. An enzyme called sortase catalyses the attachment of these surface proteins to the peptidoglycan.

Structure of Outer membrane:

The outer membrane (om) lies outside the thin peptidoglycan layer in gram-negative bacteria. It is linked to the cell in two ways. The first is by Braun's lipoprotein, the most abundant protein in the outer membrane (Fig). This small lipoprotein is covalently joined to the underlying peptidoglycan.

The outer membrane & peptidoglycan are so firmly linked by Braun's protein that they can be isolated as one unit. The second linking mechanism involves the many adhesion sites joining the om & the plasma membrane. In E. coli, 20 to 100nm areas of contact between om & PM can be seen. It has been proposed that substances can more directly pass into the cell through adhesion sites.

The most unusual constituents of the om are its lipopolysaccharides (LPSs). These large, complex molecules contain both lipid, & carbohydrate &

consist of three parts (1) Lipid A, (2) the core polysaccharide (inner core & outer core), (3) the O side chain or O antigen.

The LPS of Salmonella has been studied most. The Lipid A region contains two glucosamine sugar derivatives, each with three fatty acids & phosphate or pyrophosphate attached. The fatty acids attach the lipid A to the outer membrane, while the remainder of the LPS molecule projects from the surface. The core polysaccharide is joined to lipid A. In Salmonella, it is constructed of 10 sugars, many of them unusual in structure. The O side chain is a polysaccharide chain extending outward from the core. The sugars present in O side chain varies in composition between bacterial strains.

Functions: LPS contributes to the negative charge on the bacterial surface & helps stabilize outer membrane. LPS aids in creating a permeability barrier. It may contribute to bacterial attachment to surfaces. Interactions between neighbouring LPS molecules are thought to restrict the entry of bile salts, antibiotics & other toxic substances that may kill or injure the bacterium. LPS also protects the bacterium from host defenses.

The O side chain of LPS is also called O antigen because it elicites an

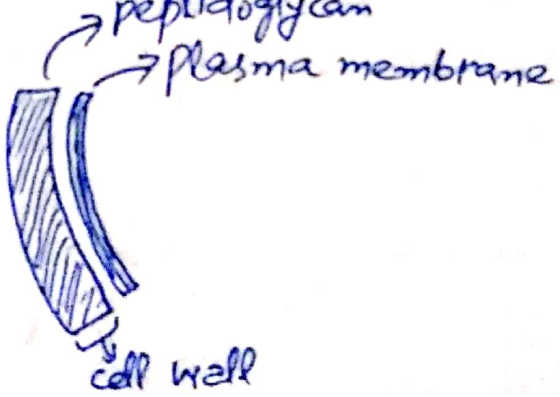
immune response. Lipid A portion of LPS often is toxic as a result the LPS can act as an endotoxin & cause some of the symptoms that arise in gram-negative bacterial infections.

Outer membrane is more permeable than the plasma membrane & permits the passage of small molecules like glucose & other monosaccharides. This is due to the presence of Porin proteins.

Structure of bacterial cell wall:

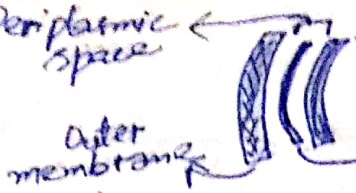
Gram staining developed by Christian Gram in 1884, divided most bacteria into two major groups, Gram +ve & Gram -ve. But the true structural difference between these two groups became clear with the discovery of transmission electron microscope.

Prokaryotic cells almost always are bounded by a chemically complex cell wall. The cell wall of both Gram-positive & Gram-negative bacteria is chemically peptidoglycan (murein). The Gram positive cell wall consists of a single 20 to 80nm thick homogenous layer of peptidoglycan lying outside plasma membrane.



In contrast, Gram-negative cell wall is quite complex. It consists of 2 to 7nm peptidoglycan layer covered by a 7 to 8nm thick outer membrane. Microbiologists often call all

peptidoglycan + outer membrane } cell wall of Gram-ve bacteria



(2)

the structures from the plasma membrane outward, the cell envelope. The space that is present between the plasma membrane and the outer membrane in gram-negative bacteria is called the periplasmic space. The substance that occupies the periplasmic space is called periplasm. The nature of the periplasmic space & periplasm differs in gram-positive & gram-negative bacteria. The periplasmic space of gram-positive bacteria when observed is smaller.

Peptidoglycan is a polymer, contains two sugar derivatives N-acetylglucosamine (NAG) & N-acetylmuramic acid (NAM) joined through β-1,4 glycosidic bond. It also contains several D- & L-aminoacids. A peptide chain of four alternating D- & L- aminoacids called tetrapeptide is connected to the lactyl group of the NAM. Aminoacids present in tetrapeptide are L-Ala, D-Glu, L-Lys or diaminopimelic acid (DAP) & D-Ala. Often the carbonyl group of the terminal D-alanine is connected directly to the amino group ($-NH_2$) of DAP. In some peptidoglycans a short peptide of 5 glycine residues, called peptide interbridge connects the tetrapeptide chains.

Gram-Positive cell walls:

Gram Positive bacteria normally have cell walls that are composed primarily of peptidoglycan. In addition, gram positive cell walls usually contain large amounts of teichoic acids (polymer of glycerol or ribotol joined by phosphate groups). Aminoacids like D-alanine or sugars such as glucose are attached to the glycerol & ribotol groups. Teichoic acids are negatively charged, & help give the cell wall its negative charge.

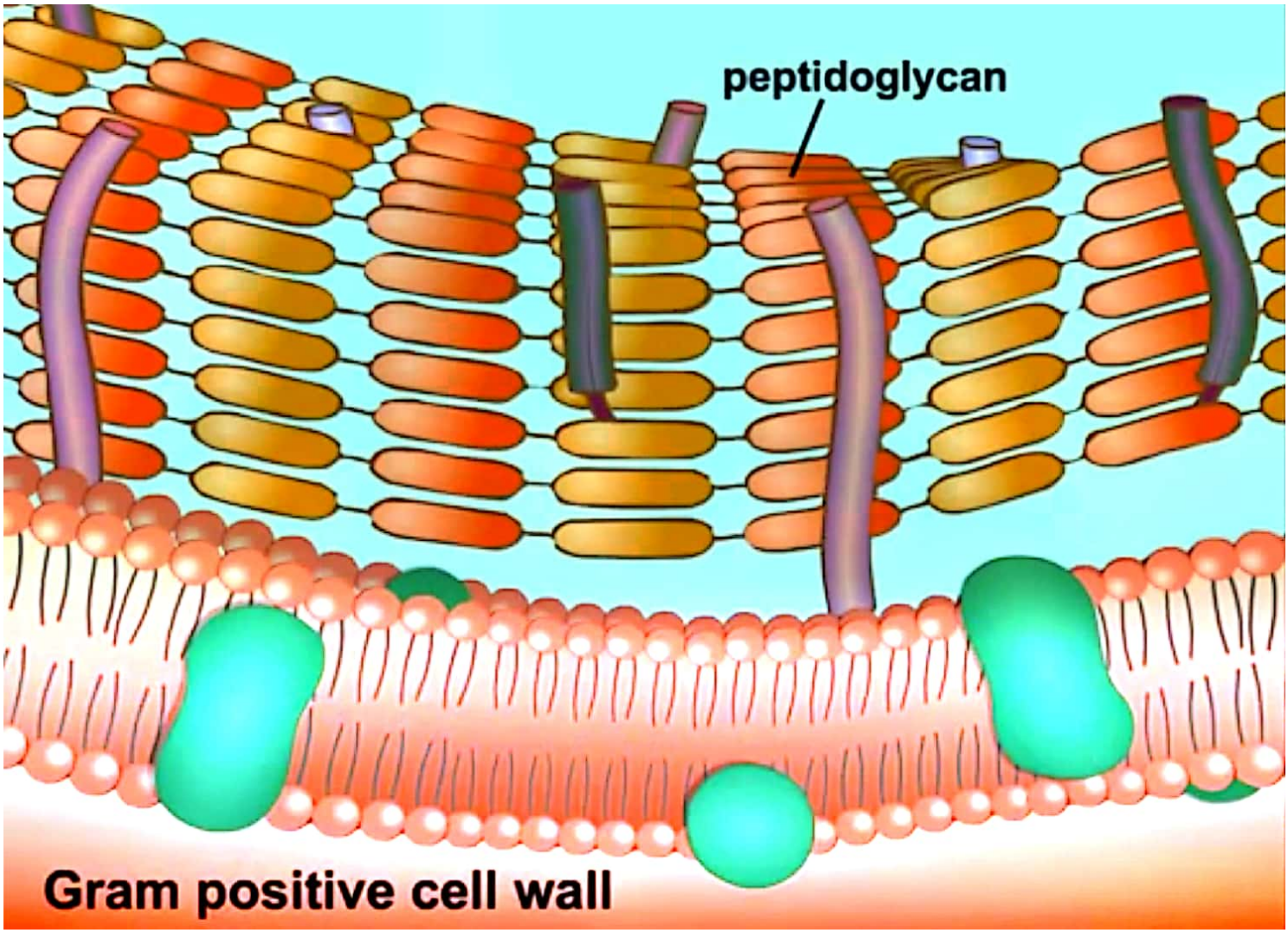
The periplasmic space in gram positive bacteria is smaller than gram-negative bacteria or absent. The periplasm has relatively few proteins. Any proteins secreted by the cell usually pass through the peptidoglycan as it is porous. Enzyme secreted by gram-positive bacteria are called exoenzymes. The role of these exoenzymes is to degrade polymeric ~~molecules~~ nutrients that would otherwise be too large for transport across the plasma-membrane. Staphylococci & most other gram-positive bacteria have a layer of proteins on the surface of their cell wall peptidoglycan. Some are non-covalently attached by binding to the peptido-glycan, teichoic acids, or other receptors. eg; S-layer proteins.

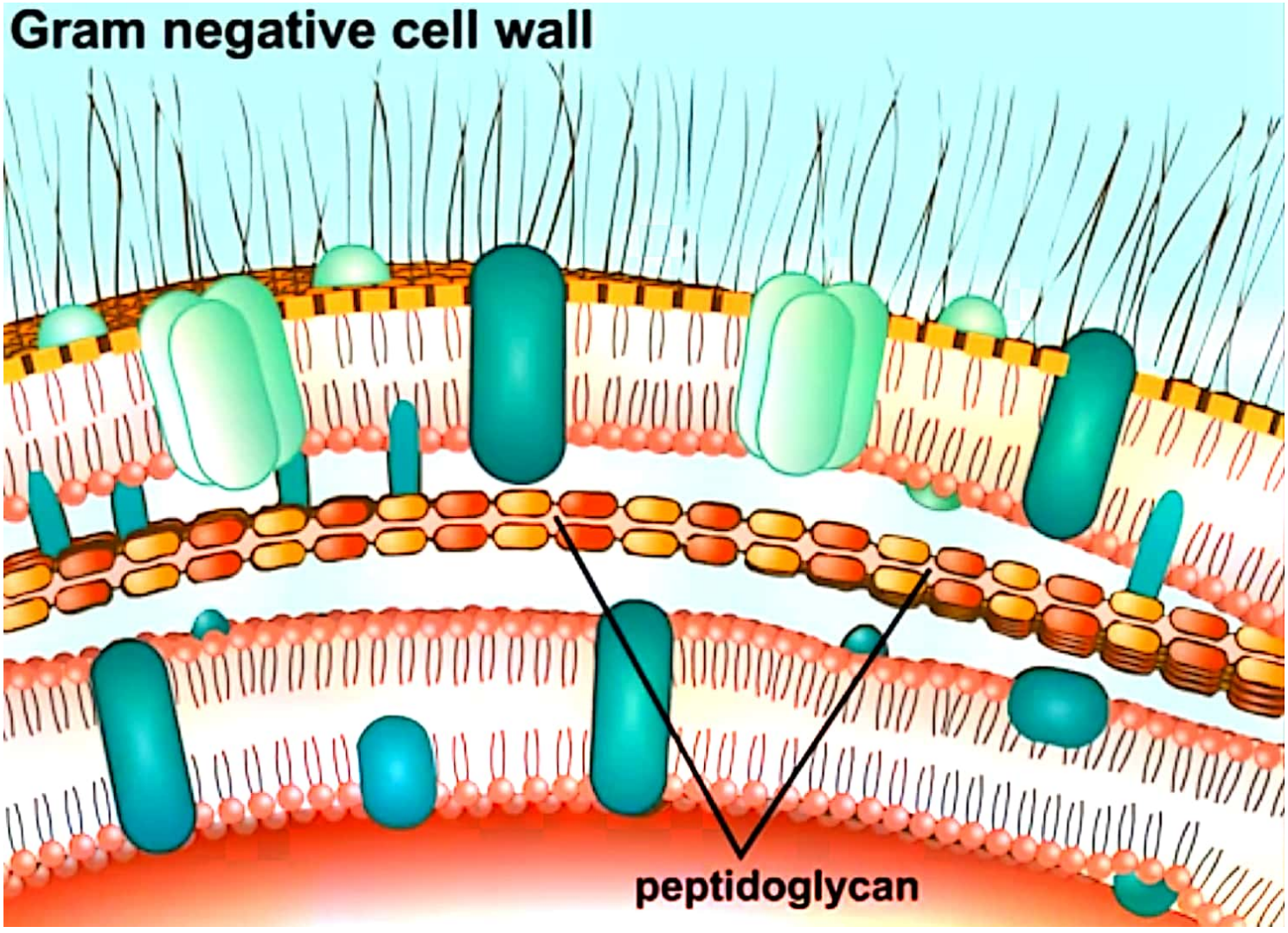
Other surface proteins are covalently attached to the peptidoglycan, such as the M protein of pathogenic streptococci have roles in virulence, such as aiding in adhesion to host tissues.

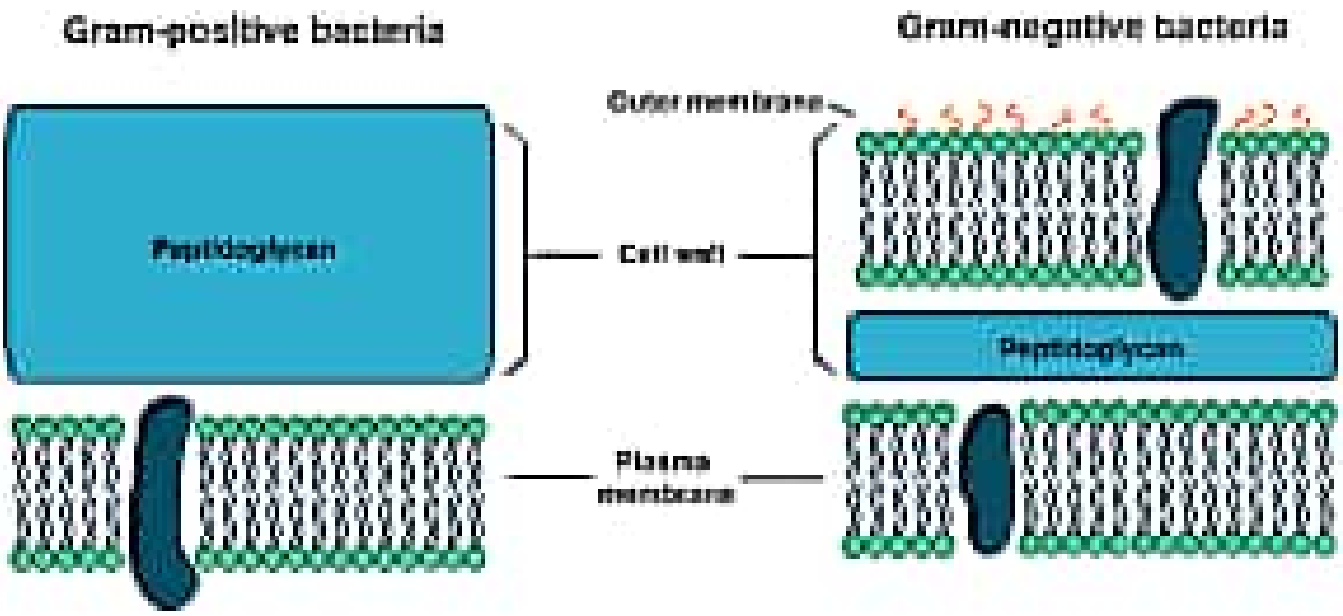
The reaction that forms the peptide cross links during peptidoglycan synthesis is called transpeptidation. Most gram-negative cell wall Peptidoglycan lacks the peptide interbridge.

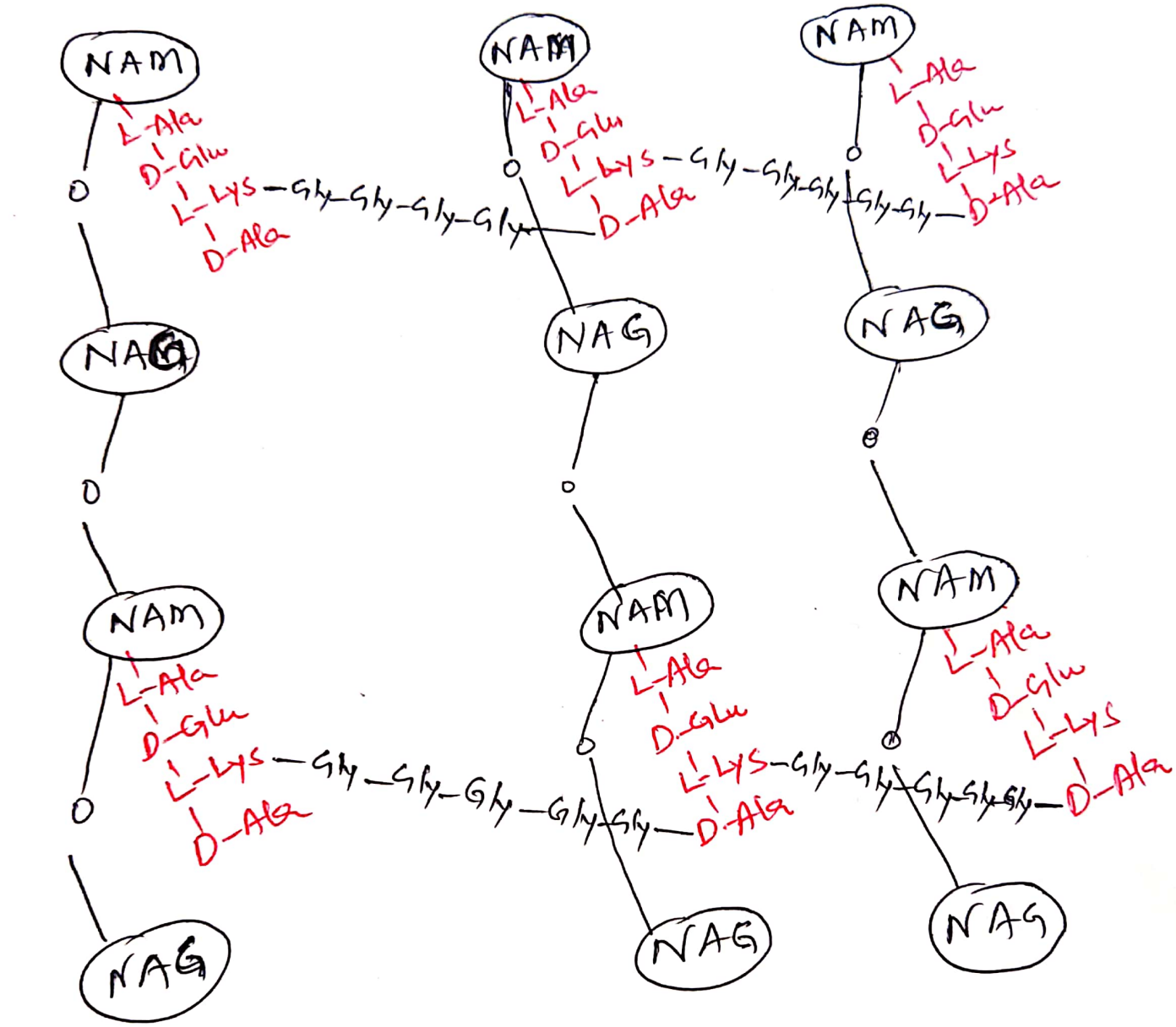
Two groups of bacteria devoid of peptidoglycan cell wall are the Mycoplasma species, which possess a surface membrane structure, & the L-forms.

Many gram-positive bacteria have acidic substances called teichoic acids in their cell wall. They can be covalently linked to NAM or to the lipids of the plasma membrane (lipoteichoic acids) or to a terminal D-alanine in the tetrapeptide. Teichoic acids are absent in Gram-negative bacteria. Teichoic acids provide rigidity to the cell wall by attracting cations such as Mg^{+2} & Na^{+} ions.









To obtain energy and construct new cellular components, organisms, must have a supply of raw materials or nutrients. **Nutrients** – are substances used in biosynthesis and energy production.

Nutrient Requirements:

Microbial cell composition shows that 95% of cell dry weight is made up of a few major elements: Carbon, oxygen, hydrogen, nitrogen, sulfur, phosphorous, potassium, calcium, magnesium and iron.

Macronutrients or macro elements:

These are required by microorganisms in relatively large amounts. Carbon, oxygen, hydrogen nitrogen, sulfurs and phosphorous are components of carbohydrates, lipids, proteins and nucleic acids. The remaining four macro elements (K, Ca, Mg and Fe) exist in the cell as cations.

K^+ - is required for the activity by a number of enzymes, including those involved in protein synthesis.

Ca^{2+} - contributes to the heat resistance of bacterial endospores. 15% of spore contains dipicolinic acid and calcium.

Mg^{2+} - serves as a cofactor for many enzymes, complexes with ATP and stabilizes ribosomes and cell membranes.

Fe^{2+} and Fe^{2+} - part of cytochromes and a cofactor for enzymes and electron-carrying proteins.

Micronutrients or Trace elements:

These are manganese, zinc, cobalt, molybdenum, nickel and copper. These are normally part of enzymes and cofactors, and they aid in the catalysis of reactions and maintenance of protein structure.

Zn^{2+} - is present at the active site of some enzymes but is also involved in the association of regulatory and catalytic subunits in *E.coli* aspartate carbomoyl transferase.

Mn^{2+} - aids many enzymes catalyzing the transfer of phosphate groups.

Mo^{2+} - required for nitrogen fixation.

Co^{2+} - is a component of Vitamin B12.

Besides macro and micro nutrients, some microorganisms may have particular requirements that reflect the special nature of their morphology or environment. Diatoms need silicic acid to construct their beautiful cell walls of silica. Bacteria growing in saline lakes and oceans depend on the presence of high concentrations of sodium ion. Microorganisms require a balanced mixture of all the above nutrients for proper growth.

Requirements for carbon, hydrogen and oxygen:

Carbon is needed for the skeleton or backbone of all organic molecules and molecules serving as carbon sources normally also contribute both oxygen and hydrogen atoms. One important carbon source that does not supply hydrogen or energy is CO_2 . **Autotrophs** – can use CO_2 as their sole or principal source of carbon. Many microorganisms are autotrophic, and most of these carry out photosynthesis and use light as their energy source. Some autotrophs oxidize inorganic molecules and derive

Besides macro and micro nutrients, some microorganisms may have particular requirements that reflect the special nature of their morphology or environment. Diatoms need silicic acid to construct their beautiful cell walls of silica. Bacteria growing in saline lakes and oceans depend on the presence of high concentrations of sodium ion. Microorganisms require a balanced mixture of all the above nutrients for proper growth.

Requirements for carbon, hydrogen and oxygen:

Carbon is needed for the skeleton or backbone of all organic molecules and molecules serving as carbon sources normally also contribute both oxygen and hydrogen atoms. One important carbon source that does not supply hydrogen or energy is CO_2 . **Autotrophs** – can use CO_2 as their sole or principal source of carbon. Many microorganisms are autotrophic, and most of these carry out photosynthesis and use light as their energy source. Some autotrophs oxidize inorganic molecules and derive energy from electron transfer. **Heterotrophs** – are organisms that use reduced pre-formed organic molecules as carbon sources. Ex. Glycolytic pathway produces carbon skeleton for use in biosynthesis and also releases energy as ATP and NADH. Actinomycetes will degrade amyl alcohol, paraffin and even rubber. *Burkholderia cepacia* can use over 100 different carbon compounds. Some microorganisms can metabolize even relatively indigestible human-made substances such as pesticides. Indigestible molecules can be oxidized and degraded in the presence of a growth promoting nutrient that is metabolized at the same time, a process called Co-metabolism. The products of this breakdown can then be used as nutrients by other microorganisms.

Nutritional types of microorganisms:

In addition to Carbon, hydrogen and oxygen all organisms require sources of energy and electrons for growth.

Carbon sources:

Autotrophs - CO_2 sole or principal biosynthetic carbon source

Heterotrophs – reduced, preformed organic molecules from other organisms.

Energy sources:

Phototrophs – use light as their energy source.

Chemotrophs – obtain energy from the oxidation of chemical compounds (either organic or in organic)

Electron sources:

Lithotrophs – use reduced inorganic substances as their electron source.

Organotrophs – extract electrons from organic compounds.

Four major nutritional classes based on their primary sources of carbon, energy and electrons is known.

Phtotolithotrophic autotrophs or photoautotrophs or photolithoautotrophs:

Source of energy – light energy

Source of electrons – Inorganic hydrogen/ electron

Carbon source - CO_2

Phtotolithotrophic autotrophs or photoautotrophs or photolithoautotrophs:

Source of energy – light energy

Source of electrons – Inorganic hydrogen/ electron

Carbon source - CO_2

Example: Algae, purple and green sulfur bacteria and cyanobacteria.

Photoorganotrophic heterotrophy or photoorganoheterotrophy:

Source of energy – light energy

Source of electrons – organic hydrogen/ electron

Carbon source – organic carbon sources (CO_2 may also be used)

Example: Purple and green nonsulfur bacteria (common inhabitants of lakes and streams)

Chemolithotrophic autotrophs or chemolithoautotrophy:

Source of energy – Chemical energy source (inorganic)

Source of electrons – Inorganic hydrogen/ electron donor

Carbon source - CO_2

Example: Sulfur-oxidizing bacteria, hydrogen bacteria, nitrifying bacteria, iron-oxidizing bacteria.

Chemoorganotrophic heterotrophs or chemoorganoheterotrophy:

Source of energy – Chemical energy source (organic)

Source of electrons – Inorganic hydrogen/ electron donor

Carbon source – organic carbon source

Example: Protozoan, fungi, most non-photosynthetic bacteria (including most pathogens)

The most common nutritional types are photolithoautotrophs and chemoorganoheterotrophs. Bacteria *Beggiatoa* rely on inorganic energy sources and organic (or sometimes CO_2) carbon sources. These microbes are sometimes called Mixotrophic because they combine chemolithoautotrophic and heterotrophic metabolic processes.

Requirements for nitrogen, phosphorous and sulfur:

Nitrogen is needed for the synthesis of amino acids, purines, pyramidines, some carbohydrates and lipids, enzyme cofactors and other substances. Most phototrophs and many nonphotosynthetic microorganisms reduce nitrate to ammonia and incorporate the ammonia in assimilatory nitrate reduction. A variety of bacteria like many Cyanobacteria and *Rhizobium* can reduce and assimilate atmospheric nitrogen using the nitrogenase systems. Phosphorous is present in nucleic acids, phospholipids, ATP, several cofactors, some proteins and other cell components. All microorganisms use inorganic phosphate as their phosphorous source and incorporate it directly. *E.coli* can use both organic and inorganic phosphate. Organophosphates such as hexose 6- phosphate can be taken up directly by transport proteins. Other organophosphates are often hydrolyzed in the periplasm by the enzyme alkaline phosphatase to produce inorganic phosphate which is then transported across the plasma membrane. When inorganic phosphate is outside the bacterium, it crosses the outer membrane by the use of a porin protein channel. Sulfur is needed for the synthesis of substances like the amino acids cysteine and methionine, some carbohydrates biotin and thiamine. Most of them use sulfate as a source of sulfur and reduce it by assimilatory sulfate reduction; a few require a reduced form of sulfur such as cysteine.

Growth factors:

Many microorganisms have the enzymes and pathways necessary to synthesize all cell components. Many lack one or more enzymes and hence require organic compounds because they are essential cell components or precursors of such components and cannot be synthesized by the organisms are called – growth factors. There are three major classes of growth factors:

Amino acids – needed for protein synthesis.

Purines and Pyramidines – for nucleic acid synthesis

Vitamins – small organic molecules that usually make up all or part of enzyme cofactors, and only very small amounts sustain growth.

Knowledge of the specific growth factor requirements of many microorganisms makes possible quantitative growth response assays for a variety of substances. The observation that many microorganisms can synthesize large quantities of vitamins has led to their use in industry. Several water-soluble and fat-soluble vitamins are produced using industrial fermentations.

Ribofalvin – *Clostridium, Candida, Ashbya, Eremothecium*

Coenzyme A – *Brevibacterium*

Vitamin B_{12} – *Streptomyces, Propionibacterium, Pseudomonas*

Vitamin C – *Gluconobacter, Erwinia, Corynebacterium*

β- Carotene – *Dunaliella*

Vitamin D - *Saccharomyces*

using industrial fermentations.

Ribofalvin – *Clostridium, Candida, Ashbya, Eremothecium*

Coenzyme A – *Brevibacterium*

Vitamin B_{12} – *Streptomyces, Propionibacterium, Pseudomonas*

Vitamin C – *Gluconobacter, Erwinia, Corynebacterium*

β- Carotene – *Dunaliella*

Vitamin D - *Saccharomyces*

REFERENCES:

Text Books:

1. Jeffery C. Pommerville. Alcamo's Fundamentals of Microbiology (Tenth Edition). Jones and Bartlett Student edition.

2. Gerard J. Tortora, Berdell R. Funke, Christine L. Case. Pearson - Microbiology: An Introduction. Benjamin Cummings.

Reference Books:

1. Lansing M. Prescott, John P. Harley and Donald A. Klein. Microbiology. Mc Graw Hill companies.

Concepts

1. Growth is defined as an increase in cellular constituents and may result in an increase in a microorganism's size, population number, or both.
2. When microorganisms are grown in a closed system, population growth remains exponential for only a few generations and then enters a stationary phase due to factors such as nutrient limitation and waste accumulation. In an open system with continual nutrient addition and waste removal, the exponential phase can be maintained for long periods.
3. A wide variety of techniques can be used to study microbial growth by following changes in the total cell number, the population of viable microorganisms, or the cell mass.
4. Water availability, pH, temperature, oxygen concentration, pressure, radiation, and a number of other environmental factors influence microbial growth. Yet many microorganisms, and particularly bacteria, have managed to adapt and flourish under environmental extremes that would destroy most higher organisms.
5. In the natural environment, growth is often severely limited by available nutrient supplies and many other environmental factors.
6. Bacteria can communicate with each other and behave cooperatively using population density–dependent signals.

The paramount evolutionary accomplishment of bacteria as a group is rapid, efficient cell growth in many environments.

—J. L. Ingraham, O. Maaløe, and F. C. Neidhardt

Chapter 5 emphasizes that microorganisms need access to a source of energy and the raw materials essential for the construction of cellular components. All organisms must have carbon, hydrogen, oxygen, nitrogen, sulfur, phosphorus, and a variety of minerals; many also require one or more special growth factors. The cell takes up these substances by membrane transport processes, the most important of which are facilitated diffusion, active transport, and group translocation. Eucaryotic cells also employ endocytosis.

Chapter 6 concentrates more directly on the growth. The nature of growth and the ways in which it can be measured are described first, followed by consideration of continuous culture techniques. An account of the influence of environmental factors on microbial growth completes the chapter.

Growth may be defined as an increase in cellular constituents. It leads to a rise in cell number when microorganisms reproduce by processes like budding or binary fission. In the latter, individual cells enlarge and divide to yield two progeny of approximately equal size. Growth also results when cells simply become longer or larger. If the microorganism is **coenocytic**—that is, a multinucleate organism in which nuclear divisions are not accompanied by cell divisions—growth results in an increase in cell size but not cell number. It is usually not convenient to investigate the growth and reproduction of individual microorganisms because of their small size. Therefore, when studying growth, microbiologists normally follow changes in the total population number. The cell cycle (pp. 87; 285–86)

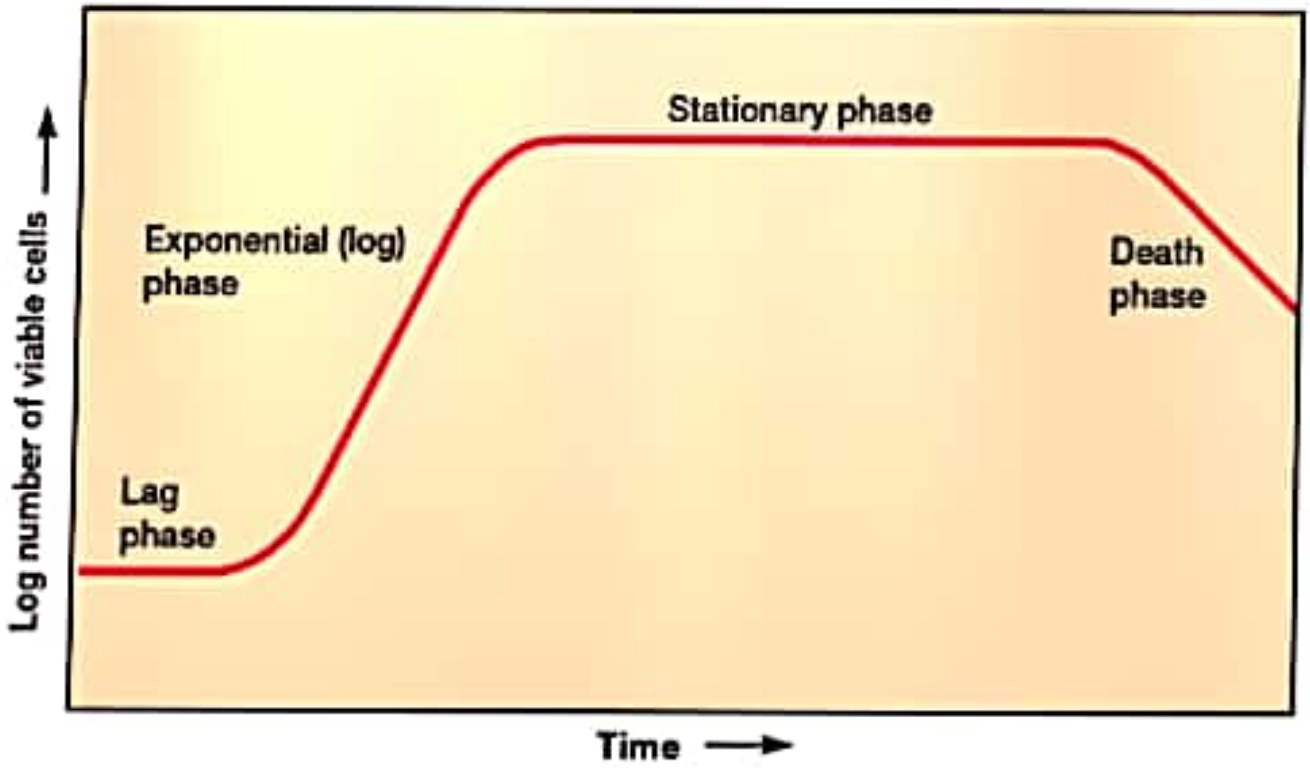


Figure 6.1 Microbial Growth Curve in a Closed System. The four phases of the growth curve are identified on the curve and discussed in the text.

6.1 The Growth Curve

Population growth is studied by analyzing the growth curve of a microbial culture. When microorganisms are cultivated in liquid medium, they usually are grown in a **batch culture** or closed system—that is, they are incubated in a closed culture vessel with a single batch of medium. Because no fresh medium is provided during incubation, nutrient concentrations decline and concentrations of wastes increase. The growth of microorganisms reproducing by binary fission can be plotted as the logarithm of the number of viable cells versus the incubation time. The resulting curve has four distinct phases (**figure 6.1**).

Lag Phase

When microorganisms are introduced into fresh culture medium, usually no immediate increase in cell number occurs, and therefore this period is called the **lag phase.** Although cell division does not take place right away and there is no net increase in mass, the cell is synthesizing new components. A lag phase prior to the start of cell division can be necessary for a variety of reasons. The cells may be old and depleted of ATP, essential cofactors, and ribosomes; these must be synthesized before growth can begin. The medium may be different from the one the microorganism was growing in previously. Here new enzymes would be needed to use different nutrients. Possibly the microorganisms have been injured and require time to recover. Whatever the causes, eventually the cells retool, replicate their DNA, begin to increase in mass, and finally divide.

The lag phase varies considerably in length with the condition of the microorganisms and the nature of the medium. This phase may be quite long if the inoculum is from an old culture or one that has been refrigerated. Inoculation of a culture into a chemically different medium also results in a longer lag phase.

On the other hand, when a young, vigorously growing exponential phase culture is transferred to fresh medium of the same composition, the lag phase will be short or absent.

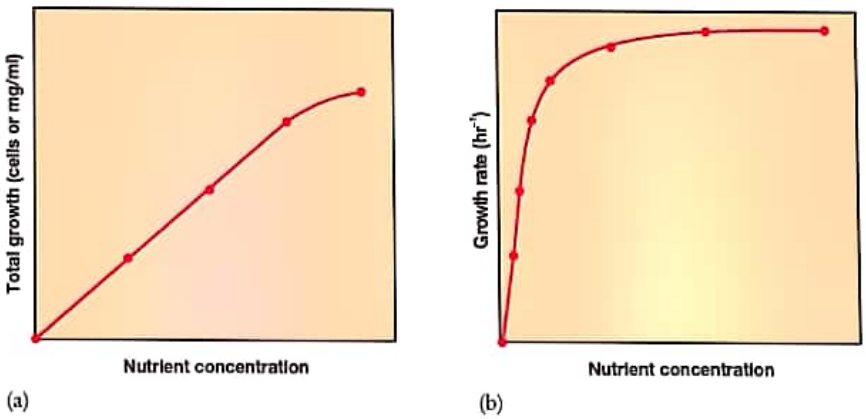
Exponential Phase

During the **exponential** or **log phase,** microorganisms are growing and dividing at the maximal rate possible given their genetic potential, the nature of the medium, and the conditions under which they are growing. Their rate of growth is constant during the exponential phase; that is, the microorganisms are dividing and doubling in number at regular intervals. Because each individual divides at a slightly different moment, the growth curve rises smoothly rather than in discrete jumps (figure 6.1). The population is most uniform in terms of chemical and physiological properties during this phase; therefore exponential phase cultures are usually used in biochemical and physiological studies.

Exponential growth is **balanced growth.** That is, all cellular constituents are manufactured at constant rates relative to each other. If nutrient levels or other environmental conditions change, **unbalanced growth** results. This is growth during which the rates of synthesis of cell components vary relative to one another until a new balanced state is reached. This response is readily observed in a shift-up experiment in which bacteria are transferred from a nutritionally poor medium to a richer one. The cells first construct new ribosomes to enhance their capacity for protein synthesis. This is followed by increases in protein and DNA synthesis. Finally, the expected rise in reproductive rate takes place. Protein and DNA synthesis (sections 11.3 and 12.2)

Unbalanced growth also results when a bacterial population is shifted down from a rich medium to a poor one. The organisms may previously have been able to obtain many cell components directly from the medium. When shifted to a nutritionally inadequate medium, they need time to make the enzymes required for the biosynthesis of unavailable nutrients. Consequently cell division and DNA replication continue after the shift-down, but net protein and RNA synthesis slow. The cells become smaller and reorganize themselves metabolically until they are able to grow again. Then balanced growth is resumed and the culture enters the exponential phase. Regulation of nucleic acid synthesis (pp. 275–83)

Figure 6.2 Nutrient Concentration and Growth. **(a)** The effect of changes in limiting nutrient concentration on total microbial yield. At sufficiently high concentrations, total growth will plateau. **(b)** The effect on growth rate.



These shift-up and shift-down experiments demonstrate that microbial growth is under precise, coordinated control and responds quickly to changes in environmental conditions.

When microbial growth is limited by the low concentration of a required nutrient, the final net growth or yield of cells increases with the initial amount of the limiting nutrient present (**figure 6.2*a***). This is the basis of microbiological assays for vitamins and other growth factors. The rate of growth also increases with nutrient concentration (figure 6.2*b*), but in a hyperbolic manner much like that seen with many enzymes (*see figure 8.17*). The shape of the curve seems to reflect the rate of nutrient uptake by microbial transport proteins. At sufficiently high nutrient levels the transport systems are saturated, and the growth rate does not rise further with increasing nutrient concentration. Microbiological assays (p. 99); Nutrient transport systems (pp. 100–4)

Stationary Phase

Eventually population growth ceases and the growth curve becomes horizontal (figure 6.1). This **stationary phase** usually is attained by bacteria at a population level of around 10^9 cells per ml. Other microorganisms normally do not reach such high population densities, protozoan and algal cultures often having maximum concentrations of about 10^6 cells per ml. Of course final population size depends on nutrient availability and other factors, as well as the type of microorganism being cultured. In the stationary phase the total number of viable microorganisms remains constant. This may result from a balance between cell division and cell death, or the population may simply cease to divide though remaining metabolically active.

Microbial populations enter the stationary phase for several reasons. One obvious factor is nutrient limitation; if an essential nutrient is severely depleted, population growth will slow. Aerobic organisms often are limited by O_2 availability. Oxygen is not very soluble and may be depleted so quickly that only the surface of a culture will have an O_2 concentration adequate for growth. The cells beneath the surface will not be able to grow unless the culture is shaken or aerated in another way. Population growth also may cease due to the accumulation of toxic waste products. This factor seems to limit the growth of many anaerobic cultures (cultures growing in the absence of O_2). For example, streptococci can produce so much lactic acid and other organic acids from sugar fermentation that their medium becomes acidic and

growth is inhibited. Streptococcal cultures also can enter the stationary phase due to depletion of their sugar supply. Finally, there is some evidence that growth may cease when a critical population level is reached. Thus entrance into the stationary phase may result from several factors operating in concert.

As we have seen, bacteria in a batch culture may enter stationary phase in response to starvation. This probably often occurs in nature as well because many environments have quite low nutrient levels. Starvation can be a positive experience for bacteria. Many do not respond with obvious morphological changes such as endospore formation, but only decrease somewhat in overall size, often accompanied by protoplast shrinkage and nucleoid condensation. The more important changes are in gene expression and physiology. Starving bacteria frequently produce a variety of **starvation proteins,** which make the cell much more resistant to damage in a variety of ways. They increase peptidoglycan cross-linking and cell wall strength. The Dps (*D*NA-binding *p*rotein from *s*tarved cells) protein protects DNA. Chaperones prevent protein denaturation and renature damaged proteins. As a result of these and many other mechanisms, the starved cells become harder to kill and more resistant to starvation itself, damaging temperature changes, oxidative and osmotic damage, and toxic chemicals such as chlorine. These changes are so effective that some bacteria can survive starvation for years. Clearly, these considerations are of great practical importance in medical and industrial microbiology. There is even evidence that *Salmonella typhimurium* and some other bacterial pathogens become more virulent when starved.

Death Phase

Detrimental environmental changes like nutrient deprivation and the buildup of toxic wastes lead to the decline in the number of viable cells characteristic of the **death phase.** The death of a microbial population, like its growth during the exponential phase, is usually logarithmic (that is, a constant proportion of cells dies every hour). This pattern in viable cell count holds even when the total cell number remains constant because the cells simply fail to lyse after dying. Often the only way of deciding whether a bacterial cell is viable is by incubating it in fresh medium; if it does not grow and reproduce, it is assumed to be dead. That is, death is defined to be the irreversible loss of the ability to reproduce.

Although most of a microbial population usually dies in a logarithmic fashion, the death rate may decrease after the population has been drastically reduced. This is due to the extended survival of particularly resistant cells. For this and other reasons, the death phase curve may be complex.

Mathematics of Growth:

Microbial growth during the exponential phase is very important and of interest to microbiologists and the analysis applies to microorganisms dividing by binary fission. The time required by a cell to divide is called the generation time or doubling time. In the laboratory, under favorable conditions, a growing bacterial population doubles at regular intervals. Growth is by geometric progression: 1, 2, 4, 8, etc. or 2^0, 2^1, 2^2, 2^3 .....2^n (where n = the number of generations). This is called exponential growth. In reality, exponential growth is only part of the bacterial life cycle, and not representative of the normal pattern of growth of bacteria in Nature. This might vary from organism to organism depending upon the environmental conditions etc. For example in *E.coli* the generation time is 20 min and hence after 20 generations a single initial cell would increase to over 1 million cells. This would require a little less than 7 hours. The population is doubling every generation; hence the increase in population is always 2^n where n is the number of generations. The resulting population increase is exponential or logarithmic.

When growing exponentially by binary fission, the increase in a bacterial population is by geometric progression. If we start with one cell, when it divides, there are 2 cells in the first generation, 4 cells in the second generation, 8 cells in the third generation, and so on. The **generation time** is the time interval required for the cells (or population) to divide.

G (generation time) = (time, in minutes or hours)/n(number of generations)

G = t/n

t = time interval in hours or minutes

B = number of bacteria at the beginning of a time interval

b = number of bacteria at the end of the time interval

n = number of generations (number of times the cell population doubles during the time interval)

$b = B \times 2^n$ (This equation is an expression of growth by binary fission)

Solve for n:

logb = logB + nlog2

n=logb-logB/log2

n= logb-logB/ .301

n = 3.3 logb/B

G = t/n

Solve for G

G= t/3.3logb/B

Example: What is the generation time of a bacterial population that increases from 10,000 cells to 10,000,000 cells in four hours of growth?

G= t/3.3logb/B

$G= 240\text{minutes}/3.3\log 10^7 /10^4$

G= 240minutes/3.3x3

G = 24 minutes

Measurement of Microbial Growth:

A number of techniques are available in order to measure growth of microbial populations. Either population number of mass may be calculated ad growth leads to increase in both.

Direct measurement of cell numbers:

Bacteria or microorganisms can be counted directly on the plate and also called as plate counting. Advantage of this method is that it measures the number of viable cells. Disadvantage is that, it is time consuming and expensive as one needs media and other conditions need to be maintained. Bacteria counted on plate counts are referred to as colony forming units as a single cell or a clump of bacterial cells can lead to a colony which contains many cells. The colonies when they are counted in plate count method are to be present sparsely for accurate counting as overcrowding can lead to incorrect counting. To solve this, one has to adapt the serial dilution method in order to get an accurate count.

Serial dilution and pour and spread plate: Supposing one has to accurately count the number of cells given in a solution, then serial dilution needs to be performed. A 1ml of the sample is taken and transferred to a tube containing 9ml of sterile water and this process can be repeated until we reach a considerable dilution (say 10^6 to 10^7). Once the original inoculum is diluted one needs to perform a pour plate or a spread plate technique in order to count the number of bacteria present in the diluted sample and then the original sample. In pour plate method the diluted sample is poured into the petriplate and then the medium which is at nearly 50^0C is poured over the inoculum and mixed by gentle agitation. With this method, colonies grow within the nutrient agar as well as on the surface of the agar plate. As certain disadvantages are encountered in this method like heat sensitive microorganisms might not grow and also bacteria when they grow within the nutrient medium might not be useful for diagnostic purposes. In order to avoid these problems, spread plate method is mostly used (Fig. 3). A 0.1ml of the diluted sample is added to the surface of the nutrient medium and spread uniformly with the help of a glass spreader and after incubation, the colonies can be counted and the concentration of the bacterial cells in the original sample is calculated as follows:

purposes. In order to avoid these problems, spread plate method is mostly used (Fig. 3). A 0.1ml of the diluted sample is added to the surface of the nutrient medium and spread uniformly with the help of a glass spreader and after incubation, the colonies can be counted and the concentration of the bacterial cells in the original sample is calculated as follows:

Number of bacteria/ml = Number of colonies on plate x reciprocal of dilution of sample

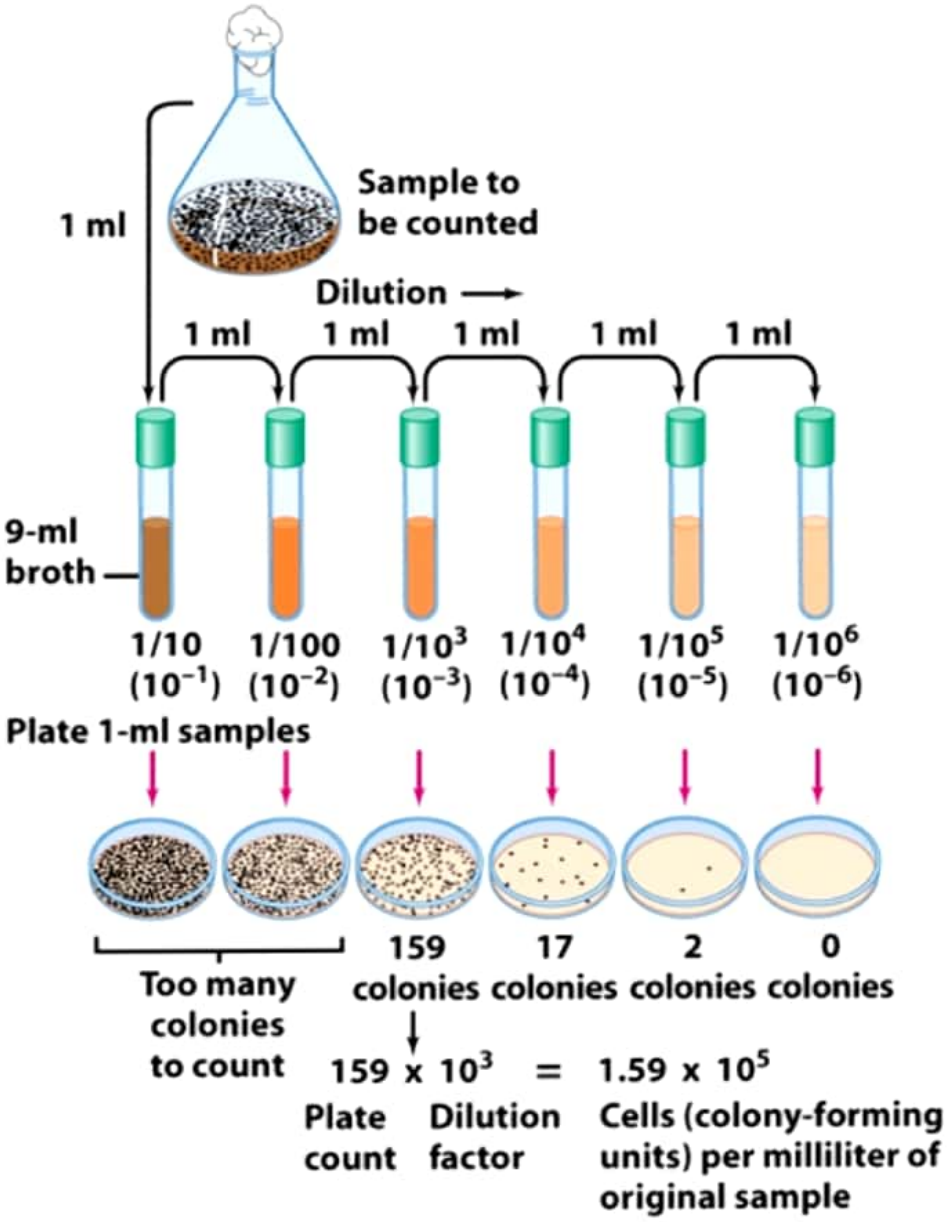


Fig. 3. Serial dilution methodology

Membrane Filtration: This method can be used in order to study if the quantity of the bacteria is very small as in aquatic samples like lakes, streams etc. Membranes with different pore sizes are used to trap different microorganisms. The sample is drawn through these special membrane filters and placed on an agar medium or on a pad soaked with liquid media. After incubation, the number of colonies can be counted and the number determined in the original sample. Selective media or differential media can be used for specific microorganisms. This is mostly used for analyzing aquatic samples.

Microscopic count: The Petroff-Hausser counting chamber or slide is easy, inexpensive and relatively quick method and also gives information about the size and morphology of the microorganisms. These specially designed slides have chamber of known depth with an etched grid on the chamber bottom (Fig.4).

Bacteria/mm^3 = (bacteria/square) (25 squares)/ (50)

Bacteria can be counted by taking into account the chamber's volume and any sample dilution. The disadvantage encountered in this method is that fairly large volume is required and also it is difficult to distinguish between living and dead cells. Microorganisms of larger sizes can be counted by using electronic counters such as coulter counter; where in the number of cells in a measured volume of liquid is counted. This method gives accurate results with larger cells and is extensively used in hospital laboratories to count red and white blood cells.

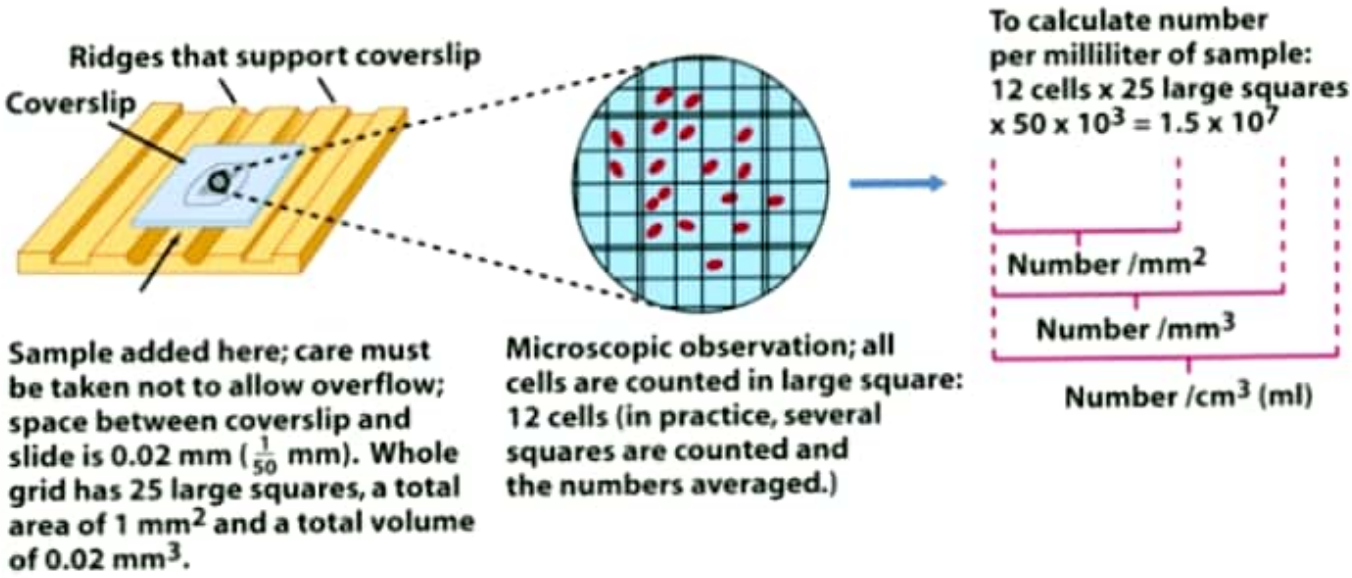


Fig. 4. Direct microscopic count of bacterial cells

Indirect methods of measurement of cell mass:

Population growth leads to increase in the total cell mass, as well as in cell numbers. The following methods can be used.

Turbidity: As bacteria grow/multiply in a liquid medium, the medium becomes turbid (Fig. 5). Spectrophotometer is used in order to measure the turbidity. A beam of light is transmitted through a bacterial suspension to a light-sensitive detector. The fact that microbial cells scatter light striking them, the amount of scattering is directly proportional to the biomass of cells present and indirectly related to cell number. The extent of light scattering can be measured and is almost linearly related to bacterial concentration at low absorbance levels.

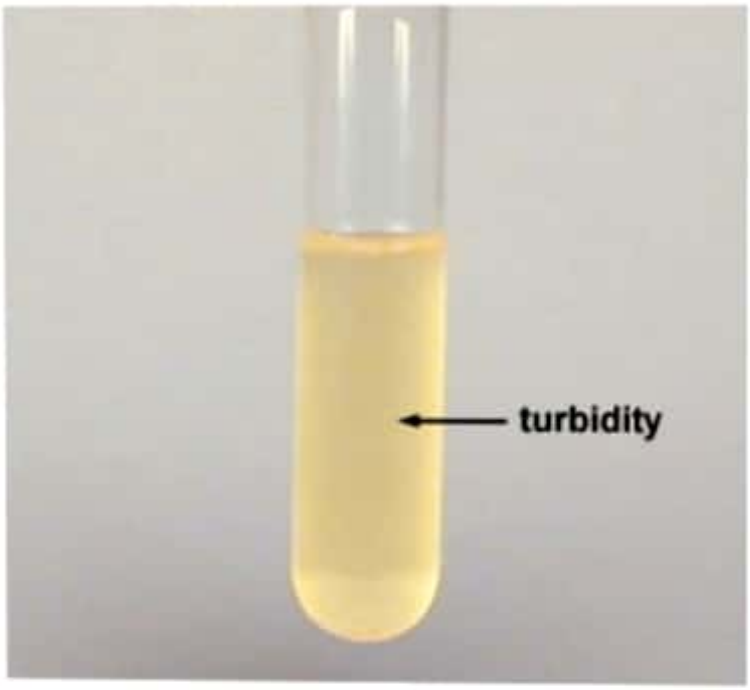


Fig. 5. Broth culture showing turbidity

Dry weight: This method is mostly used for filamentous bacteria and moulds. The microorganism is grown in liquid medium, filtered or centrifuged to remove extraneous material, and dried in an oven and then weighted. It is time consuming and hence not very sensitive.

Continuous culture of Microorganisms:

Batch cultures : Nutrient supplies are not renewed nor wastes removed.

Continuous cultures : continuous provision of nutrients and removal of wastes takes place. The population can be maintained in the exponential phase and at a constant biomass concentration for extended periods.

These can again be categorized into two types:

Chemostat : Where sterile medium is fed into the culture vessel at the same rate as the media containing microorganisms is removed.

Turbidostat : It has a photocell that measures the absorbance or turbidity of the cell culture in the growth vessel. The flow rate of media through the vessel is automatically regulated to maintain a predetermined turbidity or cell density.

Used in food and industrial microbiology.

REFERENCES:

Text Books:

1. Jeffery C. Pommerville. Alcamo's Fundamentals of Microbiology (Tenth Edition). Jones and Bartlett Student edition.

2. Gerard J. Tortora, Berdell R. Funke, Christine L. Case. Pearson - Microbiology: An Introduction. Benjamin Cummings.

Reference Books:

1. Lansing M. Prescott, John P. Harley and Donald A. Klein. Microbiology. Mc Graw Hill companies.

Measurement of Cell Numbers

The most obvious way to determine microbial numbers is through direct counting. Using a counting chamber is easy, inexpensive, and relatively quick; it also gives information about the size and morphology of microorganisms. Petroff-Hausser counting chambers can be used for counting procaryotes; hemocytometers can be used for both procaryotes and eucaryotes. Procaryotes are more easily counted in these chambers if they are stained, or when a phase-contrast or a fluorescence micro-

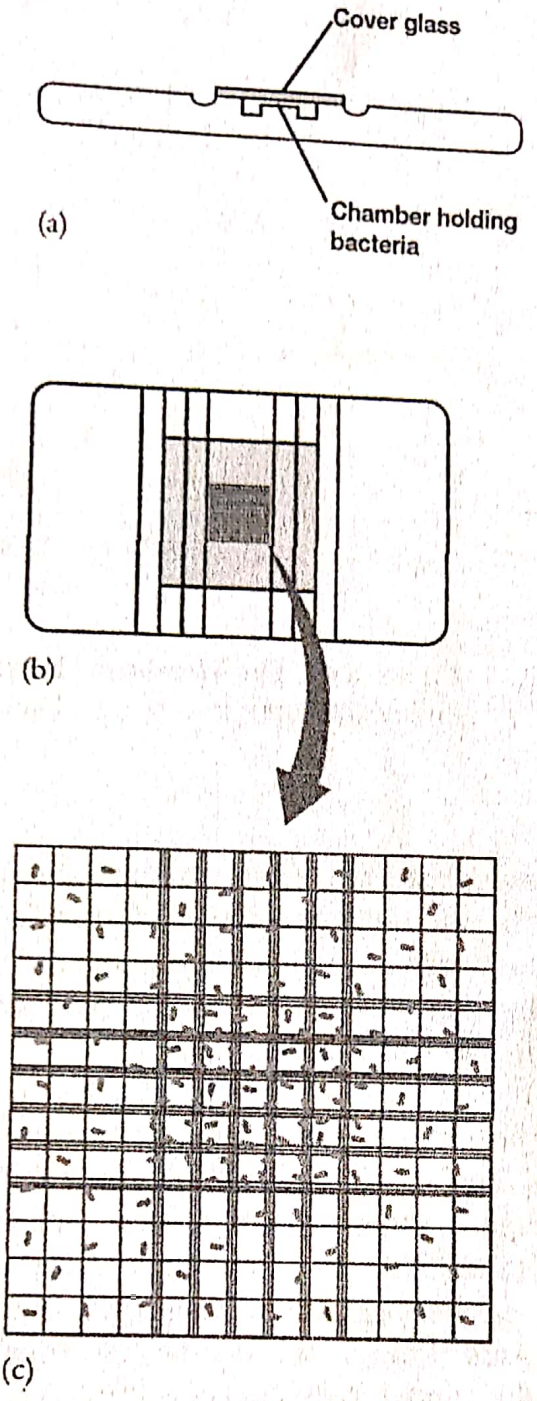


Figure 6.5 The Petroff-Hausser Counting Chamber. (a) Side view of the chamber showing the cover glass and the space beneath it that holds a bacterial suspension. (b) A top view of the chamber. The grid is located in the center of the slide. (c) An enlarged view of the grid. The bacteria in several of the central squares are counted, usually at ×400 to ×500 magnification. The average number of bacteria in these squares is used to calculate the concentration of cells in the original sample. Since there are 25 squares covering an area of 1 mm^2, the total number of bacteria in 1 mm^2 of the chamber is (number/square)(25 squares). The chamber is 0.02 mm deep and therefore,

$$\text{bacteria/mm}^3 = (\text{bacteria/square})(25\ \text{squares})(50).$$

The number of bacteria per cm^3 is 10^3 times this value. For example, suppose the average count per square is 28 bacteria:

$$\text{bacteria/cm}^3 = (28\ \text{bacteria})\ (25\ \text{squares})(50)(10^3) = 3.5 \times 10^7.$$

scope is employed. These specially designed slides have chambers of known depth with an etched grid on the chamber bottom (**figure 6.5**). The number of microorganisms in a sample can be calculated by taking into account the chamber's volume and any

sample dilutions required. There are some disadvantages to the technique. The microbial population must be fairly large for accuracy because such a small volume is sampled. It is also difficult to distinguish between living and dead cells in counting chambers without special techniques.