Volume 1

General Bacteriology

Essentials of MICROBIOLOGY for Postgraduates

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JAYPEE

Contents

CHAPTER

4 Morphology

Bacterial morphology refers to the shape, size, arrangement, and structural features of bacteria. It plays a crucial role in the identification, classification, and understanding of bacterial functions and pathogenicity.

SIZE OF BACTERIA[1, 2]

The size of bacteria varies widely depending on the species, but most bacteria fall within a typical size range. Here's a summary of bacterial sizes:

- **Average size:** Generally, majority of bacteria measure 0.2–10 µm in length and 0.2–2 µm in width. Bacteria are typically smaller than eukaryotic cells (10–100 µm in diameter), but larger than viruses (0.02–0.3 µm). Bacteria can be categorized by their size.
- **Small bacteria:** Examples include *Mycoplasma* species (~0.2–0.3 µm in diameter), which is the smallest free-living microorganism. Their small size allows them to pass through filters that typically trap bacteria. Other small bacteria include *Ureaplasma* species (~0.3 µm), *Rickettsia* species (~0.3–0.5 µm) and *Chlamydia* species (~0.3–0.6 µm).
- **Medium-sized bacteria:** The majority of bacteria fall under this category. Examples include *Escherichia coli* (~1–2 µm in length and ~0.5 µm in width), which represents the average size of many common bacteria.
- **Large bacteria:** Examples include *Bacillus* species (2–5 µm in length, 0.5–1.5 µm in width), though some species can grow up to 10 µm or more in length. *Bacillus anthracis* measures ~3–8 µm in length and ~1–1.5 µm in width.
- **Giant bacteria:** Examples include filamentous bacteria such as *Actinomycetes*. Their filaments have a width of 0.5–2 µm (comparable to many rod-shaped bacteria) and a length of several µm (often tens or hundreds of µm) as branching filaments.

SHAPE OF BACTERIA

Bacteria exhibit a diverse range of shapes, which are closely linked to their ecological roles, survival strategies, and pathogenicity. The two most common shapes are cocci and rods **(Table 4.1)**.

Table 4.1: Classification of bacteria depending on their morphology and Gram staining property.[1-3]

Bacteria	Example
Gram-positive cocci arranged in	
Cluster	*Staphylococcus*
Chain	*Streptococcus*
Pairs, lanceolate shaped	Pneumococcus
Pair or in short chain, spectacle shaped	*Enterococcus*
Tetrads	*Micrococcus*
Octate	*Sarcina*
Gram-negative cocci arranged in	
Pairs, lens shaped	Meningococcus
Pairs, kidney shaped	Gonococcus
Gram-positive bacilli arranged in	
Chain (bamboo stick appearance)	*Bacillus anthracis*
Chinese letter or cuneiform pattern	*Corynebacterium diphtheriae*
Palisade pattern	Diphtheroids
Branched and filamentous form	*Actinomyces* and *Nocardia*
Gram-negative bacilli arranged in	
Pleomorphic (various shapes)	*Haemophilus, Proteus*
Thumbprint appearance	*Bordetella pertussis*
Comma-shaped (fish in stream appearance)	*Vibrio cholerae*
Curved	*Campylobacter* (Gull-wing shaped) and *Helicobacter*
Chain	*Streptobacillus*
Spirally coiled, flexible	Spirochetes
Rigid spiral forms	*Spirillum*
Bacteria that lack cell wall	*Mycoplasma*

Cocci[1, 2]

Cocci (singular coccus) are derived from kokkos, meaning berry. They are round, oval or spherical cells. They can exist

singly or can be associated in characteristic arrangements that can be useful in their identification.

- **Diplococci** (singular, diplococcus) arise when cocci divide and remain together to form pairs. This pattern is seen in pneumococcus, meningococcus, gonococcus and sometimes in *Enterococcus.*
- **Chains of cocci** result when cells adhere after repeated divisions in one plane; this pattern is seen in the genera *Streptococcus,* and sometimes in *Enterococcus,* and *Lactococcus.*
- **Cocci in clusters:** Genus *Staphylococcus* divides in multiple random planes to generate irregular, grapelike clusters.
- **Tetrads:** Divisions in two or three planes can produce symmetrical groupings of cocci. Bacteria in the genus *Micrococcus* often divide in two planes to form square groups of four cells called tetrads.
- **Octate:** *Sarcina,* cocci divide in three planes, producing cubical packets of eight cells.

Bacilli or Rods[1, 2]

Rods, sometimes called bacilli (singular, bacillus), are cylindrical or elongated bacteria that differ considerably in their length-to-width ratio.

- **Diplobacilli:** Pairs of bacilli (e.g. *Klebsiella pneumoniae*).
- **Chains of bacilli:** Examples include *Bacillus subtilis*, and *Streptobacillus moniliformis.*
- **Coccobacilli:** They appear short, oval-shaped rods resembling cocci. Examples include *Haemophilus influenzae* and *Acinetobacter* spp.
- **Filamentous bacteria:** discussed below.

Filamentous Bacteria[1, 2]

They appear as long, thread-like structures resembling fungal filaments, which allow colonization of solid substrates and decomposition of organic materials. Examples include members of Actinomycetes.

- Anaerobic Actinomycetes such as *Actinomyces* species.
- Aerobic Actinomycetes such as *Nocardia, Actinomadura, Streptomyces,* thermophilic actinomycetes (e.g. *Saccharomonospora, Saccharopolyspora,* and *Thermoactinomyces*) and other rare members (e.g. *Gordonia, Tsukamurella, Nocardiopsis, Pseudonocardia,* etc.)

Pleomorphic (Variable Shape)[1-3]

Pleomorphic bacteria are microorganisms that can exhibit variable shapes and sizes (such as coccoid, coccobacillary, rod-shaped, or filamentous forms) rather than maintaining a single, consistent morphology. This ability to change shape is due to the absence or modification of rigid structural components, such as the cell wall, or due to environmental or genetic factors. Examples include:

- ***Mycoplasma:*** As they lack cell wall, *Mycoplasma* can change its shape and lead to pleomorphism, ranging from coccoid to filamentous to other bizarre forms.
- ***Proteus:*** The smaller, smooth (O-form) colonies of *Proteus* species represent non-swarming variants that arise after repeated subculture. On Gram stain, these colonies show highly pleomorphic cells—ranging from coccobacilli to filamentous and giant forms.
- ***Haemophilus* species:** *H. influenzae* shows pleomorphism, which is commonly seen in CSF smears, than in sputum smears.
- ***Yersinia pestis:*** Gram staining of culture smear reveals pleomorphism—coccoid, coccobacillary, bacillary, filamentous, and giant forms. **Involution forms** are seen in older cultures, hastened by the addition of 3% NaCl—a phenomenon which can be used as a means of identification of Yersiniae.
- **Nutritionally variant streptococci:** Both *Abiotrophia* and *Granulicatella* species, when cultivated in pyridoxal- or cysteine-supplemented complex media, they are pleomorphic.
- ***Corynebacterium:*** They are club-shaped, irregularly stained, gram-positive rods. *C. diphtheriae* appear highly pleomorphic, may occur singly or in pairs, often in a "V" formation resembling Chinese letters. Diphtheroids appear club-shaped, pleomorphic, gram-positive rods often with tapered ends and palisade arrangement.
- **Anaerobes:** *Bacteroides* species frequently show pleomorphism in culture smear. Some *Clostridium* species like *C. septicum,* are pleomorphic. *Bifidobacterium* can occasionally show pleomorphism. *Fusobacterium* appears as thin, pale, gram-negative rod with tapering ends (fusiform), but some species appear pleomorphic.
- **Others:** *Cardiobacterium* spp. on culture smear and *Francisella* in direct smear often show pleomorphism.
  - *Streptobacillus moniliformis* in direct and colony smear appears pleomorphic.
  - *Bartonella bacilliformis* shows pleomorphism.
  - *Pseudomonas aeruginosa* in older cultures may appear slightly pleomorphic.
  - *Vibrio parahaemolyticus* may show pleomorphism in older cultures.
  - *Helicobacter pylori:* Although *Helicobacter pylori* appear as gram-negative curved rods, after 3–5 days of incubation, they become pleomorphic, showing irregularly curved or coccoid forms.
  - *Capnocytophaga:* Staining from the colonies reveals gram-negative, fusiform in shape, and appear straight or slightly curved. Pleomorphism, with swollen or large cocci forms, can also be seen in older cultures.

Spiral-shaped Bacteria[1, 2]

They appear helical or corkscrew-like bacteria. Spiral shapes aid in motility, especially in viscous environments like mucosal surfaces. Examples include **(Fig. 4.1)**:

- ***Vibrio:*** Comma-shaped with a single curve (e.g. *Vibrio cholerae*).
- ***Mobiluncus*** appears curved and gram-variable.
- ***Spirillum:*** Rigid spiral-shaped with external flagella (e.g. *Spirillum volutans*).
- **Spirochete:** Flexible, tightly coiled bacteria with internal axial filaments (e.g. *Treponema pallidum*). They exhibit a unique corkscrew motion for penetrating tissues.

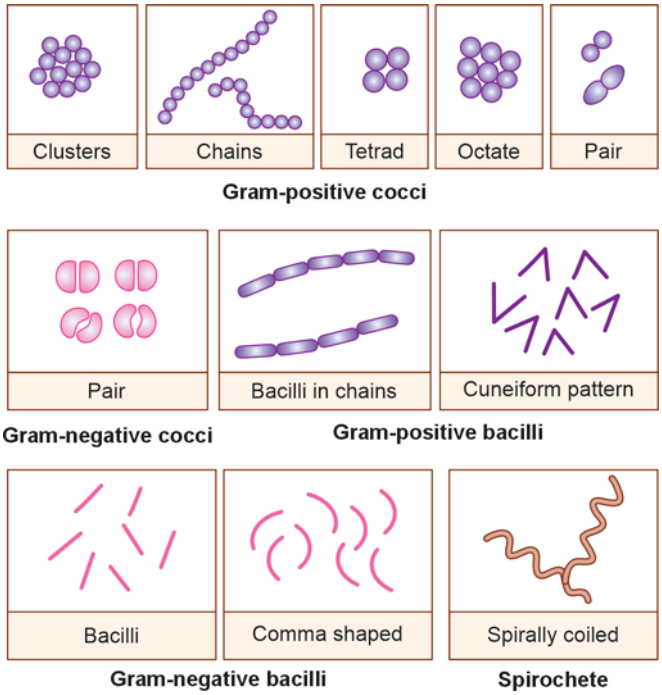


Fig. 4.1: Different morphology of bacteria and Gram-staining property.

BACTERIAL CELL ANATOMY[2-4]

Taxonomically, microorganisms of medical importance can be of two broad categories, prokaryotic and eukaryotic.

- Bacteria are prokaryotic organisms that have a simple structure and lifestyle, whereas eukaryotic organisms like fungi and parasites have a complex morphology and lifestyle.
- Though bacteria have a simple structure, they possess a complex, well-organized cell wall, which sets them apart from other eukaryotic organisms, as it confers so many unique features to the bacteria.
- Understanding the diversity in the cellular organization of these microbes is important in comprehending their functions and their implications for human health. The major differences between prokaryotes and eukaryotes are summarized in **Table 4.2**.

Bacterial cell anatomy comprises of the following structures **(Fig. 4.2)**.

- The outer layer or the **envelope** of a bacterial cell consists of (1) a rigid cell wall and (2) underlying plasma membrane.

Table 4.2: Differences between prokaryotes and eukaryotes.[2,4]

Property	Prokaryotes	Eukaryotes
Examples	Bacteria and Archaea	Parasite, fungi, plant and animals
Nucleus and genetic organization		
Nuclear membrane	Absent	Present
Nucleus	Pseudo-nuclei with a clump of DNA known as nucleoid	Well-organized complex nuclei
Nucleolus	Absent	Present
Chromosomes	Single, closed, circular double-stranded DNA	Multiple, linear chromosomes
Extra-chromosomal DNA	Usually present (plasmid)	Present in mitochondria and chloroplast
Genome	Haploid and compact	Most are diploid (except fungi, which is haploid). Has a lot of non-coding and repetitive DNA
Histone proteins	Absent	Present
Transcription/ translation	Continuous, where transcription and translation of mRNA occurs simultaneously in the cytosol	Discontinuous, where transcription occurs in the nucleus and mRNA translation occurs in the cytosol
Gene exchange	Chromosomal or horizontal gene transfer by transformation, transduction, or conjugation	It occurs through meiosis, which results in genetic recombination
Cytosol		
Cytoplasmic membrane	Made of phospholipids but no sterols (except *Mycoplasma* spp.)	Made of both phospholipids and sterols
Organelles	Ill-defined and not membrane-bound	Well-defined and membrane-bound
Cytoskeleton	Simple and uniform (e.g. FtsZ, MreB, and CreS)	Complex and diverse (e.g. actin, tubulin, etc.)
Mitochondria	Absent (but cellular respiration happens via mesosome)	Present
Golgi complex	Absent	Present
Endoplasmic reticulum	Absent	Present
Ribosomes	Present: 70S (50S + 30S)	Present: 80S (60S + 40S)
Lysosomes and peroxisomes	Absent	Present
Membrane-bound vacuoles	Absent (instead, some may have gas vacuoles (for buoyancy) and carboxysomes (for carbon fixation)	Present
Other properties		
Cell wall	Present and usually complex	Absent (but present in fungi—chitin; plants—cellulose)
Motility	Simple by means of flagella	Complex by means of intricate flagella, pseudopodia and other complex locomotory organs
Sexual reproduction	Absent	Mostly present but may be absent in some fungi and protozoa

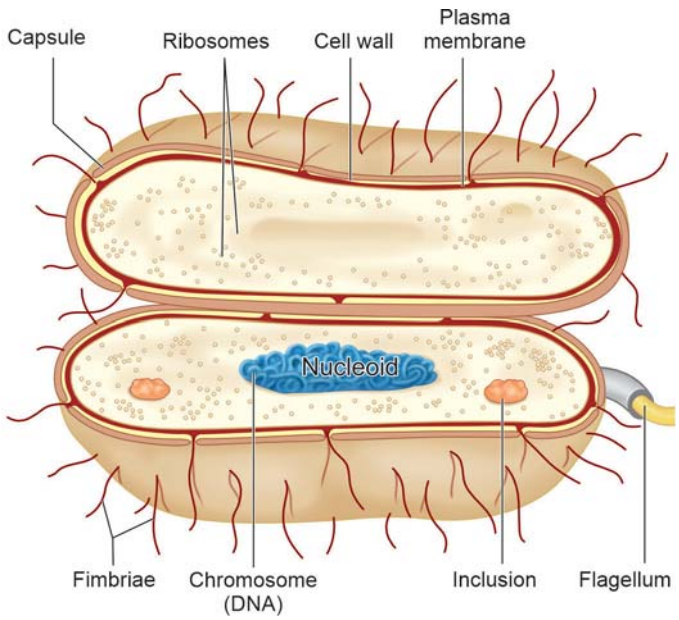


Fig. 4.2: Structure of bacterial cell.[3]

- The cytoplasm contains cytoplasmic inclusions (mesosomes, ribosomes, inclusion granules, vacuoles) and a diffuse nucleoid containing single circular chromosome.
- Some bacteria may possess additional cell wall appendages such as capsule, flagella and fimbriae.
- However, a single bacterium does not have all the above-mentioned structures, and there will always be exceptions in the structure and arrangement of these organelles.

BACTERIAL CELL WALL[5]

The cell wall is a tough and rigid structure, surrounding the bacterium. It is 10–25 nm in thickness and weighs about 20–25% of the dry weight of the cell. The bacterial cell wall has the following functions:[1, 2]

- It provides protection to the cell against osmotic lysis.
- It confers rigidity upon bacteria due to the presence of peptidoglycan layer in the cell wall.
- It accounts for the shape of the cell.
- It takes part in cell division.
- The cell wall can protect a cell from toxic substances and is the site of action of several antibiotics.
- Virulence factors: Bacterial cell wall contains certain virulence factors (e.g. endotoxin), which contribute to their pathogenicity.
- Immunity: Antibody raised against specific cell wall antigens (e.g. antibody to LPS) may provide immunity against some bacterial infections.
- Bacterial cell wall synthesis is an important target for many antibiotics.

Peptidoglycan[1, 2, 4]

Bacteria possess a cell wall with high tensile strength, which confers a range of versatile functions. The bacterial cell wall is made of a complex substance called peptidoglycan, which is also referred to as murein or mucopeptide or sometimes as peptidoglycan sacculus as it resembles an enormous mesh-like structure.[1] It is found in all bacteria except cell wall-deficient bacteria such as *Mycoplasma* and *Ureaplasma*.

- **Components:** Peptidoglycan are the building blocks of the cell wall. They are complex polymers made of three important components: the backbone, the tetrapeptide side chain, and the peptide cross-bridges.
- **Backbone:** The backbone of the peptidoglycan layer is made up of alternating units of the carbohydrate moieties N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG), which are linked to each other by a β-1,4 glycosidic linkage.
- **Tetrapeptide chain:** The tetrapeptide side chains (TSC) are short amino acid chains that are made up of a mixture of D- and L-amino acids. These chains arise from the lactyl group of C3 in the NAM molecule.
- **Amino acids in TSC:** The amino acids in peptidoglycan tetrapeptide chains are unusual because they include D-amino acids, which are rarely found in human proteins. The reason behind this is that the presence of D-amino acids protects the bacteria against the action of peptidases, which are active only on the L-form of the amino acids.
- **GP and GN TSC:** Both gram-positive and gram-negative bacteria have similar amino acids in positions 1, 2, and 4. But the amino acid in position 3 makes a major difference between gram-positive and gram-negative organisms.
  - Both groups of bacteria have L-alanine at position 1, D-glutamate at position 2, and D-alanine at position 4.
  - However, in position 3, gram-positive bacteria possess L-lysine, and on the other hand, gram-negative bacteria possess meso-diaminopimelic acid (meso-DAP), which is an immediate precursor of lysine biosynthesis in the bacteria.
- **Cross-linkage:** The peptidoglycan layers can be cross-linked with each other by two types: Direct and indirect linkage.
  - **Indirect linkage:** This is seen in gram-positive bacteria, which happens via the **pentaglycine bridge**. As the name suggests, it is a short chain of amino acids that is made of five glycine residues. It acts as a bridge and interlinks the NAM residue of one peptidoglycan chain to the other. This results in the formation of a dense, interconnected network of peptidoglycan strands **(Fig. 4.3)**.

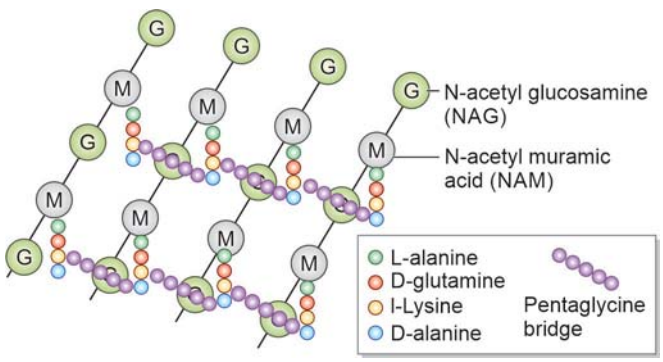


Fig. 4.3: Peptidoglycan layer of gram-positive cell wall.

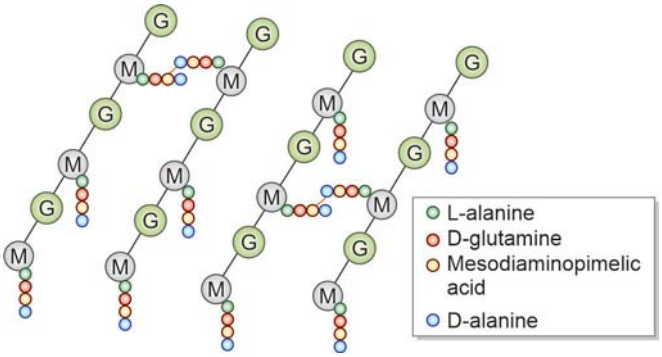


Fig. 4.4: Peptidoglycan layer of gram-negative cell wall.

- **Direct linkage:** It is seen in gram-negative organisms, where the amino group of meso-DAP at position 3 of the tetrapeptide chain of one peptidoglycan and the carboxyl group of the D-alanine at position 4 of the tetrapeptide chain of adjacent peptidoglycan are directly linked to each other **(Fig. 4.4)**.

❖ **Thickness:** There is also a significant difference in the thickness of peptidoglycan layers in gram-positive and gram-negative organisms. Gram-positive organisms can have up to 40 layers of peptidoglycan, which contributes to about 50% of the cell wall material. In contrast, gram-negative organisms can have as low as 1–2 peptidoglycan layers, thereby comprising 5–10% of the wall material.

❖ **Porosity:** Though very tough, the peptidoglycan layer is porous in nature, which can allow proteins of molecular weight up to 50,000 Daltons to pass through, depending on the stretchability of the cell wall. Exceptionally large proteins cannot pass through the cell wall directly; instead, the bacteria secrete exoenzymes, which can break these larger proteins into smaller, portable fragments.

Synthesis of Peptidoglycan

Peptidoglycan synthesis involves multiple enzymatic reactions occurring in three distinct stages: cytoplasmic synthesis of precursors, membrane-associated assembly, and extracellular polymerization **(Figs. 4.5 and 4.6)**.

I. Cytoplasmic Stage: Precursor Synthesis[6, 7]

The initial phase takes place entirely in the cytoplasm, focusing on the synthesis of the activated disaccharide-pentapeptide subunit, UDP-NAM-pentapeptide (often called Park's nucleotide).

❖ **Formation of UDP-N-acetylglucosamine (UDP-NAG):**
- Enzyme GlmS converts fructose-6-phosphate to glucosamine-6-phosphate.
- Enzyme GlmM converts glucosamine-6-phosphate to glucosamine-1-phosphate.
- Enzyme GlmU acetylates glucosamine-1-phosphate to form N-acetylglucosamine-1-phosphate and adds UDP to produce UDP-N-acetylglucosamine (UDP-NAG).

❖ **Conversion to UDP-N-acetylmuramic acid (UDP-NAM):**
- Enzyme MurA transfers an enolpyruvyl group from phosphoenolpyruvate (PEP) to UDP-NAG, forming UDP-N-acetylglucosamine-enolpyruvate.

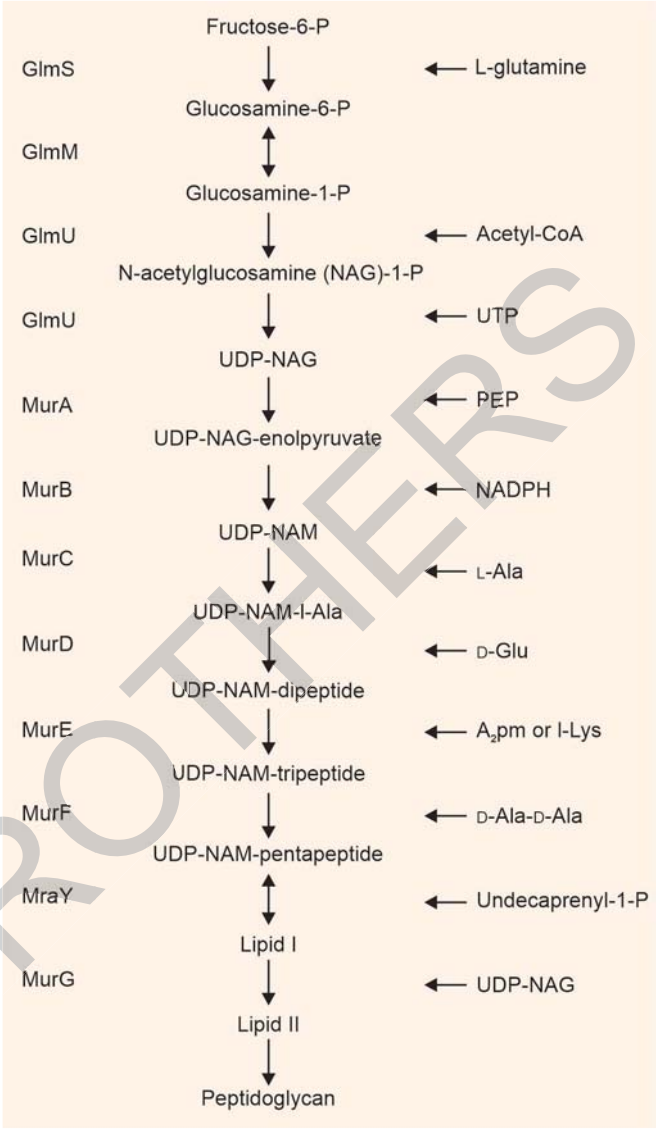


Fig. 4.5: Steps of peptidoglycan synthesis.[6]

- Enzyme MurB reduces the enolpyruvate group to form UDP-N-acetylmuramic acid (UDP-NAM).

❖ **Addition of a pentapeptide side chain to UDP-NAM:** This involves sequential addition of amino acids to UDP-NAM by enzyme Mur ligases:
- MurC enzyme: Adds L-alanine.
- MurD enzyme: Adds D-glutamic acid.
- MurE enzyme: Adds meso-diaminopimelic acid (for gram-negative bacteria) or L-lysine (for gram-positive bacteria).
- MurF enzyme: Adds a dipeptide (D-alanine-D-alanine), forming UDP-NAM-pentapeptide.

II. Membrane Stage: Lipid Intermediates Formation[6, 7]

The stage involves the transfer of peptidoglycan precursors to the lipid carrier molecules in the cell membrane.

❖ **Attachment of UDP-NAM-pentapeptide to undecaprenyl phosphate:** Enzyme MraY transfers the NAM-pentapeptide to undecaprenyl phosphate (C55-P) in the membrane, forming lipid I.

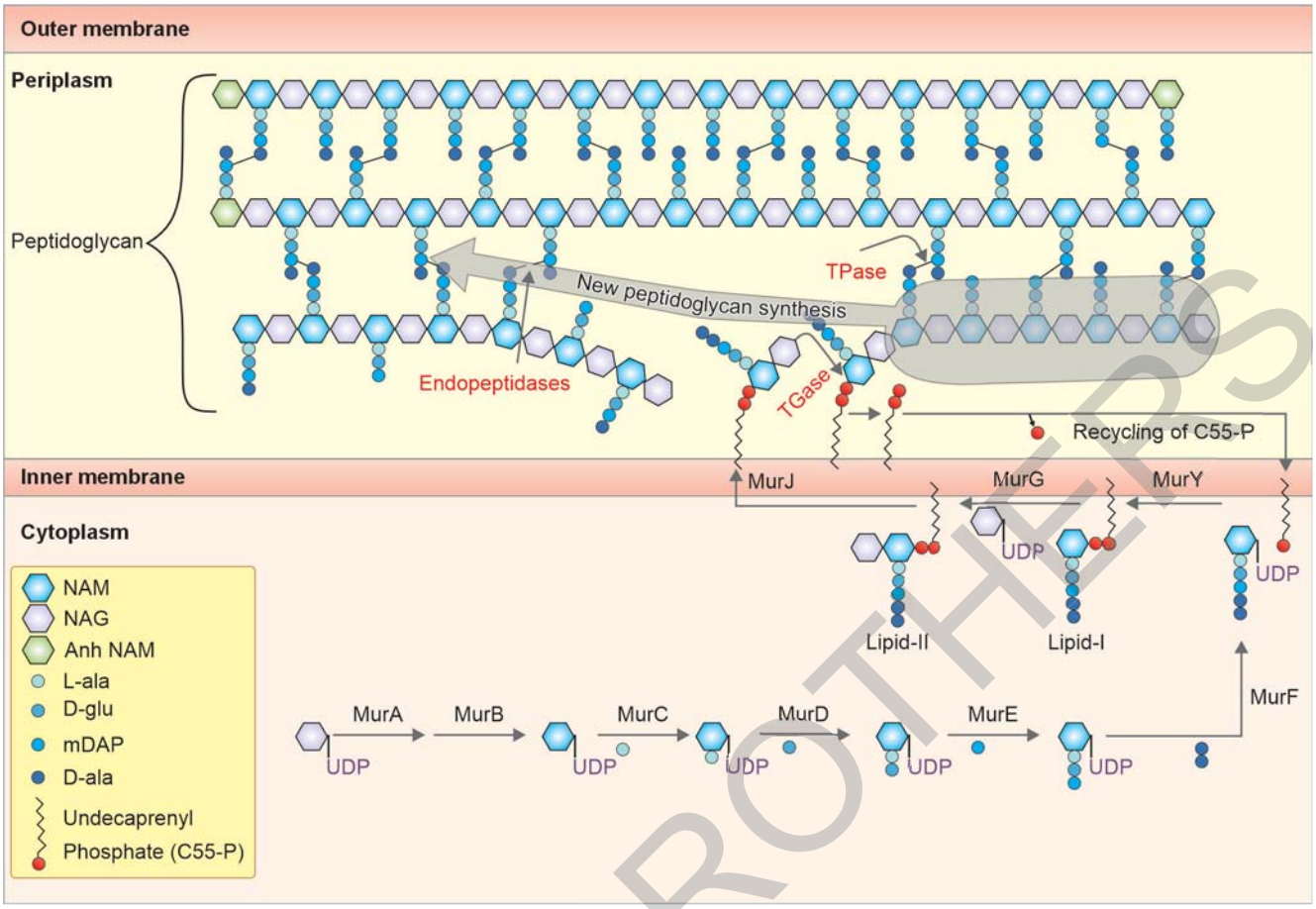


Fig. 4.6: Peptidoglycan synthetic pathway.[5]

- **Addition of NAG to lipid I:** Enzyme MurG adds a N-acetylglucosamine (NAG) residue to lipid I, forming lipid II. Lipid II contains the complete disaccharide-pentapeptide subunit.
- **Flipping of lipid II across the membrane:** Enzyme Flippase (e.g. FtsW or MurJ) flips lipid II to the inner membrane to the periplasm.

III. Extracytoplasmic Stage: Polymerization & Crosslinking[6, 7]

Lipid II carrier containing the complete disaccharide-pentapeptide subunit is now facing the outer side of the cell membrane into the periplasm, where the remaining events of peptidoglycan synthesis would take place.

- **Polymerization of glycan chains:** Enzyme transglycosylases polymerize lipid II subunits by linking NAG and NAM units to form long glycan strands; with C55-P being recycled to the cytoplasm.
- **Crosslinking of peptide chains:** Enzyme transpeptidases (penicillin-binding proteins, PBPs) catalyze crosslinking between the peptide side chains of neighboring glycan strands. Typically:
  - The terminal D-alanine from one pentapeptide is removed during crosslinking.
  - A peptide bond is formed between the meso-diaminopimelic acid (or L-lysine) of one peptide and the D-alanine of another.
- **Endopeptidase:** Hydrolysis mediated by endopeptidases leads to cleavage of existing peptide cross-links, which allow the insertion of new peptidoglycan precursors during cell wall expansion, e.g. D,D-endopeptidases cleaves between D-alanine residues in cross-linked peptides.
- **Regulation and coordination:** The synthesis process is tightly regulated to ensure cell wall integrity during growth and division. Key regulators include:
  - *FtsZ:* Organizes the division machinery and coordinates cell wall synthesis.
  - *RodA* and *MreB:* Maintain cell shape and regulate peptidoglycan synthesis in rod-shaped bacteria.

Inhibitors of Peptidoglycan Synthesis[6, 7]

Peptidoglycan (PG) synthesis is a prime target for antibiotics because it is essential for bacterial survival but absent in human cells. These inhibitors are highly specific, targeting the pathway across its three stages: the cytoplasm, the membrane, and the extracytoplasmic space.

I. Cytoplasmic Stage Inhibitors[6, 7]

These drugs prevent the initial creation of the PG precursor, UDP-NAM-pentapeptide.

- **Fosfomycin:** This antibiotic inhibits the enzyme MurA, which is responsible for the very first committed step in PG synthesis—the transfer of an enolpyruvyl group to UDP-NAG.

- **D-cycloserine:** This drug acts as a structural analog to D-alanine, competitively inhibiting two enzymes involved in forming the terminal D-alanine-D-alanine dipeptide, thereby blocking its production.

II. Membrane-Associated Inhibitors[6, 7]

These drugs block the formation or transport of the lipid II intermediate across the cell membrane.

- **Tunicamycin:** This compound inhibits the enzyme MraY, which is responsible for transferring the NAM-pentapeptide to the lipid carrier, undecaprenyl phosphate (C55–P), thus blocking the formation of lipid I.
- **CDFI/DMPI:** These compounds specifically inhibit the enzyme MurJ (a flippase), preventing the completed lipid II precursor from being flipped to the exterior of the cell membrane for polymerization.
- **Bacitracin:** This antibiotic targets the essential recycling of the lipid carrier. It binds tightly to bactoprenol pyrophosphate (C55–PP), preventing the lipid carrier molecule from being dephosphorylated and reused to transport new PG precursors.

III. Extracytoplasmic Inhibitors (Cell Wall Assembly)

These are the most well-known classes of antibiotics, targeting the final stages of polymer formation and crosslinking.

- **β-lactams (penicillins, cephalosporins, etc.):** These drugs are structural analogues of the D-Ala-D-Ala terminal and covalently bind to the active site of transpeptidases (Penicillin-Binding Proteins or PBPs). By inactivating PBPs, β-lactams prevent the final peptide crosslinking that provides the cell wall's rigidity.
- **Glycopeptides (vancomycin, teicoplanin):** This class of antibiotics works by physically binding with high affinity to the D–Ala–D–Ala terminus of the lipid II precursor. This binding physically sterically hinders both the transglycosylation (chain elongation) and transpeptidation (crosslinking) enzymes from accessing their substrate.

Penicillin-Binding Proteins[2, 8]

Penicillin-binding proteins (PBPs) are a group of bacterial enzymes involved in peptidoglycan synthesis. They are so named because they bind β-lactam antibiotics (e.g. penicillins, cephalosporins) as part of their function. PBPs are classified based on their molecular weight, enzymatic activity, and physiological role.

Native Penicillin-Binding Proteins (PBPs)[2, 8]

These are the typical PBPs found in bacterial cells that participate in peptidoglycan synthesis and are naturally sensitive to β-lactam antibiotics **(Table 4.3)**.

High-Molecular-Weight PBPs (HMW PBPs)[2, 8]

HMM PBPs are multimodular PBPs responsible for peptidoglycan polymerization and insertion into pre-existing cell wall.

- **Class A PBPs:** They are bifunctional, possess both transglycosylase + transpeptidase activities. They help in both glycan chain elongation (transglycosylation) by their N-terminal domain and peptide crosslinking (transpeptidation) by C-terminal domain. Examples include:
  - PBP1 in *Staphylococcus aureus*: Facilitates glycan chain polymerization and crosslinking.
  - PBP1a and PBP1b in *Escherichia coli*: Involved in synthesizing and repairing peptidoglycan during growth.
- **Class B PBPs:** They are monofunctional, possess only the transpeptidase activity through their C-terminal domain. They are responsible for crosslinking peptide side chains in specific locations (e.g. elongation or division zones). Examples include:
  - PBP2 in *E. coli*: Essential for cell wall elongation in rod-shaped bacteria.
  - PBP3 in *E. coli*: Required for septal peptidoglycan synthesis during cell division.

Table 4.3: Differences between normal and altered penicillin-binding proteins (PBPs).[2, 8]

Properties	Native PBPs	Altered PBPs
Function	Peptidoglycan synthesis (glycosylation and crosslinking)	Same function but altered β-lactam affinity
β-lactam binding	High affinity	Reduced affinity
Genetic origin	Native to the bacterial genome	Can be mutated, or acquired (e.g. *mecA*)
Examples	PBP1a, PBP2, PBP3 in *E. coli*	PBP2a in MRSA, PBP5 in enterococci

Low-Molecular-Weight PBPs (LMW PBPs)[2, 8]

Low-molecular-weight PBPs are the **Class C PBPs**. They are monofunctional, possess only the carboxypeptidase/endopeptidase activity. They fine-tune peptidoglycan structure by regulating peptide crosslinking and remodeling. Examples include:

- PBP4: Functions as a D,D-carboxypeptidase to remove the terminal D-alanine, regulating the extent of crosslinking.
- PBP5 and PBP6 in *E. coli*: Modify peptidoglycan to maintain structural flexibility.

Altered Penicillin-Binding Proteins[2, 8]

Altered PBPs are modified versions of native PBPs that exhibit reduced affinity for β-lactam antibiotics, enabling bacterial resistance. These alterations may result from mutations, horizontal gene transfer, or overexpression of resistant PBPs **(Table 4.3)**. Types of altered PBPs are:

- **PBPs with reduced affinity:** Point mutations in the active site or structural regions of PBPs reduce the binding efficiency of β-lactams without affecting their enzymatic function. Examples include:
  - PBP2x and PBP1a in *Streptococcus pneumoniae*: Mutated forms confer resistance to penicillin.
  - PBP3 mutations in *Haemophilus influenzae*.
- **Acquired resistant PBPs:** Horizontal gene transfer introduces PBPs with inherently low β-lactam affinity into susceptible bacteria. Examples include:

Table 4.4: Examples of penicillin-binding proteins (PBPs) in various bacteria.[8]

Bacteria	Class A	Class B	Class C
E. coli	PBP1a, PBP1b, PBP1c, MGT	PBP2, PBP3	PBP4, PBP4a, PBP5, PBP6, PBP7, and AmpH
S. aureus	PBP2, MGT	PBP1, PBP3, PBP2a	PBP4
Pneumococcus	PBP1a, PBP1b, PBP2a	PBP2b, PBP2x	PBP3
Enterococcus	PBP1a, PBP1b, PBP2a	PBP2, PBP2b, PBP4	DacF (PBP5)
Listeria	PBP1, PBP4	PBP2, PBP3	PBP5
N. gonorrhoeae	PBP1	PBP2, PBP3	PBP4

  - PBP2a in methicillin-resistant *Staphylococcus aureus* (MRSA), encoded by the *mecA* gene. It displays very low affinity for β-lactams, conferring resistance to all β-lactam antibiotics, including penicillins and cephalosporins.
  - PBP5 in *Enterococcus faecium*: Exhibits low β-lactam affinity, contributing to ampicillin resistance.
- **Hyperproduced PBPs:** Overexpression of normal PBPs dilutes the effect of β-lactam antibiotics, providing partial resistance. Examples include PBP4 overexpression in *S. aureus*—associated with vancomycin-intermediate resistance.
- **Mosaic PBPs:** Recombination between resistant and susceptible PBP genes produces hybrid proteins with reduced antibiotic binding. Examples include Mosaic PBP2x, PBP1a and PBP2b genes in *S. pneumoniae*, arise through interspecies recombination.

The total number of PBPs varies across bacterial species. For examples, *E. coli* possesses 12 PBPs, whereas *S. aureus* and *S. pneumoniae* possess 6 PBPs each **(Table 4.4)**.

Functions of Peptidoglycan Layer[1, 2, 4]

The peptidoglycan layer in gram-positive bacteria plays crucial roles in maintaining cell integrity, providing structural support, and contributing to bacterial survival and virulence. Below are the key functions of the peptidoglycan layer:

- **Structural integrity:** It provides mechanical strength to withstand high internal osmotic pressure. It determines and maintains the characteristic **shape** of the bacteria (e.g. cocci, bacilli).
- **Protection against osmotic lysis:** The thick peptidoglycan layer acts as a rigid, semipermeable barrier. It prevents the cell from bursting due to osmotic pressure differences between the cytoplasm and the external environment.
- **Elastic:** The peptidoglycan network is strong but elastic. It can stretch and contract in response to osmotic pressure. This is due to the rigidity of the backbone coupled with the flexibility of the cross-links.
- **Anchor for teichoic acids:** The peptidoglycan layer serves as a scaffold for teichoic acids and lipoteichoic acids.
- **Antibiotic target:** The peptidoglycan layer acts as a primary target for many antibiotics, including β-lactams and glycopeptides (e.g. vancomycin).
- **Immune system interaction:** Components of the peptidoglycan layer (e.g. N-acetylmuramic acid, N-acetylglucosamine) can act as pathogen-associated molecular patterns (PAMPs); recognized by immune receptors like Toll-like receptor 2 (TLR2) and triggers innate immune responses, including inflammation.
- **Defence against environmental stress:** It acts as a protective barrier against mechanical damage and environmental changes, such as pH fluctuations and desiccation. It shields the bacteria from harmful substances like detergents and enzymes.
- **Regulation of cell division:** Peptidoglycan remodelling is critical during binary fission, ensures proper septum formation and separation of daughter cells.
- **Adhesion and biofilm formation:** It provides structural support for surface-associated proteins that mediate adhesion to host tissues and biofilm formation, contributing to colonization and survival in hostile environments.
- **Virulence factor:** In pathogenic gram-positive bacteria, the peptidoglycan layer contributes to resistance against host defences, including lysozyme. It serves as a scaffold for attachment of virulence-associated proteins.

Gram-positive Cell Wall[1, 2, 9]

After the advent of transmission electron microscope, the ultrastructure of bacterial cell wall became apparently clear. Cell wall of gram-positive bacteria is simpler than that of gram-negative bacteria. As mentioned earlier, the peptidoglycan layer constitutes the majority of the gram-positive cell wall which can account for 50-80% of dry weight of the cell wall and 10% of the dry weight of the total cell.

The composition and structure of the gram-positive peptidoglycan layer is described before, under the heading "Peptidoglycan".

- Due to the presence of more layers of peptidoglycan and the pentaglycine bridges, the gram-positive cell wall is comparatively thicker and stronger than the gram-negative cell wall **(Table 4.5)**.
- Apart from peptidoglycan, the gram-positive cell wall is made up of other polysaccharides and polymers with the most predominant and important one being teichoic acid **(Fig. 4.7)**.

Periplasmic Space in Gram-positive Cell[1, 2]

The periplasmic space lies between the plasma membrane and the cell wall and is so narrow that it is often not visible by electron microscopy **(Fig. 4.7)**.

- The periplasm has relatively few proteins because most of the proteins are translocated across the plasma membrane due to the porous nature of the peptidoglycan network.
- However, those proteins that remain in the periplasmic space are usually bound. These proteins are involved in interactions of the cell with its environment. Some are noncovalently bound to teichoic acids or other cell wall polymers, while others are covalently attached to the peptidoglycan.

Table 4.5: Differences between gram-positive and gram-negative bacterial cell wall.[1,2]

Characters	Gram-positive cell wall	Gram-negative cell wall
Peptidoglycan layer	Thicker (20–80 nm) 50–100 layers thick	Thinner (2–7 nm) 1–2 layers thick
Peptidoglycan content	~40–90% of cell wall	~10% of the cell wall
At third position of tetrapeptide side chain	L-Lysine present	Meso-diaminopimelic acid present
Pentaglycine bridge	Present	Absent
Teichoic acid	Present	Absent
Lipid content	Nil or scanty (2–5%)	Present (15–20%)
Lipopolysaccharide	Absent	Present (endotoxin)
Outer membrane	Absent	Present
Variety of amino acids	Few	Several
Aromatic amino acids	Absent	Present
Periplasmic space	Typically absent or very narrow	Well-defined, containing the peptidoglycan layer

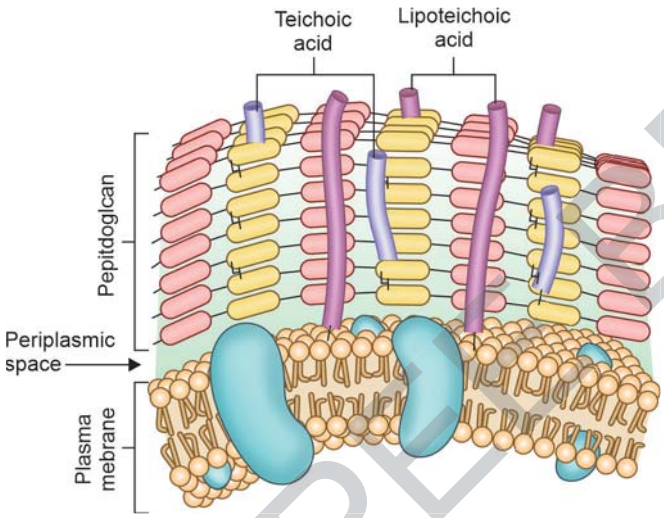


Fig. 4.7: Typical structure of a gram-positive cell wall.[4]

- Membrane-bound enzymes called 'sortases' catalyze the formation of covalent bonds that join these proteins to the peptidoglycan.

Teichoic Acid[1,2]

Gram-positive cell wall contains a significant amount of teichoic acid, which is absent in gram-negative bacteria. They are polymers of either ribitol (5-carbon) or glycerol (3-carbon) joined together by phosphodiester linkages **(Fig. 4.7)**.

- **Negative charge:** The teichoic acids are negatively charged and are partially responsible for conferring a negative charge to the cell surface as a whole.
- **Sugar substitution:** Most teichoic acids contain large amounts of D-alanine, usually attached to position 2 or 3 of glycerol and position 3 or 4 of ribitol.
  - In addition to D-alanine, certain other substituents such as glucose, galactose, N-acetylglucosamine, N-acetylgalactosamine, or succinate may also be attached to the free hydroxyl groups of glycerol and ribitol.
  - Hence, an organism can have more than one type of sugar substitute in addition to D-alanine, and whether these differences can be present in the same or different teichoic acids is also uncertain.
  - Changes in the growth medium composition (carbon, phosphate, amino acids, pH) can alter the type, length, and substitutions of teichoic acids, thereby modulating the cell wall's chemical, immunologic, and functional properties.
- **Types:** Teichoic acids are of two types based on their covalent linkage to cell wall structures.
  - **Cell wall teichoic acid (WTA):** They are ribitol teichoic acids that are covalently linked to the peptidoglycan layer via the C6 hydroxyl group of NAM molecules. They extend beyond the peptidoglycan layer, thereby protruding outwards.
  - **Lipoteichoic acid (LTA):** They are glycerol teichoic acids that are anchored to the cytoplasmic membrane via a lipid moiety. It extends into the peptidoglycan matrix.
- Together with peptidoglycan, WTA and LTA form an anionic matrix that confers various functions (discussed below). Though certain gram-positive bacteria may lack conventional WTA and LTA, such bacteria usually posses other functionally similar polymers.

Functions of Teichoic Acids[1,2]

The functions of teichoic acids are still unclear, but they may be important in maintaining the structure of the cell wall.

- **Cell wall structure and stability:** It provides rigidity and maintains the structural integrity of the thick gram-positive peptidoglycan layer. It acts as scaffolding to anchor the layers of the cell wall together.
- **Regulation of ion transport:** The negatively charged phosphate groups in teichoic acids bind divalent cations (e.g. Mg^{2+}, Ca^{2+}), playing a role in ion homeostasis.
- **Cell shape maintenance:** It contributes to the morphology and division of the bacterial cell by influencing the structure of the peptidoglycan layer.
- **Adhesion and biofilm formation:** It facilitates attachment to surfaces and host tissues, aiding in colonization and biofilm formation.
- **Virulence:** Some pathogens use teichoic acids to bind host proteins, evade immune responses, or resist antimicrobial peptides. For example, *Staphylococcus aureus* cell wall teichoic acids mediate adherence to host tissues during infection.
- **Interaction with autolytic enzymes:** It regulates autolysins, enzymes that break down peptidoglycan, to ensure controlled remodeling of the cell wall.
- **Immune activation:** Teichoic acids are recognized by Toll-like receptors (TLRs) and other pattern recognition receptors, triggering an immune response. Teichoic

acids can exacerbate inflammatory responses in certain infections, contributing to disease severity.

Target for Antibiotics (Experimental)[1, 2]

Teichoic acid biosynthesis is a promising target for antibiotics. Several investigational compounds target the enzymes or pathways involved in the synthesis of teichoic acids.

- **TarO inhibitors:** Compound 1835F03 targets TarO, the enzyme that catalyzes the first step in WTA biosynthesis (transfer of N-acetylglucosamine to undecaprenyl phosphate). It inhibits WTA production, leading to defects in cell wall maintenance and sensitivity to antibiotics like β-lactams. Targocil is another TarO inhibitor, effective against *S. aureus*.
- **LtaS inhibitors:** LtaS is the enzyme responsible for attaching glycerol phosphate-based teichoic acids to the cytoplasmic membrane (LTA biosynthesis). Experimental inhibitors of LtaS disrupt the structural integrity of the bacterial cell wall.
- **WTA inhibitor combined with β-lactams:** In combination therapies, targeting WTA biosynthesis sensitizes gram-positive bacteria to β-lactam antibiotics by impairing their ability to resist these agents.
- **Radinol and related compounds:** These inhibitors disrupt later stages of WTA biosynthesis, impairing the proper assembly and function of the cell wall.

Other Components of Gram-positive Cell Wall[2, 4]

Apart from teichoic acids, gram-positive cell walls contain other polymers such as teichuronic acids and a variety of polysaccharides.

- **Teichuronic acids:**[4] They are phosphate-free alternative to teichoic acids, which are synthesized under phosphate limitation to maintain cell wall characteristics.
  - It is made up of repeat units of sugar acids such as N-acetylmannosuronic or d-glucosuronic acid instead of phosphoric acids.
  - Though they can maintain the cell wall functions in the absence of teichoic acid synthesis, teichuronic acids are not a substitute or complete replacement for teichoic acids.
- **Virulence determinants:**[2] Cell wall of various gram-positive bacteria contains certain additional structures that play a key role in the virulence of the bacteria. Certain examples are mentioned below:
  - **M-protein** of group A streptococci is associated with lipoteichoic acids and extends out of the streptococcal cell wall as a fimbrial protein.
  - The **'group determining antigens'** of various groups of beta-hemolytic streptococci (such as groups A, B, C, F, and G) are non-teichoic acid polysaccharides present in their cell walls.
  - The **C polysaccharide** of *Streptococcus pneumoniae* is a complex lipoteichoic acid composed of ribitol and phosphate substitutions at various positions in O-glycosidic linkages and choline residues are attached through diester linkages.
- **Polysaccharides:**[4] It has been found that hydrolysis of gram-positive cell walls from certain species yielded an array of neutral sugars such as mannose, arabinose, rhamnose, glucosamine and acidic sugars such as glucuronic acid and mannuronic acid. Hence, it has been proposed that these sugars may exist as subunits of peptidoglycan or a component of teichoic or teichuronic acids. Yet the true origin of these sugars remains unknown.

Gram-negative Cell Wall[1, 2, 9]

The cell wall of gram-negative bacteria is comparatively thinner than the gram-positive cell wall, but the former is structurally more complex **(Fig. 4.8)**.

- The composition, structure, linkage and differences of the gram-negative peptidoglycan layer are the same as mentioned under the heading "Peptidoglycan".
- Due to the presence of a smaller number of peptidoglycan layers and absence of pentaglycine bridges, the gram-negative cell wall is much thinner and loosely packed.
- The structure of the gram-negative cell wall from the core to surface is as follows: Periplasm (encompasses peptidoglycan) → outer membrane → lipopolysaccharide (LPS) **(Fig. 4.8)**.

Periplasmic Space in Gram-negative Cell[1, 2]

The space between the inner cell membrane and outer membrane constitutes the periplasmic space. It encompasses the peptidoglycan. The substance that occupies the periplasmic space is the periplasm.

- **Extent:** The periplasmic space in the gram-negative cell wall is much larger than that of a typical gram-positive cell, ranging from about 30–70 nm wide and may constitute about 20–40% of the total cell volume.
- **Enzymatic content:** The periplasmic space contains various degradative enzymes such as alkaline phosphatase, proteases, nucleosidases, β-lactamases, and aminoglycoside phosphorylases.
- **Nutrient acquisition:** The periplasmic space is also a home to a variety of proteins. Some periplasmic proteins participate in nutrient acquisition—for example, hydrolytic enzymes and transport proteins.
- **Energy conservation and cell wall functions:** Some periplasmic proteins are involved in energy conservation. For instance, some bacteria have electron transport proteins in their periplasm (e.g. denitrifying bacteria, which convert nitrate to nitrogen gas). Other periplasmic proteins are involved in peptidoglycan synthesis and the modification of toxic compounds that could harm the cell.

Outer Membrane (OM)[1, 2, 4]

The outer membrane is a distinct asymmetrical lipid bilayer present outside the peptidoglycan layer. It is a bilayer structure that has two leaflets. The inner leaflet is a phospholipid layer, and the outer leaflet primarily contains the LPS. The outer membrane serves as a home to various proteins, which confer various functions to the bacterial cell. These proteins are collectively known as outer membrane proteins (OMP), and they are named based on the gene

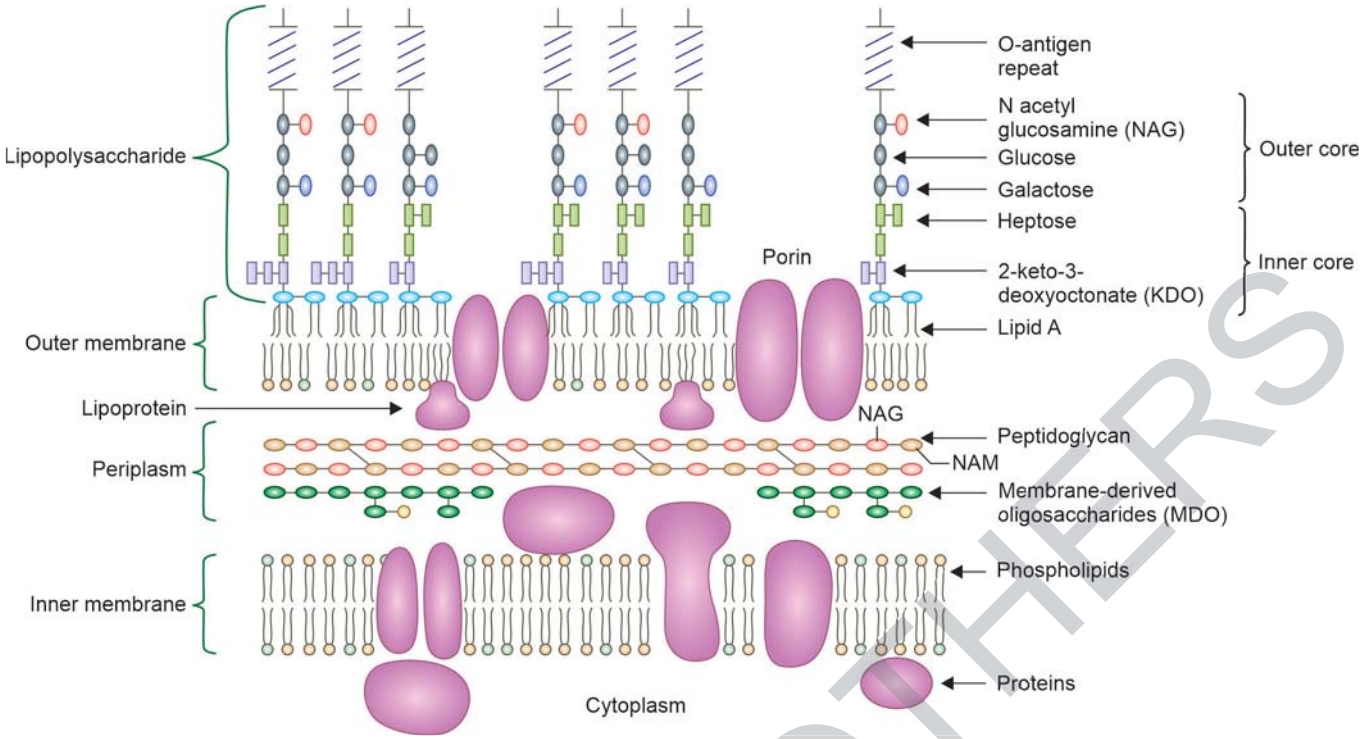


Fig. 4.8: Typical structure of a gram-negative cell wall (*E. coli* as prototype).[4]

encoding the protein and classified based on the functions they carry out. These OMPs are synthesized on ribosomes bound to the cytoplasmic surface of the cell membrane, but the exact mechanism of transport to the outer membrane still remains unclear.

Types of Outer Membrane Proteins (OMPs)[1, 2, 4]

Outer membrane proteins (OMPs) of gram-negative bacteria include structural lipoproteins (e.g. Braun's lipoprotein), structural proteins (e.g. OmpA), general diffusion porins (Group 1), substrate-specific porins (Group 2), and other transmembrane or transport proteins.

- **Braun's lipoprotein (Lpp):**[2, 4] Lpp is the most abundant outer membrane protein (OMP) of gram-negative bacteria and serves as a crucial anchor between the outer membrane and the peptidoglycan layer.
  - It is attached to the inner leaflet of the outer membrane via an N-terminal lipid moiety and covalently linked through its C-terminal lysine to the ε-amino group of meso-diaminopimelic acid (mDAP) in the peptidoglycan.
  - Lpp spans the periplasmic space as a trimeric α-helical structure, ensuring stable outer membrane–peptidoglycan attachment, maintaining cell envelope integrity, and preserving a fixed periplasmic width.
  - The other end of this lipoprotein (the N-terminal lipid moiety) is covalently attached to the protein's cysteine residue and anchored hydrophobically within the inner leaflet of the outer membrane.
- **OmpA:**[4] It is one of the major porin proteins in the OM. Apart from being a porin, it also performs other important functions such as contributing to bacterial virulence, helping in adhesion and invasion, maintaining the integrity of the outer membrane, anchoring of the outer membrane to the peptidoglycan layer (minor role), acts as the sex pilus receptor in F-mediated bacterial conjugation, etc.
- **Group 1 porin:**[4] They are the porin proteins and are trimeric in nature, which penetrate both faces of the outer membrane. The common porin proteins are OmpC, OmpD, OmpF, and PhoE of *E. coli* and *Salmonella* Typhimurium.
  - These proteins are composed of three identical proteins that form a "doughnut-shaped" pore.
  - These proteins form non-specific pores which permit the free diffusion of small hydrophilic solutes across the membrane.
  - The porins of different species exhibit different exclusion sizes ranging from molecular weights of about 600 in *E. coli* and *S.* Typhimurium to more than 3,000 in *P. aeruginosa*.
- **Group 2 porin:**[4] These are inducible, substrate-specific outer membrane proteins that form selective channels for certain nutrients. Their synthesis is regulated by environmental stimuli or substrate presence. Examples include LamB in *E. coli* (maltoporin; uptake of maltose/maltodextrins; λ-phage receptor), and stress-inducible porins such as OmpW and OmpX in *E. coli*, which are expressed under high osmolarity or membrane stress.
- **Other proteins:**[4] Apart from the above-mentioned two types, there are various other minor groups of OMPs which perform various functions.
  - *Transmembrane proteins:* These are non-porin proteins that span the outer membrane, extend through the periplasm, and are associated with the peptidoglycan layer of the cell wall. They may function in exoenzyme production and secretion, attachment to surfaces, or in

binding of the antimicrobial agents to their cell-surface targets (e.g. penicillin-binding proteins).

- *Transport proteins:* It is a minority group of proteins which are attributed to the transport of specific molecules such as vitamin B12 and iron-siderophore complexes. These proteins have high affinity for their substrates and probably function like the classic carrier transport systems of the cytoplasmic membrane. Also, these transport proteins require energy coupling for their proper function which is provided by another protein called TonB.
- The OM also houses various efflux pumps which actively expel toxic compounds and antibiotics.
- Another subset of proteins includes certain enzymes such as phospholipases and proteases.

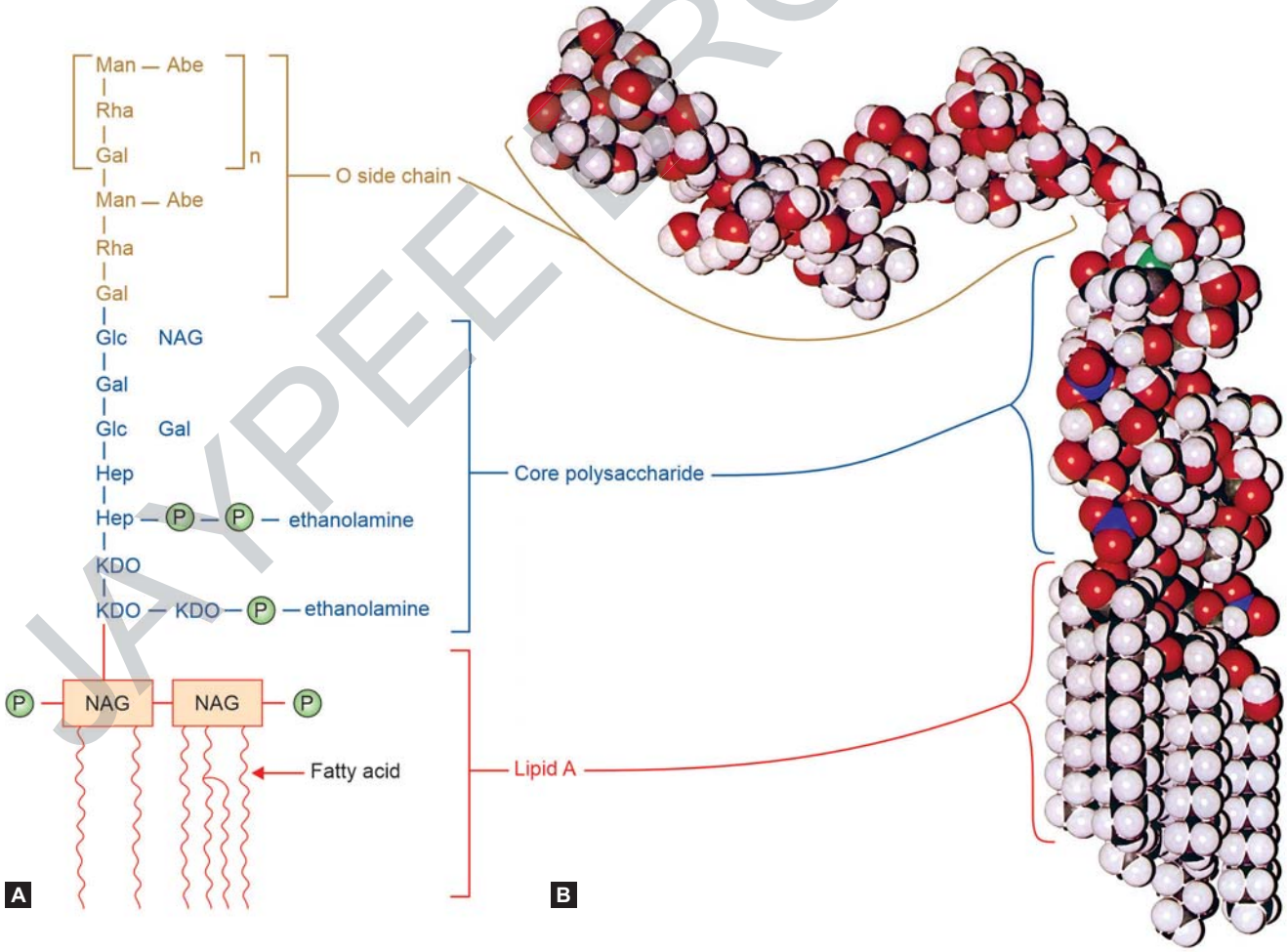
Functions of Outer Membrane[2, 4]

The outer membrane (OM) offers various functions to the bacteria.

- **Barrier:** Being lipophilic, the OM excludes the hydrophilic molecules, which is a normal surface phenomenon. However, an interesting feature noted here is that OM can also exclude hydrophobic molecules and protect the cell from lethal substances such as bile salts (especially in enteric bacteria).
- **Porins:** As mentioned above, the main function of OMPs is to aid in the passive diffusion of low-molecular-weight hydrophilic compounds such as sugars, amino acids, and certain ions.
- **Resistance:** However, an irony here is that, since the porin proteins are non-specific in nature, the large antibiotic molecules can penetrate the outer membrane relatively slowly, which accounts for the relatively high antibiotic resistance of gram-negative bacteria, as the slow progress of antibiotics can allow the bacteria to develop various resistance mechanisms.
- **Structural integrity:** Anchoring the OM to the peptidoglycan layer prevents cell lysis and maintains shape. It also prevents the loss of constituents such as periplasmic enzymes.

Lipopolysaccharide (LPS)[1, 2, 4]

The LPS of the gram-negative bacteria is the most complex and unique structure, which contributes to its virulence and endotoxic activity. It is the major surface antigenic determinants known as the somatic or 'O' antigens. The LPS of *Salmonella* species is the most extensively studied one and is usually considered as the prototype for describing the LPS of a gram-negative organism **(Figs. 4.9A and B)**.



Figs. 4.9A and B: Structure of LPS (*Salmonella* as prototype): (A) Structural component of various parts of LPS; (B) Molecular model of LPS.[4]

- **Composition:** LPS is a high molecular weight complex glycolipid that consists of three components:
  1. **Lipid A**, a complex, hydrophobic lipid portion
  2. **Core polysaccharide** that links lipid A to the external structures of the molecule and
  3. **O side chain**, which are regions of variable biochemical structure that impart unique serologic identity to gram-negative species.
- **Arrangement:** The lipid A component is embedded in the outer leaflet of the outer membrane and the core polysaccharide and the O-specific side chains project from the outer membrane surface like whiskers.
- **Interlinkage:**[2] The LPS molecules are non-covalently cross-bridged by divalent cations such as Ca^{2+} and Mg^{2+}, which provide stability. Since the integrity of the LPS primarily depends on the Ca^{2+} and Mg^{2+}, it is an absolute necessity to add these cations to the media used for antimicrobial susceptibility testing.
- **Dissociation:**[4] The LPS can be dissociated by removing the divalent cation linkage. This can be achieved by treating cell suspensions with chelating agents such as EDTA or by polycationic antibiotics such as polymyxins and aminoglycosides.
- **Synthesis:**[4] The synthesis of LPS takes place in the cytoplasm, which is then transported to its final exterior position.
- **Lipid A:** It consists of phosphorylated glucosamine disaccharide units in which the hydroxyl groups are esterified (addition of acyl groups) so that it is attached to a number of long-chain fatty acids.
  - β-Hydroxymyristic acid, a C14 fatty acid, is unique to lipid A and is always present in most of the gram-negative organisms (but absent in anaerobic gram-negative bacteria).
  - Other common fatty acids include myristomyristic acid, and lauromyristic acid.
  - Certain additional fatty acids, along with substituent groups on the phosphates, can also be present, which differ among various species of gram-negative bacilli.
- **Core polysaccharide:** It is made up of two unique carbohydrates, namely ketodeoxyoctanoic acid (KDO) (now called as—deoxy-D mannooctulosonate), an 8C sugar and heptose, a 7C sugar, along with other oligosaccharides. KDO and the heptose sugar are the most common ones.
  - *Linkage to lipid A:* The core of KDO forms covalent linkages between lipid A and heptose moieties in the core polysaccharide thereby connecting lipid A to the other structures.
  - *Other sugars:* Additional sugars such as N-acetylglucosamine, glucose, and galactose are also found in the core polysaccharide. Hence, the core polysaccharide comprises about 10–12 sugar moieties in total.
  - *Highly conserved:* The structure of the core polysaccharide is highly conserved at the genus level of the organism. However, they may vary among the species within the same genus.[2]
- **O-side chain:** They are attached to the core polysaccharide and confer antigenic specificity to the bacterial isolates.
  - It is present as repeat units containing a variable number (up to about 40) of repeating oligosaccharide units either as linear trisaccharides or branched tetra- or pentasaccharides.
  - The antigenic specificity is due to the presence of unusual or uncommon carbohydrate residues such as aminohexuronic acid, 6-deoxyhexoses, and 2,6-dideoxyhexoses, etc.
  - The hydrophilic nature of the O-side chain also covers the bacterial surfaces and protects the bacteria from exposure to hydrophobic compounds.[4]

Function of LPS[1, 2]

LPS has many important functions.

- **Membrane integrity:** LPS contributes to the structural stability of the outer membrane, maintaining its asymmetric nature and protecting the bacterial cell from mechanical stress.
- **Activation of innate immunity:** The lipid A moiety of LPS is recognized by Toll-like receptor 4 (TLR4) on host immune cells, triggering the release of pro-inflammatory cytokines (e.g. TNF-α, IL-1, IL-6).
- **Endotoxic shock:** Overactivation of the immune response by LPS (specifically lipid A) can result in septic shock, characterized by systemic inflammation, vascular leakage, and organ failure.
- **Immune evasion:** The O-antigen of LPS is highly variable between bacterial strains, helping pathogens evade host antibodies and complement-mediated killing. The O side chain of LPS (O antigen) induces the production of specific antibodies that bind the strain-specific form of LPS that elicited the response.
- **Adherence and colonization:** LPS facilitates bacterial adhesion to host cells and surfaces, promoting colonization during infection.
- **Protection from stress:** LPS shields bacteria from environmental stresses like changes in pH, desiccation, and oxidative stress.
- **Biofilm formation:** LPS can contribute to biofilm matrix development, aiding in bacterial community survival.
- **Role in horizontal gene transfer:** LPS facilitates interactions between bacterial cells and OMVs, promoting the exchange of genetic material, including antibiotic resistance genes.
- It contributes to the **negative charge** on the bacterial surface because the core polysaccharide of LPS contains negatively charged sugars and phosphate groups.
- **Permeability barrier:** LPS helps to create a permeability barrier. The geometry of LPS and interactions between neighboring LPS molecules are thought to restrict the entry of bile salts, antibiotics, detergents, and other toxic substances that might kill or injure the bacterium. Within the LPS, the negatively charged phosphate groups on the core polysaccharide interact with calcium ions to stabilize and tightly pack the LPS molecules. This results in an impermeable barrier that excludes small molecules, including many antibiotics and toxins.

Lipooligosaccharides (LOS)

Certain gram-negative bacteria contain lipid A and core polysaccharide, but instead of long-chain of O-side chain, they contain relatively short, multiantennary glycans known as lipooligosaccharides (LOS). The LOS is also known as "R-type" truncated LPS structures.

- **Organisms:** It is commonly seen in *Neisseria meningitidis*, *N. gonorrhoeae*, *Haemophilus influenzae*, *Haemophilus ducreyi,* and in rough strains of *E. coli*.
- **Antigenic variation:** Organisms possessing this LOS exhibit extensive antigenic and structural diversity even within a single strain, and the high frequency of structural modification contributes to the high degree of antigenic variation in those organisms.
- **Molecular mimicry:** LOS also acts as an important virulence factor by mimicking the host antigens. This feature contributes to biologic masking and thereby they can easily evade the host immune response.

Outer Membrane Vesicles (OMVs)[1, 10, 11]

Outer membrane vesicles (OMVs) are spherical, bilayered structures (100–300 nm) naturally shed by gram-negative bacteria as part of their normal physiology or in response to environmental stress. They are derived from the outer membrane (OM) and periplasmic components. OMVs play crucial roles in bacterial survival, communication, and interaction with hosts.

- **Structure (Fig. 4.10):** OMV is a lipid bilayer, composed of outer membrane, lipopolysaccharides, and phospholipids. It contains periplasmic contents, outer membrane proteins, lipoproteins, and sometimes nucleic acids (DNA, RNA). It can carry virulence factors, toxins, and signaling molecules.
- **Biogenesis:** OMV formation is a controlled process influenced by various factors:
  - Tension and curvature: Imbalance occurs between the inner and outer leaflets of the OM.
  - Peptidoglycan-OM decoupling: Regions where lipoproteins are not tethered to the peptidoglycan can bulge out to form vesicles.
  - Stress responses: Environmental stress (e.g. antibiotics, temperature changes, oxidative stress) can induce OMV production.
  - Enzymatic activity: Some autolysins and other enzymes promote OM detachment and vesicle formation.

Functions of OMVs[1, 10, 11]

OMVs exert several functions, including communication and competition:

- **Delivery of virulence factors:** OMVs can transport and deliver toxins, adhesins, and enzymes (e.g. proteases) directly to host cells.
  - *Pseudomonas aeruginosa:* OMVs carry elastase and other virulence factors.
  - *Escherichia coli:* Enterotoxigenic strains use OMVs to deliver heat-labile toxins.
  - *Francisella tularensis:* OMVs may modulate host immunity and deliver virulence-associated factors.

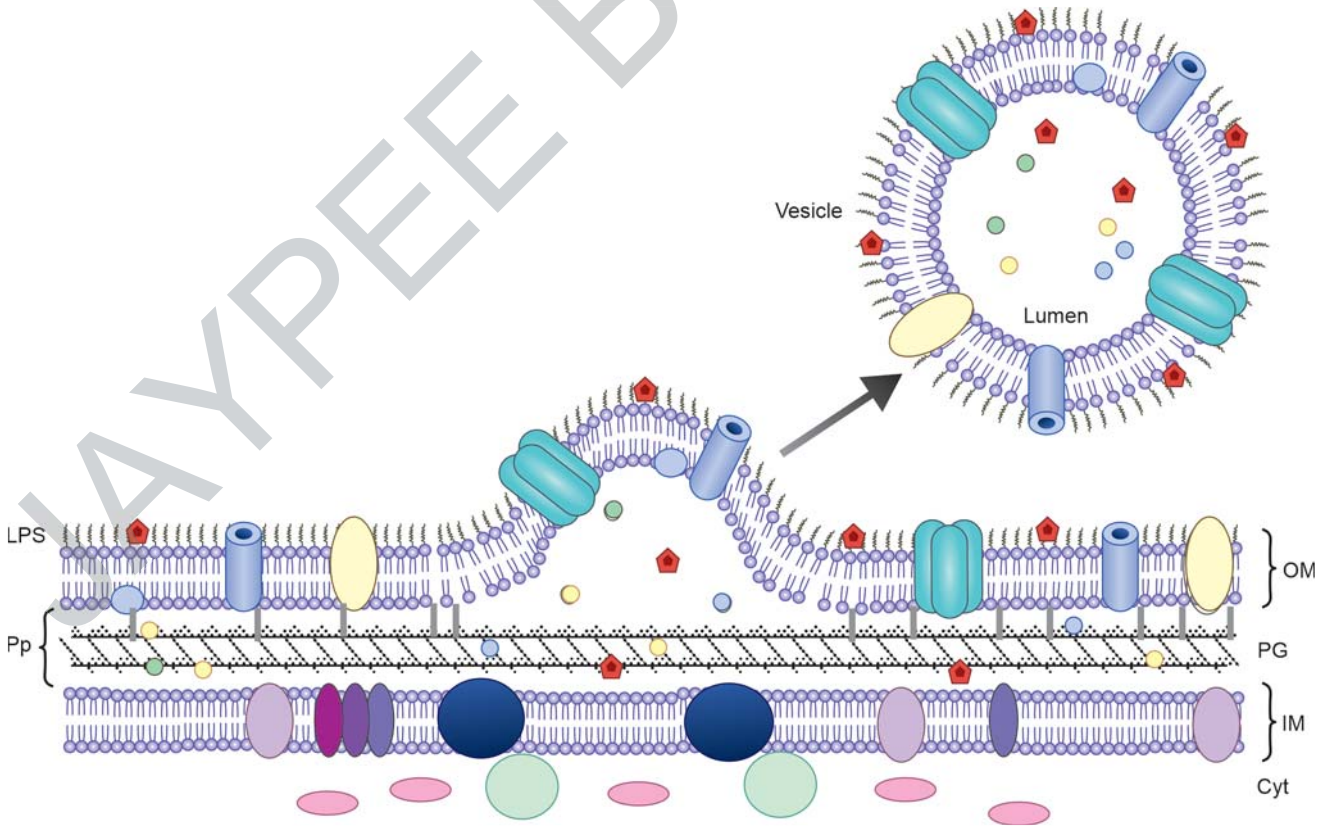


Fig. 4.10: Outer *membrane vesicle.*[9, 10]

(LPS: lipopolysaccharide; Pp: periplasm; PG: peptidoglycan; IM: inner membrane; Cyt: cytoplasm; OM: outer membrane)

- **Immune evasion:** Decoy role by absorbing antibodies or antimicrobial peptides.
- **Modulation of host immunity:** OMV components can trigger immune responses.
- **Quorum sensing:** OMVs can transport signaling molecules to coordinate bacterial behavior in biofilms.
- **Inter-bacterial warfare:** OMVs deliver antimicrobial compounds targeting competing microbes.
- **Stress response and survival:** OMVs can expel misfolded proteins and damaged molecules. They act as a buffer by capturing harmful agents (e.g. antibiotics).
- **Pro-inflammatory response:** LPS in OMVs activates host immune receptors (e.g. TLR4), inducing cytokine production.
- **Cellular uptake:** OMVs can enter host cells via endocytosis, membrane fusion, or receptor-mediated pathways.
- **Applications:** OMVs can be used for several purposes, such as a vaccine, drug delivery, etc.
  - **Vaccines:** OMVs are used as components of vaccines (e.g. *Neisseria meningitidis* B vaccine) due to their ability to elicit strong immune responses.
  - **Drug delivery:** OMVs are explored as natural nanoparticles for delivering therapeutic agents.
  - **Biotechnological tools:** Engineered OMVs can be tailored for use in diagnostics or as molecular tools.

Acid Fast Cell Wall[1, 4]

Certain gram-positive organisms such as members of the genera *Mycobacterium*, *Nocardia*, and *Corynebacterium* contain a modified gram-positive cell wall with mycolic acid. The cell wall is composed of peptidoglycan and an external asymmetric lipid bilayer where the inner leaflet contains mycolic acids linked to an arabinogalactan, and the outer leaflet contains other extractable lipids.

- **Mycolic acids:** They are large, α-substituted, β-hydroxy fatty acids that occur as esters attached to cell wall polysaccharides. They constitute 60% of the dry weight of the cell wall.
  - Carbon atoms: The number of carbon atoms present in these mycolic acids can vary among various genera.
  - *Corynebacterium* species contain 30 carbons (C30) known as corynemycolenic acids and *Nocardia* species contain 50 carbons (C50) known as nocardic acids.
  - The *Mycobacterium* genus contains 90 carbons or more. The distinct mycolic acid present in *M. tuberculosis* is 6,6′ dimycolyltrehalose, which is known as cord factor and it confers a range of virulent properties to the bacterium such as inhibition of polymorphonuclear cell migration, induction of granuloma formation, etc.
- **Cell membrane:** It is similar to other gram-positive organisms, but the cell membrane of mycobacteria consists of other additional molecules namely, phosphatidylinositol mannosides and lipoarabinomannan (LAM).
- **Tetrapeptide chain:** The tetrapeptide chain in mycobacteria is quite different from that of other gram-positive bacteria. The sequence present is L-alanine, D-isoglutamate, meso-DAP, and D-alanine.
- **Linkage:** Though pentaglycine bridges present link the NAM and NAG molecules, some of the residues are linked by phosphodiester bonds to an overlying layer of branched-chain polysaccharide macromolecules called arabinogalactans, which are made of arabinose and galactose moieties.
- **Cell wall-associated lipids:** The cell wall of mycobacteria contains certain wall-associated lipids and glycolipids to which the hydrocarbon chains of the mycolic acids are intercalated. These wall-associated lipids include trehalose sulfolipids with 2,3,6,6′-tetraacyltrehalose-2′-sulfate being the principal lipid in *M. tuberculosis*. This molecule helps the organism to prevent phagosome–lysozyme fusion following phagocytosis and survive intracellularly.

Demonstration of the Cell Wall[2, 12-14]

The cell wall cannot be seen by light microscope and does not stain with simple stains. Demonstration of cell wall can be done by methods such as:

- **Plasmolysis:** When bacteria are placed in a hypertonic saline, shrinkage of the cytoplasm occurs, while cell wall retains original shape and size.
- **Microdissection:** It uses precise instruments or lasers to manipulate or isolate microscopic structures, such as cell walls. It allows the physical separation and study of the cell wall structure. It provides insights into the composition and functionality of the cell wall by isolating it for further biochemical or structural analysis.
- **Cell wall staining:** All the methods of cell wall staining work on the same principle of differential dye affinity, where the cell wall retains the primary stain more strongly than the cytoplasmic components, allowing clear visualization of the wall structure.
  - *Chance's method:* It is a special stain used to demonstrate the bacterial cell wall without heat fixing. This staining is performed by the following steps.
    - The air-dried smear is first flooded with 0.5–1% basic fuchsin for about 3 minutes, allowing both the cell wall and cytoplasm to take up the dye.
    - Without washing, the slide is then treated with 0.5% Congo red for another 3–4 minutes, which selectively removes the dye from the cytoplasm but not from the cell wall.
    - After gentle rinsing and air drying, the cell wall appears bright red while the cytoplasm remains pale or colorless, clearly outlining the bacterial cell wall.
  - *Dyar's method:* It uses *methylene blue* followed by *iodine* and *acid alcohol* treatment. The cell wall retains the blue color while the cytoplasm is decolorized.
  - *Razin's method:* It involves staining with *crystal violet* and $CuSO_4$ solution; the cell wall remains violet, and the cytoplasm appears faint or unstained.
  - *Weigert's method:* It uses *gentian violet* and *picric acid*; the cell wall appears violet and the cytoplasm appears yellowish.
  - *Rosenow's method:* It employs *methylene blue* and *eosin*; the cell wall stains blue, and the cytoplasm pink.

- **Differential staining:** Differential staining involves using dyes to distinguish between cell components based on their chemical properties (e.g. Gram staining for bacteria).
  - Gram-positive bacteria retain crystal violet dye due to their thick peptidoglycan layer.
  - Gram-negative bacteria do not retain the dye because of their thinner peptidoglycan and outer membrane.
- **Immunological methods:** Immunological methods use antibodies that specifically bind to antigens present on the cell wall. It identifies specific proteins, polysaccharides, or other molecules present in the cell wall. It can reveal structural organization and surface antigens unique to the cell wall. Techniques like immunofluorescence or ELISA are used for this purpose. Lectins bind to specific sugar residues in the cell wall, aiding visualization and identification.
- **Electron microscopy:** It provides detailed, ultrastructural visualization of the cell wall. It reveals thickness, layers (e.g. peptidoglycan, lipopolysaccharides), and associated molecules. Scanning electron microscopy (SEM) shows surface topography, while transmission electron microscopy (TEM) reveals cross-sectional details.
- **Genetic and molecular techniques:** Gene deletion or mutation studies can be used for the disruption of genes encoding cell wall synthesis enzymes, which can reveal the cell wall's composition and importance. PCR and sequencing can identify genes associated with cell wall components, providing indirect evidence of their structure.
- **Chemical analysis:** The following methods are used for chemical analysis of the cell wall.
  - *Fourier transform infrared spectroscopy* (FTIR) analyzes chemical bonds in cell wall components like carbohydrates and proteins.
  - *Mass spectrometry* detects and quantifies cell wall molecules, such as peptidoglycan fragments.
  - *Chromatography* separates and identifies cell wall components, such as polysaccharides or lipids.
- **Enzyme sensitivity tests:** Enzymes like lysozyme (degrades peptidoglycan, zymogram analysis) or chitinase (targets fungal cell walls) are used to demonstrate structural composition.

L-FORM (CELL WALL DEFICIENT FORMS)

L-forms are the cell wall-deficient bacteria, discovered by E. Klieneberger, while studying *Streptobacillus moniliformis*. She named it as L-form after its place of discovery, i.e. Lister Institute, London (1935). Characteristics of L-form bacteria are:

- **Lack of cell wall:** Unlike typical bacteria with peptidoglycan-based cell walls, L-forms have a flexible, cell-wall-deficient structure. They are bound only by a lipid bilayer membrane. When bacteria lose their cell wall, they become spherical irrespective of their original shape.
- **Protoplasts and spheroplasts:** L-forms are derived from protoplasts (complete loss of the cell wall) or spheroplasts (partial loss of the cell wall).
  - *Protoplasts:* They are derived from gram-positive bacteria whose cell wall is entirely removed.
  - *Spheroplasts:* They are derived from gram-negative bacteria whose cell wall is partially removed.
- **Mechanism of conversion:** Transformation of bacteria into L-form can occur spontaneously or can be induced artificially.
  - It can be induced in the laboratory by exposure to cell wall-targeting agents (e.g. lysozyme, β-lactam antibiotics) or osmotic stabilizers.
  - Some bacteria naturally enter an L-form state under stressful conditions, such as exposure to antibiotics or immune system attack.
  - In isotonic environments, the absence of a cell wall does not lead to lysis, allowing the bacteria to survive in the L-form state.
- **Culture:** L-forms are difficult to cultivate in artificial media as they are extremely fragile due to lack of cell wall and require strict controlled osmoprotective conditions for their growth.
  - If these stringent conditions are not provided, the organism may fail to grow or even die if present.
  - In addition to this, L-form producing bacteria do not form visible colonies, thereby making standard culture methods ineffective.
  - Cultivation in a liquid medium is a better alternative for the culture of these organisms.
- **Reproduction:** L-forms replicate by unusual methods, such as membrane blebbing, tubulation, or vesicle formation, rather than binary fission.
- **Survival and persistence:** L-forms can survive in harsh conditions and persist in host tissues, contributing to chronic or recurrent infections.

Types of L-forms[1, 2, 3, 15]

Two types of L-forms are distinguished:

- **Unstable L-forms:** Bacteria lose the cell wall in the presence of penicillin, a mechanism of resistance shown by the bacteria against penicillin. Such L-forms are maintained only in the presence of penicillin. They are capable of dividing but can revert back to the original morphology once penicillin is removed.
- **Stable L-forms:** L-forms that are unable to revert to their original morphology are called stable L-forms.
  - Can multiply and persist without a cell wall indefinitely.
  - Retain their L-form state even after the removal of inducing conditions.
  - Mutations in genes involved in cell wall synthesis (e.g. *murA, murB*) can lead to stable L-forms.

Mycoplasma and Stable L-form[1, 2, 3, 15]

Mycoplasma does not have a true cell wall; the peptidoglycan layer is replaced by sterol. It is postulated that *Mycoplasma* may represent stable L-forms of a yet-to-be-identified parent bacteria. But many researchers do not consider *Mycoplasma* as L-forms, since they are not derived from bacteria that normally have cell walls. Although both stable L-forms and *Mycoplasma* are cell wall-deficient bacteria, they differ in origin, biology, and properties **(Table 4.6)**.

Significance of L-Forms[1-3, 15]

L forms have significant clinical relevance due to their unique biological properties and their ability to persist under hostile conditions.

Table 4.6: Stable L-form and *Mycoplasma*.[1-3, 15]

Properties	Stable L-form	*Mycoplasma*
Origin	Derived from cell walled bacteria by losing their cell wall (e.g. due to antibiotics)	Naturally cell wall-deficient bacteria; evolved without a cell wall
Cell wall synthesis genes	Retains genes for cell wall synthesis but may have mutations or suppression of their expression	Lacks genes required for peptidoglycan synthesis
Reversion	Can revert to a walled state (in unstable conditions)	Cannot revert; inherently wall-less
Genomic features	Genome is typically similar to parent walled bacteria	Genome is much smaller
Cell membrane	Contains standard bacterial membranes; no sterols	Requires sterols for membrane integrity
Replication	Through unusual methods like budding or tubulation	By binary fission
Resistance to antibiotics	Resistant to cell wall-targeting antibiotics (e.g. β-lactams, vancomycin)	Intrinsically resistant to all cell wall-targeting antibiotics
Stability	Stable L-forms can survive long-term without a cell wall but are less robust than *Mycoplasma*	Naturally stable and robust in a wall-less state
Pathogenicity	Can cause persistent chronic infections	Atypical pneumonia Urogenital infections

- **Antibiotic resistance:** L-forms exhibit resistant to cell wall-acting antibiotics such as β-lactam and glycopeptides, which target cell wall synthesis, which can contribute to treatment failures.
- **Immune evasion:** L-forms can evade host immune responses since they lack surface structures like peptidoglycan that trigger immune recognition.
- **Implications in chronic diseases:** L-forms have been implicated in persistent infections, such as urinary tract infections, pyelonephritis and other chronic infections. L-forms may play a role in inflammatory diseases like rheumatoid arthritis.
- **Survival strategy:** Transition to the L-form is a survival strategy under stressful conditions (e.g. antibiotic pressure, osmotic stress).

S-LAYER (SURFACE LAYER)

The S-layer (or surface layer) is a unique structure found on the surface of many bacteria, which has a floor tiles like pattern surrounding the bacteria. It consists of a crystalline lattice of proteins or glycoproteins that form a protective layer around the cell. The S-layer plays a crucial role in the bacterium's interaction with its environment, contributing to various aspects of bacterial physiology, such as protection against environmental stress, host immune defenses, and maintaining cell shape.

Structure of S-layer[1, 2, 16]

The S-layer is usually made up of a single type of protein or sometimes with some carbohydrate molecules attached to it. A unique property of S-layer is that it is capable of self-assembly, i.e. the proteins that form S-layer contains the necessary information required to spontaneously associate and form the S-layer without the aid of any additional enzymes or other external factors.

- **Composition:** The S-layer is typically composed of protein or glycoprotein subunits of 50–120 kDa molecular weight that self-assemble into a regular, **crystalline array**.
- **Lattice symmetry:** S-layers self-assemble on the outer surface of the organism in oblique, square, or hexagonal lattice symmetry. Depending on the lattice symmetry, the S-layer is composed of one (P1), two (P2), three (P3), four (P4), or six (P6) identical protein subunits, respectively. The proteins that form S-layer are poorly conserved and have high degree of variations in the constitution across various species.
- S-layer material may constitute up to 20% of the total cell protein. Depending on the species, the S-layers have a thickness between 5 and 25 nm and possess identical pores 2–8 nm in diameter.
- **Structure:** The S-layer forms a **two-dimensional array** of repeating units, giving it a highly ordered, crystalline appearance under electron microscopy. The lattice-like structure provides strength and flexibility, protecting the bacterium from external threats while allowing the exchange of nutrients and waste.
- **Location:** The S-layer is located on the **outermost surface** of the bacterial cell, either as a single layer directly attached to the cell membrane or cell wall, or, in some bacteria, as a separate entity outside the cell wall.
  - In **gram-positive bacteria**, the S-layer often adheres directly to the peptidoglycan layer.
  - While in **gram-negative bacteria**, it is usually found outside the outer membrane, attached by ionic interactions.
- In **Archaea**, the S-layer is the only cell wall component and, therefore, is important for mechanical stabilization.

Examples of Bacteria with S-Layers[1, 2, 16]

The S-layer is not present in all bacteria. It is more commonly found in gram-positive bacteria and some gram-negative bacteria, as well as in archaea. It is abundant in bacteria that live in harsh environments, such as high salinity, extreme pH, or environments with high osmotic pressure. The S-layer in bacteria can be visualized by transmission electron microscopy (TEM) with freeze etching technique, scanning probe microscopy, atomic force microscopy, X-ray crystallography, etc.

- **Gram-positive bacteria:** S-layer is detected in several gram-positive bacteria.
  - *Bacillus anthracis:* The S-layer plays a role in resistance to phagocytosis by immune cells. In *Bacillus anthracis*, it is now thought that the S-layer is this organism's major cell wall antigen, and it may contribute to virulence.
  - *Clostridioides difficile:* S-layer proteins are involved in resistance to bile salts and other hostile conditions in the gut.

- *Streptococcus pneumoniae:* The S-layer contributes to virulence by helping the bacteria evade immune defenses.
- *Lactobacillus*: Several species (e.g. *L. acidophilus*, *L. brevis*, *L. helveticus*) have a well-defined S-layer.

❖ **Gram-negative bacteria:** Few gram-negative bacteria also contain S-layer.
- *Pseudomonas putida:* The S-layer contributes to its ability to form biofilms and interact with various surfaces.
- *Campylobacter,* and *Aeromonas* species.
- *Aquifex aeolicus* (a thermophilic bacterium): The S-layer plays a role in stability in extreme conditions.

❖ **Archaea:** Some archaea, such as *Sulfolobus* species, also have S-layers that play similar protective roles in extreme environments like hot springs or acidic environments. In *Bdellovibrio bacteriovorous* (a primitive bacterium), S-layer protects it from the action of bacteriophages.

Functions of S-Layer[1, 16]

The S-layer can serve several functions.

❖ **Protective barrier:** The S-layer acts as a protective shield against physical damage, dehydration, and host immune responses. It also protects the cell against ion and pH fluctuations, osmotic stress, enzymes, predatory bacteria or bacteriophages.

❖ **Immune evasion:** It helps protect the bacteria from the host's immune system by preventing recognition or interaction with immune cells, such as macrophages, or by blocking the binding of antibodies and complement. It may inhibit phagocytosis in pathogenic microorganisms.

❖ **Adhesion to surfaces:** The S-layer (especially the glycosylated S-layers) can aid in bacterial **adhesion** to host tissues or environmental surfaces, which is critical for the formation of biofilms or during the initial stages of infection. The S-layer may help bacteria resist removal by mechanical forces, such as fluid flow in the bloodstream or gut.

❖ **Osmotic protection:** The S-layer plays a role in maintaining osmotic stability by protecting the bacterium from osmotic pressure changes that might otherwise disrupt cellular integrity.

❖ **Enzyme and toxin sequestration:** The S-layer may sequester enzymes or toxins, limiting their access to the cell or preventing the action of host defense proteins, such as lysozyme.

❖ **Cell shape maintenance:** In some bacteria, the S-layer helps maintain cell shape and integrity. It provides structural support by forming a scaffold that stabilizes the cell membrane.

❖ **Environmental sensing:** The S-layer may also play a role in sensing environmental conditions, such as temperature or changes in the availability of nutrients or stressors, allowing the bacterium to adapt to varying conditions.

Applications and Significance of S-Layer[1, 16]

S-layer has several important biomedical and biotechnological applications.

❖ **Vaccine development:** The S-layer has potential as a vaccine target, as it is often exposed on the surface of pathogenic bacteria. Vaccines designed to recognize S-layer proteins could help protect against bacterial infections.

❖ **Nanotechnology:** Due to their self-assembling properties and crystalline lattice structure, S-layer proteins have been explored for use in nanomaterials and biosensors.
- They can be engineered to display specific functional groups on their surface for use in various biotechnological applications such as vaccine delivery.
- S-layer proteins could be used as building blocks for the creation of technologies such as drug-delivery systems and novel detection systems for toxic chemicals or bioterrorism agents.

❖ **Bacterial biofilms:** The S-layer can aid in the formation of biofilms, which are clusters of bacteria that are difficult to treat with antibiotics. Understanding how S-layers contribute to biofilm formation could provide insights into controlling chronic bacterial infections.

CELL MEMBRANE

The cell membrane or the cell envelope is defined as the plasma membrane and all the surrounding layers external to it, which includes the plasma membrane, cell wall, and sometimes, an additional layer (e.g. capsule or slime layer). Of all these layers, the plasma membrane is the most important because it encompasses the cytoplasm and defines the cell. The plasma membrane is essential for the survival of bacteria **(Table 4.7)**.

Table 4.7: Bacterial and eukaryotic cell membrane[1, 2]

Properties	Bacterial cell membrane	Eukaryotic cell membrane
Composition	Phospholipid bilayer; no sterols (except hopanoids in some species)	Phospholipid bilayer enriched with sterols (e.g. cholesterol)
Sterols	Absent (except hopanoids in some bacteria, e.g. *Mycobacterium*)	Present (e.g. cholesterol for fluidity and stability)
Proteins	High protein-to-lipid ratio (~3:1)	Protein-to-lipid ratio vary, depending on cell type
Outer membrane	Present in gram-negative bacteria	Absent
Carbohydrates	Few glycoproteins or glycolipids	Significant glycoproteins and glycolipids for signaling and recognition
Energy production	Acts as the site for respiration and ATP synthesis (electron transport chain)	No role in energy production; this occurs in mitochondria
Transport	Selective permeability through porins, channels, and active transport systems	Similar selective permeability but more specialized transport mechanisms (e.g. endocytosis)
Cell wall synthesis	Coordinates synthesis of peptidoglycan	No such role
Membrane microdomains	Flotillin-based, smaller and less complex	Lipid rafts enriched with cholesterol, sphingolipids

Structure of Cell Membrane[1, 2]

The typical "cell membrane" is composed of phospholipids and 200 different kinds of proteins where the latter accounts for approximately 70% of the mass of the membrane. The "**fluid mosaic model**" described by Singer and Nicholson is the most widely accepted current model to describe the structure of cell membrane which describes it as membranes composed of lipid bilayers within which the proteins float **(Fig. 4.11)**.

- **Bacterial cell membrane** is 5–10 nm thick, composed of bilayered phospholipid in which several proteins are embedded, such as integral proteins and peripheral proteins.
- **Phospholipid bilayer:** It provides the basic structure and fluidity of the membrane. It is composed of asymmetric amphipathic phospholipid molecules with two types of ends.
  - Hydrophilic (polar) end: Faces outward, interacting with the aqueous environment.
  - Hydrophobic (nonpolar) end: Face inward, forming the inner region of the bilayer.
- **Membrane proteins:** Two types of membrane proteins are embedded within the lipid bilayer—integral and peripheral proteins. They identified based on their ability to be separated from the membrane. These proteins serve roles such as—transport (e.g. channels, pumps), signal transduction (e.g. receptors) and enzymatic activities.
- **Peripheral membrane proteins** are loosely connected to the membrane and can be easily removed. They are soluble in aqueous solutions and make up about 20–30% of total membrane protein.
- **Integral membrane proteins:** They account for the remaining proteins in cell membrane. These are not easily extracted from membranes and are insoluble in aqueous solutions when freed of lipids. Integral membrane proteins, like membrane lipids, are amphipathic; their hydrophobic regions are buried in membrane lipids while the hydrophilic portions project from the membrane surface outside the cell enabling the cell to interact with its environment. Integral membrane proteins carry out some of the most important functions of the membrane.
  - Many are transport proteins used to move materials either into or out of the cell.
  - Others are involved in energy-conserving processes, such as the proteins found in electron transport chains.
- **Dynamic nature:** The plasma membrane of the bacterial cell is highly dynamic where the composition of the membrane varies largely with respect to the external stimuli. This property is mainly attributed to the presence of microdomains.
- **Microdomains:** They are specialized, dynamic regions within the bacterial cell membrane where specific lipids, proteins, and other molecules are enriched and organized. These areas serve as functional compartments that concentrate and regulate cellular processes, akin to "lipid rafts" in eukaryotic membranes.
  - **Components:** Microdomains contain specific lipids—cardiolipin, phosphatidylglycerol, and hopanoids; and specialized proteins—flotillins, signal transduction proteins, and transporters.
  - **Dynamism:** Microdomains are not fixed structures; they can form, dissolve, and reorganize as needed, depending on environmental conditions and cellular processes.
  - **Size:** They are nanoscale structures (20–200 nm) and may coalesce into larger regions during certain processes.
  - **Localization:** Microdomains are frequently associated with regions of active cell wall synthesis, division sites, or poles of rod-shaped bacteria.
- **Hopanoids:** Sterol-like molecules in some bacteria that stabilize membrane fluidity, similar to cholesterol in eukaryotic membranes.
- **Flotillins:** They are integral membrane proteins present in microdomains, and they function to assemble large protein complexes like secretion systems for transporting molecules out of the cell and complexes that transmit signals from the environment to molecules in the cytoplasm.
- Bacterial cell membrane differs from eukaryotic membranes in **lacking sterols**, such as cholesterol (except in *Mycoplasma*). However, many bacterial membranes do contain pentacyclic sterol-like molecules called **hopanoids (Table 4.7)**.
- **Carbohydrate:** Some carbohydrates are often attached to the outer surface of plasma membrane proteins, involved in membrane integrity.

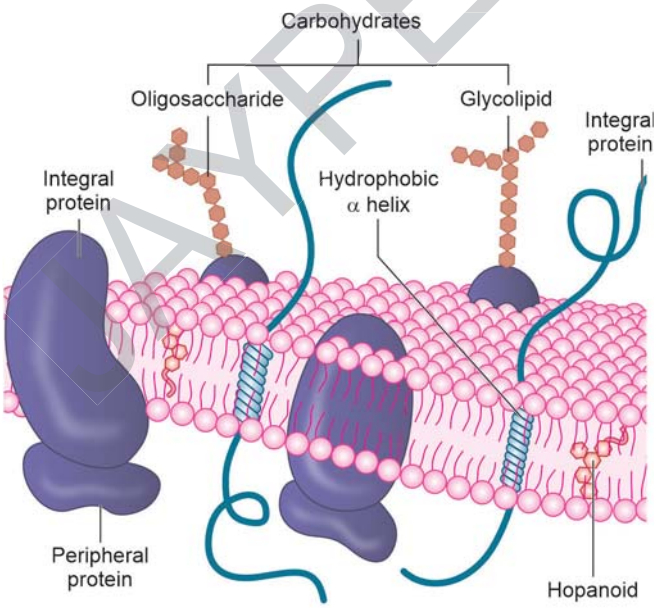


Fig. 4.11: Structure of bacterial cell membrane.

Functions of Cell Membrane[1, 2]

The bacterial cell membrane performs several essential roles.

- **Selective permeability:** It is a semipermeable membrane acting as an osmotic barrier that selectively allows only certain ions and molecules to pass, either into or out of the cell, while preventing the movement of others.
- **Transport mechanisms:** Proteins and enzymes present in cell membrane are involved in nutrient uptake, and

waste excretion. This is effectively carried out by various mechanisms such as passive diffusion, facilitated diffusion, active transport, etc.

- **Site of metabolic activities:** In bacteria, there are no membrane-bound organelles, so the cell membrane is a hub for critical metabolic functions.
- **Respiration and energy generation:** The electron transport chain (ETC) takes place on the cell membrane, which generates ATP via oxidative phosphorylation.
- **Photosynthesis:** In photosynthetic bacteria, pigments like bacteriochlorophyll are embedded in the membrane thereby enabling photosynthesis.
- **Biosynthesis:** Lipid, cell wall (e.g. peptidoglycan precursors), and polysaccharide synthesis occur at or near the membrane.
- **Signal transduction:** Membrane proteins act as receptors for environmental signals, enabling the cell to sense and respond to changes in its surroundings.
- **Structural integrity:** The membrane helps maintain cell shape by working in tandem with the cell wall.
- **Role in cell division:** During binary fission, the membrane contributes to septum formation, chromosome segregation and separation of daughter cells.
- **Secretion:** Translocation of proteins and enzymes to the periplasmic space (gram-negative) or extracellular space (gram-positive).

BACTERIAL TRANSPORT

Transport across the bacterial cell membrane is essential for nutrient uptake, waste removal, and maintaining cellular homeostasis. Bacteria use different mechanisms based on the molecules being transported, energy requirements, and environmental conditions **(Tables 4.8 and 4.9)**.

Passive Diffusion[1, 2, 4]

Passive diffusion, also known as diffusion or simple diffusion, involves movement of molecules through the lipid bilayer of the bacterial membrane down their concentration gradient, without the use of energy or specific transport proteins. Characteristics of passive diffusion are:

- **Energy requirement:** None, there is no requirement of energy source.
- **Driving force:** Simple diffusion happens only when the solute is at higher concentration outside the cell than inside.
- **Selectivity:** Non-specific; depends on the molecule's size, charge, and hydrophobicity.
- **Rate:** Proportional to the concentration gradient and the permeability of the membrane.
- **Examples of molecules** transported by passive diffusion include small, nonpolar molecules: oxygen, carbon dioxide, nitrogen and small polar molecules such as water.
- **Limitations** of passive diffusion are: (i) inefficient for larger molecules, ions, and polar substances; (ii) cannot transport against a concentration gradient.
- A large concentration gradient is required for adequate nutrient uptake by passive diffusion (i.e. the external nutrient concentration must be much higher while the internal concentration is low).
- The rate of diffusion decreases as more nutrient accumulates in the cell which occurs if the nutrient uptake does not occur immediately upon entry.

Facilitated Diffusion[1, 2, 4]

In this type of diffusion, the transport across the membrane occurs with the aid of specific membrane transport proteins, down their concentration gradient. It uses channel proteins or carrier proteins that are selective for specific substrates. Characteristics of facilitated diffusion are:

- **Energy requirement:** Similar to passive diffusion, here also there is no requirement of energy source.
- **Driving force** is the concentration gradient. Although facilitated diffusion relies on transport proteins, it is the concentration gradient spanning the membrane that drives the movement of molecules. If the concentration gradient disappears, the net inward movement ceases. The gradient can be maintained by converting the transported nutrient to another compound, as occurs when a nutrient is metabolized.
- **Selectivity:** Since specific transport proteins are involved, the transport of the molecules is selective.

Table 4.8: Comparison of passive diffusion, facilitated diffusion, and active transport[1, 2]

Properties	Passive diffusion	Facilitated diffusion	Active transport
Energy requirement	None	None	Requires energy (ATP or ion gradients)
Driving force	Concentration gradient	Concentration gradient	Against the concentration gradient
Transport mechanism	Simple movement through the membrane	Requires membrane proteins (channels/carriers)	Requires transport proteins (pumps)
Direction of transport	Down the concentration gradient	Down the concentration gradient	Against the concentration gradient
Protein involvement	No	Yes (channel or carrier proteins)	Yes (pumps or transport complexes)
Rate of transport	Linear and slow	Faster than passive diffusion, saturates at high substrate concentration	Can be rapid, depends on energy availability
Selectivity	Non-specific (small, uncharged molecules)	Specific to substrates based on the carrier	Highly specific for substrate
Substrates transported	Gases (O_2, CO_2), small hydrophobic molecules	Sugars (e.g. glucose), ions (e.g. Cl^-, Na^+)	Ions (e.g. Na^+, K^+), nutrients (e.g. amino acids, sugars)

Table 4.9: Comparison of primary active transport, secondary active transport and group translocation.[1]

Properties	Primary active transport	Secondary active transport	Group translocation
Energy source	ATP hydrolysis	Ion gradients (e.g. H^+ or Na^+ gradient)	Phosphoenolpyruvate (PEP)
Transport mechanism	Direct use of ATP to transport molecules	Coupling ion movement with substrate transport	Substrate chemically modified during transport
Substrate modification	No	No	Yes (e.g. phosphorylation)
Direction of transport	Unidirectional (in or out)	Bidirectional (symport or antiport)	Typically into the cell
Driving force	ATP provides energy	Electrochemical gradient of H^+ or Na^+	Energy from PEP's phosphate group
Examples of substrates	Ions (e.g. Na^+, K^+, Ca^{2+}), sugars, amino acids	Sugars (e.g. lactose), amino acids, ions	Sugars (e.g. glucose, mannose, fructose)
Specificity	High specificity (e.g. ATP-binding cassette)	Moderate to high, depending on transporter	Substrate-specific transport systems
Energy efficiency	Energy-intensive (direct ATP use)	Energy-efficient (uses pre-existing gradients)	Highly efficient (combines transport with metabolism)
Structural complexity	Involves ATP-binding domains and ion channels	Requires simpler transporters (symporters/antiporters)	Involves multiple enzymes and protein complexes
Representative transporters	Na^+/K^+ ATPase, ABC transporters	Lactose permease (LacY), Na^+/glucose symporter	Phosphotransferase system (PTS)

- **Types of transport proteins:** There are two types of proteins that mediate facilitated diffusion.
  - **Channel proteins:** Channels, as their name indicates, are proteins that create hydrophilic pores allowing specific ions or small molecules to pass (e.g. aquaporins for water). These channels show some specificity for the substances that pass through them, but this is considerably less than that shown by carriers proteins, which are far more substrate specific.
  - **Carrier proteins:** These types of proteins bind to the substrate, undergo conformational changes, and release the substrate on the other side. When the solute molecule binds to the outside of the carrier, it changes conformation and releases the molecule on the cell interior **(Fig. 4.12)**. The carrier subsequently changes back to its original shape and is ready to pick up another molecule.
- **Rate of diffusion** in facilitated diffusion is faster than passive diffusion due to protein involvement; exhibits saturation kinetics (limited by the number of transport proteins) **(Fig. 4.13)**.
  - The rate of diffusion depends on the size of the solute's concentration gradient (the ratio of the extracellular concentration to the intracellular concentration). This is particularly seen in facilitated diffusion mediated by carrier protein, which can be saturated. However, facilitated diffusion mediated by a channel do not exhibit a saturation effect.
  - The rate of facilitated diffusion increases with the concentration gradient much more rapidly and at lower concentrations of the diffusing molecule than that of passive diffusion.
  - When the transporter is a carrier, the diffusion rate reaches a plateau above a specific gradient value because the carrier protein is saturated; that is, it is transporting as many solute molecules as possible. The resulting curve resembles an enzyme-substrate curve and is different from the linear response seen with passive diffusion.
- **Examples of molecules** transported by facilitated diffusion include glycerol (transported by the facilitator protein **GlpF** in *E. coli*) and sugars (transported by

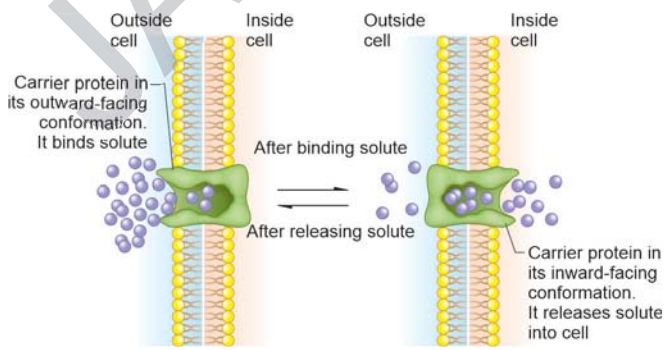


Fig. 4.12: Facilitated diffusion.[1]

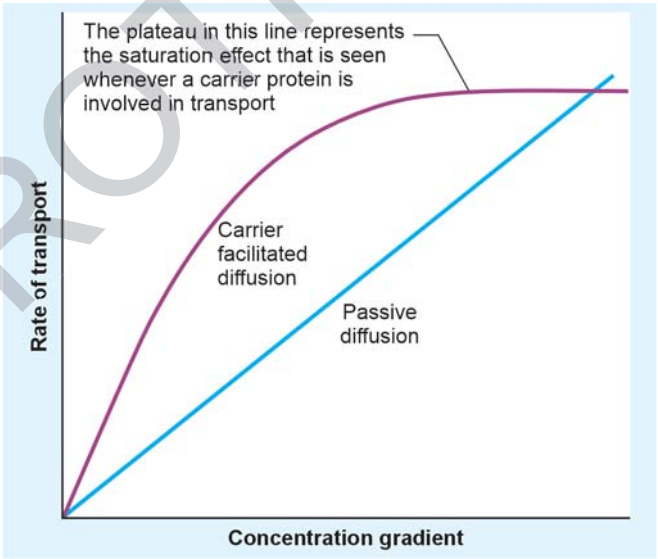


Fig. 4.13: Rate of diffusion between passive diffusion and carrier facilitated diffusion.[1]

specific permeases). However, this type of transport is more common in eukaryotes such as yeast rather than in prokaryotes.

- **Advantages** of facilitated diffusion are (i) allows hydrophilic molecules to cross the membrane efficiently; (ii) highly selective and faster than passive diffusion.
- **Limitations:** Cannot transport molecules against a concentration gradient. Therefore, facilitated diffusion has been documented in some bacteria but it does not seem to be the major uptake mechanism for these microbes.

Active Transport[1, 2]

It involves energy-dependent transport of molecules across the membrane, often against their concentration gradient. It requires energy input (e.g. ATP hydrolysis or ion gradients) and specific transport proteins. Characteristics of active transport includes:

- **Energy requirement:** Energy is required, and source of energy is either from ATP, ion gradients, or light energy.
- **Driving force:** Energy source is the driving force, not the concentration gradient.
- **Selectivity:** Highly specific, as it involves carrier proteins, like that of facilitated diffusion, which bind only to particular solutes with great specificity.
- **Saturation effect:** Active transport is also characterized by the carrier saturation effect at high solute concentrations. However, active transport differs from facilitated diffusion because it uses metabolic energy and is independent of the concentration gradient.
- **Rate of diffusion:** In active transport, the rate of diffusion is faster and independent of the concentration gradient.
- **Types:** There are three types of active transport observed in bacteria which differs based on the energy source used in the transport and whether the transported molecules undergo any further modification or not.

Primary Active Transport[1, 17]

Primary active transport is mediated by carriers called primary active transporters. It is also known as **ATP binding cassette (ABC) transport** as they use ATP hydrolysis as the energy source to move substances against a concentration gradient without modifying them.

- Primary active transporters are **uniporters**; that is, they move a single molecule across the membrane.
- **ABC transporters:** ATP-binding cassette transporters (ABC transporters) are important primary active transporters which import nutrients like maltose or vitamins.
- **Domains:** The ABC transporters consist of two pairs of distinct domains, the transmembrane domain (TMD) and the nucleotide-binding domain (NBD).
- **TMD:** The TMD is otherwise known as membrane-spanning domain (MSD) or integral membrane (IM) domain. It consists of alpha helices where its sequence and architecture are highly variable and is embedded in the lipid bilayer. It can alternate between an inward and outward facing orientation depending on the type of transport. The primary role of this domain is to recognize a variety of substrates and undergoes conformational changes to transport the substrate across the membrane.
- **NBD:** The NBD or ATP-binding cassette (ABC) domain is located in the cytoplasm and has a highly conserved sequence. This domain acts as the site for ATP binding.
- **Overall structure:**[18] Usually most ABC transporters consist of minimally of two TMDs and two NBDs where four individual polypeptide chains including two TMD and two NBD subunits, may combine to form a full transporter, e.g. *E. coli* BtuCD importer which is involved in vitamin B12 uptake. On the other hand, certain bacteria may have ABC transporters in the form of homodimers monomers of a TMD fused to an NBD, e.g. Sav1866, a multi-drug exporter of *S. aureus*.
- **Mechanism:**[19] As mentioned earlier, this type of transport is driven by the energy produced by ATP hydrolysis and/or binding to NBD which results in the conformational changes in the TMD and subsequently allows the movement of molecules across the membrane.
- **Model:** The widely accepted model to explain this phenomenon is the **alternating-access model**. It suggests that TMD alternates between an outward- and inward-facing conformations where the relative binding affinities of the two conformations for the substrate largely determines the net direction of transport. This implies that either the molecules can be imported or exported.
- **Import:**[18, 19] Here the translocation is directed from the periplasm to the cytoplasm, where the outward-facing conformation has higher binding affinity for the substrate **(Fig. 4.14)**.
  - This type of transport is crucial for the uptake of nutrients and other molecules from the environment. This process is mediated by solute binding proteins (SBP).
  - The ABC importers can be of two types: Type I (small ABC importers), e.g. ModBC-A used by *Haemophilus influenzae* for the uptake of molybdate and Type II (large ABC importers), e.g. HI1470/1 used by *Haemophilus influenzae* for the uptake of various substrates.[20]
- **Solute-binding proteins:** The import of most molecules via the ABC transporters highly rely on the high affinity solute binding proteins (SBP). These proteins are present in the periplasm of the gram-negative organisms and in the outer layer of the cell membrane in gram-positive

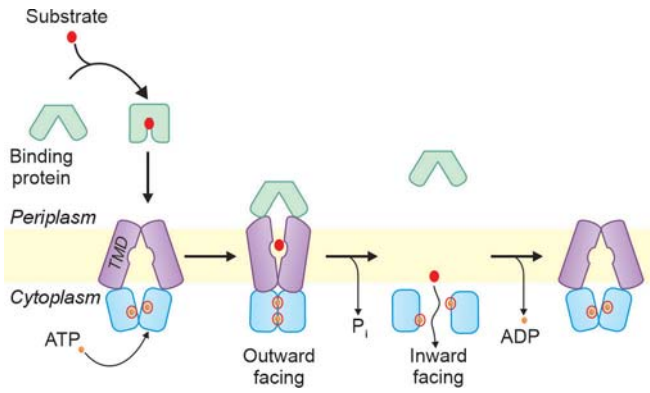


Fig. 4.14: Import of molecules via ABC transporters.
(TMD: transmembrane domain).
Source: Wikipedia/alexanderaloy and stargonzales (*with permission*).

organisms. These proteins help in the delivery of the specific solutes to the ABC transporter protein where the SBP attaches to transporter and releases solute.

- ❖ **Export:**[21, 22] In contrast to the import mechanism, here the substrate binding affinity is greater towards the inward-facing conformation which facilitates the movement of molecules from the cytoplasm to the external environment. In some instances, the exporters have an additional domain called interdomain coupling domain (ICD) which facilitates communication and interaction between the TMD and the NBD **(Fig. 4.15)**.
  - There are two classes of exporters wherein one class in involved in the export of proteins and the other is involved in drug efflux.
  - The latter class of exporter ABC proteins are of huge medical importance as they mediate the efflux of antibiotics from cytosol thereby leading to antimicrobial resistance. They are also known as multi-drug resistant (MDR) ABC transporters or hydrophobic vacuum cleaners, e.g. Sav1866, a multi-drug exporter of *S. aureus*.
  - These types of exporters are also found in human cells which can also confer drug resistance, e.g. MRP1/ABCC1, P-gp/ABCB1 lead to drug resistance in cancer cells due to reduced intracellular accumulation of drugs which subsequently leads to diminished effectivity.

Secondary Active Transport[1]

Secondary active transport in bacteria is a process where the movement of one molecule or ion across the membrane is coupled to the movement of another ion (typically H^+ or Na^+) down its electrochemical gradient. This method does not directly require ATP but instead uses the energy stored in ion gradients, which are often generated by primary active transport processes.

- ❖ **Cotransporter:** In contrast to primary active transporters which are usually uniporters, the secondary active transporters are cotransporters. They move two substances simultaneously: the ion whose gradient powers the transport and the solute to be moved across the membrane **(Fig. 4.16)**.
  - When the ion and solute move in the same direction, it is called **symport.**
  - When they move in opposite directions, it is called **antiport.**
- ❖ **Symporters:** Here the transport of two molecules occur in the same direction. They use the inward movement

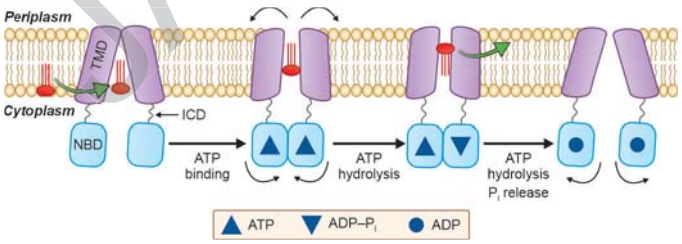


Fig. 4.15: Export of molecules via ABC transporters.

(TMD: transmembrane domin; ICD: interdomain coupling domain; NBD: nucleotide-binding domain)

Source: Wikipedia/alexanderaloy and stargonzales (*with permission*).

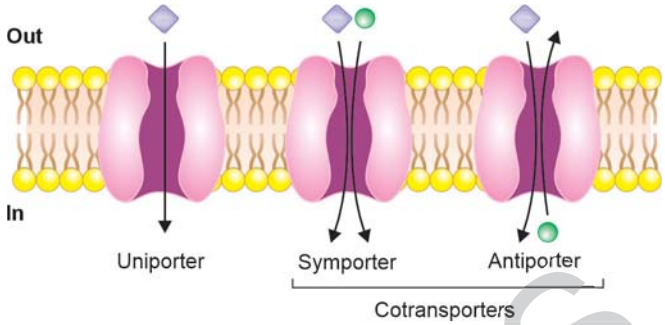


Fig. 4.16: Carrier proteins—uniporters or cotransporters.[1]

of H^+ or Na^+ to drive the uptake of solutes like sugars or amino acids. Examples include:

- Lactose-H^+ symporter (LacY): Found in *E. coli*. Couples H^+ influx to lactose uptake.
- Na^+-dependent glucose transporter: Couples Na^+ influx with glucose uptake in certain bacteria.

- ❖ **Antiporters:** Here the transport two molecules occur in opposite directions. They use the movement of ions (e.g. H^+ or Na^+) to expel waste products or balance intracellular ions. Examples include:
  - Na^+/H^+ antiporter: Exchanges extracellular Na^+ for intracellular H^+. It helps maintain pH and sodium balance.
  - Ca^{2+}/H^+ antiporter: It removes excess calcium ions by coupling with H^+ influx. The ion gradients used by secondary active transporters arise primarily in three ways.

Ion Gradients Driving Secondary Active Transport[1]

The ion gradients used by secondary active transporters arise primarily through three mechanisms that generate and maintain the electrochemical gradients across the bacterial cell membrane.

- ❖ **Proton motive force (PMF):** The proton motive force is generated by the movement of protons (H^+) across the membrane, creating an electrochemical gradient. This is achieved through electron transport systems in the membrane pump protons out of the cell during aerobic or anaerobic respiration.
  - Resulting gradient is from either: (i) chemical gradient (ΔpH)—pH difference across the membrane; (ii) electrical gradient ($\Delta\psi$)—charge difference across the membrane.
  - Symporters: Coupling proton entry with nutrient uptake (e.g. lactose/H^+ symporter in *E. coli*).
  - Antiporters: Exchanging protons for other ions (e.g. Na^+/H^+ antiporter).
- ❖ **V-type ATPase:**[23, 24] Some bacteria use specialized rotatory molecular machineries called the **Vacuolar-type ATPase** or the V-type ATPase system. This system plays a key role in bacterial ion transport, nutrient acquisition, and virulence. It hydrolyses ATP and uses the energy released to create either a proton gradient or a sodium gradient across the plasma membrane.
  - *Domains:* It has two domains, V1 and V0. The V1 domain is positioned in cytoplasm, and it performs

the ATP hydrolysis to generate energy for the transport. It contains further subunits namely A to H where A and B are the ATP binding sites. The V0 domain is a membrane-embedded domain, and it translocate protons across the membrane. It has 4 subunits, namely a, c, d, e where the c subunit forms the ring for H^+ translocation.

- *Mechanism:* As the ATP binds to the A and B subunits of the V1 domain, it undergoes hydrolysis. The ATP hydrolysis induces conformational changes in the D and F subunits which drives the rotation of central stalk components. Subsequently, this transfers the torque to the c-ring in the V0 domain. The c ring is made of multiple c-subunits which have proton binding sites. As the c-ring rotates, each subunit picks up a proton from the cytoplasmic side and carries it across the membrane. This in turn activates the a-subunit ofV0 which provides two half-channels, one opens to the cytoplasm or H^+ uptake and the other opens to the periplasm/extracellular side for H^+ release **(Fig. 4.17)**.
- *Use:* V ATPase system is primarily used for the transportation of various carbohydrates (e.g. glucose, maltose via symporters), ions (e.g. Na^+, Ca^{2+} using antiporters), metabolites and amino acids.

❖ **Sodium ion gradient:** Finally, a proton gradient can be used to create another ion gradient such as a sodium gradient.

- **Mechanism:** Sodium ion gradients are established by:
  - ♦ Sodium-proton antiporters: Exchange protons (H^+) for sodium ions (Na^+), leveraging the proton motive force to generate the Na^+ gradient.
  - ♦ Primary sodium pumps: Use ATP hydrolysis to actively pump Na^+ out of the cell (less common in bacteria).
- **Resulting gradient:** Sodium ions are more concentrated outside the cell, creating a favourable gradient for their inward movement.

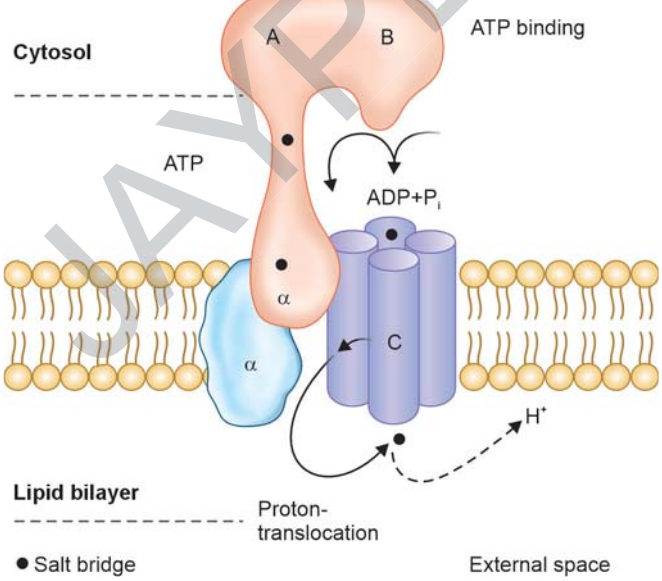


Fig. 4.17: V ATPase system in bacteria.

- **Role in transport:** Drives sodium-dependent transporters, such as symporters (e.g. Na^+/glucose symporter) and antiporters (e.g. Na^+/Ca^{2+} antiporter for ion homeostasis).

Group Translocation[1]

Group translocation is a unique form of active transport in bacteria where a substrate is chemically modified during its transport across the cell membrane. This mechanism is energy-efficient and allows bacteria to import and simultaneously prepare substrates for further metabolic processes. The phosphotransferase system (PTS) is the most well-studied example of group translocation.

❖ The PTS transports a variety of sugars while phosphorylating them, using phosphoenolpyruvate (PEP) as the phosphate donor.

❖ PEP is a high-energy molecule that can be used to synthesize ATP. However, when it is used in PTS reactions, the energy present in PEP is used to energize sugar uptake rather than ATP synthesis.

Mechanism of Group Translocation (PTS System)[1]

The phosphotransferase system (PTS) involves a series of proteins that sequentially transfer a phosphate group from PEP to the transported substrate. In *E. coli* and *Salmonella,* the PTS consists of two enzymes and a low molecular weight heat-stable protein (HPr). The steps are as follows **(Fig. 4.18)**:

❖ **PEP donates phosphate:** PEP donates a phosphate group to Enzyme I (EI), which becomes phosphorylated.

❖ **Phosphate transfer to HPr:** EI transfers the phosphate to a small heat-stable protein called **HPr**.

❖ **Substrate-specific enzymes (EII):** HPr transfers the phosphate to a substrate-specific Enzyme II complex (EII). EII consists of three subunits:
- EIIA: Receives the phosphate from HPr.
- EIIB: Transfers the phosphate to the incoming substrate.
- EIIC: Membrane-bound; facilitates the substrate's entry into the cell.

❖ **Substrate transport and modification:** As the substrate passes through EIIC, it is phosphorylated by EIIB. The

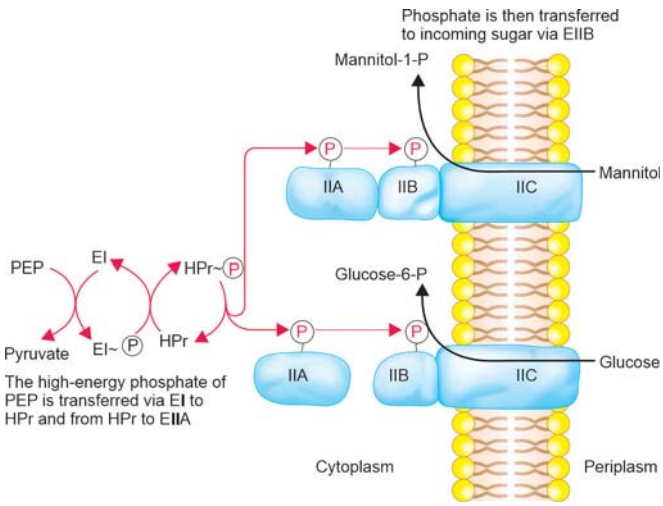


Fig. 4.18: Mechanism of group translocation.[1]

(PEP: phosphoenolpyruvate; EI: enzyme I, {IIA,IIB,IIC}, enzyme II complex, HPr: heat stable protein)

Table 4.10: Mechanisms of iron transport in bacteria.[1, 2, 4, 18]

Mechanism	Iron source	Transport proteins/systems	Example (organisms)
Siderophore-mediated uptake	Free Fe^{3+}	Siderophores, TonB-ExbB-ExbD system	*E. coli, Pseudomonas* spp.
Heme-iron uptake	Hemoglobin, myoglobin	Isd system, heme-binding proteins	*Staphylococcus aureus*
Lactoferrin/transferrin uptake	Transferrin, lactoferrin	Transferrin/lactoferrin-binding proteins	*Neisseria* spp.
Ferric iron transport	Free Fe^{3+}	Outer membrane transporters (e.g. FhuA, FepA)	*E. coli*
Ferrous iron uptake	Soluble Fe^{2+}	FeoABC system	*Helicobacter pylori*
Iron piracy	Siderophores from other bacteria	Siderophore receptors	*Pseudomonas aeruginosa*
Mineral iron uptake	Iron minerals	Reducing agents, reductases	Environmental bacteria
Hemolysin-mediated uptake	Hemoglobin in lysed RBCs	Hemolysins, heme-binding receptors	*E. coli, S. aureus, S. pyogenes*

modified substrate (e.g. glucose-6-phosphate) is released into the cytoplasm.

- **Examples** of substrates transported via PTS:
  - Glucose → Glucose-6-phosphate.
  - Mannose → Mannose-6-phosphate.
  - Fructose → Fructose-1-phosphate.
- **Specificity:** Many different PTSs exist, and they vary in terms of the sugars they transport. The specificity lies with the type of Enzyme II used in the PTS. Enzyme I and HPr are the same in all PTSs used by a bacterium.

Advantages of Group Translocation[1]

Advantages of group translocation are:

- **Energy efficiency:** Combines transport with the first step of substrate metabolism, saving ATP.
- **Concentration gradient:** Modified substrate does not accumulate outside the cell, maintaining a favorable concentration gradient for uptake.
- **Regulation:** The PTS system integrates transport and metabolism, allowing bacteria to coordinate resource usage.
- **Metabolism:** Prepares substrates for glycolysis or other pathways, enhancing metabolic efficiency.
- **Environmental adaptation:** Enables rapid and energy-efficient nutrient uptake in competitive or nutrient-scarce environments.
- **Pathogenesis:** Some pathogens utilize group translocation to import host-derived nutrients, aiding in infection.

Multiple Transporters for Same Nutrient[1]

Bacteria often have more than one transport system for a nutrient. For example, *E. coli* has at least five transport systems for the sugar galactose, three systems each for the amino acids glutamate and leucine, and two potassium transport complexes.

- When several transport systems exist for the same substance, the systems differ in such properties as their energy source, their affinity for the solute transported, and the nature of their regulation.
- This diversity gives the bacterium an added competitive advantage in a variable environment.

Mechanisms of Iron Uptake in Bacteria[1, 2]

Iron is an essential nutrient for bacteria, as it is involved in numerous biological processes, including respiration, DNA synthesis, and oxidative stress management. However, free iron is scarce in most environments due to its poor solubility of ferric iron (Fe^{3+}) and sequestration by host organisms. Bacteria have evolved several sophisticated mechanisms to acquire iron efficiently **(Table 4.10)**.

1. Siderophore-Mediated Iron Uptake

Bacteria secrete low-molecular-weight iron-chelating compounds called **siderophores** (Greek for iron bearers), which have a high affinity for Fe^{3+} ions, forming stable complexes.

- **Mechanism:** Microorganisms secrete siderophores when iron is scarce in the medium. The siderophore-Fe^{3+} complex binds to specific receptor proteins on the bacterial outer membrane.
  - The complex is transported into the periplasm via an ATP-dependent process involving the TonB-ExbB-ExbD system.
  - Fe^{3+} is then released inside the cell, often by reduction to Fe^{2+}.
- **Examples of siderophores:** Enterobactin (*E. coli*), pyoverdine (*Pseudomonas* spp.), aerobactin, salmochelin (hypervirulent *Klebsiella pneumoniae*) and mycobactin (*Mycobacterium* spp.).
- **Significance:** Siderophore enables bacteria to scavenge iron from the environment or host tissues. It is an important virulence factor in pathogenic bacteria.
- **Iron piracy:** Certain pathogens "steal" siderophores produced by other microbes. They use siderophore receptors to bind and transport these heterologous siderophores, e.g. *Neisseria meningitidis* uses enterobactin made by *E. coli*.

2. Direct Uptake of Host Iron Complexes

Bacteria can directly acquire iron from host-bound sources, such as:

- **Heme-iron uptake:** Hemoproteins (e.g. hemoglobin, heme, myoglobin) are targeted.
- Bacteria use specific heme-binding receptors (e.g. **Isd system** in *Staphylococcus aureus*).
- Once bound, heme is transported into the cell where iron is extracted by heme oxygenases.
- **Lactoferrin/transferrin uptake:** Proteins like transferrin and lactoferrin tightly bind iron in the host. Some bacteria

express transferrin-binding and lactoferrin-binding receptors to directly extract and internalize iron.

3. Other Methods of Fe Uptake

Apart from the above-mentioned methods of Fe uptake, bacteria possess various other mechanisms by which it acquires iron for its metabolism.

- **Iron binding proteins:** Bacteria possess ferric iron-binding proteins (e.g. FhuA and FepA in *E. coli*).
  - Ferric iron is transported into the periplasm via outer membrane receptors, often coupled with the TonB system.
  - Transport to the cytoplasm involves ABC transporters and periplasmic-binding proteins.
- **Ferrous iron uptake (Fe^{2+}):** In anaerobic or acidic environments, Fe^{2+} is more soluble and available. Specific transporters for Fe^{2+} include:
  - **Feo system (FeoABC):** A conserved ferrous iron uptake system in many bacteria.
  - **Nramp** (Natural resistance-associated macrophage protein): Found in some pathogens for Fe^{2+} uptake.
- **Iron acquisition from mineral sources:** Some bacteria produce reducing agents to convert Fe^{3+} from insoluble ferric hydroxides into soluble Fe^{2+}. This is common in environmental bacteria that scavenge iron from mineral deposits.
- **Reductive iron uptake:** Outer membrane reductases found in some gram-negative bacteria reduce Fe^{3+} to Fe^{2+}, which is then taken up by Fe^{2+} transport systems.
- **Hemolysin-mediated iron release:** Some bacteria (e.g. *S. aureus*, *E. coli*) produce hemolysins to lyse host red blood cells. This releases hemoglobin, which can then be targeted for heme extraction.
- **Iron uptake in iron-restricted environments:** host organisms limit bacterial access to iron through nutritional immunity (e.g. sequestration by transferrin, lactoferrin, and ferritin). Bacteria counter this by increasing siderophore production or expressing high-affinity iron receptors.

CYTOPLASMIC MATRIX

Bacterial cytoplasm, unlike that of eukaryotes, lacks membrane-bound organelles. The cytoplasmic matrix is mainly composed of water (about 70% of bacterial mass is water) and is packed with ribosomes, storage granules called **inclusions** and cell membrane invaginations called **mesosomes**. They lack true cytoskeleton but do have a cytoskeleton-like system of proteins. The plasma membrane and everything within it is called **protoplast**. The bacterial cytoplasm is estimated to be about 10 times more viscous than water.

Bacterial Cytoskeleton[1]

The bacterial cytoskeleton is a dynamic network of proteins that provides structural support, determines cell shape, and is involved in critical cellular processes like division, polarity, and intracellular organization. While cytoskeleton was once thought to be exclusive to eukaryotes, research has shown that bacteria also possess cytoskeletal elements.

Key Components of Bacterial Cytoskeleton[1]

Eukaryotes possess three major cytoskeletal elements: actin filaments, microtubules, and intermediate filaments. Homologues of all three types of eukaryotic cytoskeletal proteins have been identified in bacteria.

- **Actin-like proteins:** They are analogous to eukaryotic actin filaments, e.g. MreB, ParM and MamK.
  - MreB: It is found mostly in rod-shaped bacteria and helps to maintain their cell shape. It organizes peptidoglycan synthesis by interacting with the cell wall machinery.
  - ParM: It assists in plasmid segregation during cell division.
  - MamK: It helps in positioning magnetosomes.
- **Tubulin-like proteins:** They are the structures corresponding to eukaryotic microtubules. Examples include FtsZ and TubZ proteins.
  - FtsZ: It forms a ring-like structure (Z-ring) at the division site, guiding cell division.
  - TubZ: It is involved in plasmid stability and segregation. It is observed in multiple bacteriophages.
- **Intermediate filament-like proteins (Crescentin):** These are the equivalent structures of eukaryotic intermediate filaments. They provide shape and mechanical strength. In *Caulobacter crescentus*, crescentin localizes to one side of the cell, creating a crescent shape.
- **Min proteins** (MinC, MinD, MinE): They prevent improper placement of the division septum. MinD and MinE oscillate from pole to pole, ensuring FtsZ assembles at the center of the cell.
- **Other cytoskeletal proteins:** Include bactofilins, PilZ, FtsA and ParA.
  - **Bactofilins:** They are widely observed among bacteria. They are involved in scaffolding of proteins and chromosome segregation.
  - **PilZ:** Regulates Type IV pili, which are important for motility and adhesion.
  - **FtsA:** It helps in cell division. It works with FtsZ to recruit proteins involved in division.
  - **ParA:** It contributes to chromosome and plasmid segregation. It helps localize chemotaxis proteins and type IV pili to one pole of certain rod-shaped bacteria. It is observed in many bacteria including *V. cholerae*.

Functions of the Bacterial Cytoskeleton[1]

Bacterial cytoskeleton performs several functions.

- **Cell shape determination:** MreB helps maintain rod-shaped morphology. Crescentin induces curvature in crescent-shaped bacteria.
- **Cell division:** FtsZ forms the Z-ring at the site of septation. Other proteins (FtsA, ZapA) are recruited to complete division.

- **Polarity and spatial organization:** The cytoskeleton organizes proteins and cellular machinery spatially. Polar localization of proteins is critical for asymmetric cell division (e.g. in *Caulobacter crescentus*).
- **Intracellular transport:** ParM and TubZ facilitate plasmid segregation during cell division.
- **Adhesion and motility:** Cytoskeletal proteins regulate pili and flagellar assembly, impacting bacterial motility and host interactions.

Bacterial Ribosomes[1, 4]

Bacterial ribosomes are molecular machines responsible for protein synthesis (translation). They are smaller and structurally simpler than eukaryotic ribosomes but perform similar core functions **(Table 4.11)**.

- **Structure:** Bacterial ribosomes are composed primarily of ribosomal RNA (rRNA, (60%) and ribosomal proteins (40%).
- **Subunits:** Bacterial ribosomes are called 70S ribosomes (S for Svedberg unit) and are composed of two subunits 50S and a 30S **(Fig. 4.19)**.
- **50S (large subunit):** It comprises of 23S rRNA (2900 nucleotides) and 5S rRNA (120 nucleotides). It contains ~34 proteins (designated L1 to L34).
- **30S (small subunit):** It comprises of **16S rRNA** (1540 nucleotides) and contains ~21 proteins (designated S1 to S21).
- **rRNA role:** rRNA molecules form the structural framework of the ribosome and contribute to catalytic activity.
- The peptidyl transferase center (catalyzing peptide bond formation) resides in the 23S rRNA.
- The 16S rRNA in the 30S subunit aids in mRNA decoding and interactions with the Shine-Dalgarno sequence for translation initiation (refer Chapter 9, for detail).

Functions of Bacterial Ribosome[1]

Bacterial ribosomes provide the site for protein synthesis (translation). It decodes mRNA into a polypeptide sequence and catalyses peptide bond formation between amino acids. Steps in translation are:

Table 4.11: Bacterial and eukaryotic ribosome.[1]

Properties	Bacterial ribosome	Eukaryotic ribosome
Subunits	50S + 30S	60S + 40S
rRNA components	16S, 23S, 5S	18S, 28S, 5.8S, 5S
Size (diameter)	Smaller (20 nm)	Larger (25–30 nm)
Protein count	~55 proteins	~80 proteins
Translation initiation	Uses Shine-Dalgarno sequence and fMet-tRNA	Uses 5′ cap recognition and Met-tRNA
Sensitivity to antibiotics	Targeted by many antibiotics (e.g. tetracycline, erythromycin)	Resistant to these antibiotics

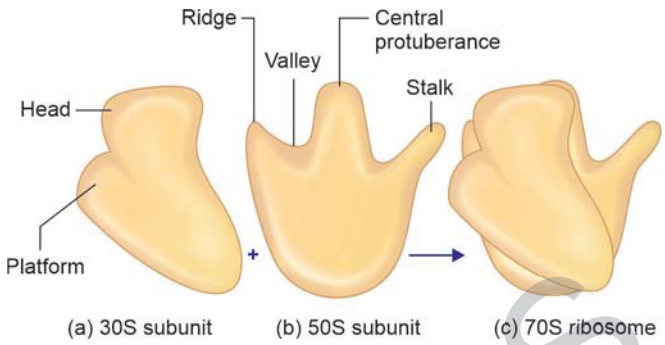


Fig. 4.19: Bacterial ribosome.[1]

- **Initiation:** 30S subunit binds mRNA at the Shine-Dalgarno sequence.
  - fMet-tRNA (initiator tRNA) binds the start codon (AUG).
  - The 50S subunit joins to form the complete 70S ribosome.
- **Elongation:** Charged tRNAs enter the ribosome at the A-site.
  - Peptide bonds are formed at the P-site.
  - Empty tRNAs exit via the E-site.
- **Termination:** Stop codons (UAA, UAG, UGA) are recognized by release factors, terminating translation. Ribosome dissociates into subunits.

Bacterial Inclusions[1]

Bacterial inclusions are intracellular insoluble storage structures found in the cytoplasm of prokaryotic cells. These structures serve as reservoirs for nutrients, metabolic intermediates, and energy, and sometimes help the cell adapt to environmental changes. They are formed by the bacteria under nutritional deficiency conditions and disappear when the deficient nutrients are supplied. They are not bound by a typical membrane but may have protein-based or lipid-based envelopes. They often appear as refractile bodies in the cytoplasm under the phase contrast microscopy.

Types of Bacterial Inclusions[1]

Bacterial inclusions can be broadly classified based on their composition and storage purpose into organic inclusions and inorganic inclusions.

- **Organic inclusions:** These inclusions store carbon, energy, or nitrogen in organic forms. Examples include glycogen granules, poly-β-hydroxybutyrate (PHB) granules, cyanophycin granules, lipid droplets and carboxysomes. Organic inclusions are typically used for nutrient storage, energy reserves, or specific metabolic pathways.
- **Inorganic inclusions:** These inclusions store inorganic molecules or ions and are often used in energy metabolism or stress response. Examples include polyphosphate (volutin) granules, sulfur granules, magnetosomes and gas vacuoles. Inorganic inclusions often play roles in structural support, magnetic navigation, energy metabolism, or adaptation to environmental stresses.

A brief description of several important inclusions is elucidated below. The most common type of bacterial

inclusions storage inclusions. The second type of inclusions which are important are referred to as microcompartments.

Storage Inclusions[1]

They act as reserve materials for times of nutrient deficiency or energy demands. Examples include:

- **Glycogen granules:** Store glycogen, a polymer of glucose. These granules are used as a carbon and energy source when the protein and nucleic acid synthesis resumes after a starvation.
- **PHA granules:** Carbon is often stored as polyhydroxyalkonate (PHA) granules, also termed carbonosomes. Several types of PHA granules have been identified, but the most common contain poly-β-hydroxybutyrate (PHB). Much of the interest in PHB and other PHA granules is due to their industrial use in making biodegradeable plastics.
- **Poly-β-hydroxybutyrate (PHB) granules:**[2] They also serve the similar purpose as that of glycogen granules. These granules store PHB, a lipid-like compound consisting of chains of β-hydroxybutyric acid units connected through ester linkages. These granules are formed where there is a shortage of nitrogen, sulfur, or phosphorous but excess of carbon in the medium. They are found in many bacteria, including *Alcaligenes* and *Pseudomonas*.
- **Polyphosphate (volutin) granules:** Polyphosphate granules store the phosphate needed for synthesis of important cell constituents such as nucleic acids.
  - They are found in certain bacteria, such as *Corynebacterium diphtheriae*.
  - In some cells, they act as an energy reserve, and polyphosphate also can serve as an energy source in some reactions, when the bond linking the final phosphate in the polyphosphate chain is hydrolyzed.
  - Also known as metachromatic granules (stainable with basic dyes). These granules are demonstrated by staining techniques such as Albert's stain, Neisser's stain and Ponder's stain (refer Chapter 16, for detail).
- **Sulfur globules:**[1, 4] These granules are found in organisms that are capable of oxidizing reduced sulfur compounds such as hydrogen sulfide and thiosulfate (e.g. *Thiobacillus*). They store elemental sulfur as an energy reserve.
  - When there is a scarcity of reduced sulfur, the sulfur stored in the granules are oxidized to commonly form sulfate which is further used for metabolic process. After usage of sulfur, the granules gradually disappear.
  - For example, some photosynthetic bacteria use hydrogen sulfide (rather than water) as an electron donor and accumulate the resulting sulfur either externally or internally.

Bacterial Microcompartment Granules[1]

Some bacterial inclusions serve functions other than simply storing substances for later use. These inclusions, called microcompartments, share several characteristics.

- Bacterial microcompartments (BMCs) are specialized, protein-bound organelle-like structures found in some bacteria.
- They are not surrounded by lipid membranes but are enclosed by a protein shell. BMCs enable compartmentalization of specific metabolic pathways, helping bacteria to localize and control biochemical reactions.

Examples of bacterial microcompartments include the ethanolamine utilization (Eut) microcompartment, the propandiol utilization (Pdu) microcompartment, and carboxysomes.

- **Carboxysomes:**[4] These inclusions are polyhedral bodies surrounded by a protein shell which contain key enzymes for $C0_2$ fixation such as ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) for carbon fixation. They are found in cyanobacteria and other autotrophic bacteria.
- **Enterosomes:** Found in enteric bacteria such as *Salmonella* and *Escherichia coli*.
  - Involved in the metabolism of specific organic compounds like ethanolamine or propanediol.
  - Function: Prevent toxic intermediates (e.g. acetaldehyde) from damaging the cell.
- **Propane diol utilization (Pdu) microcompartments:** Found in bacteria such as *Salmonella*. They contain enzymes for the degradation of 1,2-propanediol. Their main function is to sequester toxic intermediates like propionaldehyde.
- **Ethanolamine utilization (Eut) microcompartments** Found in enteric bacteria. They contain enzymes for ethanolamine metabolism. Their main function is to protect the cell from acetaldehyde, a toxic intermediate.
- **Hydroxypropionate microcompartments:** Found in certain autotrophic bacteria. They are involved in the 3-hydroxypropionate bi-cycle for carbon fixation.
- **Polyhedral organelles:** Generic term for other microcompartments that do not fit into the above categories. They may be involved in nitrogen metabolism, sulfur metabolism, or other specialized processes.

Other Inclusions[1]

Inclusions can be used for functions other than storage or as microcompartments. Two of the most remarkable inclusions are gas vacuoles and magnetosomes. Both are involved in bacterial movement.

- **Gas vacuoles:**[4] These are gas-filled structures that are essential for aquatic bacteria as they provide buoyancy for their survival. The gas vacuole's membrane is a 2-nm-thick layer of protein that is **impermeable to water and solutes but permeable to gases**. As a result, these vacuoles are filled with gas and provide the necessary buoyancy for its existence. It is found in cyanobacteria, allowing them to float to areas with optimal light for photosynthesis.
- **Magnetosomes:**[1, 4] These are special type of inclusion bodies found in aquatic magnetotactic bacteria like *Magnetospirillum* which use these to orient themselves with respect to the Earth's magnetic field.
  - Magnetosomes are intracellular chains of iron magnetite (Fe_3O_4) or greigite (Fe_3S_4) particles.
  - They are around 35–125 nm in diameter and enclosed within invaginations of the plasma membrane. The

invaginations contain distinctive proteins that are not found elsewhere in the plasma membrane.
  - For the cell to move properly within a magnetic field, magnetosomes must be arranged in a chain which is mediated by a cytoskeletal protein called MamK which establishes a framework upon which the chain can form.
  - Northern hemisphere bacteria use their magnetosome chain to determine northward and downward directions, and swim down to nutrient-rich sediments or locate the optimum depth in freshwater and marine habitats.
  - Magnetotactic bacteria in the Southern Hemisphere generally orient southward and downward, with the same result.
- **Chromatophores:** Contain pigments for photosynthesis. Found in photosynthetic bacteria, such as *Rhodospirillum*.
- **Lipid droplets:** Store lipids as an energy reserve. Found in various bacteria.
- **Cyanophycin granules:** Store nitrogen in the form of a polymer of arginine and aspartic acid. Found in cyanobacteria.
- **Proteasome-like structures:** Involved in protein degradation and regulation.

Mesosomes[1]

Mesosomes are invaginations of the plasma membrane in the shape of vesicles, tubules, or lamellae. They are generally more prominent in gram-positive bacteria. Mesosomes were first identified in the 1950s using electron microscopy.

- **Location:** Mesosomes often are found next to septa in dividing bacteria or sometimes seen attached to the bacterial chromosome.
- **Artifacts:** The existence of mesosomes is considered to be controversial. Initially mesosomes were considered to be natural, functional structures. But subsequent studies suggested that mesosomes seen in electron microscopy were actually artifacts caused by chemical fixation during sample preparation. Observations of mesosome-like structures are now considered experimental artifacts.
- **Proposed functions:** Although mesosomes are no longer considered genuine cellular structures, several roles were proposed for them (historical perspective).
  - Site of bacterial respiration: They possess respiratory enzymes and are analogous to mitochondria of eukaryotes. They are proposed to increase the surface area for enzymes involved in respiration.
  - Cell division: Mesosomes were thought to anchor the bacterial chromosome during binary fission. They may be involved in cell wall formation during division.
  - Cell wall synthesis: Suggested to play a role in the synthesis and maintenance of the bacterial cell wall.
  - Secretion: Hypothesized to assist in the secretion of extracellular substances.
- **Modern equivalent:** Although mesosomes themselves are now considered artifacts, bacteria do have functional membrane invaginations:
  - **Thylakoid membranes:** Found in photosynthetic bacteria like cyanobacteria. They contain pigments and enzymes for photosynthesis.
  - **Respiratory membranes:** Found in bacteria like *Nitrobacter*, involved in respiratory and energy-generating processes.
  - **Magnetosome membranes:** Found in magnetotactic bacteria, enclosing magnetosomes used for navigation.

Nucleoid[1]

Being a prokaryotic, bacteria do not have a true nucleus, instead it has a bundle of ill-defined genetic material called the nucleoid. There is no nuclear membrane or nucleolus.

- **Chromosome:** Bacteria possess a single haploid chromosome, comprising of super coiled circular double stranded DNA of 1 mm length. Bacterial DNA lacks basic proteins.
- **Polyploidy:** Most bacteria are **haploid** with one chromosome per cell, with some exceptions.
  - True diploidy is rare in bacteria. However, temporary diploidy occurs during bacterial replication when two copies of the chromosome are present.
  - Polyploidy is seen in certain bacteria (e.g. *Deinococcus radiodurans* and *Epulopiscium fishelsoni*) where multiple genome copies coexist for functional benefits.
- **Linear chromosome:** Although most bacteria possess circular chromosome, some bacteria have a linear chromosome, e.g. *Borrelia* species has one linear chromosome, ~900 kilobases (kb) in size. Other bacteria with linear chromosomes are *Streptomyces* species, *Agrobacterium tumefaciens, Rhodococcus fascians* and *Frankia* species.
- **Multi-chromosome bacteria:** Although most bacteria possess single chromosome, some bacteria have two chromosomes, e.g. *Vibrio cholerae, Brucella melitensis*, *Burkholderia, Rhizobium, Deinococcus* and *Paracoccus*.
- **Cell division:** Bacterial DNA divides by simple binary fission.
- **Demonstration:** The nucleoid can be seen by electron microscopy or on staining with chromosomal stains such as the Feulgen stain.
- **Packaging:** Bacterial chromosomes are longer than the length of the cell. For instance, *E. coli*'s circular chromosome measures ~1,400 μm, i.e. ~230–700 times longer than the cell. Thus, the chromosome must be organized and packaged in a manner that decreases its overall size without affecting its function.
- **Physical factors** like macromolecular crowding and supercoiling contribute to nucleoid organization.
- **Supercoiling:** The bacterial chromosome is supercoiled, meaning it undergoes additional twists beyond the natural double-helix structure. Supercoiling helps in compacting the chromosome to fit within the cell. Supercoiling is maintained by **topoisomerases**, which regulate the over- or under-winding of DNA during processes like replication and transcription.
- **Architectural proteins:** Besides physical factors, architectural proteins such as nucleoid-associated proteins also contribute to nucleoid structure.

Nucleoid-associated Proteins (NAPs)

They are small, abundant DNA binding proteins that cause the chromosome to bend and fold. These proteins help organize the DNA into a compact structure that also allows for dynamic processes like transcription and replication. Examples of NAP include HU, IHF and FIS proteins.

- **HU protein:** Binds DNA and introduces bends, playing a role in DNA packing and facilitating access to DNA.
- **IHF (integration host factor):** Another NAP that bends the DNA and is involved in processes like DNA recombination and repair.
- **FIS (factor for inversion stimulation):** Involved in DNA replication, and also in the organization of the nucleoid.
- **Functions:** NAPs have multiple functions:
  - They form bridges between one section of the chromosome and another and they participate in the many processes that occur on the chromosome.
  - Cells have several different NAPs, but HU is the only one found in almost all bacteria.
  - NAPs are particularly important during cell division, when they further compact the chromosomes.
  - This extra level of packing is important for proper segregation of daughter chromosomes during cell division.
- **Plasmids:** Possess extra-chromosomal DNA, called plasmids (refer Chapter 10, for detail).

CELL WALL APPENDAGES

The cell wall appendages of bacteria include capsule and slime layer, flagella and fimbriae.

CAPSULE AND SLIME LAYER[1, 2, 4]

Some bacteria possess a layer of amorphous viscid material lying outside the cell wall called **glycocalyx**. It is a condensed well-defined layer consisting of a network of polysaccharides extending from the surface of the cell. The term can encompass both capsules and slime layers **(Tables 4.12 and 4.13)**.

- **Capsule:** When the glycocalyx layer is well organized and not easily washed off, it is called capsule **(Fig. 4.20)**
- **Slime layer:** When the glycocalyx layer is in the form of diffuse, unorganized loose material that can be removed easily, it is called slime layer **(Fig. 4.20)**. It is usually composed of polysaccharides but is not as easily observed by light microscopy.
  - Gliding bacteria often produce slime, which in some cases has been shown to facilitate motility.
  - Some bacteria may possess both capsule and slime layer, as in *Streptococcus salivarius, Streptococcus mutans, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*
- **Composition:** Most of the bacterial capsules are polysaccharide in nature, except in *Bacillus anthracis* where it is polypeptide in nature. Capsule is also seen in fungi, e.g. *Cryptococcus neoformans*.
- **Synthesis:** In most cases, the capsule is synthesized at the level of the cell membrane.

Table 4.12: Capsulated bacteria.[2, 3, 5, 25, 26]

Common capsulated bacteria	Composition
Streptococcus pneumoniae	Polysaccharide
Neisseria meningitidis	Polysaccharide
Bacillus anthracis	Polypeptide (glutamate)
Klebsiella pneumoniae	Polysaccharide
Haemophilus influenzae	Polysaccharide
Pseudomonas aeruginosa	Alginate polysaccharide
Less common capsulated bacteria*	**Composition**
Gram-positive cocci	
Staphylococcus (microcapsule)	Polysaccharide
Streptococcus pyogenes (some strains)	Hyaluronic acid
Streptococcus agalactiae	Polysaccharide (sialic acid)
Viridans streptococci (some strains)	Polysaccharide (dextran)
Gram-negative bacilli	
Escherichia coli (extraintestinal strains)	Acidic polysaccharide
Klebsiella granulomatis (some strains)	Polysaccharide
Proteus mirabilis and *P. vulgaris*	Acidic polysaccharide
Salmonella Typhi	Vi polysaccharide capsule
Yersinia pestis	F1 glycoprotein antigen
Vibrio cholerae O139	Polysaccharide
Vibrio parahaemolyticus	Polysaccharide
Vibrio vulnificus	Polysaccharide
Acinetobacter (hypervirulent phenotype)	Polysaccharide
Burkholderia pseudomallei, B. mallei	Polysaccharide
Elizabethkingia	Polysaccharide
Campylobacter jejuni	Polysaccharide
Campylobacter fetus	Glycoprotein
Francisella tularensis	Polysaccharide
Pasteurella multocida	Polysaccharide
Capnocytophaga	Polysaccharide
Mycoplasma pneumoniae	Polysaccharide
Anaerobes	
Clostridium perfringens, C. butyricum	Polysaccharide
Bacteroides fragilis	Polysaccharide
Porphyromonas gingivalis	Polysaccharide
Prevotella melanogenica	Polysaccharide

*For these bacteria, capsule acts as a virulence factor, detectable by electron microscopy or molecular method, but not by routine microscopy.

  - Components are synthesized and exported out of the cell by an isoprenoid lipid "carrier" system, in which the components become attached to "primer" capsular material already present on the surface of the cell.
  - However, in some cases, for example in *Streptococcus mutans* the capsule is synthesized by a class of extracellular and cell wall-associated enzymes called glucosyltransferases which results in the formation of branched, insoluble glucan matrix.
- **Capsular polysaccharides** may be polymers of single monosaccharides (glucans, dextrans, levans) or

Table 4.13: Difference between slime layer and capsule.[1, 2, 4]

Feature	Slime layer	Capsule
Attachment to cell	Loosely attached; can be easily removed	Firmly attached to the cell wall or outer membrane
Structure	Amorphous, unorganized, and irregular	Organized, well-defined, and tightly bound
Thickness	Thin, diffuse, and irregular	Thick, uniform, and highly defined
Stability	Temporary; influenced by environmental conditions	Permanent under normal bacterial growth conditions
Role in pathogenicity	Less significant in direct immune evasion; aids biofilm-based infections	Major virulence factor; avoids host immune responses like phagocytosis

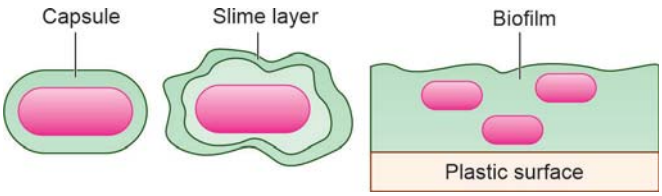


Fig. 4.20: Capsule, slime layer and biofilm.

heteropolysaccharides containing both hexose and pentose sugars, plus ribitol, glycerol, or other sugar alcohols. Phosphates are also frequently present.

- **Homopolysaccharide capsule:** It is composed of repeating units of a single type of monosaccharide (e.g. glucose, fructose). It is less effective in avoiding immune recognition due to the simple structure. It has limited functional diversity; it typically contributes to adhesion, biofilm formation, or desiccation resistance.
  - Glucans (glucose polymers): *Streptococcus mutans* (dental plaque), *Lactobacillus* spp. and *Xanthomonas campestris.*
  - Levans (fructose polymers): *Bacillus subtilis, Pseudomonas syringae, Zymomonas mobilis* and *Halomonas* spp.
  - Dextrans: *Leuconostoc mesenteroides, Enterococcus* spp. (in some strains).
- **Heteropolysaccharide capsule:** These capsules contain repeating units made of different sugars and other modifications like acetylation or phosphorylation. These heteropolysaccharides are typically highly diverse in their function.
  - *Streptococcus pneumoniae:* The capsule composition varies among the 100+ serotypes, contains common sugars such as glucose, galactose, glucuronic acid, rhamnose, N-acetylglucosamine. The type 3 capsule is composed of repeating units of glucose and glucuronic acid.
  - *Klebsiella pneumoniae:* Capsule is composed of varying monosaccharides, including glucose, mannose, galactose, and glucuronic acid.
  - *Haemophilus influenzae:* Capsule contains repeating units of ribose and ribitol, modified with phosphate groups. Type b (Hib) capsule is a polymer of polyribosyl ribitol phosphate (PRP).
  - *Escherichia coli* (*K* antigen capsule types): Capsule contains heteropolysaccharides like glucose, mannose, galactose, fucose, and sialic acid. It is found in pathogenic strains like uropathogenic *E. coli* (UPEC) and enterotoxigenic *E. coli* (ETEC). *E. coli* K1 contains sialic acid, mimicking host molecules to avoid immune recognition.
  - *Pseudomonas aeruginosa:* Capsule is composed of alginate (a heteropolysaccharide made of mannuronic acid and guluronic acid).
  - *Neisseria meningitidis:* Heteropolysaccharide capsule contains common sugars such as sialic acid, mannose, glucose, and galactose.
- ***Bacillus anthracis:***[2] The capsule of *B. anthracis* is one of a kind as it is made up of polypeptide and not polysaccharide. The polypeptide capsule is linked by β-peptide chains of D-glutamic acid ranging from 50 to 100 residues per chain. The expression of capsule is maximum when the organism is present in a medium with bicarbonate or in a >5% CO_2 environment.
- **Glucan capsule:**[4] As mentioned earlier, the glucan capsule of *S. mutans* specifically interacts with the tooth surface and strongly adheres to it which can result in dental carries.
  - Once colonized in the denture, it is capable of producing acids from dietary sugars such as sucrose which leads to decreased pH in the oral cavity and thereby leading to demineralization of the enamel. This explains the association of *S. mutans* glucan capsule and consumption of sucrose in the formation of dental carries.

Functions of Capsule[1, 2]

Capsules are not required for growth and reproduction of bacteria in laboratory cultures. However, they confer several advantages when bacteria grow in their normal habitats. The capsule contributes to bacterial virulence and protects the bacterium from the host immune system through various mechanisms.

- **Prevents phagocytosis:** The capsule shields bacteria from being engulfed and digested by host immune cells, such as macrophages and neutrophils. For example, *Streptococcus pneumoniae* and *Klebsiella pneumoniae* evade immune cells via their thick capsule.
- **Avoids complement-mediated killing:** Capsules can inhibit the activation of the complement system, which reduces opsonization (tagging of bacteria for destruction) and membrane attack complex (MAC) formation.
- **Masks antigens:** The capsule can hide antigenic components on the bacterial surface, reducing immune detection.
- **Adhesion and colonization:** The sticky nature of capsules helps bacteria adhere to host tissues, medical devices, and surfaces, facilitating colonization. For example, *Streptococcus mutans* uses its capsule to adhere to teeth, contributing to dental plaque formation.

- **Biofilm formation:** Capsules contribute to the extracellular matrix of biofilms, enabling bacteria to establish resilient communities on surfaces.
  - A biofilm is a living ecosystem made of millions of adherent bacterial cells embedded within a self-produced matrix of extracellular polymeric substance (i.e. the polysaccharide slime layer).
  - Persistent biofilms containing pathogenic bacteria are capable of adherence to damaged tissues and plastic surfaces (e.g. medical devices, such as catheters and pacemakers).
  - This is the first step in bacterial colonization and sometimes it leads to disease, e.g. prosthetic valve endocarditis and catheter related urinary tract infection **(Fig. 4.20)**.
- **Prevention of desiccation:** Capsules are hydrophilic and trap water, which protects bacteria from drying out in hostile or arid environments. This function is especially important for soil-dwelling or airborne bacteria like *Bacillus anthracis*.
- **Resistance to antimicrobials:** Capsules act as a barrier that can hinder the penetration of antibiotics and other antimicrobial agents.
- **Enzyme protection:** Protects bacteria from lytic enzymes, such as lysozyme, that degrade peptidoglycan.
- **Nutrient storage:** Capsules can serve as a reserve of nutrients, especially polysaccharides, which bacteria can metabolize during starvation or stress conditions. Capsule promotes the concentration of nutrients at the bacterial cell surface because of its polyanionic nature.
- **Protection against toxic substances:** The capsule reduces the effects of toxic chemicals, heavy metals, free radicals, and most hydrophobic toxic materials such as detergents or other environmental stresses.
- **Protection against viruses:** It protects the bacterium from the action of bacteriophages.
- **Modulation of host immune response:** Some bacteria use capsules to actively manipulate the host immune system:
  - Group B *Streptococcus* mimics the host sialic acid in its capsule to reduce immune detection.
  - Certain capsules can induce anti-inflammatory responses, reducing immune-mediated damage to the bacteria.
- **Capsule in non-pathogenic bacteria:** Even non-pathogenic bacteria produce capsules for environmental protection, nutrient acquisition, and biofilm formation, which are critical for survival in their niches.
- **Abscess formation:** Capsule of certain bacteria (e.g. *Bacteroides fragilis*) may be toxic to the host cells and induces abscess formation.
- **Source of nutrients and energy:** Capsules can be a source of nutrients and energy to microbes. *Streptococcus mutans*, which colonizes teeth, ferments the sugar in the capsule and so formed acid by-products contribute to the tooth decay.

Demonstration of Capsule[1, 2]

The capsule of bacteria like *Haemophilus influenzae* can be demonstrated using various methods. These techniques aim to visualize or detect the capsule either directly or indirectly. Here are the key methods:

- **Staining techniques** such as negative staining, Anthony's capsule stain, etc. can be used for demonstration of capsule (refer Chapter 16, for detail).
  - **Negative staining:** When stained with India ink or nigrosine, the capsule appears as a clear halo surrounding the bacterial cell against a dark background. The capsule does not take up the stain, while the background and the bacterial cell do. It is a rapid and simple demonstration of the capsule. The common negative stains used are India ink and nigrosine stains.
  - **Anthony's capsule stain:** Crystal violet stains the bacterial cell, while the capsule remains unstained, appearing as a halo.
  - **Maneval's capsule stain:** Here the capsule appears as a clear halo between the pink-stained bacterium and the bluish-gray stained background.
  - **Modified Gram stain:** Capsules are visualized as colourless regions around the stained cell.
- **Serological methods:** Capsular material is antigenic and can be demonstrated by mixing it with a specific anti-capsular serum by methods such as Quellung reaction and latex agglutination test.
  - **Capsular antigen:** It can be detected in the sample (e.g. CSF) by **latex agglutination test** by using specific anti-capsular antibodies coated on latex particles. This is available for pneumococcus, *Cryptococcus, Haemophilus influenzae* and meningococcus. It is used for detection and serotyping of encapsulated bacteria in clinical specimens.
  - **Quellung reaction** (capsular swelling test): Capsular serotypes of *Streptococcus pneumoniae* can be detected by adding antisera mixed with methylene blue. Specific antisera bind to the capsule, causing it to swell. Capsule becomes swollen, refractile and delineated when observed under a microscope. This method is also available for other capsulated bacteria like *H. influenzae*.
  - **Direct IF test:** Type b strains of *H. influenzae* can also be identified by direct immunofluorescence using fluorescein-labelled type b antiserum.
- **Molecular detection:** PCR (polymerase chain reaction) can be used to amplify genes responsible for capsule synthesis (e.g. *bexA* in *H. influenzae* type b) and to confirm the capsular genotype.
- **Cultural methods:** Encapsulated bacteria often produce mucoid colonies on agar plates due to the excretion of polysaccharides.
- **Electron microscopy:** Can be used for direct visualization of the capsule structure using electron-dense stains and to obtain high-resolution imaging.
- **Phase-contrast microscopy:** Enhances the visualization of the capsule as a refractile zone around the bacterium.
- **Capsular quantification:** Methods such as phenol-sulfuric acid assay or chromatographic techniques can be used to measure the amount and composition of capsular polysaccharides.

Capsular Serotyping[2, 25, 26]

Capsular serotyping is used to identify and classify bacterial species or strains based on the specific composition of their capsular polysaccharides. The bacterial pathogens for which capsular serotyping is done is given in the highlight box. The method employed for capsular serotyping is as follows:

- **Latex agglutination test:** It is performed by slide agglutination test, involves mixing of bacterial cells with latex beads coated with specific antibodies that bind to capsular antigens.
- **Quellung reaction:** It involves mixing bacterial cells with type-specific antisera on a microscope slide (also known as the 'capsular swelling test').
- **Multiplex PCR-based serotyping:** Molecular technique that amplifies genes encoding capsular polysaccharide synthesis can be used to simultaneously detect multiple serotypes.

Capsular Serotyping

Capsular serotyping is performed for the following bacterial pathogens.

- *Streptococcus pneumoniae*: To date, >100 capsular serotypes have been recognized, which fall into 21 serogroups, and each serogroup contains two to eight serotypes with closely related capsules.
- *Staphylococcus aureus*: It has 11 types of microcapsular serotypes
- *Streptococcus agalactiae*: The capsular polysaccharide of group B *Streptococcus* is type specific—10 capsular serotypes have been recognized so far, designated Ia, Ib, and II to IX.
- *Neisseria meningitidis*: Based on the antigenic nature of the capsule, meningococci can be typed into 13 serogroups [A–E,H,I,K,L,W–Z,]; among which only 6 serogroups—A, B, C, X, Y, and W (formerly W135)—account for the majority of cases of invasive disease.
- *Escherichia coli*: So far, >80 distinct capsular (K) antigens are known.
- *Klebsiella pneumoniae*: Based on the capsular polysaccharide (K antigen) present, *K. pneumoniae* can be classified into 80 serological types
- *Haemophilus influenzae*: It is typed into six serotypes (a to f) based on the capsular polysaccharide. However, some strains lack capsule and are referred to as nontypeable strains.

Capsular Vaccine[2, 25, 26]

Capsular polysaccharide is antigenic and anticapsular antibodies are protective in nature. Hence, capsular antigens of many bacteria are used as potential vaccine candidates.

Established Capsular Vaccine

Several capsular vaccines have been successfully developed and widely implemented, providing effective protection against major bacterial pathogens.

- **Pneumococcus:** Capsular vaccines are revolutionary to protect against invasive pneumococcal diseases.
  - 23-valent pneumococcal polysaccharide vaccine (PPSV23): It targets 23 serotypes.
  - Pneumococcal conjugate vaccine (PCV): Polysaccharides are conjugated to a protein carrier to enhance immunogenicity. It is available in various formulations such as 13-valent (PCV13), 15-valent (PCV15), 20-valent (PCV20).
- **Meningococcus:** Capsular vaccines played an important role in reducing outbreaks of meningococcal meningitis worldwide.
  - MenACWY: Quadrivalent conjugate vaccine covering serogroups A, C, W, and Y.
  - MenB vaccines: Target capsular group B with proteins due to the poor immunogenicity of MenB polysaccharides.
- ***Haemophilus influenzae*** type b (Hib): The introduction of Hib vaccines has significantly reduced the global burden of Hib-related diseases. Types of Hib vaccines include:
  - *Polysaccharide vaccine (first-generation):* It uses pure PRP polysaccharide. It is poorly immunogenic in children under 2 years due to immature T-cell-independent immune responses.
  - *Conjugate vaccines (second-generation):* PRP linked to a protein carrier (e.g. tetanus or diphtheria toxoid). It induces a robust T-cell-dependent immune response, providing long-lasting immunity.
- **Vi capsular polysaccharide vaccine:** It uses Vi capsular antigen of *Salmonella* Typhi, used to provide protection against typhoid fever.

On-going Vaccine Trials[2, 25, 26]

Several research efforts are ongoing, which focused on developing effective vaccines against several encapsulated bacterial pathogens by targeting their unique capsular structures.

- **Vaccine trials for GBS:** Capsular polysaccharide-based vaccines are under development, aiming to elicit protective immunity against various group B *Streptococcus* (GBS) serotypes. Recent clinical trials and developments are:
  - Hexavalent capsular polysaccharide conjugate vaccine (GBS6)
  - Type III capsular polysaccharide-tetanus toxoid conjugate vaccine (III-TT)
  - Virus-like particle (VLP) conjugate vaccine
- ***Klebsiella pneumoniae:*** Research focuses on targeting capsular polysaccharides (e.g. K1 and K2 serotypes) for vaccine development to prevent pneumonia and bloodstream infections.
- ***Escherichia coli:*** Capsular antigens, such as K1 polysaccharide in neonatal meningitis-causing strains, are under investigation as vaccine targets.
- ***Bacillus anthracis:*** Targeting capsule has been a focus in the development of anthrax vaccines. The poorly immunogenic poly-γ-D-glutamic acid capsule of *B. anthracis* is chemically conjugated to a protein carrier (e.g. tetanus or diphtheria toxoid) to enhance T-cell-dependent immune responses.

Microcapsule[1]

A microcapsule is a specialized form of bacterial capsule that is extremely thin and not easily detectable using conventional microscopy techniques. It is essentially a subtype of capsule with reduced thickness and subtle functionality.

- It requires advanced techniques for detection (e.g. electron microscopy).
- Functions of microcapsules are similar to that of capsule such as immune evasion, protection from environmental

stress and contribute to pathogenicity, but less robust than a regular capsule.

- Examples of bacteria with microcapsules: *Streptococcus pyogenes, Yersinia pestis* (F1 antigen), *Staphylococcus aureus,* etc.

FLAGELLA[1,2]

Flagella are thread-like appendages, protruding from the cell wall, that confer motility to the bacteria (organs of locomotion). They measure 5–20 µm in length and 0.01–0.02 µm in thickness.

- **H antigens:** Flagellar antigens in gram-negative bacilli are referred to as H antigens, from the German word "hauch," meaning "breath."
- **Stability:** H antigen is heat and alcohol labile, but resistant to the action of formaldehyde.

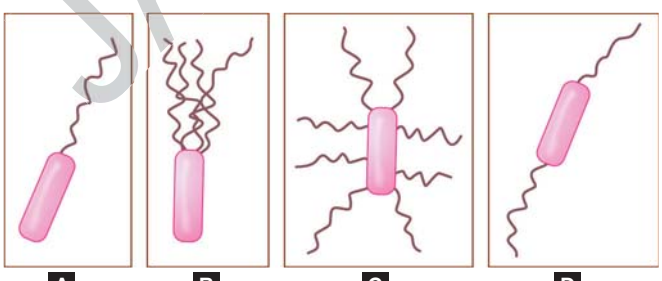
Arrangement of Flagella[1,2]

There are various patterns of arrangement of flagella with respect to the bacterial surface **(Figs. 4.21A to D)**:

- **Peritrichous** flagella are spread evenly over the whole surface of bacteria (Greek peri, around), e.g. *Salmonella* Typhi, *Escherichia coli.*
- **Monotrichous** bacteria (Greek *trikhos,* hair) have single flagellum, if it is located at an end, it is said to be a **polar flagellum**;, e.g. *Vibrio cholerae, Pseudomonas* and *Campylobacter.*
- **Lophotrichous** bacteria (Greek lopho, crest or tuft) have a cluster of flagella at one or both ends, e.g. *Spirillum* and *Helicobacter pylori.*
- **Amphitrichous** bacteria (Greek *amphi,* on both sides) have a single flagellum at each pole, e.g. *Alcaligenes faecalis.*
- **Bacteria with two types of flagella:** *V. parahaemolyticus* have two different types of flagella with distinct functions for swimming and swarming. In liquid media they produce single polar sheathed flagellum whereas on solid medium they produce unsheathed peritrichous flagella.

Ultrastructure of Flagella

Electron microscope reveals that the bacterial flagellum is composed of three parts—filament, basal body and hook **(Figs. 4.22 and 4.23)**.



Figs. 4.21A to D: Types of bacterial flagellar arrangement: (A) *Monotrichous*; (B) *Lophotrichous*; (C) *Peritrichous*; (D) *Amphitrichous*.

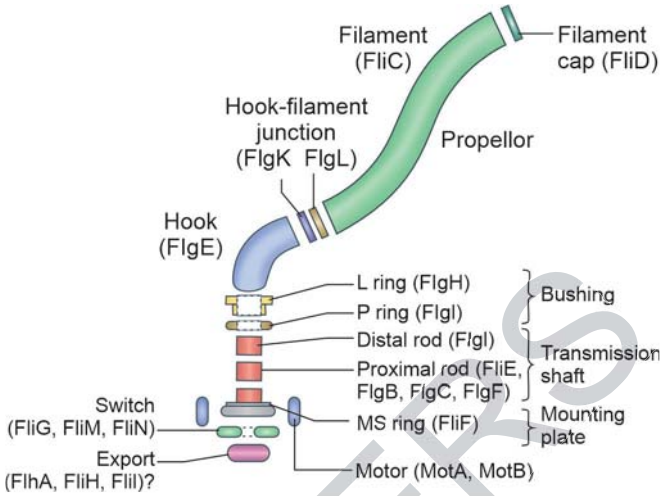


Fig. 4.22: Structure of flagella (gram-negative bacterium).[4]

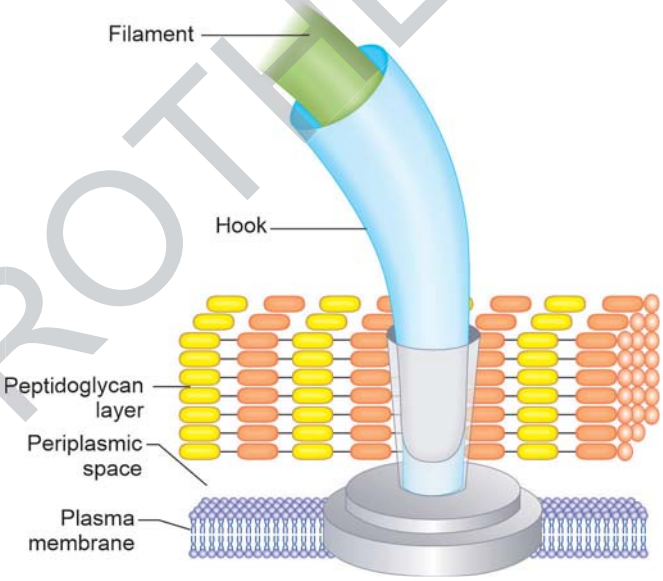


Fig. 4.23: Structure of flagella (gram-positive bacterium).

Filament[1,2]

It is the longest portion of the flagellum that extends from the cell surface to the tip.

- It is a hollow, rigid cylinder, constructed of subunits of the protein **flagellin**, which ranges in molecular mass from 30,000 to 60,000 daltons, depending on the bacterial species.
- **Flagellin:**[27] The helical N- and C-termini of flagellin form the inner core of the flagellin protein and is responsible for flagellin's ability to polymerize into a filament. The middle residues make up the outer surface of the flagellar filament. Flagellin-like structural proteins are found in other portions of the flagellum, such as the hook (flgE), the rod at the base, and the cap at the top.
- In most cases, bacteria have only one type of flagellin but in some instances two types of flagellin can be seen. The flagellins of different bacterial species presumably differ from one another in primary structure.
- The filament ends with a **capping protein**.
- Some bacteria have **sheaths** surrounding their flagella. For example, *Vibrio cholerae* flagella have lipopolysaccharide sheaths.

Hook[1, 2]

It is a short, curved flexible segment that links the filament to its basal body and acts as a flexible coupling. Slightly wider than the filament, the hook is made of different protein subunits.

Basal Body[1, 2]

This is the portion of flagellum which is embedded in the cell. It anchors the flagellum to the cell membrane and acts as a motor. It is the most complex part of a flagellum, made up of 2–4 rings, connected to a central rod.

- **Gram-negative bacteria** have four rings—L,P,MS and C **(Fig. 4.22)**, which are connected to a central rod.
  - The outer L and P rings are associated with the LPS and peptidoglycan layers, respectively. These rings evidently function as bearings, minimizing friction and leakage of materials from the cell at the points of flagellar insertion.
  - The inner MS ring lies in periplasmic space in contact with the plasma membrane and the C ring is on the cytoplasmic side of the MS ring. These rings function as flagellar motor components (discussed below).
- **Gram-positive bacteria** have only two basal body rings—an inner ring connected to the plasma membrane and an outer one probably attached to the peptidoglycan. The L and P rings are absent in gram-positive bacteria **(Fig. 4.23)**.
- **Flagellar motor components** are present within the basal body. They comprise of rotor and stator **(Fig. 4.22)**.
  - *Rotor:* It is composed of the MS-ring (FliF) and C-ring. The rotor is directly connected to the flagellar filament (FliM, FliN and FliG). The rotor ring structure rotates causing the rigid flagellar helix to turn like a propeller to generate propulsion.
  - *Stator:* It is composed of motor proteins—MotA and MotB, embedded in the inner membrane around the rotor. It forms channels that allow the flow of ions like protons (H^+) or sodium (Na^+) ions, generating torque.
  - *Torque-generating units:* The interaction between the stator and rotor converts ion flow into mechanical rotation **(Fig. 4.24)**. The peripheral rings remain firmly attached to the inner and outer bacterial membranes as well as the peptidoglycan layer (murein), thereby securing the flagellar apparatus to the cell envelope. Rotation of the flagellum is powered by the influx of protons from the periplasm into the cytoplasm, driven by the electrochemical gradient and membrane potential—collectively referred to as the proton motive force. A regulatory switch controls the direction of flagellar rotation, which determines the bacterium's movement. The motor proteins (MotA and MotB) generate torque using the proton motive force.

Synthesis of Flagella[1, 4]

The synthesis of bacterial flagella is a complex process that involves at least 20–30 genes for its assembly and maintaining the function. Besides the gene for flagellin, 10 or more genes code for hook and basal body proteins; other

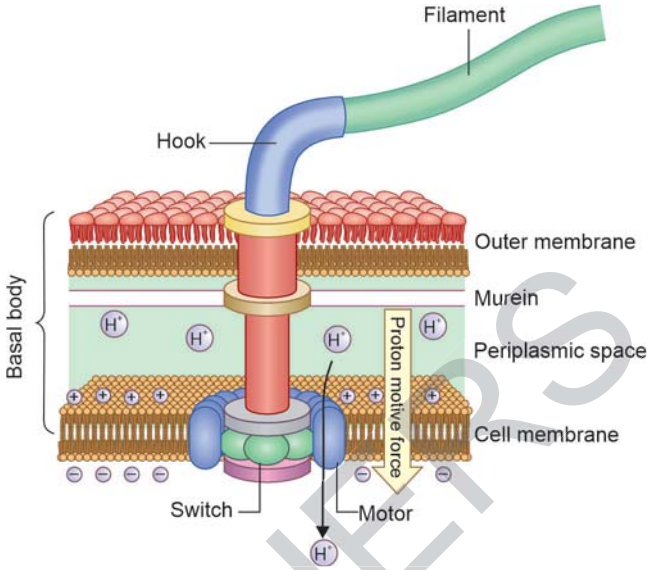


Fig. 4.24: Mechanism of motility by flagella.[4]

genes are concerned with control of flagellar construction or function. Many components of the flagellum lie outside the cell envelope and must be transported out of the cell for assembly. The synthesis of flagella happens in a phased manner. First the basal body is assembled and inserted into the cell envelope, then the hook is added and finally the filament is assembled upon which the flagellin subunits are added to the growing tip progressively.

- **Transcriptional regulation:** Flagellar assembly begins with the activation of the master regulator genes (*flhDC* in *E. coli*). These genes encode the FlhD/FlhC complex, which acts as a transcriptional activator. The *flhDC* complex regulates downstream flagellar genes in a hierarchical manner.
- **Assembly of basal body:** Interestingly, the basal body is a specialized version of the type III protein secretion system observed in typical gram-negative bacteria. Type III secretion systems have a needlelike structure through which proteins are secreted. In the flagellar type III secretion system, the filament replaces the needle.
  - The MS-ring is assembled in the inner membrane from *FliF* proteins.
  - The C-ring (*FliG*, *FliM*, and *FliN*) is attached to the cytoplasmic side of the MS-ring and interacts with the motor proteins.
  - The rod is assembled, penetrating through the cell wall with the help of P-ring (*FlgI*) and L-ring (*FlgH*), which stabilize the structure in the periplasm and outer membrane.
- **Hook assembly:** The hook (*FlgE*) is assembled after the basal body. It acts as a universal joint between the basal body and the filament. Hook length is regulated by the hook-length control protein (*FlgM*).
- **Switch to filament assembly:** Once the hook is complete, *FlgM* is secreted out of the cell, relieving its inhibition of the sigma factor σ*28* (FliA). The σ*28* then activates the transcription of late genes, including those coding for flagellin (*FliC*).

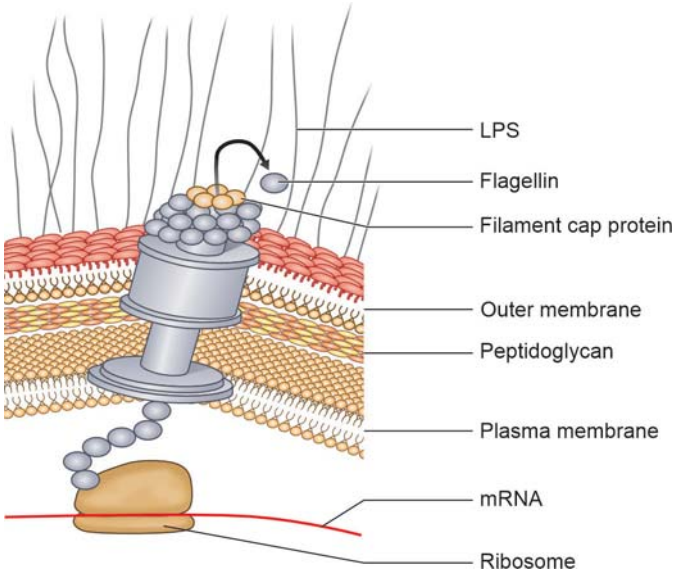


Fig. 4.25: Growth of flagellar filaments at the tip.

- **Filament assembly:** The synthesis of the filament happens via self-assembly like that of S-layer formation.
  - Flagellin subunits are transported through the hollow core of the flagellum by the export apparatus and added to the growing filament at its tip.
  - The cap protein (*FliD*) is added to the tip of the filament to prevent disassembly and facilitate the incorporation of flagellin subunits.
  - Flagellin subunits are transported through the central hollow channel of the flagellum and, upon reaching the distal tip, they assemble sequentially with previously deposited units, thereby promoting the progressive elongation of the filament **(Fig. 4.25)**.

Modified Flagellar Structures[5]

Some bacteria have slightly modified flagellar structures.

- ***Vibrio* species:** They have the typical flagellar morphology, but the flagellum is encased in a sheath derived from the outer membrane of the cell wall.
- **Spirochetes:** Here, the flagellum does not protrude into the environment but lies within a sheath that is exterior to the protoplasm. Such flagellum is called as **endoflagellum or axial filament.** It arises from one pole of the cell and wraps around the cell body internal to the sheath.

Bacterial Motility[1, 2, 26]

Several structures extending beyond bacterial cell envelopes contribute to motility. Five major methods of movement have been observed: swimming movement conferred by flagella, flagella-mediated swarming, corkscrew movement of spirochetes, twitching motility associated with type IV pili, and gliding motility **(Table 4.14)**.

- **Directional movement:** Motile bacteria do not move aimlessly. Rather, motility is used to move toward nutrients such as sugars and amino acids and away from many harmful substances and bacterial waste products.
- **Chemotaxis:** Movement toward chemical attractants and away from repellents is known as chemotaxis.

Table 4.14: Types of motility[1, 2, 26]

Type of motility	Examples of bacteria
Swimming motility	Run-tumble: Peritrichous flagella such as *E. coli, Salmonella, Bacillus spp.* and also unipolar flagellated bacteria (*Campylobacter*) Run-stop motility: *Rhodobacter sphaeroides* Run-reverse-flick motility: *Vibrio alginolyticus*
Tumbling motility	*Listeria monocytogenes* *Campylobacter jejuni, Selenomonas*
Gliding motility	*Mycoplasma, Capnocytophaga*
Stately motility	*Clostridium*
Darting motility	*Vibrio cholerae, Campylobacter jejuni*
Swarming	• All *Proteus* species • *Morganella* does not show true swarming but produces a thin film of growth over the soft agar on prolonged incubation. • *Vibrio*: Certain species such as *V. parahaemolyticus, V. alginolyticus, V. harveyi, V. diabolicus* and *V. pectenicida* • *Aeromonas*: 60% of strains can swarm (especially the mesophilic group) • *Burkholderia cenocepacia* • *Clostridium* spp. such as *C. novyi* A, *C. septicum. C. sporogenes*, and *C. tetani* • *Capnocytophaga* spp.
Corkscrew or flexion extension	Spirochetes, *Helicobacter pylori* *Anaerobiospirillum*
Sliding motility	*Legionella*
Twitching motility	*Bartonella henselae*
Twisting motility	*Bartonella bacilliformis*

- **Other types:** Motile bacteria also can move in response to environmental cues such as temperature (thermotaxis), light (phototaxis), oxygen (aerotaxis), osmotic pressure (osmotaxis), and gravity.

Swimming Motility Conferred by Flagella[1]

Swimming motility is a type of bacterial movement powered by flagella, allowing bacteria to move through liquid environments. This process is vital for nutrient acquisition, colonization, and evasion of hostile conditions.

- **Rotation of filament:** The filament of a bacterial flagellum is in the shape of a rigid helix, and the cell moves when this helix rotates like a propeller on a boat.
- **Rate of rotation:** The flagellar motor can rotate very rapidly; the rate depends up on the species.
  - *E. coli* motor rotates 270 revolutions per second (rps)
  - *Vibrio alginolyticus* rotates at an average of 1,100 rps.
- **Factors:** Motility is influenced by various mechanisms, such as glycosylation, phase variation and quorum sensing.
- **Types:** There are various patterns of swimming motility—run-tumble motility, run-stop motility and run-reverse-flick pattern.

Run-Tumble Motility[1]

For many bacteria, flagellar rotation in an aquatic environment result in two types of movement: a smooth swimming

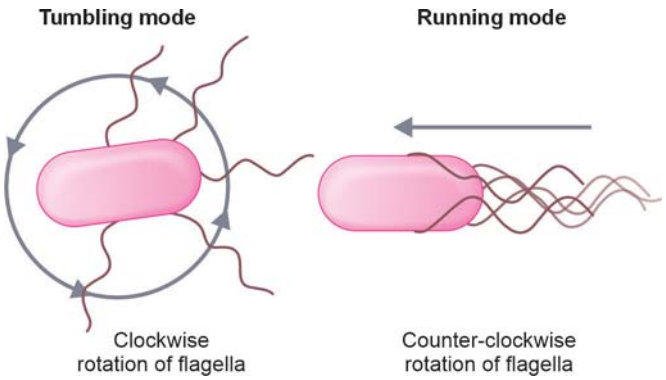


Fig. 4.26: Run and tumbling motility of peritrichous flagellated bacteria.

movement often called a run, which actually moves the cell from one spot to another, and a tumble, which serves to reorient the cell. Alternating between runs and tumbles is important for responding to environmental conditions. Often, the direction of flagellar rotation determines whether a run or a tumble occurs **(Fig. 4.26)**.

- **Run:** A run-movement is characterized by a straight locomotion, due to flagella rotate counterclockwise (CCW).
  - As flagella rotate counterclockwise, the flagella bend at their hooks to form a rotating bundle that propels the cell forward.
  - Run movement is exhibited when chemoattractant are present in the environment, which will stimulate the flagella to initiate counterclockwise rotation.
  - The environmental chemo attractants bind to a specific chemoreceptor (e.g. methyl-accepting chemotaxis proteins in *C. jejuni*).
- **Tumble:** It represents shorter, randomly oriented movements, due to the clockwise (CW) rotation of the flagella, which is initiated in a chemoattractant-free low-viscous environment. Clockwise rotation of flagella causes the bundle to disassemble and the bacterium to change direction.
- **Examples:** Swimming motility (alternating between runs and tumbles) is usually exhibited by **bacteria with peritrichous flagella** such as *E. coli, Salmonella*, *Bacillus* spp. and occasionally by monotrichous flagellar bacteria like *Campylobacter.* Tumbling-only motility is specifically demonstrated in bacteria such as *Listeria monocytogenes*, *Selenomonas,* etc.

Run-Stop Motility[1]

Not all bacteria use runs and tumbles for swimming motility. Some bacteria use run-stop mechanism, which refers to a movement pattern in which bacteria alternate between periods of smooth swimming (runs) and abrupt halts (stops). During the "stop" phase, the bacterium may reorient itself or remain stationary for a brief time before resuming forward motion. This behavior is distinct from the "run-and-tumble" motility seen in many bacteria. It is observed in bacteria such as *Rhodobacter sphaeroides.* The mechanism of run-stop motility is as follows.

- **Propulsion by flagella:** The "run" phase is driven by the rotation of flagella, typically a single polar flagellum or multiple coordinated flagella. The bacterium moves in a relatively straight trajectory.
- **Stop phase:** The "stop" occurs when the flagellar motor ceases rotation or when the flagella stop producing thrust. This can result from a cessation of motor torque, or changes in the flagellar conformation (e.g. folding or collapsing). When they resume a run, they move in a new direction.
- **Reorientation (optional):** During stops, some bacteria may use mechanical interactions with surfaces or hydrodynamic forces to reorient themselves before resuming movement.
- **Regulation:** The switch between running and stopping is often regulated by chemosensory systems, environmental cues, or intracellular signaling pathways.

Run-Reverse-Flick Motility[1]

It is a unique type of bacterial movement observed in some polar-flagellated bacteria like *Vibrio alginolyticus*. It involves three distinct phases of motion that allow bacteria to efficiently navigate their environments, especially in complex or viscous media.

- **Run:** The bacterium swims forward in a relatively straight path, propelled by its polar flagellum.
- **Reverse:** The bacterium reverses its direction by reversing the rotation of its flagellum, moving backward along its original path.
- **Flick:** Following the reverse phase, the bacterium undergoes a sharp directional change (flick), which reorients it at an angle to its original trajectory. This flick is caused by a mechanical buckling of the flagellum, enabling exploration of new directions.

Swarming Movement[1, 2]

Swarming motility is a type of coordinated bacterial movement on semi-solid or moist surfaces, where cells act collectively as a group. It is a specialized form of motility that allows bacteria to rapidly colonize new environments, respond to external stimuli, and establish biofilms.

- **Flagella-driven:** Swarming relies heavily on the bacterial flagella, which act like propellers to enable movement. Most bacteria that swarm have peritrichous flagella. During swarming, bacteria often produce more flagella (**hyper-flagellation**) or increase their activity to facilitate rapid movement. Flagellar rotation is synchronized within the swarm to produce cohesive movement.
- **Group behavior:** Swarming is a communal activity where bacteria act as a collective, coordinating their movement.
- **Cellular differentiation:** Swarming cells are often larger and multinucleated, which helps reduce drag and allows them to move more effectively as a group. Hyper-flagellation occurs to enhance motility.
- **Surface dependent:** It requires a moist environment or a low-viscosity surface, which facilitates the movement of bacterial groups. Example includes semi-solid environments with low agar concentrations (0.4–0.7%).
- **Surfactant production:** Bacteria produce biosurfactants (e.g. rhamnolipids and lipopeptides, etc.). Surfactants lower surface tension, aiding in the spreading of bacterial

groups by reducing friction and preventing cells from sticking to the surface.

- **Quorum sensing:** Swarming is regulated by quorum sensing, a cell-to-cell communication system. Quorum sensing molecules (e.g. acyl-homoserine lactones) coordinate gene expression, ensuring synchronized movement and biosurfactant production. It allows the bacterial population to act as a unit.
- **Nutrient sensing and chemotaxis:** Bacteria detect and move toward nutrient-rich areas using chemotaxis. Gradients of attractants and repellents influence the direction of swarming.
- **Extracellular matrix (ECM):** A transient ECM composed of polysaccharides, proteins, and DNA aids in adhesion and cohesion of the swarm. Unlike in biofilms, the ECM in swarming is dynamic and temporary.
- **Environmental triggers:** such as agar (0.4–0.7%), high moisture and nutrients availability, temperature and pH influence swarming.
- **Colony morphology:** When bacteria that swarm are cultured in the laboratory on appropriate solid media, they produce characteristic rapid and expansive growth pattern, spreading outward in concentric zones or irregular patterns.
- **Examples:** An increasing number of bacterial species has been found to exhibit swarming, of which the most important is *Proteus* spp. **(Table 4.14)**. For mechanism of swarming seen in *Proteus*, refer Author's Volume-2 (Chapter 11.3) book.

Corkscrew Movement of Spirochetes[1]

Spirochetes are a unique group of bacteria characterized by their helical shape and distinct mode of motility. Their motility is powered by **axial filaments**, also known as **endoflagella** are located within the periplasmic space.

- These filaments are anchored at both ends of the spirochete and extend along its length.
- The number of axial filaments varies depending on the species (e.g. *Treponema pallidum* has ~3–4 filaments, *Borrelia burgdorferi* has ~7–11 whereas Leptospira has one endoflagella at each pole).
- Spirochete motility enables movement through viscous environments, such as mucus or host tissues.

Mechanism of Spirochete Motility[1]

The endoflagella rotate, generating torque that causes the cell body to twist and rotate. This rotation does not involve the outer sheath directly but transfers torque through the periplasmic space to the cell body.

- **Corkscrew motion:** The rotation of axial filaments causes the spirochete to exhibit a characteristic **corkscrew-like motion**. This motion is highly efficient for moving through viscous environments where other forms of motility, like flagella-based swimming, would fail.
- **Components involved:** Similar to external flagella, the basal bodies of the endoflagella anchor them to the cell membrane and act as motors. It is also powered by the proton motive force (PMF). The helical shape of the spirochete and the flexibility of its outer sheath amplify the movement generated by axial filaments.

Twitching Motility[1]

Like swarming, twitching motility also occurs when the bacterial cells are on a solid surface. However, unlike swarming, it does not involve flagella.

- **Definition:** Twitching motility is described as short, intermittent, jerky motions of up to several μm in length.
- **Mechanism:** Twitching motility is mediated by **type IV pili**, which alternately extend and retract to move cells. The extended pilus contacts the surface at a point some distance from the cell body. When the pilus retracts, the cell is pulled forward. ATP hydrolysis powers the extension/retraction process.
- **Examples:** Bacteria showing twitching motility include *Haemophilus influenzae, Neisseria meningitidis* and *N. gonorrheae, Acinetobacter,* and *Bartonella henselae*. Though *P. aeruginosa* is motile by hanging drop attributed to flagella, it also shows twitching motility in culture media which is solely mediated by type IV pili. *Myxococcus xanthus* shows both twitching and gliding motility. *Legionella* shows both twitching and sliding motility.
- **Detection:** Twitching motility is expressed only on very moist surface such as agar (1.0–1.5%) or glass, but the bacteria appear non-motile when performed hanging drop. Methods to observe twitching motility are as follows:
  - *Edge spreading assays:* The strain is inoculated on a thin layer of nutrient agar (e.g. 1.0–1.5% agar) and incubated for several hours. Then the colony edges are observed under a microscope for the characteristic jerky movement.
  - *Glass surface:* A glass slide with a small amount of soft agar can be used to observe the small, jerky movements of twitching motility under phase-contrast microscopy.
  - *Time-lapse microscopy:* Time-lapse imaging on a solid surface reveals the dynamic extension and retraction of pili during twitching.

Gliding Motility[1]

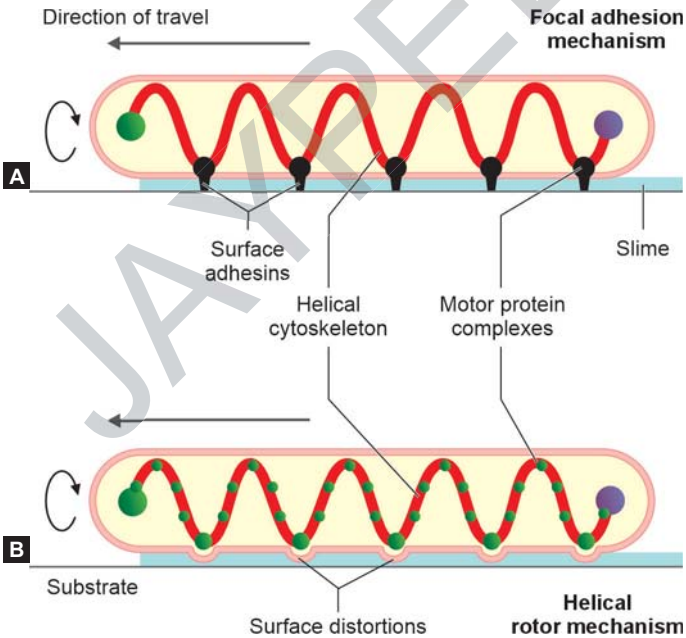
Gliding motility is another flagella-independent form of surface-associated bacterial movement. But in contrast to the jerky movement of twitching motility, gliding motility is a smooth, continuous motion that allows bacteria to move along solid surfaces. Gliding motility varies greatly in rate (2–600 μm per minute) and in the nature of the motion.

Mechanism of Gliding Motility[1]

Although first observed over 100 years ago, the mechanism by which many bacteria glide still remains a mystery. The mechanism of gliding motility varies between different bacterial species, as it is not driven by a universal structure like flagella. Instead, it involves distinct molecular systems and surface interactions. Some of the important proposed mechanisms are summarized below:

- **Type IV pili-dependent gliding:** Similar to twitching motility, gliding can involve the extension and retraction of type IV pili. The pili extends, attach to the surface, and then retract, pulling the cell forward. Example includes *Myxococcus xanthus* exhibits social (S) motility, where groups of cells move together using the pili.

- **Focal adhesion-based gliding:** Some bacteria use protein complexes located at specific points on their outer membrane to attach to the substrate, thereby pushing the body of the cell forward and generating motion **(Fig. 4.27A)**.
  - When these adhesins contact a solid surface, they attach briefly, pulling the bacterial cell forward before detaching. This repeated cycle creates a smooth, gliding movement. Cells move forward as a result of the force generated by the adhesins against cytoskeleton. Some of the examples of organisms that exhibit gliding motility, and the respective proteins involved are mentioned below.
  - Gli349 in *Mycoplasma mobile* and P1 in *Mycoplasma pneumoniae*.
  - SprB and associated motor proteins in *Capnocytophaga*.
  - *Myxococcus xanthus* also uses this mechanism for adventurous (A) motility when individual cells move independently.
- **Helical rotor mechanism:** Motor proteins (green dots) tracking on a helical cytoskeleton pull so as to create distortions in cell wall. These distortions generate drag forces between the substrate and the cell surface and result in cell movement **(Fig. 4.27B)**.
- **Slime secretion:** Bacteria secrete a layer of polysaccharide-rich slime that generates propulsion through jet-like forces. Slime extrusion from specialized nozzles pushes the bacteria forward. *Cyanobacteria* like *Oscillatoria* and *Nostoc* use this mechanism.
- **Ratchet-based surface motility:** In some bacteria, movement is thought to result from the motion of protein complexes on the inner cell membrane. These proteins interact with the substrate in a way that drives the cell forward. *Flavobacterium johnsoniae* uses a gliding motor protein complex (Gld and Spr proteins) to move along surfaces.



Figs. 4.27A and B: Focal adhesion and helical rotor mechanisms of gliding motility.

- **Helical cell movement:** Helically-shaped bacteria like spirochetes use their spiral shape to glide smoothly along surfaces. Flexing and rotating the cell body generates a gliding motion.
- **Sulfonolipids:** Studies have shown the presence of unique sulfonolipids in the outer membrane of gliding bacteria that are absent in nonmotile mutants of the same species.[2]

Bacteria Showing Gliding Motility[1]

Bacteria exhibit gliding motility are as follows:

- **Pathogenic bacteria:** Showing gliding motility are *Mycoplasma*, and *Capnocytophaga*.
- **Non-pathogenic bacteria:** Showing gliding motility are *Myxococcus xanthus, Flavobacterium johnsoniae, Cyanobacteria* (e.g. *Oscillatoria, Nostoc*), *Beggiatoa* and *Flexibacter*.
- ***Myxococcus xanthus*:** It is a soil-dwelling bacterium that exhibits two types of gliding motility:
  - Adventurous (A) motility: Individual cells move using focal adhesions.
  - Social (S) motility: Groups of cells move together using type IV pili.

Biological Roles of Gliding Motility[1]

Gliding motility helps the bacteria in many ways:

- **Surface colonization:** Helps bacteria spread across solid substrates, such as soil, host tissues, or biofilm surfaces.
- **Nutrient acquisition:** Allows access to nutrient-rich areas, particularly in competitive environments.
- **Biofilm formation:** Facilitates the initial stages of biofilm development.
- **Social behavior:** In species like *Myxococcus xanthus*, gliding motility enables coordinated predation and multicellular structures like fruiting bodies.

Darting Motility[1]

Some bacteria are actively motile with rapid, short, and erratic movement pattern and frequently changing their direction, described as darting motility (dart means a small, slender, pointed missile which shows sudden, rapid movement when thrown at a target). It is also described as shooting star or swarming gnats motility. It is produced by *Vibrio cholerae* and *Campylobacter*.

- **Flagella-driven propulsion:** Darting motility is typically driven by a single polar flagellum, which rotates rapidly, generating thrust for quick and erratic movement.
- **Lack of directional regulation:** Unlike chemotactic motility seen in run-tumble behavior, darting motility lacks tight regulation of direction. The bacterium moves in short, random trajectories.
- **Surface interaction:** In some bacteria, darting motility is enhanced by transient interactions with surfaces or the liquid-solid interface, which contribute to the erratic movement pattern.
- **Rapid speed:** The movement tends to be faster than other motility types, with frequent stops or changes in direction, making the motility appear chaotic or "darting."

Detection of Flagella[1]

Flagella can be demonstrated by either direct demonstration of flagella (staining and other methods) or indirect means by demonstrating the motility.

Flagellar Staining Methods (Light Microscopy)[1]

Flagellar staining methods are specialized techniques that enhance the visibility of bacterial flagella, which are too thin to be seen with standard light microscopy. These stains typically involve mordants or dyes that increase the diameter of the flagella, allowing visualization. Here are commonly used flagellar staining methods (refer Chapter 16, for detail).

- **Leifson's stain:** A combination of a basic dye (e.g. fuchsin) and a mordant (e.g. tannic acid) is used which adheres to the flagella and thickens them.
- **Ryu's stain:** It uses a simpler formula than Leifson's stain with crystal violet and tannic acid.
- **Silver nitrate staining:** It uses silver nitrate, which binds to flagella and deposits metallic silver for visualization.
- **Gray's stain:** It uses basic fuchsin with phenol and tannic acid as mordants.

Other Methods to Detect Flagella[1]

Apart from flagellar staining techniques, other methods to detect flagella are as follows.

- **Fluorescent staining:** It uses fluorophore-conjugated antibodies or dyes specific to flagella proteins (e.g. flagellin).
- **Electron microscopy:** Transmission electron microscopy (TEM) provides high-resolution images of flagella ultrastructure. Shadowing technique (specimen coated with a thin film of platinum at 45° angle) is particularly useful in studying virus morphology, bacterial flagella. Scanning electron microscopy (SEM) also reveals surface features of cells, including flagella arrangement.
- **Molecular methods:** PCR and RT-PCR can be performed to detect genes encoding flagellar components (e.g. *fliC* for flagellin). Next-generation sequencing (NGS) can identify and compare flagellar gene clusters among organisms.

Functional (Indirect) Assays[2]

Demonstration of the motility provides indirect evidence of the presence of flagella. However, not all types of motilities are mediated by flagella. For example, as discussed before, gliding, and twitching motility are not mediated by flagella. Similarly, not all types of motilities are demonstrable by hanging drop technique, which is the most common method to demonstrate motility. For example, motilities such as swarming, gliding, and twitching motility are demonstrable on solid surfaces, but not on handing drop. The following are the different methods to demonstrate motility, discussed in detail in Chapter 15.

- Hanging drop method (most common method)
- Craigie tube method (most reliable method)
- Semisolid medium and its modifications, e.g. mannitol motility medium.
- Dark ground or phase contrast microscopy.

Application of Flagellar Antigen

Flagellar antigens have been extensively explored for flagellar antigens are highly immunogenic, making them versatile tools in the following applications—serotyping, diagnostic marker, and vaccine candidate.

Flagellar Typing[2]

Flagellar antigens are used to differentiate bacterial strains within the same species, a process critical for epidemiological surveillance and outbreak investigation. Flagellar typing is a serological method used to classify bacteria based on the antigenic properties of their flagella (H antigens). It is performed as slide agglutination test, that uses specific antibodies (flagellar antiserum) which recognize flagellar proteins (flagellin). Below is a list of bacteria, commonly classified using flagellar typing:

- ***Salmonella:*** *S. enterica* has a complex antigenic structure with specific flagellar (H) antigens. *Salmonella* O serogroups are further divided into various serotypes based on the type of H antigen present. More than 2,500 serotypes of salmonellae have been identified.
- ***Escherichia coli:*** H typing for *E. coli* is usually limited to reference laboratories. So far, 53 H antigen types of *E. coli* have been described, numbered H1 to H56. Certain *E. coli* flagellar type are major pathogens. For example, *E. coli* O157:H7 is a major pathogenic strain responsible for foodborne illnesses.
- ***Proteus:*** About 80 H antigen types have been identified, which are used in serotyping to differentiate strains of *P. mirabilis*, *P. vulgaris*, and other species within the genus.
- **The strains of *Y. pseudotuberculosis*** can be divided into six serotypes (1 to 6) based on heat-stable O and heat-labile H antigens.
- ***Morganella morganii*** has 38 different H types.
- ***Pseudomonas aeruginosa*** flagellin shows two major antigenic types, type A (H1) and type B (H2), and within these, three principal flagellin serotypes—FlaA1, FlaA2, and FlaB—are recognized, with FlaB being the most common (40% of cases), followed by FlaA2 (30% of cases).
- ***Campylobacter jejuni:*** Based on flagellar (H) antigens and somatic (O) antigens, *C. jejuni* has over 60 and 100 serotypes, identified by the Penner serotyping scheme and Lior scheme respectively.
- ***Listeria monocytogenes:*** Based on somatic 'O' and flagellar 'H' antigens, there are 13 serovars of *L. monocytogenes* such as—1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b/4bX, 4c, 4d, 4e, and 7. Several antisera are commercially available for serotyping.
- ***C. tetani*** can be typed into ten serotypes based on agglutination tests involving flagellar H antigens.[25]

Flagellar Antigen as Diagnostic Marker[2]

Flagellar antigens are widely studied as potential diagnostic markers for detecting antibodies in various infectious diseases. Their utility stems from their high immunogenicity, which makes them effective targets for serological assays. Below is an overview of their application in antibody detection:

- **Enteric fever:** Flagellar antigen is a cornerstone in the serodiagnosis of enteric fever. H-antigen of *Salmonella* Typhi, *S.* Paratyphi A and *S.* Paratyphi B are used in the Widal test.
- **Lyme borreliosis:** In Lyme disease, caused by *Borrelia burgdorferi*, flagellar antigens (e.g. 41-kDa flagellar protein) are often targeted in serological assays (e.g. ELISA) to detect antibodies, particularly during later stages of the disease.
- ***Helicobacter pylori*:** The flagellar antigens of *H. pylori* are sometimes included in ELISA-based diagnostic assays to detect specific antibodies, helping to diagnose chronic gastritis and peptic ulcer disease.[28]

Flagellar Antigen as Vaccine Candidate[29]

Flagellar antigens have been explored as vaccine candidates in various bacterial infections due to their strong immunogenicity, ability to induce protective responses, and potential to act as adjuvants. Use of flagellar antigens as vaccine candidate induces strong immunogenicity due to their recognition by the innate immune system (via toll-like receptor 5, TLR5). Below are examples of flagellar antigens have been studied as vaccine candidates:

- ***P. aeruginosa:*** Flagella hook protein FlgE has been tried as a novel vaccine candidate of *Pseudomonas aeruginosa*.[30] *Flagellin (FliC)* has also been evaluated as potential vaccine components against *P. aeruginosa* infections.[31]
- ***Helicobacter pylori:*** Studies found that certain flagellar proteins can induce protective immunity, making them potential candidates for vaccine development.[32]
- ***Yersinia pestis*** (flagellin-adjuvanted F1/V subunit vaccine): Incorporation of flagellin as an adjuvant in a subunit vaccine against plague has been shown to enhance T-cell and antibody responses, improving protective efficacy.[33]
- **Flagellin as adjuvant:** Flagellin has been employed as a carrier and adjuvant in the development of a vaccine. The classical example is flagellin-envelope fusion protein vaccine trial for dengue virus. Here, the bacterial flagellin is fused to dengue virus envelope proteins and has demonstrated good immunogenicity and potential efficacy in eliciting protective immune responses in animal models.[34]
- **Lyme disease:** Flagellar antigens of *Borrelia burgdorferi*, particularly the FlaB protein, have been investigated as potential vaccine candidates against Lyme disease. FlaB is highly conserved and induces robust immune responses. Research has explored the use of a flagella-less mutant of *B. burgdorferi* as a live attenuated vaccine, aiming to assess its immunogenicity and protective efficacy.[29]
- ***Salmonella*:** Flagellin proteins, specifically FliC and FljB, have been investigated as components in experimental vaccines. Research has demonstrated that incorporating these flagellin proteins can enhance host immune responses and provide protective immunity against *Salmonella* infections.[35]

Phase Variation in Flagella[1, 2, 26]

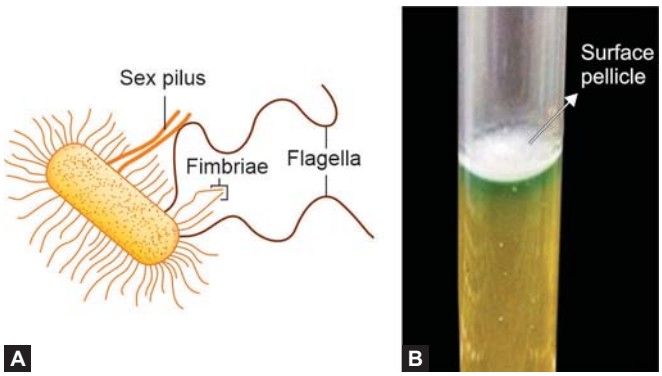
Certain flagellated bacteria can express two types of flagella alternately. This ability to alter the expressed antigenic type of flagella is known as phase variation.

- Phase variation occurs by the differential expression of chromosomal genes that code for two variously structured flagellin proteins.
- This phenomenon was first recognized in *Salmonella* species, but also occurs in other bacteria, such as *N. gonorrhoeae*.
- In most salmonellae the flagellar antigens exist in two alternative phases (phase 1 and 2). This is crucial in distinguishing serotype. At any given time point, *Salmonella* serotypes express any one type of flagellar phase. For example, *S.* Paratyphi B produces flagella of antigenic specificity 'b' when in phase 1 and of specificity '1,2' when switches to phase 2.

FIMBRIAE OR PILI[1, 2]

Pili (singular pilus; (Latin for 'hair') or fimbriae (singular fimbria; Latin for 'fringe') are short, fine, hair-like appendages that are thinner and typically shorter than flagella. They mainly help in bacterial adhesion hence called as the organ of adhesion (or adhesins).

- **Synonym:** The terms pili and fimbriae are synonymous, although certain structures are historically called pilus (e.g. sex pilus), while others are called fimbriae. Sex pilus is a special type of pilus that helps in conjugation. In this book, both the terms will be used interchangeably, except in those instances
- **Numbers:** Bacterial cell may be covered with up to 1,000 fimbriae, but they are only visible in an electron microscope due to their small size **(Fig. 4.28A)**.
- **Size:** Fimbriae are slender tubes composed of helically arranged protein subunits called pilin and are about 3–10 nm in diameter and up to several μm long.
- Several different types have been identified and most function to attach cells to solid surfaces such as rocks in streams and host tissues.
- **Antigenic:** They are antigenic; however, the antibodies against pilin antigens are not protective.



Figs. 4.28A and B: (A) Differentiation between fimbriae, sex pilus and flagella; (B) Surface pellicle (arrow showing).

Source: Department of Microbiology, JIPMER (*with permission*).

- **Motility:** Pili (with the exception of some Type IV pili) are not related to motility and can be found both in motile as well as in nonmotile organisms.

Classification of Fimbriae[36]

Fimbriae can be classified based on structure, function, adhesive properties, etc.

Based on Structural and Genetic Mechanism

Based on structural and genetic mechanisms, fimbriae can be classified into four types (Type I to IV).

Type I Fimbriae[36]

Type I fimbriae are one of the best-characterized fimbrial adhesins and are found in many members of the order Enterobacterales.

- **Structure:** Type I fimbriae appears as a peritrichous rigid, rod-like filaments made of repeating subunits of FimA, with a flexible tip containing an adhesin molecule (FimH) that confers adherence to mannose-containing glycoconjugates on host cells. These fimbriae are encoded on a gene cluster (*fim*).
- **Assembly:** Type I fimbriae are assembled by chaperone-usher pathway where periplasmic chaperones and an outer membrane usher protein guide subunit polymerization.
- **Characteristics:** Type I fimbriae bind to mannose-containing glycoproteins on host cells (mannose-sensitive adhesion). They are commonly involved in urinary tract infections (UTIs).
- **Examples:** Various kinds of type I fimbriae are found in several bacteria.
  - P fimbriae (pyelonephritis-associated pili) in uropathogenic *E. coli* (UPEC): They bind to galactose-containing receptors (e.g. P blood group antigens) in the kidney (mannose-resistant adhesion). They are encoded by *pap* genes.
  - S fimbriae in *E. coli*: They bind to sialic acid-containing receptors and are associated with neonatal meningitis. *Klebsiella pneumoniae* and *Salmonella* species also possess similar fimbriae.
  - M antigen-associated fimbriae are another example of Type I fimbriae, found in *Streptococcus pyogenes*, which contribute to adhesion and immune evasion.
  - Aggregative adherence fimbriae (AAF) found in enteroaggregative *E. coli* (EAEC).
  - Colonization factor antigens (CFA) enterotoxigenic *E. coli* (ETEC).
  - F1 fimbriae, found in *Yersinia pestis*.

Type II Fimbriae[37]

Structurally, Type II fimbriae are similar to Type I but varies in adhesin type and receptor specificity. They are encoded by *fim* genes.

- **Assembly pathway:** Chaperone-usher pathway, but with distinct structural and functional differences from Type I fimbriae.
- **Function:** Tyey mediate **mannose-resistant** adhesion to host tissues. They recognize different receptors depending on the fimbrial adhesin.

Type III Secretion-Associated Fimbriae[37]

The type 3 fimbriae are characterized by their ability to agglutinate erythrocytes treated with tannic acid in vitro, and this phenotype has been referred to as the mannose-resistant bacteria like hemagglutination (MR/K) reaction.

- **Assembly:** These fimbriae are encoded by the *mrk* operon and are predicted to also be assembled via the chaperone-usher pathway.
- **Examples:** It was first identified and characterized in *Klebsiella*, but also commonly found in several other Enterobacterales.
- **Function:** Type 3 fimbriae have shown to mediate attachment to endothelial and bladder epithelial cells and play a role in biofilm formation on abiotic surfaces, as well as surfaces coated with host-derived materials.
  - They are important in biofilm-mediated infections on indwelling devices, including CAUTIs
  - Variants of the adhesin MrkD can bind to type IV and/or type V collagen.

Type IV Pili[38]

Structurally, type IV pili are long flexible, dynamic filaments composed of pilin subunits, and capable of extension and retraction.

- **Assembly pathway:** These pili are assembled via Type IV pilus biogenesis pathway, involving specialized ATPases.
- **Function:** Type IV pili mediate several functions—mediates mannose-resistant adhesion to host cells, twitching motility and biofilm formation, involved in two methods of horizontal gene transfer—transformation and conjugation.
- **Examples:** Type IV pili are found in several bacteria.
  - *Neisseria gonorrhoeae:* Type IV pili (GC pilin, PilE) mediate adhesion and immune evasion.
  - *Pseudomonas aeruginosa:* PAK pilin (*P. aeruginosa* K pilin) is essential for biofilm formation and motility.
  - *Vibrio cholerae:* Toxin-coregulated pili (TCP) facilitate intestinal colonization. They are encoded by the *tcp* operon.
  - Bundle-forming pili (BFP): Thick, rope-like bundles of pili, found in enteropathogenic *E. coli* (EPEC). They mediate localized adherence to intestinal epithelial cells and play a critical role in the formation of microcolonies.

Types of Fimbriae Based on Adhesion Properties[1]

Based on adhesion properties, fimbriae are classified as follows:

- **Mannose-sensitive adhesion:** Mediated by fimbriae binding to mannose-containing receptors. Example includes Type 1 fimbriae in *E. coli*.
- **Mannose-resistant adhesion:** Mediated by fimbriae binding to non-mannose receptors like galactose or sialic acid. Examples include P fimbriae and S fimbriae.

Types of Fimbriae Based on Function[1, 5]

Functionally, fimbriae are classified as follows:

- **Adhesive fimbriae:** They mediate attachment to host cells, surfaces, or other bacteria. They are also called as common pili. Examples include:
  - Type 1 fimbriae (*E. coli*): Adhere to mannose residues.
  - P fimbriae (*E. coli*): Attach to uroepithelial cells.
  - S fimbriae (*E. coli*): Bind to sialic acid on host cells.
- **Conjugative (sex) pili:** They facilitate horizontal gene transfer (conjugation) by forming a bridge between bacteria. These hairlike structures differ from other pili in the following ways.
  - Sex pili often are larger (around 9–10 nm in diameter) and less numerous than adhesive fimbriae. Typically, sex pili are found 1–4 per cell, maximum up to 10 per cell.
  - They are genetically determined by conjugative plasmids and are required for conjugation.
  - Example includes F pilus (encoded by the F-plasmid in *E. coli.*
  - Some bacteriophages attach specifically to sex pili at the start of their infection cycle.
- **Biofilm-associated fimbriae:** They help in biofilm formation and environmental survival. Examples include Curli fimbriae.
- **Curli fimbriae:** They are amyloid-like fibers, involved in biofilm formation and environmental survival. They are found in *E. coli* and *Salmonella*. They are assembled via an extracellular nucleation-dependent polymerization pathway. They are encoded by the *csg* operon.

Fimbriae in Gram-positive Bacteria[36]

Although fimbriae are predominantly seen in gram-negative bacilli, certain gram-positive bacteria are recently found to harbour pili. Pili on gram-positive bacteria were first detected in *Corynebacterium renale*.

- **Types:** Fimbriae in gram-positive bacteria are structurally of two types—fibrils, longer pili.
- **Fibrils:** *Streptococcus gordonii* and *S. oralis*—are 'decorated' with short, thin rods or fibrils that extend between 0.07–0.5 µm from the bacterial surface. They bind to fibronectin adhesin receptors on host cells
- **Longer pili:** Longer pili (3 µm) that appear as flexible rods have been described in the gram-positive oral pathogens *Corynebacterium* species and pathogenic streptococci such as *S. pyogenes, S. agalactiae* and *S. pneumoniae*. They bind to collagen adhesin receptors on host cells. They are located on pathogenicity island and their synthesis is sortase-mediated.
- In gram-negative bacteria, pili are typically formed by non-covalent interactions between pilin subunits, by contrast, the recently discovered pili in gram-positive pathogens are formed by covalent polymerization of adhesive pilin subunits.

Detection of Fimbriae[1, 36-38]

Detecting fimbriae involves several techniques that allow researchers to identify their presence and study their function in bacterial cells. Electron microscopy is the only method for direct demonstration of fimbriae. However, there are some indirect methods to know the presence of fimbriae.

- **Transmission electron microscopy (TEM):** TEM is one of the most definitive methods for visualizing fimbriae on the surface of bacteria. This method provides high-resolution images of the bacterial cell surface and can clearly distinguish fimbrial structures from other surface components. TEM allows observation of fimbriae length, distribution, and density.
- **Scanning electron microscopy (SEM):** SEM can be used to examine the surface morphology of bacteria, including the presence and arrangement of fimbriae. Unlike TEM, SEM gives a 3D view of the bacterial surface. It is commonly used to assess the **density** and **pattern** of fimbriae on the bacterial surface, which can indicate whether they are involved in biofilm formation or adhesion.
- **Fluorescence microscopy:** Immunofluorescence microscopy involves using antibodies specific to fimbrial proteins conjugated to fluorescent dyes.
- **Molecular detection:** Specific primers are designed for fimbrial genes to detect and identify particular types of fimbriae (such as type I, type IV, or other specialized fimbrial types) by PCR. In DNA hybridization, a probe specific for fimbrial genes is used to detect the presence of these genes in bacterial genomes.
- **Western blotting** is used to detect fimbrial proteins in bacterial extracts. After separating proteins by SDS-PAGE, a fimbrial-specific antibody is used to bind to the target fimbrial protein, and the resulting protein bands are detected.
- **Adherence assays**, such as tissue culture adherence assay, hemagglutination assay and biofilm formation assay, etc. are indirect indicators of presence of fimbriae.
- **Tissue culture adherence assay:** In this assay, bacteria are incubated with cultured cells (e.g. epithelial or endothelial cells), and the bacteria's ability to adhere to the surface of these cells is measured. The presence or absence of fimbriae can be inferred based on **adherence patterns**—fimbriae-producing strains generally show **higher adherence rates** than non-fimbrial strains.
- **Hemagglutination assay:** Some fimbriae, especially Type 1 fimbriae, can cause hemagglutination**,** where bacteria agglutinate red blood cells of guinea pigs, fowl, horses and pigs. This property of hemagglutination is a simple method (indirect indicator) for detecting the presence of fimbriae which can be quantified. In some bacteria, the hemagglutination may be specifically inhibited by D-mannose.
- **Biofilm formation assay:** Fimbriae are important for biofilm formation, so testing the ability of bacteria to form biofilms on surfaces (using methods like **crystal violet staining**) can help detect fimbriae.
- **Surface pellicle:** Some aerobic fimbriated bacteria form a thin layer at the surface of a broth culture called as **pellicle**. The pellicle consists of many aerobic bacteria that adhere to the surface by their fimbriae **(Fig. 4.28B)**.

BACTERIAL SPORES

Spores are highly resistant resting (or dormant) stage of the bacteria formed in unfavorable environmental conditions as a result of the depletion of exogenous nutrients. Bacterial

spores formed within the parent cell, are called **endospores** and the remaining part of the bacterium is called the sporangium.

Structure[1]

Bacterial endospore comprises of several layers. From innermost towards the outermost, the layers are: core → cortex → coat → exosporium **(Fig. 4.29A)**.

- **Core:** The core is the innermost part containing the DNA material and is walled off from the cortex by an inner membrane and the germ cell wall.
  - The core has normal cell structures such as ribosomes and a nucleoid but has very low water content.
  - The core is surrounded by an inner membrane, which is in turn covered by the core wall (also called the germ cell wall, as it contains the peptidoglycan that will form the wall of the vegetative cell that grows out of the endospore following germination).
- **Cortex:** Outer to the core is the cortex which may occupy as much as half the endospore volume. It is made of peptidoglycan that is less cross-linked than that in vegetative cells. The cortex is surrounded by a phospholipid bilayer called the outer membrane.
- **Coat:** Outside of the outer membrane is the coat, a complex structure composed of several layers. It is composed of more than 70 different proteins, which are highly cross-linked to each other.
- **Exosporium:** It is the outermost layer, which is a thin covering, made up of glycoproteins.

Resistance[1]

Endospores are extraordinarily resistant to environmental stresses such as heat, ultraviolet radiation, gamma radiation, chemical disinfectants, and desiccation. In fact, some endospores have remained viable for around 100,000 years. The ability of the endospore to survive heat, radiation, and damaging chemicals requires that its enzymes and DNA be protected. The various layers of the endospore contribute to this resistance in several ways.

- **Impermeable spore coat:** The multilayered spore coat acts as a physical barrier to disinfectants, chemicals and various lytic enzymes such as lysozyme.
- **Modified inner membrane:** The inner membrane is extremely impermeable to various chemicals, including those that cause DNA damage.
- **Core:** The core has very low water content, high amounts of **dipicolinic acid** complexed with calcium ions (Ca-DPA), **small, acid-soluble DNA binding proteins (SASPs)** and a slightly lower pH, all of which contribute to the endospore's resistance to harsh conditions.
- **Low water content:** The spore core has very low water content (~10–25% of wet weight), reducing molecular mobility and enzymatic activity, which is important for endospore resistance.
  - The water content of the core is low enough to prevent rotation of enzymes and other proteins present. However, it is not low enough to prevent protein denaturation.
  - Immobilization prevents them from interacting with each other and becoming entangled. Thus, even though they may become denatured, they are able to refold to their proper active structure as the endospore germinates.

Protection of DNA

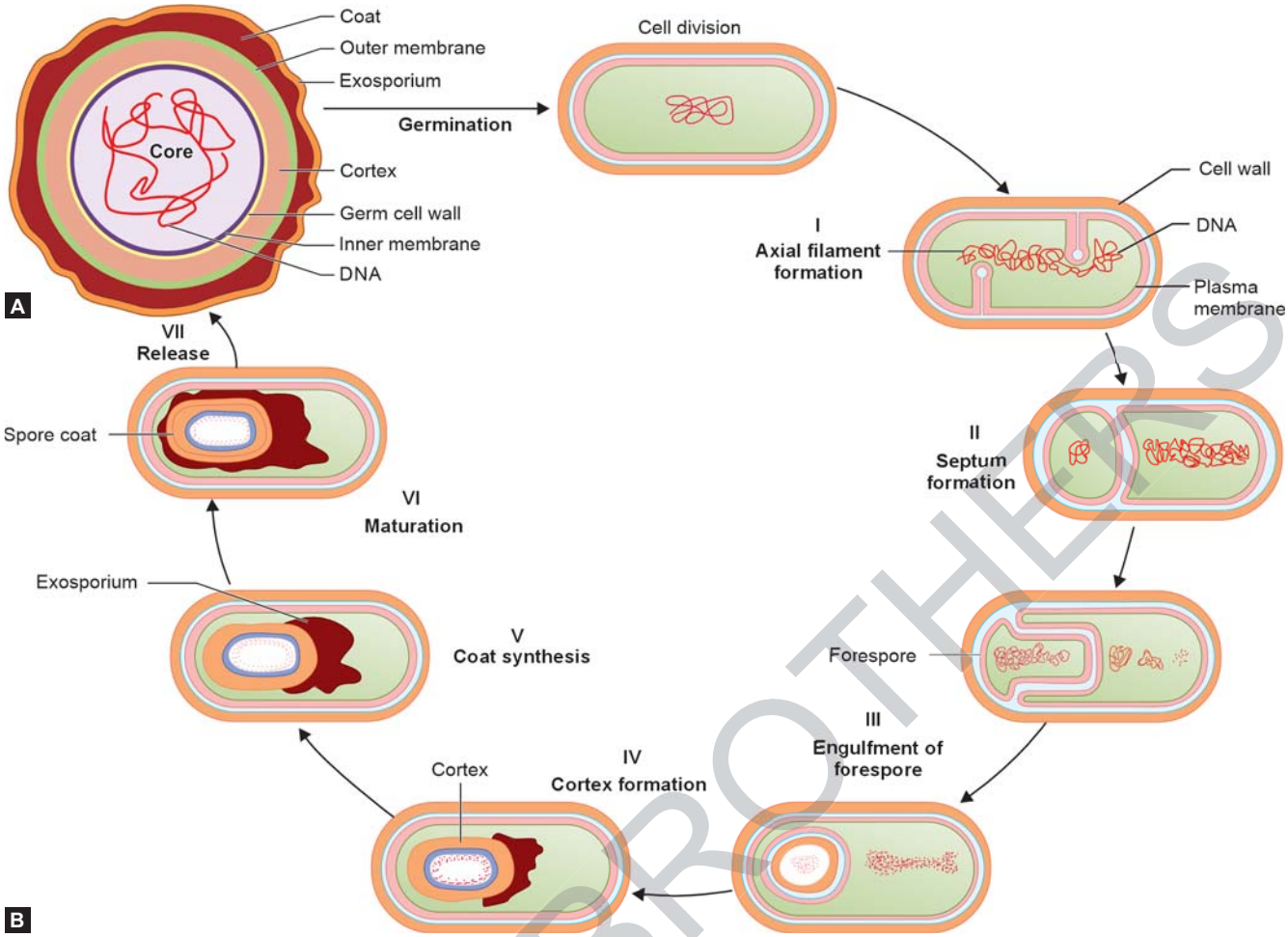
The DNA present in core is protected by two main mechanisms mediated by Ca-DPA and SASP.

- **Ca-DPA:** The dipicolinic acid calcium ion (Ca-DPA) complexes are inserted between the nitrogenous bases of DNA, which helps stabilize it.
- **SASP:** The small, acid-soluble DNA binding proteins (SASPs) saturate endospore DNA and alters the 3D structure of DNA, converting it from the common B form to A form.
- **Conversion of DNA from B form to A form:** In vegetative cells, DNA typically exists in the B form, which is a right-handed double helix with a hydrated structure.
  - In spores, SASPs induce a transition to the A form, which is also a right-handed helix but is more compact and less hydrated.
  - The A form is more resistant to damage, including UV radiation, desiccation, and enzymatic cleavage.
  - Upon germination, SASPs are degraded by specific proteases, allowing the DNA to revert to the B form, which is suitable for transcription and replication in the active vegetative state.
- **Efficient DNA repair systems:** Spores have robust repair mechanisms, such as nucleotide excision repair, which become active during germination to fix any radiation-induced damage.

Sporulation[1]

Sporulation (or sporogenesis) refers to the process of formation of spores from vegetative stage of bacteria. It is not a method of reproduction because the bacteria do not divide during sporulation. Sporulation commences when growth ceases due to lack of nutrients. It is a complex process, takes about 10 hours which may be divided into seven stages **(Fig. 4.29B)**.

- **Stage 0 (Initiation):** Sporulation begins in response to environmental stress, such as nutrient deprivation or harsh conditions. The Spo0A transcription factor becomes activated and initiates the process.
- **Stage I (Axial filament formation):** Bacterial cell division occurs and the two copies of DNA are organized along the long axis of the cell, forming an axial filament.
- **Stage II (Septum formation):** Cell membrane is folded inwards to enclose part of the DNA and to produce the forespore asymmetric septum, dividing the cell into two compartments:
  - Forespore: The smaller compartment, which will become the endospore.
  - Mother cell: The larger compartment, which nurtures the forespore.
  - DNA is translocated into the forespore via the SpoIIIE protein.
- **Stage III (Engulfment of forespore):** The membrane continues to grow and engulfs the immature spore in a



Figs. 4.29A and B: (A) Structure of bacterial spore; (B) Steps of sporulation.[1]

second membrane. The mother cell engulfs the forespore in a phagocytosis-like process, resulting in the forespore being entirely encased within the mother cell.

- ❖ **Stage IV (Cortex formation):** The cortex synthesis occurs in the space between the two membranes surrounding the forespore. This layer is critical for maintaining spore dehydration and dormancy.
- ❖ **Stage V (Coat formation):** This stage is characterized by formation of protein coat and exosporium around the cortex. The spore coat forms outside the cortex is a multilayered proteinaceous structure provides resistance to chemicals, enzymes, and radiation.
- ❖ **Stage VI (Maturation):** The spore matures, gaining heat resistance, refractility and other protective properties. The dipicolinic acid (DPA) and calcium are deposited within the core to stabilize DNA and maintain dormancy.
- ❖ **Stage VII (Release):** Finally, lytic enzymes destroy the sporangium, releasing the mature endospore into the environment. The endospore can remain dormant for long periods, awaiting favorable conditions to germinate.

Germination[1]

It is the transformation of dormant spores into active vegetative cells when grown in a nutrient-rich medium. It comprises of three stages.

1. **Activation:** It is a reversible process that prepares spores for germination and usually results from treatments like heating.
2. **Germination:** It is the process of breaking of the spore's dormant state, which begins when proteins called **germinant receptors**, located in the inner membrane and the cortex, detect small molecules such as sugars and amino acids. Upon detection of these molecules by the germinant receptors, a series of events occur.
   - These activated receptors trigger the release of the Ca-DPA complexes, breakdown of the peptidoglycan in the cortex, and water uptake.
   - Eventually water levels inside the germinating endospore reach those characteristic of vegetative cells and enzymes in the core become active.
   - This allows the endospore to begin synthesizing various molecules needed to initiate endospore outgrowth and return to a vegetative state.
   - This is associated with rupture or absorption of the spore coat, loss of resistance to heat and other stresses, loss of refractility, release of spore components, and increase in metabolic activity.
3. **Outgrowth:** The spore protoplast emerges from the remains of the spore coat and develops into an active bacterium.

Shape and Position of Spores[39]

For a given species, the precise position, shape and relative size of the spore are constant.

- **Position:** Spores may be central, subterminal or terminal **(Figs. 4.30A to F)**.
- **Shape:** They may be oval or spherical in shape.
- **Width:** The diameter of spore may be same or less than the width of bacteria (non-bulging spore, e.g. as in *Bacillus*), or may be wider than the bacillary body producing a distension or bulge in the cell (bulging spore, e.g. as in *Clostridium*).

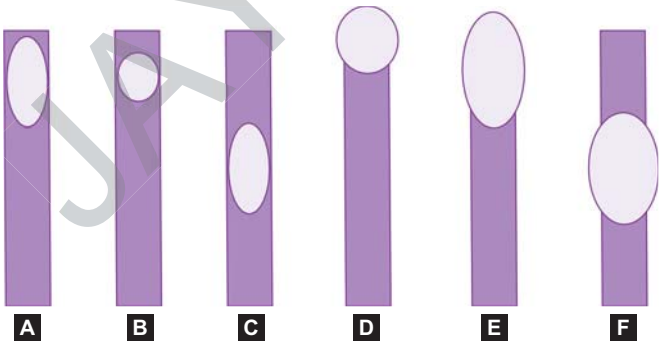
Sporicidal Agents

Spores are resistant to most of the routinely used disinfectants. Only limited sterilization methods are available to kill the spores—both physical and chemical methods.

Chemical Sporicidal Agents[39]

The following are the chemical agents to kill the spores.

- **Glutaraldehyde:** It is sporicidal at 2% concentration, but it typically requires prolonged exposure (10–14h) to achieve sporicidal activity. It is commonly employed in sterilizing medical equipment.
- **Peracetic acid:** It is highly effective against spores at low concentrations. It is used in sterilization of surgical instruments and endoscopes. It is used at 0.1–0.2%, for which a contact time of 5–15 minutes is sufficient for achieving sporicidal action.
- **Hydrogen peroxide:** Liquid H_2O_2 is sporicidal at 6–7% concentration for a contact time of 2–6 hours. It is effective for disinfecting surfaces and instruments. It is the agent used in plasma sterilization.
  - At 10–30% of liquid H_2O_2, the sporicidal action is achieved faster after a contact time of 30 minutes to 1 hour. It is common in industrial sterilization processes.
  - Vaporized H_2O_2 (VHP) requires a contact time of 10–60 minutes for sporicidal action. It is used for room or equipment decontamination. It is highly effective due to its penetration and uniform distribution.
- **Chlorine compounds:** It includes sodium hypochlorite (household bleach) and chlorine dioxide. It is effective at concentrations 5,000–6,000 ppm (0.5–0.6%) for contact time of 10–30 minutes.



Figs. 4.30A to F: Position and shape of spores: (A) Non-bulging, oval and terminal; (B) Non-bulging, round, and subterminal; (C) Non-bulging, oval and central; (D) Bulging, round and terminal; (E) Bulging, oval and terminal; (F) Bulging, oval, and central.

- **Ethylene oxide (EO) gas:** It is used for sterilizing heat-sensitive medical equipment. It is highly effective but requires careful handling due to toxicity and flammability. At ETO concentration of 700 mg/liter and 40–80% relative humidity, sterilization is achieved in 4–5 hours at 38°C or 1 hour at 55°C.
- **Formaldehyde:** It is used as a gas or solution. It is effective but has significant health risks and regulatory restrictions.
- **Ozone:** It is a gas that effectively destroys spores through strong oxidation. It is used in water treatment and specialized disinfection systems.

Physical Sporicidal Agents[39]

The following are the physical agents to kill the spores.

- **Autoclaving (steam sterilization):** 121°C for 15–20 minutes under pressure effectively kills spores.
- **Dry heat (hot air oven):** It requires higher temperatures (e.g. 170°C for 60 minutes, 160°C for 120 minutes, and 150°C for 150 minutes).
- **Gamma irradiation:** It is effective for sterilizing disposable medical supplies.

Demonstration of Spores

Demonstrating the presence of bacterial spores in a sample includes staining techniques, microscopy, and various other methods.

Staining Techniques[1]

Various staining techniques are available for the demonstration of spores. Some of the common techniques that are used are mentioned below and the detailed description of these techniques are discussed in Chapter 16.

- Dorner method
- Schaeffer-Fulton method
- Moeller staining
- Gram staining
- Modified Ziehl–Neelsen staining (0.25% sulfuric acid as decolorizer)

Microscopy[1]

Apart from bright field microscopy (staining techniques, as discussed above), bacterial spores can directly be visualized by several other microscopes.

- **Phase-contrast microscopy:** Spores can be visualized without staining due to their high refractive index, appearing bright against a darker background.
- **Fluorescence microscopy:** Stains such as acridine orange or SYTO dyes can bind to spores and fluoresce under specific wavelengths, aiding in detection.
- **Electron microscopy:** It provides high-resolution images of spores. It is useful for detailed structural studies, particularly for research purposes.

Other Methods[1]

In addition to microscopy and staining methods, several other techniques are available to demonstrate bacterial spores.

- **Turbidity and viability assay:** After heat shock, the growth of spores over time can be monitored using spectrophotometry (turbidity) or colony-forming unit (CFU) counts.
- **Dipicolinic acid assay:** DPA in spore core can be extracted and quantified using chemical or spectroscopic methods.
- **PCR-based methods:** They target specific spore-related genes (e.g. *spo* genes) to confirm the presence of spore-forming bacteria.
- **Heat shock treatment:** The heat resistance of spores to differentiate them from vegetative cells. Sample is heated at 80°C for 10–15 minutes, plated on a suitable growth medium and incubated and observed for spore-forming colonies.
- **Alcohol treatment:** Similar to heat shock, exposing samples to ethanol can inactivate vegetative cells while leaving spores viable for culture.
- **Popping method:** It is a non-staining technique that is used for the visualization of bacterial spores that is based on the production of a visible phenomenon occurring in dormant spores. A detailed description of this method is described in Chapter 16.

Applications of Spores[39]

Bacterial spores serve several important applications due to their exceptional resistance and stability under extreme conditions.

- **Sterilization control:** Spores of certain bacteria are employed as indicators for proper sterilization. Absence of the spores after autoclaving or processing in hot air oven indicates proper sterilization.
  - Spores of *Geobacillus stearothermophilus* are used as sterilization control for steam sterilizer and gas plasma (hydrogen peroxide) and liquid acetic acid sterilizer.
  - Spores of *Bacillus atrophaeus* are used as sterilization control for ethylene oxide sterilizer and dry heat sterilizer (hot air oven).
  - Spores of *Bacillus pumilus* are used as sterilization control for ionizing radiation.
- **Bioterrorism:** Spores have also been used as agents of bioterrorism, e.g. endospores of *Bacillus anthracis* were used in the 2001 anthrax bioterrorism attack.
- **Industrial microbiology:** Spores of *Bacillus subtilis, Bacillus licheniformis*, and *Bacillus amyloliquefaciens* are exploited in industrial fermentation and enzyme production (e.g., amylases, proteases) due to their stability and ease of storage.
- **Agriculture and biocontrol:** Spores of *Bacillus thuringiensis* (biopesticide against insect larvae), *Bacillus subtilis*, and *Bacillus megaterium* are used as biopesticides and biofertilizers, owing to their environmental persistence and safety.
- **Research and genetics:** Spores of *Bacillus subtilis* are widely used as model systems to study sporulation, dormancy, resistance, germination, and gene regulation under stress conditions.
- **Probiotics:** Spore-forming bacteria such as *Bacillus clausii*, *Bacillus coagulans*, and *Bacillus subtilis* are used as probiotics, as their spores survive gastric acidity and germinate in the intestine.
- **Environmental and biodefense studies:** Spores of *Bacillus anthracis* (research models, not routine use) and *Bacillus subtilis* are used to study extreme resistance, environmental persistence, aerosol survival, and biodefense preparedness.

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Dr Apurba Sastry and his team have also authored several other popular books which are extremely appreciated among faculty and students:

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