
# MICROBIOLOGY

ED.08

# MICROSCOPES

----- Active space -----

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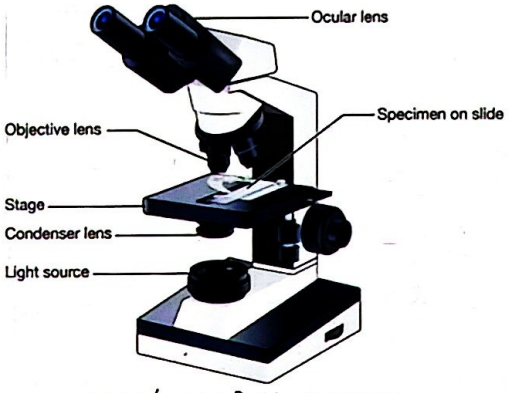
## Types and Features

00:00:15

Resolution
- Naked eye : 0.2mm
- Light microscope : 0.2µm
- Electron microscope : 0.2 nm




Light/bright field microscope

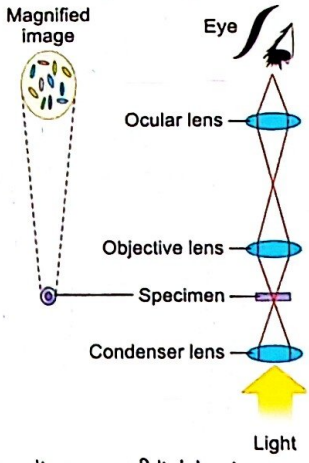
### LIGHT/BRIGHT FIELD MICROSCOPE

#### General features :

AKA : Compound microscope.

Components :
- Light source : Visible light (Wavelength : 550nm).
- Objective lens. } Magnification.
- Ocular lens. }

#### Resolution of light microscope :
- Resolution : 0.2µm.
- Depends on
  - Wavelength of light.
  - Numerical aperture of objective lens.




Ray diagram of light microscope

#### Magnification of light microscope :

| Lens | Magnification of objective lens | Magnification of ocular lens | Final magnification |
|---|---|---|---|
| Scanner lens | 4x | 10x | 40x |
| Low dry lens | 10x | | 100x |
| High dry lens | 40x | | 400x |
| Oil immersion lens | 100x | | 1000x |

### DARK FIELD MICROSCOPE

#### General features :

Advantages over light microscope :

1. Unstained/transparent specimen : Live organism.
2. Slender bacteria visible.

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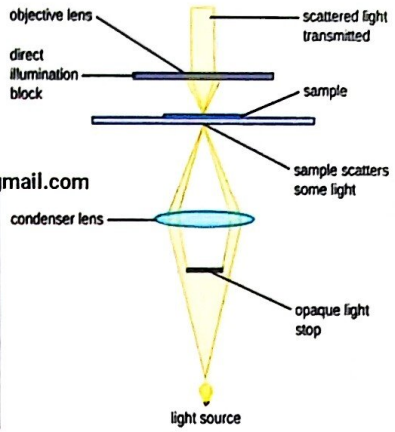
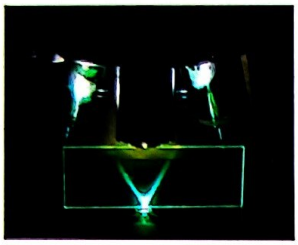
Modification :

Condenser lens has central opaque light stop :

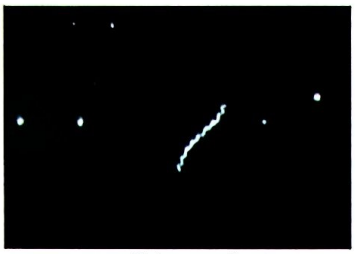
- Illuminates specimen with a hollow cone of light.
- Organism brighty lit against bright background.



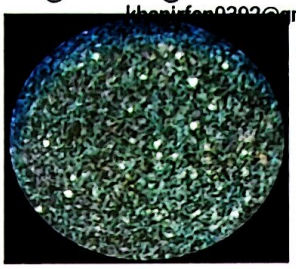

Dark field microscope



Hollow cone of light



T.pallidum under dark field microscope



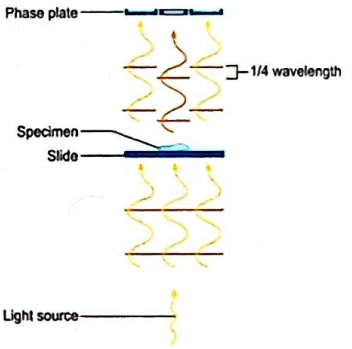
Leptospira

## PHASE CONTRAST MICROSCOPE

General features :

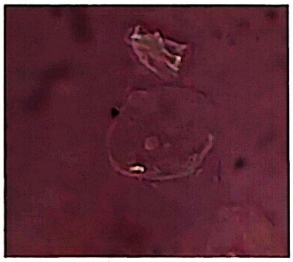
Advantages over light microscope :

1. Unstained/transparent specimen : Live organisms.
2. 3D effect and better contrast of internal structures.

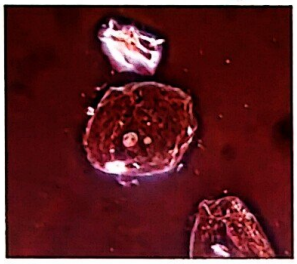



Ray diagram

Principle : Difference in amplitude of light → Differing light intensities.



Light microscope



Phase contrast microscope

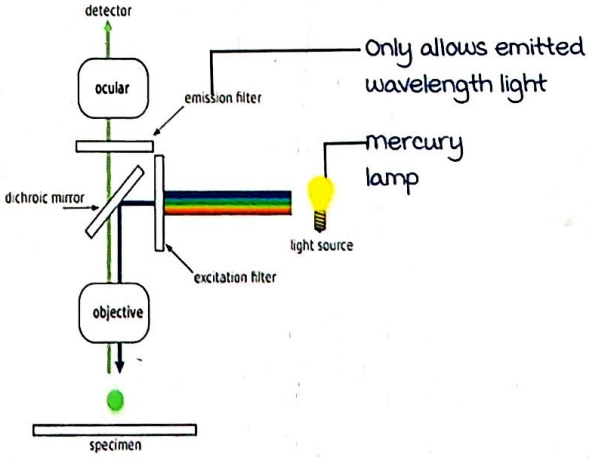
## FLUORESCENT MICROSCOPES

Fluorophores :

- Excitation wavelength : Short wavelength (UV rays).
- Emitted wavelength : Longer wavelength (Visible light).

Examples :

- Calcofluor white : Binds to chitin (Fungal cell wall).
- Acridine orange : Binds to DNA.




Ray diagram of fluorescent microscope



Feedback

- Auramine O
- Rhodamine B

} Binds mycolic acid (mycobacterial cell wall)

- Fluoresceine isothiocyanate
- Lissamine rhodamine

} Binds antibodies ($F_c$)

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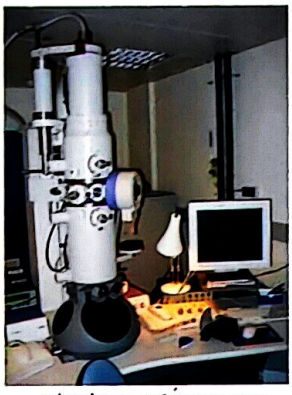
## ELECTRON MICROSCOPE

Introduced by Knoll and Ruska.

**Components :**

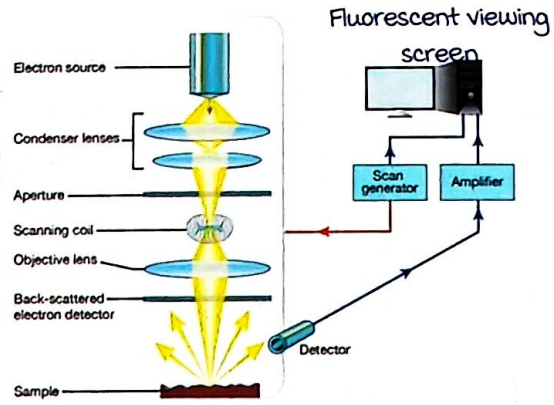
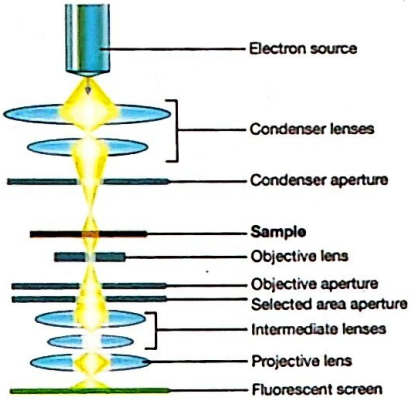
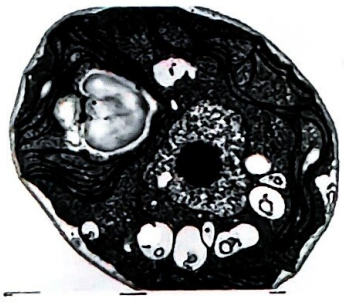
- Beam of electrons used (Wavelength : 0.005 nm).
- Only dead organisms visualised.
- Condensed lens
- Objective lens
- Projector lens

} Magnetic lens



Electron microscope

**Types :**

| | Scanning electron microscope | Transmission electron microscope |
|---|---|---|
| Stain | Vapourised gold/palladium → Secondary electrons released | Uranyl acetyl/Osmium tetroxide |
| mechanism | Secondary electrons create image | Variation in energy of transmitted electrons create image |
| Uses | Study of topography of microorganisms | Study internal structures of organisms |
| microscope |   |   |
| Image seen |  |  |

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# BACTERIAL STAINS

**Reason for staining bacteria :**

- Bacteria are colourless.
- Too small to be visualized.
- To provide contrast for better visualization.

**Types of stains :**

- Simple stains :
  - Single dye is used for staining → Every structure is stained uniformly.
  - Examples :
    a. Methylene blue : Structures appear blue.
    b. Basic fuchsin : Structures appear pink.
- Differential stains.
- Negative stains.
- Impregnation stains.
- Special stains.

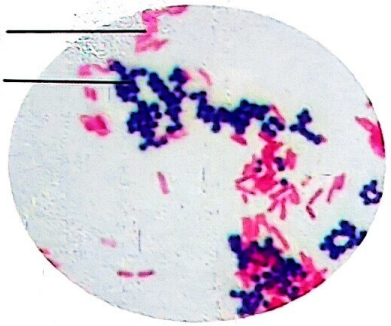


## Differential Stains

00:01:40

### I. GRAM STAIN

- m/c used stain in bacteriology.
- Introduced by : Hans Christian Gram.



Gram staining

Steps :

1. Primary stain : Crystal violet/methyl violet/gentian violet.
2. Mordant : Iodine.
   - Fixes primary stain to the structures.
3. Decolouriser : Alcohol/acetone/alcohol-acetone mixture.
   - Decolourises certain bacteria which take up primary stain.
4. Counterstain : Neutral red/carbol fuchsin/safranine (m/c used).
   - Counterstains bacteria that had undergone decolourisation.

Effects :

| | Gram positive | Gram negative |
|---|---|---|
| Primary stain | Purple | Purple |
| Mordant | Purple | Purple |
| Decolouriser | Purple | Colourless |
| Counterstain | Purple | Pink |

Note : Smear prepared is fixed by heating (Kill all bacteria).



Feedback

Principle :

----- Active space -----

1. Gram positive cytoplasm is more acidic → Better binding to primary basic stains → Slower decolourisation.
2. Gram positive cell wall is thicker → Better retaining of primary stain → Slower decolourisation.
3. Gram negative cell wall : Lipid composition is more → More pore formation while adding acetone/alcohol → Rapid decolourisation.

Exceptions :

| Bacteria | Reason for not taking Gram's stain |
|---|---|
| Mycoplasma | Small in size |
| Chlamydia<br>Rickettsia | Small in size & obligate intracellular organism |
| Mycobacteria | Lipid rich cell wall (Reagents cannot penetrate) |
| Spirochetes | Too slender |



Note : Cytoplasm is stained (Not the cell wall).

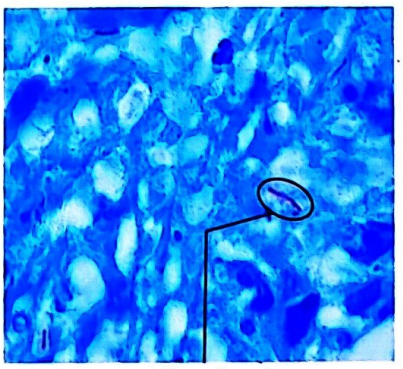
## 2. ACID FAST STAINS

### ZN staining :

- Introduced by : Paul Ehrlich (Father of chemotherapy).
- Modified by : Ziehl and Neelson.
- Used to demonstrate Mycobacterium.

Steps :

1. Primary stain : Carbol fuchsin (Basic fuchsin dissolved in phenol).
   - Heat for 3-5 minutes : Helps carbol fuchsin to enter through cell wall to stain cytoplasm.
2. Decolouriser : 20% $H_2SO_4$.
   - Retain primary stain : Acid fast.
   - Do not retain primary stain : Non acid fast.
3. Counterstain : Methylene blue/malachite green/ picric acid.
   - To demonstrate non acid fast structure.



Acid fast bacilli
(mycobacterium tuberculosis)

----- Active space -----

Effects :

| | Acid fast | Non acid fast |
|---|---|---|
| Primary stain | Pink | Pink |
| Decolouriser | Pink | Colourless |
| Counterstain | Pink | Blue/green/yellow (Based on counterstain) |

### 3. Kinyoun stain :

- AKA cold stain/Gabbet stain.
- Modification of Ziehl Neelson stain: No heating post primary stain.
  - ↑ concentration of phenol in carbol fuchsin
  - ↑ time of exposure to carbol fuchsin

} Counter omission of heating.

### Acid fast v/s partially acid fast :

| | Acid fast | Partially acid fast |
|---|---|---|
| Features | Acid fast with 20% $H_2SO_4$ | Retain primary stain with lower concentration of $H_2SO_4$ |
| Example | • Mycobacterium tuberculosis complex :<br>– Mycobacterium tuberculosis<br>– Mycobacterium bovis<br>– Mycobacterium africanum<br>– Mycobacterium caprae<br>• Atypical mycobacteria | 5% $H_2SO_4$ :<br>• Mycobacterium leprae<br>• Oocyst of cystoisospora, cyclospora & cryptosporidium (Protozoans causing diarrhoea in HIV positive pts.) |
| | | 0.5–1% $H_2SO_4$ :<br>• Nocardia • Head of sperm<br>• Rhodococcus • Legionella micdadei |
| | | 0.25%–0.5% $H_2SO_4$ :<br>• Bacterial spores |

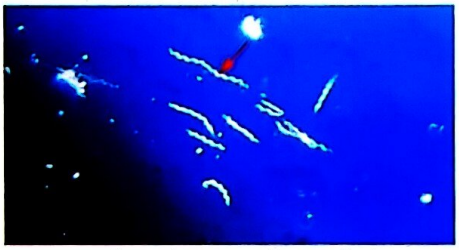
## Negative Stain

00:18:15

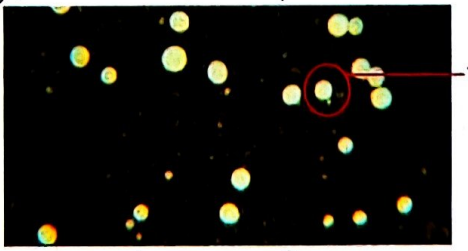
Outlining the structures against the dark background of India ink/nigrosine.

Uses :

- Demonstrate capsules of organism.
- Demonstrate very slender bacteria : Spirochetes.



Negative staining of T. pallidum.




Cryptococcus neoformans



Feedback

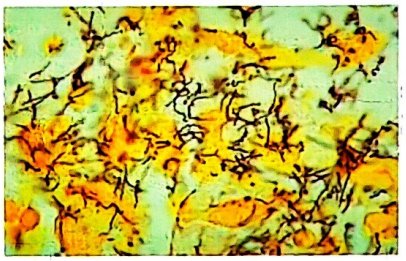
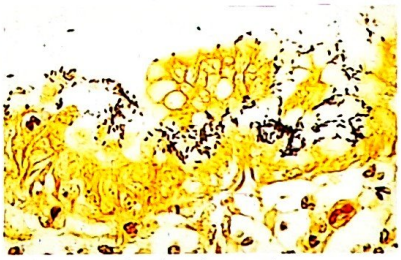
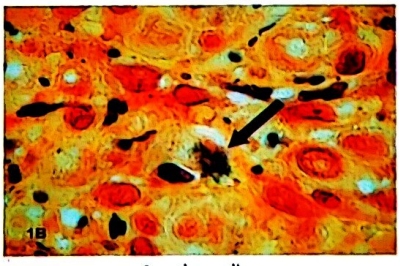
## Impregnation Staining

00:19:30 ----- Active space -----

Principle : Impregnating organism → Organism become thick enough to be visualized by light microscope.

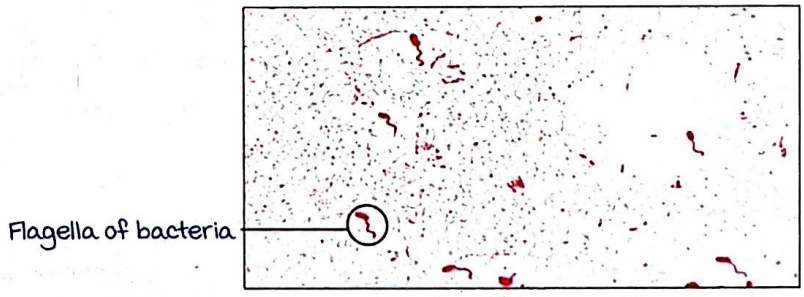
### 1. Silver stain :

Use :

| Organism | Stains used | Image |
|---|---|---|
| Spirochetes | • Levaditi stain (Tissue)<br>• Fontana stain (Fluid) |  Spirochetes |
| Helicobacter pylori | Warthin starry silver stain (Gastric biopsy) |  Helicobacter pylori |
| Bartonella | Warthin starry silver stain (Lymph node/vascular lesion biopsy) |  Bartonella |

### 2. Tannic acid :

- Stain : Leifson and Ryu impregnation stain.
- Use : Demonstrate flagella of bacteria.



Tannic acid impregnation

Feedback

----- Active space -----

## Special Stains

00:22:16

### metachromatic granules :

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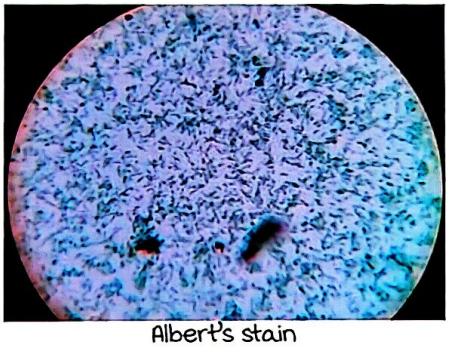
- AKA volutin granules/Babes Ernst granules/polar bodies.
- Act as energy stores.
- metachromasia : Organisms take up colour other than colour of stain used.
  - For eg : Purple red colour when stained with toluidine blue.

Seen in :

- Corynebacterium.
- Yersinia pestis.
- Bordetella pertussis.
- Gardnerella vaginalis.
- Certain mycobacterium species.



Albert's stain

Stains used :

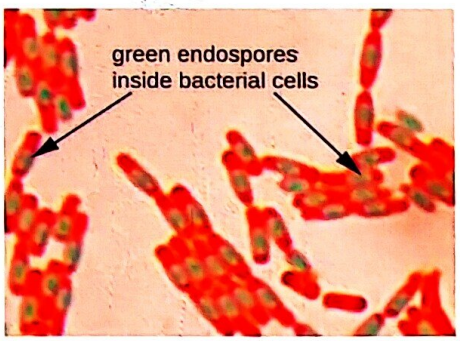
- Albert's stain : Toluidine blue + malachite green + iodine.
- Neisser's stain.
- Ponder's stain.

### Spores :

Do not take up gram stain (D/t thick cell wall) : Appear as empty spaces.

Seen in :

- Bacillus.
- Clostridium.




Ashby staining

Stain used :

- Ashby staining : Malachite green & safranine are dyes used.
- Schaeffer-Fulton stain (Modification of Ashby stain).

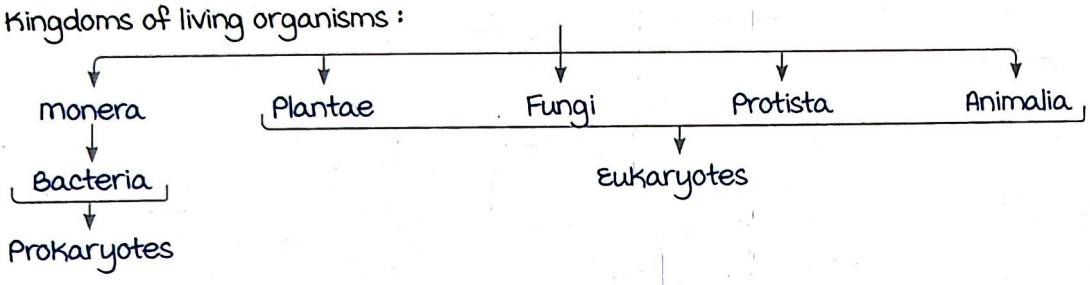
### Lipid/polysaccharide granules :

| | Lipid granule | Polysaccharide granule |
|---|---|---|
| Stain used | Sudan black B | Iodine |

# BACTERIAL ANATOMY AND TYPING



Kingdoms of living organisms :




## Prokaryotes

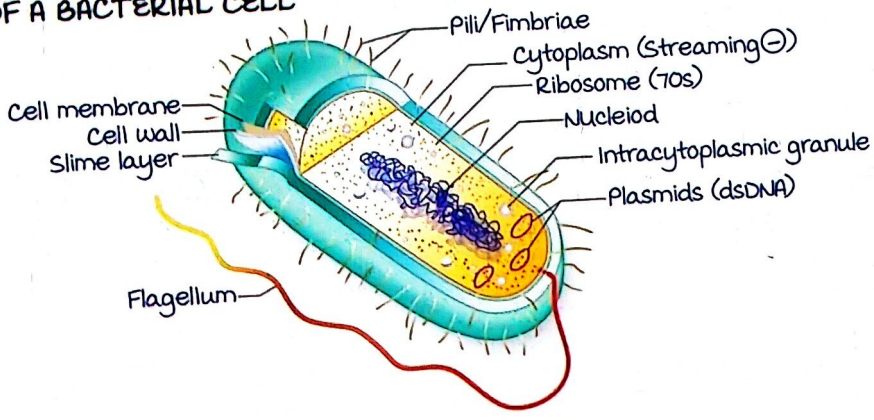
00:02:12

### PROKARYOTES VS. EUKARYOTES

| Parameter | Prokaryotes | Eukaryotes |
|---|---|---|
| 1. Nuclear membrane, nucleolus and histone proteins | ⊖ | ⊕ |
| 2. Cytoplasmic membrane-bound organelles (mitochondria, golgi body, lysosomes, endoplasmic reticulum). | ⊖ | ⊕ |
| 3. Chromosome | Single, circular (dsDNA) contained within nucleoid | multiple, linear. |
| 4. Muramic acid in cell wall | ⊕ | ⊖ |
| 5. Sterols in cell membranes | ⊖ | ⊕ |
| 6. Ribosomes | 70S → 30S, 50S | 80S → 40S, 60S |
| 7. Extrachromosomal DNA | Plasmids | mitochondria |



### STRUCTURE OF A BACTERIAL CELL



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**Exceptions :**

1. Mycoplasma :
   - Only prokaryote → Lacking a cell wall.
     → Containing sterols in cell membrane.
2. Chlamydia : Only prokaryote lacking muramic acid in cell wall.
3. Vibrio : Contains 2 chromosomes.

## Glycocalyx

00:11:30

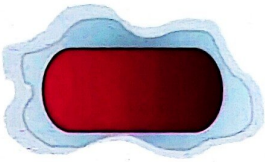
may be present/absent.

**TYPES**

**a. Slime layer :**

Loose, ill-defined, polysaccharide layer all around the cell wall.

Significance : Biofilm formation : → Antiphagocytic
→ Adhesion
→ ↓ Antibiotic activity (mechanical barrier).



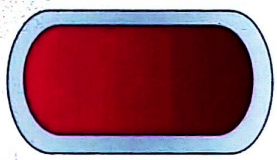
Slime layer

Examples :

- P. aeruginosa
- Streptococcus mutans (m/c cause of dental caries).
- Staphylococcus epidermidis.

**b. Capsule :**

- Well demarcated layer around cell wall.
- Composed of polysaccharides.
  - Exception : B. anthracis → polypeptide capsule (Polymer of D-glutamic acid).



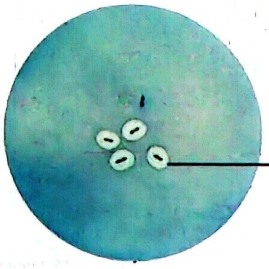
Capsule

Significance :

- Antiphagocytic : most important virulence factor.
- Protection from phages (Viruses that infect bacteria).
- Antigen : Induces antibody formation.

Gram stain : Not done (No net change).

Quellung/Neufeld reaction :

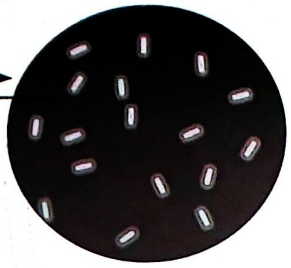


Capsule + specific anticapsular antibodies
↓
Swelling of capsule

Capsule demonstration :

Negative staining (India ink/Nigrosine).

Halo around bacteria





Feedback

----- Active space -----

Capsulated organisms :
Yersinia pestis
Streptococcus pneumoniae
Bacteroides fragilis, Bordatella pertussis.
H. influenzae.
Klebsiella.
Bacilllus anthracis.
meningococcus.
Clostridium perfringens.

Mnemonic :
Yes
Some
Bacteria
Have
Killer
And
Mean
Capsules

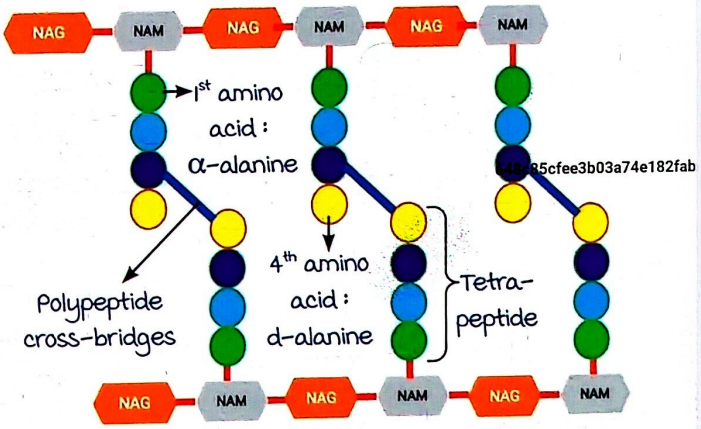
Note :
1. Negative staining also used for the demonstration of spirochetes.
2. Only capsulated fungus : Cryptococcus.

## Cell Wall

00:19:24

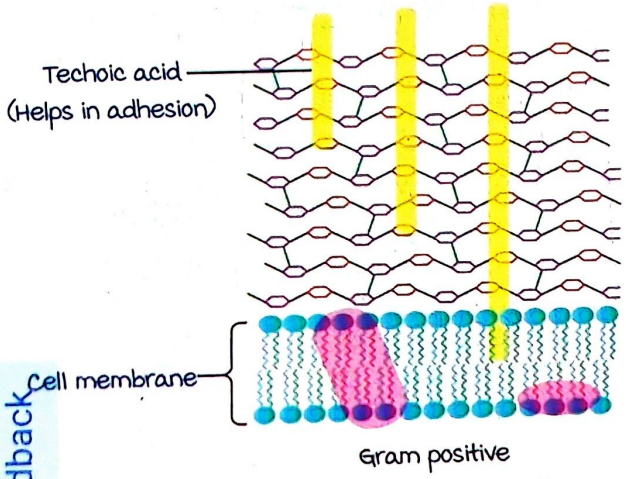
Function : Provides shape and rigidity.
Composition : Peptidoglycan.
↓
murein backbone (Carbohydrate) linked by peptides.
made up of 2 amino acids :
1. N-acetyl muramic acid (NAM)
2. N-acetyl glucosamine (NAG)

Cross linking of cell wall : mediated by
Transpeptidase Transglycolase

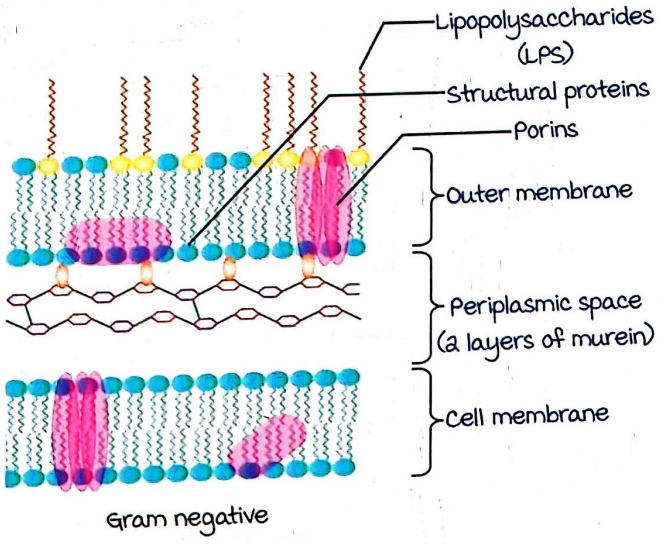


Structure of a cell wall

### GRAM POSITIVE VS. GRAM NEGATIVE



Gram positive



Gram negative

| | Gram positive | Gram negative |
|---|---|---|
| 1. Thickness | 25-80mm | 10mm |
| 2. murein layers | 50-100 layers, peptide cross linking ⊕ | 2 layers, peptide crosslinking ⊕ |
| 3. Amino acids (AA) | Aromatic & sulfur containing AA ⊖ | All types of AA ⊕ |
| 4. Techoic acids (TA) | 2 types ⊕:<br>• Lipo TA<br>• Cell wall TA | ⊖ |
| 5. Outer membrane | ⊖ | ⊕ |
| 6. Porins | | |
| 7. Periplasmic space | | |
| 8. Endotoxins (LPS) | | |

## Antibiotics acting on the cell wall :

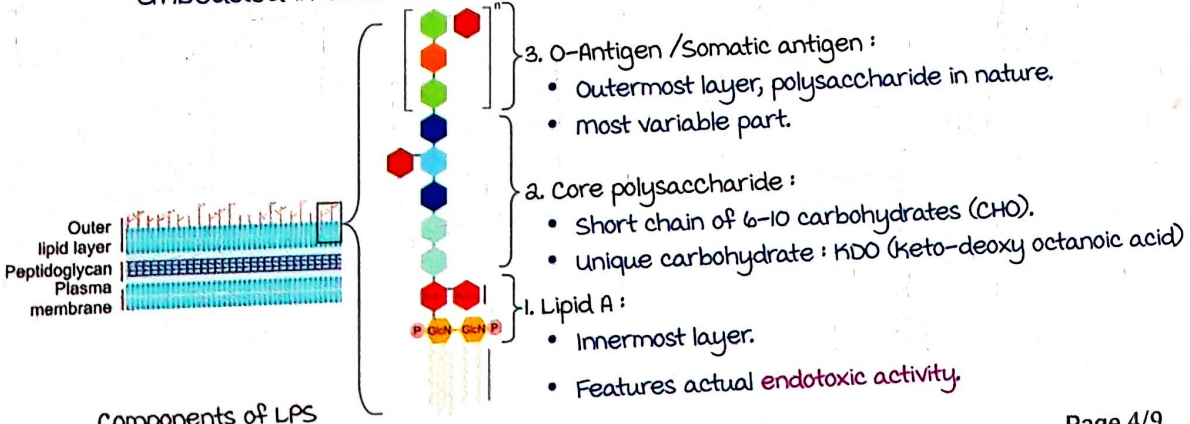
| Antibiotic | β-lactams | Vancomycin |
|---|---|---|
| mechanism | Bind to transpeptidase (AKA penicillin binding protein : PBP).<br>↓<br>Inhibit crosslinking ⟶ Lysis | Bind to D-ala D-ala moeities (cell wall precursor molecules).<br>↓<br>Disrupts action of transpeptidase |

## Demonstration of cell wall :

1. Electron microscope.
2. microdissection.
3. Plasmolysis : Bacteria placed in hypertonic solution ⟶ Water moves out, only cell wall remains.

## Endotoxin/Lipopolysaccharides (LPS) :

- Embedded in outer leaflet of outer membrane of gram negative bacteria.




Components of LPS

mechanism of action :

----- Active space -----

Lysis of bacteria
↓
Release of Lipid A

a. Recognised by TLR-4 (Toll-like receptor-4) present on macrophages & dendritic cells.
↓
Activation of NF-KB (Nuclear transcription factor Kappa B).
↓
Cytokine release
IL-1, IL-6, TNF-α

b. Activation of alternate complement pathway.

c. Activation of tissue factor

↓
Results in :

- Fever.
- Leukopenia, hypotension, intravascular coagulation, ↑ vascular permeability.
- Severe endotoxemia → DIC → Endotoxic shock with multiorgan failure.

Detection of endotoxin :

1. Rabbit pyrogenicity.
2. Limulus amoebocyte lysate (LAL) assay.

Note :

H. influenzae, Neisseria
- Lipo-oligosaccharide ⊕.
- No LPS.

**Endotoxin Vs. Exotoxin :**

| | Endotoxin | Exotoxin |
|---|---|---|
| Nature | LPS | Protein. |
| Site of release | Present in cell wall of gram negative bacteria. | Secreted by either gram positive/negative. |
| mechanism of release | Released only on lysis. | Actively secreted. Exception : Botulinum toxin → released on lysis. |
| Effect of heat | Heat stable. | Heat labile. Exception : S. aureus enterotoxin. |
| Antigenicity | Low antigenicity | Highly antigenic (Protein). Can be used for synthesis of toxoid. |
| Toxicity | Low toxicity | Highly toxic. |
| Effects | Constant effects. | Variable actions. |

Feedback

----- Active space -----

**L-forms :**

- Cell wall deficient bacteria.
- Formed spontaneously or d/t cell wall inhibitors (Eg : penicillin).
- 1st demonstrated on streptobacillus monoliformis.
- Can be formed by any gram negative/positive bacteria.
- Pleomorphic.
- Importance : → Antibiotic resistance
  → Persistance of infections.

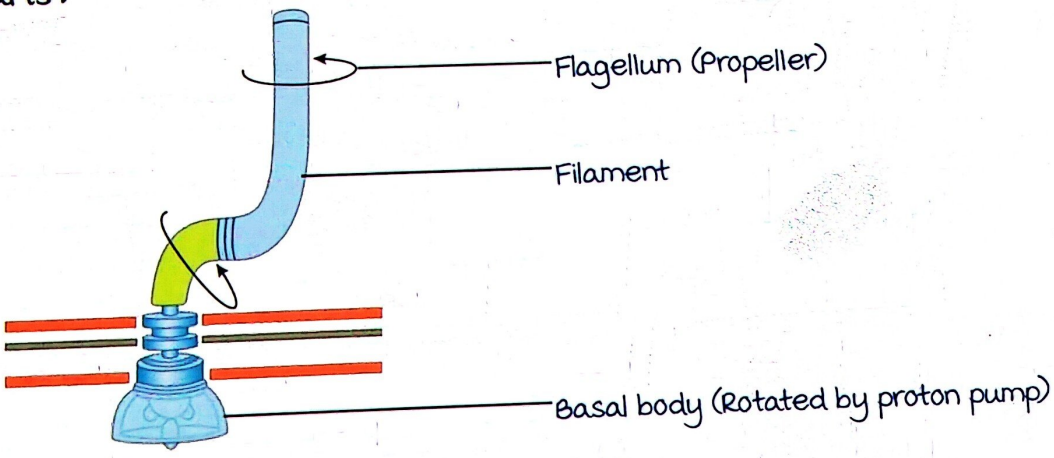
Note :
Listeria → Only gram positive bacteria containing endotoxin.

## Flagella

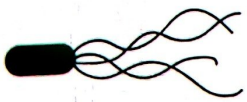
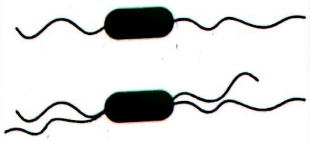
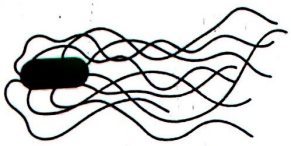
00:48:11

Organs of locomotion, help in motility.
Made up of flagellin protein : Highly antigenic → H. antigens.

**Parts :**




**Distribution :**

| Monotrichous | Lophotrichous | Amphitrichous | Peritrichous |
|---|---|---|---|
|  |  |  |  |
| Vibrio<br>P. aeruginosa | Helicobacter | Campylobacter<br>Spirillum | Enterobactericiae<br>Clostridium<br>Bacillus |

Note :
1) WIDAL test : Detection of 'H' and 'O' antibodies.
2) All pathogenic cocci are atrichous.

## DEMONSTRATION

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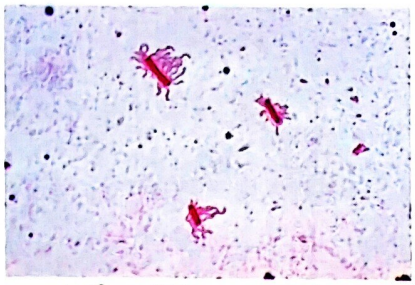
1. Electron microscope.
2. Leifson & Riu impregnation : Using tannic acid.

} Direct methods



Leifson & Riu Impregnation

**Demonstration of motility :** (Indirect methods)

1. Wet film.

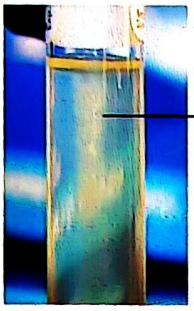
2. Hanging drop preparation.

3. Soft agar :

   a. Motility test agar : Semisolid (0.2–0.5% agar)

   b. U-tube

   c. Craig's tube



Turbidity ⊕ ↓ Indicates motile bacteria

**Types of motility :**

Corkscrew : T. pallidum.
Darting/Shooting star : Vibrio ; Campylobacter.
Gliding : Mycoplasma (Atrichous).
Stately : Clostridium ; Salmonella.
Twitching : Eikenella.
Tumbling/End-on-end : Listeria.

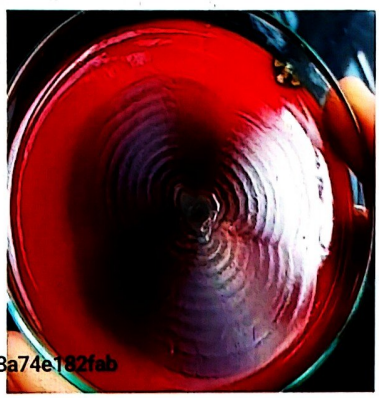
**Swarming :**

Exhibited by :

- Proteus vulgaris.
- Proteus mirabilis.
- Vibrio alginolyticus.
- Vibrio parahemolyticus.
- Clostridium tetani.
- Bacillus cereus.

Inhibition of swarming :

- MacConkey/CLED medium.
- Firm agar (5–6%).



Swarming

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## Other Bacterial Cell Components

01:01:55

### PILI/FIMBRIAE

Made up of Pilin protein.

**Types :**

a. Common pili :

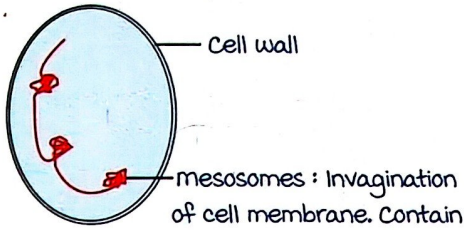
- Helps in adhesion.
- Only present on gram negative bacteria.

b. Sex pili :

- Mediate conjugation.
- Formed by both gram positive/negative bacilli (F-plasmid/'Tra' genes).

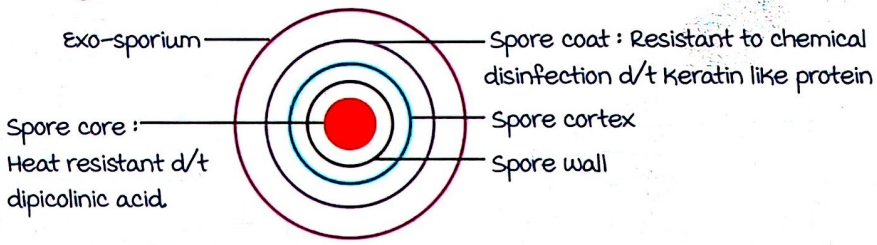
### CELL MEMBRANE

- Phospholipid bilayer.
- Site for synthesis of cell wall precursors.




### SPORES

Formed during unfavourable conditions.




Demonstration : Ashby or Schaffer-Fulton stain.

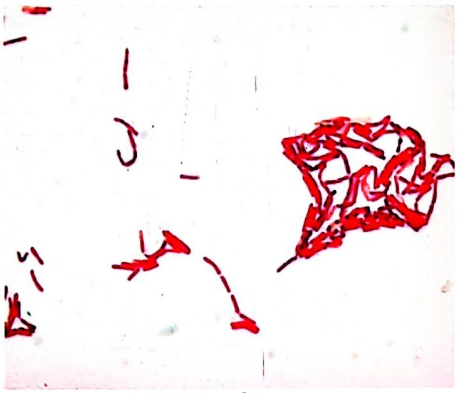
Spore forming bacteria :

a. Bacillus :

- Non-bulging.
- Only form in soil & culture.

b. Clostridium :

- Bulging spores.
- Formed in soil/culture/human body.



Staining of spores

### PLASMID

- Extra-chromosomal dsDNA.
- Not essential for bacterial survival..

- Capable of independent replication.
- All plasmids are vertically transferred.
  - Exception : Horizontally transferred → Plasmids containing 'tra' genes (encode for sex pilus).

**Types :**

| Plasmid | Encodes |
|---|---|
| Virulence plasmid | Virulence factor |
| R plasmid | Antibiotic resistance |
| Col plasmid | Bacteriocin (Antibiotic like proteins that kill related bacteria) |

## BACTERIAL TYPING

Intraspecies strain characterization → done during outbreaks.

**Types :**

### Phenotypic

- Serotyping (m/c) : On the basis of antigens.
- Biotyping : Based on biochemical reaction.
- Phage typing : Based on phage susceptibility.
- Antibiogram typing : Antibiotic susceptibility.
- Bacteriocin susceptibility.
- Multilocus enzyme electrophoresis.

### Genotypic (Preferred)

- Plasmid profile analyzer.
- RFLP (Restriction fragment length polymorphism).
- PFGE (Pulsed field gel electrophoresis).
- Ribotyping.
- Nucleic acid/DNA sequencing : Best method.
  - MLST (multi-locus sequence typing).
    i. Variant of DNA sequencing.
    ii. 7 housekeeping genes conserved.

**Principle of genomic typing :**

Genome extracted —Restriction enzymes→ Smaller fragments —Electrophoresis→ Banding patterns

----- Active space -----

# BIOCHEMICALS AND ANTIBIOTIC SENSITIVITY TESTS

## Biochemical Reactions

00:00:28

### Catalase test :

- To detect catalase enzyme.
- Bacterial suspension + $H_2O_2 \xrightarrow{\text{catalase}} H_2O + O_2$
- Catalase +ve : most pathogenic bacteria.

Except :

- All anaerobes.
- Streptococcaceae.
- Shigella dysentery type I.

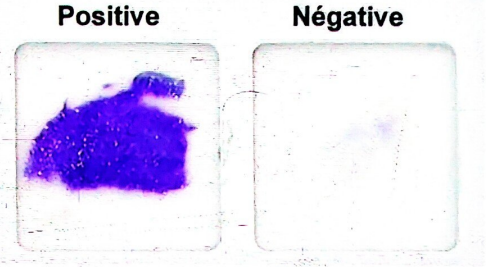
Catalase test +ve

### Oxidase test :

- To detect cytochrome c oxidase in bacteria.
- Filter paper strip impregnated with Kovac's reagent.
- Oxidase +ve : All pathogenic bacteria.

Except :

- Corynebacterium.
- Enterobacteraceae.
- Staphylococci.
- Streptococci.




Oxidase test

### Glucose utilization test :

Detect glucose utilization.

Medium : Hugh Leifson oxidative-fermentative medium (Contains glucose).

Principle :

- Glucose utilization → Produced acid : Turns yellow.
- Available as pair of test tubes — Aerobic / Anaerobic.



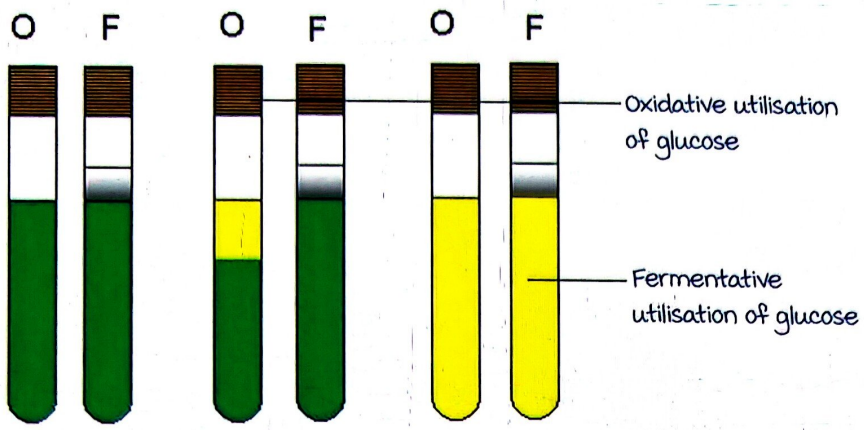
Inference :

- Color change :
  - In only aerobic test tube : Oxidative utilization of glucose.
  - In both aerobic and anaerobic test tubes : Fermentative utilization of glucose.
- Examples :
  - Facultative anaerobes : Ferment sugars.
    E.g. : Enterobacteriaceae, Staphylococci, Streptococci.

----- Active space -----

- Strict aerobes : Oxidise sugars.

  E.g : Pseudomonas, Brucella, Bordetella, Nocardia.



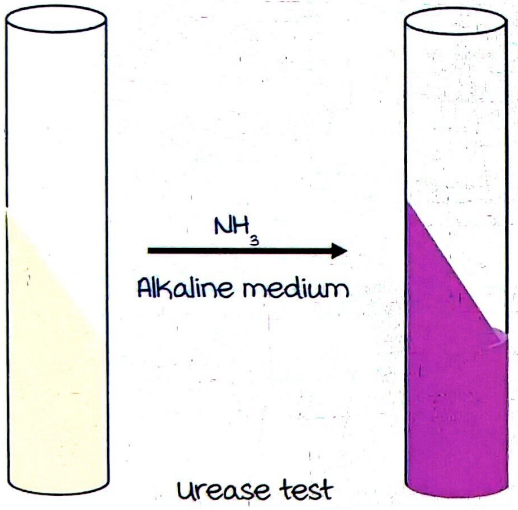

Glucose utilisation

**Urease test :**

Procedure :

Bacteria produce urease .

Urea $\xrightarrow{\text{Uricase}}$ $NH_3 + CO_2$
(Produced by bacteria)

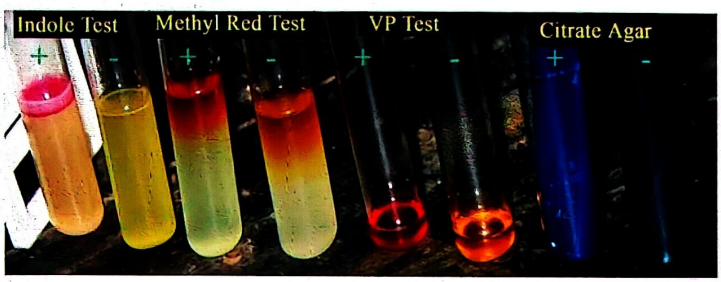



Urease test

**IMViC test :**

Indole test :

- Tryptophan $\xrightarrow{\text{Bacteria}}$ Indole.






Medium : Christensen's medium.

Urease positive organisms. :

mnemonic : PUNCH MSKB

- Proteus.
- Ureaplasma.
- Nocardia.
- Cryptococcus.
- H. pylori.
- Morganella.
- S. aureus, S. saprophyticus.
- Klebsiella.
- Brucella.