Microscopy:

Magnification: factor by which an image appears to be enlarged
[Resolution of naked eye = 200 μm]

Resolving Power (R.P): smallest distance (d) b/w 2 objects that appear as separate objects

- $d \downarrow \Rightarrow \uparrow$ R.P

$$d = \frac{0.61\,\lambda}{NA}$$

NA = numerical apeature

- RP of naked eye = 0.2 mm [200 μm]
- RP of light microscope = 0.2 μm
- RP of electron microscope = 0.2–0.5 nm

$$R.P \propto \frac{1}{d}$$

→ Magnification of compound microscope = magnification of objective lens × magnification of eye piece/ ocular lens

→ Numerical aperture (NA) = $n \sin\theta$

(n = refractive index of medium
θ = semi-vertical angle of cone formed by object at objective lens.)

→ electron microscope was introduced by Knoll & Ruska.

Staining:

- simple
- differential
- negative
- impregnation
- special

Simple Stain: use of a single dye [Basic fuchsin or Crystal Violet or Methylene blue ...]

Differential Stain: use of more than one dye (for distinguishing organisms based on their interaction with the dyes)

Gram Stain: Hans Christian Gram

Steps:

- Prepare smear ⟶ fix with heat or methanol
- Primary stain – Crystal violet / Methyl violet / Gentian violet
- Mordant – Iodine
- Decolourisation – Alcohol / acetone / mixture of alcohol-acetone
- Counterstain – Safranine / Neutral Red / Basic Fuchsin. (secondary stain)

[Come In And Stain].

{ Gram +ve bacteria appear bluish-purple
Gram -ve " " pink }

→ Acidic cytoplasm is what gets stained with Crystal violet or safranine dye (not cell wall)

Principles of Gram Stain:

Cell Wall Thickness: gram +ve cell wall is thicker ∴ Crystal violet is retained better

Cytoplasm: cytoplasm of gram +ve is more acidic ∴ slower decolourization

Cell wall lipids: gram -ve cell wall has more lipids which are dissolved by

alcohol / acetone ⟹ pore formation ⇒ rapid decolourisation

→ Most important step of Gram staining : step of decolourisation

Exceptions to Gram Stain:

- Mycoplasma (too small)
- Chlamydia, Rickettsia } minute intracellular bacteria
- Spirochetes (very slender)
- Mycobacteria (Lipid rich cell wall ∴ stain cannot pass through)

Gram +ve Cocci

Micrococcus
Enterococcus
Staphylococcus
Streptococcus

Gram -ve Cocci

Veillonella
Neisseria
Moraxella

Gram +ve Bacilli (rods)

Cory : Coryne bacterium
& Ery : Erysipelothrix
Knocked : Nocardia
Back : Bacillus
the actor's : Actinomycetes
List : Listeria
into
My : Mycobacterium
closet : Clostridium

Gram -ve rods

Enterobacteriaceae
Pseudomonas
Burkholderia
Vibrio
Legionella
Bacteroidea

Gram -ve Cocobacilli:

- Brucella
- Bordetella
- Hemophilus
- Francisella
- Chlamydia
- Rickettsia

Gram -ve Spirals

- Spirochetes
- Spirillum
- Campylobacteria
- Helicobacter

Acid Fast Stains: Mycobacteria

(Acid fast bacteria appear pink
Non-acid fast " " blue)

→ Introduced by Paul Ehrlich
→ modified by Ziehl & Neelsen (ZN)

- Heat for fixation (helps carbal fuchsin penetrate lipid layers)
- Primary Stain: Carbal Fuchsin [Basic fuchsin dissolved in phenol]
- Decolourisation: 25 % Sulphuric acid
- Counterstain: Methylene blue / picric acid / malachite green

[Read: Fite Stain]

Acid Fast Bacteria [Retain primary stain on decolourization]

- M. tuberculosis complex (MTBC)
- atypical mycobacterium (ATM)

} to differentiate in sputum of immunocompromised patients *

* MTBC → acid & alcohol fast (3% acid alcohol is decolourizer)
ATM → only acid fast

Kinyoun / Gabbet Stain: [COLD STAIN]

→ similar to Ziehl & Nielsen Stain (except - no heating)
→ increase Conc. of phenol Carbal fuschin
increase time of exposure to Carbal fuchsin.

Partially Acid-Fast Bacteria: (Modified ZN stain)

→ they retain primary stain with a lower conc. of sulphuric acid

Acid fast with 5% H_2SO_4 ⇒ M. leprae ; oocytes of cystosospora, cyclospora & cryptosporidium

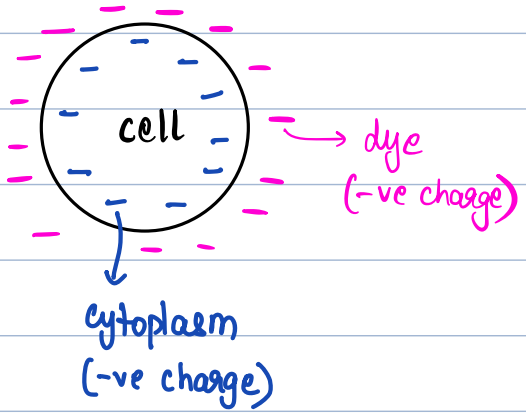
Acid fast with 0.5 - 1% H_2SO_4 ⇒ Legionella micdacei
Nocardia
Actinomycetes

Acid fast with 0.25 - 0.5% H_2SO_4 ⇒ Bacterial spores
Head of sperm

Stain - 5-10 mins
Heat - 3 times
Wash with sulphuric acid until entire smear is colourless
Wash with water
malachite green - 1 minute

Negative Stain:

→ use of acidic dye (India ink / nigrosine)



→ used for demonstrating capsule of bacteria & various slender bacteria (ex: Spirochetes) in fluid specimens

Impregnation Stain: Spirochetes

→ slender bacteria are impregnated with silver to make them thick enough

For Spirochetes

Levaditi silver Stain : for tissue sections
Fontana's " " : for films

Impregnation Stain: Flagella

↳ Tannic acid

Ashby stain / Schaeffer Fulton stain → for demonstration of endospore

Special Stains: for demonstrating special granules in bacteria

- Lipid Granules : Sudan Black B
- Polysaccharide Granules : Iodine stain
- Volutin (metachromatic / Babes Ernst / polar bodies) Granules:
  - Albert stain
  - Neisser stain
  - Ponder stain

Albert Stain:

Albert A Stain: - Toluidine blue ⇒ stains volutin granules
- Malachite green ⇒ stains cytoplasm
- Glacial acetic acid

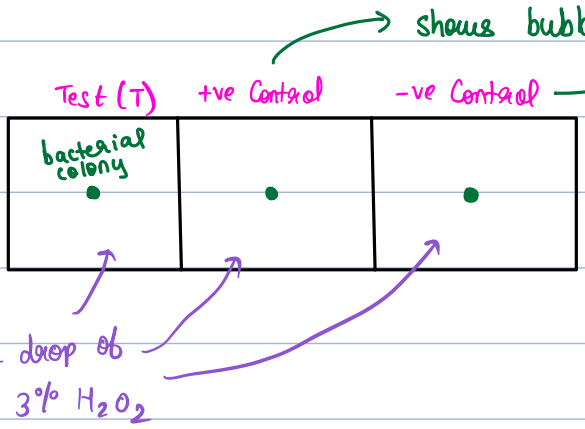
Albert B Stain: - Iodine
- Potassium iodide (mordant)

- Why are they called volutin granules? first demonstrated in Spirillum volutens
- Why " " " metachromatic granules? actually stained by toluidine blue (but they take up a bluish purple/red colour)
- What are these granules made of? Polymetaphosphate
- Do metachromatic granules (gram +ve) take up Gram stain? Yes
- Where are they present? Corynebacterium, Gardnerella vaginalis, Yersinia pestis, Bordatella pertussis, Mycobacterium species.

Biochemical Reactions: (for identification of bacteria)

Bacterial Catalase:

$$H_2O_2 \xrightarrow{\text{bacterial catalase}} 2H_2O + O_2$$



(Look for gas bubbles)
[formation of O_2]

If Test:

- shows bubbles ⇒ Catalase Test +ve
- no bubbles ⇒ Catalase Test -ve

- Most Pathogenic bacteria are catalase positive

except:

- Streptococcus
  Pneumococcus
  Enterococcus
- Anaerobes – Actinomyces
  Bacteroidea
  Clostridium
  Lactobacillus
- Shigella dysenteria - type 1.

Oxidase Test:

TMPD (tetra-methyl-paraphenylene diamine-dihydrochloride) $\xrightarrow{\text{bacterial cytochrome C}}$ Purple/Blue compound (indophenol)

Dont use — Straight wire
— Nichrome innoculating loop

} give False +ve oxidase test.

Most pathogenic bacteria are oxidase test positive except:

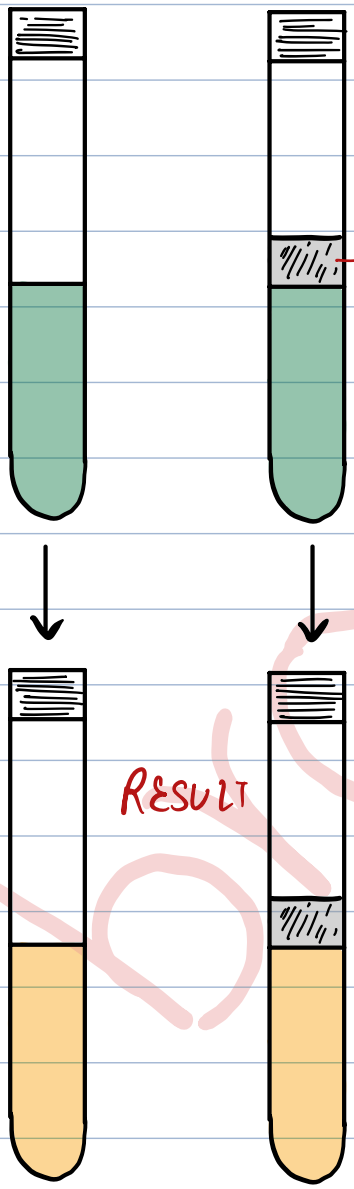
- Coryne bacterium
- Enterobacteriaceae
- Staphylococcus
- Streptococcaceae

Utilization of Sugars:

Hugh - Leifson's oxidative fermentation medium
↳ glucose + bromothymol blue (pH indicator)

If bacterial colony:

- breaks down the sugar ⇒ saccharolytic
  - only aerobic → OXIDATIVE
  - aerobic + anaerobic → FERMENTATIVE
- doesn't break down sugar ⇒ asaccharolytic



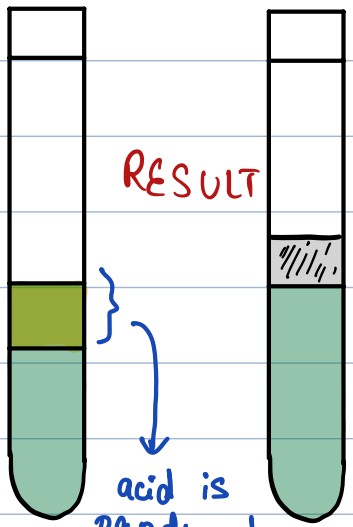
→ oil overlay ⇒ to create anaerobic conditions
(sterile petroleum jelly)

RESULT

Bromothymol blue has changed colour
⇓
acid has been produced aerobically & anaerobically
⇓
∴ bacterial colony is FERMENTATIVE

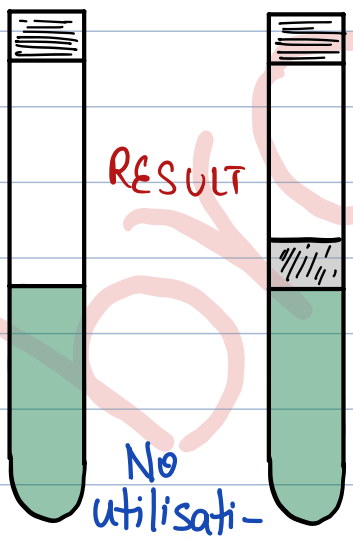
Fermentative Utilisation [Facultative Anaerobes]:

- Enterobacteriaceae
- Staphylococci
- Streptococci
- Haemophilus



Strict Aerobes:

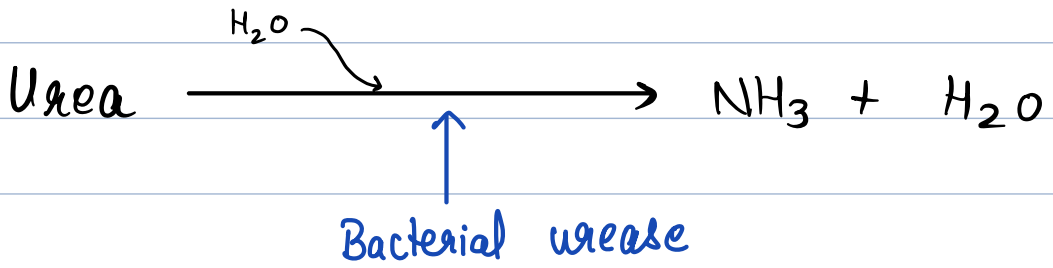
- Pseudomonas
- Brucella
- Bordetella
- Micrococcus



ASACCHAROLYTIC:

- Moraxella
- Acinetobacter
- Compylobacter
- Helicobacter

Urease Test :



Christensen's urease medium
- 2% urea
- Phenol red (indicator)

→ Medium turns pink (Alkaline pH)

Urease Positive Bacteria :

Proteus
Ureaplasma
Nocardia
Cryptococcus
Helicobacter → maximum urease producing bacteria
Morganella
Staphylus aureus & Staphylus epidermidis & Staphylus Saprophyticus
Klebsiella pneumoniaeae
Brucella

Coagulase Test	Staphylococcus aureus
Bile solubility Test	Streptococcus pneumoniae
optochin Sensitivity	〃 〃
Bile Resistance	Enterococcus
Bacitracin Sensitive	Streptococcus Pyogenes
Indole Positive	E. Coli
Indole Negative	Klebsiella

Citrate utility
Indole
Triple Sugar Iron
Black discolouration — due to H_2S & iron deposit
Mannitol motility

Bacterial Typing: intraspecies strain characterization

Purpose of typing – epidemiologic studies (outbreak studies)
– virulent & avirulent studies
– studies of transmissibility
– relapse (from same strain) vs. reinfection (from different strain)

Typing Methods

- Phenotypic
- Genotypic

Phenotypic – Serotyping :

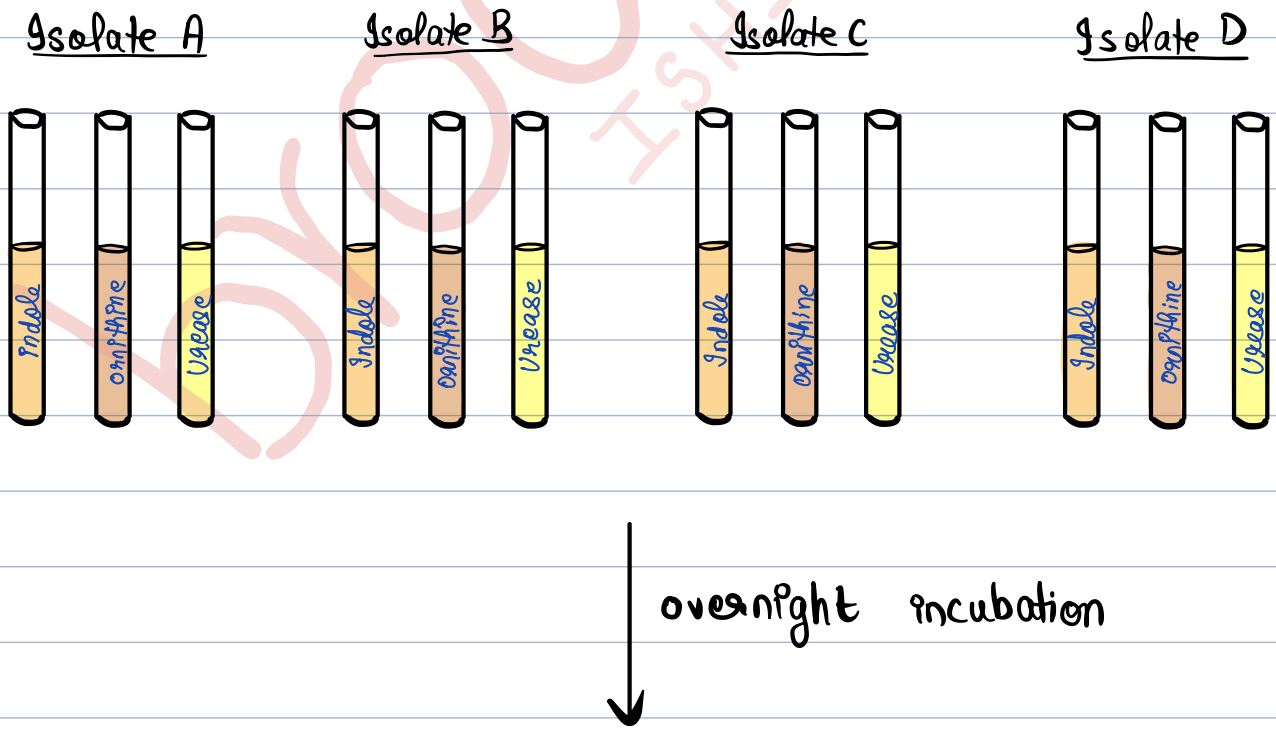
→ use of surface antigens (and their reaction with antibodies)

Staeptococcus pyogenes	Griffith Typing (M protein)	> 120 M types
Streptococcus pneumoniae	Capsular antigen	~ 95 serotypes
E. Coli	O antigen	> 170 'O' serotypes

Phenotypic – Biotyping :

→ for selected group of biochemical reactions

- I to VIII types of Haemophilus influenzae
  - ↳ chemicals used – Indole
    - Ornithine
    - Urease



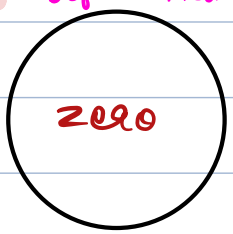
- Corynebacterium diphtheriae → 4 biotypes — gravis
  — intermedius
  — mitis
  — belfanti
- Vibrio cholerae O1 — EITor
  — Classical

Phenotypic — Auxotyping:

→ based on their ability to utilize / not utilize a specific group of substrates

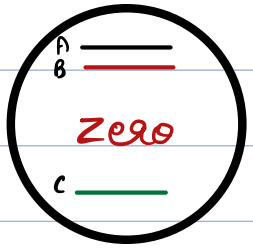
- Gonococcus:

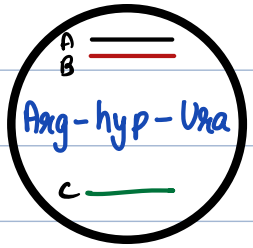
Defined medium

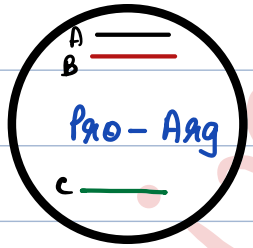


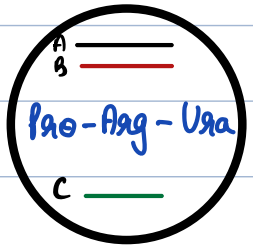
⇒ contains all essential nutrients required for growth of gonocoocus

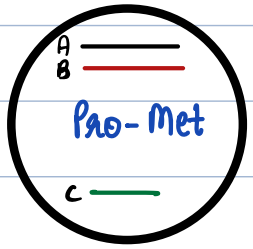
↳ many mediums were created by deleting a set of ingredients

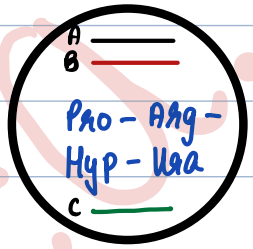


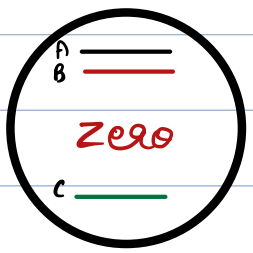


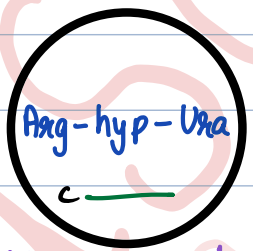




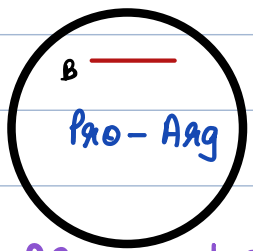




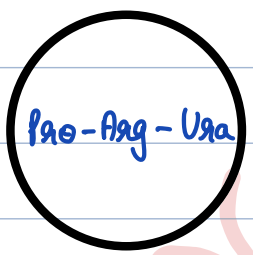


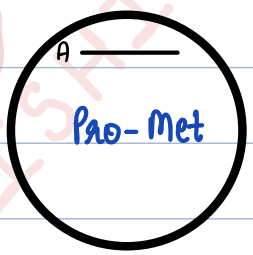


AHU- oxotype

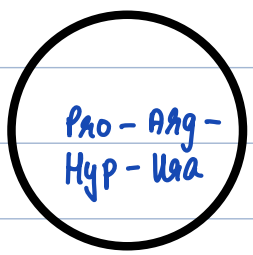


PA- oxotype



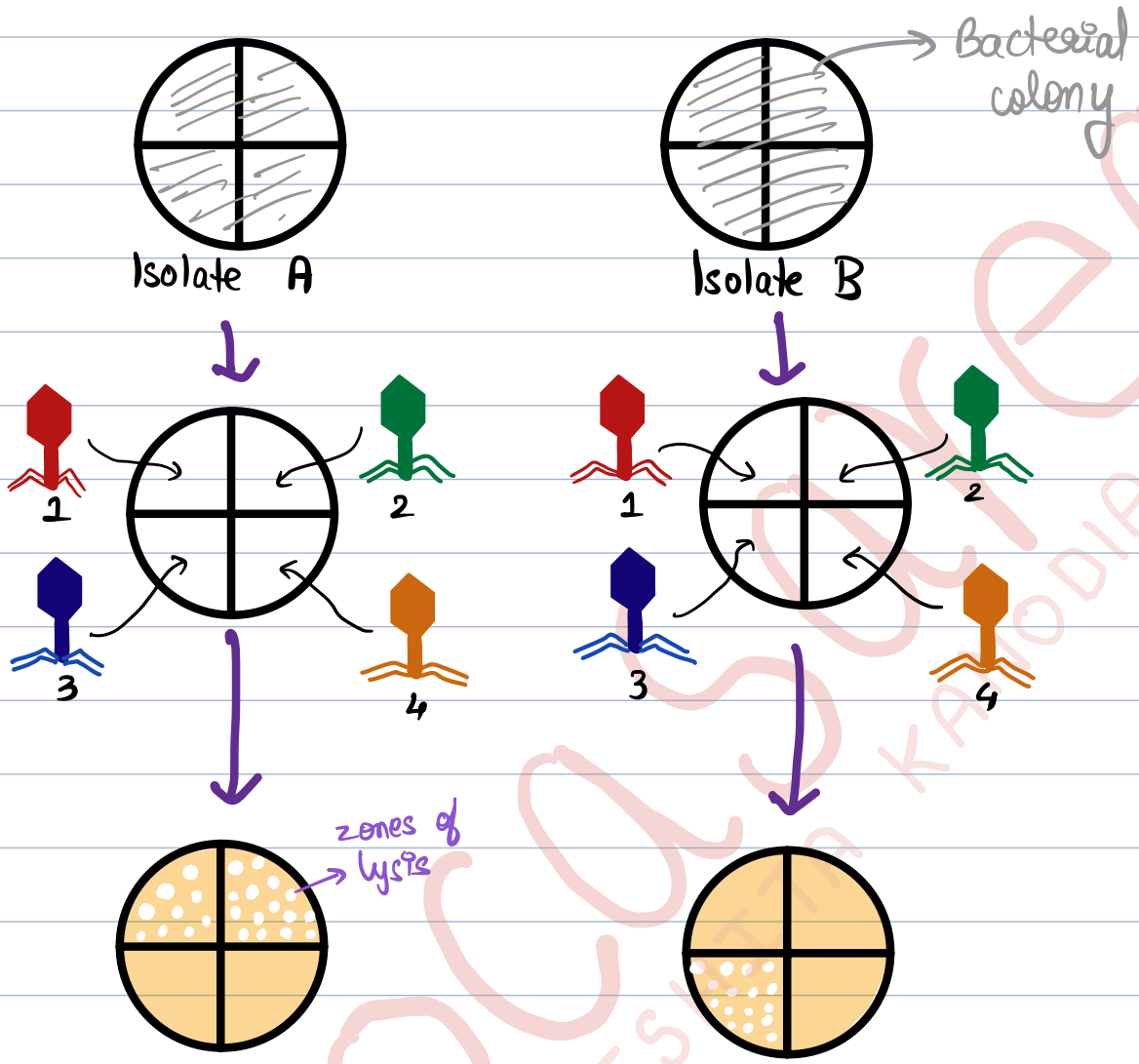


PM- auxotype



Phenotypic - Phage Typing :

→ on the basis of susceptibility to a standard set of bacteriophages.



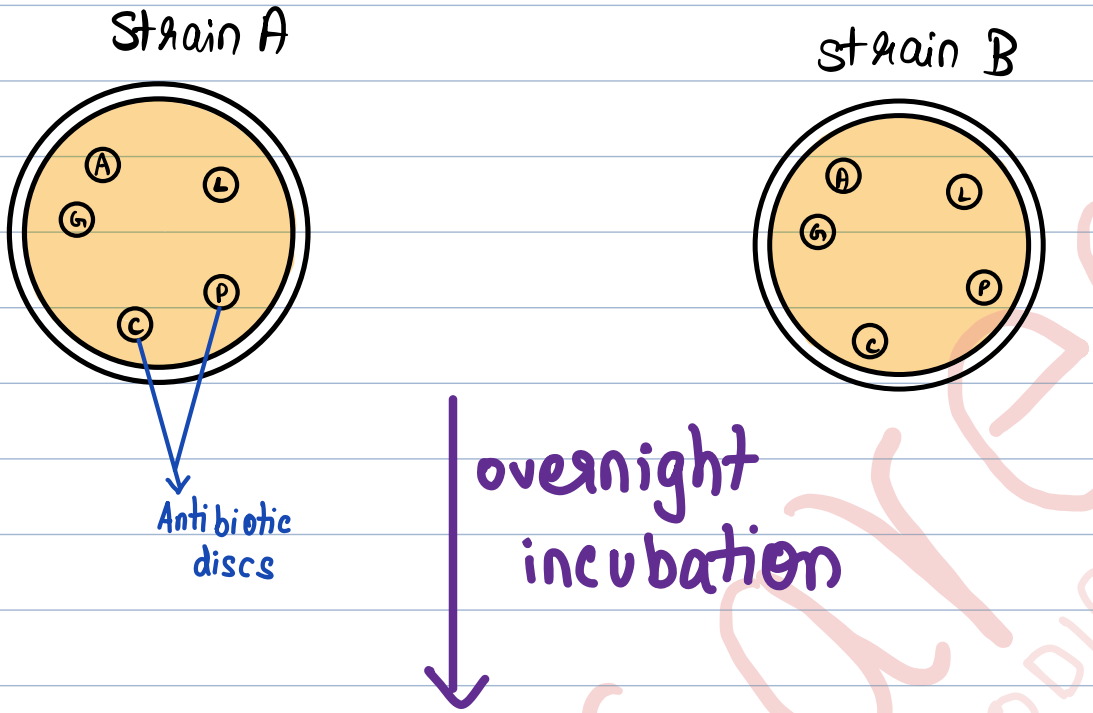
Phage Type 1, 2
(Bacterial colony is susceptible to phage 1 & 2)

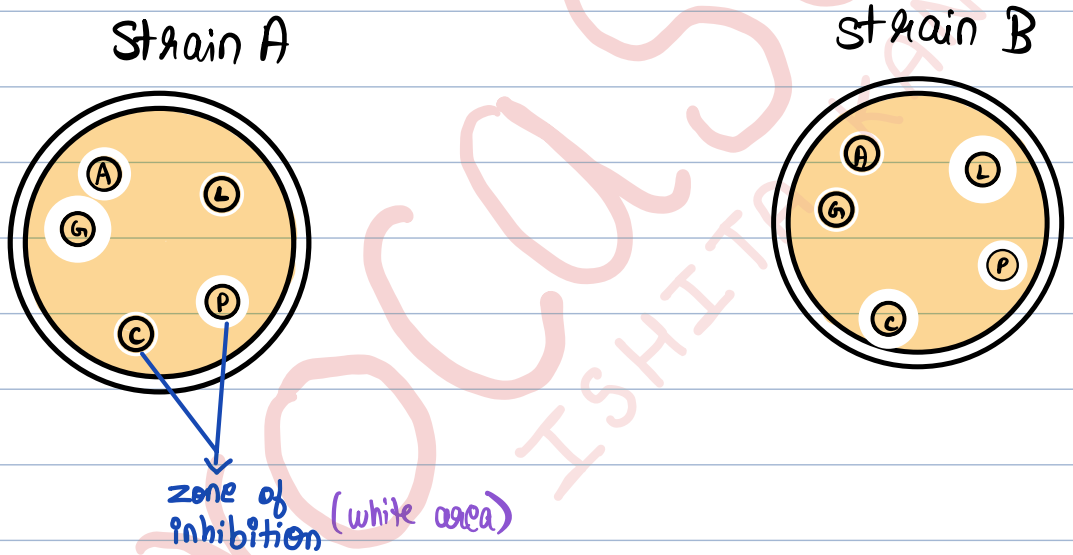
Phage type 3
(bacterial colony is susceptible to phage 3)

- Staphylococcus aureus
- Corynebacterium diphtheriae
- Salmonella
- Vibrio

Phenotypic – Antibiogram Typing :

→ based on susceptibility / sensitivity to antibiotics





- ↓ small ⇒ more resistance to that antibiotic
- ↑ large ⇒ less resistance / no resistance to that antibiotic

- Proteus vulgaris

Phenotypic – Bacteriocin Typing: susceptibility of standard strains to bacteriocin secreted by isolates

Phenotypic – whole Cell Protein Typing: Relative mobilities under electrophoresis of total extracted protein

Phenotypic – Multi Locus Enzyme Electrophoresis [MLEE]: relative mobilities under electrophoresis of specific intra-cellular enzymes

GENOTYPIC METHODS: superior to phenotypic methods

→ DNA based analysis

→ a particul part / whole DNA is extracted out ⇒ restriction endonucleases

⇓

DNA fragments ⇐ gel electrophoresis ⇐ banding pattern is checked

Plasmid Profile Analysis

Ribotyping

Pulsed field Gel Electrophoresis [PFGE]

Restriction Fragment Length Polymorphism [RFLP]

Nucleic Acid Sequencing → GOLD STANDARD METHOD FOR TYPING

↓

whole genome sequencing will take a lot of time

⇓

∴ there is —

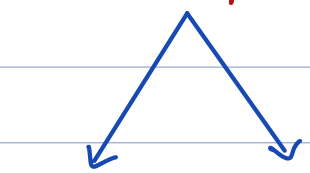
Multilocus Sequence Typing [MLST]:

→ only particular standard house keeping genes are sequenced & compared

Bacterial Cultivation:

Phototrophs: bacteria that can utilize light as a source of energy

Chemotrophs: bacteria that need to undergo chemical reactions to synthesize energy



Autotrophs: can utilize inorganic sources (in the form of atmospheric gases) of carbon & nitrogen

Heterotrophs: require pre-formed organic compounds for undergoing [chemo-organotrophs] chemical reactions

Most Human commensals & pathogens ⇒ heterotrophs (∴ culture media have to be provided for their growth)

→ **Louis Pasteur** ⇒ established bacterial growth requirements (liquid media / nutrient broth)
↳ father of medical microbiology

Advantages of Liquid Media: convenient
→ bacterial multiplication is rapid
→ originally clear solution becomes turbid ⇒ microbial growth

Disadvantages: quantitative analysis cannot be done
→ bacterial strains cannot be isolated.

Robert Koch ⇒ introduced solid media

Advantages of Solid Media: different bacterial colonies can be clearly seen

Disadvantage: slower growth than liquid media

Solidifying Agents:

Gelatin: derived from animal bone / hide

→ 15% concentration

→ **Disadvantages**: – certain bacteria can proteolyse gelatin

– liquefies above 24°C (but incubator ⇒ 37 or 38°C)

Agar - Agar / Chinese Grass: (polysaccharide) sea weeds & red algae

→ 2 % concentration

→ **inert substance** ∴ does not provide nutrition to bacteria; it neither promotes nor inhibits bacterial growth

→ Liquefies > 98°C

Solidifies < 42°C [42 - 98°C ⇒ semisolid]

→ **soft agar**: used to check motility of bacteria

– Conc. = 0.2 - 0.5 %

→ **firm agar**: used for inhibition of swarming.

– conc. = 5 - 6 %

Swarming bacteria:

- clostridium tetani } gram +ve
- Bacillus cereus }
- vibrio alginolyticus } gram -ve
- proteus vulgaris }
- proteus mirabilis }

Constituents of Culture Media:

- Peptones
- Meat / yeast extract
- Casein hydrolysate
- Electrolytes
- Water

} sources of C & N.

Culture Media:

CHEMICALLY DEFINED
→ exact composition is known

COMPLEX MEDIUM
→ exact composition not known

Simple / Basal / Basic Medium: only non-fastidious bacteria can grow
→ source of C & N only
require only C & N for their survival.

Eg:
- peptone water
- nutrient broth (peptone water + meat extract)
- nutrient agar (nutrient broth + agar)

Enriched Medium: for fastidious bacteria
→ egg / blood / serum is added

Eg:
- blood agar (autoclaved nutrient agar + 5% sterile sheep blood) cooled to 50°C
- chocolate agar (autoclaved nutrient agar + 5% sterile sheep blood) cooled to 75°C
  (RBC's get lysed at 75°C)

- Loeffler's serum [Corynebacterium diphtheriae]
- Lowenstein Jensen medium [Mycobacterium tuberculosis]

Selective Medium: (solid medium)

→ a component is added which inhibits the growth of unwanted bacteria (only allows growth of specific bacteria)

eg:
- MacConkey (mildly* selective for gram -ve bacteria)
  - → contains Na taurocholate, bile salt
  - * Exception: Staphylococcus, Enterococcus } gram +ve ⇒ can grow on MacConkey
- EMB [Eosine Methylene Blue Agar] (mildly selective for gram -ve bacteria)
- Salt Agar [selective for Staphylococci]
  (all staphylococci have the special property of being able to grow in 7-10% salt)
- crystal Violet Blood Agar [selective for Streptococcus pyogenes]
- Thayer Martin [selective for Neisseria]
  - → contains antibiotics
- TCBS [thiosulphate citrate bile salt sucrose agar]
  (selective for vibrio)
- Cetrimide agar (selective for pseudomonas)
- Potassium tellurite (selective for Corynebacterium)
- PLET (Polymyxine lysozyme EDTA thallus acetate)
  (Bacillus anthracis)
- MYPA (mannitol egg-yolk phenyl red polymyxine agar)
  (Bacillus cereus)

Enrichment Medium:

→ liquid selective medium

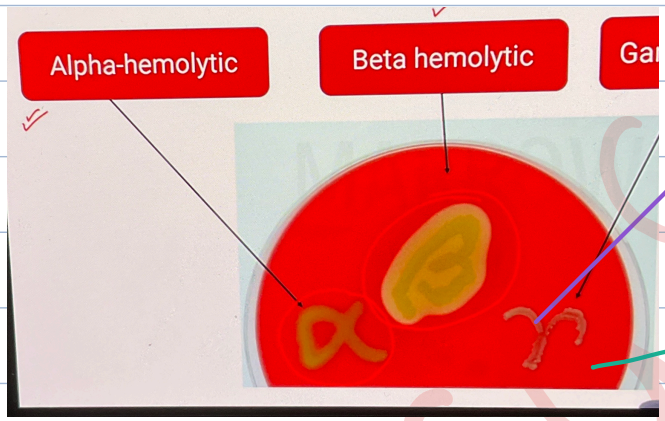
Eg:- APW (alkaline peptone water) ⇒ Vibrio

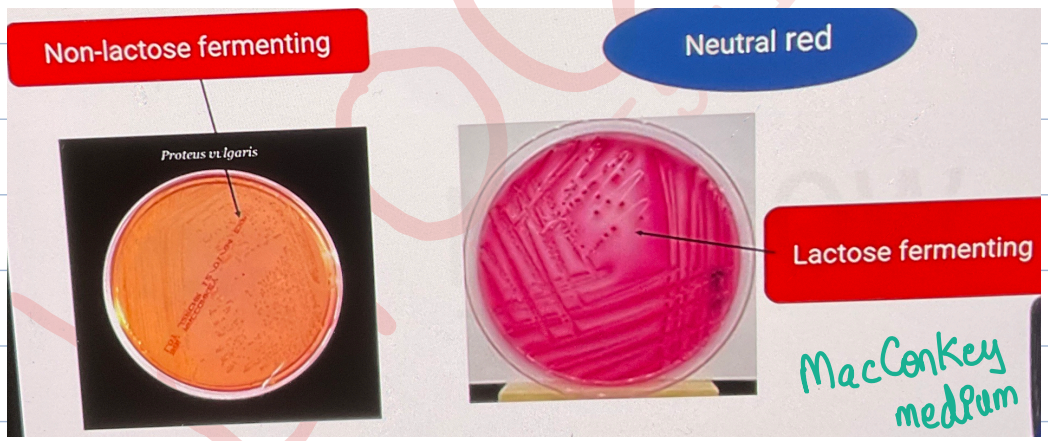
- Selenite (F) ⇒ Salmonella & Shigella
- Tetrathionate broth ⇒ Salmonella

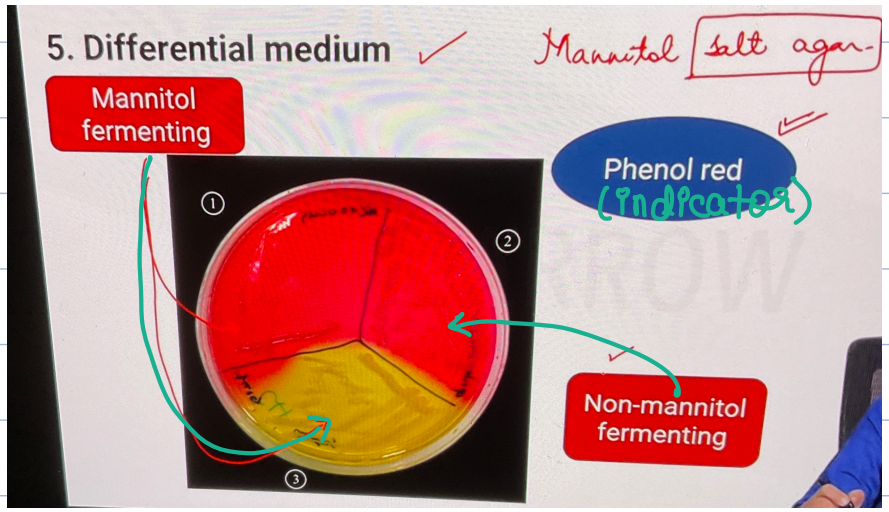
faeces

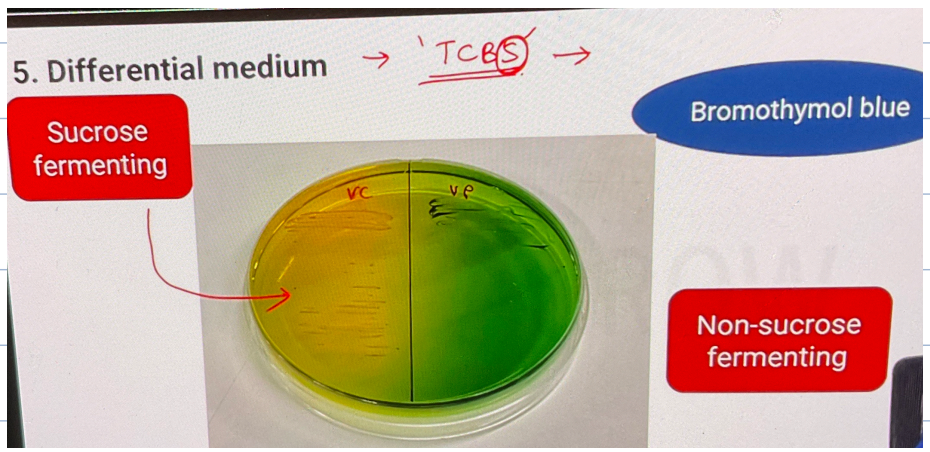
Differential Medium:

→ colony morphology / colour differentiates the bacteria.









Indicator Medium:

→ special component is added which indicates the growth of a particular group of bacteria.

Eg:
- MacConkey → Neutral red
- TCBS → Bromothymol blue
- MSA → Phenol red

Transport Medium:

→ medium which just maintains the original count of bacteria in a clinical specimen

Eg.
- Venkatraman Ramakrishnan Medium ⇒ vibrio
- Pike's ⇒ Streptococcus pyogenes
- Stuart's, Amie's } ⇒ Neisseria
- Thioglycolate ⇒ anaerobes
- Cary Blair ⇒ UNIVERSAL STOOL TRANSPORT MEDIUM

Culture Techniques:

Streak Culture:

Primary well

↓

Sterilize the innoculating loop by heating & then slightly cool it.

↓

create primary streaks

↓

sterilize the loop again

↓

turn the plate & make secondary streaks

↓

Sterilize the loop

↓

turn the plate & make tertiary streaks

↓

finally, make the tail

Lawn Culture:

take a steaile cotton swab & dip it into the innoculum

↓

swab it completely

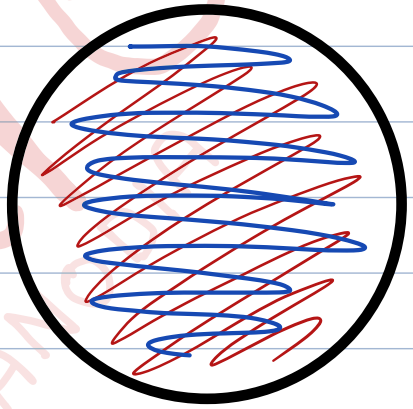
↓

make a circle at the peripheay

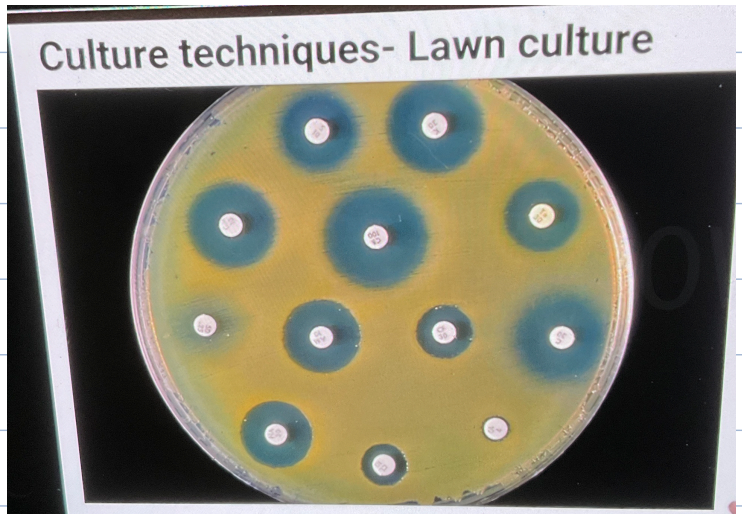
turn the plate to different angles

↓

swab completely everytime (& make a circle)

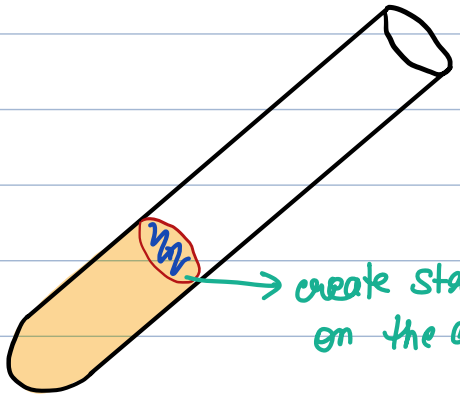


- Lawn culture is typically used for antibiotic - sensitivity testing.



Stroke Culture:

→ generally used for biochemical tests



→ create stroke with loop on the agar slant

↓

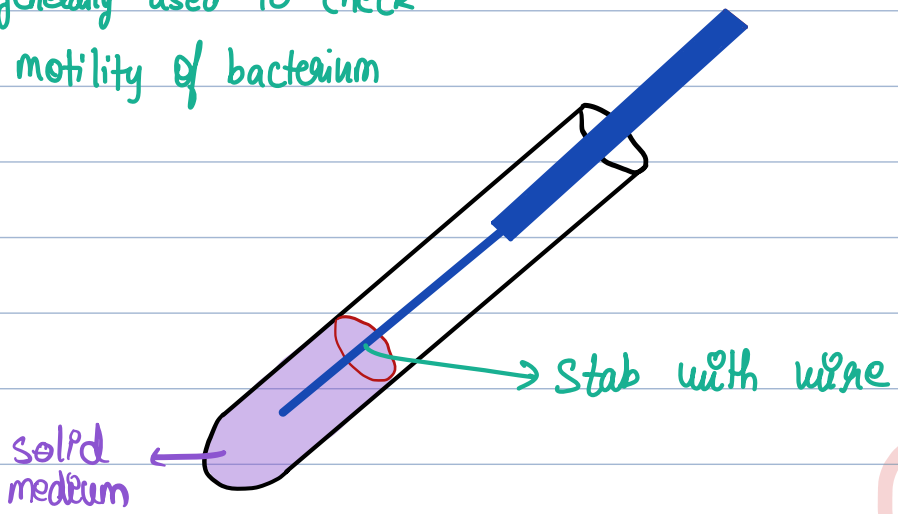
flame the test tube

↓

plug it with cotton

Stab Culture: use straight wire (not loop)

↳ generally used to check motility of bacterium

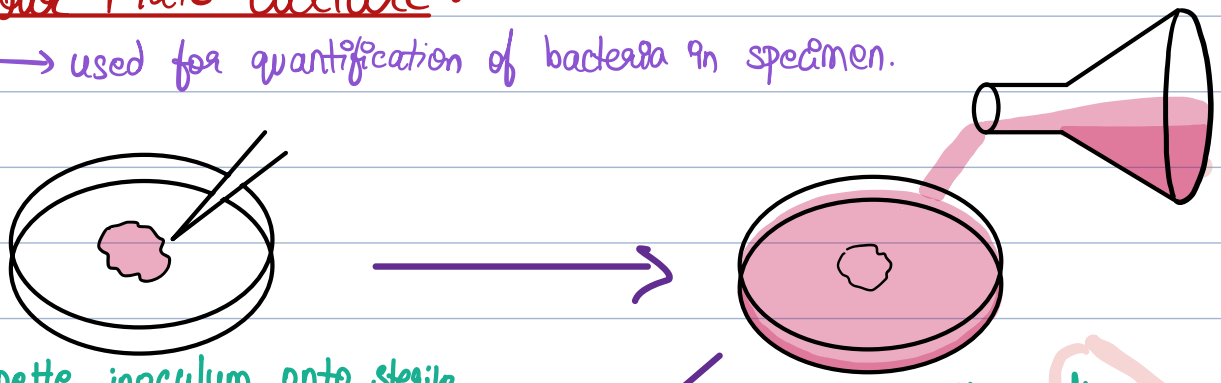


non - motile bacteria

motile bacteria

Pour Plate Culture:

↳ used for quantification of bacteria in specimen.



pipette inoculum onto sterile plate

add sterile medium

swirl to mix & incubate

Colonies grow in & on the medium

Antibiotic Susceptibility Tests (AST): (sensitivity)

→ Purpose – guideline for right antibiotic
– to study susceptibility patterns in a hospital / community

[AB = antibiotic]

Minimum Inhibitory Concentration: (MIC)

→ lowest concentration of AB that just inhibits the growth of bacteria in the medium.

Minimum Bactericidal Concentration: (MBC)

→ lowest concentration of AB, that kills all bacteria in the medium

MBC > MIC.

Methods of AST:

- Dilution (Micro broth dilution method)
- Disc diffusion
- E-test
- Automated – MICROSCAN WALKAWAY
  – PHOENIX
  – VITEK-2

→ To control the impact of environmental factors, the conditions for susceptibility testing are extensively standardised:

(a) Antimicrobial content
(b) Standard Inoculum of Test Isolate
(c) Reliable medium [Mueller Hinton Agar/Broth] *
(d) Standard temperature of incubation [35–37°C]
(e) Standard duration of incubation [16–18 hrs]
(f) Quality control strains

* Mueller-Hinton Medium ⇒ casein hydrolysate agar / broth
→ Some bacteria cannot grow on Mueller-Hinton medium

→ For those bacteria, extra supplements have to be added

Eg:- Pneumococcus ⇒ lysed horse blood / sheep blood added
- Staphylococcus aureus ⇒ 2-4% salt added

Standard Inoculum Preparation:

⇒ 0.5 McF turbidity (McF = Mac Farland's)

→ 1.5×10^8 CFU/mL.

Eg: E. Coli (urine) [CFU = colony-forming units]

→ sterile peptone water + few E. coli colonies
→ incubate for few hours
→ measure turbidity using - spectrophotometer or
- McFarland's standard media

{MHA = Mueller Hilton Agar
MHB = Mueller Hilton Broth}

[AST-1] Dilution Method: [Reference Method]

→ very cumbersome

prepare serial dilution of antibiotics in MHA / MHB.

+

fixed amount of standard inoculum

⇓

incubate at 35-37°C for 16-18 hours (overnight)

⇓

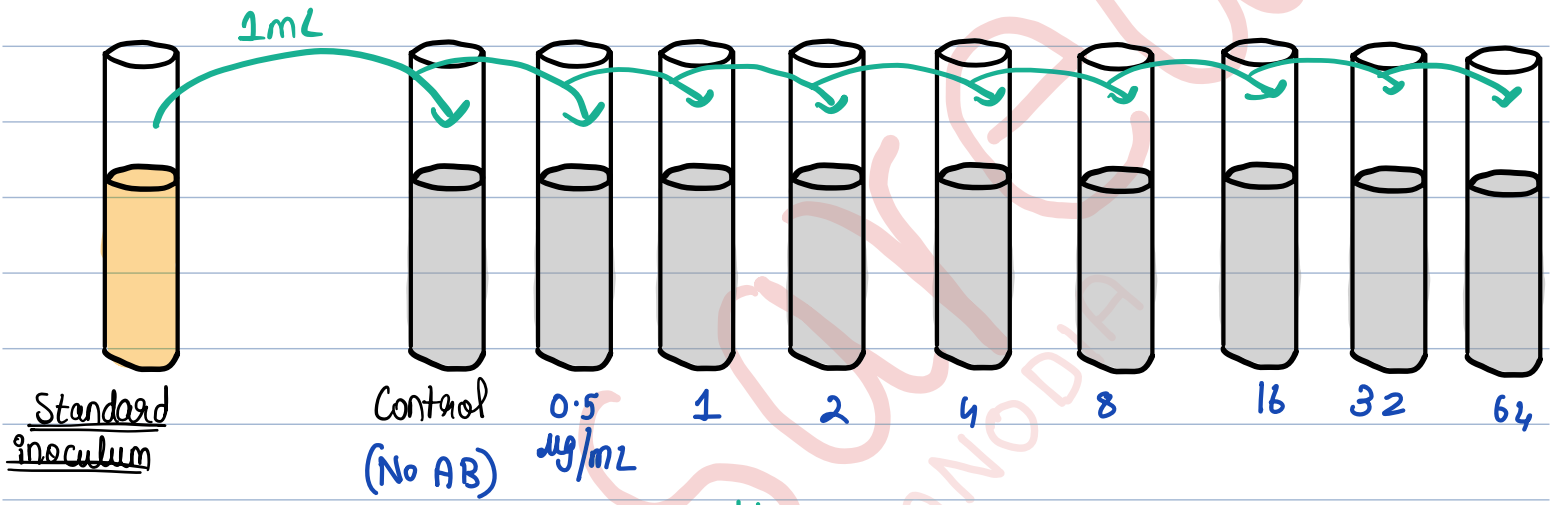
determine the **MIC**.

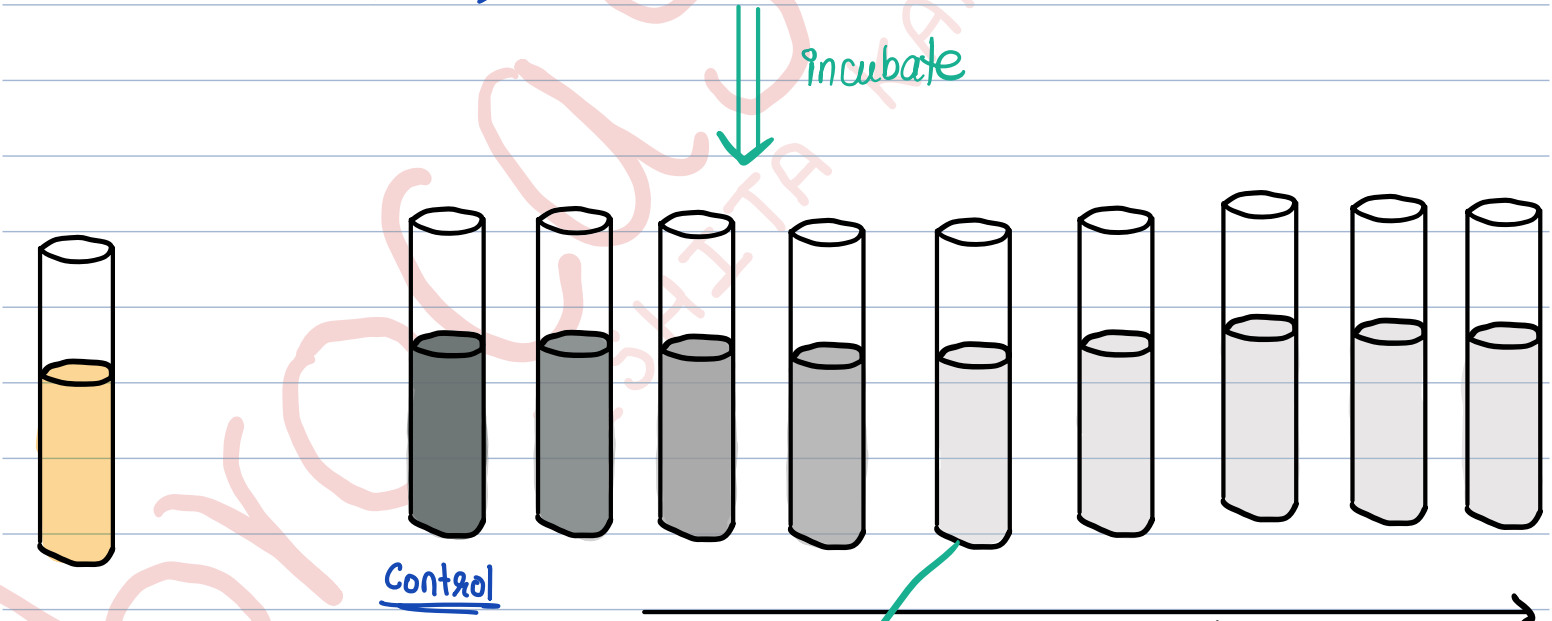
Types of Dilution Methods :

Broth Dilution : → Microbroth, → Macrobroth

Agar Dilution

Macro-broth dilution Method :



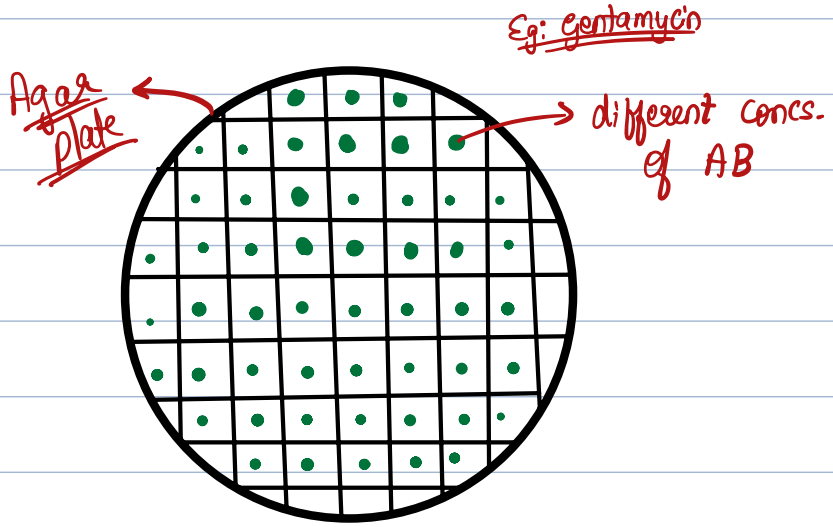


→ lots of turbidity since bacteria have multiplied freely in absence of AB

→ no turbidity

∴ MIC = 4 µg/mL.

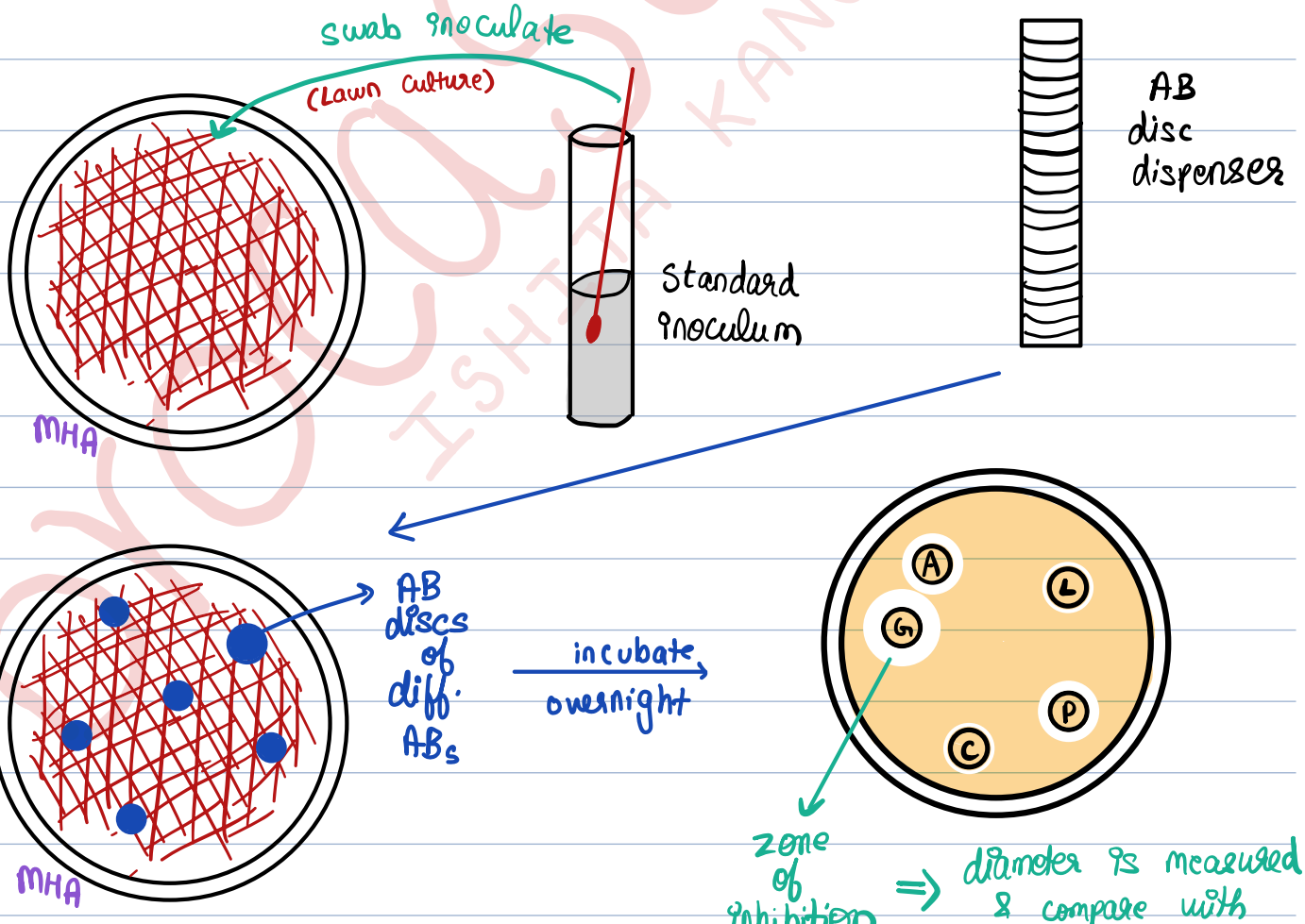
Agar Dilution Method:



[AST-2] Disc Diffusion Method: [Kirby Bauer Method]

→ AB discs impregnated with a standardised conc. of AB

→ uses MHA



Sensitive	Intermediate	Resistant
→ organism is inhibited by the recommended dose of antimicrobial agent at the infection site.	→ organism may require a higher dose of AB for a longer period of time to be inhibited	→ Organism is not inhibited by the recommended dose of the antimicrobial agent at the site of infection

Advantages of Kirby Bauer Disc Diffusion:

→ easy to do

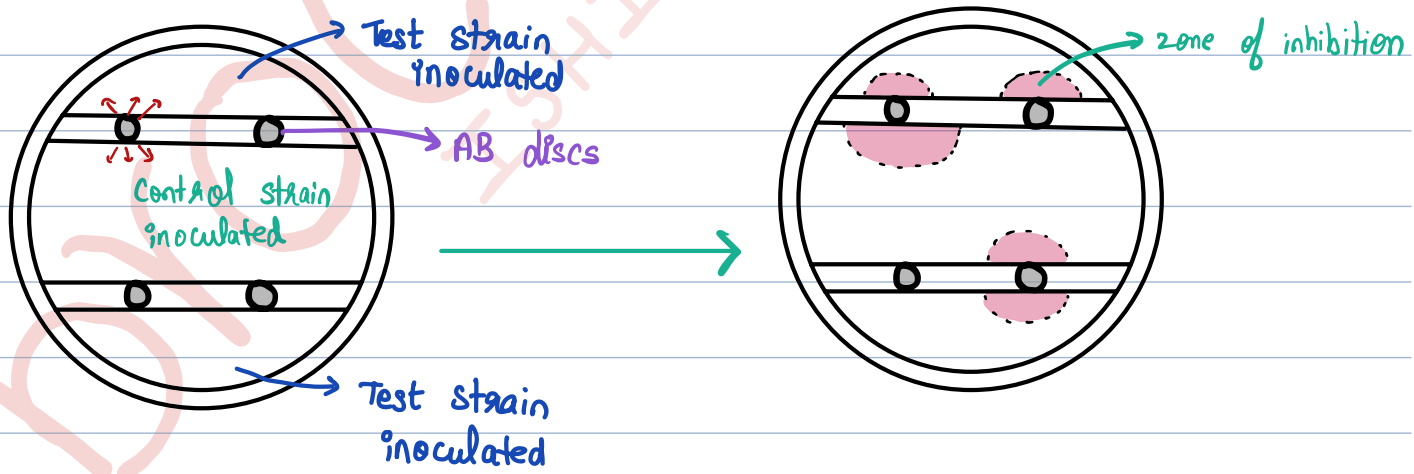
→ easy to interpret

Disadvantages of Kirby Bauer Disc Diffusion:

→ does not quantify the MIC (qualitative only)

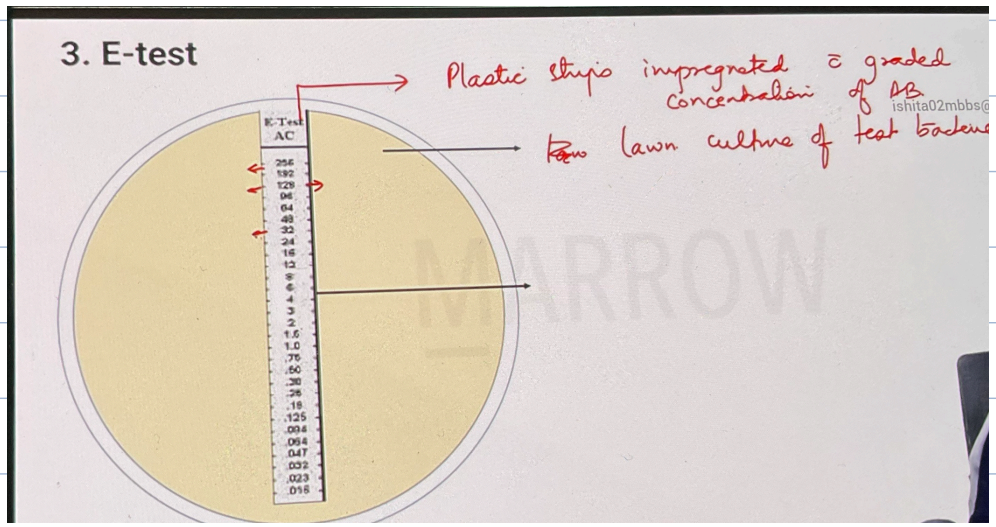
Stoke's Disc Diffusion Method:

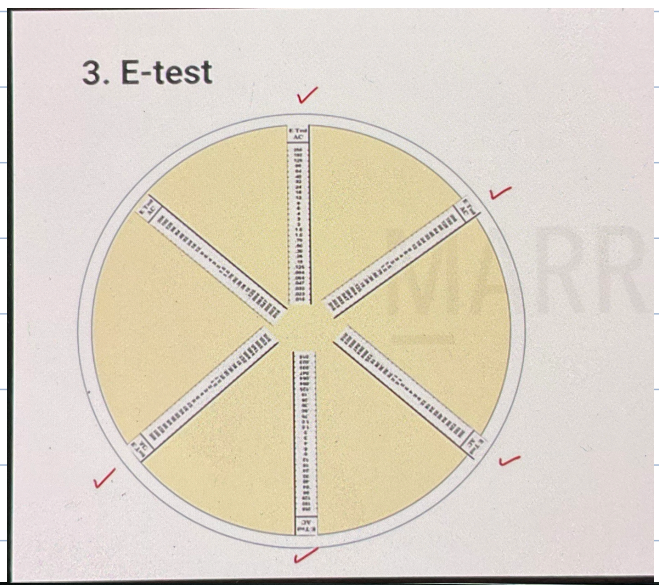
→ test & control strains are inoculated on the same petri plate.

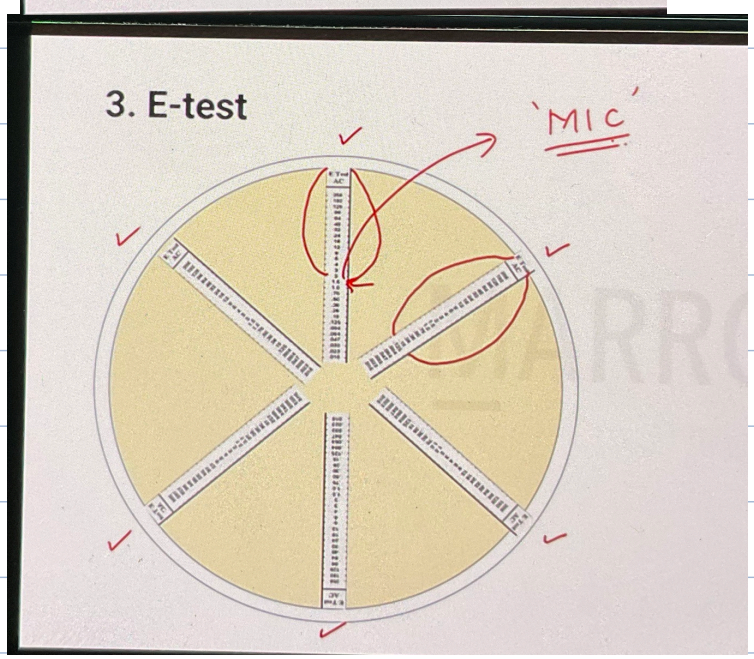


[AST-3] Epsilometer Test [ε-Test]:

→ combination of dilution method & disc diffusion method







[AST-4] Automated Methods:

→ MICROBROTH WALKAWAY

→ PHOENIX

→ VITEK-2.

Antimicrobial Resistance Mechanisms:

Beta-lactams: inhibit cell wall synthesis by binding to transpeptidases - penicillin binding proteins [PBP's]

Glycopeptides: bind to cell wall precursors [D-ala-D-ala]
∴ inhibit cell wall synthesis

Aminoglycosides
Tetracyclines
} bind to 30s ribosome subunit
∴ inhibit protein synthesis

Macrolides
Chloramphenicol
} bind to 50s ribosome subunit
∴ inhibit protein synthesis

Fluoroquinolones: inhibit DNA replication by binding to DNA gyrase / topoisomerase IV

Sulfonamide
Trimethoprim
} inhibit folate synthesis

Inherent Resistance : innate ability of bacterium to resist killing by AB by its inherent structural / functional characteristics

(a) reduced entry (bacteria is impermeable to AB)

(b) extrusion of AB by inherently encoded transporters

(c) inherent production of enzymes that inactivate the AB

Eg:

- Enterococci & Anaerobes — aminoglycosides — reduced entry
- Gram +ve bacteria — Aztreonam — lack of PBPs to which Aztreonam binds
- Gram -ve " — Vancomycin — no entry

Acquired Resistance :

→ May be due to — Mutations
— Horizontal transfer

Eg:

- Streptococcus pneumoniae — by transformation
- Staphylococcus aureus — by transduction

Most common mode of horizontal transfer in bacteria - Conjugation.

Modes of Antibiotic Resistance:

Inactivating enzymes: most common mode

Eg: A → aminoglycosides – inactivated by phosphorylation, N-acetylation
B → β lactams – hydrolysis of amide bond in β lactam ring by β lactamases
C → chloramphenicol – acetyl transferases inhibit.

Decreased Permeability:

→ absence of porins or outer membrane proteins [OMPs] which prevents entry of AB

Eg: – Resistance to β-lactams in gram -ve
– aminoglycosides
– Macrolides
– Fluoroquinolones

Antibiotic Efflux: most common mode of resistance to Tetracyclines.

Eg: – Macrolides
– Fluoroquinolones
– β-lactams

Altered Drug Target Sites: • ribosome alterations

Eg: – Aminoglycosides
– Macrolides

• Cell wall precursors Eg: – Resistance to Vancomycin

- altered enzymes Eg:- Methicillin resistant staphylococcus aureus (MRSA)
  - floroquinolones

Protection of Taget Sites: bacteria synthesize ribosomal protection proteins (RPP).
Eg: - Tetracycline resistance

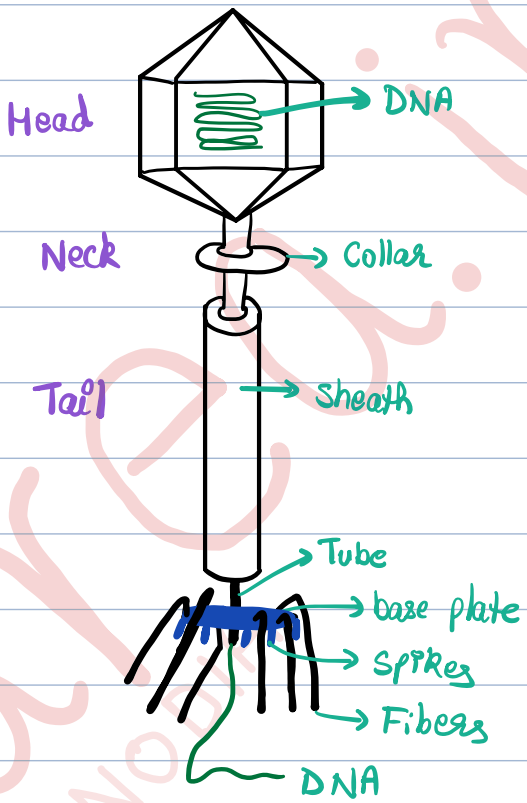
Overproduction of Drug Targets:
Eg: - β-lactam resistance in gram +ve
- sulphonamide resistance

Bypass of Antibiotic Inhibition:
Eg: - Sulfonamide resistance

MRSA

Bacteriophages: viruses that infect bacteria

Cycles of Phage: Lytic, Lysogenic



Lytic Cycle:

bacteriophage binds to bacterium

↓

injects it DNA into cytoplasm

↓

Stops bacterial metabolism

↓

Fragmentation of bacterial chromosome

↓

Synthesis of phage components using bacterial enzymes

↓

Assembly of daughter phages

↓

Induced lysis of bacterium causes the daughter phages to move out of the bacterium

↓

Infection of new bacterium

Lysogenic Cycle:

bacteriophage binds to bacterium

↓

injects its DNA into cytoplasm

↓

phage DNA gets integrated with bacterial chromosome to form prophage

↓

As bacterium undergoes binary fission, prophage is passed on to future generations of bacteria

↓

If any of these bacteria are exposed to chemicals / radiations

↓

Phage DNA disintegrates from bacterial chromosome

↓

phage DNA enters lytic cycle

Types of Phages:

- phages that can follow only lytic cycle ⇒ VIRULENT PHAGES
  Eg:- T_1, T_2 phages of E. coli.
- phages that enter lysogenic & then lytic cycle ⇒ TEMPERATE PHAGES
  Eg:- λ phage of E. coli.

Mechanisms of Gene Transfer in Bacteria:

- Transduction
- Lysogenic conversion

} via phages

- Transformation
- Conjugation

Transduction: transfer of bacterial genes from one bacterium to the other via phage

→ most common mechanism of gene transfer in bacterium

→ 1st demonstrated on *Salmonella typhimurium*.

→ Transduction types: < Generalized, specialized

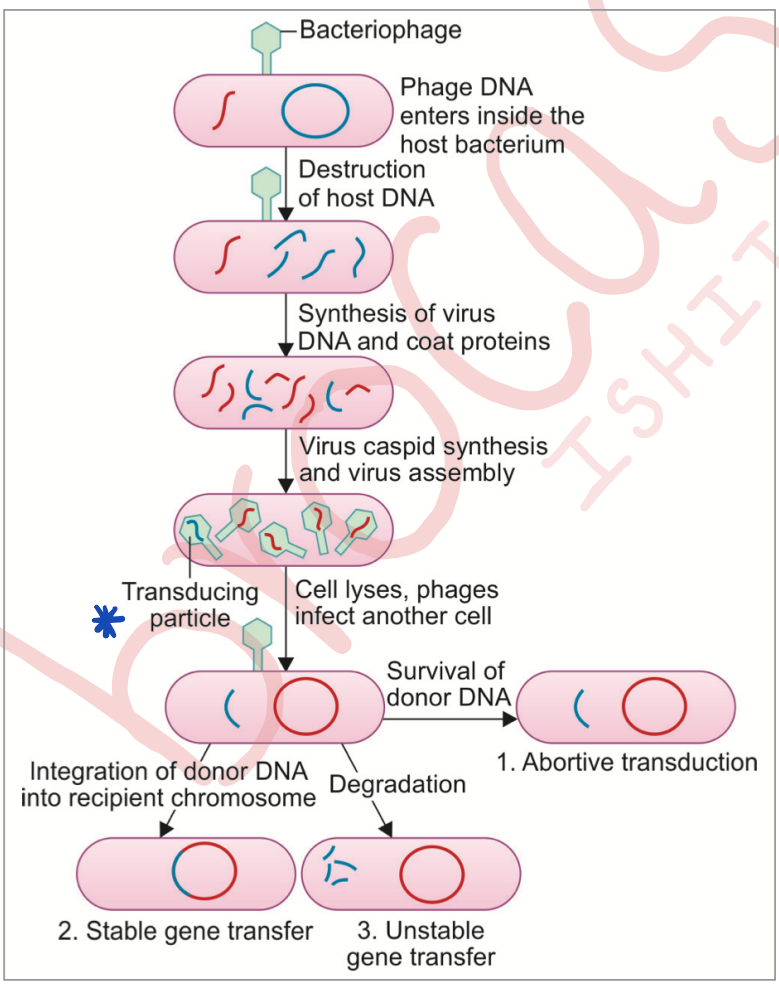


Fig. 3.4.5: Generalized transduction.

* mis-packaging of bacterial chromosome fragment into newly synthesized phage.

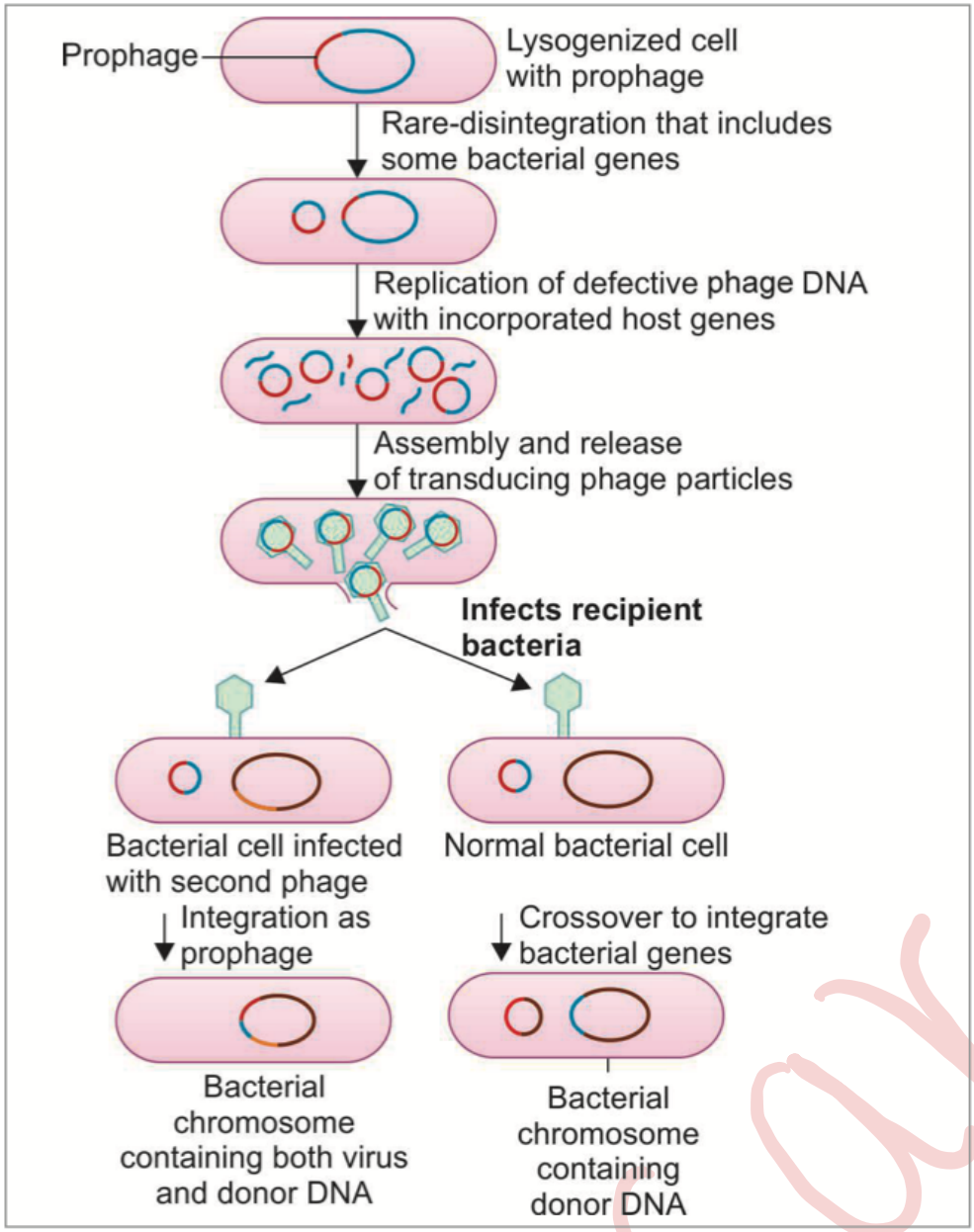


Fig. 3.4.6: Restricted transduction.

→ only restricted / specific genes of bacterium that are next to the site of integration of phage DNA are incorporated into newly synthesized phages.

Specialized transduction

Generalized	Specialized
→ occurs in lytic cycle	→ occurs following a lysogenic cycle
→ occurs due to mispackaging of chromosomal / plasmid genes during assembly	→ occurs due to defective excision during induction of prophage
→ mediated by virulent or temperate phage	→ mediated only by temperate phage

Lysogenic Conversion:

→ prophage genes (in a lysogenic cycle) themselves provide genes for a new characteristic/phenotype to the lysogenic bacterium.

→ phage-mediated toxins

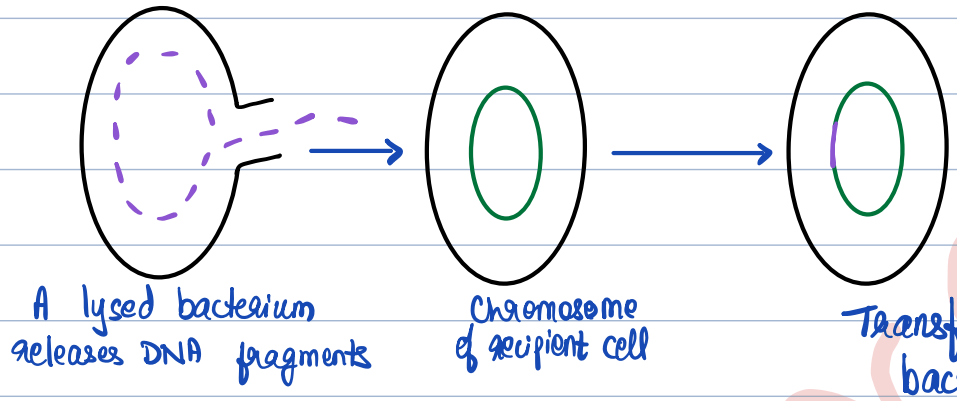
Eg: ability to produce toxins by bacteria

- Corynebacterium diphtheriae
  - non-toxigenic
  - toxigenic

Phage Mediated Toxins:

A - Pyrogenic toxin A & C of Streptococcus pyogenes → group (A) hemolytic
B - Botulinum toxin C & D
C - Cholera toxin
D - Diphtheria toxin
S - Shiga-like toxin / Verocytotoxin (produced by entero-hemorrhagic E. Coli - EHEC)

Transformation: uptake of soluble DNA fragments in the environment directly through the cell wall



Ability to transform
⇓
COMPETENCE

→ first demonstrated on Streptococcus pneumoniae by Griffith

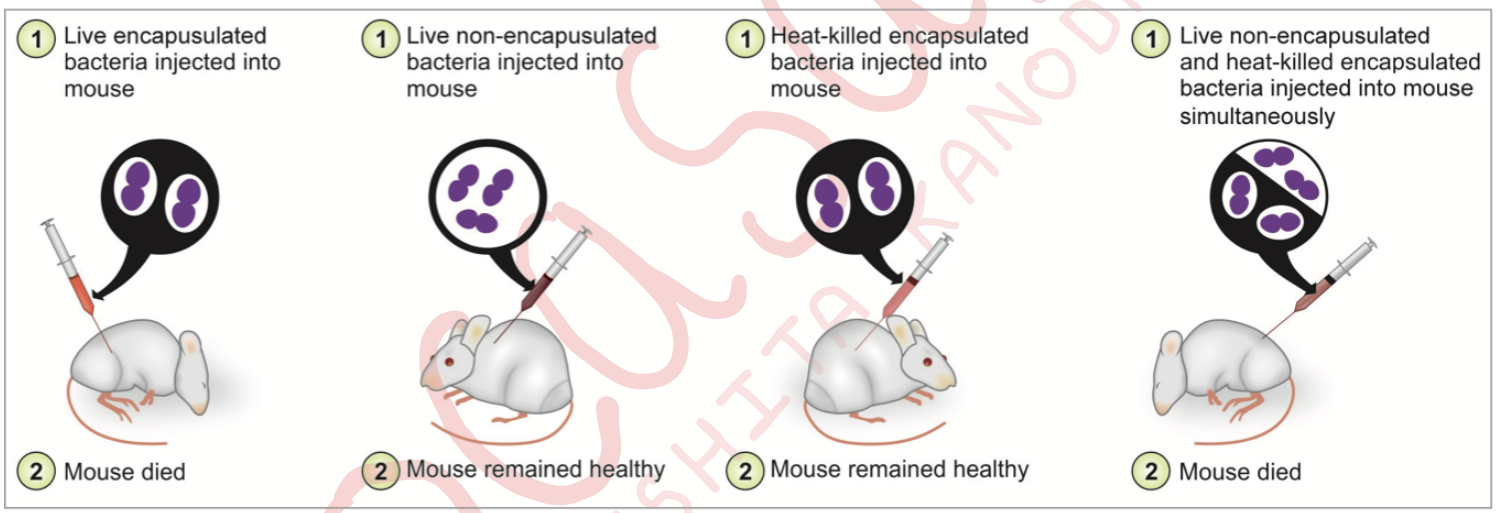


Fig. 3.4.4: Griffith experiment demonstrating transformation.

Conjugation:

→ first demonstraked by Lederberg & Tatum on E. Coli K12.

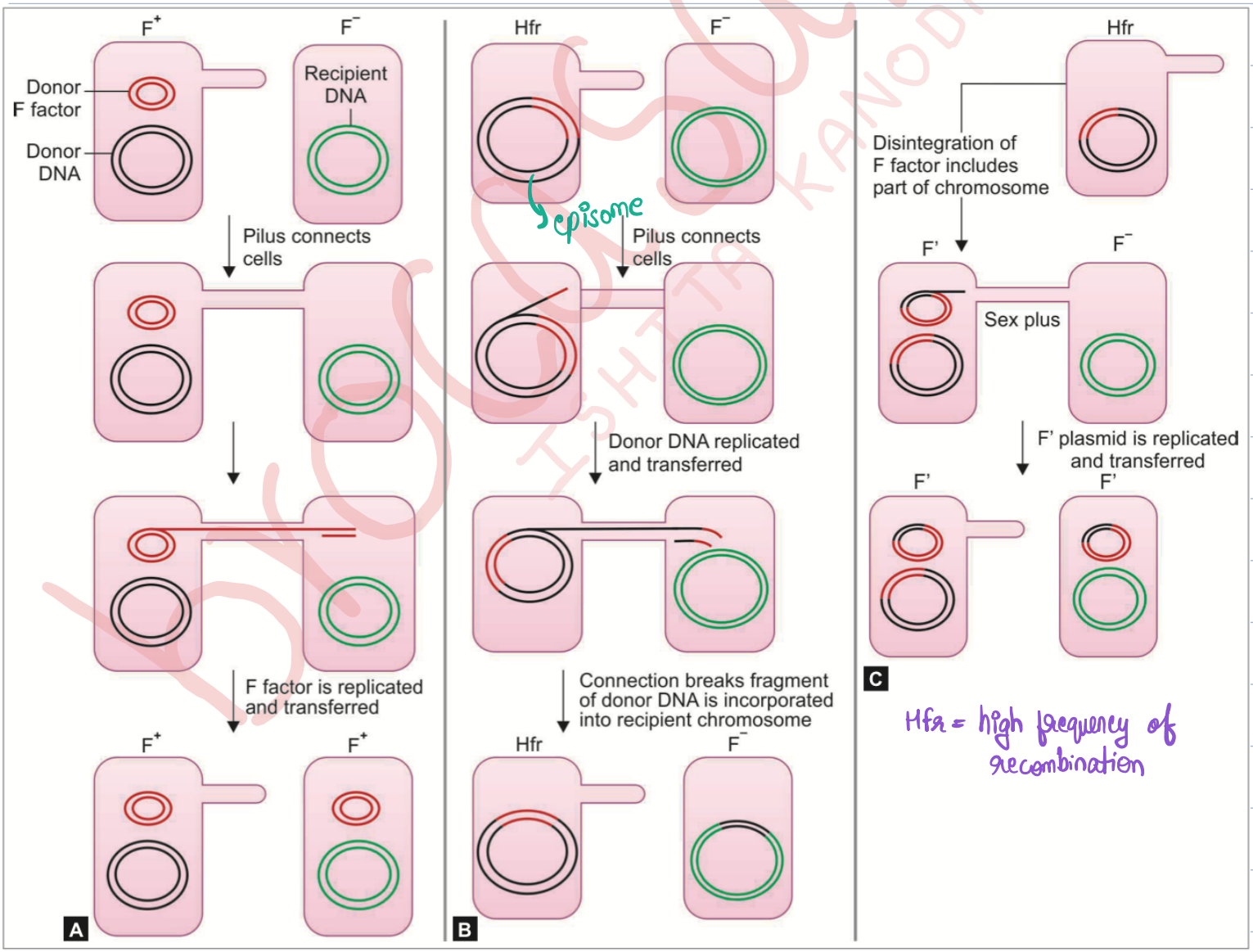
→ formation of sex pillus / conjugation tube

↓ encoded by

F - plasmid / fertility plasmid / Sex plasmid / transfer factor.

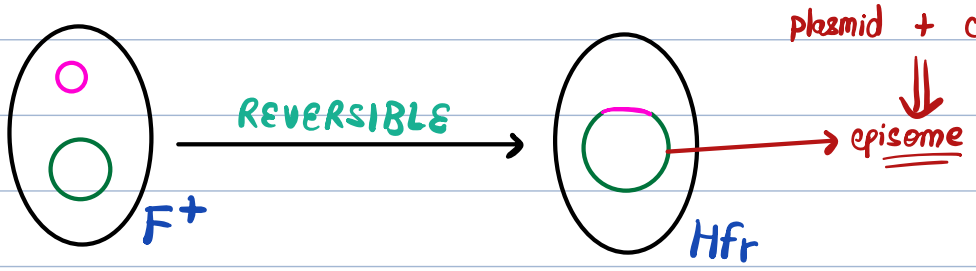
→ Bacteria which have F-plasmid ⇒ F + bacteria

" " dont " " " ⇒ F − "

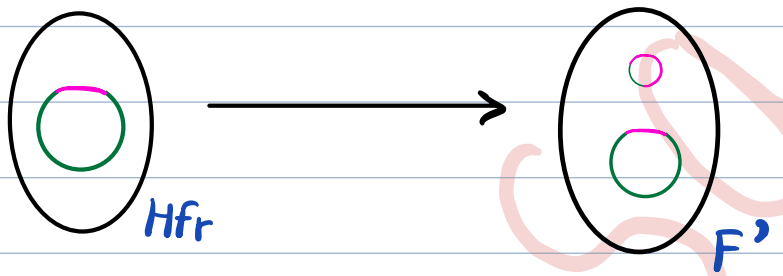


Figs 3.4.7A to C: Bacterial conjugation: **A.** F^+ X F^- mating; **B.** Hfr X F^- mating; **C.** F′ X F^- mating.

Conversion of F^+ to HfR



→ when the F plasmid reverts from integrated to free state, it may sometimes carry with it some chromosomal DNA from adjacent site of attachment
This F factor carrying some chromosomal DNA = F'



TRANSFER OF F' PLASMID = "SEX DUCTION".

R- Plasmid: F plasmid + AB resistance genes
- F plasmid → called resistance transfer factor
- AB resistance genes → called r- determinants.

→ most common mode of spread of antibiotic resistance in bacteria
→ first demonstrated in Shigella.

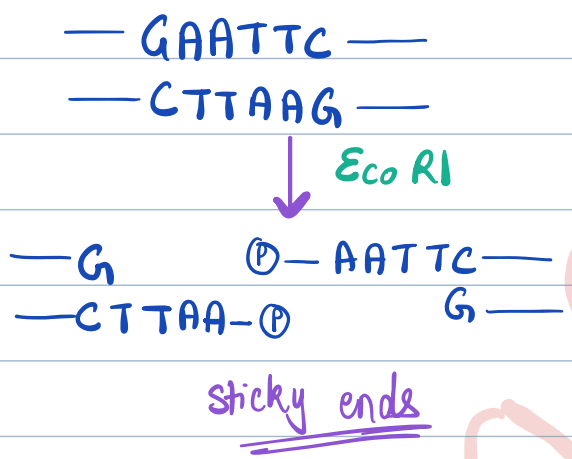
Col plasmid: F- plasmid + Bacteriocin genes

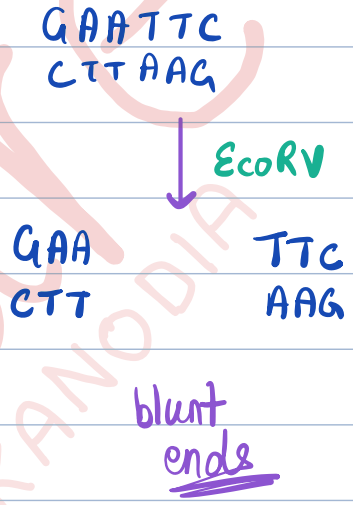
Restriction Enzymes/Endonucleases: produced by bacteria

Recognition sequence ⇒ can be palindromic or non-palindromic

Restriction site ⇒ may be a part of the recognition sequence or it may be several nucleotides away

Sticky ends
Blunt ends





→ Restriction endonuclease is a defence mechanism in bacterial systems against foreign DNA.
- But, its own DNA is protected from digestion by endonuclease due to methylation of bacterial DNA by restriction enzyme [Restriction modification systems]

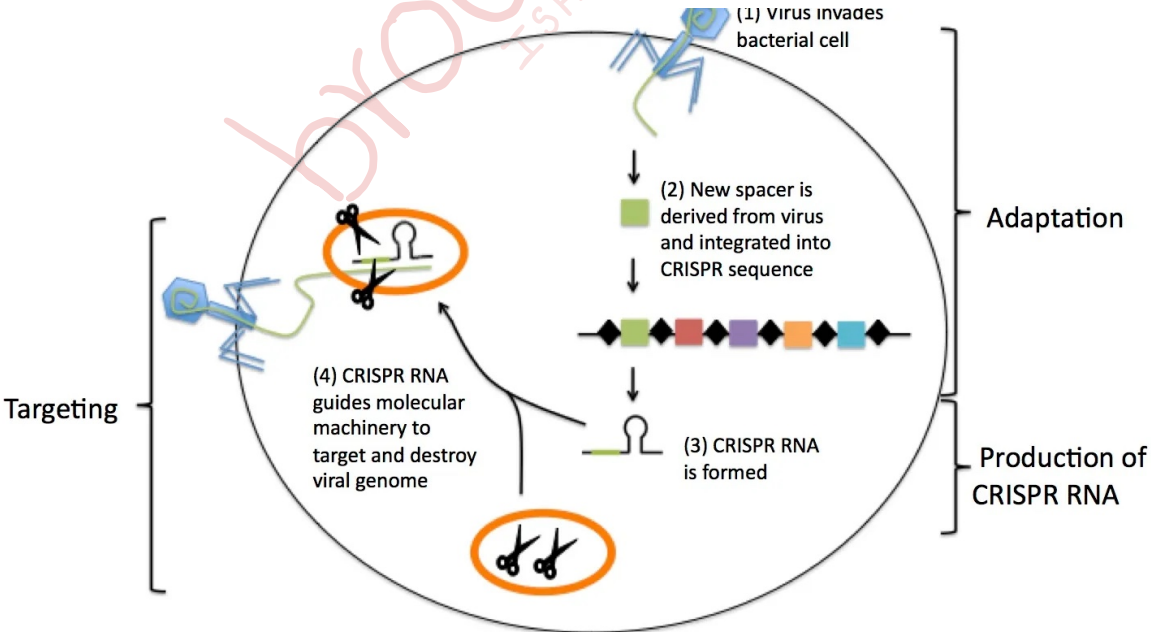
→ Type II restriction enzymes cut within recognition sites
→ all REs require Mg^{+2} cofactor.

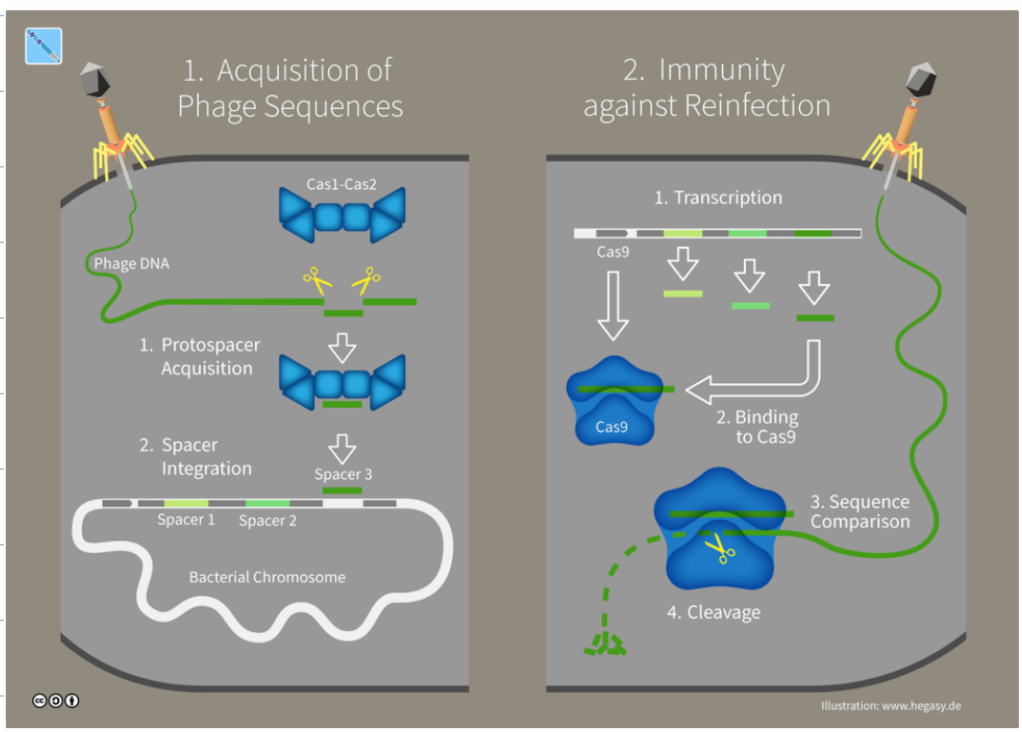
CRISPR [Clustered Regularly Interspaced Short Palindromic Repeats]:

↳ considered as an adaptive immune response of bacteria

→ bacterial genome editing method to protect from infecting viruses

R = repeat S = spacer





Typing of Bacteria: intraspecies characterization of various strains

↳ used in epidemiologic studies.

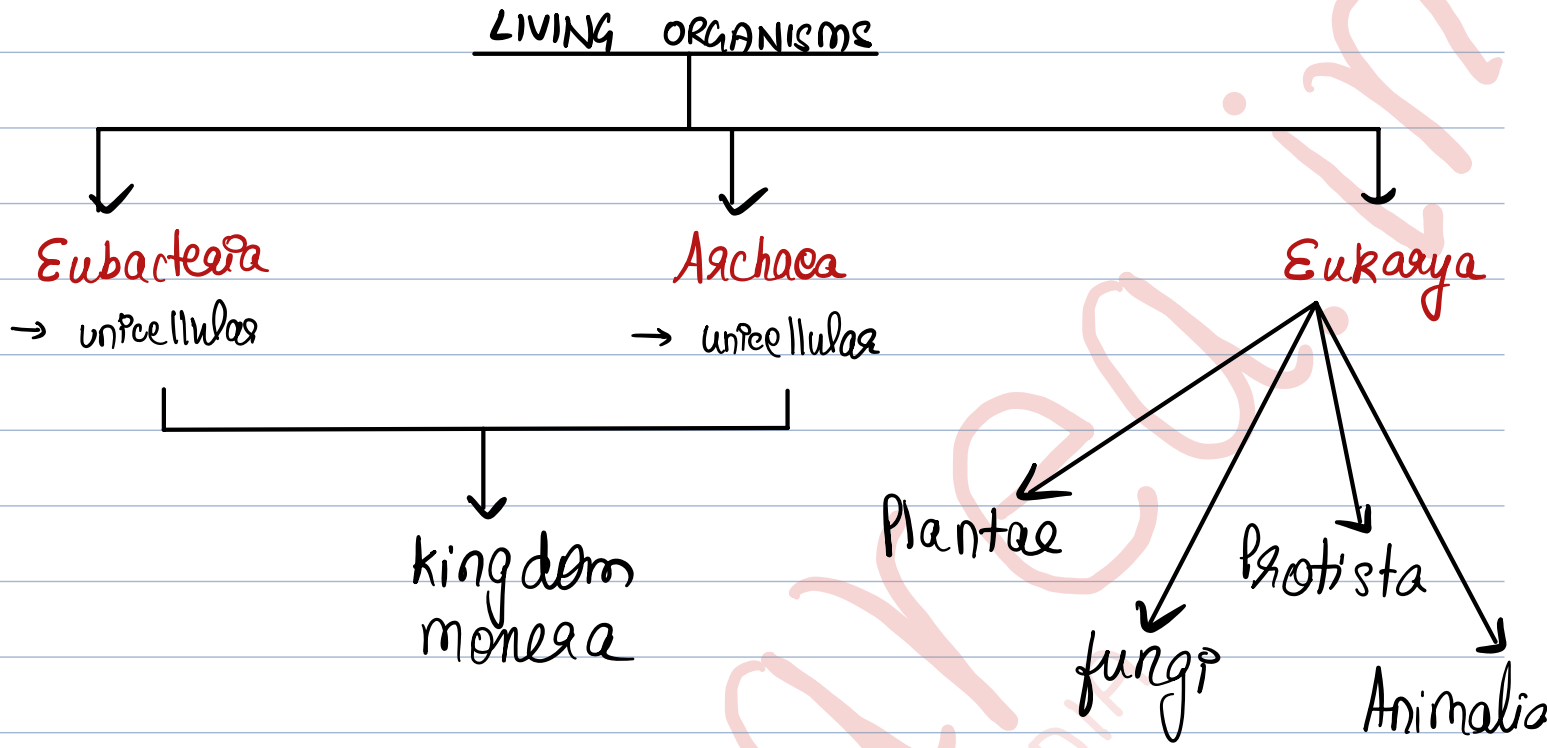
• PHOENOTYPIC METHODS

→ Serotyping (Streptococcus pneumoniae)
→ Biotyping (Hemophilus influenza)
→ Antibiogram typing
→ Bacteriocin typing
→ Phage typing
→ Whole Cell Protein Electrophoresis
→ Multilocus Enzyme Electrophoresis

• GENOTYPING METHODS

→ Plasmid profile analysis (PFGE)
→ Pulsed field gel electrophoresis
→ Restriction Fragment Length Polymorphism (RFLP)
→ Ribotyping
→ Nucleic acid sequencing
↓
Multilocus sequence typing (MLST)
⇓
only 7 house-keeping genes are sequenced out

Anatomy & Physiology of Bacteria :



	Prokaryotes	Eukaryotes
Nucleus	Nucleoid – no well defined nuclear membrane – nucleolus absent – histones absent	– well defined nuclear membrane
Chromosomes	• Single circular (ds DNA)	• Multiple linear
Extra-chromosomal DNA	plasmids	present in mitochondria
Membrane-bound organelles	mostly absent	all present

	Prokaryotes	Eukaryotes
Cell Wall	muramic acid present	No muramic acid in cell wall / complete absence of cell wall.
CELL MEMBRANE	steols absent	steols are present
Ribosomes	70s	80s
Mesosomes	present (g+ve > g-ve).	absent

EXCEPTIONS:

→ Prokaryote lacking cell wall is Mycoplasma
∴ highly pleomorphic Known as jumping jokers of microbio

→ Prokaryotes having steols in cell membrane : Mycoplasma

→ Prokaryotes having 2 chromosomes ⇒ Vibrio

→ Prokaryote that lacks muramic acid in its cell wall: Chlamydia.

Archaea: non-pathogenic bacteria

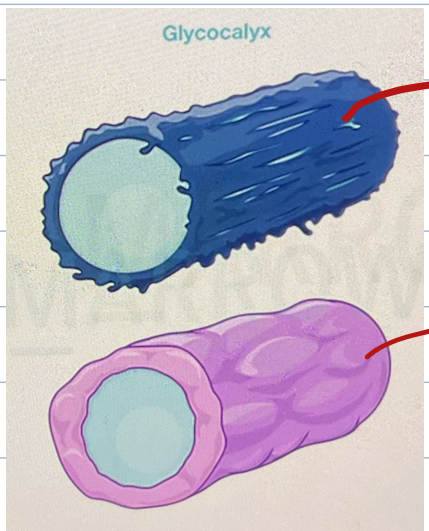
a.k.a Extremophiles (can survive extreme conditions)

Ex: Thermus aquaticus.

Eubacteria	Archaea
→ peptidoglycans in cell wall are present	→ absent
• absent	• presence of isoprenoid lipids in cell membrane (helps them survive in extreme conditions)
→ introns are always absent	→ introns are present in some genes
• absent • Eubacteria • Cyanobacteria (blue-green algae)	• characteristic rRNA sequences present

Glycocalyx: layer around the cell wall

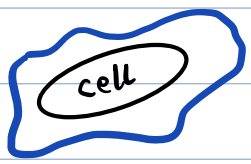
→ may or may not be present



→ Loose & flowy ⇒ Slime

→ well defined ⇒ Capsule

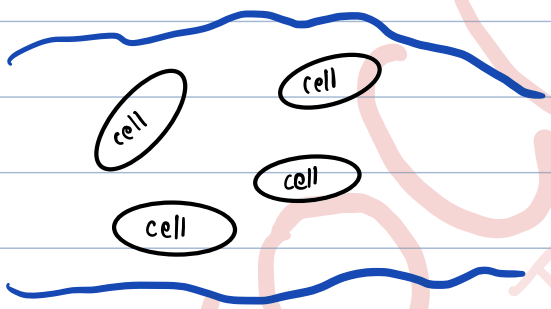
Slime: loose, ill-defined polysaccharide layer



→ group of bacteria growing together ⇒ slime collects & forms

⇓

BIOFILMS

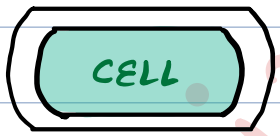


↳ Biofilm

- helps in adhesion
- anti-phagocytic
- reduces the entry of antibiotics

Eg:
- Streptococcus mutans
- Pseudomonas aeruginosa
- Staphylococcus epidermidis

Capsule: well-defined layer around the cell wall



→ generally polysaccharide in nature

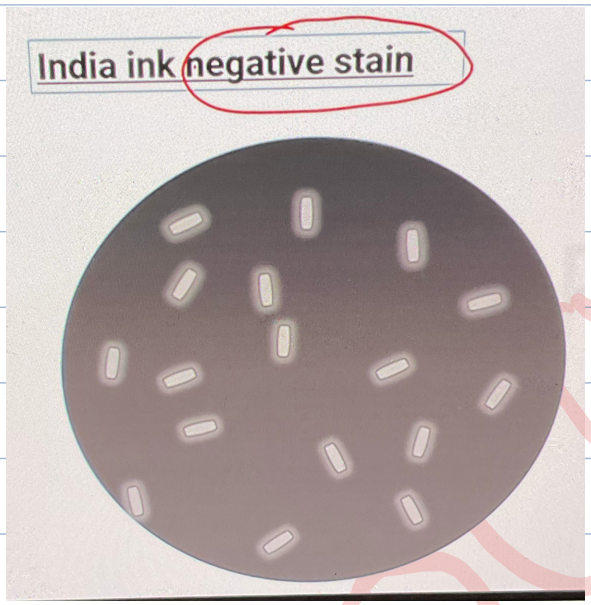
EXCEPT • Bacillus anthracis } polypeptide capsule
• Yersinia pestis }

→ capsule is anti-phagocytic

→ it has no net charge

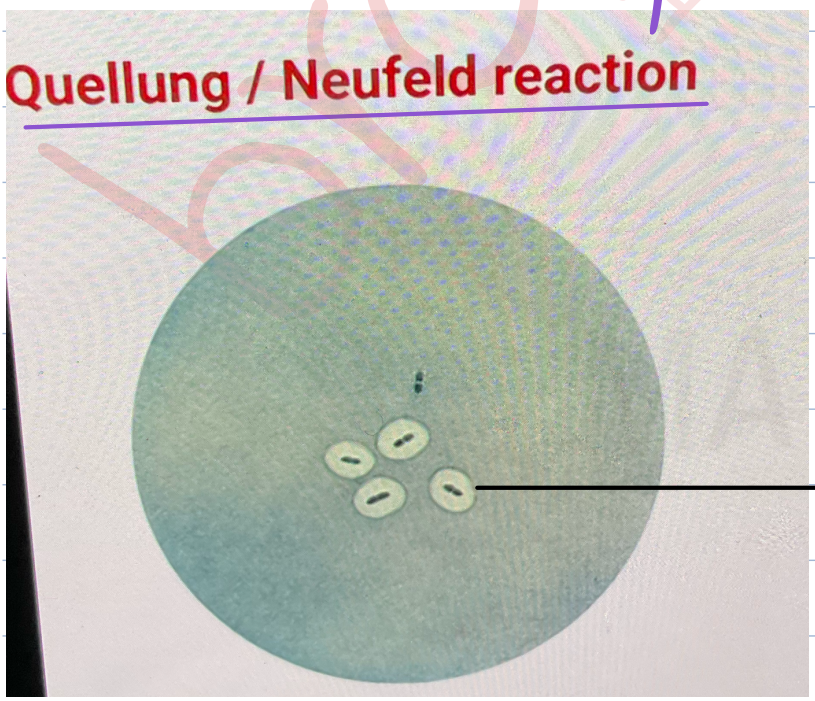
∴ cannot be gram stained

→ Capsule stains: Copper salts



(India ink or Nigrosine)

→ used for capsular typing of bacteria.



Capsule is antigenic

∴ it produces Ab's

Capsule + Anticapsular Ab's

⇓

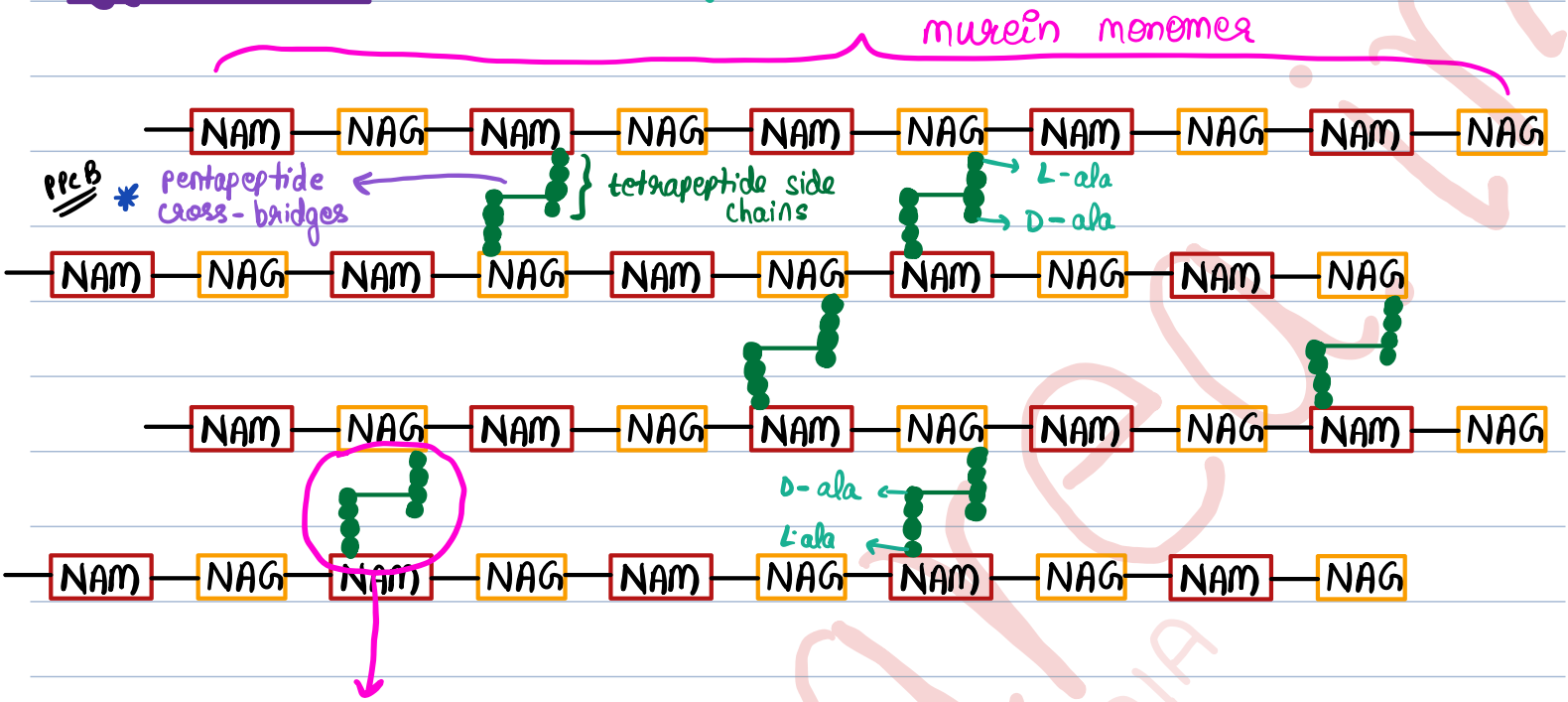
→ Swelling of capsule

Yes SSome BBacteria Have Very Killer And Mean Ccapsules

- Yersinia pestis ⇒ polypeptide capsule
- Streptococcus pneumoniae
- Staphylococcus aureus ⇒ microcapsule (very thin), zwitter ionic
- Bordatella pertussis ⇒ Non-antigenic capsule
- Bacteroides fragilis ⇒ zwitter ionic capsule
- Haemophilus influenzae
- Vibrio parahemolyticus
- Klebsiella pneumoniae
- Bacillus anthraccis ⇒ polypeptide capsule
- Meningococcus
- Clostridium perfringens ⇒ most common cause of gas gangrene
- Cryptococcus (fungus) — neoformans, gattii

Cell Wall:

NAG = N-acetyl glucosamine
NAM = N-acetyl muramic acid



CROSS-LINKING ENZYMES IN BACTERIA:

- Transpeptidases
- Transglycolases.

→ all β-lactam antibiotics bind to transpeptidases

↓

inactivation of transpeptidases

↓

no-cross linking

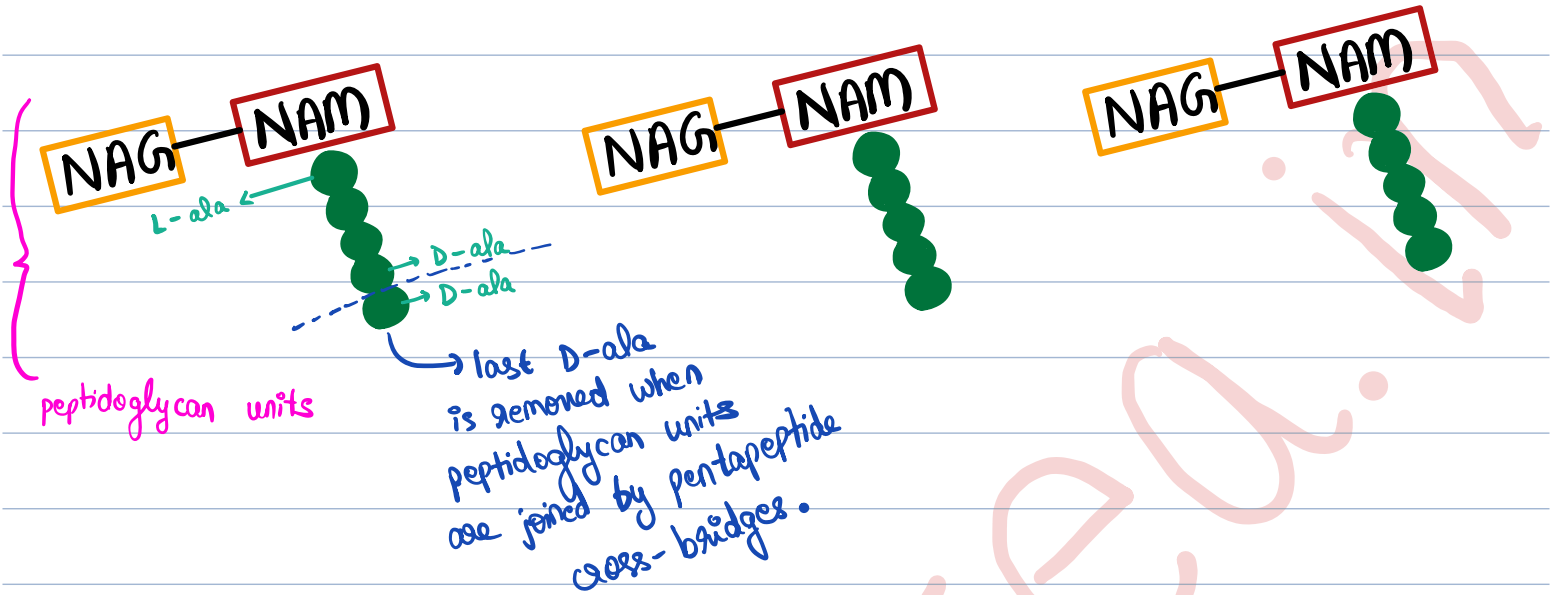
↓

impairment of cell wall synthesis

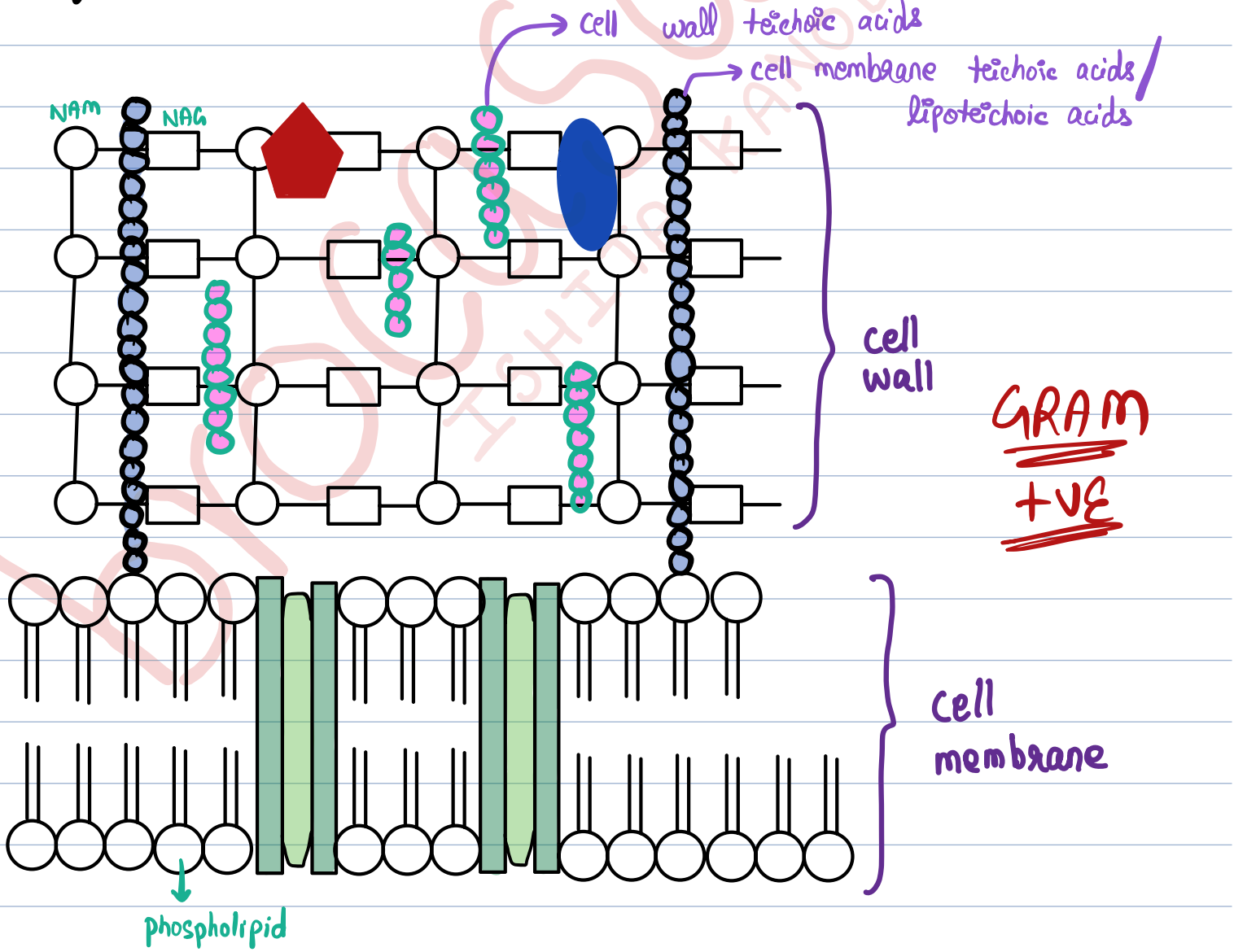
aka Penicillin Binding Proteins. (PBPs)

→ gram +ve cell wall has 50-100 layers of murein monomers

(* between 3rd ā.ā from one chain & 4th ā.ā from another
[NAM/NAG] [NAG/NAM])



Vancomycin: binds to terminal D-ala D-ala & prevents formation of PPCBs (cell wall precursor)

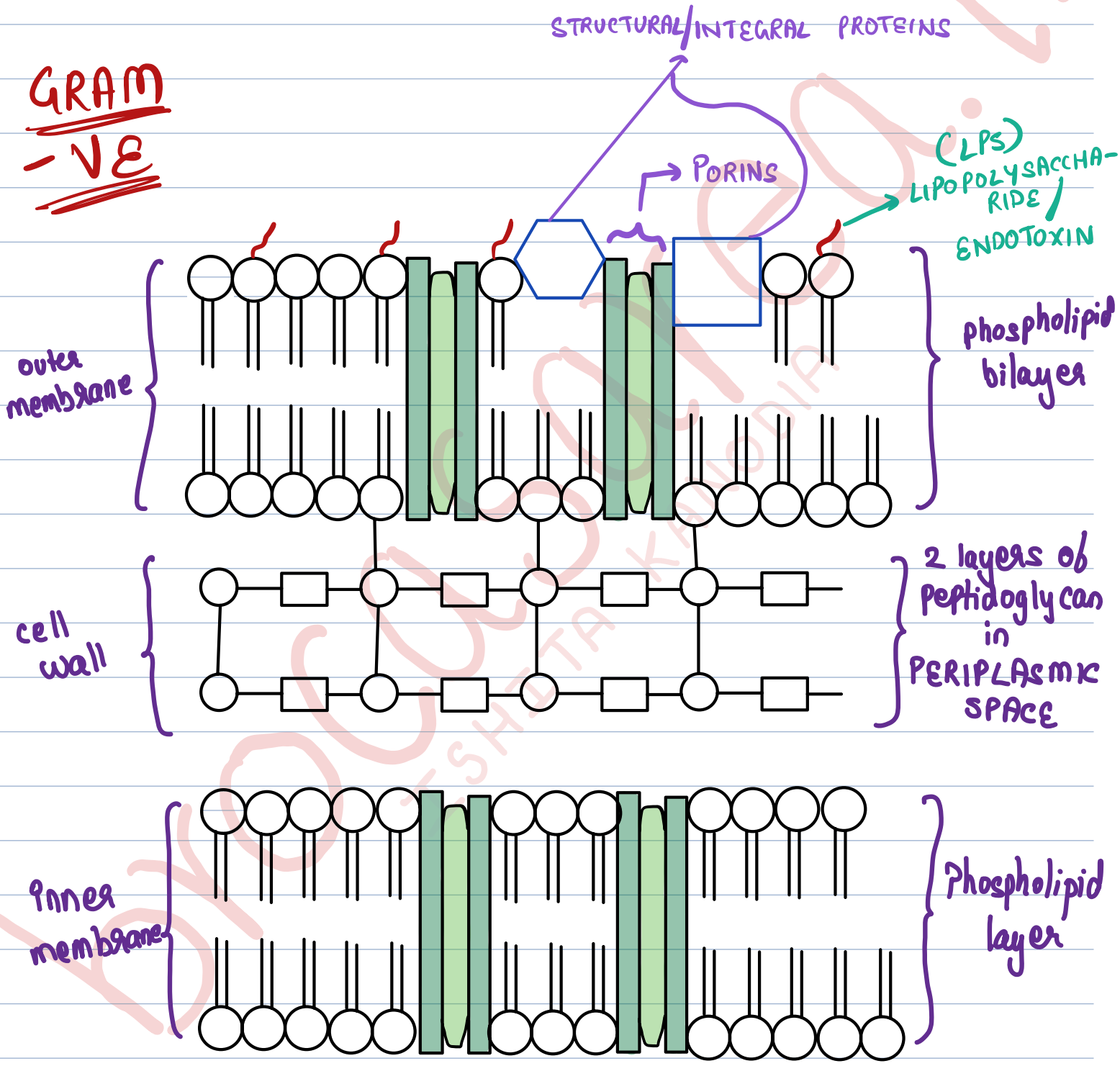


→ Cell wall teichoic acids : polymers of ribitol phosphate.

→ cell membrane teichoic acids : polymers of glycerol phosphate.

→ Teichoic acids are said to have a role in adhesion

GRAM -VE



→ Just above the phospholipid bilayer (cytoplasmic membrane) is a space known as the periplasmic space which contains the two layers of murein monomers linked by PPCBs

→ Outside this, is another phospholipid bilayer called outer membrane

→ In the outer membrane, there are some special structures:

i) Porins

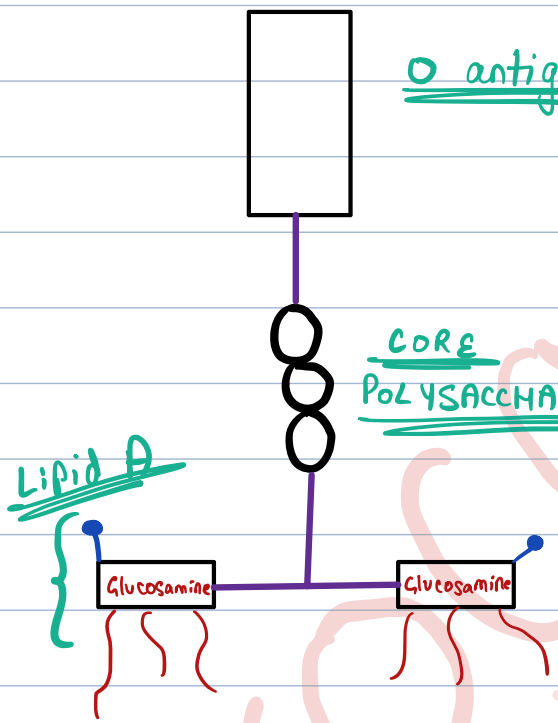
ii) Structural / integral proteins

iii) Lipopolysaccharide (LPS) / Endotoxin → embedded in the outer leaflet of the outer membrane.

	Gram +ve cell wall	Gram -ve cell wall
Thickness	~ 80 nm	10 - 25 nm
Layers of peptidoglycan	50 - 100	2 (in periplasmic space)
Amino acids in peptidoglycans	Aromatic & sulphur containing ā.ā absent.	All ā.ā present
Teichoic acids	Present	Absent
Outer membrane	Absent	Present
Lipid content	5 - 10%	30 - 50%

	Gram +ve cell wall	Gram -ve cell wall
Porins	Absent	Present
LPS (Endotoxin)	Absent Except : Listeria monocytogenes	Present

LPS / Endotoxin:

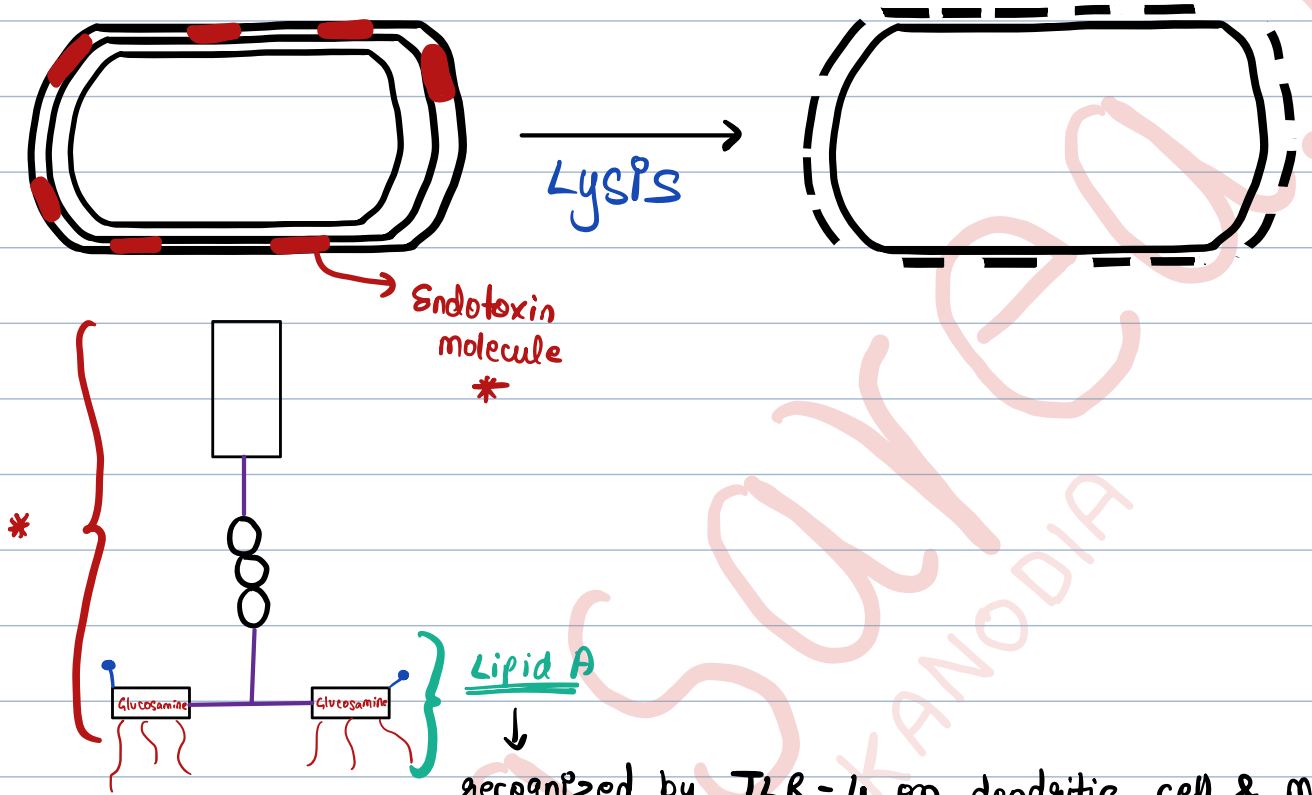


O antigen → polysaccharide
→ most variable part of LPS molecule

CORE POLYSACCHARIDE
- short chain of 6–10 $\bar{a}\cdot\bar{a}$
- contains a unique $\bar{a}\cdot\bar{a}$ ⇒ KDO [keto-deoxy octanoic acid]

Lipid A: embedded in outer membrane
↳ responsible for the actual endotoxic activity

Action of Endotoxin



↓

recognized by TLR-4 on dendritic cell & macrophages

⇓

Binding

⇓

Activation of Nuclear Transcription factor - kβ

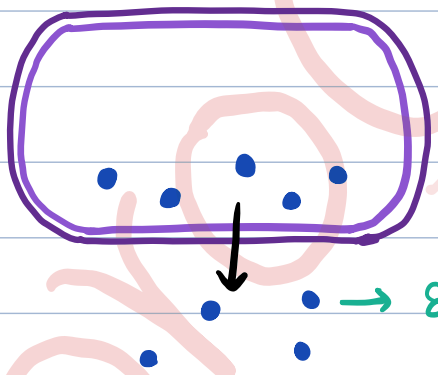
⇓

cytokine release from dendritic cell & macrophages

[IL-1,6 ; TNF-α ; IL-8,12]

Effects of Endotoxin

- Macrophage activation
  - Cytokine release from macrophages, dendritic cells
    - Fever, hypotension
- Complement Activation
  - C3a & C5a
    - Histamine release
      Neutrophil chemotaxis
      Increased vascular permeability
- Tissue factor activation
  - Activation of coagulation
    - DIC, Shock [disseminated intravascular coagulation]



→ Exotoxin

proteins in nature

Eg: Botulinum toxin,
Diptheria toxin,
Tetanospasmin

Endotoxin	Exotoxin
→ LPS	→ Protein
→ Released only on lysis	→ actively secreted Except: Botulinum
→ unique to gram -ve bacteria Except: LISTERIA	→ produced by both gram -ve & +ve
→ Heat stable	→ Heat labile Except: Staph. aureus & bacillus cereus emetic toxins
→ low antigenicity	→ highly antigenic.
→ cannot be toxoided.	→ can be toxoided. (toxin which has lost its virulence, but can still induce antibodies)
→ needs to be present in large amounts to mediate their action in humans.	→ even small amounts can mediate their action.
→ CONSTANT EFFECTS	→ VARIABLE EFFECTS in humans.

Limulus Amebocyte Lysate (LAL) Assay:

Limulus → Horse-shoe crab

Amebocyte Lysate + drop of clinical specimen (endotoxin) ⇓ gelling of lysate

→ sensitive assay for endotoxin (LPS) assay.

L forms: cell wall deficient bacteria

→ first discovered by Kleinberger Nobel at Lister Institute on *Streptobacillus moniliformis*.

→ can be formed by both Gram +ve & -ve bacteria

→ they are either formed spontaneously or in the presence of cell wall inhibitors (like penicillin) or in the presence of lysozyme.

break the bond b/w NAM & NAG.

- Gram +ve + lysosyme (in osmotically protected medium) → PROTOPLAST (L-form of gram +ve bacteria)

- Gram -ve + lysosyme (in osmotically protected medium) + EDTA → Spheroplasts. (L-form of gram -ve bacteria)

→ L-forms can be cultivated on special media on which they form TINY COLONIES

→ Non-pathogenic

→ Two types:

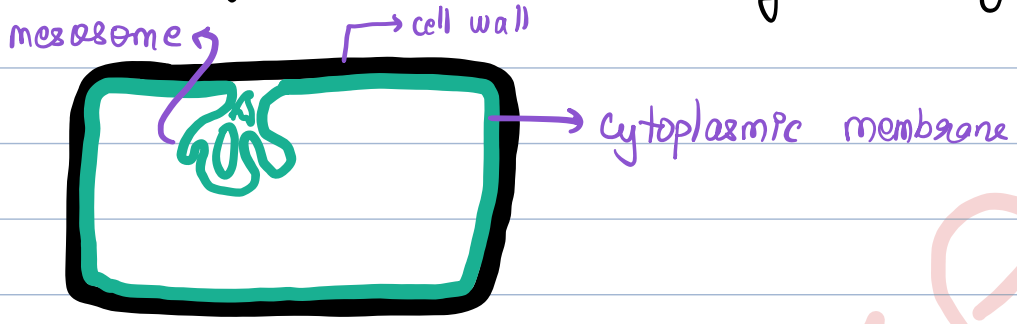
- Stable ⇒ remain cell wall deficient for the rest of their lives
- Unstable ⇒ can revert back to their cell-walled status

→ Can be responsible for persistence of infections & recurrence/relapse of infections.

Mesosomes: invaginations of cytoplasmic (cell) membrane

↳ sites for respiratory enzymes

→ site at which bacterium undergoes binary fission.



→ gram +ve have more mesosomes than gram -ve.

Flagellum:

→ helps the bacteria in locomotion

→ composed of protein subunits called flagellin.

→ highly antigenic
- Flagellum antigens = H-antigen
- corresponding Ab = H-Ab.

→ Length: 3 - 20 μm

→ Thickness: 0.01 μm

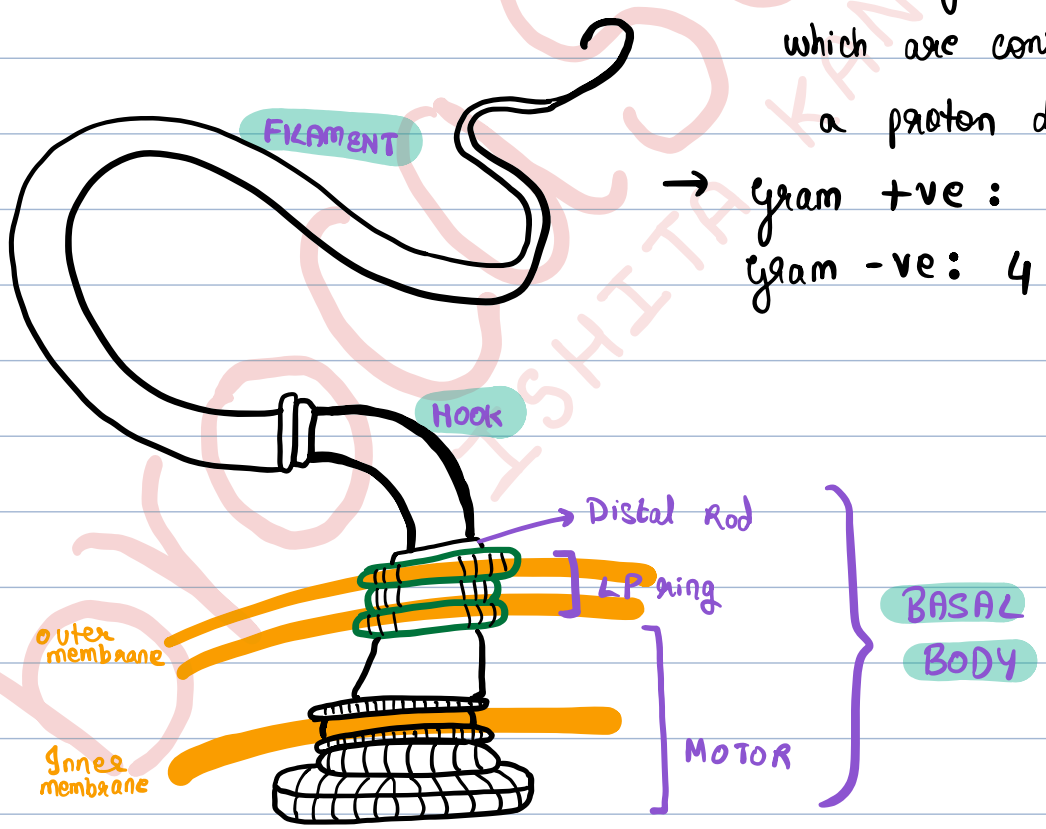
∴ flagella are too slender to be seen by light microscopy.

Flagellum Structure:

→ Basal body is made up of rings which are constantly rotated by a proton dependent pump.

→ gram +ve: 2 rings → M, S

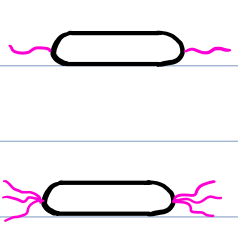
gram -ve: 4 rings → M, S, P, L



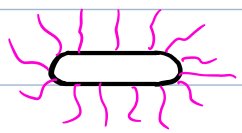
Flagellum Distribution:

→ MONOTRICHUS • Vibrio • Pseudomonas

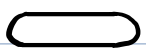
→ LOPHOTRICHUS • Helicobacter • Campylobacter (sometimes)



AMPHITRICHUS
- COMPYLOBACTER
- SPIRILLUM



PERITRICHUS
form only at room temperature (25-28°C)
- Enterobacteriaceae
- Bacillus
- Clostridium
- Listeria



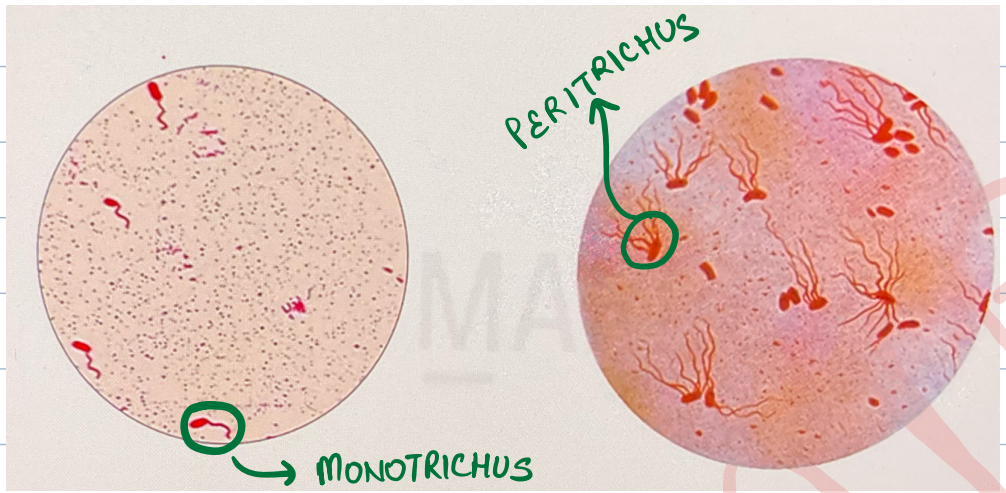
ATRICHUS

- All pathogenic cocci are atrichus.

Endoflagella: flagella in periplasmic space
- Spirochetes

Flagellum Demonstration:

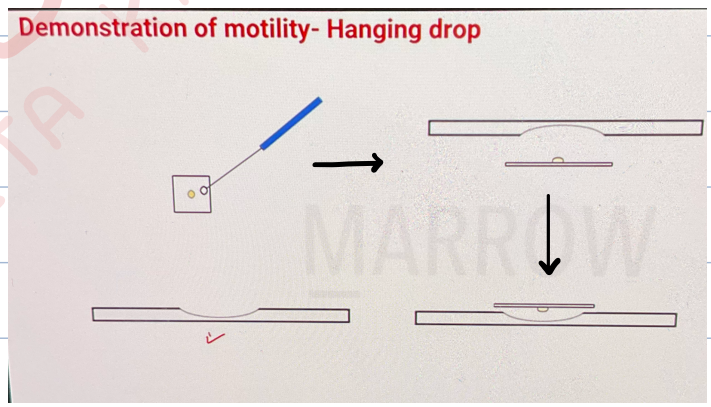
Direct Methods:
- Electron microscope
- Leifson & Ryu's impregnation stain

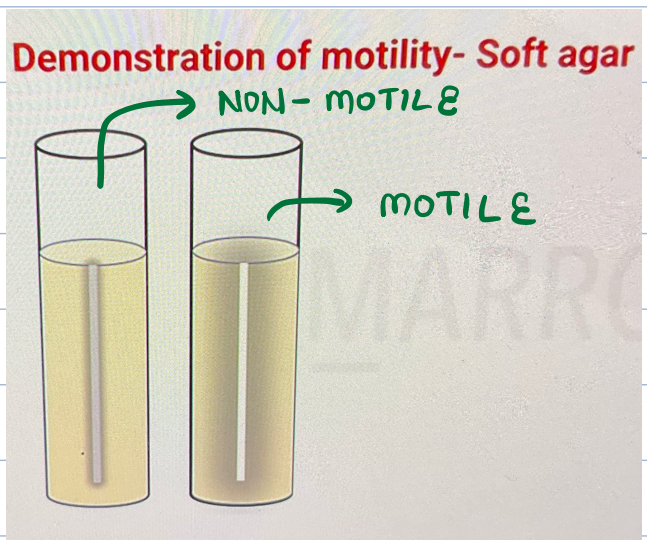


Indirect Methods: • Checking motility

Demonstration of Motility: [Methods]

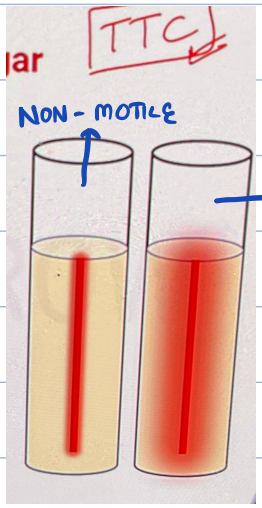
1. Wet mount
2. Hanging drop preparation
3. Soft Agar Medium. (0.2-0.5% agar)
   ⇓
   stab inoculate
   → addition of TTC *

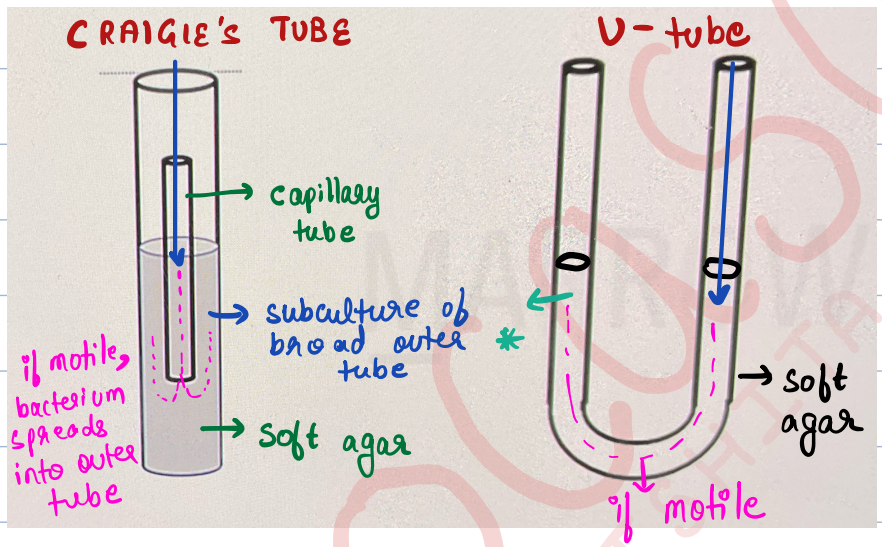




* TTC [Triphenyl tetrazolium chloride]

↳ changes colour when it comes across bacterial metabolites.

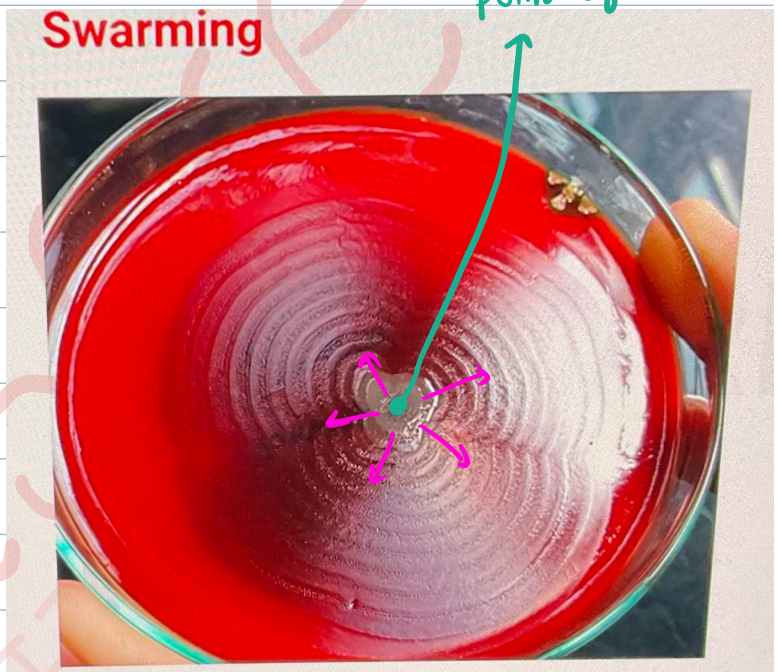




* Subculture from opposite end after overnight incubation

Types of Motility :

- Corkscrew : Treponema pallidum
- Darting / Shooting Star : • Vibrio • Campylobacter
- Gliding : Mycoplasma
- Stately : • Salmonella • Clostridium
- Tumbling / End-on-end : Listeria
- Twitching : Eikinella corrodens.



Swarming : phenomenon seen on blood agar plate

→ Swarming can be prevented by the use of FIRM AGAR (5-6% agar).

Examples :

Gram +ve : - Clostridium tetani
- Bacillus cereus

Gram -ve : - Proteus vulgaris
- Proteus mirabilis
- Vibrio alginolyticus
- Vibrio parahemolyticus.

Differential Motility :

→ motile at room temperature (25 - 28°C)

→ non-motile at 37°C

- Listeria
- Yersinia enterocolitica
- Yersinia pseudotuberculosis

Pili / Fimbriae :

→ made of repeating protein subunits : Pillin

→ shorter than flagella

① **Common Pili :** help in adhesion

↳ present only on gram -ve bacteria

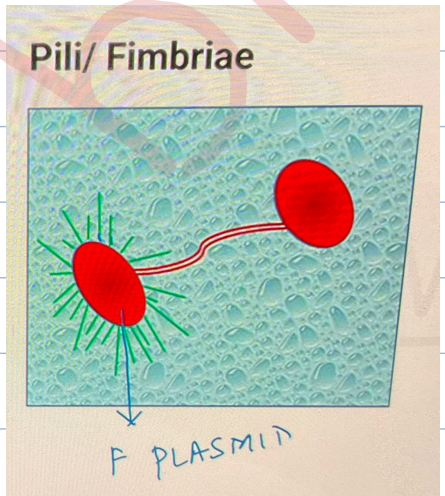
→ 1 - 1.5 μm long

→ 0.01 μm broad

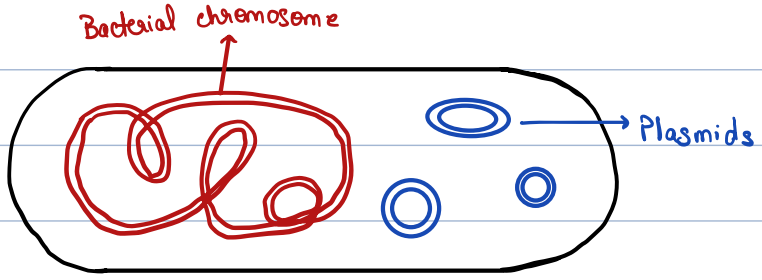
- Type IV pili in bacteria give them flagella-independent twitching motility
  - Neisseria
  - Pseudomonas.

② **Sex Pili / Fimbriae :** mediate conjugation

→ formed by both gram +ve & gram -ve bacteria, provided, they have the F-plasmid genes ['tra' genes].



Plasmids: extrachromosomal circular ds DNA molecules

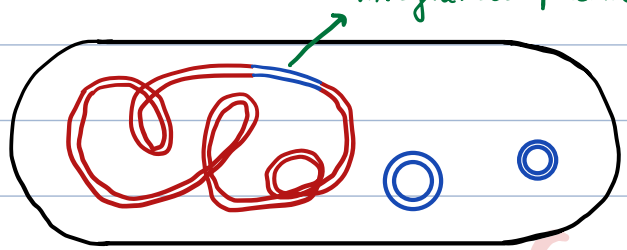


→ 1 - 40 plasmids / cell

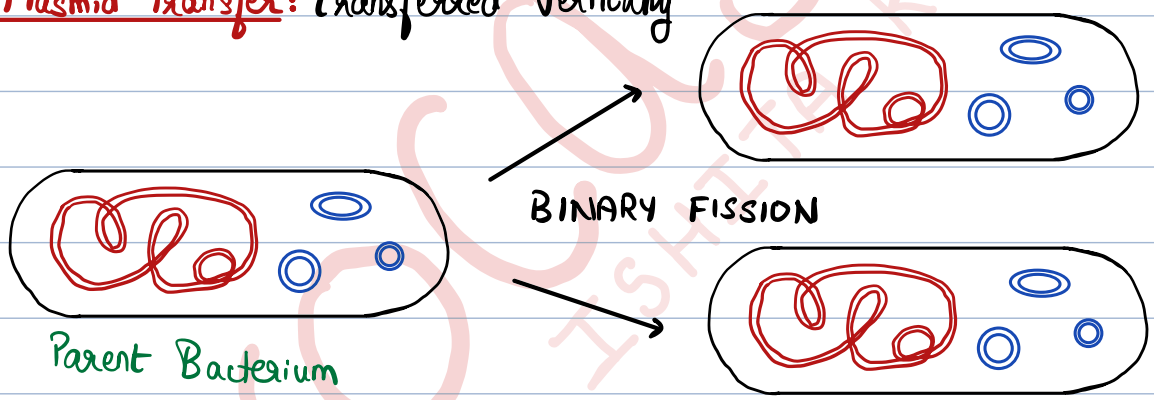
→ Plasmids are not essential for the life of the bacterium

→ Plasmids replicate independent of the chromosomes.

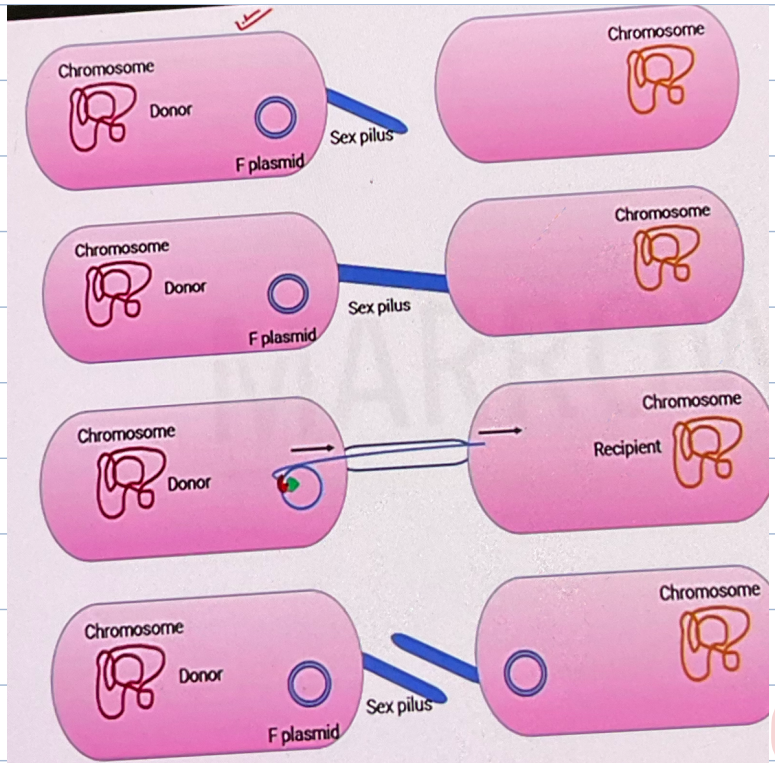
Plasmid - Episome: plasmid integrated with chromosome



Plasmid Transfer: all plasmids transferred vertically



Some plasmids are transferred horizontally: through sex pillus



→ those bacterium that have 'tra' gene (which encode sex pilli) can transfer plasmids horizontally.

Bacteriocin: Ab like small proteins which kill related bacteria.
(→ provide survival advantage to certain bacteria)

- COLICIN
- KLEBOCINS
- PYOCINS

Plasmid Types: according to mode of transferred

Conjugative: → transferred vertically & horizontally

- F plasmid (fertility)
- R plasmid (fertility + Ab resistance genes)
- Col plasmid (fertility + bacteriocin coding genes)

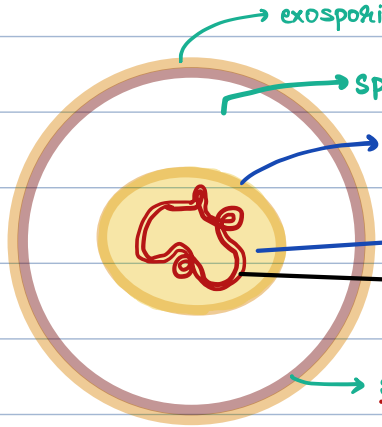
Non-conjugative: transferred vertically only

Plasmid Types: according to the genes present.

- **Virulence:** encode special virulence factors like endotoxin secretion, capsule, etc.
- **Resistance:** " Ab resistance genes.
- **Fertility:** " formation of sex pilli.
- **Metabolic:** " enzymes to metabolize special substrates.
- **Col plasmids:** " bacteriocein production.

Spore / Endospore : (helps in tiding over adverse conditions)

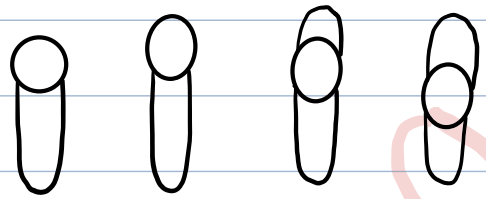
→ bacteria have the ability to form spores under environmental stress.
(eg: extreme heat / nutrient depletion / dryness)



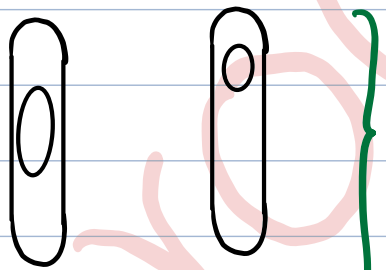
→ contains dipicolenic acid
⇓
makes the spore heat-resistant

→ Spore coat: contains a keratin like protein which resists chemical disinfection

2 pathogenic genera which form spores :



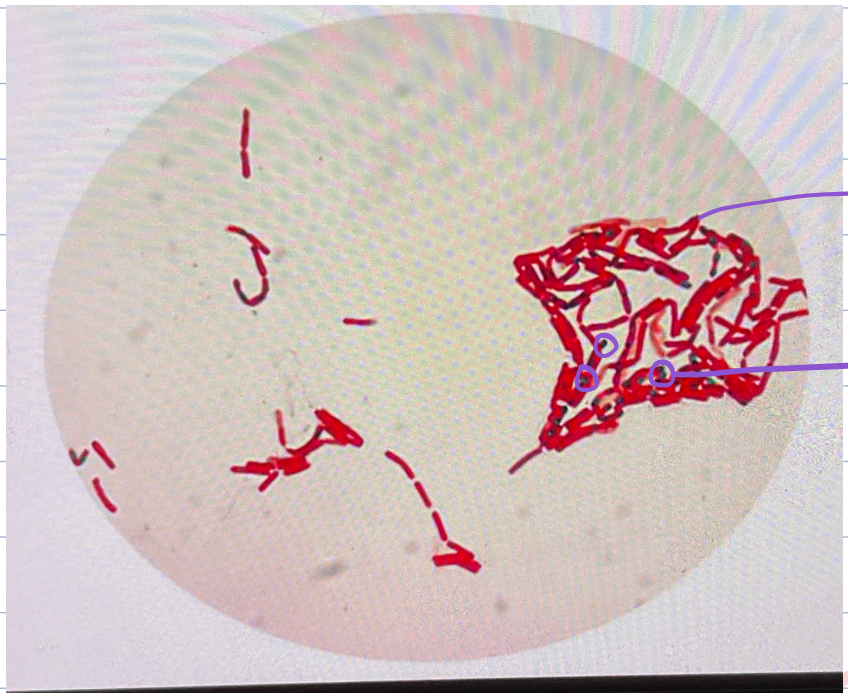
Bulging spores of • CLOSTRIDIUM
– Spores form in soil, culture & human body
Except: Clostridium perfringens
[causes gas gangrene]



• BACILLUS (Non-bulging)
→ Spores form only in soil & culture (not in human body)

Demonstration of Spores: spores do not take up grams' stain.

↳ stained by • Ashby Stain
• schaeffer Fulton Stain



bacteria (cytoplasm) stained by safranine

Spores stained by malachite green.

Bacterial Physiology :

Oxygen Requirements :

STRICT AEROBES

→ Need oxygen to grow

Nagging Pests MMust BBreathe For Life

- Nocardia
- Pseudomonas aeruginosa
- Micrococcus
- Mycobacterium tuberculosis
- Brucella
- Bordatella
- Francisella
- Leginella

STRICT ANAEROBES

→ cannot survive in the presence of oxygen ∵ they lack enzymes Catalase, peroxidase & superoxide dismutase

Losers Choke By Air

- Lactobacillus
- Clostridium
- Bacteroideae
- Actinomyces
- Porphyromonas
- Prevotella
- Bifidobacterium
- Eubacterium

FACULTATIVE ANAEROBES

→ aerobic organisms that are capable of switching to anaerobes in the absence of oxygen.

- Staphylococci
- Streptococci
- Enterobacteriaceae
- Corynebacterium
- Hemophilus

Aerotolerant : anaerobes but can grow in presence of small amounts of O_2

Microaerophilic : require oxygen, but in small amounts (2-8% oxygen)

- Campylobacter
- Helicobacter
- Mycobacterium bovis
- Cultivable spirochetes

pH Requirements:

Acidophiles
→ grow < 6
- Lactobacillus

Mesophiles / Neutrophiles
→ grow between 6-8 pH
[optimum pH: 7.2 - 7.4]
- most pathogenic bacteria

Alkaliphiles
→ grow b/w 8-10 pH
- Vibrio
- Alkaligenes

Temperature Requirements:

Thermophiles
→ best grow b/w 55 - 80°C
- Thermus aquaticus
  Taq polymerase for PCR

Mesophiles
- most pathogenic bacteria
→ grow best between 20 - 40°C
[optimum temp: 35 - 37°C]

Psychrophiles
→ Best grow < 20°C.

Psychrotrophs
→ best grow b/w 20 - 40°C
→ but they can grow < 20°C.
- Listeria
- Yersinia

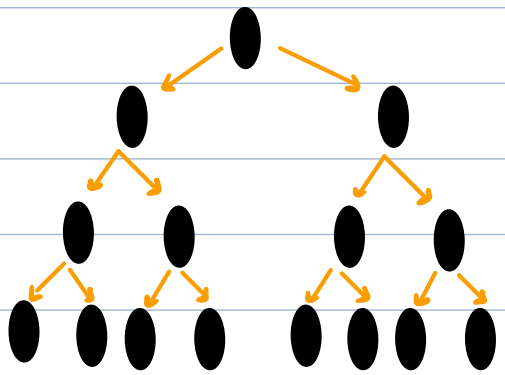
} method of cold enrichment is used to isolate them.

Bacterial Growth Curve: Plotting no. of viable cells/mL periodically

→ bacteria divide by binary fission.

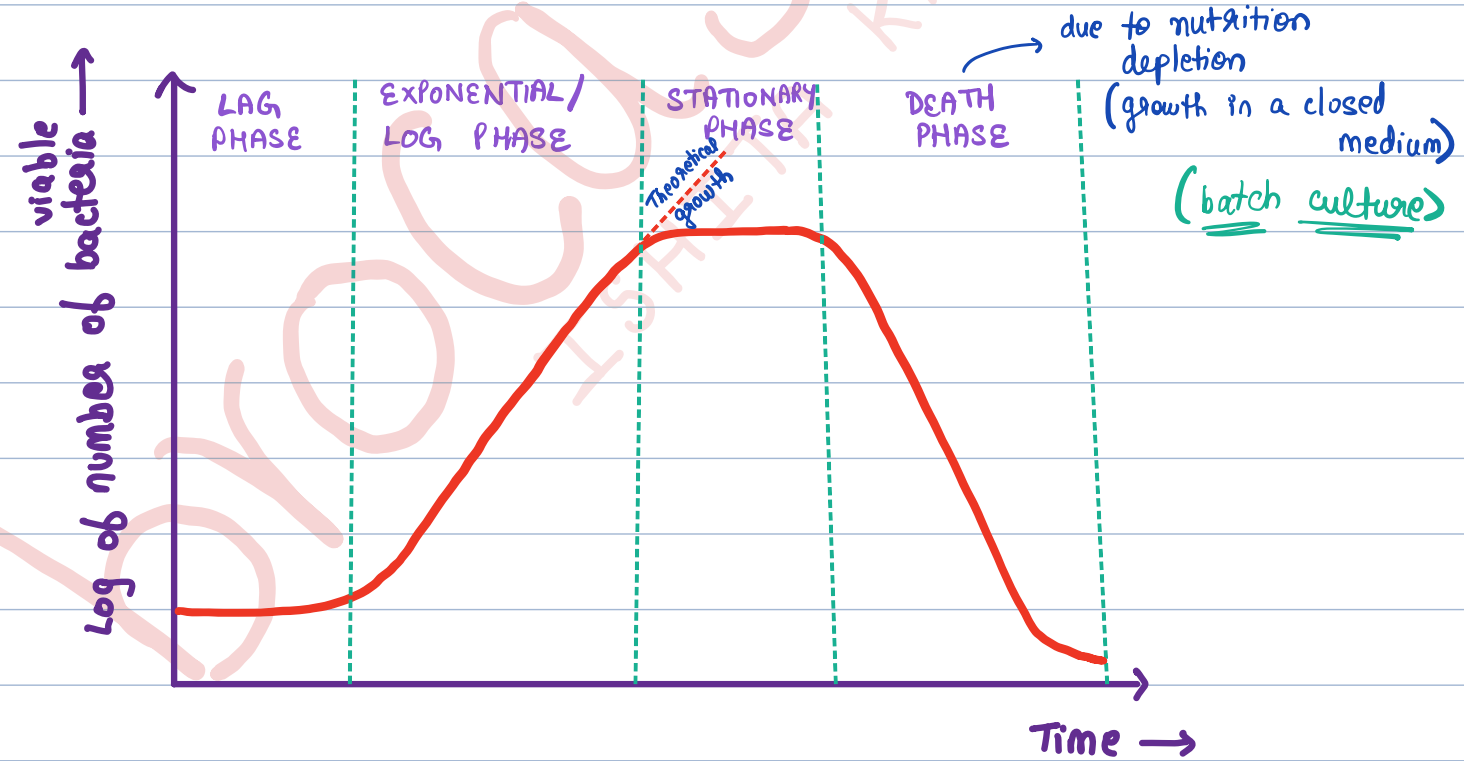
→ After n cycles of binary fission starting from a single bacterium,
no. of bacteria produced = 2^n.

Generation Time (Population Doubling Time): Time required for one binary fission



GT of:

- E. coli = 20 min
- M. tb = 20 hrs
- M. leprae = 20 days



Lag Phase: stage of adaptation

→ bacteria are metabolically active

→ no replication occurs

→ viable count & total count are constant.

→ size of bacterium is maximum just at the end of lag phase

→ variable for different bacterium.

Exponential / Log Phase: stage of active binary fission

→ stage of exponential increase in number of bacteria (2^n)

→ Increasing viable count & total count

→ size of bacterium ⇒ small

→ stains uniformly

→ Adding Ab's is going to have maximal effect.

→ Metabolically most active phase.

Stationary Phase: gradual nutrient depletion & collection of toxic metabolites

→ No. of bacteria multiplying = No. of bacteria dying

→ Viable count: constant

→ Total count: increasing

→ Sporulation occurs

→ Ab & exotoxins are secreted by bacteria.

Death / Declining Phase: total nutrient depletion

→ no further replication → involution forms

→ bacteria are dying

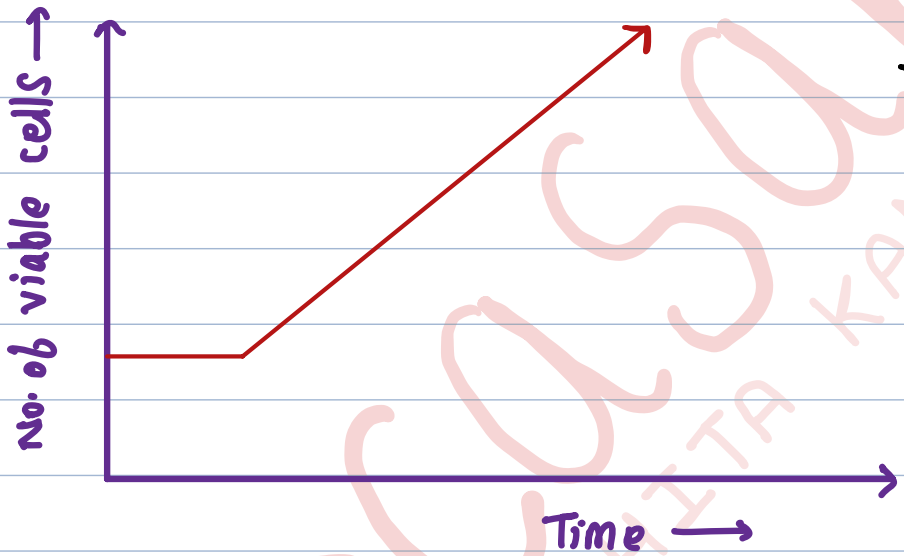
→ Viable count: falls Total count: constant

Batch Culture:

→ a closed system where all the medium components are placed at the start of cultivation

→ Microorganisms go through 4 phases
- Lag phase
- Log phase
- Stationary phase
- Death phase

Continuous Culture:



→ fresh nutrients are added & toxic metabolites are removed at regular intervals.

Quorum Sensing: a process that allows the bacteria to coordinate their gene expression according to the density of the population.

→ first demonstrated in Vibrio fischeri (light emitting)