KARPAGAM ACADEMY OF HIGHER EDUCATION

(Established Under Section 3 of UGC Act 1956)

Coimbatore – 641 021.

(For the candidates admitted from 2017 onwards)

DEPARTMENT OF MICROBIOLOGY

CLASS: I B.Sc (MB) **BATCH – 2018 -2021**

SUBJECT NAME: BACTERIOLOGY **SUB.CODE:18MBP102**

SEMESTER: I **4H – 4C**

SYLLABUS

Instruction Hours / week: L: 4 T: 0 P: 0 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 3 Hours

Unit I

Cell shape and arrangement, glycocalyx, capsule, flagella, fimbriae and pili. Cell-wall structure and composition of Gram-positive, Gram-negative and archae cell wall. Action of antibiotics and enzymes on the cell wall (sphaeroplasts, protoplasts, and L-forms). Cell Membrane- Structure, function and chemical composition of bacterial and Archaeal cell membranes. Cell organelles. Endospore: Structure, formation, stages of sporulation.

Unit II

Staining-principle and types of staining (Simple and Differential). Pure culture technique-microbial preservation- cultivation of anaerobic bacteria.

Unit III

Culture media-types, composition. Sterilization technique-Physical and Chemical methods of microbial control-types and mode of action. Bacterial growth curve-bacterial motility and cell count technique. Calculation of generation time and specific growth rate.

Unit IV

Aim and principles of classification, systematics and taxonomy, concept of species, taxa, strain; conventional, molecular and recent approaches to polyphasic bacterial taxonomy, evolutionary chronometers, rRNA oligonucleotide sequencing, signature sequences, and protein sequences. Differences between eubacteria and Archaebacteria.

Unit V

Archae bacteria and Eubacteria- General characteristics, Classification (Overview), metabolism and ecological significance. Gram Positive and Gram Negative (Low G+C and High G+C)-General characteristics with suitable examples. Cyanobacteria: An Introduction.

SUGGESTED READINGS

1. Pelczar, J.r M.J., Chan, ECS., and Krieg, N.R. (2008). Microbiology. 5th edition. Tata McGraw Hill.
2. Willey, J.M., Sherwood, L.M., and Woolverton, C.J. (2013). Prescott's Microbiology. 9th edition. McGraw Hill Higher Education.

3. Madigan, M.T., and Martinko, J.M. (2014). Brock Biology of Micro-organisms. 14th edition. Parker J. Prentice Hall International, Inc.
4. Tortora, G.J., Funke, B.R., and Case, C.L. (2008). Microbiology: An Introduction. 9th edition. Pearson Education.
5. Black, J.G. (2008). Microbiology: Principles and Explorations. 7th edition. Prentice Hall
6. Stanier, R.Y., Ingraham,J.L., Wheelis, M.L., and Painter, P.R. (2005). General Microbiology. 5th edition. McMillan.
7. Atlas, R.M. (1997). Principles of Microbiology. 2nd edition. WM.T.Brown Publishers.
8. Cappucino, J., and Sherman. N. (2010). Microbiology: A Laboratory Manual. 9th edition. Pearson Education Limited
9. Srivastava, S., and Srivastava, P.S. (2003). Understanding Bacteria. Kluwer Academic Publishers, Dordrecht.

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LECTURE PLAN
DEPARTMENT OF MICROBIOLOGY

S.No	Lecture Duration Period	Topics to be Covered	Support Material/Page Nos
		UNIT-I	
1	1	Cell shape and arrangement, glycocalyx, capsule, flagella, fimbriae.	T1: 73 to 93 T2: 39 to 78
2	1	Cell shape and arrangement, glycocalyx, capsule, flagella, fimbriae	T1: 73 to 93 T2: 39 to 78
3	1	Structure and composition of Gram Positive and Gram Negative cell wall, Archaebacteria cell wall.	T1: 99 to 114 T2: 39 to 78
4	1	Structure and composition of Gram Positive and Gram Negative cell wall, Archaebacteria cell wall.	T1: 99 to 114 T2: 39 to 78
5	1	Action of antibiotics and enzymes on cell wall – sphaeroplastas, protoplast and L-forms	T1: 115 to 120 T2: 167 to 180
6	1	Structure, composition and function of bacterial cell membrane.	T1: 151 to 170 T2: 65 to 73
7	1	Cell organells- endospore- structure, formation and sporulation stages.	R1: 13 to 42
8	1	Cell organells- endospore- structure, formation and sporulation stages.	R1: 13 to 42
9	1	Recapitulation and discussion of question	
	Total No. of Hours Planned For Unit 1=09		
		UNIT-II	
1	1	Principle and procedure of simple satining	R1: 49 to 58
2	1	Principle and procedure of differential staining –Gram stain, Negative Stain, Endospore stain and Acid fast stain.	R1: 59 to 76
3	1	Principle and procedure of differential staining –Gram stain, Negative Stain, Endospore stain and Acid fast stain.	R1: 59 to 76
4	1	Pure culture techniques used in microbial cultivation-serial dilution, spread plate, pour plate and streak plate methods.	R1: 83 to 105 T1: 99 to 110
5	1	Pure culture techniques used in microbial cultivation-serial	R1: 83 to 105

		dilution, spread plate, pour plate and streak plate methods.	T1: 99 to 110
6	1	Preservation of microbial pure culture – chilling, freezing, ultra freezing, deep freezing, lyophilization, preservation by mineral oil.	T2: 70 to 78 / W1
7	1	Preservation of microbial pure culture – chilling, freezing, ultra freezing, deep freezing, lyophilization, preservation by mineral oil.	T2: 70 to 78 / W1
8	1	Cultivation of anaerobic bacteria – anaerobic jar method	R1: 29 to 37 / W1
9	1	Recapitulation and discussion of question	
	Total No of Hours Planned For Unit II=09		
		UNIT-III	
1	1	Culture media –types and composition –simple media.	W1 R1: 101 to 116
2	1	Culture media – types and composition – complex media.	W1 R1: 101 to 116
3	1	Physical methods of microbial control.	T1: 469 to 480
4	1	Chemical methods of microbial control.	R1: 469 to 488
5	1	Bacterial growth curve and stages involved.	T2: 80 to 83
6	1	Bacterial motility analysis.	T2: 71 to 86
7	1	Cell counting techniques.	W1 R1: 119 to 142
8	1	Calculation of generation time and specific growth rate.	R1: 150 to 152
9	1	Recapitulation and discussion of question	
	Total No of Hours Planned For Uni III=09		
		UNIT-IV	
1	1	Bacterial Taxonomy.	T1: 139 to 140
2	1	Bacterial Taxonomy.	T1: 139 to 140
3	1	Concept of species, taxa and strain.	T1: 140 to 144
4	1	Molecular approach and polyphasic bacterial taxonomy.	T1: 144 to 146
5	1	Evolutionary chronometers.	T2: 70 to 133
6	1	rRNA sequences and signature sequences.	T2: 70 to 133
7	1	Protein sequencing.	R1: 33 to 146 T1: 151 to 165

8	1	Difference between eubacteria and Archaebacterial cells.	T2: 184 to 191 W1
9	1	Recapitulation and discussion of question	
	Total No of Hours Planned For Unit IV=09		
		UNIT-V	
1	1	General characteristics and classification of Archae bacteria.	T1: 151 to 152 T2: 430 to 435
2	1	General characteristics and classification of Eubacteria.	T1: 155 to 164 T2: 435 to 438
3	1	Metabolism and ecological significance of Eubacteria and Archaebacteria	T1: 164 to 165 T2: 439 to 451
4	1	Metabolism and ecological significance of Eubacteria and Archaebacteria	T3:159 – 160,165 - 168
5	1	Low G+C Gram positive bacteria and High G+C Gram positive bacteria.	T1: 156 to 157 T2: 452 to 510
6	1	Low G+C Gram negative bacteria and High G+C Gram negative bacteria.	T1: 158 to 163 T2: 510 to 511
7	1	General characteristics of Gram positive and Gram negative bacteria.	T1: 163 to 167 T2: 511 to 598
8	1	Introduction to cyanobacteria.	T1: 167 to 173 W1
9	1	Recapitulation and discussion of question	
10	1	Old question paper discussion (Last Five years)	
11	1	Old question paper discussion (Last Five years)	
12	1	Old question paper discussion (Last Five years)	
	Total No of Hours Planned for unit V=12		

SUGGESTED READINGS:

TEXT BOOK:

1. Michael J. Pelczar Jr., Chan E.C.S., Noel. R. Krieg. Microbiology (2004), Tata McGraw Hill Publishing Company Ltd, New Delhi, India.
2. Prescott, Harley and Klein's. Microbiology, (2008), McGraw Hill International, 7th edition, New York, USA.

REFERENCE:

1. Dr. N. Arumugam. Immunology and Microbiology, Saras Publication, Tamil Nadu.

WEBSITES

1. W1: www.somusbiology.com/microbiologybasics/index.php
2. W2: www.ncbi.nlm.nih.gov
3. W3: www.britannica.com

Cell shape and arrangement, glycocalyx, capsule, flagella, fimbriae and pili. Cell-wall structure and composition of Gram-positive, Gram-negative and archae cell wall. Action of antibiotics and enzymes on the cell wall (sphaeroplasts, protoplasts, and L-forms). Cell Membrane- Structure, function and chemical composition of bacterial and Archaeal cell membranes. Cell organelles. Endospore: Structure, formation, stages of sporulation

Microbiology

Micro-organisms affect every aspect of life on Earth. Some microbes cause disease but the majorities are completely harmless.

These minute life forms are essential to the cycling of nutrients in the eco-systems of the planet.

We need to understand the role of microbes in global terms, but there are many aspects of our daily lives where knowledge of microbiology can help us answer everyday questions

Biotechnology, which often uses microbes or their products, is a fast-growing area of science. Some of the techniques used, such as genetic modification, cause public concern. A good understanding of the science behind such controversial issues enables balanced judgments to be made.

Since microbes are of such biological, economic and social significance, it is important that microbiology is a part of everyone's education.

Introducing microbes

Micro-organisms (or microbes for short) play a very important role in our lives. Some microbes cause disease but the majorities are completely harmless. In fact we couldn't live without them, but they could live without us.

These microscopic organisms play a key role in maintaining life on earth, fixing gases and breaking down dead plant and animal matter into simpler substances that are used at the beginning of the food chain. Biotechnologists can also exploit the activities of microbes to benefit humans, such as in the production of medicines, enzymes and food. They are also used to breakdown sewage and other toxic wastes into safe matter. This process is called bioremediation.

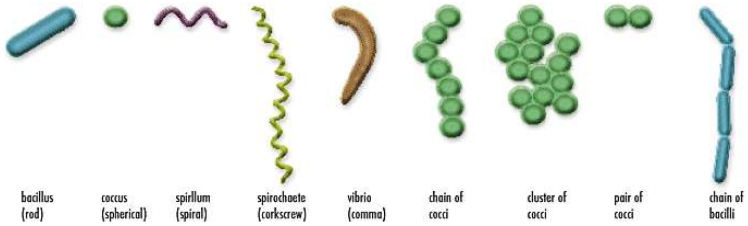
Microbes are very small living organisms, so small that most of them are invisible. The majority can only be seen with a microscope, which magnifies their image so we can see them. In fact microbes are so tiny you would find over a million in a teaspoon of soil. They make up more than 60 % of the Earth's living matter and scientists estimate that 2-3 billion species share the planet with us.

Micro-organisms occur in an amazing variety of shapes and sizes and they are divided into one of 6 groups:

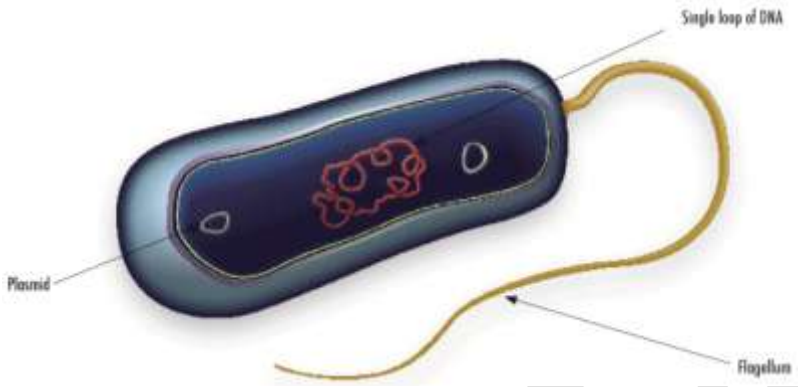
Bacteria

Bacteria are single celled microbes. The cell structure is simpler than that of other organisms as there is no nucleus or membrane bound organelles. Instead their control centre containing the genetic information is contained in a single loop of DNA. Some bacteria have an extra circle of genetic material called a plasmid. The plasmid often contains genes that give the bacterium some advantage over other bacteria. For example it may contain a gene that makes the bacterium resistant to a certain antibiotic.

Bacteria are classified into 5 groups according to their basic shapes: spherical (cocci), rod (bacilli), spiral (spirilla), comma (vibrios) or corkscrew (spirochaetes). They can exist as single cells, in pairs, chains or clusters.



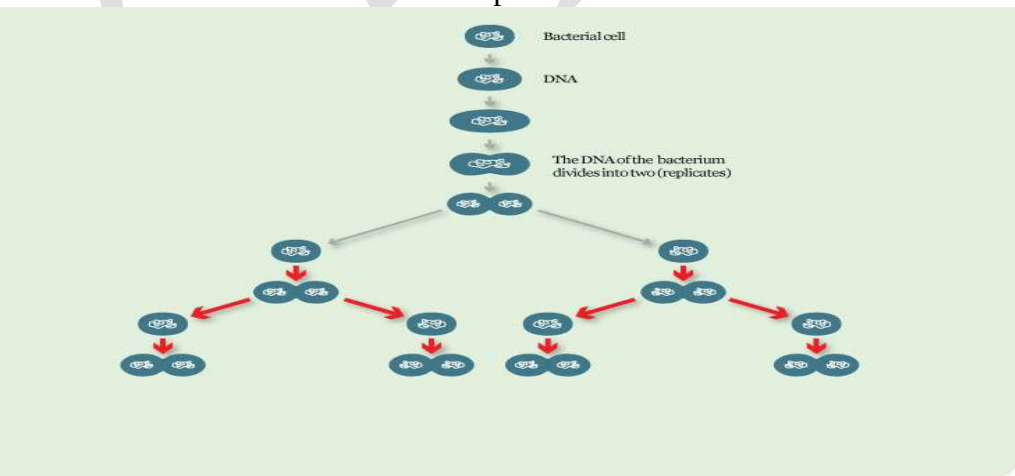
The different bacterial shapes



A typical bacterial cell

Antibiotic resistance

Artwork of bacterial cells becoming resistant to antibiotics. This resistance is acquired from a donor cell's plasmid (circular unit of deoxyribonucleic acid, DNA), which has resistance seen at upper left (red/yellow, red is resistance). Viral transmission involves a virus (pink, lower left) obtaining a resistant gene, and passing it to a bacterial cell that incorporates it into its plasmid. Bacterial cells also acquire segments of DNA released from dead cells (upper left). Mutations (not seen) may also occur, which may be antibiotic resistant and thus allow the bacteria to survive and reproduce.



Bacteria are found in every habitat on Earth: soil, rock, oceans and even arctic snow. Some live in or on other organisms including plants and animals including humans. There are approximately 10 times as many bacterial cells as human cells in the human body. A lot of these bacterial cells are found lining the digestive system. Some bacteria live in the soil or on dead plant matter where they play an important role in the cycling of nutrients. Some types cause food spoilage and crop damage but others are incredibly useful in

the production of fermented foods such as yoghurt and soy sauce. Relatively few bacteria are parasites or pathogens that cause disease in animals and plants.

How do bacteria reproduce?

Bacteria reproduce by binary fission. In this process the bacterium, which is a single cell, divides into two identical daughter cells. Binary fission begins when the DNA of the bacterium divides into two (replicates). The bacterial cell then elongates and splits into two daughter cells each with identical DNA to the parent cell. Each daughter cell is a clone of the parent cell.

When conditions are favourable such as the right temperature and nutrients are available, some bacteria like *Escherichia coli* can divide every 20 minutes. This means that in just 7 hours one bacterium can generate 2,097,152 bacteria. After one more hour the number of bacteria will have risen to a colossal 16,777,216. That's why we can quickly become ill when pathogenic microbes invade our bodies. **2/3***oli* can

Survival mechanism

Some bacteria can form endospores. These are dormant structures, which are extremely resistant to hostile physical and chemical conditions such as heat, UV radiation and disinfectants. This makes destroying them very difficult. Many endospore-producing bacteria are nasty pathogens, for example *Bacillus anthracis* is the cause of anthrax.

Archaea

Archaea can be spherical, rod, spiral, lobed, rectangular or irregular in shape. An unusual flat, square-shaped species that lives in salty pools has also been discovered. Some exist as single cells, others form filaments or clusters. Until the 1970s this group of microbes was classified as bacteria.

Other archaea

Fig: a) Sulfolobus, b)Methanosarcina rumen, c) Staphylothermus marinus, d) Halococcus salifodinae e) Merhanococcoides burtonii

Many archaea have been found living in extreme environments, for example at high pressures, salt concentrations or temperatures, and have been nicknamed extremophiles. Their cell wall differs in structure from that of bacteria and is thought to be more stable in extreme conditions, helping to explain why some archaea can live in many of the most hostile environments on Earth.

Examples of archaea habitats are boiling hot springs and geysers such as those found in Yellow Stone Park, USA and ice such as the Artic and Antarctic oceans which remain frozen for most of the year.

Comparing Of Prokaryotic And Eukaryotic Cells: An Overview

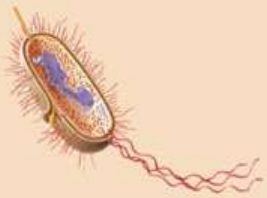
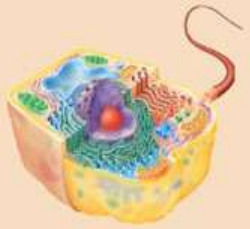
Remember, there are exceptions to every rule. We are going with statements that should begin with "For the most part..." or "As far as we know at this time..."

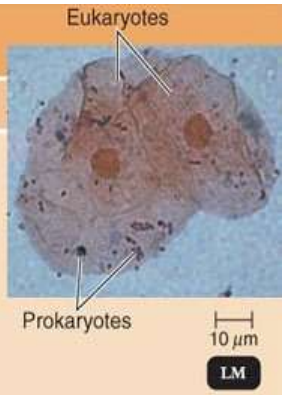
Prokaryotic cells are similar in their chemical composition and chemical reactions.

Prokaryotic cells lack membrane-enclosed organelles (including a nucleus) while eukaryotic cells have a membrane-bound nucleus and other organelles.

Peptidoglycan is found in bacterial cell walls but not in archaea or eukaryotic cell walls.

Table 4.2 Principal Differences between Prokaryotic and Eukaryotic Cells

Characteristic	Prokaryotic	Eukaryotic
		
Size of Cell	Typically 0.2–2.0 μm in diameter	Typically 10–100 μm in diameter
Nucleus	No nuclear membrane or nucleoli	True nucleus, consisting of nuclear membrane and nucleoli
Membrane-Enclosed Organelles	Absent	Present; examples include lysosomes, Golgi complex, endoplasmic reticulum, mitochondria, and chloroplasts
Flagella	Consist of two protein building blocks	Complex; consist of multiple microtubules
Glycocalyx	Present as a capsule or slime layer	Present in some cells that lack a cell wall
Cell Wall	Usually present; chemically complex (typical bacterial cell wall includes peptidoglycan)	When present, chemically simple (includes cellulose and chitin)
Plasma Membrane	No carbohydrates and generally lacks sterols	Sterols and carbohydrates that serve as receptors
Cytoplasm	No cytoskeleton or cytoplasmic streaming	Cytoskeleton; cytoplasmic streaming
Ribosomes	Smaller size (70S)	Larger size (80S); smaller size (70S) in organelles
Chromosome (DNA)	Usually single circular chromosome; typically lacks histones	Multiple linear chromosomes with histones
Cell Division	Binary fission	Involves mitosis
Sexual Recombination	None; transfer of DNA only	Involves meiosis



The Prokaryotic Cell

Bacteria are unicellular and most multiply by binary fission.

Bacterial species are differentiated by morphology, chemical composition, nutritional requirements, biochemical activities, and source of energy.

The Size, Shape, And Arrangement Of Bacterial Cells

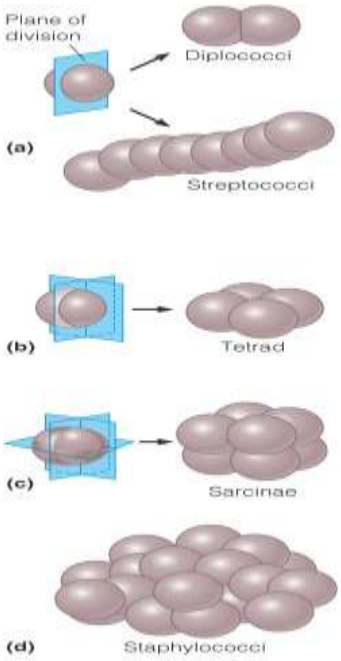
Most bacteria are 0.2 um in diameter and 2-8 um in length.

The three basic bacterial shapes are coccus (spherical), bacillus (rod-shaped), and spiral (twisted), however pleomorphic bacteria can assume several shapes.

Arrangement of cocci

Cocci may be oval, elongated, or flattened on one side.

Cocci may remain attached after cell division. These group characteristics are often used to help identify certain cocci.



Cocci that remain in pairs after dividing are called diplococci.

Cocci that remain in chains after dividing are called streptococci.

Cocci that divide in two planes and remain in groups of four are called tetrads.

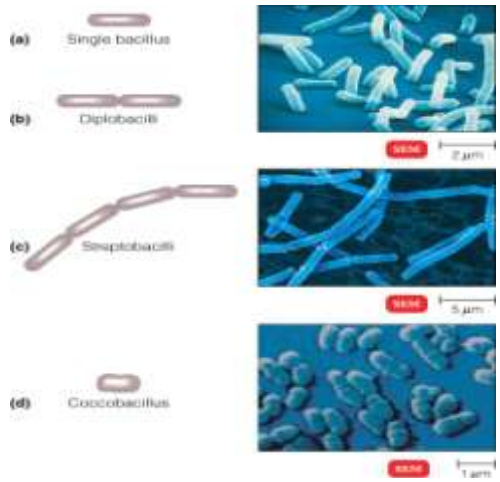
Cocci that divide in three planes and remain in groups cube like groups of eight are called sarcinae.

Cocci that divide in multiple planes and form grape like clusters or sheets are called staphylococci.

Bacilli

Since bacilli only divide across their short axis there are fewer groupings.

Bacillus is a shape (rod shaped) but there is also a genus of bacteria with the name *Bacillus*. You wouldn't confuse the two, since you know the rules for writing the genus and species names of organisms, right????



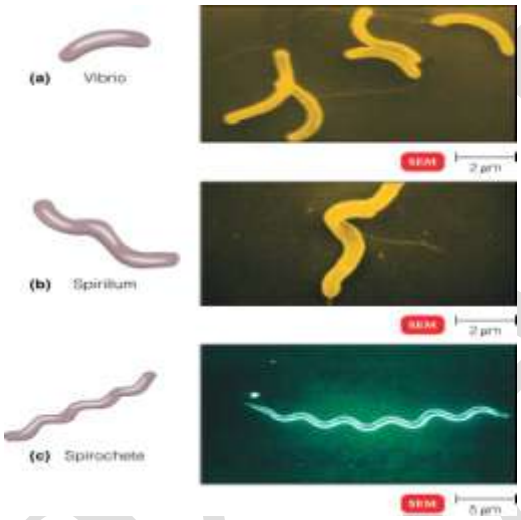
Most bacilli appear as single rods. Diplobacilli appear in pairs after division.

Streptobacilli appear in chains after division.

Some bacilli are so short and fat that they look like cocci and are referred to as coccobacilli.

Spiral bacteria

Spiral bacteria have one or more twists.

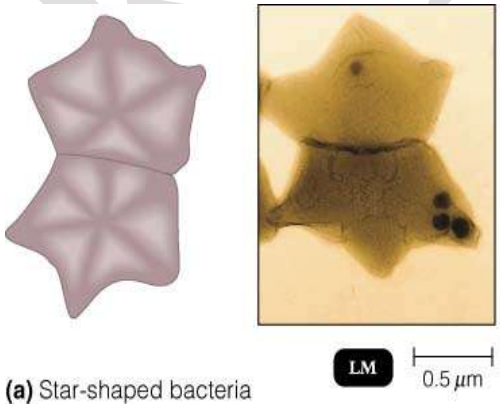


Vibrios look like curved rods.

Spirilla have a helical shape and fairly rigid bodies.

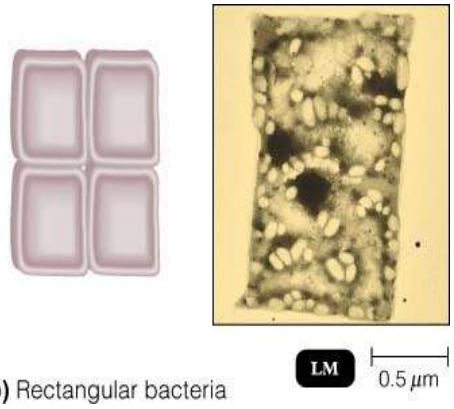
Spirochetes have a helical shape and flexible bodies. Spirochetes move by means of axial filaments, which look like flagella contained beneath a flexible external sheath.

Other shapes



(a) Star-shaped bacteria

Stella are star-shaped.



(b) Rectangular bacteria

Haloarcula, a genus of halophilic archaea, are rectangular.

Bacteria: Definition, Size, Shape and Arrangement

A. Definition:

Bacteria are prokaryotic, unicellular microorganisms, which lack chlorophyll pigments.

B. Size of Bacteria:

The average diameter of spherical bacteria is 0.5-2.0µ (Figure 2.1). For rod-shaped or filamentous bacteria, length is 1-10µ and diameter is 0.25-1 .0µ.

One group of bacteria, called the Mycoplasmas, have individuals with size much smaller than these dimensions.

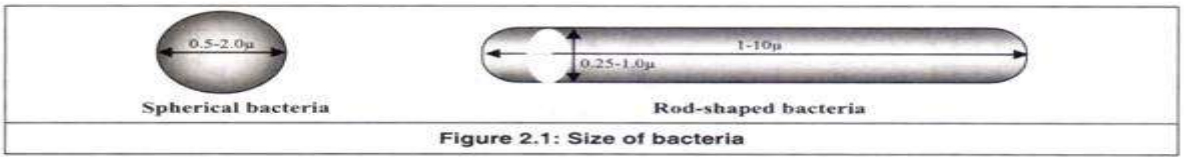


Figure 2.1: Size of bacteria

They measure about 0.25 µ and are the smallest cells known so far. They were formerly known as pleuropneumonia-like organisms (PPLO). Viruses are still smaller, but as they are particles rather than cells, Mycoplasmas are considered to be the smallest cells known. Examples: Mycoplasma laidlawii and M. gallisepticum.

C. Shape and Arrangement of Bacteria:

1. Spherical Bacteria:

Bacteria, which are spherical or ovoid in shape, are called 'coccus' (plural: cocci) (Figure 2.2). Based on the arrangement of the cells they are of the following types.

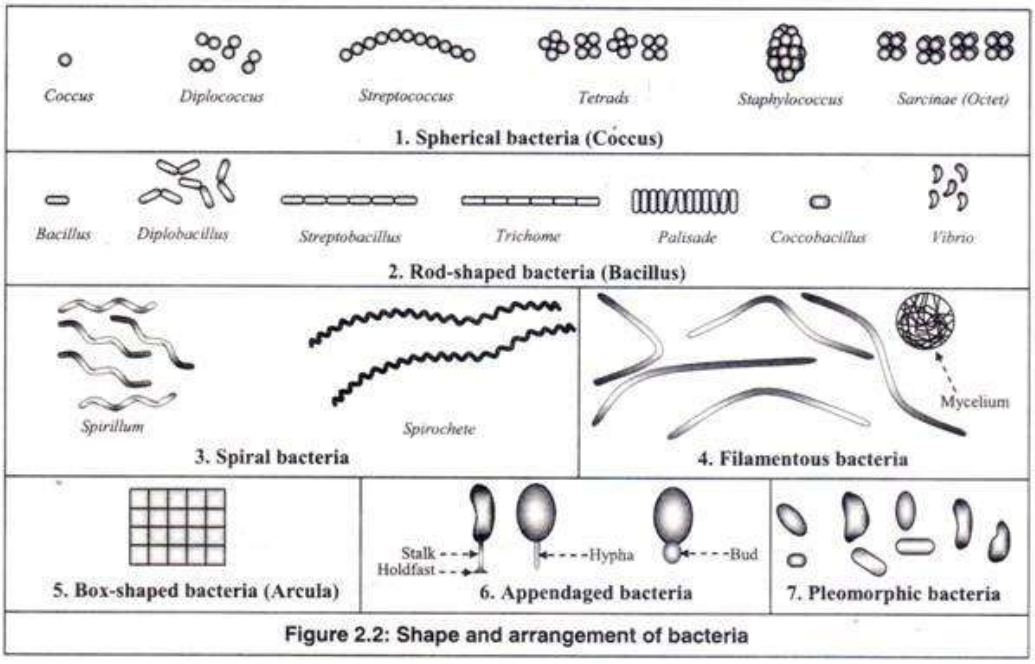


Figure 2.2: Shape and arrangement of bacteria

(a) Coccus:

The spherical bacteria cells, called cocci, are present as single individuals.

(b) Diplococcus:
The cocci are arranged in pairs.
(c) Streptococcus:
The cocci are arranged in chains, as the cells divide in one plane.
(d) Tetrads:
The cocci are arranged in packets of four cells, as the cells divide in two plains.
(e) Staphylococcus:
The cocci are arranged in grape-like clusters formed by irregular cell divisions in three plains.
(f) Sarcinae (Octet):
The cocci are arranged in a cuboidal manner, as the cells are formed by regular cell divisions in three planes.
2. Rod-shaped Bacteria:
The cylindrical or rod-shaped bacteria are called 'bacillus' (plural: bacilli).
They are of three shapes as follows:
(a) Bacillus:
They are rod-shaped bacteria. Based on arrangement they are of the following types.
(i) Bacillus:
The rod-shaped bacteria cells, called bacilli, are present as single individuals.
(ii) Diplobacillus:
The bacilli are arranged in pairs.
(iii) Streptobacillus:
The bacilli are arranged in chains, as the cells divide in one plane.
(iv) Trichomes:
The bacilli are arranged in chains with larger area of end-to-end contact between the cells.
(v) Palisades:
The bacilli bend at the points of division following the cell divisions, resulting in a palisade arrangement resembling a picket fence and angular patterns that look like Chinese letters.
(b) Coccobacillus:
These are so short and stumpy that they appear ovoid. They look like coccus and bacillus.
(c) Vibrios:
They are comma-shaped bacteria with less than one complete turn or twist in the cell.
3. Spiral Bacteria:
Unlike the vibrios, which have less than one complete turn or twist in the cell, the spiral bacteria are rod-shaped bacteria, which have more than one twist in the cell. They usually occur singly.
They are of two types as follows:
(a) Spirillum:
They have rigid spiral structure. Spirillum with many turns can superficially resemble spirochetes. They do not have outer sheath and endoflagella, but have typical bacterial flagella.
(b) Spirochetes:
They are flexible and can twist and contort their shape. They have outer sheath and endoflagella, but lack typical bacterial flagella.
4. Filamentous Bacteria:
They are very long thin filament-shaped bacteria. Some of them form branching filaments resulting in a network of filaments called 'mycelium'.

5. Box-shaped or Square-shaped Bacteria (Arcula):

They are flat, box-shaped bacteria with perfectly straight edges and sharp 90° angles at the corners. Smaller cells are usually perfectly squares (2X2µ), while larger cells are rectangular; about twice as long as they are wide (4X2µ).

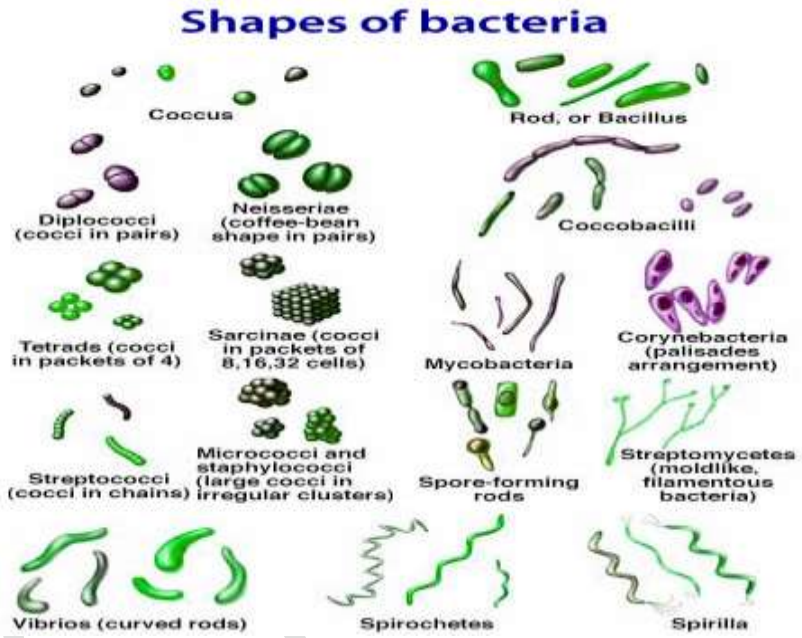
Each bacterium is a thin flexible sheet with smooth surface. After cell divisions, the cells remain attached to each other, producing large sheets of squares. It was first discovered in 1980 in natural salt ponds.

6. Appendaged Bacteria:

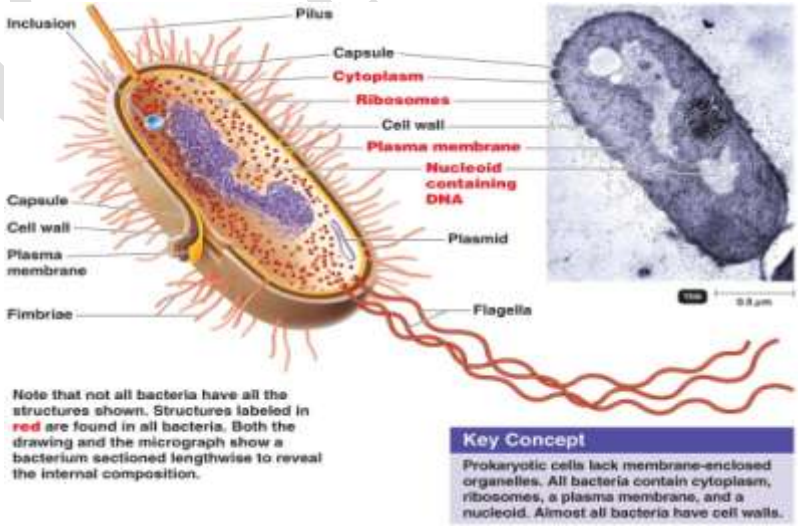
They possess extension of their cells, as long tubes in the form of stalk or hypha, or as buds.

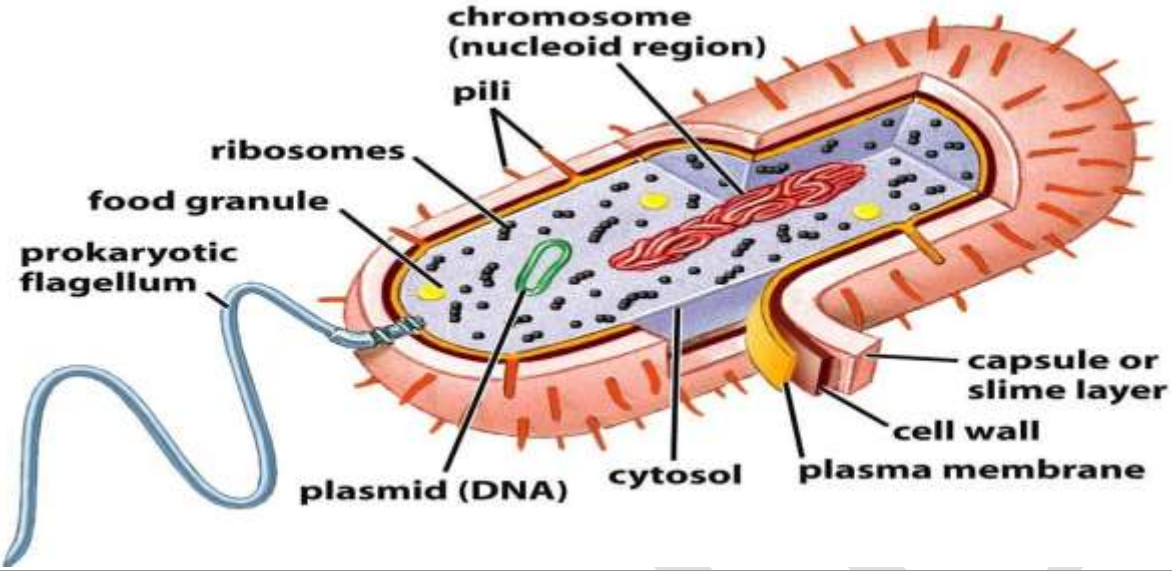
7. Pleomorphic Bacteria:

These bacteria do not have any characteristic shape unlike all others described above. They can change their shape. In pure cultures, they can be observed to have different shapes.



Typical structures of a prokaryotic cell





Structures External To The Cell Wall

Glycocalyx

The glycocalyx (capsule, slime layer, or extra cellular polysaccharide) is a gelatinous polysaccharide and/or polypeptide covering. The exact chemical composition varies depending on the species.

Capsules are organized and firmly attached to the cell wall.

Capsules may protect pathogens from phagocytosis.

Capsules enable adherence to surfaces, prevent desiccation, and may provide nutrients.

Slime layers are unorganized and loosely attached to the cell wall.

Extracellular polymeric substance (EPS) is the description of a glycocalyx that is a component of a biofilm.

Cell Wall Appendages

1) Glycocalyx

All bacteria secrete some type of glycocalyx to protext them from drying.

Polysaccharide polymer +/- peptides or polypeptides outside the cell wall

NOTE: If only contain sugars, then called Extracellular polysachharide

Can be:

Organized and thick = Capsule, firmly attached

Unorganized and thin = Slime layer, loosely attached

Regular structured, outer viscous thin layer = S-layer

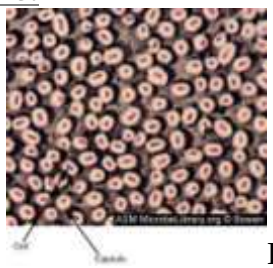
- Capsules are usually used for protection against environmental hazards and to help prevent phagocytosis.
- Slime layers are primarily used for adhesion.
- S layer can help function in protection and adhesion

The glycocalyx will form a biofilm to help surround bacterial colonies. The negative charge of this glycocalyx structure helps repel the WBCs of the immune system.

Capsule is a

- Gelatinous layer covering the entire bacterium
- Composed of polysaccharide (i.e. poly: Many, Saccharide: Means sugar)

Capsule is located immediately exterior to the murein layer of gram-positive bacteria and the outer membrane of gram-negative bacteria.



Bacterial Capsule

(Exception: The capsule of *Bacillus anthracis* is composed of polymerized D-glutamic acid)

- The sugar component of polysaccharide varies within the species of bacteria, which determines their serologic types. Example: *Streptococcus pneumoniae* has 84 different serologic types discovered so far.

Importance of Bacterial Capsule

1. **Virulence determinants:** Capsules are antiphagocytic. They limit the ability of phagocytes to engulf the bacteria. If a pathogenic bacteria lose capsule (by mutation), they wont be able to cause disease (i.e. changes to nonpathogenic bacteria).
2. **Identification of bacteria**:
    1. Using specific antiserum against capsular polysaccharide. E.g. Quellung reaction
    2. Colony characteristics in culture media: Capsulated organisms forms mucoid colonies
3. **Development of Vaccines**: Capsular polysaccharides are used as the antigens in certain vaccines. E.g. The purified capsular polysaccharides of 23 types of *S. pneumoniae* are present in current vaccine.
4. **Initiation of infection**: Capsules helps the organism to adhere to host cells.The capusle also facilitates and maintains bacterial colonization of biologic (e.g. teeth) and inanimate (e.g. prosthetic heart valves) surfaces through formation of **biofilms**.

Examples of Capsulated bacteria/yeasts:

Mneomonics to remember capsulated bacteria– **S**ome **K**illers **H**ave **P**retty **N**ice **C**apsule

1. ***S****treptococcus pneumoniae*
2. ***K****lebsiella pneumoniae*
3. ***H****aemophilus influenzae*
4. ***P****seudomonas aeruginosa*
5. ***N****eisseria meningitidis*
6. ***C****ryptococcus neoformans*

Bacterial capsule

The outer red layer in this diagram is the capsule, which is distinct from the cell envelope. This bacteria is Gram-positive, as its cell envelope comprises a single plasma membrane (orange) and a thick peptidoglycan-containing cell wall (purple).

The **cell capsule** is a very large structure of some prokaryotic cells, such as bacterial cells. It is a polysaccharide layer that lies outside the cell envelope of bacteria, and is thus deemed part of the outer envelope of a bacterial cell. It is a well-organized layer, not easily washed off, and it can be the cause of various diseases.

The capsule—which can be found in both Gram-negative bacteria and Gram-positive bacteria—should not be confused with the second lipid membrane (or bacterial outer membrane), which contains

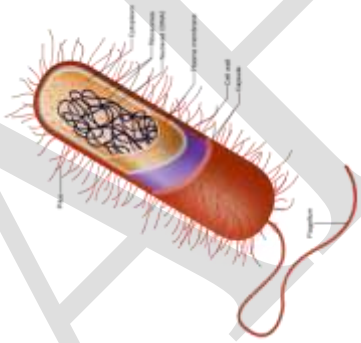
lipopolysaccharides and lipoproteins and is found only in Gram-negative bacteria. When the amorphous viscid secretion (that makes up the capsule) diffuses into the surrounding medium and remains as a loose undemarcated secretion, it is known as **slime layer**.

Composition

It usually consists of polysaccharides, but can be composed of other materials (e.g., polypeptide(D-glutamic acid) in *B. anthracis*),also peptidoglycan and muramic acid found in E.coli bacterial capsule. Because most capsules are so tightly packed, they are difficult to stain using standard stains because most stains cannot adhere to the capsule. For examination under the microscope, the bacteria and their background are stained darker than the capsule, which doesn't stain. When viewed, bacterial cells as well as the surface they are on, are stained dark, while the capsule remains pale or colorless and appears as a ring, or halo, around the cell.

Function

The capsule is considered a virulence factor because it enhances the ability of bacteria to cause disease (e.g. prevents phagocytosis). The capsule can protect cells from engulfment by eukaryotic cells, such as macrophages. A capsule-specific antibody may be required for phagocytosis to occur. Capsules also contain water which protects the bacteria against desiccation. They also exclude bacterial viruses and most hydrophobic toxic materials such as detergents. Immunity to one capsule type does not result in immunity to the other types. Capsules also help cells adhere to surfaces.



Diversity

The capsule is found most commonly among Gram-negative bacteria:

- *Escherichia coli* (in some strains)
- *Neisseria meningitidis*
- *Klebsiella pneumoniae*
- *Haemophilus influenzae*
- *Pseudomonas aeruginosa*
- *Salmonella*[*citation needed*]

However, some Gram-positive bacteria may also have a capsule:

- *Bacillus megaterium* for example, synthesizes a capsule composed of polypeptide and polysaccharides.
- *Streptococcus pyogenes* synthesizes a hyaluronic acid capsule.
- *Streptococcus pneumoniae* has at least 91 different capsular serotypes. These serotypes are the basis for the pneumococcal vaccines.
- *Streptococcus agalactiae* produces a polysaccharide capsule of nine antigenic types that all contain sialic acid (Ia, Ib, II, III, IV, V, VI, VII, VIII).

- *Staphylococcus epidermidis*

The yeast *Cryptococcus neoformans*, though not a bacterium, has a similar capsule
Capsules too small to be seen with an ordinary microscope, such as the M protein of *Streptococcus pyogenes*, are called microcapsules.

Mnemonic

A common mnemonic used to remember some encapsulated pathogens is:
"Even Some Super Killers Have Pretty Nice Big Capsules"
Escherichia coli, Streptococcus pneumoniae, Salmonella, Klebsiella pneumoniae, Haemophilus influenzae, Pseudomonas aeruginosa, *Neisseria meningitidis*, *Bacteroides fragilis*, and the yeast *Cryptococcus neoformans*.

Demonstration of Capsule

1. India ink staining: the capsule appears as a clear halo around the bacterium as the ink can't penetrate the capsule.
2. Maneval's capsule stain: the capsule appears as a clear halo between the pink-stained bacterium and the bluish-grey stained background. The background stain is the acidic stain Congo red (which changes color to bluish-grey due to the pH), and the pink stain is acid fuschin.
3. Serological methods: Capsular material is antigenic and can be demonstrated by mixing it with a specific anticapsular serum. When examined under the microscope, the capsule appears 'swollen' due to an increase in its refractivity. This phenomenon is the basis of Quellung reaction.

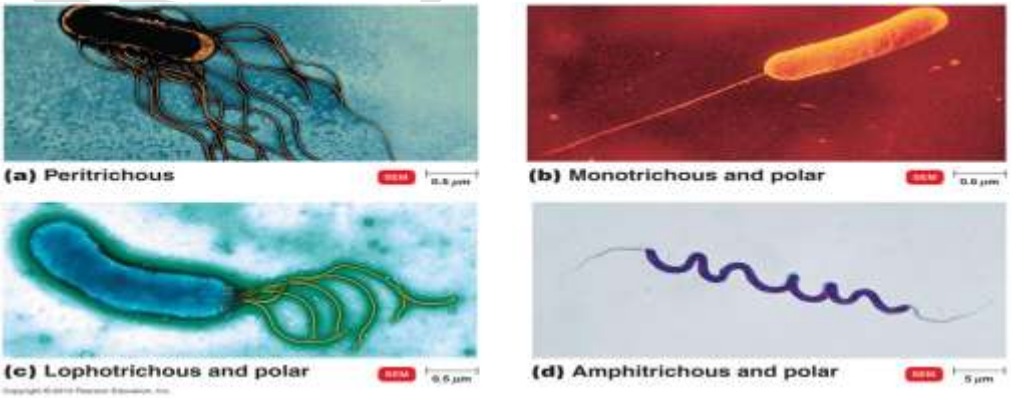
Use in vaccination

Vaccination using capsular material is effective against some organisms (e.g., *H. influenzae* type b, *S. pneumoniae*, and *N. meningitidis*). However, polysaccharides are not highly antigenic, especially in children, so many capsular vaccines contain polysaccharides conjugated with protein carriers, such as the tetanus toxoid or diphtheria toxoid. This stimulates a much more robust immune response.

Flagella

Flagella are relatively long filamentous appendages consisting of a filament, hook, and basal body.
Prokaryotic flagella rotate to push the cell.
Motile bacteria exhibit taxis; positive taxis is movement toward an attractant, and negative taxis is movement away from a repellent.

Arrangement of Bacterial Flagella



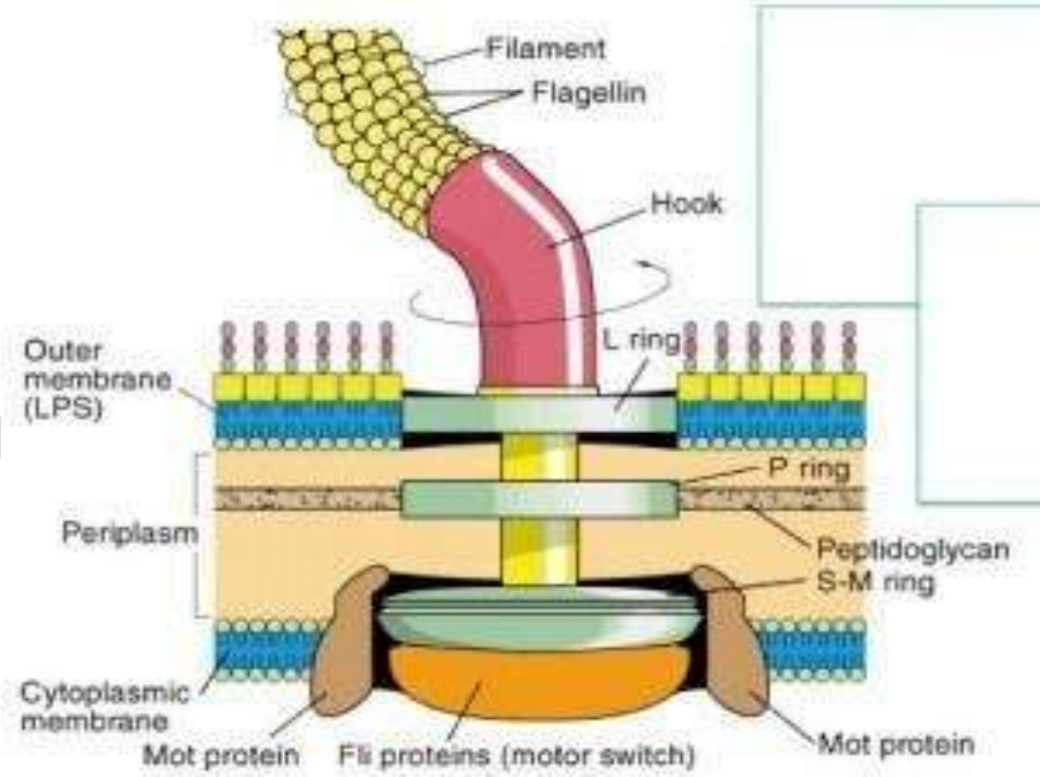
Bacterial flagella are long, thin (about 20 nm), whip like appendages that move the bacteria towards nutrients and other attractants. Flagella are free at one end and attached to the cell at the other end. Flagellum can never be seen directly with the light microscope but only after staining with special flagella

stains that increase their diameter. The long filament of flagella is composed of many subunits of a single protein, flagellin, arranged in several intertwined chains. The energy for movement, the proton motive force, is provided by ATP.

Flagellar motion in Bacterial Cells

Key Information: Most of the cocci (e.g. *Staphylococci, Streptococci* etc) don't have flagella so they are non-motile.

Flagella are helical shaped structure which is composed of subunits of a protein called **flagellin.** The wider region at the base of the flagellum is called **hook**. It is different in structure than that of the filament. Hook connects filament to the motor portion of the flagellum called **basal body**.



Structure of the prokaryotic flagellum

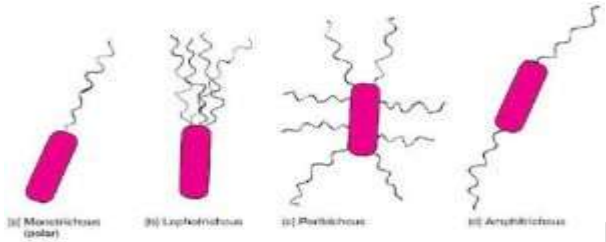
Structure of Bacterial Flagella

The basal body is anchored in the cytoplasmic membrane and cell wall. There are presence of rings which are surrounded by a pair of proteins called **Mot.** These proteins actually drive the flagellar motor causing

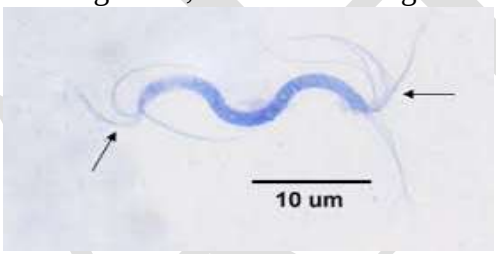
rotation of the filament . Another set of proteins called **Fli proteins** function as the motor switch, reversing rotation of the flagella in response to intra-cellular signals.

Arrangement and Types of Bacterial Flagella The number and location of flagella are distinctive for each genus. There are four types of flagellar arrangement.

1. **Monotrichous (Mono means one)**: Single polar flagellum e.g. *Vibrio cholerae, Campylobacter spp.* (polar flagella often in pairs to give a "seagull" appearance).
2. **Amphitrichous**: Single flagellum at **both ends** e.g. *Alcaligenes faecalis* (Mneomonics: Remember: the characteristics of Amphibians: live **both in land and water**)



3. **Lophotrichous:** Tuft of flagella at one or both ends e.g. *Spirilla spp*
4. **Peritrichous** (flagella in periphery): Flagella surrounding the bacterial cell. All the members of family **Enterobacteriaceae**, if motile has peritrichous flagella. e.g. *Salmonella typhi, Escherichia coli, Proteus* spp (highly motile organism; shows swarming motility)



Spirillum and its flagellar arrangement

Functions of Bacterial Flagella

Many **prokaryotes** are motile, and the majority of motile prokaryotes moves by means of flagella.

Medical Importance of Flagella

- **Role in Pathogenesis:** *Escherichia coli* and *Proteus* spp are common causes of Urinary tract infections. The flagella of these bacteria help the bacteria by propelling up the urethra into the bladder.
- **Roles in Organism identification**
  - Some species of bacteria, eg. *Salmonella* species are identified in the clinical laboratory by the use of Specific antibodies against flagellar proteins.
  - Organisms such as *Vibrio cholerae* (darting motility) and *Proteus* species (swarming growth in common culture media) are easily identified by their characteristics motility pattern.

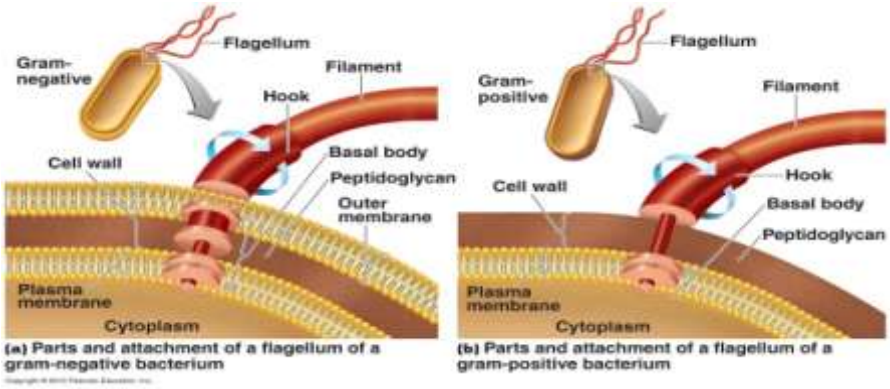
The Structure of a Prokaryotic Flagellum

Flagella are anchored by pairs of rings associated with the plasma membrane and cell wall. Gram positive bacteria have only the inner pair of rings.

The filament is composed of the globular protein flagellin, which is arranged in several intertwined chains that form a helix around a hollow core.

Flagellin can vary in structure and is used to identify some pathogenic bacteria serologically. The flagellar antigens are referred to as H antigens.

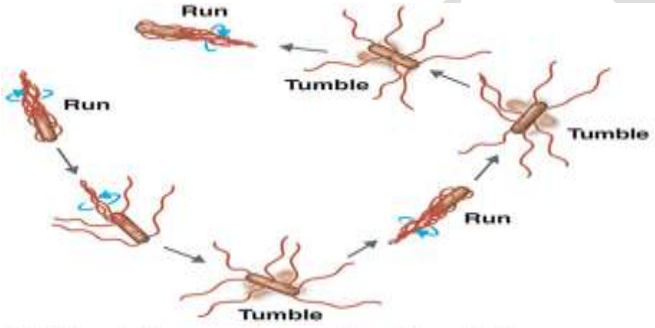
E. coli may express any of at least 50 different variants; serovars (serological variants) identified as O157:H7 are associated with food borne epidemics (O antigens are somatic antigens and are lipopolysaccharide complexes associated with the cell wall).



(a) Parts and attachment of a flagellum of a gram-negative bacterium

(b) Parts and attachment of a flagellum of a gram-positive bacterium

Flagella and Bacterial Motility



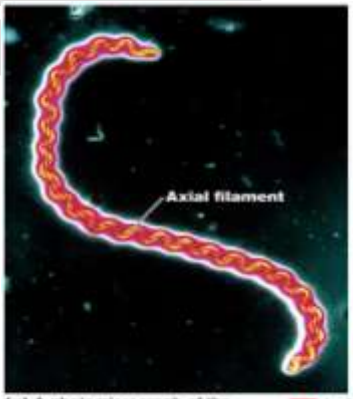
(a) A bacterium running and tumbling. Notice that the direction of flagellar rotation (blue arrows) determines which of these movements occurs. Gray arrows indicate direction of movement of the microbe.

(b) A *Proteus* cell in the swarming stage may have more than 1000 peritrichous flagella.

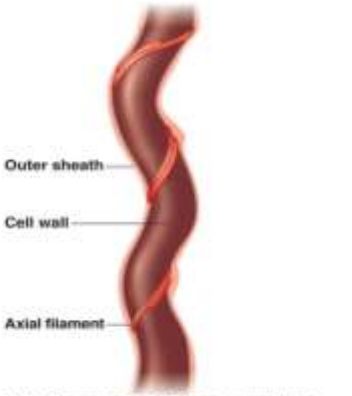
Axial Filaments

Spiral cells that move by means of an axial filament (endoflagellum) are called spirochetes.
Axial filaments are similar to flagella, except that they wrap around the cell.

Axial filaments



(a) A photomicrograph of the spirochete *Leptospira*, showing an axial filament

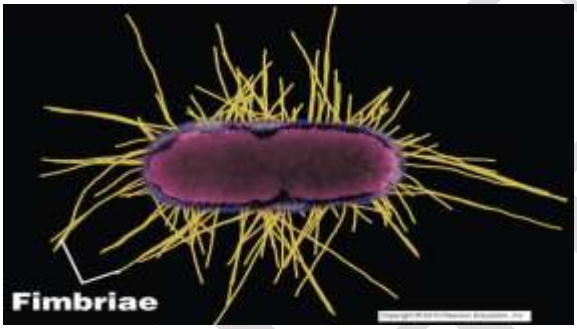


(b) A diagram of axial filaments wrapping around part of a spirochete. (See Figure 11.24a for a cross section of axial filaments)

Fimbriae And Pili

Fimbriae and pili are short, thin appendages.
Cells may have many fimbriae, which help the cells adhere to surfaces.
Cells that have pili have only one or two.
Pili join cells either for the transfer of DNA from one cell to another (sex pili) or are used for special types of movement; twitching, seen in*Pseudomonas aeurginosa*, *Neisseria gonorrhoeae* and some strains of *E. coli*, or the gliding motility of myxobacteria.

Fimbriae



Bacterial Pili (Fimbriae): Characteristics, Types and Medical Importance

Found mainly in Gram negative organisms, Fimbriae or pili (*singlular: pilus*) are hair like filaments (tiny hollow projections) that extend from the cell membrane into the external environment. A pilus is composed of subunits of the protein pilin.

Bacteria use adherence fimbriae (pili) to overcome the body's defense mechanism and cause disease. Pili are small hairs that enable some pathogens to attach and adhere easily to cell surface particularly mucous membranes. Bacteria possessing pili include *Neisseria gonorrhoeae* and some strains of *Escherichia coli*, *Salmonella* and *Shigella* species. Fimbriae (pili) are shorter, straighter and more numerous than bacterial flagella and are composed by subunits of protein called pilin.

Length: up to 2 µm

Types: Two general types of Pili are known they are:

1. Sex pili (long conjugation pili or F pili) and
2. Common pili (short attachment pili also called fimbriae).

Medical Importance of Fimbriae or Pili

1. **Common pili (Adhesins)**: They mediate the attachment of bacteria to specific receptors on the human cell surface, which is the first step in establishing infection in some organisms. They contribute to the pathogenicity of certain bacteria—their ability to produce disease—by enhancing

colonization on the surfaces of the cells of other organisms. Example: Mutants of *Neisseria gonorrhoeae* that do not form pili are nonpathogen.

2. **Sex pili (conjugation tube)**: It is a specialized kind of pili that forms the attachment between male (donor) and the female (recipient) bacteria during conjugation and acts as a conduit for the passage of DNA. This process is well characterized in the gram negative bacillus *Escherichia coli*.
3. Some pili are also involved in biofilm formation, phage transduction, DNA uptake and a special form of bacterial cell movement, known as 'twitching motility'.

Cell Wall

Most all bacteria have a semi rigid wall which surrounds the plasma membrane.

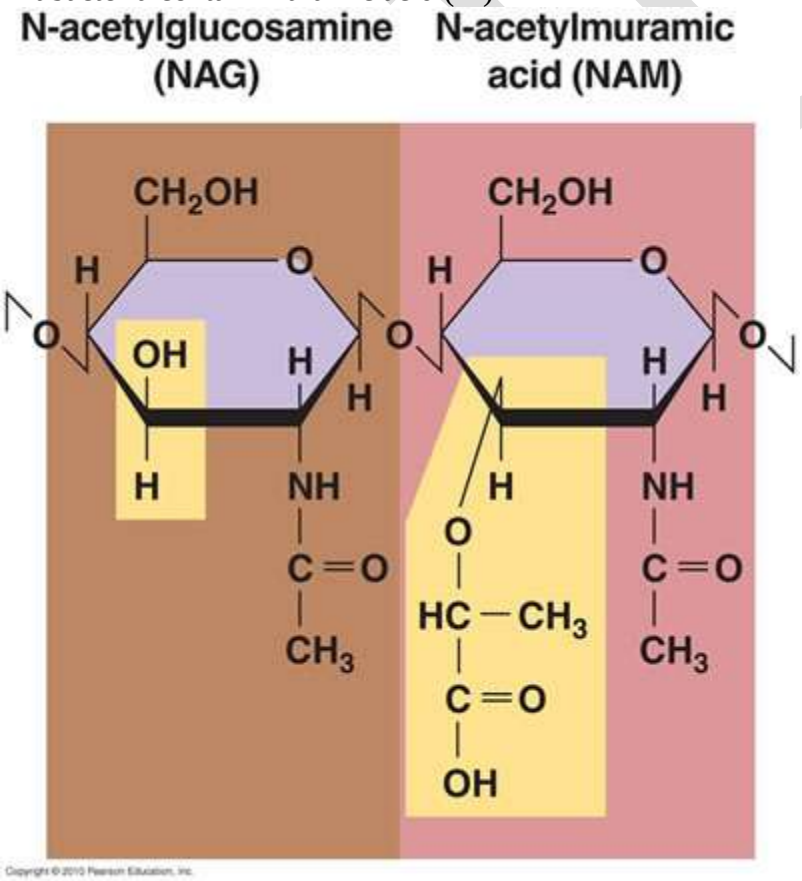
Mycoplasmas do not have a cell wall and their cell membrane has pumps to maintain proper osmotic pressure.

Their cell membranes contain different sterols that help in stabilization.

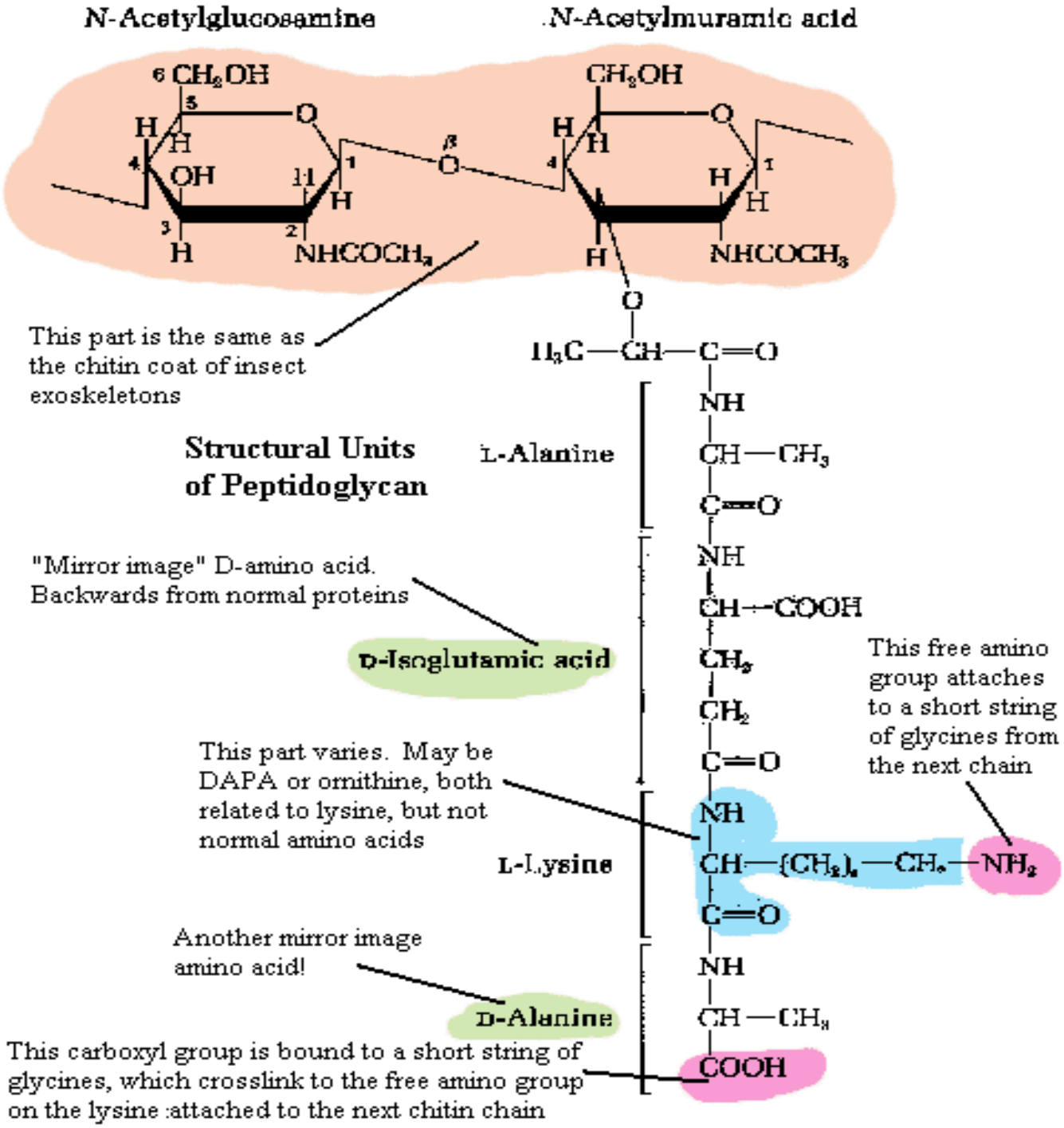
Function: to maintain shape, protect, and prevents organism from rupturing

Structure of Cell Wall :

Peptidoglycan of Eubacteria contain muramic acid (M)



Archaea bacteria do not have muramic acid



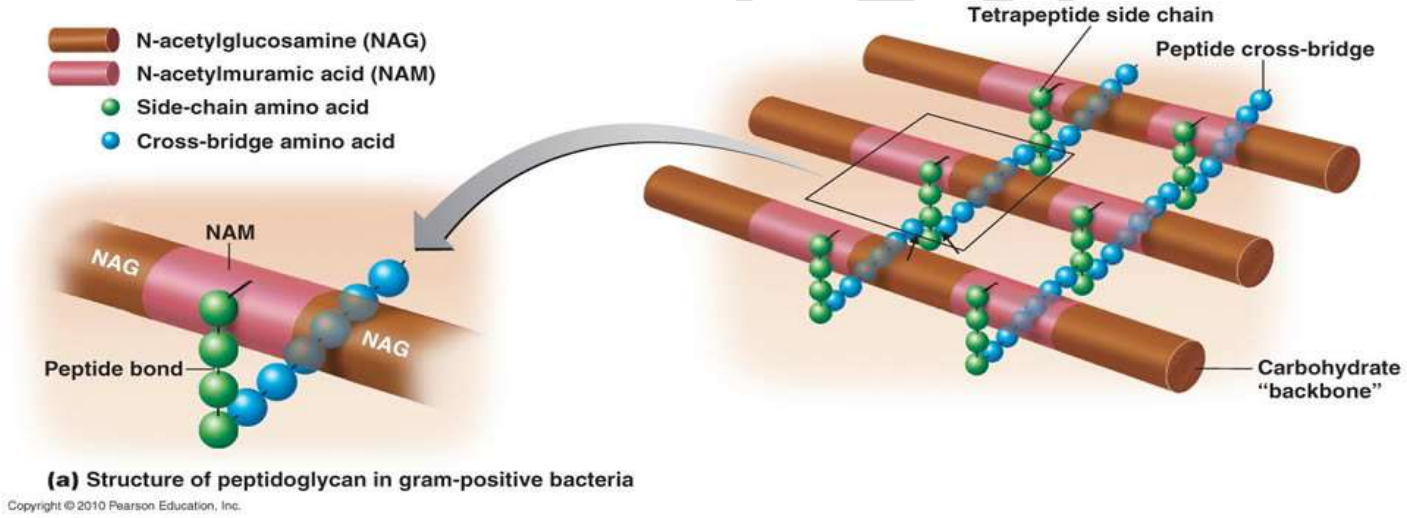
Composed of repeating & interlocking amino+disaccharides (NAG = N-acetylglucosamine and NAM = N-acetylmuramic acid) forming a carbohydrate backbone monomer [glycan part] linked by a ß 1-4 bond with an additional short tetrapolypeptide (4 AA) side chain [peptido part] that attachs to the NAM polymer.

The 4 AA side chain contains L-Alanine, D-Glutamate, mes-diaminopimelic acid (DPA), and D-alanine. Therefore, a single peptidoglycan unit is composed of NAM, NAG and the animo-side chain. The peptidoglycan unit is linked by covalent bonds to form a repeating polymer which is further strengthened by cross bridges between the amino acid 3 (D-glutamic acid) and the amino acid 4 (DPA) of the next glycan tetrapeptide. The degree of cross linkage determines the degree of rigidity for the cell wall.
In order for bacteria to increase the size following binary fission, the links in the peptidoglycan must be broken so that new polymers can be added and linked (transglycolation) and the tetrapolypeptide crosslinks resealed.

Bacterial enzymes are involved such as:

- Autolysins break the cross links in the peptidoglycan @ the ß 1-4 linkage
- Transpeptidase add new peptidoglycan monomers and reseal the wall

Interference with this process will result in a weak cell wall and lysis of the bacterium.



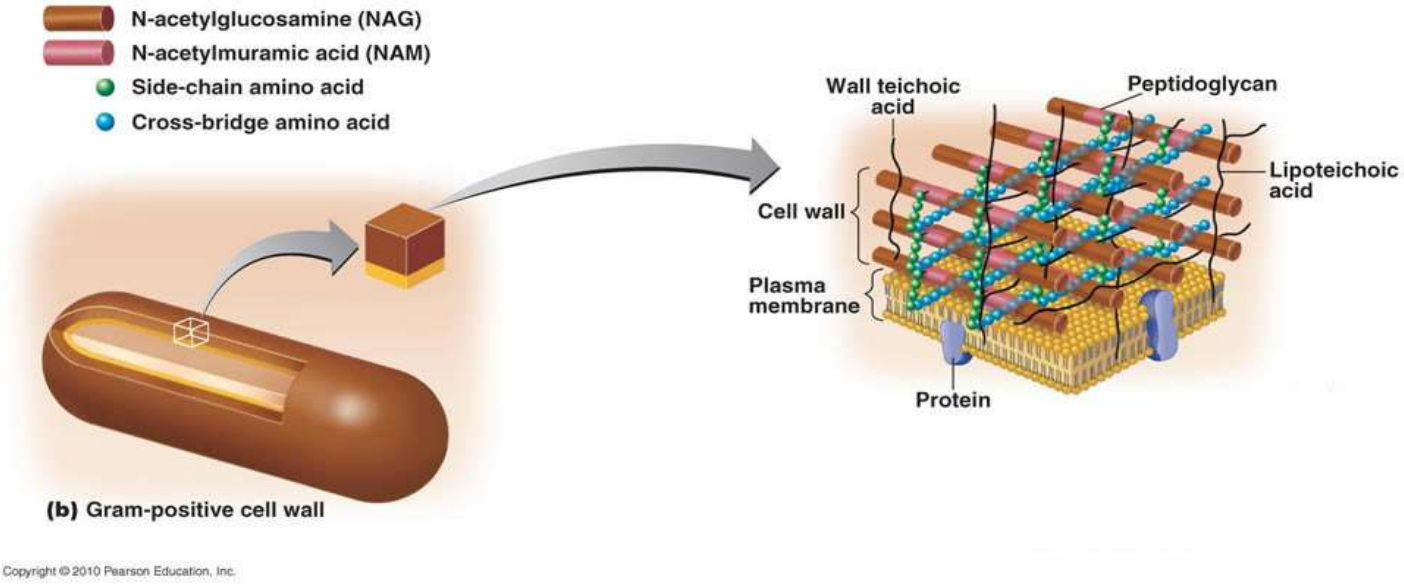
(a) Structure of peptidoglycan in gram-positive bacteria

G(+) bacteria have simple cell walls composed of many layers of peptidoglycan which form 60%-90% of their cell wall, up to 50% of the cell weight to create a 25-80nm thick structure that is heavily crosslinked. Teichoic acids are also found, composed of polymers of glycerol or ribitol joined by phosphate groups. Amino acids such as D-alanine are attached. Teichoic acid is also linked to muramic acid and helps link various layers of the peptidoglycan mesh together.

The Gram-positive Cell Wall

Gram-positive cell walls consist of many layers of peptidoglycan and also contain teichoic acids. Teichoic acids may:

- bind and regulate movement of cations into and out of the cell
- prevent extensive wall breakdown and possible cell lysis during cell growth
- provide much of the cell wall's antigenicity



G(-) bacteria have complex cell walls containing one or a few layers of peptidoglycan to create a 3nm thick structure, which makes up 10%-20% of the cell wall and only intermediately cross linked. No teichoic acids are found. The peptidoglycan layer sits in a space containing periplasm fluid between the cell membrane and the outer membrane which allows for H2O, nutrients, and protein transport. The outer membrane of the G(-) cell wall contains similar structures to the cell membrane in addition to lipoproteins and lipopolysaccharides. Porin proteins are also found that act as a channel through the outer phospholipid membrane. These channel proteins allow the passage of nutrients, vitamins, and viral attachments. The lipopolysaccharide [LPS] in the outer membrane consists of an O-polysaccharide that is antigenic (creates an immune response), a core structure, and a Lipid A, which is considered to be an endotoxin. Lipopolysaccharides [LPS] can also impede the entry of drugs into the cells and therefore, create more resistance to antibiotics.

Additional importance is placed on the capability of antibiotics such as penicillin to prevent the construction of peptidoglycan in G(+) bacteria. Lysozyme produced in an immune response (in tears) can disrupt the linkage between the carbohydrates (NAM and NAG) in the peptidoglycan layers. G(-) bacteria are more resistant to destruction due to the additional outer membrane structures described above.

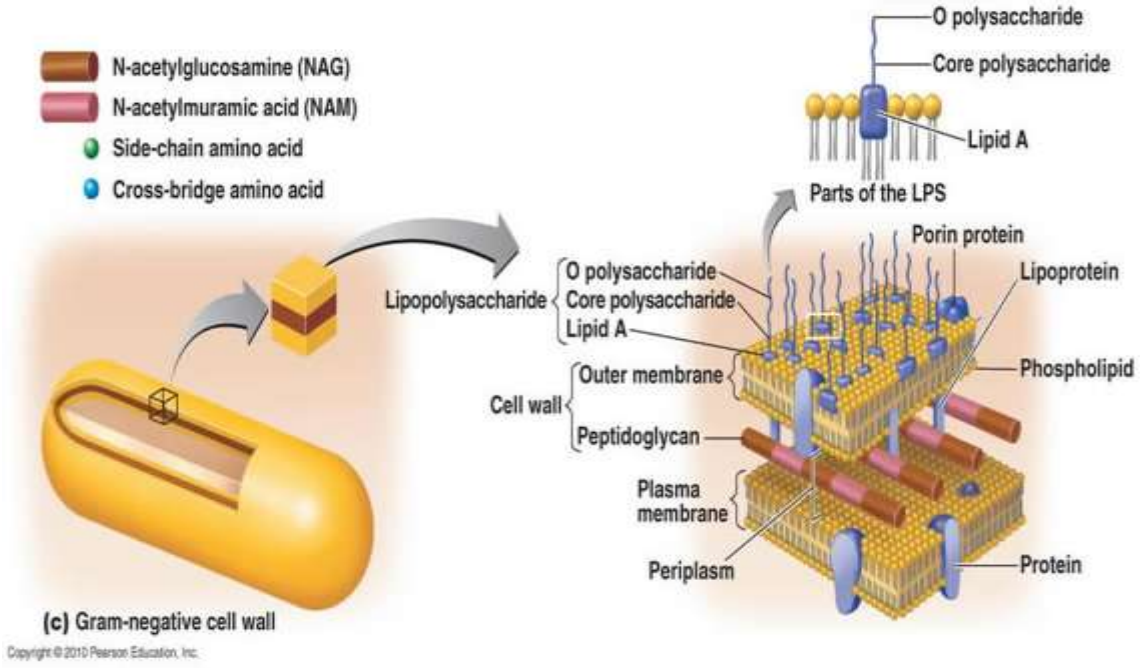
The Gram-negative Cell Wall

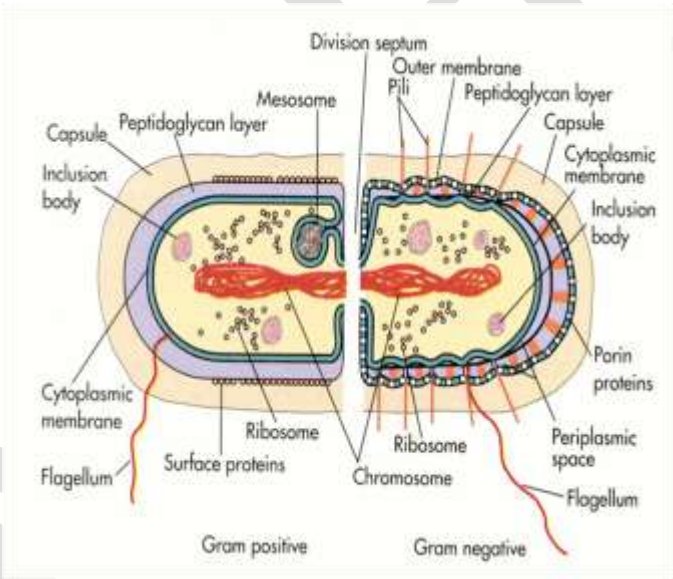
Gram-negative bacteria have a lipopolysaccharide-lipoprotein-phospholipid outer membrane surrounding a thin (sometimes a single) peptidoglycan layer. Gram-negative cell walls have no teichoic acids.

The outer membrane protects the cell from phagocytosis and from penicillin, lysozyme, and other chemicals.

Porins are proteins that permit small molecules to pass through the outer membrane; specific channel proteins allow other molecules to move through the outer membrane.

The lipopolysaccharide component of the outer membrane consists of sugars (O polysaccharides) that function as antigens and lipid A, which is an endotoxin. Endotoxin causes fever and shock.





Atypical Cell Walls

Atypical Cell Walls

No cell walls or very little material is present in

A) Mycoplasmas: plasma membrane has sterols, no cell wall

B) Archaea: cell wall has pseudomurein

C) Acid Fast organisms have a glycolipid outer membrane (instead of phospholipids) composed of mycolic acid, arabinogalactan-lipid complex, and lipoarabinomannan. They do have a thin peptidoglycan layer and a phospholipids cell (plasma) membrane (much like the G – bacteria).

Mycoplasma is a bacterial genus that naturally lacks cell walls; the presence of sterols in the plasma membrane protects from osmotic lysis.

Mycobacterium is a genus that has mycolic acids in its cell walls, giving it a "waxy" cell wall that is resistant to decolorization with acid-alcohol when stained with carbolfuschin (and so is designated "acid-fast").

Archaea have pseudomurein; they lack peptidoglycan.

Damage To The Cell Wall

In the presence of lysozyme, gram-positive cell walls are destroyed, and the remaining cellular contents are referred to as a protoplast.

In the presence of lysozyme (after disruption of the outer membrane), gram-negative cell walls are not completely destroyed, and the remaining cellular contents are referred to as spheroplasts.

Protoplasts and spheroplast are subject to osmotic lysis.

Proteus and some other genera can lose their cell walls spontaneously or in response to penicillin and swell into L forms (Lister Institute). L forms can live and divide and/or return to the normal walled state.

Antibiotics such as penicillin interfere with cell wall (peptidoglycan) synthesis.

Cell Walls and the Gram Stain Mechanism

The Gram stain was developed by Christian Gram in 1884 and can differentiate between the two types of cell walls we've just looked at.

The cells are first stained with the **primary stain**, crystal violet.

After about 1 minute of staining excess primary stain is washed off and the **mordant**, Gram's iodine, is applied for another minute.

The iodine forms a complex with the crystal violet and the crystal violet-iodine complex becomes "trapped" inside the peptidoglycan.

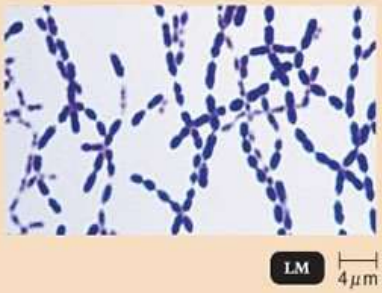
At this point cells with either type of cell wall appear purple, due to the presence of the crystal violet-iodine complex.

The next step is to **decolorize** (using acetone-alcohol) the cells for a period of time long enough to dissolve the outer membrane of Gram-negative cells and pull the crystal violet-iodine complex through the thin layer of peptidoglycan.

If done properly, Gram-positive cells retain the crystal violet-iodine complex and appear purple while Gram-negative cells are decolorized (and appear colorless, no?)

Note: decolorizing too long will result in both Gram-positive and Gram-negative cells appearing colorless, while decolorizing for too short a time will result in both cell types retaining the crystal violet-iodine complex and appearing purple. After decolorization Gram-positive cells (purple) can be differentiated from Gram-negative cells (colorless) but since colorless cells are hard to see the final step in the Gram staining process is to use a **counter-stain**, safranin, to stain the Gram-negative cells pink. Gram-positive cells will also stain with safranin but it will not be seen on top of the purple crystal violet-iodine remaining in the cells.

Table 4.1 Some Comparative Characteristics of Gram-Positive and Gram-Negative Bacteria

Characteristic	Gram-Positive	Gram-Negative
	 LM 4 μm	 LM 4 μm
Gram Reaction	Retain crystal violet dye and stain blue or purple	Can be decolorized to accept counterstain (safranin) and stain pink or red
Peptidoglycan Layer	Thick (multilayered)	Thin (single-layered)
Teichoic Acids	Present in many	Absent
Periplasmic Space	Absent	Present
Outer Membrane	Absent	Present
Lipopolysaccharide (LPS) Content	Virtually none	High
Lipid and Lipoprotein Content	Low (acid-fast bacteria have lipids linked to peptidoglycan)	High (because of presence of outer membrane)
Flagellar Structure	2 rings in basal body	4 rings in basal body
Toxins Produced	Exotoxins	Endotoxins and exotoxins
Resistance to Physical Disruption	High	Low
Cell Wall Disruption by Lysozyme	High	Low (requires pretreatment to destabilize outer membrane)
Susceptibility to Penicillin and Sulfonamide	High	Low
Susceptibility to Streptomycin, Chloramphenicol, and Tetracycline	Low	High
Inhibition by Basic Dyes	High	Low
Susceptibility to Anionic Detergents	High	Low
Resistance to Sodium Azide	High	Low
Resistance to Drying	High	Low

Cytoplasm = Cytosol + Organelles

Cytosol

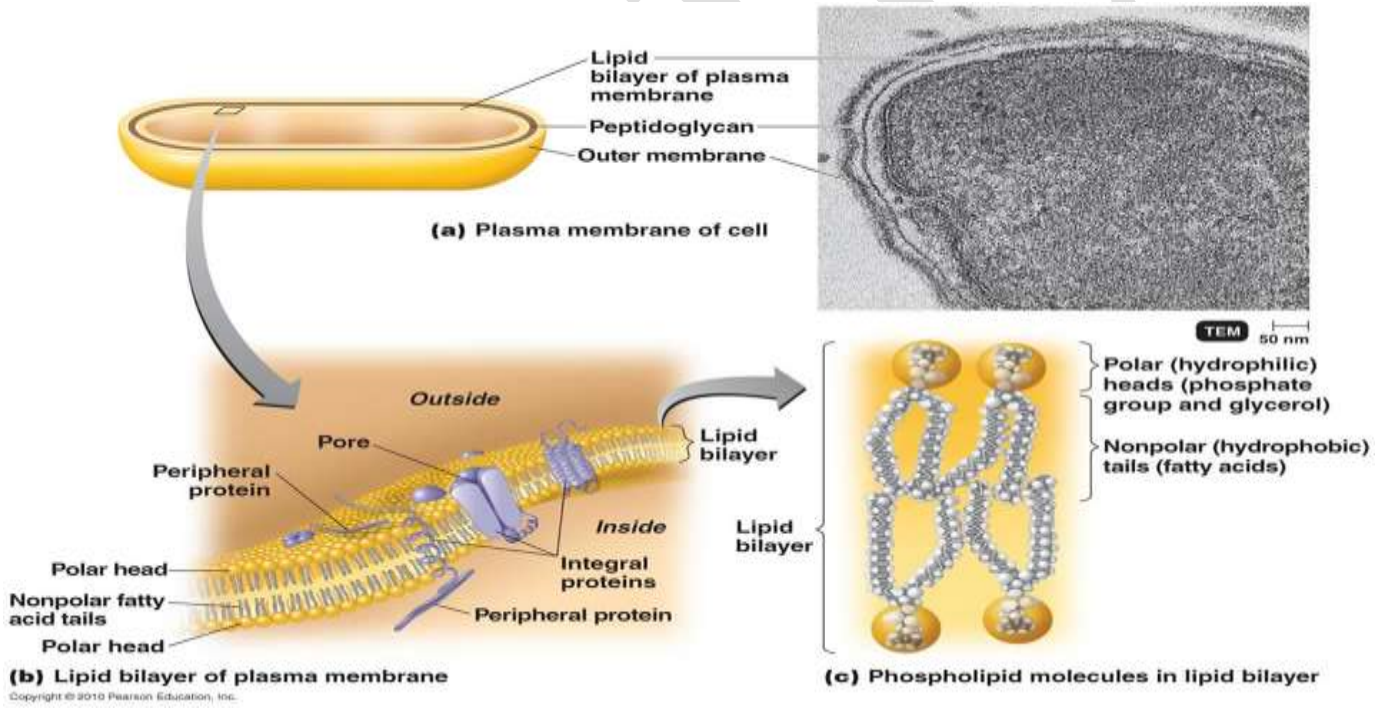
- 80% water
- proteins (primarily enzymes)
- Carbohyrates
- Lipids

- Salts / Ions

- ***Cytoplasm***
- Cytoplasm is the fluid component inside the plasma membrane.
- The cytoplasm is mostly water, which inorganic and organic molecules, DNA, ribosomes, and inclusions.

- **Structures Internal To The Cell Wall**
- **The Plasma (Cytoplasmic) Membrane**

- The plasma membrane encloses the cytoplasm and is a phospholipid bilayer with peripheral and integral proteins (**the fluid mosaic model**).
- The plasma membrane is selectively permeable.
- Plasma membranes carry enzymes for metabolic reactions, such as nutrient breakdown, energy production, DNA replication and photosynthesis in prokaryotes. These reactions take place in mitochondria, the nucleus, and chloroplasts in eukaryotic cells.



Nuclear Area (Nucleoid)

a) Single (haploid) unit of long, supercoiled, continuous closed circular (two ends covalently bonded together) double stranded DNA containing the bacterial chromosome (genome), attached to the plasma (cell) membrane.

It contains the genetic material of the bacteria.

Very little protein is associated with bacterial DNA, but there are histone-like proteins (HU, IHF, H-NS) bonded to the DNA to create about 50 domains which help make it more compact. An enzyme, DNA gyrase (topoisomerase II) helps to supercoil each domain to help cause a winding and twisting of the DNA around itself creating a compact size.

Other DNA topoisomerase enzymes are essential in unwinding, replicating, and rewinding the circular, supercoiled DNA.

REMEMBER : NO MITOSIS STAGES, NO MEIOSIS (only one chromosome) !!
The bacterial chromosome is generally 1000µm long and frequently contains as many as 3500 genes.
Bacterial DNA replication is bi-directional and leads to the replicated DNA strand created at the replication forks appearing like a Greek Letter, called a theta structure.
Protein synthesis occurs much like it does in Eukaryotes:
Genes located along the DNA are transcribed into RNA that undergo translation (mRNA, tRNA, rRNA).
Recall that the DNA base sequences determine which proteins / enzymes an organism can synthesize which therefore determines the type of chemical reactions that can occur.
NOTE: Some chemotherapeutic agents can inhibit normal nucleic acid replication.

b) Small circular rings of DNA called plasmids that contain supplemental genes:
a. Not essential for growth, are capable of autonomous replication
b. Double stranded, helical (some may have a linear form)
c. Can have anywhere from 1-700 copies of a plasmid in a cell
d. Associated with plasma membrane proteins
e. Replicate independently from bacterial chromosomes
f. Contain genes for toxins, AB resistance, other enzymes : examples include

R-plasmids in certain bacteria have the genes that code for production of sex pili and antibiotic resistance. Exchange plasmids during a process called conjugation

Other plasmids can code for exotoxins or endotoxins

c) Transponons (transposable elements or jumping genes)

a. Small pieces of DNA (1-12 genes long) that encode enzymes that move the gene from one DNA location to another.

b. May be found as part of the nucleoid or in plasmids

c. Contains genes coding for antibiotic resistance

d. Uses transposes, enzymes that cut and reseal the DNA during the exchange, so they can cut themselves out and insert themselves into another nucleoid or plasmid

e. Specific transponons called Integrons that carry multiple gene clusters that can integrate and accumulate as this unit can move from one piece of DNA to another.

The Nuclear Area

The nuclear area contains the DNA of the bacterial chromosome.

Bacteria can also contain plasmids, which are circular, extra-chromosomal DNA molecules.

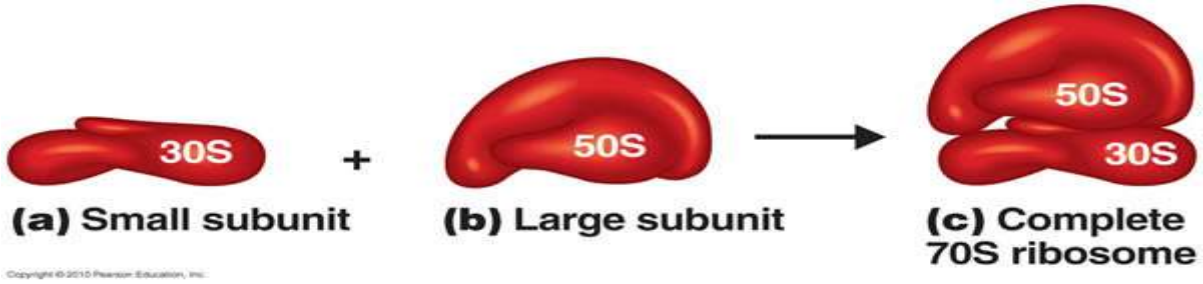
Ribosomes

- Protein syntheis: mRNA attaches to the 30s subunit and the tRNA attaches to the 50s subunit
- 2 subunits : 30s and 50s to form 70s
- contain rRNA and proteins (which are different from Eukaryotic cells)
- smaller and less dense than Eukaryotic ribosomes, 80s (40S + 60S)
- Some antibiotics alter bacterial ribosomes, thus interfering with translation

Ribosomes

The cytoplasm of a prokaryote contains numerous 70s ribosomes; ribosomes consist of rRNA and protein.

Protein synthesis occurs at ribosomes; it can be inhibited by certain antibiotics. The difference between prokaryotic (70s) and eukaryotic (80s) ribosomes allows antibiotics to selectively target the prokaryotic ribosomes while sparing eukaryotic ribosomes.



Inclusions

Inclusions are reserve deposits found in prokaryotic and eukaryotic cells.

Among the inclusions found in bacteria are metachromatic granules (inorganic phosphate), polysaccharide granules (usually glycogen or starch), lipid inclusions, sulfur granules, carboxysomes (ribulose 1,5-diphosphate carboxylase), magnetosomes (Fe3O4), and gas vacuoles.

Inclusions

Metachromic Granules

i. Volutin – reserve of PO4= for ATP
ii. Can help in ID of certain bacteria, such as Diptheria

Polysaccharided granules

i. Glycogen- store carbon and energy reserve
ii. Starch

Lipid – storage

Sulfer – store sulfur

Carboxysomes – enzymes for use of CO2 as carbon source

Gas vacuoles – buoyancy

Magnetosomes – iron magnetite to help in orientation

Poly-beta-hydroxyalkanoate (PHA) – plastic like polymer that functions as a carbon and energy storage.

Magnetosomes

- present in several gram-negative bacteria (*Aquaspirillum magnetotacticum* for example)
- act like magnets
- may be used to move downward until they reach a suitable attachment site
- have been demonstrated to decompose hydrogen peroxide in vitro so it has been suggested that magnetosomes may protect cells from hydrogen peroxide accumulation.

Chromatophores

Infoldings of the plasma membrane that contain pigments involved in photosynthesis.

Mesosomes, irregular infoldings of the plasma membrane, are artifacts, not true cell structures.

Plasma membranes can be destroyed by alcohols and polymyxins.

Endospores

Highly durable/ resistant dehydrated cells formed internal to plasma membrane.

Little or no metabolism occurs

Created by specialized vegetative cells when nutrients are depleted and are formed to help certain bacteria survive extreme environmental conditions.

Primarily seen with G(+) bacteria.

Formation called sporogenesis or sporulation.

Process: Vegetative cells are exposed to harsh environmental conditions. Cells begin sporulation by replicating their DNA and creating a septal wall that closes off the replicated DNA. This structure is called a forespore. The membrane layers can synthesize additional peptidoglycan layers to form a series of thick protein coats called the spore coat, a keratin like protein. The original cell disintegrates (lysis) and the spore is then released. Sporulation takes around 15 hours.

Arrangement of spores can help in classifying organisms:

- Central/middle
- Subterminal (near the end)
- Terminal (at the end)

The final spore structure contains

- Resistant outer coats (cortex, spore coat, exosporium)
- Core: Nucleiod region with some ribosomes, RNA, enzymes

It can remain dormant for many years and survive the harshest environments, even if using

- Chemicals
- Heat / Drying
- Radiation
- Boiling

Reversal to vegetative state is by germination and the breaking down of the protective layers which will occur once proper environmental conditions return.

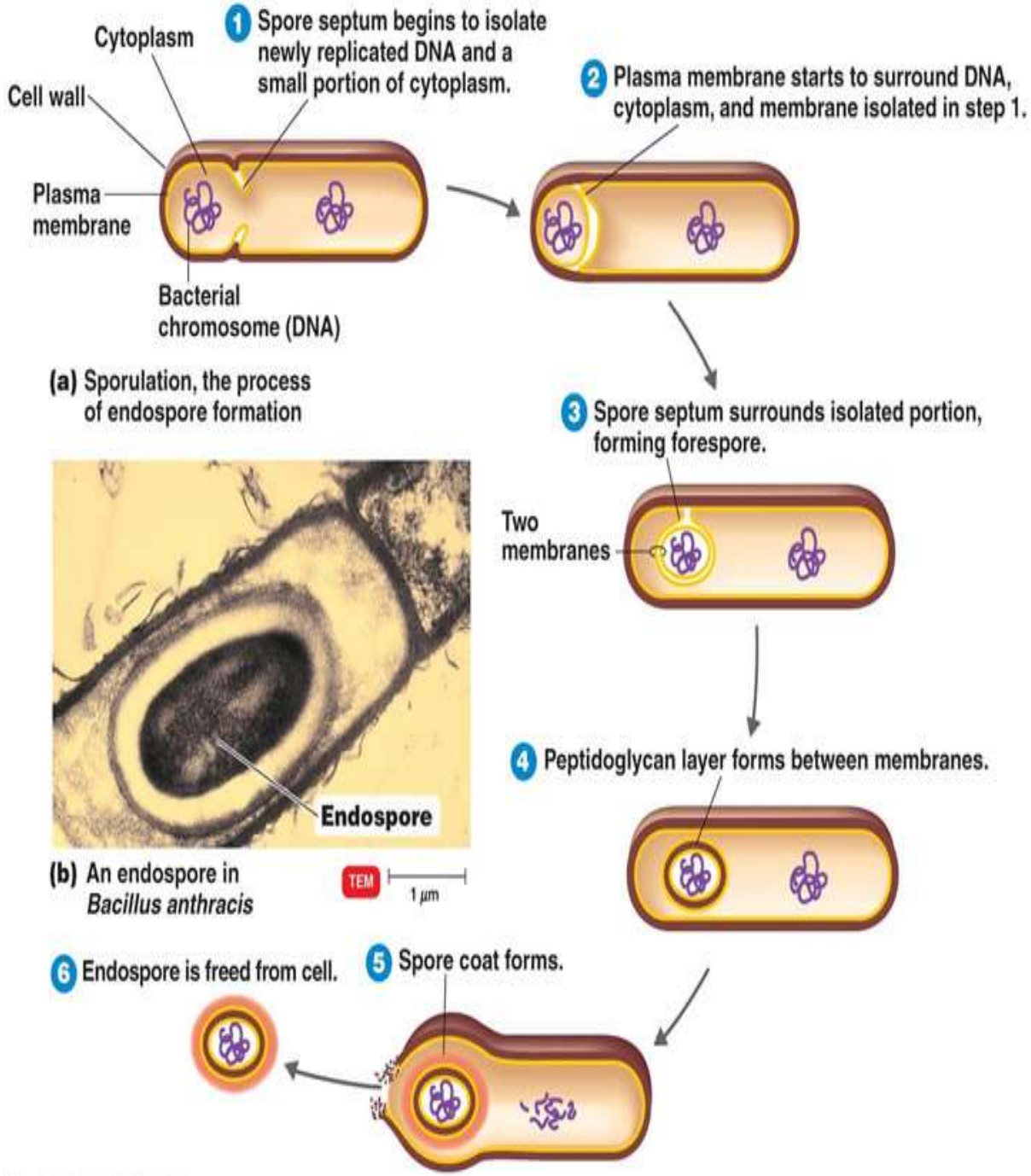
Spores require special stain to view and are the most resistant structures. Examples of bacterial genuses that forms spores are *Bacillus* and *Clostridium*.

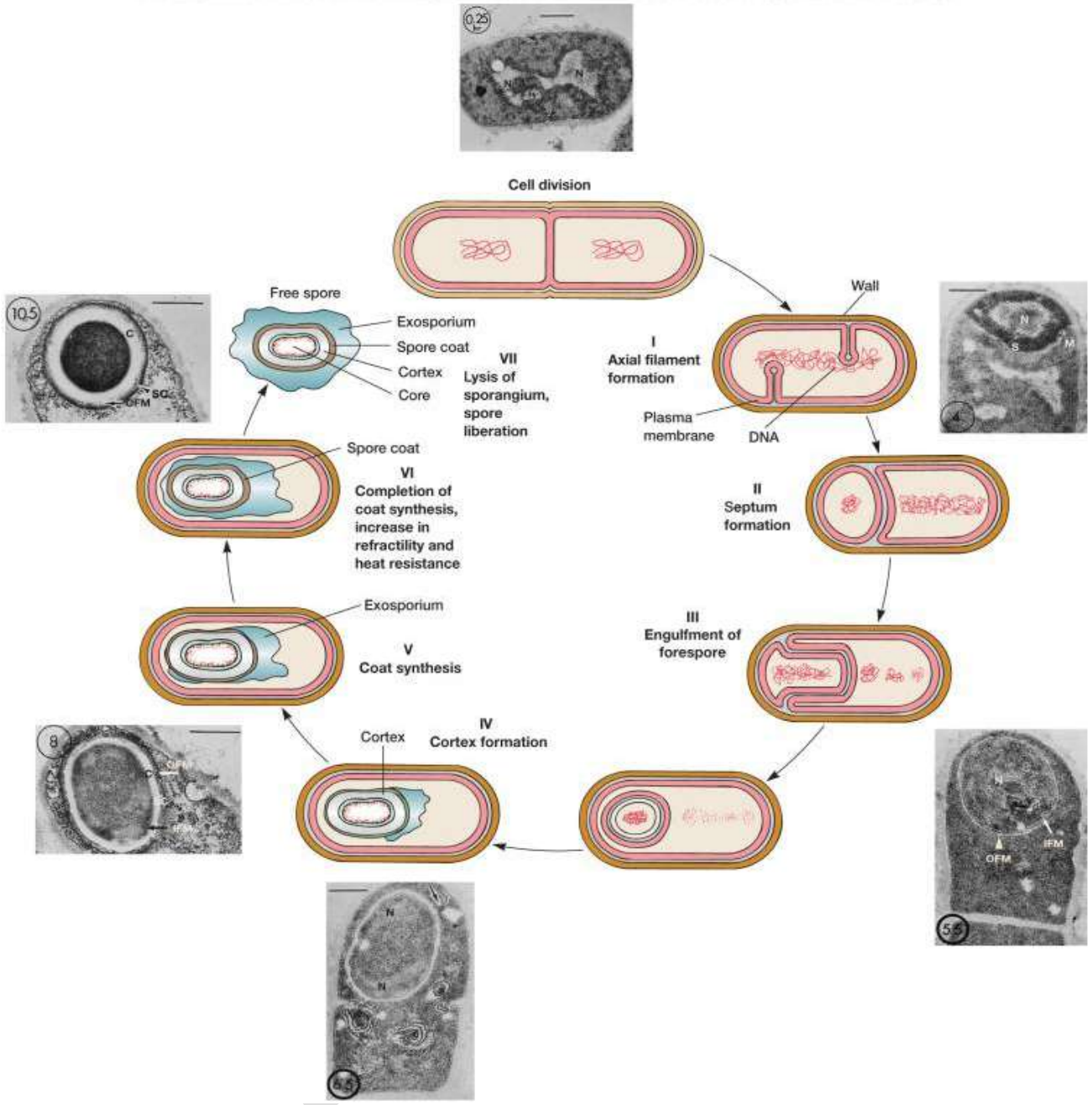
Anthrax	*Bacillus anthracis*
Tetanus	*Clostridium tetani*
Botulism	*Clostridium botulinum*
Gas Gangrene	*Clostridium perfringes*

Endospores

Endospores are resting structures formed by some bacteria for survival during adverse environmental conditions.

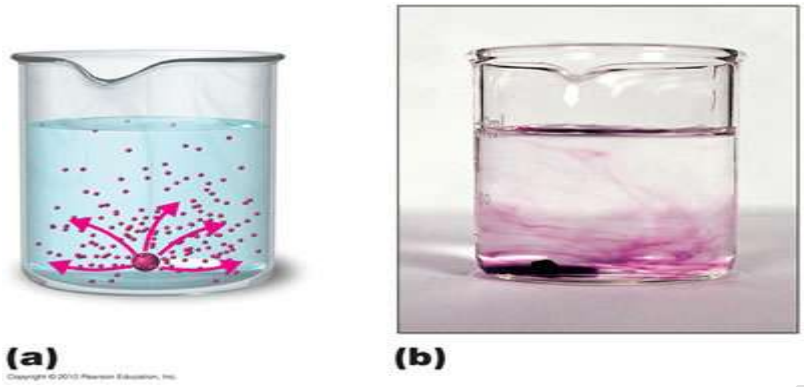
The process of endospore formation is called sporulation; the return of an endospore to its vegetative state is called germination. Two genera that commonly form endospores are *Bacillus* and *Clostridium*.



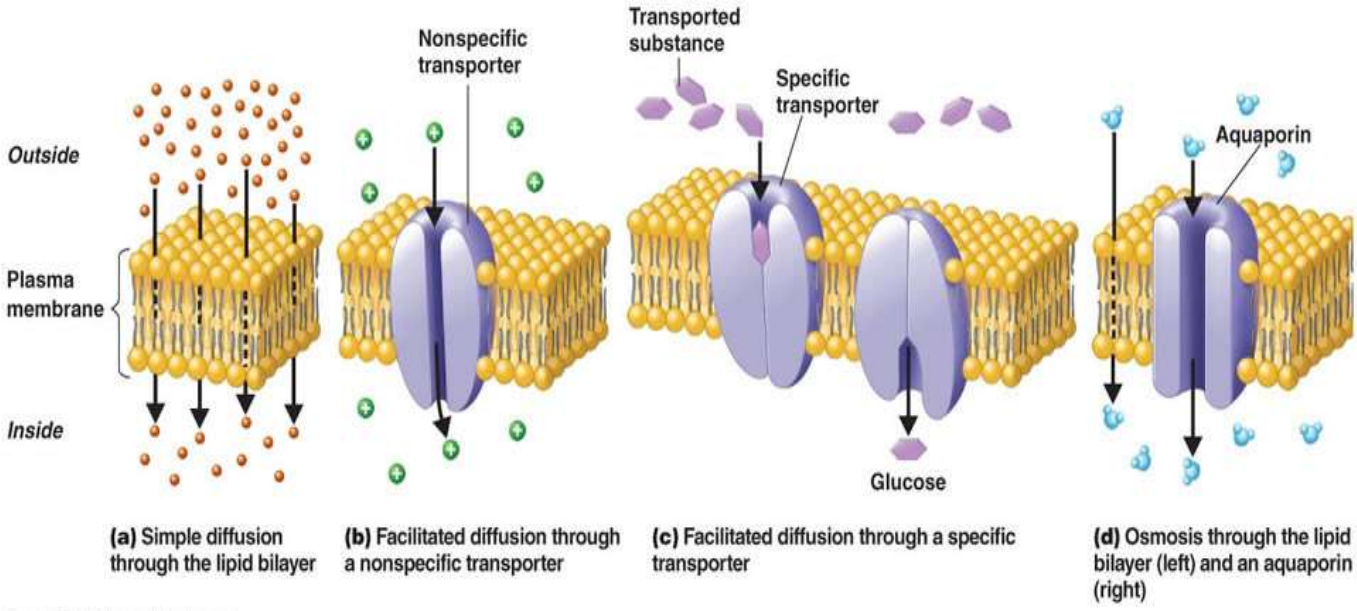


The Movement of Materials Across Membranes

Movement across the membrane may be by passive processes, in which materials move from areas of higher to lower concentration, and no energy is expended by the cell.

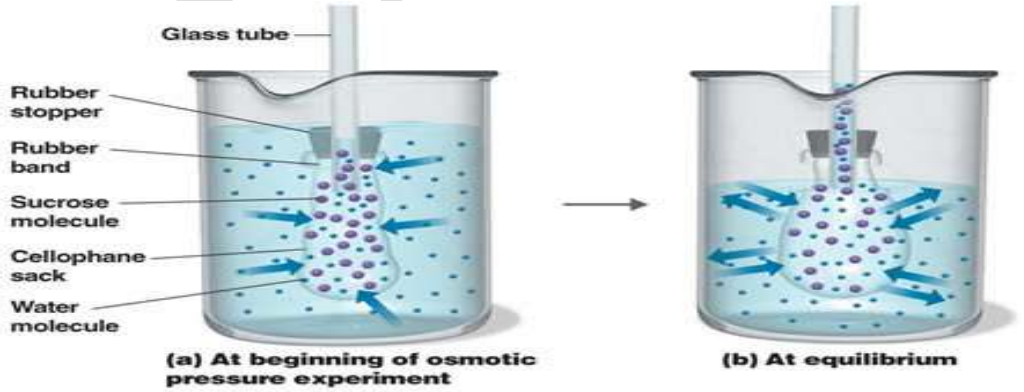


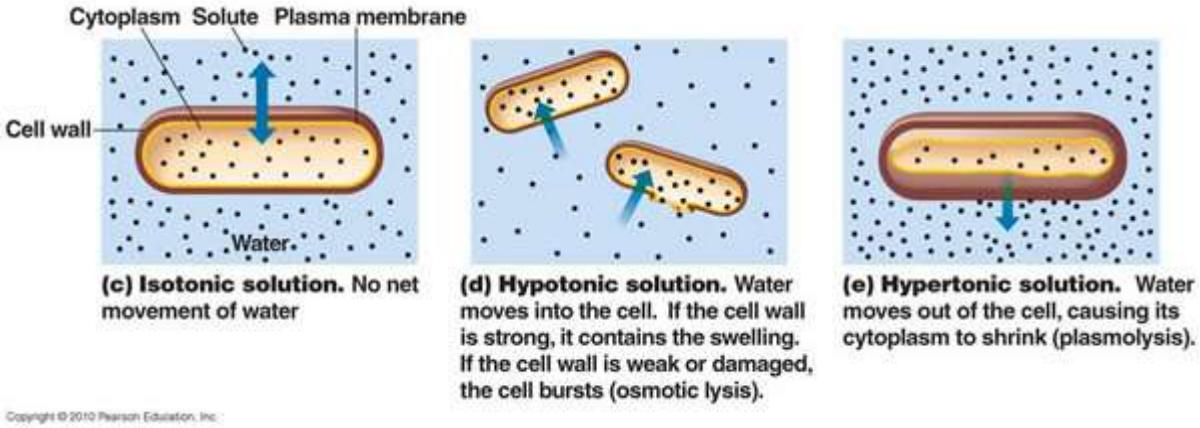
In simple diffusion, molecules and ions move until equilibrium is reached.



In facilitated diffusion, substances are transported by transporter proteins across membranes from areas of high to low concentration.

Osmosis is the movement of water from areas of high to low concentration across a selectively semi permeable membrane until equilibrium is reached.





(c) **Isotonic solution.** No net movement of water

(d) **Hypotonic solution.** Water moves into the cell. If the cell wall is strong, it contains the swelling. If the cell wall is weak or damaged, the cell bursts (osmotic lysis).

(e) **Hypertonic solution.** Water moves out of the cell, causing its cytoplasm to shrink (plasmolysis).

In active transport, materials move from areas of low to high concentration by transporter proteins, and the cell must expend energy.

In group translocation, energy is expended to modify chemicals and transport them across the membrane. Once inside the cell the chemical modification prevents the transported substance from moving out of the cell. Example: glucose is converted to glucose-6- phosphate as it passes into the cell. This traps it and allows the movement of more glucose into the cell even when extracellular concentrations may be low.

Spheroplast

A **spheroplast** is a cell from which the cell wall has been almost completely removed, as by the action of penicillin. The name stems from the fact that after a microbe's cell wall is digested, membrane tension causes the cell to acquire a characteristic spherical shape. Spheroplasts are osmotically fragile, and will lyse if transferred to a hypotonic solution.

Uses and applications

Antibiotic discovery

From the 1960s into the 1990s Merck and Co. used a spheroplast screen as a primary method for discovery of antibiotics that inhibit cell wall biosynthesis. In this screen devised by Eugene Dulaney, growing bacteria were exposed to test substances under hypertonic conditions. Inhibitors of cell wall synthesis caused growing bacteria to form spheroplasts. This screen enabled the discovery of fosfomycin, cephamycin C, thienamycin and several carbapenems.

Patch clamping

Specially prepared giant spheroplasts of Gram-negative bacteria can be used to study the function of bacterial ion channels through a technique called patch clamp, which was originally designed for characterizing the behavior of neurons and other excitable cells. To prepare giant spheroplasts, bacteria are grown in a medium containing chemicals that prevent the cells from dividing completely. This causes bacteria to form long "snakes" that share a single membrane and cytoplasm. After a period of time, the cell walls of the "snakes" are digested, and the bacteria collapse into very large spheres surrounded by a single lipid bilayer. The membrane can then be analyzed on a patch clamp apparatus to determine the phenotype of the ion channels embedded in it. It is also common to overexpress a particular channel to amplify its effect and make it easier to characterize.

The technique of patch clamping giant *E. coli* spheroplasts has been used extensively for studying the native mechanosensitive channels (MscL, MscS, and MscM) of *E. coli* since 1987. Recently, it has been extended to study other heterologously expressed ion channels and it has been shown that the giant *E. coli* spheroplast can be used as an ion-channel expression system comparable to the Xenopus oocyte.

Cell lysis

Yeast cells are normally protected by a thick cell wall which makes extraction of cellular proteins difficult. Enzymatic digestion of the cell wall with zymolyase, creating spheroplasts, renders the cells vulnerable to easy lysis with detergents or rapid osmolar pressure changes.

Transfection

Bacterial spheroplasts, with suitable recombinant DNA inserted into it, can be used to transfect animal cells. Spheroplasts with recombinant DNA are introduced into the media containing animal cells and are fused by polyethylene glycol (PEG). With this methodology, nearly 100% of the animal cells may take up the foreign DNA.

Protoplasts and spheroplasts

Protoplasts and spheroplasts are altered forms of **bacteria** or **yeast** , in which the principal shape-maintaining structure of the bacteria is weakened. Each bacterium forms a sphere, which is the shape that allows the bacterium to withstand the rigors, particularly osmotic, of the fluid in which it resides.

The term **protoplast** refers to the spherical shape assumed by Gram-positive bacteria. Spheroplast refers to the spherical shape assumed by Gram-negative bacteria. The difference is essentially the presence of a single membrane, in the case of the protoplast, and the two membranes (inner and outer) of the Gram-negative spheroplasts. It is also possible to generate a gram-negative protoplast by the removal of the outer membrane. Thus, in essence, protoplast refers to a bacterial sphere that is bounded by a single membrane and spheroplast refers to a sphere that is bounded by two membranes.

Bacteria are induced to form protoplasts or spheroplasts typically by laboratory manipulation. However, formation of the structures can occur naturally. Such bacteria are referred to as L-forms. Examples of bacterial genera that can produce L-forms include *Bacillus*, *Clostridium*,*Haemophilus*, *Pseudomonas*, *Staphylococcus*, and *Vibrio*.

The **peptidoglycan** is the main stress-bearing layer of the bacterial cell wall and the peptidoglycan also gives the bacterium its shape. In the laboratory, weakening the peptidoglycan network in the cell wall generates both protoplasts and spheroplasts.

By exposing bacteria to an enzyme called lysozyme, the interconnecting strands of the two particular sugars that form the peptidoglycan can be cut. When this is done, the peptidoglycan loses the ability to serve as a mechanical means of support.

The situation in yeast is slightly different, as other components of the yeast cell wall are degraded in order to form the protoplast.

The process of creating protoplasts and spheroplasts must be done in a solution in which the ionic composition and concentration of the fluid outside of the bacteria is the same as that inside the bacteria. Once the structural support of the peptidoglycan is lost, the bacteria are unable to control their response to

differences in the ionic composition between the bacterial interior and exterior. If the inner concentration is greater than the outer ionic concentration, water will flow into the bacterium in an attempt to achieve an ionic balance. The increased volume can be so severe that the bacteria will burst. Conversely, if the inner ionic concentration is less than the exterior, water will exit the bacterium, in an attempt to dilute the surroundings. The bacteria can shrivel to the point of death.

Preservation of ionic balance is required to ensure that bacteria will not be killed during their **transformation** into either the protoplast or the spheroplast form. Living protoplasts and spheroplasts are valuable research tools. The membrane balls that are the protoplasts or spheroplasts can be induced to fuse more easily with similar structures as well as with eukaryotic cells. This facilitates the transfer of genetic material between the two cells. As well, the sequential manufacture of spheroplasts and protoplasts in Gram-negative bacteria allows for the selective release of the contents of the **periplasm**. This approach has been popular in the identification of the components of the periplasm, and in the localization of proteins to one or the other of the Gram-negative membranes. For example, if a certain protein is present in a spheroplast population—but is absent from a protoplast population—then the protein is located within the outer membrane.

Uses for protoplasts

Protoplasts can be used to study membrane biology, including the uptake of macromolecules and viruses . These are also used insomaclonal variation.

Protoplasts are widely used for DNA transformation (for making genetically modified organisms), since the cell wall would otherwise block the passage of DNA into the cell.[1] In the case of plant cells, protoplasts may be regenerated into whole plants first by growing into a group of plant cells that develops into a callus and then by regeneration of shoots (caulogenesis) from the callus using plant tissue culturemethods.[2] Growth of protoplasts into callus and regeneration of shoots requires the proper balance of plant growth regulators in the tissue culture medium that must be customized for each species of plant. Unlike protoplasts from vascular plants, protoplasts frommosses, such as *Physcomitrella patens*, do not need phytohormones for regeneration, nor do they form a callus during regeneration. Instead, they regenerate directly into the filamentous protonema mimicking a germinating moss spore.[3]

Protoplasts may also be used for plant breeding, using a technique called protoplast fusion. Protoplasts from different species are induced to fuse by using an electric field or a solution of polyethylene glycol. Originally, non–Ti-plasmid dependent DNA uptake was demonstrated in 1985 by R. Hain and A.P. Czernilofsky et al., in the article "Uptake, integration, expression and genetic transmission of a selectable chimaeric gene by plant protoplasts" using the Ca-phosphate coprecipitation technique.[4] This technique may be used to generate somatic hybrids in tissue culture.

Additionally, protoplasts of plants expressing fluorescent proteins in certain cells maybe used for Fluorescence Activated Cell Sorting (FACS), where only cells fluorescing a chosen wavelength are retained. Among other things, this technique is used to isolate specific cell types (e.g. guard cells from leaves, pericycle cells from roots) for further investigation such as transcriptomics.

L-form bacteria, also known as **L-phase bacteria**, **L-phase variants**, and **cell wall-deficient (CWD) bacteria**, are strains of bacteria that lack cell walls. They were first isolated in 1935 by Emmy Klieneberger-Nobel, who named them **"L-forms"** after the Lister Institute in London where she was working.

Two types of L-forms are distinguished: *unstable L-forms*, spheroplasts that are capable of dividing, but can revert to the original morphology, and *stable L-forms*, L-forms that are unable to revert to the original bacteria.

Some parasitic species of bacteria, such as mycoplasma, also lack a cell wall, but these are not considered L-forms since they are not derived from bacteria that normally have cell walls.

Appearance and cell division

Bacterial morphology is determined by the cell wall. Since the L-form has no cell wall, its morphology is different from that of the strain of bacteria from which it is derived. Typical L-form cells are spheres or spheroids. For example, L-forms of the rod-shaped bacterium *Bacillus subtilis* appear round when viewed by phase contrast microscopy or by transmission electron microscopy.

Although L-forms can develop from Gram-positive as well as from Gram-negative bacteria, in a Gram stain test, the L-forms always colour Gram-negative, due to the lack of a cell wall.

The cell wall is important for cell division, which, in most bacteria, occurs by binary fission. This process usually requires a cell wall and components of the bacterial cytoskeleton such as FtsZ. The ability of L-form bacteria to grow and divide in the absence of both of these structures is highly unusual, and may represent a form of cell division that was important in early forms of life.[1] This novel mode of division seems to involve the extension of thin protrusions from the cell's surface and these protrusions then pinching off to form new cells. The lack of cell wall in L-forms means that division is disorganised, giving rise to a variety of cell sizes, from very tiny to very big.

Generation in cultures

L-forms can be generated in the laboratory from many bacterial species that usually have cell walls, such as *Bacillus subtilis* or *Escherichia coli*. This is done by inhibiting peptidoglycan synthesis with antibiotics or treating the cells with lysozyme, an enzyme that digests cell walls. The L-forms are generated in a culture medium that is the same osmolarity as the bacterial cytosol (anisotonic solution), which prevents cell lysis by osmotic shock. L-form strains can be unstable, tending to revert to the normal form of the bacteria by regrowing a cell wall, but this can be prevented by long-term culture of the cells under the same conditions that were used to produce them.

Some studies have identified mutations that occur, as these strains are derived from normal bacteria. One such point mutation is in an enzyme involved in the mevalonate pathway of lipid metabolism that increased

the frequency of L-form formation 1,000-fold.The reason for this effect is not known, but it is presumed that the increase is related to this enzyme's role in making a lipid important in peptidoglycan synthesis.

Another methodology of induction relies on nanotechnology and landscape ecology. Microfluidics devices can be built in order to challenge peptidoglycan synthesis by extreme spatial confinement. After biological dispersal through a constricted (sub-micrometre scale) biological corridor connecting adjacent micro habitat patches, L-form-like cells can be derived using a microfluifics-based (synthetic) ecosystem implementing an adaptive landscape selecting for shape-shifting phenotypes similar to L-forms.

Significance and applications

Some publications have suggested that L-form bacteria might cause diseases in humans, and other animals but, as the evidence that links these organisms to disease is fragmentary and frequently contradictory, this hypothesis remains controversial. The two extreme viewpoints on this question are that L-form bacteria are either laboratory curiosities of no clinical significance or important but unappreciated causes of disease. Research on L-form bacteria is continuing. For example, L-form organisms have been observed in mouse lungs after experimental inoculation with *Nocardia caviae*, and a recent study suggested that these organisms may infect immunosuppressed patients having undergone bone marrow transplants. The formation of strains of bacteria lacking cell walls has also been proposed to be important in the acquisition of bacterial antibiotic resistance.

L-form bacteria may be useful in research on early forms of life, and in biotechnology. These strains are being examined for possible uses in biotechnology as host strains for recombinant protein production. Here, the absence of a cell wall can allow production of large amounts of secreted proteins that would otherwise accumulate in theperiplasmic space of bacteria. The accumulation of proteins in the periplasmic space can be toxic to the bacteria, which may reduce the yield of the expressed proteins.

Possible Questions

Unit – I

Part B (Two marks)

1. Define Glycocalyx
2. Define protoplasts and sphaeroplasts.
3. Draw a neat diagram on the structure of flagella.
4. Distinguish between prokaryotes and eukaryotes.
5. Comment on the archae bacterial cell wall.

Part C (Eight marks)

1. Discuss about the structure of the gram positive cell wall.
2. Describe the stages involved in the formation of endospore.
3. Discuss on the role of endospore in the bacterial sustainability.
4. Comment on the action of penicillin on the bacterial cell wall.
5. Discuss the structure and function of pili.

KARPAGAM ACADEMY OF HIGHER EDUCATION

CLASS: I B.Sc., MB **COURSE NAME: BACTERIOLOGY**

COURSE CODE: 18MBU102 **UNIT-I** **BATCH: 2018-2021**

S.no	Question	Opt 1	Opt 2	Opt 3	Opt 4	Answer
1	Which of the following bacteria lack a cell wall and are therefore resistant to penicillin?	Cyanobacteria	Mycoplasma	Bdellovirbios	Spirochetes	Mycoplasma
2	A cluster of polar flagella is called	lophotrichous	amphitrichous	monotrichous	pertrichous	lophotrichous
3	The protein from which hook and filaments of flagella are composed of, is	keratin	flagellin	gelatin	casein	flagellin
4	The cocci which mostly occur in pairs are	Streptocci	Diplococci	Tetracoci	Pentacocci	Diplococci
5	Which of the following may contain fimbriae?	Bacillus	Streptomyces	Trichoderma	Aspergillus	Bacillus
6	Peptidoglycan accounts for _____ of the dry weight of cell wall in many gram positive bacteria	>50%	>10%	>11%	>20%	>50%
7	Bacteria having no flagella are unable to	move	reproduce	stick to tissue surface	grow in nutrient agar	move
8	Which of the following is false about cell wall of gram positive bacteria?	It consist of multiple layers	It is thicker than that associated with gram-negative bacteria	It contains teichoic acids	it is thinner than gram negative bacteria	it is thinner than gram negative bacteria
9	The cell walls of many gram positive bacteria can be easily destroyed by the enzyme known as	lipase	lysozyme	pectinase	peroxidase	lysozyme
10	For most bacteria, the optimum pH for growth lies between	6.5-7.5	3.5-4.5	4.5-5.5	5.5-6.5	6.5-7.5
11	Suspension cultures consist of cells and cell aggregates, growing dispersed in	liquid medium	solid nutrient medium	solid or liquid medium	semi solid medium	liquid medium

12	The protoplast can be used to	modifiy genetic information	create plant hybrid	study plant viral infections	study allelic information	modifiy genetic information
13	The cell wall of	gram-positive bacteria are thicker than gram-negative bacteria	gram-negative bacteria are thicker than gram-positive bacteria	both have same thickness but composition is different	both have different thickness and composition	gram-positive bacteria are thicker than gram-negative bacteria
14	Peptidoglycan is also known as	NAM	Murein mucopeptide	N acetyly glucosamine	mesodiaminopimetic acid	Murein mucopeptide
15	Which is most likely to be exposed on the surface of a gram-negative bacterium?	Pore prtoein (porin)	Protein involved in energy generation	lipothecoic acid	phospholipids	lipothecoic acid
16	The last step in synthesis of peptidoglycan is	attachment of a peptide to muramic acid	attaching two amino acids to form a cross-link	attachment of a portion of peptidoglycan to a membrane lipid	binding of penicillin to a membrane protein	attaching two amino acids to form a cross-link
17	Cytoplasmic inclusions include	ribosomes	mesosomes	fat globules	vacuoles	ribosomes
18	The cocci which forms a bunch and irregular pattern are	Staphylococci	diplococci	tetracocci	streptococci	Staphylococci
19	Chemotaxis is a phenomenon of	swimming away of bacteria	swimming towards or away of bacteria	swimming around	swimming upward	swimming towards or away of bacteria
20	The structure responsible for motility of bacteria is	pili	flagella	sheath	capsule	flagella
21	The next to last step in peptidoglycan biosynthesis is	synthesis of the NAM-	removal of the subunit from	linking the sugar of the	cross-linking the peptide side chains	cross-linking the peptide side

		peptide subunit	bactoprenol	disaccharide-peptide unit to the growing peptidoglycan chain	of peptidoglycan	chains of peptidoglycan
22	The cocci which forms a chain is	streptococci	diplococci	staphylococci	tetracocci	streptococci
23	The arrangement, in which flagella are distributed all round the bacterial cell, is known as	lophotrichous	ampphitrichous	peritrichous	monotrichous	peritrichous
24	Periplasm is	the area between the inner and outer membranes of gram-negative bacteria	the area between the inner and outer membranes of Gram-positive bacteria	the interior portion of mitochondria	the area outside the cell membrane that is influenced by the polymers	the area between the inner and outer membranes of gram-negative bacteria
25	Which of the following has peptidoglycan as a major constituent of cell wall?	Gram-negative bacteria	Gram-positive bacteria	Fungi	virus	Gram-positive bacteria
26	The common word for bacteria which are helically curved rods is	cocci	pleomorphic	bacillus	spirilla	spirilla
27	The bacteria deficient in cell wall is	Treponema	Mycoplasma	Staphylococcus	Klebsiella	Mycoplasma
28	Which of the following is not true about peptidoglycan?	It is a polymer consisting of NAM, NAG and di amino pimelic acid	It is present in prokaryotic cell wall,	It occurs in the form of a bag shaped macro molecule surrounding the cytoplasm membrane	It has lipid content in it	It occurs in the form of a bag shaped macro molecule surrounding the cytoplasm membrane
29	The common word for bacteria which	cocci	bacilli	spirilla	pleomorphic	spirilla

	are spherical in shape is					
30	Single or clusters of flagella at both poles is known as	monotrichous	peritrichous	amphitrichous	lophotrichous	amphitrichous
31	Which of the following bacterial genera (that produces endospore) have medical importance?	proteus	Bacillus	vibrio	E.coli	Bacillus
32	Bacteria are _____.	Obligate	unicellular	multicellular	seen by naked eye	unicellular
33	Who is father of Microbiology?	Leewen hoek	Twort and Felix	Edward Jenner	Louis Pasteur	Leewen hoek
34	Which year gram staining was introduced?	1884	1965	1997	1983	1965
35	Coccus means____shape	spherical	square	rectangular	irregular	spherical
36	Ribosomes are made up of _____ subunits	2	3	5	10	2
37	Bacterial motility can be performed by _____ method	hanging drop	slant	viewing drop	dry mount	hanging drop
38	Stain means_____	dye	agent	bacteria	organism	dye
39	Bacteria comes under the group _____	Monera	Portistata	Planta	Animalia	Monera
40	_____ classified bacteria into two groups	David Baltimore	Edward Jenner	Montangier	Christian Gram	Christian Gram
41	Bacteria reproduce by _____ mechanism	Fission	Own	Fusion	Direcdt	Fission
42	Bacteria are sensitive to _____	interleukins	interferon	antibiotic	antitumor	antibiotic
42	_____media is used for cultivation of bacteria	Nutrient agar	Mackonkey agar	EMB agar	Macconkey agar	Nutrient agar
43	Single bacteria will form a _____ colony	Multiple	Single	No	infinite	Single

44	Which instrument is used for sterilization above 100° C	Flame	Autoclave,	Filters,	Desiccators	Autoclave
45	_____ is the first phase in growth curve	Log	Lag	stationary,	death	Lag
46	DNA to DNA is called as	replication	biosynthesis,	translation,	transcription	replication
47	Oligonucleotide means containing	10 nucleotides	more than 10 nucleotides,	less than 10 nucleotides,	no nucleotides	10 nucleotides
48	_____ group of bacteria grows in high temperature	Halophile	Basophile	thermophile	psychrophile	thermophile
49	The group of gram positive bacteria having low G+C contents are called as	cyanobacteria	Nanobacteria	Firmicutes	Actinobacteria	Firmicutes
50	BGA expanded as	Blue Green Algae	Blue Grown Algae,	Blue non Grown Algae,	Blue Gram Algae	Blue Green Algae
51	Bacteria are _____.	Obligate,	single celled	multicellular,	seen by naked eys.	single celled
52	Who is father of Modern Microbiology.	Leewenhoek	Twort	Edward Jenner	Louis Pasteur	Louis Pasteur
53	Strain means_____	dye	agent,	bacteria	organisms	organisms
54	Prokayotic ribosomes are made up of _____ subunits	two,	three,	five,	ten	two
55	Which instrument is used for sterilization below 100° C	Flame,	Autoclave,	Filters,	Desiccators	Flame,
56	_____ is the Second phase in growth curve	Log,	Lag,	stationary,	death	Log
57	DNA to RNA is called as	replication	biosynthesis,	translation,	transcription	transcription
58	_____ group of bacteria grows in high pressure	Halophiles,	Basophiles,	thermophiles,	psychrophiles	Basophiles,
59	The group of gram positive bacteria having high G+C contents are called as	cyanobacteria	Nanobacteria,	Firmicutes,	Actinobacteria	Actinobacteria

Staining-principle and types of staining (Simple and Differential). Pure culture technique-microbial preservation- cultivation of anaerobic bacteria.

Preparation of Wet Mount of Bacteria to Observing its Natural Shape and Size

Aim: To prepare wet mount of a bacteria, for observing its natural shape, size and arrangement in living condition.

Purpose:

Bacteria cells can be seen easily and clearly, when colored by stains, but in most of the staining processes, the cells die and lose their natural shape and size due to heat-fixation as well as due to exposure to chemicals (stains, acid and alcohol).

The aim of this experiment is to observe the natural shape, size and arrangement of bacteria in living condition. Motility cannot be observed in wet mount, as the cells are pressed between the slide and the cover slip.

Principle:

In wet mount, a drop of the bacteria suspension is placed on a slide, covered with a cover slip and observed under a compound microscope or preferably under a dark-field or phase-contrast microscope using oil-immersion objective.

Materials Required:

Glass slide, cover slip, petroleum jelly, immersion oil (cedar wool oil or liquid paraffin), bacteria culture (on solid medium or in liquid suspension), loop and microscope (compound, dark-field or phase- contrast).

Procedure:

1. A slide is cleaned properly under tap water, such that water does not remain as drops on its surface.
2. The slide is dried by wiping with bibulous paper and subsequently moving over flame or keeping in the sun.
3. If the bacteria has been grown on a solid medium (as slant culture or plate culture), a drop of distilled water is put at the center of the slide. A loop is sterilized by heating it over a flame to red hot. It is cooled to room temperature. Then, aseptically a small portion of bacteria culture is scraped by the loop and transferred to the drop of water.

A suspension of bacteria is made by gently mixing the loop of bacteria in the water drop, in such a way that, the drop does not spread. If grown in a liquid medium as suspension, aseptically a drop of the bacteria suspension is placed at the center of the slide directly, using a flame-sterilized loop.

4. Petroleum jelly or similar material is applied surrounding the drop, so that, when the cover slip covers the drop, evaporation and effect of air current is minimized.
5. A cover slip is kept in a slanting position near the drop of bacteria suspension with one edge touching the slide.
6. Slowly the cover slip is lowered, till it touches the surface of the drop.
7. Then, the cover slip is released, so that the drop of bacteria suspension spreads in all directions under the cover slip. Care should be taken, so that no air bubble is formed in the suspension.
8. The slide is clipped on the stage of a compound microscope and observed using low power and high dry objectives. Then, a drop of immersion oil is put on the cover slip and observed using oil-immersion objective of the microscope. Preferably, a phase- contrast or dark-field microscope should be used for clear observation.

Observations (Under Oil-immersion Objective):

1. Shape of bacteria:

Spherical (coccus)

Rod-shaped (bacilli)

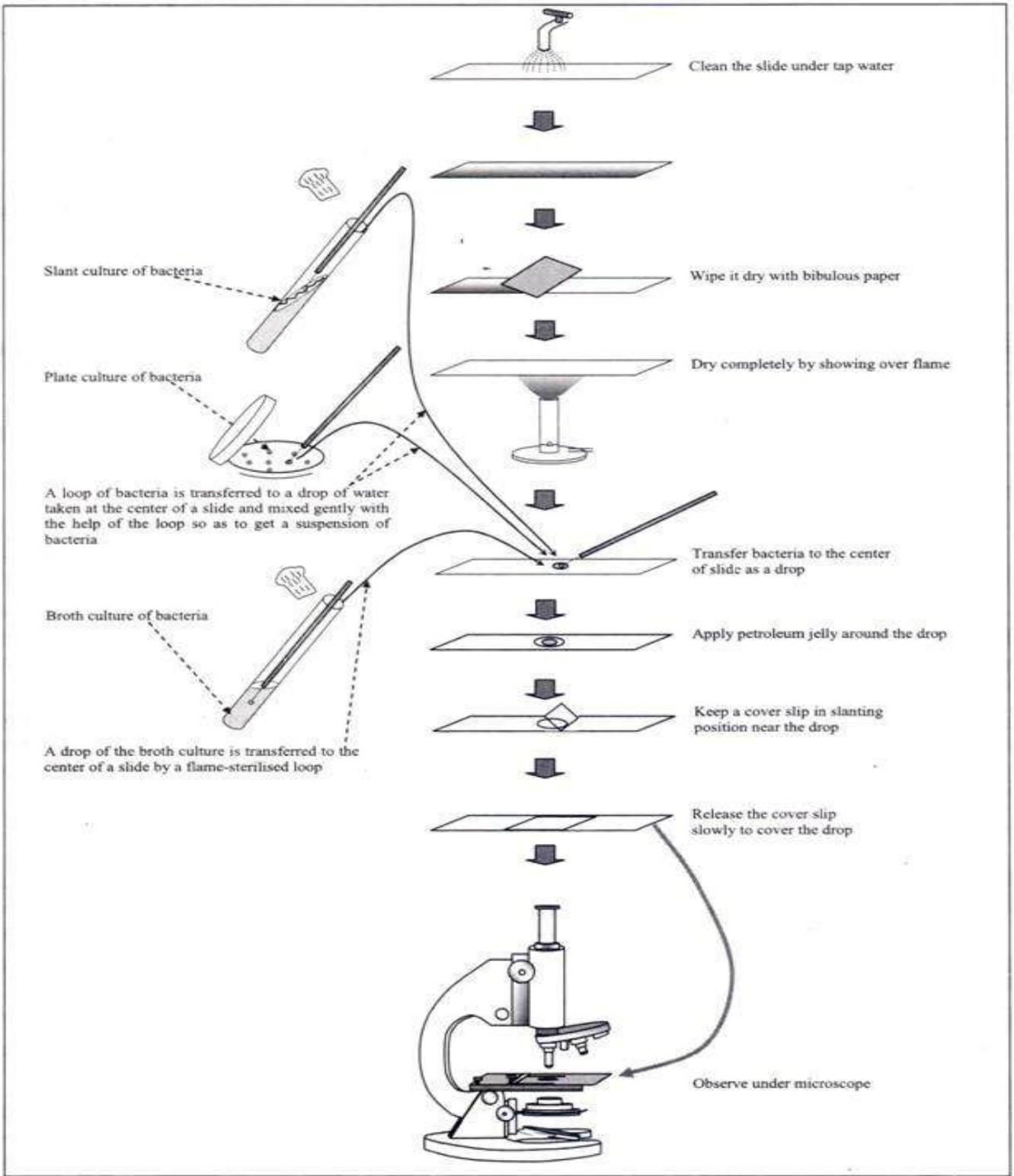


Figure 5.2: Wet mount of bacteria

2. Arrangement of bacteria:

Comma-like (vibrio)
Spiral (spirochetes)
Pairs (diplobacillus/diplococcus)
In fours (tetrads)
In chains (streptococcus/streptobacillus)
Grape-like clusters (staphylococcus)
Cuboidal (sarcinae or octet)

Hanging drop preparation

Hanging drop preparation is a special type of wet mount (in which a drop of medium containing the organisms is placed on a microscope slide), often is used in dark illumination to observe the motility of bacteria.

In this method a drop of culture is placed on a coverslip that is encircled with petroleum jelly (or any other sticky material). The coverslip and drop are then inverted over the well of a depression slide. The drop hangs from the coverslip, and the petroleum jelly forms a seal that prevents evaporation. This preparation gives good views of microbial motility.

Materials required:

1. Glass slides (glass slide with depression) or Normal glass slide with adhesive or paraffin ring
2. Paraffin wax
3. Loop
4. Coverslip
5. Microscope
6. Bunsen burner
7. Young broth culture of motile bacteria

Procedure:

1. Take a clean glass slide and apply paraffin ring, adhesive tape ring to make circular concavity. (This step is not needed if a glass slide with depression is available).
2. Hold a clean coverslip by its edges and carefully dab Vaseline on its corners using a toothpick.
3. Place a loopful of the broth culture to be tested in the center of the prepared coverslip.
4. Turn the prepared glass slide or concavity slide upside down (concavity down) over the drop on the coverslip so that the vaseline seals the coverslip to the slide around the concavity.
5. Turn the slide over so the coverslip is on top and the drop can be observed hanging from the coverslip over the concavity.
6. Place the preparation in the microscope slide holder and align it using the naked eye so an edge of the drop is under the low power objectives.
7. Turn the objective to its lowest position using the coarse adjustment and CLOSE THE DIAPHRAGM.
8. Look through the eyepiece and raise the objective slowly using the coarse adjustment knob until the edge of the drop is observed as an irregular line crossing the field.
9. Move the slide to make that line (the edge of the drop) pass through the center of the field.
10. Without raising or lowering the tube, swing the high dry objective into position (Be sure the high dry objective is clean).

11. Observe the slide through the eyepiece and adjust the fine adjustment until the edge of the drop can be seen as a thick, usually dark line.
12. Focus the edge of the drop carefully and look at each side of that line for very small objects that are the bacteria. The cells will look either like dark or slightly greenish, very small rods or spheres. Remember the high dry objective magnifies a little less than half as much as the oil immersion objective.
13. Adjust the light using the diaphragm lever to maximize the visibility of the cells.
14. Observe the cells noting their morphology and grouping and determine whether true motility can be observed.
15. Brownian movement should be visible on slides of all the organisms, but there should also show true motility.
16. Wash the depression slide and after soaking in lysol buckets or discard the prepared glass slide.

Note: While examining living organism for the property of active locomotion, it is essential to distinguish true motility, whereby the organism move in different directions and change their positions in the field, from either

- Passive drifting of the organisms in the same direction in a convectional current in the fluid or
- Brownian movement, which is an oscillatory movement about a nearly fixed point possessed by all small bodies suspended in fluid and due to irregularities in their bombardments by molecules of water.

Cultivation of Bacteria from Solid, Liquid and Swab Samples (With Figure) | Experiment

Purpose:

The main purposes of cultivation of bacteria are as follows:

1. Increasing the number of bacteria, so as to get them in visible forms, as colonies or suspensions.
2. Isolation of bacteria.
3. Maintenance of pure stock culture and standard cultures.
4. Enumeration of bacteria in samples.
5. Detection of particular bacteria of interest in a sample and its enumeration.
6. Identification of bacteria from colony characteristics, growth characteristics on slants and biochemical activities in different media.

However, the purpose of this experiment is only to cultivate bacteria from liquid, solid and surface samples in solid and liquid media, so as to get them in visible forms as colonies or suspension respectively.

Principle:

Bacteria are cultured in sterilized, nutritionally rich liquid or solid media. The liquid media, called broth, is contained in test tubes to form 'broth tubes', while the solid media, called agar media, is contained in petri dishes to form 'agar plates' or simply 'plates'. Cultivation of bacteria needs some material, which is suspected to contain bacteria.

Most of the things we see contain bacteria. They are present in teeth scrape, food, soil, water, fecal matter and even in the unsterilized microbiological media itself. A definite amount of the homogenous suspension of any of the bacteria-containing material is inoculated into the sterilized media aseptically and incubated in an incubator at 37°C for 24 hours.

In liquid broth, the bacteria grow as suspension and make it turbid. On solid agar plates, they grow as colonies; each colony growing from a single bacterium. Cultivation of bacteria is done in the following five steps.

1. Preparation of media
2. Sterilization of media and glass-wares
3. Inoculation
4. Incubation

5. Observation

1. Preparation of Media:

Usually, in preparation of liquid broth and semisolid media, the ingredients are weighed in the prescribed proportions and dissolved in the required amount of water. At present, ready-made media powders containing the ingredients in required proportions are available.

In preparation of liquid broth media, the prescribed quantity of powder (as mentioned on the label of the pack) is weighed and dissolved in the required amount of water in a conical flask. The ingredients are dissolved by warming, poured into test tubes and sterilized in autoclave.

But in preparation of solid plates, slants and deep tubes, the prescribed quantity of powder (as mentioned on the label of the pack) is weighed and dissolved in required amount of water in a conical flask. This prepared medium is first sterilized in autoclave and then allowed to cool for some time.

While still warm, before it solidifies, it is poured into sterilized Petri dishes or test tubes and allowed to solidify upon cooling to room temperature. Media, in very hot condition should never be poured into containers, as it leads to condensation of water on the wall of the containers, which drop on the surface of the media and may lead to its contamination.

2. Sterilisation:

Glasswares are sterilised in hot air oven at 180°C for 3 hours and media in autoclave at 121°C (15 psi pressure) for 15 minutes. Glasswares can also be sterilised in autoclave, but media should never be sterilised in the oven, as water escapes from the media and they dehydrate.

3. Inoculation:

Liquid samples are assumed to be homogenous suspensions of bacteria. Therefore, for cultivation in broth, a definite volume of the sample is pipetted into the broth aseptically. For cultivation on agar plates, a definite volume of the sample is pipetted onto the solid agar plate and spread on the surface of the medium aseptically.

For solid samples, a sample of definite weight is homogenized aseptically in a definite volume of normal physiological saline (0.85% sodium chloride) using a sterilized pestle and mortar or a blender. Most of the human pathogens are isotonic to human body (0.85% sodium chloride).

Usually the ratio of sample to saline is 1: 9 (1g + 9 ml; 10g + 90 ml; 25g + 225 ml or 50g + 450 ml). A definite volume of this homogenized liquid, which is assumed to be a homogenous suspension of bacteria, is pipetted aseptically to the sterilized broth in test tubes for broth culture. For culture on agar plates, the liquid suspension is pipetted onto the surface of the plates and spread aseptically.

For surface samples taken to study the microbiology of solid surfaces (table tops, body surfaces or wounds), the surface is rubbed with a sterilized swab. The swab is touched to the surface of a sterilized agar plate. From the point of touch, streaks are made by sterilized loop aseptically, so as to isolate the bacteria.

4. Incubation:

The inoculated broth tubes and plates are incubated at 37°C for 24 hours in an incubator.

5. Observation:

Turbidity in liquid broth and colonies on agar plates indicate growth of bacteria.

Material Required:

Petri dishes (6 nos.), 2-ml pipettes (5 nos.), test tubes (5 nos.), conical flasks (100 ml, 250ml and 500ml-l each), 250 ml beaker (2 nos.), glass spreader, stainless steel pipette case, craft paper, thread (or rubber band), non-absorbent cotton, small sticks, loop, ethyl alcohol, sodium chloride (NaCl), 0.1N hydrochloric acid (HCI), 0.1N sodium hydroxide (NaOH), distilled water, nutrient broth, nutrient agar, liquid sample (e.g. pond water), solid sample (e.g. soil), surface sample(e.g. table top, body surface, wound), pH paper

(or pH meter), pestle and mortar (or homogeniser), bunsen burner, hot air oven, autoclave, incubator, laminar flow chamber.

Procedure:

1. Five pipettes (in a stainless steel pipette case), six petri dishes and a pair of pestle and mortar (or one homogeniser cup) are sterilized at 180°C for 3 hours in a hot air oven. Alternatively, they can be covered with craft paper, tied with thread or rubber band and sterilized in autoclave along with the media (Figure 6.1).
2. 0.85 g of NaCl is dissolved in 100 ml of distilled water in a 250 ml beaker and 90 ml of this saline solution is poured into a 100 ml conical flask. The mouth is cotton-plugged, covered with craft paper and tied with thread or rubber band.
3. The ingredients of nutrient broth medium required for 100 ml of the broth are weighed. Alternatively, prescribed amount of ready-made nutrient broth powder (ingredient mixture) is weighed.
4. The ingredients (or the ready-made powder) are dissolved in 100 ml of distilled water in a 250 ml conical flask by shaking and swirling. Its pH is determined using a pH paper or pH meter. The pH is adjusted to 7.0 using 0.1N HCI if it is more or using 0.1N NaOH if it is less. The flask is heated, if required, to dissolve the ingredients completely.
5. The broth is distributed into 5 test tubes (approximately 10 ml each), their mouths cotton- plugged, covered with craft paper and tied with thread or rubber band.
6. The ingredients of nutrient agar medium or its ready-made powder required for 200 ml of the medium is weighed and dissolved in 200 ml of distilled water in a 500 ml conical flask by shaking and swirling. Its pH is determined using a pH paper or pH meter and adjusted to 7.0 using 0.1N HC1 if it is more or using 0.1N NaOH if it is less. The flask is heated to dissolve the agar in the medium completely. Then, it is cotton-plugged, covered with craft paper and tied with thread or rubber band.
7. Swabs are made by twisting cotton around the tips of small sticks. Few such swabs are kept in a test tube with the cotton tips downward. The test tube is cotton-plugged, covered with craft paper and tied with thread or rubber band.
8. The 100 ml conical flask with 90 ml saline, the 5 test tubes with nutrient broth, the 500 ml conical flask containing 200 ml nutrient agar medium and the test tube containing the swabs are sterilized at 121°C (15 psi pressure) for 15 minutes in an autoclave.
9. After sterilization, the sterilized materials are removed from the autoclave and allowed to cool for some time, without allowing the medium to solidify. Cooling of the medium prevents condensation and accumulation of water droplets inside the plates. If the medium has already been prepared and solidified during storage, it has to be liquefied by heating carefully till it melts completely.
10. To prepare nutrient agar plates, before the sterilised nutrient agar medium cools and solidifies, in warm molten condition, it is poured aseptically, into the 6 sterilised petri dishes (approximately 20 ml each), so that the molten medium covers the bottom of the petri dishes completely.

Then, the plates are covered with their lids and allowed to cool, so as to solidify the medium in them. Water vapour that may condense on the inner surface of the plates and lids is evaporated by keeping the plates and lids in inverted position in an incubator at 37°C for about 1 hour.

11. The liquid sample (suspected to contain bacteria, e.g. pond water) is taken. One ml each of the samples is aseptically pipetted into 2 broth tubes (Figure 6.2). The tubes are swirled. The liquid sample is also pipetted aseptically onto 2 agar plates, 0.1 ml each, and spread on the surface of the agar medium using a flame-sterilised glass spreader.

Before each spreading by the glass spreader, it is dipped in alcohol and flamed over a bunsen burner. The inoculated broth tubes and plates are incubated at 37°C for 24 hours in an incubator. The plates are incubated in inverted position, top down.

12. For the solid sample (e.g. soil), 10g of the sample is aseptically weighed and homogenised in the sterilised pestle and mortar or homogeniser cup after adding 90 ml of the sterilised saline from the 100 ml conical flask.

This homogenous bacteria suspension is aseptically pipetted into 2 broth tubes and 2 agar plates as in step 11 and incubated at 37°C for 24 hours in the incubator (Figure 6.2). The plates are incubated in inverted position, top down.

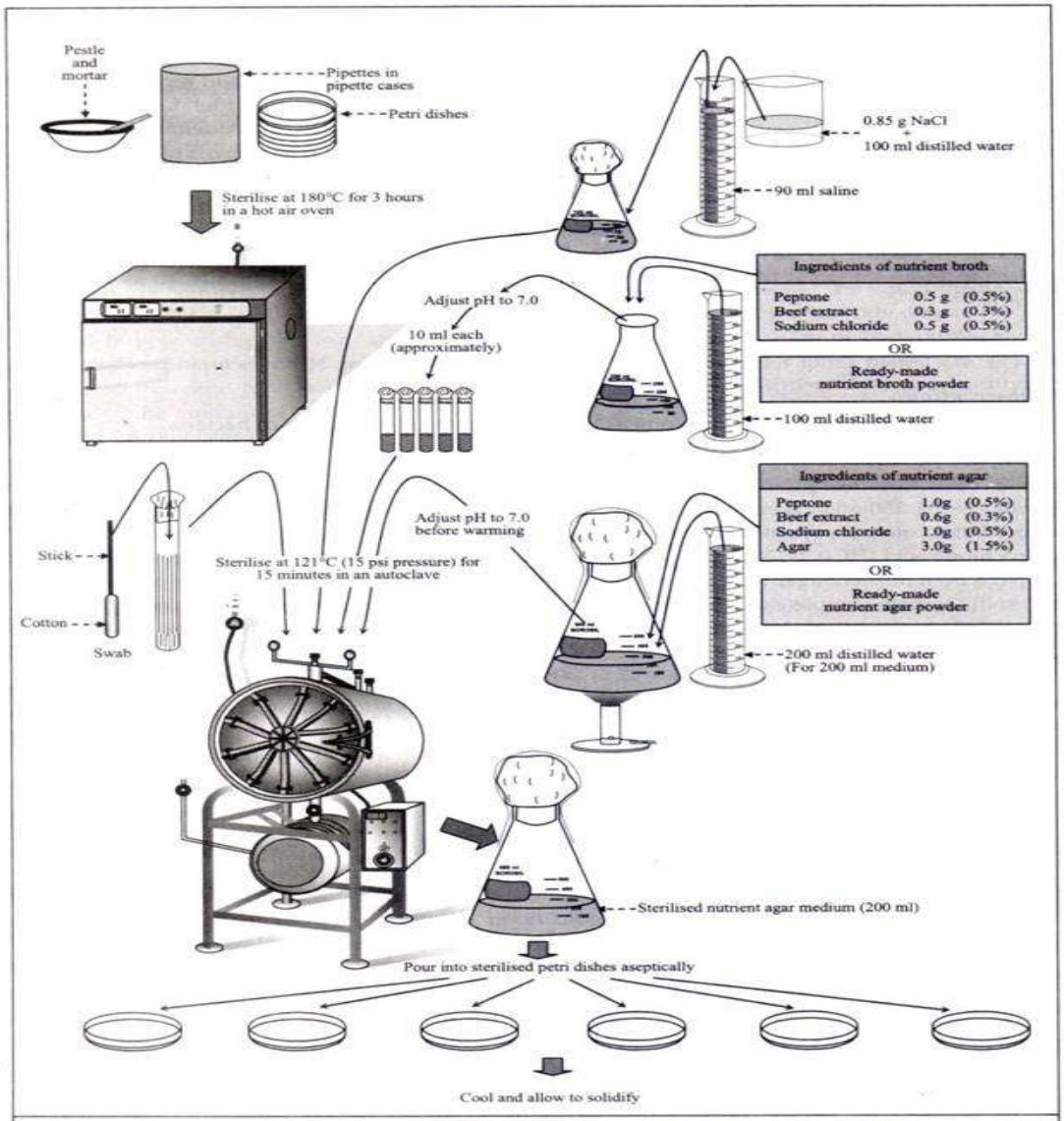


Figure 6.1: Cultivation of bacteria: Preparation and sterilisation of media

13. For surface sampling, the surface suspected to contain bacteria (table top, body surface, wound) is marked for a unit area (e.g. 1 cm^2), which is rubbed by a sterilised swab. The swab is touched onto the surface of the two left out agar plates.

Streaking is done aseptically by a flame-sterilised loop. For streaking, almost parallel lines are drawn by the loop from the swab-touch point. These form the primary streaks. After flame-sterilisation of the loop, secondary streaks are drawn almost diagonally to the primary streaks. In similar manner, tertiary and quaternary streaks are made aseptically.

14. Then, the plates are incubated in inverted position, top down, at 37°C for 24 hour in the incubator (Figure 6.2).

15. One uninoculated agar plate and one uninoculated broth tube are incubated as control to ensure proper sterilisation as indicated by no growth in them. This step is optional.

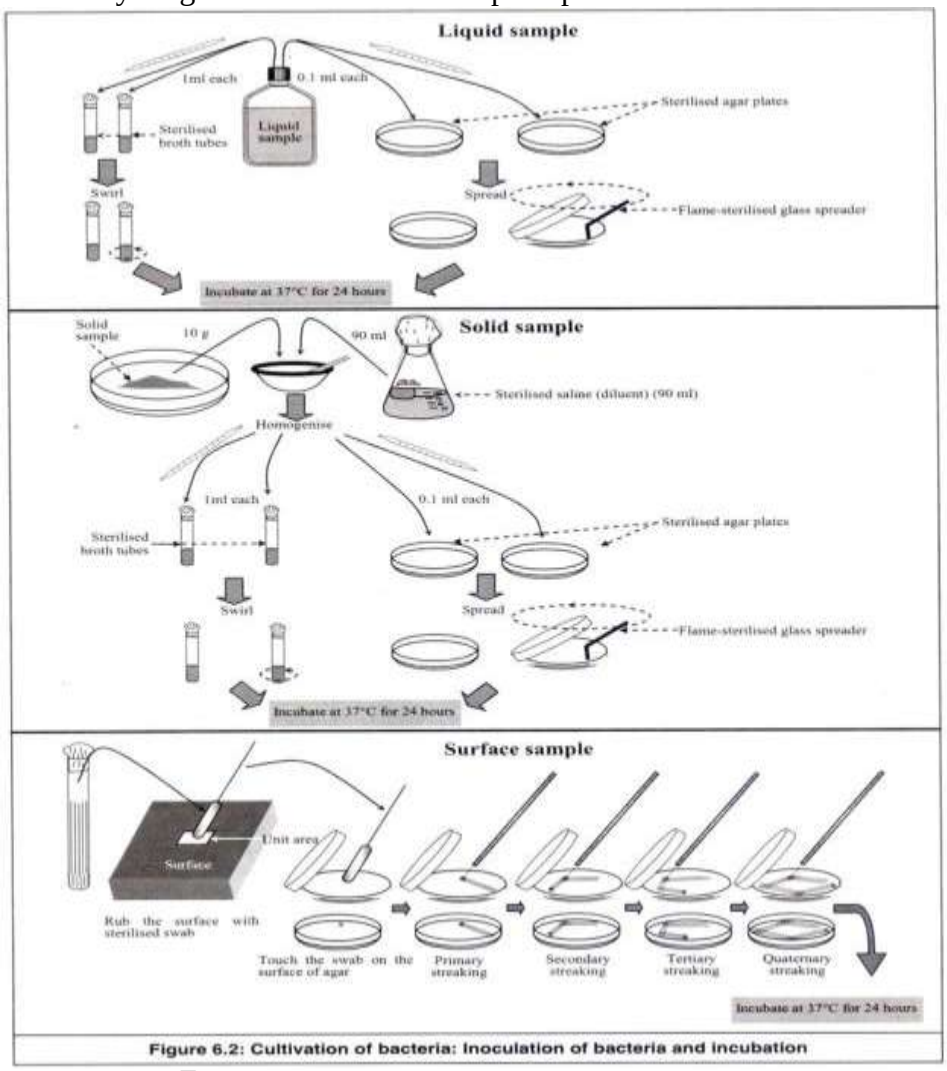


Figure 6.2: Cultivation of bacteria: Inoculation of bacteria and incubation

Observations (Cultural Characteristics):

Broth Culture:

(i) Turbidity Observed:

Growth has taken place. It is observed for growth characteristics as follows (Figure 6.3).

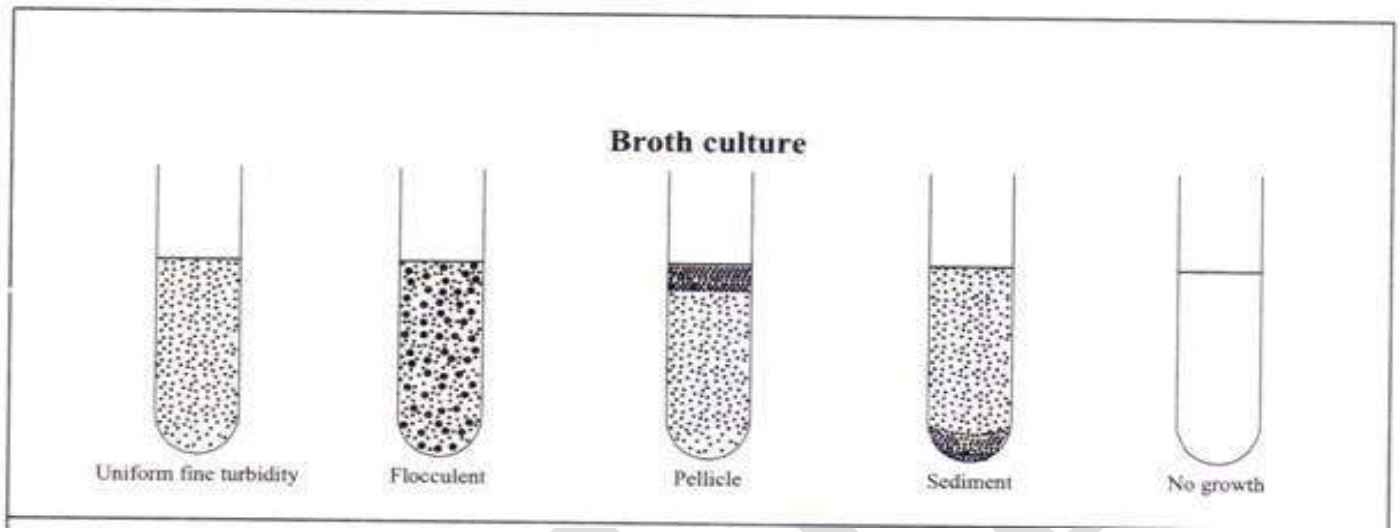
(a) Uniform Fine Turbidity: Finely dispersed growth throughout.

(b) Flocculant: Flaky aggregates dispersed throughout.

(c) Pellicle: Thick pad-like growth on surface.

(d) Sediment: Concentration of growth at the bottom of broth culture; may be granular, flaky or flocculant.

(ii) No Turbidity:
No growth has taken place. The sample used is bacteria-free.



2. Spread Plate Culture:

(i) Colonies Observed:
Growth has taken place. It is observed for colony characteristics as follows (Figure 6.3).
1. Size: Pinpoint, small, moderate or large.
2. Pigmentation: Colour of colony.
3. Form: The shape of the colony is described as follows:
(a) Circular: Unbroken peripheral edge.
(b) Irregular: Indented peripheral edge.
(c) Rhizoid: Root-like spreading growth.

4. Margin:
The appearance of the outer edge of the colony is described as follows:
(a) Entire: Sharply defined, even.
(b) Lobate: Marked indentations.
(c) Undulate: Wavy indentations.
(d) Serrate: Tooth-like appearance.
(e) Filamentous: Thread-like spreading edge.

5. Elevation:
The degree, to which colony is raised is described as follows:
(a) Flat: Elevation not discernible.
(b) Raised: Slightly elevated.
(c) Convex: Dome-shaped elevation.
(d) Umbonate: Raised with elevated convex central region.

(ii) No Colony:
No growth has taken place on the plate. The sample used is bacteria-free.

3. Streak Plate Culture:

(i) Colonies Observed:
Growth has taken place. It is observed for colony characteristics as described above.

(ii) No Colony:

No growth has taken place: The surface sample used is bacteria-free.

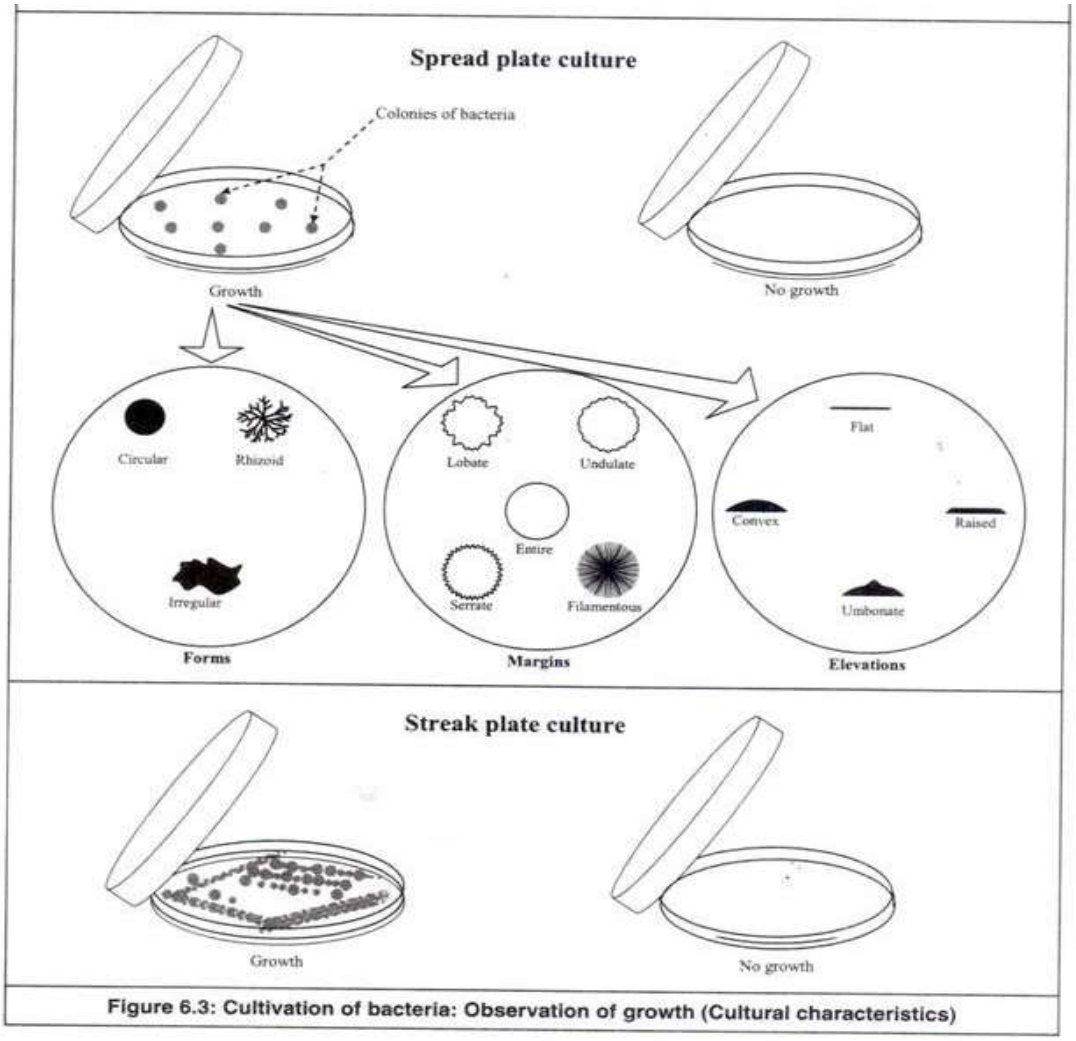


Figure 6.3: Cultivation of bacteria: Observation of growth (Cultural characteristics)

Experiment for Identification of Unknown Bacteria

Experiment for identification of unknown bacteria!

Principle:

Identification of unknown bacteria is one of the major responsibilities of the microbiologists. Samples of blood, tissue, food, water and cosmetics are examined daily in laboratories throughout the world for the presence of contaminating microorganisms.

In addition, industrial organisations are constantly screening materials to isolate new antibiotic-producing microbes or microbes that will increase the yield of marketable products, such as vitamins, solvents and enzymes. Once isolated, these unknown microbes must be identified and classified.

The science of classification is called taxonomy and deals with the separation of living organisms into interrelated groups. Bergey's Manual of Determinative Bacteriology, 8th edition, is the official taxonomic key containing the orders, families, genera and species of all known classified bacteria

With the fundamental knowledge in staining methods, isolation techniques, bacterial nutrition, biochemical activities and growth characteristics of bacteria, it becomes easier for identification of any unknown bacteria.

Characteristics of few bacteria have been given in Table 7.2. Other bacteria can be identified in a similar way based on the observations and results obtained following the experimental procedures.

Materials Required:

Slides, cavity slides, petri dishes, conical flasks, cotton plugs, inoculating loop, autoclave, bunsen burner, laminar flow chamber, dispose jar, incubator, nutrient broth, nutrient agar, gram staining reagents, media and reagents for biochemical tests, compound microscope, isolated colonies or pure cultures of bacteria.

Procedure:

1. Gram staining of the unknown bacteria is performed. Besides gram staining, its morphology and arrangement is also recorded. Motility test of the bacteria is determined by hanging drop preparation (Figure 7.25).
2. Using sterile inoculating technique, the bacteria is inoculated into two nutrient agar slants, a nutrient broth tube and to a nutrient agar plate by means of streak inoculation. Following incubation, one slant culture is used to determine the cultural characteristics of the unknown bacteria.

The second is used as a stock sub-culture, should it be necessary to repeat any of the tests. Growth characteristics are also observed in the broth tube and colony characteristics on the plate.

3. Exercising care in sterile technique, so as not to contaminate cultures and thereby obtain spurious results, the bacteria is inoculated into respective media to carry out the different biochemical tests.
4. The inoculated media are incubated at the required temperatures for the required lengths of time.

Observations:

1. In gram staining, besides gram reaction, the morphology and arrangement of the bacteria are also recorded.
2. The cultural characteristics of the bacteria in nutrient broth, on agar slant and on nutrient agar plate are noted.
3. Results of the biochemical reactions are recorded.

Interpretation of Results:

1. Using the above recorded data (For example, see Table 7.1) and with the help of the Bergey's Manual of Determinative Bacteriology, the bacteria is identified as to its genus and species. It should be kept in mind that, results might vary depending on the strains of each species used and the length of time the bacteria has been maintained in stock culture.

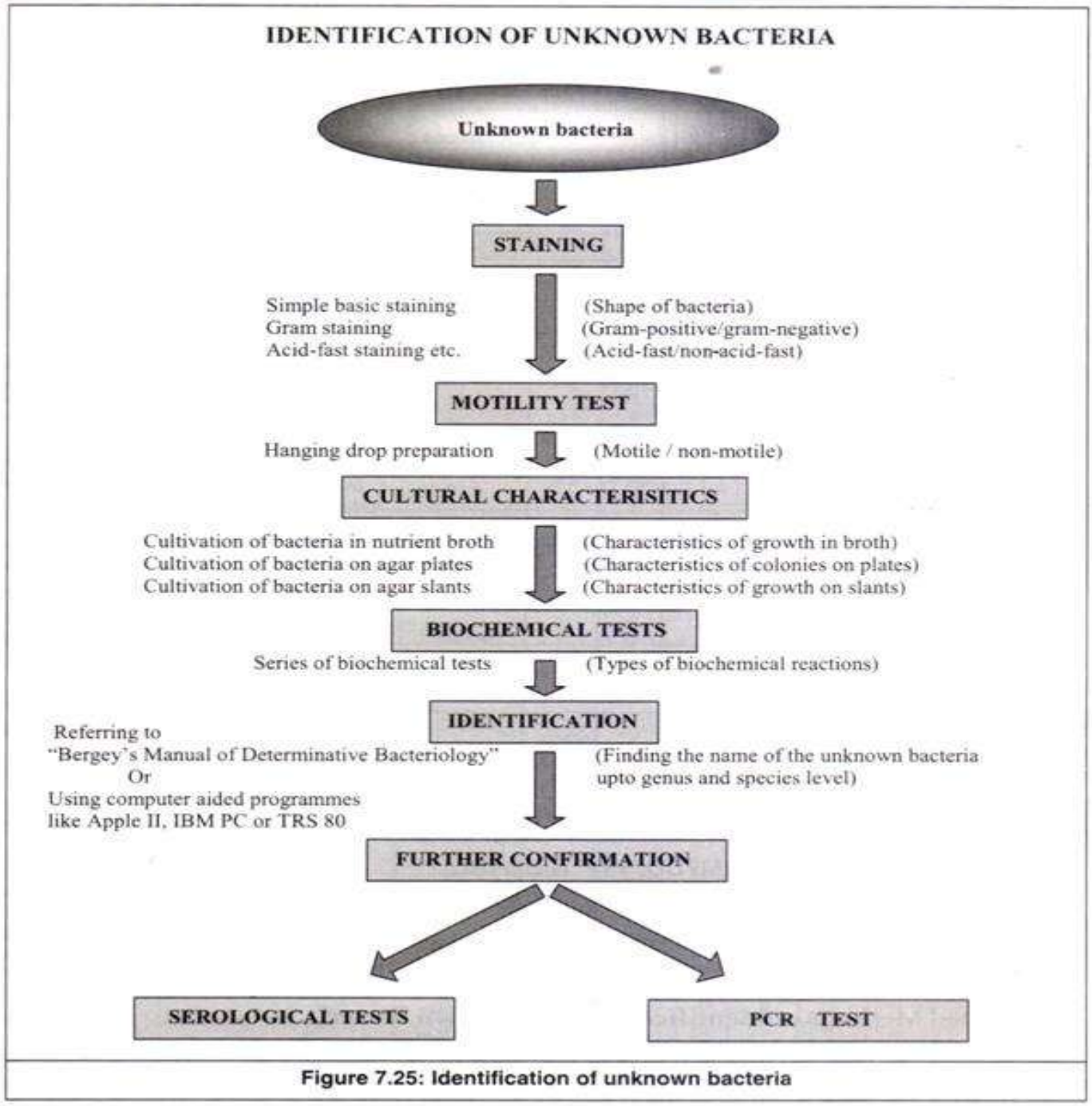


Figure 7.25: Identification of unknown bacteria

The observed results may not be completely identical to the expected results. Therefore, the bacteria that best fits the results are chosen. The identifying characteristics of few bacteria have given in Table 7.2.

Table 7.2: The identifying characteristics of few bacteria

Organism	Gram Stain	Agar slant cultural characteristics	Litmus milk reaction	Carbohydrate fermentation			H_2S production	Nitrate reduction	Indole production	MR reaction	VP reaction	Citrate utilisation	Urease activity	Catalase activity	Oxidase activity	Gelatin liquefaction	Starch hydrolysis	Lipid hydrolysis
				Lactose	Dextrose	Sucrose												
Escherichia coli	Rod –	White, moist, glistening growth	Acid, curd ±, gas ±, reduction ±	AG	AG	A ±	–	+	+	+	–	–	–	+	–	–	–	–
Enterobacter aerogenes	Rod –	Abundant, thick, white, glistening growth	Acid	AG	AG	AG ±	–	+	–	–	+	+	–	+	–	–	–	–
Klebsiella pneumoniae	Rod –	Slimy, white, somewhat–translucent, raised growth	Acid, gas, curd ±.	AG	AG	AG	–	+	–	–	±	+	+	+	–	–	–	–
Shigella dysenteriae	Rod –	Thin, even, grayish growth	Alkaline	–	A	A ±	–	+	+	+	–	–	–	+	–	–	–	–
Salmonella typhimurium	Rod –	Thin, even, grayish growth	Alkaline	–	AG ±	A ±	+	+	–	+	–	+	–	+	–	–	–	–
Proteus vulgaris	Rod –	Thin, blue–gray, spreading growth	Alkaline	–	AG	AG ±	+	+	+	+	–	–	+	+	–	+	–	–
Pseudomonas aeruginosa	Rod –	Abundant, thin, white growth, with medium turning green	Rapid peptonisation	–	–	–	–	+	–	–	–	+	–	+	+	+ Rapid	–	+
Alcaligens faecalis	Rod* –	Thin, white, spreading, viscous growth	Alkaline	–	–	–	–	–	–	–	–	–	–	+	+	–	–	–
Staphylococcus aureus	Coccus +	Abundant, opaque, golden growth	Acid, reduction, ±	A	A	A	–	+	–	+	±	–	–	+	–	+	–	+
Streptococcus lactis	Coccus +	Thin, even, growth	Acid, rapid reduction with curd	A	A	A	–	–	–	+	–	–	–	–	–	–	–	–
Micrococcus luteus	Coccus +	Soft, smooth, yellow growth	Alkaline	–	–	–	–	±	–	–	–	–	+	+	–	+ Slow	–	–
Corynebacterium xerosis	Rod +	Grayish, granular, limited growth	Alkaline	–	A ±	A ±	–	–	–	–	–	–	–	+	–	–	–	–
Bacillus cereus	Rod +	Abundant, opaque, white waxy growth	Peptonisation	–	A	A	–	+	–	–	±.	–	–	+	–	+ Rapid	+	+

2. Alternatively, using the above-recorded data, code numbers are given for the recorded results (See Table 7.3) and the bacteria identified easily with the help of computer program available for Apple II, IBM PC or TRS-80.

Table 7.3: Code numbers for computer assisted identification of unknown bacteria

Gram stain
0-not done
1-blue (Gram +)
2-red (Gram -)

Acid-fast stain
0-not done
1-red (acid-fast)
2-blue (non-acid fast)

Shape (morphology)
0-not done
1-straight rod
2-curved rod
3-spiral
4-spirochete
5-coccus
6-pleomorphic rod
7-coccobacillus

Cultural characteristics
0-not done
1-white, moist, glistening growth
2-abundant, white, thick, glistening growth
3-slimy, translucent, raised growth
4-thin, even, grayish growth
5-thin, bluish-gray, spreading growth
6-thin, transparent growth with medium turning green
7-thin, white, spreading, viscous growth
8-abundant, opaque, golden growth
9-thin, even, transparent growth
10-soft, smooth, yellow-pigmented growth
11-grayish, granular, united growth

Litmus milk reactions
0-not done
1-acid
2-acid, reduction
3-acid, reduction, curd
4-acid, reduction, curd, gas
5-peptonisation (proteolysis)
6-alkaline

Lactose fermentation
Dextrose fermentation
Sucrose fermentation
Mannitol fermentation
0-not done
1-yellow medium (acid)
2-yellow medium + air bubble (acid + gas)
3-red medium (negative)

Hydrogen sulphide
0-not done
1-medium black (positive)
2-medium clear (negative)

Nitrate reduction
0-not done
1-red colour with solutions A + B (positive)
2-no colour change with solutions A + B + Zn (positive)
3-red colour with solutions A + B + Zn (negative)

Indole production
0-not done
1-red reagent layer (positive)
2-yellow reagent layer (negative)

Methyl red test
0-not done
1-red colouration of medium (positive)
2-yellow-orange colouration of medium (negative)

Voges-Proskauer test
0-not done
1-pink colouration of medium (positive)
2-no colour change in medium (negative)

Citrate utilization test
0-not done
1-growth, medium blue (positive)
2-no growth, medium green (negative)

Urease activity
0-not done
1-medium deep pink (positive)
2-no colour change in medium (negative)

Catalase activity
0--not done
1-evolution of oxygen bubbles (positive)
2-no evolution of oxygen bubbles (negative)

Starch hydrolysis
0-not done
1-clear zone with iodine (positive)
2-no clear zone with iodine (negative)

Lipid hydrolysis
0-not done
1-zone of lipolysis (positive)
2-no zone of lipolysis (negative)

The program is designed to identify the unknown bacteria on the basis of percentage of similarity between the unknown bacteria and the known bacteria with respect to the above observations.

To initiate the program for the Apple II or IBM-PC:

(a) The disc is inserted in the left drive.
(b) The computer is turned on.
(c) The program instructions are followed by typing the responses, unknown bacteria number and code numbers as the questions appear on the screen.

To initiate the program for the TRS-80:

(a) The computer is turned on.
(b) The disc is inserted in the bottom drive.
(c) The orange button is pressed.
(d) When asked, "date (MM/DD/YY)" is entered.
(e) Orange button is pressed again.
(f) Program instructions are followed by typing in the responses, unknown bacteria number and code numbers as the questions appear on the screen.

E. Automated Methods of Identification of Unknown Bacteria:

Identification of unknown bacteria by the manual method using staining reactions, motility test, cultural characteristics and biochemical tests is a cumbersome, lengthy and time-consuming process. To make the process easy and short, automatic methods have been developed.

These methods basically use the same biochemical tests as used in the manual methods, but here it is performed in an automatic manner and the interpretation is done with a computer-aided programme.

Two such systems used at present for the automatic identification of bacteria are given as follows:

1. MINI API System
2. VITEK 2 System

F. Further Confirmation:

(1) Serological Tests:

Serological tests may be performed for further confirmation of the identified bacteria. To a suspension of the identified bacteria (say X), a drop of the antiserum (anti-X) against that bacteria is mixed. If it result in the formation of clumps or precipitates (X-anti-X complex), then the identification is confirmed.

The antisera for different bacteria are commercially available. They are produced commercially in different laboratories. The antiserum of particular bacteria is produced by injecting killed or attenuated cells of those bacteria (say X) into a laboratory animal.

As a defense mechanism, the host animal produces antibodies (anti-X) against that bacterium, which remains in the blood. The blood is collected from this animal and its serum isolated by special techniques. This serum, containing antibody against that bacteria is called the antiserum of that bacteria.

(2) PCR Test:

The Polymerase Chain Reaction (PCR) test is a rapid molecular method for confirmation of the identified bacteria. This technique is very often used for the detection of various bacteria and viruses quickly and precisely.

Spore Staining of Bacteria to Differentiate Bacterial Spore and Vegetative Cell

Purpose:

All bacteria remain in their 'vegetative forms', if the environmental conditions are favourable for their normal metabolic activities.

In this form, they take up nutrients, grow and reproduce.

On the other hand, most of them die, when the environmental conditions become adverse, such as, severe cold, extreme heat, ageing, lack of nutrients, exposure to radiation and toxic chemicals.

However, few species of bacteria, can survive under such adverse conditions by changing themselves to highly resistant, metabolically inactive forms called 'spores'. In adverse conditions, a spore is produced within a vegetative cell by dehydration and contraction of its cell contents.

This spore, formed inside the bacteria cell, is called 'endospore' (Figure 2.13). If adverse conditions worsen, the cell ruptures, releasing the endospore, which now becomes an independent dormant cell called 'spore'.

The process of spore formation is called 'sporogenesis'. The spore has thick, relatively impervious layers, such as spore cortex and spore coat, which protect the cell from any physical damage. The spore gains its resistance by several mechanisms, which have not yet been clearly explained.

When the conditions become favourable, the layers covering the spore rupture and the spore gives rise to a metabolically active vegetative cell. This process is called 'germination'.

Thus, based on the ability to form spores, bacteria can be divided into two groups as follows:

(1) Spore-forming Bacteria (Spore-formers):

A bacteria, which can produce a spore to survive under adverse environmental conditions is a spore-forming bacteria (spore-former). Mostly, the following three genera of rod-shaped bacteria can produce spores under adverse environmental conditions.

A. Aerobic rod-shaped bacteria

1. *Bacillus spp.*

B. Anaerobic rod-shaped bacteria

2. *Clostridium spp.*

3. *Desulfotomaculum spp.*

(2) Non-spore-forming Bacteria (Non-spore-formers):

A bacteria, which cannot produce a spore and therefore, dies in its vegetative form under adverse environmental conditions, is a non-spore-forming bacteria (non-spore-former).

The purpose of spore staining is to differentiate the spores and vegetative cells of a spore-former and to differentiate spore-formers from non-spore-formers. Spore staining is also helpful in identification of the bacteria belonging to the genera Bacillus, Clostridium and Desulfotomaculum.

Principle:

The spores are different from the vegetative cells in that they possess thick, relatively impervious layers around them (Figure 2.14). The primary stain, malachite green, cannot penetrate into the spores through these layers.

Therefore, penetration of the primary stain is augmented by the application of heat, which drives it through the covering layers into the spore protoplast. Now, the spores appear green. When the spores are treated with the decolourising agent, tap water, they do not undergo decolourisation, as they exclude water.

The tap water removes only the excess primary stain present in the surrounding of the spores. Thus, the spores retain the green colour. Subsequently, when counter-stained with safranin, the safranin molecules cannot enter into the spores through the covering layers. Thus, finally the spores retain the green colour of the primary stain and appear green.

On the other hand, the vegetative bacteria cells take up the primary stain, malachite green, easily, but the stain does not have strong affinity for vegetative cell components, due to which it is washed away, when decolourised under tap water.

Now, the vegetative cells appear colourless. When counter-stained with safranin, the colourless vegetative cells take up the stain and appear red unlike the spores, which appear green.

Materials Required:

Slide, loop, malachite green, safranin, spore-forming bacteria, hot plate, microscope, immersion oil.

Procedure:

1. A slide is cleaned properly under tap water, such that water does not remain as drops on its surface.
2. The adhering water is wiped out with bibulous paper and the slide is air-dried.
3. A smear of spore-forming bacteria is made at the center of a slide in two methods as given below.

(a) If spore-former grown on agar plate or agar slant is to be observed, a drop of water is placed at the center of the slide. A loop is sterilised over flame and a Clostridium loop of bacteria from an aged plate or slant culture (48-72 hours old) of spore-former, such as Clostridium supergenes or Bacillus subtilis is transferred to the water drop.

Then, by gentle rotation of the loop in the water drop, a bacteria suspension is made and the drop is spread till a smear is obtained. As the age of spore-forming bacteria on plate or slant cultures increases, they form spores.

(b) If spore-former grown in liquid broth is to be observed, the broth culture, containing only vegetative cells, is subjected to heat stress, by heating on a water bath for 15 minutes, so that some of the vegetative cells form spores to overcome the heat stress. A drop of the mixed spore-vegetative cell suspension from the broth culture is directly placed at the center of the slide by a sterile loop and a smear is made by spreading.

4. The smear is air-dried.
5. The smear is fixed by heating. Heating results in coagulation of the cellular proteins, due to which the cells stick to the slide surface and do not get washed away during staining, Heat- fixation is done by quickly passing the slide high above a flame 2-3 times, with the smear surface facing upward, so that the smear does not get heated up.
6. The smear is flooded with the primary stain, malachite green.
7. The slide is kept on a warm hot plate and the preparation allowed to steam for 2-3 minutes. The temperature of the hot plate is so adjusted that, the preparation does not boil and get evaporated quickly. The evaporation loss is replenished, so that the smear does not get dried up.
8. The slide is removed from the hot plate and cooled.
9. The smear is decolourised by washing under gently-flowing tap water, in such a way that, water does not fall directly on the smear.
10. The smear is flooded with the counter-stain, safranin, for 30 seconds.
11. Excess counter-stain is completely washed away from the smear under gently-flowing tap water, in such a way that, water does not fall directly on the smear.
12. The slide is blotted dry with bibulous paper.
13. The slide is clipped to the stage of the microscope and the smear observed under low power and high dry objectives.
14. A drop of immersion oil is put on the smear.
15. The smear is observed under oil-immersion objective.

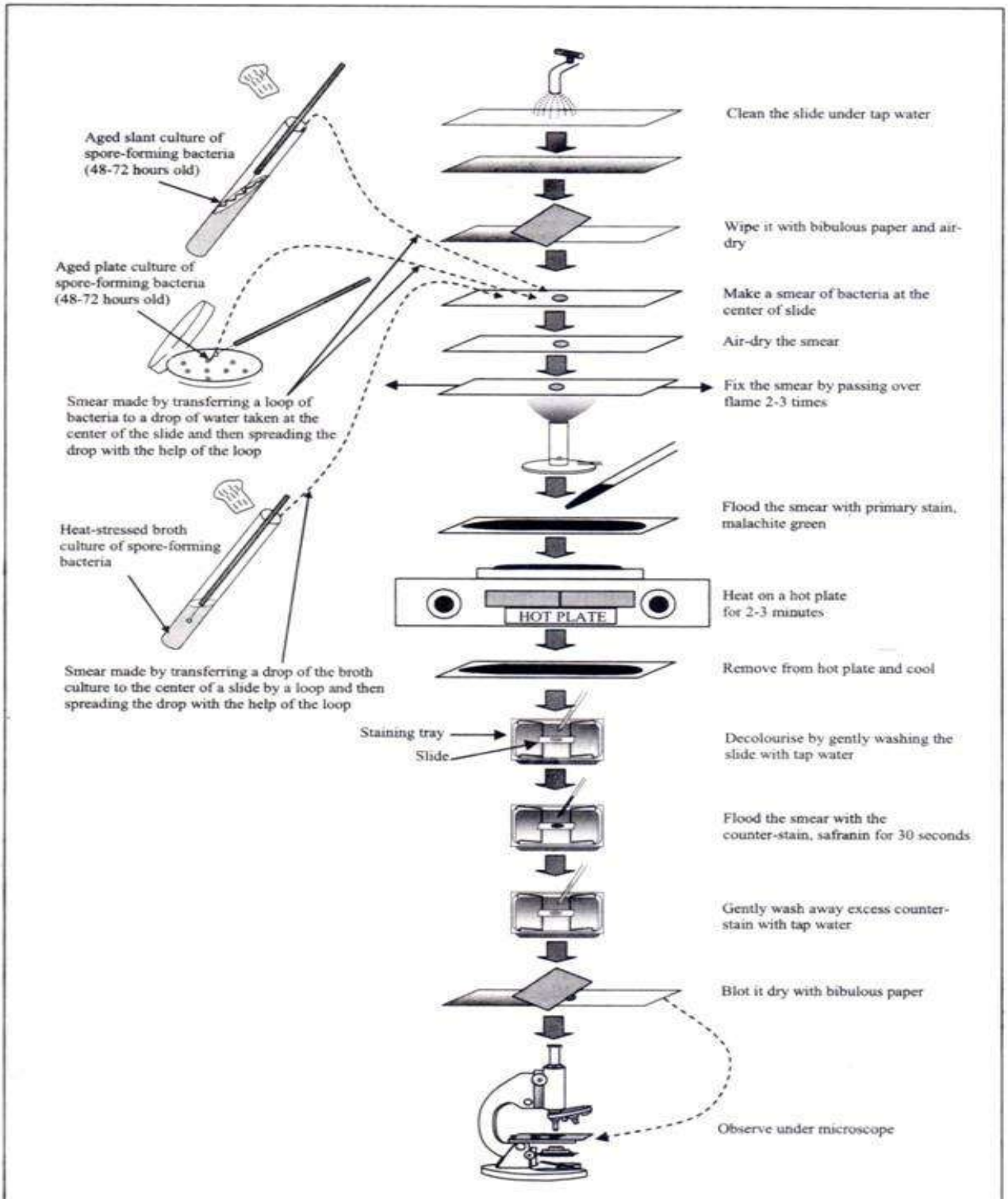


Figure 5.13: Spore staining of bacteria (Schaeffer-Fulton method)

Different Bacteria Present in a Given Sample and Maintain Their Pure Cultures

Aim: To isolate different bacteria present in a given sample and maintain their pure cultures.

Purpose:

Bacteria found in nature, do not occur as segregated species, rather they occur as mixed populations of different species.

Therefore, to study the individual species of bacteria, it is first required to segregate them from the mixed population. This process is called 'isolation of bacteria'.

It is accomplished by growing the mixed population of bacteria on the surface of a solid medium into discrete colonies. 'Colonies' are individual, macroscopically visible masses of bacteria grown on the surface of solid medium, each representing the multiplication of a single bacterium.

As each colony comes from the growth and repeated multiplication of a single bacterium, all the bacteria present in the colony belong to the same species. Thus, each colony represents a very large population of a single species of bacteria.

These colonies are transferred aseptically to separate nutrient agar slants and allowed to grow there. The isolated species of bacteria, grown separately on agar slants are called 'pure cultures'. In every 15 to 30 days, they are transferred to fresh slants, so that they do not lose their original characteristics due to over-crowding, lack of nutrients and accumulation of metabolites.

In this way, pure cultures of bacteria are maintained in the laboratory by repeated transfer to fresh slants at regular intervals. This process is known as 'maintenance of pure culture'. Pure cultures are maintained in the laboratory for their subsequent identification by various staining, biochemical, serological and molecular techniques as well as for their future use.

'Stock cultures' are standard pure cultures, whose identification has been established internationally to genus, species, sub-species, type, or sub-type level. They are maintained in internationally recognised microbiology laboratories.

Other laboratories can obtain them from these laboratories and maintain in their own laboratories as their stock cultures, by repeated transfer to fresh slants at regular intervals. They are used for confirmed identification of pure cultures obtained in these laboratories by comparison of their characteristics with those of the standard stock cultures.

Principle:

The mixed population of bacteria present in a given material (solid, liquid or surface) is grown on nutrient agar plates by spread plate or streak plate method as described previously under 'Cultivation of bacteria'. Each isolated colony found on an agar plate consists of one species of bacteria, as each colony grows from a single bacterium.

Thus, bacteria are isolated on agar plates by two techniques as follows:

(a) Spread plate technique

(b) Streak plate technique

Representative colonies (colonies having dissimilar characteristics) are selected and aseptically inoculated to separate agar slants to get the pure cultures. After each 15 to 30 days, they are transferred to fresh slants for maintenance of these pure cultures.

Materials Required:

Test tubes (5 nos.), 250 ml conical flask (1 no), 0.1N NaOH, 0.1N HCI, distilled water, nutrient agar, non-absorbent cotton, loop, craft paper, thread (or rubber band), pH paper (or pH meter), bunsen burner, autoclave, laminar flow chamber, incubator, plates containing isolated colonies of bacteria (spread plate or streak plate).

Procedure:

1. The ingredients of nutrient agar medium or its ready-made powder required for 100 ml of the medium is weighed and dissolved in 100 ml of distilled water in a 250 ml conical flask by shaking and swirling (Figure 6.4).

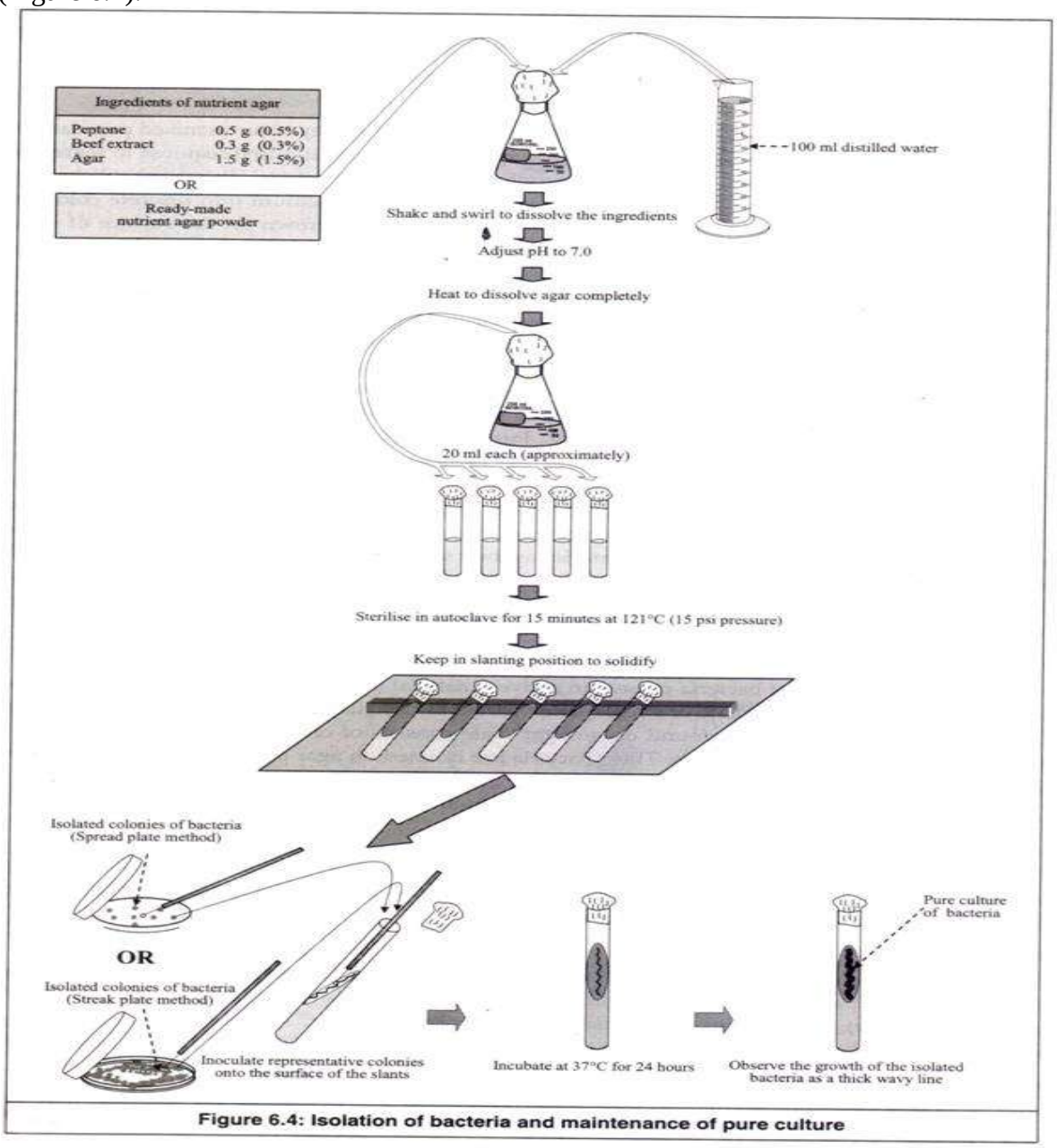


Figure 6.4: Isolation of bacteria and maintenance of pure culture

2. Its pH is determined using a pH paper or pH meter and adjusted to 7.0 using 0.1N HC1 if it is more or using 0.1N NaOH if it is less.
3. The flask is heated to dissolve the agar in the medium completely.
4. Before it solidifies, the medium in warm molten condition is distributed into 5 test tubes (approximately 20 ml each).
5. The test tubes are cotton-plugged, covered with craft paper and tied with thread or rubber band.

6. They are sterilised at 121°C (15 psi pressure) for 15 minutes in an autoclave.
7. After sterilisation, they are removed from the autoclave and kept in a slanting position to cool and solidify the medium. These test tubes containing solidified nutrient agar medium in slanting condition are called 'nutrient agar slants'.
8. Steps 10 and 11 should be performed following aseptic technique preferably in a laminar flow chamber. The mouth of the containers containing sterilised media or cultures should be shown over flame of bunsen burner after removing the cotton plug and before putting it back.
Loops, before use have to be sterilised over flame by heating to red hot and then cooling for 30 seconds to cool to room temperature. They should never be used when very hot, as they kill the microbes when touch them.
9. In the previous experiment, if isolated colonies could be observed on the spread plate or streak plate, five colonies with dissimilar characteristics are selected as representative colonies.
10. A loop is sterilised over flame and a loop of bacteria from each of the representative colonies is taken aseptically. The loop is introduced into the agar slant, so that the loop of bacteria goes to the end of the slant. The loop is moved outward in a wavy manner touching the surface of the slant, so that a wavy line is formed.
11. The loop is flame-sterilised and in similar manner the rest of the four representative colonies are inoculated separately into the left over four slants. The loop is sterilised after each inoculation.
12. The five inoculated slants are incubated at 37°C for 24 hours in an incubator.

Observations (Cultural Characteristics):

(i) A Wavy Line with Growth Along its Length Observed:

Growth has taken place.
The slant is observed for slant characteristics as follows (Figure 6.5).

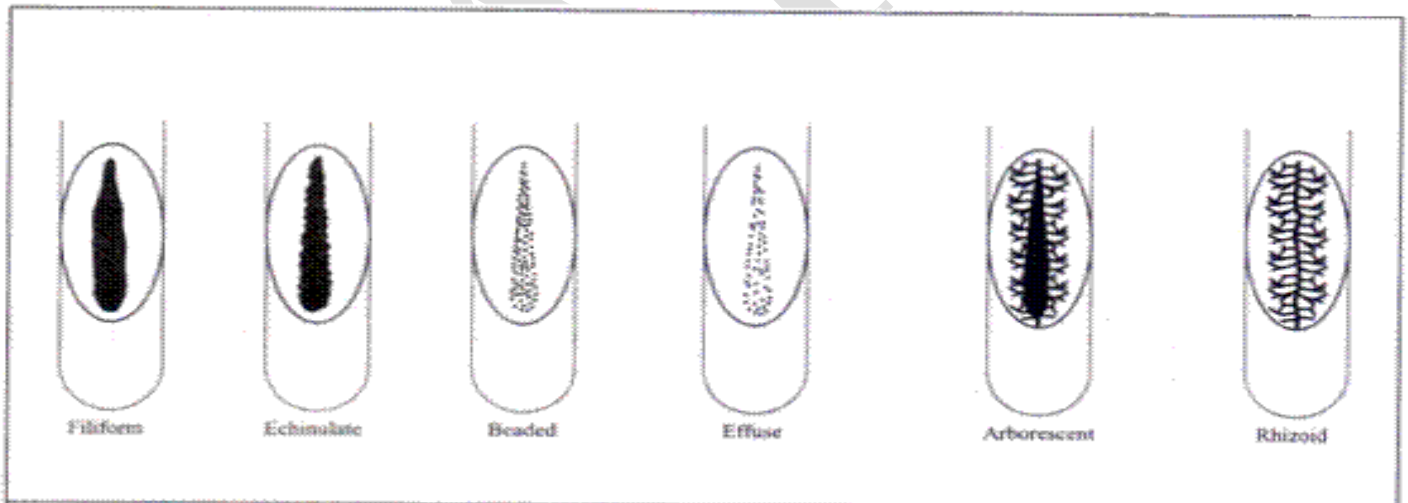


Figure 6.5: Cultural characteristics of bacteria on nutrient agar slants

1. Abundance of Growth:
The amount of growth is designated as none, slight, moderate or large.
2. Pigmentation:
Chromogenic microorganisms may produce intracellular pigments that are responsible for the coloration of the organism as seen in surface colonies. Other organisms produce extracellular soluble pigments that are

excreted into the medium and also produce a colour. Most organisms, however, are non-chromomeric and will appear white to gray.

3. Optical Characteristics:

Optical characteristics may be evaluated on the basis of the amount of light transmitted through the growth.

These characteristics are described as:

(a) Opaque: No light transmission. (b) Translucent: Partial light transmission. (c) Transparent: Full light transmission.

4. Form:

The appearance of the single line streak of growth on the agar surface is designated as follows:

(a) Filiform: Continuous, threadlike growth with smooth edges.
(b) Echinulate: Continuous, threadlike growth with irregular edges.
(c) Beaded: Non-confluent to semi-confluent colonies.
(d) Effuse: Thin, spreading growth.
(e) Arborescent: Tree-like growth.
(f) Rhizoid: Root-like growth.

(ii) No Growth along the Line of Inoculation:

Faulty technique of inoculation, Inoculation is repeated C Enumeration of Bacteria.

C. Enumeration of Bacteria:

The extent of bacterial activity in a given sample in a definite set of conditions mainly depends on the total number of bacteria present in it irrespective of their species. Therefore, it is very often required to find out the total number of bacteria present in samples of food, water, soil, air and tissue during their microbiological analysis.

Estimating the number of bacteria in a given sample is called 'enumeration of bacteria'. There are various methods of enumeration of bacteria as given below. All of these methods need bacteria to be present in a homogenous suspension.

That is why liquid samples are assumed to be homogenous suspensions of bacteria and are directly used, while solid samples are homogenized in sterile saline solutions, so as to get homogenous suspensions of bacteria.

Not used commonly:

(I) Direct Methods:

(a) Direct microscopic count
(b) Electronic cell counter

(II) Indirect Methods:

(c) Chemical methods
(d) Turbidimetric method
(e) Membrane filter count

Most widely used:

(f) Serial dilution-agar plating method or Total Plate Count Method (TPC method)

(a) Direct Microscopic Count:

In this method, the number of bacteria present in an aliquot of the homogenous suspension of bacteria is counted directly under a microscope and the total number of bacteria in the sample is determined mathematically.

The advantage of this method is that, it is very rapid. The disadvantages are that, both living (viable) and dead cells are counted and the method is not sensitive to populations of fewer than one million bacteria per milliliter.

Counting can be done in two methods as follows:

(i) Petroff-Hauser Chamber:

It is a specialised counting chamber, in which an aliquot of the homogenous suspension of bacteria is put for counting directly under a microscope.

(ii) Breed Smear:

Bacteria cells are directly counted under microscope using stained smears confined to an area of 1 mm^2 on the slide. It is mainly used for the enumeration of bacteria in milk.

(b) Electronic Cell Counter:

An electronic cell counter such as 'Coulter counter' is used to directly count the number of bacteria cells. A homogenous suspension of bacteria cells prepared in a conducting fluid is allowed to pass through a minute orifice, across which an electric current flows.

The resistance at the orifice is electronically recorded. When a cell passes through the orifice, being non-conductor, it increases the resistance momentarily. The number of times resistance increases momentarily is recorded electronically, which indicates the number of bacteria present in the suspension.

The advantage of this method is that, it is extremely rapid. The disadvantages are that, both living (viable) and dead cells are counted and the instrument cannot distinguish between bacteria cells and inert particulate matter.

(c) Chemical Methods:

These methods estimate the quantity of those substances, mainly chemicals, which increases with the increase in bacterial population.

The main parameters estimated are as follows:

(i) Protein concentration
(ii) DNA concentration
(iii) Dry weight
(iv) Carbon dioxide production
(v) Oxygen uptake
(vi) Lactic acid production

(d) Turbidimetric Method:

When bacteria are grown in liquid nutrient-rich media (e.g. nutrient broth), their profuse growth makes the media turbid, as the bacteria cells mostly remain suspended in them. The turbidity increases with increase in the number of cells in the media. As turbidity increases, the 'absorbance' or 'optical density (OD)' of the media increases.

The OD of the suspension of bacteria cells in the media is measured using a spectrophotometer at 600 nm. The number of bacteria cells in the suspension is found out by comparing the OD with a standard curve prepared by plotting the OD values for different known concentrations of bacteria. The method is rapid, but limited to bacterial suspensions of more than 10 million cells.

(e) Membrane Filter Count:

This method is used, when the number of bacteria in a liquid sample is very less. To increase the concentration of bacteria, first the liquid sample is filtered aseptically through a sterilised membrane filter apparatus using a sterile membrane filter.

The membrane filter containing the trapped bacteria is aseptically transferred to a sterile petri dish containing an absorbent pad saturated with a selective differential liquid medium. The medium oozes onto

the surface of the filter. After incubation, the number of colonies formed on the filter is counted using a simple microscope.

(f) Serial Dilution-Agar Plating Method or TPC Method:

It is the most versatile and widely used method for enumeration of bacteria.

MICROBIAL PURE CULTURE

Isolation of Pure Culture

Microorganisms are generally found in nature (air, soil and water) as mixed populations. Even the diseased parts of plants and animals contain a great number of microorganisms, which differ markedly from the microorganisms of other environments. To study the specific role played by a specific microorganism in its environment, one must isolate the same in pure culture. Pure culture involves not only isolation of individual microorganisms from a mixed population, but also the maintenance of such individuals and their progenies in artificial media, where no other microorganisms find way to grow.

However, it is not easy to isolate the individual microorganisms from natural habitats and grow them under imposed laboratory conditions. For this, great deal of laboratory manipulation is required. If inoculums from any natural habitat is taken and allowed to grow in a culture medium, a large number of diverse colonies may develop that, due to crowdedness, may run together and, thereby, may lose individuality. Therefore, it is necessary to make the colonies well-isolated from each other so that each appears distinct, large and shows characteristic growth forms. Such colonies may be picked up easily and grown separately for detailed study. Several methods for obtaining pure cultures are in use. Some common methods are in everyday-use by a majority of microbiologists, while the others are methods used for special purposes.

Common Methods of isolation of pure culture

Pure culture of microorganisms that form discrete colonies on solid media, e.g., yeasts, most bacteria, many other microfungi, and unicellular microalgae, may be most commonly obtained by plating methods such as streak plate method, pour plate method and spread plate method.

But, the microbes that have not yet been successfully cultivated on solid media and are cultivable only in liquid media are generally isolated by serial dilution method.

Streak Plate Method

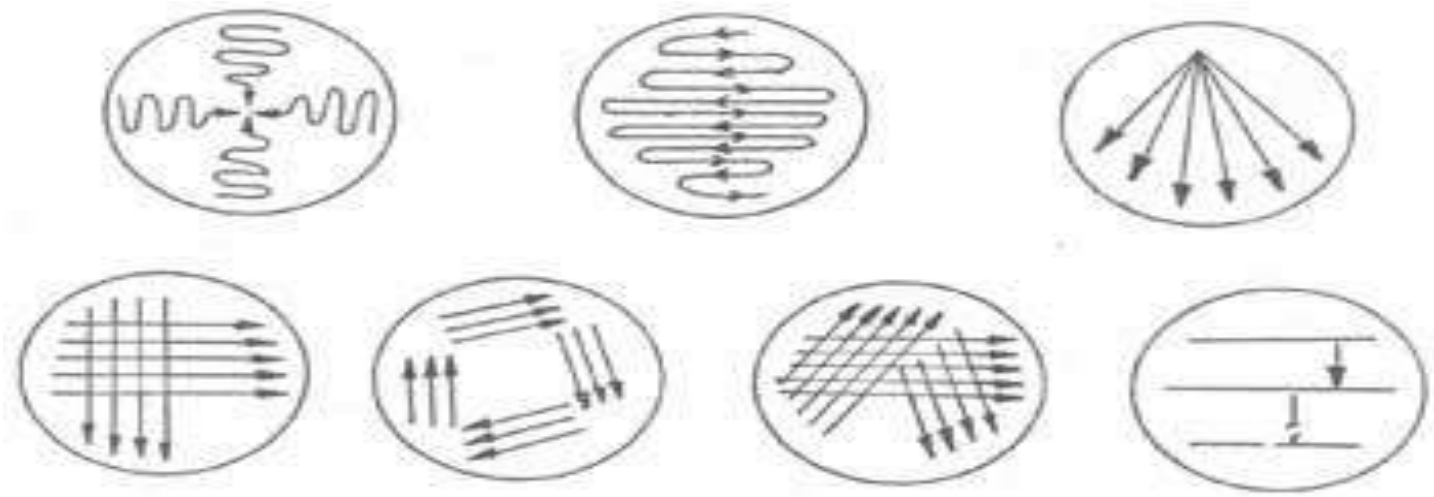
This method is used most commonly to isolate pure cultures of bacteria. A small amount of mixed culture is placed on the tip of an inoculation loop/needle and is streaked across the surface of the agar medium. The successive streaks "thin out" the inoculums sufficiently and the microorganisms are separated from each other. It is usually advisable to streak out a second plate by the same loop/needle without reinoculation. These plates are incubated to allow the growth of colonies. The key principle of this method is that, by streaking, a dilution gradient is established across the face of the Petri plate as bacterial cells are deposited on the agar surface. Because of this dilution gradient, confluent growth does not take place on that part of the medium where few bacterial cells are deposited

Various methods of streaking

Presumably, each colony is the progeny of a single microbial cell thus representing a clone of pure culture. Such isolated colonies are picked up separately using sterile inoculating loop/ needle and restreaked onto fresh media to ensure purity.

Pour Plate Method

This method involves plating of diluted samples mixed with melted agar medium. The main principle is to dilute the inoculum in successive tubes containing liquefied agar medium so as to permit a thorough distribution of bacterial cells within the medium. Here, the mixed culture of bacteria is diluted directly in tubes containing melted agar medium maintained in the liquid state at a temperature of 42-45°C (agar solidifies below 42°C).



The bacteria and the melted medium are mixed well. The contents of each tube are poured into separate Petri plates, allowed to solidify, and then incubated. When bacterial colonies develop, one finds that isolated colonies develop both within the agar medium (subsurface colonies) and on the medium (surface colonies). These isolated colonies are then picked up by inoculation loop and streaked onto another Petri plate to insure purity.

Pour plate method has certain disadvantages as follows: (i) the picking up of subsurface colonies needs digging them out of the agar medium thus interfering with other colonies, and (ii the microbes being isolated must be able to withstand temporary exposure to the 42-45° temperature of the liquid agar medium; therefore this technique proves unsuitable for the isolation of psychrophilic microorganisms.

However, the pour plate method, in addition to its use in isolating pure cultures, is also used for determining the number of viable bacterial cells present in a culture.

Pour Plate Method

A. Media/dilution

B. Pouring of the plate; and

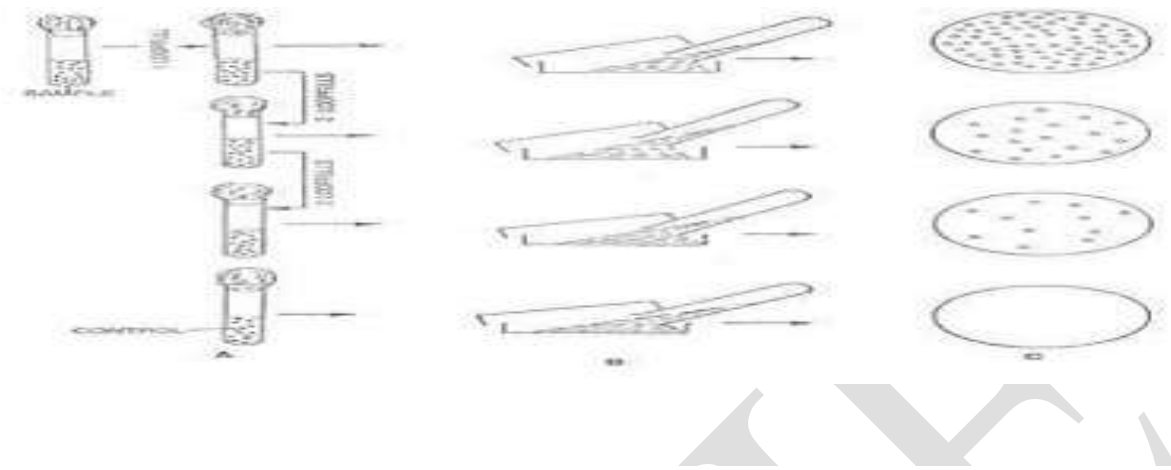
C. Colony development after incubation. Control consists of the sterilized plating medium alone

The isolated colonies are picked up and transferred onto fresh medium to ensure purity. In contrast to pour plate method, only surface colonies develop in this method and the microorganisms are not required to withstand the temperature of the melted agar medium.

Spread Plate Method

In this method the mixed culture of microorganisms is not diluted in the melted agar medium (unlike the pour plate method); it is rather diluted in a series of tubes containing sterile liquid, usually, water or physiological saline. A drop of so diluted liquid from each tube is placed on the centre of an agar plate and spread evenly over the surface by means of a sterilized bent-glass-rod.

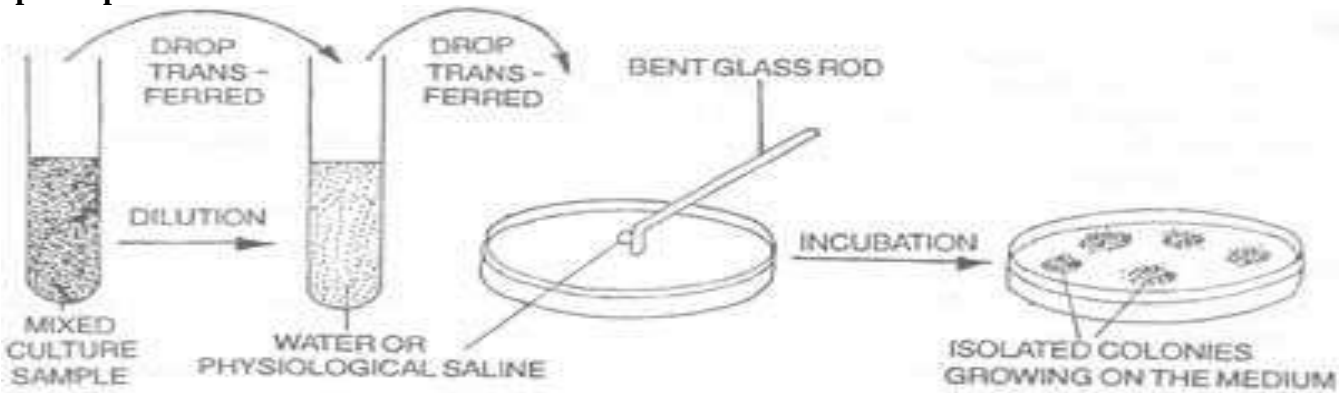
The medium is now incubated. When the colonies develop on the agar medium plates, it is found that there are some plates in which well-isolated colonies grow. This happens as a result of separation of individual microorganisms by spreading over the drop of diluted liquid on the medium of the plate.



Serial Dilution Method

As stated earlier, this method is commonly used to obtain pure cultures of those microorganisms that have not yet been successfully cultivated on solid media and grow only in liquid media. A microorganism that predominates in a mixed culture can be isolated in pure form by a series of dilutions.

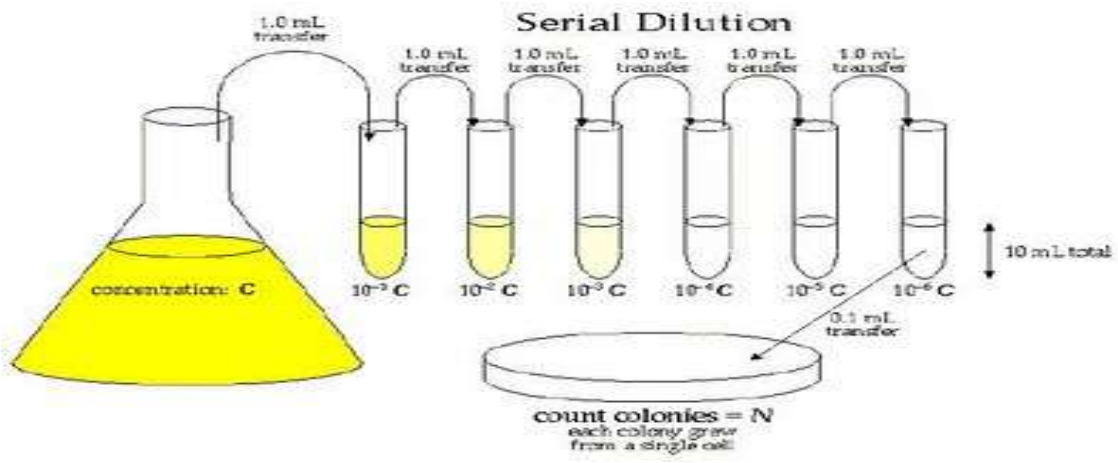
Spread plate method



The inoculum is subjected to serial dilution in a sterile liquid medium, and a large number of tubes of sterile liquid medium are inoculated with aliquots of each successive dilution. The aim of this dilution is to inoculate a series of tubes with a microbial suspension so dilute that there are some tubes showing growth of only one individual microbe. For convenience, suppose we have a culture containing 10 ml of liquid medium, containing 1,000 microorganisms i.e., 100 microorganisms/ml of the liquid medium.

Serial dilution method

If we take out 1 ml of this medium and mix it with 9 ml of fresh sterile liquid medium, we would then have 100 microorganisms in 10 ml or 10 microorganisms/ ml. If we add 1 ml of this suspension to another 9 ml. of fresh sterile liquid medium, each ml would now contain a single microorganism. If this tube shows any microbial growth, there is a very high probability that this growth has resulted from the introduction of a single microorganism in the medium and represents the pure culture of that microorganism.



Special Methods of Isolation on of Pure Culture

1. **Single Cell Isolation methods**

An individual cell of the required kind is picked out by this method from the mixed culture and is permitted to grow. The following two methods are in use.

(i) **Capillary pipette method**

Several small drops of a suitably diluted culture medium are put on a sterile glass-coverslip by a sterile pipette drawn to a capillary. One then examines each drop under the microscope until one finds such a drop, which contains only one microorganism. This drop is removed with a sterile capillary pipette to fresh medium. The individual microorganism present in the drop starts multiplying to yield a pure culture.

(ii) **Micromanipulator method**

Micromanipulators have been built, which permit one to pick out a single cell from a mixed culture. This instrument is used in conjunction with a microscope to pick a single cell (particularly bacterial cell) from a hanging drop preparation. The micro-manipulator has micrometer adjustments by means of which its micropipette can be moved right and left, forward, and backward, and up and down. A series of hanging drops of a diluted culture are placed on a special sterile coverslip by a micropipette.

Now a hanging drop is searched, which contains only a single microorganism cell. This cell is drawn into the micropipette by gentle suction and then transferred to a large drop of sterile medium on another sterile coverslip. When the number of cells increases in that drop as a result of multiplication, the drop is transferred to a culture tube having suitable medium. This yields a pure culture of the required microorganism.

The advantages of this method are that one can be reasonably sure that the cultures come from a single cell and one can obtain strains within the species. The disadvantages are that the equipment is expensive, its manipulation is very tedious, and it requires a skilled operator. This is the reason why this method is reserved for use in highly specialized studies.

2. **Enrichment Culture Method**

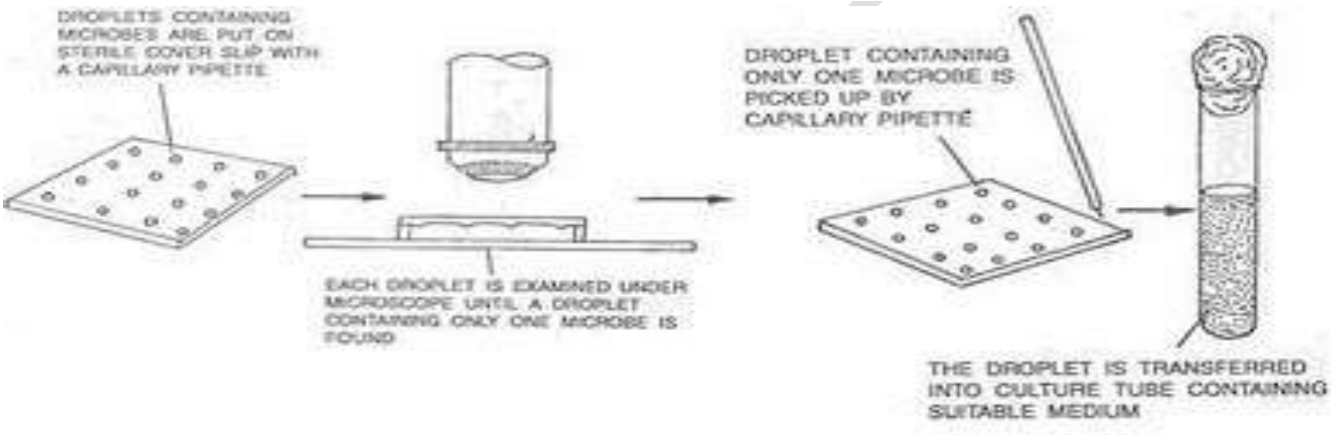
Generally, it is used to isolate those microorganisms, which are present in relatively small numbers or that have slow growth rates compared to the other species present in the mixed culture. The enrichment culture strategy provides a specially designed cultural environment by incorporating a specific nutrient in the medium and by modifying the physical conditions of the incubation. The medium of known composition and specific condition of incubation favors the growth of desired microorganisms but, is unsuitable for the growth of other types of microorganisms.

Proof of Purity of Cultures

Assuming that one has isolated a pure culture, how does one establish that it is pure? A pure culture is one in which the cells are all of one kind, i.e., demonstrate "likeness". Hence, the proof of purity of cultures consists of demonstrating the "likeness" of microorganisms in the culture. It is based on certain criteria as follows:

1.The microorganisms look alike microscopically and stain in the same fashion.
2. When plated, all the colonies formed look alike.
3. Streaks, stabs, etc. are uniform.
4. Several isolated colonies perform identically, i.e., ferment the same sugars, and so on.

Capillary method for obtaining a single microbial cell



Maintenance and Preservation of Pure Cultures

Once a microorganism has been isolated and grown in pure culture, it becomes necessary to maintain the viability and purity of the microorganism by keeping the pure cultures free from contamination. Normally in laboratories, the pure cultures are transferred periodically onto or into a fresh medium (subculturing) to allow continuous growth and viability of microorganisms. The transfer is always subject to aseptic conditions to avoid contamination.

Since repeated sub culturing is time consuming, it becomes difficult to maintain a large number of pure cultures successfully for a long time. In addition, there is a risk of genetic changes as well as contamination. Therefore, it is now being replaced by some modern methods that do not need frequent subculturing. These methods include refrigeration, paraffin method, cryopreservation, and lyophilization (freeze drying).

Refrigeration

Pure cultures can be successfully stored at 0-4°C either in refrigerators or in cold-rooms. This method is applied for short duration (2-3 weeks for bacteria and 3-4 months for fungi) because the metabolic activities of the microorganisms are greatly slowed down but not stopped. Thus their growth continues slowly, nutrients are utilized and waste products released in medium. This results in, finally, the death of the microbes after sometime.

Paraffin Method

This is a simple and most economical method of maintaining pure cultures of bacteria and fungi. In this method, sterile liquid paraffin in poured over the slant (slope) of culture and stored upright at room temperature. The layer of paraffin ensures anaerobic conditions and prevents dehydration of the medium.

This condition helps microorganisms or pure culture to remain in a dormant state and, therefore, the culture is preserved for several years.

Cryopreservation

Cryopreservation (i.e., freezing in liquid nitrogen at -196°C) helps survival of pure cultures for long storage times. In this method, the microorganisms of culture are rapidly frozen in liquid nitrogen at -196°C in the presence of stabilizing agents such as glycerol that prevent the formation of ice crystals and promote cell survival.

Lyophilization (Freeze-Drying)

In this method, the culture is rapidly frozen at a very low temperature (-70°C) and then dehydrated by vacuum. Under these conditions, the microbial cells are dehydrated and their metabolic activities are stopped; as a result, the microbes go into dormant state and retain viability for years. Lyophilized or freeze-dried pure cultures and then sealed and stored in the dark at 4°C in refrigerators. Freeze-drying method is the most frequently used technique by culture collection centers.

Culture collections

Microbial culture collections focus on the acquisition, authentication, production, preservation, catalogueing and distribution of viable cultures of standard reference microorganisms, cell lines and other materials for research in microbial systematics. Culture collection are also repositories of type strains.

Major national culture collections.

Collection Acronym	Name	Location
ATCC	American Type Culture Collection	Manassas, Virginia
NCTC	National Collection of Type Cultures	Public Health England, London, United Kingdom
BCCM	Belgium Coordinated Collection of Microorganism	Ghent, Belgium
CIP	Collection d'Institut Pasteur	Paris, France
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen	Braunschweig, Germany
JCM	Japan Collection of Microorganisms	Tsukuba, Ibaraki, Japan
NCCB	Netherlands Culture Collection of Bacteria	Utrecht, Netherlands
NCIMB	National Collection of Industrial, Food and Marine Bacteria	Aberdeen, Scotland
STCC	Spanish Type Culture Collection, Valencia University	Valencia, Spain

Maintenance and Preservation of Pure Cultures Obtained from Microbes

Once a pure culture is obtained then methods are to be devised for their maintenance and preservation so that all the characteristics can be conserved. Some of the simple methods of culture maintenance and their preservation are described below.

Use of refrigerator or cooling apparatus:

Live pure cultures can be successfully stored in their respective culture media in refrigerators or such cooling conditions at about 4°C. Generally, the metabolic activity of the organisms slows down and they become nearly inert at this temperature. However, the metabolism does not completely cease and hence, the organisms cannot be maintained for an indefinite period of time. At regular intervals, say 2-4 weeks, the culture may be taken out from the refrigerator and inoculated to fresh media, a process known as sub-culturing.

Transfer to fresh media

Periodic transfer to fresh, sterile media tubes can maintain microbial cultures. The frequency of transfer, however, varies with the organism. A culture the bacterium, Escherichia coli, for example, needs transfer at monthly intervals. After growth for 24 hours at 37°C, the slants can be stored at low temperature for 20-30 days to keep the culture viable. It is necessary to use the appropriate growth medium and proper storage temperature

Overlying with mineral oil or liquid paraffin

Covering the fresh growth in agar slants with sterile mineral oil or liquid paraffin can preserve many bacteria and fungi. The oil must be above the tip of the slanted surface. The cell viability in this method is very high as compared to frequent transfer and storage at low temperature.

Freeze drying or lyophilization

Freeze drying (lyophilization) is a rapid dehydration of organisms while they are in frozen state. In this process, the cell suspension is placed in small vials, which are, frozen by immersing in a mixture of dry ice and acetone liquid nitrogen. The vials are evacuated and dried under vacuum, sealed and stored at low temperature. Under such conditions, microbes can be stored for very long durations without upsetting their characteristics.

Storage at sub-zero temperature

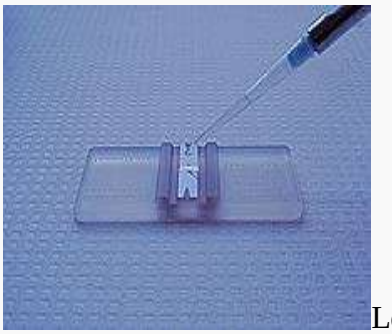
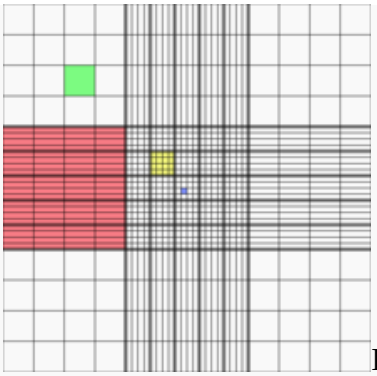
In this method, the cultures are frozen in the presence of a protective agent such as glycerol or dimethylsulphoxide in liquid nitrogen (-196° C). This method is successful in many organisms particularly those which cannot be preserved under lyophilization.

Storage in silica gel

Both bacteria and yeasts can be stored in silica gel at low temperature for 1 to 2 years. In this method, finely powdered, heat sterilized and cooled silica powder is mixed with a thick suspension of cells and stored at a low temperature. The quick desiccation at low temperature allows the cells to remain viable for a long period.

Hemocytometer

A hemocytometer. The two semi-reflective rectangles are the counting chambers.


Load a chamber

Hemocytometer grid

The **hemocytometer** is a device used to count cells. It was originally designed for the counting of blood cells.

The hemocytometer was invented by Louis-Charles Malassez and consists of a thick glass microscope slide with a rectangular indentation that creates a chamber. This chamber is engraved with a laser-etched grid of perpendicular lines. The device is carefully crafted so that the area bounded by the lines is known, and the depth of the chamber is also known. It is therefore possible to count the number of cellsor particles in a specific volume of fluid, and thereby calculate the concentration of cells in the fluid overall.

Principles

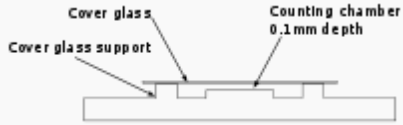
The gridded area of the hemocytometer consists of nine 1 x 1 mm (1 mm^2) squares. These are subdivided in 3 directions; 0.25 x 0.25 mm (0.0625 mm^2), 0.25 x 0.20 mm (0.05 mm^2) and 0.20 x 0.20 mm (0.04 mm^2). The central square is further subdivided into 0.05 x 0.05 mm (0.0025 mm^2) squares. The raised edges of the hemocytometer hold the coverslip 0.1 mm off the marked grid, giving each square a defined volume (see figure on the right).

Dimensions	Area	Volume at 0.1 mm depth
1 x 1 mm	1 mm^2	100 nL
0.25 x 0.25 mm	0.0625 mm^2	6.25 nL
0.25 x 0.20 mm	0.05 mm^2	5 nL
0.20 x 0.20 mm	0.04 mm^2	4 nL
0.05 x 0.05 mm	0.0025 mm^2	0.25 nL

Usage

To use the hemocytometer, first make sure that the special coverslip provided with the counting chamber is properly positioned on the surface of the counting chamber. When the two glass surfaces are in proper contact Newton's rings can be observed. If so, the cell suspension is applied to the edge of the coverslip to be sucked into the void by capillary action which completely fills the chamber with the sample. The

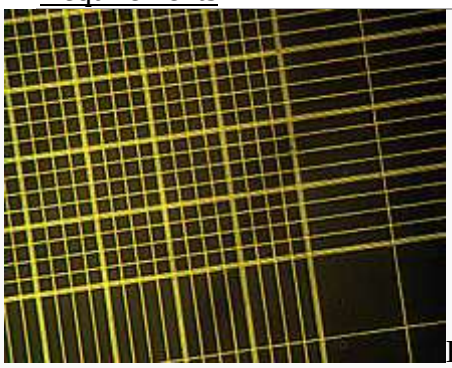
number of cells in the chamber can be determined by direct counting using a microscope, and visually distinguishable cells can be differentially counted. The number of cells in the chamber is used to calculate the concentration or density of the cells in the mixture the sample comes from. It is the number of cells in the chamber divided by the chamber's volume, which is known from the start, taking account of any dilutions and counting shortcuts:
where the volume of the diluted sample (after dilution) divided by the volume of the original mixture in the sample (before dilution) is the dilution factor. For example, if the volume of the original mixture was 20µL and it was diluted once (by adding 20µL dilutant), then the second term in parentheses is 40µL/20µL. The volume of the squares counted is the one shown in the table at the top, depending on the size (see figure on the right). The number of cells counted is the sum of all cells counted across squares in one chamber. The proportion of the cells counted applies if not all inner squares within a set square are counted (i.e., if only 4 out of the 20 in a corner square are counted, then this term will equal 0.2).



The parts of the hemocytometer (as viewed from the side) are identified.

For most applications, the four large corner squares are only used. The cells that are on or touching the top and left lines are counted, but the ones on or touching the right or bottom lines are ignored.

Requirements


Empty hemocytometer grid at 100x power.

- The original suspension must be mixed thoroughly before taking a sample. This ensures the sample is representative, and not just an artifact of the particular region of the original mixture it was drawn from.
- An appropriate dilution of the mixture with regard to the number of cells to be counted should be used. If the sample is not diluted enough, the cells will be too crowded and difficult to count. If it is too dilute, the sample size will not be enough to make stronginferences about the concentration in the original mixture.
- By performing a redundant test on a second chamber, the results can be compared. If they differ greatly, the method of taking the sample may be unreliable (e.g., the original mixture is not mixed thoroughly).

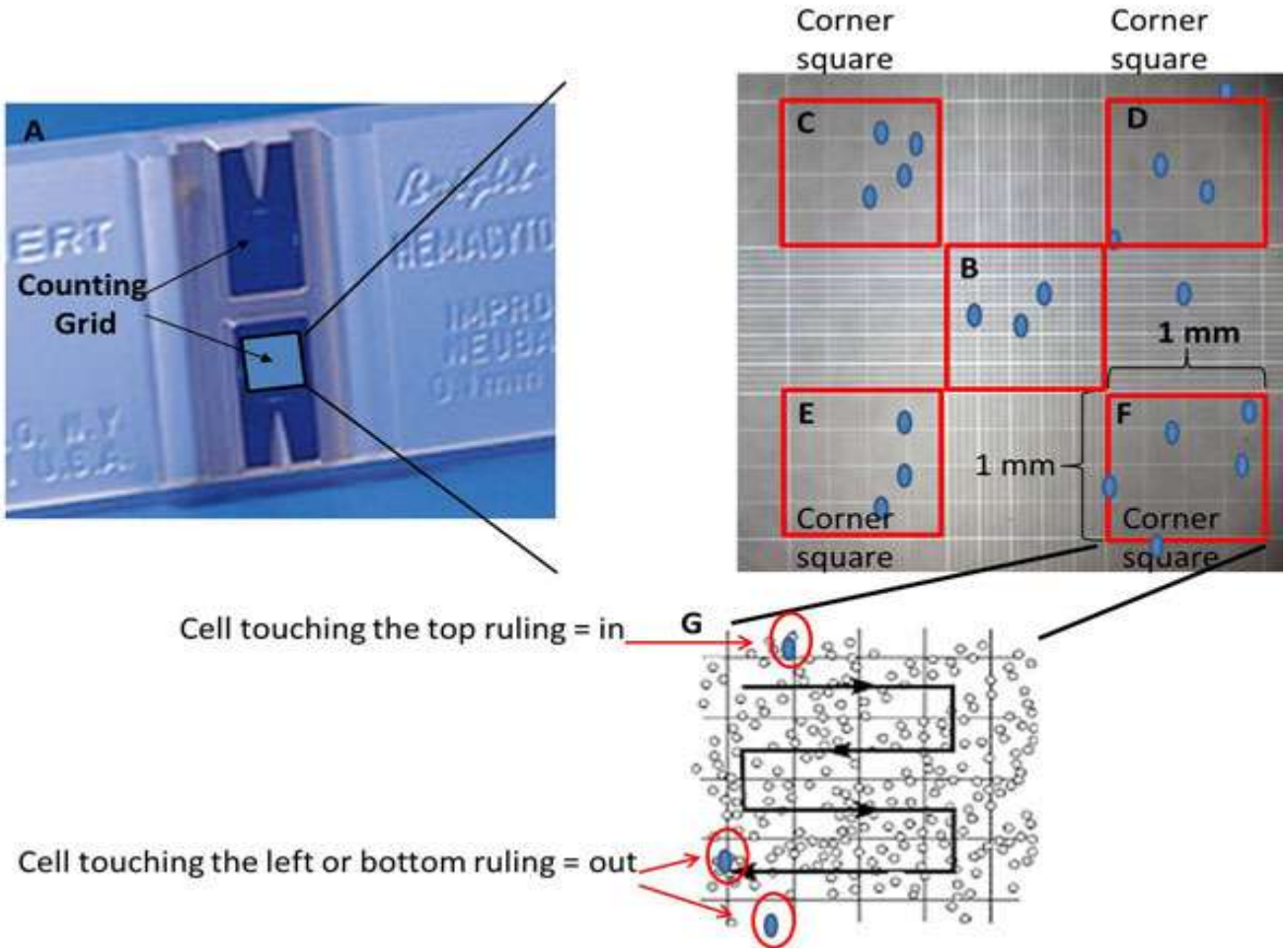
Applications

- Blood counts: for patients with abnormal blood cells, where automated counters don't perform well.
- Sperm counts
- Cell culture: when subculturing or recording cell growth over time.
- Beer brewing: for the preparation of the yeast.

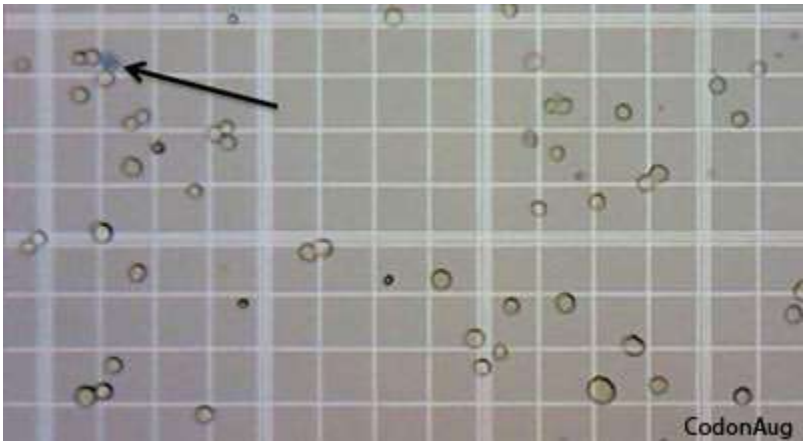
- Cell processing for downstream analysis: accurate cell numbers are needed in many tests (PCR, flow cytometry), while some others require high cell viability.
- Measurement of cell size: in a micrograph, the real cell size can be inferred by scaling it to the width of a hemocytometer square, which is known.

Counting cells in a hemocytometer

The full grid on a hemocytometer contains nine squares, each of which is 1 mm^2 (Figure 3). The central counting area of the hemocytometer (Figure 3B) contains 25 large squares and each large square has 16 smaller squares. When counting, count only those cells on the lines of two sides of the large square to avoid counting cells twice (Figure 3G). Suspensions should be dilute enough so that the cells or other particles do not overlap each other on the grid, and should be uniformly distributed.



To distinguish between dead and viable cells, the sample is often diluted with a particular stain, such as Trypan blue. This staining method, also known as dye exclusion staining, uses a diazo dye that selectively penetrates cell membranes of dead cells, coloring them blue, whereas it is not absorbed by membranes of live cells, thus excluding live cells from staining. When viewed under a microscope, dead cells would appear as dark blue.



Trypan Blue Exclusion of Live Cells on the Hemocytometer. (Arrow indicates uptake of dye across the membrane of dead cells.)

To perform the count, determine the magnification needed to recognize the desired cell type and systematically count the cells in selected squares so that the total count is approximately 100 cells, a minimum number of cells needed for a statistically significant count. For large cells, you can simply count the cells inside the four large corner squares (Figure 3C-F) and the middle one (Figure 3B). For a dense suspension of small cells you may wish to count the cells in the four outer and middle squares of the central square (Figure 3B) or make a more dilute suspension.

Remember if a cell overlaps a ruling, count it as "in" if it overlaps the top or right ruling, and "out" if it overlaps the bottom or left ruling (Figure 3G). The area of the middle (Figure 3B) and each corner square (Figure 3C-F) is 1 mm x 1 mm = 1 mm^2: the depth of each square is 0.1 mm. The final volume of each square at that depth is 100nl.

Once you have obtained the total cell count, cell concentration can be calculated from the following formula:

$$\text{Total cells/ml} = \text{Total cells counted} \times \frac{\text{dilution factor}}{\text{\# of Squares}} \times 10{,}000 \text{ cells/ml}$$

So, for example, if you diluted your sample 1:1 with Trypan blue, and you counted 325 cells in 4 corner squares plus the central big square, total cells per ml =

$$325 \text{ cells} \times \frac{2(\text{dilution factor})}{5 \text{ Squares}} \times 10{,}000 \text{ cell/ml} = 130 \times 10^4 \text{ cells/ml}$$

If you want to know how many cells you have in your original sample, just multiply the cell concentration by total sample volume. For example, if your original sample volume is 5 ml, then your sample has a total =

$$130 \times 10^4 \text{ cells/ml} \times 5\text{ml} = 650 \times 10^4 \text{ cells}$$

Cultivation of Aerobic and Anaerobic Bacteria

A. Aerobic Bacteria

Main Principle: Provide Oxygen

Atmospheric condition is generally satisfactory, for culture of aerobes or facultative anaerobes but for the growth of many aerobes, it is necessary to provide extensive aeration. Forced aeration of cultures is therefore frequently desirable and can be achieved either by vigorously shaking the flask or tube on a shaker or by bubbling sterilized air into the medium. When aerobic organisms are to be grown in large

quantities, it is advantageous to increase the exposure of the medium to the atmosphere. This can be accomplished by dispensing the medium in shallow layers or by providing aeration by constantly shaking the inoculated liquid cultures.

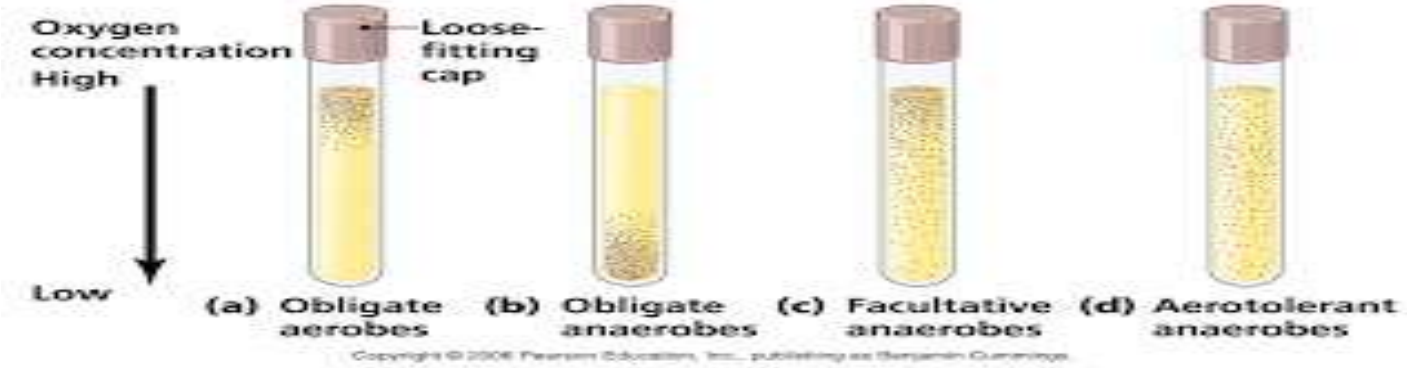
B. Cultivation of Anaerobic Bacteria

Main Principle: reduce the O_2 content of culture medium and remove any oxygen already present inside the system or in the medium.

Oxygen is ubiquitous in the air so special methods are needed to culture anaerobic microorganisms. A number of procedure are available for **reducing the O2 content** of cultures; some simple but suitable mainly for less sensitive organisms, others more complex but necessary for growth of strict anaerobes.

- Bottles or tubes filled completely to the top with culture medium and provided with tightly fitting stopper. Suitable for organisms not too sensitive to small amounts of oxygen.
- Addition of a reducing agent that reacts with oxygen and reduces it to water e.g., Thioglycolate in thioglycolate broth. After thioglycolate reacts with oxygen throughout the tube, oxygen can penetrate only near the top of the tube where the medium contacts air.

ü Obligate aerobes grow only at the top of such tubes.
ü Facultative organisms grow throughout the tube but best near the top.
ü Microaerophiles grow near the top but not right at the top.
ü Anaerobes grow only near the bottom of the tube, where oxygen cannot penetrate.



A redox indicator dye called resazurin is added to the medium because the dye changes color in the presence of oxygen and thereby indicates the degree of penetration of oxygen into the medium.

Strict anaerobes, such as methanogenic bacteria can be killed by even a brief exposure to O_2. In these cases, a culture medium is first boiled to render it oxygen free, and then a reducing agent such as H_2S is added and the mixture is sealed under an oxygen- free gas. All manipulations are carried out under a tiny jet of oxygen free hydrogen or nitrogen gas that is directed into the culture vessel when it is open, thus driving out any O_2 that might enter. For extensive research on anaerobes, special boxes fitted with gloves, called anaerobic glove boxes, permit work with open cultures in completely anoxic atmospheres.

Stringent anaerobes can be grown only by taking special precautions to exclude all atmospheric oxygen from the medium. Such an environment can be established by using one of the following methods:

Pre-reduced media

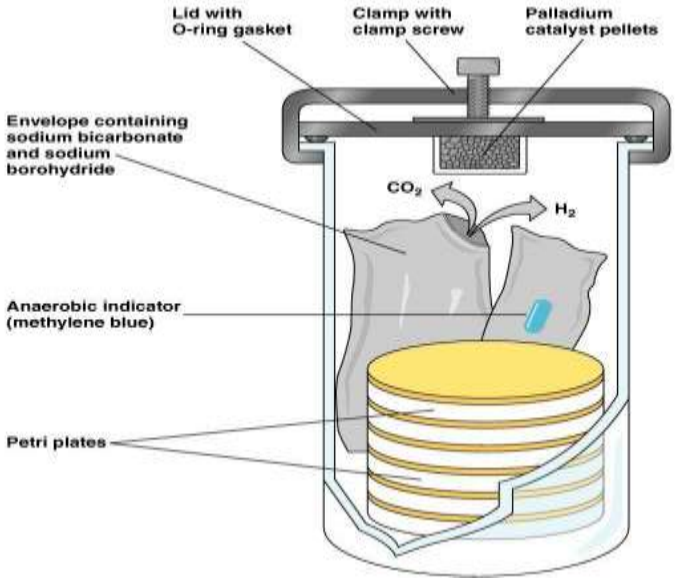
During preparation, the culture medium is **boiled** for several minutes to drive off most of the dissolved oxygen. A reducing agent e.g., cysteine, is added to further lower the oxygen content. Oxygen free N_2 is bubbled through the medium to keep it anaerobic. The medium is then dispensed into tubes which are being flushed with oxygen – free nitrogen, stoppered tightly, and **sterilized by autoclaving**. Such

tubes are continuously flushed with oxygen free CO_2 by means of a cannula, restoppered, and incubated.

Anaerobic Chambers

This refers to a plastic anaerobic glove box that contains an atmosphere of H_2, CO_2, and N_2. Culture media are placed within the chamber by means of an air lock which can be evacuated and refilled with N_2. Any oxygen in the media is slowly removed by reaction with hydrogren, forming water; this reaction is aided by a palladium catalyst. After being rendered oxygen free, the media are inoculated within the chamber (by means of the glove ports) and incubated (also within the chamber).

Anaerobic Jar



Anaerobic Jar: GasPak system

Anaerobic jar is a heavy- walled jar with a gas tight seal within which tubes, plates, or other containers to be incubated are placed along with H_2 and CO_2 generating system (GasPak system) . After the jar is sealed**oxygen** present in the atmosphere inside jar and dissolved in the culture medium, **is gradually used up**through reaction with the hydrogen in the presence of catalyst. The air in the jar is replaced with a mixture of H_2 and CO_2, thus leading to anoxic conditions.

Basic bacterial cultivation techniques

Enrichment

Enrichment media promote the growth of a particular microorganism against the others present in the sample by its specific nutrient utilisation ability or other unique metabolic properties (e.g. resistance to antibiotics or heavy metals). Only those microbes can grow in the enrichment medium that can use or tolerate the components of the selective medium.

PREPARATION OF ENRICHMENT CULTURES

Object of study:

- gasoline degrading bacteria
- cellulose degrading bacteria
- Mercury-resistant bacteria

Materials and equipment:

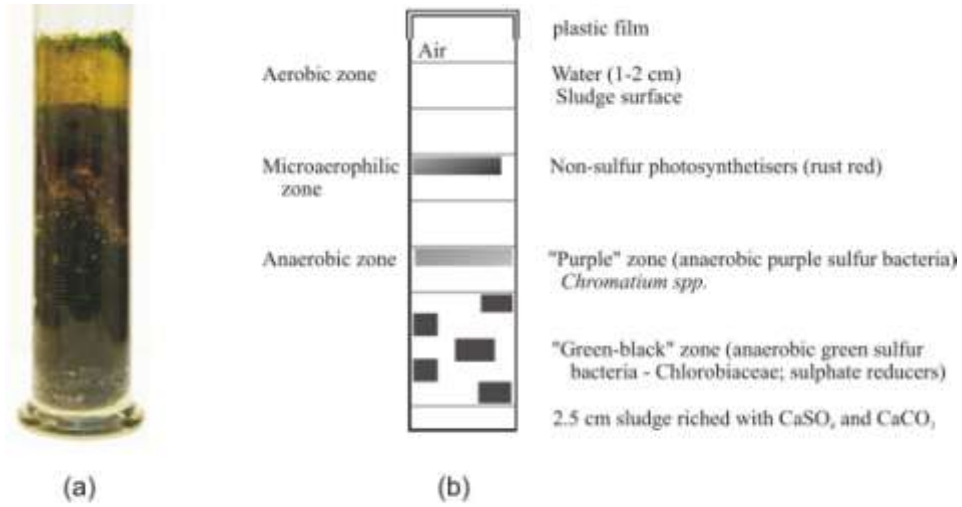
- garden soil
- gasoline-containing enrichment broth
- cellulose-containing enrichment broth
- $HgCl_2$-containing enrichment broth
- sterile chemical spoons
- laboratory scales
- shaker incubator
- glass spreader (alcohol for sterilisation)
- Bunsen burner
- incubator

Practice:

1. Measure 1-1 g of the garden soil into the flasks containing different enrichment broths.
2. For better aeration, place the inoculated flasks into a shaker incubator at 28°C for one week.
3. Prepare a five-member, 10-fold dilution series from the enrichment cultures and the original sample.
4. Spread the surface of agar plates having the same composition as the enrichment broths from the individual members of the dilution series.
5. Incubate the cultures at 28°C for one week.
6. Perform germ count estimations and colony morphology examination after the incubation period.

PREPARATION OF WINOGRADSKY COLUMN FOR THE ENRICHMENT OF PHOTOTROPHIC BACTERIA

The Winogradsky column (Fig. 19) is a small ecological system (microcosm), which models the microbial processes taking place primarily in freshwater lake benthic environments. Due to the natural selection processes occurring in the column in a few weeks, bacterial communities with diverse species composition develop at various depths, mainly according to the different oxygen sensitivity of the photosynthetic bacteria. In a Winogradsky column, presence and enrichment of purple and green anoxygenic phototrophic bacteria (e.g. *Chromatium* and *Chlorobium*), sulfate-reducing bacteria (e.g.*Desulfovibrio*), and many other anaerobic microorganisms can be observed with the naked eye and by microscopy as well.



The Winogradsky column. (a) Picture of a three-month-old column. (b) Schematic representation of the column structure.

Object of study:

- anoxygenic phototrophic bacteria
- sulfate-reducing bacteria

Materials and equipment:

- garden soil or lake mud
- $CaCO_3$
- $CaSO_4$
- laboratory scales
- chemical spoons
- bowl
- filter paper
- measuring cylinder
- plastic film (Parafilm)

Practice:

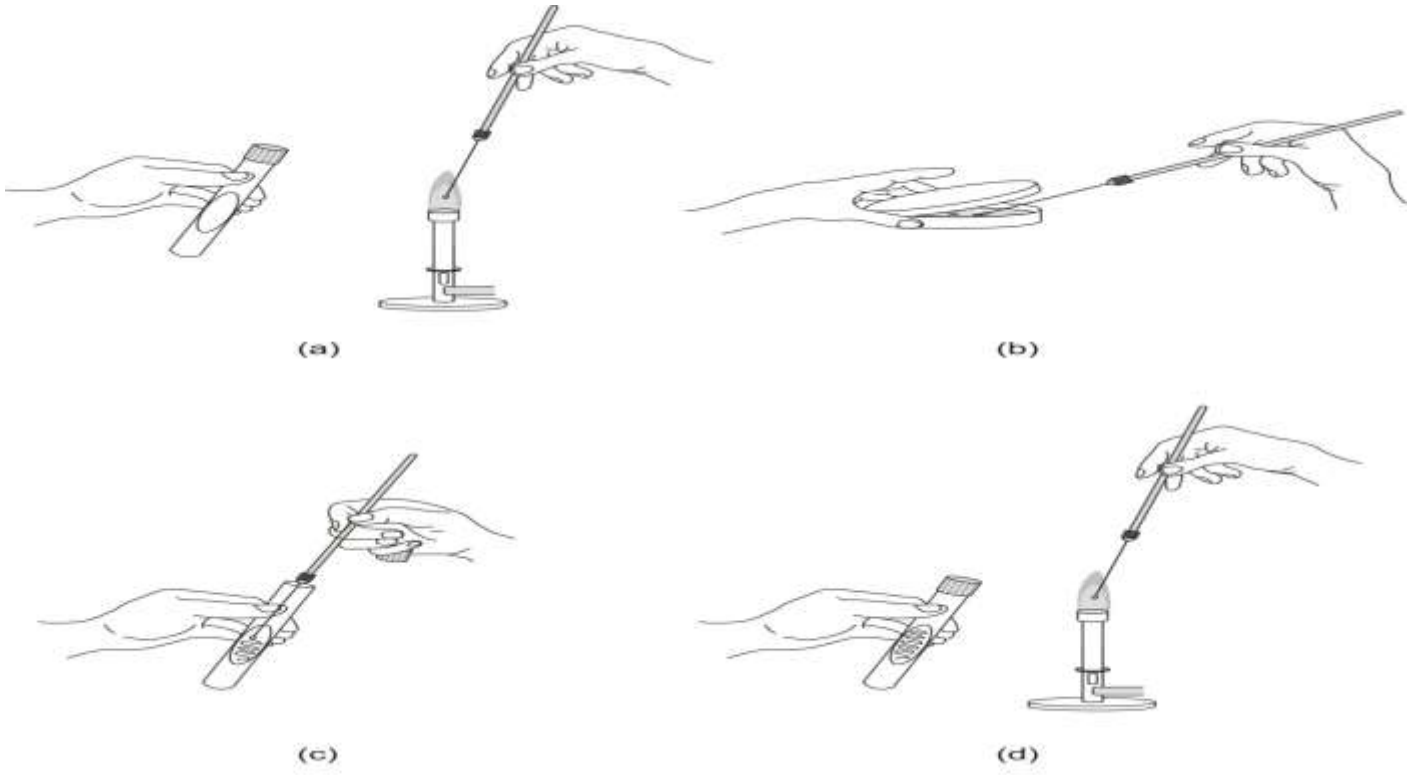
1. Mix 100 to 200 g of sieved garden soil with 3-5 g calcium carbonate and 3-5 g calcium sulphate in a bowl. Tear filter paper into small pieces and mix them with the soil. Then add tap water to the soil mixture until it reaches a cream-like consistency.
2. Apply the enriched soil to the bottom of a measuring cylinder in a thickness of about 2-5 cm; subsequently fill the column uniformly with lake mud at a height of about 15-25 cm. The column is appropriate if it has no air bubbles, and after standing for 24 hours about 0.5 cm layer of water covers the mud (pour off the excess). To avoid dehydration, close the top of the measuring cylinder with plastic film.
3. Place the column near the window for at least 4-6 weeks at room temperature (avoid direct sunlight and overheating).
4. During the incubation period, follow-up the enrichment of anoxygenic phototrophic and sulphate reducing bacteria by colour changes observed in the column. After the incubation period, carefully dissect the column and carry out microscopic examinations on anoxygenic phototrophic and sulphate reducing bacteria.

Spread plate and pour plate methods

Isolation and streak plate technique

During isolation, bacterial cells from a discrete colony that developed on the surface of an agar plate are transferred to an agar slant having the same composition. The culture developing on the surface of the agar slant after the isolation is called an isolate.

To aseptically transfer microorganisms from broth, slant or agar cultures to another medium, inoculating needles or loops are used. They are made up of a handle, a shaft, a turret and a straight or a loop-ended nickel-chromium needle.



Isolation. (a) Take the inoculating loop in one hand and hold it like a pencil. Flame the inoculating loop over a Bunsen burner until the wire becomes red-hot. (b) Make a gap on the Petri dish and choose a discrete colony to pick up a loopful of inoculum with the inoculating loop, then close the lid of the Petri dish. (c) After opening and flaming the neck of the test tube, inoculate the surface of the agar slant in zigzag streaks using the infected inoculating loop. (d) Reflame the neck of the tube, close it and sterilise the loop with reflaming as well.

ISOLATION OF CULTURES FROM THE AGAR SURFACE

Object of study:

- bacteria present in soil samples
- bacteria present in water samples

Materials and equipment:

- agar plates inoculated by spread plate technique
- agar slants
- inoculating loop
- Bunsen burner
- incubator

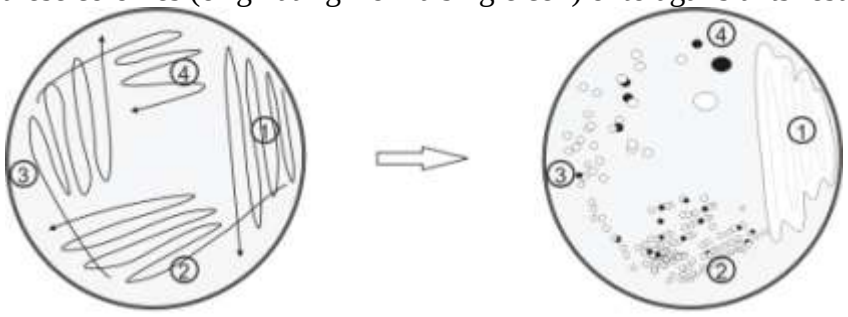
Practice:

1. Label slant to be inoculated with the date, your name and name/code/ number of isolate. Select adequate colonies from the plate culture by marking them on the bottom of the Petri dish.

2. Take the inoculating loop and hold it like a pencil. Flame the inoculating loop over a Bunsen burner until the wire becomes red-hot.
3. Open the lid of the Petri dish culture to a gap and cool the hot loop by inserting it into the agar without touching any colonies developed on the surface. Choose a discrete colony and pick a loopful of inoculum using the inoculating loop, and then close the lid of the Petri dish.
4. Using the same hand that is holding the inoculating loop, remove the cap from a test tube, hold it between your fingers, and briefly flame the neck of the tube over a Bunsen burner by passing through the flame.
5. Inoculate the surface of the agar slant in zigzag streaks using the infected inoculating loop.
6. Flame the neck of the tube again and close it with the cap.
7. Sterilise the loop again by flaming over a Bunsen burner until the wire becomes red-hot. Take care to place the infected loop first into the core of the flame, and then slowly pull it upwards until it becomes red-hot.
8. Place the tube and the inoculating loop on the rack.
9. Incubate the slant at 28°C for one week.
10. Check the growth of the isolate after the incubation period.

An isolate is not necessarily a pure culture, i.e. containing cells of the same origin (derived from a single mother cell/a clone of cells). In mixed cultures, co-multiplication of two or more microbes occurs. This can happen accidentally, but not all microbes are able to grow independently from others; e.g. one of the microbes can produce a compound that enables the growth of another microbe in the culture medium (synergism), or a substance produced by one microbe inhibits the growth of another (antagonism), or one microbe can grow faster than the other, thus growth of the latter would be limited because of the use of essential nutrients. The microbiological examination of mixed cultures generally provides confusing and misleading results due to the different metabolic properties of various microbes. Therefore, it is necessary to create pure cultures. Pure (axenic) cultures are free from other microorganisms, develop from single cells or colony forming units, and serve as the basis of species level identification of bacteria and other studies.

Pure cultures can be obtained by the streak-plate technique. This method is based on the creation of a dilution gradient on the surface of an agar plate. Due to an appropriate dilution of the inoculum (e.g. mixed culture), discrete and visible colonies can develop at the end of the inoculation line. Reisolation of bacterial cells from these colonies (originating from a single cell) onto agar slants results in pure cultures (Fig. 21).



(a) (b)

Preparation of pure cultures by streak plate method. (a) Inoculate the mixed bacterial cultures on approximately one-quarter of the surface of an agar plate with the inoculating loop (1). Sterilise the loop by reflaming, cross over the streaks of the first inoculation when streaking the second part of the agar surface (2). Repeat these steps on the third (3) and fourth quarter (4) of the agar surface. (b) Check the growth of discrete

colonies with different morphology, in this case colour, in the third and fourth quarter of the agar plate after the incubation period.

PREPARATION OF PURE CULTURES BY THE STREAK PLATE METHOD

Object of study, test organisms:

- mixed suspension of *Serratia marcescens* and *Micrococcus luteus* strains

Materials and equipment:

- nutrient agar plates
- inoculating loop
- Bunsen burner
- incubator

Practice:

1. Label a Petri plate to be inoculated with the date, your name, and the mark of the isolate to be purified.
2. Take the inoculating loop and hold it like a pencil. Flame the inoculating loop over a Bunsen burner until the wire becomes red-hot.
3. Holding the inoculating loop in one hand, take the test tube containing the suspension of mixed bacterial cultures in the other hand.
4. Using the same hand that is holding the inoculating loop, remove the cap from the test tube, hold it between your fingers, and briefly flame the neck of the tube over a Bunsen burner by passing through the flame.
5. Take a loopful of inoculum from the suspension.
6. Flame the neck of the tube again and close it with the cap. Place the tube in the rack.
7. Inoculate approximately one-third of the agar surface (at the edge) using the infected inoculating loop (without scratching the agar).
8. Sterilise the loop again by flaming until the wire becomes red-hot.
9. Cool the loop by thrusting it into the sterile agar.
10. Cross over the streaks of the first inoculation when streaking the second part of the agar surface.
11. Flame and cool the loop again before repeating the streaking process on the third part of the agar surface.
12. Sterilise the loop again by flaming and place it on the rack.
13. Incubate the culture at 28°C for one week.
14. Check the growth of discrete colonies with different morphology after the incubation period. Perform re-isolation

Anaerobic cultivation techniques

The appropriate method for the cultivation of anaerobes should be chosen with consideration of the sensitivity of the given organism to oxygen concentration and/or redox value within the media or the surrounding athmosphere. For the cultivation of bacteria sensitive to even trace amounts of oxygen (e.g. methanogens, sulphate reducing bacteria), the best technique is the use of an anaerobic system, filled with an appropriate gas, and the only contact with the outside world is through a sluice chamber that removes oxygen when materials are introduced into the system. Microbes that are not sensitive to trace amounts of oxygen and are able to survive temporarily high oxygen concentrations (e.g. sampling and sample processing) by forming endospores, can be cultivated in anaerobic jars or in semisolid media containing special reductive agents. However, colonies formed inside "anaerobic" agar deep tubes are difficult to examine and isolate. This problem can be solved by using Marino-plates (better known as Brewer's plates), a combination of a Petri-dish and an "anaerobic" agar.

CULTIVATION OF ANAEROBIC BACTERIA IN SODIUM THIOGLYCOLLATE MEDIUM

The oldest and most common method for the cultivation of anaerobic bacteria is culturing them deep in **reduced semisolid or solid media**. The most important property of these nutrient media is the suitably low redox potential. Different indicators within the culture media serve to test the appropriately low redox potential. The most common indicators are resazurin and methylene blue, which show colour only in an oxidised environment

(where resazurin is red and methylene blue is blue). To ensure that oxygen does not get into the culture media either, the inoculated media are sealed during incubation (melted and heat sterilised vaseline, or 1:1 mixture of Vaseline and paraffin is layered on top of the culture media). Semi-solid agar deep tubes are prepared with a small amount of agar, so that these media are appropriate for the cultivation of anaerobes without sealing, since agar itself can reduce convection currents, thus reoxidation; furthermore agar colloid applied in small concentrations is reductive. To lower the redox potential in semisolid media even further, reductive compounds could be used (sodium thioglycollate and cysteine).

Object of study, test organisms:

- anaerobic bacteria of soil

Materials and equipment:

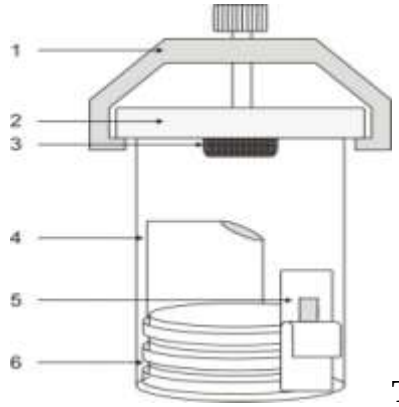
- soil sample
- 9 mL sterile distilled water in test tubes
- vortex mixer
- sodium thioglycollate containing melted agar medium
- pipette, sterile pipette tips
- Bunsen burner
- incubator

Practise:

1. Prepare a 10-fold dilution series from the soil sample and pipette 1-1 mL from each dilution to sodium thioglycollate containing melted agar medium and label the tubes.
2. Incubate the tubes at 28°C for one week.
3. Check the colonies inside the test tube and count them if possible.

CULTIVATION OF ANAEROBIC BACTERIA IN AN ANAEROBIC JAR

The anaerobic jar is usually transparent and can be sealed; contains a palladium catalyst, a disposable H_2+CO_2 generator and a redox indicator. Cultures are placed into the jar along with an envelope that includes two tablets. One of the tablets contains $NaBH_4$ that generates hydrogen when it reacts with water; the other tablet contains citric acid and sodium-hydrogen-carbonate that generates CO_2 when comes into contact with water. The CO_2 contributes to the growth of fastidious anaerobes. The jar is sealed after water is added to the envelope. In the presence of the palladium catalyst, hydrogen reacts with oxygen to form water. This reaction removes free oxygen from the inner atmosphere of the jar. The colour change of the indicator refers to the formation of an anaerobic atmosphere.



The anaerobic jar. (1) clamp (2) cover (3) palladium catalyst (4) H_2+CO_2 generator (5) redox indicator (6) culture plates.

Object of study: *Clostridium* spp. from soil

Materials and equipment:

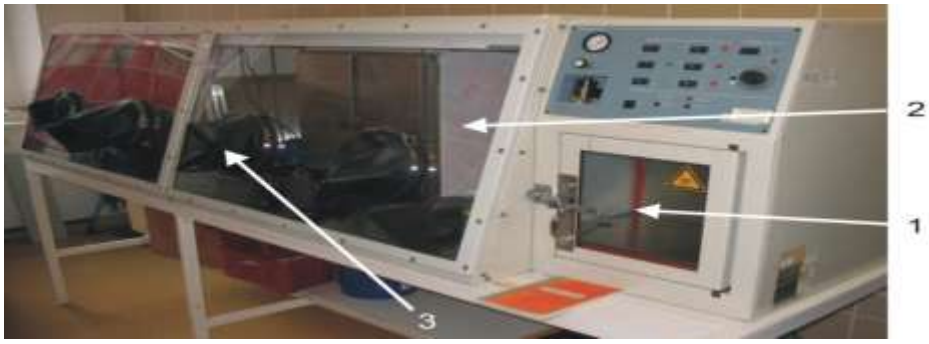
- soil or sediment sample
- 9 mL sterile distilled water in test tubes
- vortex mixer
- anaerobic jar
- redox indicator strip
- gas generator envelope
- palladium catalyst
- scissors
- pipette, sterile pipette tips
- glass spreader (alcohol for sterilisation)
- bismuth sulphite agar medium
- Bunsen burner
- incubator

Practise:

1. Prepare a 6-member 10-fold dilution series from soil sample.
2. Spread each member of the dilution series onto Wilson-Blair type agar medium.
3. Put the inoculated Petri-dishes with their surface down into the anaerobic jar (Figure). Open the redox indicator and place it next to the plates so that the indicator strip can be seen from the outside (the indicator strip will turn blue within seconds).
4. Take care that palladium catalyst has been regenerated (by incineration) or replace with a fresh one.
5. Cut the gas generator envelope with scissors, and add 8-10 mL water with a pipette (according to the manufacturers' instructions). Close the jar and screw on the clamp, then put the jar into an incubator at 28°C.
6. After one week of incubation, determine the CFU number for the original sample

DEMONSTRATION OF THE ANAEROBIC CHAMBER (GLOVE-BOX)

The anaerobic chamber is a device suitable for the cultivation of strictly anaerobic bacteria (Fig.23). Inside the system, anoxic conditions can be maintained, while microbiological operations (microscopy, isolation, inoculation, etc.) can be performed. At first step, oxygen is removed with vacuum, then a gas mixture (10% H_2, 10% CO_2, 80% N_2) is introduced into the system with slightly positive pressure. The detection of trace amounts of oxygen is performed by methylene blue or resazurin redox indicators, and the elimination of such oxygen is completed by a palladium catalyst. Active carbon inside the anaerobic chamber serves to bind catalyst poisons (e.g. H_2S) and other substances that are toxic for bacterial cells.



The anaerobic chamber (glove box). Samples are transferred through the interchange (1) into the working chamber (2), where they can be handled from outside through gloves (3).

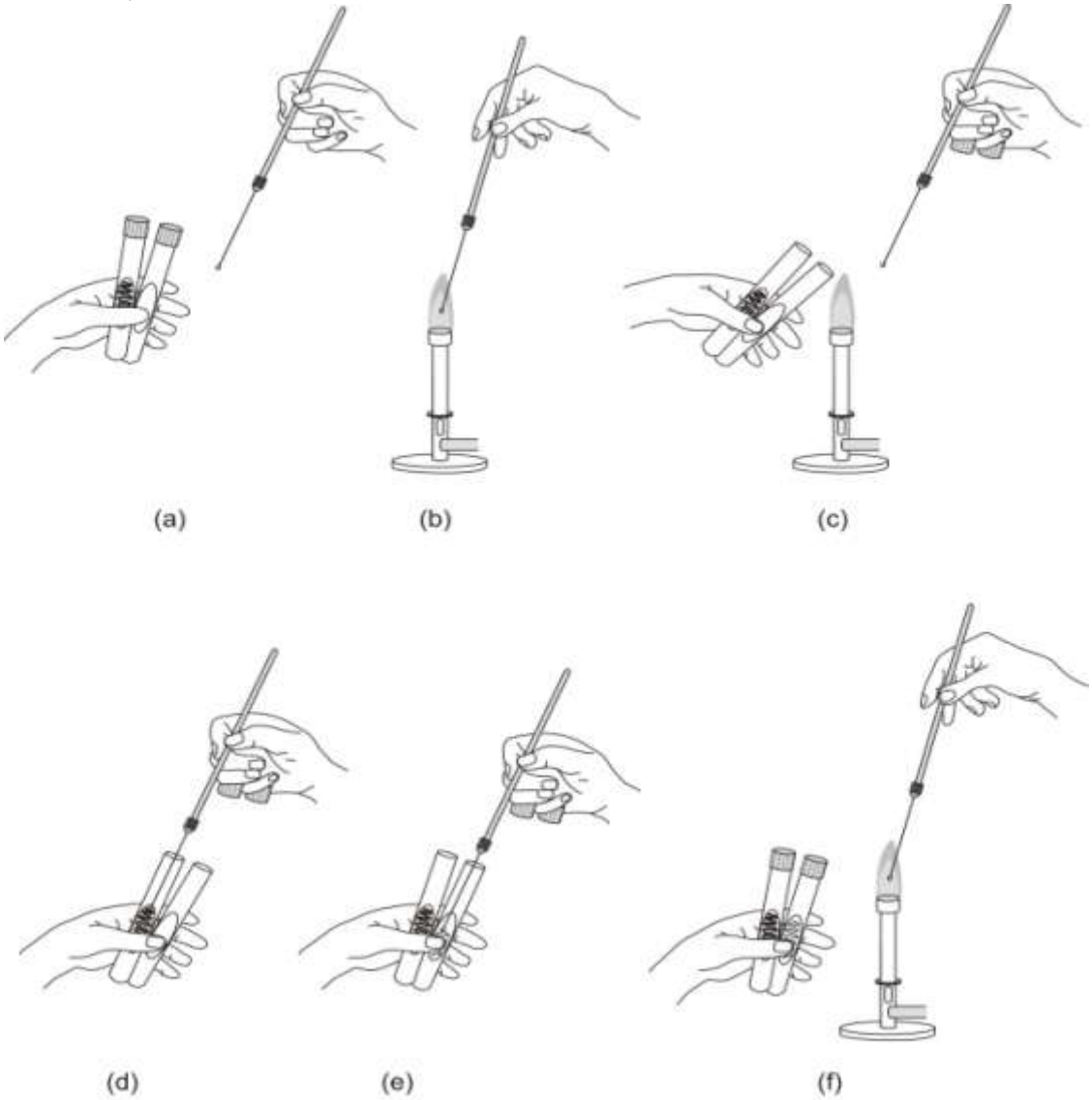
Transfer, maintenance and storage of pure cultures

The aim of strain maintenance is to keep microorganisms alive in such a way that their phenotype (and genotype) does not change relative to the original isolates. It is essential that microbial cells are not contaminated, keep their viability and their genetic material remains unchanged as much as possible during storage.

The maintenance of new environmental isolates as well as strains of described species can be done using different methods (e.g. subcultivation, lyophilisation, storage in liquid nitrogen). To select the appropriate technique, one must take into account several factors: aim of maintenance, intended duration, generation time of the microbes, etc.

PROCEDURE OF CULTURE TRANSFER (SUBCULTURING)

During the maintenance of bacterial strains by transfer (or subculturing), cultures of bacteria are transferred to fresh sterile growth medium (usually to agar slants) at appropriate intervals, and the newly developed cultures are stored at 4-6°C (in a refrigerator or a cold room). This process should be repeated periodically (every few weeks or months) depending on the characteristics of bacterial strains (Figure). The disadvantage of this method is that frequent passages may increase the risk of contaminations and mutations, and cultures may dry out during storage. In the case of storage for relatively long periods, to prevent cultures from desiccation and to reduce oxidative stress, slant surfaces should be covered with sterile mineral oil.



Subculturing microbial cultures. (a) Place both test tubes in one hand to form a V-like shape. Take the inoculating loop in the other hand (b) and flame it over a Bunsen burner. (c) Remove the cap from the test tubes, and briefly flame the neck of the tubes. (d) Pick up a loopful of bacterial cells from the culture using the sterile inoculating loop, (e) and inoculate the surface of the sterile agar slant in zigzag streaks. (f) Reflame the necks of the tubes, close them and reflame the inoculating loop.

Object of study, test organisms:

- slant culture of *Micrococcus luteus*
- slant culture of an unknown bacterial strain

Materials and equipment:

- nutrient agar slants
- inoculating loop
- Bunsen burner
- incubator

Practice:

1. Label a tube containing the sterile agar slant to be inoculated with the date, your name, and the name or mark of the test microorganism.
2. Place both test tubes (the sterile agar slant and that of containing the culture) in the palm of your hand in a V-like shape and stabilise them with your thumb. They should be held at an angle and thus not directly exposed to airborne laboratory contaminants.
3. Take the inoculating loop in the other hand and hold it like a pencil. Flame the inoculating loop along its full length over a Bunsen burner until the wire becomes red-hot.
4. Using the same hand that is holding the inoculating loop, remove the cap from the test tubes, hold it between your fingers, and briefly flame the neck of the tubes over a Bunsen burner by passing them through the flame. Do not put the caps onto the laboratory bench.
5. Cool the inoculating loop by pushing it against the top of the sterile agar slant until it stops "hissing."
6. Pick up a loopful of bacterial cells from the culture using the sterile inoculating loop, and inoculate the surface of the sterile agar slant in zigzag streaks.
7. Flame the necks of the tubes and close them with their caps.
8. Sterilise the inoculating loop again by flaming.
9. Place the tubes and the inoculating loop into the rack.
10. Incubate at 28°C for one week.
11. Check the growth of the culture.
12. For storage, place the cultures at a cool place (at 4-6°C).

The common feature of most maintenance techniques is that FREE AVAILABLE water is removed from the culture and water uptake is not allowed during storage. Several "dry maintenance" techniques are available for microorganisms, especially for fungi. In the case of fungi, cultures are mixed with sterile medium, and then dried onto silica gel, paper strips or gelatine discs. This way, microbes can preserve their viability for many years. During the process of freezing, microbial cells become dehydrated, thereby water becomes unavailable for them. If the procedure is not performed adequately, the freezing and defrosting process can damage the microbial cells. The reasons are the increased concentration of electrolyte solution within the cells and the formation of ice crystals. The harmful effects can be reduced by adding cryoprotective substances, such as dimethyl sulfoxide (DMSO) and glycerol. Freezing techniques can be classified according to the applied temperature. In general, storage above -30°C is less effective. Using deep-freezing at -70°C, a wide variety of microorganisms (e.g. bacteria, fungi, viruses) can be stored for many years. Using liquid nitrogen at -196°C or

nitrogen vapour at -140°C (ultra-freezing), the viability of such microorganisms can be successfully preserved for decades, where other methods have failed.

MAINTENANCE OF BACTERIAL CULTURES WITH FREEZE-DRYING

Lyophilisation, or freeze-drying, is one of the oldest procedures used to maintain the viability of microorganisms even for decades. In the process of freeze-drying, first a suspension is prepared by adding cryoprotective agents (e.g. blood serum, inositol, sucrose, raffinose) to the culture, and then it is suddenly frozen. Subsequently the ice is sublimated under high vacuum so that the suspension cannot melt during the process of drying. Following drying, cultures are stored in sealed sterile vials.

The basic parts of a freeze-drying equipmentare a vacuum pump and a chamber with a cooled wall to condense water. Most frequently, freezing of cell suspension takes place due to the heat loss caused by evaporation under vacuum. In this process, the samples are centrifuged in order to maximise the surface for evaporation and to avoid the formation of foam due to gas emissions. For this method, glass vials are used. The initial drying step is followed by a second, more thorough drying, also under vacuum. Alternatively, the samples can be put into glass tubes, frozen first and then dried under vacuum (the initial spin is skipped). In this case, $CoCl_2$ crystals are placed along with the samples to indicate the presence of water (e.g. if hydration takes place due to the break or rupture of the glass, the blue colour of $CoCl_2$ changes to pink)

Laboratory freeze-drying equipment. (a) Primary drying using a batch glass vial ampoule centrifuge. (b) Secondary drying, ampoules are attached to a drying manifold. (c) Lyophilised bacterial strains.

Object of study, test organisms:

- culture of *Micrococcus luteus* on agar slant

Materials and equipment:

- 5% inositol broth
- inoculating loop
- Bunsen burner
- sterile Pasteur pipette
- sterile vials
- sterile cotton plug
- metal forceps
- sterile gauze

Practice:

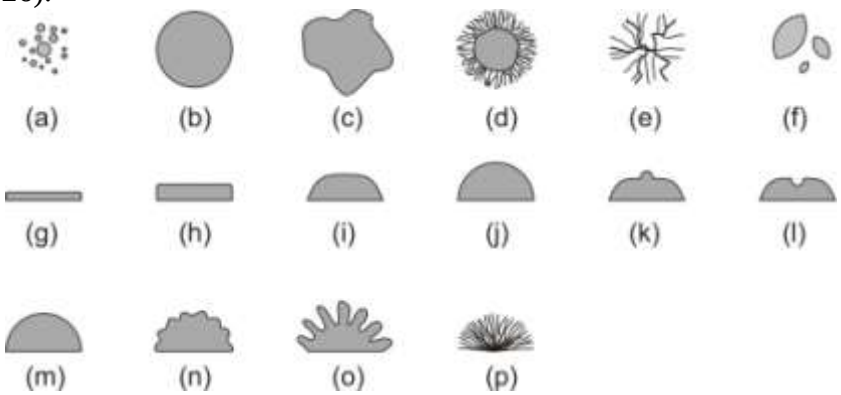
1. Culture the test organism on the most appropriate medium and temperature until its stationary phase is reached, usually for 18-24 hours.

2. Pipette 1 mL of inositol broth onto the surface of the slant culture using a sterile Pasteur pipette and suspend the cells in it. Then pipette 0.3 mL of suspension into a sterile, labelled (date and name of microorganism) 10 mL ampoule. Flame the mouth of the ampoule, and loosely place a gauze plug into it.
3. Turn on the cooling of the freeze-drying equipment, and put the ampoules into the centrifuge when the temperature of the device is -45°C for at least 10 minutes.
4. Spin for 10 minutes. Turn on the vacuum pump (the gas ballast must be completely open) immediately after spinning has started, and close the valve.
5. Following approximately 1 hour of drying time, open the valve, and place the vials onto the post-drying device. A sudden pressure drop indicates when drying has ended.
6. Carefully melt and seal (close) the neck of the vial under vacuum.
7. Open the valve and switch off the vacuum pump and then the cooling device.

Colony- and cell morphology, staining procedures

OBSERVING COLONY MORPHOLOGY ON INOCULATED PLATES

Microbes grow on solid media as colonies. A colony is defined as a visible mass of microorganisms originating from a (single) mother cell, therefore a colony constitutes a clone of bacteria all genetically identical (except mutations that occur at low frequency). The number of cells within a colony can even reach a few billion. On a given medium, a colony's shape, colour, consistency, surface appearance and size - for a given incubation time - are often characteristic, and these features are often used in the identification of particular bacterial strains (Fig. 26).



Colony morphology of bacteria. Form: (a) punctiform (b) circular (c) irregular (d) filamentous (e) rhizoid (f) spindle. Elevation: (g) flat (h) raised (i) convex (j) pulvinate (k) umbonate (l) crateriform. Margin: (m) entire (n) undulate (o) lobate (p) filamentous.

Object of study, test organisms

- agar plates with colonies originating from an environmental sample

Materials and equipment:

- ruler
- magnifying glass

Practise:

1. Select 5 different discrete colonies from the surface of a Petri plate and characterise them as follows:
    - size of the colony (diameter in mm),
    - shape or form of the colony (punctiform, circular, irregular, filamentous, rhizoid, spindle),
    - elevation of the colony (flat, convex, pulvinate, umbonate, crateriform),

- margin of the colony (entire, undulate, lobate, filamentous),
- pigmentation of the colony (diffusible water-soluble or water-insoluble pigments),
- surface of the colony (smooth, glistening, rough, dull, wrinkled),
- density of colony (transparent - clear, opaque, translucent - almost clear, but distorted vision–like looking through frosted glass, iridescent - changes colour in reflected light),
- consistency of colony by touching it with an inoculating loop (butyrous, viscid - sticks to loop, hard to get off, brittle - dry, breaks apart, mucoid),
- presence or absence of diffusible pigment in the medium around the colony.

Characteristics of cell morphology have great importance in the classification of bacteria using traditional taxonomical methods. Microorganisms cannot be identified solely by morphological characteristics, since bacterial cells can only be assigned to a limited number of categories **(Table)**. Bacteria are µm-sized organisms, where cell size is an important aspect of a thorough morphological characterisation. The size and shape of the cells are usually determined following staining. The circumstances of culturing, the age of the culture and the physiological condition of bacterial cells can alter cell size and shape. According to their shape, bacteria can usually be identified as rods, cocci or spirals. An average rod-shaped bacterium is 2-5 µm long and 0.5-0.8 µm wide in diameter. The average diameter of a sphere-shaped bacterium is 0.8 µm. The size of some bacterial groups deviates from average values: spirochetes include some extremely thin (0.2 µm) bacteria, while there are some giants: *Thiomargarita namibiensis* (100-300 x 750 µm) and *Epulopiscium fishelsoni* (50 x 600 µm).

Table. Morphology of bacterial cells

Coccus (sphere)		
Micrococcus	Following cell division, cells separate (singles)	*Micrococcus luteus*
Diplococcus	Following cell division, cells remain in pairs	*Neisseria gonorrhoeae*
Streptococcus	Chain of cocci	*Streptococcus lactis*
Staphylococcus	Grape-like cluster of cocci	*Staphylococcus aureus*
Tetragenus	Cell division on 2 planes, cocci in tetrads	*Planococcus*
Sarcina	Cell division on 3 planes, cocci in aggregates (packets) of eight	*Micrococcus luteus* (earlier *"Sarcina lutea"*)
Rod (bacillus)		
	Shape and size very variable: long-short, wide-thin, coccoid, irregular	*Bacillus megaterium* *Pseudomonas* sp. *Haemophilus influensae* *Corynebacterium* sp.
Curved rod (spiral shape)		
Vibrio	Cell with quarter or half a turn	*Vibrio cholerae*
Spirillum	Rigid cell wall, motility with flagella, cell with one or more turns	*Spirillum volutans*
Spirochaeta	Flexible cell wall, endoflagella, cell with one or more turns	*Treponema pallidum*
Filamentous		

	Actinomyces have branching cells, forming bacterial hyphae and their network (mycelium)	*Streptomyces* sp., *Nocardia* sp.
Variable		
	Intermediate forms (e.g. rod-coccus life cycle)	*Rhodococcus* sp.

Most commonly, fixed and stained smears are used for the study of cell morphology, intracellular constituents and structures. The chemistry of simple staining is based on the principle that different charges attract, while similar charges repel each other. In an aqueous environment, at pH 7, the net electrical charge produced by most bacteria is negative. Dyes applied for staining could be acidic, basic and neutral dyes according to their chemical characteristics. Each dye contains a cation (positive charge) and an anion (negative charge) and either one could be the chromophore (the part of the molecule that is coloured). Since acidic dyes carry a negative charge on their chromophore, the bacterial cells (also negatively charged) reject these dyes. Negative staining could also be conducted with dyes having a colloidal particle size that therefore cannot enter the cell (e.g. the black coloured India Ink and Nigrosine). The chromophores of basic dyes have a positive charge and result the staining of bacterial cells (positive dyes), since they bind to proteins and nucleic acids (around neutral pH carrying a negative charge). Basic dyes include safranin (red), methylene blue (blue), crystal violet (violet), malachite green (green).

Positive staining can be performed with only one dye (simple staining) or more dyes (complex staining). In case of complex staining, the first stain is called "primary stain" and the second one as "counterstain".

Generally the steps of staining are as follows: degreasing and labelling of a slide, making a smear, fixation (occasionally missing), staining (in case of complex staining also includes counterstaining), washing with water, drying and microscopic observation.

SIMPLE STAINING

Object of study, test organisms:

- *Staphylococcus aureus* slant culture
- *Bacillus cereus* slant culture
- *Pseudomonas aeruginosa* slant culture
- Optionally

Materials and equipment:

- glass slide
- glass dropper dispenser
- inoculating loop
- Bunsen burner
- wooden test tube clamps
- crystal violet dye solution
- safranin dye solution
- methylene blue dye solution
- light microscope
- immersion oil
- benzene
- wad of paper

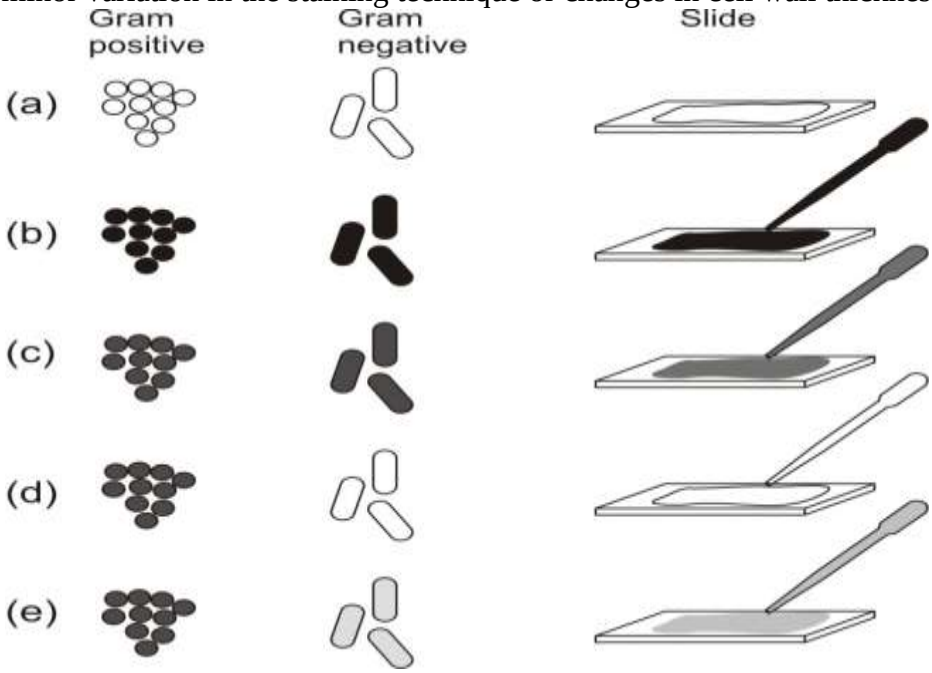
Practise:

1. Grip a glass slide with wooden test tube clamps, degrease the surface of a glass slide with alcohol over a Bunsen burner, put it down on a metal rack/staining stand with the degreased surface upwards, let it cool down.
2. Label the degreased slide adequately.

3. Put a small drop of water onto the slide (a well degreased slide will be wetted) and then mix a small loopful of bacterial culture in it. A thin suspension will be formed this way. Make a film layer (smear) with the needle of the inoculating loop and let it dry.
4. Fix your preparation with heat over the Bunsen burner.
5. Drop basic dye onto the fixed smear until it is fully covered and let it get stained for 1-2 minutes.
6. Wash the smear with tap water to remove excess dye solution.
7. Dry the slide.
8. During microscopy, first use 40x, then 100x objective lenses. In the latter case, use immersion oil. Make a drawing of the observed microscopic field.
9. After finishing microscopic observation, clean all used objective lenses with benzene (do not use alcohol for this purpose as it can dissolve the lens' adhesives).

GRAM STAINING

This important bacteriological staining procedure was discovered in 1884 by a Danish scientist, Christian Gram. The staining is based on the cell wall structure of bacteria. When bacteria are stained with crystal violet, the cells of most Gram-negative bacteria can be easily decolourised with organic solvents such as ethanol or acetone, while cells of most Gram-positive bacteria restrict decolourisation (Figure). The ability of bacteria to either retain or lose the stain generally reflects fundamental differences in the cell wall and is an important taxonomic feature. Gram staining is therefore used as an initial step in the identification of bacteria. The cells of some bacteria are strongly Gram-positive when young, but tend to become Gram-negative in ageing cultures (e.g. *Bacillus cereus, Clostridium* spp.), which may reflect degenerative changes in the cell wall. Some bacteria give a Gram-variable reaction: they are sometimes Gram-positive, sometimes Gram-negative; this could reflect minor variation in the staining technique or changes in cell wall thickness, etc.



Gram staining procedure. (a) Fix bacterial culture on a microscope slide. (b) Stain with crystal violet solution. (c) Treat with iodine solution. (d) Decolourise with 96% ethanol. (e) Counterstain with safranin solution.

Object of study, test organisms:

- *Staphylococcus aureus* slant culture
- *Bacillus cereus* slant culture

- *Pseudomonas aeruginosa* slant culture

Materials and equipment:

- glass slide
- glass dropper dispenser
- pipette
- inoculating loop
- Bunsen burner
- wooden test tube clamps
- crystal violet dye solution
- iodine solution (Lugol's)
- 95% ethanol
- safranin dye solution
- light microscope
- immersion oil

Practise:

1. Prepare a fixed smear from the strains
2. Stain with crystal violet solution (1 min).
3. Rinse with tap water.
4. Treat with iodine solution (1 min).
5. Rinse with tap water.
6. Decolourise with 96 % ethanol (drip with ethanol until the solvent runs down colorless).
7. Rinse with tap water.
8. Counterstain with safranin solution (1 min).
9. Rinse with tap water.
10. Dry the slide.
11. Examine with microscope. Gram-positive cells are purple, while Gram-negative ones are pinkish-red. Make a drawing of the observed microscopic field.

ZIEHL-NEELSEN ACID-FAST STAINING

The Ziehl-Neelsen staining is a complex and differential staining, which differentiates between acid-fast and non-acid-fast bacteria. Among acid-fast bacteria (e.g. *Mycobacterium* spp., *Nocardia* spp.), there are many pathogenic species. Acid-fast bacteria have a waxy substance, called mycolic acid, in their cell wall, which makes them impermeable to many staining procedures, including Gram staining. These bacteria are termed "acid-fast" because when stained, they are able to resist decolourisation with acid-alcohol. "Carbolfuchsin" stain contains phenol to help solubilise the cell wall. Heat is also applied during the primary staining to increase penetration. All cell types will take up the primary stain. The cells are then decolourised with acid-alcohol, which decolourises every cell except the acid-fast ones. Methylene blue is then applied to counterstain any cells that have been decolourised. At the end of the staining process, acid-fast cells will be reddish-pink, and non-acid fast cells will be blue.

Object of study, test organisms:

- *Rhodococcus rhodochrous* slant culture
- *Mycobacterium phlei* slant culture
- unknown bacterial strain slant culture
- *Nocardioides hungaricus*
- *Nocardioides daphniae*

Materials and equipment:

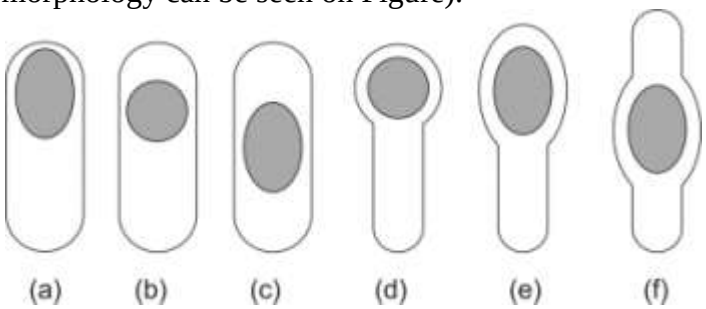
- glass slide
- glass dropper dispenser
- pipette
- inoculating loop
- Bunsen burner
- wooden test tube clamps
- carbolfuchsin dye solution (see Appendix)
- acidic ethanol (see Appendix)
- methylene blue dye solution (see Appendix)
- pieces of filter paper (2 x 4 cm)
- light microscope
- immersion oil

Practise:

1. Prepare a fixed smear from the strains
2. Cover the smear with a piece of filter paper, and drop carbolfuchsin dye solution onto it (it must cover the entire preparation). Heat the slide over the flame until the liquid starts to turn into steam (aggressive staining). Reinstate the steaming liquid permanently with dye and water. Perform aggressive staining for 10 minutes.
3. Carefully wash with tap water.
4. Wash with acidic ethanol.
5. Rinse with tap water.
6. Counterstain with methylene blue dye solution (1 min).
7. Rinse with tap water.
8. Dry the slide.
9. Examine with microscopy. Acid-fast bacteria will be violet-red, while non-acid-fast ones will be stained blue. Make a drawing of the observed microscopic field.

SCHAEFFER-FULTON SPORE STAINING

Bacterial endospores are highly resistant structures with a thick wall formed by vegetative cells during a process called sporulation. They are highly resistant to radiation, chemical agents, extremely high temperatures, desiccation and other harmful environmental effects. Several bacterial genera are capable of producing endospores; *Bacillus* and *Clostridium* are the two most common endospore-forming genera (Endospore morphology can be seen on Figure).



Morphology of endospores. Location: terminal (a, d, e), subterminal (b), central (c, f). Shape: circular (b, d), ellipsoid (a, c, e, f). Spore diameter compared with cell diameter: non-deforming (a, b, c), deforming (d, e, f).

Due to the highly resistant nature of endospores, it is necessary to steam stain into them. The most common endospore staining technique is the Schaeffer-Fulton method. Once endospores have absorbed the stain, they are resistant to decolourisation, but vegetative cells are easily decolourised with water and counterstained with safranin to aid visualisation.

Object of study, test organisms:

- *Bacillus cereus* slant culture
- *Saccharomyces cerevisiae* slant culture
- unknown bacterial strain slant culture
- *Bacillus aurantiacus*
- *Bacillus alkalisediminis*

Materials and equipment:

- glass slide
- glass dropper dispenser
- pipette
- inoculating loop
- Bunsen burner
- wooden test tube clamps
- malachite green dye solution
- safranin dye solution

- pieces of filter paper (2 x 4 cm)
- light microscope
- immersion oil

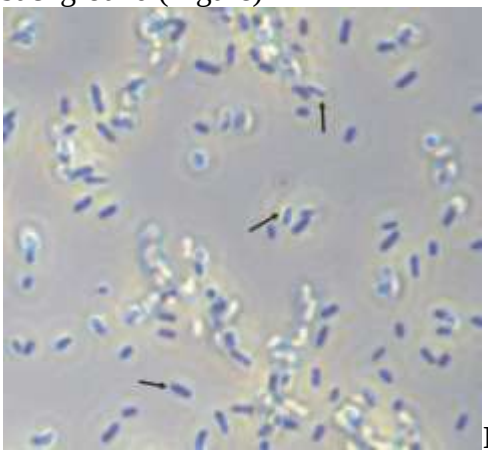
Practise:

1. Prepare a fixed smear from the bacterial strains
2. Cover the smear with a piece of filter paper, and drop malachite green dye solution onto it (it must cover the entire preparation). Heat the slide over the flame until the liquid starts to turn into steam (aggressive staining). Reinstate the steaming liquid permanently with dye and water. Perform aggressive staining for 10 min.
3. Thoroughly wash with tap water.
4. Counterstain with safranin dye solution (1 min).
5. Rinse with tap water.
6. Dry the slide.
7. Examine with a microscope. Endospores appear green meanwhile the vegetative cells are red. Make a drawing of the observed microscopic field.

CAPSULE STAINING BY LEIFSON

Several bacteria have glycocalyx/capsule outside their cell walls. This layer protects the microbe against many environmental effects: desiccation, grazing by protozoons, attachment of phages, etc. Occasionally it is a kind of nutrient storage, which helps to concentrate the excreted enzymes or helps the cell to adhere to a specific surface. For pathogenic bacteria, it gives strong protection against the antibodies and macrophages of the host.

Usually the glycocalyx is built up from polysaccharides, uronic acids or proteins. The size and consistency of this layer can vary depending on the species, occasionally even on strains. Cultivation conditions also influence the production of glycocalyx. As the glycocalyx is not dense enough to be stained with simple staining methods, usually negative staining procedure (background staining) is adequate for this purpose. In this case, the particles of the applied colloidal dye cannot enter the glycocalyx, therefore they are strongly visible against the dark background (Figure)



Micrograph of the capsules in a flocculum of *Azotobacter* sp. Arrows indicate the capsules.

Object of study, test organisms:

- *Rhizobium* sp. slant culture
- *Azotobacter vinelandii* slant culture
- *Enterobacter* sp. slant culture
- unknown bacterial strain slant culture

Materials and equipment:

- glass slide
- glass dropper dispenser

- inoculating loop
- Bunsen burner
- wooden test tube clamps
- India ink and safranin dye solution
- light microscope
- immersion oil

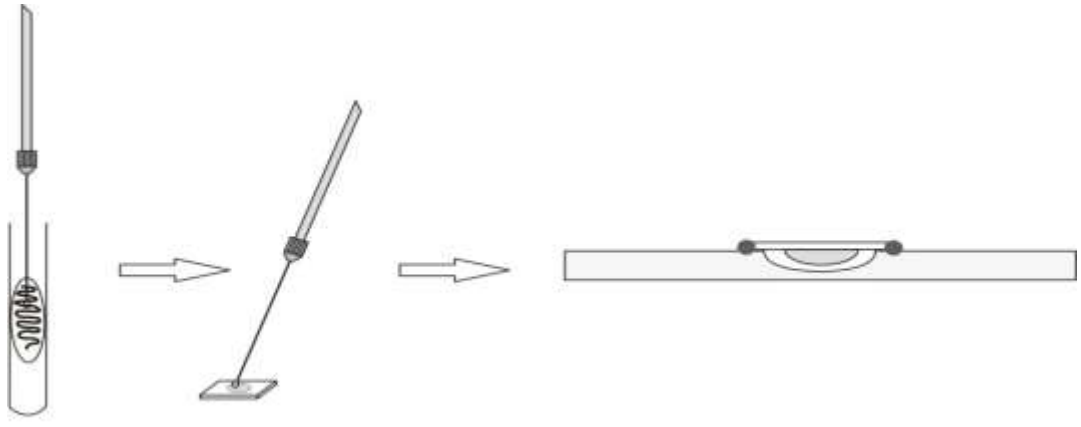
Practise:

1. Degrease a slide as described
2. Label the degreased slide.
3. Put a small drop of India ink solution onto the slide and then mix a loopful of bacterial culture in it - a thin suspension will be formed this way. Make a film layer (smear) with the needle of the loop and then let it dry.
4. Make a counter staining with safranin dye (staining the cells).
5. Examine with a microscope. Glycocalyx around bacterial cells appears as faint halo in the dark background. Make a drawing of the observed microscopic field (Figure)

HANGING DROP PREPARATION

Investigation of the movement of live bacteria by microscope is possible e. g. with hanging-drop preparation (Figure). A suspension of microorganisms is placed in the centre of a cover slip and turned over with a special glass slide with a hollow depression in the centre. When observing live bacteria, be careful not to confuse motility with Brownian motion resulting from bombardment by water molecules. In Brownian motion, organisms all vibrate at about the same rate and maintain a relatively constant spatial relationship with one another, whereas bacteria that are definitely motile progress continuously in a given direction.

Motility can be observed most satisfactorily in young cultures (24 or 48 hours), because older cultures tend to become non-motile. An old culture may become so crowded with inert living and dead bacteria that it is difficult to find a motile cell. In addition, the production of acid or other toxic products may result in the loss of bacterial motility.



Hanging drop preparation. (a) From the examined bacterial culture (b) prepare a weak suspension in a drop of water in the centre of a cover slip. (c) Put the glass slide with the hollow depression upside down over the cover slip preparation so that the drop of culture is in the centre of the depression, and then quickly turn it over.

Object of study, test organisms:

- *Pseudomonas aeruginosa* 16-24-hour slant culture
- *Proteus vulgaris* 16-24-hour slant culture
- *Staphylococcus aureus* 16-24-hour slant culture
- unknown bacterial strain slant culture

Materials and equipment:

- glass slide with hollow depression
- cover slip
- inoculating loop
- Bunsen burner
- pipette, sterile pipette tips
- light microscope
- immersion oil

Practise:

1. Prepare a weak suspension from the examined bacterial culture in a small drop of water in the centre of a cover slip.
2. Put the glass slide with the hollow depression upside down over the cover slip preparation so that the drop of culture is in the centre of the depression, and then quickly turn it over.
3. Fix the cover slip to the slide with melted paraffin wax.
4. Examine with a microscope and estimate bacterial flagellation type, based on the movement of the cells.

Preparation of microbiological culture media

The survival and growth of microorganisms depend on available and a favourable growth environment. Culture media are nutrient solutions used in laboratories to grow microorganisms. For the successful cultivation of a given microorganism, it is necessary to understand its nutritional requirements and then supply the essential nutrients in the proper form and proportion in a culture medium. The general composition of a medium is as follows:

- H-donors and acceptors (approximately 1-15 g/L)
- C-source (approximately 1-20 g/L)
- N-source (approximately 0.2-2 g/L)
- Other inorganic nutrients e.g. S, P (50 mg/L)
- Trace elements (0.1-1 µg/L)
- Growth factors (amino acids, purines, pyrimidines, occasionally 50 mg/L, vitamins occasionally 0.1-1 mg/L)
- Solidifying agent (e.g. agar 10-20 g/L)
- Solvent (usually distilled water)
- Buffer chemicals

Microbiological culture media could be classified according to:

1. **Consistency**, which could be adjusted by changing the concentration of solidifying or gelling agents, e.g. agar, gelatine (liquid media do not contain such materials)
    - Cultures in liquid media (or broth) are usually handled in tubes or flasks and incubated under static or shaken conditions. This way, homogenous conditions are generated for growth and metabolism studies,(e.g. with the control of optical density and allowing sampling for the analysis of metabolic products).
    - Semisolid media are usually used in fermentation and cell mobility studies, and are also suitable for promoting anaerobic growth.
    - Solid media are prepared in test tubes or in Petri dishes, in the latter case, the solid medium is called agar plate. In the case of tubes, medium is solidified in a slanted position, which is called agar slant, or in an upright position, which is called agar deep tube. Solid media are used to determine colony morphology, isolate cultures, enumerate and isolate bacteria (e.g. using dilutions from a mixed bacterial population in combination with spreading), and for the detection of specific biochemical reactions (e.g. metabolic activities connected with diffusing extracellular enzymes that act with insoluble substrates of the agar medium).
2. **Composition**

- Chemically-defined (or synthetic) media are composed only of pure chemicals with defined quantity and quality.
- Complex (or non-synthetic) media are composed of complex materials, e.g. yeast extract, beef extract and peptone (partially digested protein), therefore their chemical composition is poorly defined. On the other hand, these materials are rich in nutrients and vitamins.

3. **Function**
    - All-purpose media do not contain any special additives and they aim to support the growth of most bacteria.
    - Selective media enhance the growth of certain organisms while inhibit others due to the inclusion of particular substrate(s).
    - Differential media allow identification of microorganisms usually through their unique (and visible) physiological reactions. In the detection of common pathogens, most practical media are both selective and differential.
    - Enrichment media contain specific growth factors that allow the growth of metabolically fastidious microorganisms. An enrichment culture is obtained with selected media and incubation conditions to isolate the microorganisms of interest.

PREPARATION OF AGAR SLANTS

Object of study: preparation of agar slants

Materials and equipment:

- distilled water
- measuring cylinder
- flask
- bacteriological chemicals
- laboratory scales
- chemical spoons
- 1N NaOH solution
- 1N HCl solution
- pH indicator paper or pH meter
- cotton gloves
- dispenser
- test tubes
- test tube caps
- test tube basket
- slanting stage
- autoclave
- incubator

Practise:

1. Measure the components of the medium (e.g. TSA or nutrient, see Appendix) into a flask containing 9/10 volume of the solvent. Use a clean chemical spoon for every measurement. Dissolve the solid components and fill with the remaining solvent up to final volume. If the medium contains heat sensitive components (like sugars), they must be separately sterilised in solution (e.g. by filter sterilisation), and then mixed with the already sterilised and cooled agar medium.
2. Close the flask with cotton plug and cover with aluminium foil, put into the autoclave and start a sterilisation cycle. This cycle could be intermitted when the internal temperature has reached 121°C, at that temperature every component (e.g. agar-agar) will be dissolved correctly.
3. Check the pH of the medium with an indicator paper or with a pH meter and adjust to the proper value with NaOH or HCl solution.
4. Pour the 60-70°C medium into the dispenser. Add 5-6 mL medium to each test tube, close them with caps and place them into a test tube basket.
5. Place the tubes into the autoclave and complete a whole sterilisation cycle for 20 min at 121°C
6. Put the test tubes onto a slanting stage to let the medium solidify in the test tubes.
7. Label the slants according to the type of the medium and perform a sterility test: incubate the test tubes at 28°C for 24 hours, and check for sterility.
8. The prepared media can be stored for 1-2 weeks at 12-15°C, or longer in a refrigerator. (Do not store medium containing agar-agar under 4-5°C as it destroys its structure!)

PREPARATION OF AGAR PLATES

Object of study: preparation of agar plates

Materials and equipment:

- distilled water
- measuring cylinder
- flask
- bacteriological chemicals
- laboratory scales
- chemical spoons
- 1N NaOH solution
- 1N HCl solution
- pH indicator paper or pH meter
- cotton gloves
- sterile, empty Petri dishes
- Bunsen burner
- autoclave
- incubator

Practise:

1. Prepare a medium
2. Cool the sterilised medium to 55°C.
3. Take out the cotton plug and flame the mouth of the flask over a Bunsen burner, and then pour the medium into sterile, empty Petri dishes (15-20 mL into each Petri dish).
4. Keep the Petri dishes horizontally until the medium completely solidifies. Turn dishes upside-down and stack them up for storage.
5. Label the plates according to the type of the medium and perform a sterility test In case of longer storage, Petri plates must be placed into plastic bags or boxes to avoid drying out.

Possible Questions

Unit – II

Two marks

1. Define Stain.
2. What is meant by mordant?
3. Comment on acidic and basic dyes.
4. Comment on endospore staining.
5. Write a brief note on the capsule stain.

Eight marks

1. Explain the principle behind the acid fast stain.
2. Write short notes on Gram stain.
3. Explain about the principle behind pure culture techniques.
4. Discuss on the enumeration of microbes using colony count and turbidimetric method.
5. Explain in detail about cultivation of anaerobic bacteria.

S.no	Question	Opt 1	Opt 2	Opt 3	Opt 4	Answer
1	Which of the following articles cannot be sterilized in an autoclave?	Gloves	Culture media	Dressing material	sugar	Dressing material
2	Which of the following disinfectants act by disrupting microbial membranes?	Cationic detergents	Halogens	Heavy metals	Aldehydes	Cationic detergents
3	Which of the following is best to sterilize heat labile solutions?	Dry heat	Autoclave	Membrane filtration	Pasteurization	Membrane filtration
4	The time required to kill 90% of the microorganisms in a sample at a specific temperature is the	decimal reduction time	thermal death point	F value	D value	The time required to kill 90% of the microorganisms in a sample at a specific temperature is the
5	Which of the following is best used for long term storage of microbial samples when carried out properly?	Storage in a freezer at -10°C	Storage in a freezer at ultra low temperatures (-70°C)	Storage in a refrigerator on an agar slant	Storage on a petri plate at room temperature	Storage in a freezer at ultra low temperatures (-70°C)
6	Which of the antibiotic is not used as a food preservative ?	Pimaricin	Nisin	Tylosin	β-lactam antibiotic	β-lactam antibiotic
7	Which antibiotic has a beta-lactam ring?	Cephalosporin	Penicillin	Tetracycline	Streptomycin	Penicillin
8	Gram positive cells have a	second outer	multiple layer of	thick capsule that	periplasmic space	multiple layer of

		membrane that helps to retain the crystal violet stain	peptidoglycan that helps to retain the crystal violet stain	traps the crystal violet stain	that traps the crystal violet	peptidoglycan that helps to retain the crystal violet stain
9	The common word for bacteria which are straight rod in shape is	cooci	bacilli	spirilla	pleomorphic	bacilli
10	Teichoic acids are typically found in	cell walls of gram positive bacteria	outer membranes of gram positive bacteria	cell walls of gram negative bacteria	outer membranes of gram negative bacteria	cell walls of gram positive bacteria
11	In eukaryotic cells, ribosomes are	70S	80S	90S	100S	80S
12	Porins are located in	the outer membrane of gram-negative bacteria	the peptidoglycan layer of gram-positive bacteria	the cytoplasmic membrane of both gram-negative and gram-positive bacteria	the periplasmic space of gram-negative bacteria	the outer membrane of gram-negative bacteria
13	Swimming towards a chemical of bacteria is termed as	positive chemotaxis	negative chemotaxis	phototaxis	magnetotaxis	positive chemotaxis
14	Which of the following is NOT a disinfectant containing a heavy metal?	Silver nitrate	Mercurochrome	Copper sulfate	Chlorine	Copper sulfate
15	Which is the most important surface active disinfectants?	Amphoteric compounds	Cationic compounds	Non-ionic compounds	Anionic compounds	Cationic compounds
16	Which disinfectant would be most effective	Phenol	Cetylpyridinium chloride	Hexachlorophene	chlorine	Phenol

	against Staphylococcus found in a blood spill?					
17	Which of the following substances can sterilize?	Alcohol	Cetylpyridinium chloride	Ethylene oxide	Chlorine	Alcohol
18	Chemically the capsule may be	polypeptide	polysaccharide	either (a) or (b)	lipids	polysaccharide
19	Peptidoglycan is found only in the bacterial	cell membrane	glycocalyx	cell wall	spore	cell wall
20	The cell walls of Gram positive bacteria contain two modified sugar, viz. N-acetylgucosamine (NAG) and N- acetylmuramic acid (NAM). They are covalently linked by	α- 1,4-glycosidic bond	β-1,6-glycosidic bond	α- 1,6-glycosidic bond	β- 1,4-glycosidic bond	β-1,6-glycosidic bond
21	Which of the following organism has sterols in their cytoplasmic membrane?	Clostridum	Proteus	Mycoplasma	Bacillus	Mycoplasma
22	Name the component not in flagellum.	Filament	Hook	Basal body	LPS	LPS
23	Milk is pasteurized in batch method by keeping it at	63°C for 30 minutes	72°C for 60 seconds	73°C for 30 minutes	72°C for 6 minutes	63°C for 30 minutes
24	Chemotherapeutic agents must	prevent/destroy the activity of a parasite	leave unaltered the host's natural defense mechanisms	be able to come in contact with the parasite by penetrating the cells	must not infect the host and must kill the pathogen	must not infect the host and must kill the pathogen
25	Which of the following methods would be most	Dry heat sterilization	Microfiltration	Autoclaving	Desiccation	Microfiltration

	appropriate for sterilizing an antibiotic solution?					
26	The location where the bacterial chromosome concentrates is called	nucleus	nuclein	nucleoid	nucleolus	nucleolus
27	Which of the following is analogous to mesosomes of bacteria?	Mitochondria of eukaryotes	Golgi apparatus of eukaryotes	Lysosomes of eukaryotes	vaculoes	Mitochondria of eukaryotes
28	Which of the following has Chinese letter arrangement?	Bacillus anthracis	Mycobacterium tuberculosis	Clostridium tetani	Corynebacterium diphtheriae	Corynebacterium diphtheriae
29	The other name for peptidoglycan is	mucopeptide	murein	NAG	NAM	mucopeptide
30	Cyanobacteria have	a gram-positive cell wall	a gram-negative cell wall	Neither (a) nor (b)	No cell wall	a gram-positive cell wall
31	In which of the following, lipo-polysaccharide is a major constituent of outer membrane of the cellwall?	Gram-positive bacteria	Gram-negative bacteria	Fungi	virus	Gram-positive bacteria
32	Which of the following structure(s) is /are internal to cell wall?	Flagella	Stalks	Sheath	ribosomes	ribosomes
33	Which of the following may be most likely to be missing from a gram-positive bacterium?	Penicillin binding protein	Peptidoglycan	Lipopolysaccharide	Phospholipid bilayer membrane	Penicillin binding protein
34	Which of the following structure(s) is /are external to cell wall?	Flagella	cytoplasm	nucleic acid	pili	Flagella

35	Which of the following bacteria lack a cell wall and are therefore resistant to penicillin?	Cyanobacteria	Mycoplasmas	Bdellovibrios	Spirochetes	Mycoplasmas
36	A cluster of polar flagella is called	lophotrichous	amphitrichous	monotrichous	peritrichous	lophotrichous
37	The protein from which hook and filaments of fpili are composed of, is	keratin	flagellin	gelatin	pilin	pilin
38	The cocci which mostly occur in single are	Streptococci	Staphylococci	Monococci	tetracocci	monococci
39	The cocci which mostly occur in pairs are	Streptococci	Staphylococci	diplococci	tetracocci	Diplococci
40	The cocci which mostly occur in fours are	Streptococci	Staphylococci	diplococci	tetracocci	tetracocci
41	Which of the following may contain fimbriae?	Gram-positive bacteria	Gram-negative bacteria	Both gram positive and negative bacteria	No gram bacteria	Gram-negative bacteria
42	Which of the following may contain flagella?	Gram-positive bacteria	Gram-negative bacteria	Both gram positive and negative bacteria	No gram bacteria	Both gram positive and Gram-negative bacteria
42	Peptidoglycan accounts for _____ of the dry weight of cell wall in many gram positive bacteria	50% or more	About 10%	11%+ 0.22%	About 20%	50% or more
43	Bacteria having no flagella are unable to	move	reproduces	stick to tissue surfaces	grow in nutrient agar	move

44	Which of the following is false about cell wall of gram-positive bacteria	It consists of multiple layers.	It is thicker than that associated with gram-negative bacteria	It contains teichoic acids	Have mycolic acid	Have mycolic acid
45	The cell walls of many gram positive bacteria can be easily destroyed by the enzyme known as	lipase	lysozyme	pectinase	peroxidase	pectinase
46	For most bacteria, the optimum pH for growth lies between	6.5-7.5	3.5-4.5	4.5-5.5	5.5-6.5	6.5-7.5
47	Suspension cultures consist of cells and cell aggregates, growing dispersed in	liquid medium	solid nutrient medium	solid medium	Semi solid medium	liquid medium
48	The protoplast cannot be used to	modify genetic information	create plant hybrid	study plant viral infections	No modification	No modification
49	The cell wall of	gram-positive bacteria are thicker than gram-negative bacteria	gram-negative bacteria are thicker than gram-positive bacteria	both have same thickness but composition is different	gram-positive bacteria are small than gram-negative bacteria	gram-positive bacteria are thicker than gram-negative bacteria
50	Peptidoglycan is also known as	N-acetyl muramic acid	murein mucopeptide	N acetylglucosamine	mesodiaminopimetic acid	murein mucopeptide
51	Which is most likely to be exposed on the surface of a gram-negative bacterium	Pore protein (porin)	Protein involved in energy generation	Lipoteichoic acid	Phospholipids	Lipoteichoic acid

52	The last step in synthesis of peptidoglycan is	attachment of a peptide to muramic acid	attaching two amino acids to form a cross-link	attachment of a portion of peptidoglycan to a membrane lipid	binding of penicillin to a membrane protein	attaching two amino acids to form a cross-link
53	Peptidoglycan is also known as	NAM	Murein mucopeptide	N acetyly glucosamine	mesodiaminopimetic acid	Murein mucopeptide
54	Which is most likely to be exposed on the surface of a gram-negative bacterium?	Pore prtoein (porin)	Protein involved in energy generation	lipothecoic acid	phospholipids	lipothecoic acid
55	The last step in synthesis of peptidoglycan is	attachment of a peptide to muramic acid	attaching two amino acids to form a cross-link	attachment of a portion of peptidoglycan to a membrane lipid	binding of penicillin to a membrane protein	attaching two amino acids to form a cross-link
56	Cytoplasmic inclusions include	ribosomes	mesosomes	fat globules	vacuoles	ribosomes
57	The cocci which form pairs pattern are	Staphylococci	diplococci	tetracocci	streptococci	diplococci
58	thick spiral bacteria is called as	spirilla	spirocheate	helix	spirulina	spirocheate
59	Attachment of small particles or molecules to a larger particle by electric charge is called as	Adsorption	absorption	fixation	attachment	absorption

Culture media-types, composition. Sterilization technique-Physical and Chemical methods of microbial control-types and mode of action. Bacterial growth curve-bacterial motility and cell count technique. Calculation of generation time and specific growth rate.

Culture media are solutions containing nutrients and physical growth parameters necessary for microbial growth. All microorganisms cannot grow in a single culture medium and in fact many can't grow in any known culture medium.

Organisms that cannot grow in artificial culture medium are known as **obligate parasites**. *Mycobacterium leprae, rickettsias, Chlamydias,* and *Treponema pallidum* are obligate parasites. Bacterial culture media can be distinguished on the basis of **composition, consistency and purpose.**

Classification of culture media used in Microbiology laboratory on the basis of consistency

1. **Solid medium**
   solid medium contains agar at a concentration of 1.5-2.0% or some other, mostly inert solidifying agent. Solid medium has physical structure and allows bacteria to grow in physically informative or useful ways (e.g. as colonies or in streaks). Solid medium is useful for **isolating bacteria** or for determining the colony characteristics of the isolate.
2. **Semisolid media**
   They are prepared with agar at concentrations of 0.5% or less. They have soft custard like consistency and are useful for the cultivation of **microaerophilic bacteria** or for **determination of bacterial motility.**
3. **Liquid (Broth) medium**
   These media contains specific amounts of nutrients but don't have trace of gelling agents such as gelatin or agar. Broth medium serves various purposes such as propagation of large number of organisms, fermentation studies, and various other tests. e.g. sugar fermentation tests, **MR-VR broth.**

Classification of culture media based on the basis of composition

1. Synthetic or chemically defined medium
   A chemically defined medium is one prepared from purified ingredients and therefore whose exact composition is known.
2. Non synthetic or chemically undefined medium

Non-synthetic medium contains at least one component that is neither purified nor completely characterized nor even completely consistent from batch to batch. Often these are partially digested proteins from various organism sources. Nutrient broth, for example, is derived from cultures of yeasts.

TYPES OF CULTURE MEDIA

Media are of different types on consistency and chemical composition.

A. On Consistency:

1. Solid Media. Advantages of solid media: (a) Bacteria may be identified by studying the colony character, (b) Mixed bacteria can be separated. Solid media is used for the isolation of bacteria as pure culture. 'Agar' is most commonly used to prepare solid media. Agar is polysaccharide extract obtained from seaweed. Agar is an ideal solidifying agent as it is: (a) Bacteriologically inert, i.e. no influence on bacterial growth, (b) It remains solid at 37°C, and (c) It is transparent.
2. Liquid Media. It is used for profuse growth, e.g. blood culture in liquid media. Mixed organisms cannot be separated.

B. On Chemical Composition :

1. Routine Laboratory Media
2. Synthetic Media. These are chemically defined media prepared from pure chemical substances. It is used in research work.

ROUTINE LABORATORY MEDIA

These are classified into six types: (1) Basal media, (2) Enriched media, (3) Selective media, (4) Indicator media, (5) Transport media, and (6) Storage media.

1. BASAL MEDIA. Basal media are those that may be used for growth (culture) of bacteria that do not need enrichment of the media. Examples: Nutrient broth, nutrient agar and peptone water. Staphylococcus and Enterobacteriaceae grow in these media.

2. ENRICHED MEDIA. The media are enriched usually by adding blood, serum or egg. Examples: Enriched media are blood agar and Lowenstein-Jensen media. Streptococci grow in blood agar media.

3. SELECTIVE MEDIA. These media favour the growth of a particular bacterium by inhibiting the growth of undesired bacteria and allowing growth of desirable bacteria. Examples: MacConkey agar, Lowenstein-Jensen media, tellurite media (Tellurite inhibits the growth of most of the throat organisms except diphtheria bacilli). Antibiotic may be added to a medium for inhibition.

4. INDICATOR (DIFFERENTIAL) MEDIA. An indicator is included in the medium. A particular organism causes change in the indicator, e.g. blood, neutral red, tellurite. Examples: Blood agar and MacConkey agar are indicator media.

5. TRANSPORT MEDIA. These media are used when specie-men cannot be cultured soon after collection. Examples: Cary-Blair medium, Amies medium, Stuart medium.

6. STORAGE MEDIA. Media used for storing the bacteria for a long period of time. Examples: Egg saline medium, chalk cooked meat broth.

COMMON MEDIA IN ROUTINE USE

Nutrient Broth. 500 g meat, e.g. ox heart is minced and mixed with 1 litre water. 10 g peptone and 5 g sodium chloride are added, pH is adjusted to 7.3. Uses: (1) As a basal media for the preparation of other media, (2) To study soluble products of bacteria.

Nutrient Agar. It is solid at 37°C. 2.5% agar is added in nutrient broth. It is heated at 100°C to melt the agar and then cooled.

Peptone Water. Peptone 1% and sodium chloride 0.5%. It is used as base for sugar media and to test indole formation.

Blood Agar. Most commonly used medium. 5-10% defibrinated sheep or horse blood is added to melted agar at 45-50°C. Blood acts as an enrichment material and also as an indicator. Certain bacteria when grown in blood agar produce haemolysis around their colonies. Certain bacteria produce no haemolysis. Types of changes : (a) beta (p) haemolysis. The colony is surrounded by a clear zone of complete

haemolysis, e.g. Streptococcus pyogenes is a beta haemolytic streptococci, (b) Alpha (a) haemolysis. The colony is surrounded by a zone of greenish discolouration due to formation of biliverdin, e.g. Viridans streptococci, (c) Gamma (y) haemolysis, or, No haemolysis. There is no change in the medium surrounding the colony,

Chocolate Agar or Heated Blood agar. Prepared by heating blood agar. It is used for culture of pneumococcus, gonococcus, meningococcus and Haemophilus. Heating the blood inactivates inhibitor of growths.

MacConkey Agar. Most commonly used for enterobacteriaceae. It contains agar, peptone, sodium chloride, bile salt, lactose and neutral red. It is a selective and indicator medium :

(1) Selective as bile salt does not inhibit the growth of enterobactericeae but inhibits growth of many other bacteria.

(2) Indicator medium as the colonies of bacteria that ferment lactose take a pink colour due to production of acid. Acid turns the indicator neutral red to pink. These bacteria are called 'lactose fermenter', e.g. Escherichia coll. Colourless colony indicates that lactose is not fermented, i.e. the bacterium is non-lactose fermenter, e.g. Salmonella. Shigella, Vibrio.

Mueller Hinton Agar. Disc diffusion sensitivity tests for antimicrobial drugs should be carried out on this media as per WHO recommendation to promote reproducibility and comparability of results.

Lowenstein-Jensen Medium. It is used to culture tubercle bacilli. It contains egg, malachite green and glycerol. (1) Egg is an enrichment material which stimulates the growth of tubercle bacilli, (2) Malachite green inhibits growth of organisms other than mycobacteria, (3) Glycerol promotes the growth of Mycobacterium tuberculosis but not Mycobacterium bovis.

Dubos Medium. This liquid medium is used for tubercle bacilli. In this medium drug sensitivity of tubercle bacilli can be carried out. It contains 'tween 80', bovine serum albumin, casein hydrolysate, asparagin and salts. Tween 80 causes dispersed growth and bovine albumin causes rapid growth.

Loeffler Serum. Serum is used for enrichment. Diphtheria bacilli grow in this medium in 6 hours when the secondary bacteria do not grow. It is used for rapid diagnosis of diphtheria and to demonstrate volutin granules. It contains sheep, ox or horse serum.

Tellurite Blood Agar. It is used as a selective medium for isolation of Cotynebacterium diphtheriae. Tellurite inhibits the growth of most secondary bacteria without an inhibitory effect on diphtheria bacilli. It is also an indicator medium as the diphtheria bacilli produce black colonies. Tellurite metabolized to tellbrism, which has black colour.

EMB (Eosin-methylene blue) Agar. A selective and differential medium for enteric Gram-negative rods. Lactose-fermenting colonies are coloured and nonlactose-fermenting colonies are nonpigmented. Selects against gram positive bacteria.

XLD (Xylose Lysine Deoxychoiate). It is used to isolate Salmonella and Shigella species from stool specimens. This is a selective media.

SS (Salmonella-Shigella) Agar. It is a selective medium used to isolate Salmonella and Shigella species. SS Agar with additional bile salt is used if Yersinia enterocolitica is suspected.

DCA (Desoxycholate Citrate Agar). It is used for isolation of Salmonella and Shigella. The other enteric bacteria are mostly inhibited (a selective medium). It is also a differential (indicator) medium due to presence of lactose and neutral red.

Tetrathionate Broth. This medium is used for isolating Salmonella from stool. It acts as a selective medium. It inhibits normal intestinal bacteria and permits multiplication of Salmonella.

Selenite F Broth. This medium is used for isolating Salmonella from stool. It acts as a selective medium. It inhibits normal intestinal bacteria and permits multiplication of Salmonella.

Thiosulphate-Citrate-Bile-Sucrose (TCBS) Agar. TCBS agar is a selective medium used to isolate Vibrio cholerae and other Vibrio species from stool.

Charcoal-yeast agar. Used for Legionella pneumophila. Increased concentration of iron and cysteine allows growth.

Tellurite-Gelatin Agar Medium (TGAM). It may be used as transport, selective and indicator medium.

Alkaline peptone water. See under Vibrio. (Chapter 51).

Campylobacter Medium. This selective medium is used to isolate Campylobacter jejuni and Campylobacter coli from stool.

Cary-Blair Medium. It is used as a transport medium for faeces that may contain Salmonella, Shigella, Vibrio or Campylobacter species.

Amies medium is used for gonococci and other pathogens.

Peptone Water Sugar Media. These indicator media are used to study 'Sugar fermentation'. 1 % solution of a sugar (lactose, glucose, mannitol etc) is added to peptone water containing Andrade's indicator in a test tube. A small inverted Durham tube is placed in the medium. The media are colourless. After culture, change of a medium to red colour indicates acid production. Gas, if produced collects in Durham tube.

Motility Indole Urea (MIU) Medium. This is used to differentiate enterobacteria species by their motility, urease, and indole reactions.

TSI (Triple sugar iron) Agar- It has triple sugars in the composition and used in the identification of unknown bacteria by fermenting the sugars.

KIA (Kligler Iron Agar). This is a differential slope medium used in the identification of enteric bacteria. The reactions are based on the fermentation of lactose and glucose and the production of hydrogen sulphide

Christensen's Urea Medium. This is used to identify urea splitting organisms, e.g. Proteus. A purple pink colour indicates urea splitting

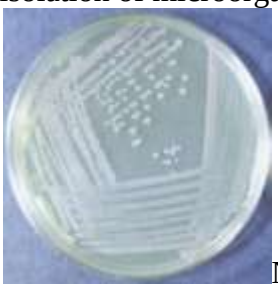
Bordet-Gengou Medium. This medium is used for culture of Bordetella pertussis. Increased concentration of blood allows growth. It contains agar, potato, sodium chloride, glycerol, peptone and 50% horse blood. Penicillin may be added to it.

Classification based on the basis of purpose/ functional use/ application

Many special purpose media are needed to facilitate recognition, enumeration, and isolation of certain types of bacteria. To meet these needs, numerous media are available.

1. General purpose media/ Basic media

Basal media are basically simple media that supports most non-fastidious bacteria. Peptone water, nutrient broth and nutrient agar are considered as basal medium. These media are generally used for the primary isolation of microorganisms.


Nutrient Agar

2. Enriched medium (Added growth factors):


Blood Agar

Addition of extra nutrients in the form of blood, serum, egg yolk etc, to basal medium makes them enriched media. Enriched media are used to grow nutritionally exacting (fastidious) bacteria. B**lood agar**, chocolate agar, Loeffler's serum slope etc are few of the enriched media. Blood agar is prepared by adding 5-10% (by volume) blood to a blood agar base. **Chocolate agar** is also known as heated blood agar or lysed **blood agar.**

3. Selective and enrichment media are designed to inhibit unwanted commensal or contaminating bacteria and help to recover pathogen from a mixture of bacteria. While selective media are agar based, enrichment media are liquid in consistency. Both these media serve the same purpose. Any agar media can be made selective by addition of certain inhibitory agents that don't affect the pathogen of interest. Various approaches to make a medium selective include **addition of antibiotics, dyes, chemicals, alteration of pH or a combination of these.**

a. Selective medium

Principle: Differential growth suppression
Selective medium is designed to suppress the growth of some microorganisms while allowing the growth of others. Selective medium are agar based (solid) medium so that individual colonies may be isolated.

Examples of selective media include:

1. Thayer Martin Agar used to recover *N.gonorrhoeae* contains antibiotics; vancomycin, colistin and nystatin.
2. **Mannitol Salt Agar** and Salt Milk Agar used to recover *S.aureus* contains 10% NaCl.
3. Potassium tellurite medium used to recover *C.diphtheriae* contains 0.04% potassium tellurite
4. **MacConkey's Agar** used for **Enterobacteriaceae** members contains bile salt that inhibits most gram positive bacteria.
5. Pseudosel Agar (Cetrimide Agar) used to recover *P. aeruginosa* contains cetrimide (antiseptic agent).
6. Crystal Violet Blood Agar used to recover *S. pyogenes* contains 0.0002% crystal violet.
7. **Lowenstein Jensen Medium** used to recover *M.tuberculosis* is made selective by incorporating malachite green.
8. Wilson and Blair's Agar for recovering *S. typhi* is rendered selective by the addition of dye brilliant green.
9. Selective media such as **TCBS Agar** used for isolating *V. cholerae* from fecal specimens have elevated pH (8.5-8.6), which inhibits most other bacteria.

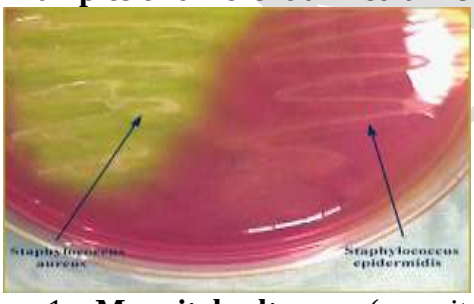
b. Enrichment culture medium

Enrichment medium is used to increase the relative concentration of certain microorganisms in the culture prior to plating on solid selective medium. Unlike selective media, enrichment culture is typically used as **broth medium**. Enrichment media are liquid media that also serves to inhibit commensals in the clinical specimen. S**elenite F broth, tetrathionate broth and alkaline peptone water (APW)** are used to recover pathogens from fecal specimens.

4. Differential/ indicator medium: differential appearance:

Certain media are designed in such a way that different bacteria can be recognized on the basis of their colony colour. Various approaches include incorporation of dyes, metabolic substrates etc, so that those bacteria that utilize them appear as differently coloured colonies. Such media are called differential media or indicator media. Differential media allow the growth of more than one microorganism of interest but with morphologically distinguishable colonies.

Examples of differential media include:



1. **Mannitol salts agar** (mannitol fermentation = yellow)
2. **Blood agar** (various kinds of hemolysis i.e. α, β and γ hemolysis)
3. **Mac Conkey agar** (lactose fermenters, pink colonies whereas non- lactose fermenter produces pale or colorless colonies.
4. **TCBS** (*Vibrio cholerae* produces yellow colonies due to fermentation of sucrose)

5. Transport media:

Clinical specimens must be transported to the laboratory immediately after collection to prevent overgrowth of contaminating organisms or commensals. This can be achieved by using transport media. Such media prevent drying (desiccation) of specimen, maintain the pathogen to commensal ratio and inhibit overgrowth of unwanted bacteria. Some of these media (Stuart's & Amie's) are semi-solid in consistency. Addition of charcoal serves to neutralize inhibitory factors.

- **Cary Blair transport medium** and Venkatraman Ramakrishnan (VR) medium are used to transport feces from suspected cholera patients.
- Sach's buffered glycerol saline is used to transport feces from patients suspected to be suffering from bacillary dysentery.
- Pike's medium is used to transport streptococci from throat specimens.

6. Anaerobic media:

Anaerobic bacteria need special media for growth because they need low oxygen content, reduced oxidation –reduction potential and extra nutrients.

Media for anaerobes may have to be supplemented with nutrients like hemin and vitamin K. Such media may also have to be reduced by physical or chemical means. Boiling the medium serves to expel any dissolved oxygen. Addition of 1% glucose, 0.1% thioglycollate, 0.1% ascorbic acid, 0.05% cysteine or red hot iron filings can render a medium reduced. Before use the medium must be boiled in water bath to expel any dissolved oxygen and then sealed with sterile liquid paraffin.

Robertson Cooked Meat (RCM) medium that is commonly used to grow *Clostridium* spps contains a 2.5 cm column of bullock heart meat and 15 ml of nutrient broth. Thioglycollate broth contains sodium thioglycollate, glucose, cystine, yeast extract and casein hydrolysate.

Methylene blue or resazurin is an oxidation-reduction potential indicator that is incorporated in the medium. Under reduced condition, methylene blue is colorless.

7. Assay media

These media are used for the assay of vitamins, amino acids and antibiotics. E.g. antibiotic assay media are used for determining antibiotic potency by the microbiological assay technique.

Other types of medium includes

Methods of Microbial Control

With the advent of the germ theory of disease, it became obvious that disease could be spread by organisms too small for the eye to see. Pioneers such as Ignaz Semmelweis and Joseph Lister utilized techniques such as the washing of hands and disinfecting of surfaces to decrease the likelihood of infection. In time, hospitals, clinics, and laboratories began to adopt these methods and improve upon them.

Methods used to control the growth of microbial growth can be placed into two broad categories, physical and chemical. **Physical methods** either exclude microbes, or reduce their numbers in a solution, or on the surface of a **fomite** (any nonliving material which might come into contact with the individual) . **Chemical methods** involve the application of specific chemical agents which inhibit growth or kill

microbes on fomites or the surface of skin. The selection of an appropriate technique is important, since many physical and chemical agents can cause damage to the cells and tissues of the individual as well as the microbe.

Agents of microbial control either sterilize or disinfect. **Sterilizing agents** kill all living things, thus removing the living source of contagion. **Disinfecting agents** kill some microbes, but inhibit the growth of others. Most techniques only provide disinfection. Also, several factors influence the effectiveness of any method of microbial control. These include population size, susceptibility of the microorganism to the agent, concentration of the dose used, and the duration of treatment.

Physical and Chemical Control of Microbes

The purpose of controlling microbial growth

To stop spreading the diseases or food spoilage

Methods:

- Physical agents
  - Heat
  - Radiation
- Chemical Agents
  - Gases
  - Liquids
- Mechanical removal
  - Filtration
  - Air
  - Liquids

Methods of Microbial Control

- Sterilization - Destruction of all forms of microbes including endospores (by steam under pressure or ethylene oxide)
- Disinfection -Destruction of vegetative cells of pathogenic microorganisms (by chemicals or physical methods)
- Pasteurization - Application of high temperature (72° C) for short period of time (15 sec) with the purpose of reducing the number of microbes
- Antiseptic - Antimicrobial agent that is sufficiently non-toxic to be applied on living tissue
- Sanitization - Lowering the number of microbes on eating and drinking utensils (by heat or chemical disinfectant)
- Decontamination
- Mechanical removal of microbes from organisms or non-living objects

Terminology

- Bactericidal (germicidal, microbicidal)- agent that destroys or kills bacteria (suffix cide - kill)
- Bacteriostatic - agent that inhibits bacterial growth (stasis - to stop)

What is Microbial Death?

- Permanent loss of reproductive capabilities
- The cell structures become dysfunctional
- Antimicrobial treatment leads to killing of microbial population at the constant rate

Factors that affect death rate:

- Time of exposure (lower temp. can be compensated with longer exposure)

- The number of microbes
- Microbial characteristics (endospore, vegetative cells)
- Agent used
- Environmental influences (suspending medium, pH)

The Mode of Action of Antimicrobial Agents

- Plasma membrane - when damaged, cell content leaks into the surrounding medium
- Proteins- enzyme active sites inactivated
  - Complete denaturation
  - Different shape
  - Blocking the active sites
- Nucleic Acid - radiation or some chemicals lethally damage the DNA or RNA (microbes can no longer replicate)
  - UV radiation causes formation of dimmers between two thymine bases

Physical Methods

Filtration

Filtration is the passing of either a solution or gasses through a device which traps microbes on one side of a container or space, preventing them from passing to the other. Filters are materials which have pores (openings) of varying sizes. Particulate matter larger than the pore size in a filter is excluded from passage and is this physically excluded. The earliest form of filter used in microbiological was cotton, a fiberous material derived from plants. Cotton fibers form a densely packed matrix which offers a torturous path for particulate matter containing microbes to pass, while still allowing air to do so. This is only true however, as long as a cotton plug, filter, or bandage remains dry, since water clings to each fiber allowing microbes unrestricted access.

In most cases, cotton has been replaced as a filter by ceramic filters and synthetic plastics such as nitrocellulose which offer very small pore sizes (0.2 m to 0.45 m) without taking up as much space. Since these materials are not fiberous, all but the very smallest microbes can be removed from a solution passing through them. This solution, called a **filtrate**, is generally free from contaminants so long as the original pre-filtered solution did not contain organisms such as mycoplasma bacteria or viruses, both of which are smaller than most filters. As a consequence, filtration should be considered an agent of disinfection rather than sterilization.

Dessication

Dessication (drying) is the removal of moisture from the body of an organism. Many bacteria are very sensitive to water loss and can be killed simply by removal of water. For example, *Treponema pallidum*, the agent of syphillis, is so intolerant to water loss that it will die within twenty seconds on the surface of a dry fomite. The physical preservation of foodstuffs by drying has been practiced by humans for thousands of years and in most cases does reduce the number of potentially pathogenic microbes. One process, called **lyophilization** or freeze-drying, is used to rapidly remove water from the body of an organism under very cold temperatures in a partial vacuum. This process does not kill organisms such as bacteria, but does inactivate their metabolic processes. Lyophilization is used to preserve living bacterial cultures for storage and transport. To restore the freeze-dried cells, an individual has only to rehydrate them in a nutrient broth solution and incubate the culture at the optimum temperature for growth of the microbe.

It is important to note, however, that not all microbes are killed or inactivated by dessication. Bacteria which form spores such as members of the genera *Bacillus* and *Clostridium*, cyst-forming protists, and viruses can withstand drying, simply becoming inactive until moisture becomes available. For this reason, dessication can only be considered a form of disinfection.

Radiation

Radiation describes a physical phenomenon which occurs when matter releases either energy, atomic particules, or both. Radiation can affect the chemical makeup of the cell by altering or disrupting the structure of biological molecules. **Ionizing radiation** strips electrons away from biological molecules. Both **gamma** and **X-radiation** are ionizing forms. **Ultraviolet radiation** is absorbed by the pyrimidine bases cytosine and thymine in DNA. When two thymine or cytosine molecules lie adjacent to one another on a nucleoside, ultraviolet radiation with wavelengths between 250 nm and 280 nm causes them to have a greater affinity for one another than for their complementary adenines on the opposite nucleoside. The two bond together, forming a **dimer**, which disrupts the normal sequence of nucleotide bases. This kind of mutation prevents the cell from producing proteins which may be necessary for normal metabolism to occur. Some cells can repair this damage if exposed to visibile light through a process called **photoreactivation** (light repair), wherein the dimer is nicked by a restriction endonuclease, then cut away and replaced by DNA polymerase. The new thymine or cytosine bases are then bonded to their complementary adenines or guanines by DNA ligase. Since light repair can occur, the use of ultraviolet radiation has only disinfecting activity and cannot be considered a sterilizing agent.

Temperature

Excess heat energy can cause proteins to become **denatured**, meaning that they lose their normal three-dimensional shape. Effective temperature for the reduction of microbes is measured as the **thermal death point** (**TDP**) of each organism, which is the temperature at which all growth stops. **Thermal death time** (**TDT**) is the amount of time it takes to kill all of the microbes in a sample, and the **decimal reduction factor** (**DRF**) is the amount of time at a specific heat necessary to reduce the population of microbes in a sample tenfold.

The most common methods of applying excess heat energy are **flaming** and **incineration**, which completely destroy all life. Flaming of inoculating loops and needles, as well as the tops of glass culture tubes and flasks insures that no contaminating microbes can infect sterile media. Applying **dry heat** by forcing hot air onto the surface of an object can be used in a similar fashion, though many spore formers are capable of withstanding this.

The application of **moist heat**, such as **boiling**, **steaming**, and **pasteurization** (application of high heat to a solution for a short period of time), is also commonly used. These methods work well for most microbes, but are incapable of killing organisms which are **thermoduric** (capable of withstanding elevated temperatures), or are spore formers. For example, the spores of *Clostridium botulinum*, the bacterium which causes botulism, can be boiled for up to five hours and still remain viable. The most effective application of moist heat is through the use of a device called an **autoclave**. The autoclave works on the principle of **saturated steam**. The inner chamber is raised to an air pressure of 15 lb/inch2, then steam at a temperature of 121^{o} C is injected. The steam strikes the surface of the object to be sterilized and condenses into water as its excess heat energy is released. This condensation creates a partial vacuum which draws more steam to the object. Saturated steam is extremely effective as a sterilizing agent, at least 1500 times more effective than the application of dry heat. Autoclaves are usually operated in cycles between 15 and 90 minutes, and can be used to sterilize glassware, surgical implements, soil, water, and microbiological media such as broths and agars. They cannot, however, be used to sterilize hydrophyllic

powders which would clump, or hydrophobic oils since microbes suspended in oils would only be subjected to dry heat. Also, while contaminated bandages can be placed in an autoclave, the toxins or exoenzymes left behind by killed microorganisms such as *Clostridium perfringens* (the agent of gas gangrene) may still be capable of causing host cell damage, so these should be rinsed thoroughly with sterile water prior to reuse.

All of the above physical means of control can be checked for effectiveness utilizing various bacteria as quality control agents. Devices which emit ionizing radiation can be tested with *Micrococcus radiouridans*, U.V. devices with *Bacillus pumilis*, and heat disinfecting and sterilizing units such as hot-air ovens, pressure cookers, and autoclaves with *Bacillus stearothermophilus*. These organisms are generally supplied to laboratories live or in ampules or tape strips, which can be placed in the control device. After a normal operating cycle, the organisms are incubated in microbiological media. If growth occurs, the device is not operating properly and should be repaired. Quality control checks and maintenance are vital to the effective microbiological laboratory or health-care facility, and should be performed on a regular basis to prevent contamination and the spread of disease.

Physical methods of microbial control

Heat

- Moist heat and dry heat
- Mechanism: denaturing the enzymes
- Most commonly used method of killing the microbes
- Thermal death point - the lowest temp at which all the microbes are killed in 10 min
- Thermal death time
- the minimal length of time needed to kill all bacteria at given temperature

Moist heat

- nonpressurized steam
- Mechanism: coagulation of proteins
- Boiling (100^0C) for 10 min kills vegetative cells of bacteria, viruses, and fungi
- Hepatitis virus can survive up to 30 min of boiling; some bacterial spores can survive more than 20 h.

Tyndalization

- boiling the medium for 60 min repeatedly for 3 day

Autoclaves

- steam under pressure
- Provide high temp. and high pressure (Pressure: 1 atm, temp.: 121^0 C)
- All microbes are killed in 15 min
- Steam should contact all surfaces
- Time is different for larger volumes
- Used for sterilization of:
  - Culture media
  - Equipment
  - Biological waste

Pasteurization

- Original pasteurization: 63^0 C for 30 min

- Today's pasteurization – *high temperature short-time pasteurization*: 72^0 C for 15 sec. or
- Ultra-high-temperature treatment - Exposure to 134^0 C for 3 sec. then rapidly cooled

Dry heat sterilization

- Mechanism: oxidation
- Flaming - inoculating loops
- Hot-air sterilization— Oven - 170^0 C for 2h

Desiccation

- In the absence of water microbes cannot grow but can survive
- Bacterial spores can survive for centuries
- Survival depends on microbial type and organism's environment (embedded in mucus - better survival)
- *Mycobacterium tuberculosis* – long survival
- *Neisseria gonorrhoeae* – dies after a few hours of air drying

Low temperatures

- Effect depends on the microbial type
- Ordinary refrigeration (0-7^0 C) - bacteriostatic effect
- Psychotrophs grow slowly
- Pathogenic bacteria will not grow
- Rapid freezing – microbes become dormant
- Lyophilization – frozen samples (bacterial cultures) dried in vacuum

Slow freezing – more harmful

Can Microbes Survive Millions of Years Traveling in Space? Experts Say "Yes"

Radiation

- Ionizing radiation (gamma rays, X rays) -radiation ejects electrons – ions are formed
- Non-ionizing radiation (UV light)

Ionizing radiation

- Short wavelength, high energy
- Emitted by radioactive elements (Co)
- Mechanism of action: ionization of water which forms hydroxyl radicals which react with DNA
- Used for sterilization of
  - Medical supplies (plastic syringes, Petri plates etc.)
  - Certain food (spices, meat, vegetables)

Non-ionizing radiation

- UV light, germicidal light – 260 nm – used for disinfection
- Mechanism of action:
  - damage of DNA – formation of thymine dimmers
  - Toxic free radicals are formed
- Sterilization of the air (hospital rooms, operating rooms, cafeteria)
- Disadvantage
  - Poor penetration
  - Harmful for human eyes, skin

Filtration

- Removal of microbes from a solution
- Membrane filters (pore size 0.2 or 0.45 um)

Osmotic pressure

- High concentration of salt causes water to leave the cell
- Used in preservation of food (high sugar concentration - fruit preserve)

Physical Methods of Microbial Control

Heat

Heat is frequently used to eliminate microorganisms.

Moist heat kills microbes by denaturing enzymes.

- Heat works better at low pH.

Thermal death point (TDP) is the lowest temperature at which all the microbes in a liquid culture will be killed in 10 minutes.

Thermal death time (TDT) is the length of time required to kill all bacteria in a liquid culture at a given temperature.

Decimal reduction time (DRT) is the length of time in which 90% of a bacterial population will be killed at a given temperature.

Boiling (100º C) kills many vegetative cells and viruses within ten minutes.

Autoclaving (steam under pressure) is the most effective method of moist heat sterilization. The steam must directly contact the material to be sterilized.

- 15 psi, 121° C for 15 minutes kills all vegetative bacteria and endospores.

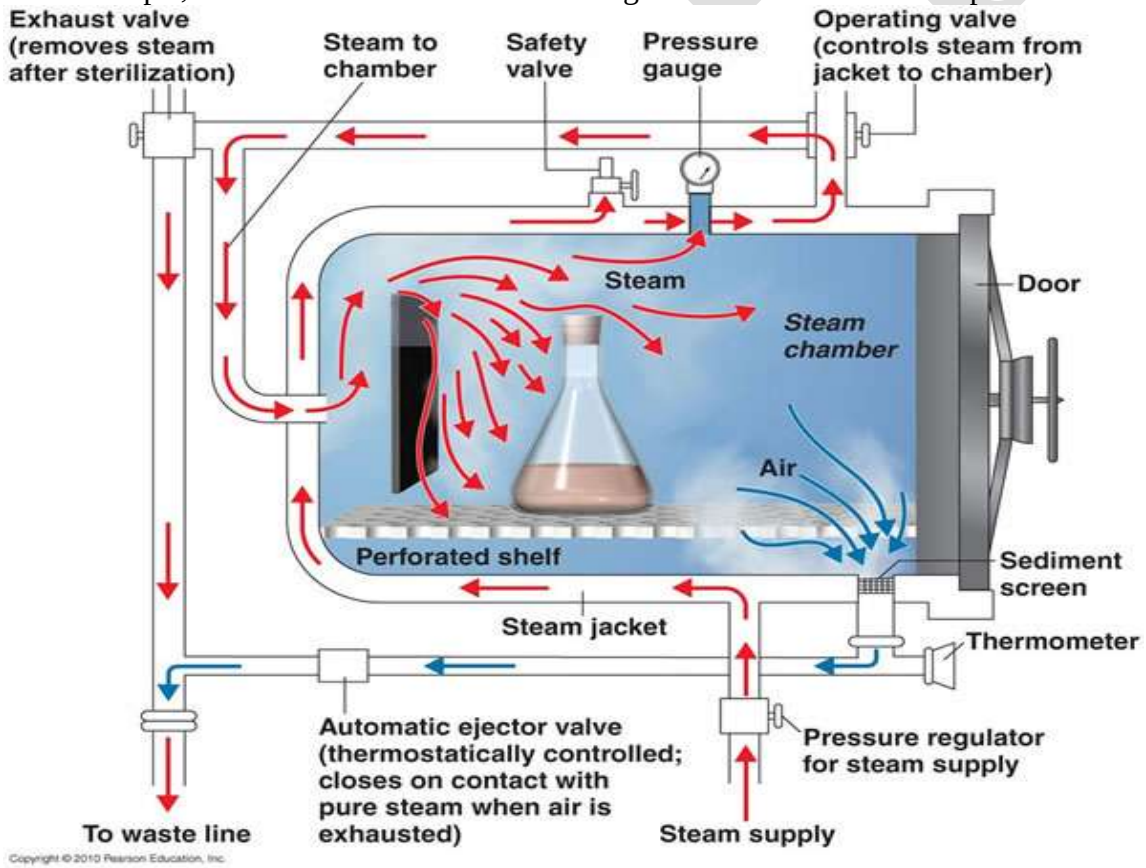


Table 7.3 The Relationship between the Pressure and Temperature of Steam at Sea Level*

Pressure (psi in Excess of Atmospheric Pressure)	Temperature (°C)
0	100
5	110
10	116
15	121
20	126
30	135

*At higher altitudes, the atmospheric pressure is less, a phenomenon that must be taken into account in operating an autoclave. For example, to reach sterilizing temperatures (121°C) in Denver, Colorado, whose altitude is 5280 feet (1600 meters), the pressure shown on the autoclave gauge would need to be higher than the 15 psi shown in the table.

Table 7.4 The Effect of Container Size on Autoclave Sterilization Times for Liquid Solutions*

Container Size	Liquid Volume	Sterilization Time (min)
Test tube: 18 × 150 mm	10 ml	15
Erlenmeyer flask: 125 ml	95 ml	15
Erlenmeyer flask: 2000 ml	1500 ml	30
Fermentation bottle: 9000 ml	6750 ml	70

*Sterilization times in the autoclave include the time for the contents of the containers to reach sterilization temperatures. For smaller containers, this is only 5 min or less, but for a 9000-ml bottle it might be as much as 70 min. A container is usually not filled past 75% of its capacity.

Dry heat sterilization (killing or removal of all microorganisms, including bacterial spores) technique requires longer exposure time (1.5 to 3 hours) and higher temperatures than **moist heat sterilization**. Various available methods of dry heat sterilization are; **hot air oven**, **incineration**, **flaming** (wire loop) etc.

Dry heat ovens are used to sterilize items that might be damaged by moist heat or that are impenetrable to moist heat (e.g., powders, petroleum products, sharp instruments).

Principle of Dry heat sterilization using HOT AIR OVEN

Sterilizing by dry heat is accomplished by **conduction**. The heat is absorbed by the outside surface of the item, then passes towards the centre of the item, layer by layer. The entire item will eventually reach the temperature required for sterilization to take place.

Dry heat does most of the **damage by oxidizing molecules.** The essential cell constituents are destroyed and the organism dies. The temperature is maintained for almost an hour to kill the most difficult of the resistant spores.

Closed view of Hot Air Oven

The most common time-temperature relationships for sterilization with hot air sterilizers are

1. **170^{o}C (340^{o}F) for 30 minutes,**
2. **160^{o}C (320^{o}F) for 60 minutes**, and
3. 150^{o}C (300^{o}F) for 150 minutes or longer depending up the volume.
4.

Bacillus atrophaeus spores should be used to monitor the sterilization process for dry heat because they are more resistant to dry heat than the spores of ***Geobacillus stearothermophilus*.** The primary lethal process is considered to be oxidation of cell constituents.

There are two types of dry-heat sterilizers:

1. the static-air type and
2. the forced-air type.

The **static-air type** is referred to as the oven-type sterilizer as heating coils in the bottom of the unit cause the hot air to rise inside the chamber via gravity convection. This type of dry-heat sterilizer is much slower in heating, requires longer time to reach sterilizing temperature, and is less uniform in temperature control throughout the chamber than is the forced-air type.

The **forced-air or mechanical convection** sterilizer is equipped with a motor-driven blower that circulates heated air throughout the chamber at a high velocity, permitting a more rapid transfer of energy from the air to the instruments.

Advantages of dry heat sterilization

1. A dry heat cabinet is easy to install and has relatively low operating costs;
2. It penetrates materials
3. It is nontoxic and does not harm the environment;
4. And it is **noncorrosive** for metal and sharp instruments.

Disadvantages for dry heat sterilization

1. Time consuming method because of **slow rate of heat penetration** and microbial killing.

2. High temperatures are not suitable for most materials.

Tyndallization

Tyndallization is a process dating from the nineteenth century for sterilizing substances, usually food, named after its inventor, scientist John Tyndall. It is still occasionally used. A simple, effective, sterilizing method used today is to heat the substance being sterilized to 121 °C for 15 minutes in a pressured system. If sterilisation under pressure is not possible because of lack of equipment, or the need to sterilize something that will not withstand the higher temperature, unpressurized heating at a temperature of up to 100 °C, the boiling point of water, may be used. The heat will kill the bacterial cells, but bacterial spores capable of later germinating into bacterial cells may survive. Tyndallization can be used to destroy the spores

Tyndallization essentially consists of heating the substance to boiling point (or just a little below boiling point) and holding it there for 15 minutes, three days in succession. After each heating, the resting period will allow spores that have survived to germinate into bacterial cells; these cells will be killed by the next day's heating. During the resting periods the substance being sterilized is kept in a moist environment at a warm room temperature, conducive to germination of the spores. When the environment is favourable for bacteria, it is conducive to the germination of cells from spores, and spores do not form from cells in this environment.

The Tyndallization process is usually effective in practice. But it is not considered totally reliable—some spores may survive and later germinate and multiply. It is not often used today, but is used for sterilizing some things that cannot withstand pressurized heating, such as plant seeds.

Pasteurization

- A high temperature is used for a short time to destroy pathogens and food spoilage organisms without altering the flavor of the food.
- Classic pasteurization was done at 63° C for thirty minutes.
- HTST pasteurization is done at 72º C for fifteen seconds.
- Ultra-high-temperature (UHT) treatment (140º C for three seconds) is used for dairy products.
- Dry Heat Sterilization
- Methods of dry heat sterilization include direct flaming, incineration, and hot -air sterilization (170° C for two hours).
- Dry heat kills by oxidation.
- Different methods that produce the same effect (reduction in microbial growth) are called equivalent treatments.

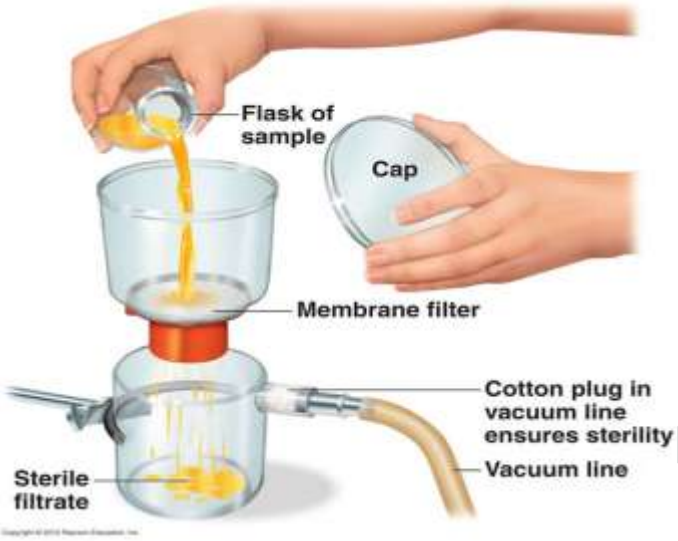
Filtration

Filtration is the passage of a liquid or gas through a filter with pores small enough to remain microbes.
Microbes can be removed from air by high -efficiency particulate air filters.

- HEPA – 0.3 um pore size
- Decreases numbers

Membrane filters composed of nitrocellulose or cellulose acetate are commonly used to filter out bacteria, viruses, and even large proteins.

- Range from 0.22 um – 0.45 um for bacteria but sometimes mycoplasma or spirochetes can pass through.
- Sizes can go as low as 0.01 um to filter viruses and proteins.



Low Temperatures

- The effectiveness of low temperatures depends on the particular microorganism and the intensity of the application.
- Most microorganisms do not reproduce at ordinary refrigerator temperatures (0-7º C).
- Many microbes survive (but do not grow) at subzero temperatures used to store foods.

High Pressure

- High pressure denatures proteins in vegtetative cells.
- Endospores aren't usually harmed, but can be allowed to grow out and the vegetative form killed with high pressure.
- Used in Japan and the U.S. to preserve fruit juices – preserves the flavor, color, and nutrient values.

Desiccation

- In the absence of water, microorganisms can grow but cannot remain viable.
- Viruses and endospores can resist desiccation.
- *Mycobacterium tuberculosis* is fairly resistant to desiccation but the gonorrhea bacterium is very sensitive.
- Environmental conditions effect efficiency of desiccation – pus, feces, and mucus are protective

Osmotic Pressure

- Microorganisms in high concentrations of salts and sugars undergo plasmolysis.
- Molds and yeasts are more capable than bacteria of growing in materials with low moisture or high osmotic pressure.

Radiation

The effects of radiation depend on its wavelength, intensity and duration.

Ionizing radiation (gamma rays, X-rays and high-energy electron beams) has a high degree of penetration and exerts its effect primarily by ionizing water and forming highly reactive hydroxyl radicals.

- Destroys DNA.

Non-ionizing radiation causes damage to DNA.

- Ultraviolet causes formation of thymine dimers.

- Microwaves heat water but it is possible to isolate vegetative forms from microwave ovens after usage and uneven heating in solid foods may leave live organisms in the food after microwave cooking.

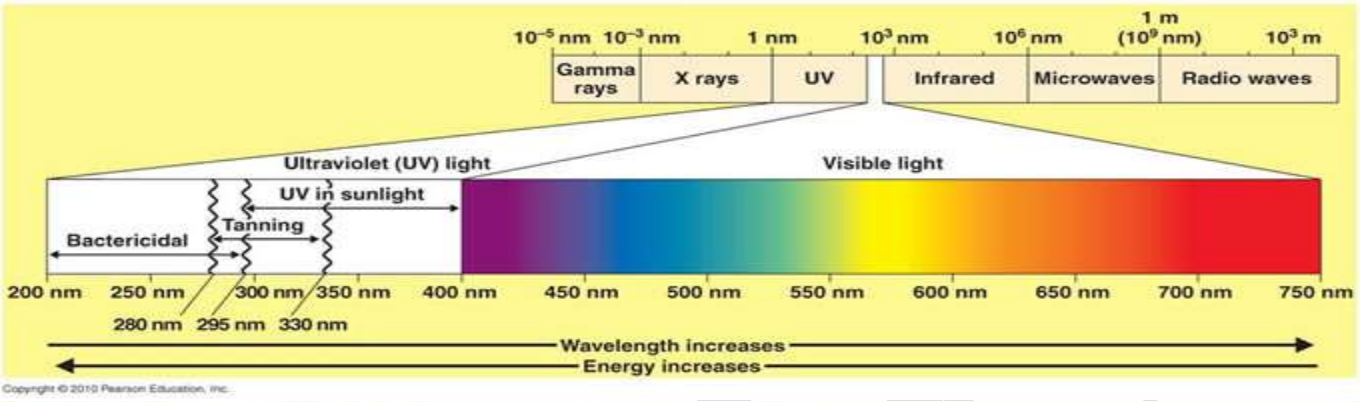


Table 7.5 Physical Methods Used to Control Microbial Growth

Methods	Mechanism of Action	Comment	Preferred Use
Heat			
1. Moist heat			
a. Boiling or flowing steam	Protein denaturation	Kills vegetative bacterial and fungal pathogens and almost all viruses within 10 min; less effective on endospores	Dishes, basins, pitchers, various equipment
b. Autoclaving	Protein denaturation	Very effective method of sterilization; at about 15 psi of pressure (121°C), all vegetative cells and their endospores are killed in about 15 min	Microbiological media, solutions, linens, utensils, dressings, equipment, and other items that can withstand temperature and pressure
2. Pasteurization	Protein denaturation	Heat treatment for milk (72°C for about 15 sec) that kills all pathogens and most nonpathogens	Milk, cream, and certain alcoholic beverages (beer and wine)
3. Dry heat			
a. Direct flaming	Burning contaminants to ashes	Very effective method of sterilization	Inoculating loops
b. Incineration	Burning to ashes	Very effective method of sterilization	Paper cups, contaminated dressings, animal carcasses, bags, and wipes
c. Hot-air sterilization	Oxidation	Very effective method of sterilization but requires temperature of 170°C for about 2 hr	Empty glassware, instruments, needles, and glass syringes

Filtration	Separation of bacteria from suspending liquid	Removes microbes by passage of a liquid or gas through a screenlike material; most filters in use consist of cellulose acetate or nitrocellulose	Useful for sterilizing liquids (enzymes, vaccines) that are destroyed by heat
Cold			
1. Refrigeration	Decreased chemical reactions and possible changes in proteins	Has a bacteriostatic effect	Food, drug, and culture preservation
2. Deep-freezing (see Chapter 6, page 170)	Decreased chemical reactions and possible changes in proteins	An effective method for preserving microbial cultures, in which cultures are quick-frozen between −50° and −95°C	Food, drug, and culture preservation
3. Lyophilization (see Chapter 6, page 170)	Decreased chemical reactions and possible changes in proteins	Most effective method for long-term preservation of microbial cultures; water removed by high vacuum at low temperature	Food, drug, and culture preservation
High Pressure	Alteration of molecular structure of proteins and carbohydrates	Preservation of colors, flavors, nutrient values	Fruit juices
Desiccation	Disruption of metabolism	Involves removing water from microbes; primarily bacteriostatic	Food preservation
Osmotic Pressure	Plasmolysis	Results in loss of water from microbial cells	Food preservation
Radiation			
1. Ionizing	Destruction of DNA	Not widespread in routine sterilization	Sterilizing pharmaceuticals and medical and dental supplies
2. Nonionizing	Damage to DNA	Radiation not very penetrating	Control of closed environment with UV (germicidal) lamp

Chemical methods of microbial control

- Effectiveness of the disinfectant depends on:
- Type of the chemical agent
- Type of microbes
- Concentration of a disinfectant
- Time of contact
- pH of the medium
- Temperature

Types of Disinfectants

Halogens

- Fluorine, bromine chlorine, and iodine
- Iodine is the oldest antiseptic
- Iodine tincture → skin disinfection
- Chlorine - gas (Ca-hypochlorite; Na-hypochlorite- bleach)
- Mode of action: oxidizing agent - alters cellular components
- Disinfection of drinking water, swimming pools, household (bleach)

Phenolics (derivatives of phenol)

- Used first time by Lister → carbolic acid

- Mechanism of action: damages the plasma membrane, enzyme inactivation
- Advantage: active even in the presence of organic compounds
- Hexachlorophene (bisphenol) used in antimicrobial soaps

Is antibacterial soap any better than regular soap?

- The antibacterial components of soaps need to be left on a surface for about two minutes in order to work.

Alcohols

- Ethanol or isopropanol 60% - 95%
- Kills vegetative cells of bacteria and fungi (not spores and nonenvelope viruses)
- Mechanism of action: protein denaturation

Is pure ethanol a better disinfectant than 70% ethanol? Why?

- 100% ethanol coagulates proteins in the cell wall
- 70% ethanol penetrates the cell wall and coagulates the proteins inside the cell

Hydrogen Peroxide

- 3% solution used as an antiseptic
- Skin and wound cleansing
- Mouthwash
- Contact lens
- Surgical implants
- Endoscopes

Chemicals with surface action: Detergents / Soaps

- Detergents are polar molecules - surfactants
- Decrease the surface tension among molecules and water
- Soaps and Detergents are not antiseptics they break the oily film on the surface of skin
- They have microbicidal power when mixed with quaternery ammonium compounds

Heavy metals

- Silver, mercury, copper, gold, arsenic
- Only mercury and silver have germicidal significance
- Mechanism of action: ions combine with sulfhydril groups - protein denaturation
- 1% Silver nitrate - antiseptic
- Copper sulfate - controls algal growth
- Can be toxic to humans

Evaluation of a disinfectant

- Filter paper method
  - Paper disks are soaked in a solution of disinfectant and placed on a agar previously inoculated with a test organism
  - Observe the inhibition zone around the disk

Aldehydes (formaldehyde, glutaraldehyde)

- Most effective antimicrobials
- Formalin - used for preservation of biological specimens
  - High level disinfectant
  - Toxic - carcinogenic
- Glutaraldehyde

- Used for disinfection of hospital instruments
- Mode of action: forms covalent cross-links with functional groups of proteins
- Kills bacterial spores, fungal spores and viruses

Chemical Methods

Chemical agents for the control of microbial growth are either **microbiocidal** or **microbiostatic**. Microbiocidal agents are **sterilants** which kill all living cells. Microbiostatic agents kill some cells and inhibit the growth of others. The spectrum of activity exhibited by any microbiocidal or microbiostatic agent is an important factor in choice, and should be considered, along with potential harmful effects on the user. An agent which kills staphylococci may be totally ineffective against mycobacteria, and would be useless in a tuberculosis ward. Also, a broadly killing sterilant may release gasses which are toxic to patients and staff. Most often, chemical agents which disinfect are utilized by clinics, hospitals, and laboratories. While these agents do not sterilize, their toxicity is usually much less than that of a sterilant, and prevention of infection is stressed.

There are four large categories for agents of chemical control. **Antibiotics** are produced by microorganisms to kill or inhibit the growth of other microbes. These agents are generally selectively toxic, and can be naturally produced, synthesized, or or semisynthetic. **Antiseptics** are synthetic compounds which kill or inhibt the growth of microbes on the surface of the skin. **Disinfectants** are chemical compounds which kill or inhibit microbes on the surface of fomites. **Preservatives**, such as sugars, salt, nitrates, nitrites, sulfate, and sulfites inhibit microbial growth in food, usually by producing osmotic environments which are unfavorable to microbial growth. These can be further subdivided as high-, intermediate, or low level agents. **High-level germicides** sterilize fomites, but are toxic to skin and mucus membranes. **Intermediate-level disinfectants and antiseptics** kill and inhibit on fomites and skin, but can be toxic to the user at medium to high concentrations. Examples include phenolics and halogens. **Low-level disinfectants**, such as alcohols, hydrogen peroxide, heavy metals, and soaps kill some microbes but inhibit the growth of most.

High-Level Germicides

These are called **agents of cold sterilization**, since no heat needs to be applied to increase their activity. These are generally alkylating agents, which kill by adding ethyl or methyl groups to nucleic acids or proteins. While the agents are capable of killing vegetative cells, spores, and inactivating viruses, some take up to several hours to complete their germicidal activity.

Aldehydes, such as **formaldehyde** and **gluteraldehyde**, fix tissues by alkylating and forming cross-links between adjacent proteins. They are commonly used as fixitive compounds for electron microscopy, preservatives of specimens and cadavers, in some synthetic plastic compounds, and can be used to sterilize anesthesia tubing and surgical implements. Aldehydes can fix living tissues such as mucus membranes and have the ability to vaporize or **outgas** from compounds containing them, so they should be handled with caution. □**-propiolactone** is a liquid alkylating sterilant with a high boiling point (155^{o}C). It is generally used to sterilize bone used in grafts. It quickly breaks down into nontoxic compounds when it comes into contact with organic matter, but can burn skin.

Ethylene oxide (carboxide) kills vegetative cells and spores. It is a liquid at temperatures below 10.8^{o} C, but rapidly sublimates into a highly inflammable gaseous state above this temperature. It is generally used in a chamber similar to an autoclave at 60^{o} C for 1-10 hours, where it is mixed in a 9:1 ratio with carbon dioxide (90% CO_2, 10% ethylene oxide), which reduces its toxicity, but also its inflammability.

Carboxide can be used to sterilize surgical implements and glassware, but these fomites must be allowed to degass before use, since residues can stimulate mutations in bacteria.

Ozone (O_3) occurs naturally in the upper atmosphere, where it serves to shield the surface of the earth from solar radiation, and is produced as an exhaust gas by vehicles and industry, acting as a pollutant in the lower atmosphere. Applied properly in a chamber, ozone is a powerful oxidizing agent which kills cells and spores on the surface of glassware, surgical implements, and bandages. An advantage of sterilization with this compound is that it outgasses quickly, leaving no toxic residues as can ethylene oxide and b-propiolactone.

Intermediate-Level Disinfectants and Antiseptics

Phenol (carbolic acid) is one of the earliest disinfectant compounds to be used in health care facilities and laboratories. Joseph Lister used atomized phenolic compounds in the 1860's to disinfect his surgery during invasive procedures as a means of cutting down on postoperative septic infections. Phenol kills microorganisms by denaturing proteins and destabilizing cell membranes, is bacteriocidal, fungicidal, and virucidal at high concentrations, but is not effective against bacterial endospores, and is effective against many potential pathogens, including mycobacteria, staphylocci, streptococci, and gram-negative coliforms, such as *E. coli*. It can be used to disinfect garbage cans, surgical operating facilities, laboratory equipment, feces, urine, and sputum, but it is very corrosive at higher concentrations and its fumes can be lethal. Because of its toxicity, this compound is generally used as a solution between 2% to 5% in concentration, and many less toxic derivitives have been produced. The most common of th phenolic derivatives are called **cresols** and **bisphenols**. Cresols are formed by adding methyl groups to phenol, and are used for the preservation of wood products, as compounds such as creosote (para-cresol). Bisphenols are produced by combining two phenol molecules. Lysol™ is a combination of cresol and soap, which has about the same spectrum of activity as phenol, but is much less toxic to skin. Other cresols include resorcinol, hexylresorcinol, and hexachlorophene. Hexachlorophene soaps such as pHizoHex were once widely utilized as antiseptic soaps by health-care personnel for hand washing and the bathing of newborn infants, but have been discarded, since it was found that this phenolic could be absorbed through the skin and potentially cause birth defects.

Since phenol has such a broad-spectrum of activity, it is used as a standard by which to judge how well other disinfecting compounds work. The **phenol coefficient** (**P.C.**) is a mathematical value used to compare the effectiveness of a test disinfectant to that of phenol, and is derived from the following formula:

$$\text{P.C.} = \frac{\text{dilution of a test disinfectant necessary to kill a standard population of bacteria}}{\text{dilution of phenol which has the same effect}}$$

For example, a 1:250 dilution of a test reagent kills a standard population of *S. aureus*. A 1:60 dilution of phenol kills the same size population. To derive the P.C., we divide 250/60:

P.C. = 250/60 =4.2 Therefore the test disinfectant is 4.2 times as effective as phenol.

Halogens are a family of elements with a high affinity for electrons. This affinity makes them very reactive with biological molecules, and they can serve to disrupt enzyme activity, break down lipid structure, and produce oxidizing agents such as singlet oxygen (O). The halogens most commonly used as disinfectants are chlorine and iodine. **Chlorine** is used as a disinfectant only, either as a gas or in liquid form which is effective against many vegetative forms of microbes as well as some viruses such as HIV and hepatitis. Commonly, chlorine is supplied in **sodium hypochlorite** (NaOCl) bleach, which is a combination of 94.75% water and 5.25% NaOCl. It is used as a bleaching compound as well as a

disinfectant for used hypodermic needles, in swimming pools, toilets, water supplies, and in sewage treatment plants. It is inactivated by organic matter, and produces toxic fumes (the "mustard gas" used in World War I because of its yellow color) which can cause considerable damage to skin and mucus membranes with direct contact. **Iodine** is lethal to all vegetative forms of microorganisms, can inactivate viruses, and is fairly effective in higher concentrations against endospores. Pure iodine is caustic to tissues, so it is diluted with other compounds. Tincture of iodine is produced by dissolving crystaline iodine in alcohol. This solution is a good antiseptic for most minor cuts and scrapes, but it excites pain receptors at the site of an injury, and as the alcohol component dries, the iodine may concentrate and damage exposed tissues. **Iodophore compounds**, such as Betadine™ and Wescodine™ are composed of iodine dissolved in mild detergent and alcohol. These do not excite pain receptors as readily and can be used to clean and disinfect large areas of skin prior to invasive surgical procedures.

Low-Level Disinfectants

Hydrogen peroxide is a good low-level disinfectant agent when used in concentrations of 3% or lower. Higher concentrations are caustic to human skin, and cannot be used. This compound is used as an antiseptic for the treatment of minor cuts and scrapes and as a bleaching agent. When placed on the surface of an injury, hydrogen peroxide bubbles due to the release of the enzyme catalase from tissues, which break it down into water and oxygen. This breakdown also releases the peroxide ion (O_2^{-2}), a strong oxidizing agent, and that the water released provides hydroxide ions which strip hydrogen from biological molecules. Obligate anaerobic microbes are especially sensitive to hydrogen peroxide, since they do not produce catalase and the rapid release of oxygen gas inhibits their growth.

Alcohols such as ethanol and isopropanol are effective antiseptics and disinfectants when used in concentrations between 70% and 80%. Alcohols kill microbes by denaturing proteins, dehydrating (100% concentration), and as nonpolar solvents which disrupt the phospholipid structure of the cell membrane, but are relatively ineffective against spores and viruses. Also, dehydration may actually be beneficial to some microbes, enhancing their survival by extracting extracellular water, so alcohols such as ethanol are normally used in lower concentrations. Isopropanol is used as an antiseptic and to clean the epidermis prior to syringe and I.V. needle use.

Heavy metals such as mercury, silver, and copper tend to combine with sulfur groups in the proteins of microbes, causing them to denature. This **oligodynamic activity** makes the heavy metals useful in small concentrations. These are some of the earliest used agents for the control of microorganisms. Mercury, in the form of mercuric chloride, was used by the Greeks and Romans to disinfect skin. This element is toxic in high concentrations, so it is commonly blended with other compounds such as iodine and alcohol in mercurochrome. Silver, applied as silver nitrate ($AgNO_3$) in a 1% solution, is commonly used to inhibit the growth of *Neisseria gonorrhoeae* in the eyes of newborn infants, a condition called **neonatorum opthamalia** (though many hospitals now use antibiotic ointments such as erythromycin or tetracycline), which can lead to blindness and is acquired as the organisms are transmitted to the infant as it passes through the birth canal. Copper, in the form of copper sulfate, is used to limit the growth of algaes in ponds and lakes, and as an antifungal compound for use on plants.

Detergents and soaps are composed of lipids and compounds having basic pH, such as sodium hydroxide. These break up surface tension, act as wetting agents which release particles attached to the surface of objects, and destabilize the phosphate portions of the plasma membrane of microorganisms. Detergents are either anionic or cationic, releasing negatively or positively- charged ions into solution. Anionic forms are weakly active against gram-positive bacteria but tend to repel negatively-charged cells,

thus they are generally used in the production of iodophore compounds. Cationic forms are attracted to bacterial cells and are bacteriostatic, while remaining relatively mild to the surface of skin. **Quartenary ammonium compounds** (**QUATS**) are cationic detergents which contain one or more long-chain akyl groups. Quats such as benzalkonium chloride (Zephiran™) have broad-spectrum inhibitory activity against bacteria, fungi, and protozoa, are mildly antiseptic and disinfecting when used as cleaning agents for laboratory fomites and on the surface of skin, and remain active after drying, but lose much of their activity when mixed with soaps. Cetylpyridium chloride (ceepryn) is the quat in Cepacol™ which is mildly antiseptic and safe for use on the mucus membranes of the oral cavity.

Some **dyes** can not only be used to stain microorganisms, but also have antimicrobial activity. Crystal violet (used in very low concentrations as gentian violet) can be used to treat oral infections by bacteria such as *Rochlaemia quintana*, the agent of trenchmouth, and fungal infections such as *Candida albicans*, which causes oral thrush.

Chemical Methods of Microbial Control

- Chemical agents are used on living tissue (as antiseptics) and on inanimate objects (as disinfectants).
- Few chemical agents achieve sterility.

Principles of Effective Disinfection

- Properties of the disinfectant – how it works determines what it will be effective against.
- Concentration of the disinfectant - requires proper hydration.
- The presence of organic matter can act as a buffer
- The degree of contact with microorganisms – if the surface needs cleaning or is porous microorganisms can escape contact with the disinfectant.
- Temperature should also be considered, since increased temperatures usually enhance the efficacy of disinfectants.
- Time left in contact is also an important consideration.

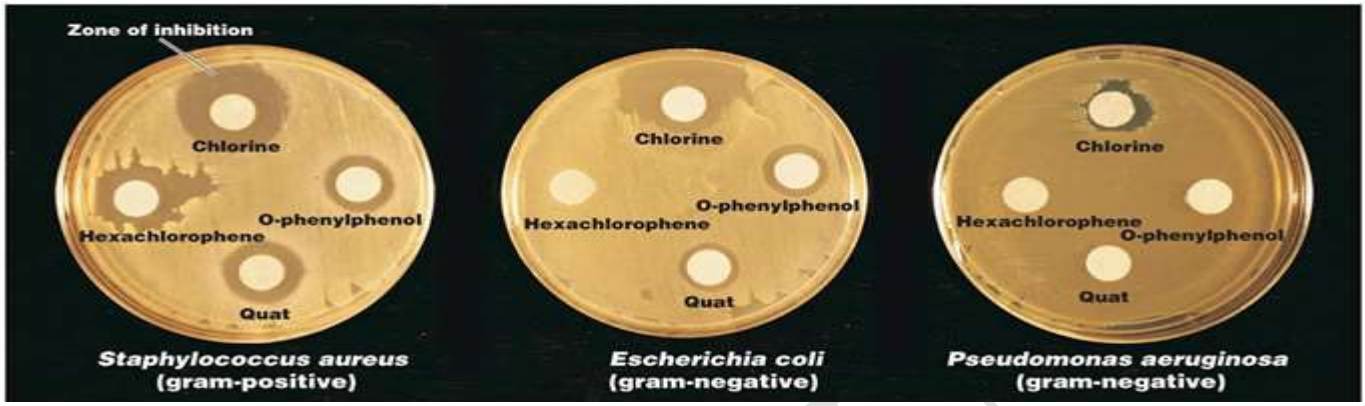
Evaluating a Disinfectant

- Phenol coefficient – compares activity of a disinfectant to phenol, was once the standard to evaluate the effectiveness of a disinfectant.
- The current standard is the American Official Analytical Chemist's use-dilution test. In this test bacterial (*Salmonella choleraesuis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*) survival in the manufacturer's recommended dilution of a disinfectant is determined.

Procedure:

- Dip metal rings into broth cultures of the organisms.
- Dry the rings briefly at 37° C.
- Place rings in disinfectant at manufacturer's recommended concentration for 10 minutes at 20° C.
- Transfer rings to culture media, effectiveness correlates with the numbers of cultures that grow.
- Viruses, endospore-forming bacteria, mycobacteria, and fungi can also be used in the use-dilution test.

In the disk-diffusion method, a disk of filter paper is soaked with a chemical and placed on an inoculated agar plate; a clear zone of inhibition indicates effectiveness.



Types of Disinfectants

Phenol and Phenolics

Phenolics are derivatives of phenol that have been altered to reduce irritating qualities or increase antimicrobial activity when combined with detergents.

Phenolics exert their action by injuring plasma membranes.

- Can also denature proteins – enzyme inactivation

Qualities:

- Not inactivated by organic compounds
- Stable for long periods
- Persist for long periods after application
- Good for disinfecting things like pus, saliva, and feces

Cresols are phenolics that are derived from coal tar.

O-phenylphenol is a cresol that is the the main ingredient in Lysol.

Bisphenols

Bisphenols have two phenolic groups and include triclosan (over the counter) and hexachlorophene (prescription).

Hexachlorphene is used in pHisoHex

- Particularly good against gram-positive strep and staph (cause skin infections in infants)
- Can cause neurological damage if exposure is high (bathing several times per day)

Triclosan is used in soaps, toothpaste, and incorporated into plastic kitchenware.

- Broad spectrum, especially against gram-positive bacteria and fungi
- Resistant strains of bacteria have arisen and triclosan may contribute to antibiotic resistance as well

Biguanides

Chlorhexidine

- Binds well to skin and mucus membranes
- Low toxicity
- Surgical hand scrubs and pre-op skin prep
- Can damage eyes
- Not sporocidal

Halogens

Some halogens (iodine and chlorine) are used alone or as components of inorganic or organic solutions.

Iodine may combine with certain amino acids to inactivate enzymes and other cellular proteins.

Iodine is available in a tincture (in solution with alcohol) or an iodophor (combined with an organic molecule).

- Betadine, Isodine (povidone-iodines)
- Povidone is surface active – improves wetting actions.

The germicidal action of chlorine is based on the formation of hypochlorous acid (HOCl) when chlorine is added to water.

- Good oxidizing agent.
- Chlorine is used as a disinfectant in gaseous form (Cl_2) or in the form of a compound, such as calcium hypochlorite (Semmelweis's lime water), sodium hypochlorite (NaOCl, Clorox), sodium dichloroisocyanurate, and chloramines.
- Used to disinfect drinking water and swimming pools
- Two drops Clorox per liter (four if cloudy) and let sit 30 minutes

Alcohols

Alcohols exert their action by denaturing proteins and dissolving lipids.

- Not good for wound disinfection because proteins coagulate and form a protective coat around bacteria.

In tinctures, they enhance the effectiveness of other antimicrobial chemicals.

Aqueous ethanol (60-95%) and isopropanol (lower concentrations required, typically 62 - 65%) are used as disinfectants. Alcohol evaporates quickly and leaves no residue behind.

- Denaturation requires H_2O, which is why aqueous preparations are better than pure.
- 70% is best ethanol concentration

Table 7.6 Biocidal Action of Various Concentrations of Ethanol in Aqueous Solution against *Streptococcus pyogenes*

Concentration of Ethanol (%)	Time of Exposure (sec) 10	20	30	40	50
100	G	G	G	G	G
95	NG	NG	NG	NG	NG
90	NG	NG	NG	NG	NG
80	NG	NG	NG	NG	NG
70	NG	NG	NG	NG	NG
60	NG	NG	NG	NG	NG
50	G	G	NG	NG	NG
40	G	G	G	G	G

NOTE:
G = growth
NG = no growth

Heavy Metals and Their Compounds

Silver, mercury, copper, and zinc are used as germicidals.

They exert their antimicrobial action through oligodynamic action. When heavy metal ions combine with sulfhydryl (—SH) groups, proteins are denatured.

Silver

Used as 1% silver nitrate solution to prevent opthalmia neonatorum before being replaced by antibiotics. Ironically, silver impregnated wound dressings are protective against antibiotic-resistant bacteria.

Silver sulfadiazine - topical cream for burns.

Surfacine - water-insoluble silver iodide in a polymer carrier, persistent for at least 13 days, can be used on animate and inanimate objects.

Silver nanoparticle infused commericial products - plastic food storage containers keep food fresher, minimizes odors in athletic clothing.

Mercury

Primarily bacteriostatic but broad range of activity. Some question of toxicity (har) and autism (BS).

Mercuric chloride is one formulation that was once used to treat syphylis. It is so toxic that poisoning was common, the symptoms of which were often confused with those of syphilis.

Other antiseptic mercury based compounds include the former parental favorites Mercurochrome and Merthiolate.

Mercurochrome is a trade name for merbromin, a compound containing mercury and bromine.

Merthiolate is a trade name for thimerosal, a compound containing mercury and sodium, which ~~is~~ was also available as a tincture.

They both "sting" upon application and the merthiolate tincture (as I remember) feels such that it conjures the thought that the application of battery acid with a red-hot poker would be preferable.

Thimerosal is still in use but the primary applications of mercury use are industrial and as an inhibitor of mildew in paint.

Copper

Copper sulfate is used to control green algae growth in ponds, pools, reservoirs and fish tanks and copper compounds are sometimes used in paint to prevent mildew.

Zinc

Zinc chloride is used in some mouthwashes, zinc pyrithione in antidandruff shampoos.

Surface -Active Agents

Surface-active agents decrease the surface tension among molecules of a liquid; soaps and detergents are examples.

Soaps have limited germicidal action but assist in the removal of microorganisms through scrubbing.

- Emulsify fats

Acid-anionic detergents are used to clean dairy equipment.

- Interact with plasma membrane and may cause damage to enzymes.

Quanternary Ammonium Compounds (Quats)

Quats are cationic detergents attached to NH_4^+.

By disrupting plasma membranes, they allow cytoplasmic constituents to leak out of the cell.

- Also denature proteins (inhibit enzymes) and are surface active.

Quats are most effective against gram-positive bacteria.

- Also good against fungi, amoeba, and enveloped viruses but pseudomonads can grow in them.

Inactivated by anions, soaps, detergents, and organic material.

Chemical Food Preservatives

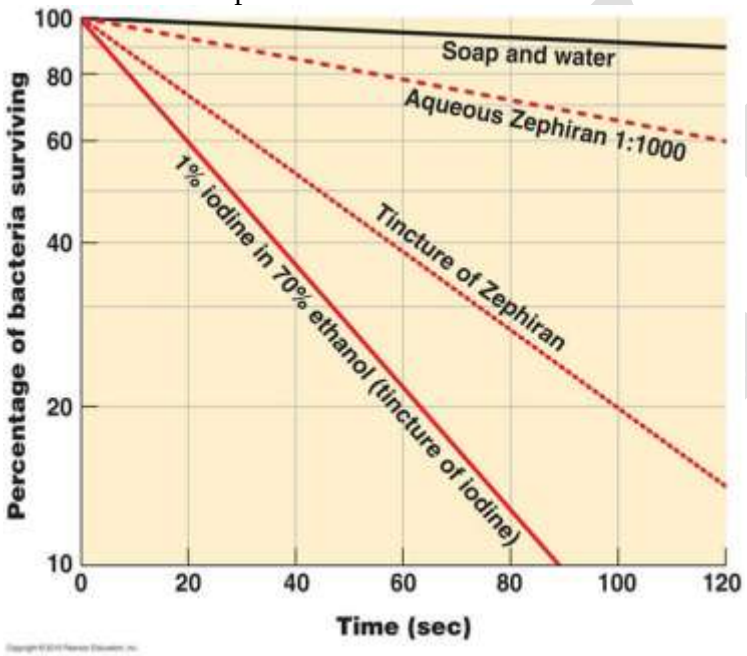
SO_2, sorbic acid, benzoic acid, and propionic acid inhibit fungal metabolism and are used as food preservatives.

Good to prevent mold growth in acidic foods (molds grow well at low pH).

Nitrate and nitrite salts prevent germination of *Clostridium botulinum* endospores in meats and preserve red color.

- Nitrites combine with amino acids to produce nitrosamines, which may be carcinogenic.
- Nitrosamines are produced from other things in the body and may not be as big a deal as once thought.

Effectiveness comparison



Antibiotics

Nisin and natamycin are antibiotics used to preserve foods, especially cheese.

Not used for treatment of disease.

Aldehydes

Aldehydes such as formaldehydes and glutaraldehyde exert their antimicrobial effect by inactivating proteins.

They are among the most effective chemical disinfectants.

TABLE 7.8 Chemical Agents Used to Control Microbial Growth

Chemical Agent	Mechanism of Action	Preferred Use	Comment
Phenol and Phenolics			
Phenol	Disruption of plasma membrane, denaturation of enzymes.	Rarely used, except as a standard of comparison.	Seldom used as a disinfectant or antiseptic because of its irritating qualities and disagreeable odor.
Phenolics	Disruption of plasma membrane, denaturation of enzymes.	Environmental surfaces, instruments, skin surfaces, and mucous membranes.	Derivatives of phenol that are reactive even in the presence of organic material; O-phenylphenol is an example.
Bisphenols	Probably disruption of plasma membrane.	Disinfectant hand soaps and skin lotions.	Triclosan is an especially common example of a bisphenol. Broad spectrum, but most effective against gram-positives.
Biguanides (Chlorhexidine)	Disruption of plasma membrane.	Skin disinfection, especially for surgical scrubs.	Bactericidal to gram-positives and gram-negatives; nontoxic, persistent.
Halogens	Iodine inhibits protein function and is a strong oxidizing agent; chlorine forms the strong oxidizing agent hypochlorous acid, which alters cellular components.	Iodine is an effective antiseptic available as a tincture and an iodophor; chlorine gas is used to disinfect water; chlorine compounds are used to disinfect dairy equipment, eating utensils, household items, and glassware.	Iodine and chlorine may act alone or as components of inorganic and organic compounds.
Alcohols	Protein denaturation and lipid dissolution.	Thermometers and other instruments. When the skin is swabbed with alcohol before an injection, most of the disinfecting action probably comes from a simple wiping away (degerming) of dirt and some microbes.	Bactericidal and fungicidal, but not effective against endospores or nonenveloped viruses; commonly used alcohols are ethanol and isopropanol.
Heavy Metals and Their Compounds	Denaturation of enzymes and other essential proteins.	Silver nitrate may be used to prevent ophthalmia neonatorum; silver-sulfadiazine is used as a topical cream on burns; copper sulfate is an algicide.	Heavy metals such as silver and mercury are biocidal.
Surface-Active Agents			
Soaps and detergents	Mechanical removal of microbes through scrubbing.	Skin degerming and removal of debris.	Many antibacterial soaps contain antimicrobials.
Acid-anionic sanitizers	Not certain; may involve enzyme inactivation or disruption.	Sanitizers in dairy and food-processing industries.	Wide spectrum of activity; nontoxic, noncorrosive, fast-acting.
Quaternary ammonium compounds (cationic detergents)	Enzyme inhibition, protein denaturation, and disruption of plasma membranes.	Antiseptic for skin, instruments, utensils, rubber goods.	Bactericidal, bacteriostatic, fungicidal, and virucidal against enveloped viruses. Examples of quats are Zephiran and Cepacol.
Chemical Food Preservatives			
Organic acids	Metabolic inhibition, mostly affecting molds; action not related to their acidity.	Sorbic acid and benzoic acid effective at low pH; parabens much used in cosmetics, shampoos; calcium propionate used in bread.	Widely used to control mold and some bacteria in foods and cosmetics.
Nitrates/nitrites	Active ingredient is nitrite, which is produced by bacterial action on nitrate. Nitrite inhibits certain iron-containing enzymes of anaerobes.	Meat products such as ham, bacon, hot dogs, sausage.	Prevents growth of *Clostridium botulinum* in food; also imparts a red color.
Aldehydes	Protein denaturation.	Glutaraldehyde (Cidex) is less irritating than formaldehyde and is used for disinfecting medical equipment.	Very effective antimicrobials.
Chemical Sterilization			
Ethylene oxide and other gaseous sterilants	Inhibits vital cellular functions.	Mainly for sterilization of materials that would be damaged by heat.	Ethylene oxide is the most commonly used. Heated hydrogen peroxide and chlorine dioxide have special uses.
Plasma sterilization	Inhibits vital cellular functions.	Especially useful for tubular medical instruments.	Usually hydrogen peroxide excited in a vacuum by an electromagnetic field.
Supercritical fluids	Inhibits vital cellular functions.	Especially useful for sterilizing organic medical implants.	Carbon dioxide compressed to a supercritical state.
Peroxygens and Other Forms of Oxygen	Oxidation.	Contaminated surfaces; some deep wounds, in which they are very effective against oxygen-sensitive anaerobes.	Ozone is widely used as a supplement for chlorination; hydrogen peroxide is a poor antiseptic but a good disinfectant. Peracetic acid is especially effective.

Chemical Sterilization

Gas

Ethylene oxide is a gas commonly used for sterilization.

Penetrates most materials and kills all microorganisms (and destroys endospores) by cross linking proteins and nucleic acids.

Requires long exposure times and is explosive and toxic in pure form but works well at low (room) temperatures

Chlorine dioxide is a commonly used gas in water treatment to prevent or reduce formation of carcinogens during chlorination; although it is far more stable in aqueous solution, it has also been used (in gaseous form) to fumigate enclosed building areas contaminated with anthrax endospores.

Plasmas

A state of matter that occurs when a gas is excited (commonly with an electromagnetic field) to produce a mixture of nuclei with various charges and free electrons.

Good for sterilizing plastic surgical instruments and long hollow tubes with a small diameter.

Done in a chamber where conditions are generated by an electromagnetic field in a vacuum. The chemical that forms the plasma is often H_2O_2. The free radicals generated kill vegetative cells and endospores and the process can be carried out at a low temperature. But expensive.

Supercritical Fluids

At ordinary pressures (1 atmosphere or so) substances above their critical temperature are gases and under their critical temperature become liquids. If you increase the pressure enough a substance will become both gas-like and liquid like; it will expand to fill a container and still have the density of a liquid.

These supercritical liquids make great solvents and are used in a number of industrial applications (decaffienating coffee, dry-cleaning, etc.).

It turns out that they can be used for food preservation and are now used to sterilize tissues (bones, ligaments, tendons) that are to be surgically implanted.

CO_2 is often used; it requires a pressure of 73 atmospheres (a fair amount of pressure) but can be done at a temperature of roughly 31º C.

Peroxygens (Oxidizing Agents)

Peroxygens exert their effect by oxidizing molecules inside the cell.

Hydrogen peroxide (H_2O_2) - not a good antiseptic because it is rapidly detoxified by catalase released by injured cells in a wound but does make a good disinfectant on non-living surfaces.

At high concentrations it is sporocidal and overcomes the catalase/peroxidase of aerobes and facultative anaerobes. Is used in the food industry for aseptic packaging and for contact lens disinfection.

Can be used as a gas but doesn't penetrate as well as ethelene oxide and can't be used to sterilize liquids and textiles.

Peracetic acid - very good liquid disinfectant, highly sporocidal, isn't affected much by the presence of organic material, and leaves no toxic byproducts behind.

Used for sterilizing food processing and medical equipment and is approved for washing fruits and vegetables.

Ozone (O_3) - highly reactive, forms when oxygen is passed through an electrical field.

Good for eliminating odors and tastes; used to supplement chlorination of water (more effective than chlorine but unstable in water) and to keep Hemmingway's from smelling like last week's socks.

Benzoyl peroxide - over the counter acne medication

Reproduction in Bacteria - Vegetative, Asexual and Sexual Methods

Bacteria are the simplest, the smallest, and the most successful microorganisms. They were first discovered by **Anton Leeuwenhoek** (1676). In the five kingdom classification, they are placed in Kingdom Monera.

Bacteria reproduce by **Vegetative, asexual and sexual** methods.

Vegetative reproduction includes **Budding, Fragmentation** and **Binary fission**

Budding:

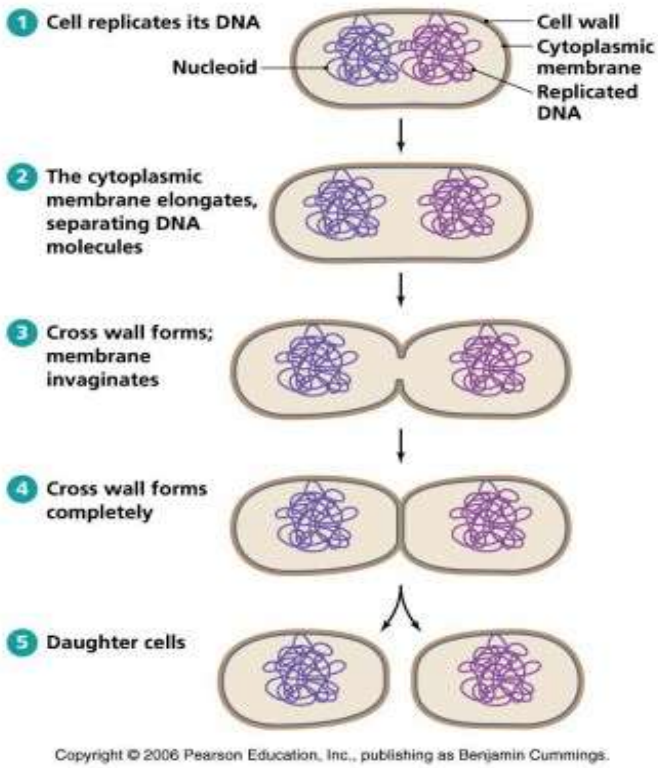
- In this case, a small protuberance, called bud, develops at one end of the cell. Genome replication follows, and one copy of the genome gets into the bud. Then the bud enlarges, eventually become a daughter cell and finally gets separated from the parent cell.

Fragmentation:

- Mostly during unfavorable conditions, bacterial protoplasm undergoes compartmentalization and subsequent fragmentation, forming minute bodies called **gonidia.** Under favorable conditions, each gonidium grows to a new bacterium. It becomes apparent that prior to fragmentation the bacterial genome has to undergo repeated replication so that each fragment gets a copy of it.

Binary fission:

- It is the commonest type of reproduction under favorable conditions in which cell divides into two similar daughter cells. During the process, the bacterial chromosomes get attached to the cell membrane and replicates to the bacterial chromosomes. As the cell enlarges the daughter chromosomes gets separated. A cross wall is formed between the separating daughter chromosomes. It divides the cell into two daughter cells. The daughter cells soon grow to maturity within 20-30 minutes. Under favorable conditions many bacteria divide once in 20-30 minutes.

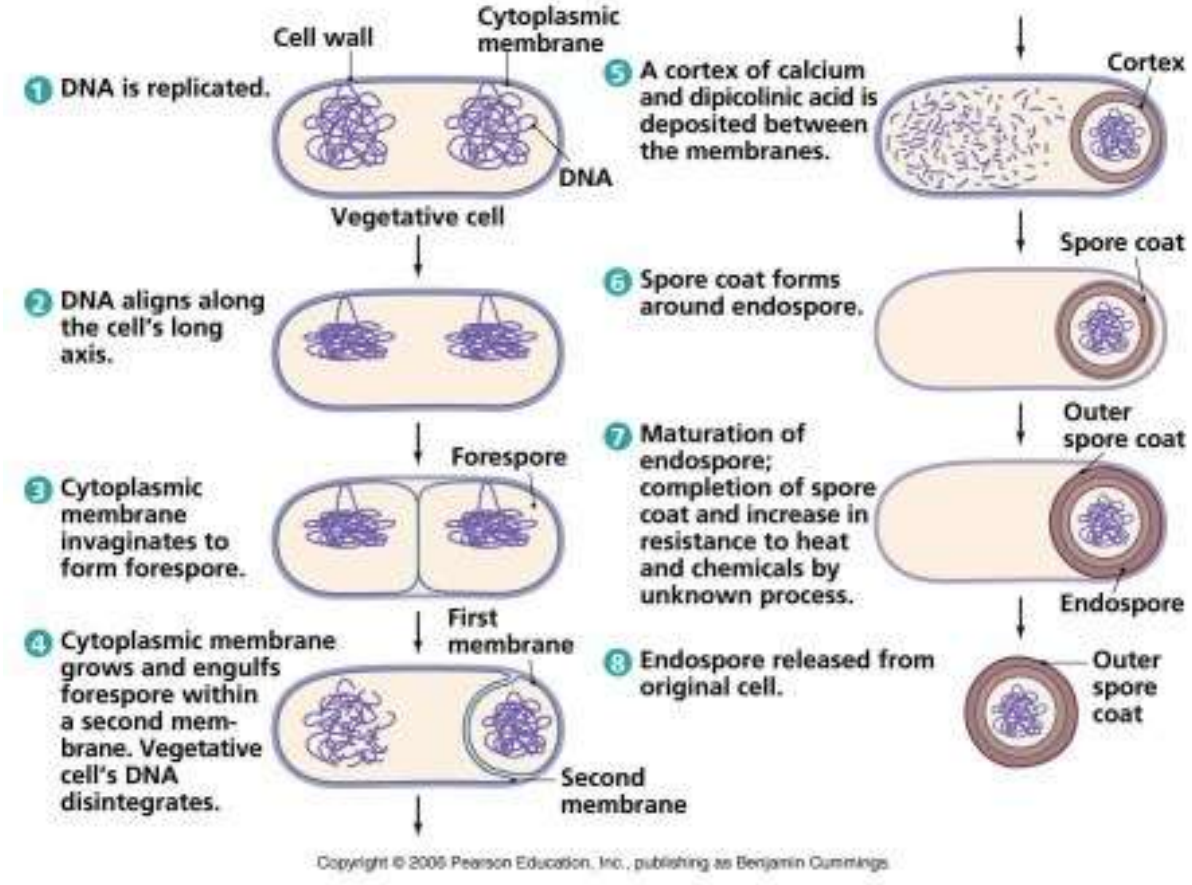


Binary Fission

Asexual reproduction

takes place by endospore formation, conidia and zoo spores.

- **Endospore formation:** Endospore are resting spores formed in some gram positive bacteria (Bacillus and Clostridium) during unfavourable conditions. They are formed within the cells. During this process a part of the protoplast becomes concentrated around the chromosome. A hard resistant wall is secreted around it. The rest of the bacterial cell degenerates; Endospore are very resistant to extreme physical conditions and chemicals. During favourable conditions the spore wall gets ruptured and the protoplasmic mass gives rise to a new bacterium.



Endospore Formation

Sexual reproduction occurs in the form of **genetic recombination.** There are three main methods of Genetic Recombination: Transformation, Transduction and Conjugation.

- **Transformation:** Here genetic material of one bacterial cell goes into another bacterial cell by some unknown mechanism and it converts one type of bacterium into another type (non capsulated to capsulated form). This was first studied by Griffith (1928) in *Diplococcus pneumonia.*
- **Transduction:** In this method, genetic material of one bacterial cell goes to other bacterial cell by agency of bacteriophages or phages (viruses, infecting bacteria). It was first of all reported in Salmonella typhimeurium by Zinder and Lederberg (1952).

- **Conjugation:** It was first reported by Lederberg and Tatum (1946) in E.coli bacteria. Cell to cell union occurs between two bacterial cells and genetic material (DNA) of one bacterial cell goes to another cell lengthwise through conjugation tube which is formed by sex pili.

Generation Time

The time required for a cell to divide or a population to double is known as the generation time.

Most bacteria have a doubling time of 1-3 hours, although some may be greater than 24 hours.

E. coli may have a doubling time of 20 minutes; get 20 generations in 7 hours, going from one cell to one million cells.

Logarithmic Representation of Bacterial Populations

Bacterial division occurs according to a logarithmic progression (two cells, four cells, eight cells, etc.).

Numbers of Cells	Numbers Expressed as a Power of 2	Visual Representation of Numbers
1	2^0	●
2	2^1	●●
4	2^2	●●●●
8	2^3	●●●●●●●●
16	2^4	●●●●●●●●●●●●●●●●
32	2^5	●●●●●●●●●●●●●●●●●●●●●●●●●●●●●●

(a) Visual representation of increase in bacterial number over five generations. The number of bacteria doubles in each generation. The superscript indicates the generation, that is, $2^5 = 5$ generations.

Generation Number	Number of Cells	Log_{10} of Number of Cells
0	$2^0 = 1$	0
5	$2^5 = 32$	1.51
10	$2^{10} = 1{,}024$	3.01
15	$2^{15} = 32{,}768$	4.52
16	$2^{16} = 65{,}536$	4.82
17	$2^{17} = 131{,}072$	5.12
18	$2^{18} = 262{,}144$	5.42
19	$2^{19} = 524{,}288$	5.72
20	$2^{20} = 1{,}048{,}576$	6.02

(b) Conversion of the number of cells in a population into the logarithmic expression of this number. To arrive at the numbers in the center column, use the y^x key on your calculator. Enter 2 on the calculator; press y^x; enter 5; then press the = sign. The calculator will show the number 32. Thus, the fifth-generation population of bacteria will total 32 cells. To arrive at the numbers in the right-hand column, use the log key on your calculator. Enter the number 32; then press the log key. The calculator will show, rounded off, that the log_{10} of 32 is 1.51.

Figure 6.12 - Overview

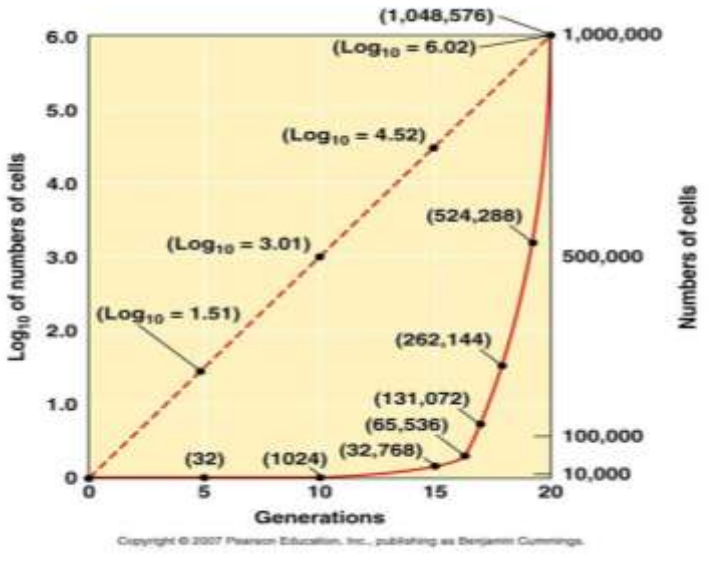


Figure 6.13

Phases of Growth

During the lag phase, there is little or no change in the number of cells, but metabolic activity is high.

- DNA and enzyme synthesis occurs; may last from 1 hour to several days.

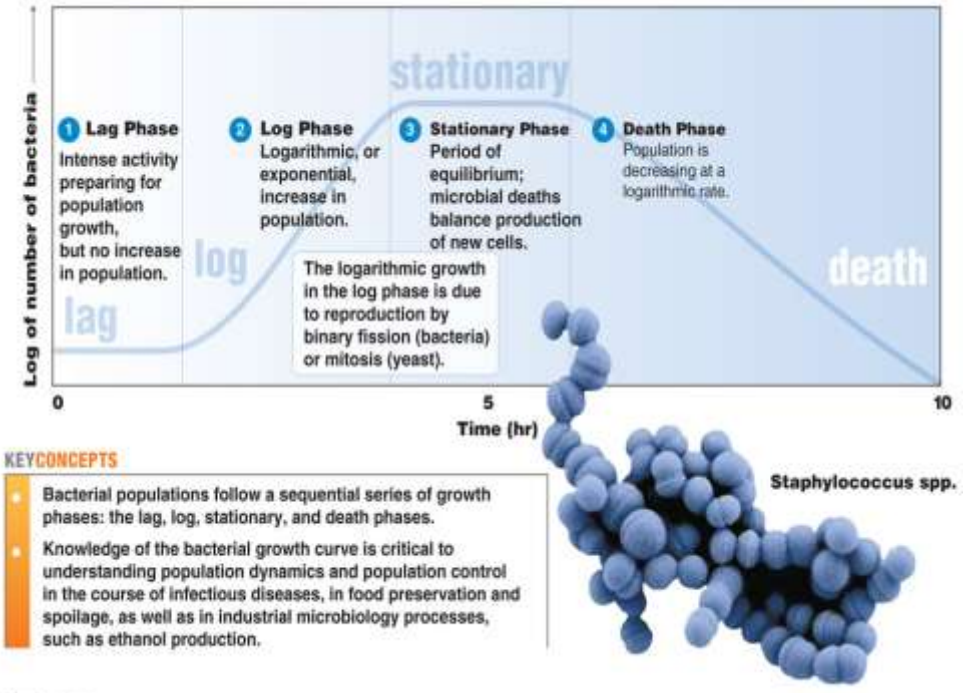
During the log phase, the bacteria multiply at the fastest rate possible under the conditions provided.

- Maintained by use of a chemostat – constant supply of fresh media

During the stationary phase, there is an equilibrium between cell division and death.

- Nutrients are exhausted and waste products build up; pH increases.

During the death phase, the number of deaths exceeds the number of new cells formed.



The different phases of bacterial growth.

The increase in the cell size and cell mass during the development of an organism is termed as growth. It is the unique characteristics of all organisms. The organism must require certain basic parameters for their energy generation and cellular biosynthesis. The growth of the organism is affected by both physical and Nutritional factors. The physical factors include the pH, temperature, Osmotic pressure, Hydrostatic pressure, and Moisture content of the medium in which the organism is growing. The nutritional factors include the amount of Carbon, nitrogen, Sulphur, phosphorous, and other trace elements provided in the growth medium. Bacteria are unicellular (single cell) organisms. When the bacteria reach a certain size, they divide by binary fission, in which the one cell divides into two, two into four and continue the process in a geometric fashion. The bacterium is then known to be in an actively growing phase. To study the bacterial growth population, the viable cells of the bacterium should be inoculated on to the sterile broth and incubated under optimal growth conditions. The bacterium starts utilising the components of the media and it will increase in its size and cellular mass. The dynamics of the bacterial growth can be studied by plotting the cell growth (absorbance) versus the incubation time or log of cell number versus time. The curve thus obtained is a sigmoid curve and is known as a standard growth curve. The increase in the cell mass of the organism is measured by using the Spectrophotometer. The Spectrophotometer measures the turbidity or Optical density which is the measure of the amount of light absorbed by a bacterial suspension. The degree of turbidity in the broth culture is directly related to the number of microorganism present, either viable or dead cells, and is a convenient and rapid method of measuring cell growth rate of an organism. Thus the increasing the turbidity of the broth medium indicates increase of the microbial cell mass (Fig 1) .The amount of transmitted light through turbid broth decreases with subsequent increase in the absorbance value.

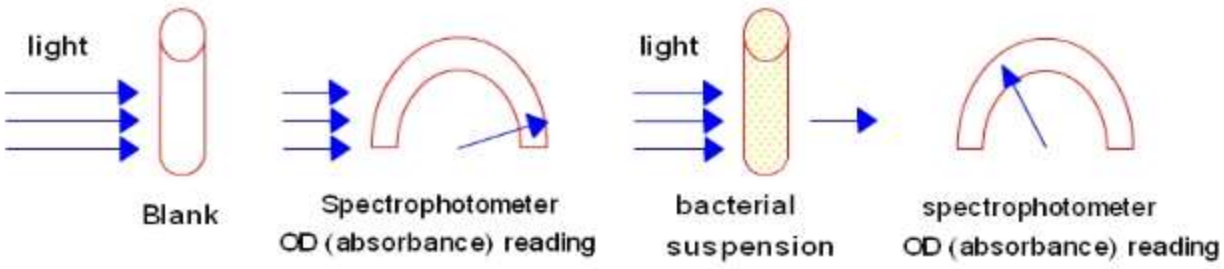


Fig 1: Absorbance reading of bacterial suspension

The growth curve has four distinct phases

1. Lag phase

When a microorganism is introduced into the fresh medium, it takes some time to adjust with the new environment. This phase is termed as Lag phase, in which cellular metabolism is accelerated, cells are increasing in size, but the bacteria are not able to replicate and therefore no increase in cell mass. The

length of the lag phase depends directly on the previous growth condition of the organism. When the microorganism growing in a rich medium is inoculated into nutritionally poor medium, the organism will take more time to adapt with the new environment. The organism will start synthesising the necessary proteins, co-enzymes and vitamins needed for their growth and hence there will be a subsequent increase in the lag phase. Similarly when an organism from a nutritionally poor medium is added to a nutritionally rich medium, the organism can easily adapt to the environment, it can start the cell division without any delay, and therefore will have less lag phase it may be absent.

2. Exponential or Logarithmic (log) phase

During this phase, the microorganisms are in a rapidly growing and dividing state. Their metabolic activity increases and the organism begin the DNA replication by binary fission at a constant rate. The growth medium is exploited at the maximal rate, the culture reaches the maximum growth rate and the number of bacteria increases logarithmically (exponentially) and finally the single cell divide into two, which replicate into four, eight, sixteen, thirty two and so on (That is 2^0, 2^1, 2^2, 2^3.....2^n, n is the number of generations) This will result in a balanced growth. The time taken by the bacteria to double in number during a specified time period is known as the generation time. The generation time tends to vary with different organisms. *E.coli* divides in every 20 minutes, hence its generation time is 20 minutes, and for *Staphylococcus aureus* it is 30 minutes.

3. Stationary phase

As the bacterial population continues to grow, all the nutrients in the growth medium are used up by the microorganism for their rapid multiplication. This result in the accumulation of waste materials, toxic metabolites and inhibitory compounds such as antibiotics in the medium. This shifts the conditions of the medium such as pH and temperature, thereby creating an unfavourable environment for the bacterial growth. The reproduction rate will slow down, the cells undergoing division is equal to the number of cell death, and finally bacterium stops its division completely. The cell number is not increased and thus the growth rate is stabilised. If a cell taken from the stationary phase is introduced into a fresh medium, the cell can easily move on the exponential phase and is able to perform its metabolic activities as usual.

4. Decline or Death phase

The depletion of nutrients and the subsequent accumulation of metabolic waste products and other toxic materials in the media will facilitates the bacterium to move on to the Death phase. During this, the bacterium completely loses its ability to reproduce. Individual bacteria begin to die due to the unfavourable conditions and the death is rapid and at uniform rate. The number of dead cells exceeds the number of live cells. Some organisms which can resist this condition can survive in the environment by producing endospores.

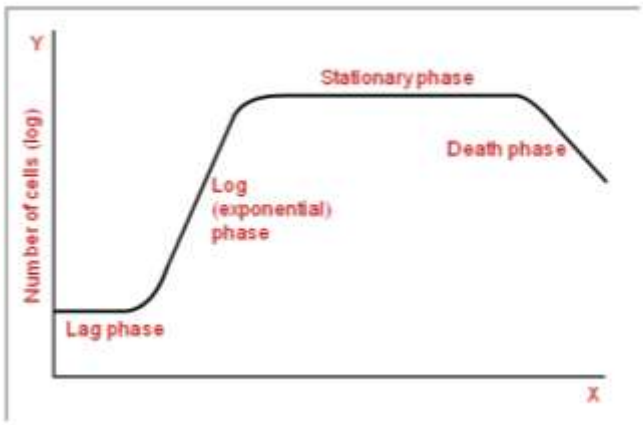
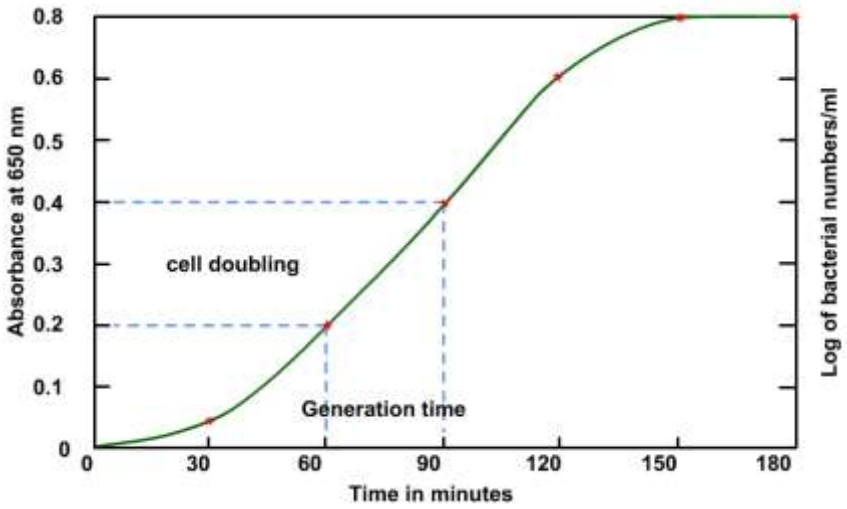


Fig 2: Different phases of growth of a bacteria

CALCULATION:

The generation time can be calculated from the growth curve



Calculation of generation time

The exactly doubled points from the absorbance readings were taken and, the points were extrapolated to meet the respective time axis.

Generation Time = (Time in minutes to obtain the absorbance 0.4) – (Time in minutes to obtain the absorbance 0.2)
=90-60
= 30 minutes

Let No = the initial population number

Nt = population at time t

N = the number of generations in time t

Therefore,

$$Nt = No \times 2^n \ldots\ldots\ldots (1)$$

$$\log Nt = \log No + n \log 2$$

Therefore,

$$n = (\log Nt - \log No) / \log 2$$

$$n = (\log Nt - \log No) / 0.301 \ldots\ldots\ldots (2)$$

The growth rate can be expressed in terms of mean growth rate constant (k), the number of generations per unit time.

$$k = n / t$$

$$k = (\log Nt - \log No) / (0.301 \times t) \ldots\ldots\ldots\ldots (3)$$

Mean generation time or mean doubling time (g), is the time taken to double its size.

Therefore,

$$Nt = 2No \ldots\ldots\ldots\ldots (4)$$

Substituting equation 4 in equation 3

$$k = (\log Nt - \log No) / (0.301 \times t)$$
$$= (\log 2N0 - \log No) / (0.301 \times t)$$
$$= \log 2 + (\log No - \log No) / 0.301\ g$$

(Since the population doubles t= g)

Therefore,

$$k = 1 / g$$

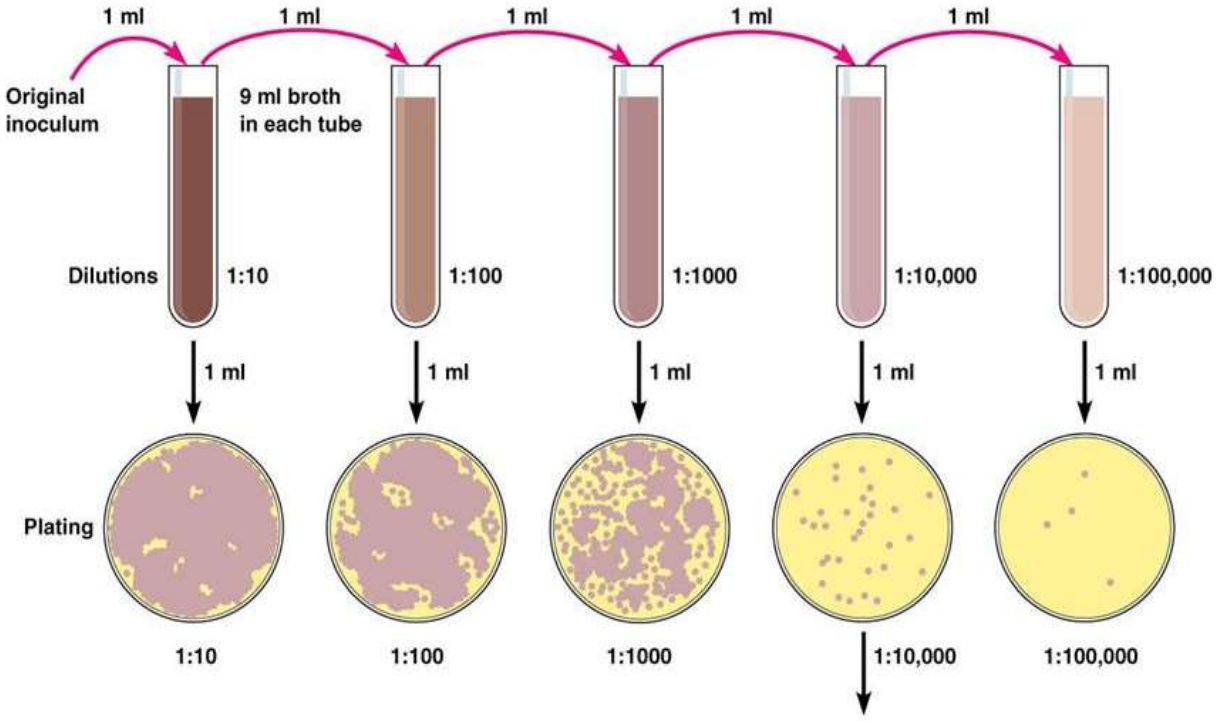
Mean growth rate constant, $k = 1 / g$

Mean generation time, $g = 1 / k$

The Growth Of Bacterial Cultures

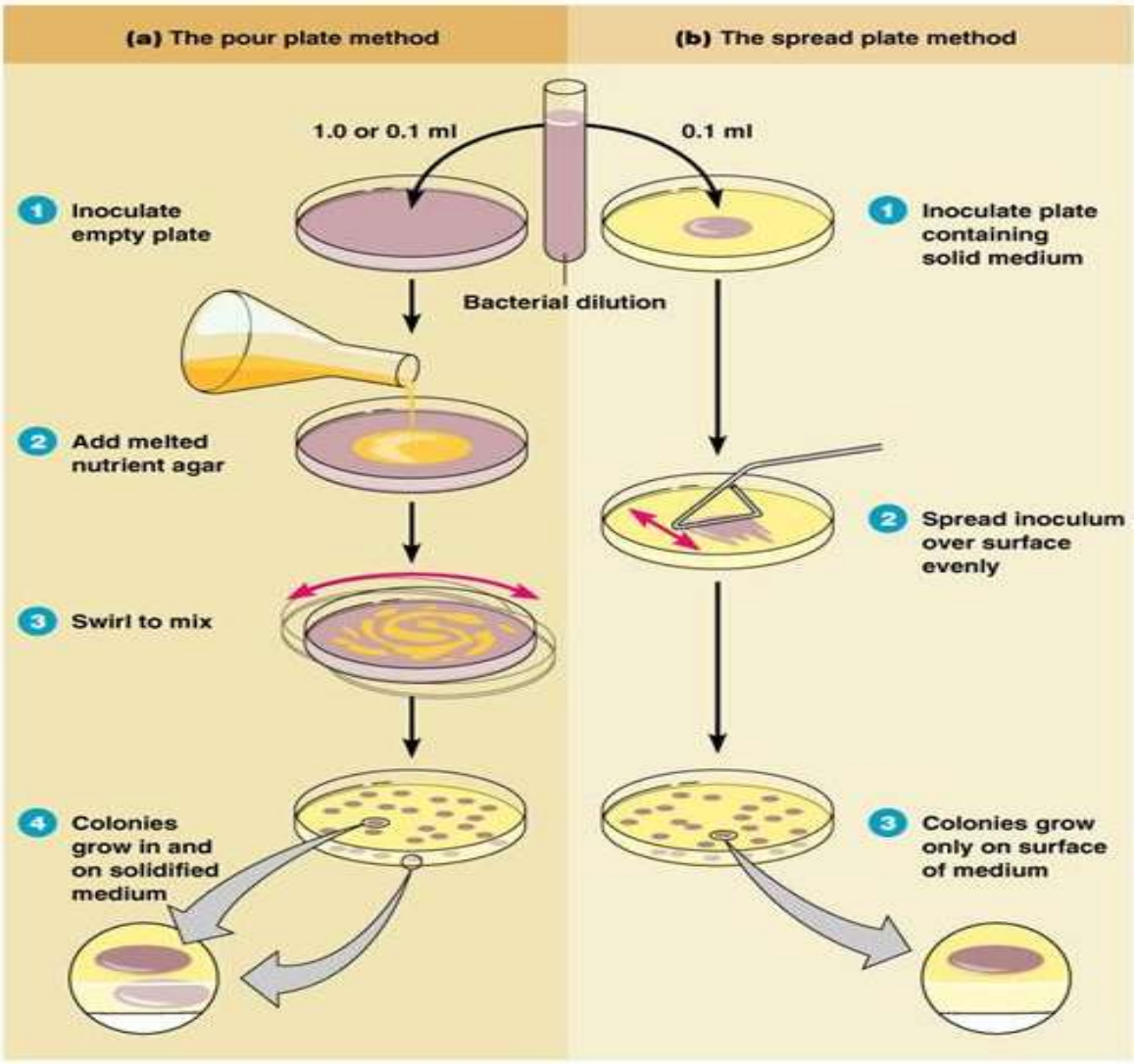
Direct Measurement of Microbial Growth

A standard plate count reflects the number of viable microbes and assumes that each bacterium grows into a single colony.
Because it is impossible to say that each colony actually arose from an indivual cell (cells clump, fact of life) plate counts are reported as the number of colony-forming units (CFU) instead of the number of cells.
If the concentration of bacteria is too great the colonies will grow into each other and the plate will be uncountable.
To insure a countable plate a series of dilutions should be plated. The serial dilutions should give at least one countable plate in the series (25-250 or 30-300, depending on preference of the individual lab).



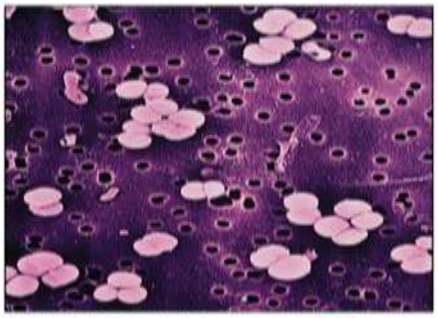
Calculation: Number of colonies on plate × reciprocal of dilution of sample = number of bacteria/ml
(For example, if 32 colonies are on a plate of $^{1}/_{10,000}$ dilution, then the count is 32 × 10,000 = 320,000 bacteria/ml in sample.)

A plate count may be done on plates prepared by either the pour plate method or the spread plate method.

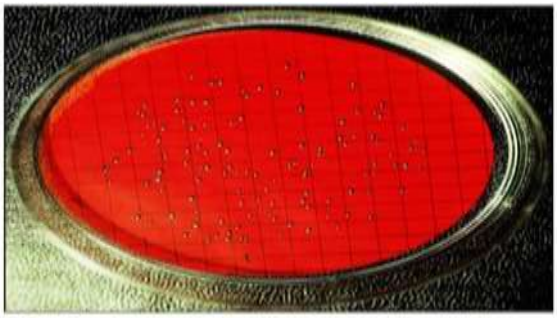


Some samples may be very dilute to begin with, so a plate prepared with undiluted inoculum may still have too few colonies to be countable. In this case the concentration of bacteria can be increased by filtering the sample.

In filtration, bacteria are retained on the surface of a membrane filter and then transferred to a culture medium to grow and subsequently be counted.



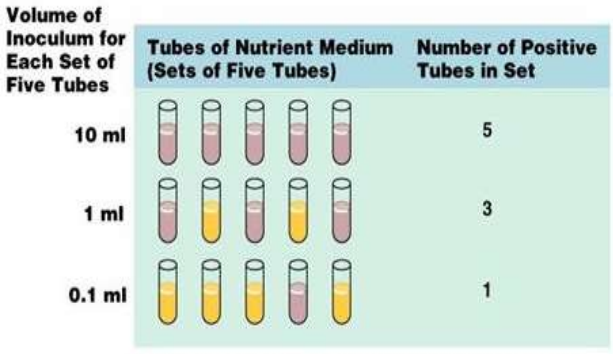
(a) The bacteria in 100 ml of water were sieved out onto the surface of a membrane filter.



(b) A filter such as shown in photo (a), with the bacteria much more widely spaced, was placed on a pad saturated with liquid Endo medium, which is selective for gram-negative bacteria. The individual bacteria grew into visible colonies. One hundred twenty-four colonies are visible, so we would record 124 bacteria per 100 ml of water sample.

The most probable number (MPN) method can be used for microbes that will grow in a liquid medium; it is a statistical estimation.

- A dilution series to no growth is prepared and the combination of positives is used to look the most probable number up in a table (see ***(b) MPN Table*** below).
- Used for microbes that won't grow on solid media or are grown in differential liquid media for identification purposes.

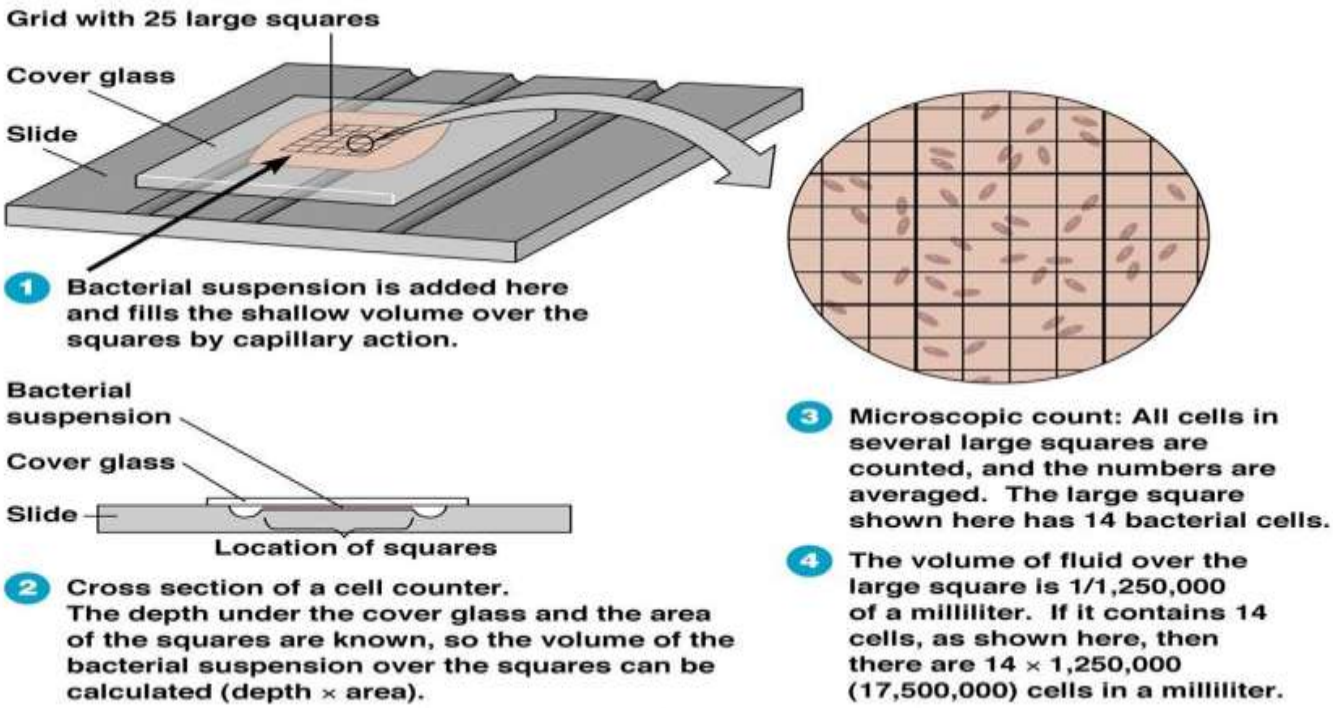


(a) Most probable number (MPN) dilution series. In this example, there are three sets of tubes and five tubes in each set. Each tube in the first set of five tubes receives 10 ml of the inoculum, such as a sample of water. Each tube in the second set of five tubes receives 1 ml of the sample, and the third set, 0.1 ml each. There were enough bacteria in the sample so that all five tubes in the first set showed bacterial growth and were recorded as positive. In the second set, which received only one-tenth as much inoculum, only three tubes were positive. In the third set, which received one-hundredth as much inoculum, only one tube was positive.

Combination of Positives	MPN Index/ 100 ml	95% Confidence Limits	
		Lower	Upper
4-2-0	22	9	56
4-2-1	26	12	65
4-3-0	27	12	67
4-3-1	33	15	77
4-4-0	34	16	80
5-0-0	23	9	86
5-0-1	30	10	110
5-0-2	40	20	140
5-1-0	30	10	120
5-1-1	50	20	150
5-1-2	60	30	180
5-2-0	50	20	170
5-2-1	70	30	210
5-2-2	90	40	250
5-3-0	80	30	250
5-3-1	110	40	300
5-3-2	140	60	360

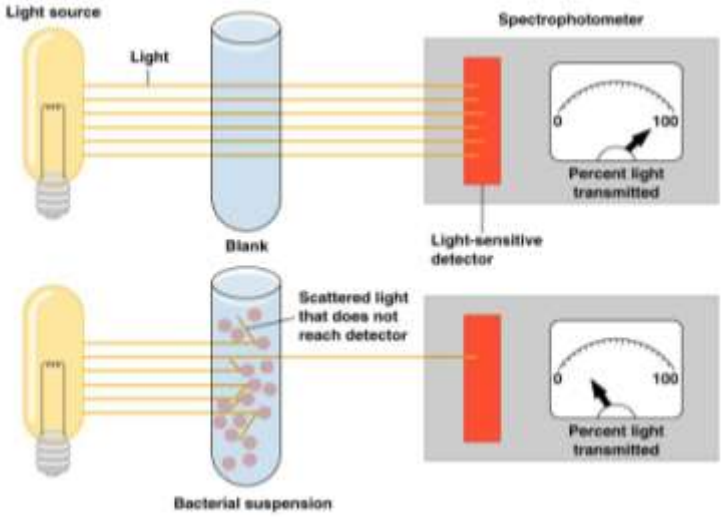
(b) MPN table. MPN tables enable us to calculate for a sample the microbial numbers that are statistically likely to lead to such a result. The number of positive tubes is recorded for each set: in the shaded example, 5, 3, and 1. If we look up this combination in an MPN table, we find that the MPN index per 100 ml is 110. Statistically, this means that 95% of the water samples that give this result contain 40–300 bacteria, with 110 being the most probable number.

In a direct microscopic count, the microbes in a measured volume of a bacterial suspension are counted with the use of a specially designed slide (a Petroff-Hausser cell counter). It can be done automatically with an electronic cell counter (Coulter counter).
Hard to count motile cells
Dead cells look the same as live cells
No incubation time is required



Estimating Bacterial Numbers by Indirect Methods

A spectrophotometer is used to determine turbidity by measuring the amount of light that passed through a suspension of cells.



An indirect way of estimating bacterial numbers is measuring the metabolic activity of the population (for example, acid production or oxygen consumption).

For filamentous organisms such as fungi, measuring dry weight is a convenient method of growth measurement.

You won't know how the measurement of turbidity with a spectrophotometer or measurement of metabolic activity or any other indirect measurement correlates to cell number unless you do a standard curve

Specific growth rate:

It is defined as the increase in cell mass per unit time, e.g., grams cells (g) per gram cells (g) per hour. The specific growth rate is commonly given by the symbol, μ (mu), and the most common units are in reciprocal hours (h−1); however, it can also be expressed in reciprocal seconds (s−1) or minutes (min−1) or any other units of time.

Specific growth rate constant:

Specific growth rate constant is a way of measuring how fast the cells are dividing in a culture. It is defined on the basis of doubling rate.

Exponential phase and calculating growth rates:

The growth rate of a microbial population is a measure of the increase in biomass over time and it is determined from the exponential phase. Growth rate is one important way of expressing the relative ecological success of a species or strain in adapting to its natural environment or the experimental environment imposed upon it. The duration of exponential phase in cultures depends upon the size of the inoculum, the growth rate and the capacity of the medium and culturing conditions to support microbial growth. Biomass estimates need to be plotted over time, and logistical constraints determine their frequency but once every one to two days is generally acceptable. Cell count and dry weight are common units of biomass determination.

Possible Questions

Unit – III

Two marks

1. Define culture media
2. Define growth.
3. Define generation time. How are bacterial growth classified?
4. Define simple and complex media.
5. Describe about turbidostat and chemostat.

Eight marks

1. Write short notes on factors affecting microbial growth.
2. Distinguish between moist and dry heat sterilization with apt examples.
3. Write short notes on the use of chemical methods for controlling microbes.
4. Write short notes bacterial motility and cell count techniques.
5. Write short notes on calculation of specific growth rate.

S.no	Question	Opt 1	Opt 2	Opt 3	Opt 4	Answer
1	TVC means _____	Total Viable Counts	Total NonViable Counts	Time Variable Counts	Time nonVariable Counts	Total Viable Counts
2	_____ is Mass of living matter present.	biodiverse	Biomass	biogroup	bioaccumulation	Biomass
3	_____ is derived from an environment other than that in which it is found.	autothonous	Allochthonous	hetrothonous	xenothonous	autothonous
4	_____are organism which grows at high pressure rather than at atmospheric pressure.	Barophile	halophile	thermophile	neutrophil	Barophile
5	_____are organism which grows at high salinity.	Barophile	halophile	thermophile	neutrophil	halophile
6	Living together of two organisms with mutual advantage and without losing their identity is called as	Antagonism	Commensalism	Symbiosis	Mutualism	Commensalism
7	Chemotaxis is a phenomenon of	swimming away of bacteria	swimming towards bacteria	swimming away or towards of bacteria in presence of chemical compound	Swimming in media	swimming away or towards of bacteria in presence of chemical compound
8	The structure responsible for movement of bacteria is	pilli	flagella	sheath	capsule	flagella

9	The structure responsible for conjuction of bacteria is	pilli	flagella	sheath	capsule	pilli
10	The structure responsible for protection of bacteria is	pilli	flagella	sheath	capsule	capsule
11	BGA expanded as	Blue Green Algae	Blue Grown Algae	Blue non Grown Algae	Brown Green Algae	Blue Green Algae
12	The unifying feature of the archaea that distinguishes them from the bacteria is	habitats which are extreme environments with regard to acidity	absence of a nuclear membrane temperature	presence of a cell wall containing a characteristic outer membrane	cytoplasmic ribosomes that are 70S	habitats which are extreme environments with regard to acidity
13	The Archaea include all of the following except	methanogens	halophiles	thermoacidophiles	cyanobacteria	cyanobacteria
14	Cytoplasmic inclusions exclude	ribosomes	mesosomes	fat globules	capsule	capsule
15	_____ of an atom is a measure of its power to attract electrons that it is sharing in a covalent bond.	electronegativity	electronaffinity	electropositivity	electroavidity	electroavidity
16	The distance between two atomic nuclei in a covalent molecule is called _____.	Bond angle	Bond circle	Bond distance	Bond area	Bond distance
17	_____ Cytochrome oxidase is	a3	aa3	a	n	aa3
18	Ionization of water can be described by an _____ constant.	molecular	ionization	equilibrium	ionizable	ionization
19	The number of H+ ions	Alkalinity	basicity	acidity	avidity	acidity

	present in a solution is a measure of _____ of the solution.					
20	The _____ of a solution is dependent upon the number for hydroxyl ions present.	basicity	acidity	alkalinity	neutrality	alkalinity
21	_____ is defined as the negative logarithm of hydrogen ion concentration.	pH	[–H]	[-OH]	H+	pH
22	The pH of pure water at 25°C is _____.	6	8	7	10	7
23	A condition called _____ occurs when pH of the blood is higher than normal.	acidosis	alkalosis	basidosis	avidosis	alkalosis
24	A ____ is defined as a substance that has a greater tendency to lose its proton and completely dissociates.	strong acid	weak acid	strong base	weak base	strong acid
25	A compound which can accept a pair of electrons from a base is called _____.	electrophile	nucleophile	extremophile	acidophile	electrophile
26	_____ is used to determine the amount of an acid in a given solution.	centrifugation	separation	titration	neutralization	titration
27	_____ resists changes in pH on the addition of acid or base.	buffer	pH paper	acidohile	electrophile	buffer
28	The pK_a of the weak acid is	Lowry-Bronsted	Lowry-	Henderson-	Hasselbach	Henderson-

	given by a simple expression called _____ equation.		Hasselbach	Hasselbach		Hasselbach
29	The principal buffer for media is _____.	bicarbonate	phosphate	protein	water	water
30	The pH ofmedia is maintained at _____.	7.8	7.4	6.4	7.1	7.4
31	The branch of microbiology that deals with the study of bacteria is called _____	parasitology	bacteriology	myology	fungyology	bacteriology
32	The study and effect of toxins and their effects is called _____	toxicology	Mycotoxin	Mycotoxicology	bacteriotoxin	toxicology
33	A character that promotes the pathogenic potential of fungus is called as _____	toxin	enzyme	byproducts	virulence factor	virulence factor
34	Isolation of bacteria in pure is not possible in	solid media	Liquid media	Differential media	Selective media	Liquid media
35	_____Used as an indicator in Mcintosh anaerobic jar for preparation of anaerobic culture.	Methylene blue	Phenol red	Bromothymol blue	Neutral red	Methylene blue
36	_____ is a simple media	Blood agar	Robertson's medium	Mac conkey's agar	Nutrient agar	Nutrient agar
37	_____ media contain additives that enhance the growth of the desired organism by inhibiting other organisms.	Simple	Transport	Selective	Indicator	Selective

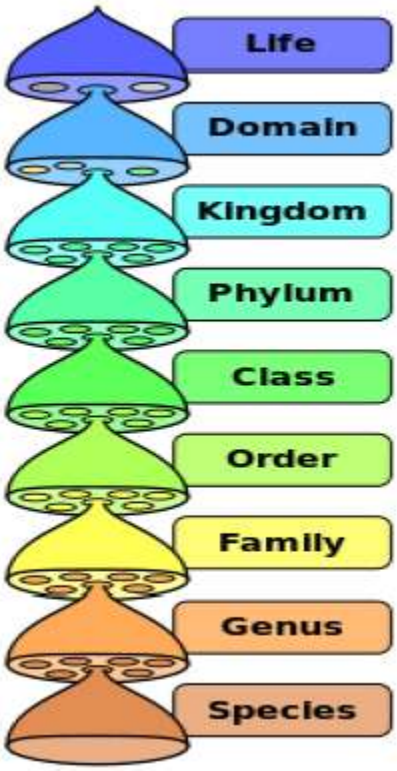
38	When S.typhi is grown in Wilson and Blair medium, containing sulphite, the bacterial colonies become due to reduction of sulphite.	Black	Green	Pink	Yellow	Black
39	Mac Conkey's agar is a _____ medium	Selective	Differential	Both a and b	Enrichment	Both a and b
40	_____ is a transport medium	Mac Conkey	Blood agar	Stuart's Media	Nutrient agar	Stuart's Media
41	In Mac conkey's Medium, lactose fermenters produce _____ colonies.	Pink	Yellow	Black	Green	Pink
42	Assay medium is also known as _____	Selective media	Complex media	Production media	Indicator media	Production media
42	Assay medium is used to estimate the quantity of	Oxygen	Vitamins	Gas	Bacteria	Vitamins
43	Petri dishes were invented by	Robert Koch	Louis Pasteur	Richard Petri	Burnet	Richard Petri
44	Culture media can be solidified by the addition of	Blood	Agar	Sugar	Peptone	Agar
45	The function of agar in culture medium is	Carbon source	Nitrogen source	Buffering agent	Solidifying agent	Solidifying agent
46	Crystal violet inhibit the growth of _____ bacteria	Aerobic	Anaerobic	Gram positive	Gram negative	Gram positive
47	The differential medium used for isolation of Escherichia coli.	Mac conkey agar	Eosin methylene blue agar	Nutrient agar	Stuart's medium	Eosin methylene blue agar
48	Anaerobic bacteria can be	Methylene blue	Thioglycolate	Palladium	Serum	Thioglycolate

	isolated by pour plate technique using a medium containing a reducing agent such as					
49	Cultures of organism that are maintained in the laboratory for stud and reference are Called ----------	Stab culture	Stock culture	Broth culture	Mixed culture	Stock culture
50	Media that contain some ingredients of unknown chemical composition are	Defined media	Synthetic media	Complex media	Natural media	Complex media
51	Solidifying used other than agar is	Thio glycollate	Silica gel	Soya meal	Casein	Silica gel
52	_____ is an enriched media	Nutrient agar	Tryptic soy agar	Blood agar	Macconkey agar	Blood agar
53	All of the following are true about agar except it	liquefies at 100°C	is a polysaccharide derived from a red alga	Solidifies at approximately 40°C	is metabolized by many bacteria	is metabolized by many bacteria
54	For photolithotrophic autotroph; in addition to light, _____would be most essential to maintaining its growth	a continual supply of abundant oxygen	a nutrient medium containing glucose	a source of CO2	a source of O2	a source of CO_2
55	For chemolithotrophic autotroph; _____ would be most essential to maintaining growth of the organism	a nutrient medium containing glucose	nutrient medium containing glucose	a source of reduced inorganic compound such as NH4	a source of carbon compounds	a source of reduced inorganic compound such

						as NH4
56	Which of the following uses radiant energy as their energy source?	Chemotroph	Lithotroph	Autotroph	Phototroph	Phototroph
57	Addition of blood to a culture medium only is an example of a _____	Differential media	Chemically defined media	Simple media	Selective media	Differential media
58	Vitamins are _____	the building blocks of proteins	a part of an enzyme cofactor	used for transfer of energy and information within the cell	a major energy source for bacteria	a part of an enzyme cofactor
59	Addition of salt to a culture medium is an example of a	Differential media	Chemically defined media	Simple media	Selective media	Selective media

Aim and principles of classification, systematics and taxonomy, concept of species, taxa, strain; conventional, molecular and recent approaches to polyphasic bacterial taxonomy, evolutionary chronometers, rRNA oligonucleotide sequencing, signature sequences, and protein sequences. Differences between eubacteria and Archaebacteria.

Bacterial taxonomy



The hierarchy of biological classification's eight major taxonomic ranks. Intermediate minor rankings are not shown.

Bacterial taxonomy is the taxonomy, i.e. the rank-based classification, of bacteria. In the scientific classification established by Carl von Linné,[1] each species has to be assigned to a genus (binary nomenclature), which in turn is a lower level of a hierarchy of ranks (family, suborder, order, subclass, class, division/phyla, kingdom and domain). In the currently accepted classification of life, there are three domains (Eukaryotes, Bacteria and Archaea), which, in terms of taxonomy, despite following the same principles have several different conventions between them and between their subdivisions as are studied by different disciplines (botany, zoology, mycology and microbiology), for example in zoology there are type specimens, whereas in microbiology there are type strains.

Diversity

Prokaryotes share many common features, such as lack of nuclear membrane, unicellularity, division by binary-fission and generally small size. The various species differ amongst each other based on several characteristics, allowing their identification and classification. Examples include:

Phylogeny: All bacteria stem from a common ancestor and diversified since, consequently possess different levels of evolutionary relatedness)

Metabolism: Different bacteria may have different metabolic abilities

Environment: Different bacteria thrive in different environments, such as high/low temperature and salt Morphology: There are many structural differences between bacteria, such as cell shape, Gram stain (number of lipid bilayers) or bilayer composition Pathogenicity: Some bacteria are pathogenic to plants or animals

Classification history

Bacteria were first observed by Antonie van Leeuwenhoek in 1676, using a single-lens microscope of his own design. He called them "animalcules" and published his observations in a series of letters to the Royal Society O. F. Müller (1773, 1786) described eight species of the genus *Vibrio* (in Infusoria), three of which were spirilliforms. The term *Bacterium* (a genus) was introduced much later, by Christian Gottfried Ehrenberg in 1838.

Classical classification

Placement

Bacteria were first classified as plants constituting the class *Schizomycetes*, which along with the *Schizophyceae* (blue green algae/*Cyanobacteria*) formed the phylum *Schizophyta*.

Haeckel in 1866 placed the group in the phylum *Moneres* (from μονήρης: simple) in the kingdom *Protista* and defines them as completely structureless and homogeneous organisms, consisting only of a piece of plasma. He subdivided the phylum into two groups:

die Gymnomoneren (no envelope)

o *Protogenes*—such as *Protogenes primordialis*, now classed as a eukaryote and not a bacterium

o *Protamaeba*—now classed as a eukaryote and not a bacterium

o *Vibrio*—a genus of comma shaped bacteria first described in 1854

Bacterium—a genus of rod shaped bacteria first described in 1828, that later gave its name to the members of the Monera, formerly referred to as "a moneron" (plural "monera") in English and "eine Moneren"(fem. plural "Moneres") in German

o *Bacillus*—a genus of spore-forming rod shaped bacteria first described in 1835

o *Spirochaeta*—thin spiral shaped bacteria first described in 1835

o *Spirillum*—spiral shaped bacteria first described in 1832

o etc.

die Lepomoneren (with envelope)

o *Protomonas*—now classed as a eukaryote and not a bacterium. The name was reused in 1984 for an unrelated genus of Bacteria

o *Vampyrella*—now classed as a eukaryote and not a bacterium

The group was later reclassified as the *Prokaryotes* by Chatton

The classification of *Cyanobacteria* (colloquially "blue green algae") has been fought between being algae or bacteria (for example, Haeckel classified *Nostoc* in the phylum Archephyta of Algae). In 1905 Erwin F. Smith accepted 33 valid different names of bacterial genera and over 150 invalid names, and in 1913 Vuillemin in a study concluded that all species of the *Bacteria* should fall into the genera *Planococcus, Streptococcus, Klebsiella, Merista, Planomerista, Neisseria, Sarcina, Planosarcina, Metabacterium,*

Clostridium, *Serratia*, *Bacterium* and *Spirillum*. Ferdinand Cohn recognized 4 tribes: Spherobacteria, Microbacteria, Desmobacteria, and Spirobacteria. Stanier and van Neil recognized the Kingdom Monera with 2 phyla, Myxophyta and Schizomycetae, the latter comprising classes Eubacteriae (3 orders), Myxobacteriae (1 order), and Spirochetae (1 order). Bisset distinguished 1 class and 4 orders: Eubacteriales, Actinomycetales, Streptomycetales, and Flexibacteriales. Migula, which was the most widely accepted system of its time and included all then-known species but was based only on morphology, contained the 3 basic groups, Coccaceae, Bacillaceae, and Spirillaceae but also Trichobacterinae for filamentous bacteria; Orla-Jensen established 2 orders: Cephalotrichinae (7 families) and Peritrichinae (presumably with only 1 family).

Bergey et al presented a classification which generally followed the 1920 Final Report of the SAB(Society of American Bacteriologists) Committee (Winslow et al), which divided Class Schizomycetes into 4 orders: Myxobacteriales, Thiobacteriales, Chlamydobacteriales, and Eubacteriales, with a 5th group being 4 genera considered intermediate between bacteria and protozoans: *Spirocheta*, *Cristospira*, *Saprospira*, and *Treponema.*

However, different authors often reclassified the genera due to the lack of visible traits to go by, resulting in a poor state which was summarised in 1915 by Robert Earle Buchanan By then, the whole group received different ranks and names by different authors namely

Schizomycetes (Naegeli 1857

Bacteriaceae (Cohn 1872,

Bacteria (Cohn 1872b,

Schizomycetaceae (DeToni and Trevisan 1889,

Furthermore the families into which the class was subdivided, changed from author to author and for some such as Zipf the names where in German and not in Latin. The first edition of the Bacteriological Code in 1947 sorted several problems out. A.R. Prévot's system had 4 subphyla and 8 classes as follows: Eubacteriales (classes Asporulales and Sporulales) Mycobacteriales (classes Actinomycetales, Myxobacteriales, and Azotobacteriales) Algobacteriales (classes Siderobacteriales and Thiobacteriales) Protozoobacteriales (class Spirochetales)

Linnaeus	Haeckel	Chatton	Copeland	Whittaker	Woese et al.	Cavalier-Smith
1735	1866	1925	1938	1969	1990[1]	1998

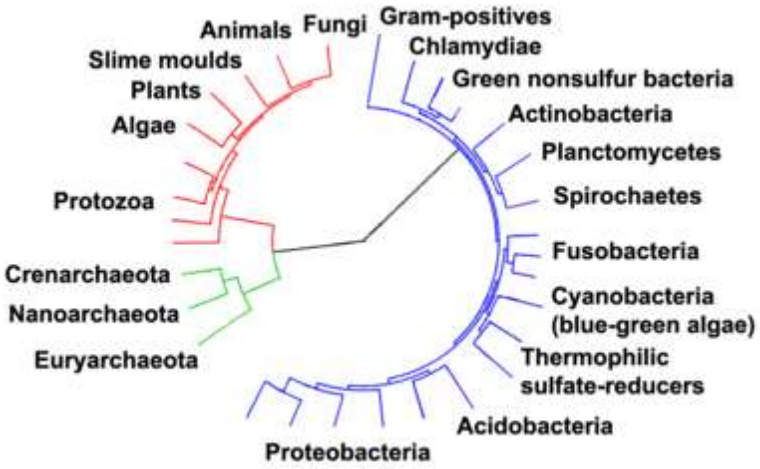
2 kingdoms	3 kingdoms	2 empires	4 kingdoms	5 kingdoms	3 domains	6 kingdoms
(not treated)	Protista	Prokaryota	Monera	Monera	Bacteria	Bacteria
					Archaea	
		Eukaryota	Protoctista	Protista	Eucarya	Protozoa
						Chromista
Vegetabilia	Plantae		Plantae	Plantae		Plantae
				Fungi		Fungi
Animalia	Animalia		Animalia	Animalia		Animalia

Subdivisions based on Gram staining
Despite there being little agreement on the major subgroups of the *Bacteria*, Gram staining results were most commonly used as a classification tool. Consequently, until the advent of molecular phylogeny, the Kingdom *Prokaryotae* was divided into four divisions, A classification scheme still formally followed by Bergey's manual of systematic bacteriology for tome order
Gracilicutes (gram-negative)

Photobacteria (photosynthetic): class *Oxyphotobacteriae* (water as electron donor, includes the order *Cyanobacteriales*=blue-green algae, now phylum *Cyanobacteria*) and class *Anoxyphotobacteriae* (anaerobic phototrophs, orders: *Rhodospirillales* and *Chlorobiales*

o *Scotobacteria* (non-photosynthetic, now the *Proteobacteria* and other gram-negative nonphotosynthetic phyla)
Firmacutes [sic] (gram-positive, subsequently corrected to *Firmicutes*)
o several orders such as *Bacillales* and *Actinomycetales* (now in the phylum *Actinobacteria*)
Mollicutes (gram variable, e.g. *Mycoplasma*)

Mendocutes (uneven gram stain, "methanogenic bacteria", now known as the *Archaea*)
Molecular era
"Archaic bacteria" and Woese's reclassification



Phylogenetic tree showing the relationship between the archaea and other forms of life. Eukaryotes are colored red, archaea green and bacteria blue. Adapted from Ciccarelli *et al.*

Woese argued that the bacteria, archaea, and eukaryotes represent separate lines of descent that diverged early on from an ancestral colony of organisms. However, a few biologists argue that the Archaea and Eukaryota arose from a group of bacteria. In any case, it is thought that viruses and archaea began relationships approximately two billion years ago, and that co-evolution may have been occurring between members of these groups. It is possible that the last common ancestor of the bacteria and archaea was a thermophile, which raises the possibility that lower temperatures are "extreme environments" in archaeal terms, and organisms that live in cooler environments appeared only later.[48] Since the Archaea and Bacteria are no more related to each other
than they are to eukaryotes, the term *prokaryote*'s only surviving meaning is "not a eukaryote", limiting its value.

With improved methodologies it became clear that the methanogenic bacteria were profoundly different and were (erroneously) believed to be relics of ancient bacteria thus Carl Woese, regarded as the forerunner of the molecular phylogeny revolution, identified three primary lines of descent: the *Archaebacteria*, the *Eubacteria*, and the *Urkaryotes*, the latter now represented by the nucleocytoplasmic component of the *Eukaryotes*. These lineages were formalised into the rank Domain (*regio* in Latin) which divided Life into 3 domains: the *Eukaryota*, the *Archaea* and the *Bacteria*. This scheme is still followed today.

Subdivisions

In 1987 Carl Woese divided the *Eubacteria* into 11 divisions based on 16S ribosomal RNA (SSU) sequences, which with several additions are still used today.

Opposition

While the three domain system is widely accepted, some authors have opposed it for various reasons. One prominent scientist who opposes the three domain system is Thomas Cavalier-Smith, who proposed that the *Archaea* and the *Eukaryotes* (the *Neomura*) stem from Gram positive bacteria (*Posibacteria*), which in turn derive from gram negative bacteria (*Negibacteria*) based on several logical arguments, which are highly controversial and generally disregarded by the molecular biology community (*c.f.* reviewers' comments on, *e.g.* Eric Bapteste is "agnostic" regarding the conclusions) and are often not mentioned in reviews due to the subjective nature of the assumptions made. However, despite there being a wealth of statistically supported studies towards the rooting of the tree of life between the *Bacteria* and the *Neomura*

by means of a variety of methods, including some that are impervious to accelerated evolution—which is claimed by Cavalier-Smith to be the source of the supposed fallacy in molecular methods—there are a few studies which have drawn different conclusions, some of which place the root in the phylum *Firmicutes* with nested archaea.

Radhey Gupta's molecular taxonomy, based on conserved signature sequences of proteins, includes a monophyletic Gram negative clade, a monophyletic Gram positive clade, and a polyphyletic Archeota derived from Gram positives. Hori and Osawa's molecular analysis indicated a link between Metabacteria (=Archeota) and eukaryotes. The only cladistic analyses for bacteria based on classical evidence largely corroborate Gupta's results.

James Lake presented a 2 primary kingdom arrangement (Parkaryotae + eukaryotes and eocytes + Karyotae) and suggested a 5 primary kingdom scheme (Eukaryota, Eocyta, Methanobacteria, Halobacteria, and Eubacteria) based on ribosomal structure and a 4 primary kingdom scheme (Eukaryota, Eocyta, Methanobacteria, and Photocyta), bacteria being classified according to 3 major biochemical innovations: photosynthesis (Photocyta), methanogenesis (Methanobacteria), and sulfur respiration (Eocyta) He has also discovered evidence that Gram-negative bacteria arose from a symbiosis between 2 Gram-positive bacteria.

Authorities

Classification is the grouping of organisms into progressively more inclusive groups based on phylogeny and phenotype, while nomenclature is the application of formal rules for naming organisms.[71]

Nomenclature authority

Despite there being no official and complete classification of prokaryotes, the names (nomenclature) given to prokaryotes are regulated by the International Code of Nomenclature of Bacteria (Bacteriological Code), a book which contains general considerations, principles, rules, and various notes, and advises[72] in a similar fashion to the nomenclature codes of other groups.

Classification authorities

The taxa which have been correctly described are reviewed in *Bergey's manual of Systematic Bacteriology*, which aims to aid in the identification of species and is considered the highest authority. An online version of the taxonomic outline of bacteria and archaea is available. LPSN is an online database which currently contains over two thousand accepted names with their references, etymologies and various notes.

Description of new species

The *International Journal of Systematic Bacteriology/International Journal of Systematic and Evolutionary Microbiology* (IJSB/IJSEM) is a peer reviewed journal which acts as the official international forum for the publication of new prokaryotic taxa. If a species is published in a different peer review journal, the author can submit a request to IJSEM with the appropriate description, which if correct, the new species will be featured in the Validation List of IJSEM.

Distribution

Microbial culture collections are depositories of strains which aim to safeguard them and to distribute them. The main ones being:

Collection Acronym	Name	Location
ATCC	American Type Culture Collection	Manassas, Virginia
NCTC	National Collection of Type Cultures	Public Health England, United Kingdom
BCCM	Belgium Coordinated Collection of Microorganisms	Ghent, Belgium
CIP	Collection d'Institut Pasteur	Paris, France
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen	Braunschweig, Germany
JCM	Japan Collection of Microorganisms	Saitama, Japan
NCCB	Netherlands Culture Collection of Bacteria	Utrecht, Netherlands
NCIMB	National Collection of industrial, Marine and food bacteria	Aberdeen, Scotland
ICMP	International Collection of Microorganisms from Plants	Auckland, New Zealand

Analyses

Bacteria were at first classified based solely on their shape (vibrio, bacillus, coccus etc.), presence of endospores, gram stain, aerobic conditions and motility. This system changed with the study of metabolic phenotypes, where metabolic characteristics were used. Recently, with the advent of molecular phylogeny, several genes are used to identify species, the most important of which is the 16S rRNA gene, followed by 23S, ITS region, gyrB and others to confirm a better resolution. The quickest way to identify to match an isolated strain to a species or genus today is done by amplifying it's 16S gene with universal primers and sequence the 1.4kb amplicon and submit it to a specialised web-based identification database, namely either Ribosomal Database Project, which align the sequence to other 16S sequences using infernal, a secondary structure bases

global alignment or ARB SILVA, which aligns sequences via SINA (SILVA incremental aligner), which does a local alignment of a seed and extends it Several identification methods exists;

Phenotypic analyses

o fatty acid analyses

o Growth conditions (Agar plate, Biolog multiwell plates)

Genetic analyses

o DNA-DNA hybridization

o DNA profiling

o Sequence

o GC ratios

Phylogenetic analyses

o 16S-based phylogeny

o phylogeny based on other genes

o Multi-gene sequence analysis

o Whole-genome sequence based analysis

New species

The minimal standards for describing a new species depend on which group the species belongs to. *c.f*

Candidatus

Candidatus is a component of the taxonomic name for a bacterium that cannot be maintained in a Bacteriology Culture Collection. It is an interim taxonomic status for noncultivable organisms. e.g. "Candidatus Pelagibacter ubique"

Species concept

Bacteria divide asexually and for the most part do not show regionalisms ("Everything is everywhere"), therefore the concept of species, which works best for animals, becomes entirely a matter of judgement. The number of named species of bacteria and archaea (approximately 13,000) is surprisingly small considering their early evolution, genetic diversity and residence in all ecosystems. The reason for this is the differences in species concepts between the *bacteria* and macro-organisms, the difficulties in growing/characterising in pure

culture (a prerequisite to naming new species, *vide supra*) and extensive horizontal gene transfer blurring the distinction of species.

he most commonly accepted definition is the polyphasic species definition, which takes into account both phenotypic and genetic differences.[81] However, a quicker diagnostic *ad hoc* threshold to separate species is less than 70% DNA–DNA hybridisation,[82] which corresponds to less than 97% 16S DNA sequence identity. It has been noted that if this were applied to animal classification, the order primates would be a single species.

Pathology vs. phylogeny

Ideally, taxonomic classification should reflect the evolutionary history of the taxa, i.e. the phylogeny. Although some exceptions are present when the phenotype differs amongst the group, especially from a medical standpoint. Some examples of problematic classifications follow.

Escherichia coli: overly large and polyphyletic

In the Enterobacteriaceae family of the class Gammaproteobacteria, the species in the genus *Shigella* (*S. dysenteriae*, *S. flexneri*, *S. boydii*, *S. sonnei*) by an evolutionary point of view are strains of the species *Escherichia coli* (polyphyletic), but due to genetic differences cause different medical conditions in the case of the pathogenic strains., Escherichia coli is a badly classified species as some strains share only 20% of their genome. Being so diverse it should be given a higher taxonomic ranking. However, due to the medical conditions associated with the species, it will not be changed to avoid confusion in medical context.

Bacillus cereus group: close and polyphyletic

In a similar way, the *Bacillus* species (=phylum *Firmicutes*) belonging to the "*B. cereus* group" (*B. anthracis*, *B. cereus*, *B . thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis* and *B. medusa*) have 99-100% similar 16S rRNA sequence (97% is a commonly cited adequate species cut-off) and are polyphyletic, but for medical reasons (anthrax *etc.*) remain separate.

Yersinia pestis: extremely recent species

Yersinia pestis is in effect a strain of *Yersinia pseudotuberculosis*, but with a pathogenicity island that confer a drastically different pathology (Black plague and tuberculosis-like symptoms respectively) which arose 15,000 to 20,000 years ago.

Nested genera in *Pseudomonas*

In the gammaproteobacterial order *Pseudomonadales*, the genus *Azotobacter* and the species *Azomonas macrocytogenes* are actually members of the genus *Pseudomonas*, but were misclassified due to nitrogen fixing capabilities and the large size of the genus *Pseudomonas* which renders classification problematic.[74][88][89] This will probably rectified in the close future.

Nested genera in *Bacillus*

Another example of a large genus with nested genera is the *Bacillus* genus, in which the genera *Paenibacillus* and *Brevibacillus* are nested clades.[90] There is insufficient genomic data at present to fully and effectively correct taxonomic errors in *Bacillus*.

Agrobacterium: resistance to name change

Based on molecular data it was shown that the genus *Agrobacterium* is nested in *Rhizobium* and the *Agrobacterium* species transferred to the *Rhizobium* genus (resulting in the following comp. nov.: *Rhizobium radiobacter* (formerly known as *A. tumefaciens), R. rhizogenes, R. rubi, R. undicola* and *R. vitis*)[91] Given the plant pathogenic nature of *Agrobacterium* species, it was proposed to maintain the genus *Agrobacterium*[92] and the latter was counter-argued.

Nomenclature

Taxonomic names are written in italics (or underlined when handwritten) with a majuscule first letter with the exception of epithets for species and subspecies. Despite it being common in zoology, tautonyms (e.g. *Bison bison*) are not acceptable and names of taxa used in zoology, botany or mycology cannot be reused for Bacteria (Botany and Zoology do share names). Nomenclature is the set of rules and conventions which govern the names of taxa. The difference in nomenclature between the various kingdoms/domains is reviewed in.

For Bacteria, valid names must have a Latin or Neolatin name and can only use basic latin letters (w and j inclusive, see History of the Latin alphabet for these), consequently hyphens, accents and other letters are not accepted and should be transliterated correctly (e.g. ß=ss). Ancient Greek being written in the Greek alphabet, needs to be transliterated into the Latin alphabet.

When compound words are created, a connecting vowel is needed depending on the origin of the preceding word, regardless of the word that follows, unless the latter starts with a vowel in which case no connecting vowel is added. If the first compound is Latin then the connecting vowel is an -i-, whereas if the first compound is Greek, the connecting vowel is an -o-.

For etymologies of names consult LPSN.

Rules for higher taxa

If a new or amended species is placed in new ranks, according to Rule 9 of the Bacteriologocal Code the name is formed by the addition of an appropriate suffix to the stem of the name of the type genus. For subclass and class the recommendation from is generally followed, resulting in a neutral plural, however a few names do not follow this and instead keep into account graeco-latin grammar (e.g. the female plurals *Thermotogae, Aquificae* and *Chlamydiae*, the male plurals *Chloroflexi*, *Bacilli* and *Deinococci* and the greek plurals *Spirochaetes*, *Gemmatimonadetes* and *Chrysiogenetes*).

Rank	Suffix	Example
Genus		*Elusimicrobium*
Subtribe (disused)	-inae	(*Elusimicrobiinae*)
Tribe (disused)	-inae	(*Elusimicrobiieae*)
Subfamily	-oideae	(*Elusimicrobioideae*)
Family	-aceae	*Elusimicrobiaceae*
Suborder	-ineae	(*Elusimicrobineae*)
Order	-ales	*Elusimicrobiales*
Subclass	-idae	(*Elusimicrobidae*)
Class	-ia	*Elusimicrobia*
Phylum	see text	*Elusimicrobia*

Phyla endings

Phyla are not covered by the Bacteriological code, however, the scientific community generally follows the Ncbi and Lpsn taxonomy, where the name of the phylum is generally the plural of the type genus, with the of the *Firmicutes*, *Cyanobacteria* and *Proteobacteria*, whose names do not stem from a genus name. The higher taxa proposed by Cavalier-Smith are generally disregarded by the molecular phylogeny community (*vide supra*).

For the *Archaea* the suffix -archaeota is used. For bacterial phyla it was proposed that the suffix -bacteria be used for phyla. Consequently for main phyla the name of the phyla is the same as the first described class:

Acidobacteria (from *Acidobacterium*)

Actinobacteria (from *Actinomyces*)

Caldisericia (from *Caldisericum*)

Elusimicrobia (from *Elusimicrobium*)

Fusobacteria (from *Fusobacterium*)

Thermodesulfobacteria (from *Thermodesulfobacterium*)

Thermotogae (from *Thermotoga*)

Aquificae (from *Aquifex*)

Chlamydiae (from *Chlamydia*)

Chloroflexi (from *Chloroflexus*)

Chrysiogenetes (from *Chrysiogenes*)

Gemmatimonadetes (from *Gemmatimonas*)

Deferribacteres (from *Deferribacter*)

Whereas for others where the -ia suffix for class is used regardless of grammar they differ:
phylum *Bacteroidetes* vs. class *Bacteroidia* from *Bacteroides*

phylum *Chlorobi* vs. class *Chlorobia* from *Chlorobium*

phylum *Verrucomicrobia* vs. class *Verrucomicrobiae* from *Verrucomicrobium* (anomalous class name)

phylum *Dictyoglomi* versus class *Dictyoglomia* from *Dictyoglomus*

phylum *Fibrobacteres* versus class *Fibrobacteria* from *Fibrobacter* (c.f. the suffix -bacter, note the difference with *Deferribacteres*)

phylum *Lentisphaerae* versus class *Lentisphaeria* from *Lentisphaera*

phylum *Nitrospira* or *Nitrospirae* versus class *Nitrospira* from *Nitrospira*

phylum *Spirochaetes* versus class *Spirochaetae* from *Spirochaeta*

phylum *Synergistetes* versus class *Synergistetia* from *Synergistes*

phylum *Planctomycetes* versus *Planctomycea* from *Planctomyces*
An exception is the phylum *Deinococcus-Thermus*, which bears a hyphenated pair of genera —only non accented latin letters are accepted for valid names, but phyla are not officially recognised.[100]
Names after people
Many species are named after people, either the discoverer or a famous person in the field of microbiology, for example *Salmonella* is after D.E. Salmon, who discovered it (albeit as "Bacillus typhi" For the generic epithet, all names derived from people must be in the female nominative case, either by changing the ending to -a or to the diminutive -ella, depending on the name.
For the specific epithet, the names can be converted into either adjectival form (adding -nus (m.), -na (f.), -num (n.) according to the gender of the genus name) or the genitive of the latinised name.[96]
Names after places
Many species (the specific epithet) are named after the place they are present or found (e.g. Thiospirillum jenense). Their names are created by forming an adjective by joining the locality's name with the ending -ensis (m. or f.) or ense (n.) in agreement with the gender of the genus name, unless a classical Latin adjective exists for the place. However, names of places should not be used as nouns in the genitive case.
Vernacular names
Despite the fact that some hetero/homogeneus colonies or biofilms of bacteria have names in English (e.g. dental plaque or Star jelly), no bacterial species has a vernacular/trivial/common name in English.
For names in the singular form, plurals cannot be made (singulare tantum) as would imply multiple groups with the same label and not multiple members of that group (by analogy, in English, chairs and tables are types of furniture, which cannot be used in the plural form "furnitures" to describe both members), conversely names plural form are pluralia tantum. However, a partial exception to this is made by the use of vernacular names. However, to avoid repetition of taxonomic names which break the flow of prose, vernacular names of members of a genus or higher taxa are often used and recommended, these are formed by writing the name of the taxa in sentence case roman ("standard" in MS Office) type, therefore treating the proper noun as an English common noun (e.g. the salmonellas), although there is some debate about the

grammar of plurals, which can either beregular plural by adding -(e)s (the salmonellas) or using the ancient Greek or Latin plural form (irregular plurals) of the noun (the salmonellae); the latter is problematic as the plural of - bacter would be -bacteres, while the plural of myces (N.L. masc. n. from Gr. masc. n. mukes) is mycetes.

Customs are present for centain names, such as those ending in -monas are converted into -monad (one pseudomonad, two aeromonads and not -monades).

Bacteria which are the etiological cause for a disease are often referred to by the disease name followed by a describing noun (bacterium, bacillus, coccus, agent or the name of their phylum) e.g. cholera bacterium (*Vibrio cholerae*) or Lyme disease spirochete (*Borrelia burgdorferi*) , note also rickettsialpox (*Rickettsia akari*)

Treponema is converted into treponeme and the plural is treponemes and not treponemata.

Some unusual bacteria have special names such as Quin's oval (*Quinella ovalis*) and Walsby's square (*Haloquadratum walsbyi*). Before the advent of molecular phylogeny, many higher taxonomic groupings had only trivial names, which are still used today, some of which are polyphyletic, such as Rhizobacteria.

Some higher taxonomic trivial names are:

Blue-green algae are members of the phylum *Cyanobacteria*

Green non-sulfur bacteria are members of the phylum *Chloroflexi*

Green sulfur bacteria are members of the *Chlorobi*

Purple bacteria are some, but not all, members of the phylum Proteobacteria

Purple sulfur bacteria are members of the order *Chromatiales*

low G+C Gram-positive bacteria are members of the phylum *Firmicutes*, regardless of GC content

high G+C Gram-positive bacteria are members of the phylum *Actinobacteria*, regardless of GC content

Rhizobacteria are members of various genera of proteobacteria

Rhizobia are members of the order *Rhizobiales*

Lactic streptococci are members of the genus *Lactococcus*

Coryneform bacteria are members of the family *Corynebacteriaceae*

Fruiting gliding bacteria or myxobacteria are members of the order *Myxococcales*

Enterics are members of the order Enterobacteriales, although the term is avoided if they do not live in the intestines, such as *Pectobacterium*

Acetic acid bacteria are members of the family *Acetobacteraceae*

Terminology

The abbreviation for species is sp. (plural spp.) and is used after a generic epithet to indicate a species of that genus. Often used to denote a strain of a genus for which the species is not known either because has the organism has not been described yet as a species or insufficient tests were conducted to identify it. For example *Halomonas* sp. GFAJ-1

If a bacterium is known and well-studied but not culturable, it is given the term Candidatus in its name

A basonym is original name of a new combination, namely the first name given to a taxa before it was reclassified

A synonym is an alternative name for a taxa, i.e. a taxa was erroneusly described twice

When a taxon is transferred it becomes a new combination (comb. nov.) or nomina nova (nom. nov.)

paraphyly, monophyly and polyphyly

Microbial Taxonomy A. Taxonomy is the science of biological classification B. Classification is the arrangement of organisms into groups (taxa) C. Nomenclature refers to the assignment of names to taxonomic groups D. Identification refers to the determination of the particular taxon to which a particular isolate belongs E. Systematics is the study of organisms with the ultimate object of characterizing and arranging them in an orderly manner F. New molecular techniques are being used in classifying microorganisms but the traditional approaches still have value Microbial Evolution and Diversity A. Fossilized remains of bacterial cells around 3.5 to 3.8 billion years old have been found in stromatolites and sedimentary rocks B. Stromatolites are layered or stratified rocks that are formed by incorporation of mineral sediments into microbial mats C. The earliest bacteria were probably anaerobic
D. Aerobic cyanobacteria probably developed 2.5 to 3.0 billion years ago
E. The work of Carl Woese and his collaborators suggests that organisms fall into one of three domains (empires) into which the traditional kingdoms are distributed 1. Eucarya - contains all eucaryotic organisms 2. Bacteria (Eubacteria) - contains procaryotic organisms with eubacterial rRNA and membrane lipids that are primarily diacyl glycerol ethers 3. Archaea - contains procaryotic organisms with archaeobacterial rRNA and membrane lipids that are primarily isoprenoid glycerol diether or diglycerol tetraether derivatives A. Modern eucaryotic cells appear to have arisen from procaryotes about 1.4 billion years ago B. One hypothesis for the development of chloroplasts and mitochondria involves invagination of the plasma membrane and subsequent compartmentalization of function C. The alternative is the endosymbiotic hypothesis which suggests the following 1. The first event in the development of eucaryotes was the formation of the nucleus (possibly by fusion of ancient eubacteria and archaea) 2. Chloroplasts were formed from free-living photosynthetic bacteria that entered into a symbiotic relationship with the primitive eucaryote (cyanobacteria and Prochloron have been suggested as possible candidates) 3. Mitochondria may have arisen by a similar process (ancestors of Agrobacterium, Rhizobium, and the rickettsias have been suggested) The endosymbiotic hypothesis has received support from the discovery of an endosymbiotic Cyanobacterium that inhabits the biflagellate protist Cyanophora paradoxa and acts as its chloroplast; the endosymbiont is called a cyanelle I. Taxonomic Ranks A. The taxonomic ranks (in ascending order) are: species, genus, family, order, class and kingdom B. Microbiologists often use less formal group (section) names that are descriptive (e.g., methanogens, purple bacteria, lactic acid bacteria, etc.) C. The basic taxonomic group is the species D. Bacterial species are defined on the basis of sexual reproductive compatibility (as for higher organisms) but rather are based on phenotypic and genotypic differences A bacterial species is a collection of strains that share many stable properties and differ significantly from other groups of strains
A strain is a population of organisms that descends from a single organism or pure culture isolate
a. Biovars - strains that differ biochemically or physiologically b. Morphovars - strains that differ morphologically c. Serovars - strains that differ in antigenic properties 1. The type strain is usually the first studied (or most fully characterized) strain of a species; it does not have to be the most representative member A. A genus is a well-defined group of one or more species that is clearly separate from other

genera B. The binomial system of nomenclature devised by Carl von Linne (Carolus Linnaeus) is used in which the genus name is capitalized while the specific epithet is not; both terms are italicized (e.g., Escherichia coli). After first usage in a manuscript the first name will often be abbreviated to the first letter (e.g., E. coli) C. Bergey's Manual of Systematic Bacteriology focuses on the classification and biology of bacteria but often is more detailed than is necessary for identification D. Bergey's Manual of Determinative Bacteriology is a single volume that is intended for use in identifying bacteria I. Classification Systems A. Natural classification - arranges organisms into groups whose members share many characteristics and reflects as much as possible the biological nature of organisms B. Phenetic systems group organisms together based on overall similarity 1. Frequently a natural system is based on shared characteristics 2. Not dependent on phylogenetic analysis 3. Use unweighted traits 4. Best system compares as many attributes as possible A. Numerical Taxonomy 1. Information abouth the properties of an organism is converted to a form suitable for numerical analysis 2. Compared by means of a computer 3. The presence or absence of at least 50 (preferably several hundred) characters should be compared a. Morphological, biochemical and physiological characters should be included b. Determine an association coefficient between characters possessed by two organisms

1. Simple matching coefficient-proportion that match whether present or absent
2. Jaccard coefficient - ignores characters that both organisms lack a. Arrange to form a similarity matrix b. Organisms with great similarity are grouped together into phenoms 1. A treelike diagram called a dendrogram is used to display the results of numerical taxonomic analysis 2. The significance of the phenoms is not always obvious but phenoms with an 80% similarity often are equivalent to bacterial species A. Phylogenetic (phyletic) systems group organisms together based on probable evolutionary relationships 1. Has been difficult for bacteria because of the lack of a good fossil record 2. Direct comparison of genetic material and gene products such as rRNA and proteins overcomes this problem I. Major Characteristics Used in Taxonomy A. Classical Characteristics 1. Morphological characteristics are easy to analyze, genetically stable and do not vary greatly with environmental changes; often are good indications of phylogenetic relatedness 2. Physiological and metabolic characteristics are directly related to enzymes and transport proteins (gene products) and therefore provide an indirect comparison of microbial genomes 3. Ecological characteristics include life-cycle patterns, symbiotic relationships, ability to cause disease habitat preferences and growth requirements 4. Genetic analysis includes the study of chromosomal gene exchange through transformation and conjugation; these processes only rarely cross genera; one must take care to avoid errors that result from plasmid-borne traits A. Molecular Characteristics 1. Comparison of proteins is useful because it reflects the genetic information of the organism; analysis is by:

a. Determination of the amino acid sequence of the protein

b. Comparison of electrophoretic mobility c. Determination of immunological cross-reactivity d. Comparison of enzymatic properties 1. Nucleic acid base comparison (G + C content) a. Can be determined by determination of the melting temperature (Tm) which is related to the temperature at which the two strands of a DNA molecule separate from one another as the temperature is slowly increased b. Taxonomically useful because variation within a genus is usually less than 10% but variation between genera is quite variable ranging from 25% to 80% 1. Nucleic acid hybridization a. Determines the degree of sequence homology b. The temperature of incubation controls the degree of sequence homology needed to form a stable hybrid 1. Nucleic acid sequencing a. rRNA gene sequences are most ideal for comparisons because they contain both evolutionarily stable and evolutionarily variable sequences b Recently, complete bacterial genomes have been sequenced; direct comparisons of complete genome sequences undoubtedly will become important in bacterial taxonomy I. Assessing Microbial Phylogeny A. Molecular Chronometers - based on the assumption of a constant rate of change, which is not a correct assumption;

however, the rate of change may be constant within certain genes B. Phylogenetic Trees 1. Made of branches that connect nodes, which represent taxonomic units such as species or genes 2. Rooted trees provide a node that serves as the common ancestor for the organisms being analyzed 3. Developed by comparing molecular sequences and differences are expressed as evolutionary distance 4. Organisms are then clustered to determine relatedness; alternatively, relatedness can be estimated by parsimony analysis assuming that evolutionary changes occurs along the
shortest pathway with the fewest changes to get from ancestor to the organism in question
A. rRna, DNA, and Proteins as Indicators of Phylogeny 1. Association coefficients from rRna studies are a measure of relatedness 2. Oligonucleotide signature sequences occur in most or all members of a particular phylogenetic group and are rarely or never present in other groups even closely related ones; useful at kingdom or domain levels 3. DNA similarity studies are most effective at the species and genus level 4. Protein sequences are less affected by organism-specific differences in G + C content 5. The three types of molecules do not always produce the same evolutionary trees I. The Major Divisions of Life A. Empires (Domains) 1. Eubacteria - comprise the vast majority of procaryotes; peptidoglycan contains muramic acid; membrane lipids contain ester-linked straight-chain fatty acids 2. Archaea - procaryotes that lack muramic acid and have lipids with ether-linked branched aliphatic chains, tRNAs lack thymine, RNA polymerase is distinctive, ribosomes have a different composition and shape when compared to the Eubacteria 3. Eucarya - have a more complex membrane-delimited organelle structure 4. Several different phylogenetic trees have been proposed relating the major domains and some trees do not even support a three-domain pattern A. Kingdoms 1. Five Kingdom system a. Animalia - multicellular, nonwalled eucaryotes with ingestive nutrition b. Plantae - multicellular, walled eucaryotes with photoautotrophic nutrition c. Fungi - multicellular, and unicellular, walled eucaryotes with absorptive nutrition d. Protista - unicellular eucaryotes with various nutritional mechanisms e. Monera (Procaryotae) - all procaryotic organisms 1. Six Kingdom system - separate Monera into Eubacteria and Archaeobacteria 2. Eight Kingdom system (two empires) a. Separates procaryotes into Eubacteria and Archaeobacteria
b. Redefines protists into several better-defined kingdoms
I. Bergey's Manual of Systematic Bacteriology - A detailed work that contains descriptions of all procaryotic species currently identified A. The First Edition of Bergey's Manual of Systematic Bacteriology - primarily phenetic 1. 33 sections in 4 volumes 2. Each section contains bacteria that share a few easily determined characteristics and bears a title that describes these properties or provides the vernacular names of the bacteria included 3. There is considerable disagreement between the phenetic system in Bergey's and phylogenetic relationships as determined by a variety of means 4. Despite limitations it is the most widely accepted system for identifying bacteria A. The Second Edition of Bergey's Manual of Systematic Bacteriology 1. Twice the number of species with 170 newly described genera 2. Largely phylogenetic rather than phenetic 3. Will not be available for some time yet 4. Pathogenic species are not grouped together but rather are scattered throughout the five volumes according to their phylogenetic relationships IX. A Survey of Bacterial Phylogeny and Diversity - based on the 2nd edition of Bergey's A. Volume 1: The Archaea, Cyanobacteria, Phototrophs and Deeply Branching Genera 1. Archaea - divided into two kingdoms a. Crenarchaeota - diverse kingdom that contains thermophilic and hyperthermophilic 1. organisms as well as some organisms that grow in oceans at low temperatures as 2. picoplankton 3. b. Euryarchaeota - contains primarily mathanogenic and halophilic bacteria and also 4. thermophilic, sulpher-reducing bacteria 1. Eubacteria - complex with several small groups of phototrophs, cyanobacteria, and deeply branching eubacteria A. Volume 2 - Gram negative proteobacteria (purple bacteria) - complex group B. Volume 3 - Gram positive bacteria with low G + C content (< 50%)
C. Volume 4 - Gram positive bacteria with high G + C content (> 50-55%)

D. Volume 5 - An assortment of deeply branching phylogenetic groups that are not necessarily related to one another although all are Gram negative • the techniques involved in the sequencing of a gene. • To learn the importance of 16S ribosomal RNA in the identification of bacteria. Ribosomes are complex structures found in all living cells which functions in protein synthesis machinery. Basically ribosome's consists of two subunits, each of which is composed of protein and a type of RNA, known as ribosomal RNA (rRNA). Prokaryotic ribosomes consist of 30S subunit (small sub unit) and 50S subunit (large sub unit) which together make up the complete 70S ribosome, where S stands for Svedberg unit non-SI unit for sedimentation rate. 30S subunit is composed of 16S ribosomal RNA and 21 polynucleotide chains while 50S subunit is composed of two rRNA species, the 5S and 23S rRNAs. The presence of hyper variable regions in the 16S rRNA gene provides a species specific signature sequence which is useful for bacterial identification process. 16S Ribosomal RNA sequencing is widely used in microbiology studies to identify the diversities in prokaryotic organisms as well as other organisms and thereby studying the phylogenetic relationships between them. The advantages of using ribosomal RNA in molecular techniques are as follows:

1. Ribosomes and ribosomal RNA are present in all cells.

2. RNA genes are highly conserved in nature.

3. Culturing of microbial cells is absent in the sequencing techniques.

Signature sequences are some specific base sequences which are always found in all groups of organisms. These unique DNA sequences are about 5–10 bases long and found specifically in the 16S rRNA location, and are unique to many major groups of prokaryotic organisms, archaea and Eukarya. The average lengths of the structural rRNA genes are 1,522 bp, 2,971 bp, and 120 bp respectively for 16S, 23S, and 5S rRNAs. Conventional microbiology techniques such as culturing of microorganisms, biochemical tests and other related methods are used worldwide to identify most of the bacteria, fungi and other pathogens, still it takes about 8 to 20 hours for an accurate result. New diagnostic techniques have been developed to overcome the limitations of conventional microbiological methods for identifying etiological agents of infections. Nucleic acid based detection methods help in the detection of genomic materials and thus many genetic or infectious diseases can now be diagnosed by performing a study of relevant DNA sequence by nucleic acid-based techniques.

Steps in Ribosomal RNA Sequencing:

Extraction of DNA

The genetic material of all living organisms contains information that is crucial for heredity. The DNA segments that carry this genetic information are called genes which are necessary for genetic analysis, which is used for scientific, medical, or forensic purposes. DNA is not free inside the nucleus of a cell. It is usually associated with different proteins and encased in a cellular membrane. Presence of these proteins, lipids, polysaccharides and some other organic or inorganic compounds in the DNA preparation can interfere with DNA analysis methods. Factors affecting the methods of DNA isolation are the age, source, and size of the sample. The presence of proteins, lipids, polysaccharides etc. during DNA preparation can interfere with DNA analysis methods by reducing the quality of DNA. The extraction methods to efficiently purify DNA from various sources have to be adapted depending on factors such as sample size, the freshness of the sample, and the biochemical content of the cells from which DNA is being extracted. The isolation method must vary depending on the size of sample. In the case of bacteria, the main biochemicals present in a cell extract are protein, DNA and RNA.

Action of Different Chemicals in DNA Extraction

1. TE buffer: It is a buffer used for the storage of nucleic acids (DNA and RNA), and also to prevent it from degradation.

2. Lysozyme: Enzyme that is used for degrading the cell wall of the organism.

3. SDS: SDS is strong anionic detergent that helps in solubilizing the proteins and lipids present in the membranes. It exposes the chromosomes that contain the DNA, also helps in releasing DNA from histones and other DNA binding proteins by denaturing them.

4. Proteinase K: Degrades most of the protein impurities in the DNA(Deproteination).

5. Phenol: Helps in removing most of the protein impurities from the DNA.

6. Chloroform: Prevents the shearing effect of DNA during the extraction process.

7. Isoamyl alcohol: Reduce the formation of the foams in extraction techniques.

8. Sodium acetate: Precipitates DNA in the solution

9. Absolute ethanol: Precipitate the DNA out of solution.

Polymerase Chain Reaction

PCR is a rapid, automated technique used for the amplification of specific DNA sequences, invented by Kary B Mullis in 1983, and for which he won the Nobel Prize in Chemistry in 1993. PCR has gained over nucleic acid based detection techniques due to its simplicity, specificity, rapidity and sensitivity. In this technique only the DNA of the organism is examined, not the entire viable microorganism, as a result, the pathogenic microorganism can also be evaluated. Valuable genetic information about the microorganisms can be obtained quickly. PCR has become an essential tool in research laboratories and is also creating an impact in diagnostic laboratories.

Steps in the PCR process

A technique for amplification of a specific fragment of DNA of interest by a series of successive cycles. By this process, a single molecule of template DNA can generate over a billion copies of itself after 30 cycles of exponential replication. There are three phases in the cycle, each of which occurs at a different temperature. The phases can be performed in an instrument known as thermocycler, which provides these different temperatures.

1. Denaturation: During this process, the double helical arrangement of the sample DNA (template DNA) is denatured at a temperature of about 94°C- 95°C. The two strands get separated out.

2. Annealing: In this step, the primers, which are the sequences of DNA added to the reaction mixture anneal with the complementary (similar or matching) sequences in the template DNA. This occurs at different temperatures.

3. Extension: This is the final stage of the PCR cycle, occurs at 72°C when the enzyme Polymerase added to the reaction mixture, make the primers extend along the length of the DNA strand.

The enzyme polymerase (mainly Taq Polymerase) was isolated from a thermophilus organism, so that it can function optimally at temperature around 72°C and thus allowing the DNA synthesis step to be performed at higher temperatures.

Agarose Gel Electrophoresis

Electrophoresis is a technique used in the laboratory for separating charged molecules. DNA is negatively charged and it can be moved through an agarose matrix by means of electric current. Shorter molecules migrate more easily and move faster than longer molecules through the pores of the gel and this process is called sieving. The gel might be used to look at the DNA in order to quantify it or to isolate a particular band. The DNA can be visualized in the gel by the addition of ethidium bromide. It is an intercalating agent whichintercalates between nucleic acid bases and allows the convenient detection of DNA fragments in gel. When exposed to UV light, it will fluoresce with an orange color. After the running of DNA through an EtBr-treated gel, any band containing more than ~20 ng DNA becomes distinctly visible under UV light. The migration rate of the linear DNA fragments through agarose gel is proportional to the voltage applied to the system. As voltage increases, the speed of DNA also increases. But voltage should be limited because it heats and finally causes the gel to melt.

Elution of DNA

Elution describes the extraction of specific bands of DNA from agarose gels in which they are separated through electrophoresis. The first step in extracting DNA is identifying the DNA band which is to extract, by illuminating under UV light. Recovery of DNA from agarose gels by electrophoresis onto DEAE-cellulose membrane is one of the rapid and effective methods. Electro elution is a rapid method for the successful isolation of DNA especially for larger DNA fragments, where the gel fragment containing the DNA band is cut out of the gel and placed into a dialysis bag with some buffer. The bag is then kept into a gel box, which contains the same buffer, and then subjected to an electric current. The extracted DNA precipitates out from the solution. Low melting point agarose is widely employed for the separation of DNA from agarose. Low melting point agarose melts at a lower temperature than standard agarose since it does not denature DNA structure.

Radiolabeling Technique The ability to label nucleic acids is one of the most fundamental tools in molecular biology techniques. Radiolabeling is one of the best methods of choice for the most sensitive when it would be difficult to visualize a nonradioactive label, such as the gel mobility shift assay, where the probe remains within the gel matrix. Radioactive tracers have the ability to detect small quantities of substances of interest. In case of radio labeling 16S ribosomal sequence, the specific sequence is in tiny amount compared to the large genomic size of the organism. The direct measurement methods such as ultraviolet absorption, staining with specific dyes are not applicable in most cases due to the limited sensitivities of the methods. Modern techniques such as autoradiography, phosphor imaging and liquid scintillation counting techniques are recently applied for detecting the radioactive tracers.

Restriction Digestion Restriction enzymes are endonucleases which cleave double-stranded DNA at specific oligonucleotide sequences. The specific sites at which they cleave the nucleic acids in order to generate a set of smaller fragments are called restriction sites. The natural function of restriction enzymes in bacteria may be the destruction of foreign DNA that may enter the bacterial cell. But the cells own DNA is not cleaved by these restriction enzymes. This self protection is achieved by the help of the specific DNA methyltransferase enzyme which will methylates the specific DNA sequence for its respective restriction enzymes by transferring methyl groups to adenine or cytosine residues to produce N6-methyladenine or 5-methylcytosine. An interesting feature of restriction endonuclease is that they commonly recognize recognition sequences that are mostly palindromes - they shows the same forward (5' to 3' on the top strand) and backward (5' to 3' on the bottom strand) sequences. The DNA fragments of varying length can be separated by gel electrophoresis and stained with ethidium bromide and can be photographed for future studies.

Southern Blotting DNA fragments obtained by restriction digestion and separation on gel can be transferred from the gel by blotting to nitrocellulose or nylon membrane that binds the DNA. The DNA thus bound to the nitrocellulose membrane is converted to the single-stranded forms (denaturation) and then treated with radioactive single-stranded DNA probes. These will hybridize with the homologous DNA, if present in the sample, to form radioactive double stranded segments. Finally the bands are visualized by autoradiography with x-ray film or by phosphor imaging techniques. This highly sensitive technique for identifying DNA fragments by DNA-DNA hybridization is called Southern blotting technique. Steps in Southern blotting technique

1. The DNA to be analyzed is first digested to completion with a restriction enzyme.

2. The complex mixture of fragments is subjected to gel electrophoresis

3. The restriction fragments present in the gel are denatured with alkali.

4. Transfer the gel onto a nitrocellulose filter or nylon membrane by blotting.

5. The filter is then incubated under hybridization conditions with a specific radiolabeled DNA probe.

6. The probe then hybridizes to the complementary DNA restriction fragment.

7. Excess probe is washed away and the probe bound to the filter is detected by autoradiography.

Autoradiography

Autoradiography is a technique used to detect radioactive compounds with the aid of photographic emulsion, which is basically a piece of X-ray film. The radioactive DNA fragments on a gel is then placed in contact with X-ray film and kept it in the dark area for few hours, or even days. The radioactive emission from the bands of the DNA exposes the film. When the film is developed dark bands appear corresponding to the DNA bands present in the gel. In other words, the DNA bands in the agarose gel take a picture of themselves, and hence the name autoradiography. To enhance the autoradiography sensitivity, intensifying screen can be used which is coated with a compound that fluoresces when it is excited by β-rays at low temperature. The electrophoresed radioactive DNA fragments are seen as parallel lanes and it position depends on the size of the fragments. But the DNA bands are invisible but the positions can be identified with some dotted lines. This gel is then placed in contact with a piece of X-ray film. Leave it for several hours to days. Finally, develop the film to see where the radioactivity has exposed the film, which shows the position of DNA bands on the gel. The large, slowly migrating bands are thought to be more radioactive, and thus the bands corresponds to them on the autoradiogram are darkest than the others.

Applications of 16S Ribosomal RNA in Microbiology

1. 16S rRNA gene sequencing has been established as the ―gold standard‖ for identification and taxonomic classification of bacterial species.

2. Comparison of the bacterial 16S rRNA sequence has been emerged as a valuable genetic technique and can lead to the recognition of novel pathogens such as *Mycobacterium* species.

3. The hyper variable regions of 16S rRNA gene sequences provide species-specific signature sequences useful for bacterial identification.

4. In medical microbiology, 16S rRNA sequencing serves as a rapid and cheap alternative to phenotypic methods of bacterial identification.

5. It is also capable of reclassifying bacteria into completely new species, or even genera.

6. The sequencing techniques can be used to describe new species that have never been successfully cultured in laboratories.

In addition to all its applicability, sequencing 16S ribosomal RNA lacks widespread use due to the technical and cost issues. The future work is to translate sequence information from 16S rRNA into suitable biochemical testing process, thereby improving the accuracy and efficiency of genotypic identification phenomenon for better advances even in smaller and routine clinical microbiology laboratories.

Study of ribosomal RNA led to the definition of three separate ―Domains‖ of life; Eukaryotes, Bacteria, and Archaea

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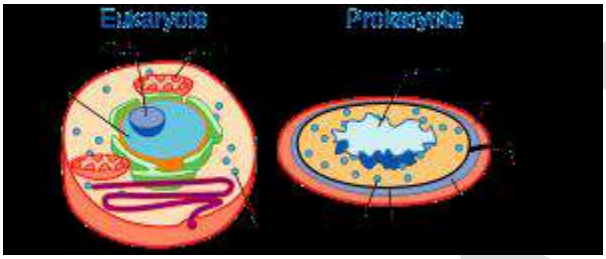
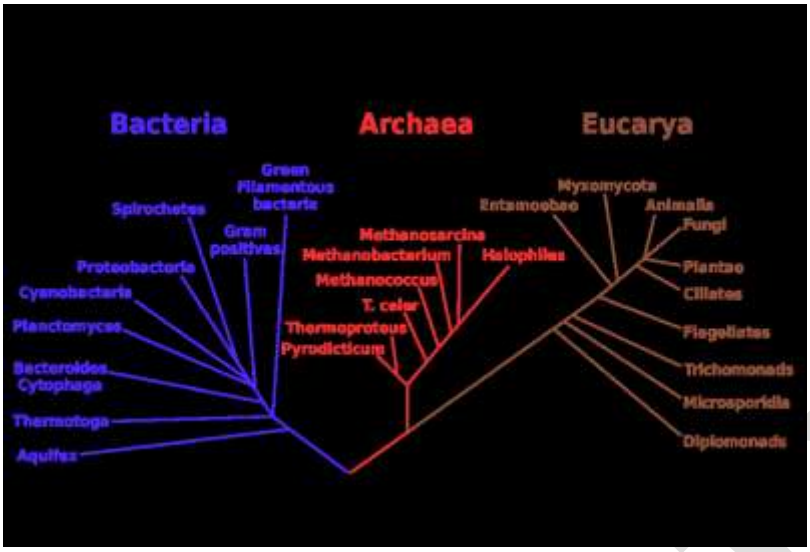


The division of life before the study of rRNA

The introduction of DNA-based studies made a tremendous impact on evolutionary biology. It changed the basic shape of our constructed ―tree of life‖, which, until the advent of sequencing, biologists had based on comparative morphology. For most of the last 150 years, biologists divided organisms into two main groups. The eukaryotes had their DNA contained within a special membrane bound compartment (known as the nucleus). Any organism without a nucleus was known as a prokaryote (bacteria, mostly).

However, in the 1970s Carl Woese began studying the evolution of organisms by comparing the sequences of their ribosomal RNA. At the time, it was difficult to read the sequence of the region of the DNA that encoded the ribosomal RNA, but it was possible (though still hard) to read bits and pieces of the sequence of the RNA molecules themselves. He started sequencing ribosomal RNA from a diversity of organisms, and found a striking result: the organisms lumped together into the prokaryotes formed two distinct and ancient groups. One corresponded to the standard bacteria many people were familiar with. The other defined a collection of highly unusual and little studied organisms.

This group, originally named Archaeabacteria, is now known as Archaea. Woese's work showed that there were three main lineages of organisms on the planet – the Eukaryotes, the Bacteria, and the Archaea. These lineages are now generally known as the ―Three Domains.‖



rRNA-based Tree of Life showing the Three Domains

DNA-RNA-Protein

Summary/Key Points

DNA is the genetic material of all cellular organisms.

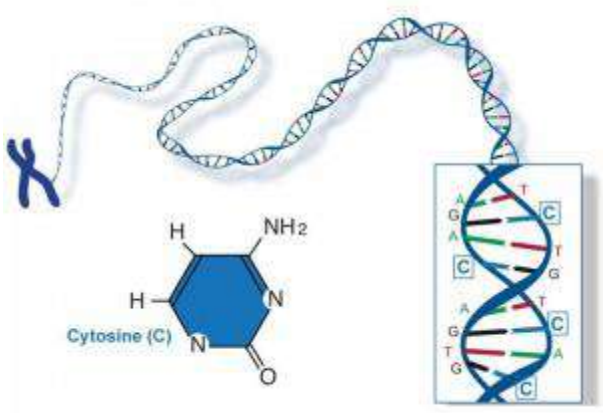
RNA functions as an information carrier or ―messenger‖.

RNA has multiple roles.

Ribosomal RNA (rRNA) is involved in protein synthesis.

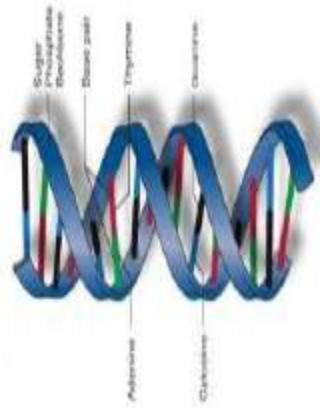
Introduction

At their core, all organisms on the planet have very similar mechanisms by which they handle their genetic information and use it to create the building blocks of a cell. Organisms store information as DNA, release or carry information as RNA, and transform information into the proteins that perform most of the functions of cells (for example, some proteins also access and operate the DNA library). This ―central dogma‖ of molecular biology is an extremely simplistic model, but useful for following the flow of information in biological systems. Among the core features: 1. DNA is the genetic material of all cellular organisms.



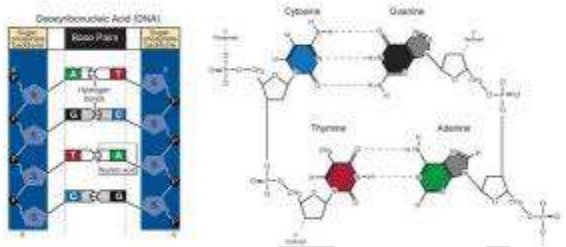
Cytosine, a nucleotide

Deoxyribonucleic acid (DNA) is the material substance of inheritance. All cellular organisms use DNA to encode and store their genetic information. DNA is a chemical compound that resembles a long chain, with the links in the chain made up of individual chemical units called nucleotides. The nucleotides themselves have three components: a sugar (deoxyribose), phosphate, and a nucleobase (frequently just called a base). The bases come in four chemical forms known as adenine, cytosine, guanine, and thymine, which are frequently simply abbreviated as A, C, G and T. The order, or ―sequence‖, of bases encodes the information in DNA.



DNA double helix

All living organisms store DNA in a safe, stable, duplex form: the famous ―double helix‖, in which two chains (also known as strands) of DNA wrap around each other. The two DNA strands are arranged with the bases from one lining up with the bases of the other. The sugar and phosphate components run up the outside like curving rails, with the matched bases forming ladder-like rails in the center. (Note – some viruses have their genetic material in the form of a single strand of DNA).

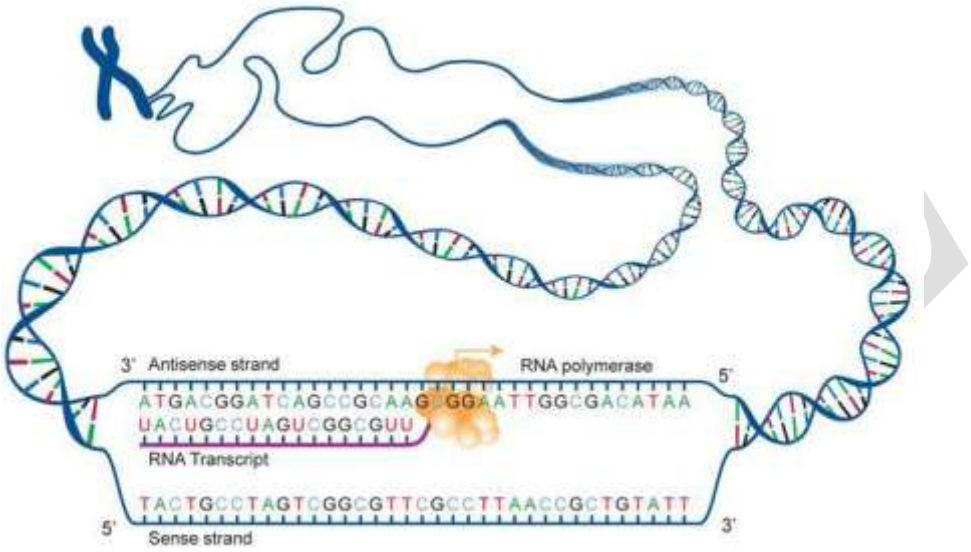


Nucleotide Base Pairing

The shape and charge of the bases cause A to bond weakly to T, and C to bond weakly to G. The bases from one strand of a DNA helix are in essence a mirror image of the bases in the other strand — when there is an A in one strand there is a T in the other; when there is a C in one strand there is a G in the other. These ―base pairing‖ rules are the key to understanding how DNA carries information and is copied into a new DNA strand (a cell must copy its DNA before it divides into two cells). When organisms copy their genomes, enzymes separate the two strands of the double helix, pulling apart the paired bases. Other enzymes start new DNA strands, using the base pairing rules to make a new mirror image of each of the original strands. Mistakes in this process can lead to mutations (changes in the genomic sequence between generations). Many organisms possess error checking mechanisms that scan through the newly replicated

DNA for mistakes and correct them, thus greatly limiting the number of mutations that arise due to replication errors.

2. RNA ‖carries‖ information DNA holds information, but it generally does not actively apply that information. DNA does not *make* things. To extract the information and get it to the location of cellular machinery that can carry out its instructions (usually the blueprints for a protein, as we will see below) the DNA code is ―transcribed‖ into a corresponding sequence in a ―carrier‖ molecule called ribonucleic acid, or RNA. The portions of DNA that are transcribed into RNA are called ―genes‖.



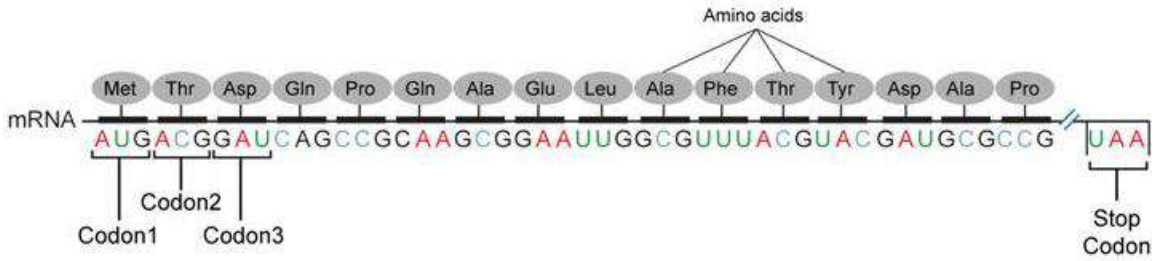
DNA is transcribed to RNA

RNA is very similar to DNA. It resembles a long chain, with the links in the chain made up of individual nucleotides. The nucleotides in RNA, as in DNA, are made up of three components – a sugar, phosphate, and a base. The sugar in RNA is ribose instead of the more stable dexoyribose in DNA, which helps to make RNA both more flexible and less durable.

As in DNA, in RNA the bases come in four chemical forms, and the information in RNA is encoded in the sequence in which these bases are arranged. As in DNA, in RNA one finds adenine (A), cytosine (C), and guanine (G). However, in RNA uracil (abbreviated U) takes the place of thymine (T) (the switch allows RNA some special properties that we won't go into here, at the cost of making it less stable than DNA). Cells make RNA messages in a process similar to the replication of DNA. The DNA strands are pulled apart in the location of the gene to be transcribed, and enzymes create the messenger RNA from the sequence of DNA bases using the base pairing rules. 3. RNA molecules made in a cell are used in a variety of ways.

For our purposes here, there are three key types of RNA: messenger RNA, ribosomal RNA, and transfer RNA. Messenger RNA (mRNA) carries the instructions for making proteins. Like DNA, proteins are polymers: long chains assembled from prefab molecular units, which, in the case of proteins, are amino acids. A large molecular machine* called the ribosome translates the mRNA code and assembles the proteins. Ribosomes readthe message in mRNA in three letter ―words‖ called codons, which translate to specific amino acids, or an instruction to stop making the protein. Each possible three letter arrangement of

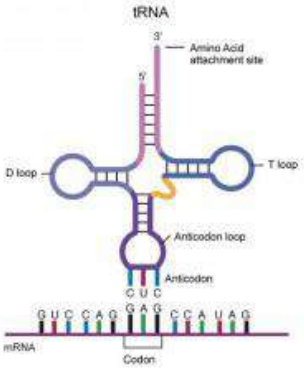
A,C,U,G (e.g., AAA, AAU, GGC, etc) is a specific instruction, and the correspondence of these instructions and the amino acids is known as the ―genetic code.‖ Though exceptions to or variations on the code exist, the standard genetic code holds true in most organisms.



Ribosomes are found in all cellular organisms and they are incredibly similar in their structure and function across all of life. In fact, the extreme similarity of ribosomes across all of life is one of the lines of evidence that all life on the planet is descended from a common ancestor. **Biologists often refer to proteins, especially large complexes of proteins, that move, turn, lever, or generally use energy to perform work, as "machines". Biologists do not mean to imply that such molecules are designed. "Machine" is a useful metaphor for such functions, and simpler and more illuminating than "complex of large molecules that translates chemically stored energy into moving parts".*

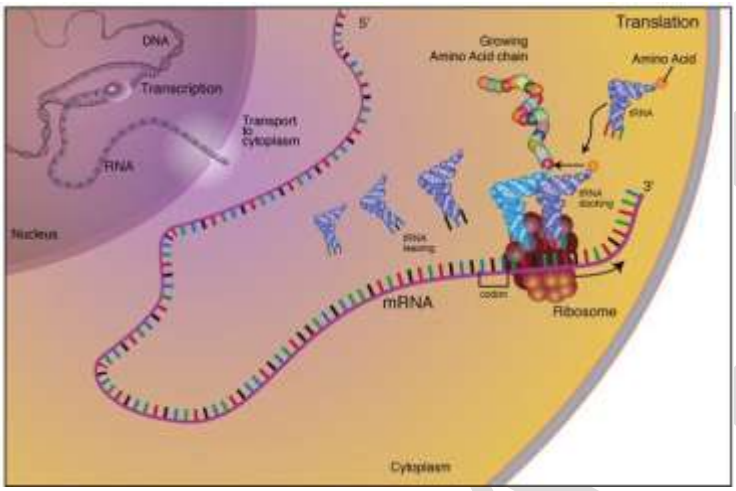
4. Ribosomes make proteins using ribosomal RNA (rRNA). The ribosome reads the instructions found in the messenger RNA molecules in a cell and builds proteins from these mRNAs by chemically linking together amino acids (these are the building blocks of proteins) in the order defined by the mRNA. Messenger RNA molecules are longer than the encoded protein sequence instructions, and include instructions to the ribosome to ―start‖ and ―stop‖ building the protein. Within any particular organism, there can be hundreds to thousands to tens of thousands of distinct mRNAs that lead to distinct proteins. The diversity of form and function in organisms is determined in a large part by the types of proteins made as well as the regulation of where and when these proteins are made.

The ribosome that converts mRNA into proteins is large and complex. It has more than fifty proteins (the exact number varies by species) in two major subunits (known generally as the large and small subunit). In addition to proteins, each subunit includes special RNA molecules, known as ribosomal RNAs (rRNA) because they function in the ribosome. They do not carry instructions for making a specific protein (i.e., they are not messenger RNAs) but instead are an integral part of the ribosome machinery that is used to make proteins from mRNAs.



Transfer RNA (tRNA)

Ribosomes do not read the instructions present in mRNA directly — they need help from yet another type of RNA in cells. Transfer RNAs (tRNA) couple amino acids to their RNA codes. Each codon is supposed to be converted into either a specific amino acid in a protein or a specific instruction to the ribosome (e.g., start, stop, pause, etc). At one end, a transfer RNA presents a three-base codon. At the other, it grasps the corresponding amino acid. Transfer RNAs ―read‖, or ―translate‖, the messenger RNA through base pairing, the chemical attraction of A for T and C for G, just as the RNA sequence is ―transcribed‖ from DNA by base pairing. The ribosome acts like a giant clamp, holding all of the players in position, and facilitating both the pairing of bases between the messenger and transfer RNAs, and the chemical bonding between the amino acids. The making of proteins by reading instructions in mRNA is generally known as ―translation.‖



mRNA is translated into protein

Archaebacteria are not actually bacteria as they are in the Domain "Archaea". The other two Domains being "Bacteria" and "Eukarya" (which includes us humans). Archaebacteria are characterized by having different cell call components, coenzymes and RNA Sequences compared to bacteria. It is believed that they evolved separately from Bacteria. The usual examples of Archaebacteria include many groups that today live in niche environments such as the methanogens (live in anaerobic environments and produce methane), halophiles (live in high salt environments), and thermophiles (live in high temperature environments). However, they are also found in soil as well as on and in the human body. Eubacteria are members of the Family Eubacteriaceae, which are part of the Class Clostridia within the Domain "Bacteria". Eubacteria have a rigid cell wall. However, they can be both gram positive and gram-negative. They can be motile or non motile. Archaea possess several metabolic pathways that are more in common with the Eukarya. The cellular

membranes of Archaea contain ether lipids, while Bacteria generally do not. Archaea use a broader spectrum of compounds for energy sources, ranging from organic compounds (e.g., sugars) to inorganic molecules (e.g., iron, hydrogen, ammonia). Finally, unlike the Bacteria, no Archaea have been found to form spores. Cell Wall Archaebacteria - Variety of types, not containing muramic acid Eubacteria - Variety with one types, contain muramic acid Membrane Lipids Archaebacteria - Ether linked,branched aliphatic chains Eubacteria - Ester linked straight aliphatic chain Thymine in Common arm of t-RNA Archaebacteria - Absent Eubacteria - Present in most t-RNA's of most species Sensitivity to chloramphenicol Archaebacteria - Insensitive Eubacteria - Sensitive Structure of RNA polymerase core Archaebacteria - Ten Subunits Eubacteria - Four Subunits Pathogenicity Archaebacteria - None Eubacteria - some are

methanogenesis Archaebacteria - can obtain energy through methanogenesis Eubacteria - cannot Cellular respiration Archaebacteria - obligate anaerobes Eubacteria - there are obligate anaerobes and aerobes
Difference Between: Eubacteria and Archaebacteria

No	Character	Eubacteria	Archaebacteria
1.	Habitat	Present every where	Mostly inhabit in extreme environmental conditions.
2	Cell wall	Peptidoglycan with muramic acid.	Variety of types, no muramic acid.
3	Membrane lipids	Ester linked, straight -chained fatty acids are present containing L-glycerol phosphate	Ether linked branched aliphatic chains are present containing D-glycerol phosphate.
5	DNA Dependent RNA polymerase	Simple subunit pattern	Complex subunit pattern similar to eukaryotic enzyme
6	tRNA	Thymine present in most tRNAs N-formylmethionine (f met) carried by initiator tRNA	No thymine in T□ C arm of tRNA methionine (met) carried by initiator tRNA
7	Intron	Introns are absent	Introns are present

Differences Between Eubacteria and Archaea
Comparative analysis of the characteristic features among Bacteria and Archaea

Morphological and Genetic		
Characteristic	Bacteria	Archaea
Prokaryotic cell structure	Yes	Yes
Covalently-closed and circular DNA	Yes	Yes
Histone proteins	No	Yes
Membrane-bound nucleus	Absent	Absent
Cell-wall	Muramic-acid present	Muramic-acid present
Membrane-lipids	Ester-linked	Ester-linked
Ribosomes	70S	70S
Initiator tRNA	Formylmethionine	Methionine
Plasmids	Yes	Yes
Ribosome sensitivity to diphtheria toxin	No	Yes
RNA polymerase	One (4 subunits)	Several (8-12 subunits each)
Sensitivity to chloramphenicol, streptomycin and kanamycin	Yes	No

Physiological		
Characteristic	Bacteria	Archaea
Methanogenesis	No	Yes
Nitrification	Yes	No
Denitrification	Yes	Yes
N_2 fixation	Yes	yes
Chlorophyll-based photosynthesis	Yes	No
Gas vesicles	Yes	Yes

Possible Questions

Unit – IV

Two marks

1. Define taxonomy.
2. Define genus and species
3. Write any two points about principle of taxonomy.
4. What is meant by monophasic and polyphasic taxonomy.
5. Distinguish between eubacteria and archae bacteria.

Eight marks

1. Write short notes on the concept of species, taxa, and strain.
2. What is meant by 16S rRNA gene sequencing.
3. Comment on the mechanism of gene sequencing and ribotyping.
4. Write in detail about the signature sequences.
5. Discuss in detail about reverse sequencing methods.
6. What is meant by evolutionary chronometers?.
7. Discuss on RAPD.

KARPAGAM ACADEMY OF HIGHER EDUCATION

CLASS: I B.Sc., MB **COURSE NAME: BACTERIOLOGY**

COURSE CODE: 18MBU102 **UNIT- IV** **BATCH: 2018-2021**

S.no	Question	Opt 1	Opt 2	Opt 3	Opt 4	Answer
1	_____ consists of a population of only one species of micro organism, all derived from a single parent micro organism.	Pure Culture	Inoculum	Mixed Culture	Broth Culture	Pure Culture
2	Agar was suggested as a substitute for gelatin in pure culture technique by	Robert Koch	Mrs. W. Hesse	Joseph Lister	Louis Pasteur	Mrs. W. Hesse
3	Agar, is a polysaccharide derived from _____ species	Microspore	Spirochete	gelidium	Mycoplasma	gelidium
4	Pure culture technique is used to	Isolate bacteria	Obtain sufficient growth for tests	Maintain stock cultures	Decontaminate	Isolate bacteria
5	Inoculation loops are usually made of	Plastic	Rubber	Nichrome	Steel	Nichrome
6	Mac Conkey's agar is a _____ medium	Selective	Differential	Both a and b	Enrichment	Both a and b
7	_____ is a transport medium	Mac Conkey	Blood agar	Stuart's Media	Nutrient agar	Stuart's Media
8	In Mac conkey's Medium, lactose fermenters produce _____ colonies.	Pink	Yellow	Black	Green	Pink
9	Petri dishes were invented by	Robert Koch	Louis Pasteur	Richard Petri	Burnet	Richard Petri
10	Culture media can be solidified by the addition of	Blood	Agar	Sugar	Peptone	Agar
11	The function of agar in	Carbon source	Nitrogen source	Buffering agent	Solidifying agent	Solidifying

	culture medium is					agent
12	The differential medium used for isolation of Escherichia coli.	Mac conkey agar	Eosin methylene blue agar	Nutrient agar	Stuart's medium	Eosin methylene blue agar
13	Solidifying used other than agar is	Thio glycollate	Silica gel	Soya meal	Casein	Silica gel
14	_____ is an enriched media	Nutrient agar	Tryptic soy agar	Blood agar	Macconkey agar	Blood agar
15	All of the following are true about agar except it	liquefies at 100°C	is a polysaccharide derived from a red alga	Solidifies at approximately 40°C	is metabolized by many bacteria	is metabolized by many bacteria
16	Endospores were first discovered by	Tyndall	Pasteur	Robert Koch	Ferdinand Cohn	Ferdinand Cohn
17	The principle of pour plate technique is _____ of the culture in the tubes of liquefied or melted agar.	Dilution	Reduction	Concentration	Fraction	Dilution
18	_____ is considered as Father of Modern Microbiology	Fanny Hesse	Rous	Louis Pasteur	Twort.	Louis Pasteur
19	The role of microorganisms in the fixation of atmospheric nitrogen was first stated by	Beijerinck and Metchnihoff	Winogradsky and Beijerinck	Winogradsky and Pasteur	Pasteur and Koch	Winogradsky and Beijerinck
20	Cell theory was proposed by	Schleiden and Schwann	Schleiden and Robert	Robert Hooke	Leeuwenhock	Robert Hooke
21	Who discovered the penicillin	Alexander Flemming	Alexander Francis	Leewenhoek	Roberthook	Alexander Flemming
22	Who proposed one- gene-	Avery	Stanley	Beadle and Tatum	Leader berg	Beadle and

	one –enzyme hypothesis					Tatum
23	Who showed that lacticacid fermentation is due to a micro organism?	Robert koch	Robert hook	Louis pasture	Francisco redi	Louis pasture
24	Who were the first to introduce the idea ofusing cotton plugs for pluging microbial culture tubes	Franz sehulze & Theodar schwan	George Schroeder & Theodar von Dush	Beadle&Tatum	Robert koch	George Schroeder & Theodar von Dush
25	_____ was the first to observe and report microorganisms.	Watson, Crick	Wasserman	Leeuwen hoek	Robert hook	Leeuwen hoek
26	Germ theory of disease was proved by _____	Robert Hook	Robert Koch	Louis Pasteur	Leeuwen hoek	Robert Koch
27	_____ provide the germ theory of fermentation	Fanny Hesse	Jaco Henle	Pasteur	Leeuwen hoek	Pasteur
28	To disprove spontaneous generation theory Pasteur did an experiment in _____ flask.	Round	Swan neck	Narrow	Broad	Swan neck
29	_____ for the first time proved that a bacterium was the cause of animal disease.	Robert Koch	Fanny Hesse	Wasserman	Richard	Robert Koch
30	Concept of aseptic technique was developed by _____	Robert Koch	Leeuwen hoek	Jaco Henk	Lister	Lister
31	Tubercle bacilli were first isolated by	Boardet	Robert Koch	Ehrlich	Twort	Robert Koch

	_____					
32	Complement fixation test for Syphilis was introduced by _____	Wasserman	Fleming	Ricketts	Bordet	Wasserman
33	Vaccination againt Rabies was first introduced by _____	Ricketts	Louis Pasteur	Bordet	Ehrtich	Louis Pasteur
34	Filament synthesis is an excellent example of	self assembly	Mutualism	Parasitism	Commensalism	self assembly
35	The inclusion bodies of procaryotic cells are present in the .	plasma membrane	cytoplasmic matrix	nucleus	ribosomes	cytoplasmic matrix
36	is needed for peptidoglycan synthesis.	lactose	mannitol	glucose	sucrose	glucose
37	are present in many cyanobacteria, nitrifying bacteria and thiobacilli	carboxyzomes	nitrates	pigment	pili	carboxyzomes
38	is the typical example of bacterium with rod shape.	Bacillus megaterium	Streptococcus	Corynebacterium	Proteus	Bacillus megaterium
39	Some bacteria are variable and lack a single characteristic form are called .	atrichous	pleomorphic	irregular	diploid	pleomorphic
40	The length of Oscillatoria is about in diameter.	7micrometer	2micrometer	5micrometer	1micrometer	7micrometer
41	The most widely accepted current model for membrane	Direct model	Varied model	Fluid mosaic model	Common model	Fluid mosaic model

	structure is the .					
42	contains beta hydroxybutyrate molecules joined by ester bonds between the carboxyl and hydroxyl groups.	Poly beta hydroxybutyrate	magnetosomes	Esters	phosphate granules	Poly beta hydroxybutyrate
42	Many bacteria store phosphate as .	iron	phosphate	polyphosphate granules	butyrate	polyphosphate granules
43	The inorganic inclusion bodies magnetosome contain iron in the form of .	iron	magnetite	Phosphate	esters	magnetite
44	The S in 70S ribosome and similar values stands for .	Svedberg unit	Simple unit	Sample unit	Sigma unit	Svedberg unit
45	The chromosome in the procaryotic cell is located in an irregularly shaped region called .	ribosome	cytoplasm	nucleoid	cell wall	nucleoid
46	The peptidoglycan layer lying outside the plasma membrane is otherwise called as .	Murine layer	Glycan layer	outer layer	cell wall	Murine layer
47	The gram positive cell wall usually contains large amounts of .	Calcium ions	Iron	teichoic acid	Lipopolysaccharide	teichoic acid
48	A is a zone of diffuse, unorganised material that is	Murine layer	Slime layer	outer layer	Flagellin	Slime layer

	removed easily.					
49	bacteria have a single flagellum at each pole.	Pertrichous	Atrichous	Amphitrichous	Polar flagella	Amphitrichous
50	The filament of flagella consist of a single protein called .	insulin	Flagellin	Thymine	Pectin	Flagellin
51	Cyanobacteria undergo a different type of motility called .	Gliding motility	Twisting motility	Dwelling	Moving	Gliding motility
52	The special resistant dormant structure produced by gram positive bacteria is called an .	Exospore	Outer coat	Spore coat	Endospore	Endospore
53	As much as 15% of the endospores dry weight consist of .	Magnesium ions	Lipopolysaccharide	Dipicolinic acid	Peptidoglycan	Dipicolinic acid
54	The endospores spore coat is made up of .	Lipopolysaccharide	Peptidoglycan	teichoic acid	Dipicolonic acid	Peptidoglycan
55	Movement toward chemical attractants and away from repellents is known as .	Chemoreceptors	Chemotaxis	Chemical repellents	Repellents	Chemotaxis
56	Attractants and repellents are detected by a special proteins called .	Chemoreceptors	Chemotaxis	Chemical repellents	Repellents	Chemoreceptors
57	links the filament to its basal body and act as a flexible coupling.	Neck	Hook	Filament	Hair	Hook
58	The meaning of trichous is	Head	Thin	Long	Hair	Hair

	.					
59	aid in the attachment of bacteria to objects .	pili	Fimbriae	Flagella	cell wall	Fimbriae

Archae bacteria and Eubacteria- General Characteristics, Classification (Overview), metabolism and ecological significance. Gram Positive and Gram Negative (Low G+C and High G+C)-General characteristics with suitable examples. Cyanobacteria: An Introduction

Nanoarchaeum

Nanoarchaeota is a phylum of very small parasitic Archaea that branches closest to the root of the archaeal phylogentic tree. Cells of *Nanoarchaeum*, the only genus in this phylum, are small coccoids that live as parasites, or possibly as symbionts, of the crenarchaeota *Ignicoccus*. Cells of*Nanoarchaeum* are about 0.4 µm in diameter and replicate only when attached to the surface of*Ignicoccus*. *Nanoarchaeum* is hyperthermophilic, with an optimal growth of about 90°C. The metabolism of *Nanoarchaeum* is unknown, but its host is an autotroph, growing with H2 as electron donor and elemental sulfur as electron acceptor. Isolates from *Nanoarchaeum* have been obtained from submarine hydrothermal vents as well as terrestrial hot springs. The genome of *Nanoarchaeum*is only 0.49 Mbp, the smallest genome known. It lacks identifiable genes for most known metabolic functions, including the synthesis of monomers, such as aminoacids, nucleotides, and coenzymes.

Ignicoccus sp. with four *Nanoarchaeum* cells attached. Scale bar = 1.0um.

Classification

Higher order taxa:

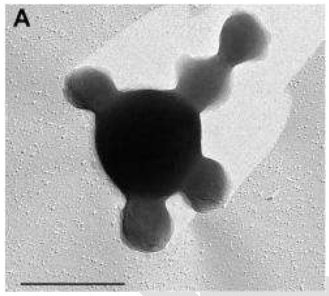
Archaea; Nanoarchaeota

Species:

Nanoarchaeum equitans

NCBI: Taxonomy

Genome



Five *N. equitans* cells attacted to *Ignicoccus* cell. bar = 1um.

Discovered as being tiny dots on another organism, *Nanoarchaeum* was published as being an organism in need of a new phylum among the Archaea. They have not been able to grown on their own and while a parasitic lifestyle cannot be excluded at present, there have been several observations that promote a symbiotic mode of life. Since the original publication, there have been nanoarchaeotal 16 S rDNA sequences found in hightemperature biotopes all over the world that are related to *Nanoarchaeum* but need to be classified in different families.

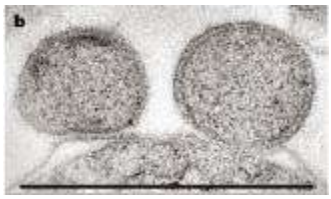
Genome Structure

Nanoarchaeum has a tiny genome with only 490 kb, which represents the smallest archaeal genome to date. Comparing ss rRNA sequences, it was noted that sequence identities were more like archaeon than

bacterial species. There was no difference, however, in the sequence identity to the Crenarchaeota, Euryarchaeota, and 'Korarchaeota', indicating it represents a new archaeal phylum. It is very common for parasitic and symbiotic bacteria to have small genomes. They can develop these by the elimination of unneeded genes and the acquisition of new genes by lateral gene transfer. It is possible that *N. equitans* had a size reduction during adaptation to *Ignicoccus* or it could be an ancient genome since it is assumed that the genomes of the first microorganisms were small.

Recently, the genome of *Nanoarchaeum equitans Kin4-M* was sequenced.

Cell Structure and Metabolism



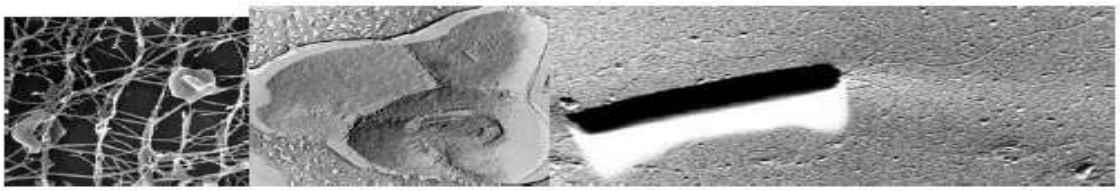
N. equitans attached to the outer membrane of*Ignicoccus*. Scale bar = 1.0um These coccus cells are only 400 nm in diameter and are covered by an S-layer. They require cell-cell contact with an actively growing *Ignicoccus* cell in order to grow. No negative effects have been observed on *Ignicoccus* cells with the addition of *Nanoarchaeum*, hinting at a possible non-parasitic lifestyle. This new *Ignicoccus* species that has been found as a *Nanoarchaeum* host, is an autotrophic sulfur-reducing thermophile.

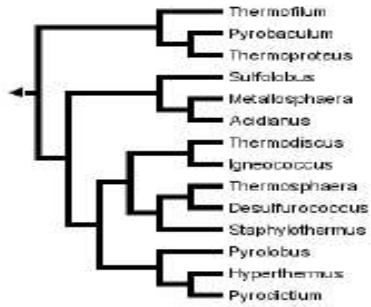
Ecology

This new microorganism was first identified from hot rocks taken at Kolbeinsey ridge, north of Iceland. This hydrothermal system is located at the subpolar Mid-Atlantic Ridge at a depth estimated at 106m. *N. equitans* can grow in the same temperature range as its host, which is 70-98oC, a pH around 6.0, and salt concentrations of about 2% NaCl. They grow on *Ignicoccus* cells in what is either a symbiotic or parasitic relationship.

Other nanoarchaeotal 16S rRNA genes have been obtained from the East Pacific Rise (pH 6.5), the Obsidian Pool in Yellowstone National Park (80oC, pH 6.0), and Caldera Uzon in Kamchatka, Russia (85oC, pH 5.5).

Crenarchaeota





Tree from Burggraf et al., 1997

Tree from Burggraf et al., 1997
Containing group: Archaea

Introduction

The kingdom Crenarchaeota has the distinction of including microbial species with the highest known growth temperatures of any organisms. Although they are microscopic, single-celled organisms, they flourish under conditions which would quickly kill most "higher" organisms. As a rule, they grow best between 80° and 100°C (100°C = 212°F, the boiling point of water at sea level), and several species will not grow below 80°C. Several species also prefer to live under very acidic conditions in dilute solutions of hot sulfuric acid. Approximately 15 genera are known, and most of the hyperthermophilic species have been isolated from marine or terrestrial volcanic environments, such as hot springs and shallow or deep-sea hydrothermal vents. Recent analyses of genetic sequences obtained directly from environomental samples, however, indicate the existence of low temperature Crenarchaeota, which have not yet been cultivated.

Crenarchaeota comprise one kingdom in the larger domain of Archaea. Although they are simple, microscopic organisms, Archaea are quite distinct from more commonly encountered Bacteria, having branched off from the latter very early in evolutionary history (probably >3.5 billion years ago.) In fact, Archaea are more similar to humans than to Bacteria in many important ways, and are probably more closely related to us as well!

Characteristics

The Kingdom Crenarchaeota has been defined phylogenetically, based on comparative molecular sequence analyses, and its members are therefore primarily defined by sequence similarity. However, like all Archaea, Crenarchaeota are prokaryotic, and are bounded by ether-linked lipid membranes which contain isoprinoid side chains instead of fatty acids. Cells range in size from cocci <1μm in diameter to filaments over 100μm in length. Species display a wide range of cell shapes, including regular cocci clustered in grape-like aggregates (Staphylothermus), irregular, lobed cells (Sulfolobus), discs

(Thermodiscus), very thin filaments (<0.5µm diameter; Thermofilum), and almost rectangular rods (Thermoproteus, Pyrobaculum). Most species possess flagella and are motile. A few members of the Crenarchaeota exhibit strange morphologies: Pyrodictium produces disk-shaped cells connected by extensive networks of proteinaceous fibers which may help it to attach to sulfur granules. Metabolically, Crenarchaeota are quite diverse, ranging from chemoorganotrophs to chemolithoautotrophs. They are anaerobes, facultative anaerobes or aerobes, and many utilize sulfur in some way for energy metabolism. Several species are primary producers of organic matter, using carbon dioxide as sole carbon source, and gaining energy by the oxidation of inorganic substances like sulfur and hydrogen, and reduction of sulfur or nitrate. Others grow on organic substrates by aerobic or anaerobic respiration or by fermentation. The most spectacular feature of the Crenarchaeota, however, is their tolerance to, and even preference for, extremes of acidity and temperature. While many prefer neutral to slightly acidic pH ranges, members of the crenarchaeal order Sulfolobales flourish at pH 1-2 and die above pH 7. Optimum growth temperatures range from 75° to 105°C, and the maximum temperature of growth can be as high as 113°C (Pyrobolus). Most species are unable to grow below 70°C, although they can survive for long periods at low temperatures.

Two types of environments were Crenarchaeota thrive: Left. Obsidian Pool, in the Mud Volcano area of Yellowstone National Park, is a neutral-pH hot spring which contains an unusually wide diversity of Crenarchaeota. Note yellow deposits of sulfur on banks. (Photograph by Norm Pace, © 1997.) Right. Hot, sulfur-rich, acidic habitats, like this pool in Yellowstone, are often home to species of Sulfolobus. (Photograph by S. Barns, © 1997.)

Surprisingly, recent rRNA sequence-based analyses indicate that Crenarchaeota also may be widely distributed in low-temperature environments such as ocean waters and terrestrial sediments and soils (Bintrim 1997, DeLong 1994, Furhman 1992, and Hersberger 1996). Although none of these organisms have been cultivated tostrongly suggest that these organisms are mesophilic (or even psychrophilic). Nothing is known of their physiology. Quantitation of rRNA abundance in Antarctic ocean waters indicates that these novel species may constitute a significant portion of the marine bacterioplankton. This, together with the fact that crenarchaeal rRNA sequences have been obtained from every low-temperature environment in which they were sought, indicates that what were once thought to be obscure organisms living in extreme conditions may instead be globally distributed, important players in the biosphere. For more information about low-temperature Crenarchaeota, have a look at the CrenPage, a Web site of Norm Pace's lab at UC Berkeley.

Crenarchaeota aren't just for microbiologists...

These unusual properties of Crenarchaeota have attracted the attention of a wide range of scientists, including evolutionary biologists, exobiologists and biotechnology companies. The extreme conditions under which Crenarchaeota live today may be similar to those which existed on the early Earth at the time that life first arose. This, together with information about their geneology, suggests that these organisms may be much like the earliest lifeforms on earth. Photographs of some regions of the surface of Mars suggest that large hot spring systems, perhaps containing microbial life, may have once existed there. As a result, NASA exobiologists may study these features for chemical and fossil remnants of organisms resembling Crenarchaeota. Finally, the extreme resistance of crenarchaeal cellular enzymes to heat and acid make them very attractive to biotechnology companies, several of which are currently developing such enzymes for industrial and research uses. To see what one biotech company, Diversa, is doing with Crenarchaeota, check out the Diversa Web site.

Discussion of Phylogenetic Relationships

Evolutionary relationships between cultivated members of the Crenarchaeota have been inferred in several studies utilizing small and large subunit ribosomal RNA sequences (Barns 1996, Kjems 1992, Burggraf 1997) Unfortunately, few sequences for genes other than rRNA from more than one crenarchaeal species have been determined to date, thus no alternative molecular sequence-based hypotheses are currently available. However, where analyses overlap, topologies of rRNA-based trees are largely concordant, and show division of the kingdom into three main lineages. The earliest branch within the kingdom contains the genera Thermoproteus, Thermofilum and Pyrobaculum, organisms distinguished by a rod-shaped morphology, neutrophily and anaerobiasis or facultative anaerobiasis. A second lineage contains the Sulfolobus, Stygiolobus, Acidianus and Metallosphaera genera, whose members share coccoid morphology and thermoacidophilic growth. The remaining Crenarchaeota cluster into a group comprised of several genera, including Pyrodictium, Desulfurococcus, Staphylothermus, Thermodiscus, Aeropyrum, Igneococcus and Thermosphaera, all of which are coccoid, neutrophilic hyperthermophiles.

Intriguingly, most of the currently available crenarchaeal rRNA sequences have been obtained from uncultivated organisms through PCR-mediated cloning and sequencing of rDNAs directly from mixed-population DNAs extracted from sediments, soils and water samples (Barns 1996, Bintrim 1997, DeLong 1994, Furhman 1992, McInerney 1995, Hershberger 1996). Most of these sequences branch more deeply from the crenarchaeal line of descent than do those of cultivated species. Addition of such sequences to phylogenetic analyses of Crenarchaeota do not substantially change apparent relationships between cultivated species. However, analysis of these environmental rDNA sequences do reveal considerably greater phylogenetic breadth than was previously known for the kingdom. To see a phylogenetic tree containing many of these environmental sequences, have a look at the Pace lab's "Cren Tree".

Major Groups of the Archaea. Right now the Archaea are being broken up into 2 groups based on biochemical similarities and sequence data. The 2 groups are: 1) **Euryarchaeota** Methanogens, Extreme Halophiles 2) **Crenarchaeota** Extreme Thermophiles. Mesophilic Crens. Please note however that there are thermophiles distributed throughout the phylogenetic tree of the Archaea and that the halophiles are closely related to some of the methanogens (see Figure 20.7). 1) **Euryarchaeota Methanogens** (= methane generators) Methane (CH4) or "natural gas" is an odorless gas that is used widely today in industry and the home. Much of this gas was probably created over the eons by methanogens. Methane is also a greenhouse gas that may be contributing to global warming because it is increasing in concentration in the atmosphere. The methanogens are the most widely distributed of the Archaea and have been found almost everywhere that humans have looked. They are most abundant in anaerobic environments (guts, swamps, sediments etc.) About 30% of adult humans have high populations of methanogens in their guts (all of us have some methanogens inside) and this may explain why some folks emit more gas than others. Methane is odorless

so the foul smell of some flatulence comes from microbial fermentations of organic compounds, especially proteins.

Table 20.2 shows some of the huge diversity of methanogens. Note that they come in all shapes and have a wide variety of cell-wall types - ranging from pseudopeptidoglycan("pseudomurein" in Figure 20.2) to protein or glycoprotein cell walls. Also note that they all have "Methano" as part of their generic names. The % G+C of the methanogens ranges from 26 to 62, again indicating that this is a very diverse group. The diversity of methanogenic metabolic types is given in Table 20.2. Methanogens all live in very reducing environments where e- acceptors like oxygen, nitrate and sulfate have been depleted and where even fermentable substrates have been mostly used up. Most of the metabolic pathways used by these guys are still not well understood but we know quite a bit about the methanogens that use carbon dioxide and hydrogen to make methane. In general these organisms are using H2 as their e- donor and CO2 as their e- acceptor. 4 H2 + CO2 -----> CH4 + 2 H2O

See overhead; note that methanogens use a bunch of novel co-enzymes ("vitamins") in their metabolism.

See overhead for a synopsis of role that methanogens play in the breakdown of complex molecules like cellulose in anaerobic environments. Note that they are just one part of complex anaerobic food webs. **Extreme Halophiles**. These organisms require high concentrations of salt in order to live. Their optimal NaCl concentrations for growth range from 2 to 4.5 M. For reference the salt concentration of sea water is about 0.5 M. Thus, they are found in habitats like the Great Salt Lake, Dead Sea and salterns (= evaporation basins for obtaining salt; you can see these in the San Francisco bay) etc. They also colonize some salted foods but no halophiles have been shown to cause food-borne illnesses. The % G+C of the halophiles ranges from 60 to 71 and the best studied extreme halophile, *Halobacterium*, has a glycoprotein cell wall. One other unique aspect of some extreme halophiles is that they have a novel form of photosynthesis found in no other living organisms. They have a trans-membrane protein called bacteriorhodopsin (box 20.1) which acts as a light driven proton pump (unlike regular photosynthesis, no electron transport is involved). When oxygen is present *Halobacterium salinarium* cells are red-pigmented and grow chemoheterotrophically. Under anaerobic conditions, they produces patches of purple membrane. Purple membrane expresses a protein called bacteriorhodopsin (Box 20.1). This is a protein with a retinal molecule attached. Light causes the protein to undergo a conformational change that pumps protons across the membrane. This mechanism is similar to how our eyes transduce light signals. The proton gradient is used to generate ATP, which allows *Halobacterium*to survive temporary oxygen limitation. 2) **Crenarchaeota** All cultivated representatives are sulfur-dependent and thermophilic. However, recent data indicate that uncultivated mesophilic Crenarchaeota (see below) are present in many environments. Metabolic diversity of studied Crenarchaeota is limited. **Extreme Thermophiles**. As noted above, thermophiles are distributed throughout the Archaea, but many of them group on a main branch of the Archaeal tree (see Figures 19.3 and 19.12). Most of the extreme thermophiles have temperature optima for growth above 80 degrees C whereas most thermophilic Eubacteria have temperature optima of 70 degrees or lower (see overhead). This niche separation is further indication that the Archaea are adapted to environmental extremes similar to those that may have been present on earth when life evolved. It is also of ecological significance that the extreme thermophiles exist in environments that are too hot for photosynthesis to take place. Thus, it is not surprising that many of them have unique forms of metabolism that involve sulfur compounds etc.. (see Table 20.1). Metabolic diversity of studied Crenarchaeota is limited: H2/S *Pyrodictium, Pyrobaculum*, probably*Sulfolobus*). Some will also oxidize organics, using a variety e- acceptors

Because of their high thermostability, enzymes from thermophiles are being used in many commercial applications. For example, proteases from moderate thermophiles are used in laundry detergents and DNA polymerases from extreme thermophiles are used in the polymerase chain reaction (PCR) and other molecular techniques that are carried out at high temperatures.

Mesophilic Crenarchaeota Not much is known about these guys since none have been cultured in the laboratory, but rRNA gene abundance studies indicates that they are a few percent of most environments and in some places much higher (e.g. mesophilic Crens are approx. 50% of marine microbes below 100 meters depth in the oceans, so they may be among the dominant life forms on earth!! Recent studies also indicate that they very common in soils, but no one has any good ideas for what they are doing there (or in the oceans). A crenarchaeote has been detected as a common symbiont in sponges, but what it is doing there is not known either.

Sulfolobus is a genus of microorganism in the family Sulfolobaceae. It belongs to the archaea domain.

Sulfolobus species grow in volcanic springs with optimal growth occurring at pH 2-3 and temperatures of 75-80 °C, making themacidophiles and thermophiles respectively. *Sulfolobus* cells are irregularly shaped and flagellar.

Species of *Sulfolobus* are generally named after the location from which they were first isolated, e.g. *Sulfolobus solfataricus* was first isolated in the Solfatara volcano. Other species can be found throughout the world in areas of volcanic or geothermal activity, such as geological formations called mud pots, which are also known as *solfatare* (plural of solfatara).

Sulfolobus as a model to study the molecular mechanisms of DNA replication

When the first Archaeal genome, *Methanococcus jannaschii*, had been sequenced completely in 1996, it was found that the genes in the genome of *Methanococcus jannaschii* involved in DNA replication, transcription, and translation were more related to their counterparts in eukaryotes than to those in other prokaryotes. In 2001, the first genome sequence of *Sulfolobus*, *Sulfolobus solfataricus* P2, was published. In P2's genome, the genes related to chromosome replication were likewise found to be more related to those in eukaryotes. These genes include DNA polymerase, primase (including two subunits), MCM, CDC6/ORC1, RPA, RPC, and PCNA. In 2004, the origins of DNA replication of *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius* were identified. It showed that both species contained two origins in their genome. This was the first time that more than a single origin of DNA replication had been shown to be used in a prokaryotic cell. The mechanism of DNA replication in archaea is evolutionary conserved, and similar to that of eukaryotes. *Sulfolobus* is now used as a model to study the molecular mechanisms of DNA replication in Archaea. And because the system of DNA replication in Archaea is much simpler than that in Eukaryota, it was suggested that Archaea could be used as a model to study the much more complex DNA replication in Eukaryota.

Role in biotechnology

Sulfolobus proteins are of interest for biotechnology and industrial use due to their thermostable nature. One application is the creation of artificial derivatives from *S. acidocaldarius* proteins, named *affitins*. Intracellular proteins are not necessarily stable at low pH however, as*Sulfolobus* species maintain a significant pH gradient across the outer membrane. *Sulfolobales* are metabolically dependent on sulfur:heterotrophic or autotrophic, their energy comes from the oxidation of sulfur and/or cellular respiration in which sulfur acts as the final electron acceptor. For example, *S. tokodaii* is known to oxidize hydrogen sulfide to sulfate intracellularly.

Genome status

The complete genomes have been sequenced for *S. acidocaldarius* DSM 639 (2,225,959 nucleotides), *S. solfataricus* P2 (2,992,245 nucleotides), and *S. tokodaii* str. 7 (2,694,756 nucleotides).

Genome structure

The archaeon *Sulfolobus solfataricus* has a circular chromosome that consists of 2,992,245 bp. Another sequenced species, *S. tokodaii*has a circular chromosome as well but is slightly smaller with 2,694,756 bp. Both species lack the genes ftsZ and minD, which has been characteristic of sequenced crenarchaeota. They also code for citrate synthase and two subunits of 2-oxoacid:ferredoxin oxidoreductase, which plays the same role as alpha-ketoglutarate dehydrogenase in the TCA cycle. This indicates that Sulfolobus has a TCA cycle system similar to that found in mitochondria of eukaryotes. Other genes in the respiratory chain which partake in the production of ATP were not similar to what is found in eukaryotes. Cytochrome c is one such example that plays an important role in electron transfer to oxygen in eukaryotes. This was also found in A. pernix K1. Since this step is important for an aerobic microorganism like Sulfolobus, it probably uses a different molecule for the same function or has a different pathway.

Cell structure and metabolism

Sulfolobus can grow either lithoautotrophically by oxidizing sulfur, or chemoheterotrophically using sulfur to oxidize simple reduced carbon compounds. Heterotrophic growth has only been observed, however, in the presence of oxygen. The principle metabolic pathways are a glycolytic pathway, a pentose phosphate pathway, and the TCA cycle. All Archaea have lipids with ether links between the head group and side chains, making the lipids more resistant to heat and acidity than bacterial and eukaryotic ester-linked lipids. The Sulfolobales are known for unusual tetraether lipids.In Sulfolobales, the ether-linked lipids are joined covalently across the "bilayer," making tetraethers. Technically, therefore, the tetraethers form a monolayer, not a bilayer. The tetraethers help Sulfolobus species survive extreme acid as well as high temperature.

Ecology

S. solfataricus has been found in different areas including Yellowstone National Park, Mount St. Helens, Iceland, Italy, and Russia to name a few. Sulfolobus is located almost wherever there is volcanic activity. They thrive in environments where the temperature is about 80 °C with a pH at about 3 and sulfur present. Another species, S. tokodaii, has been located in an acidic spa in Beppu Hot Springs, Kyushu, Japan. Sediments from ~90m below the seafloor on the Peruvian continental margin are dominated by intact archaeal tetraethers, and a significant fraction of the community is sedimentary archaea taxonomically linked to the crenarchaeal Sulfolobales (Sturt, *et al.*, 2004).

DNA damage response

Exposure of *Sulfolobus solfataricus* or *Sulfolobus acidocaldarius* to the DNA damaging agents UV-irradiation, bleomycin or mitomycin C induced cellular aggregation. Other physical stressors, such as pH or temperature shift, did not induce aggregation, suggesting that induction of aggregation is caused specifically by DNA damage. Ajon et al. showed that UV-induced cellular aggregation mediates chromosomal marker exchange with high frequency in *S. acidocaldarius*. Recombination rates exceeded those of uninduced cultures by up to three orders of magnitude. Wood et al. also showed that UV-irradiation increased the frequency of recombination due to genetic exchange in *S. acidocaldarius*. Frols et al. and Ajon et al. hypothesized that the UV-inducible DNA transfer process and subsequent homologous recombinational repair represents an important mechanism to maintain chromosome integrity in *S. acidocaldarius* and *S. solfataricus*. This response may be a primitive form of sexual interaction, similar to the more well-studied bacterial transformation that is also associated with DNA transfer between cells leading to homologous recombinational repair of DNA damage.

The *ups* operon

The *ups* operon of *Sulfolobus* species is highly induced by UV irradiation. The pili encoded by this operon are employed in promoting cellular aggregation, which is necessary for subsequent DNA exchange between cells, resulting in homologous recombination. A study of the *Sulfolobales acidocaldarius ups* operon showed that one of the genes of the operon, *saci-1497*, encodes an endonuclease III that nicks UV-damaged DNA; and another gene of the operon, *saci-1500*, encodes a RecQ-like helicase that is able to unwindhomologous recombination intermediates such as Holliday junctions.[12] It was proposed that Saci-1497 and Saci-1500 function in an homologous recombination-based DNA repair mechanism that uses transferred DNA as a template.[12] Thus it is thought that the *ups* system in combination with homologous recombination provide a DNA damage response which rescues *Sulfolobales* from DNA damaging threats.

Sulfolobus as a viral host

Lysogenic viruses infect *Sulfolobus* for protection. The viruses cannot survive in the extremely acidic and hot conditions that *Sulfolobus* lives in, and so the viruses use *Sulfolobus*as protection against the harsh elements. This relationship allows the virus to replicate inside the archaea without being destroyed by the environment. The Sulfolobus viruses are temperate or permanent lysogens. Permanent lysogens differ from lysogenic bacteriophages in that the host cells are not lysed after the induction of Fuselloviridae production and eventually return to the lysogenic state. They are also unique in the sense that the genes encoding the structural proteins of the virus are constantly transcribed and DNA replication appears to be induced. The viruses infecting archaea like Sulfolobus have to use a strategy to escape prolonged direct exposure to the type of environment their host lives in, which may explain some of their unique properties.

Thermoproteus

Thermoproteus	
Scientific classification	
Domain:	Archaea
Kingdom:	Crenarchaeota
Phylum:	Crenarchaeota
Class:	Thermoprotei
Order:	Thermoproteales
Family:	Thermoproteaceae
Genus:	*Thermoproteus* Zillig & Stetter, 1982
Species	
• *T. neutrophilus*	
• *T. tenax*	
• *T. uzoniensis*	

In taxonomy, ***Thermoproteus*** is a genus of the Thermoproteaceae. These prokaryotes are thermophilic sulphur-dependent organisms related to the genera *Sulfolobus*, *Pyrodictium* and *Desulfurococcus*.[1] They are hydrogen-sulphur autotrophs and can grow at temperatures of up to 95 °C.

Description and significance

Thermoproteus is a genus of anaerobes that grow in the wild by autotrophic sulfur reduction. Like other hyperthermophiles, *Thermoproteus* represents a living example of some of Earth's earliest organisms, located at the base of the Archaea.

Genome structure

Genetic sequencing of *Thermoproteus* has revealed much about the organism's modes of metabolism. Total genome length is 1.84 Mbp, and the DNA is double-stranded and circular. Genes are arranged in co-transcribed clusters called operons. The *Thermoproteus tenax* genome has been completely sequenced.

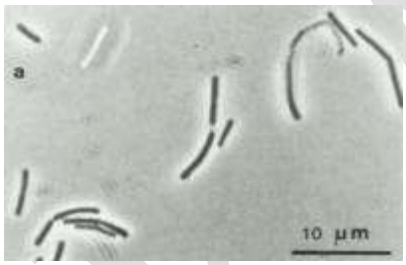
Cell structure and metabolism

A significant amount of research has been done on the metabolism of *Thermoproteus* and other hyperthermophiles as well. *Thermoproteus* metabolizes autotrophically through sulfur reduction, but it grows much faster by sulfur respiration in cultivation. In *T. tenax*, a number of metabolic pathways allow the cell to select a mode of metabolism depending on the energy requirements of the cell (depending, for example, on the cell's developmental or growth stage). Like all archaea, *Thermoproteus* possesses unique membrane lipids, which are ether-linked glycerol derivatives of 20 or 40 carbon branched lipids. The lipids' unsaturations are generally conjugated (as opposed to the unconjugation found in Bacteria and Eukaryota). In *Thermosphaera*, as in all members of the Crenarchaeota, the membranes are predominated by the 40-carbon lipids that span the entire membrane. This causes the membrane to be composed of monolayers with polar groups at each end. The cells are rod-shaped with diameters of up to 4 micrometres and up to 100 micrometres in length, and reproduce by developing branches on the end of the cell which grow into individual cells. They are motile by flagella.

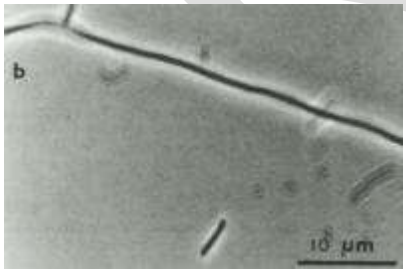
Ecology

Members of *Thermoproteus* are found in acidic hot springs and water holes; they have been isolated in these habitats in Iceland, Italy, North America, New Zealand, the Azores, and Indonesia. Their optimal growth temperature is 85 °C

Methanobacterium palustre



A microscopic view of*Methanobacterium palustre*.



Another photograph displaying*Methanobacterium palustre'*s cell shape.

Classification

Domain: *Archaea*--- Kingdom: *Euryarchaeota*--- Phylum: *Euryarchaeota*--- Class: *Methanobacteria*--- Order: *Methanobacteriales*--- Family: *Methanobacteriaceae*--- Genus: *Methanobacterium*--- Species: *Palustre*

Species NCBI:

Taxonomy

Methanobacterium palustre

Description and Discovery

photographs a typical peat bog environment where *M. palustre*inhabits. *Methanobacterium palustre* was discovered in 1989 in a location in Germany known as the Sippenauer Moor. The environmental habitat dominating in this area is known as a peat bog. Others often refer to it as a marshland. Due to its ability to anaerobically produce methane through a process known as methanogenesis, researchers thus named it's genus "*Methanobacterium*". Ironically the term used for the species identification, "*palustre*", is French for the word: marshland. Therefore the microorganism could be described as an Archaea that thrives in a marshland habitat via producing methane metabolically. (Zellner, G., et al.)

Cell Morphology

Methanobacterium palustre has a thin, rod-like shape and has been characterized as Strain F. This bacillus microorganism has an average cell length of anywhere between 2.5µm – 5µm. The cell body occasionally has filamentous appendages protruding outwards. These are used mostly as a means for cellular reproduction. In comparison, the appendages are over 10x the length of the actual cell body measuring to about 65µm. A Gram stain revealed that it is in fact Gram Positive meaning that it lacks a peptidoglycan layer outside of its cytoplasmic membrane. After viewing this microorganism using the wet mount technique, motility was not observed. Therefore, *M. palustre* must rely on water currents to move about its anoxic environment. (Zellner, G., et al.)

Ideal Living Requirements and Metabolism

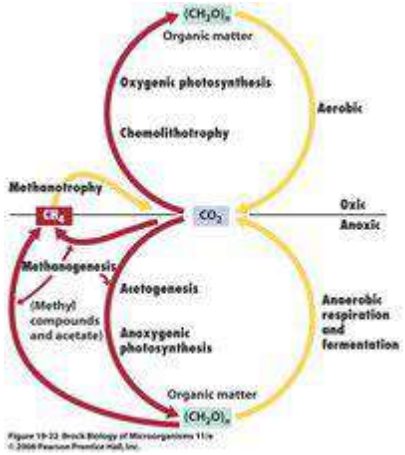
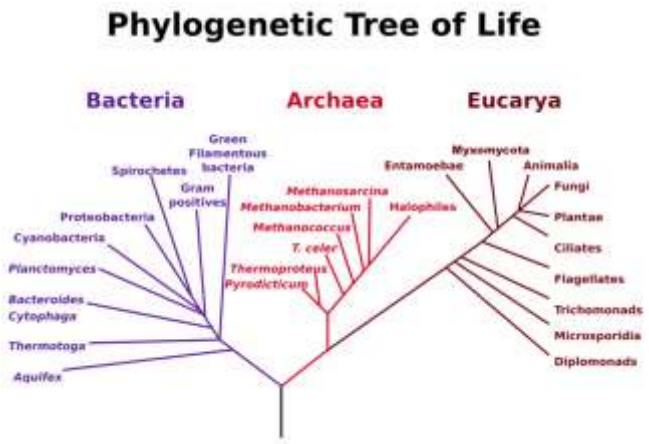


Diagram portraying the Carbon Cycle and Methanogenesis. *Methanobacterium palustre* is a mesophilic organism, best suited for temperatures between 33 degrees and 37 degrees Celsius. The minimum and maximum growth temperatures are between 20 degrees and 45 degrees, respectively. Optimal pH for growth is approximately 7 and a pH value as low as 6 is known to be tolerable. The microbe is sensitive to salinity. Growth is impeded at 18 g/L NaCl and growth completely halts at 30 g/L NaCl. (Zellner, G., et al.) When focusing on *Methanobacterium palustre*'s metabolism, it is an autrophic microorganism that undergoes methanogenesis. In order to utilize this process, the conditions must be anoxic. This means that *M. palustre* is anaerobic and found at low depths within its peat bog environment. The microorganism's metabolism is dictated via 2 processes: the Carbon Cycle and Alcohol Dehydrogenase enzyme. When following the Carbon Cycle pathway, carbon dioxide oxidizes hydrogen gas to produce methane and water. In this case, hydrogen gas is the electron donor and carbon dioxide is the electron acceptor. The alternate pathway, as stated above, utilizes an alcohol dehydrogenase enzyme to convert carbon dioxide and 2-propanol. This reaction produces the products methane and acetone. In this case, the hydrogens bound in the 2-propanol molecule are the electron donors and carbon dioxide is the electron acceptor. (Zellner, G., et al.)

Significance in Today's World

Current research studies of electrically probing *M. palustre* to achieve renewable "Green Methane". Methane is known to be a major greenhouse gas, over 25 times more potent than carbon dioxide. The methane becomes trapped within the Earth's atmosphere and causes increased global warming. Although the exact impact of *M. palustre* is unknown, wetland microbes alone emit 164 teragrams of CH4 per year (*"Overview of Greehouse Gases..."*) *M. palustre* also has potential for use in microbial fuel cells. Researchers at Stanford University and Penn State University have developed advanced cathodes that fuel

the microbes' metabolism with the use of electricity. They achieved this by using a reverse battery with positive and negative electrodes. They then placed these probes into a beaker of nutrient enriched water. The resulting methane is captured then combusted as fuel. If the electricity used to power the reaction was obtained by emissions free sources, then the net carbon output is zero. The carbon released by the combustion of the methane originally came from atmospheric carbon dioxide. The conversion of electrical currents to methane is in fact very efficient with yield levels up to 80%. These efficiency levels plummeted greatly when the strains were isolated alone on media plates when compared to when they were probed as a whole biofilm community. This shows the high necessity of *M. palustre* to live in a microbial community. (Schwartz, M)



Thermococcus

Thermococcus chitonophagus
Classification
Higher order taxa:
Archaea; Euryarchaeota; Thermococci; Thermococcales; Thermococcaceae
Species:
Thermococcus acidaminovorans, T. aegaeus, T. aggregans, T. alcaliphilus, T. atlanticus, T. barophilus, T. barossii, T. celer, T. chitonophagus, T. coalescens, T. fumicolans, T. gammatolerans, T. gorgonarius, T. guaymasensis, T. hydrothermalis, T. kodakarensis, T. litoralis, T. marinus, T. mexicalis, T. pacificus, T. peptonophilus, T. profundus, T. radiotolerans, T. sibiricus, T. siculi, T. stetteri, T. waimanguensis, T.

waiotapuensis, T. zilligii

NCBI: Taxonomy Genome

Description and Significance

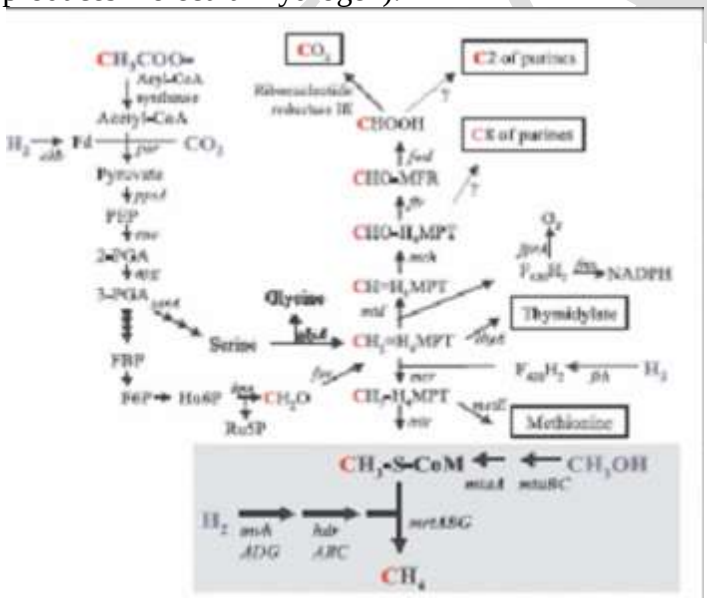
Thermococcus is an obligate heterotroph, strictly anaerobic archaeon which grows on organic substrates, primarily in the presence of elemental sulfur (S°), which is reduced to hydrogen sulfide. The genus contains the highest number of characterized isolates. It is highly motile with a polar tuft of multiple flagella. The archaeon grows between 60-100°C, with an optimum temperature of approximately 85°C, and between 5-9 pH (optimum = 6.5). As more genomes of the species are sequenced, it is expected to encode genes responsible for various cellular functions that provide an advantage for these organisms natural high-temperature habitats.

Genome Structure

T. kodakaraensis has been reported as *Pyrococcus* sp; however, its complete DNA sequence clearly shows its distiction from its relative.Its circular genome is 2,088,737 bp in length with no exochromosomal element, and contains 2,358 coding sequences (CDSs), of which 2,306 have been identified.Of those CDSs identified, 261 are unique to *T. kodakaraensis* with no significant homology to any known sequences up to date. Also, its G+C content (52%) is much higher than that of *Pyrococus* species (40-44%), which is a well-known difference between the two genera.

Cell Structure and Metabolism

The cells of *Thermococcus kodakaraensis* are irregular cocci of 1-2 μm in diameter, and multiply by constriction. Its cell envelope consists of two layers. Elemental sulfur (as a terminal electron acceptor) is required for heterotrophic growth on yeast extract, tryptone, peptone, and free amino acids (which produces hydrogen sulfide), but not in the presence of sodium thiosulfate pentahydrate, sodium sulfate, sodium nitrate, sodium nitrite or ferric chloride hexahydrate. However, it is able to utilize maltooligosaccharides, cyclodextrins, starch, or pyruvate in the absence of elemental sulfur (which produces molecular hydrogen).



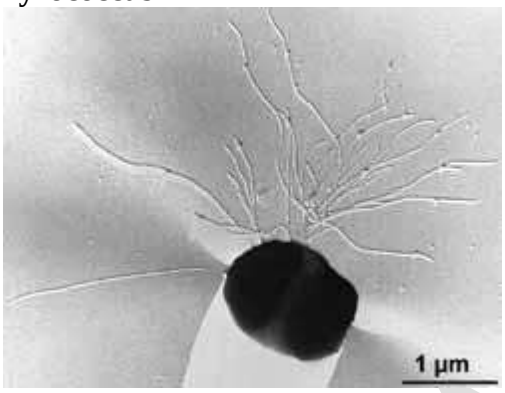
Predicted pyruvate metabolism in *Thermococcus kodakaraensis* The figure shows an overview of pyruvate metabolism by *T. kodakaraensis*. Abbreviations: ACS = acetyl-CoA synthetase; AlaAT = alanine aminotransferase; AspAT = aspartate aminotransferase; Fd = ferre-doxin; GDH = glutamate dehydrogenase; MAE = malic enzyme; PCK = phosphoenolpyruvate carboxykinase; POR =

pyruvate:ferredoxin oxidoreductase; PPS = phosphoenol pyruvate synthase; and PYK = pyruvate kinase. The study by Fukuda et al] raises the possibility of the involvement of malic enzyme in carbon metabolism. Currently, it is understood that malate and fumarate are metabolically isolated in the metabolism of *T. kodakaraensis* because linkages between malate and OAA (malate oxido-reductase) are missing in the pathways deduced from the whole genome analysis.

Ecology

The strain of *T. kodakaraensis* was isolated from a solfatara (102°C, pH 5.8) on Kodakara Island, Kagoshima, Japan. However, some of the other species, such as *T. peptonophilus*, are isolated from deep-sea hydrothermal areas. In either case, the genus is considered to play a major role in the ecology and metabolic activity of microbial consortia within hot-water ecosystems.

Pyrococcus



Pyrococcus furiosus. Copyright K.O.Stetter and R.Rachel, Univ. Regensburg, Germany.

Classification

Higher order taxa:

Archaea; Euryarchaeota; Thermococci; Thermococcales; Thermococcaceae

Species:

Pyrococcus horikoshii, P. abyssi, P. furiosus, P. endeavori

NCBI: Taxonomy Genome *P. abyssi P. furiosus P. horikoshii*

Three of the *Pyrococcus* species have been sequenced. *P. furiosus* is the largest containing 1908256 bp followed by *P. abyssi* with 1765118 bp and *P. horikoshii* with 1738505 bp. The genomes encode for many different metabolic enzymes which gives themselves a wider spectrum of living conditions because they can transport and metabolize a wide range of organic substances. Variation was detected between species as well.

Cell Structure and Metabolism

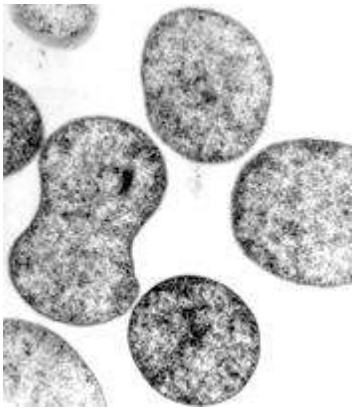
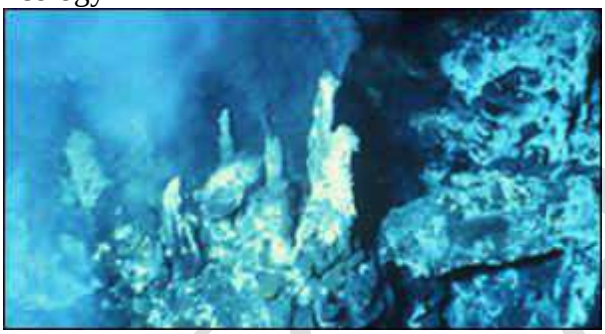


Image of *P. furiosus*. Courtesy of Henry Aldrich. The cells of *Pyrococcus* are about 0.8- 2um and are slightly irregular cocci in shape. They show a polar grouping of flagella and are enveloped by an S-layer enclosing a periplasmic space around the cytoplasmic membrane. *Pyrococcus* species are anaerobic but vary slightly concerning their metabolism. Peptide fermentation is the principle metabolic pathway however, growth has been observed for *P. furiosus* and *P. abyssi* on starch, maltose, and pyruvate but not for *P. horikoshii*. While the presence of elemental sulfur is not needed for growth, growth is enhanced with the addition of So.

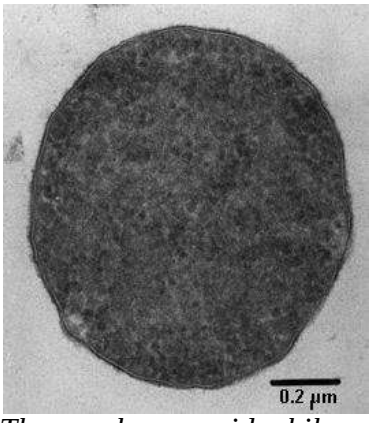
Ecology



A deep sea vent where *P. abyssi* has been located. *Pyrococcus* species inhabit environments with extremely high temperatures such as undersea hot vents. Optimal growth conditions include a pH level of about 7, a salt concentration around 2.5%, and a temperature around 98oC. Growing in temperatures this high, it is easy to see why they are anaerobic since at these boiling temperatures hardly any oxygen will be available. In the example of undersea hot vents, where *P. abyssi* has been found, there is no sunlight and the pressure is around 200 atm in addition to the extremely high temperature.

Thermoplasma

A Microbial Biorealm page on the genus *Thermoplasma*



Thermoplasma acidophilum.

Classification

Higher order taxa:

Archaea; Euryarchaeota; Thermoplasmata; Thermoplasmatales; Thermoplasmataceae

Species:

Thermoplasma acidophilum, T. volcanium **NCBI: Taxonomy *T. acidophliumT. volcanium***

Description and Significance

Thermoplasma was first isolated in the 1960s, in a coal pile in Indiana. *Thermoplasma* is a thermophile, meaning that it grows best in hot environments, usually between 55 and 60 degrees Celsius. This genus is most famous for its acidophilia, preferring pH range of 0.5-4. *Thermoplasma* cells lyse at a neutral pH.

Genome Structure

In 2001, the genome strucutres of both *Thermoplasma acidophilum DSM 1728* and *Thermoplasma volcanium GSS1* were sequenced. Until the genome was sequenced, *Thermoplasma* was believed to belong to the Eukaryotes. *T. acidophilum* contains 1,564,905 base pairs; it is one of the smallest genomes ever sequenced. Despite its size, there are 68 proteins that do not exist in any other archaeal genome. This genome lacks some characteristics as well. For example, although *Thermoplasma* have flagella, no chemotaxis protein has been found. In addition, *Thermoplasma* genes that reduce sulfur resemble those of bacteria instead of other archaea.

Cell Structure and Metabolism

Like all archaea, *Thermoplasma* are unicellular. Despite living in acidic environments, *Thermoplasma* lack cell walls. They are contained only by plasma membranes which consists of diether and tetraether lipids. In addition, *Thermoplasma* are not nucleated. The shape of these cells can be filamentous or coccoid, or they can take the form of a disc or club. *Thermoplasma* are flagellated. *Thermoplasma* show an irregular form when placed in water, and become spheres at room temperature. *Thermoplasma* are heterotrophic organisms. They are capable of obtaining energy in anaerobic environments via sulphur respiration.*Thermoplasma* are also scavengers, eating organisms that cannot survive in a highly acidic environment.

Ecology

"Pisciarelli Solfatara," a solfataric area in Naples, Italy, which harbors*Thermoplasma*. *Thermoplasma* are found in self-heating coal waste piles, although this is not a natural habitat. They are typically found in hot springs, as well as solfataric fields. *Thermoplasma acidophilum* can be employed as a model organism for scientific research, partially because this species has a strong evolutionary relationship to eukaryotes.

Halococcus

Halococcus	
Scientific classification	
Domain:	Archaea
Kingdom:	Euryarchaeota
Phylum:	Euryarchaeota
Class:	Halobacteria
Order:	Halobacteriales
Family:	Halobacteriaceae

Genus: *Halococcus*
Binomial name
Halococcus Schoop 1935
Species
• *H. dombrowskii* • *H. hamelinii* • *H. morrhuae* • *H. qingdaogense*

- *H. saccharolyticus*
- *H. salifodinae*
- *H. thailandensis*

Halococcus is a genus of the Halobacteriaceae.

Description and significance

Halococcus is a genus of extreme halophilic archaea, meaning that they require high salt levels, sometimes as high as 32% NaCl, for optimal growth. Halophiles are found mainly in inland bodies of water with high salinity, where their pigments (from a protein called rhodopsinprotein) tint the sediment bright colors. Rhodopsinprotein and other proteins serve to protect *Halococcus* from the extreme salinities of their environments. Because they can function under such high-salt conditions, *Halococcus* and similar halophilic organisms have been utilized economically in the food industry and even in skin-care production.

Genome structure

Halococcus' genome has not been sequenced yet, although studies of its 16s rDNA have demonstrated its placement on the phylogenetic tree. Due to the organisms' potential longevity, *Halococcus* may be a good candidate for exploring taxonomic similarities to life found in outer space.

Cell structure and metabolism

Halococcus is able to survive in its high-saline habitat by preventing the dehydration of its cytoplasm. To do this they use a solute which is either found in their cell structure or is drawn from the external

environment. Special chlorine pumps (see diagram) allow the organisms to retain chloride to maintain osmotic balance with the salinity of their habitat. The cells are cocci, 0.6–1.5 micrometres long with sulfated polysaccharide walls. The cells are organtrophic, using amino acids, organic acids, or carbohydrates for energy. In some cases they are also able to photosynthesize

Ecology

Halococcus is found in environments with high salt levels, mainly inland bodies of salt water, but some may be located in highly salted soil or foods. The pigmented proteins in some species cause the reddish tint found in some areas of the Dead Sea and the Great Salt Lake, especially at the end of the growing season. When under cultivation, the organisms grew best under high salinity conditions.

Halobacterium

Halobacterium sp. strain NRC-1, each cell about 5 µm in length.

Scientific classification	
Domain:	Archaea
Kingdom:	Euryarchaeota
Phylum:	Euryarchaeota
Class:	Halobacteria
Order:	Halobacteriales
Family:	Halobacteriaceae

Genus:	*Halobacterium*

Binomial name

Halobacterium
Elazari-Volcani 1957

Species

- *H. jilantaiense*
- *H. noricense*
- *H. salinarum*
- *H. piscisalsi*

Synonyms

- *Flavobacterium (subgen. Halobacterium) Elazari-Volcani 1940*
- *Halobacter Anderson 1954*
- *Halobacter*
- *not "Halobacterium" Schoop 1935 (nomen nudum)*

Note: The word "halobacterium" is also the singular form of the word "halobacteria".

In taxonomy, ***Halobacterium*** is a genus of the Halobacteriaceae.

The genus ***Halobacterium*** ("salt" or "ocean bacterium") consists of several species of archaea with an aerobic metabolism which require an environment with a high concentration of salt; many of their proteins will not function in low-salt environments. They grow on amino acids in their aerobic conditions. Their cell walls are also quite different from those of bacteria, as ordinary lipoprotein membranes fail in high salt concentrations. In shape, they may be either rods or cocci, and in color, either red or purple. They reproduce using binary fission (by constriction), and are motile. *Halobacterium* grows best in a 42 °C environment. The genome of an unspecified *Halobacterium* species, sequenced by Shiladitya DasSarma, comprises 2,571,010 bp (base pairs) of DNA compiled into three circular strands: one large chromosome with 2,014,239 bp, and two smaller ones with 191,346 and 365,425 bp. This species, called *Halobacterium* sp. NRC-1, has been extensively used for post-genomic analysis. *Halobacterium* species can be found in the Great Salt Lake, the Dead Sea, Lake Magadi, and any other waters with high salt concentration. Purple *Halobacterium* species owe their color to bacteriorhodopsin, a light-sensitive protein which provides chemical energy for the cell by using sunlight to pump protons out of the cell. The resulting proton gradient across the cell membrane is used to drive the synthesis of the energy carrier ATP. Thus, when these protons flow back in, they are used in the synthesis of ATP (this proton flow can be emulated with a decrease in pH

outside the cell, causing a flow of H+ ions). The bacteriorhodopsin protein is chemically very similar to the light-detecting pigment rhodopsin, found in the vertebrate retina.

Species of *Halobacterium*

Halobacterium cutirubrum > *Halobacterium salinarum*
Halobacterium denitrificans > *Haloferax denitrificans*
Halobacterium distributum > *Halorubrum distributum*
Halobacterium halobium > *Halobacterium salinarum*
Halobacterium jilantaiense
Halobacterium lacusprofundi > *Halorubrum lacusprofundi*
Halobacterium mediterranei > *Haloferax mediterranei*
Halobacterium noricense
Halobacterium pharaonis > *Natronomonas pharaonis*
Halobacterium piscisalsi
Halobacterium saccharovorum > *Halorubrum saccharovoru*
Halobacterium salinarum
Halobacterium sodomense > *Halorubrum sodomense*
Halobacterium trapanicum > *Halorubrum trapanicum*
Halobacterium vallismortis > *Haloarcula vallismortis*
Halobacterium volcanii > *Halobacterium volcanii*

Description and significance

Halobacteria are halophilic microorganisms, which means they grow in extremely high salinity environments. This archaeon can act as a good model for some aspects of eukaryotic biology, such as DNA replication, transcription, and translation. Comparing a halophile genome to that of other prokaryotes should give insight into microbial adaptation to extreme conditions.

Genome structure

The *Halobacterium* NRC-1 genome is 2,571,010 bp compiled into 3 circular replicons. More specifically, it is divided into one large chromosome with 2,014,239 bp and 2 small replicons pNRC100 (191,346 bp) and pNRC200 (365,425 bp). While much smaller than the large chromosome, the two plasmids account for most of the 91 insertion sequences and include genes for a DNA polymerase, seven transcription factors, genes in potassium and phosphate uptake, and cell division. The genome was discovered to contain a high G+C content at 67.9% on the large chromosome and 57.9% and 59.2% on the two plasmids. The genome also contained 91 insertion sequence elements constituting 12 families, including 29 on pNRC100, 40 on pNRC200, and 22 on the large chromosome. This helps explain the genetic plasticity that has been observed in *Halobacteria*. Of the archaea,*Halobacteria* are viewed as being involved in the most lateral genetics (gene transfer between domains) and a proof that this transfer does take place.

Cell structure and metabolism

Halobacterium species are rod shaped and enveloped by a single lipid bilayer membrane surrounded by an S-layer made from the cell-surface glycoprotein. *Halobacteria* grow on amino acids in aerobic conditions. Although *Halobacterium* NRC-1 contains genes for glucose degradation as well as genes for enzymes of a fatty acid oxidation pathway, it does not seem able to use these as energy sources. Even though the

cytoplasm retains an osmotic equilibrium with the hypersaline environment, the cell maintains a high potassium concentration. It does this by using many active transporters. Many *Halobacterium* species possess proteinaceous organelles called gas vesicles.

Ecology

Halobacteria can be found in highly saline lakes such as the Great Salt Lake, the Dead Sea, and Lake Magadi. *Halobacterium* can be identified in bodies of water by the light-detecting pigment bacteriorhodopsin, which not only provides the archaeon with chemical energy, but adds to its reddish hue as well. An optimal temperature for growth has been observed at 37 °C.

On an interesting note, however, *Halobacteria* are a candidate for a life form present on Mars. One of the problems associated with the survival on Mars is the destructive ultraviolet light. These microorganisms develop a thin crust of salt that can moderate some of the ultraviolet light. Sodium chloride is the most common salt and chloride salts are opaque to short-wave ultraviolet. Their photosynthetic pigment, bacteriorhodopsin, is actually opaque to the longer wavelength ultraviolet (its red color). In addition, *Halobacteria*make pigments called bacterioruberins that are thought to protect cells from damage by ultraviolet light. The obstacle *Halobacteria* would need to overcome is being able to grow at a low temperature during a presumably short time span when a pool of water could be liquid.

Recombination and mating

UV irradiation of Halobacterium sp. strain NRC-1 induces several gene products employed in homologous recombination.[2] For instance, a homolog of the *rad51*/*recA* gene, which plays a key role in recombination, is induced 7-fold by UV. Homologous recombination may rescue stalled replication forks, and/or facilitate recombinational repair of DNA damage.[2] In its natural habitat, homologous recombination is likely induced by the UV irradiation in sunlight.

Halobacterium volcanii has a distinctive mating system in which cytoplasmic bridges between cells appear to be used for transfer of DNA from one cell to another.[3] In wild populations of *Halorubrum*, genetic exchange and recombination occur frequently.[4] This exchange may be a primitive form of sexual interaction, similar to the more well studied bacterial transformation that is also a process for transferring DNA between cells leading to homologous recombinational repair of DNA damage

Eubacteria

The Eubacteria, also called just "bacteria," are one of the three main domains of life, along with the Archaea and the Eukarya. Eubacteria are prokaryotic, meaning their cells do not have defined, membrane-limited nuclei. As a group they display an impressive range of biochemical diversity, and their numerous members are found in every habitat on Earth. Eubacteria are responsible for many human diseases, but also help maintain health and form vital parts of all of Earth's ecosystems.

Structure

Like archeans, eubacteria are prokaryotes, meaning their cells do not have nuclei in which their DNA is stored. This distinguishes both groups from the eukaryotes, whose DNA is contained in a nucleus. Despite this structural resemblance, the Eubacteria are not closely related to the Archaea, as shown by analysis of their RNA Eubacteria are enclosed by a cell wall. The wall is made of cross-linked chains of peptidoglycan, a **polymer** that combines both amino acid and sugar chains. The network structure gives the wall the strength it needs to maintain its size and shape in the face of changing chemical and**osmotic** differences outside the cell. Penicillin and related antibiotics prevent bacterial cell growth by inactivating an enzyme that builds the cell wall. Penicillin-resistant bacteria contain an enzyme that chemically

modifies penicillin, making it ineffective. Some types of bacteria have an additional layer outside the cell wall. This layer is made from lipopolysaccharide (LPS), a combination of **lipids** and sugars. There are several consequences to possessing this outer layer. Of least import to the bacteria but significant for researchers, this layer prevents them from retaining a particular dye (called Gram stain) that is used to classify bacteria. Bacteria that have this LPS layer are called Gram-negative, in contrast to Gram-positive bacteria, which do not have an outer LPS layer and which do retain the stain. Of more importance to both the bacteria and the organisms they infect is that one portion of the LPS layer, called endotoxin, is particularly toxic to humans and other mammals. Endotoxin is partly to blame for the damage done by infection from *Salmonella* and other Gram-negative species. Within the cell wall is the plasma membrane, which, like the eukaryotic plasma membrane, is a phospholipid bilayer studded with proteins. Embedded in the membrane and extending to the outside may be flagella, which are whiplike protein filaments. Powered by molecular motors at their base, these spin rapidly, propelling the bacterium through its environment. Within the plasma membrane is the bacterial cytoplasm. Unlike eukaryotes, bacteria do not have any membrane-bound organelles, such as mitochondria or chloroplasts. In fact, these two organelles are believed to have evolved from eubacteria that took up residence inside an ancestral eukaryote. Bacterial cells take on one of several common shapes, which until recently were used as a basis of classification. Bacilli are rod shaped; cocci are spherical; and spirilli are spiral or wavyshaped. After division, bacterial cells may remain linked, and these form a variety of other shapes, from clusters to filaments to tight coils.

Metabolism

Despite the lack of internal compartmentalization, bacterial metabolism is complex, and is far more diverse than eukaryotic metabolism. Within the Eubacteria there are species that perform virtually every biochemical reaction known (and much bacterial chemistry remains to be discovered). Most of the vitamins humans require in our diet can be synthesized by bacteria, including the vitamin K humans absorb from the *Escherichia coli* (**E. coli**) bacteria in our large intestines. The broadest and most significant metabolic distinction among the Eubacteria is based on the source of energy they use to power their metabolism. Like humans, many bacteria are heterotrophs, consuming organic (carbon-containing) high-energy compounds made by other organisms. Other bacteria are chemolithotrophs, which use inorganic high-energy compounds, such as hydrogen gas, ammonia, or hydrogen sulfide. Still others are phototrophs, using sunlight to turn simple low-energy compounds into high-energy ones, which they then consume internally. For all organisms, extraction of energy from high-energy compounds requires a chemical reaction in which electrons move from atoms that bind them loosely to atoms that bind them tightly. The difference in binding energy is the profit available for powering other cell processes. In almost all eukaryotes, the ultimate electron acceptor is oxygen, and water and carbon dioxide are the final waste products. Some bacteria use oxygen for this purpose as well. Others use sulfur (forming hydrogen sulfide, which has a strong odor), carbon (forming flammable methane, common in swamps), and a variety of other compounds. Bacteria that use oxygen are called aerobes. Those that do not are called anaerobes. This distinction is not absolute, however, since many organisms can switch between the two modes of metabolism, and others can tolerate the presence of oxygen even if they do not use it. Some bacteria die in oxygen, however, including members of the **Gram positive** *Clostridium* genus.*Clostridium botulinum* produces botulinum toxin, the deadliest substance known. *C. tetani*produces tetanus toxin, responsible for tetanus and "lockjaw," while other *Clostridium* species cause gangrene.

Life Cycle

When provided with adequate nutrients at a suitable temperature and pH,*E. coli* bacteria can double in number within 20 minutes. This is faster than most species grow, and faster than *E. coli* grows under

natural conditions. Regardless of the rate, the growth of a bacterium involves synthesizing double the quantity of all its parts, including membrane, proteins, **ribosomes** , and DNA. Separation of daughter cells, called binary fission, is accomplished by creating a wall between the two halves. The new cells may eventually separate, or may remain joined. When environmental conditions are harsh, some species (including members of the genus*Clostridium*) can form a special resistive structure within themselves called an endospore. The endospore contains DNA, ribosomes, and other structures needed for life, but is metabolically inactive. It has a protective outer coat and very low water content, which help it survive heating, freezing, radiation, and chemical attack. Endospores are known to have survived for several thousand years, and may be capable of surviving for much longer, possibly millions of years. When exposed to the right conditions (presence of warmth and nutrients), the endospore quickly undergoes conversion back into an active bacterial cell.

DNA

Most eubacteria have DNA that is present in a single large circular chromosome. In addition, there may be numerous much smaller circles, called **plasmids** . Plasmids usually carry one or a few genes. These often are for specialized functions, such as metabolism of a particular nutrient or antibiotic. Despite the absence of a nucleus, the chromosome is usually confined to a small region of the cell, called the **nucleoid** , and is attached to the inner membrane. The bacterial genome is smaller than that of a eukaryote. For example, *E. coli* has only 4.6 million base pairs of DNA, versus three billion in humans. As in eukaryotes, the DNA is tightly coiled to fit it into the cell. Unlike eukaryotes, however, the DNA is not attached to histone proteins. Much of what we know about DNA replication has come from study of bacteria, particularly *E. coli*, and the details of this process are discussed elsewhere in this encyclopedia. Unlike eukaryotic replication, prokaryotic replication begins at a single point, and proceeds around the circle in both directions. The result is two circular chromosomes, which are separated during cell division. Plasmids replicate by a similar process.

Gene Transfer

While bacteria do not have sex like multicellular organisms, there are several processes by which they obtain new genes: **conjugation** , transformation, and transduction. Conjugation can occur between two appropriate bacterial strains when one (or both) extends hairlike projections called pili to contact the other. The chromosome, or part of one, may be transferred from one bacterium to the other. In addition, plasmids can be exchanged through these pili. Some bacteria can take up DNA from the environment, a process called transformation. The DNA can then be incorporated into the host chromosome. Some bacterial viruses, called phages, can carry out transduction. With some phages, the virus temporarily integrates into the host chromosome. When it releases itself, it may carry some part of the host DNA with it. When it goes on to infect another cell, this extra DNA may be left behind in the next round of integration and release. Other phages, called generalized transducers, package fragments of the chromosome into the phage instead of their own**genome** . When the transducing phage infects a new cell, they inject bacterial DNA. These phages lack their own genome and are unable to replicate in the new cell. The inserted bacterial DNA may recombine (join in with) the host bacterial chromosome.

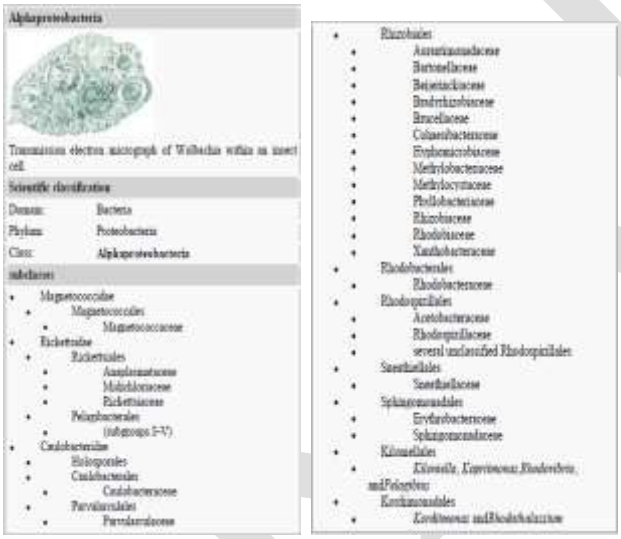
Gene Regulation and Protein Synthesis

Gene expression in many bacteria is regulated through the existence of operons. An operon is a cluster of genes whose protein products have related functions. For instance, the *lac* operon includes one gene that transports lactose sugar into the cell and another that breaks it into two parts. These genes are under the control of the same **promoter** , and so are transcribed and translated into protein at the same time. RNA polymerase can only reach the promoter if a repressor is not blocking it; the *lac* repressor is dislodged by lactose. In this way, the bacterium uses its resources to make lactose-digesting **enzymes** only when lactose

is available. Other genes are expressed constantly at low levels; their protein products are required for "housekeeping" functions such as membrane synthesis and DNA repair. One such enzyme is DNA gyrase, which relieves strain in the double helix during replication and repair. DNA gyrase is the target for the antibiotic ciproflaxin (sold under the name Cipro), effective against *Bacillus anthracis*, the cause of anthrax. Since eukaryotes do not have this type of DNA gyrase, they are not harmed by the action of this antibiotic. As in eukaryotes, translation (protein synthesis) occurs on the ribosome. Without a nucleus to exclude it, the ribosome can attach to the messenger RNA even while the RNA is still attached to the DNA. Multiple ribosomes can attach to the same mRNA, making multiple copies of the same protein.

The ribosomes of eubacteria are similar in structure to those in eukaryotes and archaea, but differ in molecular detail. This has two important consequences. First, sequencing ribosomal RNA molecules is a useful tool for understanding the evolutionary diversification of the Eubacteria. Organisms with more similar sequences are presumed to be more closely related. The same tool has been used to show that Archaea and Eubacteria are not closely related, despite their outward similarities. Indeed, Archaea are more closely related to eukaryotes (including humans) than they are to Eubacteria. Second, the differences between bacterial and eukaryotic ribosomes can be exploited in designing antibacterial therapies. Various unique parts of the bacterial ribosome are the targets for numerous antibiotics, including streptomycin, tetracycline, and erythromycin.

Alpha proteobacteria



Alphaproteobacteria is a class of bacteria in the phylum *Proteobacteria* (See also bacterial taxonomy). Its members are highly diverse and possess few commonalities, but nevertheless share a common ancestor. Like most members of the *Proteobacteria*, most of its members are Gram-negative and some of its intracellular parasitic members lack peptidoglycan and are consequently gram variable.

Characteristics

The *Alphaproteobacteria* is a diverse taxon and comprises several phototrophic genera, several genera metabolising C1-compounds (*e.g.*,*Methylobacterium spp.*), symbionts of plants (e.g., *Rhizobium spp.*), endosymbionts of arthropods (*Wolbachia*) and intracellular pathogens (*e.g. Rickettsia*). Moreover, the class includes (as an extinct member) the protomitochondrion, the bacterium that was engulfed by the eukaryotic ancestor and gave rise to the mitochondria, which are organelles in eukaryotic cells (See endosymbiotic theory). A species of technological interest is *Rhizobium radiobacter* (formerly *Agrobacterium*

tumefaciens): scientists often use this species to transfer foreign DNA into plant genomes. Aerobic anoxygenic phototrophic bacteria, such as *Pelagibacter ubique*, are alphaproteobacteria that are a widely distributed marine plankton that may constitute over 10% of the open ocean microbial community.

Evolution and genomics

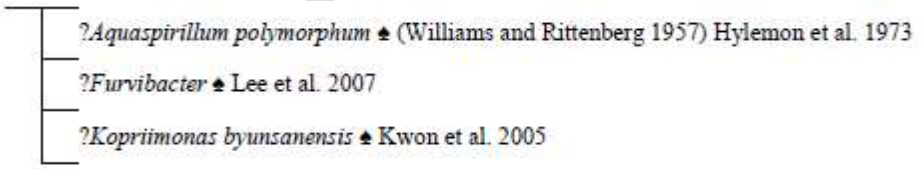
There is some disagreement on the phylogeny of the orders, especially for the location of the *Pelagibacterales*, but overall there is some consensus. This issue stems form the large difference in gene content (*e.g.* genome streamlining in *Pelagibacter ubique*) and the large difference in GC-richness between members of several order. Specifically,*Pelagibacterales*, *Rickettsiales* and *Holosporales* contains species with AT-rich genomes. It has been argued that it could be a case of convergent evolution that would result in an artefactual clustering. However, several studies disagree,. Furthermore, it has been found that the GC-content of ribosomal RNA, the traditional phylogenetic marker, little reflects the GC-content of the genome: for example, members of the *Holosporales* have a much higher ribosomal GC-content than members of the *Pelagibacterales* and *Rickettsiales*, which have similarly low genomic GC-content, because they are more closely related to species with high genomic GC-contents than to members of the latter two orders. The Class *Alphaproteobacteria* is divided into three subclasses *Magnetococcidae*, *Rickettsidae* and *Caulobacteridae*.

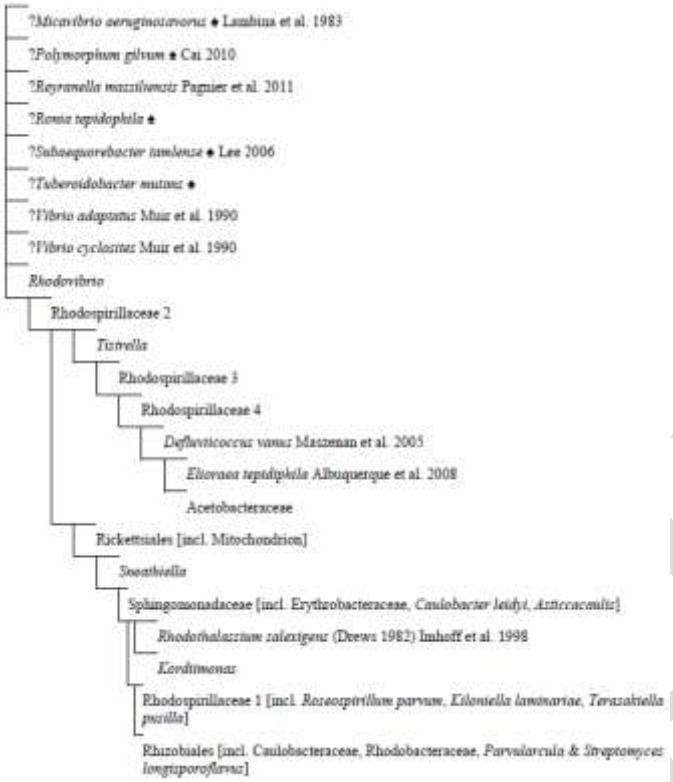
The basal group is*Magnetococcidae*, which is composed by a large diversity of magnetotactic bacteria, but only one is described, *Magnetococcus marinus*. The *Rickettsidae* is composed of the intracellular *Rickettsiales* and the free-living *Pelagibacterales*. The *Caulobacteridae* is composed of the *Holosporales*, *Rhodospirillales*, *Sphingomonadales*, *Rhodobacterales*, *Caulobacterales*, *Kiloniellales*, *Kordiimonadales*,*Parvularculales* and *Sneathiellales*.

Comparative analyses of the sequenced genomes have also led to discovery of many conserved indels in widely distributed proteins and whole proteins (i.e. signature proteins) that are distinctive characteristics of either all *Alphaproteobacteria*, or their different main orders (viz. *Rhizobiales*, *Rhodobacterales*, *Rhodospirillales*, *Rickettsiales*, *Sphingomonadales* and *Caulobacterales*) and families viz.*Rickettsiaceae*, *Anaplasmataceae*, *Rhodospirillaceae*, *Acetobacteraceae*, *Bradyrhiozobiaceae*, *Brucellaceae* and *Bartonellaceae*). These molecular signatures provide novel means for the circumscription of these taxonomic groups and for identification/assignment of new species into these groups.[13] Phylogenetic analyses and conserved indels in large numbers of other proteins provide evidence that*Alphaproteobacteria* have branched off later than most other phyla and Classes of Bacteria except *Betaproteobacteria* and*Gammaproteobacteria*.

Phylogeny

The currently accepted taxonomy is based on the List of Prokaryotic names with Standing in Nomenclature (LPSN) and National Center for Biotechnology Information (NCBI) and the phylogeny is based on 16S rRNA-based LTP release 106 by 'The All-Species Living Tree' Project





Notes: ♠ Strains found at the National Center for Biotechnology Information (NCBI) but not listed in the List of Prokaryotic names with Standing in Nomenclature (LSPN)

Betaproteobacteria

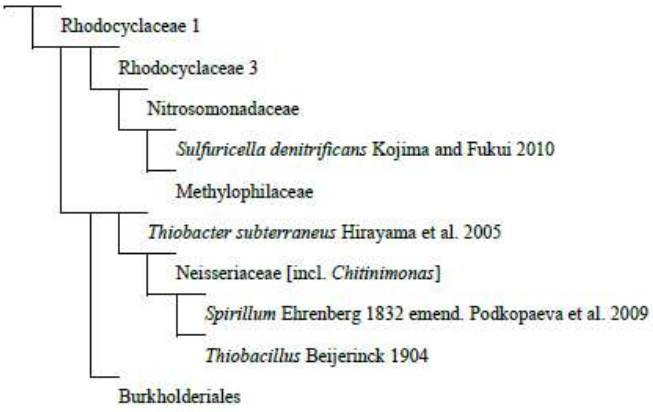
- Rhodocyclales
  - Rhodocyclaceae

Betaproteobacteria are a class of Proteobacteria. Betaproteobacteria are, like all Proteobacteria, Gram-negative.

The Betaproteobacteria consist of several groups of aerobic or facultative bacteria that are often highly versatile in their degradation capacities, but also contain chemolithotrophic genera (e.g., the ammonia-oxidising genus *Nitrosomonas*) and some phototrophs (members of the genera *Rhodocyclus* and *Rubrivivax*). Betaproteobacteria play a role in nitrogen fixation in various types of plants, oxidizing ammonium to produce nitrite – an important chemical for plant function. Many of them are found in environmental samples, such as waste water or soil.Pathogenic species within this class are the Neisseriaceae (gonorrhea and meningitis) and species of the genus *Burkholderia*.

Phylogeny

The currently accepted taxonomy is based on the List of Prokaryotic names with Standing in Nomenclature and National Center for Biotechnology Information and the phylogeny is based on 16S rRNA-based LTP release 106 by 'The All-Species Living Tree' Project



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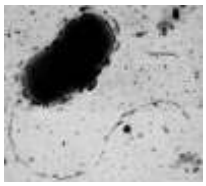
Deltaproteobacteria



Deltaproteobacteria is a class of Proteobacteria. All species of this group are, like all Proteobacteria, gram-negative.

The *Deltaproteobacteria* comprise a branch of predominantly aerobic genera, the fruiting-body-forming *Myxobacteria* which releasemyxospores in unfavorable environments, and a branch of strictly anaerobic genera, which contains most of the known sulfate-(*Desulfovibrio*, *Desulfobacter*, *Desulfococcus*, *Desulfonema*, etc.) and sulfur-reducing bacteria (e.g. *Desulfuromonas* spp.) alongside several other anaerobic bacteria with different physiology (e.g. ferric iron-reducing *Geobacter* spp. and syntrophic *Pelobacter* and *Syntrophus* spp.). A pathogenic intracellular Deltaproteobacteria has recently been identified.

Phylogeny

The currently accepted taxonomy is based on the List of Prokaryotic names with Standing in Nomenclature (LPSN) [3] and National Center for Biotechnology Information (NCBI)[4] and the phylogeny is based on 16S rRNA-based LTP release 111 by 'The All-Species Living Tree' Project [5]

Epsilonproteobacteria

Epsilonproteobacteria are a class of Proteobacteria.[1] All species of this class are, like all Proteobacteria, Gram-negative.

The Epsilonproteobacteria consist of few known genera, mainly the curved to spirilloid *Wolinella* spp., *Helicobacter* spp., and*Campylobacter* spp. Most of the known species inhabit the digestive tracts of animals and serve as symbionts (*Wolinella* spp. in cattle) orpathogens (*Helicobacter* spp. in the stomach, *Campylobacter* spp. in the duodenum).

Numerous environmental sequences and isolates of Epsilonproteobacteria have also been recovered from hydrothermal vents and cold seep habitats. Examples of isolates include *Sulfurimonas autotrophica*,[2] *Sulfurimonas paralvinellae*,[3]*Sulfurovum lithotrophicum*[4] and*Nautilia profundicola*.[5]

A member of the class Epsilonproteobacteria occurs as an endosymbiont in the large gills of the deepwater sea snail *Alviniconcha hessleri*.[6]

The Epsilonproteobacteria found at deep-sea hydrothermal vents characteristically exhibit chemolithotrophy, meeting their energy needs by oxidizing reduced sulfur, formate, or hydrogen coupled to the reduction of nitrate or oxygen.[7] Autotrophic Epsilonproteobacteria use the reverse Krebs cycle to fix carbon dioxide into biomass, a pathway originally thought to be of little environmental significance. The oxygen sensitivity of this pathway is consistent with their microaerophilic or anaerobic niche in these environments, and their likely evolution in the Mesoproterozoic oceans,[8] which are thought to have been sulfidic with low levels of oxygen available from cyanobacterial photosynthesis.[9]
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The currently accepted taxonomy is based on the List of Prokaryotic names with Standing in Nomenclature (LPSN) [10] and National Center for Biotechnology Information (NCBI)[11] and the phylogeny is based on 16S rRNA-based LTP release 106 by 'The All-Species Living Tree' Project [12]
Notes: Prokaryotes where no pure (axenic) cultures are isolated or available, i.e. not cultivated or can not be sustained in culture for more than a few serial passages
Proteobacteria

P. Srinivasan, Asst. Professor, Dept. of Microbiology, KAHE The Epsilonproteobacteria found at deep-sea hydrothermal vents characteristically exhibit chemolithotrophy, meeting their energy needs by oxidizing reduced sulfur, formate, or hydrogen coupled to the reduction of nitrate or oxygen.[7] Autotrophic Epsilonproteobacteria use the reverse Krebs cycle to fix carbon dioxide into biomass, a pathway originally thought to be of little environmental significance. The oxygen sensitivity of this pathway is consistent with their microaerophilic or anaerobic niche in these environments, and their likely evolution in the Mesoproterozoic oceans,[8] which are thought to have been sulfidic with low levels of oxygen available from cyanobacterial photosynthesis.[9]
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Proteobacteria **Proteobacteria**
The **Proteobacteria** are a major group (phylum) of Gram-negative bacteria. They include a wide variety of pathogens, such as*Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*, *Yersinia*, and many other notable genera.[3] Others are free-living (nonparasitic), and include many of the bacteria responsible for nitrogen fixation.
Carl Woese established this grouping in 1987, calling it informally the "purple bacteria and their

relatives".[4] Because of the great diversity of forms found in this group, the Proteobacteria are named after Proteus, a Greek god of the sea capable of assuming many different shapes; it is not named after the genus *Proteus*.[1][5]

Alphaproteobacteria grow at very low levels of nutrients and have unusual morphology such as stalks and buds. They include agriculturally important bacteria capable of inducing nitrogen fixation in symbiosis with plants. An example of Alphaproteobacteria is*Wolbachia*, which is the most common infectious bacterial genus in the world that lives only inside the cells of their hosts, usually insects.

Betaproteobacteria often use nutrient substances that diffuse away from areas of anaerobic decomposition of organic matter (hydrogen gas, ammonia, methane) and includes chemoautotrophs. An example of Betaproteobacteria is *Bordetella pertussis*, which causes pertussis (whooping cough).

Gammaproteobacteria are the largest subgroup which includes *Acinetobacter*, *Pseudomonas*, *Escherichia*, *Salmonella*, and *Serratia*species.

Deltaproteobacteria include bacteria that are predators on other bacteria and are important contributors to the sulfur cycle. An example is *Desulfovibrio*, which is found in anaerobic sediments and in the intestinal tracts of humans and animals.

Epsilonproteobacteria are slender, Gram-negative rods that are helical or curved. They are also motile by flagella and are microaerophilic. An example is *Helicobacter*, which has been identified as the most common cause of peptic ulcers in humans and a cause of stomach cancer.

Characteristics

All proteobacteria are Gram-negative, with an outer membrane mainly composed of lipopolysaccharides. Many move about using flagella, but some are nonmotile or rely onbacterial gliding. The last include the myxobacteria, a unique group of bacteria that can aggregate to form multicellular fruiting bodies. Also, a wide variety in the types ofmetabolism exists. Most members are facultatively or obligately anaerobic, chemoautotrophs, and heterotrophic, but numerous exceptions occur. A variety of genera, which are not closely related to each other, convert energy from light through photosynthesis. These are called purple bacteria, referring to their mostly reddish pigmentation.

Proteobacteria are associated with the imbalance of microbiota of the lower reproductive tract of women. These species are associated with inflammation.[6]

Taxonomy

The group is defined primarily in terms of ribosomal RNA (rRNA) sequences. The Proteobacteria are divided into six sections, referred to by the Greek letters alpha through zeta. These were previously regarded as subclasses of the phylum, but they are now treated asclasses. The alpha, beta, delta, and epsilon classes are monophyletic. The genus *Acidithiobacillus*, part of the*Gammaproteobacteria* until it was transferred to Class *Acidithiobacillia* in 2013,[10] is paraphyletic to *Betaproteobacteria* according tomultigenome alignment studies.[11]

Proteobacterial clades include some prominent genera,[12] e.g.:

Alphaproteobacteria: *Brucella, Rhizobium, Agrobacterium, Caulobacter, Rickettsia, Wolbachia*, etc.

Betaproteobacteria: *Bordetella, Ralstonia, Neisseria, Nitrosomonas*, etc.

Gammaproteobacteria: *Escherichia, Shigella, Salmonella, Yersinia, Buchnera, Haemophilus, Vibrio, Pseudomonas*, etc.

Deltaproteobacteria: *Desulfovibrio, Geobacter, Bdellovibrio*, etc.

Epsilonproteobacteria: *Helicobacter, Campylobacter, Wolinella*, etc.

Zetaproteobacteria: *Mariprofundus ferrooxydans*

Zetaproteobacteria

The class **Zetaproteobacteria** is the sixth and most recently described class of the Proteobacteria.[1] Zetaproteobacteria can also refer to the group of organisms assigned to this class. The Zetaproteobacteria are represented by a single described species, *Mariprofundus ferrooxydans*,[2] which is an iron-oxidizing neutrophilic chemolithoautotroph originally isolated from Loihi Seamount in 1996 (post-eruption).[1][3]Molecular cloning techniques focusing on the small subunit ribosomal RNA gene have also been used to identify a more diverse majority of the Zetaproteobacteria that have as yet been unculturable.[4]

Regardless of culturing status, the Zetaproteobacteria show up worldwide in estuarine and marine habitats associated with opposing steepredox gradients of reduced (ferrous) iron and oxygen, either as a minor detectable component or as the dominant member of the microbial community.[5][6][7][8][9][10] Zetaproteobacteria have been most commonly found at deep-sea hydrothermal vents,[4] though recent discovery of members of this class in near-shore environments has led to the reevaluation of Zetaproteobacteria distribution and significance.

Microbial mats encrusted with iron oxide on the flank of Loihi Seamount, Hawaii. Microbial communities in this type of habitat can harbor microbial communities dominated by the iron-oxidizing Zetaproteobacteria.

Significance

The Zetaproteobacteria are distributed worldwide in deep sea and near shore environments at oxic/anoxic interfaces. With this wide distribution, the Zetaproteobacteria have the potential to play a substantial role in biogeochemical cycling, both past and present. Ecologically, the Zetaproteobacteria play a major role in the engineering of their own environment through the use of the controlled deposition of mineralized iron oxides, also directly affecting the environment of other members of the microbial community. Prevalence of the Zetaproteobacteria in near-shore metal (e.g. steel) coupon biocorrosion experiments highlights the impact of these marine iron oxidizers on expensive problems such as the rusting of ship hulls, metal pilings, and pipelines.

Discovery

The Zetaproteobacteria were first discovered in 1991 by Craig Moyer, Fred Dobbs, and David Karl as a single rare clonein a mesophilic, or moderate temperature, hydrothermal vent field known as Pele's Vents at Loihi Seamount, Hawaii. This particular vent was dominated by sulfur-oxidizing Epsilonproteobacteria. With no close relatives known at the time, the clone was initially labeled as Gammaproteobacteria.[16]

Subsequent isolation of two strains of *M. ferrooxydans*, PV-1 and JV-1,[3] along with the increasing realization that a phylogenetically distinct group of Proteobacteria (the Zetaproteobacteria) could be found globally as dominant members of bacterial communities led to the suggestion for the creation of this new class of the Proteobacteria.

Cultivation

Neutrophilic microaerophilic Fe-oxidizing bacteria are typically cultivated using an agarose-stabilized or liquid culture with an FeS or FeCO3 plug. The headspace of the culture tube is then purged with air or a low concentration of oxygen (often 1% or less O2). Fe-oxidizers have also successfully been cultivated in liquid culture with FeCl2 as the Fe source. These cultivation techniques follow those found in Emerson and Floyd (2005).[17]

Recently, researchers have been able to culture the Zetaproteobacteria using graphite electrodes at a fixed voltage.[18]Researchers have also aimed to improve cultivation techniques using a high-biomass batch culturing technique.[19]

Morphology

One of the most distinctive ways of identifying circumneutral iron oxidizing bacteria visually is by identifying the structure of the mineralized iron oxyhydroxide product created during iron oxidation.[3][20] Oxidized, or ferric iron is insoluble at circumneutral pH, thus the microbe must have a way of dealing with the mineralized "waste" product. It is thought that one method to accomplish this is to control the deposition of oxidized iron.[21][22][23] Some of the most common morphotypes include: amorphous particulate oxides, twisted or helical stalks (figure),[21] sheaths,[24] and y-shaped irregular filaments.

These morphologies exist both in freshwater and marine iron habitats, though common freshwater iron-oxidizing bacteria such as *Gallionella* sp. (twisted stalk) and *Leptothrix ochracea* (sheath) have only extremely rarely been found in the deep sea (not significant abundance). One currently published morphotype that has been partially resolved is the twisted stalk, which is commonly formed by *M. ferrooxydans*. This bacteria is a gram negative kidney-bean-shaped cell that deposits iron oxides on the concave side of the cell, forming twisted stalks as it moves through its environment.[21][22]

Another common Zetaproteobacteria morphotype is the sheath structure, which has yet to be isolated, but has been identified with Fluorescence in situ hybridization (FISH).[24] Iron oxidation morphotypes can be preserved and have been detected in ancient hydrothermal deposits preserved in the rock record Some current work is focused on how the Zetaproteobacteria form their individual biominerals in the modern environment so that scientists can better interpret Fe biominerals found in the rock record.

Ecology

Biodiversity

An operational taxonomic unit, or an OTU, allows a microbiologist to define a bacterial taxa using defined similarity bins based on a gene of interest. In microbial ecology, the small subunit ribosomal RNA gene is generally used at a cut off of 97% similarity to define an OTU. In the most basic sense, the OTU represents a bacterial species.

For the Zetaproteobacteria, 28 OTUs have been defined.[4] Of interest were the two globally distributed OTUs that dominated the phylogenetic tree, two OTUs that seemed to originate in the deep subsurface,[10] and several endemic OTUs, along with the relatively limited detection of the isolated Zetaproteobacteria representative.

Classification

Zetaproteobacteria OTUs can now be classified according to the naming scheme used in McAllister et al. (2011).[4] The program ZetaHunter uses closed reference binning to identify sequences closely related to the established OTUs in addition to identifying novel Zetaproteobacteria OTUs. ZetaHunter's feature list continues to grow, but includes: 1) stable OTU binning, 2) sample comparison, 3) database and mask management options, 4) multi-threaded processing, 5) chimera checking, 6) checks for non-database-related sequences, and 7) OTU network maps.

Habitats

Deep-sea hydrothermal vents associated with:
hotspots
back arc spreading centers/troughs[0][37][38][39]
Island arcs[6][8]
Near-shore venting associated with a coral reef ecosystem

Spreading centers (on- and off axis)
Inactive sulfides along the East Pacific Rise (spreading center)[46]
Flooded caldera[9]

Guaymas Basin[47]
Massive sulfide deposits [48]
Altered deep-sea basalts[49]
Levantine Basin and continental margin[50]
Antarctica continental shelf sediment[51]
Brine/seawater interface[52]
Stratified Chesapeake Bay estuary[43]
Intertidal mixing zone of a beach aquifer[13][43]
Salt marsh sediment[11][53]
Oxygenated worm burrows or bioturbated beach sands[13][54][55]
Near-shore metal biocorrosion experiments[11][14]
Tsunami impacted soils[56]
Mangrove soils[57]
Deep subsurface CO2-rich springs[58][59]
Subsurface flow reactor in the Äspö Hard Rock Laboratory[60]
Rimicaris exoculata (shrimp) gut at the MAR[61][62]

Ecological Niche

All of the habitats where Zetaproteobacteria have been found have (at least) two things in common: 1) they all provide an interface of steep redox gradients of oxygen and iron.[63] & 2) they are marine or brackish.[44]

Reduced hydrothermal fluids, for instance, exiting from vents in the deep-sea carry with them high concentrations of ferrous iron and other reduced chemical species, creating a gradient upward through a microbial mat of high to low ferrous iron. Similarly, oxygen from the overlying seawater diffuses into the microbial mat resulting in a downward gradient of high to low oxygen. Zetaproteobacteria are thought to live at the interface, where there is enough oxygen for use as an electron acceptor without there being too much oxygen for the organism to compete with the increased rate of chemical oxidation, and where there is enough ferrous iron for growth.[20][63]

Iron oxidation is not always energetically favorable. Reference[39] discusses favorable conditions for iron oxidation in habitats that otherwise may have been thought to be dominated by the more energy yielding metabolisms of hydrogen or sulfur oxidation. Note: Iron is not the only reduced chemical species accociated with these redox gradient environments. It is likely that Zetaproteobacteria are not all iron oxidizers.

Metabolism

Iron oxidation pathways in both acidophilic and circumneutral freshwater iron oxidation habitats, such as acid mine drainage or groundwater iron seeps, respectively, are better understood than marine circumneutral iron oxidation.

In recent years, researchers have made progress in suggesting possibilities for how the Zetaproteobacteria oxidize iron, primarily through comparative genomics. With this technique, genomes from organisms with similar function, for example the freshwater Fe-oxidizing Betaproteobacteria and the marine Fe-oxidizing Zetaproteobacteria, are compared to find genes that may be required for this function. Identifying the iron oxidation pathway in the Zetaproteobacteria began with the publication of the first described cultured representative, *M. ferrooxydans* strain PV-1. In this genome, the gene neighborhood of a molybdopterin oxidoreductase protein was identified as a place to start looking at candidate iron oxidation pathway genes.[64] In a follow up analysis of a metagenomic sample, Singer et al. (2013) concluded that this molybdopterin oxidoreductase gene cassette was likely involved in Fe oxidation.[65] Comparative

analysis of several single cell genomes, however, suggested an alternative conserved gene cassette with several cytochrome c and cytochrome oxidase genes to be involved in Fe oxidation.[66] For further reading on Fe oxidation pathways see reference.[67]

The phylogenetic distance between the Zetaproteobacteria and the Fe-oxidizing freshwater Betaproteobacteria suggests that Fe oxidation and the produced biominerals are the result of convergent evolution.[24] Comparative genomics has been able to identify several genes that are shared between the two clades, however, suggesting that the trait of Fe oxidation could have been horizontally transferred, possibly virally-mediated.[68][69]

Fe mats associated with the Zetaproteobacteria, in addition to oxidizing Fe have been found to have the genetic potential for denitrification, arsenic detoxification, Calvin-Benson-Bassham (CBB) cycle, and reductive tricarboxylic acid (rTCA) cycles. Novel primers have been designed to detect these genes in environmental samples.[70] It is difficult at this point to speculate on the metabolism of the entire class of Zetaproteobacteria (with at least 28 different OTUs/species) with the limited sample size.

Low G+C Gram Positive Bacteria

This is an ecologically and industrially important group of microorganisms. The group name refers to a phylum of Bacteria, also known as the Firmicutes, its members share a common evolutionary history. Many have certain distinct cellular characteristics. Gram-positive organisms stain purple with a differential staining procedure developed in 1884 by Christian Gram. This procedure identifies cells that have a thick cell wall of peptidoglycan. While many Firmicutes stain Gram-positive, some do not. In fact, some Firmicutes have no cell wall at all! They are called "low G+C" because their DNA typically has fewer G and C DNA bases than A and T bases as compared to other bacteria. Exceptions have been identified and some Firmicutes have G+C content as high as 55% (e.g. *Geobacillus thermocatenulatus*). Certain Firmicutes make resistant progeny called endospores, while others can only reproduce through binary fission. It is evident that Firmicutes are as diverse as they are important.

The typical Firmicutes cell envelope consists of a layer of peptidoglycan, which is a polymer of protein and carbohydrate that gives structure and shape to the cell and protects the bacterium from osmotic stress. Underneath the peptidoglycan there is a phospholipid bilayer and its associated proteins that act as a selective barrier. Many members of the Firmicutes have an outermost envelope layer of protein called the S layer. The function of the S layer is not known but it is believed to prevent predation in the environment.

The Firmicutes and Gram-negative Proteobacteria cell envelopes differ most notably by the presence or absence of an outer membrane. Other distinguishing features of these cell envelopes include porins, S layers, teichoic and lipoteichoic acids, and lipoproteins. Groups of Firmicutes have been classified based on characteristics like type of cell envelope, endospore formation and aerotolerance (how well they live and grow in oxygen). Currently, there are seven recognized Classes of Firmicutes: the Erysipelotrichia, the Negativicutes, the Limnochordia, the Tissierellia, the Thermolithobacteria, the Clostridia and the Bacilli. Mollicutes such as the Mycoplasma are very small cells lacking a peptidoglycan cell wall. Without a cell wall these bacteria tend to be very delicate. Therefore Mollicutes are often closely associated with a host that can provide them with a stable environment. They also have special lipids called sterols and lipoglycans, to help protect against osmotic stress. This group contains several very important human pathogens that can cause pneumonia, urethritis, carditis, and gingivitis, to name a few.

The DNA of all living things is made up of four nucleotide bases Adenine (A), Cytosine (C), Guanine (G) and Thymidine (T). In a double helix of DNA, Adenine pairs with Thymidine and Guanine pairs with Cytosine. Therefore the number of Cytosine bases equals the number of Guanine bases and likewise A=T. The percentage of G+C is one of many general features used to characterize bacterial genomes

Most Firmicutes have cell walls, and these bacteria can be found in a great variety of habitats. They are grouped in the Class Bacilli or Class Clostridia. Diverse Firmicutes include *Staphylococcus, Micrococcus, Streptococcus* and *Lactobacillus*. Some staphylococci and micrococci are commonly found on human skin and mucosal surfaces. *Streptococcus* is most famous for causing "strep throat" but many benign streptococci are normally found in the mouth and throat. *Lactobacillus* is common in the making of yogurt and cheese products. Some *Lactobacillus* species are associated with mucosal surfaces of humans. These resident *Lactobacillus* species help maintain our health by preventing colonization by disease-associated bacteria.

Some Firmicutes can form an endospore, a resistant differentiated cell produced under special, usually stressful, conditions. Endospore-forming bacteria such as *Bacillus* and*Clostridium* species can be classified by their aerotolerance. Many anaerobic organisms fall under the*Clostridium* banner. These organisms have very diverse ways of getting energy without using oxygen, but almost all are fermenters. Some *Clostridium* species are used by industry to produce solvents, an end product of their fermentation activity. Others produce toxins. One famous application of a*Clostridium* toxin is the use of *Clostridium botulinum* toxin, also known as BoTox, to paralyze muscles of the face to reduce skin wrinkles. *Epulopiscium* is closely related to *Clostridium*species.

Bacilli prefer to live in oxygen-rich environments but some are capable of survival without it. Members of this group are commonly found in soil. Some are responsible for the disease anthrax while others produce antibiotics or insecticides. *Bacillus subtilis* is one of the primary model organisms used by researchers to understand topics ranging from cell differentiation to iron storage and DNA replication. The organisms described above represent only a tiny part of the diversity found within the group of Firmicutes. Their huge impact on fields as diverse as agriculture, medicine, food production and ecology make them a vital subject of inquiry. **Life in Extreme Environments Dry environments** Imagine a desert and a feeling of dehydration follows. In the absence of water, lipids (fats) , proteins and nucleic acids (DNA, RNA) suffer structural damage. The Atacama desert located on the high northern Andean plains of Chile is one of the oldest, driest hot deserts on the Earth, while the Antarctic dry valleys are the coldest, driest places on Earth. In both cases, despite environmental extremes, life exists in the form of microbes: cyanobacteria, algae, lichens, and fungi.

Anhydrobiosis is a strategy organisms use to survive dry spells. During anhydrobiosis their cells come to contain only minimal amounts of water. No metabolic activity is performed. A variety of organisms can become anhydrobiotic, including bacteria, yeast, fungi, plants, insects, the aforementioned tardigrades, mycophagous (fungi-eating) nematodes, and the brine shrimp *Artemia salina* (also known as "Sea Monkeys" when marketed to school age children). During the drying out process (desiccation), less available water forces substances to increase in their concentration. Such increases lead to stressful responses within a cell that are similar to those of a cell experiences when exposed to high salt environments. The ultimate dry environment is the "desert" of space. Adaptations to desiccation are critical for organisms to survive in interplanetary space. One organism in particular (described below) is a natural born space traveler. **Salinity** As airplanes descend into the San Francisco area, red patches on the eastern shore of the South Bay are conspicuous. These are evaporation ponds of Cargill Salt Company. The cause of the red color is halophilic (salt-loving) microbes that produce red pigments called carotenoids. The microbes involved are either members of the Archaea, a major group of microbes superficially similar to bacteria, or the green alga Dunaliella salina. At a bit lower (25-33%) salinity, bacteria, cyanobacteria, other green algae, diatoms and protozoa are found. Some Archaea, cyanobacteria, and *Dunaliella salina* can even survive periods in saturated sodium chloride - about as salty an environment as one can imagine. Salt water can evaporate leaving deposits ("evaporite deposits")

consisting of salts such as sodium chloride (halite) and calcium sulfate (gypsum). Within evaporates are fluid inclusions - small trapped pockets of water - which can provide a refuge for microbes for at least six months. Our research group showed that cyanobacteria trapped within dry evaporite crusts can continue to have low levels of metabolic function such as photosynthesis. These deposits also form nice fossils of the organisms trapped within. Although highly controversial, others claim that bacteria might survive for millions of years in the fluid inclusions of salt deposits including evaporates. Tantalizingly, such deposits have been found on Mars. So how do cells adapt to this potentially deadly environment? To prevent an exodus of water from the cell, halophiles offset the high salt in the environment by accumulating such compounds as potassium and glycine-betaine. This allows a balance of salts inside and outside of the cell preventing water from flowing outward as would be the case if lower salt levels existed within the cells.

Acidity and Alkalinity Yellowstone National Park has bubbling acid hotsprings that would make a witch's cauldron seem benign. They also teem with life. Once again we have been astounded that such environments harbor life. Acidity and alkalinity are measures of the concentration of protons, the units used are pH units. The lower the number (down to zero), the higher the acidity. The higher (up to 14), the more alkaline. A neutral pH near 7 is optimal for many biological processes, although some - such as the light reactions of photosynthesis - depend on pH gradients. In nature, pH can be high, such as in soda lakes or drying ponds, or as low as 0 and below. Organisms that live at either extreme do this by maintaining the near-neutral pH of their cytoplasm (i.e.) the liquid and materials within their cells.

Low pH is the realm of acidophiles - "acid lovers". If you are looking for champion acid lovers, forget fish and cyanobacteria which have not been found below pH 4, or even plants and insects which don't survive below pH 2 to 3. The extreme acidophiles are microbes. Several algae, such as the unicellular red alga *Cyanidium caldarium* and the green alga *Dunaliella acidophila*, are exceptional acidophiles both of which can live below pH 1. Three fungi, *Acontium cylatium, Cephalosporium sp.,* and *Trichosporon cerebriae*, grow near pH 0. Another species, *Ferroplasma acidarmanus*, has been found growing at pH 0 in acid mine drainage in Iron Mountain in California. These polyextremophiles (tolerant to multiple environmental extremes) thrive in a brew of sulfuric acid and high levels of copper, arsenic, cadmium, and zinc with only a cell membrane and no cell wall.

High Temperature

Temperature is a critical parameter because it determines whether liquid water is present. If temperature is too low, enzymatic activity slows, membrane fluidity decreases. Below freezing ice crystals form that slice through cell membranes. High temperatures can irreversibly alter the structure of biomolecules such as proteins, and increase membrane fluidity. The solubility of gasses in water is correlated with temperature, creating problems at high temperature for aquatic organisms requiring oxygen or carbon dioxide.

As it happens, organisms can outwit theory. Geysers, hotsprings, fumaroles and hydrothermal vents all house organisms living at or above the boiling point of water. The most hyperthermophilic (VERY hot loving) organisms are Archaea, with *Pyrolobus fumarii* (of the Crenarchaeota), a nitrate-reducing chemolithotroph (an organism that derives energy from minerals), capable of growing at up to 113°C, is the current champion. As such, these hyperthermophiles are able to prevent the denaturation and chemical modification (breakdown) of DNA which normally occurs at or around a comparatively cool 70°C. The stability of nucleic acids is enhanced by the presence of salts which protect the DNA from being destroyed. Thermophily (living in hot places) is more common than living in scalding, ultra hot locales, and includes phototrophic bacteria (i.e., cyanobacteria, and purple and green bacteria who derive energy from photosynthesis), eubacteria (i.e., *Bacillus, Clostridium, Thiobacillus, Desulfotomaculum, Thermus,* lactic acid bacteria, *actinomycetes, spirochetes,* and numerous other genera), and the Archaea (i.e.,

Pyrococcus, Thermococcus, Thermoplasma, Sulfolobus, and the methanogens). In contrast, the upper limit for eukaryotes is ~ 60°C, a temperature suitable for some protozoa, algae, and fungi. The maximum temperature for mosses is another 10° lower, vascular plants (house plants, trees) about 48°C, and fish 40°C. **Low Temperature**

Representatives of all major forms of life inhabit temperatures just below 0°C. Think winter, think polar waters. While sperm banks and bacterial culture collections rely on the preservation of live samples in liquid nitrogen at -196°C, the lowest recorded temperature for active microbial communities and animals is substantially higher at -18°C. Freezing of water located within a cell is almost invariably lethal. The only exception to this rule known from nature is the nematode *Panagrolaimus davidi* which can withstand freezing of all of its body water. In contrast, freezing of extracellular water - water outside of cells - is a survival strategy used by a small number of frogs, turtles and one snake to protect their cells during the winter. Survival of freezing must include mechanisms to survive thawing, such as the production of special proteins or "cryoprotectants" (additives that protect against the cold) called "antifreeze" proteins. The other method to survive freezing temperatures is to avoid freezing in the first place. Again "antifreeze" molecules are produced which can lower the freezing point of water 9 to 18°C. Fish in Antarctic seas manage to employ these mechanisms to their advantage. Other changes with low temperature include changes in the structure of a cell's proteins - most notably their enzymes - so as to allow them to function at lower temperatures. The fluidity of cell membranes decreases with temperature. In response, organisms that are able to adapt to cold environments simply increase the ratio of unsaturated to saturated fatty acids thus retaining the required flexibility of membranes. **Radiation** Radiation is a hazard even on a comfortable planet like Earth. Sunlight can cause major damage unless mechanisms are in place to repair - or at least limit - the damage. Humans lacking the capacity to repair ultraviolet (UV) damage have xeroderma pigmentosa. This disease is so serious that suffers cannot leave their house during the day unless completely covered, and must even shade the windows in their homes. Once you leave the protected surface of Earth, things can get more hostile. One of the major problems that organisms might face during interplanetary transfer (inside a rock blasted off of a planet by a large impact event for example), living on Mars, or even at high altitudes on Earth is the high levels of UV (ultraviolet) radiation.

In space there is cosmic and galactic radiation to contend with as well. The dangers of UV and ionizing radiation range from inhibition of photosynthesis up to damage to nucleic acids. Direct damage to DNA or indirect damage through the production of reactive oxygen molecules creates can alter the sequence or even break DNA strands.

Several bacteria including two Rubrobacter species and the green alga *Dunaliella bardawil,* can endure high levels of radiation. *Deinococcus radiodurans,* on the other hand is a champ and can withstand up to 20 kGy of gamma radiation up to 1,000 joules per s02. meter of UV radiation. Indeed, *D. radiodurans* can be exposed to levels of radiation that blow its genome into pieces only to have the organism repair its genome and be back to normal operations in a day.

This extraordinary tolerance is accomplished through a unique repair mechanism which involves reassembling damaged (fragmented) DNA. Scientists at the Department of Energy are looking to augment *D. radiodurans* genome such that it can be used to clean up mixed toxic and radioactive spills. So eager are biotechnologist to understand just how *D. radiodurans* does what it does that its genome was among the first organisms to be fully sequenced. **Gravity** Gravity is a constant force in our lives; who has not imagined what it would be like to be an astronaut escaping gravity even temporarily? The universe offers a variety of gravitational experiences, from the near absence of gravity's effects in space (more accurately referred to as microgravity) to the oppressive gravitational regimes of planets substantially larger than

ours.

Gravitational effects are more pronounced as the mass of an organism increases. That being said, flight experiments have revealed that even individual cells respond to changes in gravity. Cell cultures carried aboard various spacecraft including kidney cells and white blood cells showed marked alterations in their behavior, some of which is directly due to the absence of the effects of a strong gravity field. Indeed, recent work conducted aboard Space Shuttle missions has shown that there is a genetic component (as yet understood) to kidney cell responses to microgravity exposure.

Pressure

Pressure increases with depth, be it in a water column or in rock. Hydrostatic (water) pressure increases at a rate of about one-tenth of an atmosphere per meter depth, whereas lithostatic (rock) pressure increases at about twice that rate. Pressure decreases with altitude, so that by 10 km above sea level atmospheric pressure is almost a quarter of that at sea level.

The boiling point of water increases with pressure, so water at the bottom of the ocean remains liquid at 400¡C. Because liquid water normally does not occur above ~100°C, increased pressure should increase the optimal temperature for microbial growth, but surprisingly pressure only extends temperature range by a few degrees suggesting that it is temperature itself that is the limiting factor.

The Marianas trench is the world's deepest sea floor at 10,898 m, yet it harbors organisms that can grow at temperature and pressure we experience everyday. It has also yielded obligately piezophilic species (i.e. organisms that are pressure loving and can only grow under high pressure) that can only grow at the immense pressures found at the ocean's greatest depths.

Other extreme conditions

A bit of creative thinking suggests other physical and chemical extremes not considered here, including unusual atmospheric compositions, redox potential, toxic or xenobiotic (manmade) compounds, and heavy metal concentration. There are even organisms such as *Geobacter metallireducens* that can survive immersion in high levels of organic solvents such as those found in toxic waste dumps. Others thrive inside the cooling water within nuclear reactors. While these organisms have received relatively little attention from the extremophile community, the search for life elsewhere may well rely on a better understanding of these extremes.

Possible Questions

Unit – V

Two marks

1. Write any two ecological significance of the archae bacteria.
2. What is meant by thermophiles?.
3. What are cyanobacteria?
4. Define halophile
5. What are methanogens?
6. What is meant by biological cycle?

Eight marks

1. How are archae bacteria classified? Explain.
2. Write a brief note on halophiles
3. Write in detail about the general characteristics about archae.
4. Write short notes on barophiles and pshyrophiles.
5. Write about the archae bacterial cell wall.
6. How are cyanobacteria culture in lab?
7. Explain in detail about the role of phytoplankton in microbial kingdom.

S.no	Question	Opt 1	Opt 2	Opt 3	Opt 4	Answer
1	aids in the motility of gliding bacteria.	Slime	Fimbriae	Flagella	Pili	Slime
2	Sporulation takes place for 10hrs in .	*Streptococcus*	*Bacillus megaterium*	*Bacillus anthraces*	*Corynebacterium*	*Bacillus megaterium*
3	The synthesis of flagella involves genes	20-30	40-50	15-30	30-40	20-30
4	The information required for flagella construction is present in the structure of .	Flagellin	hook	filament	basal body	Flagellin
5	Freeze drying is otherwise called as	Lyophilization	Tyndallisation	Drying	Air drying	Lyophilization
6	Nitrogen storage is otherwise called as	Lyophilization	Cryopreservation	Sterilization	Tyndallisation	Cryopreservation
7	MTCC is located at	Ranchi	Delhi	Chandighar	Coimbatore	Chandighar
8	The substance used in preservation of anaerobic cultures in slant is	Glycol	Alcohol	Liquid paraffin	Tween 80	Liquid paraffin
9	Cryopreservation is _____	Heating in liquid nitrogen	Freezing in liquid nitrogen	Drying in liquid nitrogen	Steaming in liquid nitrogen	Freezing in liquid nitrogen
10	In Cryopreservation, the microorganisms of culture are rapidly frozen in liquid nitrogen at_____	-72°C	-86°C	-196°C	-96°C	-196°C
11	The stabilizing agents used in cryopreservation is	Glycerol	Phenol	Terpenol	Lysol	Glycerol

	_____					
12	_____that prevent the formation of ice crystals and promote cell survival.	Glycerol	Phenol	Terpenol	Lysol	Glycerol
13	Lyophilization is otherwiseknown as _____(Freeze-Drying)	Freeze etching	Freeze drying	Freeze shadowing	Freeze liquid nitrogen	Freeze drying
14	In Lyophilization, the culture is rapidly frozen at _____	-196°C	-86°C	-70°C	-96°C	-70°C
15	The metabolic activities of microbial cells are stopped in lyophilization method by_____	dry dehydration	vacuum dehydration	Spray dehydration	Dry heat dehydration	vacuum dehydration
16	Lyophilized or freeze-dried pure cultures sealed and stored in the dark at_____	1°C	8°C	7°C	4°C	4°C
17	_____ method is the most frequently used technique by culture collection centres.	Lyophilization	Cryopreservation	Sterilization	Tyndallisation	Lyophilization
18	In oil overlaying method paraffin oil is used in specific gravity of _____.	0.743 -0.780	0.801-0.825	0.901-0.925	0.865-0.890	0.865-0.890
19	Oil overlaying method is first used by _____.	Brell	Bacon	Dulaney	Nakayama.	Brell
20	Bacterial species are	10-15 years	15-20 years	5-10 years	5 years.	15-20 years

	preserved by oil overlaying method for _____.					
21	Freezing mixture used in lyophilization is _____.	Ice and methanol	Ice and alcohol	Only dry ice	Nitrogen and ice	Ice and alcohol
22	Nitrogen storage is also called as _____.	Freezing	Cooling	Cryogenic storage	Lyophilization	Cryogenic storage
23	Fungal species are stored by _____.	Freezing	Soil cultures	Nitrogen storage	Silica gel cultures	Soil cultures
24	Lyophilization is first used by _____.	N.Appert	Davis	Alexander	Pepler	Alexander
25	The specific gravity of British Pharmacopoeia Medicinal Paraffin oil is_____ .	0.665	0.865	0.965	0.765	0.865
26	Agar slants with cultures when covered with sterile mineral oil will prevent _____	Dressing	Heating	Drying	Freezing	Drying
27	Crowded plate technique is used to isolate organism producing _____	Enzyme	Aminoacids	Antibiotics	Toxin	Toxin
28	Culture grown on agar slopes are stored in a refrigerator at _____	10ºC	5ºC	1ºC	0ºC	10ºC
29	Cryoprotective agent is _____	10% Glycerol	20% Glycerol	30% Glycerol	40% Glycerol	10% Glycerol
30	Storage at Liquid nitrogen reduces _____ activity of microorganisms.	cationic	metabolic	enzymatic	proliferative	cationic

31	Temperature at which microorganisms are stored using liquid nitrogen is _____	–20º	–200º	–196º	-350º	–196º
32	Freeze drying method is otherwise known as _____	Dehydrated storage	Glycerol Storage	Liquid nitrogen storage	Lyophilization	Lyophilization
33	An example for protective medium is _____	Water	Skimmed Milk	Butter	Sewage	Sewage
34	N2 storage is otherwise known as _____ storage.	Dry	Pyrogenic	Sand	Cryogenic	Dry
35	NCTC is _____	National Collection of Type Cultures	North Collection of Type Cultures	National Cultures Type Collection	North Cultures Type Collection	North Cultures Type Collection
36	IFO is located at _____	America	Italy	Korea	Japan	Italy
37	ATCC is _____	American Type Collection Cultures	African Type Collection Cultures	American Type Culture Collection	African Type Culture Collection	American Type Culture Collection
38	The function of paraffin is to prevent ----------	Dehydration	Decarboxylation	Loss of viability	Hydroxylation	Dehydration
39	Freeze drying was first introduced by ----------	Alexanander & Raper	Raper & Pasteur	both a& b	Alexander & Pasteur	Alexanander & Raper
40	In lyophilization special type of flask is used which named as ----------	Conical flask	plastic flask	dewar flask.	Swan neck flask	dewar flask.
41	Vial is made up of ----------	.Aluminium	Asbestose	Copper	steel	Asbestose

	-----					
42	The bacterial suspension are kept in lyophilizer in ---------- ------	Glass tube.	Petriplates	Ampoule	plastic tube	Ampoule
42	Dewar flask containg the mixture of ----------	dry ice & alcohol	Ethanol &H2O	dry ice & H2O	methanol & formaldehyde.	dry ice & alcohol
43	Lyophilization process was successfully done in year -- ----------	1946	1948	1942	1947	1942
44	A chemical that absorbes O2 is ----------	salicylic acid	.aldehyde	farmaldehyde	alkaline pyrogallol.	alkaline pyrogallol.
45	Catalyst used in Mcintyosh jar is ----------	palladium	copper rod	iron rod	pallanised asbestose	pallanised asbestose
46	Indicator used in the gas pak system is ---------- -	phenol red	oxidized methylene blue	reduced methylene blue	bromophenol blue	reduced methylene blue
47	Lyophilization cause ---------- ---------- of culture.	drying	heating	sublimation	breaking	sublimation
48	The vials with cell suspension frozen at ----------	-55to -78c̊	-60to -78c̊	-65to -78c̊	-70to -78c̊	-60to -78c̊
49	Cultivation of anaerobes by - ----------	nutrient medium	differential medium	Robertsons cooked meat medium	blood agar medium	Robertsons cooked meat medium
50	In lyophilization the vials are scaled off under ---------- ---	open tube	water contains flask	pressure	vaccum.	vaccum.
51	In mineral overlaying method allow the diffusion of ----------	gases	cells	minerals	.colony	gases

	----------					
52	The first preservation technique used by the microbiologist is ----------	preservation under oil	preservation in soil	lyophilization	serial transfer	serial transfer
53	Which is the preservation process successful for algae	Lyophilization	glycerol storage	serial transfer	cryopreservation	serial transfer
54	Who introduced the concept of preservation in distilled water	Taylor	wheaton	castellani	Martine	castellani
55	Preservation of culture in distilled water is mainly used for the conservation of ----------	Bacteria	Algae	fungai	protozoa	fungai
56	----------is used for the conservation of non sporulating strains	preservation under oil	preservation in soil	serial transfer	cryopreservation	preservation under oil
57	The mineral oil which used for preservation of culture have a specific gravity of ----	0.3 to 0.5	1.3 to 1.5	.0.3 to 1	0.8 to 0.9	0.8 to 0.9
58	The culture preserved in mineral oil preserved at a temperature of ----------	10˚C	50˚C	-4˚C	5˚C	10˚C
59	The most commonly used natural cryopreservative agents are ----------	Glucose	Lactose	soy bean oil	skim milk	skim milk

60	Moisture indicator consisting of filterpaper strips used in freeze drying is impregnated with --------- ---------	Cacl2	Cocl2	Zncl2	.Kcl	Cocl2
61	Which organism is successfully maintained over silica gel ?	Neurospora	Dermatophytes	.Aspergillus	Penicillium.	Neurospora
62	Preserved culture over silica gel is at a temperature of about ----------	. 20˚C	. 10˚C	15˚C	. 25˚C	. 25˚C
63	The temperature to thaw samples rapidly at ----------	. 50˚C	.20˚C	37˚C	52˚C	37˚C

Reg. No. : ----------

[18MBU102]

KARPAGAM ACADEMY OF HIGHER EDUCATION

(Under Section 3 of UGC Act 1956)

COIMBATORE – 641 021

DEPARTMET OF MICROBIOLOGY

FIRST INTERNAL EXAMINATION, AUGUST – 2018

FIRST SEMESTER

BACTERIOLOGY

Time: 2 hours **Maximum: 50 marks**

Date: / /2018 **Class: I B.Sc., MB**

PART-A – (20 x 1 = 20 marks)

(Answer all the questions)

1. Bacteria are _____.
   a. Intracellular b. Unicellular
   c. Multicellular d. Extracellular.
2. Who is father of Microbiology?
   a. Leuwenhoek b. Twort and Felix
   c. Edward Jenner d. Louis Pasteur
3. Gram staining was introduced in the year _____.
   a. 1884 b. 1965
   c. 1997 d. 1983
4. Coccus means _____.
   a. Spherical shape b. Square shape
   c. Rectangular shape d. Irregular shape
5. _____media is used for cultivation of bacteria.
   a. Nutrient agar b. Mackonkey agar
   c. EMB agar d. MSA
6. Bacterial motility can be performed by _____method.
   a. Hanging drop b. Slant
   c. Viewing drop d. Dry mount
7. The term mordant refers to _____.
   a. Acidic dye b. Basic Dye
   c. Dye fixing solution d. Dye removing solution
8. Bacteria is classified under the _____.
   a. Monera b. Protista
   c. Animalia d. Plantae
9. _____ classified bacteria into two groups based on the stain uptake.
   a. David Baltimore b. Edward jenner
   c. Montangier d. Christian Gram
10. Bacteria move by rotatory mechanism of _____.
    a. Flagella b. Pili
    c. Fimbriae d. Basal body
11. Which of the following is a beneficial bacteria _____.
    a. *Lactobacillus* sps. b. *Escherichia coli*
    c. *Virbio cholerae* d. *Bacillus anthracis*

12. A cluster of polar flagella is called_____

a. lophotrichous b. amphitrichous

c. monotrichous d. pertrichous

13. Cocci in chains are called as _____.

a. Streptococci b. Diplococci

c. Pneumococci d. Staphylococci

14. Sterilization above 100° C using steam is performed in _____.

a. Incinerator b. Autoclave

c. Hot air oven d. Desiccators

15. _____ is the first phase in growth curve

a. Log b. Lag

c. Stationary d. Death

16. Bacteria having flagella distributed around the cells is called _____.

a. Amphitrichous b. Monotrichous

c. Peritrichous d. Lophotrichous

17. In Gram positive bacterial cell wall _____ acid is observed.

a. Diaminopimelic acid b. Hyaluronic acid

c. Teichoic acid d. Dipicolinic acid

18. Gram negative cell wall is characterized by the presence of _____.

a. GlycoLipids b. Phospholipid

c. Glycopolysaccharide d. Lipopolysaccharide

19. Flagella is made up of _____protein.

a. Globulin b. Fibrin

c. Flagellin d. Actin

20. _____is the resistant structure formed by bacteria during unfavourable condition.

a. Exospore b. Conidiospore

c. Endospore d. Clamydiospore

PART – B (3x2 = 6 marks)

(Answer all the questions)

21. What is Peptidoglycan?
22. Draw a neat diagram on the structure of flagella.
23. What are Mordant solutions? Give example.

PART – C (3x8 = 24marks)

Answer all the questions either a or b. (All question carry equal marks)

24. a) Write short notes on the classification of bacteria based on cell shape.

(or)

b) Write in detail about the gram positive cell wall.

25. a) Give a detailed account on cell organelles of bacteria.

(or)

b) Explain the principle and procedure of Gram staining.

26. a) Discuss on the endospore staining.

(or)

b) Give a brief account on the maintenance of pure culture.

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[18MBU102]

KARPAGAM ACADEMY OF HIGHER EDUCATION

(Under Section 3 of UGC Act 1956)

COIMBATORE – 641 021

DEPARTMET OF MICROBIOLOGY

SECOND INTERNAL EXAMINATION, AUGUST – 2018

FIRST SEMESTER

BACTERIOLOGY

Time: 2 hours **Maximum: 50 marks**

Date: / /2018 **Class: I B.Sc., MB**

PART-A – (20 x 1 = 20 marks)

(Answer all the questions)

1. _____Used as an indicator in Mcintosh anaerobic jar for preparation of anaerobic culture.
   a. Methylene blue b. Phenol red
   c. Bromothymol blue d. Neutral red.
2. _____ is a simple media
   a. Blood agar b. Robertson's medium
   c. Mac conkey's agar d. Nutrient agar
3. _____ media contain additives that enhance the growth of the desired organism by inhibiting other organisms.
   a. Simple b. Transport
   c. Selective d. Indicator
4. Mac Conkey's agar is a _____ medium.
   a. Selective b. Selective & Differential
   c. enriched media d. Differential
5. Assay medium is also known as _____.
   a. Selective media b. Complex media
   c. Production media d. Indicator media
6. Petri dishes were invented by_____.
   a. Robert Koch b. Louis Pasteur
   c. Richard Petri d. Burnet
7. The function of agar in culture medium is _____.
   a. Carbon source b. Nitrogen source
   c. Buffering agent d. Solidifying agent
8. Anaerobic bacteria can be isolated by pour plate technique using a medium containing a reducing agent such as _____.
   a. Methylene blue b. Thioglycolate
   c. Palladium d. Serum
9. The differential medium used for isolation of Escherichia coli.
   a. Mac conkey agar b. Eosin methylene blue agar
   c. Nutrient agar d. Stuart's medium
10. _____ is an enriched media.
   a. Nutrient agar b. Tryptic soy agar
   c. Blood agar d. Macconkey agar
11. Addition of salt to a culture medium is an example of a _____.
   a. Differential media b. Chemically defined media
   c. Simple media d. Selective media

12. Media that contain some ingredients of unknown chemical composition are

a. Defined media b. Synthetic media

c. Complex media d. Natural media

13. _____ is a transport medium.

a. Mac conkey agar b. Eosin methylene blue agar

c. Nutrient agar d. Stuart's medium

14. Sterilization above 100° C using steam is performed in _____.

a. Incinerator b. Autoclave

c. Hot air oven d. Desiccators

15. In Mac conkey's Medium, lactose fermenters produce _____ colonies.

a. Pink b. Yellow

c. Black d. Green

16. Isolation of bacteria in pure is not possible in_____.

a. solid media b. Liquid media

c. Differential media d. Selective media

17. The branch of microbiology that deals with the study of bacteria is called _____.

a. Parasitology b. Bacteriology

c. Myology d. Phycology

18. The number of H+ ions present in a solution is a measure of _____ of the solution.

a. Alkalinity b. Basicity

c. Acidity d. Avidity

19. The _____ of a solution is dependent upon the number for hydroxyl ions present.

a. Basicity b. Acidity

c. Alkalinity d. Neutrality

20 _____ is defined as the negative logarithm of hydrogen ion concentration.

a. pH b. [–H]

c. [-OH] d. H+

PART – B (3x2 = 6 marks)

(Answer all the questions)

21. How Autoclave works?
22. Write about the uses of EMB agar.
23. Write about Streak plate method.

PART – C (3x8 = 24marks)

Answer all the questions either a or b. (All question carry equal marks)

24. a) Give a detailed note on pure culture method.

(or)

b) What are acid fast bacilli? Write short notes on its staining method.

25. a) What is culture media? Write brief about its types.

(or)

b) Give a brief note on dry and wet methods of sterilization.

26. a) What is Sterilization? Add a note on hot air oven.

(or)

b) Give a brief note on Chemical method of sterilization.

Reg. No. :_____

[18MBU102]

KARPAGAM ACADEMY OF HIGHER EDUCATION

(Under Section 3 of UGC Act 1956)

COIMBATORE – 641 021

DEPARTMET OF MICROBIOLOGY

THIRD INTERNAL EXAMINATION, OCTOBER – 2018

FIRST SEMESTER

BACTERIOLOGY

Time: 2 hours **Maximum: 50 marks**

Date: /10/2018 **Class: I B.Sc., MB**

PART-A – (20 x 1 = 20 marks)

(Answer all the questions)

1. Which one of the following belongs to Monera?
   a. *Ameoba* b. *E.coli*
   c. *Gelidium* d. *Spirogyra*
2. The main diference between the Gram (+) ve and Gram (-) ve bacteria resides in their
   a. Cell wall b. Cell membrane
   c. Cytoplasm d. flagella
3. Cyanobacteria undergo a different type of motility called_____.
   a. Gliding motility b. Twisting motility
   c. Dwelling d. Moving
4. Movement toward chemical attractants and away from repellents is known as _____.
   a. Chemoreceptors b. Chemotaxis
   c. Chemical repellents d. Repellents
5. _____links the filament to its basal body and act as a flexible coupling.
   a. Neck b. Hook
   c. Filament d. Hair
6. Some bacteria are variable and lacks single characteristic forms are called _____.
   a. atrichous b. pleomorphic
   c. irregular d. diploid
7. The inclusion bodies of procaryotic cells are present in the _____.
   a. plasma membrane b. cytoplasmic matrix
   c. nucleus d. ribosomes
8. _____ are present in many cyanobacteria, nitrifying bacteria and thiobacilli.
   a. carboxyzomes b. nitrates
   c. pigment d. pili
9. The most widely accepted current model for membrane structure is the_____.
   a. Direct model b. varied model
   c. Common model d. Fluid mosaic model
10. A non-photosynthetic aerobic nitrogen fixing soil bacterium is _____.
    a. *Rhizobium* b. *Clostridium*
    c. *Azotobacter* d. *Klebsiella*
11. A few organisms are known to grow and multiply at temperature of 100 -105°C. They belongs to_____.
    a. Thermophilic Sulphur Bacteria b. Hot Spring BGA
    c. Methanogenic archae bacteria d. Marine archae bacteria

12. What is true for archaebacteria?

a. All Halophiles b. All Photosynthetics

c. all fossils d. Oldest living beings

13. Choose the correct sequence of stages of growth curve for bacteria.

a. Lag, Log, Stationary, Decline b. Log, Lag, Stationary, Decline

c. Lag, Log, Decline, Stationary d. Stationary, Decline, Log, Lag

14. Which of the following definition covers a greater number of organisms?

a. Class b. Genus

c. Order d. Family

15. The Five Kingdom Classification of microorganisms was proposed by_____.

a. Whittaker b. John Ray

c. Robert Koch d. Carl Woese

16. First step in taxonomy

a. Naming b. Description

c. Identification d. Classification

17. A system of classification based on all important morphologically character is termed as____.

a. Artificial System b. Natural system

c. Genetic System d. Both a and b

18. Taxon is

a. A genus b. A species

c. A taxonomic unit d. A taxonomic category of any rank

19. Modern classification is based on _____.

a. Physiology b. Fossils

c. Phylogeny d. Morphology

20 Basic taxonomic unit is.

a. Kingdom b. Genus

c. Species d. Order

PART – B (3x2 = 6 marks)

(Answer all the questions)

21. What is meant by Thermophiles?
22. What are cyanobacteria?
23. Define halophiles.

PART – C (3x8 = 24marks)

Answer all the questions either a or b. (All question carry equal marks)

24. a) Write short notes on the concept of species, taxa, and strain.

(or)

b) Write short notes on halophiles,, barophiles and pshyrophiles.

25. a) Write about the archae bacterial cell wall.

(or)

b) Write in detail about the general characteristics about eubacteria.

26. a) What is meant by evolutionary chronometers?

(or)

b) Write in detail about the general characteristics about archae.