BACTERIOLOGY

M.Sc. MICROBIOLOGY

SEMESTER-I, PAPER-III

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BACTERIOLOGY

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FOREWORD

Since its establishment in 1976, Acharya Nagarjuna University has been forging ahead in the path of progress and dynamism, offering a variety of courses and research contributions. I am extremely happy that by gaining 'A+' grade from the NAAC in the year 2024, Acharya Nagarjuna University is offering educational opportunities at the UG, PG levels apart from research degrees to students from over 221 affiliated colleges spread over the two districts of Guntur and Prakasam.

The University has also started the Centre for Distance Education in 2003-04 with the aim of taking higher education to the door step of all the sectors of the society. The centre will be a great help to those who cannot join in colleges, those who cannot afford the exorbitant fees as regular students, and even to housewives desirous of pursuing higher studies. Acharya Nagarjuna University has started offering B.Sc., B.A., B.B.A., and B.Com courses at the Degree level and M.A., M.Com., M.Sc., M.B.A., and L.L.M., courses at the PG level from the academic year 2003-2004 onwards.

To facilitate easier understanding by students studying through the distance mode, these self-instruction materials have been prepared by eminent and experienced teachers. The lessons have been drafted with great care and expertise in the stipulated time by these teachers. Constructive ideas and scholarly suggestions are welcome from students and teachers involved respectively. Such ideas will be incorporated for the greater efficacy of this distance mode of education. For clarification of doubts and feedback, weekly classes and contact classes will be arranged at the UG and PG levels respectively.

It is my aim that students getting higher education through the Centre for Distance Education should improve their qualification, have better employment opportunities and in turn be part of country's progress. It is my fond desire that in the years to come, the Centre for Distance Education will go from strength to strength in the form of new courses and by catering to larger number of people. My congratulations to all the Directors, Academic Coordinators, Editors and Lesson-writers of the Centre who have helped in these endeavors.

Prof. K. Gangadhara Rao
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SEMESTER-I

M.SC. MICROBIOLOGY

1O3MB24 -BACTERIOLOGY

UNIT-I

Outline classification of microorganisms - Different types of kingdom systems (Haeckel's three kingdom concept, Whittaker's five kingdom concept); three domain concept of Carl Woese. Principles of bacterial taxonomy and classification of bacteria - Numerical taxonomy, Identification characters - morphological, staining, physiological, biochemical and genetical (mol% G*C, Nucleic acid hybridization, 165 rRNAsequencing) characters. Bacterial classification as per the latest edition of Bergey's Manual of Systematic Bacteriology. Ultrastructure of typical bacterial cell - Surface appendages, Cell envelope and Cytoplasmic components. Sporulation in bacteria.

UNIT-II

Sterilization methods to control bacterial growth - Physical (Heat, Filtration, Radiation) and Chemical methods. General methods of isolation of bacteria from soil (Plating methods, Serial dilution technique, MPN technique, Contact slide technique, Winogradsky column) and water (Multiple tube fermentation test, Membrane filter technique); anaerobic culture methods. Maintenance and Preservation of bacterial cultures - Sub-culturing, Oil over laying, Lyophilization, Cryo- preservation. Techniques for staining bacteria - Negative, Simple and Differential staining methods.

UNIT-III

Bacterial Nutrition - Nutritional classification of bacteria, Essential macronutrients, micronutrients and growth factors. Bacterial Growth - Growth characteristics of bacteria on solid medium, Kinetics of growth, Typical bacterial growth curve, Diauxie growth curve, Batch culturing, Continuous culturing chemostat and turbidostat, synchronous culturing. Factors affecting the bacterial growth; Methods for measurement of bacterial growth. Bacterial Homeostasis.

UNIT-IV

Classification, General characters, Reproduction and significance of Archaeobacteria. Classification, General characters, Reproduction and significance of Cyanobacteria. Classification, General characters, Reproduction and significance of Actinomycetes.

UNIT-V

Taxonomy and characteristic features of the following bacterial genera-Agrobacterium, Bacillus, Clostridium, Escherichia, Mycoplasma, Nitrosomonas, Pseudomonas, Rhizobium, Rickettsia. Spirochaete, Staphylococcus, Streptococcus.

REFERENCE BOOKS

l. Brock, T.D.and Madigam, M.T. - Biology of Micoroorgansims (1999)

2. Prescott, L.M., Harley, J.P. Klein, D.A. - Microbiology (2008)

3. Stanier, R.Y., Ingraham, J.L., Wheelis, M.L., Painter, P.R. - The Microbial World (1988)

4. Pelczar, M.J, Chan, E.C.S., Kreign, N.R. - Microbiology (2006)

5. Black, J.G.- Microbiology - Principles and Explorations (1999)

6. Atlas, R.M. - Priniciples of Microbiology (1996)

7. Salle AJ. Fundamental principles of Bacteriology (2001)

8. Birge-Modern Microbiology

9. Schlegel HG. General Microbiology (2003)

10. Sneath, P.H.A., Mair, N.S., Elizabeth,M. -Bergey's Bergey's Manual of systematic Bacteriology

I l. Dubey RC and Maheswari DK. - A Text Book of Microbiology (2010)

12. Alcamo E. - Fundamentals of Microbiology (2001)

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CENTRE FOR DISTANCE EDUCATION

ACHARYA NAGARJUNA UNIVERSITY

M.Sc. Degree Examination

MICROBIOLOGY- I SEMESTER

BACTERIOLOGY

Model Question Paper

Time: 3 hours **Maximum Marks: 70**

Answer ALL Questions **(5x14 = 70 marks)**

UNIT-I

1. a) Give an account on different types of kingdom systems and three-domain concept of classification of microorganisms.

OR

b) Explain the Bacterial classification as per the second edition of Bergey's Manual of Systematic Bacteriology.

UNIT-II

2. a) Write an account on physical and chemical methods of sterilization to control bacterial growth.

OR

b) Describe the different techniques used for staining bacteria.

UNIT-III

3. a) Give an account on bacterial growth characters and its growth curve.

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OR

b) Explain the nutritional classification of bacteria and a note on essential macro and micro nutrients.

UNIT-IV

4. a) Write an account on general characters and reproduction of Archaeobacteria.

OR

b) Describe the general characters and significance of cyanobacteria.

UNIT-V

5. a) Explain the taxonomic and characteristic features of *Bacillus* and *Rhizobium* genera.

OR

b) Describe the characteristic features of bacterial genera *Mycoplasma* and *Rickettsia*.

CONTENTS

LESSON – 1

OUTLINE CLASSIFICATION OF MICROORGANISMS

OBJECTIVE OF THE LESSON

Students will understand the basis of classification of microorganisms into different kingdom systems based on various criteria.

STRUCTURE OF THE LESSON

1.1 INTRODUCTION

Classification is the arrangement of organisms into taxonomic groups known as taxa on the basis of similarities or relationships. Organisms that are closely related or having similar characteristics are placed into the same taxon. Basing on similarities and differences, the organisms are categorized into larger groups. Different classification schemes existed at different time because the classification of organisms is a complex and controversial subject. Linnaeus, in his classification, recognized only two kingdoms of living things viz., Animalia and Plantae. Classifying larger organisms into kingdoms is usually easy, but in a microenvironment it can be complex. As the knowledge of the properties of various groups of microbial life exploded, it became apparent that at this level of biological knowledge a division of the living world into two kingdoms cannot really be maintained on a logical basis and consistent ground. So, later on the organisms are grouped into different kingdom-systems. Kingdoms are divided into categories called Phyla; each phylum is divided into Classes, each class into Orders, each order into families, each family into Genera and each genus into Species. While the concept of kingdoms continues to be used by some taxonomists, there has been a movement away from traditional kingdoms, as they are no

longer seen as providing a cladistics classification, where there is emphasis in arranging organisms into natural groups. Further basing on rRNA studies, Carl Woese opined that life could be divided into three large domains and referred the classification as 'Three Domain System'. The three domains are named as Bacteria, Archaea and Eukarya.

1.2 TYPES OF KINGDOMS

1.2.1 Two Kingdom System

At the time of initiation of classification system, Carl Linnaeus grouped all living forms into two kingdoms namely Plantae and Animalia. The Plantae include the living forms that did not move or eat and which continue the growth throughout the life. Animalia include every living form that move, ate and grew only to a certain size and stop growing. But it became very difficult to group some living things either into Plantae or Animalia. Then a proposal of three kingdom system came into picture.

1.2.2 Three Kingdom System

In 1980s, Ernst Haeckel who is a German scientist proposed and put forward the three kingdom system of classification in order to overcome the objections and limitations of the Two Kingdom System of classification. Haeckel suggested that the inconsistencies of the two kingdom system could be avoided by introducing a third kingdom and he proposed a new third kingdom namely Protista. He accommodated the organisms exhibiting the characters either common to both plants and animals, or unique to their own into the new kingdom. Accordingly, the Haeckel's three kingdoms are Plantae, Animalia and Protista. The arrangement of kingdoms was done on the basis of morphological complexities and tissue system, division of labor and mode of nutrition. Based on the lack of tissue differentiation the unicellular animals, algae and fungi are separated from other organisms. The organisms lacking morphological complexities, tissue system, division of labor, having diversified modes of nutrition are segregated and placed in the new kingdom, Protista. So, all the known microorganisms are recognized as protists, neither plants nor animals.

Organisms having diverse tissue system with well-defined division of labor and maximum morphological complexities are segregated from protists and bifurcated into two categories. Organisms with autotrophic mode of nutrition are considered as plants and placed in kingdom Plantae. Others having entirely holophagic or phagotrophic mode of nutrition are considered as animals and placed in kingdom Animalia. However, Haeckel's system was not widely accepted due to some objections and limitations. One objection is that nucleated and anucleated organisms are kept together in Protista. The second one is that heterotrophic bacteria and fungi are placed along with autotrophic algae.

1.2.3 Four Kingdom System

The development of microscopy revealed important distinctions between those organisms whose cells do not have a distinct nucleus (Prokaryotes) and organisms whose cells do have a distinct nucleus (Eukaryotes). In 1937, Edouard Chatton introduced the terms 'prokaryote' and 'eukaryote' to differentiate these organisms. In 1938, Herbert F. Copeland proposed a four kingdom system of classification by creating a novel kingdom Monera of prokaryotic forms and included the organisms that are now classified as Bacteria and Archaea. As per this classification, the four kingdoms created are **Monera**, **Protista** or **Protoctista**, **Plantae** and

Animalia. Later in 1960s, Roger Stanier and C.B. van Niel promoted and popularized the Chatton's work.

1.2.4 Five Kingdom System

The first classification system to gain popularity is the Five Kingdom system which is suggested by Robert H. Whittaker in 1960s. Whittaker placed the organisms into five kingdoms based on at least three major criteria viz., (1) cell type (prokaryotic or eukaryotic), (2) level of organization (solitary and colonial unicellular organization or multicellular, and (3) nutritional type. According to this system, the five kingdoms are Monera or prokaryotae, Protista, Fungi, Animalia and Plantae (Fig. 1.1).

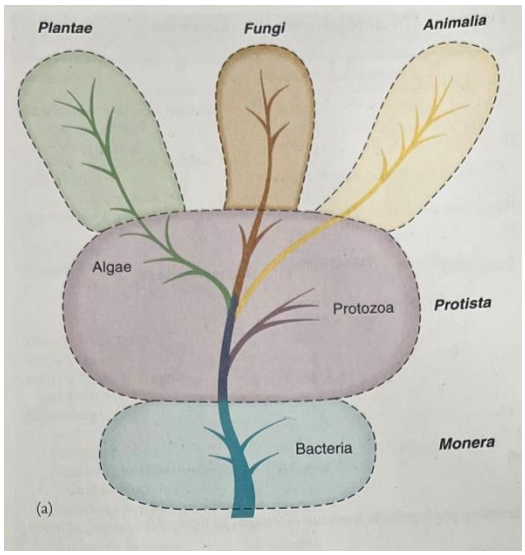


Figure-1.1: Five-kingdom classification system of Whittaker
(Source – Microbiology – Prescott et. al.)

Kingdom Monera: this includes all the prokaryotic organisms. The individuals of this kingdom are single-celled, may or may not move, have a cell wall, have no chloroplasts or other organelles and have no distinct nucleus.

Kingdom Protista: it is the least homogenous and hardest to define. Protists are the eukaryotes with unicellular organization, either in the form of solitary cells or colonies of cells lacking true tissues. They may have ingestive, absorptive, or photoautotrophic nutrition. This kingdom includes many of the algae, protozoa and majority of simpler fungi.

Kingdom Fungi: this Fungi kingdom contains eukaryotic and predominately multinucleate organisms, with nuclei dispersed in a walled and often septate mycelium. Their size ranges from microscopic to very large. The members have absorptive mode of nutrition.

Kingdom Plantae: this is composed of multicellular plants and most of them do not move, although gametes of some plants move using cilia or flagella. Members contain cell wall and the mode of nutrition is primarily photoautotrophic and so carryout the photosynthesis by using sunlight.

Kingdom Animalia: this kingdom contains multicellular animals with wall-less eukaryotic cell and move with the aid of cilia or flagella. They have primarily ingestive mode of nutrition.

Many of the biologists have not initially accepted the five-kingdom system of classification. One major problem is its lack of distinction between archaeobacteria and eubacteria. The kingdom Protista also may be too diverse to be taxonomically useful. Additionally, the boundaries between the kingdoms Protista, Plantae, and Fungi are not well defined. For example, the brown algae are probably not closely related to the plants but the five-kingdom system placed them in the kingdom Plantae. Because of these problems with the five-kingdom system, various alternatives have been proposed by some biologists.

1.2.5 Six-Kingdom System

As a simplest option, a six-kingdom system was developed by Carl Woese, an American scientist, in which the kingdom Monera is divided into two kingdoms namely **Eubacteria** and **Archaeobacteria** (Fig. 1.2). Many attempts have been made to divide the protists into several better defined kingdoms. In that process eight kingdom system was proposed.

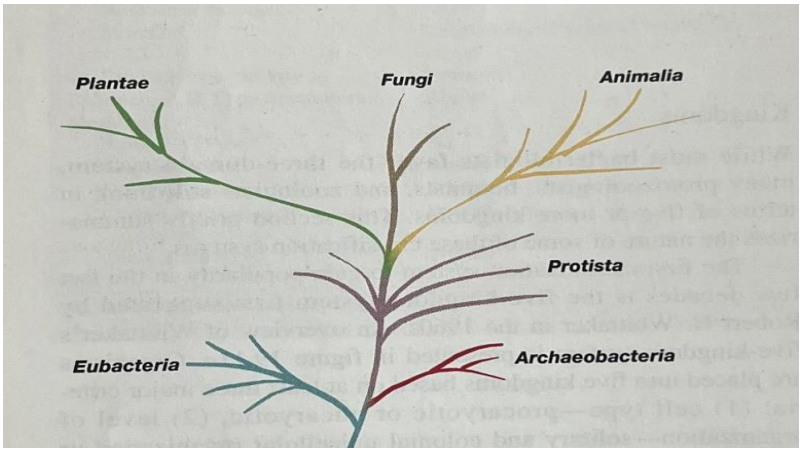


Figure-1.2: Six-kingdom classification system of Carl Woese
(Source – Microbiology – Prescott et. al.)

1.2.6 Eight-kingdom system

Cavalier-Smith has developed the eight-kingdom system in 1993 (Fig. 1.3) believing that differences in cellular structure and genetic organization are exceptionally important in determining phylogeny. So, Cavalier-Smith used the ultrastructural characteristics as well as rRNA sequences and other molecular data in developing his classification. He divided all the living organisms in two empires with a total of eight kingdoms. The **Empire Bacteria** contains two kingdoms namely **Eubacteria** and **Archaeobacteria**. The second **Empire Eukaryorta** contains six kingdoms of eukaryotic organisms. He introduced two new kingdoms of eukaryotes viz., **Archezoa** and **Chromista.**

The kingdom Archezoa consists primitive eukaryotic unicellular organisms such as *Giardia* that have 70S ribosomes and lack of Golgi apparatuses, mitochondria, chloroplasts, and peroxisomes. Whereas, the kingdom Chromista contains mainly photosynthetic organisms that have their chloroplasts within the lumen of the rough endoplasmic reticulum rather than in cytoplasmic matrix. The diatoms, brown algae, cryptomonads, and oomycetes are all placed in the Chromista. And the boundaries of the remaining four kingdoms – Plantae,

Fungi, Animalia, and Protozoa have been adjusted to better define each kingdom and distinguish it from others.

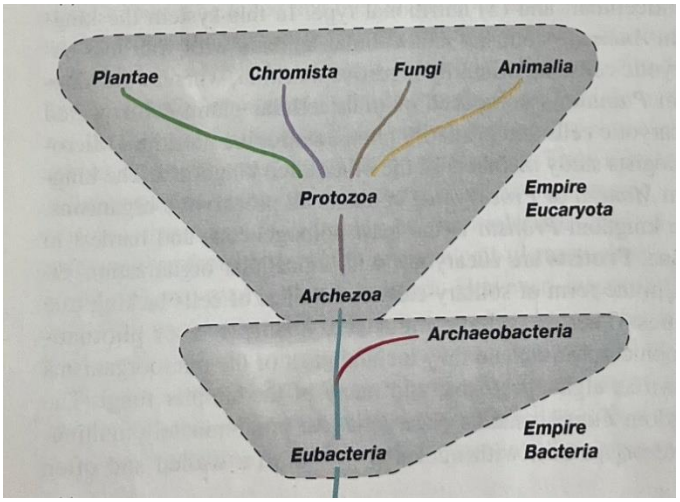


Figure-.3: Eight-kingdom classification system of Cavalier-Smith)
(Source – Microbiology – Prescott et. al.)

1.3 Three Domain System

Carl Woese and his collaborators studied the rRNA sequences in prokaryotic cells and suggested that prokaryotes divided into two distinct groups at very early on. They noted that bacteria are distant from plants and animals and, by contrast, plants and animals are not so far from each other. The archaea and bacteria first diverged, and then the eukaryotes developed. Therefore, they established a new superior concept of domains over the kingdom, and proposed three domains namely **Bacteia**, **Archaea** and **Eukarya** (Fig. 1.4). The domains differ markedly from one another. Eukaryotic organisms with primarily glycerol fatty acyl diester membrane lipids and eukaryotic rRNA belong to the eukarya. The domain bacteria contains prokaryotic cells with eubacterial rRNA and membrane lipids are primarily diacyl glycerol diesters. The domain archaea consists the prokaryotes having isoprenoid glycerol diether or diglycerol tetraether lipids in their membranes and archaeobacterial rRNA.

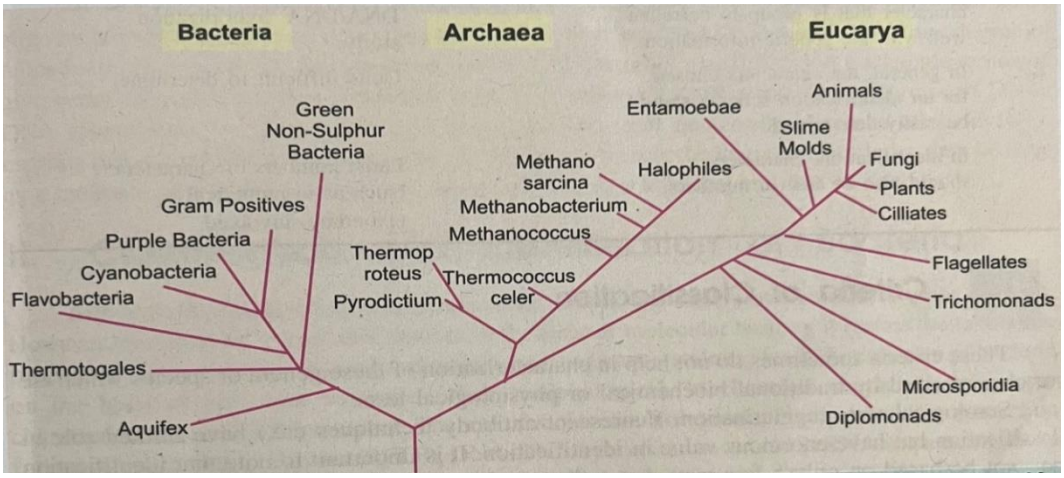


Fig. 1.4: Three Domain classification of Carl Woese
(Source – Microbiology – Prescott et. al.)

So, there are two quite different groups of prokaryotes, the bacteria or eubacteria and the archaeobacteria. The eubacteria or true bacteria comprise the vast majority of bacteria. Among the other properties, eubacteria either have cell wall peptidoglycan containing

muramic acid or are related to bacteria with such cell walls, and have membrane lipids with ester linked, straight-chained fatty acids that resemble eukaryotic membrane lipids. The second group, archaeobacteria differ from eubacteria in many respects and resemble eukaryotes in some ways.

Archaeobacteria differ from eubacteria in lacking muramic acid in their cell wall and in possessing (1) membrane lipids with ether-linked branched aliphatic chains, (2) transfer RNAs without thymidine in the TψC arm, (3) distinctive RNA polymerase enzymes, and (4) ribosomes of different composition and shape. Though archaeobacteria resemble eubacteria in their prokaryotic cell structure, they vary considerably at molecular level. But both the eubacteria and archaeobacteria differ from eukaryotes in their cell ultrastructure and many other properties. However, both of them do share some biochemical properties with eukaryotic cells. For example, eubacteria and eukaryotes have ester linked membrane lipids; archaeobacteria and eukaryotes are similar with respect to some components of the RNA and protein synthetic systems.

Domain Archaea

Archaea are the prokaryotic cells which are typically characterized by membranes that are composed of branched hydrocarbon chains (many also containing rings within the hydrocarbon chains) attached to glycerol by ether linkages. The cell walls of Archaea contain no peptidoglycan. Archaea are not sensitive to some antibiotics that affect the Bacteria, but are sensitive to some antibiotics that affect the Eukarya. Archaea contain rRNA that is unique to the Archaea as indicated by the presence of some molecular regions distinctly different from the rRNA of Bacteria and Eukarya. Archaea often live in extreme environments and include methanogens, extreme halophiles, and hyperthermophiles. One reason for this is that the ether-containing linkages in the Archaea membranes are more stable than the ester-containing linkages in Bacteria as well as Eukarya. The presence of this ether containing linkages in Archaea adds to their ability to withstand the extreme temperature and highly acidic conditions. Extreme halophiles i.e. organisms which thrive in highly salty environment and hyperthermophiles i.e. the organisms which survive in extremely hot environment are the best and exclusive examples of Archaea.

Domain Bacteria

Like that of Archaea, though bacteria are also prokaryotic cells but their membranes are made of unbranched fatty acid chains attached to glycerol by ester linkages. There is a great diversity in this domain, such that it is next to impossible to determine how many species of bacteria exist on the planet. The cell walls of Bacteria members contain peptidoglycan, unlike the Archaea and Eukarya. Bacteria are sensitive to traditional antibacterial antibiotics but are resistant to most antibiotics that affect Eukarya. Bacteria contain rRNA that is unique to the Bacteria as indicated by the presence of molecular regions distinctly different from the rRNA of Archaea and Eukarya. Bacteria importantly include the Gram-positive and Gram-negative bacteria, Cyanobacteria etc.

Domain Eukarya

As the name suggests, the Eukarya includes eukaryotic cell containing organisms which have membranes that are pretty similar to that of bacteria. So, their membranes are also composed of unbranched fatty acid chains attached to glycerol by ester linkages. Not all eukaryotes have a cell wall, and even if they do contain, peptidoglycan is absent. While cells are organized into tissues in case of kingdoms Plantae and Animalia, the presence of cell walls is

only restricted to the members of Plantae kingdom. Eukarya are resistant to traditional antibacterial antibiotics but are sensitive to most antibiotics that affect eukaryotic cells. Eukarya contain rRNA that is unique to the Eukarya as indicated by the presence of molecular regions distinctly different from the rRNA of Archaea and Bacteria. The Eukarya is further divided into kingdom Protista (algae, protozoans, etc.), kingdom Fungi (yeast, mold, etc.), kingdom Plantae (flowering plants, ferns, etc.) and kingdom Animalia (insects, vertebrates, etc.).

Kingdom Protista – Protista are simple, predominately unicellular eukaryotic organisms and includes slime molds, euglenoids, algae and protozoans as an examples.

Kingdom Fungi– fungi are unicellular or multicellular organisms with eukaryotic cell types. The cells have cell walls but are not organized into tissues. They do not carry out photosynthesis and obtain nutrients through absorption. The sac fungi, club fungi, yeasts and molds are the examples.

Kingdom Plantae– plants are multicellular organisms composed of eukaryotic cells. The cells are organized into tissues and have cell walls. They obtain nutrients by photosynthesis and absorption. The examples include mosses, ferns, conifers, and flowering plants.

Kingdom Animalia– animals are multicellular organisms composed of eukaryotic cells. The cells are organized into tissues and lack cell walls. They do not carry out photosynthesis and obtain nutrients primarily by ingestion. Examples – sponges, worms, insects, and vertebrates. The major differences among the three domains are given in Table 1.1.

Table-1.1: Major differences among the domains Bacteria, Archaea and Eukarya

Property	*Bacteria* (Eubacteria)	*Archaea* (Archaeobacteria)	*Eucarya* (Eucaryotes)
Membrane-Enclosed Nucleus with Nucleolus	Absent	Absent	Present
Complex Internal Membranous Organelles	Absent	Absent	Present
Cell Wall	Peptidoglycan containing muramic acid	Variety of types, no muramic acid	No muramic acid
Membrane Lipid	Have ester-linked, straight-chained fatty acids	Have ether-linked, branched aliphatic chains	Have ester-linked, straight-chained fatty acids
Gas Vesicles	Present	Present	Absent
Transfer RNA	Thymine present in most tRNAs	No thymine in T or TψC arm of tRNA	Thymine present
	N-formylmethionine carried by initiator tRNA	Methionine carried by initiator tRNA	Methionine carried by initiator tRNA
Polycistronic mRNA	Present	Present	Absent
mRNA Introns	Absent	Absent	Present
mRNA Splicing, Capping, and Poly A Tailing	Absent	Absent	Present
Ribosomes			
Size	70S	70S	80S (cytoplasmic ribosomes)
Elongation factor 2	Does not react with diphtheria toxin	Reacts	Reacts
Sensitivity to chloramphenicol and kanamycin	Sensitive	Insensitive	Insensitive
Sensitivity to anisomycin	Insensitive	Sensitive	Sensitive
DNA-Dependent RNA Polymerase			
Number of enzymes	One	Several	Three
Structure	Simple subunit pattern (4 subunits)	Complex subunit pattern similar to eucaryotic enzymes (8–12 subunits)	Complex subunit pattern (12–14 subunits)
Rifampicin sensitivity	Sensitive	Insensitive	Insensitive
Polymerase II Type Promoters	Absent	Present	Present
Metabolism			
Similar ATPase	No	Yes	Yes
Methanogenesis	Absent	Present	Absent
Nitrogen fixation	Present	Present	Absent
Chlorophyll-based photosynthesis	Present	Absent	Present[a]
Chemolithotrophy	Present	Present	Absent

a Present in chloroplasts (of bacterial origin).

(Source – Microbiology – Prescott et. al.)

1.4 SUMMARY

While most bacteriologists favor the three-domain system, many protozoologists, botanists, and zoologists still think in terms of five or more kingdoms. The first classification system to gain popularity in the last few decades is the five-kingdom system that was suggested in 1960s by Robert H. Whittaker. Because of some lacuna in the boundary distinctions among the kingdoms, various alternative systems have been suggested by several other scientists. The three-domain system, which classifies life on the planet into three different domains – Archaea, Bacteria and Eukarya, was proposed and put forward by American microbiologist and Physicist Carl Woese in the year 1990. Basically, it is a biological classification of the three domains of life based on the differences in their 16S rRNA genes. Before the concept of three domains of cellular life came into existence, life on planet was grouped into two categories namely Prokaryotae of Monera which comprised bacteria and Eukaryotae which comprised animals, plants, fungi and protists.

In his biological system of classification, Carl Woese divided the Prokaryotae into two groups – Archaea and Bacteria, and thus came into the existence the 'three-domain system' or the concept of 'three domains of life'. The division of Prokaryotae into Archaea and Bacteria can be attributed to the fact that neither of the two are ancestors of each other, and even though they share a few common characteristic traits, they have some peculiar traits of their own as well.

1.5 TECHNICAL TERMS

Three Domain system, Carl Woese, Bacteria, Archaea, Eukarya, Protista, Plantae, Animalia, Fungi, Ether linkages, Ester linkages, 16S rRNA,

1.6 SELF ASSESSMENT QUESTIONS

Q.1 Explain the different kingdom systems and basis for their classification.

Q.1 Describe the Haeckel's three-kingdom system and Whittaker's five-kingdom system of classifications.

Q.3 Give a detailed account on Carl Woese three-domain system of classification and its priorities in grouping the life forms.

1.7 SUGGESTED READINGS

1. Microbiology - Lansing M. Prescott, John P. Harley, Donald A.Klein

2. Principles of Microbiology - Ronald M. Atlas, McGraw Hill, 2nd Edition.

3. Biology of Microorganisms - Thomas D. Brock, Michael P. Madigan, Prentice Hall

4. Microbiology - Michael J. Pelczar, Jr., E.C.S.Chan, Noel R. Krieg, 5th Edition.

Prof. A. Amruthavalli

LESSON - 2

PRINCIPLES OF BACTERIAL TAXONOMY AND CLASSIFICATION

OBJECTIVE OF THE LESSON

To understand the classification and taxonomy of bacteria and different approaches used in classification of microorganisms.

STRUCTURE OF THE LESSON

2.1 INTRODUCTION

Because of the bewildering diversity of living organisms, it is desirable to classify or arrange them into groups based on their mutual similarities. Taxonomy (Greek *taxis,* arrangement or order, and *norms, law,* or *nemein,* to distribute or govern) is defined as the science of biological classification. In a broader sense, it consists of three separate but interrelated parts: Classification, Nomenclature, and Identification. Classification is the arrangement of organisms into groups or taxa (singular - taxon) based on mutual similarity or evolutionary relatedness. Nomenclature is the branch of taxonomy concerned with the assignment of names to taxonomic groups in agreement with published rules. Identification is the practical side of taxonomy, the process of determining that a particular isolate belongs to a recognized taxon.

Taxonomy is important for several reasons. Firstly, it allows organizing huge amounts of knowledge about organisms because all members of particular group share many characteristics. Secondly, taxonomy allows making predictions and framing hypotheses for further research based on knowledge of similar organisms. Thirdly, taxonomy places microorganisms in meaningful and useful groups with precise names so that microbiologist can work with them and communicate efficiency. Fourth, taxonomy is essential for accurate identification of microorganisms.

2.2 CLASSIFICATION SYSTEMS

Once taxonomically relevant characteristics of microorganisms have been collected, they may be used to construct a classification system. The most desirable classification system, called as **Natural Classification**, arranges organisms into groups, whose members share many characteristics and warrant into groups and reflects as much as possible the biological nature of organisms. There are two general ways in which classification systems can be constructed. Organisms can be group together based on overall similarity to form a Phenetic system or they can be grouped based on probable evolutionary relationships to produce Phylogenetic system.

Phenetic Classification

Many taxonomists maintain that the most natural classification is the one with the greatest information content or predictive value. A good classification should bring an order to biological diversity and may even clarify the function of a morphological structure. For example, if motility and flagella are always associated in particular microorganisms, it is reasonable to suppose that flagella are involved in at least some types of motility. The best natural classification system may be a Phenetic system, one that groups the organisms together based on the mutual similarity of their phenotypic characteristics. Although phenetic studies can reveal possible evolutionary relationships, they are not dependent on phylogenetic analysis. They compare many traits without assuming that any features are more phylogenetically important than others-that is, unweight traits are employed in estimating general similarity. Obviously the best phenetic classification is one that constructed by comparing as many attributes as possible. Organisms sharing many characteristic make up a single group or taxon.

Numerical taxonomy

The development of computers has made it possible for quantitative approach known as numerical taxonomy. Peter H.A., Sneath and Robert Sokal have defined numerical taxonomy as "the grouping by numerical methods of taxonomic units into taxa on the basis of their character states".

The process beings with the determination of presence or absence of selected characters in the group of organisms under study. A character usually is defined as an attribute about which a single statement can be made. Many characters, at least 50 and preferably several hundreds, should be compared for an accurate and reliable classification. It is best to include many different kinds of data: morphological, biochemical and physiological.

After character analysis, as association coefficient, a function that measures the agreement between characters possessed by two organisms is calculated for each pair of organisms in the group. The simple matching coefficient (S_{SM}), the most commonly used coefficient in bacteriology, is the proportion of characters that match regardless of whether the attribute is present or absent (Table-2.1). Sometimes the Jaccard coefficient (S_J) is calculated by ignoring any character that is absent in both organisms. Both coefficients increase linearly in value from 0.0 (no matches) to 1.0 (100% matches).

Table 2.1 The Calculation of Association Coefficients for Two Organisms

In this example, organisms A and B are compared in terms of the characters they do and do not share. The terms in the association coefficient equations are defined as follows:

		Organism B: 1	Organism B: 0
Organism A	1	A	b
	0	C	d

a = number of characters coded as present (1) for both organism
b and c = numbers of characters differing (1,0 or 0,1) between the two organisms d = number of characters absent (0) in both organisms
Total number of characters compared = a + b + c + d

The simple matching coefficient $(S_{SM}) = \frac{a + b}{a + b + c + d}$

The Jaccard coefficient $(S_J) = \frac{a}{a + b + c}$

(Source: Microbiology – Prescott et al.)

The simple matching coefficients or other association coefficients are then arranged to form a similarity matrix (Fig. 2.1). In this matrix, rows and columns represent the organisms and each one is compared with other one. Organisms with great similarity are grouped together and separated from dissimilar organisms and such groups of organisms are called phenons or phenoms. The results of numerical taxonomy analysis are also often summarized in a tree-like diagram known as dendrogram (Fig. 2.2). This diagram is usually graduated with units of % similarity on one side and each branch point is at the similarity value relating the two branches. The organisms in the two branches share many characteristics. The two groups are seen to be separate only after the thorough examination of coefficient association greater than the magnitude of the branch point value. Below the branch point value, the two groups appear to be one.

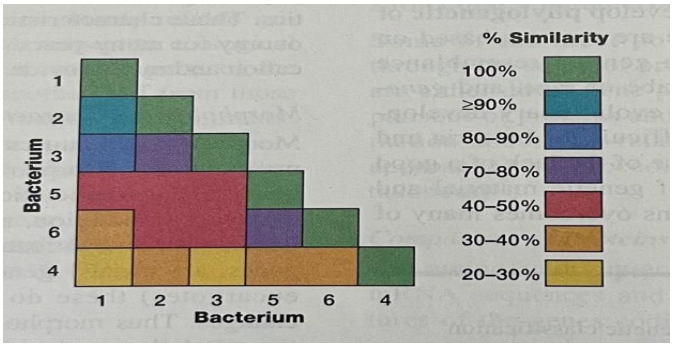


Fig.2.1: Similarity index between bacteria
(Source: Microbiology – Prescott et al.)

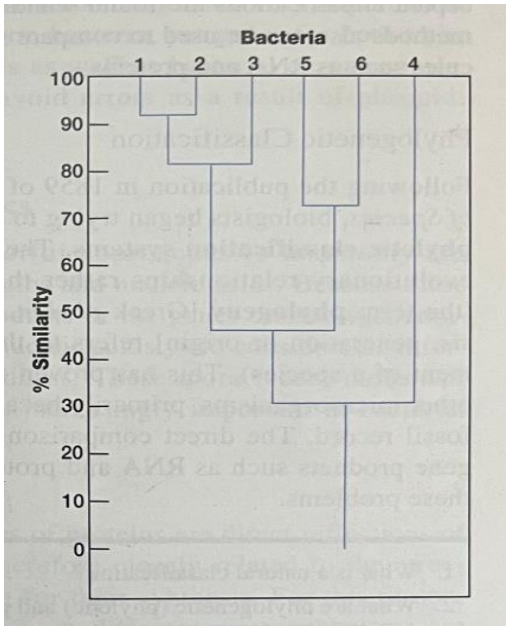


Fig. 2.2: Dendrogram
(Source: Microbiology – Prescott et al.)

Phylogenetic Classification

Following the publication of Darwin's, *On the Origin of Species* in 1859, biologists began trying to develop phylogenetic or phyletic classification systems. These are the systems based on evolutionary relationships rather than general resemblance (the term phylogeny [Greek phylon, tribe or race, and genesis, generation or origin] refers to the evolutionary development of a species) has proven difficult for prokaryotes and other microorganisms, primarily because of the lack of a good fossil record. The direct comparison of genetic material and gene products such as RNA proteins overcomes many of these problems.

2.3 MAJOR CHARACTERISTICS USED IN TAXONOMY

Many characteristics are used in classifying and identifying microorganisms that have been divided in two groups, Classical and Molecular.

Classical Characteristics

Classical approaches to taxonomy make use of morphological, physiological, biochemical, ecological, and genetic characteristics. These characteristics have been employed in microbial taxonomy for many years. They are quite useful in routine identification and may provide phylogenetic information as well.

Morphological Characteristics

Morphological features are important in microbial taxonomy for many reasons. Morphology is easy to study and analyze, particularly in eukaryotic microorganisms and the more complex prokaryotes. In addition, morphological comparisons are valuable because the structural features do not vary greatly with environmental changes. Thus, morphological

similarity is often considered as a good indication of phylogenetic relatedness. Many different morphological features are employed in the classification and identification of microorganisms (Table-2.2). Although the light microscope has always been a very important tool, its resolution limit of about 0.2µm reduces its usefulness in viewing smaller microorganisms and structures. The transmission and scanning electron microscopes, with their greater resolution, have immensely aided the study of all microbial groups.

Table 2.2 Some morphological features used in classification and identification.

Feature	**Microbial Groups**
Cell shape	All major groups
Cell size	All major groups
Colonial morphology	All major groups
Ultrastructural characteristics	All major groups
Staining behavior	Bacteria, some fungi
Cilia and flagella	All major groups
Mechanism of motility	Gliding bacteria, spirochetes
Endospore shape and location	Endospore forming bacteria
Spore morphology and location	Bacteria, algae, fungi
Cellular inclusions	All major groups
Color	All major groups

Physiological and metabolic characteristics

Physiological and metabolic characteristics are very useful because of their direct relation with the nature and activity of microbial enzymes and transport proteins. Since proteins are the gene products, analysis of these characteristics provides an indirect comparison of microbial genomes. The most important physiological and metabolic characteristics used in classification include carbon and nitrogen sources, cell wall constituents, energy sources, fermentation products, general nutritional types, growth temperatures, pH growth range, luminescence, energy conversion mechanisms, motility, oxygen relationships, osmotic tolerance, photosynthetic pigments, salt requirement and tolerance, storage inclusions etc.

Ecological Characteristics

In nature, many ecological properties affect the relation of microorganisms to their environment. Often these are taxonomically valuable because even very closely related microorganisms can differ considerably with respect to ecological characteristics. Microorganisms living in various parts of the human body markedly differ from one another and from those growing in freshwater, terrestrial, and marine environments. Some examples of taxonomically important ecological properties are life cycle patterns; the nature of symbiotic relationships; the ability to cause disease in a particular host; and habitat preferences such as requirements for temperature, pH, oxygen, and osmotic concentration.

Genetic analysis

Because most eukaryotes are bale to reproduce sexually, genetic analysis has been of

considerable use in the classification of these organisms. As mentioned earlier, the species is defined in terms of sexual reproduction possibly. Although prokaryotes do not reproduce sexually, the study of chromosomal gene exchange through transformation and conjugation is sometimes useful in their classification.

Transformation can occur between different prokaryotic species but only rarely between genera. The demonstration of transformation between two strains provides evidence of a close relationship since transformation cannot occur unless the genomes are fairly similar. Transformation studies have been carried out with several genera: *Bacillus, Micrococcus, Haemophilus, Rhizobium,* and others. Despite the usefulness of transformation, its results are sometimes hard to interpret because the absence of transformation may result from factors other than major differences in DNA sequence. Conjugation studies also yield taxonomically useful data, particularly with the enteric bacteria. For example, *Escherichia* can undergo conjugation with the genera *Salmonella* and *Shigella* but not with *Proteus* and *Enterobacter*. These observations fit with other data showing that the first three genera are more closely related to one another than to *Proteus* and *Enterobacter*.

Plasmids are undoubtedly important in taxonomy because they are present in most bacterial genera, and many carry genes coding for phenotypic traits. Because plasmids could have a significant effect on classification, if they carried the gene for a trait of major importance in the classification scheme, it is best to base on many characters for classification. When the identification of a group is based on a few characteristics and some of these are coded for by plasmid genes, errors may result. For example, hydrogen sulfide production and lactose fermentation are very important in the taxonomy of the enteric bacteria, yet genes for both traits can be borne on plasmids as well as bacterial chromosomes. One must take care to avoid errors as a result of plasmid-borne traits.

Molecular characteristics

Some of the most powerful approaches to taxonomy are through the study of proteins and nucleic acids. As these are the either direct gene products or the genes themselves, comparison between proteins and nucleic acids yield considerable information about true relatedness. These recent molecular approaches have become increasingly important in prokaryotic taxonomy.

Comparison of Proteins

The amino acid sequences of proteins are direct reflections of mRNA sequences and therefore closely related to the structures of the genes coding for their synthesis. For this reason, comparison of proteins from different microorganisms is taxonomically very useful. There are several ways to compare proteins. The most direct approach is to determine the amino acid sequence of proteins with the same function. The sequences of proteins with dissimilar functions often changes at different rates; some sequences change quite rapidly, whereas others are very stable. Nevertheless, if the sequences of proteins with the same function are similar, the organisms possessing them are probably closely related. The sequences of cytochromes and other electron transport proteins, histones, heat-shock proteins, transcription and translation proteins, and a variety of metabolic enzymes have been used in taxonomic studies. Because the protein sequencing is slow and expensive, more indirect methods of comparing proteins frequently have been employed. The electrophoretic mobility of proteins is useful in studying relationships at the species and subspecies levels. Antibodies can

discriminate between very similar proteins, and immunologic techniques are used to compare proteins from different microorganisms. The physical, kinetic and regulatory properties of enzymes have been employed in taxonomic studies. Since enzyme behavior reflects amino acid sequence, this approach is useful in studying some microbial groups, and group specific patterns of regulation.

Nucleic Acid Base Composition

Microbial genomes can be directly compared, and taxonomic similarity can be estimated in different ways. The first, and possibly the simplest, technique to be employed is the determination of DNA base composition. DNA contains four purine and pyrimidine bases: adenine (A), guanine (G), cytosine (C), and thymine (T). In double stranded DNA, 'A' pairs with 'T', and 'G' pairs with 'C'. Thus, the (G + C) / (A + T) ratio or the percent of G + C in DNA, reflects the base sequence and varies with sequence changes.

$$\text{Mol \% G + C} \frac{\text{G + C}}{\text{G + C + A + T}} \text{ X } 100$$

The base composition of DNA can be determined in several ways. Although the G + C content can be ascertained after hydrolysis of DNA and analysis of its bases with High Performance Liquid Chromatography (HPLC) technique, physical methods are easier and more often used. The G + C content is determined from the melting temperature (T_m) of DNA. In double stranded DNA three hydrogen bonds join GC base pairs, and two bonds connect AT base pairs. As a result, DNA with a greater G + C content will have more hydrogen bonds, and its strands will separate only at higher temperatures – that is, it will have a higher melting point. DNA melting can be easily monitored spectrophotometrically as the absorbance of 260 nm UV light by DNA increase during strand separation. When a DNA sample is slowly heated, the absorbance increases as hydrogen bonds are broken and reaches a plateau when all the DNA has become single stranded, the midpoint of the rising curve gives the melting temperature, a direct measure of the G + C content. Since the density of DNA also increases linearly along with G + C content, the percent G + C can be obtained by centrifuging DNA in a CsCl density gradient.

The G + C content of DNA from animals and higher plants averages around 40% and ranges between 30 and 50%. In contrast, the DNA of both eukaryotic and prokaryotic microorganisms varies greatly in G + C content; prokaryotic G + C content is the most variable, ranging from around 25 to almost 80%. Despite such a wide range of variation, the G + C content of strains within a particular species is constant. If two organisms differ in their G + C content by more than about 10%, their genomes have quite different base sequences. On the other hand, it is not correct to assume that organisms with very similar G + C contents also have similar DNA base sequences because two very different base sequences can be constructed with same proportions of AT and GC base pairs. The G + C content data is taxonomically valuable in at least two ways. First, they can confirm a taxonomic scheme developed using other data. If organisms in the same taxon are too dissimilar in G + C content, the taxon probably should be divided. Second, G + C content appears to be useful in characterizing prokaryotic genera since the variation within a genus is usually less than 10% even though the content may vary greatly between genera. For example, *Staphylococcus* has a G + C content of 30 to 38%, whereas *Micrococcus* DNA has 64 to 75% G + C; yet these two genera of Gram- positive cocci have many other features in common.

Nucleic Acid Hybridization

The similarity between genomes can be compared more directly by using nucleic acid hybridization studies. If a mixture of single stranded DNA, formed by heating ds DNA, is cooled and held at a temperature about 25^0C below the Tm, strands with complementary base sequences will reassociate to form stable ds DNA, whereas non-complementary strands will remain single. Because strands with similar, but not identical, sequences associate to form less temperature stable dsDNA hybrids, incubation of the mixture at 30 to 50^0C below the Tm will allow hybrids of more diverse ssDNA to form. Incubation at 10 to 15^0C below the Tm permits hybrid formation only with almost identical strands.

In one of the more widely used hybridization techniques, nitrocellulose filters with bound non-radioactive DNA strands are incubated at the appropriate temperature with single stranded DNA fragments made radioactive with ^{32}P, ^{3}H, or ^{14}C. After radioactive fragments are allowed to hybridize with the membrane-bound ssDNA, the membrane is washed to remove any non-hybridized ssDNA and its radioactivity is measured. The quantity of radioactivity bound to the filter reflects the amount of hybridization and thus the similarity of the DNA sequences. The degree of similarity or homology is expressed as the percent of experimental DNA radioactivity retained on the filter compared with the percent of homologous DNA radioactivity bound under the same conditions. Two strains whose DNAs show at least 70% relatedness under optimal hybridization conditions and less than 5% difference in Tm often are considered as members of the same species. If DNA molecules are very different in sequence, they will not form a stable, detectable hybrid. Therefore DNA-DNA hybridization is used to study only closely related microorganisms.

Molecular (rRNA) based classification

Although classical numerical taxonomic approaches are useful for classification of micro-organisms at the species and sub species levels, reliable phylogenetic classification of higher level groupings of evolutionary divergent microorganisms is not feasible using such taxonomic methods. Classification systems that relay on phenotypic features have resulted in the assignment of bacteria to supra-generic groups, many of which were subsequently shown to be heterogeneous. Only by using molecular analysis, classification systems could be developed in which higher order phylogenetic groups were properly classified. Genetic measures such as rRNA analysis and DNA hybridization provide the measures of higher-level taxonomic groupings.

The bacterial systematics, which begins as largely intuitive subject, has become objective with the introduction and application of new molecular methods. This became clear with the recognition that bacteria and archaea represent phylogenetically distinct prokaryotes. This major breakthrough in determining evolution and phylogeny of prokaryotes occurred with the introduction of rRNA sequencing techniques.

The RNA components of ribosomes (rRNAs) are among the most evolutionarily conserved macromolecules in all living systems. Their functional roles in primitive information processing systems must have been well established in the earliest common ancestors of bacteria, archaea and eukaryotes. Because of the functional constraints, large portions of ribosomal RNA genes are well conserved and the sequence can be used to measure phylogenetic distances between even the most distantly related organisms. The changes in RNA nucleotide sequence

are indices of evolutionary change. The comparison of rRNA molecules isolated from different organisms is useful for determining the evolutionary relationships among the living things. Any similarity in two nucleotide sequences of RNA suggests some phylogenetic relationship between these nucleotide sequences and the organisms that contain them. In particular, the 16s rRNA of bacteria and archaea is used to determine the phylogenetic relationship among these microorganisms. For Eukaryotes, 18s rRNA is analyzed. The advantages of using 16s rRNA and 18s rRNA are that they found in all organisms, and large enough molecules to provide a significant number of nucleotides to compare sequences and yet they are small enough to analyze easily.

Carl Woese who has started phylogenetic studies with 5S rRNA, which has only 120 nucleotides, switched his studies to the larger 16s rRNA which has 1500 nucleotides. He argued that 16S and 18S rRNAs make excellent molecular chronometers because they 1) occur universally in all organisms, 2) have long highly conserved regions to asses close relationships, and 3) have sufficient variable reasons to asses close relationships, and 4) never prone to rapid sequence change due to selection because of their central function in gene expression. The first complete rRNA gene sequence was determined for *E. coli*. A comparison of this sequence with the oligonucleotide catalogue data reviled the universally conserved elements that are distributed along the entire length of the *E. coli* rRNA. Similar sequence analysis of rRNA coding regions from *saccharomyces cerevisiae* and *Xenopuslaevis, Dictyostelium, Disoideum, Halobactrium*, and from several mitochondrial and chloroplast genomes confirmed this observation and identified the existence of these conserved elements. At the same time these analyses showed that the rRNA of bacteria, archaea and eukarya domains were specific and each has its own characteristic rRNAs with diagnostic sequences and characteristic secondary structures. Analyzing these rRNA, forms the basis for the phylogenetic analysis of organisms in all the three domains of life.

Several methods can be used to analyze the rRNA molecules. One approach for analyzing the rRNA sequence is to extract the rRNA from the cells and to analyze it directly or to use the rRNA as a template for making cDNA and then using the polymerase chain reaction (PCR) to produce sufficient DNA for analysis. Typically, if rRNA is to be captured for analysis, cells are ruptured in the presence of Dnase to degrade all DNA. The RNA is then extracted with phenol and water whereby the large RNA molecules get into the aqueous phase. After precipitation of RNA with alcohol and salt, a DNA primer that is complementary to the conserved region of the 16s rRNA is added. Reverse transcriptase can then be used to generate cDNAs. The cDNAs can be amplified using PCR and complete sequences of the nucleotide in the cDNAs are determined so that the nucleotide sequence in the rRNAs can be deduced from this analysis. Comparison of nucleotide sequences of 16s rRNA allows the calculation of evolutionary distances and the construction of phylogenetic trees that show relative evolutionary positions and relationships. The resultant phylogeny based on 16s rRNA analysis revealed the separate domains viz., bacteria, archaea and eukarya.

Nucleic Acid Sequencing

Despite the usefulness of G + C content determination and nucleic acid hybridization studies, genome structures can be directly compared only by sequencing DNA and RNA. Techniques for rapid sequencing of both DNA and RNA are now available; thus far RNA sequencing has been used more extensively in microbial taxonomy. Most attention has been given to sequences of the 5S and 16S rRNA isolated from the 50S and 30S subunits, respectively, of

prokaryotic ribosome. The rRNAs are almost ideal for studies of microbial evolution and relatedness since they are essential to a critical organelle found in all microorganisms. Their functional role is same in all ribosomes. Furthermore, their structure changes very slowly with time presumably because of their constant and critical role. Because rRNA contains variable and stable sequences, both closely related and very distantly related microorganisms can be compared.

2.4 SCHEME OF CLASSIFICATION

Classification, an aspect of taxonomy, is a process of arranging the organisms into groups or taxa basing on established procedures and rules, their interrelationships and boundaries between groups of organisms. In addition to classification, taxonomy is concerned with nomenclature (assign names to the units described in a classification system), and identification (applying the system of classification and nomenclature to assign the proper name to an unknown organism and to place it in its proper position within the classification system).

Classification attempts to differentiate microbial taxa into structured groups so that the members of a group are more closely related to each other than they are to members of any other group. Classification is a coherent scheme by which a collection of organisms are arranged to reflect the relationships between individuals and groups basing on similarities. Historically, many classification systems used for microorganisms were artificial rather than natural and they are based on observable phenotypic features and not on evolutionary (genetic) relatedness. Taxa based on observed phenotypic characteristics may not accurately reflect the genetic similarities and such a classification may not correspond to the evolutionary flow of events. It is possible for genetically dissimilar bacteria to produce yellow pigments, and a characteristic scheme based on such a phenotypic characteristic could produce a taxonomic group of genetically unrelated bacteria. In fact, classification systems are filled with errors made by using such phenotypic characteristics. Various groups of bacteria that have been defined on the basis of their apparent phenotypic relationship are now considered to be “groups of uncertain taxonomic affinity” because the taxonomic group may not be homologously similar and therefore may not accurately have genetic similarities.

2.5 TAXONOMIC HIERARCHY

In the scheme of taxonomic hierarchy, microorganism is placed within a small, homogenous group that is itself a member of larger groups in a non-overlapping hierarchical arrangement. A category in any rank unites groups in the level below it based on shared properties (Table 2.3). Ideally, each defined level of a taxonomic hierarchy represents a coherent degree of homologous similarity in genetic and evolutionary relationships. Each taxonomic group should be monophyletic, that is the members of each taxa should have the same evolutionary history. A genus, for example should contain only species that evolved from the same ancestral species that first evolved in that genus. Classification systems based on molecular analyses aimed at directly assessing phylogeny tend to meet this condition. In contrast, taxa defined on phenotypic characteristics often are polyphyletic, that is taxa defined by such systems often include organisms with different evolutionary histories that represent varying degrees of analogous (phenotypic) similarity.

The levels of a taxonomic hierarchy, from the highest to the lowest, are domain or empire, kingdom, phylum or division, class, order, family, genus and species. By assuming similarity between species, they may be arranged into genera which may in turn, be fused into higher

taxa such as families until the whole range of variation is accounted for in the hierarchical system. The hierarchical separation of microorganisms into taxonomic grouping of species, genera, families and so forth can be defined at the molecular level.

Table -2.3: Taxonomic Ranks (Example)

Rank	**Example**
Domain	Bacteria
Kingdom	Proteobacteria
Section	γ- Proteobacteria
Class	Zymobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	*Shigella*
Species	*S. dysenteriae*

The basic taxonomic group in microbial taxonomy is the species. 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. Although species are the basic taxonomic units, the genetic variability of microorganisms permits a further division into subspecies or types that describe the specific clone of cells. The subspecies or type may differ biochemically or physiologically (biovar), morphologically (morphovar), or antigenically (serovar). It is often important to differentiate the subspecies of a given microorganisms. For example one strain of a bacterial species may produce a toxin and be a virulent pathogen, and other strains of the same species may be non-pathogenic. A strain is a population of cells that are descendants of a single cell. The ability to distinguish correctly between such strains and subspecies of a particular bacterial species is of obvious importance in medical and industrial microbiology. Pure cultures grown in the microbiology laboratory represent individual strains of a species. After organisms are defined as representing a new species of strain, a culture generally is deposited in an appropriate culture collection as the type culture. That type culture and its description become the foundation for future reference.

An alternate method of describing species today is based on molecular analyses. The nucleotide sequence of informational molecules can be determined even for microorganisms that have yet to be cultured. New taxa can be defined based on finding nucleotide sequences that are sufficiently divergent to merit placing them into new groups. Thus many have never been cultured. Some of these can be observed under a microscope and specially tagged using labeled gene probes so that at least their appearance can be described. In many cases, however little or nothing is known about the physiologies of these newly discovered taxonomic groupings, it is to say that there are tens of thousands of diverse microbial species that have yet to be classified and described.

Microbiologists name the microorganisms by following the binomial nomenclature system of Swedish botanist namely Carolus Linnaeus. The Latinized, italicized name consists of two parts. The capitalized first part is the generic name and uncapitalized second part is the specific epithet (e.g., *Escherichia coli*). The specific epithet is stable and the oldest epithet

for a particular organism takes precedence and must be used. In contrast, a generic name can change if the organism is assigned to another genus because of new information. As an example, the genus *Streptococcus* has been divided into 2 genera, *Enterococcus* and *Lactococcus* basing on rRNA sequence analysis and various other characteristics. Thus, *Streptococcus faecalis* is now *Enterococcus faecalis*. Often the name of the organism may be shortened by abbreviating the genus name with a single capital letter – e.g., *E. coli*.

2.6 SUMMARY

Taxonomy, the science of biological classification, is composed of three parts viz., classification, nomenclature, and identification. The two major types of natural classification are phylogenetic system and phonetic system. Classifications may be constructed by means of numerical taxonomy, in which the general similarity of organisms is determined using a computer to calculate and analyze association coefficients. In microbial taxonomy, morphological, physiological, metabolic and ecological characters are widely used. The G+C content of DNA, which is an indirect reflection of the base sequence, is taxonomically valuable tool. Nucleic acid hybridization and nucleic acid sequencing studies are used to determine genetic relatedness and genome comparisons, respectively. The sequences of rRNA, DNA and proteins are used to construct the phylogenetic trees which give information on genetic relatedness and differences among the members of the taxonomic groups. A bacterial species is a large collection of strains that have many stable properties in common and differ significantly from other groups of stains. And microorganisms are named according to the binomial nomenclature.

2.7 TECHNICAL TERMS

Phenetic system, Phylogenetic system, Numerical taxonomy, Similarity index, Similarity coefficient, Jaccard coefficient, Taxonomic ranks, Phenons, Binomial nomenclature.

2.8 SELF ASSESSMENT QUESTIONS

Q.1 Describe the principles of bacterial taxonomy and classification.

Q.2 Explain the different identification characters used in bacterial taxonomy.

Q.3 What is the role of molecular characteristics used in bacterial classification.

Q.4 What is the numerical taxonomy and how it is useful in taxonomic classification of bacteria.

2.9 SUGGESTED READINGS

1) Principles of microbiology - Ronald M. Atlas, McGraw Hill, 2nd Edition.
2) Biology of microorganisms - Thomas D. Brock, Michael P. Madigan, Prentice Hall Englewood Cliffs, 5th Edition.
3) Microbiology - Michael J. Pelczar, Jr., E.C.S.Chan, Noel R. Krieg, 5th Edition.
4) Microbiology - Lansing M. Prescott, John P. Harley, Donald A.Klein

Dr. J. Madhavi

LESSON – 3

BERGEY'S MANUAL OF SYSTEMATIC BACTERIOLOGY FOR BACTERIAL CLASSIFICATION (SECOND EDITION)

OBJECTIVE OF THE LESSON

Students will know the different groups of bacteria, their classification and arrangement into different volumes based on their characteristic features according to Second edition of Bergey's Manual of Systematic Bacteriology.

STRUCTURE OF THE LESSON

3.1 INTRODUCTION

In 1923, David Bergey, professor of bacteriology at University of Pensylvania and four colleagues published a classification of bacteria that could be used for identification of bacterial species, the Bergey's Manual of Determinative Bacteriology. Bergey's Manual of Systematic Bacteriology focuses on the classification and biology of bacteria, but often is more detailed than required for identification. The first edition of Bergey's Manual of Systematic Bacteriology is largely phenotypic and divides the bacterial into groups based on easily determined characteristics viz., shape, Gram staining properties, oxygen relationships and motility. Whereas, the second addition is designed with five volumes and thirty sections. In this, the prokaryotes are organized phylogenetically and distributed among two domains and at least 14 kingdoms.

3.2 FIRST EDITION OF BERGEY'S MANUAL OF SYSTEMATIC BACTERIOLOGY

Because it has not been possible in the past to classify prokaryotes satisfactorily based on phylogenetic relationship, the system given in the first edition of Bergey's Manual of Systematic Bacteriology is primarily phenetic. Each of the 33 sections in the four volumes contains bacteria that share a few easily determined characteristics and bears a title that either describes these properties or provides the vernacular names of the prokaryotes included. The characteristics used to define sections are normally features such as general shape and morphology, Gram staining properties, O_2 relationship, motility, presence of endospores, mode of energy production, and so forth. Bacterial groups are divided among the four volumes in the following manner: (1) Gram-negative bacteria of general, medical or industrial importance (2) Gram-positive bacteria other than actinomycetes (3) Gram-negative bacteria with distinctive properties, cyanobacteria, and archaeobacteria, and (4) Actinomycetes.

Gram staining properties play a significant role in the phonetic system of classification as it usually reflects fundamental differences in eubacterial wall structure. Gram-staining properties also are correlated with many other properties of bacteria. Typical Gram-negative bacteria, Gram-positive bacteria, and mycoplasmas (bacterial lacking walls) differ in many characteristics. For these and other reasons, bacteria traditionally have been classified as Gram positive or Gram negative. This approach is retained to some extent in more phylogenetic classifications and is useful in understanding the bacterial diversity.

Many phonetically defined taxa are not phylogenetically homogenous and have members distributed among two or more different phylogenetic groups. Often the characteristics given great weight and importance in Bergey's manual do not appear to be phylogenetically significant. For example, photosynthetic bacteria are found in several different phylogenetic groups together with very closely related non-photosynthetic bacteria. Similarly, the Mycoplasmas are placed in a separate division, Mollicutes in Bergey's manual, but the rRNA studies show that they are closely related to Gram positive bacteria though they do not contain cell walls. Despite the uncertainties with the classification, the Bergey's manual is the most widely accepted and used system for the identification of bacteria.

3.3 SECOND EDITION OF BERGEY'S MANUAL OF SYSTEMATIC BACTERIOLOGY

There has been enormous progress in prokaryotic taxonomy since the publication of the first volume of Bergey's Manual of Systematic bacteriology in 1984. In particular, the sequencing of RNA, DNA, and proteins has made phylogenetic analysis of prokaryotes feasible. As a consequence, the second edition of Bergey's Manual will be largely phylogenetic rather than phonetic and thus quite different from the first edition.

The second edition is published in five volumes. It will have more ecological information about individual taxa. The second edition will not group all the clinically important prokaryotes together as in the first edition. Instead, pathogenic species are placed phylogenetically and thus scattered throughout the following five volumes.

Volume 1 – The Archaea, and the deeply branching and Phototrophic Bacteria
Volume 2 – The Proteobacteria

Volume 3 – The Low G + C Gram-Positive Bacteria
Volume 4 – The High G + C Gram-Positive Bacteria
Volume 5 – The Planctomycetes, Spirochaetes, Fibrobacteres, Bacteroidetes and Fusobacteria (Volume 5 also contain a section that updates descriptions and phylogenetic arrangements that have been revised since publication of volume 1).

The second edition's five volumes will have a different organization than the first edition. The greatest change in organization of the volumes will be with respect to the Gram-negative bacteria. The first edition describes all Gram-negative bacteria in two volumes. Volume 1 contains the Gram negative bacteria of general, medical or industrial importance; Volume 3 describes the Archaea, Cyanobacteria, and remaining Gram-negative groups. The second edition describes the Gram-negative bacteria in three volumes, with Volume 2 reserved for the Proteobacteria. The two editions treat the Gram-positive bacteria more similarly. Although volume 2 of the first edition does not have some G+C bacteria, much of its coverage is equivalent to the new Volume 3. The Volume 4 of the first edition describes the actinomycetes and is similar to Volume 4 of the second edition (high G+C Gram-positive bacteria), although the new Volume 4 will have broader coverage. For example, *Micrococcus* and *Corynebacterium* are in Volume 2 of the first edition and is in Volume 4 of the second edition.

The second edition did not group all the clinically important prokaryotes together as the first edition did instead pathogenic species will be placed phylogenetically and thus scattered throughout the five following volumes. The second edition is divided into a total of 30 sections accommodated in five volumes.

3.3.1 Volume 1. Archaea

This volume contains a wide diversity of bacteria in two domains: the Archaea and the Eubacteria. The Archaea comprise organisms that evolved as separate domain often retaining specialized phenotypic characteristics. Archaea has several features relative to their cell structure that permit them to live in extreme habitats and to function under conditions considered inhospitable to life. The Archaea differ from Eubacteria in many ways. Archaea are divided into 2 kingdoms based on rRNA sequences. The kingdom **Crenarchaeota** contains thermophilic and hyperthermophylic sulfur-metabolizing organisms of the orders Thermoproteales and Sulfolobales. However, recently many other crenarchaeota have been discovered. Some are inhibited by sulfur; others grow in the oceans at low temperatures as picoplankton. This kingdom is clearly more diverse than first thought. The second kingdom, Euryarchaeota contains primarily methanogenic bacteria and halophilic bacteria; thermophilic, sulfur-reducing organisms (thermoplasmas, thermococci) also are in this kingdom. The two kingdoms are divided into three classes and 10 orders, and described in the first five sections – thermoprotei; sulfolobi and barophiles; methanogens; halobacteria; thermoplasmas; and thermococci.

The cytoplasmic membrane of archaea are unique in structure and chemical composition they have high protein content and diverse lipids including are phospholipids, sulpholipids, glycolipids and a non-polar isoprenoid lipid. The structure of membrane of many archaea is a lipid bilayer composed of glycerol diether lipids. But some archaeal membranes are monolayers composed of glycerol tetraether lipids. These monolayers are heat stable The archaeal cell wall is distinct in chemical composition then the other organisms, some Archaea

stain Gram-negative and some Gram-positive, no Achaean has a true bacterial Gram-negative or Gram-positive cell wall structure and all archaea lack peptidoglycan in their cell walls.

The archaeal chromosomes resemble bacterial chromosomes in that it is circular however there is a difference in the organization of archaeal chromosomes. The chromosome of archaea is associated with proteins which make is similar someway to eukaryotic chromosomes. Histone-like proteins are involved in maintaining the structure of Archaeal chromosomes. Introns occur with the archaeal chromosomes. Archaeal introns found in the stable RNA genes of thermophilic and halophilic archaea sliced by archaeal specific mechanism. The promoter sequences of some Archaea indicates the presence of two conserved regions one conserved region occurs at the sight of transcription. The second sequence occurs 25 base pairs of stream of transcriptional start sight resemble the TATA box of eukaryotic cell genomes. The ribosomes of the archaea are of 70S type composed of 30s and 50s subunits resembling 70S ribosomes of bacterial cells.

The eubacteria are an extraordinarily diverse assemblage of prokaryotes with well over a dozen distinct groups as per the rRNA analysis. In volume 1, deeply branching eubacterial groups, several small groups of phototrophs, and cyanobacteria are placed and described through sections VI to XIV.

3.3.2 Volume 2

The volume 2 of the second edition is devoted completely to the Gram negative proteobacteria, often called as purple bacteria and accommodated into five sections (15 to 19). The proteobacteria are a coherent deep evolutionary branch on the bacterial tree encompassing a phenotypically very diverse group of Gram-negative bacteria. The kingdom proteobacteria is a large and extremely complex group that currently contains over 1,300 species in 332 genera. Even though they are all related, the group is quite diverse in morphology, physiology, and life-style. All major nutritional types are represented – phototrophy, heterotrophy, and chemolithotrophy of several varieties. Many species important in medicine, industry, and biological research are proteobacteria. The best examples are *Escherichia*, *Neisseria*, *Pseudomonas*, *Rhizobium*, *Rickettsia*, *Salmonella*, and *Vibrio* genera. The kingdom is divided into five groups based on rRNA data and each is described in a different section in Bergey's manual. Because the photosynthetic bacteria are found in α, β, and γ groups of the proteobacteria, may believe that the whole group arose from a photosynthetic ancestor. Even though they have a common evolutionary history, which has been confirmed by multiple measures including DNA-rRNA hybridizations, 16S rRNA cataloging and 16S or 5S sequencing the proteobacteria confront us with a bewildering range of phenotype features, apparently indicating independent and uncoordinated evolutionary modifications.

Apparently the proteobacteria have common underlying biosynthetic and cellular housekeeping functions that reflect their common ancestry metabolic capabilities of the recent evolutionary changes that have increased diversity within this group. This could have occurred as a result of genetic exchange; Gram negative bacteria are sometimes described as promiscuous because of their frequent exchange of plasmids and recombination that crosses species and genus boundaries. It may also be that the phenotypic metabolic diversity is the centers of

relatively minor changes in the active centers of the enzymes that have underlying homologous proteins.

The proteobacteira have been divided into five separate lineages based on rRNA sequences; these lineages are designated the alpha, beta, gamma, delta and epsilon subgroups. These subgroups of proteobacteria have characteristic differences. For example, an unusual polyamine- 2hydroxyputresceine occurs as a specific component within members of the beta subgroup of the proteobacteria. In the alpha subgroup, a triamine, such as spermidine or symhomospermidine, is found as a characteristic biochemical component. Even these subgroups of proteobacteria contain very diverse genera species. The diversity of phenotypic features brings into question the evolutionary linkage of the proteobacteria and the course of evolution, especially of the processes involved in cellular energy generation.

The occurrence of bacteriochlorophyll dependent photosynthesis in three of the subgroups of the proteobacteria (alpha, beta, and gamma) suggests that the proteobacteira are all derived from photosynthetic ancestors. The proteobacteria originally were named the purple bacteria to reflect this relationship to the purple bacteria to reflect this relationship to the purple photosynthetic bacteria. Chemoautotrophic bacteria, such as *E. coli* appear to have lost their photosynthetic capabilities. Other non-photosynthetic bacteria in the proteobacteria retain metabolic traces of their photosynthetic past. Bacteriochlorophyll a, for example, neither of which are anaerobic phototrophs.

3.3.3 Volume 3

Low Mole % G + C Gram-positive Bacteria

The volume 3 includes the Gram positive bacteria with low G + C content in their DNA. The dividing line is about 505 G + C; bacteria with a mol% of lower than this value are placed in volume 3. Most of these bacteria are Gram positive and heterotrophic bacteria. However, because of their close relationship to low G + C Gram positive bacteria, the mycoplasmas are placed in this volume even though they lack cell walls and stain Gram negative. There is a considerable variation in morphology – some are rods, others are cocci, and mycoplasmas are pleomorphic. Endospores may be present. This volume contains three sections – XX, XXI, and XXII.

Based on comparisons of 16S rRNA catalogs and sequences, the Gram-positive bacteria can be divided into two major evolutionary lines of descent: gram-positive bacteria with a low mole % G + C (clostridial lineage) and gram positive bacteria with a high mole % G + C (actinomycete lineage). The dividing line between these independent lineages is at about mole % G + C 50. Phylogenetic analyses based on 16S rRNA support the division of the Gram-positive bacteria into these two distinct evolutionary branches. The low mole% G + C Gram-positive group generally include most Gram-positive bacteria: endospore forming genera, including *Lactobacillus, Listeria, Pediococcus, Mycoplasma, Heliobacterium* and others. All of the lactic acid bacteria and staphylococci are in this group. Based on oligonucleotide catalogs of 16S rRNA, the lactic acid bacteria, *Bacillus* and *Streptococcus* form a super cluster within the clostridial lineage. The *Lactobacillus* lineage diverges at about the same similarly coefficient as the *Bacillus* and *Streptococcus* lineages.

Comparisons of 16S rRNA sequences, 5S rRNA sequences, and rRNA homologies have divided the clostridia into three phylogenetic groups. *Clostridium* represents one of the largest genera of all the bacteria because they are defined by relatively few criteria: anaerobic, endospore

forming, non-sulfate reducing Gram-positive bacteria. There are over 100 species within this group. The members of groups I and II are fairly homogenous in their intragroup relationships, whereas the members of group III show little or no rRNA sequence similarly. *Bacillus* essentially differs from *Clostridium* species in that the former are aerobic and the latter are anaerobic. This divergence corresponds to the appearance of high concentrations of oxygen in the Earth's atmosphere about 700 to 800 million years ago. This hypothesis is further supported by the appearance of other Gram-negative and Gram-positive aerobic lineages at about the same time.

The fact that the low mole % G + C Gram-positive group contains both endospore-forming and non-endospore forming genera leads to some speculation about when endospore formatting has been highly conserved in bacillus and Clostridium, which have been studied most extensively. This conservation suggest that the mechanism of sporulation developed only once during evolutionary history. Since *Clostridium* is phylogenetically more ancient than Bacillus, the ancestral progenitor of this group was probably an anaerobic spore forming bacterium. Later divergence from this ancestor would have resulted in the loss of the ability to sporulate. It follows that *Pediococcus, Lactobacillus* and other members of the low G + C gram-positive bacterial group must have lost the ability of differentiate into spores.

Mycoplasma species appear to be the descendants of the *Bacillus – Lactobacillus – Streptococcus* lineage. Mycoplasmas are unusual bacteria both in terms of their phenotype-they lack cell walls and genotype, which appears to have evolved more rapidly than other bacteria. The more rapid the rate of evolution at the genotypic level one manifestation of which is a lineage on a phylogenetic tree that is abnormally long the more unusual and atypical the resulting phenotypic changes. The clearest examples of such rapid genotypic change and unusual phenotype so far encountered among the bacteria are the mycoplasmas. Phenotypically, mycoplasmas constitute a separate bacterial class; their uniqueness includes lack of a cell wall and the small size of their genomes. Based on rRNA sequence analysis, which is a measure of genotype, the mycoplasmas represent a typical bacterial group. Thus, mycoplasmas represent a relatively superficial branching within bacterial phylogeny. What is unusual about the mycoplasmas by this genotypic measure is that their individual lineages tend to be evolving more rapidly than normal bacteria. Mycoplasmas have the smallest genomes among self reproducing organisms. Genome sizes for members of the Mollicutes appear to fall into two clusters: one composed of *Mycoplasma* and *Ureaplasma* species have genomes of about 750 kb, and the other of *Acholeplasma, Spiroplasma, Anaeroplasma* and *Asteroplasma* species have genomes of about twice that size. Given that the mycoplasmas evolved from Gram-positive bacteria with much larger genomes, it appears that their evolution involved an unexpected streamlining of genetic information needed to enhance efficiency for survival and reproduction.

Perhaps as the surprising revelation that the mycoplasmas are descendants of the low mole% G + C Gram-positive bacteria is the discovery that *Epulopiscium fishelsoni* the largest of all bacteria is closely related to the endospore formers. *E. fishelsoni* is a descendant of *Clostridium lentocellus,* a cellulolytic, endospore forming anaerobe. Since *E. fishelsoni* lives in the gut of the herbivorous surgeonfish it is likely that it can grow using cellulose as a substrate. Although *E. fishelsoni* does not form endospores it exhibits an unusual form of reproduction that resembles the initial stages of sporulation. This bacterium produces multiple daughter cells, which are released through a slit in the mother cell.

Analyses of 16S rRNA indicate that *E. fishelsoni* is closely related to *Metabacterium polyspora*, which forms multiple endospores. There are physical similarities between the appearances of inclusions within cells of *E. fishelsoni* and *M. polyspora*, except the inclusions in *E. fishelsoni* are metabolically active daughter cells and those within *M. polyspora* are resting endospores. The origin of the live daughter cells involved in the reproduction of *Epulopiscium* appear to have evolved as a modification of the sporulation process in a predecessor to this most unusual bacterium.

3.3.4 Volume 4

High Mole% G + C Gram-positive Bacteria

The volume 4 is devoted to the high G + C Gram positive bacteria with mol% values above 50 to 55%. There is enormous morphological variety among these prokaryotes. Some are cocci, others are regular to irregular rods. High G + C Gram positives called actinomycetes form complex branching. Although none of these bacteria produce true endospores, many genera do form a variety of asexual spores and some have complex life cycles. There is considerable variety in cell wall chemistry among the high G + C Gram positives as the composition of peptidoglycan varies greatly. Mycobacteria produce large mycolic acids that distinguish their walls from those of other bacteria.

The taxonomy of these bacteria is very complex and quite different from that given in the first edition. There is one class, Actinobacteria, five subclasses, six orders, nine suborders, and 35 families. Genera such as *Actinomyces*, *Arthrobacter*, *Corynebacterium*, *Micrococcus*, *Mycobacterium*, and *Propionibacterium* were formerly placed in volume 2 along with Gram positive bacteria other than the actinomycetes. They are now in the volume 4 within the suborders Actinomycineae, Micrococcineae, and Corynebacterineae because rRNA studies have shown them to be related to actinomycetes. The volume also contains genera often classified as nocardioform actinomycetes (*Nocardia*, *Rhodococcus*) and a variety of actinomycete groups such as the actinoplanates and streptomycetes. Although many genera are still familiar, the larger taxonomic categories have been extensively revised. As before, the largest and most complex genus is *Streptomyces*, which contains over 500 species.

Gram-positive bacteria with a high mole% G + C (>55) comprise a morphologically diverse group that is phylogenetically related. This lineage of high mole% G+ C Gram-positive bacteria includes the actinomycetes, actinobacteria (*Arthrobacter*, *Micrococcus*, *Brevibacterium* and *Actinomyces*) Among other genera, corynebacteria, mycobacteria, bifidobacteria and propionibacteria. Within this phenotypically diverse group that branched from the low mole% G + C Gram-positive bacterial lineage, there has been a continuation of cell wall evolution as seen in variations in the biochemical compositions of the peptidoglycan molecules. Several of these bacteria, most notably the mycobacteria, produce mycolic acids that make their cell walls biochemically distinct from other bacteria. There also has been the evolution of branching hyphae as a means of cells growth. Many of the actinobacteria exhibit irregular morphologies but the true actinomycetes from mycelia that bear a variety of spores. Complex life cycles with various spores and the production of growth regulators, many of which are antibiotics, characterize the actinomycetes.

Actinomycetes have been separated into six subgroups based on oligonucleotide sequences: Actinoplanetes. Maduromuycetes, Nocardioforms, Streptomycetes, Thermomonospora and those with multilocular sporangia; the actinobacteria, and propionobacteria form separate related groups. Based on 16S rRNA analyses, *Corynebacterium* species are placed into their own family – corynebacteriaceae and *Mycobacterium* species are grouped together with *Nocardia* and *Rhodococcus* species in the family Mycobacteriaceae. Detailed comparisons of 16S rRNA nucleotide sequences indicates that the genus Mycobacterium is most closely related to *Streptomyces lividans* (93% similarly) and less so to *Bacillus subtilis* (74% similarity). Interestingly, phylogenetic classifications of the mycobacteria based on these 16S rRNA analyses support the traditional phonetic separation of fast growing mycobacteria, such as *Mycobacterium phlei*, and slow growing mycobacteria such as *Mycobacterium tuberculosis*.

Analyses of 16S rRNA sequences from several cyanobacteria strains show that the diversity within the cyanobacteria is much less than that seen in other bacterial groups. Although the cyanobacteria as a group display extensive morphological and physiological diversity, they are relatively closely related to one another on a phylogenetic level. Also, the diverse branching of the five orders within the cyanobacteria (Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales and Stigonematales) show similar branching depths. The small change in sequence diversity and multiple fanlike branch arrangements within each order makes it difficult to assess the order of branching during evolution. Therefore taxonomic classifications of cyanobacteria based principally on morphology do not necessary reflect phylogenetic relationship.

3.3.5 Volume 5

The fifth volume describes an assortment of deeply branching phylogenetic groups that are located here for convenience: the kingdoms Planctomycetes, Spirochaetes, Fibrobacteres, Bacteroides, and Fusobacteria. The inclusion of these groups in volume 5 does not imply that they are directly related. Although they are all Gram negative bacteria, there is considerable variation in morphology, physiology, and life cycle pattern. Several genera are of considerable biological or medical importance. The volume has seven sections (from 24 to 30) – The Planctomycetes, Chlamydia and relatives; The Spirochetes; The Fibrobacters; The Bacteroides; The Flavobacteria; The Sphingobacteria, Flexibacteria, and Cytophage; The Fusobacteria.

The organization of second edition of Bergey's Manual of Systematic Bacteriology is given in table 3.1.

Taxonomic Rank and Section	Representative Genera
Volume 1. The *Archaea*, Cyanobacteria, Phototrophs, and Deeply Branching Genera	
The *Archaea*	
Kingdom *Crenarchaeota*	
Section I—Thermoprotei, Sulfolobi, and Barophiles	*Thermoproteus, Sulfolobus*
Kingdom *Euryarchaeota*	
Section II—The Methanogens	*Methanobacterium*
Section III—The Halobacteria	*Halobacterium, Halococcus*
Section IV—The Thermoplasms	*Thermoplasma, Picrophilus*
Section V—The Thermococci	*Archaeoglobus, Thermococcus*
Deeply Branching Genera	
The Bacteria (Eubacteria)	
Section VI—*Aquifex* and Relatives	*Aquifex, Hydrogenobacter*
Section VII—Thermotogas and Geotogas	*Thermotoga, Geotoga, Thermodesulfobacterium*
Section VIII—The Deinos	*Deinococcus*
Section IX—Thermi	*Thermus, Magnetobacterium*
Section X—*Chrysiogenes*	*Chrysiogenes*
Section XI—The Chloroflexi and Herpetosiphons	*Chloroflexus, Herpetosiphon*
Section XII—Thermomicrobia	*Thermomicrobium*
Section XIII—*Prochloron* and Cyanobacteria	*Prochloron, Synechococcus, Pleurocapsa, Oscillatoria, Anabaena, Nostoc, Stigonema*
Section XIV—*Chlorobia*	*Chlorobium, Pelodictyon*
Volume 2. The Proteobacteria	
The *Bacteria*	
Kingdom *Proteobacteria*	
Section XV—The α-Proteobacteria	*Rhodospirillum, Rickettsia, Caulobacter, Rhizobium, Brucella, Nitrobacter, Methylobacterium, Beijerinckia, Hyphomicrobium*
Section XVI—The β-Proteobacteria	*Neisseria, Burkholderia, Alcaligenes, Comamonas, Nitrosomonas, Methylophilus, Thiobacillus*
Section XVII—The γ-Proteobacteria	*Chromatium, Leucothrix, Legionella, Pseudomonas, Azotobacter, Vibrio, Escherichia, Klebsiella, Proteus, Salmonella, Shigella, Yersinia, Haemophilus*
Section XVIII—The δ-Proteobacteria	*Desulfovibrio, Bdellovibrio, Myxococcus, Polyangium*
Section XIX—The ϵ-Proteobacteria	*Campylobacter, Helicobacter*
Volume 3. The Low G + C Gram Positives	
Section XX—The Clostridia and Relatives	*Clostridium, Peptostreptococcus, Eubacterium, Epulopiscium, Desulfotomaculum, Veillonella, Haloanaerobium*
Section XXI—The Mollicutes	*Mycoplasma, Ureaplasma, Spiroplasma, Acholeplasma*
Section XXII—The Bacilli and Lactobacilli	*Bacillus, Caryophanon, Paenibacillus, Thermoactinomyces, Lactobacillus, Streptococcus, Enterococcus, Listeria, Staphylococcus*
Volume 4. The High G + C Gram Positives	
Section XXIII—Class *Actinobacteria*	*Actinomyces, Micrococcus, Arthrobacter, Corynebacterium, Mycobacterium, Nocardia, Actinoplanes, Propionibacterium, Streptomyces, Thermomonospora, Frankia, Actinomadura, Bifidobacterium*
Volume 5. The Planctomycetes, Spirochaetes, Fibrobacters, Bacteroides and Fusobacteria	
Section XXIV—The Planctomycetes, Chlamydia and Relatives	*Planctomyces, Chlamydia*
Section XXV—The Spirochetes	*Spirochaeta, Borrelia, Treponema, Serpulina, Leptospira*
Section XXVI—The Fibrobacters	*Fibrobacter*
Section XXVII—The Bacteroides	*Bacteroides, Porphyromonas, Prevotella*
Section XXVIII—The Flavobacteria	*Flavobacterium*
Section XXIX—The Sphingobacteria, Flexibacteria, and *Cytophaga*	*Sphingobacterium, Flexibacter, Cytophaga*
Section XXX—The Fusobacteria	*Fusobacterium*

3.4 SUMMARY

Bergey's Manual is the principal resource in bacterial taxonomy and is used by microbiologists around the world. The Bergey's Manual of Systematic Bacteriology gives the accepted system of bacterial taxonomy. The first edition of Bergey's Manual of Systematic Bacteriology provides a primarily phonetic system of classification, and many taxa are not phylogenetically homogenous. Easily determined features such as cell shape, Gram staining characters, oxygen relationships, motility and the mode of energy production are used to classify the bacteria. But the comparisons of nucleic acid sequences, particularly 16S rRNA sequences, are the foundation of the second edition of Bergey's Manual of Systematic Bacteriology. This second edition is published in five volumes. The first volume includes the Archaea, Cyanobacteria, Phototrophs, and Deeply branching genera. Volume 2 contains the Proteobacteria. All the proteobacteria included in this volume has been divided into 5 major groups basing on rRNA sequences and other characters. The third volume encompasses the Low G + C Gram positive bacteria. Volume 4 consist of the High G + C Gram positive bacteria. And the last fifth volume comprises Planctomycetes, Spirochaetes, Fibrobacters, Bacteroids, and Fusobacteria. This volume has a variety of different Gram negative eubacterial groups.

3.5 TECHNICAL TERMS

David Bergey, Bergey's Manual of systematic Bacteriology, Archaea, Cyanobacteria, Phototrophs, Proteobacteria, Low G + C, High G + C.

3.6 SELF ASSESSMENT QUESTIONS

Q.1 Give a detailed account on Bergey's Manual of Systematic Bacteriology.
Q.2 Describe the characteristics of the five groups of proteobacteria.

3.7 SUGGESTED READINGS

1. Principles of microbiology - Ronald M. Atlas, McGraw Hill, 2nd Edition.
2. Biology of microorganisms - Thomas D. Brock, Michael P. Madigan, Prentice Hall Englewood Cliffs, 5th Edition.
3. Microbiology - Michael J. Pelczar, Jr., E.C.S.Chan, Noel R. Krieg, 5th Edition.
4. Microbiology - Lansing M. Prescott, John P. Harley, Donald A.Klein

Dr. K. Nagaraju

LESSON – 4

ULTRA STRUCTURE OF TYPICAL BACTERIAL CELL

OBJECTIVE OF THE LESSON

To understand the different structural components of a typical bacterial cell and their functional aspects to survive in different environmental habitats.

STRUCTURE OF THE LESSON

4.1 INTRODUCTION

The ultrastructure of bacteria is a typical example for the prokaryotic cell or organism that lacks the membrane bound nucleus and other complex structures. Bacteria are small and simple in structure when compared with eukaryotes, yet they often have characteristic shapes and sizes. Bacteria are one of the most important microbial groups by any criterion like numbers of organisms, general ecological importance and practical importance for humans. Indeed much of the understanding of phenomena in biochemistry and molecular biology comes from research on bacteria.

4.2 MORPHOLOGY OF BACTERIAL CELL

Typically, bacteria display three basic shapes viz., spherical, rod like and spiral but variations abound. A spherical bacterium is called a coccus. These cocci can exist as individual cells, but also are associated in characteristic arrangements that are frequently useful in bacterial identification. Different shapes of bacteria are given in Fig. 4.1.

Diplococci : arise when cocci divide and remain together to form pairs. Eg: *Neisseria*
Streptococci: long chains of cocci result when cells adhere after repeated divisions in one plane. Eg: *Streptococcus, Enterococcus, Lactococcus*

Staphylococci: form when cocci divide in random planes to generate irregular grape-like clumps. Eg: *Staphylococcus*
Tetrads : cocci divide in two planes to form square groups of four cells. Eg: *Micrococcus*
Sarcinae : cocci divide in three planes producing cubical packets of eight cells. Eg: *Sarcina*

A rodlike bacterium is called a bacillus and the typical example of this shape is *Bacillus megaterium*. Bacilli differ considerably in their length-to-width ratio and the coccobacilli (Eg: *Brucella*) are the short rods intermediate in shape between cocci and bacilli. The shape of the rod's end often varies between species and may be flat, rounded, cigar-shaped or bifurcated. Although many rods do occur singly, they may remain together after division to form pairs or chains.

A few rod shaped bacteria are curved to form distinctive comma shaped bacteria called as vibrios. Many bacteria are shaped like long rods twisted into spirals or helices. If helices are rigid then called as spirilla and if helices are flexible called as spirochetes. Some bacteria may show some rare shapes like oval-to-pear shape, square shape and star shape. Bacteria that are variable in shape and lack a single characteristic form are called as pleomorphic (Eg: *Corynebacterium*) forms. The actinomycete group of bacteria are filamentous in nature.

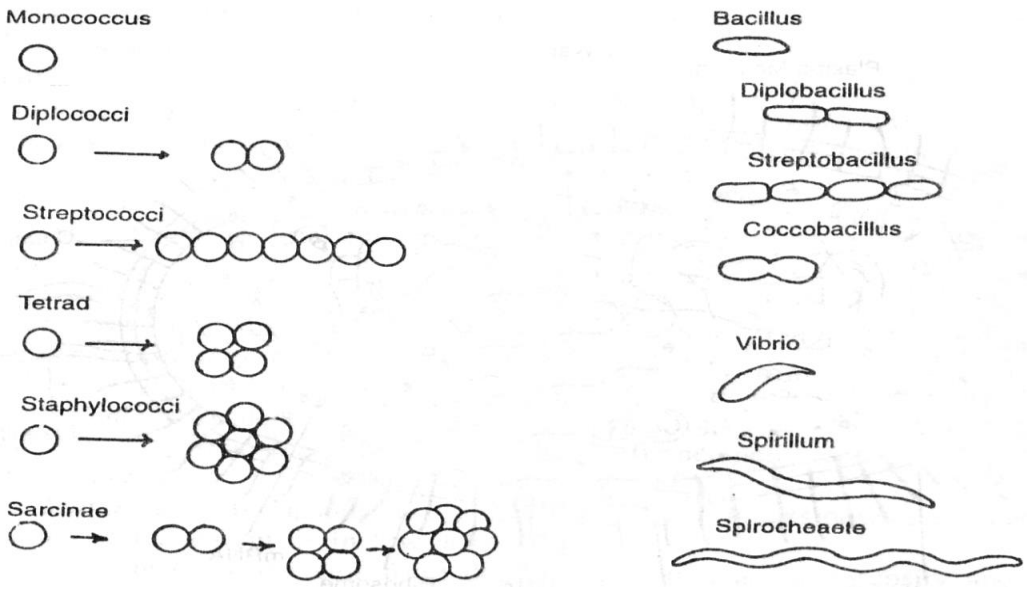


Figure-4.1: Different shapes of bacteria

Bacteria vary in size as much as in shape. The smallest (Eg: some members of *Mycoplasmas*) are about 0.3 μm in diameter, approximately the size of the largest viruses. However, the recently discovered nanobacteria or ultramicrobacteria appear to range from around 0.2 μm to less than 0.05 μm in diameter. The model bacillus bacterium, *Escherichia coli*, is of about average size of 1.1 – 1.5 μm wide by 2.0 – 6.0 μm long. A few bacteria are fairly large. For example, some spirochetes occasionally reach 500 μm in length, and the cyanobacterium *Oscillatoria* is about 7 μm in diameter. Very recently, a huge bacterium namely *Epulopiscium fishelsoni* has been discovered in the intestine of the brown surgeonfish, *Acanthurus nigrofuscus*. This bacterium gorws as large as 600 by 80 μm.

4.3 ULTRA STRUCTURE OF BACTERIAL CELL

Structurally bacterial cells consist the following—
-- Components external to the cytoplasmic membrane which include surface appendages (flagella, pili, fimbriae), glycocalyx layer and cell wall

-- Cell membrane or plasma membrane
-- Components internal to the cytoplasmic membrane

The structure of typical bacterial cell is given in Fig. 4.2 below.

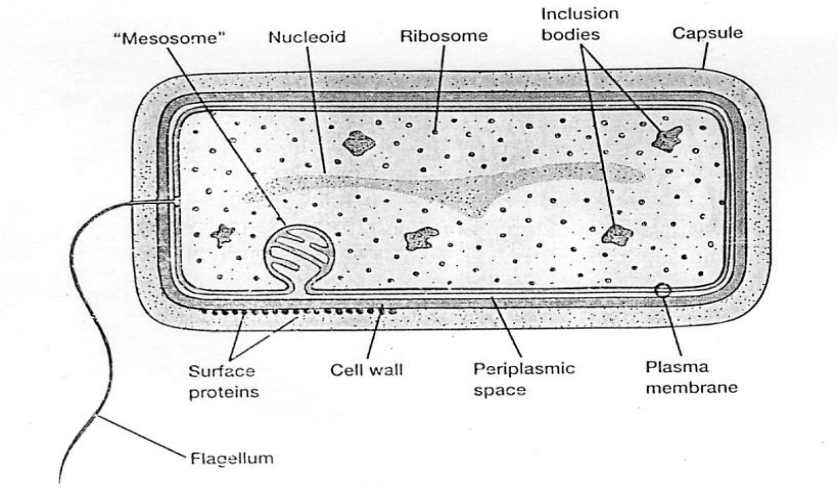


Figure-4.2: Structure of a typical bacterial cell
(Source: Microbiology – Prescott et al.)

4.3.1 Structures external to the plasma membrane

Surface appendages: The surface appendages that extend from the cell membrane through the cell wall and to the outer surface of the cell. These appendages are of two main types – appendages involve in locomotion (flagella) and appendages do not involve in locomotion (pili and fimbriae).

Flagella

About half of all known bacteria are motile and move by the use of flagella. The flagella are the long, thin, thread-like, helical, slender and rigid locomotor appendages with about 20 nm diameter and up to 15 to 20 μm length. The diameter of the bacterial flagellum is about one-tenth that of a eukaryote's flagellum. Flagella are so thin they cannot be observed directly with a bright-field microscope, but must be stained with special techniques designed to increase their thickness. The detailed structure of a flagellum (Fig. 4.3) can only be seen in the electron microscope. Bacterial species often differ distinctively in their number and pattern of distribution of flagella.

Monotrichous bacteria: bacteria with a single polar flagellum located at one end or pole Eg: *Pseudomonas*

Amphitrichous bacteria: bacteria with two flagella, one at each end Eg: *Spirillum*

Lophotrichous bacteria: bacteria with a cluster of tuft of flagella at one end or both ends Eg: *Spirillum*

Peritrichous bacteria: bacteria with many flagella spread fairly evenly all over the surface Eg: *Proteus, Salmonella*

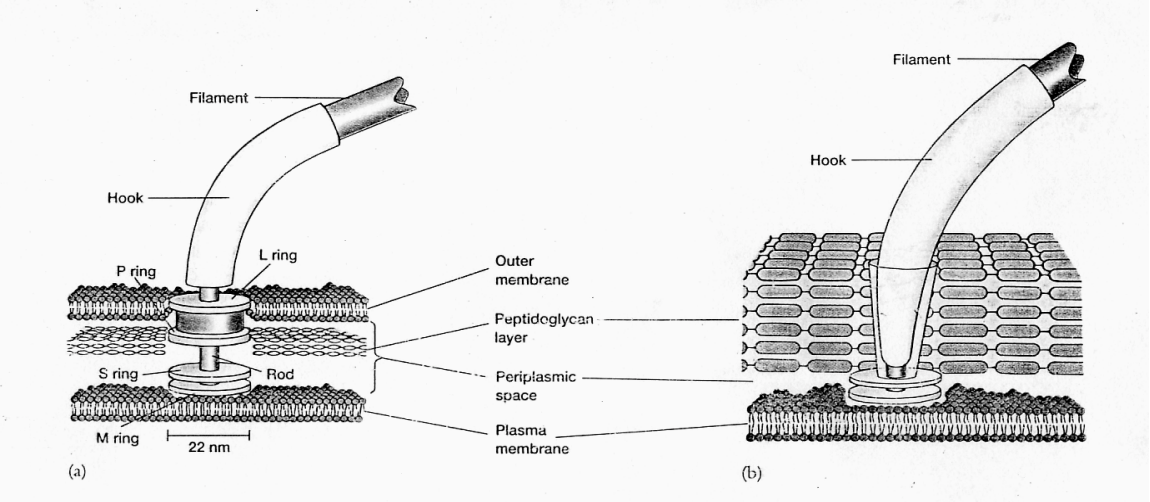


Figure-4.3: a) Gram-ve bacterial flagellum b) Gram +ve bacterial flagellum (Source: Microbiology – Prescott et al.)

The bacterial flagellum is composed of three main parts namely (i) **Filament** – the longest and most obvious portion of the flagellum that extends from the cell surface to the tip, (ii) **Basal body** – portion of the flagellum that is embedded within the cell, and (iii) **Hook** – a short, curved segment that links the filament to its basal body and acts as a flexible coupling. The filament is a hollow, rigid cylinder constructed of a single protein called flagellin which ranges in molecular weight from 30,000 to 60,000 daltons. The hook and basal body are quite different from the filament. The hook is slightly wider than the filament and is made of different protein subunits. The basal body is the most complex part of a flagellum and consists of a central rod or shaft surrounded by a set of rings. Gram –ve bacteria have two pairs of rings named as outer pair and inner pair. The inner pair of rings (S and M rings) embedded in the cell membrane and outer pair of rings (L and P rings) associated with the peptidoglycan and lipopolysaccharide layers of the cell wall. In Gram +ve bacteria, the outer pair of rings is absent and only inner pair of rings (S and M rings) is found associated with cell membrane and cell wall.

The structure of the bacterial flagellum allows it to spin like a propeller, with the basal body acting like a motor to rotate the flagellum, and thereby to propel the bacterial cell. Rotation of the flagellum requires energy which is supplied by the proton gradient across the cytoplasmic membrane. Approximately 2506 protons must cross the cytoplasmic membrane to power a single rotation of the flagellum. The flagellum can rotate at speeds of up to 1,200 revolutions per minute, thus enabling bacterial cells to move at speeds of 100 μm /second.

The direction of flagellar rotation determines the nature of bacterial movement. Monotrichous, polar flagella rotate counterclockwise during normal forward movement, whereas the cell itself rotates slowly clockwise. The rotating helical flagellar filament thrusts the cell forward with the flagellum trailing behind. Monotrichous bacteria stop and tumble randomly by reversing the direction of flagellar rotation. Peritrichously flagellated bacteria operate in a somewhat similar way. To move forward, the flagella rotate counterclockwise and during this they bend at their hooks to form a rotating bundle that propels them forward

and the bacteria run or move in a straight line. When flagella rotate clockwise, the flagellar bundle disrupts and the cell tumbles or twiddle. Both the runs and twiddles are generally random movements. Runs last an average of 1 second during which the bacteria swim about 10-20 times of its body length. Twiddles last about 0.1 second and no forward progress is made. The flagellar motion of monotrichous and peritrichous bacteria are given in Fig. 4.4.

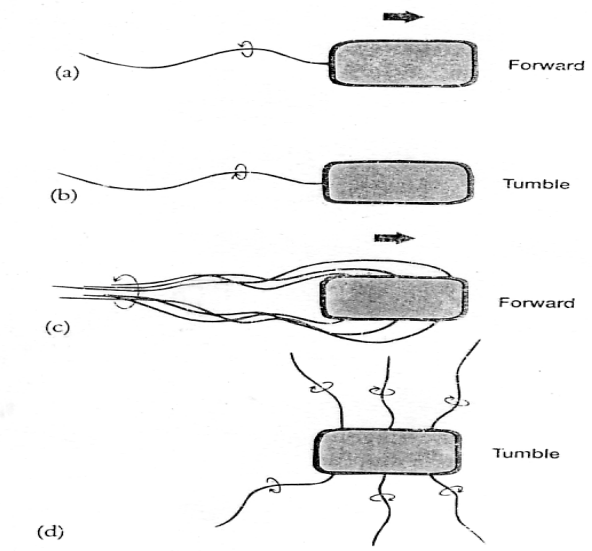


Figure- 4.4: Flagellar motility a & b – motion of monotrichous bacteria
c & d – motion of peritrichous bacteria
(Source: Microbiology – Prescott et al.)

Chemotaxis

The movement of the bacteria toward a chemical attractant or away from a chemical repellent is known as chemotaxis. This behavior is of obvious advantage to bacteria. The movement towards the attractant is referred as positive chemotaxis and the movement away from the repellent is named as negative chemotaxis. This chemotaxis behavior of bacteria is mediated by some membrane bound chemosensor proteins called as Methyl-accepting chemotaxis proteins (MCPs). The MCPs are transmembrane proteins that interact with the chemorepellents and chemoattractants on the outside the cytoplasmic membrane or indirectly with receptors in the periplasm. The MCPs alternate with methylation and demethylation events and result in the tumbling and runs, respectively.

Pili and Fimbriae

Many Gram –ve bacteria have short, fine, hair-like appendages that are thinner than flagella and not involved in motility. These are usually called fimbriae or attachment pili. These attachment pili or fimbirae help the bacteria adhere to surfaces such as cell surfaces and in the interface of water and air. They contribute to the pathogenicity of certain bacteria by enhancing the colonization on the surfaces of the cells of other organisms. These fimbriae are also responsible for the formation of pellicles or scums on the surface of the broth medium.

Sex pili or conjugation pili are the similar appendages found only in certain groups of bacteria. These pili are often larger than fimbriae with a diameter around 9 to 10 nm. These pili are made up of by specific protein subunits or monomers called as pilins. These sex pili involve in the transfer of genetic material from one bacterium to the other. Some bacterial viruses attach specifically to receptors on sex pili at the start of their reproductive cycle.

Glycocalyx: Many bacteria synthesize and secrete large amounts of viscous, organic polymer material that surrounds the bacterial cell. This slimy or gummy material is generally termed as glycocalyx. The glycocalyx may vary in its composition among different organisms but usually composed of glycoproteins and a large number of different polysaccharides. However, an exception is seen in *Bacillus anthracis* where the glycocalyx is made up of poly-D-glutamic acid which is a polypeptide. The glycocalyx may be thick or thin, rigid or flexible depending upon the chemical nature in a specific organism. If the glycocalyx forms a rigid, condensed, well defined and organized layer that tightly and closely surrounding the cell, the layer is termed as Capsule. If the glycocalyx is disorganized and loosely surround the cell, it is referred as slime layer. The capsule made up of by single kind of sugars is termed as homopolysaccharide capsule (*Streptococcus mutans*) and the capsule with more than one kind of sugars is known as heteropolysaccharide capsule (*Streptococcus pneumoniae*).

Functions of Glycocalyx layer includes—

- Helps in attachment of certain pathogenic bacteria to their hosts
- Gives protection to bacteria from phagocytosis
- Provides resistance to bacteria against desiccation
- During emergency serves as a nutrient source

Cell Wall

The cell wall is an external structure or layer that surrounds the cell membrane in all bacteria except for the mycoplasmas and some archaeobacteria. Cell wall is firm, rigid in nature and gives protection to the cell from osmotic lysis, provide solid support for flagella and maintain the characteristic shape of the organism. Cell wall accounts to 20-40% of the dry weight of the bacterial cell. The cell walls of many pathogens have components that contribute to their pathogenicity. Basing on the response to the Gram stain developed by Christian Gram in 1884, bacteria could be divided into two major groups namely Gram +ve and Gram –ve bacteria. The nature of the cell wall also contributes to this differential response to Gram stain by bacteria.

Peptidoglycan

The most important component of cell wall is the peptidoglycan of murein layer which is common to both Gram +ve and Gram –ve bacteria. In Gram +ve bacteria as many as 40 sheets of murein forms the peptidoglycan whereas in Gram –ve bacteria the number of murein sheets is usually two. Peptidoglycan is homogenous layer of 20-80 nm thickness in Gram +ve bacteria and accounts to 40-90% of cell wall dry weight but it is only 2-7 nm thickness in Gram –ve bacteria accounting to only 5-20% of total cell wall dry weight. Peptidoglycan is made of two parts, a peptide portion composed of amino acids connected by peptide linkages and a glycan or sugar portion. Structurally the peptidoglycan can be

divided into three components viz., backbone, tetrapeptide side chain and peptide interbridge or cross link.

Backbone

The glycan portion of the peptidoglycan polymer forms the backbone. This backbone is composed of alternately repeating units of the amino sugars N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) linked to each other by β 1-4 glycosidic bonds. Each strand contains 10-65 disaccharide units.

Tetrapeptide side chain

This component of peptidoglycan contains four amino acids which includes L-alanine, D-glutamic acid or its derivative, L-lysine or Diaminopimelic acid (DAP) and D-alanine. Diaminopimelic acid is found in all Gram –ve bacteria and in few Gram +ve bacteria. Most Gram +ve cocci have lysine instead of DAP. The tetrapeptide side chain is connected to the carboxyl group of N-acetylmuramic acid but not to N-acetylglucosamine residue (Fig. 4.5).

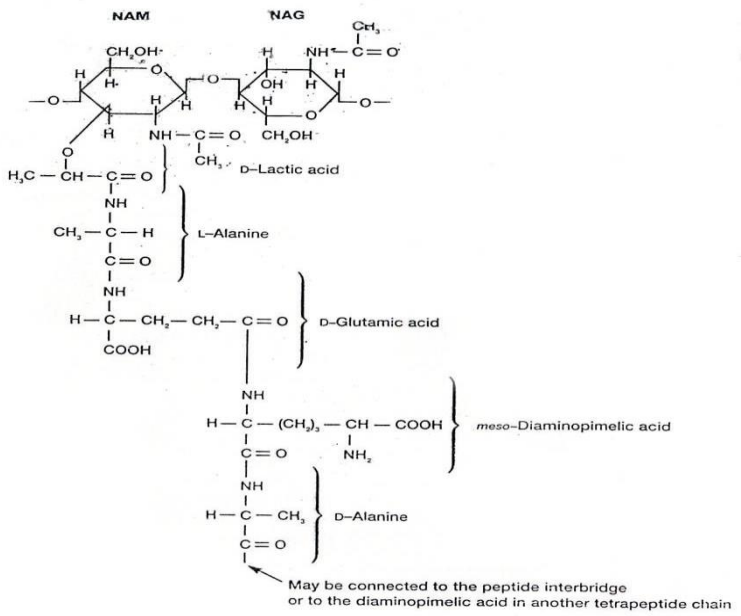


Figure-4.5: Tetrapeptide side chain
(Source: Microbiology – Prescott et al.)

Peptide interbridge or Cross-link

Two tetrapeptide side chains of two adjacent murein stands or of the same strand are connected by cross-links or inter-bridges. In Gram –ve bacteria the cross-link is direct between amino group of DAP of one tetrapeptide side chain and carboxyl group of terminal D-alanine of other tetrapeptide side chain (Fig. 4.6). In Gram +ve bacteria the cross-link is by a peptide interbridge composed of amino acids. For example, the interbridge in *Staphylococcus aureus* is composed of five glycine amino acids (Fig. 4.7).

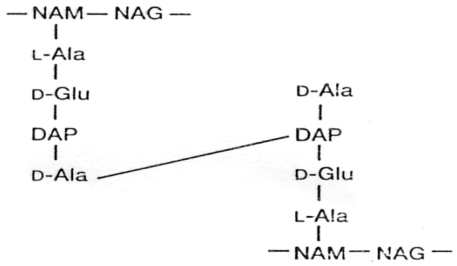


Figure-4.6: Cross linkage in Gram –negative bacteria

(Source: Microbiology – Prescott et al.)

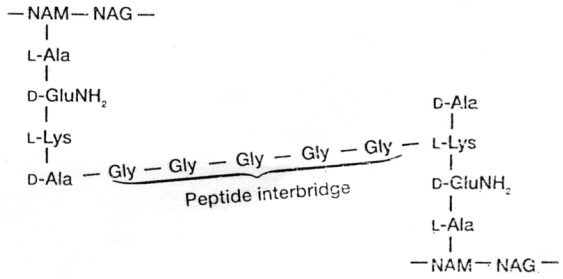


Figure-4.7: Cross linkage in Gram –positive bacteria
(Source: Microbiology – Prescott et al.)

The shape of cell depends on the lengths of the peptidoglycan chains and manner and extent of cross- linking the chains. True peptidoglycan, NAM and DAP are found exclusively in bacteria. The greatest variation in the chemical composition of the peptidoglycan occurs due to the variation in cross-linkages.

Gram-positive cell wall

The cell wall of the Gram +ve bacteria is thick, homogenous and composed primarily of peptidoglycan, which often contains a peptide interbridge. The cell walls of most Gram +ve bacteria also have teichoic acids, polymers of glycerol or ribitol joined by phosphate groups. Amino acids such as D-alanine or sugars like glucose are attached to the glycerol and ribitol groups. The teichoic acids are connected usually to the peptidoglycan itself by a covalent bond with the six hydroxyl of N-acetylmuramic acid. These teichoic acids are called as lipoteichoic acids when they are attached to lipids of plasma membrane. Teichoic acids are exclusive to Gram +ve bacteria and absent in Gram –ve bacteria. Functionally teichoic acids can bind to protons thereby maintain the cell wall at a relatively low pH which may prevent the autolysins from degrading the cell wall. Teichoic acids also bind cations such as Ca^{2+} and Mg^{2+} and act as receptor sites for some viruses. When phosphate concentrations are low, Gram +ve bacteria replace the phosphate-rich teichoic acids of the cell wall with teichuronic acids. This enables them to conserve phosphate that is essential for ATP, DNA, and other cellular components. Teichuronic acids are polysaccharide chains of uronic acids and N-acetylglucosamine, which fulfill the cell's requirement for an acidic, anionic polysaccharide in the cell wall.

Gram-negative cell wall

The Gram –ve cell wall (Fig. 4.8) is far more complex than the Gram +ve cell wall. Outside the thin peptidoglycan in Gram –ve bacteria an outer membrane is present. The most abundant membrane protein is Braun's lipoprotein, a small lipoprotein covalently joined to the underlying peptidoglycan by its protein portion and associate with the hydrophobic portion of the outer membrane by its fatty acid end. The outer membrane and peptidoglycan are so firmly linked by this lipoprotein.

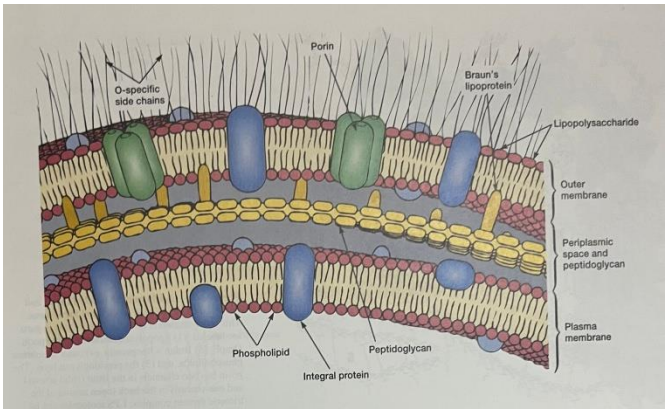


Figure-4.8: Gram-negative envelope
(Source: Microbiology – Prescott et al.)

Outer membrane

The outer membrane is the exclusive component of Gram –ve cell wall and it is a lipid bilayer containing phospholipids, proteins, lipoproteins and lipopolysaccharides. Unlike the cytoplasmic membrane, outer membrane is relatively permeable to most small molecules. The outer membrane contains lipopolysaccharides (LPS), which are not found in cytoplasmic membranes. LPS is often called as endotoxin as it may result in shock and death in some animals. LPS is a complex molecule composed of distinct regions (Fig. 4.9). The innermost portion of LPS is a lipid, called Lipid A, that anchors the LPS to the hydrophobic portion of the outer membrane. Lipid A consists of N-acetylglucosamine disaccharide linked via ester and amide bonds to unusual fatty acids such as β-hydroxymyristic acid, caproic acid and lauric acid. The toxic portion of LPS lies in the lipid A. The polysaccharide portion of the LPS, which is external to lipid A, consists of a core polysaccharide and a repeat polysaccharide called the O-antigen or O-polysaccharide. The core polysaccharide is fairly consistent fro most Gram –ve bacteria and contains glucose, galactose, N- acetylglucosamine, and unusual sugars such as the 8-carbon sugar ketodeoxyoctulosonic acid (KDO) and heptoses (7-carbon sugars). The repeat polysaccharide consists of 3-5 sugars whose sequence is repeated up to about 25 times. The O-polysaccharide typically contains glucose, galactose, rhamnose, mannose, and several dideoxy sugars such as abequose, colitose, paratose and tyvelose.

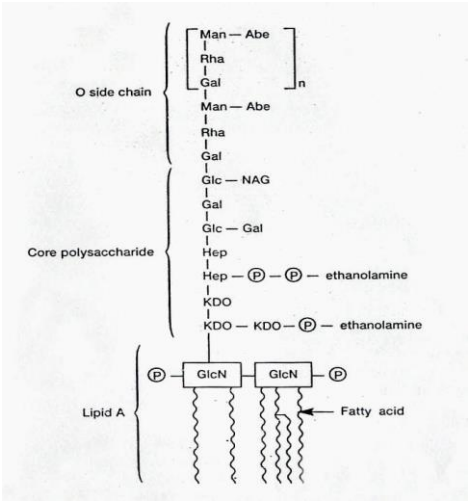


Figure-4.9: Structure of LPS
(Source: Microbiology – Prescott et al.)

The composition of the sugars and their arrangement varies from one Gram –ve bacterium to another or even from one subspecies to another. The important functions of the outer membrane are

- Acts as permeability barrier to toxic lysozyme, betalysin, leukocyte proteins
- Prevents leakage of periplasmic proteins
- Protect enteric bacteria from bile salts
- Shows –ve charge and so evade phagocytosis

The outer membrane contains some proteins which can be categorized into Porin proteins and Non- porin proteins. Porin proteins are the aggregates of three porin molecules that form cross-membrane channels through which some low molecular weight molecules can diffuse. These porin proteins may be specific, for example the maltoprotein that allows only maltose and maltodextrans and or non- specific. The non-porin proteins include the Omp A protein for anchoring and minor proteins that function in vit B_{12} transportation.

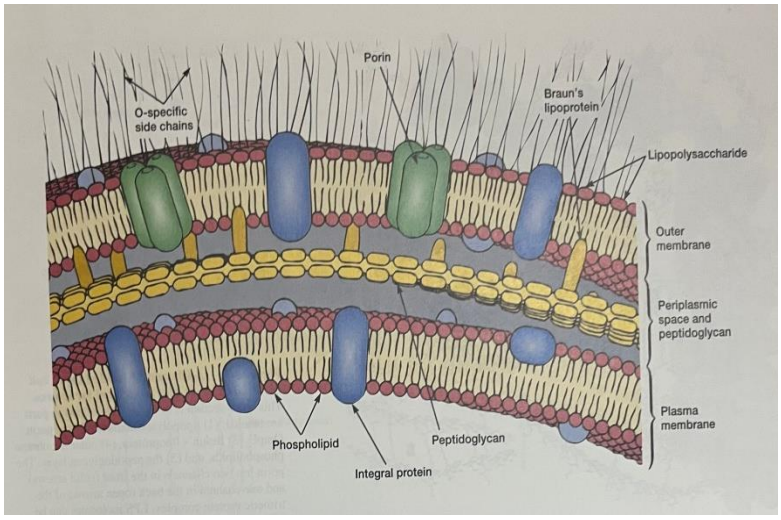
Periplasmic space

The region between the cytoplasmic and outer membranes is known as periplasmic space or periplasmic gel. The substance that occupies the periplasmic space is the periplasm. This is an important region in Gram –ve bacteria where diverse chemical reactions occur, including oxidation-reduction reaction, osmotic regulation, solute transport, protein secretion, and hydrolytic activities. Several proteins such as binding proteins, chemoreceptors, and enzymes (oxidases and dehydrogenases) are found in the periplasmic space. The binding proteins facilitate the transport of substances into the cell by delivering substances to carriers that are bound to the cytoplasmic membrane. The hydrolytic enzymes in the periplasm break down larger molecules to smaller products for easy transportation across the cytoplasmic membrane. The chemoreceptors by binding with the substances direct the cell's movement toward or away from those substances. Oligosaccharides present in the periplasmic region involves in osmoregulatory function.

An outline comparison of Gram +ve and Gram –ve bacterial cell walls:

Component	Gram-positive cell wall	Gram-negative cell wall
Peptidoglycan	Always present; occurs as a thick layer	Always present; occurs as a thin layer
Peptidoglycan Tetrapeptide	Most contain lysine in 3rd amino acid position	All contain diaminopimelic acid in 3rd amino acid position
Peptidoglycan cross linkage	Generally pentapeptide, for example, entirely glycine	Direct bonding of diamino-pimelic acid of one chain to the terminal D-alanine of another chain
Teichoic acid	Present	Absent
Teichuronic acid	Present	Absent
Lipoproteins	Absent	Present
Lipopolysaccharide (LPS)	Absent	Present
Outer membrane	Absent	Present
Periplasmic space	Absent	Present

4.3.2 Plasma membrane: The boundary layer that surrounds the cytoplasmic contents of the bacterial cell is known as plasma membrane or cell membrane. This plasma membrane constitutes 8- 15% of the cell dry weight. It is distinct from the cell wall by its shrinkage nature under high osmotic pressure. It is a critical barrier that separates the inside of the cell from its environment. Structurally it is a tri-laminar unit membrane with a thickness of 7-8 nm. In electron microscopy, membrane appears as outer and inner electron-dense layers with middle electron-transparent space. The plasma membrane structure is said to be fluid-mosaic model (Fig. 4.10) proposed by S.J. Singer and G. Nicholson.



Figure–4.10: Structure of Plasma membrane
(Source: Microbiology – Prescott et al.)

The cell membrane is largely lipoprotein in nature with about 20-30% phospholipids such as phosphatidyl ethanolamine, phosphatidyl serine and phosphatidyl choline and about 60-70%

proteins. The phospholipids are structurally asymmetric and are amphipathic with polar and non- polar ends. These phospholipids form a lipid bilayer. The lipids contain hydrophobic fatty acid groups directed inward and hydrophilic glycerol groups directed outwards and associate with water. The proteins embedded in fluid matrix of lipid are known as integral or intrinsic proteins which account to 70-80% of total proteins. These integral proteins are insoluble and cannot be removed easily. The proteins that are loosely attached to membrane are called as peripheral or extrinsic proteins which account to 20-30% and can be easily freed from membrane.

Some of the functional roles of these membrane proteins are i) Energy transformation ii) Transport of molecules iii) Protein export iv) Association of DNA with membrane v) Chemotaxis vi) Electron and proton transport vii) Penicillin-binding proteins and viii) Flagellar activity. In the absence of sterols, stability to the plasma membrane in bacteria is provided by the presence of some penta-cyclic sterol-like molecules called hopanoids. The plasma membrane invaginate inwardly here and there to form mesosomes.
The major functions of plasma membrane are

- Selective permeability and transport of solutes
- Electron transport and oxidative phosphorylation in aerobic species
- Excretion of hydrolytic exoenzymes
- Involves in biosynthesis of DNA, cell wall polymers and membrane lipids as contain the required enzymes and carrier molecules
- Involves in sensory transduction systems

4.3.3 Structures internal to the plasma membrane

Mesosomes

A simple membrane system present in the bacterial cell comprise these mesosomes. Plasma membrane of bacteria invaginate to form vesicle or tubular or lamellar structures called mesosomes. These invaginations are present in both Gram +ve bacteria and Gram –ve bacteria, but more prominent in Gram +ve bacteria. Mesosomes often found adjacent to septa or cross walls in dividing bacteria and sometimes attached to the bacterial chromosome. In some photosynthetic bacteria such as purple bacteria or nitrifying bacteria that exhibit high respiratory activity, the mesosome system is extensive and complex. The main function of this membrane system is to provide a larger membrane surface for greater metabolic activity.

Genetic material

Bacterial cells lack a membrane delimited nucleus. A single, closed, circular, double stranded DNA material is located in an irregularly shaped region called nucleiod or nuclear body or nuclear region. Nucleiod is composed of about 60% DNA, some RNA and a small amount of protein. The nucleiod can be stained with Feulgen stain. In addition to this main chromosomal DNA material, many bacteria possess extra-chromosomal material referred as plasmids. These plasmids are circular, double stranded DNA that can exist and replicate independently or integrated with chromosome. Plasmids are not necessary for growth and reproduction of the bacteria but they confer special characteristic features like drug resistant to the bacteria and thereby give new metabolic activities to their hosts.

Ribosomes

Cytoplasmic matrix of the cell is often packed with ribosomes in bacteria. Ribosomes also found loosely attached to plasma membrane. The number of ribosomes per cell may be 10,000 or more and they are the sites of protein synthesis. Mg^{2+} ions and chemical energy are required for the function of ribosomes. The ribosomes of bacteria are commonly referred as 70S and are smaller than eukaryotic ribosomes. The ribosomes are complex made up of both protein and ribonucleic acid. The 70S ribosome of bacteria is composed of two components namely 30S and 50S subunits. The smaller 30S subunit is composed of 16S rRNA of 1540 ntd length and 21 proteins. Whereas, the larger 50S subunit is composed of 23S rRNA of 2900 ntd length, 5S rRNA of 120 ntd length and 34 proteins.

Gas vacuoles

The prokaryotic organisms like cyanobacteria, purple and green photosynthetic bacteria and few aquatic forms (*Halobacterium, Thiothrix*) that exhibit a floating existence in lakes and sea produce these gas vacuoles for buoyancy. Due to the presence of these gas vesicles, organisms come to the surface waters against the gravitational pull referred to as buoyancy phenomenon. Gas vacuoles are the aggregates of gas vesicles. Each vesicle is spindle shaped, hollow but rigid with a constant diameter of 70 nm and varying lengths of 200-1000 nm. Gas vesicle is bounded by a protein layer of 2 nm thick and gives rigidity to the structure. Gas vesicles are impermeable to water and solutes, but permeable to gases. Gas vesicles lose their buoyancy by collapsing due to high hydrostatic pressure.

Chlorosomes

These chlorosomes are the pigments that are housed in a series of cigar shaped vesicles. These vesicles are arranged in a cortical layer that immediately underlies the cell membrane. Chlorosomes are the part of the photosynthetic apparatus in green photosynthetic bacteria. Chlorosomes are 50 nm wide and 100-150 nm long with an enclosed simple membrane of 3-5 nm thick.

Polyhedral bodies

Members of cyanobacteria, certain purple bacteria and chemoautotrophic bacteria possess some structures known as polyhedral bodies with granular content. These bodies are also called carboxysomes as they contain carboxydismutase, a key enzyme in CO_2 fixation process.

Magnetosomes

R.P.Blackmore, in 1975, described a remarkable group of bacteria that possess magnetotactic nature. The organelles responsible for this property are termed as magnetosomes. When the organisms are placed in a magnetic field as weak as 0.2 guass, they orient and swim towards one or another of the magnetic poles due to the presence of these magnetic power sensing organelles. The magnetosomes are uniformly shaped, enveloped with magnetite (Fe_3O_4) crystals. These magnetosomes are best seen in *Aquaspirillum magnetotacticum.*

Inclusion bodies

In prokaryotic cells, a variety of cellular reserve materials or granules or inclusion bodies are

seen. The nature of these inclusion bodies may differ in different organisms but almost always function in the storage of energy or serve as structural building blocks. These inclusion bodies are either organic or inorganic in nature and present in cytoplasmic matrix. PHB granules and glycogen granules are important organic forms and phosphate granules and sulphur granules are the important inorganic forms.

Poly β-hydroxybutyric acid (PHB) granules

PHB are the most common inclusion bodies in prokaryotic cells. PHB is a lipid-like compound containing a number of monomeric β- hydroxybutyric acid units. These monomeric units are linked by ester bonds between the carboxyl and hydroxyl groups of adjacent units to form poly- β-hydroxybutyric acid molecule. These polymers aggregate together to form PHB granules. These granules can be stained with sudan black. PHB granules serve as a storage depot for carbon and energy.

Glycogen granules

These are starch-like polymer granules with glucose subunits. Long chains of this polymer is formed by the α-(1-4) glycosidic bonds between adjacent glucose units and the branched chains connect to long chains by α-(1-6) glycosidic bonds. These polymeric molecules aggregate together to form the glycogen granules. These granules are smaller than PHB granules. Glycogen granules are evenly dispersed throughout the cytoplasmic matrix and can be stained to reddish-brown colour with iodine. Glycogen granules serve as carbon storage reservoirs.

Polyphosphate granules

Many bacteria that grow in phosphate rich environments accumulate phosphate as polyphosphate granules. Polyphosphates are linear polymers of orthophosphates joined by ester bonds. Aggregation of these polymers form the polyphosphate granules which function as storage reservoirs for phosphate, an important component of cell constituents such as nucleic acids. Volutin granules is the other name for these polyphosphate granules. They can be stained with either toulidine blue or methylene blue. During the staining, as they exhibit metachromatic effect (color change effect) they are also be called as metachromatic granules.

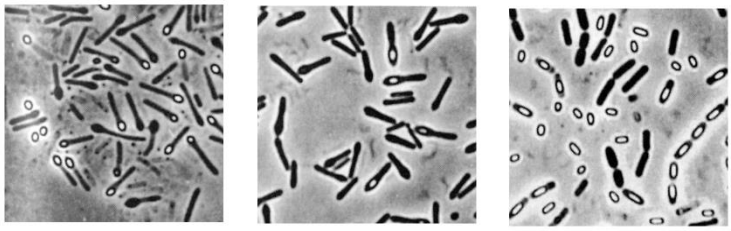
Sulphur granules

A variety of bacteria like purple photosynthetic bacteria are capable of oxidizing the reduced sulphur compounds such as H_2S, thiosulphate and accumulate the resulting elemental sulphur in cells in the form of granules. During the reactions of energy metabolism or biosynthesis, elemental sulphur frequently accumulates inside the cell in large readily visible granules. These granules of elemental sulphur remain in cells as long as the source of reduced sulphur is available. When the reduced sulphur source becomes limiting, the sulphur in granules is oxidized to sulphate and the granules ultimately disappear.

4.4 SPORULATION IN BACTERIA

During the unfavourable environmental conditions, some Gram-positive bacteria such as *Bacillus, Clostridium* and few others produce a special resistant, dormant structure called an

endospore. These endospores are extraordinarily resistant to environmental stresses such as heat, ultraviolet radiation, chemical disinfectants and desiccation. They can remain viable for a number of years. Formation of spore from the vegetative cell is known as sporogenesis or sporulation. Spore position in the mother cell frequently differs among species. Spores may be centrally located, close to one end (sub- terminal) or definitely terminal (Fig. 4.11).



Figure–4.11: Terminal, Sub-terminal and centrally located spores
(Source: Microbiology – Prescott et al.)

The spore (Fig. 4.12) is often surrounded by a thin, delicate covering called the exosporium. A spore coat lies beneath the exosporium, is composed of several protein layers and may be fairly thick. It is impermeable and responsible for the spore's resistance to chemicals. The cortex, which may occupy as much as half of spore volume, rests beneath the spore coat. It is made of a peptidoglycan that is less cross-linked than that in vegetative cells. The spore cell wall or core wall is inside the cortex and surrounds the protoplast or core. The core has the normal cell structures such as ribosomes and a nucleoid. The dipicolinic acid forming complex with calcium ions in the core is believed to responsible for the heat resistance of endospores. When the environmental conditions become more favorable, endospores germinate into vegetative cells.

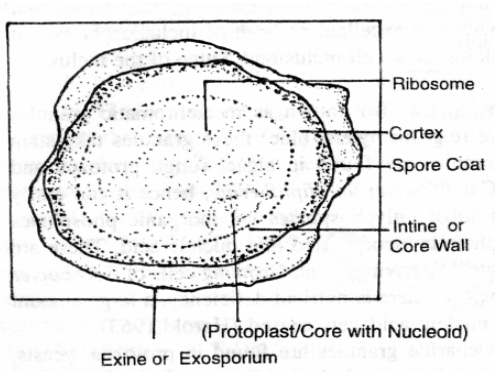


Figure-4.12: Structure of bacterial endospore
(Source: Microbiology – Prescott et al.)

4.5 SUMMARY

Bacteria may be spherical, rod-shaped, spiral or filamentous in shape. Some form buds and stalks, and some are pleomorphic without any characteristic shape. Bacterial cells can remain together after division to from pairs, chains, and clusters of various sizes and shapes. All bacteria are prokaryotes and structurally much simpler than eukaryotes. The plasma membrane and most other membranes are composed of a lipid bilayer in which integral

proteins are buried. Peripheral proteins are more loosely attached to membranes. The plasma membrane may invaginate to form some simple structures such as membrane systems containing photosynthetic and respiratory assemblies and possibly mesosomes. The cytoplasmic matrix contains inclusion bodies and ribosomes. The genetic material is located in an area called the nucleoid and it is not enclosed by a membrane.

Most bacteria have a cell wall outside the plasma membrane to give them shape and protect from osmotic lysis. Bacterial walls are chemically complex and usually contain peptidoglycan or murein. Bacteria often are classified as either Gram-positive or Gram-negative based on differences in cell wall structure and their response to Gram staining. Gram +ve walls have thick, homogenous layers of peptidoglycan and teichoic acids. Gram –ve bacteria have a thin peptidoglycan layer surrounded by a complex outer membrane containing lipopolysaccharides and other components. Structures such as capsules, fimbriae, and sex pili are found outside the cell wall. Many bacteria are motile, usually by means of threadlike locomotory organelles called flagella. Bacterial species differ in the number and distribution of their flagella. The flagellar filament is a rigid helix and rotates like a propeller to push the bacterium through the water. Motile bacteria can respond to gradients of attractants and repellents by a phenomenon known as chemotaxis. Some bacteria survive adverse environmental conditions by forming endospores, dormant structures resistant to heat, desiccation and many chemicals.

4.6 TECHNICAL TERMS

Bacterial cell, Gram +ve, Gram –ve, Flagella, Cell wall, Peptidoglycan, Cell membrane, Genetic material, Ribosomes, Chlorosomes, Gas vacuoles, Magnetosomes, Inclusion granules.

4.7 SELF ASSESSMENT QUESTIONS

Q-1. Give an account on ultra structure of the bacterial cell.

Q-2. Describe the physical and chemical structure of cell wall in Gram-positive and Gram-negative bacteria.

Q-3. Compare the characters of prokaryotic cell and eukaryotic cell.

4.8 SUGGESTED READINGS

1. Microbiology - Prescott, L.M., Harley, J.P., and Klein, D.A. (4th edition) 1999 – WCB McGraw-Hill Publishers
2. Brock Biology of Microorganisms - Michael T. Madigan, John M. Martinko and Jack Parker (8th edition) 1997 – Prentice Hall International, Inc.
3. Principles of Microbiology - Ronald M. Atlas (2nd edition) 1997 – WCB McGraw-Hill Publishers
4. The Physiology and Biochemistry of Prokaryotes - David White 1995 – Oxford Univ. Press
5. Microbiology-Principles and Explorations - Jacquelyn G. Black (4th edition) 1999 – Prentice Hall International, Inc.
6. Microbiology - M.J. Pelczar Jr., E.C.S.Chan and Noel R. Krieg (5th edition) 1995 – Tata McGraw Hill Publishing Company Ltd.

Prof. V.Umamaheswara Rao

LESSON - 5

STERILIZATION METHODS TO CONTROL BACTERIAL GROWTH

OBJECTIVE OF THE LESSON

To study about the various physical and chemical sterilization techniques that are used for complete elimination of microorganisms.

STRUCTURE OF THE LESSON

5.1 INTRODUCTION

Sterilization is defined as a process of complete elimination or killing of all microorganisms including their spores and making the material free from them. According to sterilization aspect, the term control is used to refer the reduction in numbers and activity of microflora. The importance involved in controlling the microorganisms is to prevent transmission of disease and infection, to prevent contamination, to inhibit the growth of undesirable microorganisms and to prevent deterioration and spoilage of materials by microorganisms. In the process of sterilization all living cells, spores, viruses and viroids are killed, inactivated or removed from specific object or environment. For obtaining pure cultures of microorganisms, sterilization of culture media and other instruments is essential. Sterilization can be accomplished by various physical and chemical procedures.

5.2 PHYSICAL METHODS

Several physical agents can be used to control microbial populations, such as high temperature and ionizing radiations, which kill microorganisms by damaging essential cell components. These physical agents often disrupt enzymes, DNA and Cytoplasmic membranes. Other physical treatments such as filtration remove microorganisms without

killing them. The physical treatments are widely used in microbiology laboratories for sterilization of culture media and lab ware. They are also used in medicine and dentistry for sterilization of instruments and materials contaminated with microorganisms. The sterilization by physical methods which involves the exposure of the material to physical agents those are lethal to microorganisms. Different physical methods can be divided mainly into 3 heads viz., (1) Sterilization by heat, (2) Sterilization by radiation and (3) Sterilization by filtration.

Sterilization by heat

Heat is the most often employed as it is the simplest and most reliable means of sterilization. Heat can be applied in two different forms for sterilization purpose. They are (i) Dry heat and (ii) Moist heat. Of these two, sterilization by dry heat requires greater duration and intensity, because heat conduction is less rapid in dry air than in moist air. Moist heat sterilization is more effective than dry heat, especially at lower temperatures in a given time.

Dry Heat

Dry heat sterilization is the general application in laboratories mainly for the sterilization of glass materials, which are used in laboratory. The penetrating power of dry heat is less and hence it requires more time and high temperature when compared to moist heat. The principle involved in the dry heat is denaturation of proteins. Dry heat can be applied for sterilization purpose in different ways –

Flaming: The most rapid sterilization method is "Flaming method". The flame of the Bunsen burner is employed to sterilize the bacterial loop before removing the sample from the culture tube and after preparing the smear. Small articles like inoculating loops, points of forceps, scalpels, spatulas and glass rods are sterilized by holding them in a burner flame, till they become red hot. The mouth of the test tube, flasks and other containers are also routinely passed through the flame of a Bunsen burner to destroy the microorganisms. This method however prevents some bacteria but it is unsatisfactory.

Incineration: It is a method of destruction of microorganisms by simple burning into ashes. This is an excellent method used for the destruction of materials like dressings, carcasses; infected laboratory animals, beddings and clothing of patients having contagious infection.

Hot air oven sterilization: The dry heat or hot-air sterilization is carried out in an electrical appliance called as Hot-air Oven (Fig. 5.1). The oven is usually heated by electricity and has a thermostat that maintains the chamber air constantly at the chosen temperature. The dry heat is believed to kill microorganisms by promoting a destructive oxidation of essential cell constituents. Spores of *Bacillus subtilis* are more resistant to dry heat process. This bacterium is used as a biological indicator for evaluating the efficiency of hot air oven for sterility. Dry heat sterilization is employed mainly for glassware like test tubes, Petri dishes, flasks, pipettes and instruments such as forceps, scalpels, scissors, syringes, metal instruments and paper-wrapped goods which are not spoiled by the high temperature and required to be dry. It is also used for anhydrous fats, oils and powders which are impermeable to moisture and thus are incapable of sterilization by moist heat. The glassware should be perfectly dry before the sterilization in hot-air oven, because the wet glassware is liable to crack. The materials such as rubber washers, corks are not suitable for sterilization by dry heat. The normal duration required for dry heat sterilization is 2 hours at 160^{o} C or 60 minutes at 170^{o} C temperature. However, these high temperatures cause charring of paper, cotton and other organic materials.

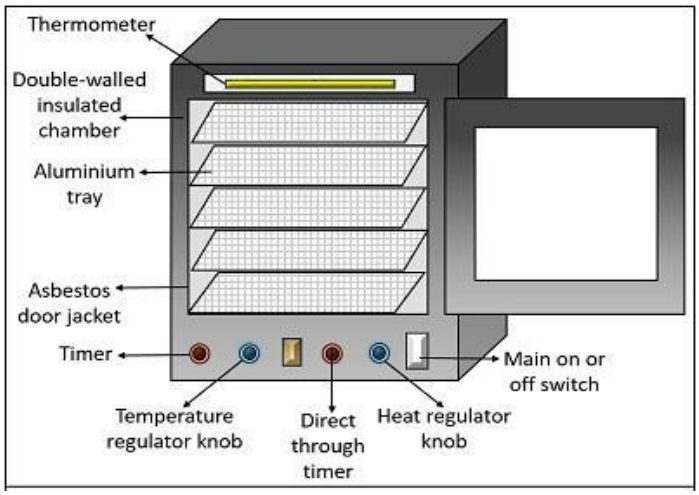


Figure-5.1: Hot-air Oven

Moist heat

The bactericidal action of moist heat was first demonstrated and used by "Robert Koch". Moist heat is more effective than dry heat. The penetrating power of moist heat is more than dry heat. The time required for sterilization by using moist heat is less when compared with dry heat. The temperatures required for sterilization by using moist heat are also less than dry heat. The moist heat kills microorganisms by coagulating or denaturing their proteins and it is much more effective than dry heat.

Sterilization using moist heat can be achieved at different temperatures

a) Temperature below 100^0C
b) Temperature at 100^0C
c) Temperature above 100^0C

a) Temperature below 100^0C

Pasteurization

It is a mild heating process employing temperatures below 100^0C to destroy spoilage causing organisms and other types of pathogens. This process was discovered by "Louis Pasteur"; who discovered the technique of heating food without altering its composition. In 1860s, the French wine industry was plagued by the problem of wine spoilage, which made wine storage and shipping difficult. Pasteur examined the spoiled wine under microscope and detected microorganisms that looked like the bacteria responsible for lactic acid and acetic acid fermentations. He then discovered that a brief heating at 55 to 60^o C temperature would destroy these microorganisms and preserve wine for long periods. Pasteurization does not sterilize a beverage, but it does kill any pathogens present and drastically slows spoilage by reducing the level of nonpathogenic spoilage microorganisms. Later, the same pasteurization technique was adapted for preserving the milk. The milk can be pasteurized in different ways.

Low temperature long time (LTLT) method
High temperature short time (HTST) method
Ultra high temperature (UHT) method

Low temperature long time method: In this method, milk is sterilized at a temperature of 63^oC for 30 minutes.

High temperature short time method: This method is also known as flash pasteurization. In this method, milk is subjected to quick heating at the temperature of 71.2^{o}C for 15

seconds followed by rapid cooling.

Ultra high temperature method: In this method, milk and milk products are heated at 140 to 150^{o} C temperature for 1 to 3 seconds. UHT-processed milk does not require refrigeration and can be stored at room temperature for about 2 months without any changes in flavor.

b) Temperature at 100^{0}C

Boiling

Boiling in water is a simple method of sterilization. Contaminated materials (or) objects exposed to boiling water cannot be sterilized with certainty. All vegetative cells will be destroyed within minutes by exposure to boiling water. But some bacterial spores can with stand this condition for many hours. The practice of exposing instruments for short periods of time in boiling water is more likely to bring about disinfection i.e., destruction of vegetative cells of disease producing microorganisms rather than sterilization. Sterilization by boiling can be enhanced by the addition of 2% sodium bicarbonate to the water. Boiling water cannot be used in laboratory as a method of sterilization.

Tyndallization (or) Fractional steam sterilization

Some microbiological media, solutions of chemicals and biological materials cannot be heated above 100^{o} C without being damaged. Then the alternative method applied to sterilize the materials is "tyndallization". This method involves the heating of material at 90 to 100^{o} C for 30 minutes on each of three consecutive days with incubation at 37^{o} C in between. The first heating will destroy all the vegetative cells of the microorganisms, but not the bacterial endospores. The resistant spores germinate during the incubation period and on subsequent exposure to heat, the vegetative cells will be destroyed.

c) Temperature above 100^{0}C (or) Autoclave

This is also called as steam heat sterilization. Heat in the form of saturated steam under pressure is the most practical and dependable agent for sterilization than hot air. Steam under pressure provides temperatures above those obtainable by boiling. Moist heat kills microorganisms by coagulating and denaturing the enzymes and structural proteins, a process in which water participates. The laboratory apparatus designed to use steam under regulated pressure is called an autoclave. The process of sterilization of materials in autoclave is called as autoclaving.

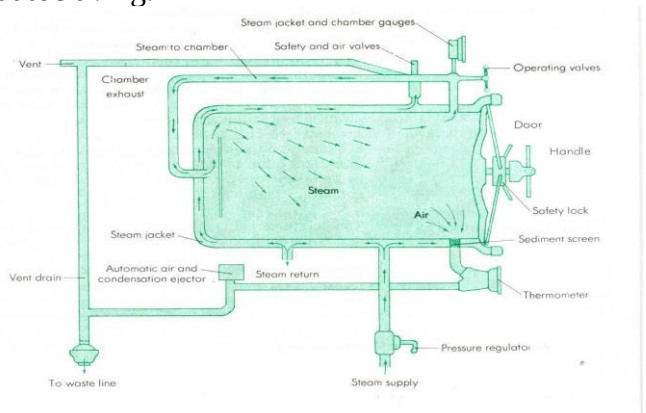


Figure-3.1: Autoclave unit

(Source: Microbiology – Pelczar et. al.)

Autoclave is essentially a double-jacketed steam chamber. It is usually equipped with devices for the complete discharge of air from the chamber and filling the chamber with saturated steam. It device also consist options to maintain required temperature and pressure for any period of time. When the chamber is completely filled with steam, without any air, it attains

121^{o} C temperature and 15lbs pressure. If air is present in the chamber, it reduces the temperature even at 15lbs pressure. Temperature of the steam is that kills the organisms but not the pressure. This steam sterilization is especially suitable for culture media and aqueous solutions, since the atmosphere of the steam prevents the loss of water by evaporation during heating. In autoclave, small volumes of liquids need 20 minutes sterilization and larger volumes require more time. Even the more resistant spores can be killed within 15-30 minutes by autoclaving process.

Sterilization by Filtration

Fluids and gases that cannot be sterilized by heat (or) by chemicals may be sterilized by allowing them to pass through specially constructed filters; without altering their physical and chemical properties. This process is generally used for the sterilization of serum, bacterial toxins, cell extracts, enzymatic solutions, antibiotics and carbohydrate solutions etc.

This procedure involves a simple passage of liquid to be sterilized through filters having small pores. These small pores retain the bacterial cells back in the filters. The nature of the fluid, pore size of the filter, charge of filter can influence the efficiency of filtration. There are various types of filters made up of by different materials. Mainly there are two broad categories of filters viz., Depth filters and Membrane filters.

Depth filters

These filters are fibrous sheets (or) mats made from paper, asbestos (or) glass fibers constructed at a random array of overlapping fibers into a thick layer filled with twisting channels of small diameter. The solution containing microorganisms is sucked through this layer under vacuum, and microbial cells are removed by physical screening or entrapment and also adsorption to the surface of the filter material. During filtration particles get trapped in the paths created throughout the depth. The common filters that are used in laboratory are:

1) Pasteur-Chamberland filters made up of porcelain material.
2) Berkefeld filters made up of diatomaceous material.
3) Seitz filters made up of asbestos material.
4) Sintered glass filters made up of ground glass material.

Membrane filters

These are the new type of filters which are also called as molecular filters which replaced the depth filters for many purposes. These are the circular, porous membranes, a little over 0.1mm thick, and generally made of cellulose acetate, cellulose nitrate, polycarbonate, polyvinylidene fluoride or any other synthetic materials. Although a wide variety of pore sizes are available, membranes with pores about 0.2 μm in diameter are used to remove vegetative cells from solutions ranging in volume from 1ml to many liters. The membranes are held in special holders and often preceded by depth filters made of glass fibers to remove larger particles that might clog the membrane filters. The solution is pulled or forced through the filter with a vacuum or with pressure and collected in previously sterilized containers. They are extensively used in laboratories and in industries to sterilize pharmaceuticals, culture media, oils, antibiotics, and other heat-sensitive fluid materials.

Air also can be sterilized by filtration. The development of “HEPA” (High Efficiency Particulate Air) filters has made it possible to deliver clean air to an enclosure such as a cubicle (or) a room. These HEPA filters which remove 0.3 μm diameter particles with an

efficiency of 99.97% are one of the most important air filtration systems. The best example is the "Laminar airflow", which is a biological safety cabinet, used to produce dust and bacteria free air. They are usually employed in research laboratories and industries, such as pharmaceutical industry, when a sterile working place is needed for conducting assays, media preparation, examination of tissue cultures, etc.

Sterilization by Radiation

Materials like gasses and liquids, which are heat labile, also can be sterilized by employing different types of radiation. Sometimes the sterilization by radiation is also referred to as 'cold sterilization'. There are two main types of radiation viz., (i) non-ionizing radiation, and (ii) ionizing radiation.

Sterilization by using non-ionizing radiation

Ultraviolet light is the best example for non-ionizing radiation. The UV light rays with 290-220 nm wavelengths are lethal to various microorganisms. But the rays with wavelength of 253.7 nm are the most effective radiation. Though lethal to microorganisms, UV rays exhibit very little ability of penetration through glass, dirt film, water, and other substances. So, only the microbes on the surfaces of an object which are directly exposed to UV rays are susceptible to destruction. Because of this disadvantage, UV radiation is used as a sterilizing agent only in a few specific situations. UV lamps are sometimes placed on the ceilings of rooms or in biological safety cabinets to sterilize the air and any exposed surfaces. Because UV radiation burs the skin and damage eyes, people working in such areas must be careful and certain of switching off the UV lamps while working in those areas. UV light is mostly absorbed by nucleic acid, in which it forms pyrimidine dimers and ultimately causes damage to the cell.

Sterilization by using ionizing radiation

X-rays, γ-rays and cathode rays are included in the category of ionizing radiation. Ionizing radiation is a good sterilization agent with greater penetration but the technique is very expensive. Bacterial spores are more resistant and Gram-negative bacteria are more sensitive to ionizing radiations. X-rays are lethal to microorganisms as well as higher forms of life. Gamma rays are also the best examples of ionizing radiation. Gamma radiation from cobalt 60 source is used in the cold sterilization of antibiotics, hormones, sutures, and plastic disposable supplies such as syringes. Also has been used to sterilize and pasteurize meat and other foods. This radiation causes damage to cells by producing hyper reactive ions. However, at higher doses, complete sterilization is difficult without causing color and flavour changes in the material. Hence, this method is ideal and commonly used for the sterilization of disposable materials made of plastic, wood, cotton etc.

Chemical Sterilization

Disinfection is the process of killing, inhibition (or) removal of microorganisms that may cause disease. Though objects are sometimes disinfected with physical agents, chemicals are more often employed in disinfection and antisepsis. The chemical agents that are used for this disinfection are called as chemical disinfectants. Disinfectants are potent and toxic in destroying pathogenic microorganisms but not necessarily in killing the resistant spores. Disinfectants are suitable for applications on inanimate objects. Many factors such as the kinds of microorganisms potentially present, the concentration and nature of the disinfectant used, length of the treatment given influence the effectiveness of chemical disinfectants and antiseptics. Dirty surfaces must be cleaned before a disinfectant or antiseptic is applied. Proper use of chemical agents is essential in hospitals and laboratories for safety purpose.

There are several chemicals which are often employed in disinfection process. The major disinfectants that are commonly used include (1) Phenol and phenolic compounds, (2) Alcohols, (3) Halogens, (4) Heavy metals, (5) Dyes (6) Detergents (7) Aldehydes, and (8) Gaseous agents.

Phenol and Phenolic Compounds

Phenol is the first disinfectant used by "Joseph Lister" in 1880's. Phenol and phenolic compounds are very effective disinfectants. A 5% aqueous solution of phenol rapidly kills the vegetative cells of microorganisms. Phenol and phenolic compounds such as bisphenols, cresols, xylenols, orthophenyl phenols are used as disinfectants in most of the laboratories and hospitals. Lysol is a commercial disinfectant prepared by a mixture of phenolics. Depending on the concentration in which they are used, phenolic substances are either bactericidal or bacteriostatic. Some phenolics are highly fungicidal. Bacterial spores and viruses are more resistant than vegetative cells. Phenols and phenolic compounds by their actions inactivate and denature the enzymes, proteins and ultimately disrupt the cell membrane to irreversible state.

Alcohols

Alcohols are one of the most widely used disinfectants. They are bactericidal, fungicidal but not sporicidal. The most widely used and popular disinfectants are ethyl alcohol or ethanol and isopropyl alcohol isopropanol. Ethyl alcohol in concentration between 50-90% is effective against vegetative and non-spore forming cells. For practical application generally 70% ethyl alcohol is used. The higher alcohols like propylalcohol, butyl alcohol and amyl alcohol and others are more germicidal than ethyl alcohol. Alcohols are effective in reducing the microbial flora of skin and for the disinfection of clinical oral thermometers. In laboratories, they are used as common disinfectants. Alcohols act as disinfectants by denaturing proteins, inactivating the enzymes and dissolving the membrane lipids.

Halogens

Chlorine and iodine are the important and most generally used disinfectants than the other halogens. Chlorine is an important and excellent disinfectant, and it may be applied as chlorine gas, sodium hypochlorite or calcium hypochlorite. It is generally used in municipal waters, swimming pools, dairy and food industries. Using the halogen tablets can successfully disinfect small quantities of water, which slowly releases chlorine when added to water. The antimicrobial action of chlorine and its derivatives is due to the formation of hypochlorous acid. It causes the oxidation of proteins and enzymes and thereby destroys the vegetative cells.

Iodine is one of the oldest and most effective disinfectants. At higher concentrations it may even kill some spores. Iodine is normally applied in the form of tincture of iodine and iodophores. Iodophores are the preparatives of iodine, complexed with some organic carriers. They are used in hospitals for pre-operative skin damaging. The mode of action of iodine and its derivatives is due to the inactivation of essential metabolic compounds such as proteins with sulfhydryl groups as they are highly oxidizing agents.

Heavy Metals

The ions of heavy metals like mercury, zinc, arsenic, silver and copper are used as disinfectants. They are used either alone or in combination with certain compounds. The most effective heavy metals are mercury, silver and copper. These heavy metals and their compounds act by interacting with cellular proteins and inactivating them. Higher concentrations of mercury, copper and silver coagulate cytoplasmic proteins and result in

the damage or death of the cells. Silver nitrate solution of 1% concentration is often used to prevent ophthalmic gonorrhea in infants. Silver sulfadiazine is used on burns. Copper sulfate is an effective algicide in lakes and swimming pools.

Dyes

Two classes of dye compounds are used as disinfectants. They are (i) Triphenylmethane dyes and (ii) Acridine dyes.

Triphenylmethane Dyes

The best dyes of this category are malachite green, brilliant green and crystal violet. Gram positive organisms are more susceptible to lower concentration of these compounds than Gram negative ones. Crystal violet can be used as bactericide as well as fungicide. The concentrations of these dyes to inhibit the organisms vary from one organism to another. These dyes interfere with cellular oxidation processes and inhibit the growth of the organisms.

Acridine dyes

Acriflavin and tryptoflavin are the two best examples of acridine dyes. These compounds exhibit selective inhibition against bacteria, particularly staphylococci and gonococci. They are used to some extent for the treatment of burns and wounds and for ophthalmic application. However, their application is reduced at present due to some side effects.

Detergents

Detergents are the organic molecules that serve as wetting agents and emulsifiers because they have both polar hydrophilic and non-polar hydrophobic ends. Due to their amphipathic nature, detergents solubilize otherwise insoluble residues and are very effective cleansing agents. The most common example is soap. But the soap is a poor detergent in hard waters. The synthetic detergents called as “surfactants” are the more efficient cleaning agents. They are extensively used in laundry and dish washing powders, shampoos etc. Chemically, detergents are mainly of three types.

1) Anionic detergents
2) Cationic detergents
3) Non-ionic detergents

Though anionic detergents have some antimicrobial properties, only cationic detergents are the effective disinfectants. The most popular of these disinfectants are quaternary ammonium compounds which act as germicides and characterized by positively charged quaternary nitrogen and a long hydrophobic aliphatic chain. They disrupt microbial membranes and may also denature proteins. The cationic detergents are widely used as skin disinfectants as a preservative in ophthalmic solutions and in preparation of cosmetics. These are also used in hospitals, nursing homes, hotels, and at other public places to sanitize the food, utensils and places etc. Cationic detergents like benzalkonium chloride (trade name – Zephiran) and cetylpyridinium chloride (trade name – Ceepryn) kill most bacteria but not the endospores. They do have the advantages of being stable, non-toxic but they are inactivated by hard water. The non-ionic detergents are not significant in disinfectant activity.

Aldehydes

Several aldehydes having low molecular weight are used as disinfectants. The two important and widely used aldehydes are formaldehyde and glutaraldehyde. Aldehydes are very reactive molecules and act as microbicidal as well as sporicidal. They combine with proteins

and inactivate them. Formaldehyde is the simplest compound of aldehydes. It is usually dissolved in water or alcohol before used. The fumes of formaldehyde are irritating to tissues and eyes, and used to disinfect the closed areas. Formalin is one of the aqueous solutions available commercially in the market. Glutaraldehyde is as an effective disinfectant and used as 2% buffered solution. It causes less irritation than formaldehyde. It exhibits wide spectrum of antimicrobial activity against bacteria, fungi, spores and viruses. Glutaraldehydes disinfect the objects within ten minutes of time. It is used for sterilizing biological instruments, lensed instruments, and respiratory therapy equipment in medical fields.

Gaseous Agents

Many heat sensitive surgical instruments and disposable Petri dishes are disinfected by using certain gases. Ethylene oxide (EtO) and Betapropiolactone (BPL) are the two best sterilizing gases. Ethylene oxide is both microbicidal and sporicidal. It is so effective, as it penetrates rapidly through packing materials and through plastic wrappers also. Sterilization by EtO is carried out in a special ethylene oxide sterilizer, that resembles the autoclave in appearance, that controls the EtO concentration, temperature, and humidity. As pure EtO is explosive, it is usually supplied in a 10 to 20% concentration mixed with either CO_2 or dichlorodifluoromethane. The ethylene oxide concentration, humidity, and temperature influence the rate of sterilization. Ethylene oxide combines with organic compounds such as enzymes and proteins and inactivates them by alkylation reactions. Removal of residual ethylene oxide is necessary as it is so toxic. Extensive aeration of sterilized materials removes the residual of ethylene oxide.

Betapropiolactone is occasionally employed as sterilizing gas. In liquid form, it is used to sterilize vaccines and sera. It destroys microorganisms more rapidly than ethylene oxide, but the penetration power is less than ethylene oxide. BPL decomposes to an inactive form after several hours and is therefore not as difficult to eliminate as EtO. It also destroys microorganisms more readily than EtO but does not penetrate materials well and may be carcinogenic. For these reasons, BPL has not been used as extensively as EtO.

5.3 Summary

Sterilization is the complete killing of all microorganisms. Sterilization can be accomplished by both physical and chemical methods. In physical methods of sterilization, the most widely used method is the application of heat. The temperature for heat sterilization is selected to eliminate the most heat resistant organisms in the material usually bacterial endospores. Heat can be applied in two forms for sterilization purpose. They are dry heat and moist heat. The most widely used method of sterilization by dry heat is hot air oven. This is mainly used for sterilization of glassware. But the heat sterilization by moist heat is more advantageous than dry heat. For routine sterilization in various laboratories, an autoclave is used. This permits application of steam heat under pressure at temperatures above the boiling point of water. Filter sterilization involves the removal of living microorganisms from liquids. Membrane filters are widely used for sterilization of heat sensitive liquids in laboratory. Materials which are heat labile also can be sterilized by employing different types of “Radiation”.

Chemicals are often used to control microbial growth. Chemicals that kill microorganisms are called cidal agents; and those that inhibit growth are called static agents. The value of a chemical agent is assessed by determining the minimum concentration necessary to kill (or) inhibit growth and by determining whether it exhibits selective toxicity. Disinfectants are

chemical compounds used to decontaminate (or) sterilize inanimate objects. Antiseptics can be used to decontaminate living tissues. These compounds are used in many commercial, health care and industrial applications.

5.4 TECHNICAL TERMS

Sterilization, Disinfection, Dry heat, Wet heat, Tyndallization, Autoclave, Hot air oven, Radiation, Filtration, Alcohols, Phenols, Aldehydes, Heavy metals, Detergents, Dyes.

5.5 SELF ASSESSMENT QUESTIONS

Q.1 Define the term sterilization and explain different physical methods of sterilization?

Q.2 What is sterilization? Write the different methods of sterilization.

Q.3 Define disinfection and write a detailed account about the chemical disinfectants?

Q.4 For cultivation of a microorganism explain the sterilization and disinfection methods?

5.6 SUGGESTED READINGS

1) Microbiology - Pelczar, M.J., Chan, E.C.S., Kreig, N.R. Tata McGraw Hill Publishing Company, (1996).
2) Biology of Microorganisms - Brock T.D., Madigan, M.T., Prentice Hall Englewood Cliffs Publications.
3) Microbiology - Prescott, L.M., Harley, J.P., Klein, D.A., WCB – McGraw Hill Publications, (1999).
4) Principles of Microbiology - Atlas R.M., WCB McGraw Hill Publications, (1996)
5) Text Book of Microbiology - Ananthanarayan. R and C.K.Jayaram Paniker, Orient Longman Publications.

Prof. A. Amruthavalli

LESSON – 6

GENERAL METHODS OF ISOLATION OF AEROBIC BACTERIA AND ANAEROBIC CULTURE METHODS

OBJECTIVE OF THE LESSON

Students will know how to isolate various kinds of bacteria by using different isolation methods based on the purpose and also know to test the water quality.

STRUCTURE OF THE LESSON

6.1 INTRODUCTION

In natural habitats, microorganisms usually grow in complex, mixed populations containing several species. Different habitats of environment like air, water and soil consist of a number of bacteria and other microorganisms in mixed populations. A study of the microorganisms of these habitats requires knowledge of the particular microbes present in them. To study the morphological as well as physiological characteristics of any individual species of these habitats, that particular species should be isolated or separated from its mixed population. The isolated species is then cultivated in laboratories to get the pure culture. So, a pure culture is necessary to characterize and study the physiological, immunological and other characters of a particular species.

The development of techniques for growing microorganisms on solid media and efficiently obtaining pure cultures was due to the efforts of the German bacteriologist Robert Koch and his associates. Culture media are needed to grow microorganisms in the laboratory and to carry out specialized procedures like microbial identification, water and food analysis, and the isolation of particular microorganisms. Many different media are available for these and other purposes. Pure cultures can be obtained through the use of spread plates, streak plates, or pour plates and are required for careful study of an individual microbial species.
By definition, a pure culture is a population of cells that arises from a single cell and is free from contamination with others. And, isolation is a process of separation of a single cell or species from a mixture of cells or species. Pure cultures are so necessary and important for various reasons – (1) Identification of the species, (2) Analyses of food, water and industrial wastes and products, (3) Sterility testing of products destined for human use, (4) Assay of antibiotics and vitamins, (5) Determination of antibiotic sensitivity of pathogens isolated

from patients, and (6) Preparation of biological products or materials used for immunizations and etc.

6.2 METHODS FOR ISOLATION OF BACTERIA FROM SOIL

As bacteria show physiological variations, these properties can be used to isolate specific groups of microorganisms. Isolation can be carried easily by incorporating different dyes, chemicals or antibiotics into the growth medium to inhibit the growth of undesirable microorganisms. Before the advent of different types of culture media, natural materials such as potato slices, bread slices etc., are used to isolate the microorganisms from different sources. But, with the introduction of successful culture media, a variety of techniques have been developed for the isolation to accomplish pure cultures of bacteria. However, each technique has certain advantages and limitations of its own. Hence, no single isolation method can be commonly used for the isolation of all bacteria. Several methods are available by which a selective group of organism can be encouraged to grow.

Plating Techniques

Among the different isolation procedures, plating techniques namely (i) Streak plate, (ii) Spread plate, and (iii) Pour plate methods are important and are widely used. These methods are almost indispensable to the bacteriologist to isolate pure cultures. The effectiveness of any isolation technique can be greatly increased by using differential or selective or enrichment media.

Streak plate technique

This is the most practical method for the isolation of pure cultures. It is developed for the first time by two bacteriologists, Friedrich Loeffler and George Gaffky in the laboratory of Robert Koch. The principle involved in this technique is the thinning or dilution of the inoculum along the successive streaks which results in the development of isolated colonies. In this method, a small amount of sample from a mixed inoculum is transferred to the surface of suitable agar medium with the help of inoculating loop. Then, the inoculum is streaked on the surface of agar medium and incubated. After incubation, isolated colonies appear on the agar surface at the end of the streaks. Depending on the mode of streaking, there are different types of streak plate techniques. Some of them are a) Radiant streak method, b) Spiral streak method, c) Zig Zag streak method, and d) Quadrant streak method. Of these methods, quadrant streak method is the most commonly and widely used method as it has many advantages over the other methods. Unlike the spread plat and pour plate methods, streak plate method is not useful in quantitative studies.

Quadrant streak method

This method is also called as ‘clock-wise streak plate technique’ (Fig. 6.1). In this method, a small amount of inoculum is placed at one place on the agar medium surface with the help of inoculation loop. Then the inoculum is streaked over a small portion of the medium surface. Then, inoculating loop is flamed to destroy the residual bacteria. The plate is then rotated approximately to one quarter of a turn and again inoculum is streaked starting from the first set of streaks. This sequence of flaming the loop, rotating the plate and streaking is repeated further for two times to dilute the inoculum. However, while streaking the new set, previous set of streaks should crossed only once by the loop. As the loop is heated in between the streakings, the inoculum is diluted to a greater extent. A confluent growth may occur along the initial streaks but well isolated colonies will appear in the last set of streaks, if this technique is properly performed.

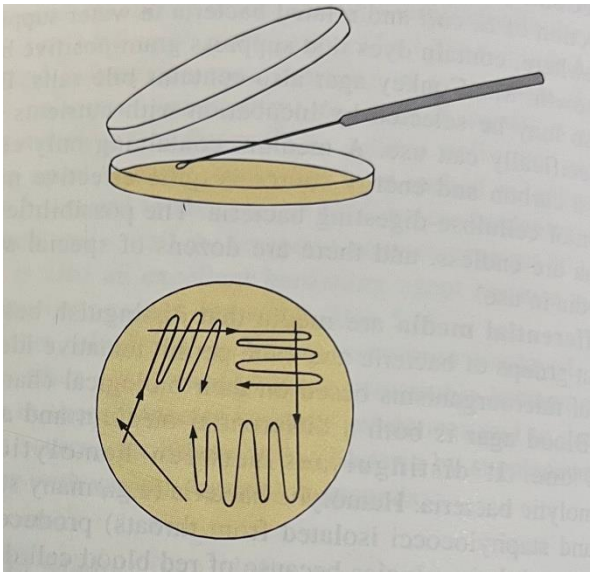


Figure-6.1: Quadrant streak plate technique

Spread plate technique

The spread plate technique (Fig. 6.2) is an easy and direct method for achieving isolated colonies on agar surface. In this method, the original inoculum is successively diluted. A small volume of inoculum from the sufficiently diluted sample is placed on the surface of agar medium. Then the inoculum is spreaded over the surface by using sterile bent glass rod. On incubation, the dispersed cells develop into isolated colonies. Unlike in pour plate technique, only surface colonies develop in this method. Like the pour plate method, this technique is also useful in quantitative studies. But in contrast to the pour plate technique, psychrophilic bacteria can be isolated by employing this spread plate technique.

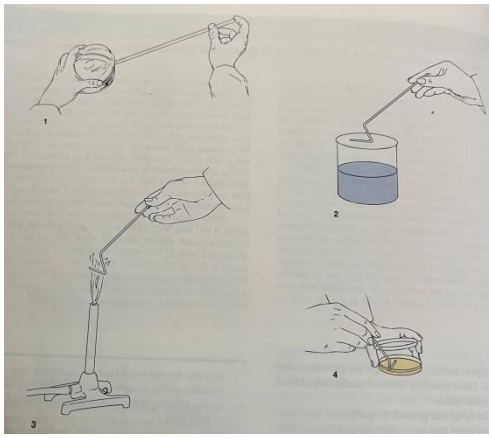


Figure-6.2: Spread plate technique

Pour plate technique

The basic method of this pour plate technique (Fig. 6.3) is developed in the laboratory of Robert Koch, a famous bacteriologist. In this method, the original sample of inoculum is diluted several times to reduce the microbial population in the sample. This successive dilution ensures the development of separate colonies upon plating on agar surface. A small volume of diluted sample is mixed thoroughly with molten state agar medium which is

cooled to about 45^{o} C temperature after sterilization. Then the mixture is poured into sterile Petri plates and allowed to solidify. As the medium solidifies, the microbial cells distributed throughout the medium get fixed in position. These trapped cells develop into individual colonies on incubation. Growth of colonies occurs both at surface and sub-surface areas, as the medium is soft enough to permit the growth of the cells. This pour plate method is also useful for quantitative studies to enumerate the microbial cells in the sample in addition to qualitative studies. However, the main disadvantage in this method is the difficulty of removing sub-surface colonies for further study. This method is also not suitable for the isolation of psychrophilic microorganisms, as they do not withstand the temperature of molten agar.

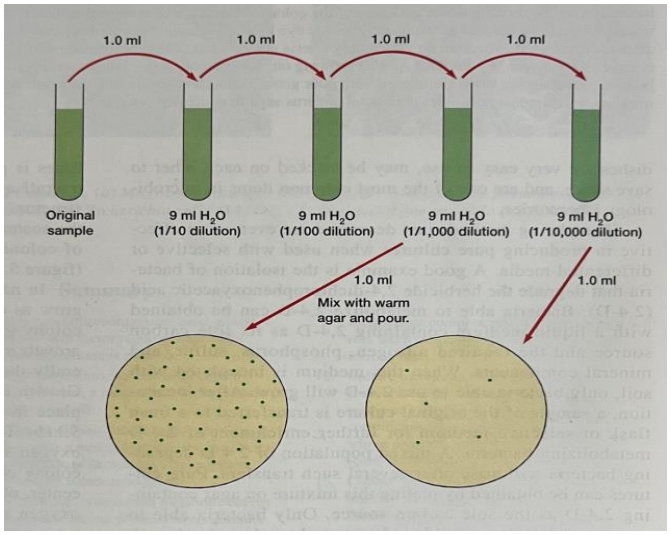


Figure-6.3: Pour plate technique

Serial dilution technique

In this method, the original mixed culture sample is serially diluted in a sterile liquid medium. Due to serial dilution of the microbial population, the final dilution will contain one or none of the microbial cells. The growth in the last tube or penultimate tube of dilution series may be presumed to have resulted from a single cell. Thus pure culturing can also be obtained by serial dilution technique. This method is useful especially for the isolation and purification of bacteria and yeasts.

Micromanipulator technique

This technique is generally used when neither plating nor dilution methods are not applicable for isolation. This technique involves the isolation of a single cell from the mixed microbial population. Sometimes, this method is also called as "Single-cell isolation" technique. For the isolation of a single cell by this method, a device namely micromanipulator is used. The micromanipulator is equipped with a microscope and a micropipette or microprobe (a fine needle). With the help of this microprobe, a single cell is picked up and separated from the mixed culture while looking through the microscope. This technique requires a great skill to operate the microprobe. The technical difficulty in this method is its inverse relation to the size of the microbial cells. So, this technique is more convenient to isolate large celled microorganisms such as algae and protozoa and difficult to isolate bacteria.

Winogradsky Column

This column is devised by Russian scientist, S. Winogradsky in 1880 for the isolation of

purple and green phototrophic bacteria and other anaerobes. The column is a miniature anaerobic ecosystem that can be an excellent long-term source of all types of prokaryotes involved in nutrient cycling. A major factor in aquatic environments is the movement of materials, whether they may be gaseous, solid, or in the dissolved phase. The mixing and movement of nutrients, O_2 and waste products that occur in fresh water and marine environments are the dominant factors controlling the microbial community. Winogradsky column is a model ecosystem used to study soil and sediment microorganisms. In this microcosm, microorganisms and nutrients interact over a vertical gradient. The height of the column allows the development of an aerobic zone at the surface and microaerophilic and anoxic zones below the surface. Fermentation products and sulfide migrate up from the reduced lower zone and O_2 penetrates from the upper surface. This creates conditions which are similar to those in a lake with nutrient-rich sediments. Light is provided to stimulate the penetration of sunlight into the anaerobic lower region, which allows photosynthetic microorganisms to develop. This column illustrates the co-existence and interdependence of different ecological niches and reflects the interactions and gradients that occur in aquatic environment (Fig. 6.4).

A layer of reduced mud is mixed with sodium sulfate or calcium sulfate, sodium carbonate or calcium carbonate, shredded newspaper (as cellulose source), shredded leaves and roots, ground meat/boiled eggs and even dead material and also some additional soil is placed or placed at the bottom of column up to 1/3 of the column. Then water is slowly poured into the column along the sides without disturbing the packed material and incubated in diffused light. As the column begins to mature, a series of reactions occurs and particular members of the microbial community develop in specific micro environments in response to the chemical gradients. In the bottom of the column, cellulose is degraded to fermentation products by the genus *Clostridium*. With these fermentation products available as reductants and using sulfate as oxidant, *Desulfovibrio* produces hydrogen sulfide. The hydrogen sulfide diffuses upward towards the oxygenated zone, creating a stable hydrogen sulfide gradient. In this gradient, the phototrophs, *Chloribium* and *Chromatium* develop as visible olive green and purple or red zones. These microorganisms use H_2S as electron source and CO_2, from sodium carbonate, as carbon source. Above this region, the purple non-sulfur bacteria of the genera *Rhodospirillum* and *Rhodopseudomonas* can grow. These photoheterotrophs use organic matter as an electron donor under anaerobic conditions and function in a zone where sulfide level is lower.
Both O_2 and H_2S may be present higher in the column, allowing specially adapted microorganisms to function. These include *Beggiatoa*, *Thiothrix* which use reduced sulfur compounds as a reductant and O_2 as an oxidant. In the upper portion of the column, diatoms and cyanobacteria may be visible. These commensalistic microorganisms, that develop sequentially, are dependent on the reductant originally provided as cellulose or plant debris. When this reductant is exhausted, the column gradually becomes oxidized and the sulfide-dependent photosynthetic microorganisms and other anaerobes can no longer maintain themselves in the microcosm.

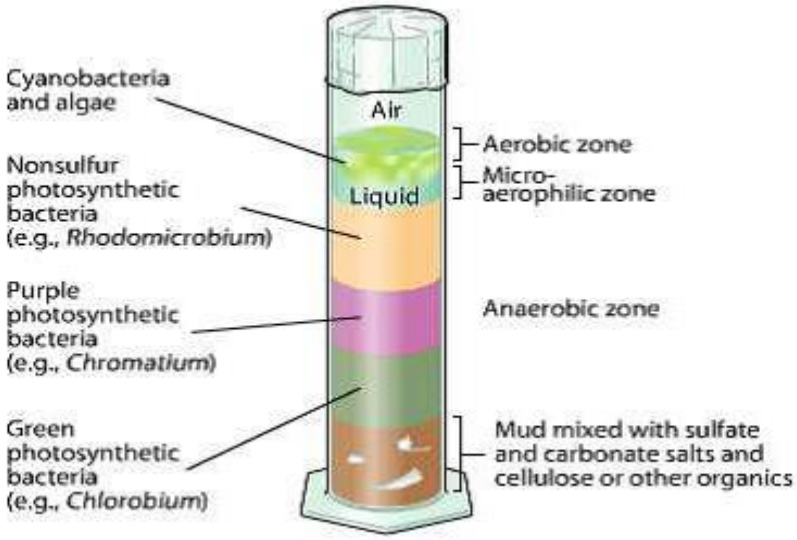


Figure-6.4: Winogradsky column

Contact slide technique

This technique is also called as Rossi-Cholodny method or Buried slide method. It is one of the direct methods used in soil microbiology to visualize the distribution and relative preponderances of microorganisms in intact soil portions. Also used to study the qualitative changes in soil micro flora under the influence of soil amendments and in different types of soils. Slides allow the physical posture of microorganisms typifying their normal position and associations in relation with other microorganisms. Two clean glass slides, in intact position, are inserted into the soil which is moistened with sufficient water. Ensure that the slides are in direct contact with the soil particles. The setup is incubated for a period of 2-3 weeks with intermittent water addition. During incubation, the slides act as substratum and allow the microorganisms to colonize the slide surface. After the incubation, the slides are carefully removed, stained the positive side (the side which is in contact with the soil) of the slides with phenolic rose bengal or 1% erythromycin and examined under a microscope to observe the occurrence of different microbial populations, bacteria, fungi and actinomycetes, in relation to each other within that soil. So, this technique primarily provides information about the presence and distribution of microorganisms but not their exact numbers.

MPN technique

Most Probable Number method is sometimes referred to as Ultimate or Extinction dilution method used for the enumeration of *Nitrosomonas* population in soils. This method uses statistical analyses and successive dilution of sample and gives statistically based estimate of microorganisms. Generally, nitrifying bacteria are difficult to isolate but their presence or absence in the soil can be detected by this MPN method. This gives the population density without the actual count of single cells or colonies. The principle involved in this is 'determination of presence or absence of microbes in several individual aliquots of each of several consecutive dilutions of soil sample'. The main prerequisite is that 'the microbial population to be determined must be able to bring about some characteristic and readily recognizable transformation in the medium into which it is inoculated or else the microorganism itself must be easily recognizable in the substrate after its multiplication. The positive reading means the presence or absence of those microbes initially in that aliquot. When culture medium is not suitable or inadequate, microbial antagonism occurs during incubation period and MPN underestimate the actual population density.

The prepared Ammonium-Calcium carbonate medium as per the composition is dispersed in 9 ml aliquots into the test tubes. After sterilization, the tubes cooled to room temperature. Prepared the soil suspensions up to 10^{-9} dilutions by using 10-fold serial dilution technique and added 1 ml of the soil suspensions of different dilutions into separate test tubes containing the sterilized medium. Maintained five replicates for each dilution and incubated the inoculated tubes for 10 days. After incubation, few ml of Griess-Illosvay reagent is added to the incubated test tubes and observed for the reddish colour development. To the test tubes not developed the colour, added a pinch of Zn-Cu-MnO_2 mixture and observed for the purplish colour. Marked the tubes that developed colour after the addition of Griess-Illosvay reagent alone or both the reagents as positive tubes and remaining tubes as negative tubes. Selected P_1, the number of all or maximum positive tubes in least concentrated dilution, and P_2 and P_3 as the number of positive tubes in the next two higher dilutions. Considering the P_1, P_2 and P_3 values from the data, selected the table value from the 'Cochran' table. Multiplied the table value with dilution value of P_2 to get the final populations of *Nitrosomonas* per gram soil.

Table of most probable numbers for use with 10-fold dilutions and 5 tubes per dilution (Cochran, 1950)

P_1	P_2	Most probable number for indicated values of P_3					
		0	1	2	3	4	5
0	0	-	0.018	0.036	0.054	0.072	0.090
0	1	0.018	0.036	0.055	0.073	0.091	0.11
0	2	0.037	0.055	0.074	0.092	0.11	0.13
0	3	0.056	0.074	0.093	0.11	0.13	0.15
0	4	0.075	0.094	0.11	0.13	0.15	0.17
0	5	0.094	0.11	0.13	0.15	0.17	0.19
1	0	0.020	0.040	0.060	0.080	0.10	0.12
1	1	0.040	0.061	0.081	0.10	0.12	0.14
1	2	0.061	0.082	0.10	0.12	0.15	0.17
1	3	0.083	0.10	0.13	0.15	0.17	0.19
1	4	0.11	0.13	0.15	0.17	0.19	0.22
1	5	0.13	0.15	0.17	0.19	0.22	0.24
2	0	0.045	0.068	0.091	0.12	0.14	0.16
2	1	0.068	0.092	0.12	0.14	0.17	0.19
2	2	0.093	0.12	0.14	0.17	0.19	0.22
2	3	0.12	0.14	0.17	0.20	0.22	0.25
2	4	0.15	0.17	0.20	0.23	0.25	0.28
2	5	0.17	0.20	0.23	0.26	0.29	0.32
3	0	0.078	0.11	0.13	0.16	0.20	0.23
3	1	0.11	0.14	0.17	0.20	0.23	0.27
3	2	0.14	0.17	0.20	0.24	0.27	0.31
3	3	0.17	0.21	0.24	0.28	0.31	0.35
3	4	0.21	0.24	0.28	0.32	0.36	0.40
3	5	0.25	0.29	0.32	0.37	0.41	0.45
4	0	0.13	0.17	0.21	0.25	0.30	0.36
4	1	0.17	0.21	0.26	0.31	0.36	0.42
4	2	0.22	0.26	0.32	0.38	0.44	0.50
4	3	0.27	0.33	0.39	0.45	0.52	0.59
4	4	0.34	0.40	0.47	0.54	0.62	0.69
4	5	0.41	0.48	0.56	0.64	0.72	0.81
5	0	0.23	0.31	0.43	0.58	0.76	0.95
5	1	0.33	0.46	0.64	0.84	1.1	1.3
5	2	0.49	0.70	0.95	1.2	1.5	1.8
5	3	0.79	1.1	1.4	1.8	2.1	2.5
5	4	1.3	1.7	2.2	2.8	3.5	4.3
5	5	2.4	3.5	5.4	9.2	16.00	-

6.3 METHODS FOR BACTERAIL ANALYSIS OF WATER

Multiple Tube Fermentation Test

The multiple-tube fermentation test has been used for many years for the sanitary analysis of water for the presence of coliforms in water. This method involves the three routine standard tests – a) the presumptive test, b) the confirmed test, and c) the complete test (Fig. 6.5).

a) Presumptive test

A series of fermentation tubes each containing lactose broth or lauryl tryptose broth of known concentration, are inoculated with known amount of water. These tubes are incubated for 24 to 48 hours at 35^{o} C temperature. Generally, five fermentation tubes containing single or double strength broth are inoculated with 10 ml of water, 5 tubes with 1 ml of water and 5 with 0.1 ml of water. At the end of the 24 hours of incubation, the tubes indicate that the coliforms are absent. These tubes are incubated for an additional 24 hours to be sure for the absence of coliforms i.e. gas production.

b) Confirmed test

If a positive test of gas production is obtained, it does not mean that coliforms are present. The other organisms too also give false positive presumptive test because they are also capable of fermenting lactose with the formation of acid and gas. The positive presumptive test is resulted due to synergistic action of two microbes on a carbohydrate with the production of gas which is not formed if both are grown separately. In addition, if yeasts, *Clostridium* species and some other microorganisms are present, gas is also produced. Therefore, a confirmed test is performed for the presence of coliforms. All fermentation tubes showing gas within 48 hours at 35^{o} C are used for confirmed test and it is done in two ways.

The positive presumptive fermentation tube is gently shaken and a drop of its culture is transferred to brilliant green lactose bile broth fermentation tube. The tubes are incubated for 48 hours at 35^{o} C. The appearance of gas within this period indicates for positive confirmed test. The brilliant green dye inhibits the Gram positive bacteria and synergistic reactions of Gram positive and Gram negative bacteria for a common food base. The second confirmed test is done by eosine methylene blue (EMB) agar or endo agar method. In EMB agar method, a definite amount of two stains (eosin and methylene blue) is added to a melted lactose agar. The medium is poured into Petri plates. Over the surface of EMB agar medium, a loopful culture from each positive fermentation tube is streaked. The plates are incubated at 35^{o} C for 24 hours keeping them in inverted position. Then three types of colonies may develops: a) typical colonies – nucleated, with or without metallic sheen

b) atypical colonies – opaque, non-nucleated mucoid, pink colour

c) negative colonies – all other types

The development of typical colonies shows that the confirmed test is positive.

c) Completed test

In the last, the completed test is performed to ascertain about the presence of coliforms in water sample. The purpose of the completed test is to determine whether (i) the colonies growing on EMB or endo agar are again capable of fermenting lactose and forming acid and gas, and (ii) the organisms transferred to agar slants show the morphological appearance of coliform group. Each colony that gives positive confirmed test is transferred to lactose fermentation tube and to

nutrient agar slants. The tubes are incubated at 35° C for 48 hours. Production of gas in fermentation tubes and, demonstration of Gram negative, non-spore forming rods on the agar slants constitute a positive completed test for coliforms. The absence of gas production confirms for negative test of coliforms.

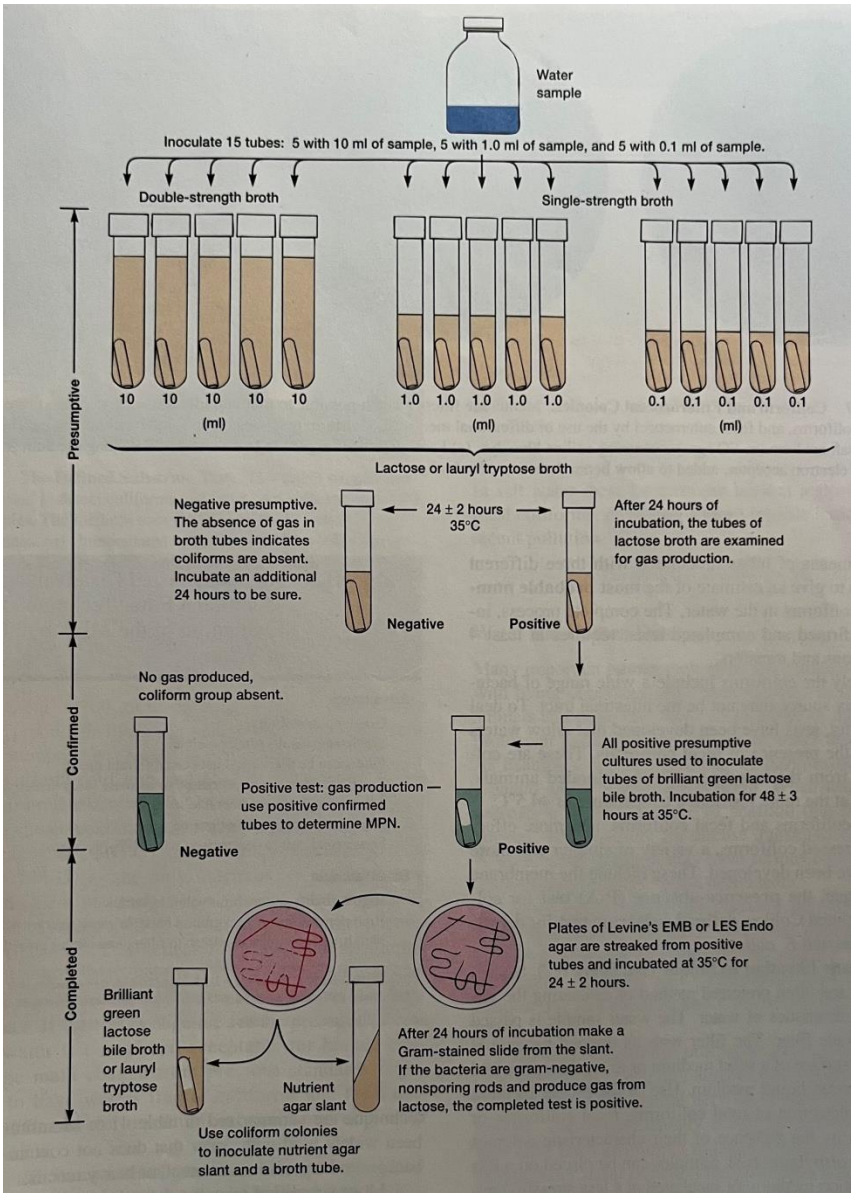


Figure-6.5: Multiple tube fermentation test

Membrane filter technique

In 1951, Goetz and Tsuneishi described a new method for enumeration of coliform microorganisms in water and named it as ‘Millipore filter technique’. However, it is often referred to as ‘Membrane filter technique’ (Fig. 6.6). This technique has been accepted as the standard method for the microbiological examination of sewage, water, etc. The filtering apparatus consists of a glass or stainless steel funnel, and a flask. The funnel of stainless steel is

clamped to a base containing a molecular filter. The stem of base is inserted into a filter flask through a rubber stopper. A sterile membrane filter disc of 0.45 µm is placed in the sterilized holding apparatus. A volume of water is passed through the filter disc. Bacteria present in water sample are retained on filter disc. Thereafter, the membrane filter disc is aseptically removed by a sterile forceps and placed on absorbent disc saturated with culture medium and contained in Petri plate. The medium passes through the pores of membrane and nourishes the bacteria present on it. After proper incubation at 35^{o} C for 24 hours, each bacterial cell multiplies to form a visible colony on the membrane. The colonies are easily counted. This method has some advantages and disadvantages.

Advantages

1. Method shows good reproducibility.
2. Single step results are often possible.
3. Filters can be transferred between different media.
4. Large volumes of samples can be processed to increase sensitivity.
5. Ability to complete filtration process at site itself.
6. Cost effective when compared to MPN method.

Disadvantages

1. High turbidity waters limit volumes sampled.
2. High populations of background bacteria cause over growth.
3. Metals and phenols can absorb to filters and inhibit the bacterial growth.
4. Water should not be loaded with algae or colloidal particles which clog the filters.

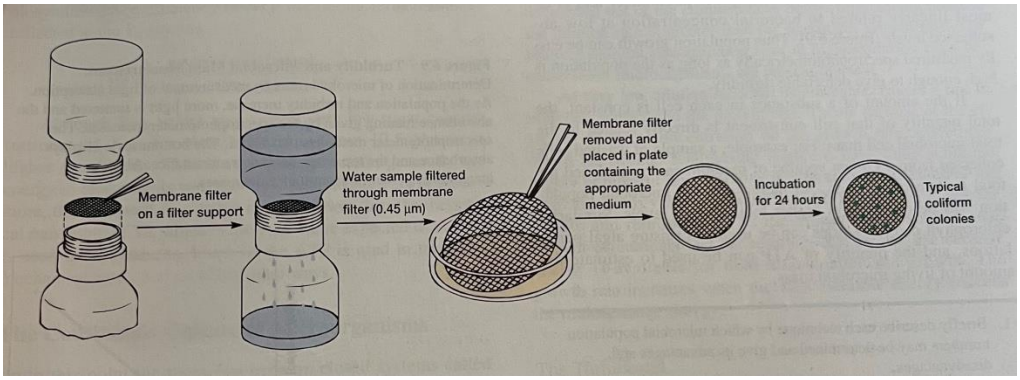


Figure-6.6: Membrane filter technique

6.4 CULTURE METHODS FOR ANAEROBIC BACTERIA

Anaerobes are sensitive to O_2 and all oxygen must be excluded for their growth. There are number of methods for achieving anaerobiosis condition with different principles like

1) Exclusion of oxygen or vacuum production,
2) Displacement of oxygen with other gases,
3) Absorption of oxygen by chemical, and 4) Reduction of oxygen.

Cultivation in vacuum

This method involves the incubation of cultures in a vacuum desiccator. Desiccators are the

sealable enclosures containing desiccants like calcium chloride or silica gel to preserve moisture sensitive items. However, this method is unsatisfactory as some O_2 always remain behind.

Displacement of O_2 with other gases

This method involves the displacement of oxygen with other gases like H_2, N_2, CO_2 and Helium etc. Anaerobiosis is seldom complete by this method alone. One of the methods under this principle is the 'Candle Jar' method which is the oldest and popular method but not so effective. Culture inoculated plates are placed inside a large air-tight container and a lighted candle kept inside before sealing the container. In this, scented candles should not be used as oils may inhibit the bacterial growth. Burning candle uses up all O_2 and release CO_2. When CO_2 extinguishes the candle, conditions may be created for anaerobic growth.

Absorption of O_2 by chemical

Buchner (1888) introduced a method namely Pyrogallol method that works under this principle, which was later applied with some modifications.

1. Pyrogallic acid or pyrogallol to NaOH in a large test tube and placed inside an air-tight jar, which can create anaerobiosis. However, small amount of 'CO' that formed may be inhibitory to some bacteria.
2. In another method, a disc of filter paper having same diameter as a Petri dish is place on top half of Petri plate and a mixture of pyrogallol plus sodium carbonate, in dry powder form, spread on it. Inoculated plate is inverted on it over filter paper and tightly sealed with molten wax. Dry pyrogallol mixture is activated by moisture within closed system and complete anaerobiosis is achieved within about 2 hours of time. This method is in common use for the growth of anaerobic bacteria.

McInosh and Fildes' anaerobic jar method

This method is most reliable and widely used one. Here, the glass or metal jar, having dimensions of 8 X 5 inches with a metal lid which can be clamped air-tight with a screw. Metal jar is more preferable to avoid the risk of explosion. The lid consists of two electrical terminals connected to electrical supply. The lid has one inlet tube and one outlet tube for the passage of gas. Leading from the terminals on underside of lid, palladinized asbestos wrapped as a layer over porcelain spool is suspended by stout wires. The inoculated agar plates and indicator are placed inside the jar. The outlet is connected to vacuum pump and air is evacuated. Then the outlet tap is closed and inlet tube is connected to H_2 supply. After the jar is filled with H_2, electrical terminals are connected to electrical supply to heat the palladinized asbestos. This act as catalyst for the combination of H_2 and residual O_2. This provides complete anaerobiosis.

Gaspak Jar method

This is the best choice method of present day for anaerobic culturing. Gaspak is commercially available as disposable envelope or packet of aluminum foil containing the pellets of chemicals like sodium borohydride, cobalt chloride, citric acid, and sodium bicarbonate that generate H_2 and CO_2 on addition of water. Palladium pellets present in the envelope acts as cold catalyst and permits H_2 to combine with O_2 generating anaerobiosis. Reduced methylene blue is used as indicator in jars. It remains colorless anaerobically, but turns blue on exposure to O_2. The released CO_2 promotes more rapid growth of the anaerobic bacteria (Fig. 6.7).

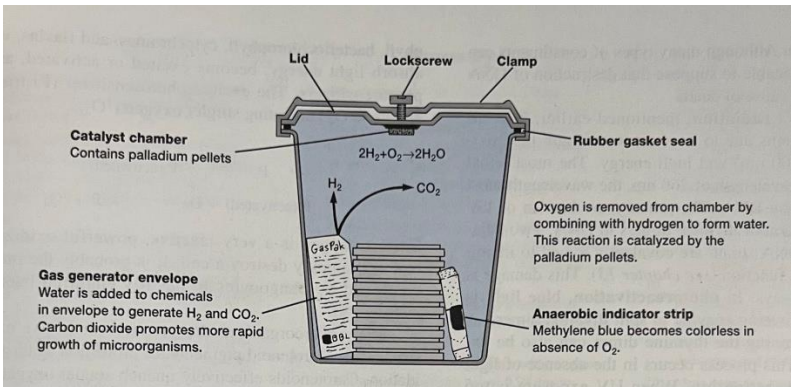


Figure-6.7: Gas Pak Jar

Agar shake tube method

The agar shake tube technique is employed for the isolation of anaerobic bacteria into pure culture. This method involves the dilution of a mixed culture in tubes of molten agar. This result in the development of colonies embedded in the agar medium rather than on the surface of a plate. The shake tube method is found useful in purifying particular types of microorganisms such as phototrophic sulfur bacteria and sulfur reducing bacteria.

Roll-tube technique

The roll-tube method is used for isolation of stringent or obligate anaerobes. The inner walls of anaerobic culture tube is coated with a pre-reduced agar medium and stoppered. Initially, the tube consists an atmosphere of oxygen free N_2. The stopper is removed and tube is kept anaerobic by continuously flushing the oxygen-free CO_2 from a gas cannula. Inoculum is taken up with a loop and streaked on the surface of agar medium present in anaerobic culture tube. After inoculation, the tube is re-stoppered and incubated. As the culture tube contains oxygen free CO_2 and provide anaerobic condition, only anaerobic bacteria show growth after sufficient period of incubation.

6.5 SUMMARY

The pure cultures usually are obtained by isolating individual cells with any of three plating techniques namely the spread plate, streak plate and pour plate methods. Microorganisms growing on solid surfaces tend to form colonies with distinctive morphology. Colonies usually grow most rapidly at the edge where larger amounts of required resources are available. Spread plate, pour plate and membrane filter technique can be employed for viable counting of bacteria. Indicator organisms are used to assess the presence of pathogenic microorganisms. Most probable number (MPN) and Membrane filtration procedures are employed to estimate the number of indicator organisms present.

6.6 TECHNICAL TERMS

Streak plate method, Spread plate method, Pour plate method, Micromanipulator technique, MPN technique, Multiple tube fermentation test, Winogradsky column, Gaspak jar, Membrane filter technique.

6.7 SELF ASSESSMENT QUESTIONS

Q.1 Explain the different plating techniques for the isolation of bacteria and their importance.

Q.2 Describe the Most Probable Number technique for the enumeration of *Nitrosomonas* bacteria from soil.

Q.3 Give an account on Winogradsky column and its significance.

Q.4 Write an account on Multiple tube fermentation test for the presence of coliforms in water.

Q.5 Describe the important methods for culturing anaerobic bacteria.

6.8 SUGGESTED READINGS

1. Microbiology - Pelczar, M.J., Chan, E.C.S., Kreig, N.R. Tata McGraw Hill Publishing Company, (1996).
2. Biology of Microorganisms - Brock T.D., Madigan, M.T., Prentice Hall Englewood Cliffs Publications.
3. Microbiology - Prescott, L.M., Harley, J.P., Klein, D.A., WCB – McGraw Hill Publications, (1999).
4. Principles of Microbiology - Atlas R.M., WCB McGraw Hill Publications, (1996)
5. Text Book of Microbiology - Ananthanarayan. R and C.K.Jayaram Paniker, Orient Longman Publications.

Prof. V. Umamaheswara Rao

LESSON – 7

MAINTENANCE AND PRESERVATION OF BACTERIAL CULTURES

OBJECTIVE OF THE LESSON

To understand the different methods of maintenance and preservations of bacterial cultures which is essential from research and application points of view.

STRUCTURE OF THE LESSON

7.1 INTRODUCTION

Microorganisms are usually grown or multiplied in laboratories by using artificial media to study the various aspects of scientific and research interest. This multiplication process of microorganisms is known as ‘culturing’ and the population of cells thus cultured is called as a ‘culture’. The culture may be either mixed culture, if it contains a population of more than one

type of species. If a culture contains a population of single type of species, it is called as 'pure culture or monoculture'. So, the pure culture of a species is defined as 'a population of identical cells that arise from a single cell'. As the pure cultures are so important for research and other purposes, they have to be maintained and preserved in laboratories. Microbiologists are concerned with the maintenance and preservation of the cultures with which they work. It is necessary to have convenient methods to keep cultures alive and in genetically stable form.

The choice of method depends on the nature of the microorganism, facilities available and on the preservation objectives. The method to be used depends on whether the culture is to be preserved for a few days until a positive identification is made, for the duration of a research project or for future long term reference. The preservation method also reflects the difference in the biological properties of the bacteria and in their ability to survive in natural and artificial environments. Preservation methods have a common objective of reducing the organism's metabolic rate as low as possible while still maintaining the viability. A high recovery or survival rate with a minimum of damage or change to the surviving organisms is also highly desirable.

Often, valuable cultures are deposited in centralized culture collections and it is important to deposit all the new microbial species in such culture collections to ensure their indefinite preservation and to make them available for scientific study. Some of the centers recognized for this purpose include—

1. American Type Culture Collection (ATCC), USA
2. Central Bureau Voor Schimmel Cultures, The Netherlands
3. Commonwealth Mycological Institute (CMI), England
4. Culture Collection of Algae and Protozoa, England
5. Institute Pasteur, France
6. Microbial Type-Culture Collection, Japan
7. USSR Antibiotic Research Institute, USSR
8. National Collection of Industrial Bacteria, Scotland
9. Microbial Type Culture Collection and Gene Bank (MTCC), India
10. Indian Type Culture Collection (ITCC), India

Culture collections occupy a central and essential position in microbiology because effective identification, research and training demand reliable sources of microorganisms. Culture collections consist of three main types viz., service collections whose function and organization is chiefly for the preservation and supply of cultures on demand; institutional collections whose supply is mainly for internal use in the institution concerned; and private collections chiefly for personal research purposes. These private collections may be highly specialized and important to other scientists, and often are the only available source of particular strains or kinds of microorganisms.

7.2 PRESERVATION METHODS

The methods that are used for maintenance and preservation should conserve all the characteristics of the organisms. There are different methods for maintenance and preservation of the cultures viz.,

(a) Periodic Subculture
(b) Preservation by Freeze-Drying
(c) Preservation by Liquid Drying,
(d) Cryogenic Storage
(e) Storage under Mineral Oil
(f) Preservation in Sterile Soil
(g) Storage in Sterile Distilled Water
(h) Preservation on Porcelain Beads
(i) Preservation in Gelatin Discs
(j) Preservation over Phosphorus Pentoxide in vacuum

7.2.1Periodic Subculture

Sub-culturing is the transfer of inoculum culture on to a fresh medium (Fig. 7.1). Periodic transfer or subculture is the traditional method used by microbiologists to maintain isolates in the laboratory. Generally, agar slants are used for sub-culturing. These agar slants are prepared by arranging the test tubes having sterile and molten agar medium in a slant position. After solidification, agar medium takes slanting shape and provide more surface area for sub-culturing. The culture medium, storage temperature and the period of transfer vary with the species. The temperature and the chosen medium should support a slower growth rather than rapid growth. This facilitates the period of transfer to be as long as possible. As an example, *E. coli* culture needs a monthly interval transfer. Many heterotrophic bacteria can be successfully maintained on nutrient agar medium with 20 – 30 days period of sub-culturing.

Apart for some cultures for which no long term preservation methods are yet available, periodic subculture is not recommended for long term preservation. Genetic change through selection of variants is likely to occur, the chances of contamination and mislabeling are high and the risk of culture loss is greater than in other methods. Many bacteria and fungi are able to survive for ten or more years on sealed agar slopes or in agar stabs at room temperature or in refrigerators by this method. Longevity of such cultures is influenced by the nature of the organism itself, the composition and pH of the medium, the degree of aeration, and the temperature of storage. Low temperatures are usually preferred. However, some strains of *Pseudomonas cepacia* prefer storage at room temperature. The degree of hydration is also an important factor and slow uncontrolled dehydration often leads to a loss of viability.

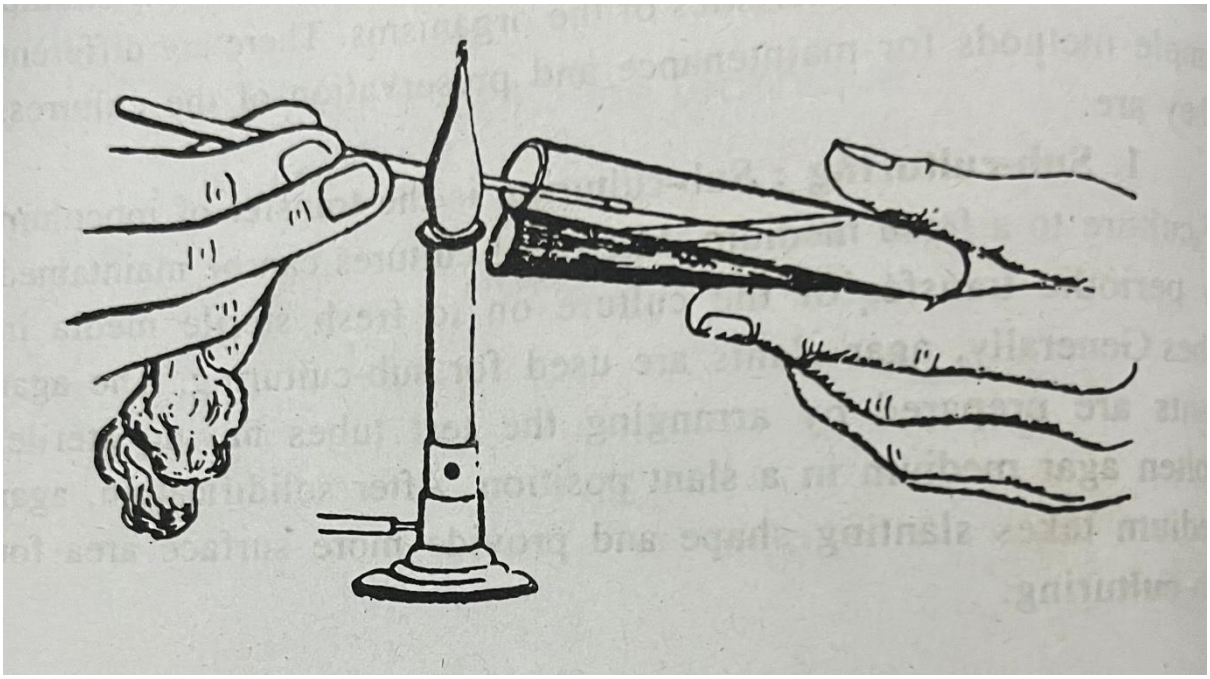


Figure-7.1: Process of Sub-culturing

7.2.2 Preservation by Freeze-Drying

Freeze-drying or Lyophilization has been widely used as the preferred method for long term preservation for many years. Many bacterial species that are killed by ordinary drying can be preserved even more than 30 years by following the freeze-drying process known as Lyophilization (Fig. 7.2). The method is suitable for many types of microorganisms including most bacteria, yeasts, sporing fungi but generally unsuitable for non-sporing fungi, algae and protozoa. Freeze - drying combines two of the most successful long term preservation methods, freezing and drying. In this process, a dense cell suspension is placed in small vials and then allowed to freeze at -60 to -78^{o} C temperature. The overall process involves the removal of water vapor by vacuum sublimation from the frozen state. This results in dehydration of the bacteria with a minimum damage to delicate cell structures. The method thereby overcomes the problems associated with drying from the liquid state and the dried ampoules may be stored at room temperature in the dark, although long term survival is improved by storage in refrigerators.

Freeze-drying is the most technologically complex of the preservation methods in use, requiring the highest level of technical skill and high capital expenditure for equipment. Freeze-dryers use one of the two methods for freezing the cell suspension prior to the drying process. In the pre-freezing method, the cell suspensions are frozen in the ampoules before being dried under vacuum, freezing being achieved using a mixture such as dry-ice in ethanol. The alternative method is known as centrifugal freeze drying where the cell suspension is frozen by evaporative cooling under vacuum while the ampoules are spun in a low speed centrifuge to minimize foaming. It is essential to use a suitable preservative suspended medium to protect the living organisms from damage during the freezing and drying stages. The functions of such preservatives include stabilization of protein, prevention of freezing damage and protection

against over drying. The choice of preservation depends on the organism and it must maintain the organism in a viable state and allow good recovery from the dried state. The main advantages of this method of preservation are (1) occupation of less space for storage and (2) easy transportation to other places. This method will not cause any changes in characteristics of the strain. However, loss of vacuum in the vials during storage leads to inactivation of cultures.

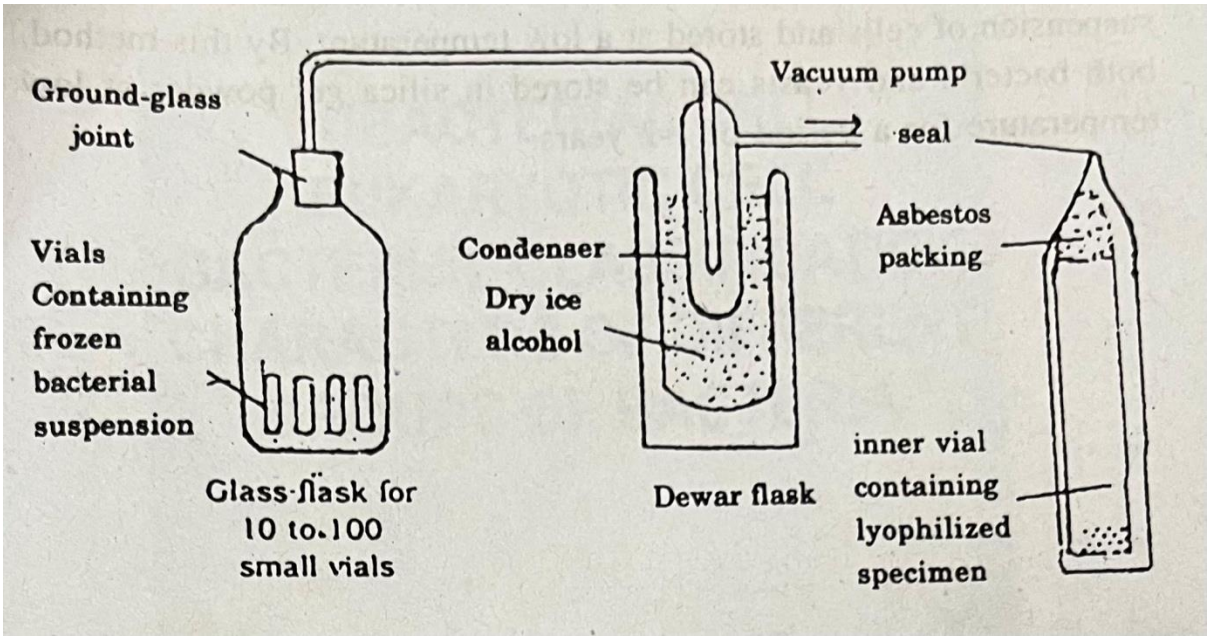


Figure-7.2: Lyophilization process

7.2.3 Preservation by Liquid Drying

Some strains of bacteria which are sensitive to freeze-drying can be preserved by drying from the liquid state rather than the frozen state. The method was developed by Annear and has been used successfully to preserve bacteria, yeasts, fungi and viruses. Small quantity of dense suspension of cells dispensed into ampoules. Then ampoules are constricted in a fine gas-air flame and attached to a vacuum pump. The lower half of the ampoules is immersed into a water bath controlled at –25° C. Ampoules are degassed and sealed under vacuum.

7.2.4 Cryogenic storage

Most microorganisms including bacteria, yeasts, fungi, viruses, bacteriophages, some algae and protozoa can survive long term storage in the frozen state by markedly reducing their metabolic rate. This method is also suitable for the preservation of animal as well as human cells. Microorganisms have been stored in freezers at temperatures around –20° C and –70° C. The lower the temperature the less is the loss of viability of most microorganisms and temperatures higher than –70° C should not be used for long term storage but may be satisfactory for period of up to 1 year.

The use of ultra-low temperatures obtained by freezing in liquid nitrogen at –196^{o}C has been used successfully to preserve a wide range of microorganisms and mammalian cells with a much reduced viability loss and a high degree of genetic stability. Though many bacteria survive freezing in their growth medium, the addition of cryoprotectants such as 5-10% glycerol or dimethyl-sulphoxide affords some protection from the stresses of freezing. Other cryoprotectants such as methanol, sugars, starch and polyvinyl- pyrrolidone have been used by various workers. Some cryoprotectants at concentrations required to afford protection during freezing and thawing may be toxic and affect the recovery of the organism unless they are diluted out or removed completely. The rate of cooling should be slow and controlled down to the temperature range of –20^{o} C to –40^{o}C and then rapid to the final freezing temperature. The rate of thawing should be as rapid as possible. Rapid freezing rates may lead to intracellular ice crystal formation and electrolyte imbalance and cause lethal cell damage. In general, bacteria, yeasts and fungi are less sensitive to freezing damage than algae and protozoa. This method is mainly useful in the case of bacterial cultures that cannot be preserved by lyophilization. However, this method is relatively expensive as it involves the use of liquid nitrogen. The bacterial cultures can be viable for 10-30 years, when stored by this method.

7.2.5 Storage under Mineral Oil

This is a simple method for preserving the cultures of many bacteria and yeasts by storing them on agar slants covered with sterile mineral oil. The oil used is medicinal grade liquid paraffin. In this way of protection, the interval between sub-culturing may often be extended for several years, and up to 20 years for some fungi. Long term preservation is often further improved if the oil-layered cultures are stored at –4^{o}C. The microorganism to be preserved is simply grown on its usual maintenance medium on agar slants, or in an agar deep or broth culture, and then covered with sterile paraffin oil to a depth of approximately 20 mm, or at least 10 mm above the top of the slant. Addition of the sterile paraffin should be carried out without disturbing the growth on the agar slant surface. To avoid the cross contamination, the sterile paraffin is either stored in individual layering quantities and poured onto the culture, or transferred by means of a sterile 10 ml pipette after each transfer is made. Cultures are routinely grown on slants but to economize the oil, slants cultures are often grown on “short” slants. With this method, some microorgansims can be preserved for more than 15-20 years.

This technique is simple, but for purposes of transport is not as convenient as the drying methods and for longevity and subsequent ease of handling, not as convenient as freeze- drying or freezing in liquid nitrogen. However, in the absence of these facilities, storage under oil affords protection to many bacterial and fungal cultures for a period of several years. The disadvantage with this method is that there is a slow diffusion of oxygen through the oil which allows growth to continue at a slow rate and therefore genetic stability may be poor.

7.2.6 Preservation in Sterile Soil

Many bacteria and fungi survive well in dried soil for long time periods of 20 years or more when stored at room temperature. The method is particularly useful for spore forming fungi, *Streptomyces* and also for spore-forming bacteria such as *Bacillus* and *Clostridium.* Excellent survival has also been experienced with *Rhizobium*. The advantages of the method are its low material cost, room temperature storage and genetic stability, which would be expected to be much improved when compared with storage under sterile mineral oil. A suitable selected

loamy soil is pulverized and autoclaved at 121^{o}C and 15 lbs pressure for 1 hr on 3 successive days. Autoclaved soil is then heated to dryness in an oven at 105^{o}C. The cell suspension is prepared in 2% sterile peptone water and small quantities are added to the dried soil and stored in desiccator till the further use. Bacterial cultures maintained by following this procedure are found to be viable for 70-80 years.

7.2.7 Storage in Sterile Distilled Water

Some bacteria, particularly the Gram-negative rods such as *Pseudomonas* may be preserved for considerable periods of time when stored as a dense suspension in sterile distilled water. Storage may be at room temperature or slightly at reduced 10-15^{o}C, if facilities are available. The method is applicable to a narrow range of bacteria like *Pseudomonas, Agrobacterium* and *Corynebacterium* and fungi, and as slow growth may continue, genetic stability would not be expected in the long term. As storage is in liquid, contamination is often a problem and one which is compounded when working with a range of similar strains. This method is best used as a source of working stock cultures in conjunction with stocks preserved by other more stable methods where contamination is less likely. Distilled water in screw-capped bottles is sterilized by autoclaving. Dense cell suspension prepared is transferred to the sterilized distilled water in the screw-capped bottles and stored at room temperature or preferably at 10-15^{o}C.

7.2.8 Preservation on Porcelain Beads

This method is a simple one for preserving many microorganisms involving the drying of cell suspension on porcelain beads, using silica gel as the drying agent. A layer of silica gel is placed in the bottom of a screw capped bottle. The silica gel is covered by a layer of "slag wool" and porcelain beads are mixed with a dense suspension of cells and placed on top of the slag wool. The bottle is tightly capped and the moisture is removed from the beads by the silica gel. Excess silica gel maintains the beads in a dried state. The method is suitable for the long term preservation of many bacteria and fungi. Perforated glass beads may be used in place of porcelain beads. The culture may be absorbed directly onto silica gel without indicator. But when water is added to silica gel, heat is produced. So, care should be taken to keep the silica gel cool while the suspension is being absorbed in order to avoid damage to the cells. By this method, both bacteria and yeasts can be stored in silica gel powder at low temperature for a period of 1-2 years.

7.2.9 Preservation in Gelatin Discs

A simple but very effective method for the preservation of bacteria is to store them in dried gelatin discs. The method involves the preparation of single drops of a dense suspension of cells in nutrient gelatin supplemented with ascorbic acid, and drying these by evacuation over phosphorus pentoxide. The method is suitable for the long term preservation of bacteria but little data is available on survival rates. Sterilized paraffin wax is allowed to set as a layer in a sterile glass petri dish. A dense cell suspension is prepared in 10% nutrient gelatin containing 0.25% ascorbic acid. Using a sterile Pasteur pipette single drops of the gelatin cell suspension on the sterile paraffin surface are prepared and allowed the drops to set. Petri dish with gelatin drops is placed in a vacuum desiccator containing phosphorus pentoxide and evacuated until the drops form dry discs. Aseptically, the discs are transferred to sterile tubes and stored over phosphorus pentoxide at 4^{o}C.

7.2.10 Preservation over Phosphorus Pentoxide in vacuum

This method has been successfully used in the National Collection of Type Cultures to preserve a wide variety of bacteria. The culture is preserved by placing loopful of dense cell suspension prepared in sterile horse serum in a small glass tube. This small tube is then placed in an outer tube containing a small quantity of phosphorus pentoxide at the bottom. A constriction is made in the neck of the outer tube using a fine gas-air flame. Then it is attached to a vacuum pump and subjected to evacuation for 5 minutes. Outer tube is sealed and the ampoules are stored at room temperature or in refrigerator. The method is suitable for the long term preservation of bacteria, yeasts and fungi and good survival has been observed for periods of 5-28 years for some strains.

7.3 SUMMARY

Once a microorganism has been isolated and grown in pure culture, it is necessary to maintain the viable culture, free from contamination, for some period of time. There are several methods available for maintaining and preserving pure cultures. The organisms may simply be sub-cultured periodically onto or into a fresh medium to permit continued growth and to ensure the viability of a stock culture. Although proper aseptic technique must be used each time the organism is transferred, there is always a risk of contamination. Furthermore, repeated sub-culturing is extremely time consuming, making it difficult to maintain large numbers of pure cultures successfully for indefinite periods of time. Additionally, genetic changes are like to occur when cultures are repeatedly transferred.

Therefore various methods besides sub-culturing have been developed for preserving pure cultures of microorganisms. These methods include refrigeration at 0^o to 5^o C for short storage times, freezing in liquid nitrogen at -196^o C for prolonged storage, and lyophilization or freeze-drying to dehydrate the cells. In lyophilization, the culture is frozen at a very low temperature and placed under a high vacuum. Under these conditions, the water in the culture and microbial cells goes directly from the frozen solid state to the gaseous state through a process of sublimation thereby drying the cells without disrupting them. By sufficiently lowering the temperature or by removing water, microbial growth is precluded but viability in a dormant state is maintained, permitting preservation of microorganisms for extended period of time.

The choice of the preservation method depends on the nature of the culture and the facilities available. When freezing is used to preserve microorganisms, the rates of freezing and thawing must be carefully controlled to ensure the survival of the microorganisms because ice crystals formed during freezing can disrupt membranes. Glycerol is often employed as an antifreeze agent to prevent damage due to ice crystals and to ensure the ability to recover viable microorganisms when frozen cultures are thawed.

Culture collections have played a fundamental role in the development of microbiology by ensuring that most of the types of microbes that have been described have been safely maintained for the present and future generations. Culture collections thus provide permanent laboratories where strains can be preserved and made available to scientists who wish to repeat, compare or extend work described in the literature.

7.4 TECHNICAL TERMS

Cultures, Preservation, Sub-culturing, Freeze-drying, Lyophilization, Cryogenic, Mineral oil, Glycerol, Porcelain beads, Gelatin discs, phosphorus pentoxide,

7.5 SELF ASSESSMENT QUESTIONS

Q.1. Describe the methods usually employed in microbial culture preservation.
Q.2. Write an essay on Maintenance and Preservation of microbial cultures.
Q.3. Write notes on periodic sub-culturing and cryogenic storage of cultures.
Q.4. Discuss the merits and demerits of various microbial preservation methods.

7.6 SUGGESTED READINGS

1. Microbiology - Prescott, L.M., Harley, J.P., and Klein, D.A.
2. Biology of Microorganisms - Brock, T.D. and Madigan, M.T
3. Principles of Microbiology - Atlas, R.M
4. The Physiology and Biochemistry of Prokaryotes - White, D
5. Bacterial Metabolism - Gottschalk, G

Prof. A. Amruthavalli

LESSON – 8

TECHNIQUES FOR STAINING BACTERIA

OBJECTIVE OF THE LESSON

The students will become familiar with staining of bacteria and understand the morphological shapes and arrangements of bacterial cells. And also will be able to differentiate the two principle groups of bacteria and also the different components of a bacterial cell.

STRUCTURE OF THE LESSON

8.1 INTRODUCTION

The resolution and magnification are important in microscopy, but the degree of contrast between structures to be observed and their backgrounds is equally important. Nothing can be seen without contrast, so special techniques have been developed to enhance contrast. Staining (dyeing) is the method used to increase the contrast between the specimen and the background. Smear preparation from a loopful of bacterial culture on glass slide followed by air drying and heat fixing is needed before staining the smear. Heat fixation helps in three ways: kill the organisms; make the organisms to adhere to the slide; and alters the organisms so that they accept stains readily. If the slide is exposed to high flame, the organisms will be charred and destroyed. If heat fixing is too little, the organisms may not stick and they may wash off during next steps. For capsule staining, only air-drying is to be followed but not heat fixing.

Some of the microorganisms cannot be visualized properly as they are transparent, colourless and difficult to see when suspended in an aqueous medium. Specimens are stained to increase

visibility, which help to identify microbes. Several stains and staining procedures, that employ dyes of different colors, are available to study the properties of various microorganisms. Staining techniques help the investigator to group major categories of microorganisms, examine structural and chemical differences in cellular structures and look at the parts of the cell. The chemical substances commonly used to stain bacteria are known as dyes or stains.

Staining

Staining is a process of making the microorganisms readily visible by the application of certain colored substances called as dyes. Although the living microorganisms can be directly examined with the light microscope, they often must be fixed and stained to increase the visibility, accentuate specific morphological features, and preserve them for future study. The stained cells seen in a microscope should resemble living cells as closely as possible. In the staining procedure, fixation is a process by which the internal and external structures of cells and microorganisms are preserved and fixed in position. It inactivates enzymes that might disrupt cell morphology and toughens cell structures so that they do not change during staining and observation. During fixation, a microorganism is usually killed and attached firmly to the microscope slide.

There are two fundamentally different types of fixation –

(1) Heat-fixation: Bacterial smears are heat-fixed by gently flame heating an air-dried film of bacteria. This adequately preserves overall morphology but not the structures within the cells.

(2) Chemical fixation: Used to protect fine cellular substructures and the morphology of larger, more delicate microorganisms. Chemical fixatives penetrate cells and react with cellular components, usually proteins and lipids, to render them inactive, insoluble, and immobile. The common fixative mixtures contain such components as ethanol, acetic acid, mercuric chloride, formaldehyde and glutaraldehyde.

Dyes

Dyes are classified as natural or synthetic. Synthetic dyes are used for bacterial stain preparations. Chemically a dye is defined as an organic compound containing a benzene ring plus a chromatophore and auxochrome group. Chromatophore gives colour to the dye and they bind the cells by ionic, covalent or hydrophobic bonding. Based on the ionic charge, dyes can be divided into acidic, basic and neutral.

Basic dyes: These dyes are cationic and have positively charged groups usually some form of pentavalent nitrogen, and are generally sold as chloride salts. They bind to negatively charged molecules like nucleic acids and many proteins. Because the surfaces of bacterial cells also are negatively charged, basic dyes are most often used in bacteriology. Ex: Methylene blue, Basic fuchsin, Crystal violet, Safranin and Malachite green.

Acidic dyes: These dyes are anionic and possess negatively charged groups such as carboxyls (-COOH) and phenolic hydroxyls (-OH). Acid dyes, because of their negative charge, bind to positively charged cell structures.
Ex: Eosin, Rose bengal and Acid fuchsin.

Neutral stains: These are formed by mixing together aqueous solutions of certain acidic and basic dyes. The coloring matter in neutral stains is present in both negatively and positively charged components.
The pH may alter the staining effectiveness since the nature and degree of the charge on cell

components change with pH. Thus anionic dyes stain best under acidic conditions when proteins and many other molecules carry a positive charge; and basic dyes are most effective at higher pHs.

There are different kinds of staining procedures, Negative staining, Simple staining, and Differential staining.

8.2 NEGATIVE STAINING

In negative staining technique, a simple stain is used that does not stain the bacteria but stains the background. This staining requires the use of an acidic stain such as Nigrosin or India ink. In negative staining procedure for light microscopy, the stain which is acidic, with its negatively charged chromogen repelled by the microorganisms with the negative charge on their surfaces. The unstained bacterial cells appear transparent upon examination against the stained background. Negative staining is advantageous for two reasons. Cells appear less shriveled or distorted because heat fixing is not done, their natural size and shape can be seen, capsulated bacteria and some spirilli can be observed by this technique. For electron microscopy, negative stains include heavy metal salts such as uranyl acetate, Uranyl formate, Phosphotungstic etc., are used.

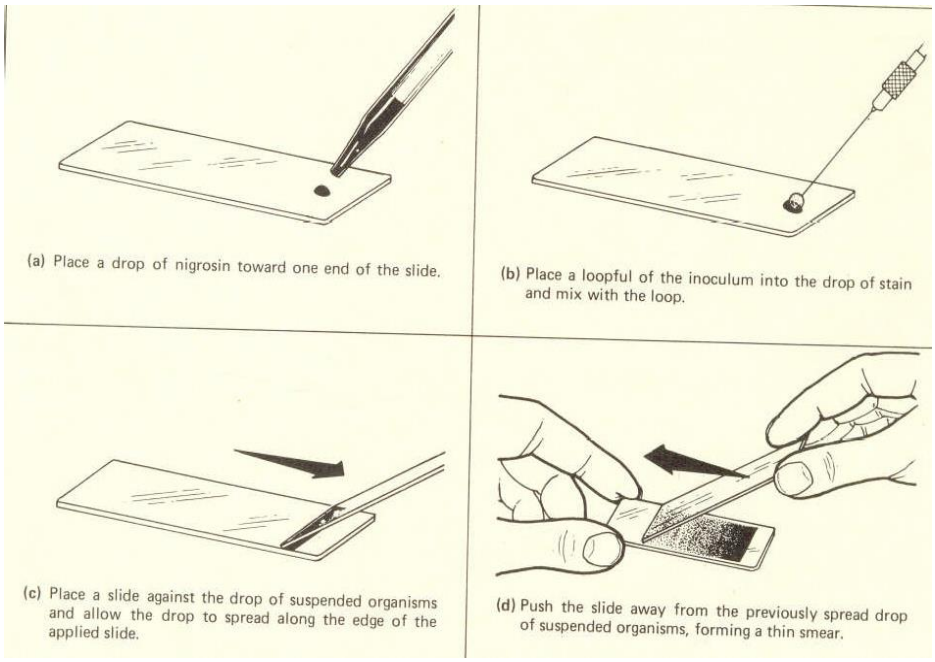


Figure-8.1: Procedure of Negative staining

Procedure

With the help of a sterile inoculating loop, transfer a loopful of inoculum from the broth culture on to the slide at one end. Then add one drop of nigrosin stain to the inoculum and mix it with the loop. Never heat-fix the slide. Take another clean slide, place it against the drop of mixture at 30^{o} angle and spread the mixture (suspension + culture) into a thin wide smear by pushing top slide to the left in the forward direction along the entire surface of the bottom slide (Fig. 8.1). The thickness of the film should be uniform. Air dry the smear and observe the slide for bacterial cells under the microscope.

8.3 SIMPLE STAINING

In simple staining, the smear of cells is stained by the application of a single staining reagent and all cells and structures generally stain the same colour, regardless of type. The staining procedure may be positive, in which the stain is attracted to the cells and take on the colour. This technique is used to determine cell morphology, size and arrangement of bacterial cells. In positive staining procedures for light microscopy, basic stains with positively charged chromophore (from Greek ***Chroma*** meaning colour; coloured portion of the stain molecule) that is attracted to the negatively charged structures like nucleic acids and cell wall components that carry a negative charge. A stain such as methylene blue has a blue chromophore, resulting in positive blue staining of the microorganisms. Exposure time varies among bacterial species for example crystal violet-2-60 seconds, carbol fuchsin-15-30 seconds and methylene blue-15-120 seconds.

Procedure

Take a clean glass slide, wash and dry it. Prepare bacterial suspension and place a loopful of suspension on the slide. Heat-fix the smear and apply about 5 drops of a stain for the designated period. Pour off the stain and wash the smear gently under slow running tap water. Blot dry the slide with blotting paper and examine the slide under oil-immersion objective.

8.4 DIFFERENTIAL STAINING

Differential staining procedures divide bacteria into separate groups based on staining properties. In differential staining procedures, multiple staining reactions are involved and specific types of microorganisms and particular structures of a microorganism exhibit different staining reactions that can be readily distinguished by their different colours. They distinguish between structures within a cell and types of cells by staining them different colours. This differential staining separates microorganisms into Gram positive and Gram negative, capsulated and non-capsulated, spore formers and non- spore formers and acid fast and non-acid fast bacteria. It is also useful to visualize the structures like capsule, spore, flagella etc.

8.4.1 Gram staining

The Gram staining, developed in 1884 by the Danish Physician Dr. Christian Gram, is the most widely employed staining method in bacteriology. It divides bacteria into two major groups, Gram positive and Gram negative. The Gram staining technique uses four different reagents that are applied to a heat fixed smear (Fig. 8.2). In the first step, the bacterial smear is stained with the basic dye crystal violet, the primary stain. It is followed by treatment with an iodine solution functioning as a ‘mordant’ which forms an insoluble complex with primary stain which is called as ‘crystal violet-iodine (CV-I)’ complex. This CV-I complex increases the interaction between the cell and the dye, so that the cell is stained more strongly. In the next step, smear is decolorized by washing with ethanol (decolorizing agent). This step generates the differential aspect of the Gram stain; the Gram positive bacteria retain the crystal violet, whereas Gram negative bacteria lose their crystal violet and become colorless. Finally, the smear is counterstained with a basic dye, safranin which colors Gram negative bacteria pink or red and leaves Gram positive bacteria as dark purple.

The difference in staining responses to Gram stain is related to chemical and physical differences in their cell walls. This decolorizing agent serves a dual function as a lipid solvent and as a protein-dehydrating agent. Its action is determined by the lipid concentration of the

microbial cell walls. The Gram negative bacterial cell wall is thin, complex multilayered structure with high lipid contents in addition to protein and mucopeptides. The high lipid concentration found in outer layers of the cell wall is dissolved by alcohol, creating large pores in the cell wall that do not close on dehydration of cell wall proteins. This facilitates release of the unbound CV-I complex, leaving these cells colourless or unstained.

In Gram positive cells, the low lipid concentration is important for the retention of the CV-I complex. The small amount of lipid content is readily dissolved by the action of alcohol. This causes formation of minute pores in cell wall. These are closed by the dehydrating action of alcohol. Because of this, the tightly bound primary stain is difficult to remove and the cells remain purple. Safranin, the final counter stain, gives red color to the decolorized cells of Gram negative bacteria. However, Gram-positive cells retain the purple colour of the primary stain.

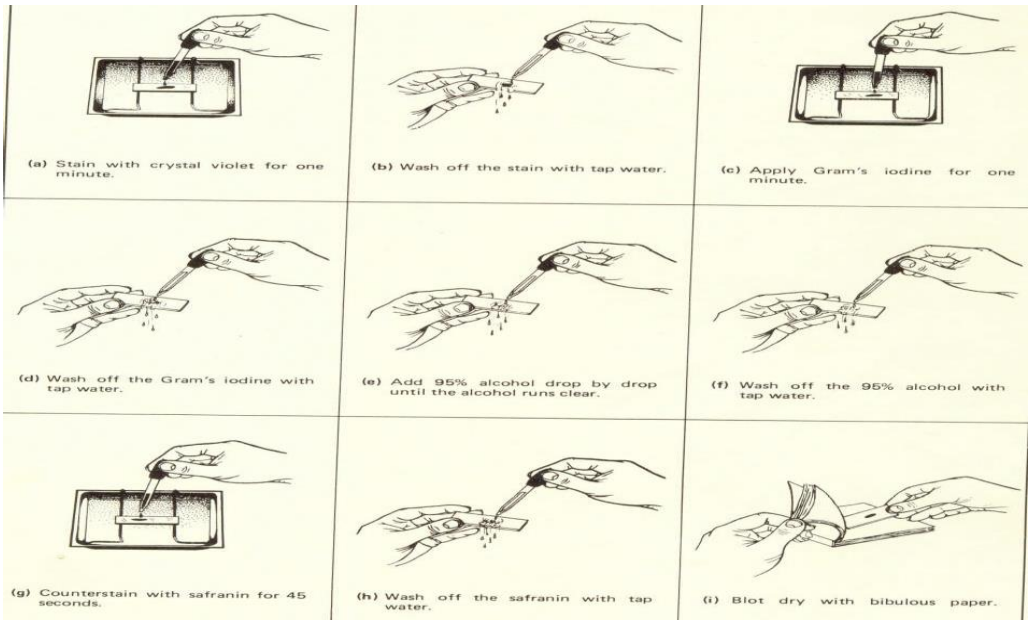
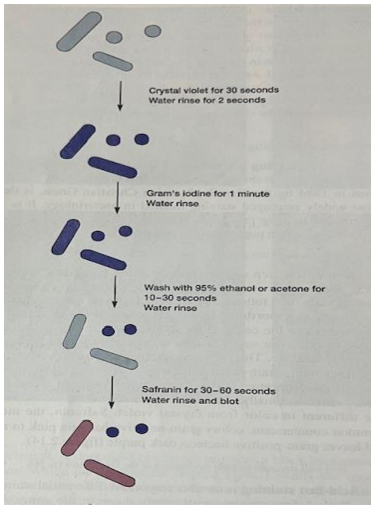
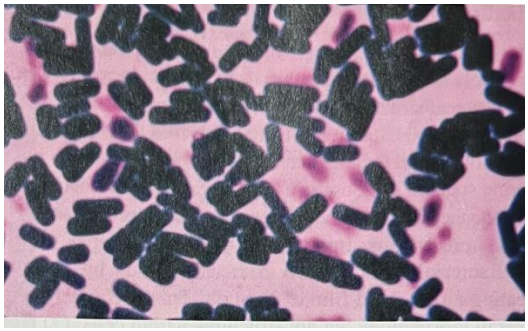


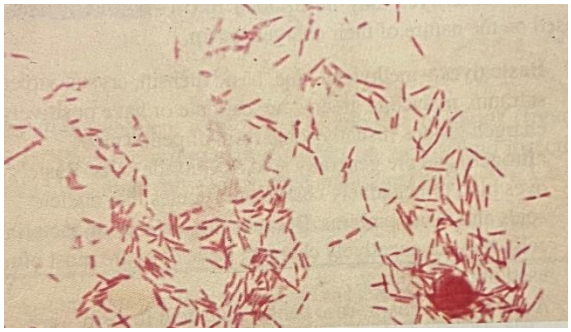
Figure-8.2: Gram's staining procedure



Steps in Gram staining procedure and appearance of cells at each step



Gram positive bacterial cells after staining



Gram negative bacterial cells after staining

8.4.2 Acid-fast staining or Ziehl-Neelsen staining

Acid-fast staining is another important differential staining procedure (Fig. 8.3). The characteristic difference between *Mycobacterium* and other bacteria is the presence of a thick waxy wall that makes the penetration of stains extremely difficult. In this procedure, once the carbol- fuchsin stain has penetrated with the aid of heat and phenol, acid-fast cells are not easily decolorized by an acid-alcohol (3% HCl + 95% ethanol) wash and hence remain red. This is due to the high lipid content of acid-fast cell walls; in particular, 'mycolic acid'. This branched chain of hydroxyl lipids appears responsible for acid-fastness. The non-acid-fast bacteria are decolorized by acid-alcohol and thus are stained blue by methylene blue counter stain. This method is used particularly to identify *Mycobacterium tuberculosis* and *M. leprae*, the pathogens responsible for tuberculosis and leprosy, respectively.

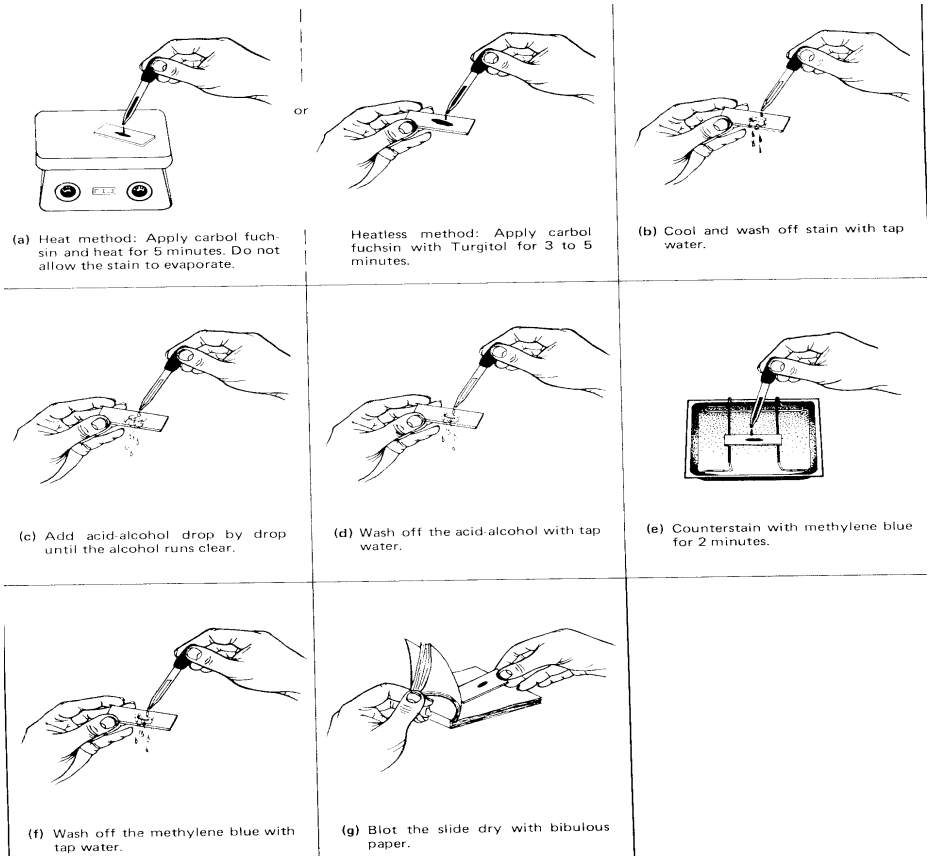


Figure-8.3: Acid-fast staining procedure

Procedure

On a clean glass slide, bacterial smear is prepared and allow the smear to air dry and heat fix. The smear stained with carbol fuchsin and place on a warm hot plate, allowing the preparation to steam for 5 minutes. Do not allow stain to evaporate and prevent stain from boiling by adjusting the hot plate temperature. After cooling the slide, decolorize the smear with acid-alcohol. Then add the carbol fuchsin reagent drop by drop till fails to wash from smear. Wash the smear with tap water and counter stain with methylene blue for 2 minutes. Again wash the smear with tap water, blot dry the slide and examine under microscope with oil immersion.

Staining with the fluorescent dye, ammonia – rhodamine also has become important in the clinical microbiology laboratory for the detection of acid-fast mycobacteria. This stain binds to the mycolic acids of the cell walls of mycobacteria and resists decolourization with acid-alcohol. When viewed with a fluorescence microscope, acid-fast bacteria fluoresce orange- yellow against a black background.

8.4.3 Endospore staining

Another key differential staining procedure reveals the presence or absence of bacterial endospores. Members of the anaerobic genera *Clostridium* and *Desulfotomaculum* and the aerobic genus *Bacillus* are examples of organisms that have metabolically active vegetative cells or metabolically inactive and highly resistant cell types called spores. Spores that are produced inside a parent cell are called endospores. Bacterial endospore is a heat resistant structure even to boiling water. For example, *C. botulinum* sometimes survives the heat treatment of canning and causes a food poisoning disease known as botulism, when contaminated food is eaten.

The spores are differentially stained by using special procedures that help dyes penetrate the spore wall. Endospores are not easily stained and in normal simple staining procedures the endospore remains colourless while the rest of the cell is stained. Primary stain in spore stain is Malachite Green. Endospores can be stained using malachite green and steam to drive the stain into the endospore. Spore will not accept the primary stain easily. To further penetration, application of heat is required. Once the spore accepts the malachite green, it cannot be decolorized by tap water. In a typical endospore staining procedure, water is used as decolorizing agent. This removes only excess staining. The spores remain green and vegetative cells become colourless as water washes the primary stain out of vegetative cells but not the endospores. The counter stain is Safranin, which gives colour to the decolourized vegetative cells. At the end of endospore staining procedure, the spores retain green and the vegetative cells absorb counterstain and appear red, permitting differentiation of the endospore from the vegetative cell (Fig. 8.3).

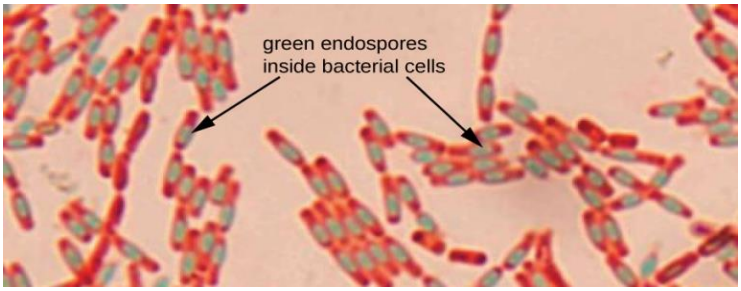


Figure-8.3: Bacterial endospores after staining

Procedure

Made a smear of bacterial culture on a clean glass slide and air dried. Flooded the smear with malachite green. Slide is heated under steaming for 5 minutes adding more stain to the smear from time to time. Slide is washed under slow running tap water. Counter stained the smear with safranin for 30 seconds. Washed the slides with distilled water and blot dried the slide with blotting paper. Examined the slide under oil-immersion objective. Endospores stain green and the vegetative cells stain red (Fig. 8.4).

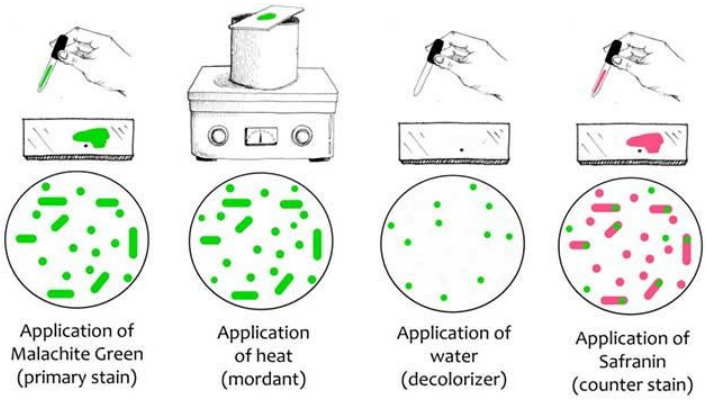


Figure-8.4: Procedure of bacterial endospore staining

8.4.4 Capsule staining

Some bacterial cells are surrounded by a mucilaginous, viscous layer around the cell wall and is called as capsule. It is not common to all organisms. Cells that have a heavy capsule are generally virulent and produce disease. Capsule gives protection against phagocytic activities of host cells. Chemically, the capsular material is a polysaccharide, a glycoprotein or a polypetide and the composition vary among species of bacteria. Capsules occur in pneumonia causing strains of *Streptococcus pneumoniae***,** *Haemophilus influenzae* and *Klebsiella pneumoniae.* Some ***Bacillus*** species, in contrast, produce capsules composed of glutamic acid, largely in the D form, rather than polysaccharide capsules.

Capsule staining is more difficult than other types of differential staining procedures because the capsular materials are water-soluble and may be removed with vigorous washing. Smear should not be heated, because the resultant cell shrinks and creates a clear zone around the organism that is an artifact that is mistaken for the capsule. The capsule stain uses two reagents, Crystal Violet and Copper Sulfate (20%). Crystal Violet is applied to a non-heat-fixed smear. The cell and the capsule will take on the dark colour. Copper Sulfate (20%) is the decolourizing agent and also the counterstain. The capsule is non-ionic; the primary stain adheres to the capsule without binding to it. Since the capsule is water-soluble, copper sulfate is used to wash the purple primary stain out of the capsular material without removing the stain that is bound to the cell wall. At the same time, it acts as a counterstain as it is absorbed into the decolorized capsular material. The capsule will now appear light blue in contrast to the deep purple colour of the cell. The presence of a capsule in an ***Acinetobacter*** species can also be demonstrated by negative staining with india ink observed by Phase contrast microscopy. The india ink does not penetrate the capsule and so it is revealed in outline as a light structure on a dark background (Fig.8.5).

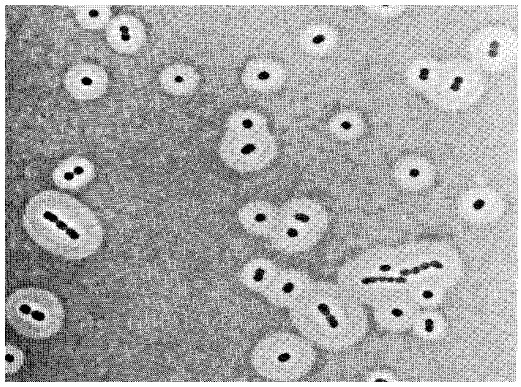


Figure-8.5: Negative staining of bacterial capsules

8.4.5 Flagella staining

Many bacteria are motile as they have one or more very fine thread like, filamentous appendages called flagella. Bacterial flagella are long, thin appendages free at one end attached to the cell at the other end. The presence, location and the number of flagella are useful in identification and classification of bacteria. As they are very thin (about 20 nm) a single flagellum can never be seen directly with the light microscope but only after staining with special flagella stains that increase their diameter. So they are made thick first by using some chemicals called mordents. After mordent's treatment, bacteria are stained with dye. Flagella staining reveal the number and arrangement of flagella on bacteria, which is vital information for identifying many species. Successful flagellum staining is an art that develops only with practice.

Procedure

Bacterial suspension is prepared and a loopful of the suspension is placed to prepare bacterial smear. Allowed the smear to air dry at room temperature without heat fixation. Smear is flooded with mordant, tannic acid or potassium alum, for 10 minutes which will coat the flagella. Then slide is washed with distilled water and flooded with basic fuchsin for 15 minutes. Again washed the slide with distilled water, air dried and examined under microscope with immersion oil. This method is referred as Gray method. In another method, Leifson method, pararosaline stain is used instead of basic fuchsin.

8.5 SUMMARY

Staining increases the contrast between a specimen and its background so it can be seen under the microscope. Stains are dyes used to increase contrast. Most stains are effective only after microorganisms are fixed / killed and attached to a microscope slide. Basic dyes are composed of positively charged ions, and acidic dyes are composed of negatively charged ions. A mordant is a compound that increases a specimen's affinity for a dye or that coasts a structure to make it larger. A simple stain used only one dye. A differential stain involves two stains, a primary stain and a counter stain. The Gram stain, a type of differential stain, distinguishes between Gram-positive and Gram-negative bacteria, reflecting differences in their outer surfaces. The acid-fast stain or Ziehl-Neelsen stain is a differential stain that colours mycobacteria and actinomycetes red and all other bacteria blue. The flagella or Leiflson flagella stain uses stains and mordents to thicken flagella, thread like appendages used for motility. Negative staining is used to reveal the protective capsule some bacteria have. Spore formers can be differentiated from non-spore formers by spore staining procedure. After endospore staining, vegetative cells appear red and spores appear green.

8.6 TECHNICAL TERMS

Negative staining, Simple staining, Gram staining, Acid-fast staining, Primary stain, Counter stain, Mordant, Malachite green.

8.7 SELF ASSESSMENT QUESTIONS

Q. 1 Give a detailed explanation on Negative staining and Simple staining.
Q. 2 What is Gram staining? Explain the procedure of staining and its significance.
Q. 3 Explain differential staining methods for staining the structures of a bacterial cell.
Q. 4 Describe the acid-fast staining technique and its significance.

8.8 SUGGESTED READINGS

1) Microbiology - L. M. Prescott, John P. Harley and Donald A. Klein, Wm. C. Brown Publishers.
2) Brock Biology of microorganisms - Prentice Hall International, Inc.
3) Text Book of Microbiology - R. Anathanarayan and C.K. Jayaram Panider, Orient Longman Ltd.
4) Microorganisms in our world - Ronald M. Atlas, Mosby-Year, Inc.
5) Introduction to Microbiology - John L. Ingraham and Catherine A. Ingraham, Thomson Books/Cole.
6) General Microbiology - Roger Y. Stanier, John L. Ingraham, Mark L. Wheelis and Page R. Painter International Edition.

Prof. A. Amruthavalli

LESSON - 9

BACTERIAL NUTRITION

OBJECTIVE OF THE LESSON

To understand the nutritional types of bacteria based on their nutritional requirements, the various nutrient elements required by them and the modes of nutritional transport into their cells form the external environment.

STRUCTURE OF THE LESSON

9.1 INTRODUCTION

Bacteria need a variety of chemical substances to obtain energy and to construct the new cellular components. The basic elements or constituents of a cell come from the natural environment and are transformed by the cell into characteristic constituents of the cell. The substances used in biosynthesis and energy production by the cell are called nutrients and therefore required for growth. The process of building of cell constituents by the cell from the nutrients obtained from its environment is called Anabolism or Biosynthesis. Anabolism is an energy-required process and the cell acquires this energy either directly from the light or from the breakdown of organic compounds or inorganic compounds into simpler substances. This process of breakdown of organic or inorganic chemicals into simpler constituents is known Catabolism.

9.2 NUTRITIONAL CLASSES OF BACTERIA

Besides the nutrient elements, bacteria also require sources of energy, hydrogen and electrons for their growth. Basing on the sources from which these requirements are available, bacteria

can be grouped into different nutritional classes.

Energy sources

1) Light ---------- Phototrophs
2) Organic or Inorganic compounds ---------- Chemotrophs

Hydrogen or Electron sources

1) Reduced inorganic substances ---------- Lithotrophs
2) Organic molecules ---------- Organotrophs

Principal Carbon source

1) Carbon di-oxide ---------- Autotrophs
2) Reduced, preformed, organic molecules --- Heterotrophs

Bacteria exhibit a great diversity in metabolism. Basing on the primary sources of energy, hydrogen and/or electrons and carbon used, bacteria are categorized into four main nutritional classes viz., Photolithotrophic autotrophs, Photoorganotrophic heterotrophs, Chemolithotrophic autotrophs and Chemoorganotrophic heterotrophs. Of these, photolithotrophic autotrophs and chemoorganotrophic heterotrophs include large majority of the organisms that are studied well. The other two classes include fewer organisms but are ecologically very important. In response to environmental changes, a particular species belonging to a particular nutritional class may alter its nutritional or metabolic pattern.

9.2.1 Photolithotrophic autotrophs

Also called simply as Photoautotrophs. Organisms use the light as energy source and CO_2 as carbon source. Inorganic molecules like hydrogen, hydrogen sulfide and elemental sulfur are used as electron donors. E.g.: Purple and green sulfur bacteria and Cyanobacteria.

9.2.2 Photoorganotrophic heterotrophs

Organisms use light as energy source and organic matter as electron donor as well as carbon source. These organisms are common inhabitants of polluted lakes and streams. E.g.: Purple non-sulfur bacteria and Green non-sulfur bacteria.

9.2.3 Chemolithotrophic autotrophs

Organisms derive both energy and electrons for biosynthesis from reduced inorganic compounds such as iron, nitrogen and sulfur molecules. The carbon source is CO_2. These chemolithotrophs greatly contribute to the chemical transformations of elements that continually occur in ecosystem. The best known examples of this class are Sulfur- oxidizing bacteria, Hydrogen bacteria, Nitrifying bacteria and Iron bacteria.

9.2.4 Chemoorganotrophic heterotrophs

This class is also referred as chemoheterotrophs and sometimes even as heterotrophs. They use the organic compounds as sources of energy, hydrogen, electron and carbon for biosynthesis. In most of the cases, the same organic nutrient will satisfy all these requirements. This group includes most non-photosynthetic bacteria.

9.3 NUTRITIONAL ELEMENTS

The approximate elementary composition of the bacterial cell is 50% carbon, 20% oxygen, 14% nitrogen, 8% hydrogen, 3% phosphorus, 1% sulfur, 1% potassium, 1% sodium, 0.5% calcium, 0.5% magnesium, 0.5% chlorine, 0.2% iron and others account to 0.3%. This cell composition shows that over 95% of cell dry weight is made up of a few major elements.

Basing on the amounts in which required, the elements are grouped into two main categories viz., Macroelements and Microelements.

Macroelements

Also be called major elements or macronutrients. This category includes elements such as carbon, oxygen, nitrogen, hydrogen, sulfur, phosphorus, potassium, magnesium, calcium and iron and contributes to over 95% of the cell dry weight. The first six (C, O, H, N, S, and P) are components of carbohydrates, lipids, proteins, and nucleic acids. The remaining four macroelements exist in the cell as cations and play a variety of roles.

Element	**Usual form in nature**	**Chemical form in culture media**	**Functions**
Carbon	CO_2; Organic compounds	Glucose, malate, acetate, pyruvate etc; complex mixtures (yeast/peptone)	Backbone for all cell organic molecules
Hydrogen	H_2O; Organic compounds	H_2O; Organic compounds	pH maintenance, Hydrogen bonds in macromolecules, prime force in oxidation-reduction reactions
Oxygen	H_2O; O_2; Organic compounds	H_2O; O_2; Organic compounds	Major component in carbohydrates, lipids, proteins
Nitrogen	NH_3, NO_3^-, N_2, Organic Nitrogen compounds	Inorganic: NH_4Cl, $(NH_4)_2SO_4$, KNO_3, N_2 Organic: Amino acids, nitrogen bases, N-containing organic compounds	Major constituents of proteins and nucleic acids; present in peptidoglycan of cell wall
Phosphorus	PO_4^{3-}(inorganic), Organic phosphates	KH_2PO_4, Na_2HPO_4	Present in nucleic acids, phospholipids, ATP, several cofactors, some proteins and other cell component
Sulphur	H_2S, SO_4^{2-}, Organic S compounds, Metal sulphides	Na_2SO_4, $Na_2S_2O_3$, Na_2S, Cysteine, other organic sulphur compounds	Play a structural role in cysteine and methionine amino acids; present in a number of vitamins like thiamine, biotin, lipoic acid

Potassium	K^+ solution /various K salts	KCl, KH_2PO_4	Important in enzyme action and maintain osmotic potential and electrical potential within the cell
Magnesium	Mg^{2+}solution/various Mg salts	$MgCl_2$, $MgSO_4$	For stabilization of ribosomes, cell membranes and nucleic acids; for the activity of many enzymes that involve in phosphate transfer; integral part of chlorophyll molecule
Calcium	Ca^{2+}solution/$CaSO_4$/other Ca salts	$CaCl_2$	Actually not essential for growth of many bacteria, in certain higher bacteria it forms deposits of calcium carbonate and calcium oxalate; gives stability to some extracellular enzymes and to cell
Iron	Fe^{2+}/Fe^{3+}solution /FeS, $Fe(OH)_3$/other Fe salts.	$FeCl_3$, $FeSO_4$, various chelated iron solutions	Plays a major role in cellular respiration; key component of cytochromes, ferridoxins and iron-sulphur proteins that involves in electron transport

Microelements: These are also called micronutrients or trace elements. These trace elements are normally a part of enzymes and cofactors, and they aid in the catalysis of reactions and maintenance of protein structure. These microelements such as manganese, zinc, cobalt, molybdenum, nickel and copper are needed by most of the cells.

Element	**Function**
Zinc	Plays a structural role in many enzymes including carbonic anhydrase, alcohol dehydrogenase, RNA and DNA polymerases, alkaline phosphatase, aldolase; in DNA binding proteins; holds the protein subunits together in proper configuration for enzyme activity
Manganese	Activator of many enzymes; component in certain superoxide dismutases; component in water-splitting enzyme of photosystem II in oxygenic phototrophs
Molybdenum	Present in certain enzymes viz., molybdoflavoproteins; in nitrogenases; in formate dehydrogenases

Copper	Play a role in certain enzymes involved in respiration; present in cytochrome oxidase and oxygenases; in some superoxide dismutases; involves in synthesis of melanin
Cobalt	Constituent of B_{12}
Nickel	Present in urease and hydrogenases; part of coenzyme F_{430} of methanogens; required for autotrophic growth of hydrogen-oxidizing bacteria

Growth factors

Many photoautotrophic microorganisms often grow well and reproduce when minerals and sources of energy, carbon, nitrogen, phosphorus and sulfur are supplied. These organisms have the enzymes and pathways necessary to synthesize all cell components. But many microorganisms lack one or more essential enzymes to build up their organic cell constituents as they cannot synthesize them. Any organic compound, other than the carbon and energy source, required essentially but cannot be synthesized by organisms is called growth factor. So, these substances must be provided as nutrients.

There are three major classes of growth factors viz., (1) amino acids, (2) purines and pyrimidines, and (3) vitamins. The amino acids are required for protein synthesis whereas purines and pyrimidines are needed for nucleic acid synthesis. Vitamins are the small organic molecules that usually make up all or part of enzyme cofactors, and very small amounts sustain growth. Some microorganisms like *Enterococcus faecalis*, a lactic acid bacterium, require as many as eight different vitamins for growth. Knowledge of the specific growth factor requirements of many microorganisms makes possible quantitative growth response assays for a variety of substances.

Vitamin	**Functions**	**Example**
Biotin	Carboxylation (CO_2 fixation); Carboxyl transfer; Fatty acid biosynthesis	*Leuconostoc mesenteroides*
Cobalamin (B_{12})	Molecular rearrangements; reduction and transfer of single carbon fragments; synthesis of deoxyribose	*Lactobacillus* spp.
Folic acid	One-carbon metabolism; methyl group transfer	*Enterococcus faecalis*
Thiamine (B_1)	Pyruvate decarboxylation; α-keto acid oxidation	*Bacillus anthracis*
Riboflavin (B_2)	Precursor of FMN, FAD in flavoproteins involved in electron transport	*Caulobacter vibrioides*
Pyridoxine (B_6)	Amino acid and keto acid transformations	*Lactobacillus* spp.
Pantothenic acid	Precursor of coenzyme A; activation of acetyl and other acyl derivatives; pyruvate oxidation; fatty acid metabolism	*Proteus morganii*

Nicotinic acid (Niacin)	Precursor of NAD and NADP; electron transfer in oxidation-reduction reactions; and Dehydrogenations	*Brucella abortus*
Lipoic acid	Transfer of acyl groups in decarboxylation of pyruvate and α-ketoglutarate	*Lactobacillus casei*

9.4 NUTRIENT TRANSPORT IN BACTERIA

Nutrients and other substances needed by the cell must penetrate the cell boundary layers before they can be metabolized. The cell wall does not present much of a barrier to small molecules and ions, but it excludes large molecules with a relative molecular mass above 600 daltons. The cell boundary component that governs the uptake of most substances into the cell is the cytoplasmic membrane. Transport of nutrients through the cytoplasmic membrane is usually specific; only those nutrients are taken up by the cell for which transport systems are available. With few exceptions, the transport mechanism is dependent on specific permeases and translocases. These are the membrane proteins, and the names indicate that they exhibit some of the properties of enzymes.

The term transport can have several quite different meanings in cell biology. Two main types of transport processes that occur in or through the cytoplasmic membrane such as primary transport and secondary transport are distinguishingly considered. Primary transport consists of those processes that lead to the transfer of ions like $H^{+,}$ Na^{+}, K^{+}, and hence to alterations in the electrochemical potential. The term secondary transport is applied to all processes resulting in the uptake (influx) or outflow (efflux) of cellular metabolites, which are driven by electrochemical potential gradients. In view of the enormous variety of nutrients and the complexity of the task, it is not surprising that microorganisms make use of several different transport mechanisms. The most important of these are simple diffusion, facilitated diffusion, active transport, and group translocation.

Simple or Passive diffusion

The non-specific penetration of substances into the cell is called simple or passive diffusion (Fig- 9.1). It is a process in which molecules move from a region of higher concentration to one of lower concentration because of random thermal agitation. The rate of passive diffusion is dependent on the size of the concentration gradient between a cell's exterior and its interior. A fairly large concentration gradient is required for adequate nutrient uptake by passive diffusion and the rate of uptake decreases as more nutrient is acquired unless it is used immediately. Uptake of sugars by passive diffusion has never been demonstrated. Apparently water, non-polar toxins, inhibitors and other substances that are not part of the normal intracellular milieu are taken up by passive diffusion. Thus passive diffusion is an inefficient process and is not employed extensively by microorganims.

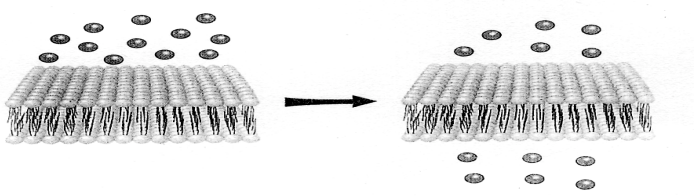


Figure-9.1: Simple diffusion

Facilitated diffusion

The transportation of a substance into the cell along its concentration gradient, i.e. towards equilibrium between the external and internal concentrations. In most of the cases, this process is mediated by a substrate-specific permeases which are embedded in the plasma membrane. Because a carrier aids the diffusion process, it is called facilitated diffusion (Fig-9.2). The rate of this transport is governed, over a wide range, by the substrate concentration in the medium. The rate of facilitated diffusion increases with the concentration of the diffusing molecule than that of passive diffusion. Facilitated diffusion is independent of metabolic energy and the nutrient cannot accumulate inside the cell against a concentration gradient. Carrier proteins are selective and specific to transport only closely related solutes. It appears that carrier protein complex spans the membrane. After the solute molecule binds to the outside, the carrier may change conformation and release the molecule on the cell interior. Subsequently the carrier molecule change back to its original shape and be ready to pick up another molecule. This facilitated diffusion does not seem to be important in prokaryotes. Glycerol is transported by facilitated diffusion in *E. coli*, *Salmonella typhimurium*, *Pseudomonas* spp., *Bacillus* spp. and many other bacteria.

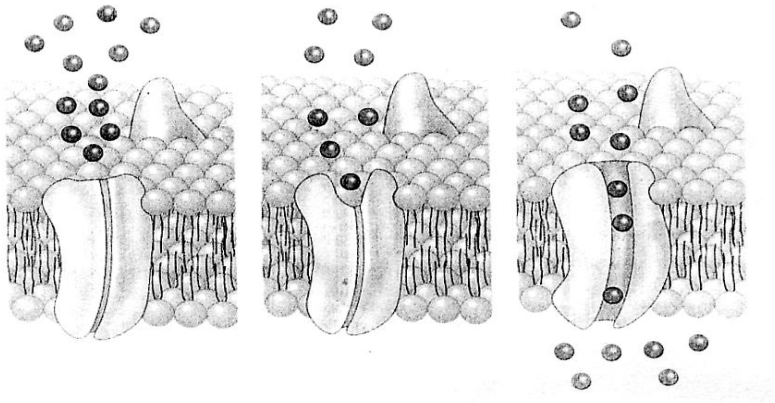


Figure-9.2: Facilitated diffusion

Active transport

The transportation of solute molecules to higher concentrations, or against a concentration gradient, with the use of metabolic energy input is referred as active transport (Fig-9.3). Active transport resembles the facilitated diffusion in some ways as it involves the protein carrier activity. The carrier proteins bind particular solutes with great specificity for the molecules transported. Active transport is characterized by the carrier saturation effect at high solute concentrations. The active transport differs from facilitated diffusion in its use of metabolic energy and in its ability to concentrate substances. Metabolic inhibitors that block energy production will inhibit active transport but will not affect facilitated diffusion, at least for some time.

Bacteria use proton motive force to drive active transport. The membrane transport proteins responsible for this process lack special periplasmic solute-binding proteins. The lactose permease in *E. coli* transports lactose molecules inward as a proton simultaneously enters the cell. Such linked transport of two substances in the same direction is called symport. *E. coli* also uses proton symport to take up amino acids and organic acids like succinate and malate. Protonmotive force can power active transport indirectly, often through the formation of a sodium ion gradient. In this case, the sodium transport system pumps sodium outward in

response to the inward movement of protons. Such linked transport in which the transported substances move in opposite directions is termed antiport. The sodium gradient generated by this proton antiport system then drives the uptake of sugars and amino acids.

It seems reasonable for a microorganism to have only one transport system for each nutrient. But the bacterium, *E. coli*, has at least five transport systems for the sugar galactose, three systems each for the amino acids glutamate and leucine, and two potassium transport complexes. When there are several transport systems for the same substance, the systems differ in their energy source, in their affinity for the solute transported, and in their nature of regulation. Figure 9.4 shows the uniport, antiport and symport systems.

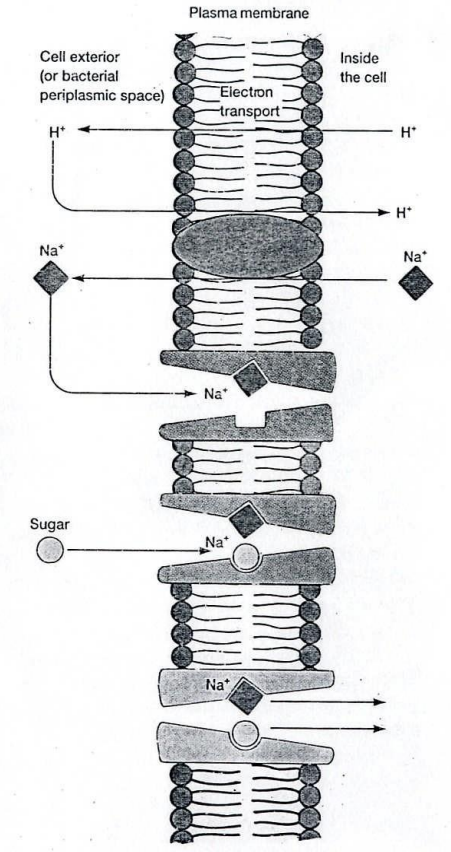


Figure-9.3 Active transport mechanism

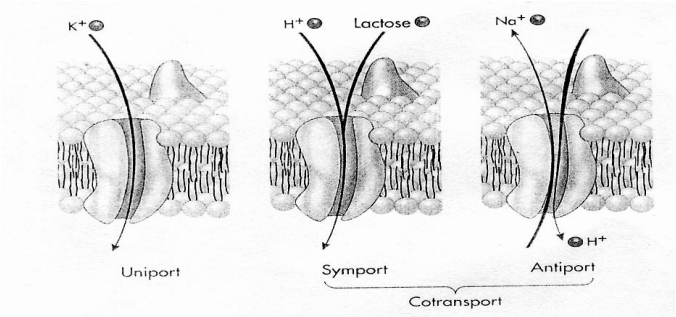


Figure- 9.4: Transport across the Cytoplasmic Membrane- Uniport, Symport, Antiport

Group Translocation

The process in which a molecule is transported into the cell while being chemically altered is called group translocation (Fig.-9.5). The best known group translocation system is the phosphoenolpyruvate: sugar phosphotransferase system (PTS). This system transports a variety of sugars into prokaryotic cells while phosphorylating them using phosphoenolpyruvate (PEP) as the phosphate donor. The PTS system is quite complex and consists of two enzymes and a low molecular weight heat-stable protein (HPr) in *E. coli* and *Salmonella typhimurium*. HPr and enzyme I (EI) are cytoplasmic. Enzyme II (EII) is more variable in structure and often composed of three subunits or domains. EIIA is cytoplasmic and soluble. EIIB is hydrophilic but frequently is attached to EIIC, a hydrophobic protein that is embedded in the membrane. A high-energy phosphate is transferred from PEP to enzyme II with the aid of enzyme I and HPr. Then, a sugar molecule is phosphorylated as it is carried across the membrane by enzyme II. With PTS enzyme II varies and transports only specific sugars, whereas enzyme I and HPr are common to all PTSs.

PTS are widely distributed in prokaryotes. Except for some species of *Bacillus* that have both the Embden-Meyerhof pathway and phosphotransferase systems, aerobic bacteria seem to lack PTSs. Members of the genera *Escherichia*, *Salmonella*, *Staphylococcus*, and other facultatively anaerobic bacteria have phosphotransferase systems. Some obligate anaerobic bacteria like *Clostridium* also have PTSs. *E. coli* takes up glucose, fructose, mannitol, sucrose, N-acetylglucosamine, cellobiose and other carbohydrates by group translocation.

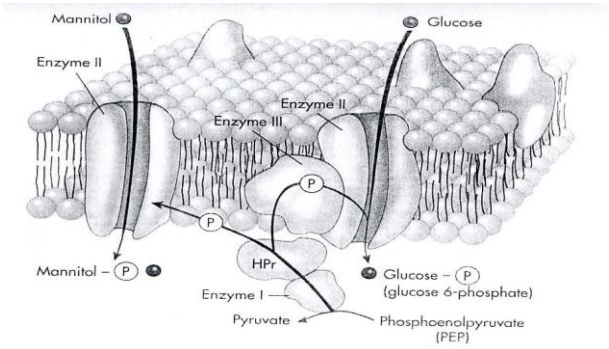


Figure-9.5: Group translocation mechanism

9.5 SUMMARY

Bacteria require nutrient materials that are used in biosynthesis and energy production. These materials are drawn by the bacteria from their surrounding environments. Bacteria can be categorized into nutritional classes based on the sources from which they acquire the carbon, energy and electrons. Accordingly the nutritional types of bacteria are photolithotrophic autotrophs, photoorganotrophic heterotrophs, chemolithotrophic autotrophs and chemoorganotrophic heterotrophs. Several elements require by bacteria in varying quantities as nutrients. Basing on the amounts or quantities in which they are required the elements are Macroelements (needed in larger quantities) and Microelements (needed in smaller quantities).

Various amino acids are also required by some bacteria as essential growth factors. The nutritional requirements of a bacterium are determined by the kind and number of its enzymes. So, the nutritional complexity reflects a deficiency in biosynthetic enzymes. Some

nutrients can enter the bacterial cells by simple passive diffusion. But a membrane carrier protein is usually required for the uptake of some nutrients. In facilitated diffusion the transport protein carries a molecule across the plasma membrane of the cell. Active transport systems use metabolic energy and membrane carrier protein for the movement of molecules into the cell. Bacteria also transport the organic molecules by modifying them in the process of group translocation.

9.6 TECHNICAL TERMS

Photolithotrophic autotrophs, Photoorganotrophic heterotrophs, Chemolithotrophic autotrophs, Chemoorganotrophic heterotrophs, Major elements, Minor elements, Growth factors, Simple diffusion, Facilitated diffusion, Active Transport, Group translocation.

9.7 SELF ASSESSMENT QUESTIONS

Q.1. Define nutrition and explain the types and functions of elements required by bacteria as nutrients.
Q.2. Explain the mechanisms of nutrient transport by the bacteria.
Q.3. Write a note on different nutritional classes of bacteria.
Q.4. Give short notes on growth factors.

9.8 SUGGESTED READINGS

1. Microbiology - Prescott, L.M., Harley, J.P., and Klein, D.A.
2. Biology of Microorganisms - Brock, T.D. and Madigan, M.T
3. Principles of Microbiology - Atlas, R.M
4. The Physiology and Biochemistry of Prokaryotes - White, D
5. Bacterial Metabolism - Gottschalk, G

Prof. V. Umamaheswara Rao

LESSON -10

BACTERIAL GROWTH AND ITS MEASUREMENT METHODS

OBJECTIVE OF THE LESSON

To study the pattern of bacterial growth in culture medium and also the methods employed for the measurement of growth in terms of cell numbers and cell mass.

STRUCTURE OF THE LESSON

10.1 INTRODUCTION

Growth in a biological system is defined as 'an increase in mass or size accompanied by the synthesis of macromolecules, leading to the production of new organized structure'. In the case of multinucleate, coenocytic microorganisms the nuclear divisions are not accompanied by cell divisions, so growth results in an increase in cell size but not in cell number. But in the case of many unicellular microorganisms, like bacteria, which multiply or divide by binary fission, 'growth' leads to a rise in cell number. So, in microbiology the growth is defined as an increase in the number of cells. Growth is an essential component of microbial function because of the finite life span of microbes in nature. Because of their small size, it is usually not convenient to investigate the growth and reproduction of individual microorganisms. Therefore, usual practice of investigation of growth is to follow the changes in the total population number from time to time.

10.2 BACTERIAL GROWTH

10.2.1 Growth Curve

The bacterial population generally shows a characteristic growth pattern when grown in a batch culture system or closed system. In this system, cells are incubated in a closed culture vessel with a single batch of the medium without the addition of fresh medium into vessel. In this set up, nutrient concentrations decline and concentrations of wastes increase. The growth pattern of bacteria reproducing by binary fission in the culture system follows a typical curve when a graph is plotted between the logarithm of cell number versus incubation time. The resulting curve is called bacterial growth curve which consists four distinct phases namely Lag phase, Exponential phase, Stationary phase and Death phase (Fig. 10.1).

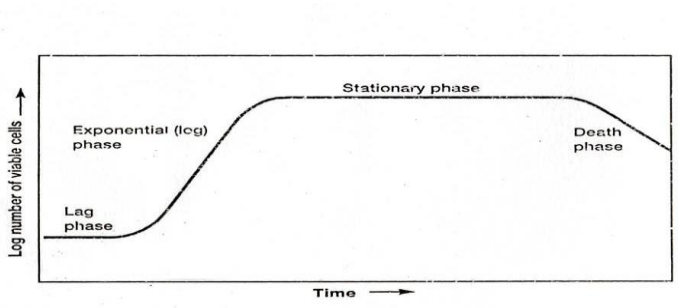


Figure-10.1: Bacterial growth curve

Lag Phase

When bacterial population is inoculated into fresh liquid culture medium, usually no immediate growth will occur. This period is called as lag phase and the increase in cell number of bacteria takes place only after this phase. During this phase, cells adjust to the new environment and undergo the synthesis of new components like essential cofactors, various enzymes which are required for the growth. This lag phase is an essential phase for a population prior to its cell division. The lag phase varies considerably in length with the condition of the microorganisms and the nature of the medium. This phase may be quite long if the inoculum is from an old culture or one that has been refrigerated. Inoculation of a culture into a chemically different medium also results in a longer lag phase. On the other hand, when a young, vigorously growing exponential phase culture is transferred to fresh medium of the same composition, the lag phase will be short or absent.

Log phase or Exponential phase

This phase is also called Log phase. During this phase, bacteria grow and divide at the maximum possible rate and the number of cells increases exponentially in a geometrical progression. The growth rate in this phase is dependent on the genetic potential of the organism, nature and composition of the medium and conditions of culturing. The exponential growth rate of one organism differ with that of the another organism. For a given organism, the growth rate during this exponential phase is constant as the cells divide and double in number at regular intervals. The growth curve rises smoothly rather than in discrete jumps in this phase. The population is most uniform in terms of chemical and physiological properties during this phase, therefore exponential phase cultures are usually used in biochemical and physiological studies.

Mathematical expression of growth

During the exponential phase each cell divides at constant intervals and thus population will double in number during a specific length of time called the generation time or doubling time. When culturing is started with the inoculation of one cell, increase in population occurs in a simple geometric progression of the number 2 as the cell number doubles for every generation. This increase can be expressed as a geometric progression as the following

$1 - 2^1 - 2^2 - 2^3 - 2^4 - 2^5 \text{--------} 2^n$

the exponent 'n' is the number of generations

Let N_0 = the initial population number
N_t = the final population number at the time 't'
n = the number of generations in the time 't'

So, $N_t = N_0 \times 2^n$

Solving for the number of generations 'n' by converting the cell number into logarithms of base 10

$$\text{Log}_{10} N_t = \text{Log}_{10} N_0 + n.\log_{10}2$$

$$n = \frac{\log_{10}N_t - \log_{10}N_0}{\log_{10}2}$$

$$n = \frac{\log_{10}N_t - \log_{10}N_0}{0.301} \quad \ldots \text{ as the value of } \log_{10}2 \text{ is } 0.301$$

$$n = 3.3 (\log_{10}N_t - \log_{10}N_0)$$

By using this formula the number of generations can be calculated if initial and final populations are known.

Generation time --- the time required for completion of one generation is called the generation time. Also be called doubling time as the cell number becomes double in one generation. It is denoted by letter 'g' and g = t/n where, t is the time of incubation and 'n' is the number of generations occurred during the incubation time of 't'.

Growth rate is the change in cell number or mass per unit time and denoted by 'R'. During exponential growth phase the growth rate is reciprocal to the generation time.
So, R = 1/g = n/t

Stationary Phase

In a batch culture the exponential growth cannot continue for a long period or cannot occur indefinitely. At some point, the growth of population ceases and the growth curve comes horizontal. This phase is called as stationary phase where the total number of viable cells remains constant without any net increase or decrease in the cell number due to the balance between cell division and cell death, but cells remain metabolically active. The stationary phase for bacteria is attained when the population level reaches to a level of 10^9 cells per ml of broth. Bacterial populations enter into the stationary phase for several reasons like (i) limitation and depletion of an essential nutrient in the culture medium (ii) accumulation of some toxic waste products to an inhibitory level.

Death Phase

This phase is also called as decline phase. When the culturing is continued after the stationary phase, cells may die due to the occurrence of detrimental environmental changes like nutrient deprivation and the buildup of toxic wastes. The death of a microbial, like its growth during exponential phase, is usually logarithmic.

10.2.2 Continuous culturing

Exponential growth of organisms occurs for only a few generations and soon reaches the stationary phase in the batch culture system. The growth of bacterial population at a particular rate in the exponential phase can be maintained through continuous culturing system in which the constant environmental conditions are maintained through continual provision of nutrients and removal of wastes. There are two major types of continuous culture systems in common use – 1) The Chemostat and 2) The Turbidostat.

Chemostat

A chemostat is constructed so that the sterile medium is fed into the culture vessel at the same rate as the medium containing microorganisms is removed. The culture medium for a chemostat possesses an essential nutrient like an amino acid in limiting quantities. Because of the presence of a limiting nutrient, the growth rate is determined by the rate at which new medium is fed into the growth chamber, and the final cell density depends on the concentration of the limiting nutrient. So, the concentration of the limiting nutrient in substrate of the culture vessel controls the growth rate. The concentration of the substrate is in turn controlled by the dilution rate.

$$\text{The dilution rate 'D'} = \frac{\text{flow rate (f)}}{\text{vessel volume (v)}}$$

Both the microbial population level and the generation time are related to the dilution rate. As the dilution rate increases the generation time decreases. If the dilution rate rises too high, the microorganisms can actually be washed out of the culture vessel before reproducing because the dilution rate is greater than the maximum growth rate. The limiting nutrient concentration raises at higher dilution rates because fewer microorganisms are present to use it. By adjusting the flow rate, growth rate can be controlled. The chemostat apparatus is shown in Fig.10.2.

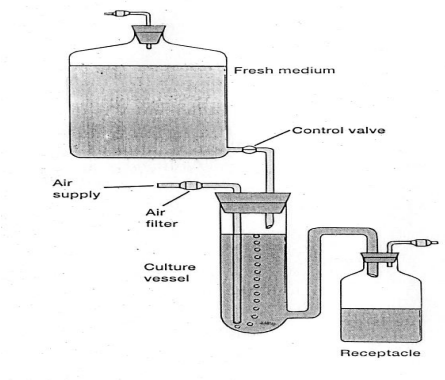


Figure-10.2: Chemostat apparatus

Turbidostat

Turbidostat is the second type of continuous culture system. This device (Fig. 10.3) has a photocell that measures the absorbance or turbidity of the culture in the growth vessel. The flow rate of media through the vessel is automatically regulated to maintain a predetermined turbidity of cell density. The turbidostat differs from the chemostat in several ways. The dilution rate in turbidostat varies rather than remaining constant, and its culture medium lacks a limiting nutrient. The turbidostat operates best at high dilution rates whereas the chemostat is most stable and effective at lower dilution rates.

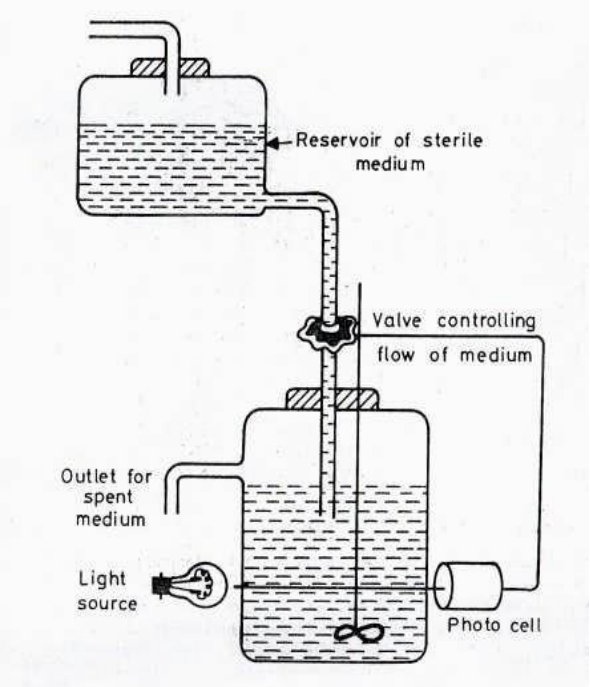


Fig. – 10.3 Turbidostat apparatus

10.2.3 Synchronous culturing

The bacterial population, at any given instant during log phase consists of cells in various stages of division viz., about-to-divide cells, just-divided cells, and cells in the different physiological stages of preparation to division. So, the population is said to be heterogeneous in behavior. To eliminate this heterogeneity synchronous culturing is used in which all the cells provoked to divide at the same time. In this synchronous culture since all the cells are physiologically identical, cell division occurs periodically at constant intervals (Fig. 10.4). This can be achieved by the methods that provoke the entire log-phase culture into the division process simultaneously. Such methods involve the arrest of cell division but not of cytoplasmic growth by chemical or physical means for a period followed by the sudden relief of this inhibition. With this sudden relief, a marked degree of synchronous division of an entire culture is obtained.

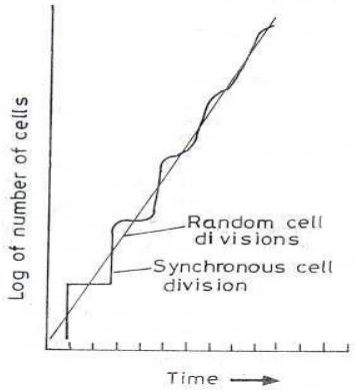


Fig.– 10.4 Synchronous growth curve

A population can be synchronized by manipulating the physical environment or the chemical composition of the medium. For example, when cells inoculated into a medium at sub-optimal temperature and maintained the same condition for some time, they will metabolize slowly but not divide. When the temperature is subsequently raised, the cells will undergo a synchronized division. Similarly, *E. coli* growth can be synchronized by changing the chemical nature of the medium. When a thymine requiring mutant is starved for thymine by placing it in a thymine deficient medium, it is incapable to grow. Then the addition of thymine in the culture medium causes the surviving of cells to undergo several synchronous divisions.

10.2.4 Biphasic growth curve

A biphasic growth curve reflects the preferential utilization of substrates and this is referred as diauxie phenomenon. A combination of catabolite repression and operon control mechanisms results in a biphasic growth curve (Fig.-10.5). In this diauxie phenomenon, when two carbon sources are present in the medium the organism preferentially utilizes one source completely before the use of other. For example, cultures of *E. coli* exhibit the biphasic growth curve when inoculated into a medium containing both glucose and lactose as substrate. While growing on glucose *E. coli* exhibits the normal lag, log and stationary phases of growth. Rather than exhibiting a prolonged stationary phase, *E. coli* enters a second lag phase when the glucose is no longer readily available in concentrations that suppress disaccharide utilization by catabolite repression. During this second lag phase, allolactose acts as an inducer to derepress the lac operon system. The enzymes that are necessary for lactose metabolism are synthesized, and the bacteria begin to grow exponentially by using the lactose substrate. When the lactose is also utilized, the bacterium enters into the secondary stationary phase.

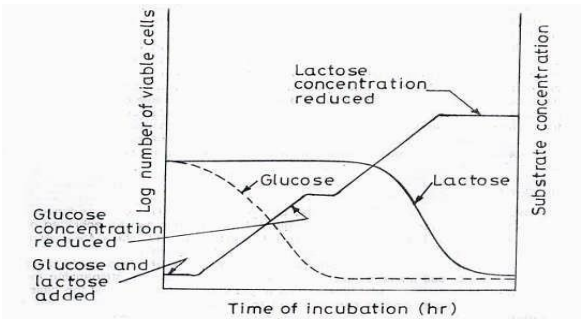


Figure-10.5: Biphasic growth curve

10.3 MEASUREMENT OF BACTERIAL GROWTH

The growth of bacteria can be measured in two different ways – measurement of cell numbers and measurement of cell mass.

10.3.1 Measurement of cell numbers

Counting chamber

The most obvious way to determine bacterial numbers is through direct counting. Using a counting chamber for the purpose is easy, inexpensive and relatively quick. It also gives information about the size and morphology of bacteria. The most widely and extensively used counting chamber is Petroff-Hausser counting chamber (Fig. 10.6). This chamber is a specially designed slide with chamber of known depth with an etched grid on the chamber bottom. The grid is located in the center of the slide and it consists 25 squares covering an area of. The depth of the chamber is 0.02 mm. The number of bacteria can be calculated by taking the chamber's volume into account. The total number of bacteria in 1 mm^2 area is number of bacteria per square x 25 squares. To get the number of bacteria per mm^3

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

The number of bacteria per cm^3 is 10^3 times of mm^3 value,

so

Bacteria/cm^3 = (bacteria/square) (25 squares)(50)(10^3)

Counting chambers yield counts of both live and dead cells.

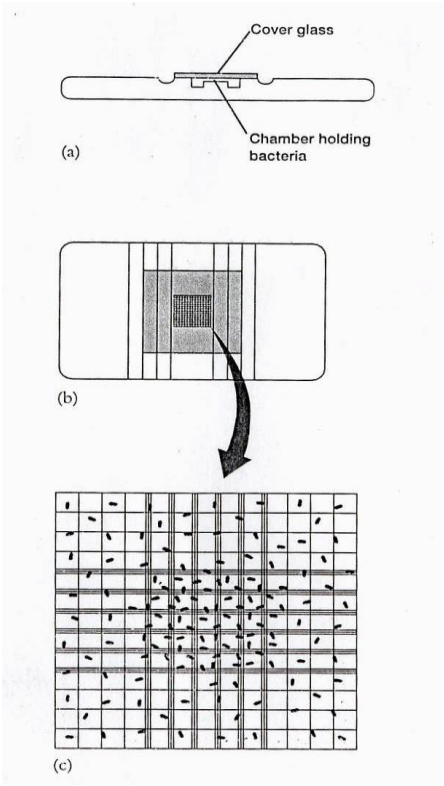


Figure-10.6: Petroff-Hausser counting chamber

Plating method

Plating techniques such as spread plate and pour plate methods which are simple and sensitive are also widely used to measure the viable count of bacteria in the samples of food, water and soil. In spread plate method, bacterial sample is diluted by 10-fold serial dilution technique to the required dilution. A small amount of bacterial sample from the appropriate dilution is spread or dispersed over a solid agar medium surface and incubated for the development of the colonies. In pour plate method also sample is diluted to the desired level of dilution. But here, a known volume of the sample is dispersed first into the sterilized empty petri plate and then the sterilized molten agar medium is poured. The contents of the plate are mixed well by rotation of the plate and incubated for the development of the colonies. In both the methods, the original number of viable cells in the sample can be calculated from the number of colonies formed and the sample dilution. The cell count can be measured more accurately by the use of special colony counter. For best results, the samples used should yield between 25 and 250 colonies per plate.

Membrane filter technique

This technique can be routinely used for the enumeration of bacteria from the liquid samples. Bacterial numbers are determined from counts of colonies growing on special membrane filters having pores small enough to trap bacteria. In this technique, the sample is drawn through a special membrane filter. Then filter is placed on an agar medium or on a pad soaked with liquid media and incubated until each cell forms a separate colony. The colony count gives the number of bacteria in the filtered sample and some special media can be used to select for specific bacteria (Fig. 10.7)

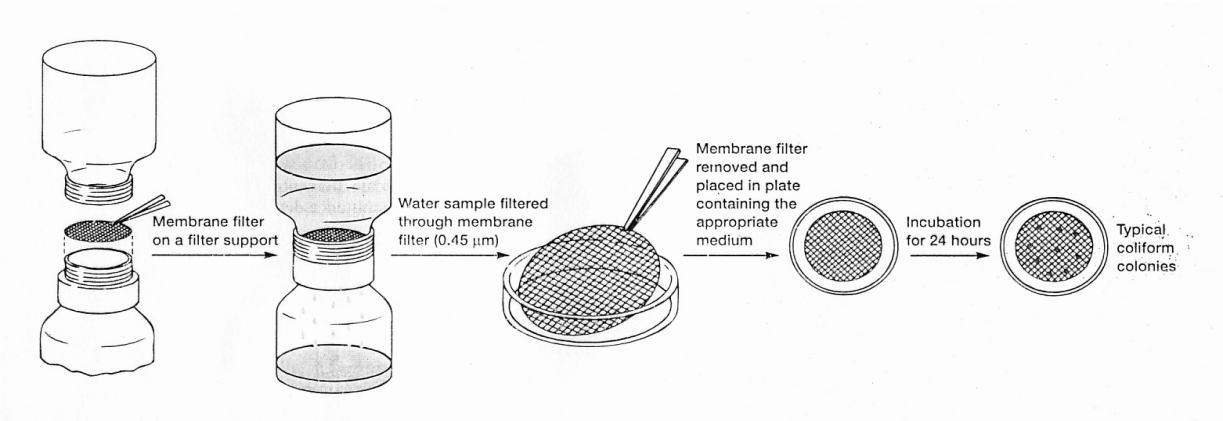


Figure-10.7: Membrane filter technique

10.3.2 Measurement of Cell Mass

Dry weight method

The most direct approach in the measurement of cell mass of bacteria is the determination of bacterial dry weight. Cells growing in liquid medium are collected by centrifugation, washed, dried in hot-air-oven to a constant weight and then weighed. This method is time consuming and not very sensitive. Because bacteria weigh so little, it is necessary to centrifuge several hundred milliliters of culture to collect a sufficient quantity. So, this method is not so preferable for bacteria.

Turbidity measurement method

More rapid and sensitive method for the measurement of cell mass is the determination of the turbidity developed in the liquid medium due to the increased cell density during incubation. This technique depends on the fact that microbial cells scatter the light that strikes them. Because the microbial cells in a population are more or less of constant size, the amount of scattering is proportional to the concentration of cells present. When the concentration of bacteria reaches about 10^7 cells per ml, the medium appears slightly cloudy or turbid. Further increase in concentration result in greater turbidity and less light is transmitted through the medium. The extent of light scattering can be measured by a spectrophotometer and is almost linearly related to the bacterial concentration at low absorbance levels. Thus population growth can be easily measured by spectrophotometer as long as the population is high enough to give detectable turbidity.

Estimation of cell constituents

If the amount of a substance in each cell is constant, the total quantity of that cell constituent is directly related to the total microbial cell mass. In this mode of estimation, a known volume of sample is centrifuged and washed cells are collected. This sample of cells is analyzed for total protein or ATP. An increase in the microbial population will be reflected in higher total protein levels or ATP.

Protein is a reasonable measure of growth since it normally constitutes the majority (50- 70%) of the organic cellular dry weight. The method by which cell protein is assessed depends upon the purpose and the amount of material involved. There are three methods that are commonly used for protein measurement. They are 1) the biuret method, 2) Folin reagent method, and 3) the Coomassie blue dye-binding method. Each procedure is useful under different conditions.

The biuret procedure depends upon the interaction of the cupric ammonium ion with the peptide bond. The procedure is considered by many to be the most unbiased protein measurement, since it depends only on the peptide bond and is independent of the amino acid composition. Reaction of copper with the peptide bond yields a complex that is blue- colored and absorbs broadly over a range between 500 and 650 nm. At an appropriate wavelength, a linear relationship is found between the copper-protein complex and protein concentration. Relatively large amounts of protein are required to obtain substantial absorbance readings in the visible wavelength range.

The Folin reaction method which is relatively sensitive depends upon interaction of protein with the cupric ion followed by oxidation of the complex by the Folin reagent. The oxidation of the complex is coupled with the reduction of a mixture of phosphotungstic and phosphomolybdic acids in the reagent, producing a blue color that is proportional to the amount of copper-protein complex.

Coomassie blue method is an alternate sensitive procedure for protein measurement. It takes the advantage of the fact that, in highly acidic solution, interaction of protein with Coomassie blue dye leads to a change in the dye's absorption from 465 nm to 595 nm that is proportional to the concentration of the protein-dye complex. When carefully standardized, the procedure is highly sensitive, rapid and less subject to interferences than the Folin procedure.

10.4 SUMMARY

Growth is an increase in cellular constituents and results in an increase in cell size, cell number of both. When bacteria are grown in a closed system or batch culture, the resulting growth curve usually has four phases viz., lag phase, exponential or log phase, stationary phase and death phase. In lag phase, the cells adapt to the new environment and prepare itself to undergo multiplication. During the exponential phase, the population doubles at a constant interval called the doubling or generation time. The mean growth rate constant is the reciprocal of the generation time. In stationary phase, the cell number remains constant due to the depletion of nutrients. During death or decline phase of growth, bacterial cells die due to the accumulation of toxic substances to lethal levels. Bacteria can be grown in an open system in which nutrients are constantly provided and wastes are removed. A continuous culture system is an open system that can maintain a bacterial population in the log phase for desired period. There are two types of these systems namely chemostat and turbidostat.

Bacterial populations can be counted directly with counting chambers, electronic counters or fluorescence microscopy. Viable counting techniques such as spread plate technique pour plate technique and the membrane filter method can be employed. Population changes also can be followed by determining variations in microbial mass through the measurement of dry weight, turbidity, or the amount of a cell component. The amount of microbial mass produced from a nutrient can be expressed in terms of the growth yield.

10.5 TECHNICAL TERMS

Growth curve, Lag phase, Log phase, Stationary phase, Death phase, Chemostat, Turbidostat, Synchronous growth, Biphasic growth, Membrane filter technique

10.6 SELF ASSESSMENT QUESTIONS

Q.1. What is the growth curve? Explain the growth pattern of bacteria in a batch culture system.
Q. 2. Describe the various methods used to measure the bacterial growth.
Q. 3. Write notes on biphasic growth curve.
Q. 4. Write notes on synchronous culturing.

10.7 SUGGESTED READINGS

1. Microbiology - Prescott, L.M., Harley, J.P., and Klein, D.A.
2. Biology of Microorganisms - Brock, T.D. and Madigan, M.T
3. Principles of Microbiology - Atlas, R.M
4. The Physiology and Biochemistry of Prokaryotes - White, D
5. Bacterial Metabolism - Gottschalk, G

Prof. V. Umamaheswara Rao.

LESSON – 11

FACTORS AFFECTING BACTERIAL GROWTH AND BACTERIAL HOMEOSTASIS

OBJECTIVE OF THE LESSON

To know the impact and influence of various environmental factors of a habitat on the growth of bacteria.

STRUCTURE OF THE LESSON

11.1 INTRODUCTION

Microorganisms are found in nearly every environment on earth, including environments in which no other life forms can survive. The growth of these microorganisms is greatly affected by the chemical and physical nature of their surroundings. So, the kinds of organisms found in a given environment and the rates at which they grow can be influenced by a variety of factors, both physical and biochemical. The physical factors include pH, temperature, oxygen concentration, moisture hydrostatic pressure, osmotic pressure and radiation.

11.2 PHYSICAL FACTORS

11.2.1 pH

pH is a measure of the hydrogen ion activity of a solution and is defined as the negative logarithm of the hydrogen ion concentration. The pH scale is invented by the Danish Chemist, Soren Sorenson to describe the limits of growth of microorganisms in various media. The pH scale extends from 0.0 to 14.0 and each pH unit represents a tenfold change in hydrogen ion

concentration. Bacteria, as they grow, usually cause changes in pH of their environments. So, to maintain the pH of the medium constant several buffers are used and each type of buffer work only for a narrow pH range. pH dramatically affects the microbial growth. Each species has a definite pH growth range with an optimum pH value. Most natural environments have pH values between 5.0 and 9.0 and the organisms with optima in this range are common.

Basing on the tolerance for acidity or alkalinity, the bacteria can be categorized into Acidophiles, Neutrophiles and Alkaliphiles. However, no single species can tolerate the full pH range of any of these categories, and many bacteria tolerate a pH range that overlaps two categories. Acidophiles or acid-loving bacteria have their growth optimum between pH 0.0 and 5.5. Lactobacillus, which produces lactic acid is an acidophile but it tolerates only mild acidity. Neutrophiles exist between pH 5.5 and 8.0. Most of the bacteria that cause disease in humans are neutrophiles. The third category alkaliphiles or alkali-loving or base-loving bacteria prefer the pH range of 8.5 to 11.5. *Vibrio cholerae*, the causative agent of the disease Asiatic cholera, grows best at a pH of about 9.0. The alkaliphiles having the growth optima at pH 10.0 or higher are called as Extreme alkaliphiles. Most bacteria prefer the near neutral pH for their optimum growth.

Despite wide variations in habitat pH, the internal pH of most bacteria is close to neutrality. This may be due to the relatively impermeability of plasma membrane of bacteria to protons. Neutrophiles appear to exchange potassium for protons using an antiport transport system. Extreme alkaliphiles maintain their internal pH closer to neutrality by exchanging internal sodium ions for external protons. Although bacteria often grow over wide ranges of pH, there are limits to their tolerance. Drastic variations in pH can harm bacteria by disrupting the plasma membrane or inhibiting the activity of enzymes and membrane transport proteins. Bacterial death occurs if the internal pH drops much below 5.0 to 5.5. Changes in the external pH also might alter the ionization of nutrient molecules and thus reduce their availability to the organism. Bacteria often must adapt to environmental pH changes to survive. In bacteria, potassium/proton and sodium/proton antiport systems probably correct small variations in pH. If the pH becomes too acidic, other mechanisms come into play. When the pH drops below 5.5 to 6.0, bacteria like *Salmonella typhimurium* and *E. coli* synthesize an array of new proteins as part of their acidic tolerance response. This protective response is contributed by a proton-translocating ATPase either by making more ATP or by pumping protons out of the cell. In the case of a decrease in external pH to 4.5 or lower, chaperons such as acid shock proteins and heat shock proteins are synthesized that may prevent the acid denaturation of proteins.

11.2.2 TEMPERATURE

Environmental temperature is one of the most important environmental factors influencing the growth and survival of organisms. Microorganisms are particularly susceptible because they are unicellular and '*poikilothermic'* i.e., their temperature varies with that of external temperature. A most important factor influencing the effect of temperature on growth is the temperature sensitivity of enzyme-catalyzed reactions. Temperature affects the organisms in either of two opposing ways. At low temperatures, the rate of enzymatic reactions in the cell will become roughly double for every 10^{o}C rise in temperature. As a result the growth of the organism becomes faster. But above a certain point of temperature, a further increase in temperature slows down the growth and sufficiently high temperatures are lethal.

High temperatures damage the microorganisms by denaturing the enzymes, transport carriers,

and other proteins. Other affects include the disruption of microbial membranes and simple melting and disintegration of lipid bilayer. Because of these opposing influences of temperature, each type of microorganism possesses three characteristic temperatures to explain their dependence on temperature. These are minimum temperature, optimum temperature and maximum temperature that are collectively called as 'cardinal temperatures'. The cardinal temperatures for a particular species are not rigidly fixed but often depend to some extent on other environmental factors such as pH and the available nutrients. And the cardinal temperatures vary greatly among the microorganisms. To define these temperatures

- Minimum temperature below which no growth occurs
- Optimum temperature at which growth rate is rapid
- Maximum temperature above which growth is not possible

The microbial growth can occurs at temperatures extending from –20^{o}C to over 100^{o}C, but no single organism shows growth over this whole temperature range. Usually a given microorganism exhibits a span of about 30^{o} – 40^{o}C temperature range. Species having a small range of growth temperature are called as 'Stenothermal' (e.g.: *Neisseria gonorrhea*) and that can grow over a wide range are known as 'Eurythermal' (e.g.: *Enterococcus faecalis*).

Basing on their temperature ranges, microbes can be categorized into different classes (Fig. 11.1).

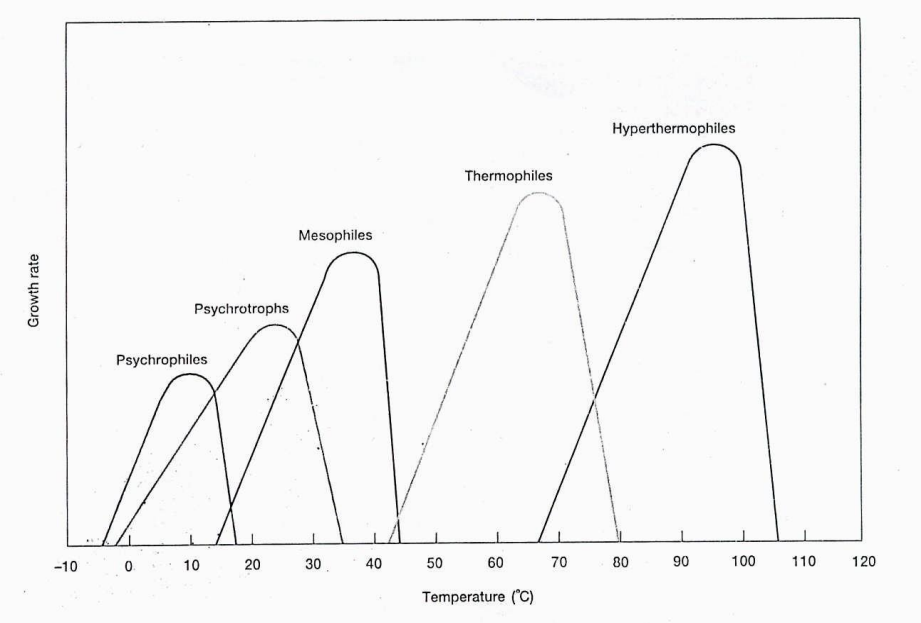


Figure-11.1: Temperature ranges of bacteria

Psychrophiles

These are cold-living organisms showing low temperature optima and are found in environments that are constantly cold like Arctic and Antarctic habitats. They grow well at 0^{o}C with an optimum growth temperature of 15^{o}C or below and maximum temperature is around 20^{o}C. Some members of *Pseudomonas*, *Flavobacterium*, *Achromobacter* and *Alcaligenes* are best known examples of psychrophiles. The enzymes, transport systems and protein synthetic mechanisms of these psychrophiles are adapted to function well at low temperatures. The cell membranes of these organisms have high levels of unsaturated fatty acids and remain semifluid when cold. Many species can grow at 0^{o} C even though they have

optima between 20º and 30ºC, and maxima at about 35ºC. These are called as psychrotrophs or facultative psychrophiles or psychrotolerants and are mainly responsible for the spoilage of the refrigerated foods.

Mesophiles

Organisms with mid-range temperature optima are referred as mesophiles. Their growth optima is around 20º to 45ºC, minimum is of 15º to 20ºC and the maximum temperature is about 45ºC. Most of the microorganisms probably fall within this category. Almost all the human pathogens are included in this category as they grow best near human body temperature of 37ºC. Some mesophiles that can withstand to the exposure of higher temperatures for short periods are called as thermoduric organisms which are responsible for the canned food spoilage.

Thermophiles

These organisms are heat loving in nature and exhibit relatively high temperature optima. Their growth minimum is around 45ºC and has optimum temperature between 55 and 65ºC. Some bacteria have growth optima between 80ºC and about 113ºC and are called hyper thermophiles (*Pyrococcus* spp. and *Pyrodictium* spp.). Thermophilic bacteria flourish in many habitats including composts, self-heating hay stacks, hot water lines, and hot springs. The molecular adaptation of these thermophiles or thermophily is mainly due to the presence of heat-stable enzymes and protein synthesis systems that are able to function at higher temperatures. The membrane lipids of thermophiles are also more saturated than those of mesophiles and have higher melting points which enable the thermophile membranes to remain intact at higher temperatures.

11.2.3 Oxygen Concentration

Oxygen is a peculiar environmental factor as it can be both essential and toxic. Depending on the effect and requirement of oxygen, bacteria can be divided into different groups (Fig. 11.2).

Obligate aerobes: Require the presence of oxygen for growth. Oxygen serves as the terminal electron acceptor for the electron-transport chain in aerobic respiration. Eg. *Pseudomonas* spp.

Obligate anaerobes: These organisms do not tolerate O_2 and die in the presence of oxygen. These are also termed as strict anaerobes. Examples include species of *Clostridium, Bacteroides, Fusobacterium* and *Methanococcus.*

Facultative anaerobes: Do not require O_2 for growth but grow better in its presence. These forms carry on aerobic metabolism in the presence of O_2 and shift to anaerobic metabolism in the absence of oxygen. E.g.: *Escherichia coli*

Aerotolerant anaerobes: These forms simply ignore the presence of O_2 and grow equally well whether it is present or not. E.g.: *Enterococcus faecalis* and *Lactobacillus* spp.

Microaerophiles: Few aerobes such as *Campylobacter* spp. require O_2 levels below the range of 2 to 10% and are damaged by the normal atmospheric level of O_2 i.e. 20%.

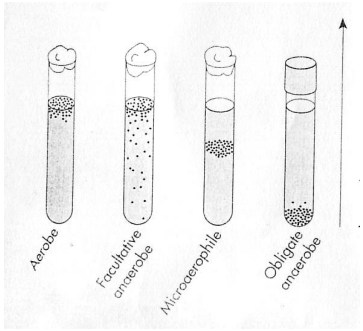


Figure-11.2: Oxygen relationship with growth of bacteria

These different O_2 relationships are due to either inactivation of proteins or effect of toxic O_2 derivatives. During the reduction of oxygen to water, several toxic intermediates like hydroxyl radical (OH), hydrogen peroxide (H_2O_2) and superoxide (O_2^-) are formed. This reduction reaction involves

$O_2 + e$ ---------- $O_2^{-}\cdot$ (superoxide radical)
$O_2^{-}\cdot + 2H^+$ ---------- H_2O_2 (hydrogen peroxide)
$H_2O_2 + e^- + H^+$ ---------- $H_2O + OH\cdot$ (hydroxyl radical)

These products of oxygen reduction are extremely toxic because they are powerful oxidizing agents and rapidly destroy cellular constituents. Superoxide radical is potentially toxic and highly reactive that can cause oxidative destruction of lipids and other biochemical components. This intermediate may even pass from one cell to another cell. Hydrogen peroxide is produced in small amounts by almost all aerobic organisms. Hydroxyl radical is the most reactive of all the O_2 intermediates. It is the most potent oxidizing agent and capable of attacking any organic substance in cells. A microorganism must be able to protect itself against such oxygen products or it will be killed.

Many bacteria possess enzymes that afford protection against toxic O_2 products. The important enzymes in this regard are 'catalase', 'peroxidase' and superoxide dismutase. Both the catalase and peroxidase enzymes act on H_2O_2, whereas superoxide dismutase acts on superoxide radical. Superoxide dismutase and catalase together can bring about the conversion of superoxide back to O_2. No enzymatic system exists to deal with hydroxyl radicals. Removal of H_2O_2 from cells, however protect the cells in part by preventing the formation of hydroxyl radicals. Obligate aerobes and facultative anaerobes usually contain superoxide dismutase and catalase enzymes. Aerotolerant bacteria may lack catalase but always possess superoxide dismutase. Obligate anaerobes lack both catalase and superoxide dismutase or possess in very low concentrations and so cannot tolerate O_2.

11.2.4 Water Activity

In natural environments, water availability is one of the most important factors affecting the growth of microbes as they all require water for life. The amount of water available to microorganisms can be reduced by interaction with solute molecules (the osmotic effect) or by adsorption to the surfaces of solids (the matric effect). Water availability is generally expressed in physical terms such as water activity or water potential. Water activity is denoted as a_w and expressed as a ratio of the vapor pressure of the air in equilibrium with the substance or solution divided by the vapor pressure of pure water at the same temperature.

$$a_w = \frac{P_{soln}}{P_{water}}$$

The values of a_w vary between '0' and '1'.
The movement or diffusion of water across a selectively permeable membrane from a dilute solution (higher water concentration) to a more concentrated solution (low water concentration) is called as Osmosis. Microorganisms can be affected by changes in the osmotic concentration of their surroundings as they are separated by a selectively permeable plasma membrane from their environment. If a microorganism is placed in a hypotonic solution i.e., solution with low osmotic concentration, water will enter the cell and cause it to burst unless something is done to prevent the influx. When microorganisms with rigid cell walls are placed in a hypertonic environment, water leaves the cell and plasma membrane shrinks away from the wall, a process known as plasmolysis. This condition dehydrates the cell and may damage the plasma membrane and the cell usually becomes metabolically inactive and ceases to grow.

Most of the bacteria, to maintain the shape and integrity of their cells, keep the osmotic concentration of their protoplasm above that of the habitat by the use of solutes referred to be as Compatible solutes. These compatible solutes are the substances that are compatible with metabolism and growth and always maintain the plasma membrane pressed firmly against the cell wall. Most bacteria increase their internal osmotic concentration through the synthesis or uptake of choline, betaine, proline, glutamic acid, and other amino acids. Sometimes elevated levels of potassium ions are also used for the purpose.

Bacteria that grow or live in environments that are high in sugar concentration are known as Osmophiles. Bacteria greatly differ in their ability to adapt to habitats with low water activity. To grow in a low a_w habitat, the bacteria should expend extra effort to maintain a high internal solute concentration. The bacteria having this capacity of growing over a fairly wide range of water activity or solute concentration are called Osmotolerants. The bacteria that require moderate to large quantities of sodium chloride are referred to as salt-loving bacteria or halophiles. The halophiles are typically found in ocean, where the salt concentration is optimum for their growth. The membrane transport systems of these halophiles actively transport sodium ions out of the cells and concentrate potassium ions inside them. This high level of potassium in the cells of halophiles is required for the stability and activity of various enzymes, ribosomes and transport proteins. Bacteria that are able to live in very dry environments are known as Xerophiles and they use glycerols as compatible solutes.

11.2.5 Radiation

On the earth sunlight is the major source of radiation which includes visible light, ultraviolet light, infrared rays and radio waves. The wavelength of these radiations decreases with the increase in the energy. Many forms of electromagnetic radiation particularly ionizing radiation are very harmful to microorganisms due to their shorter wavelength and high energy that cause atoms to lose electrons or ionize. The two major forms of ionizing radiations are X-rays and γ-rays. X-rays are artificially produced and the γ-rays are emitted during radioisotope decay. Low levels of ionizing radiation will produce mutations and may indirectly result in cell death. But the higher levels of ionizing radiation are directly lethal and cause death. Though microorganisms are comparatively resistant to ionizing radiation, they will be destroyed by sufficiently large doses. A variety of changes in cells occur due to ionizing radiation. Of these, breaking of hydrogen bonds, oxidization of double bonds, destruction of

ring structures and polymerization of some molecules are important.
Ultraviolet radiation is the non-ionizing radiation having short wavelength and high energy. This UV radiation can kill all kinds of microorganisms. The most lethal wavelength of UV radiation that is absorbed effectively by DNA of the cell is 260 nm. The principal effect of ultraviolet radiation is the formation of thymine dimers in which the two adjacent thymines of the same DNA strand are covalently joined. This thymine dimer formation inhibits the DNA replication. This UV damage can be repaired by either photo reactivation mechanism or dark reactivation mechanism.

11.2.6 Pressure

Most organisms that spend their lives on land or on the surface of water, always subjected to a pressure of 1 atmosphere and never affected significantly by pressure. However, the standing waters in oceans exert a pressure called as hydrostatic pressure in proportion to its depth. Such pressure doubles with every 10 m increase in depth and it may reach to a pressure of 600-1100 atms in deep sea. Despite these extremes, some bacteria survive and adapt. These bacteria are not much affected as the others and are known as barotolerant bacteria. Some bacteria in the gut of deep-sea invertebrates grow more rapidly at high pressures and are referred as barophilic bacteria or simply barophiles. The membranes and enzymes of these barophiles require pressure to function properly. The high pressure is necessary to keep their enzyme molecules in the proper three-dimensional configuration without which the enzymes lose their shape and denature ultimately resulting in the death of organism.

11.3 BACTERIAL HOMEOSTASIS

Homeostasis is defined as the ability of any living organism to maintain a constant internal environment despite the changes that occur in external environmental conditions. In bacteria, it is the capability of the bacterial cell to maintain a steady and constant intracellular pH a constant osmotic differential across the cell membrane, in spite of the fluctuations that occur in external pH and osmolarity.

pH Homeostasis

In nature, bacteria can grow in a wide range of environmental habitats that vary in pH from a low of pH 1-2 (eg. Acid springs) to as high as pH 11 (soda lakes and alkaline soils). Irrespective of the external pH, the bacterial cell maintains the internal pH within 1-2 units on either side of neutral pH which is essential to perform normal physiological functions. In acidophiles, which grow optimally between p H 1-4 maintains the internal pH of about 6.5 - 7.0, so they maintain a pH gradient greater than 2.5 units. However, neutrophiles manage a pH gradient of 0.5 - 1.5 units. Whereas, alkaliphiles that grow oprimally in the range of pH 10 - 12 have an internal pH of 8.4 - 9.0. So, alkaliphiles maintain a negative pH gradient of about 1.5 - 2.0 units

Regulation of intracellular pH is mainly due to the flow of protons across the cell membrane. In acidic environment, when cell cytosol is too acid, protons are pumped out and brings K^+ ions into the cell. In alkaline environment, cell cytosol becomes too basic and then protons are brought in through exchange with outgoing K^+ or Na^+. In this, a feedback mechanism exists where the intracellular pH can signal for proton pumps and antiporters.

In case of alkaliphiles, the main problem is to keep the cytoplasmic pH more acidic than the external pH. This can be overcome by the bacterial cell by bringing in protons with simultaneous exclusion of Na^+ by Na^+/H^+ antiporters. In acidophiles, the external pH is several units lower than the cytoplasmic pH. Maintenance of larger pH gradient requires an inverted membrane potential. This may be due to the inward flux of K^+ greater than an outward flux of protons. Maintenance of pH gradient in acidophiles and neutrophils may be similar in relying on K^+ influx to depolarize the membrane.

Osmolarity Homeostasis

The physiological significance osmotic homeostasis is the maintenance of an internal turgor pressure necessary for growth. Bacteria shows the ability to adjust their internal osmolarity in accordance to the changes in external osmolarity thereby maintain the cell turgor pressure. The bacteria shows adaptation to high osmolarity. When the bacterial cells are placed in high osmolarity medium, spontaneous increase in intracellular concentration of certain solutes which are referred as osmolytes will occur and thereby cell turgor is maintained. These osmolytes used by bacteria are called as Compatible Solutes. These compatible solutes are non-toxic and either synthesized or taken up from the medium through transport. The compatible solutes that are taken by the cells from external medium are generally referred as Osmoprotectants. The most important compatible solute used by most bacteria is K^+, whose intracellular concentration is sufficiently high to maintain the internal osmolarity. Other compatible solutes used by bacteria include amino acids, glutamate, glutamine, proline, quaternary amino betaine and certain sugars like trehalose. Many bacteria prefer betaine as osmoprotectant as it suppresses the uptake of the other osmoprotectants which facilitate excretion of K^+ and catabolism of trehalose.

Many bacteria are capable of adjusting to the low osmolarity of the medium and thereby limit the cell turgor pressure. This may be achieved by decreasing the concentration of cytoplasmic osmolytes due to the excretion of specific osmolytes. Eg. *Escherichia coli* excretes K^+ through special transporters that respond to cell turgor.

In Gram negative bacteria, periplasm remains as an important component and shows an osmolarity which is isosmotic in nature with that of cytoplasm. These Gram negative bacteria respond to low osmolarity by raising the osmolarity of periplasm, so the cytoplasm never experience of effect of low osmolarity of the external medium. The Gram negative bacteria maintain a higher osmolarity in periplasm than that of external medium by the synthesis and/or accumulation of periplasmic osmolytes. Eg. *E. coli*, *Salmonella typhimurium*, *Pseudomonas* sp., *Rhizobium* sp., etc.

Heat Shock response

When exposed to elevated temperatures, bacteria quickly respond and adapt to new conditions by synthesizing a group of special proteins called as heat-shock proteins which are not usually present under normal conditions. The heat-shock proteins produced differ from one organism to another organism. Synthesis of these heat-shock proteins is mediated by the formation of particular σ factor namely σ 32 which allow the DNA-dependent RNA polymerase to identify particular promoters of genes that code for heat-shock proteins.

1.4 SUMMARY

The bacterial growth will be influenced by various environmental factors of their habitats either directly or indirectly. Most bacteria have rigid cell walls and are hypertonic to the habitat because of solutes such as amino acids, polyols, and potassium ions. Different bacteria exhibit their respective optimum pH for growth and can be classified as an acidophiles, neutrophiles and alkaliphiles. Bacteria can alter the pH of their surroundings, and most culture media must be buffered to stabilize the pH.

Bacteria have distinct temperature ranges for growth with minima, maxima and optima collectively referred as cardinal temperatures. These ranges are determined by the effects of temperature on the rates of catalysis, protein denaturation, and membrane disruption. Basing on their tolerance and requirement of temperature for the growth, bacteria can be classified majorly to Psychrophiles, Mesophiles and Thermophiles.

Bacteria can be placed at least into five different categories basing on their response to the presence of oxygen namely obligate aerobes, facultative anaerobes, aerotolerant anaerobes, obligate anaerobes and microaerophiles. The amount of water actually available to bacteria is expressed in terms of the water activity. Though most bacteria will not grow well at water activities below 0.98 due to plasmolysis and associated effects, osmotolerant bacteria survive and even flourish at low water activities.

High-energy or short-wavelength radiation harms organisms in several ways. Ionizing radiation which includes X-rays and gamma rays ionizes molecules and destroys DNA and other cell components. Ultraviolet (UV) radiation induces the formation of thymine dimmers and strand breaks in DNA and cause the cell death. Most deep-sea bacteria are barotolerant, but some are barophilic and require high pressure for optimal growth.

11.5 TECHNICAL TERMS

Acidophiles, Neutrophiles, Basophiles, Cardinal temperatures, Psychrophiles, Mesophiles, Thermophiles, Aerobes, Anaerobes, Radiation, Homeostasis, Osmolytes, Heat-shock proteins.

11.6 SELF ASSESSMENT QUESTIONS

Q. 1. Explain the influence of different environmental factors on the growth of bacteria.
Q. 2. What are the physical factors of an environment? How these factors affect the bacterial growth.
Q. 3. Describe the types of bacteria basing on their relationship O_2 requirement for growth.
Q. 4. Write a note on the types of bacteria in relation to the effect of temperature on their growth.
Q. 5. Explain the effects of pH and water activity on the growth of bacteria.

11.7 SUGGESTED READINGS

1. Microbiology - Prescott, L.M., Harley, J.P., and Klein, D.A.
2. Biology of Microorganisms - Brock, T.D. and Madigan, M.T
3. Principles of Microbiology - Atlas, R.M
4. The Physiology and Biochemistry of Prokaryotes - White, D
5. Bacterial Metabolism - Gottschalk, G

Prof. V. Umamaheswara Rao

LESSON – 12

ARCHAEBACTERIA

OBJECTIVE OF THE LESSON

Students will be able to understand what Archaea are and also to differentiate Archaea from other microorganisms along with knowing their general characters, classification, mode of reproduction and significance.

STRUCTURE OF THE LESSON

12.1. INTRODUCTION

The group Archaea or archaeobacteria [Greek archaios, ancient, and bakterion, a small rod] are quite diverse in morphology, physiology, reproduction and ecology. Archaea were formerly believed to be bacteria and so, the term 'Archaebacteria' was coined. This was because of their physical similarities, but according to genetic studies, the Archaea are different from both bacteria and eukaryotes, and were placed in their own domain, Archaea, in the Three Domain Classification, which was first put forth by Carl Woese in 1977. They can stain either Gram positive or Gram negative and show a great variation in their shape. The Archaea are unicellular, microscopic, organisms, devoid of nuclei and membrane bound organelles similar to bacteria. They can be aerobic, facultatively anaerobic, or strictly anaerobic. Nutritionally, they range from chemolithoautotrophs to organotrophs. Some are mesophiles and others are hyperthermophiles. They exhibit primitive features like intake of nutrients, metabolism and able to withstand

extreme environmental conditions. They can grow in anaerobic, hypersaline, high temperature, and also cold environments. They constitute up to 34% of the prokaryotic biomass in coastal Antarctic surface waters. A few members are symbionts in animal digestive systems. The cell wall composition, membrane lipids, tRNA, ribosomes, elongation factors, metabolism of archaea differ from other eubacteria and eukaryotes.

12.2. GENERAL CHARACTERS

Habitat

Initially, most of the Archaea were isolated from extreme conditions like acidic, hypersaline and thermophilic environments, so they were called as extremophiles (organisms that love to grow in extreme conditions). Archaea inhabit a wide range of environments, including extreme conditions that were once thought to be sterile. These conditions often feature high temperatures exceeding 100°C, such as those found in geothermal springs, hydrothermal vents on the ocean floor, black smokers, oil wells, and extremely acidic or basic lakes, as well as hypersaline areas like the Dead Sea. Hyperthermophilic archaea, such as *Methanopyrus kandleri*, thrive at temperatures of 122º C, marking the highest tolerance for any known living organism. Some archaea inhabit highly acidic conditions, like *Picrophilus torridus*, which exists at nearly pH 0, while others are found in cold oceanic environments, including polar seas. Now-a-days, they are found everywhere like in soil, water surface, on humans, etc.

Cell Structure

The cells of archaea may be spherical, rod-shaped, spiral, lobed, plate-shaped, and irregularly shaped or pleomorphic. Some are single cells, whereas others form filaments or aggregates. They range in diameter from 0.1 to over 15μm, and some filaments can grow up to 200 μm in length. Generally, the rod shaped archaea measures about 1 to 2μm width by 1 to 5μm length; cocci measure about 1 to 3μm in diameter. Some archaea show chain like arrangement of cells, some cocci exhibit clusters (Fig. 12.1a), some rod shaped cells are curved, some are spiral shaped and some show branching also (Fig. 12.1b). Pleomorphism is also observed in Archaea. It is to be noted that there are extremely small and extremely large archaea. Symbiotic nanoarchaeotes (0.2μm in diameter) are smaller and live as symbionts that require host cells for nutrition. On the other hand, there exists a giant archaeon that is long and made of filaments up to 30 mm in length. The shapes of archaea differ widely from cocci, rods, to branched rods. The importance of shapes in archaea is to increase the surface area to volume ratio, thereby increasing the nutrient uptake efficiently by easy diffusion of molecules.

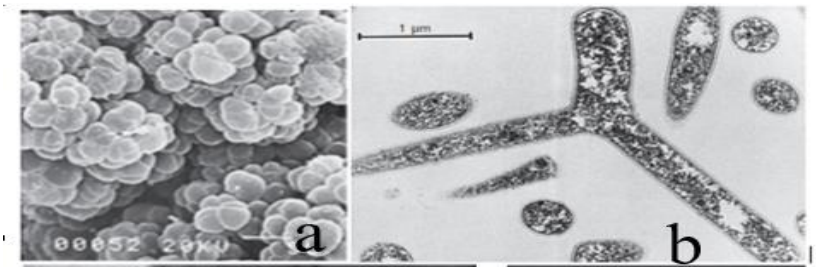


Figure-12.1: Morphological features of some archaea

a) Clusters form of *Methanosarcina mazei,* b) Branched form of *Thermoproteus tenax,*
(Courtesy: Prescott's Microbiology 12th Edition)

Special structures in Archaea

They have structures that are very similar to bacteria like pili, flagella, plasmids and inclusion bodies. Rarely, capsules are also found in archaea. Recent studies on archaea reveal that there are many distinct features in archaea that are responsible for their categorization in a new domain. Some structures those are unique to archaea, some of which are found to be partially similar to bacteria, with some modifications. They include:

Cannulae (sing. cannulus): It is a unique structure in archaea, discovered in some marine strains of archaebacteria. They are like hollow tubes that are found to connect with adjacent cells after the cell division, making a network of cells and tubes that help them in maintaining a community of cells to float on surface, as they anchor each other.

Hami (sing. hamus): It is an another unique structure with three hooks in archaebacteria, which is a long helical tube and possess hooks at the end, that helps in attachment of the cells to make cell communities.

Pili (sing. pilus): It was observed that archaeal pili were made of modified form of pilin proteins, which are present in bacteria.

Flagella (sing. flagellum): Both the bacterial as well as archaeal flagellum help in locomotion of the cell, but there is a marked difference in the mechanism of the motility. Proton motive force (PMF) is used by bacterial flagella, on the other hand, Adenosine tri-phosphate (ATP) is utilized by archaeal flagella, due to which, the term 'Archaellum' was proposed for archaeal flagellum. Moreover, the proteins that are composed in archaeal flagellum are not exactly similar to the flagellin proteins of eubacteria.

Spinae: Some marine archaea produce tube like structures called as 'Spinae' which forms a network to connect cells over distances about many micrometres to help exchange of signals between the adjacent cells.

Cell wall

Although archaea can stain either Gram positive or Gram negative, their cell wall structure and chemistry differ from that of eubacteria. There is a considerable variation in archaeobacterial cell wall structure. Many Gram positive archaeobacteria have a wall with a single thick homogenous layer like Gram positive eubacteria (Fig. 12.2a). Gram negative archaeobacteria lack the outer membrane and complex peptidoglycan network or saccules of Gram negative eubacteria. Instead, they usually have a surface layer of protein or glycoprotein subunits (Fig. 12.2b).

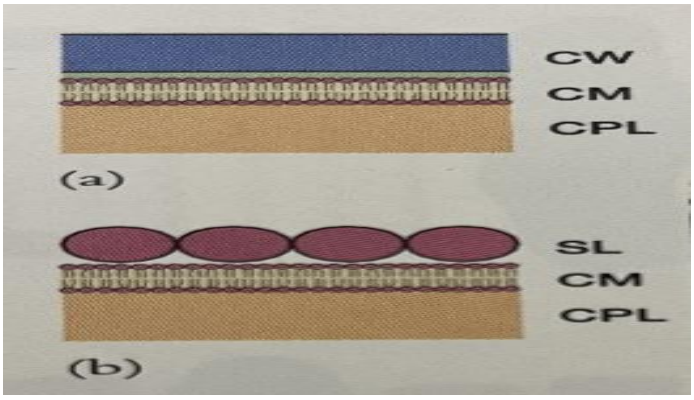


Figure-12.2: Cell envelopes of archaeobacteria
a) *Methanobacterium formicicum* (Gram positive archaeobacterium)
b) *Thermoproteus tenax* (Gram negative archaeobacterium)
CW – Cell wall; SL – Surface layer; CM – Cell membrane; CPL – Cytoplasm
(Source: Microbiology - Prescott et. al.)

The cell wall chemistry of archaea is also quite different from that of the eubacteria. The muramic acid and D-amino acids, characteristic of eubactrial peptidoglycan, are absent in archaea. But all the archaea resist the attack by lysozyme and β-lactam antibiotics such as pencillin. Gram positive arachaea can have a variety of complex polymers in their walls. Methanobacterium and some other methanogens have walls containing 'pseudomurein' (Fig. 12.3), a peptidoglycan-like polymer that has L-amino acids in its cross-links, N-acetyltalosaminuronic acid instead of N-acetlymuramic acid, and β-(1-3) glycosidic bonds instead of β-(1-4) glycosidic bonds. *Methanosarcina* and *Halococcus* contain complex polysaccharides similar to the chondroitin sulphate of animal connective tissue.

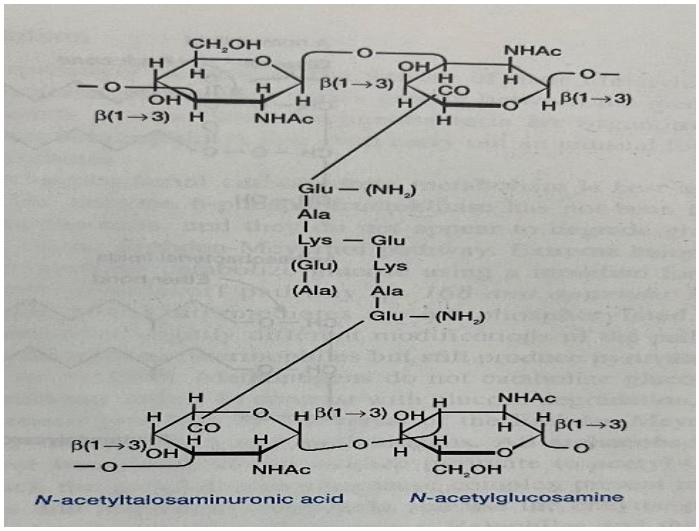


Figure-12.3: Structure of Pseudomurein
(Source: Microbiology - Prescott et. al.)

Gram negative archaea have a layer of protein or glycoprotein outside their plasma membrane. The layer may be as thick as 20 to 40 nm. Sometimes there are two layers, a sheath surrounding an electron-dense layer. Some methanogens (*Methanolobus*), *Halobacterium*, and several other extreme thermophiles (*Sulfolobus*, *Thermoproteus*, and *Pyrodictium*) have glycoproteins in their walls. In contrast, other methanogens (*Methanococcus*, *Methanomicrobium* and *Methanogenium*) and the extreme thermophile, *Desulfurococcus* have protein walls.

Cell membrane

There are some distinct features of the plasma membrane which set archaea apart from other domains. An example of this is the chirality of the bond that connects the side chain and the glycerol part of the phospholipid head. Eukaryotes and eubacteria have the D-isomeric form, while archaea have the L-isomeric form. Another distinction is that there is a side chain-glycerol bond that is ether-linked, which is contrary to the ester-linked lipids in eubacteria and eukaryotes. Sometimes two glycerol groups are linked to form an extremely long tetraether. Usually, diether side chains are 20 carbons in size and the tetraether chains are of 40 carbos. Due to ether-linkage, the membrane exhibits more chemical stability. The other differences are related to the side chains which are unbranched fatty acids in bacteria and eukaryotes, while in archaea they are isoprenoid chains which may have some branching at the side chains. Polar lipids are also present in archaea membranes such as phospholipids, sulfolipids, and glycolipids. About 7 to 30% of the membrane lipids are nonpolar lipids, which are usually the derivatives of squalene. Of course, the archaeal membranes may contain a mix of diethers, tetraethers and other lipids. The detailed structure of archaeal phospholipid and comparison of membrane lipids of archaeal, eubacterial and eukaryotic cells are given in figures 12.4 and 12.5, respectively.

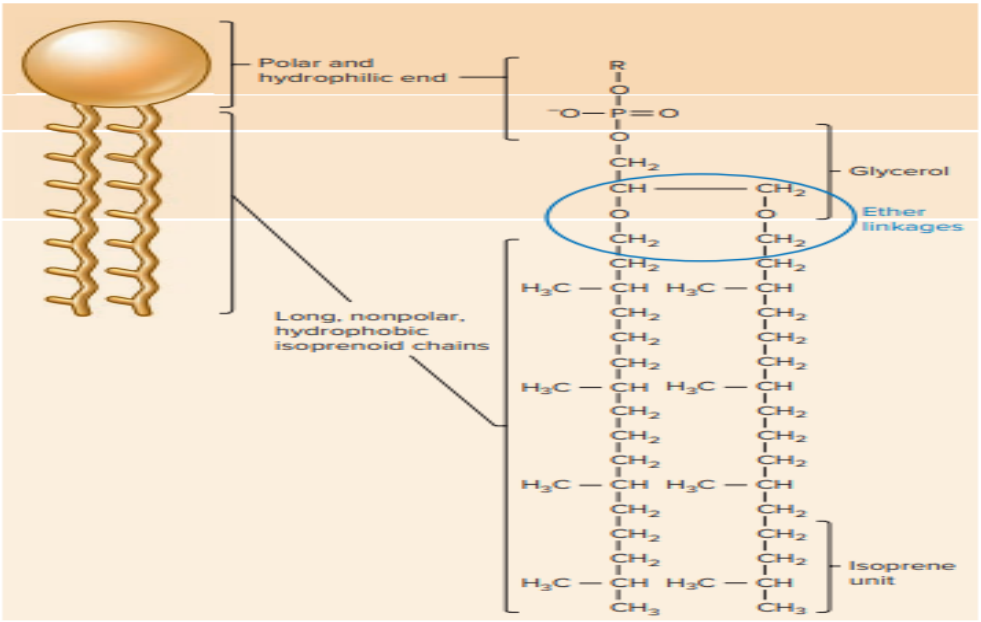


Figure-12.4: Detailed structure archaeal phospholipid
(Source: Microbiology – Prescott et. al.)

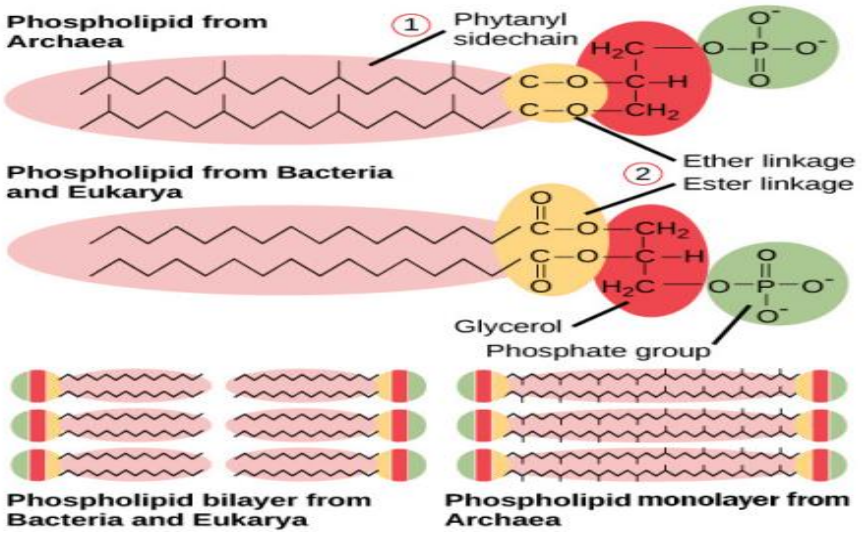


Figure- 12.5: Comparison of membrane lipids of Archaea with Bacteria and Eukarya
(Source: General Microbiology - Linda Bruslind)

Ribosomes

Even though there are 70s ribosomes, the same as those of the bacteria, scientists were able to show that archaea must have a separate domain due to differences in rRNA nucleotides. In addition, the shape of archaeal ribosomes is different from that of bacterial ribosomes, and they possess proteins which are different to that of archaea. As a result, they are immune to the drugs like Chloramphenicol that act by blocking the action of bacterial ribosomes.

Genetics

Some features of archaeobacterial genes are similar to those in eubacteria. Their chromosome is a single, closed and circular DNA. The genomes of some archaea are significantly smaller than the normal eubacterium. The variation in G + C content is great, from about 21 to 68 mol%, is another sign of archaeobacterial diversity. Archaeobacterial mRNA appears similar to that of eubacteria rather than to eukaryotic mRNA. Polygenic mRNA was found and there is no evidence of mRNA splicing. Unlike of eubacteria and eukaryotes, the TψC arm of archaeobacterial tRNA lacks thymine and contains pseudouridine or 1-methylpseudouridine. Some archaea, such as many methanogens in the kingdom Euryarchaeota , differ from other prokaryotes in having histone proteins that associate with DNA to form nucleosome-like structures. The archaeobacterial DNA-dependent RNA polymerases resemble the eukaryotic enzymes but not that of eubacterial RNA polymerase.There are some unique genes in archaea that code for proteins which are found in them alone. The t-RNA and r-RNA gene sequences in archaea are very different from bacteria and other organisms. It was observed that few archaeal DNA has introns, which is a feature of eukaryotes. Apart from asexual reproduction, horizontal genetic transfer among archaea may occurs. Moreover, the RNA polymerase in archaea is very similar to eukaryotes.

Metabolism

In view of their variety of life-styles, archaeal metabolism varies greatly between the members of different groups. Archaeal species are efficient in methanogenesis (production of methane gas) which is unique to archaea which are called as methanogens. In addition to this, they are chemolithotrophs (use chemicals and grow on rocks), photoautotrophs (use sun light and carbondioxide to synthesize food), and hyperthermophiles (high temperature loving organisms). Because of the absence of the enzyme 6-phosphofructokinase in archaea, they do not degrade glucose by the way of Embden-Meyerhof pathway. Extreme halophiles and thermophiles catabolize the glucose by a modified form of Entner-Doudoroff pathway. Halophiles and the extreme thermophile (*Thermoplasma*) have a functional tricarboxylic acid cycle. The synthetic pathways for amino acids, purines and pyrimidines are similar to those in other organisms. Some methanogens can fix atmospheric dinitrogen. Autotrophy is widespread among the methanogens and extreme thermophiles, and CO_2 fixation occurs in more than one way. *Thermoproteus* and possibly *Sulfolobus* incorporate CO_2 by reductive tricarboxylic acid cycle and methanogenic bacteria and most extreme thermophiles incorporate CO_2 by reductive acetyl-CoA pathway.

12.3 REPRODUCTION

Reproduction in archaea is carried out asexually by various methods. They include: fragmentation, binary fission and budding, but there are no reports on formation of endospores as seen in bacteria. Mitosis is never found in archaea as there is no nuclear membrane; rather they reproduce by binary fission. In the process, archaeal DNA replicates by pulling apart of the both strands while cell grows in size. In few cases, the number of daughter chromosomes formed is more than two and pulled apart, this process is called as multiple fission. Unlike, bacterial origin of replication, multiple origins of replication is formed in archaeal chromosomes. In addition to this, DNA polymerases used by archaea are similar to counterpart enzymes of eukaryotes. On the other hand, FtsZ proteins that help in cell division forms contractile ring around the cell. The septum constructed in the middle of the cell and its components resemble that of bacteria.

12.4 CLASSIFICATION

Archaea are classified into different phyla namely Crenarchaeota, Euryarchaeota, Korarchaeota.

12.4.1 Crenarchaeota

The designation Crenarchaeota signifies "scalloped archaea." These organisms frequently exhibit irregular morphological structures. All crenarchaeotes are characterized by the ability to synthesize an unique tetraether lipid, identified as crenarchaeol. Initially, this group included thermophilic and hyperthermophilic archaea, acidophiles and sulphur dependent. The sulphur may be used either as an electron acceptor in anaerobic respiration or as an electron source by lithotrophs. Almost all are strict anaerobes and grow in geothermally heated water of soils that contain elemental sulphur which are referred to as sulphur-rich hot springs or solfatara. This phylum consists three orders namely Igneococcales, Thermoproteales and Sulfolobales and at least 12 genera. The best studied genera of this crenarchaeota are *Thermoproteus* and *Sulfolobus*. Recent investigations have revealed that newly identified Crenarchaeota are inhibited by sulfur and exhibit growth at reduced temperatures. These organisms exhibit a Gram-negative staining

characteristic and display considerable morphological diversity, including rod-shaped, coccoid, filamentous, and irregularly shaped cells.

A well notable representative of the Crenarchaeota phylum is *Sulfolobus solfataricus*, which has been isolated from geothermally heated sulphuric springs located in Italy and exhibits optimal growth at a temperature of 80° C and a pH range of 2-4. Other examples include *Pyrolobus fumarii* and *Sulfolobus acidocaldarius*.

12.4.2 Euryarchaeota

It is a well-recognized and the largest phylum of archaea containing diverse genera that can survive in extremely alkaline, extremely saline, and extreme thermophilic conditions. Several methanogens are also grouped under this phylum. Though initially Euryarchaeota were thought to be extremophiles only, several genera were isolated from a normal environment like water springs, soil, water, rhizosphere, intestines, etc. This phylum exhibits considerable diversity, encompassing seven distinct classes: Methanococci, Methanobacteria, Halobacteria, Thermoplasmata, Thermococci, Archaeglobi, and Methanopyri. This phylum comprises nine orders and fifteen families. Based on the habitat, these organisms can be categorized into the following groups: methanogens, extreme halophiles, sulfate reducers, and numerous extreme thermophiles characterized by sulfur-dependent metabolic processes.

Halophiles: Halo signifies salt and "phil" denotes loving. Organisms classified as halophiles necessitate a saline environment for their sustenance. They inhabit salt lakes and regions where the evaporation of seawater occurs, such as the Great Salt Lake in the United States and the Dead Sea. These organisms are capable of thriving in aquatic environments with salinity levels surpassing 15%, whereas the ocean's salinity is approximately 4%. For example, *Halobacterium* is one of the halophilic archaea, which includes several species, found in salt lakes and high saline ocean environments. *Halobacterium salinarum*, *H. denitrificans* and *H. halobium* are found in The Great Salt Lake in Utah.

Methanogens: These are the single-celled microorganisms that generate methane as by-product in environments with no presence of oxygen. They are strictly anaerobic and oxygen-sensitive organisms, which die when exposed to oxygen. They produce methane gas in oxygen-poor environments like swamps and marshes, by reducing carbon dioxide using hydrogen. Methanogens usually occur in oxygen-free conditions such as mud at the bottom of lakes and swamps, some animal intestines like cows and humans, and dead and decaying organic matter, etc. Methanogens are added to biogas reactors to produce methane gas for cooking and sewage treatment plants. Examples of methanogens include *Methanofollis aquaemaris*, *M. ethanolicus*, *M. formosanus* and *M. liminatans.*

Sulphur reducing and Thermophilic archaea: In general, all the thermophilic organisms need heat to grow. In addition, some archaea need calcium or sulphur and few love to live in environments with alkaline pH.

Thermoacidophiles: These are the archaea, that include both aerobic and anaerobic species. They love to thrive in both acidic and hot environments.

Hyperthermophilic archaea: These are the unique archaea, which loves to grow in extreme high temperatures. Eg: *Pyrolobus fumarii*, which holds the record of growing in the highest temperature of about 113^{o}C.

12.4.3 Korarcheota (also called as Xenarchaeota)

The greek work 'Kore' or 'Koros' means 'young man' or 'young woman', which was used to name this group of archaea. In addition, the greek adjective 'archaios' means ancient. These are thermophilic, acidophilic and alkanophilic. This group of archaea have only found in hydrothermal environments ranging from terrestrial, including hot springs to marine, including shallow hydrothermal vents and deep-sea hydrothermal vents. However, the greater diversity of Korarchaea found in terrestrial hot springs compared to marine environments. They were found in high temperature hydrothermal environments which can survive up to 128^{o}C. *Korarchaeum cryptofilum* is the organism of this group that was isolated from Yellowstone National Park, which has pH of about 5.7 to 7.0. This group of archaea can tolerate alkalinity up to 11pH. Korarchaeota organism is an obligate anaerobe and grows heterotrophically using peptide and amino acid degradation pathways. The genome of Korarchaeota contains genes for dissimilatory sulphur metabolism in addition to anaerobic methane metabolism.

12.5. SIGNIFICANCE

They are microorganisms that are very diverse, found everywhere on earth, especially withstanding extreme environments, play very important roles in the nature.

Methanogenesis
Production of methane gas is one of the significant things that was explored for the welfare of humans these days. In general, these archaea are the only cause of natural production of methane gas in the nature.

Nutrient recycling
Due to the ability of archaeal cells to feed on chemicals or rocks, synthesize food using light and carbondioxide, they are able to survive in many harsh environments or extreme climatic conditions. Moreover, as they have diverse and more complex biochemistry and metabolism, they can decompose (breakdown) and utilise diverse forms of inorganic and organic compounds, which helps the nature in nutrient recycling and in establishing of an ecosystem with diversity.

Biogeochemical Cycling
Biogeochemical cycles are very important in the nature for the sustenance of living organisms. As archaea are found everywhere, even in the extreme conditions, they take part actively in all biogeochemical cycles like Carbon, Sulphur, Nitrogen and other cycles.

Symbiotic Relationship
Archaea are symbiotically associated with plant roots for nutrition and space and return some nutrients to plants. They are also associated symbiotically with coral reefs. Some archaea are found to be associated with the gut of animals.

Marine Ecosystem

Twenty percent of marine microbes are archaea and play a significant role in the production of organic matter, decomposition, nutrient recycling and maintenance of marine ecosystems.

Human Welfare Applications

As archaea are very diverse, they have diverse applications for the welfare of mankind.

1. Some archaea which are extremophiles have enzymes that can withstand different extreme environmental conditions, which can be utilized in food, detergent, textile and leather industries.
2. These days, biogas plants are established in houses which produce organic matter as waste material. These waste materials are converted by methanogenic bacteria in the synthesis of Biogas.
3. Bioremediation of harmful and toxic substances is very difficult in these days, due to human intervention and almost all the natural resources are polluted by heavy metals, toxic substances and xenobiotics. The only way to get rid of those substances from the nature, especially crop fields or from polluted ground water is by the use of these diverse archaea, which can breakdown or bioconvert most of the substances to make them less harmful to living organisms.
4. Archaea are also employed in cheese making, probiotics and in preparation of few fermented foods, which are used in food industry and add commercial value to the food products.
5. Some archaea produce chemical substances which are called as polyhydroxyalkanoates that are biodegradable plastic like chemicals that are used to replace plastics to save our environment.
6. As archaea are very diverse in their ability to breakdown many kinds of organic matter, they are also used in sewage treatment. They are methanogenic archaea, which are able to anaerobically digest the sewage and produce biogas as the by-product.
7. Archaea are also employed in the extraction of metals. Acidophilic archaea are used in the extraction of cobalt, gold and copper from their ores.

12.5 SUMMARY

Archaea are single celled, prokaryotic cells that are given a separate domain ‘Archaea’, due to their distinctive and special characters from bacteria and eukaryotes. Most of the archaea are extremophiles that live in extreme conditions like thermophilic or halophilic conditions. Some measures about 400 nm while others measure 3cms in length. There is great diversity of size and shape in archaea. In addition to this, archaea exhibit special structures like Cannula, Pili, Archellum, Hami and Spinae, which make them distinct from other groups of microbes. Moreover, archaea differ in their cell wall and cell membrane from bacteria. There is a marked difference in archaea in terms of their genetics, metabolism and in their cell organelles like ribosomes.

The archaea are classified into three important phyla viz., Crenarchaeota, Euryarchaeota, and Korarchaeota. Their mode of reproduction is also distinct, from eukaryotes as they do not show sexual reproduction as well as mitosis, because they lack nuclear membrane. It is to be noted that they also differ from bacteria in having replication machinery that is similar to eukaryotes. The archaea have significant role in every aspect of human life as well as every biological and chemical process that take place in nature. As most of them are extremophiles, they have great roles in nutrient recycling, biogeochemical cycles like carbon, sulphur and nitrogen cycles,

methanogenesis, maintenance of marine ecosystems, nutrient recycling, and decomposition of complex organic matter and deterioration of toxic chemicals. Their significance is extended in everyday life of humans, in having symbiotic relationship with human gut, in production of food, industrial enzymes, chemicals like detergents, mining of metals, sewage treatment, as well as in biogas production for cooking. Thus archaea play a very important role in the environment, as well as in human life.

12.6 TECHNICAL TERMS

Domain, Extremophiles, Prokaryotes, Pleomorphism, Cannulae, Pili, Archellum, Spinae, Pseudomurein, Peptidoglycan, Hyperthermophiles, Halophiles, Methanogens, Binary fission, Crenarchaeota, Euryarchaeota, Korarchaeota.

12.7SELF ASSESSMENT QUESTIONS

Q.1 What are Archaea and how are they different from all other living organisms?

Q.2 Discuss the general characters and classification of Archaea.

Q.3 How are Archaea classified? Add a note on the significance of Archaea.

12.8 SUGGESTED READINGS

1. Microbiology – Prescott et. al. 12th edition.
2. General Microbiology - Linda Bruslind
3. Brock Biology of Microorganisms – Madigan et. al.

Dr. J. Madhavi

LESSON - 13
CYANOBACTERIA

OBJECTIVE OF THE LESSON

To make the students to understand the general characters, classification, reproduction and significance of Cyanobacteria and also to differentiate cyanobacteria from other groups of microorganisms.

STRUCTURE OF THE LESSON

13.1. INTRODUCTION

Cyanobacteria are also called as Blue-green algae. These are prokaryotic autotrophs which are unicellular, filamentous as well as colonial forms that are pigmented and generally live in aquatic habitats. In blue green algae, there are both motile and non-motile forms, which are both aquatic as well as terrestrial forms. In aquatic environments, Blue green algae are the main cause of blooms and scums, which have negative effect on water quality (due to the release of algal toxins) in terms of taste, odour and also cause foaming. Cyanobacteria are differentiated from algae in having prokaryotic cells and differ from fungi in having photosynthetic apparatus. It is difficult to see individual blue green algal cells, but can be seen as concentrated clumps which appear as green flakes and bundles or brown dots on the surface of the water. These organisms can be free living or attached to the substratum. The members of cyanobacteria that are in

contact with the substratum show active gliding movement. While others that don't have contact with the substratum, actively swim. Some members show phototaxis, which means moving towards or away from the source of light, while others show chemotaxis, which means moving towards or away from the chemical substances or nutrients.

Cyanobacteria are thought to have converted the early atmosphere from reduced state to the oxidized state, because of their ability of performing oxygenic photosynthesis. These are one of the primitive organisms to possess two types of photosystems which use photosynthesis to synthesize food and release oxygen into the environment. These organisms usually possess photosynthetic pigments like chlorophyll-a and biliproteins which harness light energy. On the other hand, photosynthetic bacteria do not produce oxygen as they possess different pigments. Photosynthetic pigments that are generally seen in Cyanobacteria include Chlorophyll-a, Phycobilins, Xanthophylls and Carotenoids. Chloroplast is absent (photosynthetic pigments are located in thylakoids, which move freely in the cytoplasm). The Phycobilins that are present are of 3 types. They are phycoerythrin (red coloured), phycocyanin (blue green colour - shorter wavelengths) and allophycocyanin (blue green colour - longer wavelengths). A variety of carotenoids are seen, some are very specific to algae, while others are as seen in plants and algae. Apart from these pigments, there is a very special pigment called as 'Scytonemin', which is an extracellular pigment that shields ultraviolet radiations.

13.2. GENERAL CHARACTERS

13.2.1. Habitat

Cyanobacteria are cosmopolitan, which can grow everywhere. Their habitat ranges from water bodies to volcanoes. Some members grow on moist rocks, soil and in deserts also. Most of the members of cyanobacteria grow along with algae in aquatic environments. They include both marine and fresh water cyanobacteria. They are found in all water bodies like seas, rivers, ponds, lakes and even in water storage tanks. Also they grow on snow or hot springs. Some members grow in acidic or alkaline environments also. They contribute about 40 percent of global primary food production along with algae. Furthermore, they grow as endophytes and also in endosymbiotic association with animals. They also live as constituents of lichens.

13.2.2 Thallus Organization

The Blue green algae don't have leaves, roots, stems or branches. Instead, they have a structure called 'Thallus' (Pl. thalli). These organisms have a range of diversity from unicellular to multicellular and filamentous forms (Fig. 13.1). Unicellular forms can either be single cells or colonies. These single cells can either be free living or attached. Multi cellular or filamentous forms can either be uniseriate trichome or multiseriate trichome. Some blue- green algae show true branching, while others show false branching. The cell protoplast is clearly visible as two parts. The peripheral pigmented region called as chromoplasm and a central colourless region, called as centroplasm.

Non-filamentous forms are mainly coccoid forms, with spherical, cylindrical or fusiform shapes. Colonies are made by many cells due to repeated divisions and the colonies are enveloped by mucilaginous sheath. Filamentous forms are formed by long series of cells present one over the

other forming a long trichome. Trichome secretes mucilaginous sheath with different consistency, either firm or flexible sheath. Trichome can be either straight, or somewhat bent. In some species, whole trichome is spirally coiled. Cells in trichomes are either uniform, called as homocystous or interrupted at some intervals by heterocysts (special thick walled cells). Morphological structures of some cyanobacteria are given in Fig. 13.2.

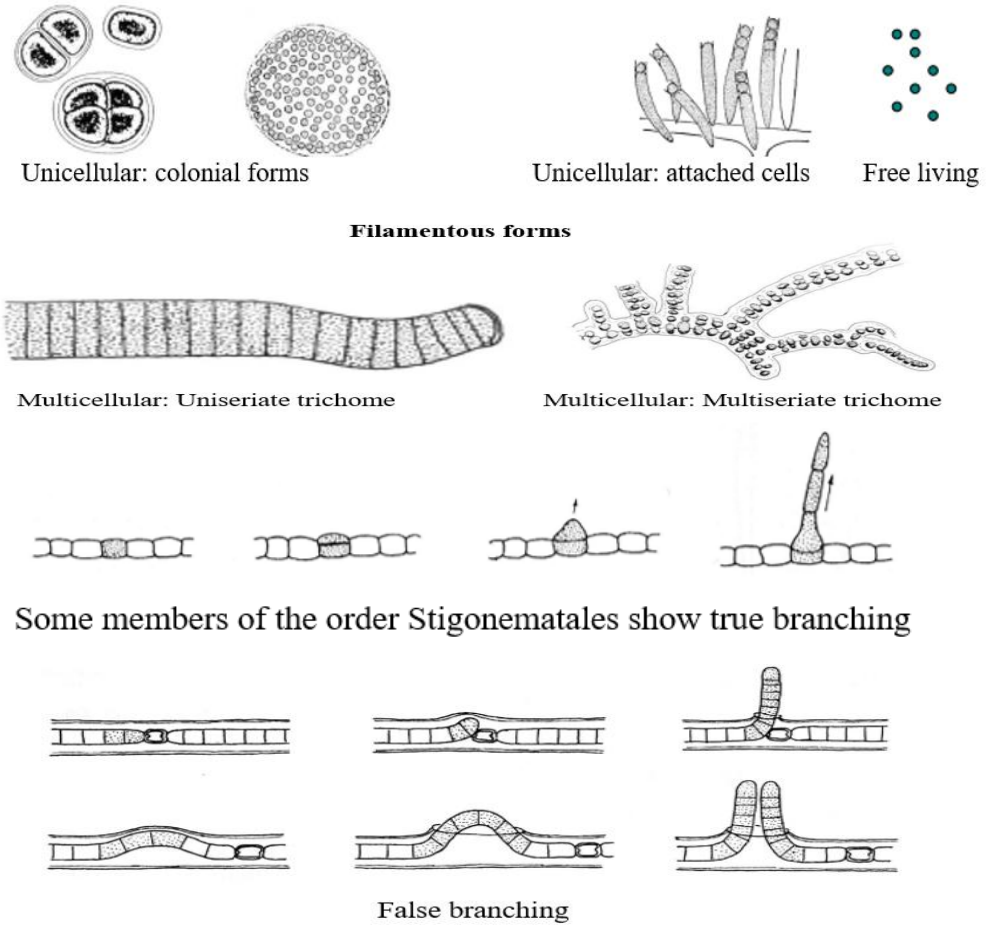


Figure-13.1: Structural diversity in cyanobacteria

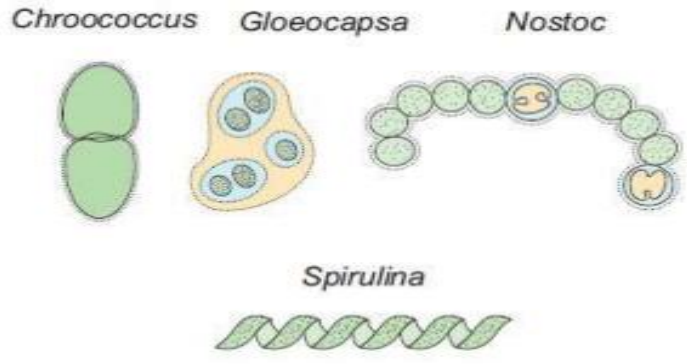


Figure-13.2: Morphological structures of some cyanobateria

13.2.3. Cell Structure

As prokaryotes, blue-green algae, lack organelles like Nucleus, Mitochondria, Chloroplasts, Golgi apparatus, and Endoplasmic reticulum. Glycogen granules, Phycobilisomes, Cyanophycin, Nucleoid, Cell wall, Plasma membrane, Gas vesicles, Ribosomes, Carboxysomes, Lipid granules, Polyphosphate granules and Thylakoids are present (Fig. 13.3)

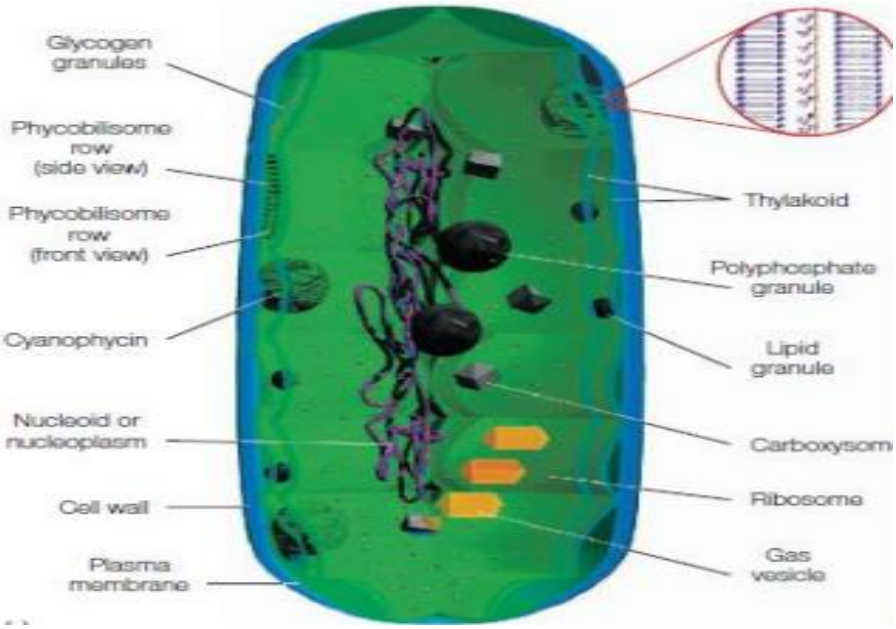


Figure-13.3: Cyanobacterial cell structure
(Source: Hartwell T. Crim 1998.)

Cell wall: The cell wall is Gram negative and consists of murein (peptidoglycan). The cells are embedded in sheath of mucilage. Polypeptides and polysaccharides are present as a thick layer outside the cell wall, which form a brownish pigmented sheath called as 'scytonemin'. This sheath helps the cell in absorbing ultraviolet radiation.

Plasma membrane: The plasma membrane or cell membrane is selectively permeable as in most living organisms and encloses the cytoplasm. It is composed of lipids and proteins.

Cytoplasm: Cytoplasm lies just beneath the plasma membrane which has some structures with different functions. At the periphery, some structures are located, called as lamellae that have pigments, which are not organised as plastids. These membranes or lamellae are plasma membrane derived, which contain pigments like Xanthophylls, Chlorophylls, c-phycocyanin, c-erythrocyanin and Carotenoids. Presence of c-phycocyanin and c-erythrocyanin is the characteristic feature of cyanobacterial members only. Cytoplasm also shows some membrane bound vesicles, sometimes are present in layers which are stacked vertically. Ribosomes are also found in the cytoplasm, which are scattered everywhere.

Nucleoplasm (Nuclear material): The DNA containing region is called as nucleoid, as in bacteria. There is no distinct nuclear membrane, but nuclear material is located at the centre of the cell. Nucleolus is absent and no spindle fibres are formed during cell division, which also makes them different from eukaryotes.

13.2.5. Unique features

Heterocyst

Heterocyst is a thick-walled cell that is larger than the adjacent cells, which help in fixing atmospheric nitrogen. It is also called as diazotroph. Heterocysts are photosynthetically inactive as they lack photosystem II, but they are related to nitrogen fixation. These cells are larger, but they are found to be empty under the microscope (akinetes are differentiated with storage products). Heterocysts create anoxygenic environment for the organism which is necessary for the enzyme nitrogenase. This structure is surrounded by glycolipid cell wall that is thick and laminated, which helps in not allowing atmospheric gases into the heterocyst.

Symbiotic association

Lichens: It is the symbiotic association between algal members and fungi. But there are also some cyanobacteria that occur in about 8% of all the species of lichens.

Anabena-Azolla: This is one of the remarkable symbiotic relationships that occur between *Anabena* (cyanobacteria) and water fern (*Azolla*), which grows widely in all water logged paddy fields. It is a natural fertilizer for paddy fields that provide rich nitrogen source, as *Anabena* has nitrogen fixing ability. It is well documented that the yields of rice greatly increase when farmers use *Azolla* as a natural fertilizer in paddy fields.

Other symbiotic relationships: Cyanobacteria are also known to have symbiotic relationships with other organisms like protozoa, amoeba, green algae, diatoms, liverworts, vascular plants, as well as with water molds.

Anabena-Azolla Symbiosis

Production of Toxins

Cyanobacteria are well known to produce and release some toxins into their habitat, especially in the water bodies, which cause illness to the animals or humans when they consume the water. They include neurotoxins and hepatotoxins. Toxin production distinguishes some cyanobacterial members easily from green algae, as none of the members of green algae were reported to have produced toxins.

Neurotoxins: These toxic substances are alkaloids in nature which target the nervous system in animals and humans. They include saxitoxin and anatoxin, which cause symptoms like gasping, twitching of muscles, convulsions and staggering. The cyanobacterial members that produce these toxins include *Aphanizomenon*, *Oscillatoria* and *Anabena*.

Hepatotoxins: These substances are large compounds which are confronted by liver and causes liver damage due to their remarkable size. The symptoms include diarrhoea, vomiting and weakness. The cyanobacterial members that are responsible for the production of these toxins are *Nostoc*, *Oscillatoria*, *Nodularia*, *Anabena* and *Microcystis*.

13.3. CLASSIFICATION

Phylum Cyanophyta has a class Cyanophyceae, which is divided into 5 orders. They include: Chroococcales, Chamaesiphonales, Pleurocapsales, Nostocales and Stigonematales.

Order Chroococcales - unicellular or colonial forms, but never trichome, no base or apex is seen, no exospores are formed.
Family Chroococcaceae - Unicellular or colonial forms are present. Eg. *Microcystis, Gleocapsa*.
Family Entophysalidaceae - Pseudofilamentous forms are seen. Eg. *Entophysalis*, *Placoma*.

Order Chamaesiphonales – Unicellular cells, these are organized with base/apex, endo/ exospore is present.

A. Family Cylindiaceae - The cells are spherical Eg. *Chroococcidiopsis*
B. Family Chamesiphonaceae – Unicellular cyanobacteria, attached with base/apex, Exospores are present Eg. *Chamaesiphon*
C. Family Dermocarpaceae - Unicellular, attached with base/apex, endospores are present. Eg. *Dermocarpa*

Order Pleurocapsales - These are distinctly filamentous, attached, no hormogones or Heterocysts are present.
Family Pleurocapsaceae – They have firm gelatinous membrane, filamentous in nature, endospores are formed Eg. *Myxosarcina*
Family Hyellaceae - Filaments without hormogones, di/tetrachotomous, endospores Eg. *Hyella*

Order Nostocales - They are filamentous, homogonalen are seen, akinetes, heterocysts,exospores or endospores are formed. This order shows no true branching.

A. Family Oscillatoriaceae - Trichome shows single row of uniform broad cells, sometimes tapering, unbranched with firm mucilage sheath. Heterocyst or spores are absent, trichome may be spirally coiled Eg. *Lyngbya*, *Oscillatoria*, *Spirulina*, *Phormidium*, *Trichodesmium*.
B. Family Nostocaceae - Filaments are either in single or in a definite colony, heterocyst is present which is positioned terminally or intercalary. Heterocysts can be in single or more than one. Eg. *Anabaena, Nostoc, Nodularia.*
C. Family Scytonemataceae - Filamentous forms are present with thick firm sheath, lamellated, showing false branching. Heterocyst is intercalary; many trichomes are present in a sheath. Eg. *Plectonema*, *Scytonema*, *Tolypothrix.*
D. Family Microchaetaceae - Trichome with differentiation of base and apex is seen, these are unbranched, sheath can be seen with single trichome and a heterocyst is present. Eg. *Microchaete*.
E. Family Rivulariaceae - Trichome with tapering apex is seen, which is unbranched possessing basal heterocyst. Hormogones are also present. Eg. *Calotrix*, *Homoeothrix, Dichothrix, Rivularia, Gloeotrichia*.

Order Stigonematales - Filamentous cells with hormogonalen, the structures like heterocysts, akinetes, exo/endospores, with true branching and dichotomy is seen, with prostrate and erect arrangements in this order.

A. Family Capsosiraceae - Thallus is attached and hemispherical, free irregular branches are present, filament with series of one or two cells are seen, heterocyst can either be present or absent. Eg. *Stauromatonema*

B. Family Nostochopsidaceae - Thallus is made up of erect, but many bent filaments which are branched with two types of branching. Some are long and some with limited growth. Terminal heterocyst are present. Eg. *Nostochopsis*

C. Family Mastigocladaceae - Trichome with reverse V shaped branching is seen, intercalary heterocyst is present and endospores are formed. Eg. *Brachytrichia*, and *Mastigocladus*.

D. Family Mastogocladopsidaceae - Trichome with V shaped and lateral branching is seen, Heterocyst is lateral, terminal and intercalary Eg. *Mastigocladopsis*

E. Family Stigonemataceae - Thallus with free bent filaments are present, which are irregularly branched, often prostrate and erect, there might be a lateral or an intercalary heterocyst, Eg. *Hapalosiphon* and *Stigonema*.

13.4. REPRODUCTION

Reproduction in cyanobacteria is carried out by vegetative and asexual methods. Sexual reproduction is not observed in Cyanobacteria.

Vegetative Reproduction: The vegetative reproduction is reported through the following methods (Fig. 13.4):

Hormogonia or Pseudohormogones - short sections of a trichome gets detached and forms a new thallus.

Fragmentation: The cyanobacteria filament breaks into 2 parts by any injury or by mechanical stress. Both the separated fragments form new thalli.

Endospores and Exospores: The internal division of the protoplast results in a mass of spores which can be exospores or endospores.

Nanocytes: These are very small cells formed due to the environment that has very less nutrients.

Planococci or Akinetes: These are resting spores; these are formed by the cells that are resistant to unfavorable conditions like heat or desiccation.

Asexual reproduction: The asexual reproduction is reported through the method called as binary fission.

Binary fission: It is the division of an individual single cell to two new individual single cells (Fig. 13.5), this is similar to that of bacterial binary fission.

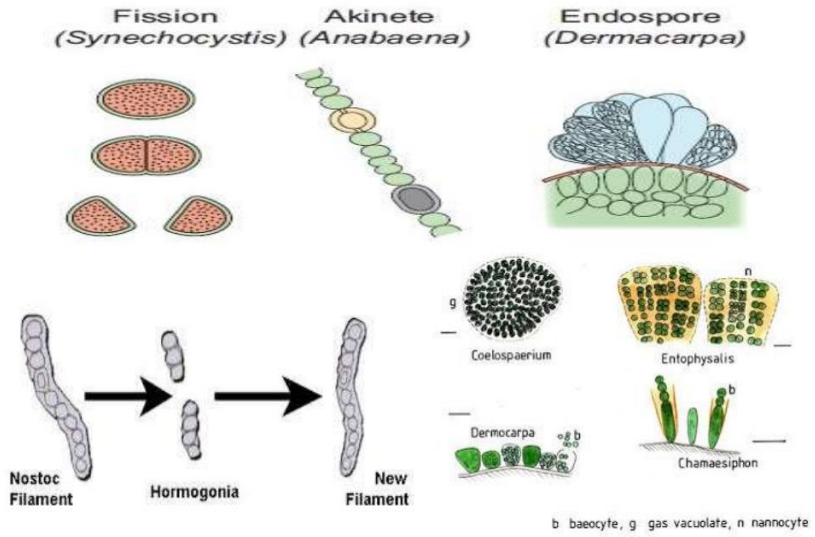


Figure-13.4: Vegetative reproduction in cyanobacteria

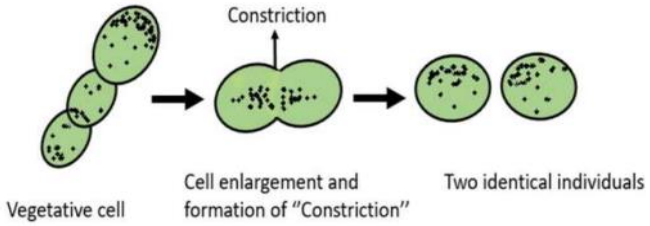


Figure-13.5: Asexual reproduction by binary fission in Cyanobacteria

13.5. SIGNIFICANCE

Cyanobacteria have numerous roles in almost all aspects of human life as well as in nature. It ranges from ecology, extending to food industry, medicine, agriculture, food chain, biotechnology, etc. The applications of Cyanobacteria are numerous and they are increasing as research is carried out more on Cyanobacteria. Some of the applications of Blue green algae are discussed below.

Ecological Importance

Cyanobacteria are one of the very important organisms that help in ecological balance and maintenance of ecosystems. These colonize soil and rocks on their surface, which lead to the formation of mats that help in reduction of soil erosion. Moreover, they help in the formation of coral reefs on the sea bed. Also they precipitate limestone ($CaCO_3$) from waters and helps in the formation of rocks in tropical waters. In addition, one of the very important functions in the environment is carbon sequestering from the atmospheric carbondioxide. This is performed by cyanobacteria that help in the decrease of global warming, which is a great threat to the planet earth these days.

Importance in Agriculture

As cyanobacterial members are efficient in nitrogen fixation, these can be used as bio fertilizers that help the crop plants crow without chemical contamination. If chemical fertilizers are used, they pollute soil and underground water, so cyanobacteria can be used in the crop fields, which help increase the soil fertility of the field without causing chemical pollution. Moreover, these bio fertilizers grow in the field, making it cost effective for the farmers. Eg: *Anabena-Azolla* symbiosis in paddy fields.

Importance in Food chain

Cyanobacteria are very important in the food chain as they contribute a great portion along with algae as primary producers. They grow as planktonic forms like phytoplankton and zooplankton in marine and fresh water habitats, which are consumed by secondary producers. This feature in cyanobacteria is also utilized by cattle breeders, bird keepers, breeders of molluscs, fish and shrimp who provide cyanobacteria derived feed and fodder to the cattle for providing better protein content and vitamins. Generally, *Nostoc*, *Anabena*, *Calothrix* and *Spirulina* are used as food for these animals. In India, *Phormidium valderianum* is used as feed for fish due to its non-toxic nature.

Importance in Food industry

The fact that cyanobacteria can be consumed as food has revolutionized the food industry these days. In fact, this practice is followed from long time. People in Japan, Thailand, China and other countries consume *Nostoc* as food, due to its rich composition of fiber proteins and other nutrients, which have good role in human diet. Members include: *N. commune*, *N.elude*, *N. pruniforme*, etc. In Oregon, USA, *Aphanizomenon* sp. is used as healthy food, which is collected from natural blooms in Lake Klamath. *Spirulina platensis* and other species are consumed by people in Kanembu near Lake Chad, located in North-central Africa from many centuries. *Spirulina* has vitamins and varying protein contents of about 50-70%, which is one of the remarkable sources of protein. In addition, *Spirulina* contains good amount of fats, carbohydrates, minerals and many vitamins like thiamine, riboflavin, β-carotene (Beta carotene) and richest source of vitamin B12.

Importance in Biotechnology

Blue green algae or cyanobacteria have gained importance due to their valuable applications. Some members accumulate PHA (Polyhydroxyalkanoates) that are similar to polyethylene, but produced by cyanobacteria and can be easily degraded by microorganisms. These are the biodegradable plastics produced that do not cause damage to the environment. In addition, cyanobacteria produce many enzymes like nitrogenase, hydrogenase, phosphatase, β-lactamase, elastase, hydroperoxide lyase, arylsulfatase, chitinase, L-asparaginase, L-glutaminase, amylase, protease, lipase, cellulase, urease and lactamase. Cyanobacteria are also employed by researchers to produce biofuels like biodiesel, bioethanol, biomethane and bioalcohol.

Importance in Medicine

Antibiotics and other potential drugs: *Nostoc* was used to treat diseases like fistula, gout and cancers of different types. Natural products from cyanobacteria are very useful to treat many diseases without side effects as in chemically synthesized drugs. Some examples include: Anti-HIV, anticancer, antifungal, antimalarial and antimicrobial agents against, *E. coli*, *Bacillus* sp.

and *Staphylococcus* sp. A natural product named Norharmane is produced by *Nodularia harveyana* which has anti-cyanobacterial activity which can be used in controlling harmful Cyanobacterial toxins. Antifungal activity of *Nostoc* sp. is found to be effective against many fungi like Cryptococcus sp. a cause of secondary fungal infections in AIDS patients.

Cyanobacteria are found to produce antiviral natural products that are effective against many types of viruses. Some natural products are isolated from *Nostoc* and *Cyanothece* which show inhibitory activity against HIV-1, HIV-2, simian immunodeficiency virus (SIV), feline immunodeficiency virus, and measles virus. Also, they were found to inhibit Influenza and Ebola viruses. In addition to this, Cyanobacteria are known to produce antimalarial drugs like Symplocamide (obtained from *Symploca* sp.) was found to be active against *Plasmodium falciparum*. One of the cyanobacterial natural products named as 'Curacin-A', isolated from *L.majuscula* was found promising to be effective against many cancers like colon, renal and breast cancers. Borophycine is a boron containing polyketide compound, that is produced by *Nostoc linkia* which is another promising anticancer compound. Consumption of cyanobacteria also conferred immunity against many inflammatory diseases like colitis, arthritis, etc.

Apart from all the above useful applications, cyanobacteria are also significant in having harmful effects. They kill great number of marine fish every year by forming dense concentrations of blooms in the marine as well as in fresh water resources. By releasing toxins into the waters in reservoirs, they cause gastro-intestinal tract illnesses in cattle and in humans.

13.6. SUMMARY

Blue green algae or cyanobacteria are photosynthetic prokaryotes, which are the only prokaryotes apart from bacteria. They are different from algae and fungi and have prokaryotic cell structure. They have a variety of photosynthetic pigments like Chlorophyll-a, Phycobilins, Xanthophylls, Carotenoids and Phycobilins. Some of which are unique to cyanobacteria like Scytonemin. They are cosmopolitan in nature, but most of the members live in aquatic habitats, in all types of waterbodies. They can grow in a variety of environments like hyperthermophilic, marine, fresh water, terrestrial, desert environments and even in volcanic eruptions. They live in symbiotic relationship with a variety of living organisms like fungi, algae and animals. Along with algae, they account for some portion of Lichens. These organisms have a range of diversity from unicellular to multicellular and filamentous forms, which show branching as well as false branching. Cyanobacteria lack organelles like Nucleus, Mitochondria, Chloroplasts, Golgi apparatus, and Endoplasmic reticulum. Glycogen granules, Phycobilisomes, Cyanophycin, Nucleoid, Cell wall, Plasma membrane, Gas vesicles, Ribosomes, Carboxysomes, Lipid granules, Polyphosphate granules and Thylakoids are present. Cell wall contains of murein (peptidoglycan). Some members form a brownish pigmented sheath called as Scytonemin, which helps the organism in shielding them from Ultraviolet radiation. Nuclear membrane is absent and the nuclear material is present in the cytoplasm as nucleoid. Nucleolus is absent and during cell division, no spindle fibres are formed. Some unique features in Cyanobacteria include: formation of heterocysts, having symbiotic relationships and production of toxins in the blooms. Based on different characters, all the cyanobacterial members are classified into five orders. They include: Chroococcales, Chamaesiphonales, Pleurocapsales, Nostocales and Stigonematales.

Cyanobacteria reproduce in a variety of ways. They include vegetative mode that comprises of hormogonia, fragmentation, spore formation, nanocytes and planococci or akinetes. These organisms also reproduce asexually by binary fission. Cyanobacteria are very significant in every realm of life. They play very important role in ecology, by preventing soil erosion, limestone formation, formation of coral reefs and decreasing greenhouse effect by carbon sequestering. Blue green algae like *Anabena* are very important in agriculture as they are the best biofertilizers, which can fix atmospheric nitrogen. Cyanobacteria are one of the best sources of enzymes, bioplastics, biofuels and other industrial products in biotechnology. Moreover, they have great prominence in human health as they are the source of almost all types of essential drugs like antimicrobials, anti-inflammatory, anticancer, antimalarial, antiviral drugs. Not only good, but there are some members of cyanobacteria that produce toxins in blooms, which cause illness to animals and humans. Thus, cyanobacteria are very significant in all the areas of human life.

13.7. TECHNICAL TERMS

Photosynthesis, Chlorophyll, Phycobilins, Carotenoids, Scytonemin, Lichen, Thallus, Trichome, Heterocyst, Thylakoid, Nitrogenase, hormogonia, Nanocytes, Akinetes, Primary producers, Plankton, *Spirulina*, *Nostoc*, Polyhydroxyalkanoates, Biofuels.

13.8. SELF ASSESSMENT QUESTIONS

Q.1 Discuss the general characters of Cyanobacteria with special focus on its cell structure.
Q.2 Describe the classification and reproduction in Cyanobacteria
Q.3 Explain in detail about the significance of Blue-green algae.

13.9. SUGGESTED READINGS

1. Microbiology – Prescott et. al. 12th edition.
2. General Microbiology - Linda Bruslind
3. Brock Biology of Microorganisms – Madigan et. al.
4. Blue green algae and its application (http://www.phytojournal.com)

Dr. J. Madhavi

LESSON - 14
ACTINOMYCETES

OBJECTIVE OF THE LESSON

Students will be able to understand the cell structure, reproduction and significance of actinomycetes and also differentiate these actinobacteria from eubacteria, fungi and other microorganisms.

STRUCTURE OF THE LESSON

14.1. INTRODUCTION

Actinomycetes are the prokaryotic filamentous Gram positive bacteria with united cylindrical cells that resemble mycelium of the fungus. They are also called as 'Ray fungi'. They are thin, aseptate and shows branching. In some species, filaments break and appear as small coccoid cells, which are non-motile. Young filaments show homogenous cytoplasm, but after maturity cytoplasm shows vacuoles, fat droplets, granules, etc. As they are prokaryotic, they do not have distinct nucleus but many chromatin granules are present. After maturation, the cell wall becomes fragile, allowing the fully grown filaments to easily break into pieces. Actinobacteria is one of the largest groups among other major groups within the domain Bacteria. They play a crucial role in soil ecology and are significant in the production of antibiotics and other bioactive compounds. These actinobacteria are significant in medicine, veterinary sciences, ecology and biotechnology as they are genetically very diverse. Actinomycetes are known for their filamentous structure and complex life cycles. Moreover, these can be easily grown in the laboratory using growth medium. They produce a variety of coloured spores and powder like textures on the surface of the medium. In nature, these organisms are responsible for the pleasant earthy smell observed during the start of monsoons, by producing spores and a volatile chemical substance, called as Geosmin.

14.2. GENERAL CHARACTERS

Distinct characters: Though all actinobacteria are Gram positive, they exhibit a unique morphology that distinguishes them from other bacteria:
Filamentous Structure: Long filamentous structures with branching are formed which are called hyphae and an aggregate of such hyphae forms mycelium.
Cell Wall Composition: The cell wall of actinomycetes is composed of Peptidoglycan as in Gram positive bacteria, but it also contains mycolic acids which help them resist certain antibiotics.

Formation of Spores: In most of the actinomycetes, spores (conidia) are produced. These spores can withstand stress in the environment around them, thus supports them in their survival and the dispersal of spores.

Physiology and Metabolism

The metabolic abilities of actinomycetes are very diverse. This ability is the key for them to survive in a variety of conditions. Actinomycetes are generally aerobes, which need oxygen for growth, but there are some anaerobic actinomycetes too. They are able to degrade like chitin, cellulose and other complex organic chemical compounds. Thus, they have great role in decomposition of complex substances in the soil. Actinomycetes can withstand and grow in a range of temperatures like from 20^{o} C - 60° C. The Hydrogen ion concentration (pH) levels actinomycetes can grow at range from pH4 - pH10, this feature enables them to colonize and live in varied habitats.

Cell Structure

The cell structure in actinomycetes is very diverse. They appear in many forms, which include cocci, small rods, long rods, and some exhibit filament or hyphae like cell structures (Fig. 14.1). Their cell organelles are very similar to that of bacteria, but have certain distinct features in their cell wall, cell membrane and other fungi like special structures called as sporangia, that bear spores until they mature and disperse.

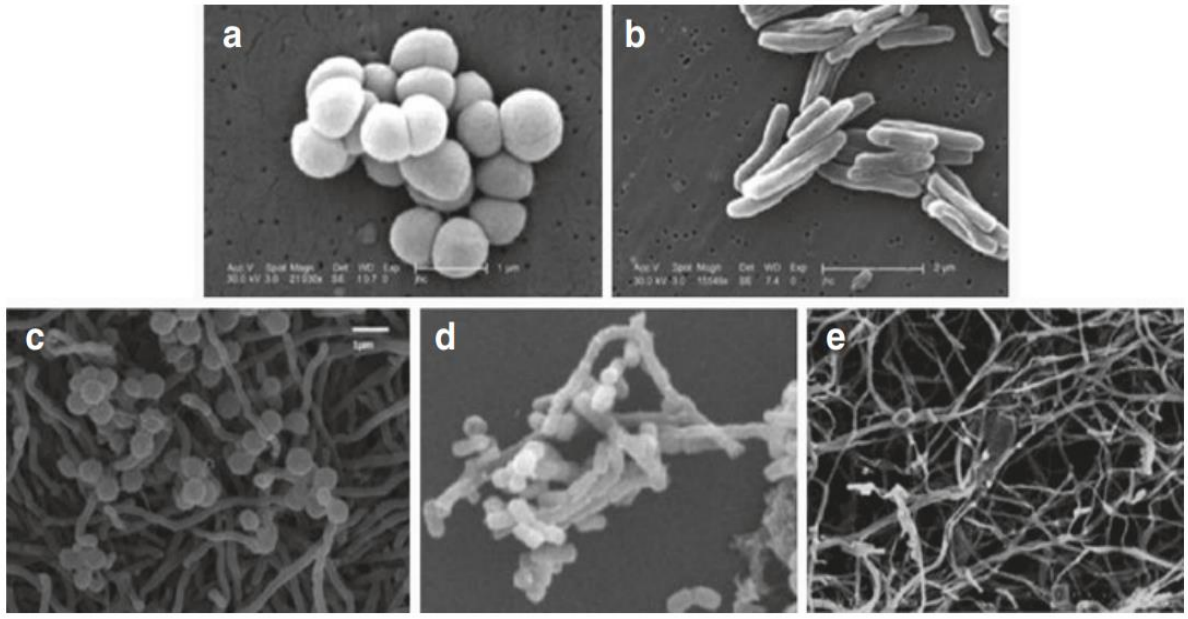


Figure-14.1: Scanning Electron Microscope images of some actinomycetes
(a) cocci of *Micrococcus luteus* (b) rods of *Mycobacterium tuberculosis* (c) branched hyphae of *Micromonospora schwarzwaldensis* (d) fragmenting mycelia of *Nocardia asteroides* and (e) branched aerial hyphae of *Streptomyces mangrovisoli*.

The actinomycetes exhibit similarity with bacteria in terms of cell organelles; on the other hand, they show some specialized structures like spores which have some similarity with fungi. However, they exhibit some unique characters that are not seen in eubacteria as well as in fungi.

Components present outside cytoplasm

The differentiating features of cell wall and cell membrane of *Streptomyces, Corynebacterium* and *Mycobacterium* are given in Fig. 14.2. In *Streptomyces*, as in other Gram-positive bacteria, the cell wall consists of a peptidoglycan layer that covers the cytoplasmic membrane. *Corynebacterium* and *Mycobacterium* contain more complex cell walls including an additional layer of arabinogalactan and an outer membrane, which is composed of mycolic acids, trehalose monomycolate, trehalose dimycolate and free lipids. The mycobacterial cell wall has a high proportion of covalently attached mycolic acid residues, which constitute a permeability barrier contributing to antibiotic resistance and pathogenicity. Phosphatidylinositol-mannosides are also present. In other actinobacterial genera, wall teichoic acids are covalently attached to peptidoglycan. Although, lipoteichoic acid is suggested to be available merely in *Firmicutes*, they are reported in two actinobacterial genera, namely, *Agromyces* and *Streptomyces*, as well as in *Thermobifida fusca* which was suggested to have roles in the maintaining the homeostasis associated with cell envelope.

Another major compartment of actinobacterial cell wall is teichuronic acids, the heteropolymeric polysaccharides composed of an uronic acid along with amino sugars and neutral monosaccharides which are linked to a polymer of either amino acids or glycerol phosphates. These are of common cell wall components of actinobacteria such as *Propionibacterium, Corynebacterium, Catellatospora, Actinoplanes, Streptomyces,* and *Kribbella*.

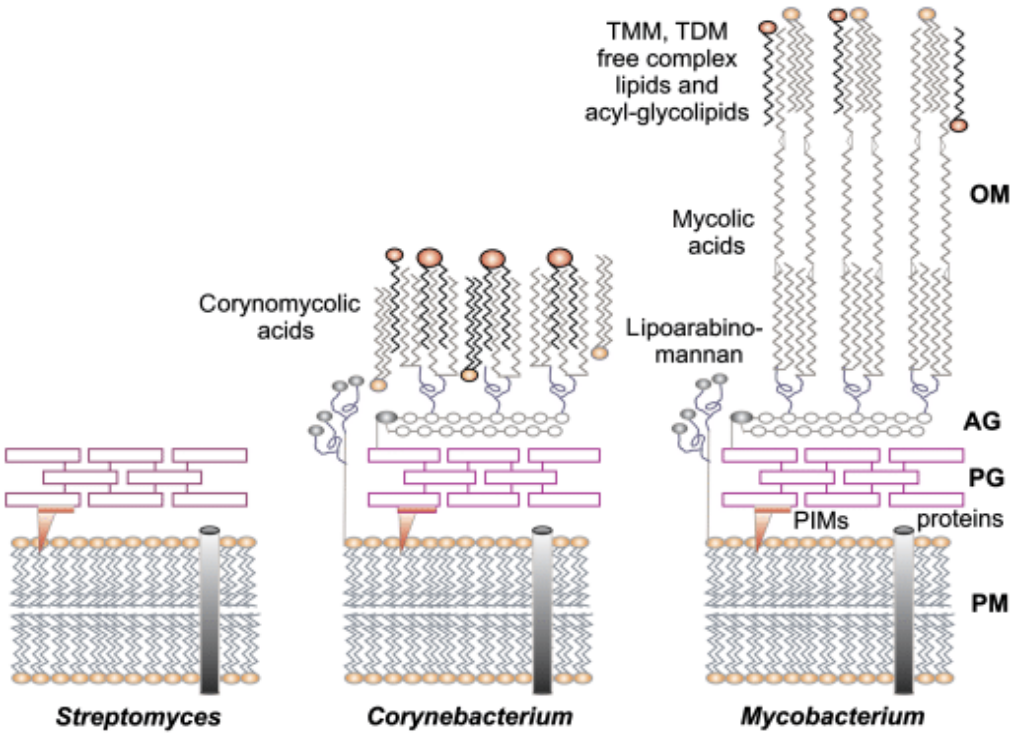


Figure-14.2: Structures outside the cytoplasm of *Streptomyces*, *Corynebacterium* and *Mycobacterium*: PG- Peptidoglycan, AG – arabinogalactan, OM- Outer membrane, PM- Plasma membrane, PIMs- phosphatidylinositol-mannosides, TMM- trehalose monomycolate, TDM- trehalose dimycolate

Special features and structures

Spores

One of the morphological features that distinguish the actinomycetes from eubacteria is sporulation, forming powdery texture and different coloured spores. They exhibit great diversity in terms of their size, shape and colours. Generally, the shape of actinobacterial spores is often spherical, although other shapes may be observed such as cuboid in *Chainia*, oval in *Actinomadura*, or claviform spores of *Dactylosporangium*. Moreover, mature spores usually show a variety of colours such as white, pink, grey, blue, and so on. Sporangia, the bag-like structures for the development and release of spores, also vary vastly on the basis of shape and size.

Pigments present in Actinomycetes

There are a number of pigments that can be seen in actinobacteria, produced by different genera. Some of those pigments are given in the following table.

S.No.	Pigment	Source Organism
1	Anthracyclin glycoside	Streptomyces galilaeus, Streptomyces melanogenes, Streptomyces peucetius
2	Carotenoids	Streptomyces griseus, Streptomyces setonii, Streptomyces coelicolor
3	Melanin	Streptomyces
4	Naphthoquinone	Streptomyces coelicolor
5	Prodigiosin	Serratia, S. treptoverticillium rubrireticuli, Streptomyces longisporus
6	Phenoxazinone	Streptomyces parvullus
7	Violacein	Chromobacterium violaceum

Sporangia

These are the bag-like structures for the development and release of spores, also vary vastly on the basis of shape and size. They are formed whether on substrate or aerial mycelium and can be globose (*Spirillospora*, *Streptosporangium*), cylindrical (*Planomonospora*, *Planobispora*), claviform (*Dactylosporangium*), and in other shapes while they are 2–50 μm with most of them being 10 μm in size. Just like the spores, sporangia have different types based on the number of spores. Sporangia with few spores may be called oligosporous sporangia while polysporous ones contain numerous spores as the name denotes. Most of the actinobacterial members forming sporangium produce planospores although exceptions exist as for *Stretosporangium* and *Kutzneria*. Morphological sporangia of different actinomycetes are given in figure 14.3.

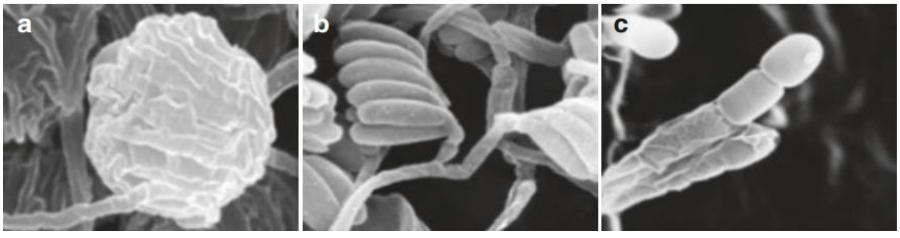


Figure-14.3: Morphology of Sporangia in some actinobacteria.

(a) *Spirillospora albida*
(b) *Planomonospora parontospora*
(c) *Dactylosporangium fulvum*

Other reproductive structures

Finally, there are other less-studied types of reproductive structures reported in actinobacteria such as columnar hyphal structures called synnemata, which bear chains of conidia in *Actinosynnema* and sclerotia in some *Streptomyces* and *Chainia* (Fig. 14.4)

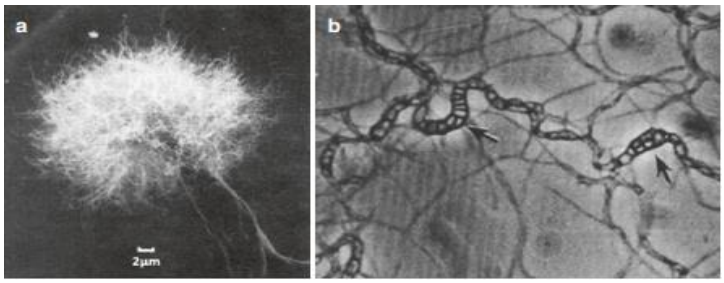


Figure-14.4: Sporangia-like structures.
(a) Synnemata in *Actinosynnema*
(b) Sclerotia (arrows) in *Chainia barodensis*
(Source: Ganju and Iyengar 1974 and Hasegawa et al. 1978)

Reproduction

Reproduction in actinomycetes is carried out by asexual mode. Sexual reproduction is not seen in them.

Asexual methods

Fragmentation: The mycelium can fragment into smaller pieces, each capable of growing into a new organism.

Spore formation: Aerial hyphae produce spores that can be dispersed by wind or water, leading to the establishment of new colonies.

Life cycles of sporulating actinobacteria

The following steps are seen in the lifecycle of actinobacteria (Fig. 14.5).

Vegetative growth

In this stage, actinomycetes grow into the substrate, with the help of substrate mycelium as well as reproductive aerial hyphae forming colonies on the surface of the medium or substratum. Actinomycetes need all essential nutrients from the substrate or medium at this stage to grow. After attaining enough growth, at this stage, secondary metabolites like antibiotics are produced by these organisms which are of great value in the industry and medicine.

Coiling

After the enough growth of aerial hyphae is attained, due to unfavourable conditions or nutrient depletion, the hyphae coils, such that the surface area is increased and the space occupied becomes less when coiled. In addition, when hyphae are coiled, this gives some support or balance to the hyphae.

Septation and Chromosome segregation

Generally, once coiling is finished, the septa are formed, which ensures that there is genetic material in each compartment between adjacent septa.

Spore maturation

Then, the cell that is formed due to septa gets thickened cell wall, that can withstand harsh environmental conditions like high temperature or desiccation. As the spore undergoes dormancy, there will be no need for the spore to have active metabolism or supply of food till it is germinated.

Spore release

Then the septa become fragile to get broken down to individual spores, which help the spores disperse freely in the air to reach appropriate places to germinate, where there is availability of necessary nutrients.

Germination

After the dispersal of spores, they can withstand few years, which helps them to survive till the food or favourable conditions are available. The availability of all the essential nutrients in the spore vicinity is of prime importance for its germination.

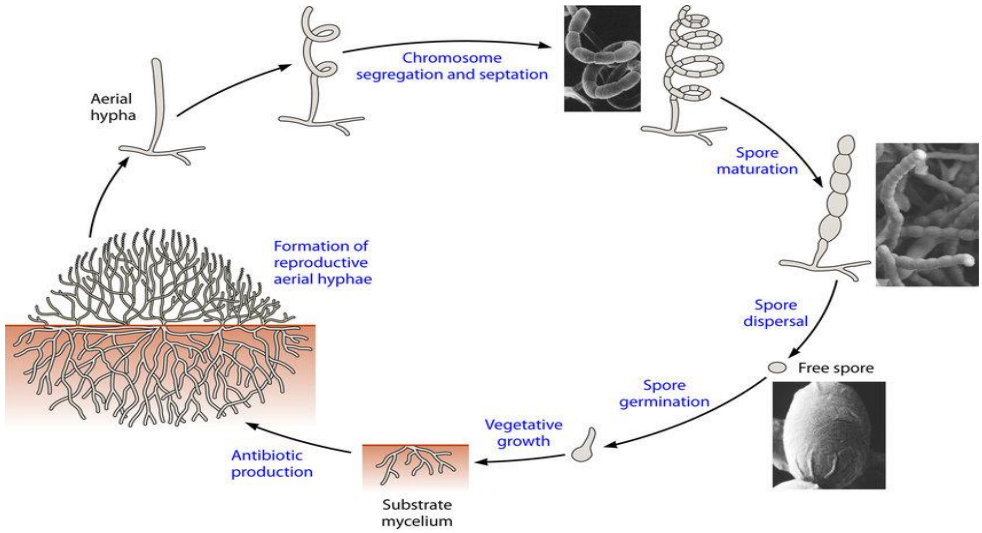


Figure-14.5: The lifecycle of sporulating actinobacteria

14.3 CLASSIFICATION

The classification of actinomycetes is detailed in Bergey's Manual of Determinative Bacteriology, which provides a systematic approach to identifying and categorizing these microorganisms. The classification of actinomycetes is essential for understanding their ecological roles, potential applications in biotechnology, and their significance in medicine. All the actinomycetes are categorized into one class, which were divided into 5 subclasses. Further they are divided into 6 orders, which are Actinomycetales, Rifidobacteriales, Acidinderobiales, Cariobactenales, Sphaerobacterales and Rabrobacterales. They have their suborders as well. Altogether, they were divided into 44 families and their respective genera.
Within these families, actinomycetes can be further classified into various genera and species, based on their morphological, physiological, and biochemical characteristics. Some key features used for classification include filamentous growth patterns, spore formation, cell wall composition, metabolic capabilities and antibiotic production. Some of the important genera include, *Actinomyces, Streptomyces, Corynebacterium, Micrococcus, Propionibacterium,* etc. The largest genus among all actinobacteria is *Streptomyces*, which include about 150 species.

Some of the genera are efficient in secondary metabolite production like *Streptomyces*. Few others in the genus *Mycobacterium* are disease causing bacteria which are known to cause leprosy and tuberculosis, they have acid fast nature which helps them easily get stained and differentiated from other bacteria by Acid fast staining, also called as Zheil-Neelson staining. Also the genus *Corynebacterium* are known to cause diphtheria (*Corynebacterium diphtheriae*) and other bacterial species from other genus are efficient in decomposition of complex chemicals, which help in nutrient supply to plants. The classification is very important in understanding different groups of these organisms, based on their characters.

Families and genera of the class Actinobacteria

Family	Genus	*Family*	Genus
Acidimicrobiaceae	*Acidimicrobium*	*Actinosynnemaceae*	*Actinosynnema*
Rubrobacteraceae	*Rubrobacter*		*Lenzea*
Sphaerobacteraceae	*Sphaerobacter*		*Kutzneria*
Coriobacteraceae	*Coriobacterium*		*Streptoalloteichus*
	Atopobium		*Saccharothrix*
	Slackia	*Streptomycetaceae*	*Streptomyces*
	Egerthella		*Kitasatospora*
Actinomycetaceae	*Actinomyces*		"Trichotomospora"
	Actinobaculum	*Streptosporangiaceae*	*Streptosporangium*
	Arcanobacterium		*Microbispora*
	Mobiluncus		*Planomonospora*
Micrococcaceae	*Micrococcus*		*Planotetraspora*
	Arthrobacter		*Herbidospora*
	Renibacterium		*Planobispora*
	Kocuria		"Sebekia"
	Stomatococcus		"Cathayosporangium"
	Nesterenkonia	*Nocardiopsaceae*	*Nocardiopsis*
	Rothia		
Cellulomonadaceae	*Cellulomonas*		*Thermobifida*
	Oerskovia		*Prauserella*
Promicromonosporaceae	*Promicromonospora*	*Thermomonosporaceae*	*Thermomonospora*
Dermatophilaceae	*Dermatophilus*		*Spirillospora*
Dermacoccaceae	*Dermacoccus*		*Actinomadura*
	Kytococcus		
	Demetria		*"Parvopolysora"*
			"Streptomycoides"
Brevibacteriaceae	*Brevibacterium*	*Actinocoralliaceae*	*Actinocorallia*
Dermabacteraceae	*Dermabacter*		"Sarraceniospora"
	Brachybacterium	*Micromonosporaceae*	*Micromonospora*
Intrasporangiaceae	*Intrasporangium*		*Actinoplanes*
	Janibacter		*Catenuloplanes*
	Terrabacter		*Couchioplanes*
	Terracoccus		*Catellatospora*
Bogoriellaceae	*Bogoriella*		*Dactylosporangium*
Sanguibacteraceae	*Sanguibacter*		*Pilimelia*
Rarobacteraceae	*Rarobacter*		*Spirilliplanes*
Jonesiaceae	*Jonesia*		*Verrucosispora*
Microbacteriaceae	Microbacterium	Frankineae	*Frankia*
	Agrococcus	*Geodermatophilaceae*	*Geodermatophilus*
	Agromyces		*Blastococcus*
	Clavibacter		*Modestobacterium*
	Curtobacterium	*Microsphaeraceae*	*Microsphaera*
	Leucobacter	*Sporichtyaceae*	*Sporichtya*
	Rathayibacter	*Acidothermaceae*	*Acidothermus*
Corynebacteriaceae	*Corynebacterium*	*Cryptosporangiaceae*	*Cryptosporangium*
Mycobacteriaceae	*Mycobacterium*	*Propionibacteraceae*	*Propionibacterium*
Nocardiaceae	*Nocardia*		*Friedmaniella*
	Rhodococcus		*Luteococcus*
Gordoniaceae	*Gordonia*		*Microlunatus*
	Skermania		*Propioniferax*
Tsukamurellaceae	*Tsukamurella*		*Tessaracoccus*
Dietziaceae	*Dietzia*	*Nocardioideaceae*	*Nocardioides*
Pseudonocardiaceae	*Pseudonocardia*		*Aeromicrobium*
	Actinobispora		"Hongia"
	"Actinoalloteichus"	*Glycomycetaceae*	*Glycomyces*
	Actinopolyspora	*Bifidobacteraceae*	*Bifidobacterium*
	Amycolatopsis		*Gardnerella*
	Saccharomonospora		
	Kibdelosporangium	*Kineococcaceae*[a]	*Kineococcus*
	Saccharopolyspora		*Kineosporia*
	Thermocrispum		

Significance of Actinobacteria

Soil health and fertility: They contribute to soil fertility by decomposing dead and decaying organic matter, which helps in recycling of nutrients. This makes nutrients available for the plants.

Plant Growth Promotion: Some species form symbiotic relationships with plants, enhancing nutrient uptake and growth, as they also can produce different secondary metabolites that protect the plants from different fungal and bacterial infections.

Antibiotic Production: Actinomycetes, particularly the genus *Streptomyces*, are known for the production of a wide range of antibiotics, including streptomycin and tetracycline, which are crucial in medicine.

Pharmaceuticals: They are a primary source not only for natural antibiotics, but also for anticancer compounds and other bioactive compounds that are used in medicine.

Biotechnology: Actinomycetes are utilized in the production of enzymes, biofuels, bioactive metabolites, and other bioproducts.

Thus, actinomycetes are a diverse and ecologically important group of bacteria with unique morphological and physiological characteristics. Their ability to decompose organic matter, produce antibiotics, and form beneficial relationships with plants highlights their significance in both natural ecosystems and human applications. Understanding their biology and ecology is essential for harnessing their potential in medicine, agriculture, and biotechnology.

14.4 SUMMARY

Actinomycetes, called as actinobacteria are one of the unique and very diverse and one of the very successful microbes on our planet. They share both the characteristics of bacteria and fungi and possess uniqueness in their cell wall and cell membrane. Moreover, they have special structures called spores, for their survival in harsh conditions. They exhibit a variety of pigmentation on their spores, which makes them identify by their texture.

Actinomycetes are categorized into 1 class, further divided into 5 subclasses. They are divided into 6 orders, which are Actinomycetales, Rifidobacteriales, Acidinderobiales, Cariobactenales, Sphaerobacterales and Rabrobacterales and they have their subclasses.
Altogether, they were divided into 44 families and their respective genera.
They reproduce asexually, by fragmentation and by the formation of spores. Sexual reproduction is not seen in Actinomycetes. There are also some special reproductive structures like synnemata, (that bear chains of conidia in Actinomycetes) reported in Actinomycetes. The lifecycle of actinomycetes is very complex. It has stages like vegetative growth, coiling, Septation, spore maturation, spore release and germination.

14.5 TECHNICAL TERMS

Actinomycetes, Geosmin, Ray fungi, Sporangia, Arabinogalactan, Mycolic acids, Synnemata, Sclerotia, Septation, Antibiotics, Bioactive metabolites, Enzymes.

14.6 SELF ASSESMENT QUESTIONS

Q.1 What are actinobacteria? Discuss their general characters and significance.
Q.2 How are actinobacteria classified, give details on their families and genera.
Q.3 Discuss the reproduction, life cycle and significance of actinobacteria.

14.7 SUGGESTED READINGS

1. Microbiology – Prescott et. al. 12th edition.
2. General Microbiology - Linda Bruslind
3. Brock Biology of Microorganisms – Madigan et. al.
4. Biology and Biotechnology of Actinobacteria – Wink et. al. (10.1007/978-3-319-60339-1, Springer publication).

Dr. J. Madhavi

LESSON - 15

BACTERIAL GENERA – BACILLUS, CLOSTRIDIUM, STAPHYLOCOCCUS, RHIZOBIUM, AGROBACTERIUM AND ESCHERICHIA

OBJECTIVE OF THE LESSON

To know the characteristic features of different bacterial genera which is very important and significant in identifying the bacterial genera.

STRUCTURE OF THE LESSON

15.1 INTRODUCTION

Bacteria are prokaryotic organisms and can be distinguished from eukaryotic organisms but in some instances it may be difficult, especially the hyphae formed by actinomycetes might be confused with the hyphae formed by molds. Some eukaryotic cells are as small as bacteria, and some bacteria are as large as some eukaryotes. Characterizing tests for the identification of various bacterial genera isolated from different sources range from the descriptive through simple

biochemical tests for the detection of metabolic products or enzyme action, to highly specialized techniques required for the estimation of the GC percentage of the bacterial DNA. The desirability of careful morphological examination of strains as a first step in identification cannot be overemphasized. Simpler biochemical and physiological tests which are frequently useful in identification of bacteria at the species level include oxides, catalase, urease, nitrate reduction, H_2S production, acid or gas production from sugars, tests of amino acid metabolism, temperature range of growth, response to NaCl and antibiotic sensitivity. In some genera, the nutritional requirements, ability to utilize specific substrates and production of polyhydroxybutyrate are also useful. For a practical point of view, it is important to identify a bacterium by its characters.

15.2 BACILLUS

Characteristics

The genus Bacillus was erected by Cohn in 1872 to accommodate a bacterium described by C.G. Ehrenberg in 1835 as Vibrio subtilis. Bacillus (Latin "stick") is a genus of Gram positive, rod shaped bacteria. It belongs to the phylum Bacillota, class Bacilli, order Bacillales and family Bacillaceae with 266 named species. The term is also used to describe the shape (rod) of other so-shaped bacteria, and the plural Bacilli is the name of the class of bacteria to which this genus belongs. Distributed in a wide range of habitats, a few species are pathogenic to vertebrates or invertebrates. Members of the genus Bacillus can be easily isolated from soil or air. They are the most common organisms to appear when soils are streaked on agar plates. Morphologically the cells are rod shaped (Fig. 15.1) and straight, with a size range of 0.5-2.5 μm x 1.2 – 10 μm. The cells are often arranged in pairs or chains with rounded or squared ends. Cells stain Gram-positive and are motile by peritrichous flagella.

Members of the genus grow in the temperature range of –5ºC to 75ºC and in the pH range of 2.0 to 8.0. They tolerate the salt concentration of 2-25% range. Members are aerobic or facultatively anaerobic. They exhibit a great diversity in physiological abilities and biochemical properties. Most of the members are chemoorganotrophs with fermentative or respiratory metabolism. Members usually grow well on synthetic media containing sugars, organic acids, alcohols and so as sole carbon source, and ammonium as the sole nitrogen source. A few isolates require vitamins for their growth. Many bacilli produce extracellular hydrolytic enzymes that can break down the polysaccharides, nucleic acids and lipids permitting the organisms to use these products as carbon sources. Species of this genus produce endospores which are oval or sometimes round or cylindrical in

shape. These endospores are highly resistant to many adverse conditions. Sporulation process is not repressed by the exposure to air and not more than one spore is produced per cell. Because the spores of many Bacillus species are resistant to heat, radiation, disinfectants, and desiccation, they are difficult to eliminate from medical and pharmaceutical materials and are a frequent cause of contamination. Members of the genus Bacillus produce the antibiotics like bacitracin, gramicidin and polymyxin. Some Bacillus species can synthesize and secrete lipopeptides, in particular surfactins and mycosubtilins. Bacillus species are also found in marine sponges. Marine sponge associated B. subtilis (strains WS1A and YBS29) can synthesize several antimicrobial peptides and can develop disease resistance in Labeo rohita.

Bacillus species are ubiquitous in nature and occur in extreme environments such as high pH (B. alcalophilus), high temperature (B. thermophiles) and high salt concentrations (B. halodurans). They also very commonly found as endophytes in plants where they can play a critical role in their immune system, nutrient absorption and nitrogen fixing capabilities. B. thuringiensis produces a toxin that can kill insects and thus has been used as insecticide. B. siamensis has antimicrobial compounds that inhibit plant pathogens, such as the fungi Rhizoctonia solani and Botrytis cinerea, and they promote plant growth by volatile emissions. Some species of Bacillus are naturally competent for DNA uptake by transformation. Two Bacillus species namely B. anthrasis (causes anthrax disease) and B. cereus (causes food poisoning) are medically important. Bacillus subtilis is an important model organism and a notable food spoiler causing ropiness in bread and related foods. Some environmental and commercial strains of B. coagulans may play a role in food spoilage of highly acidic, tomato-based products.

Many Bacillus species are industrially significant and are able to secrete large quantities of enzymes. B. amyloliquefaciens is the source of a natural antibiotic protein barnase (a ribonuclease), alpha amylase used in starch hydrolysis, the protease subtilisin used in detergents, and the BamH1 restriction enzyme used in DNA research. The capacity of selected Bacillus strains to produce and secrete large quantities (20-25g/L) of extracellular enzymes has placed them among the most important industrial enzyme producers. The ability of different species to ferment in the acid, neutral, and alkaline pH ranges, combined with the presence of thermophiles in the genus, has led to the development of a variety of new commercial enzyme products with the desired temperature, pH activity, and stability properties to address a variety of specific applications. Classical mutation and/or selection techniques, together with advanced cloning and protein engineering strategies, have been exploited to develop these products.

So, Bacillus subtilis is one of the best understood prokaryotes, in terms of molecular and cellular biology. Its superb genetic amenability and relatively large size have provided the powerful tools required to investigate a bacterium from all possible aspects. Research on B. subtilis has been at the forefront of bacterial molecular biology and cytology, and the organism is a model for differentiation, gene or protein regulation, and cell cycle events in bacteria.

Species

Bacillus subtilis ----- small rods, occur singly, lateral flagella, stains uniformly, produce endospores. Cell size is 0.8 μm x 1.5-1.8 μm. Colonies on agar medium are round or irregular, surface is dull, become thick and opaque, may be wrinkled. May be cream or brown in colour. Species produce 2,3- butanediol and glycerol during glucose fermentation.

Bacillus cereus --- cells tend to occur in chains. Polyhydroxy butyric acid granules and volutin granules are the reserve food materials. Some strains produce red pigment, some produce yellow green fluorescent pigment, and some produce pinkish brown pigment. On fermentation of glucose, 2,3-butanediol and glycerol are produced by this species. Causes some forms of food poisoning and can infect humans.

Bacillus licheniformis– – colonies are opaque on agar medium. Hair like outgrowths are common. Many strains form red pigment on media containing sufficient iron. Produce 2,3-butanediol and glycerol on fermentation of glucose.

Bacillus thuringiensis- - - can be used as insecticide as it produces a solid protein crystal called as parasporal body next to their endospores during sporulation. This B.T. parasporal body contains protein toxin that kill over 100 species of moths by dissolving in the alkaline gut contents of caterpillars and destroying their gut epithelium. The solubilized toxin proteins are cleaved by midgut proteases to smaller toxic polypeptides that attack the epithelial cells. The alkaline gut contents escape into the blood causing paralysis and death. This species is pathogenic to larvae of Lepidoptera.

Bacillus sphaericus --- produce parasporal body that contains proteins toxic to larvae of mosquito.

Bacillus anthracis --- well known for causing anthrax disease in both animals and humans.

Bacillus polymyxa ----- multiplication occurs in decomposing vegegation, participlate in retting of flax, produce 2,3-butanediol ethanol and H_2S, spores are widely spreaded, produce the antibiotic, polymyxin.
The other important species of this genus include B. macerans, B. brevis, B. coagulans, B. stearothermophilus etc.

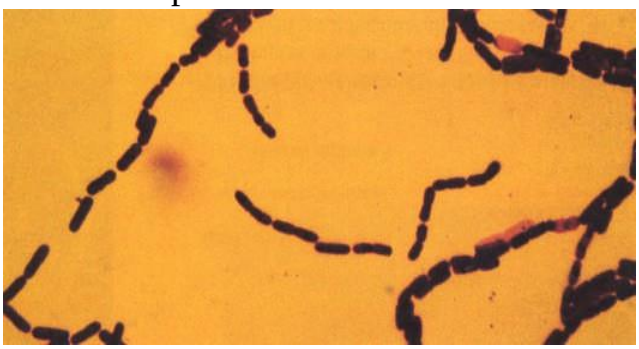


Figure-15.1: Cell structure of Bacillus megaterium

15.3 CLOSTRIDIUM

Characteristics

The genus Clostridium was established by Prazmocoski in 1880 to accommodate butyric acid fermenting organism and was named as Clostridium butyricum. Clostridium genus belongs to phylum Firmicutes, class Clostridia, order Clostridiales and family Clostridiaceae. More than 60 species were described in this genus. Soil is the main habitat of clostridia where they live primarily in anaerobic pockets made by facultative organisms acting upon various organic compounds present. They are also found in fresh water and marine waters especially in the anaerobic zones. In addition, a number of clostridia have adopted to the anaerobic environment of the mammalian intestinal tract. Many clostridia cause spoilage of canned foods. Some soil clostridia cause serious diseases in man and animals mainly by their ability to produce toxins. Tetanus of humans, gas gangrene in humans and domestic animals and botulism in sheep and ducks are caused by members of Clostridium genus.

Morphologically, the cells are straight or slightly curved rods. In size, they measure 0.3-2.0 μm x 1.5-8.0 μm. Mostly occur as single cells, may be arranged in pairs or short chains with rounded or sometimes pointed ends. Some are known to be pleomorphic. They are Gram-positive. Most of them are motile by peritrichous flagella, a few are non-motile. Produce highly resistant, heat stable endospores which may be spherical or oval placed in the center or eccentric or distal (Fig. 15.2) or sub-terminal positions.

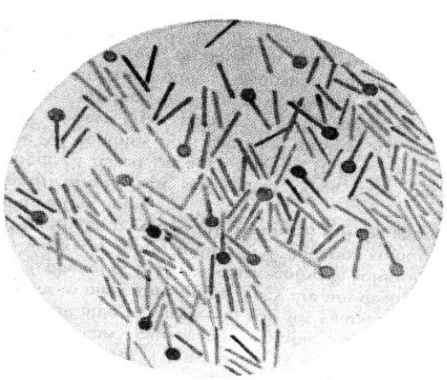


Figure-15.2: Endospore of Clostridium tetani

On nutrient agar members show little or no growth, show good growth on glucose agar. Colonies are circular to irregular, spreading type with white to cream colour. In glucose broth, they show diffuse turbid growth and ropy sediment. A red coloured pigment is produced by C. rubrum. Most species are chemo-organotrophic. Members may be saccharolytic, proteolytic, neither or both. Usually they produce mixtures of organic acids and alcohols from carbohydrates or peptones. Species do not carry dissimilatory sulfate reduction. They are catalase negative and obligately anaerobic. If at all growth occurs in air, it is scanty and sporulation is inhibited. Metabolically they are very diverse with optimum temperature of 10-65^{o}C. Biochemically clostridia show much variation and this property is commercially exploited to produce organic acids.

Various species of this genus are known to be capable of fermenting different substrates like cellulose, sugar, starch, pectin, proteins, amino acids, carbohydrates, purines, ethanol to yield different end products like acetic acid, lactic acid, succinic acid, ethanol, H_2, CO_2, acetone, butanol, butyric acid, fatty acids, formate, acetate, isobutyric acid, isovaleric aicds, butyric acid. The pathogenic species of this genus produce highly potent neurotoxin namely Botulinum toxin and tetanospasmin.

Species

Clostridium butyricum ------ Straight or slightly curved rods, 0.6-1.2 by 3.0-7.0 μm, with rounded ends; occurring singly, in pairs, in short chains and occasionally long filaments. Motile with peritrichous flagella. Spores are oval and eccentric to subterminal, with no exosporium and no appendages. Gram-positive becoming negative in old cultures. Cell wall contains diaminopimelic acid and glucose is the only cell wall sugar. Little or no growth on nutrient agar and good growth on

glucose agar. Surface colonies circular to slightly irregular, 1-3 mm in diameter, slightly raised, white to cream color, glossy to matt surface. Good growth and gas production in broth media with fermentable carbohydrate. Fermentation products include acetic acid, butyric acid and butanol. Casein and gelatin are not hydrolyzed. Milk becomes acid with early coagulation and often with stormy fermentation. Does not require amino acids or vitamins, other than biotin, for growth. Optimum temperature for growth is 25°-37°C. found in soil, animal feces, cheese, naturally soured milk. The G+C content of the DNA is 27-28 moles %.

15.4 STAPHYLOCOCCUS

Characteristics

First observed in human pyogenic lesions by Von Recklinghausen in 1871 and the genus Staphylococcus was erected by Rosenbach in 1884. It belongs to phylum Firmicutes, class Bacilli, order Bacillales, and family Staphylococcaceae. Derived the name as so because of the arrangement of berry like cells in a grape bunch fashion (Fig. 15.3). Most of the strains are pathogenic. Mainly associated with skin, skin glands and mucous membranes of warm-blooded animals. They also serve as hosts for a wide range of bacteriophages.

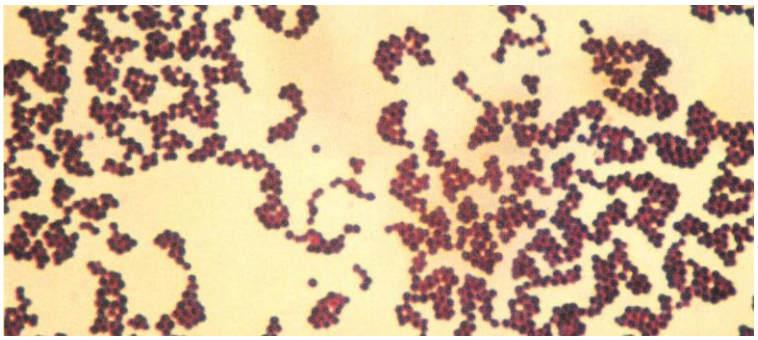


Figure-15.3: Grape bunch structure of Staphylococcus aureus

Cells are spherical with a diameter in the range of 0.5 μm -1.5 μm. They occur singly or in pairs or in characteristic irregular clusters. They are non-motile. Resting stages are not known in the genus. Members are Gram-positive with normal peptidoglycan and teichoic acids in the cell wall. They can grow over a temperature range of 6.5°C – 46°C with optimum temperature between 35°C to 40°C. The range of pH in which the organism grows is 4.2 – 9.3, but the optimum pH is between 7.0 to 7.5. Most strains grow in the presence of 15% NaCl or 40% bile. Members are chemoorganotrophs with respiratory and fementative metabolisms. They show a positive catalase reaction. Menaquinones and

Cytochromes a, b, and O form electron transport system. They employ O_2 as the universal terminal electron acceptor. Amino acids and vitamins are required for aerobic growth. Uracil and fermentable carbon source are required for anaerobic growth.

A wide range of carbohydrates may be utilized, particularly in the presence of air with the production of acid with no detectable gas. However, acid is not usually produced from arabinose, cellobiose, inositol, inulin and raffinose. Under anaerobic conditions, the main product of glucose fermentation is lactic acid. Main products of glucose fermentation in aerobic conditions are acetic acid and small amounts of CO_2. Starch is usually not hydrolyzed but a variety of protein and fat-containing substrates are hydrolyzed. Some members of the genus produce extracellular enzymes and toxins. Staphylococcus spp. are usually sensitive to heat and moderately resistant to γ-radiation. They are sensitive to antibiotics like β –lactams, macrolides, tetracyclines, novobiocin and chloramphenical, and also to antibacterials such as phenols and their derivatives, salicylanilides, carbanilides, halogens and their derivatives. But they are resistant to lysozyme, polymyxin and polyenes.

Species

Staphylococcus aureus ----- Cell walls contain organic phosphorus, ribitol, glucosamine, muramic acid, glycine, lysine, aspartic acid, serine, glutamic acid, alanine and small amounts of threonine, proline, valine and leucine. Cell membranes contain the glycolipids, mono and diglucosyl- diglyceride and the phospholipids, lysyl-phospatidyl-glycerol, phosphatidyl-glycerol and cardiolipin. Colonies are smooth, low-convex, glistening with entire edge. Colonial pigmentation is extremely variable, hence the variety of specific epithets such as aureus, albus and citreus have been applied to this species. Colonies of most strains are orange in color although certain antibiotic resistant strains are commonly yellow pigemented.

Chemoorganotrophs with respiratory and fermentative metabolism. Acid is produced aerobically and anaerobically from glucose, lactose, maltose and mannitol. In air a wider range of carbohydrates are used as carbon and energy sources and hexoses, pentoses, disaccharides and sugar alcohols are metabolized with the production of acid. Acetoin is produced as an end-product of glucose metabolism. Nitrates reduced by nitrate reductases. Ammonia produced from arginine by arginine dihydrolase. Proteases, lipases, phospholipases, lipoprotein lipases, esterases and lyases are produced. Coagulases are produced by virtually all strains. At least three hymolysins are produced (alpha, beta and delta), distinguished by type and range of hemolysis on sheep, rabbit and human erythrocytes.

Facultative anaerobes growing best under aerobic conditions. Most strains grow best at optimum temperature of 30º-37ºC and optimum pH of 7.0 – 7.5 and in 15% sodium chloride or 40% bile. The G+C content of the DNA is 30.7-39 moles %. Potential pathogens causing a wide range of infections and intoxications.

Originally isolated from pus in wounds but also found in nasal membranes, hair follicles, skin and perineum of warm-blooded animals. Other important species in the genus are Staph. epidermidis, Staph. Hominis, Staph. saprophyticus and Staph. capitus.

15.5 RHIZOBIUM

Characteristics

Rhizobium is placed under the phylum Proteobacteria, class Alphaproteobacteria and family Rhizobiaceae. Cells are rod shaped with a size range of 0.5-0.9 μm width and 1.2-3.0 μm length. Commonly pleomorphic under adverse growth conditions. Cells stain Gram-positive and motility occurs by one polar flagellum or sub-polar flagellum or by 2-6 peritrichous flagella. Few strains possess fimbriae. Usually contain polyhydroxy butyric acid granules which are refractile by phase contrast microscopy.

Colonies are circular, convex, semi-translucent, raised and mucilaginous, 2-4 mm in diameter within 3-5 days on yeast-mannitol-mineral salts agar medium. In agitated broth a pronounced turbidity develops after 2 or 3 days. The optimum temperature for a good growth range from 25ºC to 30ºC and the optimum pH for growth range from 6.0 to 7.0. Members of the genus are aerobic with respiratory metabolism and use O_2 as terminal electron acceptor. Often grow well under O_2 tensions less than 1.0 kPa.

Mostly chemoorganotrophic and utilize a wide range of carbohydrates and salts of organic acids as carbon source without any gas formation. They are not capable of utilizing cellulose and starch. In mineral salts medium containing mannitol and other carbohydrates, they produce an acidic reaction. On carbohydrate media, growth is usually accompanied by copious extracellular polysaccharide slime. Ammonium salts, nitrite, nitrate and most amino acids can serve as nitrogen sources. Some strains will grow in a simple mineral salts medium with vitamin-free casein hydrolysate as the sole source of both carbon and nitrogen. Peptone is poorly utilized. Casein and agar are not hydrolyzed. Some strains require biotin or other water soluble vitamins for their growth.

Species of Rhizobium are able to invade the root hairs of temperate-zone and some tropical-zone leguminous plants and incite production of root nodules (Fig. 15.4). The bacteria are present in root nodules in the form of bacteroids. These bacteroides are pleomorphic, swollen and misshapen rhizobial cells which involves in the fixation of atmospheric nitrogen into ammonia that can be utilized by host plants. This conversion is catalyzed by the enzyme nitrogenase. All strains of the genus that nodulate the plants exhibit host range affinities referred as host specificity.

Figure-15.4: Root nodule formed by Rhizobium species

Species

Rhizobium leguminosarum ---- Motile by two to six peritrichous flagella. Fimbriae described on a few strains. Some forms are encapsulated. Colonies are circular, covex, semitranslucent, raised and mucilaginous; usually 2-4 mm in diameter within 3-5 days on yeast mannitol mineral salts agar. Pronounced turbidity develops after 2-3 days in agitated broth. Utilize a wide range of carbohydrates, glucose, mannitol or sucrose are usually preferred. Some strains require biotin or other water-soluble vitamins. Generally cause nodule formation on temperate zone leguminous plants. The G+C content of the DNA ranges from 59.1-63.1 moles %.

Important other species of the genus include – R. loti and R. meliloti among the others.

15.6 AGROBACTERIUM

Characteristics

The genus Agrobacterium was erected by Cohn in 1942 to accommodate three

species into it. A fourth species was added in 1947. The genus with four important species was placed in the phylum Pseudomonadota, class Alphaproteobacteria, order Hyphomicrobiales and family Rhizobiaceae. Cells are rods in shape with a size range of 0.6 μm –1.0 μm in width and 1.5 μm - 3.0 μm in length. Cells occur either singly or in pairs. They are Gram-negative and non-spore forming. Motility occurs by 1-6 peritrichous flagella. The optimum temperature for the growth of organism is between 25ºC to 28ºC. Colonies are usually convex, circular, smooth and non-pigmented. The growth on carbohydrate containing media is usually accompanied by copious amounts of extracellular polysaccharide slime. Members of the genus are positive to catalase, urease and oxidase activities.

Nutritionally Agrobacterium species are chemorganotrophs capable of utilizing a wide range of carbohydrates, salts of organic acids and amino acids as carbon source. Cellulose, starch, and galactose cannot be utilized. Ammonium salts and nitrates can serve as nitrogen sources for strains of some species and biovars, whereas, some species require amino acids and other growth factors.
Except the A. radiobacter, other members of this genus invade the crown, roots and stems of a great variety of dicotyledenous and some gymnosperms via wounds causing the transformation of the plant cells into autonomously proliferating tumor cells. The induced plant disease are commonly known as crown gall, hairy root and cane gall. Some strains possess a wide host range and some others possess a very limited host range. The tumor (Fig.15.5) induction by Agrobacterium tumefaciens is due to the presence of a large tumor inducing plasmid called Ti-plasmid in the bacterial cells. Following infection, a part of Ti-plasmid called transfer DNA called T-DNA is integrated into the genome of the plants. This T-DNA carries genetic information for tumor formation and for the production of a number of modified amino acids called opines. Octopine and nopaline are the two most common opines.

Figure-15.5: Tumour growth formed by Agrobacterium tumefaciens

Opines are produced by plant cells transformed by T-DNA and serve as a source of carbon and nitrogen for Agrobacterium cells, but not essential for tumor formation. Ti-plasmid also encodes genes for several virulence factors and for two phytoharmones, auxin and cytokinin. The ratio of these harmones affect the final morphology of the tumor. The T-DNA portion of the Ti-plasmid moves from bacterium to plant and integrate into plant genome and subsequently transcribed and translated. Agrobacterium tumefaciens has been used as a significant vector to introduce foreign DNA into plants to generate transgenic plants through rDNA technology.

Species

Agrobacterium tumefaciens----- Exhibit rapid growth on meat extract or yeast extract peptone media. A range of simple carbohydrates, organic acids and amino acids serve as carbon and energy sources. On mannitol nitrate glycerophosphate agar medium, colonies show mucoid growth with halo or browning and often with a white precipitate. Causes galls of plants in more than 40 families. Gall tissues are ill-defined consisting of disorganized masses of hyperplastic and hypertrophic tissues interspersed with badly organized groups of elements resembling trachea.

Agrobacterium rhizogenes ----- Causes hairy root or woolly knot disease whereby masses of intertwined fleshy and fibrous roots are produced on nursery stock.

Agrobacterium rubi ------ Causes the formation of small spherical growths or elongated ridges described as beading, corralling or knotting on black and purple cane raspberries and to a lesser extent on red raspberries.

15.7 ESCHERICHIA

Characteristics

Genus Escherichia is placed in phylum Proteobacteria, class Gammaproteobacteria, order Enterobacteriales and the family Enterobacteriaceae. E.coli was first isolated by the German Bactriologist Theodar Escherich in 1885 from the intestinal contents and described it as Bacterium coli. In 1919, Castellani and Chalmers erected the genus Escherichia in honour to Escherich. E.coli is the universal inhabitant of large intestine i.e., colon of humans and warm blooded animals. It occurs in polluted waters, sewage etc. and the presence of it is taken as an indicator of faecal matter contamination. Few strains of E. blattae occurs in the hind gut of cockroaches. The pathogenic forms cause intestinal disorders or diarrohoea and urogenital tract diseases.

Cells of Escherichia are straight rods which measure between 1.1 μm to 1.5 μm in width and 2.0 μm to 6.0 μm in length. They may occur singly or in pairs. Many strains possess capsules or microcapsules. Cells are motile or non-motile, if motile they move by peritrichous flagella. Escherichia possesses frimbriae or pili for attachment. They grow well at an optimum temperature of 37ºC and pH of 7.0. Colonies on nutrient agar may be smooth, low convex, moist shiny surface, gray and easily emulsifiable in saline or colonies may be rough and do not emulsify in saline. In broth medium, growth is shown by a general turbidity and a heavy deposit in S-forms and a clear supernatant with granular deposit in R-form.

Members are facultatively anaerobic and chemoorganotrophs having both respiratory and fermentative types of metabolism. Nutritionally so versatile and readily grow on simple media. Biochemically, organisms are negative for oxidase, Voges-Proskauer and citrate tests and positive for catalase and MR tests. Serologically possess O-antigen or somatic antigen, K-antigen or capsular antigen and H-antigen or flagellar antigen. Some strains produce enterotoxins which are of two types namely heat-stable toxins and heat-labile toxins. Some strains of E. coli produce colicins, an antibiotic like substances. The important pathogenic strains of E. coli are EPEC, EHEC, ETEC and E1EC strains.

E. coli is undoubtedly the best studied bacterium and experimental organism of choice for various studies because of its rapid growth ability, simple nutritional requirements besides the easiness to work with the organism. The ability of E. coli to support the growth of a whole range of bacterial viruses made it possible to study the details of nature and multiplication of viruses.

Species

Escherichia coli --- Many strains have capsules or similar less well developed structures. Fimbriae on many strains; subdivided by their direct hemagglutinating capacity or differences in morphology into several fimbrial types; the sex (or F) fimbrial type can be detected by its affinity for special male phages and by its antigenic properties. In broth, growth is shown by a general turbidity and a heavy deposit which disperses completely on shaking (S form); the extreme R form shows a clear supernatant and a granular deposit which does not disintegrate completely on shaking.

Found in the lower part of the intestine of warm blooded animals. Many, if not all, members may show opportunistic pathogenicity like urinary tract infections in man, mastitis in cows etc. A limited number of well defined serotypes is closely associated with certain infectious enteric diseases in human infants and young of

other animals. Hemolytic strains are found in high frequency in pigs.
Other species of the genus include E. blattae, E. fergusorii, E. hermanii and E. vulneris.

15.8 SUMMARY

Different genera of bacteria possess their own characteristic features besides some common characters. Each bacterial genus may sometimes differ with other genus in their ecological and medical significance. Bacillus and Clostridium are the two important genera belonging to Gram- positive endospore forming bacteria. Bacillus species are aerobic or facultatively anaerobic Gram- positive rods that include the etiologic agent of anthrax (Bacillus anthracis). Another species is used as an important biological insecticide (Bacillus thuringiensis). The members of Clostridium are obligate anaerobic Gram-positive rods whose members include the etiologic agent of tetanus (Clostridium tetani), botulism (Clostridium botulinum), and gas gangrene (Clostridium perfringens). These three diseases are among the most significant causes of death in humans.

One of the most important Gram-negative rods in agriculture is the genus Rhizobium. The Rhizobium species are symbiotic, that is, they live on the roots of legume plants and perform nitrogen fixation. In this process they trap nitrogen from the air and fix it into nitrogen compounds that can be used by plants in their metabolism. Another important Gram-negative rod bacterium in agriculture is the Agrobacterium tumefaciens. This bacterium is a plant pathogen and infects the plants causing tumor-like growth called crown gall. DNA technologists have used the ability of Agrobacterium to insert its genes in the plant and have isolated its plasmids to deliver foreign genes to a plant.

The most familiar bacterium Escherichia coli is a type of enteric bacterium. It is a Gram-negative, facultatively anaerobic bacterium that inhabits the human intestine. Most strains of E. coli live as harmless commensals in the human intestine, but there are certain strains that are considered pathogenic as they invade the tissues and produce toxins. These strains are said to be enteroinvasive and enterotoxic strains, respectively. One strain, E. coli 0157:H7, has been implicated in food-related outbreaks of intestinal disease in recent years.

One of the Gram-positive cocci important to the humans is the genus Staphylococcus. Members of this genus occur in grape-like clusters and include the organism S. aureus. This organism may be the cause of abscesses, boils, and carbuncles, as well as toxic shock syndrome. Species of Staphylococcus can be aerobic, facultatively anaerobic or anaerobic.

15.9 TECHNICAL TERMS

Aerobic, Anaerobic, Endospores, Rhizobium, Agrobacterium, Bacillus, Clostridium, Escherichia, Staphylococcus.

15.10 SELF ASSESSMENT QUESTIONS

Q.1 Describe the characteristic features of the genera Bacillus and Clostridium
Q.2 Give an account on the salient features of Rhizobium and Agrobacterium
Q.3 Write an essay on the characters and significance of Staphylococcus and Escherichia

15.11 SUGGESTED READINGS

1. Bergey's Manual of Determinative Bacteriology (8^{th} edition) - R.E. Buchanan and N.E. Gibbons (Co-editors)1974 – The Williams & Wilkins Company
2. Bergey's Manual of Determinative Bacteriology (9^{th} edition) --John G.Holt et. al. 1994 – Williams & Wilkins Company
3. Bergey's Manual of Systematic Bacteriology (1^{st} edition) -- Noel R. Krieg and John G.Holt (editors) –1984 – Williams & Wilkins
4. Text book of microbiology (5^{th} edition) -- R. Ananthanarayan and C.K. Jayaram Paniker 1998 – Orient Longman Ltd.

Dr. K. Nagaraju

LESSON - 16

BACTERIAL GENERA - *MYCOPLASMA*, *RICKETTSIA*, AND *SPIROCHAETA*

Objective of the lesson

To make the students to understand the characteristic features of bacterial genera *Mycoplasma, Rickettsia* and *Spirochaeta*.

Structure of the lesson

16.1 INTRODUCTION

Mycoplasma species are among the smallest free-living organisms (about 0.2 - 0.3 μm in diameter). They have been found in the pleural cavities of cattle suffering from pleuropneumonia. These organisms are often called MLOs (mycoplasma-like organisms) or, formerly, PPLO (pleuropneumonia-like organisms). The genus *Rickettsia* is placed in the order Rickettsiales and family Rickettsiaceae of the Proteobacteria. The family Rickettsiaceae comprises of two genera, *Rickettsia* and *Orientia*. S**pirochetes** belong to a phylum of distinctive Gram-negative bacteria, which have long, helically coiled (spiral-shaped) cells. Spirochetes are chemoheterotrophic in nature, with lengths between 5 and 250 μm and diameters around 0.1-0.6 μm.

16.2 *MYCOPLASMA*

The class Mollicutes has five orders and six families. The best studied genera are found in the orders Mycoplasmatales *(Mycoplasma, Ureaplasma),* Entomoplasmatales *(Entomoplasma, Mesoplasma, Spiroplasma),* Acholeplasmatales *(Acholeplasma),* and Anaeroplasmatales *(Anaeroplasma, Asteroleplasma).*

Members of the class Mollicutes are commonly called mycoplasmas. Although they evolved from ancestors with Gram positive cell walls, they now lack cell walls and cannot synthesize peptidoglycan precursors. Thus they are penicillin resistant but susceptible to lysis by osmotic

shock and detergent treatment. Because they are bounded only by a plasma membrane, these prokaryotes are pleomorphic and vary in shape from spherical or pear-shaped organisms, about 0.3 to 0.8 µm in diameter, to branched or helical filaments. Some mycoplasmas (e.g., *M. genitalium*) have a specialized terminal structure that projects from the cell and gives them a flask or pear shape. This structure aids in attachment to eukaryotic cells. They are among the smallest bacteria capable of self-reproduction. Although most are non-motile, some can glide along liquid-covered surfaces. Most species differ from the vast majority of bacteria in requiring sterols for growth, which are incorporated into the plasma membrane. Here sterols may facilitate osmotic stability. Most are facultative anaerobes, but a few are obligate anaerobes. When growing on agar, most species form colonies with a "fried-egg" appearance because they grow into the agar surface at the center while spreading outward on the surface at the colony edges (Figure- 16.1). Their genomes are among the smallest found in prokaryotes, ranging from 0.7 to 1.7 Mb; the G-C content ranges from 23 to 41%. The complete genomes of the human pathogens *Mycoplasma genitalium, M. pneumoniae,* and *Ureaplasma urealyticum* have been sequenced. These genomes are characteristically small with less than 1,000 genes; it seems that not many genes are required to sustain a free-living existence. Mycoplasmas can be saprophytes, commensals, or parasites, and many are pathogens of plants, animals, or insects.

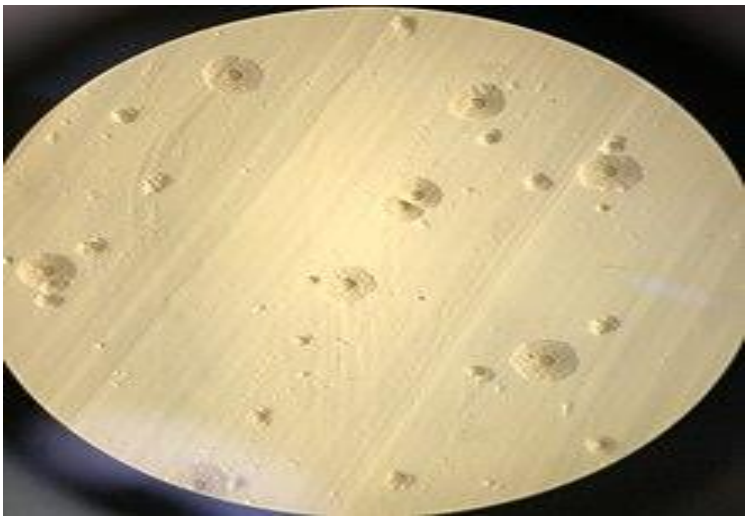


Figure-16.1: Fried egg appearance of *Mycoplasma* colonies on Hay flick agar medium

Due to the lack of a rigid cell wall, *Mycoplasma* species can contort into a broad range of shapes, from round to oblong. So, they are identified as pleomorphic organisms and therefore cannot be identified as rods, cocci or spirals. The plasma membrane forms the outer boundary layer of the cell. They also lack the membrane bound nucleus and other membrane bound organelles. The genetic material is a single duplex DNA which is naked and the ribosomes are of 70S type. The molecular structure of the *Mycoplasma* cell is given in Fig. 16.2. They possess a replicating disc at one end which assists replication process and also the separation of the genetic material. Majority of the *Mycoplasma* species are parasitic in nature due to their inability to synthesize the required growth factors. Over 100 species have been included in the genus *Mycoplasma* are parasites or commensals of humans, animals, and plants. *Mycoplasma* species are among the smallest free-living organisms (about 0.2 - 0.3 µm in diameter). They have been found in the

pleural cavities of cattle suffering from pleuropneumonia. These organisms are often called MLOs (mycoplasma-like organisms) or, formerly, PPLOs (pleuropneumonia-like organisms).

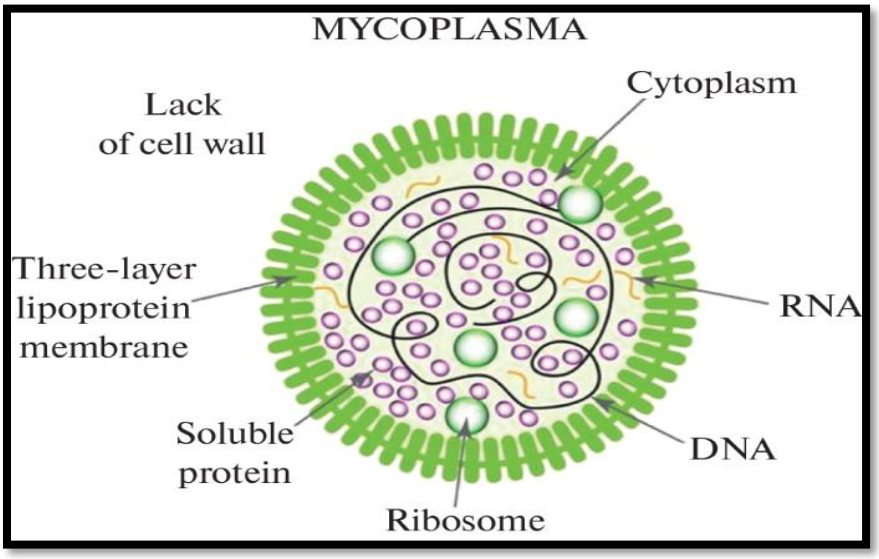


Figure-16.2: Molecular structure of *Mycoplasma*

In 1954, using phase-contrast microscopy, continual observations of live cells have shown that *Mycoplasma* species and L-form bacteria (previously also called L-phase bacteria) do not proliferate by binary fission, but by uni- or multi-polar budding mechanism.

Metabolically, the mycoplasmas are incapable of synthesizing a number of macromolecules. In addition to requiring sterols, they also need fatty acids, vitamins, amino acids, purines, and pyrimidines. Some may produce ATP by the Embden-Meyerhof pathway and lactic acid fermentation. Others catabolize arginine or urea to generate ATP. The pentose phosphate pathway seems to be functional in at least some mycoplasmas; none appear to have the complete tricarboxylic acid cycle.

Mycoplasmas are remarkably widespread and can be isolated from animals, plants, the soil, and even compost piles. Although their complex growth requirements can make their growth in pure (axenic) cultures difficult, about 10% of the mammalian cell cultures in use are probably contaminated with mycoplasmas. This seriously interferes with tissue culture experiments. In animals, mycoplasmas colonize mucous membranes and joints and often are associated with diseases of the respiratory and urogenital tracts. Mycoplasmas cause several major diseases in livestock, for example, contagious bovine pleuropneumonia in cattle (*M. mycoides*), chronic respiratory disease in chickens (*M. gallisepticum*), and pneumonia in swine (*M. hyopneumoniae*). *M. pneumonia* causes primary atypical pneumonia in humans. *Ureaplasma urealyticum* is commonly found in the human urogenital tract. It is now known to be associated with premature delivery of newborns, as well as neonatal meningitis and pneumonia. Spiroplasmas have been isolated from insects, ticks, and a variety of plants. They cause disease in citrus plants, cabbage, broccoli, corn, honey bees, and other hosts. Arthropods may often act as vectors and carry the spiroplasmas between plants. It is likely that more pathogenic mollicutes will be discovered with improvement in techniques for their isolation and detection.

16.3 *RICKETTSIA*

The genus *Rickettsia* is placed in family Rickettsiaceae and the order Rickettsiales of the Proteobacteria. Family Rickettsiaceae possess two genera namely *Rickettsia* and *Orientia*. The term *Rickettsia* is sometimes also used to refer to organisms of the order Rickettsiales.

These bacteria are rod-shaped, coccoid, or pleomorphic with typical gram-negative walls and no flagella. Although their size varies, they tend to be very small. For example, *Rickettsia* is 0.3 to 0.5-m in diameter and 0.8 to 2.0m long (Fig. 16.3). All species are parasitic or mutualistic. The parasitic forms grow in vertebrate erythrocytes, macrophages, and vascular endothelial cells. Often they also live in blood-sucking arthropods such as fleas, ticks, mites, or lice, which serve as vectors or as primary hosts. They are natural parasites of certain arthropods (notably lice, fleas, mites, and ticks) and can cause serious diseases—usually characterized by acute self-limiting fevers—in humans and other animals. As these genera include important human pathogens, their reproduction and metabolism have been intensively studied. Virtually all rickettsiae can reproduce only within animal cells. Rickettsiae are usually transmitted to humans by a bite from an arthropod carrier. Because certain species can withstand considerable drying, transmission of rickettsia can also occur when arthropod feces are inhaled or enter the skin through abrasion. Most rickettsiae normally infect animals other than humans, who become involved as dead-end hosts only accidentally. Epidemic typhus and trench fever are exceptions, since humans are the only host of proven importance. The other rickettsial infections occur primarily in animals, which serve as reservoirs from which bloodsucking arthropods acquire the rickettsial bacteria and in turn transmit them to other animals and, occasionally, humans.

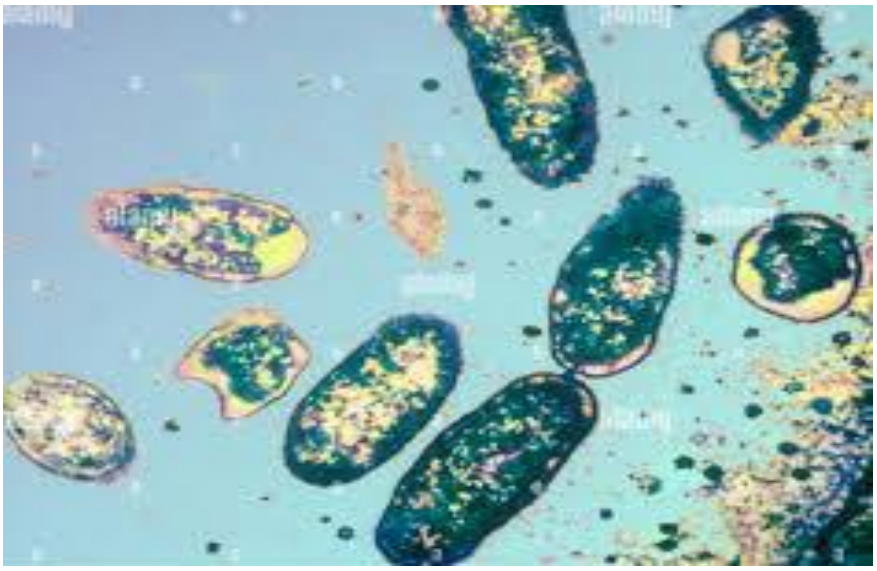


Figure-16.3: Molecular structure of *Rickettsia*

Rickettsias enter the host cell by inducing phagocytosis. Members of the genus *Rickettsia* immediately escape the phagosome and reproduce by binary fission in the cytoplasm. In contrast, Rickettsias are very different from most other bacteria in physiology and metabolism. They lack glycolytic pathways and do not use glucose as an energy source, but rather oxidize

glutamate and tricarboxylic acid cycle intermediates such as succinate. The rickettsial plasma membrane has carrier-mediated transport systems, and host cell nutrients and coenzymes are absorbed and directly used. For example, rickettsias take up both NAD and uridine diphosphate glucose. Their membrane also has an adenylate exchange carrier that exchanges ADP for external ATP. Thus host ATP may provide much of the energy needed for growth.

This metabolic dependence explains why many of these organisms must be cultivated in the yolk sacs of chick embryos or in tissue culture cells. Genome sequencing shows that *R. prowazekii* is similar in many ways to mitochondria. Possibly mitochondria arose from an endosymbiotic association with an ancestor of *Rickettsia*. The largest rickettsial genus, *Rickettsia*, is generally subdivided into the typhus group, the spotted fever group, and the scrub typhus group. This genus alone is responsible for a number of highly virulent diseases, including Rocky Mountain spotted fever, epidemic typhus, Brill-Zinsser disease (a type of epidemic typhus) and scrub typhus. *Rickettsia prowazekii* and *R. typhi* are associated with typhus fever, and *R. rickettsii* with Rocky Mountain spotted fever. *Coxiella burnetii* causes Q fever in humans. Rickettsias are also important pathogens of domestic animals such as dogs, horses, sheep, and cattle.

Several *Rickettsia* species present in Australia are capable of causing disease in people. These species include: *Rickettsia australis* - Queensland tick typhus, *Orientia tsutsugamushi* - scrub typhus, *Rickettsia honei* - Flinders Island spotted fever and *Rickettsia typhi* - murine typhus. *Rickettsia prowazekii* (epidemic typhus) is spread by human body lice and can result in outbreaks of disease, but is only seen in conflict settings and refugee camps and is not naturally occurring in Australia. Rickcttsiae are readily inactivated by physical and chemical agents. They are rapidly destroyed at 56^{o} C and at room temperature when separated from host components, cultured in skimmed milk or a suspending medium containing sucrose, potassium phosphate and glutamate (SPG medium).

Signs and symptoms

Rickettsiae are transmitted to humans by arthropod vectors through their bite or feces. Commonly a small, hard, black sore appears first at the bite site where the infection was introduced. Fever, headache, muscle aches, swollen lymph glands, cough and rashes. These infections are not common and usually mild they can be difficult to diagnose. While signs and symptoms can suggest the diagnosis, a definite diagnosis is made with a blood test or skin biopsy (for example a sample of skin) of the bite site for rickettsial infections

Treatment and Prevention

Treatment is usually with the tetracycline antibiotic doxycycline which reduces the duration and severity of infection. Exclusion of people with rickettsial infections from childcare, preschool, school and work is not necessary. There is no vaccine available to prevent infection. Wear long sleeved protective clothing and a broad brimmed hat to reduce the risk of infection when undertaking activities where human contact with ticks, lice, mites or fleas may occur, such as bushwalking and camping in infected areas. Use an insect repellent containing DEET or picaridin and examine your skin for possible bites (especially behind the ears, on the back of the head, in the groin, armpits and behind the knees) following these activities.

16.4 *SPIROCHAETA*

Spirochaetes are spiral-shaped bacteria that are part of the phylum Spirochaetota. They are Gram-negative, motile, with a double membrane. S**pirochaetes** belong to a phylum of distinctive Gram-negative bacteria, which are long and helically coiled (spiral-shaped) cells. Spirochetes are chemoheterotrophic in nature, with length between 5 and 250 µm and diameter around 0.1-0.6 µm. Spirochaetes are distinguished from other bacteria by the location of their flagella, sometimes called axial filaments which run lengthwise. Some spirochetes are part of the normal flora, while others are pathogenic. These cause a twisting motion which allows the spirochaete to move about. When reproducing, a spirochaete will undergo asexual transverse binary fission. Most spirochaetes are free-living and anaerobic, but there are numerous exceptions. The spirochetes are divided into three families: (Brachyspiraceae, Leptospiraceae, and Spirochaetaceae), all placed within a single order (Spirochaetales). Some of the disease-causing members include the following:

1. *Leptospira* sp., which causes leptospirosis;
2. *Borrelia recurrentis*, which causes relapsing fever;
3. *Treponema pallidum* - causes syphilis;
4. *Treponema pertenue*, which causes yaws.

Characteristic features

The shape of the cell is spiral or flat-wave with a size range of 3–500 µm in length and 0.09–3 µm in diameter (Fig. 16.4). It shows swim character by rolling or undulation of the cell body. These are found in water, soil, decaying organic matter, plants, animals, and humans. Spirochetes have a unique structure, and as a result their motility is different from that of other bacteria. These organisms can swim in a highly viscous, gel-like medium, such as that found in connective tissue, that inhibits the motility of most other bacteria. In spirochetes, the organelles for motility, the periplasmic flagella, reside inside the cell within the periplasmic space also called as OS (outer sheath). A given periplasmic flagellum is attached only at one end of the cell, and depending on the species, may or may not overlap in the center of the cell it was enclosed in the protoplasmic cylinder (PC) by the attachment of Axial fibril (AF) (Fig. 16.5A1 and A2). The number of periplasmic flagella varies from species to species. These structures have been shown to be directly involved in motility and function by rotating within the periplasmic space (Fig.16.5 B). Backward moving, propagating waves enable these bacteria to swim and translate in a given direction (A1).

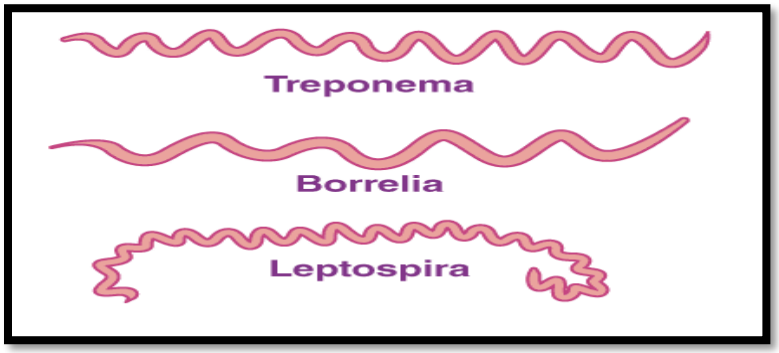


Figure-16.4: Morphology of some Spirochaetes

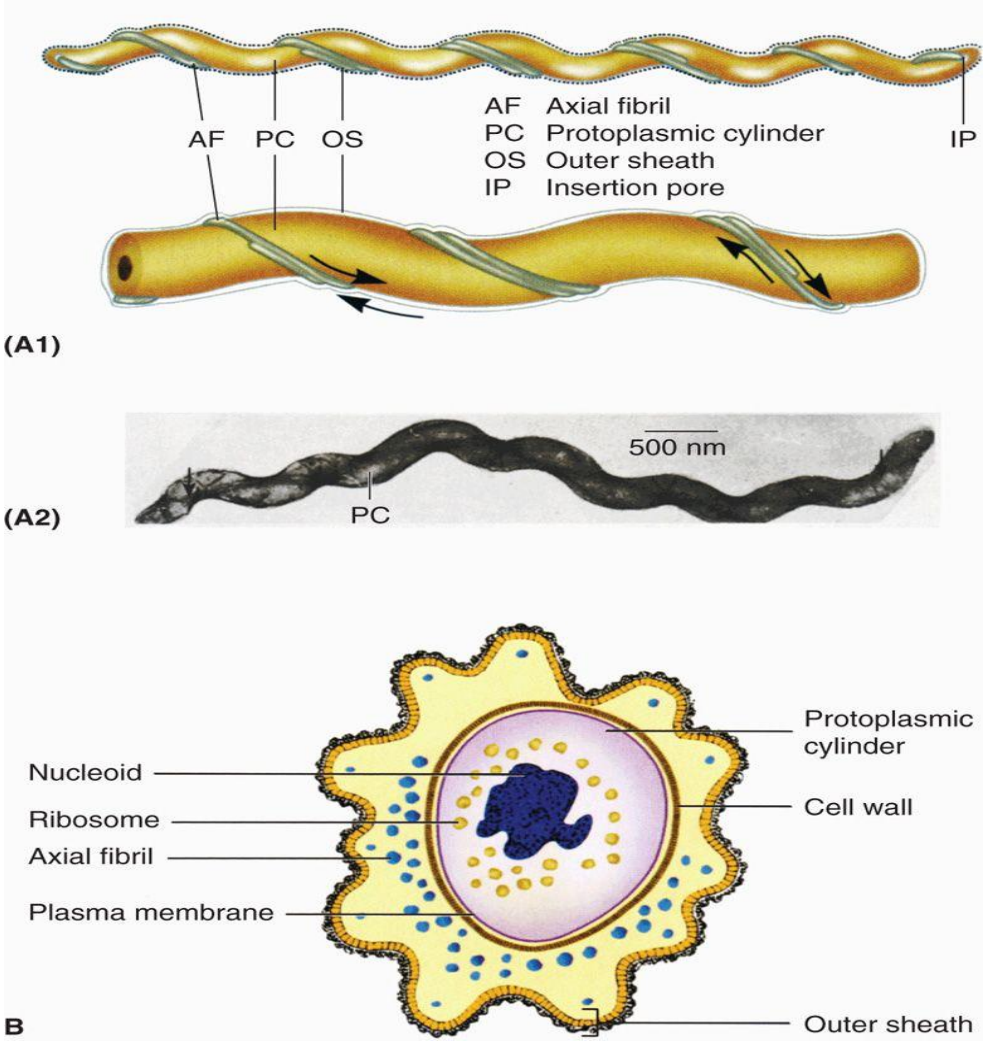


Figure-16.5: Structure and motility of Spirochaetes

16.5 SUMMARY

Mycoplasmas cause several major diseases in livestock, for example, contagious bovine pleuropneumonia in cattle (*M. mycoides*), chronic respiratory disease in chickens (*M. gallisepticum*), and pneumonia in swine (*M. hyopneumoniae*). *M. pneumonia* causes primary atypical pneumonia in humans. *Ureaplasma urealyticum* is commonly found in the human urogenital tract. It is now known to be associated with premature delivery of newborns, as well as neonatal meningitis and pneumonia. Spiroplasmas have been isolated from insects, ticks, and a variety of plants. They cause disease in citrus plants, cabbage, broccoli, corn, honey bees, and other hosts. Rickcttsiae are readily inactivated by physical and chemical agents. They are rapidly destroyed at 56^{o} C and at room temperature when separated from host components, cultured in skimmed milk or a suspending medium containing sucrose, potassium phosphate and glutamate (SPG medium). Spirochaetes are distinguished from other bacterial phyla by the location of their flagella, sometimes called axial filaments which run lengthwise. Some spirochetes are part of the normal flora, while others are pathogenic.

16.6 TECHNICAL TERMS

Mycoplasma, Ureaplasma, Entomoplasma, Mesoplasma, Spiroplasma, Acholeplasmatales*, Anaeroplasma,Asteroleplasma*, Brachyspiraceae, Leptospiraceae, Spirochaetaceae, *Rickettsia australis, Orientia tsutsugamushi, Rickettsia honei, Rickettsia typhi, Rickettsia prowazekii*.

16.7 SELF ASSESSMENT QUESTIONS

Q.1 Explain the important characters of *Mycoplasma*.
Q.2 Write an account on *Rickettsia*.
Q.3 Discuss about *Spirochaeta*
Q.4 Write in detail about Rickettsia and Spirochaetes.

16.8 SUGGESTED READINGS

1. Biology of Microorganisms(1999) - Brock, T.Dand M.T.
2. Microbiology(2008) - Prescott, LM. Harley, J.P Klein, D.A
3. Microbiology(2006) - Pelczar,M.J, Chan, E.C.S., Kreign,N.R
4. Principles of microbiology (1996) - Atlas, R.M
5. General Microbiology - Schlegel HG.
6. A text Book of Microbiology (2010) - Dubey RC and Maheswari DK

Dr. K. Nagaraju

LESSON- 17

BACTERIAL GENERA - *PSEUDOMONAS*, *NITROSOMONAS*, AND *STREPTOCOCCUS*

Objective of the lesson

To aware the students about the general characters of *Pseudomonas, Nitrosomonas* and *Streptococcus* are in detail.

Structure of the lesson

17.1 INTRODUCTION

The Pseudomonads have a great practical impact in several ways**.** *Pseudomonas aeruginosa, P. fluorescens, P. putida* and *P. syringae* are the important members of this group. *Nitrosomonas* is a genus of Gram-negative bacteria, belonging to the Betaproteobacteria. It is one of the five genera of ammonia-oxidizing bacteria and, as an obligate chemolithoautotroph, uses ammonia as an energy source and carbon dioxide as a carbon source in the presence of oxygen. *Nitrosomonas* species are important in the global biogeochemical nitrogen cycle, since they increase the bioavailability of nitrogen to plants and in denitrification, which is important for the release of nitrous oxide, a powerful greenhouse gas. Streptococci are Gram-positive, non-motile, non-spore forming, catalase-negative cocci that occur in pairs or chains. Older cultures may lose their Gram-positive character. Most streptococci are facultative anaerobes, and some are obligate (strict) anaerobes. Most of the species require enriched media (blood agar) for their growth.

17.2 *PSEUDOMONAS*

The genus *Pseudomonas* is an exceptionally heterogeneous taxon currently composed of about 60 species. Many can be placed in one of seven rRNA homology groups. The three best characterized groups are subdivided according to properties such as the presence of poly-hydroxybutyrate (PHB), the production of a fluorescent pigment, pathogenicity, the presence of arginine dihydrolase, and glucose utilization. For example, the fluorescent subgroup does not accumulate PHB and produces a diffusible, water-soluble, yellow-green pigment that fluoresces under UV radiation.

Characteristics

Members of the genus display these defining characteristics: Rod-shaped, Gram-negative, Flagellum one or more, providing motility, Aerobic, Non-spore forming, Catalase-positive and Oxidase-variable. Other characteristics that tend to be associated with *Pseudomonas* species (with some exceptions) include secretion of pyoverdine, a fluorescent yellow-green siderophore under iron-limiting conditions. *Pseudomonas* is the most important genus in the family Pseudomonadaceae of order Pseudomonadales. These bacteria are straight or slightly curved rods, 0.5 to 1.0 µm by 1.5 to 5.0 µm in length and are motile by one or several polar flagella. These chemoheterotrophs usually carry out aerobic respiration. Sometimes nitrate is used as the terminal electron acceptor in anaerobic respiration. All pseudomonads have a functional tricarboxylic acid cycle and can oxidize substrates completely to CO_2.

Pseudomonas aeruginosa, P. fluorescens, P. putida and *P. syringae* are the most important members of this group. Certain *Pseudomonas* species may also produce additional types of siderophores, such as pyocyanin by *P. aeruginosa* and thioquinolobactin by *P. fluorescens*. *Pseudomonas* species are positive to the oxidase test, negative to gas formation from glucose, glucose is oxidised in oxidation/fermentation test using Hugh and Leifson O/F test, beta hemolytic (on blood agar), negative to indole , methyl red, and Voges–Proskauer tests, but citrate positive. *Pseudomonas* may be the most common nucleator of ice crystals in clouds, thereby being of utmost importance to the formation of snow and rain around the world. The genus *Pseudomonas* is recognized for its remarkable metabolic diversity, enabling it to thrive in a wide range of environments. These bacteria produce a vast array of including antibiotics, siderophores, and biosurfactants, which contribute to their ecological versatility and biotechnological potential.

The pseudomonads have a great practical impact in several ways, including these: **1**. Many can degrade an exceptionally wide variety of organic molecules. Thus they are very important in the mineralization process (the microbial breakdown of organic materials to inorganic substances) in nature and in sewage treatment. The fluorescent pseudomonads can use approximately 80 different substances as their carbon and energy sources. **2**. Several species (e.g., *P. aeruginosa*) are important experimental subjects. Many advances in microbial physiology and biochemistry have come from their study. For example, the study of *P. aeruginosa* has significantly advanced our understanding of how bacteria form biofilms and the role of extracellular signalling in bacterial communities and pathogenesis. The genome of *P. aeruginosa* has an unusually large number of genes for catabolism, nutrient transport, the efflux of organic molecules, and metabolic regulation. This may explain its ability to grow in many environments and resist antibiotics. **3**. Some pseudomonads are major animal and plant pathogens. *P. aeruginosa* infects people with low resistance such as cystic fibrosis patients. It also invades burns, and causes urinary tract infections. *P. syringae* is an important plant pathogen. **4**. Pseudomonads such as *P. fluorescens* are involved in the spoilage of refrigerated milk, meat, eggs, and seafood because they grow at 4°C and degrade lipids and proteins.

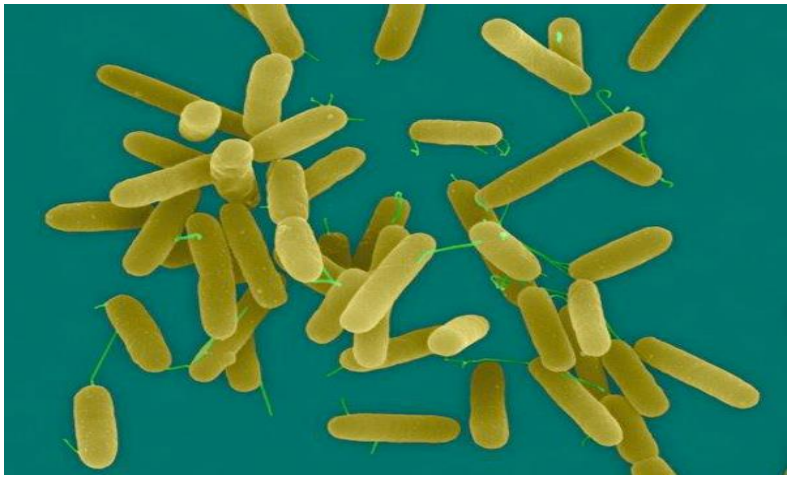


Figure-17.1: Morphology of *Pseudomonas*

Biological applications

Biofilm formation

All species and strains of *Pseudomonas* have historically been classified as strict aerobes. Exceptions to this classification have recently been discovered in *Pseudomonas* biofilms. A significant number of cells can produce exopolysaccharides associated with biofilm formation. Secretion of exopolysaccharides such as alginate makes it difficult for pseudomonads to be phagocytosed by mammalian white blood cells. Exopolysaccharide production also contributes to surface-colonizing biofilms that are difficult to remove from food preparation surfaces. Growth of pseudomonads on spoiling foods can generate a "fruity" odor.

Antibiotic resistance

Most *Pseudomonas* spp. are naturally resistant to penicillin and the majority of related beta-lactam antibiotics, but a number are sensitive to piperacillin, imipenem, ticarcillin, or ciprofloxacin. Aminoglycosides such as tobramycin, gentamicin, and amikacin are other choices for therapy. This ability to thrive in harsh conditions is a result of their hardy cell walls that contain proteins known as porins. Their resistance to most antibiotics is attributed to efflux pumps, which pump out some antibiotics before they are able to act.

Pseudomonas aeruginosa is increasingly recognized as an emerging opportunistic pathogen of clinical relevance. One of its most worrying characteristics is its low antibiotic susceptibility. This low susceptibility is attributable to a concerted action of multidrug efflux pumps with chromosomally encoded antibiotic resistance genes (e.g., *mexAB-oprM*, *mexXY*, etc.) and the low permeability of the bacterial cellular envelopes. Besides intrinsic resistance, *P. aeruginosa* easily develops acquired resistance either by mutation in chromosomally encoded genes or by the horizontal gene transfer of antibiotic resistance determinants. Development of multidrug resistance by *P. aeruginosa* isolates requires several different genetic events that include acquisition of different mutations and/or horizontal transfer of antibiotic resistance genes. Hypermutation favours the selection of mutation-driven antibiotic resistance in *P. aeruginosa* strains producing chronic infections, whereas the clustering of several different antibiotic resistance genes in integrons favours the concerted acquisition of antibiotic resistance determinants. Some recent studies have shown phenotypic

resistance associated to biofilm formation or to the emergence of small-colony-variants, which may be important in the response of *P. aeruginosa* populations to antibiotic treatment.

Pathogenicity

Infectious species include *P. aeruginosa*, *P. oryzihabitans*, and *P. plecoglossicida*. *P. aeruginosa* flourishes in hospital environments, and is a particular problem in this environment, since it is the second-most common infection in hospitalized patients (nosocomial infections). This pathogenesis may in part be due to the proteins secreted by *P. aeruginosa*. The bacterium possesses a wide range of secretion systems, which export numerous proteins relevant to the pathogenesis of clinical strains. *P. syringae* is a prolific plant pathogen. It exists as over 50 different pathovars, many of which demonstrate a high degree of host-plant specificity. Numerous other *Pseudomonas* species can act as plant pathogens, notably all of the other members of the *P. syringae* subgroup, but *P. syringae* is the most widespread and best-studied. *P. tolaasii* can be a major agricultural problem, as it can cause bacterial blotch of cultivated mushrooms. Similarly, *P. agarici* can cause drippy gill in cultivated mushrooms.

Use as biocontrol agents

Since the mid-1980s, certain members of the genus *Pseudomonas* have been applied to cereal seeds or applied directly to soils as a way of preventing the growth or establishment of crop pathogens. This practice is generically referred to as biocontrol. The biocontrol properties of *P. fluorescens* and *P. protegens* strains (CHA0 or Pf-5 for example) are currently best-understood, although it is not clear exactly how the plant growth-promoting properties of *P. fluorescens* are achieved. Theories include: the bacteria might induce systemic resistance in the host plant, so it can better resist attack by a true pathogen; the bacteria might outcompete other (pathogenic) soil microbes, e.g. by siderophores giving a competitive advantage at scavenging for iron; the bacteria might produce compounds antagonistic to other soil microbes, such as phenazine-type antibiotics or hydrogen cyanide. Experimental evidence supports all of these theories. Other notable *Pseudomonas* species with biocontrol properties include *P. chlororaphis*, which produces a phenazine-type antibiotic active agent against certain fungal plant pathogens, and the closely related species *P. aurantiaca*, which produces di-2,4-diacetylfluoroglucylmethane, a compound antibiotically active against Gram-positive organisms.

Use as bioremediation agents

Some members of the genus are able to metabolise chemical pollutants in the environment, and as a result, can be used for bioremediation. Notable species demonstrated as suitable for use as bioremediation agents include: *P. alcaligenes*: which can degrade polycyclic aromatic hydrocarbons. *P. mendocina*: which is able to degrade toluene. *P. pseudoalcaligenes*: which is able to use cyanide as a nitrogen source. *P. resinovorans*: which can degrade carbazole. *P. aeruginosa*, *P. putida*, *P. desmolyticum*, and *P. nitroreducens* can degrade chlorpyrifos. *P. veronii*: which has been shown to degrade a variety of simple aromatic organic compounds. *P. putida*: which has the ability to degrade organic solvents such as toluene.

17.3 *NITROSOMONAS*

It is usually found in all types of waters, globally distributed in both eutrophic and oligotrophic freshwater and saltwater, emerging especially in shallow coastal sediments and under the upwelling zones, such as the Peruvian coast and the Arabian Sea, but can also be found in fertilized soils.

Nitrosomonas is a genus of Gram-negative bacteria, belonging to the Betaproteobacteria. It is one of the five genera of ammonia-oxidizing bacteria and, as an obligate chemolithoaututroph, uses ammonia as an energy source and carbon dioxide as a carbon source in the presence of oxygen. *Nitrosomonas* are important in the global biogeochemical nitrogen cycle, since they increase the bioavailability of nitrogen to plants and in the denitrification, which is important for the release of nitrous oxide, a powerful greenhouse gas. This microbe is photophobic, and usually generate a biofilm matrix, or form clumps with other microbes, to avoid light.

Nitrosomonas is generally found in highest numbers in all habitats in which there is abundance of ammonia (environment with plentiful protein decomposition or in wastewater treatment), thrive in a pH range of 6.0–9.0, and a temperature range of 20–30 °C (68–86 °F). Some species can live and proliferate on a monuments' surface or on stone buildings' walls, contributing to erosion of those surfaces.

Some *Nitrosomonas* species, such as *N.europaea,* possess the enzyme urease (which catalyzes the conversion of urea into ammonia and carbon dioxide) and have been shown to assimilate the carbon dioxide released by the reaction to make biomass via the Calvin cycle, and harvest energy by oxidizing ammonia (the other product of urease) to nitrite. This feature may explain enhanced growth of AOB in the presence of urea in acidic environments.

Characteristics

All species included in this genus have ellipsoidal or rod-shaped cells which have extensive intracytoplasmic membranes displaying as flattened vesicles (Fig.17.2). Most species are motile with a flagellum located in the polar region of the cell. Three basic morphological types of *Nitrosomonas* were studied, which are: short rods *Nitrosomonas,* rods Nitrosomonas, and *Nitrosomonas* with pointed ends. *Nitrosomonas* species cells have different criteria of size and shape: *N. europaea* cells appear as short rods with pointed ends, with a size of 0.8–1.1 x 1.0–1.7 μm; motility has not been observed. *N. eutropha* cells present as rod to pear shaped cells with one or both ends pointed, with a size of 1.0–1.3 x 1.6–2.3 μm. *N. halophila* cells have a coccoid shape and a size of 1.1–1.5 x 1.5–2.2 μm. Motility is possible because of a tuft of flagella. *N. communis* has large rods with rounded end cells with a size of 1.0–1.4 x 1.7–2.2 μm. Motility has not been observed in this species.

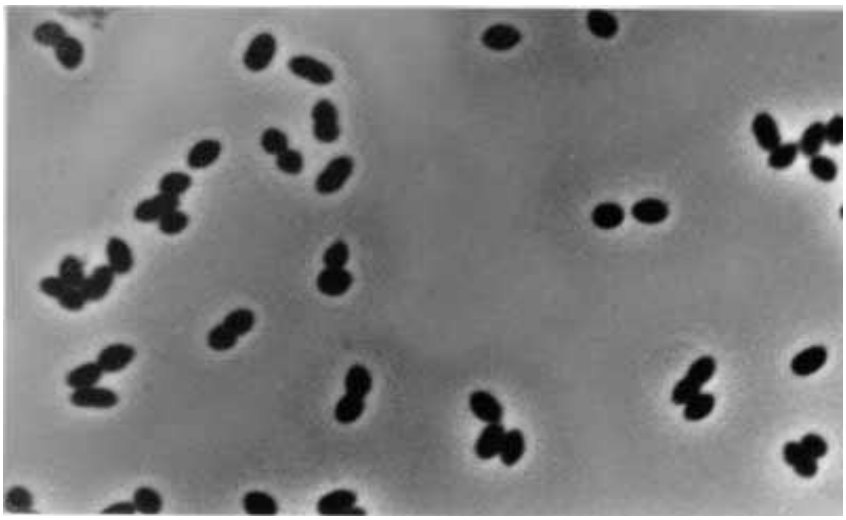


Figure-17.2: Morphology of *Nitrosomonas*

Nitrosomonas is used in activated sludge in aerobic wastewater treatment; the reduction of nitrogen compounds in the water is given by nitrification treatment in order to avoid environmental issues, such as ammonia toxicity and groundwater contamination. Nitrogen, if present in high quantities can cause algal development, leading to eutrophication with degradation of oceans and lakes. Employing as wastewater treatment, biological removal of nitrogen is obtained at a lower economic expense and with less damage caused to the environment compared to physical-chemical treatments. *Nitrosomonas* has also a role in biofilter systems, typically in association and collaboration with other microbes, to consume compounds such as or and recycle nutrients.

Biological applications: Genome sequencing of *Nitrosomonas* species has been important to understand the ecological role of these bacteria. Among the various species of *Nitrosomonas* that are known today, the complete genomes of *N. ureae* strain Nm10 and *N. europaea*, *N.sp. Is79* have been sequenced.

Ammonia-oxidation genes: The presence of the genes for ammonia oxidation characterizes all these species. The first enzyme involved in the ammonia oxidation is ammonia monooxygenase (AMO), which is encoded by the *amoCAB* operon. The AMO enzyme catalyzes the oxidation from (ammonia) to (hydroxylamine). The *amoCAB* operon contains three different genes: *amoA, amoB* and *amoC*. While *N. europaea* presents two copies of the genes, *N. sp. Is79* and *N. ureae* strain Nm10 have three copies of these genes.

Denitrification genes: The discovery of genes that encode for enzymes involved in the denitrification process includes the first gene *nirK* which encodes for a nitrite reductase with copper. This enzyme catalyzes the reduction form (nitrite) to (nitric oxide). While in *N. europaea, N. eutropha,* and *N. cryotolerans, nirK* is included in a multigenetic cluster; in *Nitrosomonas* sp. Is79 and *N.* sp. AL212, it is present as a single gene. A high expression of the *nirK* gene was found in *N.ureae* and this has been explained with the hypothesis that the NirK enzyme is also involved in the oxidation of in this species.[22] The second gene involved in denitrification is *norCBQD* which encodes a nitric-oxide reductase that catalyze the reduction from (nitric oxide) to (nitrous oxide). These genes are present in *N. sp. AL212, N.cryotolerans, and N. communis strain Nm2*. In *Nitrosomonas europaea,* these genes are included in a cluster.[23] These genes are absent in *N. sp. Is79 and N. ureae*.[15] Recently, it was found that the *norSY* gene encodes for a nitric-oxide reductase with copper in *N. communis strain Nm2* and *Nitrosomonas AL212.*

Carbon fixation genes: *Nitrosomonas* uses the Calvin-Benson cycle as a pathway for Carbon fixation. For this reason, all of the species have an operon that encodes for the RuBisCO enzyme. A peculiarity is found in *N. sp Is79* in which the two copies of the operon encode for two different forms of the RuBisCO enzyme: the IA form and the IC form, where the first one has a major affinity with the Carbon dioxide. Other species present different copies of this operon that encodes only for the IA form.[15] In *N. europaea,* an operon is characterized by five genes (*ccbL*, *ccbS*, *ccbQ*, *ccbO*, and *ccbN*) that encode for the RuBisCO enzyme. *ccbL* encodes for the major subunit while *ccbS* encodes for the minor subunit; these genes are also the most expressed within the operon. *ccbQ* and *ccbO* genes encode for a number of proteins involved in the mechanisms of processing, folding, assembling, activation, and regulation of the RuBisCO enzyme. Instead, *ccbN* encodes for a protein of 101 amino acids, whose function is not known yet. A putative regulatory gene, *cbbR*, was found 194 bases upstream of the start codon of *cbbL* and is transcribed in the opposite direction of other genes).

Transporter genes: Since *Nitrosomonas* are part of the ammonia-oxidizing bacteria (AOB), ammonia carriers are important to them. Bacteria adapted to high concentrations of ammonia can absorb it passively by simple diffusion. Indeed, *N. eutropha,* that is adapted to high levels of ammonia, does not present genes that encode for an ammonia transporter. Bacteria adapted to low concentrations of ammonia have a transporter (transmembrane protein) for this substrate. In *Nitrosomonas,* two different carriers for ammonia have been identified, differing in structure and function. The first transporter is the Amt protein (amtB type) encoded by *amt* genes and was found in *Nitrosomonas sp. Is79*. The activity of this ammonia carrier depends on the membrane potential. The second was found in *N. europaea,* wherein the *rh1* gene encodes an Rh-type ammonia carrier. Its activity is independent from the membrane potential.

Ammonia-oxidation: *Nitrosomonas* oxidizes ammonia into nitrite in a metabolic process, known as nitritation (a step of nitrification). This process occurs with the accompanying reduction of an oxygen molecule to water (which requires four electrons), and the release of energy. The oxidation of ammonia to hydroxylamine is catalyzed by ammonia monooxygenase (AMO), which is a membrane-bound, multisubstrate enzyme. In this reaction, two electrons are required to reduce an oxygen atom to water:

$$NH_3 + O_2 + 2\,H^+ + 2\,e^- \rightarrow NH_2OH + H_2O$$

Since an ammonia molecule only releases two electrons when oxidized, it has been assumed that the other two necessary electrons come from the oxidation of hydroxylamine to nitrite, which occurs in the periplasm and it is catalyzed by hydroxylamine oxidoreductase (HAO), a periplasm associated enzymes.

$$NH_2OH + H_2O \rightarrow NO_2^- + 5\,H^+ + 4\,e^-$$

Two of the four electrons released by the reaction, return to the AMO to convert the ammonia in hydroxylamine. 1,65 of the two remaining electrons are available for the assimilation of nutrients and the generation of the proton gradient. They pass through the cytochrome c552 to the cytochrome caa3, then to O_2, which is the terminal acceptor; here they are reduced to form water. The remaining 0,35 electrons are used to reduce NAD+ to NADH, to generate the proton gradient. Nitrite is the major nitrogen oxide produced in the process, but it has been observed that, when oxygen concentrations are low, nitrous oxide and nitric oxide can also form, as by-products from the oxidation of hydroxylamine to nitrite. The species *N. europaea* has been identified as being able to degrade a variety of halogenated compounds including trichloroethylene, benzene, and vinyl chloride.

17.4 *STREPTOCOCCUS*

Characteristics

Streptococci are Gram-positive, non-motile, non-spore forming, catalase-negative cocci that occur in pairs or chains (Fig.17.3). Older cultures may lose their Gram-positive character. Most streptococci are facultative anaerobes, and some are obligate (strict) anaerobes. They are divided into three groups by the type of hemolysis on blood agar: β-hemolytic (clear, complete lysis of red cells), α hemolytic (incomplete, green hemolysis), and γ hemolytic (no hemolysis). Serologic grouping is based on antigenic differences in cell wall carbohydrates (groups A to V), in cell wall pili-associated protein, and in the polysaccharide capsule in group B streptococci. The individual cocci are spherical or ovoid, 0.5-1 μm in diameter and are arranged in chains. The length of chain varies widely with cultural conditions. Larger

chains are formed in liquid or broth than in solid agar. Streptococci are gram positive but they may become gram negative in ageing when bacteria die.

Streptococci are aerobic or facultative anaerobes. They grow best at temperature 37°. Streptococci grow well in media containing blood and sugar. 10 % CO_2 promotes growth and haemolysis. Selective media containing aminoglycosides or 1: 500,000 crystal violet selectively permit growth of Streptococci by inhibiting other bacteria. **On blood agar medium, the** colonies are small, circular, semi-transparent, and convex with small clear zone of hemolysis. Growth and hemolysis increases y 10% CO2. Streptococci are classified on the basis of hemolysis, α-hemolysis, β-hemolysis and no-hemolysis group. Streptococci are non-halophiles but can tolerate up to 6.5% NaCl (halotolerant). Tolerates 0.1% methylene blue and bile esculin agar. Streptococci ferment most sugars such as sorbitol. Trehalose, lactose maltose, mannitol etc., with the production of acid but no gas. Catalase (–) negative.

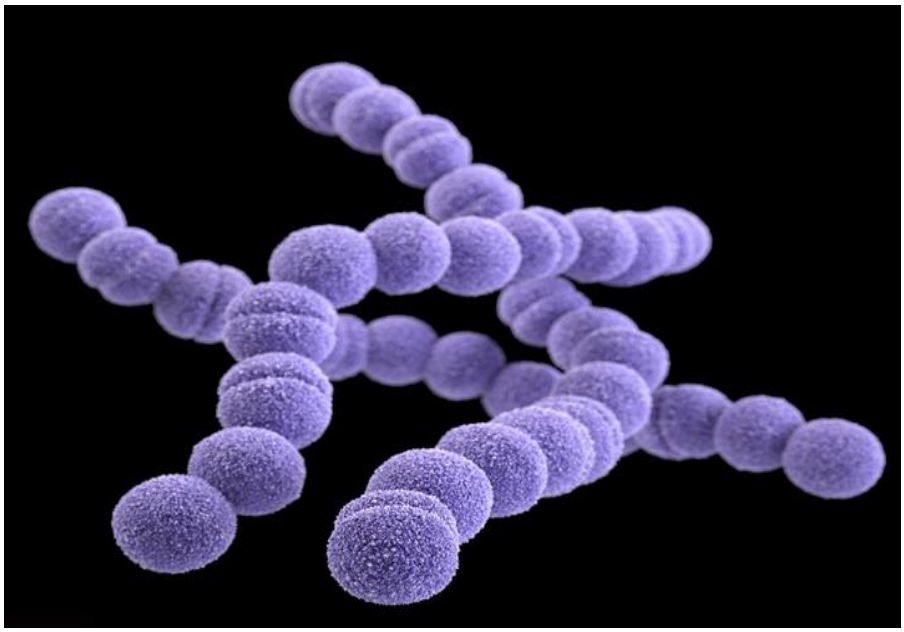


Figure-17.3: Morphology of Streptococci

Pathogenesis

Streptococci are members of the normal flora. Virulence factors of group A streptococci include (1) M protein and lipoteichoic acid for attachment; (2) a hyaluronic acid capsule that inhibits phagocytosis; (3) other extracellular products, such as pyrogenic (erythrogenic) toxin, which causes the rash of scarlet fever; and (4) streptokinase, streptodornase (DNase B), and streptolysins. Some strains are nephritogenic. Immune-mediated sequelae do not reflect dissemination of bacteria. Nongroup A strains have no defined virulence factors. Antibody to M protein gives type-specific immunity to group A streptococci. Antibody to erythrogenic toxin prevents the rash of scarlet fever. Immune mechanisms are important in the pathogenesis of acute rheumatic fever. Maternal IgG protects the neonate against group B streptococci.

Epidemiology and Diagnosis

Group A: β-hemolytic streptococci are spread by respiratory secretions and fomites. The incidence of both respiratory and skin infections peaks in childhood. Infection can be

transmitted by asymptomatic carriers. Acute rheumatic fever was previously common among the poor; susceptibility may be partly genetic. Group B: streptococci are common in the normal vaginal flora and occasionally cause invasive neonatal infection.

Diagnosis is based on cultures from clinical specimens. Serologic methods can detect group A or B antigen; definitive antigen identification is by the precipitin test. Bacitracin sensitivity presumptively differentiates group A from other β-hemolytic streptococci (B, C, G); group B streptococci typically show hippurate hydrolysis; group D is differentiated from other viridans streptococci by bile solubility and optochin sensitivity. Acute glomerulonephritis and acute rheumatic fever are identified by anti-streptococcal antibody titers. In addition, acute rheumatic fever is diagnosed by clinical criteria. Prompt penicillin treatment of streptococcal pharyngitis reduces the antigenic stimulus and therefore prevents glomerulonephritis and acute rheumatic fever. Vancomycin resistance among the enterococci is an emerging microbial threat.

The genus *Streptococcus* is large and complex. These bacteria have been clustered into three groups: pyogenic streptococci, oral streptococci, and other streptococci. Many bacteria originally placed within the genus have been moved to two other genera, *Enterococcus* and *Lactococcus.* Many characteristics are used to identify these cocci. One of their most important taxonomic characteristics is the ability to lyse erythrocytes when growing on blood agar, an agar medium containing 5% sheep or horse blood. In α-hemolysis, a 1 to 3 mm greenish zone of incomplete hemolysis forms around the colony; α-hemolysis is characterized by a zone of clearing or complete lysis without a marked color change. In addition, other hemolytic patterns are sometimes seen. Serological studies are also very important in identification because these genera often have distinctive cell wall antigens. Polysaccharide and teichoic acid antigens found in the cell wall or between the wall and the plasma membrane are used to identify these cocci, particularly pathogenic α-hemolytic streptococci. Biochemical and physiological tests are essential in identification (e.g., growth temperature preferences, carbohydrate fermentation patterns, acetoin production, reduction of litmus milk, sodium chloride and bile salt tolerance, and the ability to hydrolyze arginine, esculin, hippurate, and starch). Sensitivity to bacitracin, sulfa drugs, and optochin (ethylhydrocuprein) also are used to identify particular species. Some of these techniques are being replaced by molecular genetic approaches such as multilocus sequence typing (MSLT).

Pyogenic streptococci usually are pathogens and associated with pus formation (pyogenic means pus producing). Most species produce α-hemolysis on blood agar and form chains of cells. The major human pathogen in this group is *S. pyogenes,* which causes streptococcal sore throat, acute glomerulonephritis, and rheumatic fever. The normal habitat of oral streptococci is the oral cavity and upper respiratory tract of humans and other animals. In other respects oral streptococci are not necessarily similar. *S. pneumoniae* is α-hemolytic and grows as pairs of cocci. It is associated with lobar pneumonia and otitis media (inflammation of the middle ear). *S. mutans* is associated with the formation of dental caries.

The genus *Streptococcus*, a heterogeneous group of Gram-positive bacteria, has broad significance in medicine and industry. Various streptococci are important ecologically as part of the normal microbial flora of animals and humans; some can also cause diseases that range from subacute to acute or even chronic. Among the significant human diseases attributable to streptococci are scarlet fever, rheumatic heart disease, glomerulonephritis, and pneumococcal pneumonia. Streptococci are essential in industrial and dairy processes and as indicators of pollution.

The nomenclature for streptococci, especially the nomenclature in medical use, has been based largely on serogroup identification of cell wall components rather than on species names. For several decades, interest has focused on two major species that cause severe infections: *S pyogenes* (group A streptococci) and *S. pneumoniae* (pneumococci). In recent years, increasing attention has been given to other streptococcal species, partly because innovations in serogrouping methods have led to advances in understanding the pathogenetic and epidemiologic significance of these species. A variety of cell-associated and extracellular products are produced by streptococci, but their cause-effect relationship with pathogenesis has not been defined. Some of the other medically important streptococci are *S. agalactiae*, an etiologic agent of neonatal disease; and the viridans streptococci. Particularly for the viridans streptococci, taxonomy and nomenclature are not yet fully reliable or consistent. Important members of the viridans streptococci, normal commensals, include *S. mutans* and *S. sanguis* (involved in dental caries), *S. mitis* (associated with bacteremia, meningitis, periodontal disease and pneumonia), and "*S. milleri*" (associated with suppurative infections in children and adults).

17.5 SUMMARY

Pseudomonas is the most important genus in the order Pseudomonadales, the family Pseudomonaceae. These are chemoheterotrophs usually carry out aerobic respiration. Sometimes nitrate is used as the terminal electron acceptor in anaerobic respiration. All pseudomonads have a functional tricarboxylic acid cycle and can oxidize substrates completely to CO_2. *Nitrosomonas* is used in activated sludge in aerobic wastewater treatment; the reduction of nitrogen compounds in the water is given by nitrification treatment in order to avoid environmental issues, such as ammonia toxicity and groundwater contamination. Nitrogen, if present in high quantities can cause algal development, leading to eutrophication with degradation of oceans and lakes. Employing as wastewater treatment, biological removal of nitrogen is obtained at a lower economic expense and with less damage caused to the environment compared to physical-chemical treatments. The genus *Streptococcus*, a heterogeneous group of Gram-positive bacteria, has broad significance in medicine and industry. Various streptococci are important ecologically as part of the normal microbial flora of animals and humans; some can also cause diseases that range from sub-acute to acute or even chronic. Among the significant human diseases attributable to streptococci are scarlet fever, rheumatic heart disease, glomerulonephritis, and pneumococcal pneumonia. Streptococci are essential in industrial and dairy processes and as indicators of pollution.

17.6 TECHNICAL TERMS

Pseudomonas aeruginosa, P. fluorescens, P. putida, P. syringae, Nitrosomonas europea, Nitrosomonas eutropha, Nitrosomonas halophila, Nitrosomonas mobilis, eutrophication, *Streptococcus*, α-hemolysis, β-hemolysis and γ hemolysis.

17.7 SELF ASSESSMENT QUESTIONS

Q.1 Write an account on the genus *Pseudomonas*.
Q.2 Discuss about Biological applications of *Pseudomonas*.
Q.3 Write an account on *Streptococcus*.
Q.4 Discuss about biological applications of *Nitrosomonas*.
Q.5 Write in detail about *Nitrosomonas*.

17.8 SUGGESTED READINGS

1. Biology of Microorganisms - Brock, T. Dand M.T. (1999)
2. Microbiology- Prescott, LM. Harley, J.P Klein, D.A. (2008)
3. The Microbial World - Stainer, R,Y., J.L., Wheelis,M.L. Painter, P.R. (1988)
4. Microbiology - Pelczar,M.J, Chan, E.C.S., Kreign,N.R. (2006)
5. Black,J.G- Microbiology- principles and Explorations (1999)
6. Principles of microbiology- Atlas, R.M. (1996)
7. Fundamental principles of Bacteriology - Salla AJ. (2001)
8. Modern Microbiology – Birge (2008)
9. General Microbiology - Schlegel HG.
10. A text Book of Microbiology - Dubey RC and Maheswari DK (2010)

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