SCHOOL OF DISTANCE EDUCATION

BHARATHIAR UNIVERSITY

POST GRADUATE DIPLOMA COURSE IN MICROBIAL BIOTECHNOLOGY

STUDY MATERIALS

PAPER I
FUNDAMENTALS OF MICROBIOLOGY

PAPER I FUNDAMENTALS OF MICROBIOLOGY

PREAMBLE

Scope

Scope

This paper deals with various types of classification of microbes. The paper also throws light on multifarious habitats of microbes and provides information about all the microbial cellular functions and various metabolic pathways in microbes.

Objective

To impart knowledge on classification of microbes. This paper is also designed to provide knowledge on metabolic function and biochemical reaction going on inside the microbial cell

Goal

This paper enables the students to identify any microorganisms. The students will be able to understand and predict the intermediate metabolism of any microbe used in Industrial production processes

CONTENTS

UNIT I

CLASSIFICATION AND MOLECULAR SYSTEMATICS: Taxonomy – Classification of viruses, bacteria and fungi. Molecular systematics – Classical, numerical, polyphasic and molecular (G+C analysis, DNA-DNA hybridization, 16s rRNA sequencing and construction of phylogenetic tree) taxonomy

UNIT II

MICROBIAL CELL BIOLOGY AND METABOLISM: General structural organization of bacteria, viruses, Actinomycetes. Molecular architecture of nucleus, mitochondria, chloroplast, cell wall, ribosome, cilia, flagella, vacuole and other microbodies. Metabolic pathways and bioenergetics. Aerobic and anaerobic growth – product formation and substrate utilization – endogenous and maintenance metabolism.

UNIT III

MICROBIAL GENOMICS AND REPLICATION: Fine structure of gene, genetic code; Genetic rearrangement – organization of coding sequences and repetitive sequences. Genetic system of bacteria – transformation, transduction, recombination; plasmids and transposons; Genetic systems of viruses – phage I, RNA viruses and retroviruses. Genetic system of fungi – Yeast and Neurospora. Genetic system of protozoa and mycoplasma. Multiplication of bacteriophages, bacteria and differentiating organisms such as yeast, fungi and actinomycetes. Sexual and asexual reproduction in bacteria and fungi.

UNIT IV

MICROBIAL ECOLOGY: Soil, aquatic and aerobiology; Influence of environment on microbial physiology – chemical factors; nutrients – water, C, H, O, N, P, S, growth factors-amino acids, purines, pyrimidines, nucleosides, nucleotides, vitamins, lipids, inorganic nutrients, antimicrobial compounds, metabolic inhibitors. Physical factors – radiations, temperature, pH and pressure. Response to environment – growth and reproduction; growth inhibition and death, movement, differentiation, modification to the environment – changes in chemical composition, changes in physical properties

UNIT V

MICROBIAL TECHNIQUES: Isolation of microbes from various sources, Serial dilution technique, pure culture techniques and culture preservation techniques. Microbial culture collection centres. Staining techniques – Gram, endospore, negative, flagellar and methylene blue staining. Inoculum development – Development of inocula for yeast, bacterial, mycelial and vegetative fungal processes; aseptic inoculation of the fermentor

Sterilization methods: Moist heat; dry heat, flame, filter, gas (ethylene oxide), Richards' rapid method - HTST (high temperature/short time) treatments – continuous sterilizers and pasteurizers - Sterility, asepsis, Uses of UV and non-ionizing radiation. Sterilization methods – medium sterilization, batch sterilization, contiuous sterilization, filter sterilization

Microbiological media: Types of media, composition of media – carbon sources, nitrogen sources, vitamins and growth factors, mineral, inducers, precursors and inhibitors. Selection and optimization of media

Strain improvement methods; Recombinant cell culture process – guidelines for choosing host, vector systems, plasmid sterility in recombinant cell culture, limits to over expression

REFERENCES

1. Microbiology by Pelczar, Reid and Chan, McGraw Hill Book Company.
2. Microbiology, Fundamental and Applications by R.A. Atlas, McMillan Publishers.
3. General Microbiology by Powar and Daginawala, Himalaya Publishing House.
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UNIT-1 CLASSIFICATION AND MOLECULAR SYSTEMATICS

CONTENTS

LESSON – 1

TAXONOMY AND CLASSIFICATION OF BACTERIA, VIRUS AND FUNGI

Contents

1.0. AIMS AND OBJECTIVES

The chapter discusses the taxonomy, classification of bacteria, virus and fungi.

1.1. INTRODUCTION

The science of naming and classifying organisms is called taxonomy. The word comes from the Greek taxis, 'order', nomos, 'law' or 'science'. Three separate but interrelated disciplines are involved in taxonomy

- Identification - characterizing organisms
- Classification - arranging into similar groups
- Nomenclature - naming organisms

Organizing larger organisms based on morphology is often quite simple such as fins, legs, feathers, fur, etc. But with prokaryotes, it is not as simple. Prokaryote Classification involves technologies used to characterize and ID prokaryotes viz. microscopic examination,

culture characteristics, biochemical testing, nucleic acid analysis, combination of the above is most accurate.

Taxonomic Classification Categories are arranged in hierarchical order and species is basic unit

Domain
Kingdom
Phylum or Division
Class
Order
Family
Genus
Species

Organisms are ranked and a category in any rank unites groups in the level below it, based on shared properties.

For eg.

Domain	Bacteria
Phylum	Proteobacteria
Class	g- Proteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	Shigella
Species	dysenteriae

Microbial taxonomy is a means by which microorganisms can be grouped together. Organisms having similarities with respect to the criteria used are in the same group, and are separated from the other groups of microorganisms that have different characteristics.

1.1.1. IMPORTANCE OF TAXONOMY

1. It allows us to organize huge amounts of knowledge about the organisms (Acts like a filing system).
2. Taxonomy allows for predictions & frame hypothesis for further research based on knowledge of similar organisms.
3. It places microorganisms in meaningful, useful groups with precise names so that microbiologist can work with them & communicate efficiently.
4. It is essential for accurate identification of microorganisms (For example, the need to know the pathogen for a clinical test.
5. Microbial Evolution & Diversity
6. Universal Phylogenetic Tree

1.2. CLASSIFICATION OF BACTERIA

Bacteria are classified and identified to distinguish one organism from another and to group similar organisms by criteria of interest to microbiologists or other scientists. Bacteria may be the most significant group of organisms on earth. They are responsible for much of the decomposition of dead organisms, they convert nitrogen for plants, they help many

animals digest food, they produced oxygen in the early atmosphere, and they make certain foods (yogurt, cheese, etc.).
Bacteria can be classified by:
1. colony shape in culture
2. motility
3. morphological characteristics other than shape...eg multiple flagella
4. metabolic activity eg. sugars they ferment
5. DNA sequence

1.2.1. BACTERIA CLASSIFICATION BASED ON SHAPES AND COLONY MORPHOLOGY

Before the advent of DNA sequencing, bacteria were classified based on their shapes and biochemical properties. Most of the bacteria belong to three main shapes: rod (rod shaped bacteria are called bacilli), sphere (sphere shaped bacteria are called cocci) and spiral (spiral shaped bacteria are called spirilla). Some bacteria belong to different shapes, which are more complex than the above mentioned shapes.

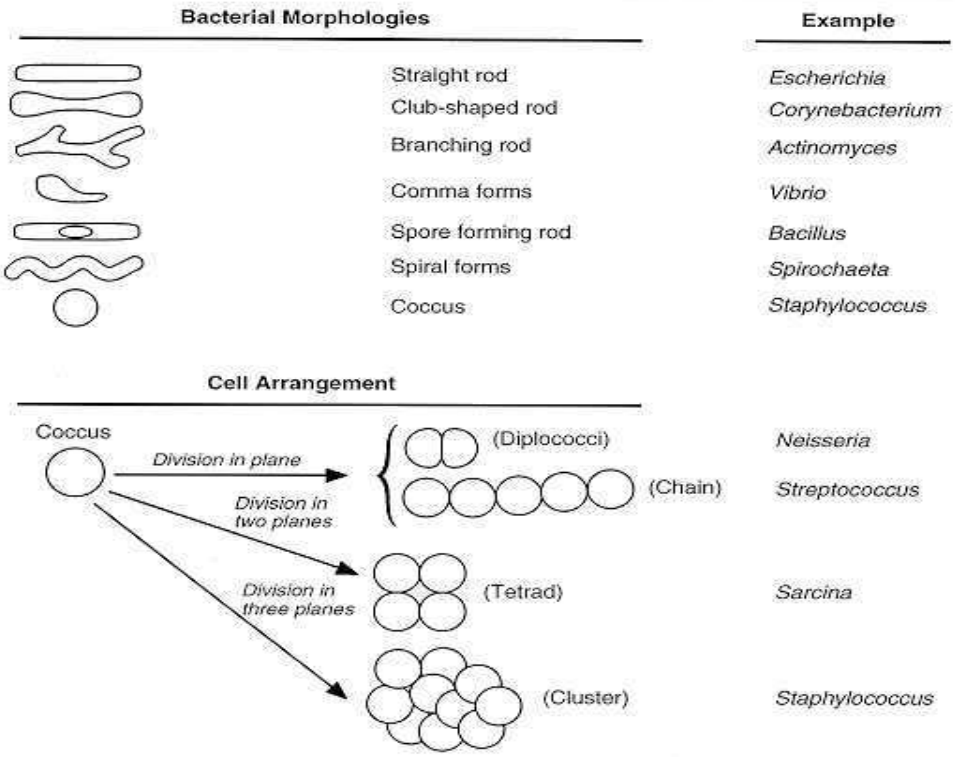


Fig. 1 morphological classification of bacteria

Bacteria show characteristic type of growth on solid media under appropriate cultural conditions and the **colony morphology** can be used in presumptive identification. The colonies can be varying in size and diameter, in outline (circular, wavy, rhizoid etc.) elevation (flat, raised, convex, etc.) and translucency (transparent, opaque, and translucent). The colour of the colony or the changes that they bring about in their surroundings is also used as diagnostic tools in the tentative identification of the bacteria. For example, colonies of streptococci on blood agar medium are small, beadlike and have a opalescent grey colour with smooth or slightly rough edges.

1.2.2. AEROBIC AND ANAEROBIC BACTERIA

Bacteria are also classified based on the requirement of oxygen for their survival. Bacteria those need oxygen for their survival are called Aerobic bacteria and bacteria those do not require oxygen for survival. Anaerobic bacteria cannot bear oxygen and may die if kept in oxygenated environment (anaerobic bacteria are found in places like under the surface of earth, deep ocean, and bacteria which live in some medium).

1.2.3. GRAM POSITIVE AND GRAM NEGATIVE BACTERIA

For more than a century bacteria have been classified according to their "Gram reaction" - named after Christian Gram who devised the protocol for his staining process in 1884. Bacteria are grouped as ‘Gram Positive’ bacteria and ‘Gram Negative’ bacteria, which is based on the results of Gram Staining Method (in which, an agent is used to bind to the cell wall of the bacteria) on bacteria.

1.2.4. AUTOTROPHIC AND HETEROTROPHIC BACTERIA

This is one of the most important classification types as it takes into account the most important aspect of bacteria growth and reproduction. Autotrophic bacteria (also known as autotrophs) obtain the carbon it requires from carbon-dioxide. Some autotrophs directly use sun-light in order to produce sugar from carbon-dioxide whereas other depend on various chemical reactions. Heterotrophic bacteria obtain carob and/or sugar from the environment they are in (for example, the living cells or organism they are in).

1.2.5. CLASSIFICATION OF BACTERIA BY NUTRITIONAL REQUIREMENT

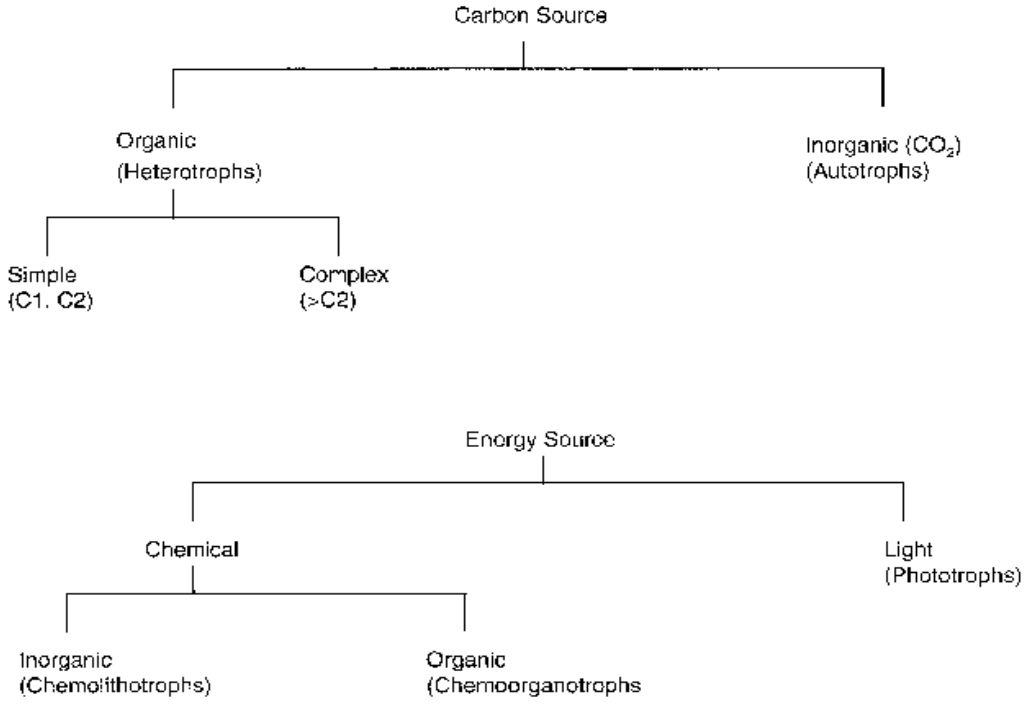


Fig. 2 classification of bacteria based on nutritional requirement

1.2.6. CLASSIFICATION BASED ON PHYLA

Based on the morphology, DNA sequencing, conditions required and biochemistry, scientists have classified bacteria into phyla:

1) Aquificae
2) Xenobacteria
3) Fibrobacter
4) Bacteroids
5) Firmicutes
6) Planctomycetes
7) Chrysogenetic
8) Cyanobacteria
9) Thermomicrobia
10) Chlorobia
11) Proteobacteria
12) Spirochaetes
13) Flavobacteria
14) Fusobacteria
15) Verrucomicrobia

Each phylum further corresponds to number of species and genera of bacteria. The bacteria classification includes bacteria which are found in various types of environments such as sweet water bacteria, ocean water bacteria, bacteria that can survive extreme temperatures (extreme hot as in sulfur water spring bacteria and extreme cold as in bacteria found in Antarctica ice), bacteria that can survive in highly acidic environment, bacteria that can survive highly alkaline environment, aerobic bacteria, anaerobic bacteria, autotrophic bacteria, heterotrophic bacteria, bacteria that can withstand high radiation etc.

1.2.7. THE BERGEY CLASSIFICATION OF BACTERIA

One of the more comprehensive bacterial classification manuals has been Bergey's manual of determinative Bacteriology. Because of on going taxonomic studies new species are continuously being described and changes are made.

- Bergey Division I = The Cyanobacteria (formerly the blue-green alga) - These bacteria can use light as their energy source under aerobic conditions. The use carbon dioxide and produce oxygen.
- Bergey Division II = The Bacteria (includes the photo bacteria and all other classical bacteria).
- The archaeabacteria were mixed within the 19 parts of the book

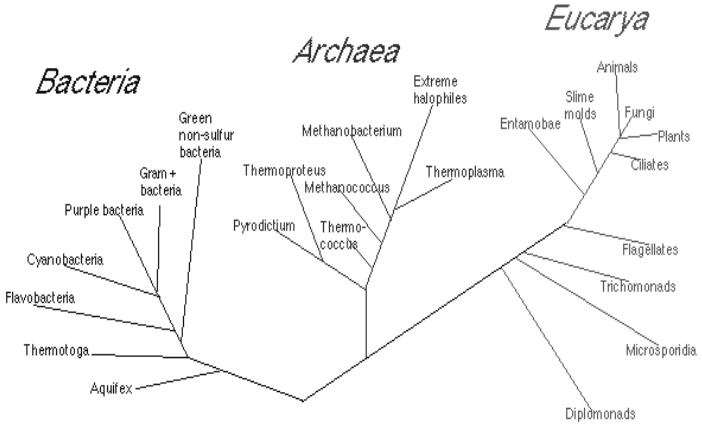


Fig. 3 Bergey's classification of bacteria

The Bergey Classification of Bacteria into 19 parts.

- **Phototrophic Bacteria:** *Rhodospirillum - Rhodopseudomonas - Chromatium*
- **Gliding Bacteria:** *Myxococcus - Beggiatoa - Simonsiella - Leucothrix*
- **Sheathed Bacteria:** *Sphaerotilus - Leptothrix*
- **Budding / Appendaged Bacteria:** *Caulobacter - Gallionella*
- **Spirochetes:** *Spirochaeta - Treponema - Borrelia*
- **Spiral and Curved Bacteria:** *Spirillum - Auqaspirillum - Oceanospirillum - Bdellovibrio*
- **Gram-negative Aerobic Rods and Cocci:** *Pseudomonas - Xanthanomonas - Zoogloea - Gluconobacter - Azotobacter - Rhizobium - Agrobacterium - Halobacterium - Acetobacter*
- **Gram-Negative Facultative Anaerobic Rods:** *Escherichia - Citrobacter - Salmonella - Shigella - Klebsiella - Enterobacter - Serratia - Proteus - Yersinia - Erwinia - Vibrio - Aeromonas - Zymomonas - Chromobacterium - Flavobacterium*
- **Gram-negative anaerobes:** *Bacteriodes - Fusobacterium - Desulfovibrio - Succinimonas*
- **Gram-Negative cocci:** *Nisseria - Branhamella - Acinetobacter - Paracoccus*
- **Gram-negative anaerobic cocci:** *Veillonella - Acidaminococcus*
- **Gram-Negative Chemolithotrophic:** *Nitrobacter - Thiobacillus - Siderocapsa*
- **Methane producing:**
- **Gram-Positive Cocci:** *Micrococcus - Staphylococcus - Streptococcus - Leuconostoc - Pediococcus - Aerococcus - Peptococcus - Ruminococcus - Sarcina*
- **Endospore-forming Rods and cocci:** *Bacillus - Clostridium - Sporosarcina*
- **Gram-positive, non-sporing rods:** *Lactobacillus - Listeria - Erysipelothrix - Caryophanon*
- **Actinomycetes and Related:** *Corynebacterium - Arthobacter - Brevibacterium - Cellumonas - Kurthia - Propionibacterium - Eubacterium - Actinomyces - Archina - Bifidiobacterium - Rothia - Mycobacterium - Frankia - Streptosporangia - Nocardia - Streptomyces - Streptoverticillium - Micromonospora*
- **Rickettsias:** *Rickettsia - Erhlichia - Wollbachia - Bartonella - Chlamydia*

1.3. VIRUS CLASSIFICATION

Viruses are not usually classified into conventional taxonomic groups but are usually grouped according to such properties as size, the type of nucleic acid they contain, the structure of the capsid and the number of protein subunits in it, host species, and immunological characteristics. It also means that when a new species of known virus family or genus is investigated it can be done in the context of the information that is available for other members of that group. Virus classification involves naming and placing viruses into a taxonomic system. Like the relatively consistent classification systems seen for cellular organisms, virus classification is the subject of ongoing debate and proposals. This is largely due to the pseudo-living nature of viruses, which are not yet definitively living or non-living. As such, they do not fit neatly into the established biological classification system in place for cellular organisms, such as plants and animals, for several reasons. Virus classification is based mainly on phenotypic characteristics, including morphology, nucleic acid type, mode of replication, host organisms, and the type of disease they cause.

1.3.1. BASED ON MORPHOLOGY

Viruses are grouped on the basis of size and shape, chemical composition and structure of the genome, and mode of replication. Helical morphology is seen in nucleocapsids of many filamentous and pleomorphic viruses. Helical nucleocapsids consist of a helical array of capsid proteins (protomers) wrapped around a helical filament of nucleic acid. Icosahedral morphology is characteristic of the nucleocapsids of many "spherical" viruses. The number and arrangement of the capsomers (morphologic subunits of the icosahedron) are useful in identification and classification. Many viruses also have an outer envelope.

1.3.2. BASED ON GENETIC MATERIAL

1.3.2.1. DNA VIRUSES

Group I: viruses possess double-stranded DNA and include such virus families as Herpesviridae (examples like HSV1 (oral herpes), HSV2 (genital herpes), VZV (chickenpox), EBV (Epstein-Barr virus), CMV (Cytomegalovirus)), Poxviridae (smallpox) and many tailed bacteriophages. The mimivirus was also placed into this group.
Group II: viruses possess single-stranded DNA and include such virus families as Parvoviridae and the important bacteriophage M13.

Virus Family	Virus Genus	Virion- naked/ enveloped	Capsid Symmetry	Type of nucleic acid
1.Adenoviridae	Adenovirus	Naked	Icosahedral	ds
2.Papovaviridae	Papillomavirus	Naked	Icosahedral	ds circular
3.Parvoviridae	B 19 virus	Naked	Icosahedral	ss
4.Herpesviridae	Herpes Simplex Virus, Varicella zoster virus, Cytomegalovirus, Epstein Barr virus	Enveloped	Icosahedral	ds
5.Poxviridae	Small pox virus, Vaccinia virus	Complex coats	Complex	ds
6.Hepadnaviridae	Hepatitis B virus	Enveloped	Icosahedral	ds circular
7.Polyomaviridae	Polyoma virus (progressive multifocal leucoencephalopathy)	?	?	ds

TABLE:1. Classification of DNA virus

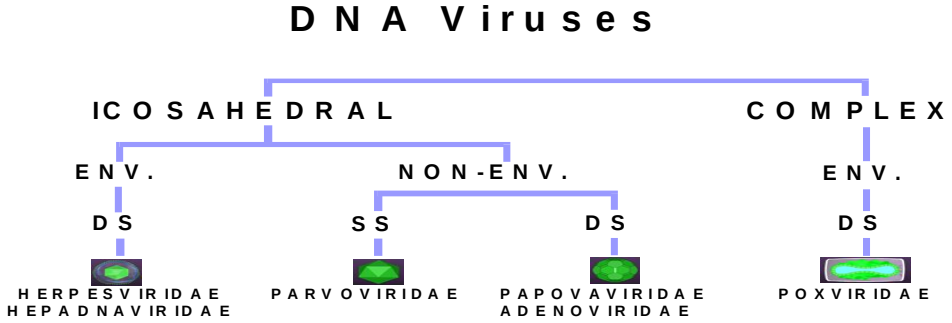


Fig 4. DNA virus classification

1.3.2.2. RNA VIRUSES

Group III: viruses possess double-stranded RNA genomes, e.g. rotavirus. These genomes are always segmented.

Group IV: viruses possess positive-sense single-stranded RNA genomes. Many well known viruses are found in this group, including the picornaviruses (which are a family of viruses that includes well-known viruses like Hepatitis A virus, enteroviruses, rhinoviruses, poliovirus, and foot-and-mouth virus), SARS virus, hepatitis C virus, yellow fever virus, and rubella virus.

Group V: viruses possess negative-sense single-stranded RNA genomes. The deadly Ebola and Marburg viruses are well known members of this group, along with influenza virus, measles, mumps and rabies.

Virus Family	Virus Genera	Virion-naked/ enveloped	Capsid Symmetry	Type of nucleic acid
1.Reoviridae	Reovirus, Rotavirus	Naked	Icosahedral	ds
2.Picornaviridae	Enterovirus, Rhinovirus, Hepatovirus, Cardiovirus, Aphthovirus, Parechovirus, Erbovirus, Kobuvirus, Teschovirus	Naked	Icosahedral	ss
3.Caliciviridae	Norwalk virus, Hepatitis E virus	Naked	Icosahedral	ss
4.Togaviridae	Rubella virus	Enveloped	Icosahedral	ss
5.Arenaviridae	Lymphocytic choriomeningitis virus	Enveloped	Complex	ss
6.Retroviridae	HIV-1, HIV-2, HTLV-I	Enveloped	Complex	ss
7.Flaviviridae	Dengue virus, Hepatitis C virus, Yellow fever virus	Enveloped	Complex	ss
8.Orthomyxoviridae	Influenzavirus A, Influenzavirus B, Influenzavirus C, Isavirus, Thogotovirus	Enveloped	Helical	ss
9.Paramyxoviridae	Measles virus, Mumps virus, Respiratory syncytial virus	Enveloped	Helical	ss
10.Bunyaviridae	California encephalitis virus, Hantavirus	Enveloped	Helical	ss
11.Rhabdoviridae	Rabies virus	Enveloped	Helical	ss
12.Filoviridae	Ebola virus, Marburg virus	Enveloped	Helical	ss
13.Coronaviridae	Corona virus	Enveloped	Complex	ss
14.Astroviridae	Astrovirus	Naked	Icosahedral	ss
15.Bornaviridae	Borna disease virus	Enveloped	Helical	ss

TABLE: 2.Classification of RNA virus

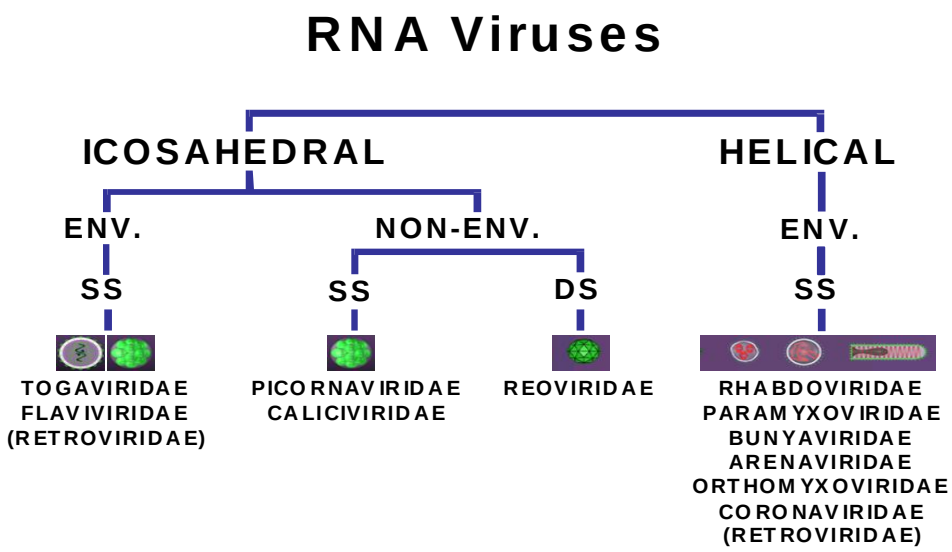


Fig 5. RNA virus classification

1.3.2.3. REVERSE TRANSCRIBING VIRUSES

Group VI: viruses possess single-stranded RNA genomes and replicate using reverse transcriptase. The retroviruses are included in this group, of which HIV is a member.

Group VII: viruses possess double-stranded DNA genomes and replicate using reverse transcriptase. The hepatitis B virus can be found in this group.

1.3.3. THE BALTIMORE CLASSIFICATION

The Baltimore system of virus classification provides a useful guide with regard to the various mechanisms of viral genome replication. The central theme here is that all viruses must generate positive strand mRNAs from their genomes, in order to produce proteins and replicate themselves. The precise mechanisms whereby this is achieved differ for each virus family. These various types of virus genomes can be broken down into seven fundamentallyhttp://www.nlv.ch/Virologytutorials/graphics/Baltimoretotal.gif different groups, which obviously require different basic strategies for their replication. **David Baltimore**, who originated the scheme, has given his name to the so-called "Baltimore Classification" of virus genomes. By convention the top strand of coding DNA written in the 5' - 3' direction is + sense. mRNA sequence is also + Sense. The replication strategy of the virus depends on the nature of its genome. Viruses can be classified into seven (arbitrary) groups:

I: Double-stranded DNA (Adenoviruses; Herpes viruses; Poxviruses, etc)

Some replicate in the nucleus e.g adenoviruses using cellular proteins. Poxviruses replicate in the cytoplasm and make their own enzymes for nucleic acid replication.

II: Single-stranded (+) sense DNA (Parvoviruses)

Replication occurs in the nucleus, involving the formation of a (-) sense strand, which serves as a template for (+)strand RNA and DNA synthesis.

III: Double-stranded RNA (Reoviruses; Birnaviruses)

These viruses have segmented genomes. Each genome segment is transcribed separately to produce monocistronic mRNAs.

IV: Single-stranded (+) sense RNA (Picornaviruses; Togaviruses, etc)

a) Polycistronic mRNA e.g. Picornaviruses; Hepatitis A. Genome RNA = mRNA. Means naked RNA is infectious, no virion particle associated polymerase. Translation results in the formation of a polyprotein product, which is subsequently cleaved to form the mature proteins.

b) Complex Transcription e.g. Togaviruses. Two or more rounds of translation are necessary to produce the genomic RNA.

V: Single-stranded (-)sense RNA (Orthomyxoviruses, Rhabdoviruses, etc)

Must have a virion particle RNA directed RNA polymerase.
a) Segmented e.g. Orthomyxoviruses. First step in replication is transcription of the (-)sense RNA genome by the virion RNA-dependent RNA polymerase to produce monocistronic mRNAs, which also serve as the template for genome replication.
b) Non-segmented e.g. Rhabdoviruses. Replication occurs as above and monocistronic mRNAs are produced.

VI: Single-stranded (+)sense RNA with DNA intermediate in life-cycle (Retroviruses)
Genome is (+)sense but unique among viruses in that it is DIPLOID, and does not serve as mRNA, but as a template for reverse transcription.

VII: Double-stranded DNA with RNA intermediate (Hepadnaviruses)
This group of viruses also relies on reverse transcription, but unlike the Retroviruses, this occurs inside the virus particle on maturation. On infection of a new cell, the first event to occur is repair of the gapped genome, followed by transcription.

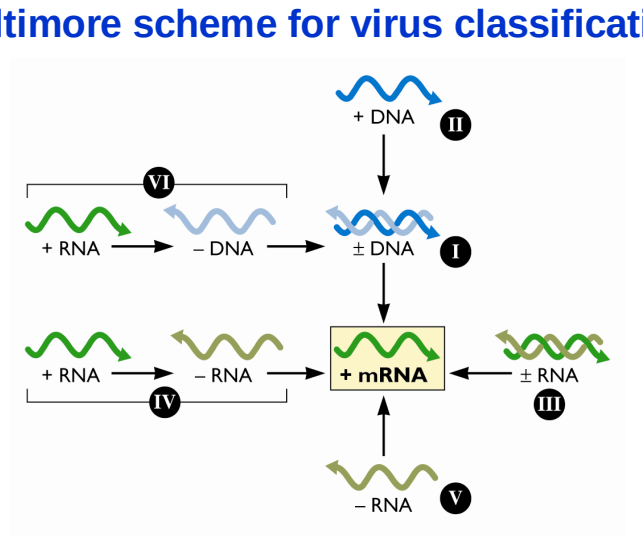


Fig 5 Baltimore Scheme of Virus Classification

1.4. CLASSIFICATION OF FUNGI

The organisms of the fungal lineage include mushrooms, rusts, smuts, puffballs, truffles, morels, molds, and yeasts, as well as many less well-known organisms. The Kingdom Fungi includes some of the most important organisms, both in terms of their ecological and economic roles. By breaking down dead organic material, they continue the cycle of nutrients through ecosystems. More than 70,000 species of fungi have been described; however, some estimates of total numbers suggest that 1.5 million species may exist

1.4.1. PROPOSED BY AINSWORTH

A more natural system of classification of fungi was proposed by G.C.Ainsworth(1996),which was used in the ***Dictionary of the Fungi*** (6^{th} edition 1971).The kingdom fungi , was divided into two divisions:the Myxomycota, for plasmodal forms, and Eumycota (true fungi) for non plasmodial forms, which are usually mycelial. The outline of this classification is given below.

Division-I Eumycota(true fungi)
Sub-division-1.Ascomycotina
Class (i) Discomycetes:
-Fruiting body perithecium type ,asci unitunicate,inoperculate with apical pore or slit.
Class (ii) Plectomycetes
-Asci unitunicate,Evanescent; fruiting body, cleistothecium type.
Class (iii) Pyrenomycetes
-Fruiting body perithecium type, asci unitunicate, inoperculate with apical pore or slit.
Class (iv)Hemiascomycetes
-Asci naked; no ascorp and ascogenous hypae.
Class (v)Laboulbeniomycetes
-Fruiting body perithecium type,asci unitunicate, inoperculate; exoparasitesof arthropods.
Class (vi)Loculoascomycetes
-asci bitunicate; ascocarp an ascostroma.

Sub-division-2.Zygomycotina
Class (i) Tricomycetes
-Mycelium not immersed in host tissue; often parasitic on orthropods.
Class (ii) Zygomycetes
-Mycelium immersed in host tissue, usually saprophytic, parasitic or predacious.

Sub-division-3.Mastigomycotina
Class (i) Oomycetes
-usually mycelial; zoospores biflagellate with posterior flagellum whiplash and anterior flagellum tinsel type.
Class (ii) Hyphochytridiomycetes
-Often unicellular, zoospores with single, anterior,tinsel flagellum.
Class (iii) Chytridiomycetes

-Often unicellular, zoospores with singe, posterior, whiplash flagellum.

Sub-division-4.Deuteromycotina

Class (i) Hyphomycetes

-mycelial,sterile or asexual spores producing directly on hypae or conidiophores..

Class (ii) Coelomycetes

- Mycelial; asexual spores on pycnidium or acervuli

Class (iii) Blastomycetes

- True mycelium lacking; budding cells with or without promycelium.

Sub-division-5.Basidiomycotina

Class (i) Hymenomycetes

- Basidiocarp present;basidia arranged in hymenium,completely or partly exposed at maturity.

Class (ii) Teliomycetes

-Basidiocarp lacking;teliospores grouped in sori or scattered within the host tissue.

Class (iii) Gasteromycetes

-Basidiocarp present;basidia arranged in hymenium,enclosed within the basidiocarp, basidai aseptate.

Division-II Myxomycota (slime mould)

Sub-division-1.Ascomycotina

Class (i) Hydromyxomycetes

-Plasmodium forms a slimy network; mostly parasitic on marine plants.

Class (ii) Ascrasiomycetes(cellular slime mould)

-assimilative phase free-living amoebae that aggregate to form a pseudoplasmodium before reproduction.

Class (iii) Plasmodiophoromycetes

-Plasmodium parasitic within the cells of the host plants.

Class (iv)Myxomycetes

-a true plasmodium; free living, saprophytic.

PROPOSED BY ALEXOPOULOS

C.J. Alexopoulos (1962) placed all fungi (including slime molds) in a separate division Mycota, and it was divided into two sub-divisions-*Myxomycotina* and *Eumycotina* on the basis of the absence and presence of cell wall respectively. Slime molds were included in the subdivision *Myxomycotina* and the true fungi in *Eumycotina*. His classification is as follows.

Division Mycota

Thallus microscopic, unicellular or fillamentous; nucleus with a distinct nuclear membrane and nucleolus; cell wall chitinous or cellulosic; reproduction by asexual means.

Sub-division-1.Eumycotina

Vegetative phase is represented by unicellular or branced siphonaceous mycelium,cell possesses distinct cell wall;hyphae are aseptate and multinucleate or septate;cells are uni-, or

multinucleate;reproduction by spores or gametes.the sub-division Eumycotina was divided into eight classes:

Class (i) Trichomycetes

-thallus simple or branched and multinucleate; often parasitic on arthropods.

Class (ii) Oomycetes

-mycellium well developed and multinucleate during vegetative phase;motile cells biflagellate (one flagellum whiplash and the other tinsel type); flagella are arranged in opposite directions.

Class (iii) Ascomycetes

-Hyphae septate; ascospores produced endogenously in specialized sporangium,known as ascus.

Class (iv)Deuteromycetes

-Hyphae septate;reproduces only by asexual spores; sexual phase lacking.

Class (v)Chytridiomycetes

-Motile cells with solitary posterior whiplash flagellum.

Class (vi)Zygomycetes

-parasitic or saprophytic fungi;mycelium well developed and multinucleate;motile structure absent.

Class (vii)Basidiomycetes

-Hyphae septate;basidiospores exogenously on basidium..

Class (viii)Hyphochytridiomycetes

-Motile cells with solitary anterior tinsel flagellum;includes aquatic fungi.

Class (ix)Plasmodiophoromycetes

-parasitic fungi;cell wall lacking;multinucleate thallus remains inside the host tissue; motile cells with two unequal anterior tinsel flagella.

Sub-division-2.Myxomycotina

Plant body in the form of naked protoplast known as plasmodium.

Class (i) Myxomycetes

-Vegetative phase is represented by a solitary large multinucleate naked protoplast plasmodium); reproduction by minute multinucleate walled spores.

1.5. LET US SUM UP

- Taxonomy is the science dealing with description, identification, nomenclature, and classification of living things
- Bacteria is classified based on various criteria such as shape, colony morphology, morphological characteristics,genomic characteristics., etc. but Bergey's manual is widely accepted and used by broad range of people.
- Classification of virus is mainly based on the genetic material it possesses. The broad classification is DNA viruses and RNA viruses.
- In natural system of classification by G.C. Ainsworth the kingdom fungi , was divided into two divisions: the Myxomycota and Eumycota.

1.6. LESSON END ACTIVITIES

1. Write down the features of Baltimore classification of viruses. (Refer 1.3.3)
2. Compare the approaches by Ainsworth and Alexopoulos who divided fungi.
3. Elucidate few examples for bacteria based on its nutritional requirements.

1.7. POINTS FOR DISCUSSION

1. **Give the significance of taxonomy?**

Taxonomy helps in identification, classification and naming of an organism. It helps in grouping of organisms with the similar characteristics. It also helps in to assess the extent of diversity of different types of organisms.

2. **Which classification of virus is frequently used?**

Viruses are mainly classified mainly based on genetic material

- DNA viruses and
- RNA viruses.

1.8 REFERENCES

1. Microbiology by Pelczar, Reid and Chan, McGraw Hill Book Company.
2. Microbiology, Fundamental and Applications by R.A. Atlas, McMillan Publishers.
3. General Microbiology by Powar and Daginawala, Himalaya Publishing House.
4. Microbial genetics by David friefelder

LESSON – 2

CLASSICAL AND NUMERICAL TAXONOMY

Contents

2.0. AIMS AND OBJECTIVES

The chapter discusses the classical and numerical taxonomy of microorganisms.

2.1. INTRODUCTION

Biological classification has two historical roots

1. Pre-Darwinian(phenetic) and
2. Darwinian (evolutionary)

Though both produce natural systems, two are natural in totally different senses. the phonetic classification organizes phenomena or organisms into types (classes) according to perceived similarity. Linnaean classification was an example of phonetic classification. On the other hand, Darwinian classification reflecting as if does, the evolutionary process, centre about the natural relationships among organisms.

The two modes of classification often tend to converge because similarity among organisms is fundamentally the result of common ancestry; phonetic grouping, based on similarity, should therefore amount to grouping based on genealogical relationship.

2.2 CLASSICAL TAXONOMY

Since a large array of microbiologists study the characteristics of organisms (morphological, physiological, biochemical, genetical , molecular), sometimes, it is difficult to assign an organism based on all the characters because the character may be important to a particular microbiologist may not be that important to another, hence different taxonomists may arrive at very different groupings. Sometimes this approach may be found to be useful.

2.2.1. CLASSICAL IDENTIFICATION METHODS

- most analysis require a pure culture
- phenotypic criteria are used to classify . e.g., growth substrates,
- metabolic products,
- biochemical characteristics

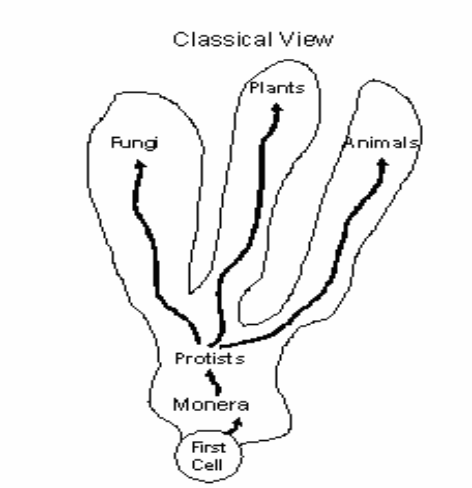


Fig 1. CLASSIFICATION OF MICROORGANISMS

2.2.2. CHARACTERISTICS USED (PHENOTYPE APPROACH)

- **Microscopic characteristics:**
  Morphology (cell shape, size, arrangement; flagellar arrangement; endospores
  Staining reactions (gram stain, acid fast stain)
- **Growth characteristics**:
  Appearance in liquid culture; colony morphology, pigmentation; habitat;
  Symbiotic relationships
- **Biochemical characteristics:**
  Cell wall chemistry; pigments; storage inclusions; antigens
- **Physiological characteristics:**
  Temperature range, optimum; O2 relationships; pH range; osmotic tolerance;
  Salt requirements, tolerance; antibiotic sensitivity
- **Nutritional characteristics:**
  energy sources; carbon sources; nitrogen sources; fermentation products;
  Modes of metabolism (autotrophic, heterotrophic, fermentative, respiratory)
- **Genetic characteristics:** DNA (%G+C)

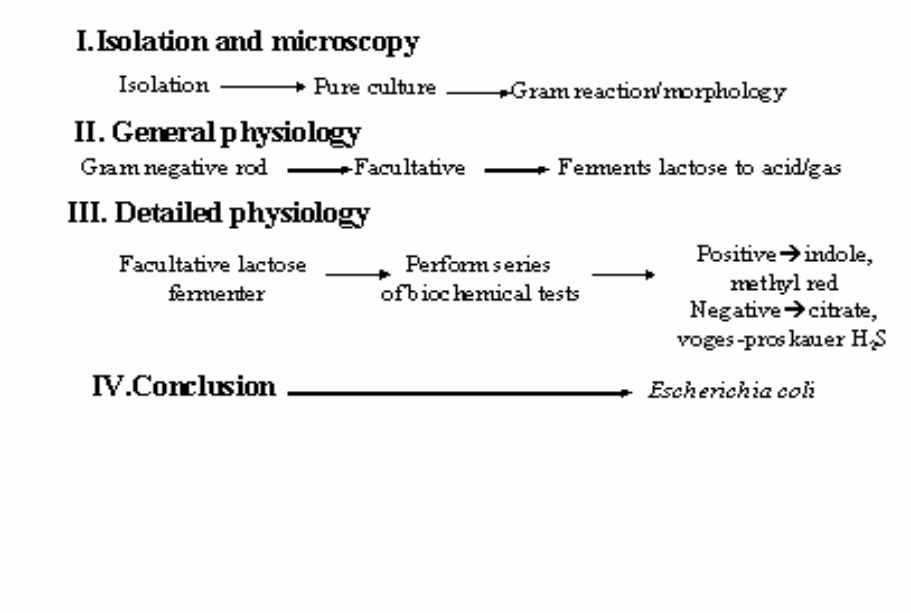


Fig 2. PHENOTYPE CHARACTERIZATION FOR CLASSIFICATION

2.3. NUMERICAL TAXONOMY

Phenetics, also known as numerical taxonomy, was proposed by Sokal and Sneath in the 1950s. Although very few modern taxonomists currently use phenetics, Sokal and Sneath's methods clearly revolutionized taxonomy by introducing computer-based numerical algorithms, now an essential tool of all modern taxonomists.

Phenetics classifies organisms based on their overall similarity. First, many different characteristics of a group of organisms are measured. These measurements are then used to calculate similarity coefficients between all pairs of organisms. The similarity **coefficient** is a number between 0 and 1, where 1 indicates absolute identity, and 0 indicates absolute dissimilarity. Finally, the similarity coefficients are used to develop a classification system.

Critics of phenetic classification have argued that it tends to classify unrelated organisms together, because it is based on overall morphological similarity, and does not distinguish between analogous and homologous features. Pheneticists have responded that they ignore the distinction between analogous and homologous features because analogous features are usually numerically over-whelmed by the larger number of homologous features. Most evolutionary biologists would consider this response questionable, at best.

2.3.1. CRITERIA FOR USED IN NUMERICAL TAXONOMY

Numerical taxonomy typically invokes a number of these criteria at once. The reason for this is that if only one criterion was invoked at a time there would be a huge number of taxonomic groups, each consisting of only one of a few microorganisms. The purpose of grouping would be lost. By invoking several criteria at a time, fewer groups consisting of larger number of microorganisms result.The groupings result from the similarities of the members with respect to the various criteria. A so-called similarity coefficient can be calculated. At some imposed threshold value, microorganisms are placed in the same group

2.3.2. GROUPING OF ORGANISMS BY NUMERICAL METHOD

The grouping by numerical methods of taxonomic units based on their character states. The application of numerical methods to taxonomy, dating back to the rise of biometrics in the late nineteenth century, has received a great deal of attention with the development of the computer and computer technology. Numerical taxonomy provides methods that are objective, explicit, and repeatable, and is based on the ideas first put forward by M. Adanson in 1963. These ideas, or principles, are that the ideal taxonomy is composed of information-rich taxa based on as many features as possible, that *a priori* every character is of equal weight, that overall similarity between any two entities is a function of the similarity of the many characters on which the comparison is based, and that taxa are constructed on the basis of diverse character correlations in the groups studied.

In the early stages of development of numerical taxonomy, phylogenetic relationships were not considered. However, numerical methods have made possible exact measurement of evolutionary rates and phylogenetic analysis. Furthermore, rapid developments in the techniques of direct measurement of the homologies of deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) between different organisms now provide an estimation of "hybridization" between the DNAs of different taxa and, therefore, possible evolutionary relationships. Thus, research in numerical taxonomy often includes analysis of the chemical and physical properties of the nucleic acids of the organisms the data from which are correlated with phenetic groupings established by numerical techniques.

2.3.3. STEPS INVOLVED IN GROUPING

- Uses a variety of characteristics: e.g., Gram stain, cell shape, motility, size, aerobic/anaerobic capacity, nutritional capabilities, cell wall chemistry, immunological characteristics, etc.
- Relies on similarity coefficients
- If use 10 characteristics, then match organisms.
- Ex. A and B share 8 characters out of 10: similarity coefficient S_{ab} is 8/10 = 0.8
- Can use many such values to establish similarity matrix
- Dendrograms help display this information clearly.

Fig.3. Dendrogram of species

Dendrogram is just a graphical display of similarity coefficients; but one often assumes that these are representative of a deeper evolutionary relationship. This may or may not be legitimate conclusion, depending on the traits used. Fig.4 is a hypothetical evolutionary diagram, superficially similar to a dendrogram but actually quite different, since it seeks to

portray an accurate picture of how and when organisms diverged from common ancestors over time.

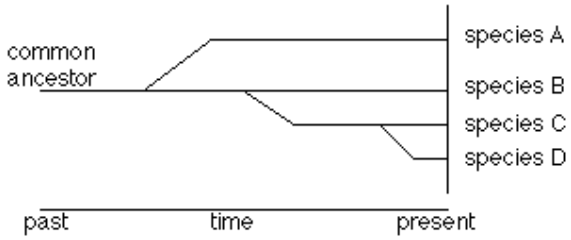


Fig.3. Divergence of species

To get accurate phylogeny, must decide which characteristics give best insight. DNA and RNA sequencing techniques are considered to give the most meaningful phylogenies.

2.3.4 PERCENTAGE SIMILARITY FOR NUMERICAL TAXONOMY

This calculation is based on several characteristics for each strain and each character is given equal weight age

%S = NS/ (NS+MD)

Where,

NS= number of characteristics for each strains which are similar or dissimilar.

ND= number of characteristic that are dissimilar or different.

On the basis of %S, S = similarity if it is high to each other, placed into groups larger and so on.

2.4. LET US SUM UP

- In Classical taxonomy the organisms are grouped based on their morphological, physiological, biochemical, genetical, molecular characteristics.
- Classical taxonomy is simple and easy to perform. The criteria used are based on the need of the microbiologists.
- This makes difficult to assign the group of the organism as different microbiologists will prefer different characteristics.
- Numerical taxonomy is also called as phenitics and it takes number of criteria for grouping at once.
- Percentage similarity of number of characters is taken into account and grouping is done manually or by computer.

2.5. LESSON END ACTIVITIES

Compare classical and numerical taxonomies.

2.6. POINTS FOR DISCUSSION

1. Disscuss the disadvantages of classical taxonomy?

- Does not include all characters.
- Evolution based studies is not carried out.
- Some times the closely related organisms are placed into different groups characters

2. How many criteria are used for numerical taxonomy and how the organisms are grouped?

In numerical taxonomy maximum numbers of criteria's that can be accessed are used to avoid grouping of large number of in organisms into a single group.

Percentage similarity of all the results will be calculated and dendogram is drawn showing the similarity and diversity with one another.

2.7 REFERENCES

1. Microbiology by Pelczar, Reid and Chan, McGraw Hill Book Company.
2. Microbiology, Fundamental and Applications by R.A. Atlas, McMillan Publishers.
3. General Microbiology by Powar and Daginawala, Himalaya Publishing House.
4. Microbial genetics by David friefelder

LESSON – 3

MOLECULAR TAXONOMY

Contents

3.0. AIMS AND OBJECTIVES

The chapter discusses the molecular taxonomy of microorganisms.

3.1. INTRODUCTION

Molecular taxonomy is also called chemotaxonomy. This approach employs molecular analysis to identify and classify organisms. The three main methods are DNA - DNA hybridization, ribotyping, and lipid analysis. Effective habitat conservation, bioprospecting and sustainable use of biodiversity on a global basis, require taxonomic decisions and expertise on a scale not presently available. It is always desirable to carry out detailed genetic analysis, in addition to morphological studies, to segregate taxonomically difficult taxa particularly at or below the species level, and also the understand the evolutionary processes and to reconstruct phylogenetic relationships in groups of plants that are significant from any standpoint. Recent advances in biochemical genetics and molecular biology have made it possible to analyze divergence at particular genes of different species without having to do genetic crosses. Modern molecular techniques provide powerful tools to determine the genetic closeness of wild relatives to the plant genomes, and to clarify the confusing state in taxonomic classifications of some crop groups. Diagnostic molecular markers are used directly for distinguishing cultivars, tagging agronomic traits in marker selected breeding, and for identifying seed contaminants and weed biotypes. The integration of molecular studies with other biosystematics data will enable information on these germplasms to be readily retrieved and utilized in breeding programs and weed management strategies.

Early in its history, taxonomy relied exclusively on phenotypic characteristics. For higher organisms, this usually meant morphology, whereas, for microorganisms, biochemical characteristics were used together with such morphological features as were discernible. Biologically, species were defined as “a group of organisms that can interbreed with one another”. However, for many microorganisms, and particularly a number of groups of yeasts and fungi, no sexual stage could be discovered in their life cycles, so the biological definition of a species proved unworkable. The availability of facile methods to sequence first proteins

and then genes led to the rapid growth of molecular phylogenetics and the use of sequence comparisons to both define and identify species

This molecular phylogeny allows the twin aims of the taxonomist to be achieved. Gene sequences permit the construction of phylogenetic trees that reflect the evolutionary history of the species in a given clade and thus provide a natural taxonomy. Moreover, the ready acquisition of such sequences from individual specimens, using the Polymerase Chain Reaction (PCR), means that this approach also facilitates identification. The problem is that these phylogenies are based on a single sequence, either a protein-encoding gene or (more frequently) a sequence encoding all or part of a ribosomal RNA. This means that molecular taxonomy is subject to the same variation as were the biochemical tests previously used to discriminate between species. The problem can be seen when several trees, each for the same group of species but based on a different gene sequence, are compared. The variation in tree structure is typical of single-sequence analysis, and it has been suggested that a larger group of core genes could be used for a more accurate reference. Those ORFs contained within the genome that are known to be transferred or rearranged, such as the structural genes in bacteria or those telomeric or highly repeated sequences in yeast, would be avoided in these core groups. Whole-genome methods of phylogeny reconstruction are generally considered to avoid the limitations of constructing phylogenies from sequence data from just a few loci. Gene duplication followed by divergence in sequence between the duplicate gene copies is usually cited as a typical mechanism whereby a "gene tree" constructed from any single gene may differ considerably from the "species tree". At the level of genotype, it may not necessarily make sense to talk of a species tree because one would expect portions of the genome to have different evolutionary histories. However, as a means of classifying a species within a phylogeny, genome-wide methods have the advantage of taking into account all of the differences between species.

For microbial eukaryotes, such as the yeasts, the best method would be to construct phylogenetic trees from complete (or near-complete) genome sequences. This is currently being done by using techniques such as whole-genome shotgun sequencing. Although this method allows robust phylogenies to be constructed, it will not be a comprehensive approach for any taxonomic group in the near future and does not address the second aim of the taxonomist—that of facile identification of species. Similarly, the sequencing of a core set of less than or equal to 20 genes is not a practicable approach to routine species identification. An alternative method is Comparative Genomic Hybridization (CGH), which is the comparison of whole genomic DNA to reference DNA, by hybridization. The term CGH is usually applied to the cytogenetic method of screening genetic changes in immobilized metaphase chromosomes from tumor samples by comparison to a reference. However, with the advent of microarray technology, this term now incorporates studies based on hybridizations to microarrays). In addition to tissue analysis, this method has recently been applied, as a taxonomic tool, to several microorganisms in order to compare intra- and interspecific genetic diversity. An advantage of this method of taxonomy is that it "circumvents the need for sequencing multiple closely related genomes" and could be used for routine identification of specimens.

Under the Molecular Taxonomy following projects can be carried out:

- Studies on complete diversity characterization and ascertaining the Center of origin.
- Proper identification and resolving taxonomical problems at family, genera, species and subspecies level.

- Complete characterization of collections of important crops from different geographical locations and based on different traits.

3.2. DETERMINATION OF GC CONTENT

GC-content (or guanine-cytosine content), in molecular biology, is the percentage of nitrogenous bases on a DNA molecule which are either guanine or cytosine .This may refer to a specific fragment of DNA or RNA, or that of the whole genome. When it refers to a fragment of the genetic material, it may denote the GC-content of part of a gene (domain), single gene, group of genes (or gene clusters) or even a non-coding region. G (guanine) and C (cytosine) undergo a specific hydrogen bonding whereas A (adenine) bonds specific with T (thymine).

Microbial genomes can be directly compared, and taxonomic similarity can be estimated in many ways. the first, and possibly the simplest, technique to be employed is the determination of DNA based composition.

DNA contains 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. 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), physical methods are easier and more often used. The G+C content often is determined from the melting temperature Tm of DNA. In double stranded DNA three hydrogen bonds join GC base pairs, and two bonds connect AT basepairs. As a result DNA with a greater GC content will have more hydrogen bonds, and its strands will separate only at high temperatures. That is, it will have a higher melting point. DNA melting can be easily followed spectrophotometrically because of the absorbance of 250 nm UV light by the DNA increases during strand separation. When a DNA sample is slowly heated the absorbance increases as hydrogen bonds are broken and reaches a plateau when the entire 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 with linearly with G+C content, the percent G+C can be obtained by centrifuging DNA in cesium chloride 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 prokaryotic and eukaryotic microorganisms vary greatly in G+C content; prokaryotic G+C content is the most variable, ranging from around 25 to almost 80%. Despite such a wide variation, the G+C content of strains within a particular species is constant. If two organisms differ in their G+C content for more than about 10%, their genomes have quite different base sequences. On the other hand, it is not safe to assume that the organisms with very similar G+C contents also have similar DNA base sequences because two very different base sequences can be constructed from the same proportions of AT and GC base pairs. Only if two microorganisms also are alike phenotypically does their similar G+C content suggest close relatedness.

G+C content data are taxonomically valuable in atleast two ways. First, they confirm 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 the genus

is usually less than 10% even though the content may vary greatly between genera. For e.g., staphylococcus has a G+C content of 30-38%, whereas micrococcus has 64 -75% G+C; yet these two genera of gram positive cocci many features in common.

GC content is usually expressed as a percentage value, but sometimes as a ratio (called **G+C ratio** or **GC-ratio**). GC-content percentage is calculated as

G+C/G+C+A+T

whereas the G+C ratio is calculated as

A+T/G+C

GC content is found to be variable with different organisms, the process of which is envisaged to be contributed by variation in selection, mutational bias and biased recombination-associated DNA repair . The species problem in prokaryotic taxonomy has led to various suggestions in classifying bacteria and the *ad hoc committee of on reconciliation of approaches to bacterial systematics* has recommended use of GC ratios in higher level hierarchical classification.For example, the Actinobacteria are characterised as "high GC-content bacteria". In "Streptomyces coelicolor" A3(2) it is 72%. The GC-content of Yeast (*Saccharomyces cerevisiae*) is 38%, and that of another common model organism Thale Cress (*Arabidopsis thaliana*) is 36%. Because of the nature of the genetic code, it is virtually impossible for an organism to have a genome with a GC-content approaching either 0% or 100%. A species with an extremely low GC-content is *Plasmodium falciparum* (GC% = ~20%), and it is usually common to refer to such examples as being AT-rich instead of GC-poor

3.3. DNA HYBRIDIZATION

In DNA hybridization, the double strands of DNA of each of two organisms are split apart, and split strands from the two organisms are allowed to combine. The strands from different organisms will anneal by base pairing – A with T and G with C. the amount of annealing is directly proportional to the quantity of identical base sequences in the two DNAs. High degree of homology exist when both organisms has long identical sequences of bases. Close DNA homology indicates that the two organisms are closely related and that they probably evolved from a common ancestor. A small degree of homology indicates that the organisms are mot very closely related. Ancestors of such organisms probably diverged from each other thousands of centuries ago and have since evolved from along separate lines.

In the comparison of human and kangaroo cytochrome c, a single molecule provides only a narrow window for glimpsing evolutionary relationships.

The technique of DNA-DNA hybridization provides a way of comparing the **total genome** of two species. Let us examine the procedure as it might be used to assess the evolutionary relationship of **species B** to **species A**:

- The total DNA is extracted from the cells of each species and purified.
- For each, the DNA is heated so that it becomes denatured into single strands (**ssDNA**).
- The temperature is lowered just enough to allow the multiple short sequences of repetitive DNA to rehybridize back into double-stranded DNA (**dsDNA**).
- The mixture of ssDNA (representing single genes) and dsDNA (representing repetitive DNA) is passed over a column packed with hydroxyapatite. The **dsDNA sticks** to the hydroxyapatite; **ssDNA does not** and flows right through. The purpose of

this step is to be able to compare the information-encoding portions of the genome — mostly genes present in a single copy — without having to worry about varying amounts of noninformative repetitive DNA.

- The ssDNA of **species A** is made radioactive.
- The radioactive ssDNA is then allowed to rehybridize with nonradioactive ssDNA of the same species (**A**) as well as — in a separate tube — the ssDNA of species **B**.
- After hybridization is complete, the mixtures (**A/A**) and (**A/B**) are individually heated in small (2°–3°C) increments. At each higher temperature, an aliquot is passed over hydroxyapatite. Any radioactive strands (**A**) that have separated from the DNA duplexes pass through the column, and the amount is measured from their radioactivity.
- A graph showing the percentage of ssDNA at each temperature is drawn.
- The temperature at which 50% of the DNA duplexes (dsDNA) have been denatured ($T_{50}H$) is determined.

As the figure shows, the curve for **A/B** is to the left of **A/A**, i.e., duplexes of **A/B** separated at a lower temperature than those of **A/A**. The sequences of A/A are precisely complementary so all the hydrogen bonds between complementary base pairs (A-T, C-G) must be broken in order to separate the strands. But where the gene sequences in **B** differ from those in **A**, no base pairing will have occurred and denaturation is easier.

Thus DNA-DNA hybridization provides genetic comparisons integrated over the entire genome. Its use has cleared up several puzzling taxonomic relationships.

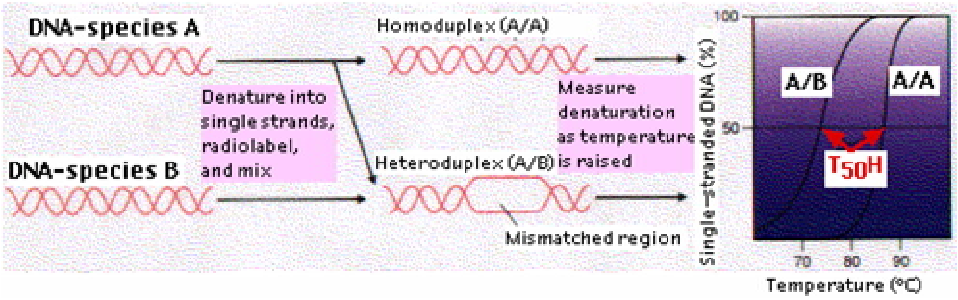


Fig.1 DNA HYBRIDIZATION

3.4. PHYLOGENETIC TREES

Phylogenetic tree also called Dendrogram, a diagram showing the evolutionary interrelations of a group of organisms derived from a common ancestral form. The ancestor is in the tree "trunk"; organisms that have arisen from it are placed at the ends of tree "branches." The distance of one group from the other groups indicates the degree of relationship; i.e., closely related groups are located on branches close.

jA phylogenetic tree is a graphical representation of the evolutionary relationship between taxonomic groups. The term phylogeny refers to the evolution or historical development of a plant or animal species, or even a human tribe or similar group. Taxonomy is the system of classifying plants and animals by grouping them into categories according to their similarities. A phylogenetic tree is a specific type of cladogram where the branch

lengths are proportional to the predicted or hypothetical evolutionary time between organisms or sequences. Cladograms are branched diagrams, similar in appearance to family trees that illustrate patterns of relatedness where the branch lengths are not necessarily proportional to the evolutionary time between related organisms or sequences. Bioinformaticians produce cladograms representing relationships between sequences, either DNA sequences or amino acid sequences. However, cladograms can rely on many types of data to show the relatedness of species. In addition to sequence homology information, comparative embryology, fossil records and comparative anatomy are all examples of the types of data used to classify species into phylogenic taxa.

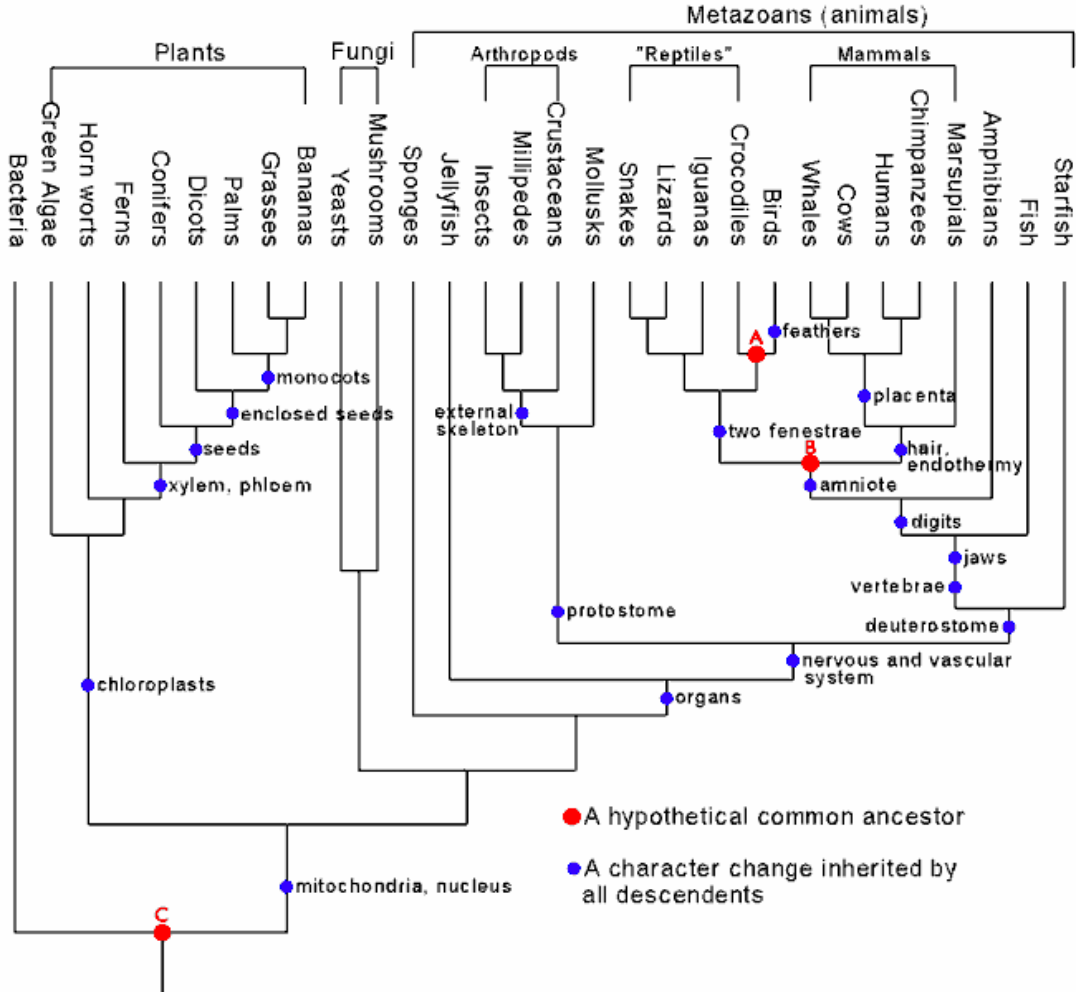


Fig. 2. PHYLOGENETIC TREE

Phylogenetic tree is not the best model for

- **For individuals within a species.** The genetic material of an individual doesn't derive from a single earlier existing individual. Animals and plants that multiply by sexual reproduction receive half their genetic material from each of two parents, so a tree like this is inappropriate. For species that multiply asexually, a tree is appropriate. Even for species that usually multiply asexually—such as many one-celled creatures—the occasional exchange of genetic material through conjugation is so important that trees are inappropriate.

- **For closely related species.** Individuals do occasionally mate between closely related species, and their progeny survive to contribute to the gene pool of one or both of the parent species. As the species diverge, such intermixing of genetic material becomes rarer. One solution is to treat closely related species as one larger variable species. Another is simply not to consider closely related species.
- **Hybrid species.** In the plant world it occasionally happens that a new tetraploid species arises from two diploid species. The two parent species need to be somewhat related for this to happen.
- **Distant interaction.** There are a couple of ways that genetic material from one species can find its way into a distantly related or unrelated species. Among bacteria, sometimes a bacterium of one species can ingest the genetic material of a bacterium of another species and incorporate part of it into its own genetic material. Rare as this may be, the effects are significant. Sometimes viruses can inadvertantly transport genetic material from one species to another. When some viruses break out of cells of one species, they may infect other species and carry that material to them.

The application of these requirements results in the following terms being used to describe the different ways in which groupings can be made:

- A **monophyletic** grouping is one in which all species share a common ancestor and all species derived from that common ancestor are included. This is the only form of grouping accepted as valid by cladists. (For example, turtles, lizards, crocodilians and birds are all derived from a shared common ancestor. Thus a monophyletic grouping would place all of these together, rather than placing birds into a separate group.)
- A **paraphyletic** grouping is one in which all species share a common ancestor, but not all species derived from that common ancestor are included (for example, grouping turtles, lizards and crocodiles as "reptiles" and separating that grouping from the birds).
- A **polyphyletic** grouping is one in which species that do not share an immediate common ancestor are lumped together, while excluding other members that would link them (for example, a hypothetical group the "lizmams" made by grouping together the lizards and the mammals).

Thus, in cladistics, no matter how divergent in appearance B might be from C, relative to A, if B and C share a common ancestor that is not shared by A, then B and C must be grouped together and separated from lineage A. In the cladogram of the reptiles and birds Fig-2, you can see an example of such a situation.

3.4.1. TYPES OF PHYLOGENETIC TREE

In *rooted trees* the root is the position in the tree occupied by the common ancestor of all the sequences (A - E in the figure). Branch lengths indicate amount of divergence/change: longer branches indicate more changes. *Unrooted trees* contain no information about a hypothetical common ancestor of all the sequences but branch lengths still reflect degree of divergence.

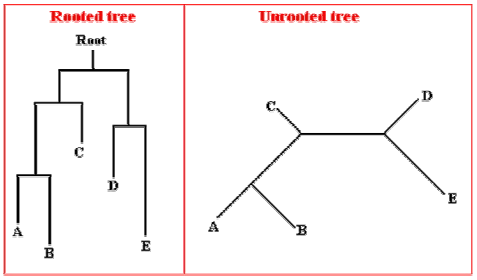
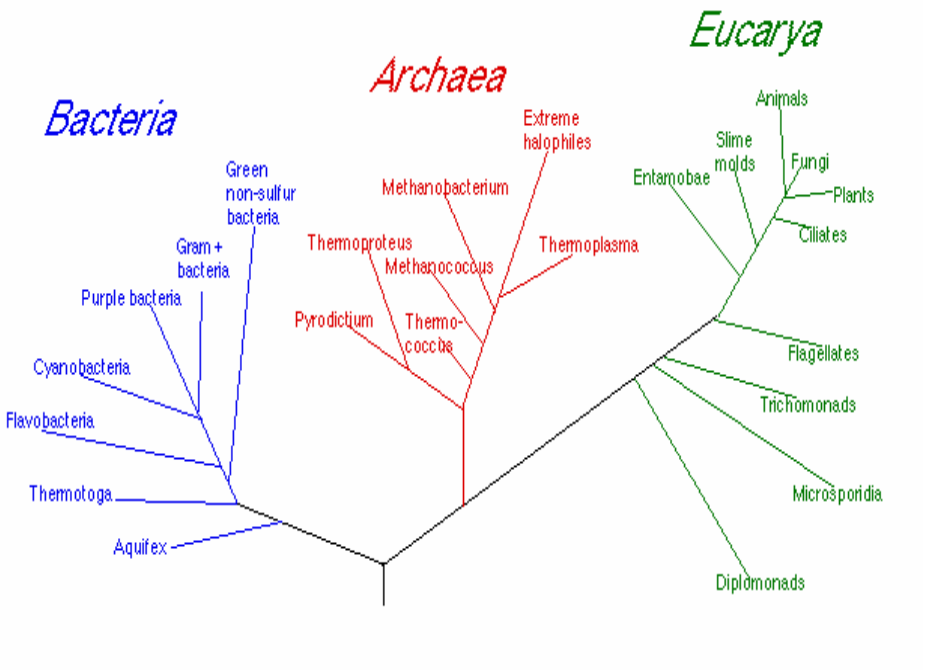


FIG.3. ROOTED AND UNROOTED TREE



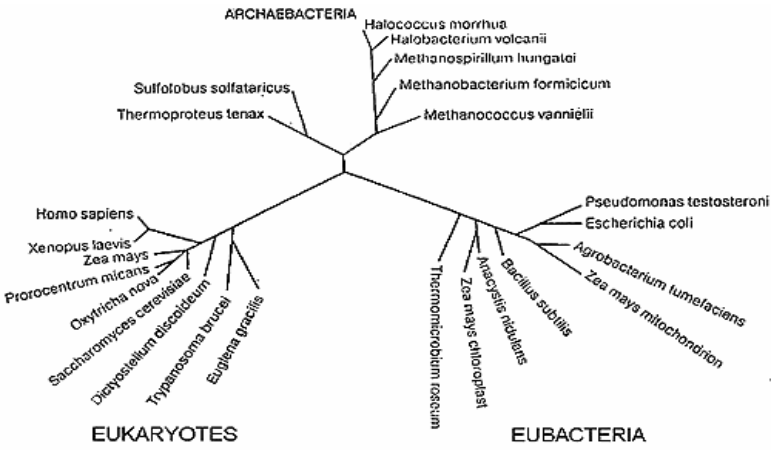


FIG.4. EXAMPLE OF ROOTED AND UNROOTED TREE

In describing a tree:

leaves represent contemporary sequences;

nodes are leaves plus internal branch points;

edges are lines connecting nodes;

topology is the branching pattern.

Two trees can have the same topology but differing edge lengths

Some tree generation methods produce rooted trees whereas others produce unrooted trees. Roots can be added to un-rooted trees in two main ways:

(1) Using an out group,

(2) Assuming a molecular clock.

Using an out-group

From other information we may know that one sequence or set of sequences is more distantly related to form an out-group. An example would be bacterial sequence in a set of mammalian ones. The root then goes between the out-group and the other sequences.

Assuming a molecular clock

The assumption is that the rate of evolution is the same on the longest branches of the tree. This implies that the root lies at the midpoint of the longest chain of consecutive edges.

3.4.2. METHODS OF GENERATING TREES

There are four main methods of generating trees:

(1) Maximum parsimony

(2) UPGMA (clustering method)

(3) Neighbor joining

(4) Maximum likelihood

(1) Maximum parsimony

Maximum parsimony is based on finding the tree with the minimal number of substitutions. It involves a best guess at evolution. A sequence is stored at each internal node (the ancestor of the sequences below it). The method uses branch-and-bound algorithm to search all possible tree topologies. The method is expensive because of the number of possible topologies.

(2) UPGMA (clustering method)

UPGMA stands for Unweighted Pair Group Method using Arithmetic averages. It is based on a set of pair-wise distances between sequences (a distance matrix): d_{ij} is distance between sequences i and j.

There are several ways of calculating distances, of which the most common is based on the

Percentage identity of the sequences.

In summary the steps in the UPGMA algorithm are:

1. Begin with N sequences
2. Find two closest sequences i,j and define them as a cluster (in the first round we now have N-2 sequences and a cluster)
3. Recalculate distances (Distances to a cluster are averages for seqs in the cluster)
4. Create a new internal node with daughter nodes i,j at height $d_{ij}/2$.
5. Iterate (2-5) until only two clusters remain
6. Place root midway between them the two remaining clusters

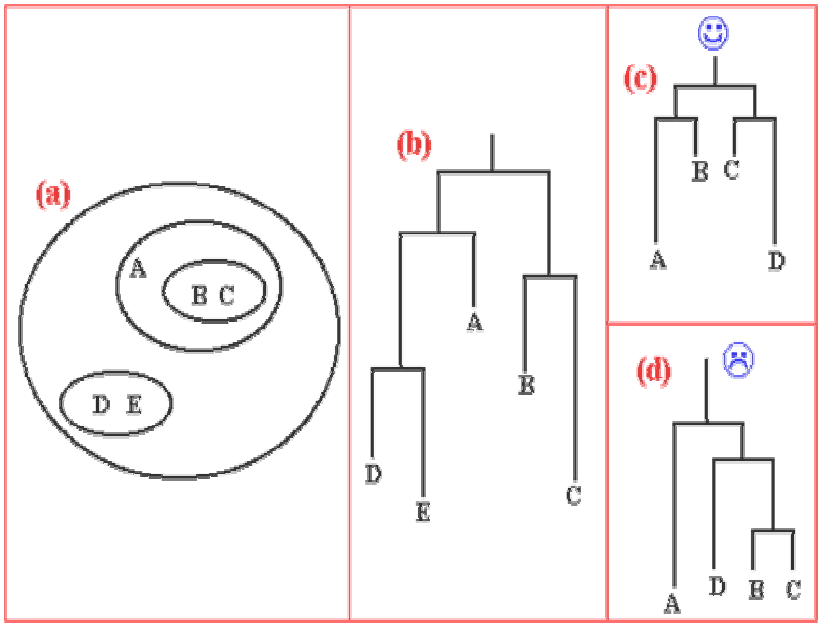


Fig.4. UPGMA (clustering method)

(a) is a diagram for sequences A-E (the distance between sequences is proportional to separation on the page).

(b) Is the resulting tree.

Unhappily UPGMA can lead to errors: a tree that should look like (c) might end up like (d).

(3) Neighbour joining

Neighbour joining is an algorithm which gets round the pitfalls of UPGMA. No assumption is made about a constant mutation rate.

Distances are corrected to account for fast evolving edges:

$D_{ij} = d_{ij} - r_i - r_j$

where r_i is the average distance of sequence i from other sequences.

(4) Maximum likelihood

Maximum likelyhood is based on finding tree which maximises the likelihood P(data|tree). Maximum likelyhood needs a probabilistic model of evolution (e.g. PAM matrices for proteins), is computationally expensive. Not many implementations are available.

General considerations

Maximum likelihood and parsimony algorithms are intellectually most satisfying but computationally expensive. In practice, neighbour joining is almost always a good compromise.

The commonest method of establishing the statistical significance of the tree that is generated is the *bootstrap*. The tree calculation is repeated many times. Each time change the multiple alignments by removing some columns and duplicating others. For each node report the percentage of times those sequences were found to cluster together. A rule of thumb is that for reliability a node should have a bootstrap value of 70% or more.

3.5. LET US SUM UP

- Molecular taxonomy employs molecular analysis for classifying the organisms
- It can be carried out with the - G + C content, Hybridization studies,Direct sequence analysis,Analysis of DNA restriction fragment patterns
- Nucleic Acid Hybridization - The process of forming a hybrid double-stranded DNA molecule using a heated mixture of single stranded DNA from two different sources; if the sequences are fairly complementary, stable hybrids will form.
- Nucleic Acid base sequencing – allows the direct comparison of DNA & RNA. In microbiology mostly RNA is used.
- Phylogenetic Trees – a graph made of nodes & branches, much like a tree in shape, which shows organisms and sometimes also indicates the evolutionary development of groups.
- A phylogenetic tree, a graph made of nodes & branches, is a graphical representation of the evolutionary relationship between taxonomic groups.

3.6. LESSON ENDACTIVITIES

Give the importance of DNA hybridization.

3.7. POINTS FOR DISCUSSION

1. **G+C % as a classification criteria- discuss.**
    - G+C % varies widely in microbes than in plants and animals
    - These values are useful as they vary less within a species than between a genus.
    - Closely related organisms have similar G+C %
2. **Give the steps involved in DNA hybridization.**
    - Double stranded DNA from different sources is taken and strands are separated by melting.
    - Separated strands are mixed to anneal.
    - They anneal in regions where the sequences are similar/same creating a hybrid molecule.
    - The extent of annealing is a quantitative index of the similarity of base sequences in the DNA from different sources.

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LESSON – 4

POLYPHASIC TAXONOMY

Contents

4.0. AIMS AND OBJECTIVES

The chapter discusses the polyphasic taxonomy.

4.1. INTRODUCTION

For a long time, bacterial taxonomy was considered one of the dullest fields in microbiology, not immediately the preferred discipline of young or ambitious scientists. Recent developments have changed this attitude, mainly because of the spectacular developments witnessed in the last 10 years in the field of sequencing of rRNA and genes coding for rRNA (rDNA) and their contribution to bacterial phylogeny and in molecular fingerprinting techniques. These techniques revolutionized our insights in the phylogeny and taxonomy of all living organisms. Taxonomy of bacteria finally also could be assigned a place in phylogeny. Another development of bacterial taxonomy, polyphasic taxonomy, arose 25 years ago and is aiming at the integration of different kinds of data and information (phenotypic, genotypic, and phylogenetic) on microorganisms and essentially indicates a

consensus type of taxonomy. The term “polyphasic taxonomy” was coined by Colwell and is used for the delineation of taxa at all levels. Also, the terms “polyphasic classification” and “polyphasic identification” can be validly used in this context. The recent developments of polyphasic taxonomy and phylogeny clearly constitute milestones in modern bacterial taxonomy. There will never be a definitive classification of bacteria. It is only the illustration of a rule, valid in all experimental sciences, stating that scientific progress is linked to and made possible through technological progress.

4.2. POLYPHASIC TAXONOMY

Taxonomy is generally taken as a synonym of systematics or biosystematics and is traditionally divided into three parts:

(i) Classification, i.e., the orderly arrangement of organisms into taxonomic groups on the basis of similarity;
(ii) Nomenclature,i.e., the labelling of the units defined in (i); and
(iii) Identification of unknown organisms, i.e., the process of determining whether an organism belongs to one of the units defined in (i) and labeled in

Two additional parts are needed to completely define modern biosystematics: phylogeny and population genetics. In the last decade, it became generally accepted that bacterial classification should reflect as closely as possible the natural relationships between bacteria, which the phylogenetic relationships are as encoded in 16S or 23S rRNA sequence data. The species is the basic unit of bacterial taxonomy and is defined as a group of strains, including the type strain, sharing 70% or greater DNA-DNA relatedness with 5°C or less ΔTm (*Tm* is the melting temperature of the hybrid as determined by stepwise denaturation; ΔTm is the difference in *Tm* in degrees Celsius between the homologous and heterologous hybrids formed under standard conditions. Phenotypic and chemotaxonomic features should agree with this definition. The designated type strain of a species serves as the name bearer of the species and as the reference specimen.

The bacterial species definition given above is founded upon whole genomic DNA-DNA hybridization values. Practical problems exist, however, because different methods are used to determine the level of DNA-DNA hybridization. These methods do not always give the same (quantitative) results, and the value of 70% DNA relatedness seems only to be indicative rather than absolute. Although not available, an alternative phylogenetic species concept could delineate a species in a phylogenetic framework as determined by percent 16S rRNA similarities. Within the present manuscript, all the attention will be focused on the taxonomic ranks of species, genus, and family. The species is certainly the most important and at the same time the central element of bacterial taxonomy, but the hierarchical structure of taxonomy requires us to consider at least the higher taxa of genus and family. Much more than the species, they are difficult to define and represent agglomerates of nodal species and internodal strains and agglomerates of genera, respectively.

4.2.1. DIFFERENT TYPES OF INFORMATION USED IN BACTERIAL POLYPHASIC TAXONOMY

In principle, all genotypic, phenotypic, and phylogenetic information may be incorporated in polyphasic taxonomy. Genotypic information is derived from the nucleic acids (DNA and RNA) present in the cell, whereas phenotypic information is derived from proteins and their functions, different chemotaxonomic markers, and a wide range of other expressed features(Fig: 1**)**. The number of different molecules which have been applied in

taxonomic studies is large, and their applications as markers are manifold. Several of the methods described briefly below (e.g., determinations of the moles percent G+C content and DNA-DNA hybridization studies) became classic and were applied in taxonomic analysis of virtually all bacteria. Others, such as amino acid sequencing, were performed on a limited number of taxa only, because they are laborious, time-consuming, or technically demanding or because they were applicable to only one particular taxon. Working one's way through lists of methods, it is of primary interest to understand at which level these methods carry information and to realize their technical complexity, i.e., the amount of time and work required. The taxonomic information level of some of these techniques is illustrated in Fig. 2. Obviously,typing methods such as restriction enzyme patterning, multilocus enzyme electrophoresis, and serological analysis are not useful for phylogenetic studies, whereas rRNA or protein sequencing is, in general, not adequate to type large numbers of strains. Chemotaxonomic methods such as fatty acid analysis are fast methods, which allow us to compare and group large numbers of strains in a minimal period, whereas DNA-DNA hybridization studies, for example, will be restricted to a minimal but representative set of strains.The list of methods given below is not meant to be complete or to contain a description of all of their aspects. It comprises the major categories of taxonomic techniques required to study bacteria at different taxonomic levels and will roughly describe their general concept and applications.

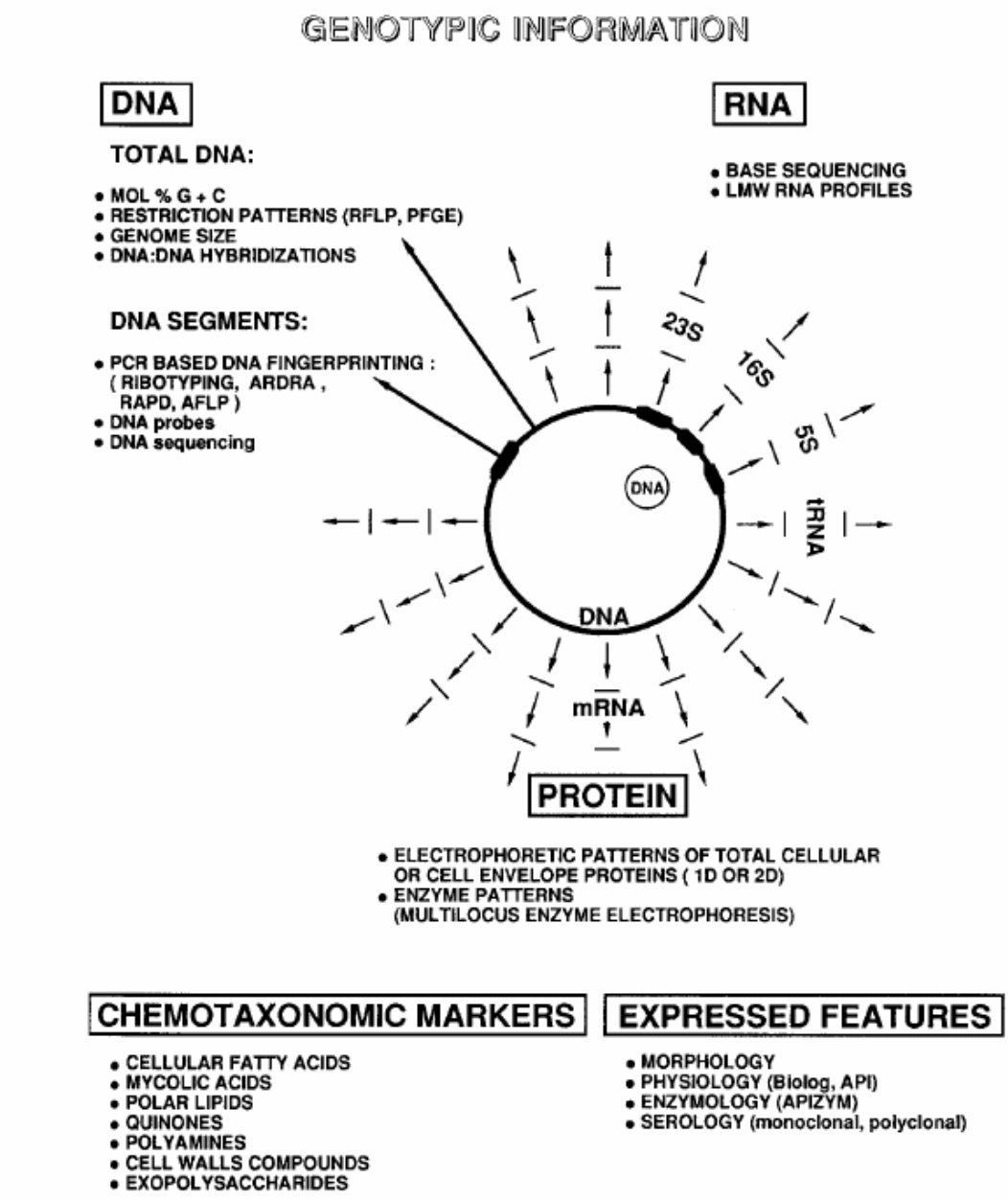

Fig: 1 Schematic overview of various cellular components and techniques used

4.2.2. GENOTYPIC METHODS

Genotypic methods are those that are directed toward DNA or RNA molecules. Undoubtedly, these methods presently dominate modern taxonomic studies as a consequence of technological progress, but primarily because our present view on classification is that it should reflect the natural relationships as encoded in the DNA. In fact, we are only substantiating our own dogma.

4.2.2.1. DETERMINATION OF THE DNA BASE RATIO (MOLES PERCENT G+C)

Determination of the moles percent guanosine plus cytosine is one of the classical genotypic methods and is considered part of the standard description of bacterial taxa. Generally, the range observed is not more than 3% within a well-defined species and not more than 10% within a well-defined genus. It varies between 24 and 76% in the bacterial world.

4.2.2.2. DNA-DNA HYBRIDIZATION STUDIES

The percent DNA-DNA hybridization and the decrease in thermal stability of the hybrid are used to delineate species. The percent DNA binding or the DNA-DNA hybridization value or the relative binding ratio is an indirect parameter of the sequence similarity between two entire genomes. It has been established that thermal stabilities decrease from 1 to 2.2% for each 1% of mispairing . It is, however, highly debatable whether data which were obtained with short oligonucleotides and experimentally induced mispairing can be extrapolated to entire genomes. At present, it therefore remains impossible to convert a percent DNA-binding or DNA-DNA hybridization value into a percentage of whole-genome sequence similarity. Different methods have been described: the hydroxyapatite method, the optical renaturation rates method, and the S1 nuclease method are the most common.

The advantage of the optical renaturation rates method is that the DNA needs no label, but it has the inconvenience of not allowing ΔTm *determinations* and of being taxonomically insignificant below approximately 30%. The hydroxyapatite method and the two procedures of the S1 nuclease method allow determination of the ΔTm and have been compared. It has been shown that the results obtained by these methods give different relative binding ratios but similar ΔTm values. The most similar relative binding ratios were obtained by the S1-DE81 procedure and the hydroxyapatite method when hybridized at 75°C.

These classical techniques, however, need considerable amounts of DNA and are time consuming. New, quick methods consuming less DNA have been described and are promising to replace the classical methods provided that they are further compared with them under strictly comparable conditions. Indeed, a large number of DNA-DNA hybridization protocols have been described, and it is often not clear whether if hybridizations are performed under optimal, stringent, or suboptimal conditions. The stringency of the reaction is determined by the salt and formamide concentrations and by the temperature and the moles percent G+C of the DNAs used. DNA-DNA hybridizations are often performed under standard conditions that are not necessarily optimal or stringent for all bacterial DNAs. Generally, optimal conditions for hybridizations are preferred, because the optimal temperature curve for hybridization is rather broad (about 5°C). As a rule, renaturation or hybridization under optimal conditions requires a temperature of 22 to 26°C (mean, 24°C) below the melting temperature, measured or calculated at equal salt concentration. The melting temperature (*Tm*) can be calculated from the salt concentration and the DNA base ratio by using different equations. The melting temperature and the optimal hybridization temperature decrease by 0.6°C for each percentage of formamide added to the melting or hybridization mixture.

4.2.2.3. rRNA HOMOLOGY STUDIES

It is now generally accepted that rRNA is the best target for studying phylogenetic relationships because it is present in all bacteria, is functionally constant, and is composed of highly conserved as well as more variable domains . The components of the ribosome (rRNA and ribosomal proteins) have been the subject of different phylogenetic studies for several decades. The gradual development of new molecular techniques enabled the microbiologist to focus on the comparative study of the rRNA molecules. Indirect comparison by either hybridization studies or rRNA cataloging of RNase T1-resistant oligonucleotides of 16S rRNA,) have already revealed the natural relationships with and within a number of bacterial lineages. Later, sequencing of the rRNA molecules gradually resulted in an rRNA sequence database of 5S rRNA, which was the first rRNA molecule to be sequenced for numerous bacteria because of its less complex primary and secondary structures. A limited number of 16S rRNA gene sequences became available by direct sequencing after cloning of the genes from the bulk of the DNA. Sequencing of 16S rRNA with conserved primers and reverse transcriptase was a very important advance in bacterial phylogeny and resulted in a spectacular increase in 16S rRNA sequences. Nowadays, these techniques have mostly been replaced by direct sequencing of parts or nearly entire 16S or 23S rDNA molecules by using the PCR technique and a selection of appropriate primers. They provide a phylogenetic framework which serves as the backbone for modern microbial taxonomy.

The results obtained and the dendrograms constructed with data obtained from the above methods are more or less equivalent, taking into account the specific resolution of each method. However, it is obvious that the larger the conserved elements, the more information they bear and the more reliable the conclusions become. The cataloging method and the DNA-rRNA hybridization experiments have gradually disappeared, although the latter method had the important advantage that multiple strains could easily be included. International databases comprising all published and some unpublished partial or complete sequences have been constructed

.

4.2.2.4. DNA-BASED TYPING METHODS

DNA-based typing methods generally refer to techniques which allow us to subdivide species into a number of distinct types. Classically, subtyping of species was performed by means of phenotypic analysis such as biochemical (hence biotyping) or serological (hence serotyping) tests, antibiotic susceptibility patterning, phage or bacteriocin typing, and many others. During the last few years, a battery of DNA-directed typing methods has been developed. Ideally, these techniques are universally applicable (The number of nontypeable strains, if any, is mostly small), they are reproducible and simple to perform, and they are highly discriminatory. Although several of the techniques listed below do not conform to this general description, genotyping has replaced classical typing in many laboratories and will most probably continue to do so.

The first-generation DNA-based typing methods included whole-genome restriction fragment analysis and plasmid DNA analysis. In the former, whole-genome DNA is extracted and digested with restriction enzymes. The resulting array of DNA fragments is separated and visualized by agarose gel electrophoresis, and restriction fragment length polymorphisms are established. The technique has the disadvantage those often very complex patterns of DNA fragments are generated, which are very difficult to compare. The disadvantages of plasmid

analysis are obvious. Strains do not always contain or keep their plasmids, and most strains often belong to only a few types. Restriction fragment analysis of plasmids combines the two techniques and generates more simple banding patterns; it is necessary to establish the identity of plasmids with equal molecular weight. Methods that were elaborated subsequently have reduced the number of DNA fragments compared with the former method and enhanced the reliability and discriminatory power. The number of DNA fragments can be reduced by selecting restriction enzymes which only rarely cut DNA, recognizing a specific combination of six to eight bases. The technique is referred to as low-frequency restriction fragment analysis. The fragments, however, are too large to be separated by conventional agarose gel electrophoresis.

The technique of low-frequency restriction fragment analysis has therefore been dependent on the development of special electrophoretic techniques, generally known as pulsed-field gel electrophoresis, and is now often considered to be the most discriminatory DNA-based typing method. Alternatively, the complex DNA patterns generated after restriction enzyme digestion can be transferred to a membrane and then hybridized with a labeled probe, which allows us to reveal the hybridized fragments. A typical example of one of these developments is the ribotyping method, which uses rRNA as probe. Since its initial description, many ariants have been presented, but the general principle has remained the same. The rRNA probe may vary in both the labeling technique and sequence. For example, 16S or 23S rRNA or both, with or without the spacer region, or a conserved oligonucleotide part of the rRNA can be used. Also, DNA sequences corresponding to elongation factor Tu, ribosomal protein S12, and flagellar proteins have all been used as probes.

The introduction of the PCR methodology into the microbiology laboratory has opened a vast array of applications. Among others, a battery of different typing methods was developed. PCR-based DNA-typing methods attracted much interest because of their universal applicability, simplicity, and rapidity. Different methods in which short arbitrary sequences were used as primers in the PCR assay were described: oligonucleotides of about 20 bases are used in arbitrarily primed PCR; oligonucleotides of about 10 bases are used in randomly amplified polymorphic DNA analysis; and oligonucleotides of about 5 bases are used in DNA-amplified fingerprinting. Alternatively, consensus motifs complementary to fragments of repetitive elements dispersed throughout the genomes of gram-positive or gram-negative bacteria or to tRNA gene fragments may be used as primers. The latter PCR-based method was reported to allow differentiation at the species and infraspecific levels depending on the stringency of the PCR conditions. PCR assays have also been used to amplify the rDNA genes (with or without spacer regions) by means of universal rDNA primers. The polymorphisms between the different rRNA operons generate simple arrays of DNA fragments with different lengths.

PCR-based DNA typing was combined with restriction enzyme analysis in the so-called amplified-rDNA restriction analysis method. The PCR product, being 16S or 23S rDNA or parts of both genes with or without the spacer region, is amplified by using universal primers located in the conserved regions of the rRNA genes. The amplicon is subsequently digested with a selected combination of restriction enzymes. In contrast to most other DNA-based methods, amplified-rDNA restriction analysis generates mostly species-specific patterns, which is not unexpected considering the conserved character of the rRNA genes. Another combination of the PCR and restriction enzyme methodologies yielded the AFLP (amplified fragment length polymorphism) technique. The basic principle of AFLP is restriction fragment length polymorphism analysis but with a PCR-mediated amplification to

select particular DNA fragments from the pool of restriction fragments. AFLP screens for amplified fragment length polymorphisms by selective amplification of restriction fragments. The restriction is performed by using two restriction enzymes, which yield DNA fragments with two different types of sticky ends, combined randomly. To these ends, short oligonucleotides (adapters) are ligated to form templates for the PCR. The selective amplification reaction is performed by using two different primers, containing the same sequence as the adapters but extended to include one or more selective bases next to the restriction site of the primer. Only fragments which completely match the primer sequence are amplified. The amplification process results in an array of about 30 to 40 DNA fragments, some of which are group specific while others are strain specific. The technique can therefore be used simultaneously for identification purposes and typing purposes. Apart from the application of tRNA sequences in the PCR based typing methods mentioned above; the tRNA gene pool can be used in a so-called low-molecular-weight RNA profiling method. These fingerprints comprise the 5S rRNA and the total tRNA pool, which appear on one-dimensional gels as a set of bands belonging to three different classes . The rRNA fraction of the profiles allows us to discriminate between some of the major eubacterial groups, while the tRNA Fraction reveals more specific taxonomic information.

4.2.3. PHENOTYPIC METHODS

Phenotypic methods comprise all those that are not directed toward DNA or RNA; therefore, they also include the chemotaxonomic techniques. As the introduction of chemotaxonomy is generally considered one of the essential milestones in the development of modern bacterial classification, it is often treated as a separate unit in taxonomic reviews. The term “chemotaxonomy” refers to the application of analytical methods to collect information on various chemical constituents of the cell to classify bacteria. As for the other phenotypic and the genotypic techniques, some of the chemotaxonomic methods have been widely applied on vast numbers of bacteria whereas others were so specific that their application was restricted to particular taxa.

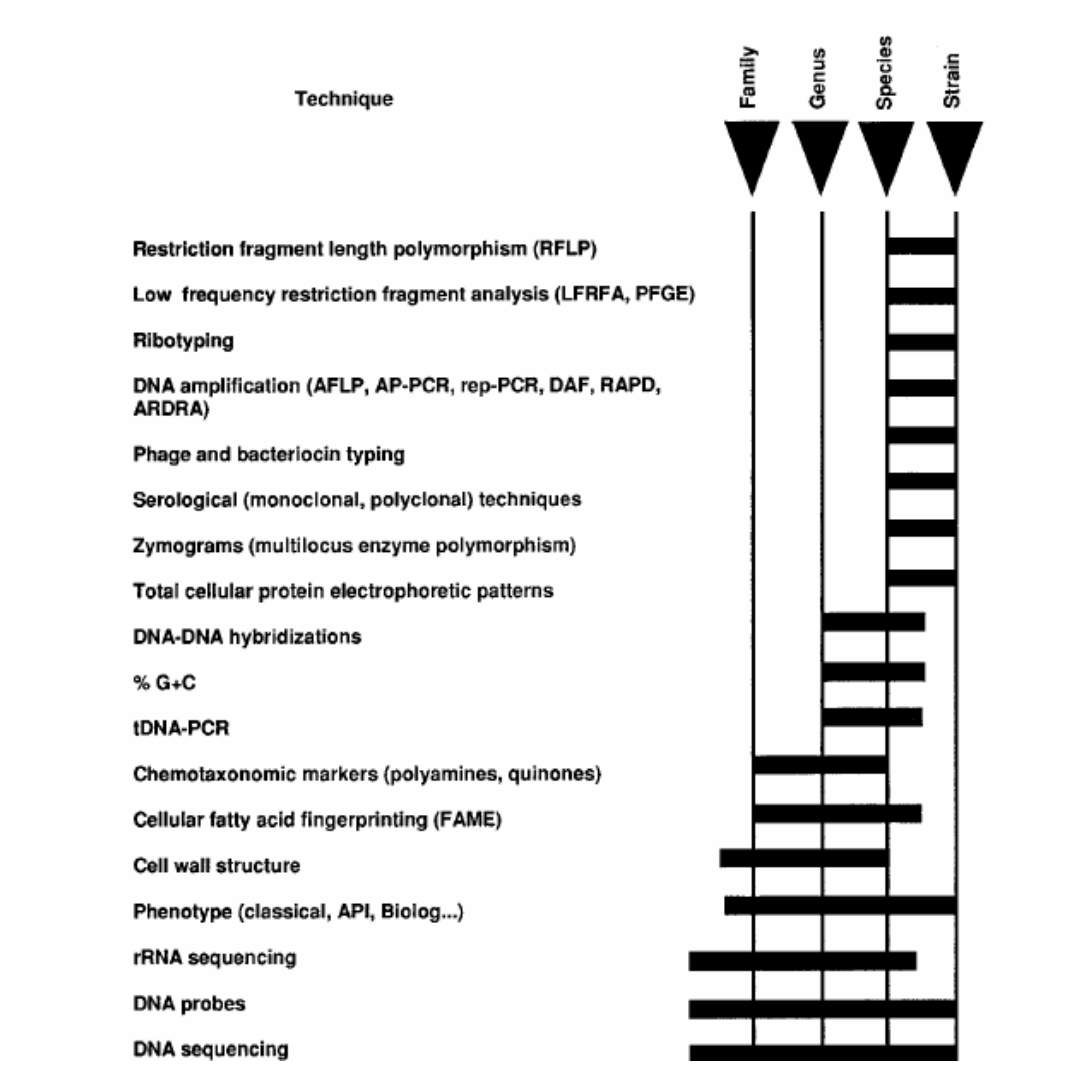

Fig.2 TAXONOMIC RESOLUTION OF SOME OF THE CURRENTLY USED TECHNIQUES

4.2.3.1. CLASSICAL PHENOTYPIC ANALYSIS

The classical or traditional phenotypic tests are used in identification schemes in the majority of microbiology laboratories. They constitute the basis for the formal description of taxa, from species and subspecies up to genus and family. While genotypic data are used to allocate taxa on a phylogenetic tree and to draw the major borderlines in classification systems, phenotypic consistency is required to generate useful classification systems and may therefore influence the depth of a hierarchical line. The paucity of phenotypic characteristics in particular bacterial groups often causes problems in describing or differentiating taxa. A typical example concerns the genus *Campylobacter* and allied bacteria. For such bacteria, alternative chemotaxonomic or genotypic methods are often required to re-liably identify strains. In addition, the phenotype of endosymbionts or unculturable bacteria is beyond the reach of our present methods. The classical phenotypic characteristics of bacteria comprise morphological, physiological, and biochemical features. Individually, many of these characteristics have been shown to be irrelevant as parameters for genetic relatedness, yet as

a whole, they provide descriptive information enabling us to recognize taxa. The morphology of a bacterium includes both cellular (shape, endospore, flagella, inclusion bodies, Gram staining) and colonial (color, dimensions, form) characteristics. The physiological and biochemical features include data on growth at different temperatures, pH values, salt concentrations, or atmospheric conditions, growth in the presence of various substances such as antimicrobial agents, and data on the presence or activity of various enzymes, metabolization of compounds, etc. Very often, highly standardized procedures are required to obtain reproducible results within and between laboratories.

4.2.3.2. NUMERICAL ANALYSIS

Phenotypic data were the first to be analyzed by means of computer-assisted numerical comparison. In the 1950's, numerical taxonomy arose in parallel with the development of computers and allowed comparison of large numbers of phenotypic traits for large numbers of strains. Data matrices showing the degree of similarity between each pair of strains and cluster analysis resulting in dendrograms revealed a general picture of the phenotypic consistency of a particular group of strains. Obviously, such large numbers of data reflect a considerable amount of genotypic information, and it soon became evident, by comparing the results of such cluster analysis with those of other taxonomic approaches, that analysis of large numbers of phenotypic characteristics was indeed taxonomically relevant.

4.2.3.3. AUTOMATED SYSTEMS

Miniaturized phenotypic fingerprinting systems have been introduced and may be in the future replace classical phenotypic analysis. These systems mostly contain a battery of dehydrated reagents, and addition of a standardized inoculum initiates the reaction (growth, production of enzymatic activity, etc.). The results are interpreted as recommended by the manufacturer and are readily available with a minimal input of time. The outcome of a particular test with a commercial system is sometimes different from that with a classical procedure, but the same is often true for two classical procedures in the same test. Clearly, phenotypic tests must be performed under well-standardized conditions to obtain reproducible results.

4.2.3.4. TYPING METHODS

Many of the cellular compounds which belong to the bacterial phenotype have been used in typing systems to characterize strains at the intraspecific level. Simple biotyping systems were used which involved a number of tests yielding variable results within species. Serotyping is based on the presence of variability in the antigenic constituents of the cells. Structural components such as capsules, cell envelopes, flagella, or fimbriae and intracellular molecules or secretion products such as enzymes and toxins have all been used in serological studies. Antigens may be proteins or carbohydrates and may be thermostable or thermolabile. Different kinds of serological reactions, including simple precipitation or agglutination tests and reactions requiring one or more additional components, such as complement fixation tests, have been described. Many of the described typing techniques are suitable for only some organisms and are performed by only a few reference laboratories. As their application in taxonomy is restricted, they will not be discussed here. One of the phenotypic typing methods which is still used for various bacteria is multilocus enzyme electrophoresis. In this technique, native enzymes are electrophoretically separated and stained for enzyme activity and their mobilities are compared. The mobility is an indicator for the existence of multiple

polymorphisms of the encoding gene. Multilocus enzyme electrophoresis has been extensively used in population genetics, allowing us to establish the overall genetic relatedness of bacterial strains

4.2.3.5. CELL WALL COMPOSITION

Determination of the cell wall composition has traditionally been important in gram-positive bacteria. The peptidoglycan type of gram-negative bacteria is rather uniform and provides little information. Cell walls of gram-positive bacteria, in contrast, contain various peptidoglycan types, which may be genus or species specific. The procedure is time-consuming, although a rapid screening method has been proposed. Membrane-bound teichoic acid is present in all gram-positive species, whereas cell wall-bound teichoic acid is present in only some gram-positive species. Teichoic acids can easily be extracted and purified and can be analyzed by gas-liquid chromatography.

4.2.3.6. CELLULAR FATTY ACIDS

A variety of lipids are present in bacterial cells. Polar lipids are the major constituents of the lipid bilayer of bacterial membranes and have been studied frequently for classification and identification purposes. Other types of lipids, such as sphingophospholipids, occur in only a restricted number of taxa and were shown to be valuable within these groups. The lipopolysaccharides present in the outer membranes of gram-negative bacteria can be analyzed by gel electrophoresis, giving typical lipopolysaccharide ladder patterns which are interpreted as variants in the O-specific side chains. Fatty acids are the major constituents of lipids and lipopolysaccharides and have been used extensively for taxonomic purposes. More than 300 different chemical structures of fatty acids have been identified. The variability in chain length, double-bond position, and substituent groups has proven to be very useful for the characterization of bacterial taxa. Mostly, the total cellular fatty acid fraction is extracted, but particular fractions such as the polar lipids have also been analyzed . Cellular fatty acid methyl ester content is a stable parameter provided that highly standardized culture conditions are used. The method is cheap and rapid and has reached a high degree of automation.

4.2.3.7. ISOPRENOID QUINONES

Isoprenoid quinones occur in the cytoplasmic membranes of most prokaryotes and play important roles in electron transport, oxidative phosphorylation, and, possibly, active transport. Two major structural groups, the naphthoquinones and the benzoquinones, are distinguished. The former can be further subdivided into two main types, the phylloquinones, which occur less commonly in bacteria, and the menaquinones. The large variability of the side chains (differences in length, saturation, and hydrogenation) can be used to characterize bacteria at different taxonomic levels.

4.2.3.8. WHOLE-CELL PROTEIN ANALYSIS

The comparison of whole-cell protein patterns obtained by highly standardized sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has proven to be extremely reliable for comparing and grouping large numbers of closely related strains. Numerous studies have revealed a correlation between high similarity in whole-cell protein content and DNA-DNA hybridization. The use of SDS-PAGE for general identification

purposes is hampered by the fact that it yields only discriminative information at or below the species level.

POLYAMINES

Although the role of polyamines in the bacterial cell is not entirely clear, they seem to be important in bacterial metabolism. The observation of their universal character and quantitative and qualitative variability turned them into a suitable chemotaxonomic marker that can be determined by gas chromatography or high-performance liquid chromatography. Depending on the group of organisms studied, polyamine patterning has been used to trace relatedness at and above the genus level and at the species level.

4.2.3.9. PYROLYSIS MASS SPECTROMETRY, FOURIER TRANSFORMATION INFRARED SPECTROSCOPY, AND UV RESONANCE RAMAN SPECTROSCOPY

Pyrolysis mass spectrometry, Fourier transform infrared spectroscopy, and UV resonance Raman spectroscopy are sophisticated analytical techniques which examine the total chemical composition of bacterial cells. These methods have been used for taxonomic studies of particular groups of bacteria.

4.3. LET US SUM UP

- In polyphasic taxonomy all the genotypic, phenotypic, and phylogenetic information are taken into account for classifying the organisms
- In genotypic methods, DNA or RNA of the organisms are analyzed by various means
  - By Determining the base ratio of DNA
  - By studying the rRNA homology
  - DNA- DNA hybridization study
  - DNA based typing methods
- Phenotypic methods comprises all the techniques which are not directed towards genetic materials.
  - Classical phenotypic analysis- morphological, physiological, and biochemical features
  - Numerical analysis
  - Automated systems
  - Typing methods
  - Cell wall composition
  - Cellular fatty acids
  - Isoprenoid quinones
  - Whole-cell protein analysis
- Phylogenetic tree is drawn for grouping and to study the evolutionary relationship of the organisms
- It is widely used as it fulfills all the disadvantages and problems in taxonomy

4.4. LESSON END ACTIVITIES

Write down the features of polyphasic taxonomy.

4.5. POINTS FOR DISCUSSION

1. Significance of polyphasic taxonomy as a better classification method.
   Polyphasic taxonomy includes all the genotypic, phenotypic, and phylogenetic information into account for classifying the organisms; hence it's the most appropriate method for classifying organisms.
2. **Give the outline of criteria used in polyphasic taxonomy.**
   - By Determining the base ratio of DNA
   - By studying the rRNA homology
   - DNA- DNA hybridization study
   - DNA based typing methods
   - Classical phenotypic analysis- morphological, physiological, and biochemical features
   - Numerical analysis
   - Automated systems
   - Typing methods
   - Cell wall composition
   - Cellular fatty acids
   - Isoprenoid quinones
   - Whole-cell protein analysis

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UNIT II – MICROBIAL CELL BIOLOGY AND METABOLISM

CONTENTS

LESSON – 5

GENERAL ORGANIZATION OF BACTERIA, VIRUS AND ACTINOMYCETES

Contents

5.0. AIMS AND OBJECTIVES

The chapter deals with the general structural organization of bacteria, virus and actinomycetes.

5.1. INTRODUCTION

Ubiquitous in nature, unicellular, light microscopic - 0.2 - 2 µm in diameter; 2 - 8 µm in length, Prokaryotic in cell structure. All bacteria, both pathogenic and saprophytic, are unicellular organisms that reproduce by binary fission. Most bacteria are capable of independent metabolic existence and growth, but species of Chlamydia and Rickettsia are obligately intracellular organisms. Bacterial cells are extremely small and are most conveniently measured in microns (10-6 m). They range in size from large cells such as *Bacillus anthracis* (1.0 to 1.3 µm X 3 to 10 µm) to very small cells such as *Pasteurella tularensis* (0.2 X 0.2 to 0.7 µm) Mycoplasmas (atypical pneumonia group) are even smaller, measuring 0.1 to 0.2 µm in diameter. Bacteria therefore have a surface-to-volume ratio that is very high: about 100,000.

5.2. BACTERIA

Bacteria have characteristic shapes. The common microscopic morphologies are cocci (round or ellipsoidal cells, such as *Staphylococcus aureus* or *Streptococcus* respectively); rods, such as Bacillus and Clostridium species; long, filamentous branched cells, such as Actinomyces species; and comma-shaped and spiral cells, such as *Vibrio cholerae* and *Treponema pallidum*, respectively. The arrangement of cells is also typical of various species or groups of bacteria. Some rods or cocci characteristically grow in chains; some, such as *Staphylococcus aureus*, form grapelike clusters of spherical cells; some round cocci form cubic packets. Bacterial cells of other species grow separately. The microscopic appearance is therefore valuable in classification and diagnosis.

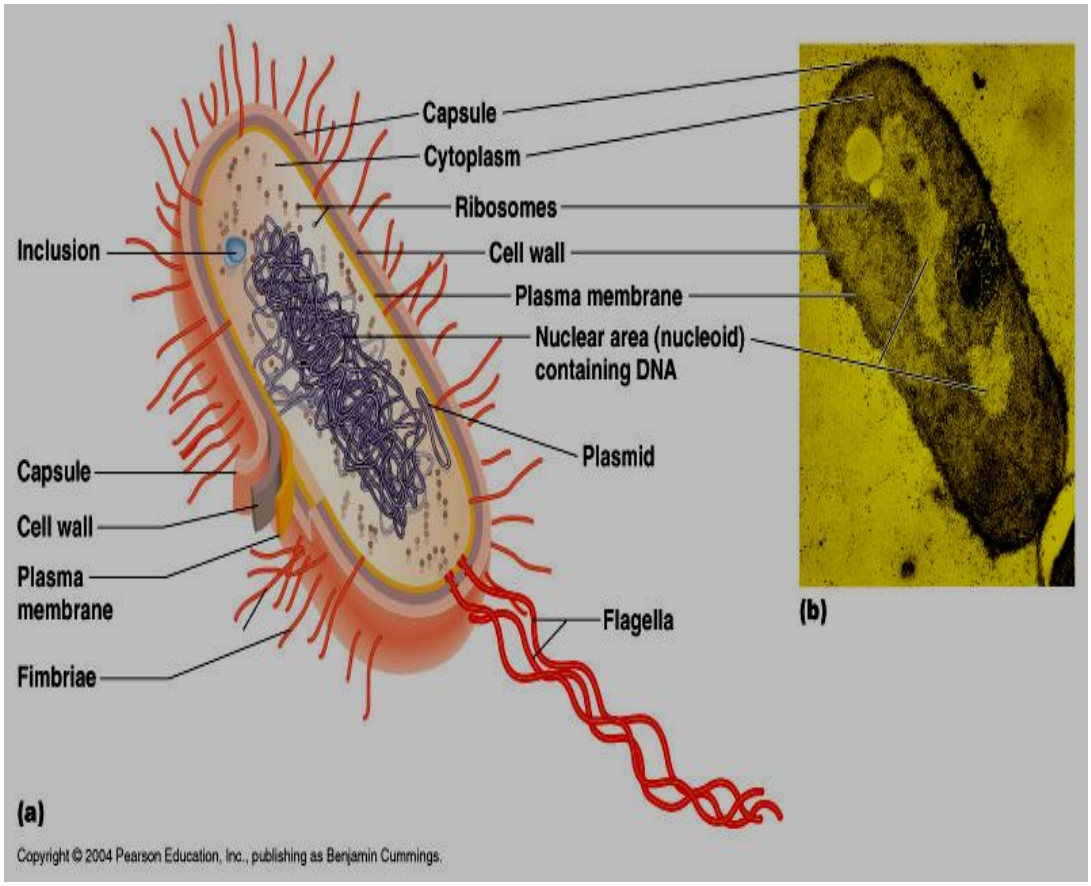

Fig.1 ANATOMY OF A TYPICAL BACTERIUM

Cell shapes & arrangements: They are established by cell wall but environmental conditions can change them.

Coccus (plural: cocci): cells that are spherical in shape.

Streptococcus: arranged in chains, like beads on a string.

Diplococcus: arranged in pairs. (e.g.: *Streptococcus pneumoniae*, *Neisseriae*)

*Staphylococcus***:** arranged like clusters of grapes. Random planes of division.

Tetrad: arranged in a group of 4, looking almost like a square under the microscope. 2 planes of division.

Sarcina (plural: sarcinae): arranged in a group of 8. Sarcinae look like small cubes and may be difficult to distinguish from tetrads. 3 planes of division.

Bacillus (plural: bacilli): rod-shaped cells. A single plane of division.

Diplobacillus: arranged in pairs.

Streptobacillus: arranged in end-to-end chains (e.g.: *Mycobacteria*, *Bacillus anthracis*)

Coryneform bacillus: arranged at angles to form V- and L-shaped arrangements (e.g. *Corynebacterium*)

Coccobacillus (e.g.: *Gardnella vaginalis*): intermediate to coccus and bacillus

Spiral-shaped & no arrangement.

Spirillum (plural: spirilla): rigid

Spirocheta: flexible

Vibrio: a bacterium with curved or comma-shaped cells. No arrangement.

Pleiomorphic: do not display a constant shape even during growth in an otherwise unchanging, homogeneous environment

Fruiting body: macroscopic reproductive structure produced by some bacteria, including *Myxobacteria*. Fruiting bodies are distinctive in size, shape, and coloration for each species.

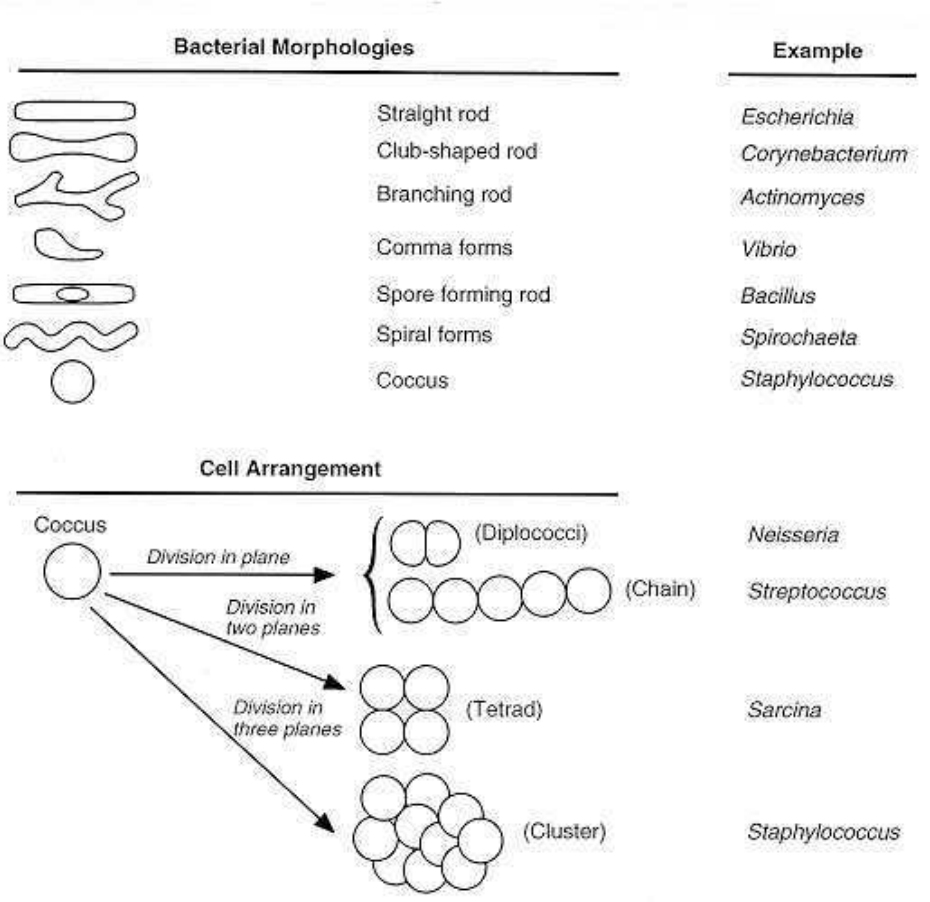

Fig.2 MORPHOLOGIES OF BACTERIA

5.3. VIRUS

Viruses do not fall strictly into the category of unicellular microorganisms as they do not posses a cellular organization. Viruses do not have a cellular organization and contain only one type of nucleic acid either DNA or RNA. They are obligate intracellular parasites. They lack the enzymes necessary for protein and nucleic acid synthesis and are dependent for replication on the synthetic machinery of host cells.

The extracellular infectious virus particle is called virion. The virion consists essentially of a nucleic acid surrounded by a protein coat, the capsid. The capsid with the enclosed nucleic acid is known as the nucleocapsid. The function of capsid is to protect the nucleic acid from inactivation by nucleases and other deleterious agents in the environment. The capsid is composed of a large number of capsomers which form its morphological units. The chemical units of the capsid are polypeptide molecules which are arranged symmetrically to form an impenetrable shell around the nucleic acid core. One of the major functions of the capsid is to introduce viral genome into host cells by adsorbing readily to the cell surfaces.

Two kinds of symmetry are encountered in the capsid, icosahedral and helical. An icosahedron is a polygon with 12 vertices or corners and 20 facets or sides. Each facet is in the shape of an equilateral triangle. Two types of capsomers constitute the icosahedral capsid. They are the pentagonal capsomers making up the facets. There are always 12 pentons but the number of hexons varies with the virus group. In the nucleocapsids with helical symmetry, the capsomers and nucleic acid are wound together to form a helical or spiral tube. The tube my be rigid, as in tobacco mosaic virus but in the case of animal viruses, the tubular nucleocapsid is pliable and may be coiled on itself. Not all viruses show the typical icosahedral or helical symmetry. Some, like the poxviruses, exhibit complexes symmetry.

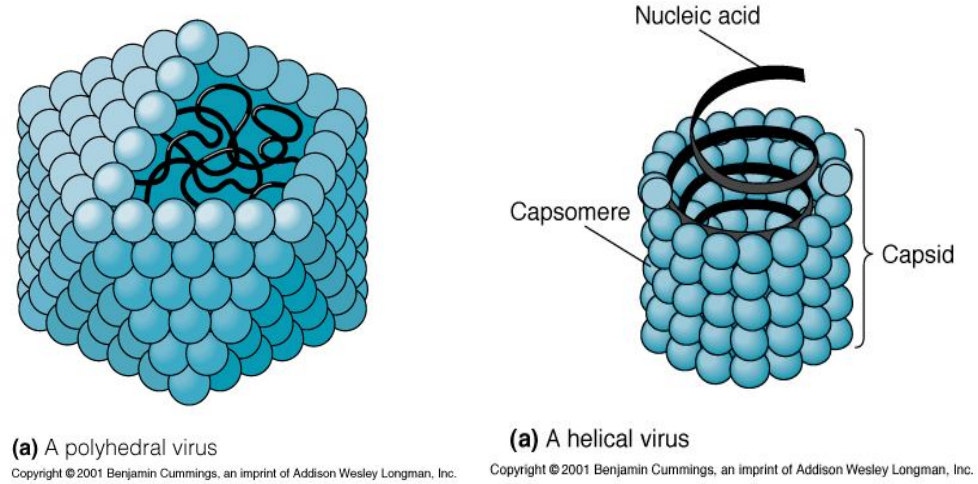

Fig.3 STRUCTURE OF POLYHEDRAL AND HELICAL VIRUS

Virions may be enveloped or non enveloped. The envelope or outer covering of viruses is derived from the host cell membrane when the progeny virus is released by budding. The envelope is lipoprotein in nature. The lipid is largely of host cell origin while the protein is virus coded. Protein subunits may be seen as projecting spikes on the surface of the envelope. These structures are called peplomers. A virus may have more than one type of peplomer. The influenza virus carries two kinds of peplomers – the hemagglutinin which is triangular spike and the neuraminidase which is a mushroom shaped structure. Envelopes confer chemical, antigenic and biological properties on viruses. Enveloped viruses are

susceptible to the action of lipid solvents like ether, chloroform and bile salts. Specific neutralization of virus infectivity depends on antibodies to the surface antigens. Biological properties such as attachment to host cell surface or hemagglutination depend on the envelope. Fibrils protrude from the vertices in case of adenovirus. Most animal viruses are roughly spherical. Some are irregular and pleomorphic. The rabies virus is bullet shaped, Elbovirus filamentous and poxviruses are brick shaped; the tobacco mosaic virus is rod shaped.

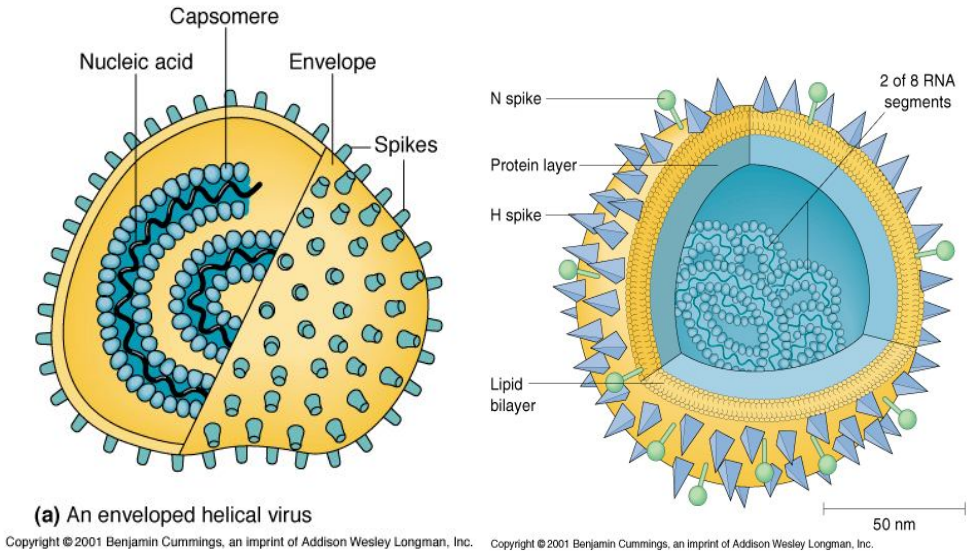

Fig.4 STRUCTURE OF ENVELOPED VIRUS

Bacterial viruses have a complex morphology.

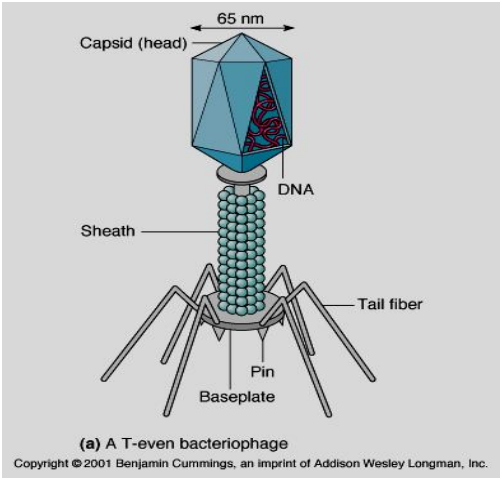

Fig.5 STRUCUTURE OF BACTERIOPHAGE

5.4. ACTINOMYCETES

Actinomycetes are traditionally considered to be transitional forms between bacteria and fungi. Like fungi they form a mycelial network of branching filaments but, like bacteria, they are thin, possess cell walls containing muramic acid, have prokaryotic nuclei and are susceptible to antibacterial antibiotics. They are therefore true bacteria, bearing a superficial resemblance to fungi. Actinomycetes are related to mycobacteria and corynebacteria. They are Gram positive, non motile, non sporing, non capsulated filaments that break up into

bacillary and coccoid elements. Most are free-living, particularly in the soil. Actinomycetes include many genera of medical interest such as the anaerobic *Actinomyces, Arachnia, Bifidobacterium, Rothia* and aerobic *Nocardia, Actinomadura, Dermatophilus* and *Streptomyces.*

5.5. LET US SUM UP

- Bacteria, virus and actinomycetes are microscopic in structure having their own structural organizations unlike eukaryotes.
- Bacteria have numerous structures like coccoid, rods, and coccobacillus and are classified into various groups based on their morphology.
- Virus has a genetic material and an outer envelope as their general structural organization.
- Actinomycetes are filamentous bacteria having similarities to bacteria in their structure and other characteristic features.

5.6. LESSON END ACTIVITIES

Take small quantity of curd and try to isolate microorganisms from the sample.
Observe for the various forms of bacteria from the curd.
What are the forms of bacteria?
Describe the structure and forms of virus
What are actinomycetes? How they are different from other microorganisms

5.7. POINTS FOR DISCUSSION

Observe the general structures of bacteria and write a note on them.
Explain the difference between bacteria and actinomycetes.

5.8 REFERENCES

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LESSON – 6

MOLECULAR ARCHITECHTURE OF MICROORGANISM

Contents

6.0. AIMS AND OBJECTIVES

The chapter deals with the various inner and outer structures of bacteria in detail.

6.1. INTRODUCTION

In higher organisms there is a distinction between physiology and biochemistry. Physiology is the study of the relation between structure and function, e.g. how does the mammalian intestine fulfill its function of digesting food? Biochemistry is the study of life processes at the molecular level. Because bacteria are small, single-celled organisms with no distinct organs or tissues there is no valid distinction between anatomy, physiology, and biochemistry. For example, the cell wall of most bacteria consists of a single peptidoglycan molecule and bacterial nutrition involves uptake of discrete molecules, not large lumps of food.

6.2. PROKARYOTES VERSUS EUKARYOTES

Living cells are divided into two major types - PROKARYOTES and EUKARYOTES, on the basis of their genetic organization. Higher organisms together with microorganisms such as fungi, algae and protozoa are eukaryotes. Bacteria (including the blue green "algae") are prokaryotes and comprise the subject of this course.

In eukaryotes the genetic information is separated from the rest of the cell in a membrane bound compartment - the nucleus. In prokaryotes the DNA is free in the cytoplasm. The presence or absence of a separate nucleus defines the difference between the prokaryotic and eukaryotic cell. The presence of a separate nucleus allows eukaryotes to accumulate much more DNA per cell than prokaryotes. Consequently eukaryotic cells are usually much larger than prokaryotes. In addition the DNA of eukaryotes is generally partitioned among several chromosomes. The greater genetic complexity of eukaryotes facilitates the differentiation of cells into the complex tissues and organs seen in higher organisms.

The presence of a nucleus brings problems as well as advantages. The major problem is to divide the genome equally upon cell division. This is performed by the complex process of mitosis. Mitosis involves the temporary dissolution of the nuclear membrane and the creation of the mitotic spindle. The spindle is made up of microtubules projecting from structures known as centrioles. Between cell divisions, microtubules, with associated microfilaments, form an internal scaffolding system for the eukaryotic cell - the cytoskeleton. The centrioles also function as the basal bodies of eukaryotic flagella and cilia.

Eukaryotic cells contain several membrane-bound organelles and membrane systems. By increasing the surface to volume ratio, these allow eukaryotic cells to be larger. Note that growth of a cell is limited by the surface area available to take up oxygen, nutrients, etc. [Many bacteria have infoldings of the cell membrane known as mesosomes. These may be involved in cell division, though they are poorly characterized and have had almost every possible function attributed to them at some time.] The two most prominent organelles of eukaryotes are the mitochondria and chloroplasts. Both possess their own DNA, their own ribosomes, which are of the prokaryotic 70S type, and are in several respects semi-autonomous. New mitochondria and chloroplasts arise only by the division of pre-existing mitochondria or chloroplasts. Thus these organelles are in some ways equivalent to prokaryotic cells. The endosymbiont theory proposes that mitochondria and chloroplasts evolved from symbiotic bacteria which were trapped inside larger eukaryotic cells and lost their independence.

CHARACTER	PROKARYOTES	EUKARYOTES
a) Size	1-10 microns	10-100 microns
b) Complexity	unicellular, rarely small clusters or filaments	sometimes unicellular more often multicellular
c) Membrane bound organelles	none (mesosome is infolding of cytoplasmic membrane)	nuclei, mitochondria, chloroplasts, lysosomes, endoplasmic reticulum, golgi, & vacuoles
d) Nucleus	no	yes

e) Chromosomes	single & circular	usually several & linear
f) Introns & Exons	occasionally	frequent
g) Histones	no	yes
h) Ploidy	haploid	diploid
i) Mitosis & Meiosis	absent	present
j) Sexual reproduction	none, or unidirectional from donor to recipient	usually, involves fusion of haploid gametes
k) Ribosomes	70s (50s + 30s subunits)	80s (60s + 40s) in cytoplasm (mitochondria & chloroplasts have prokaryotic ribosomes)
l) Cytoskeleton	absent	microtubules and microfilaments
m) Cell wall	usually present, contains peptidoglycan	absent in animals present in fungi (chitin) & plants (cellulose)
n) Motility	simple, prokaryotic, flagella, gliding motion	complex "9+2" flagella or cilia with centrioles
o) Endocytosis & cytoplasmic streaming	absent	present
p) Differentiation	usually absent	cells differentiate to form tissues & organs
q) Energy metabolism	many diverse pathways in various bacteria	glycolysis in cytoplasm, Krebs Cycle and ETC in mitochondria
r) Oxygen	aerobic and/or anaerobic	usually aerobic
s) Sterols	usually absent	used as hormones and in plasma membrane

TABLE 1. STRUCTURAL COMPARISON OF PROKARYOTES AND EUKARYOTES

Differentiation is mostly confined to eukaryotes; however, there are a few prokaryotic examples. Some blue-green bacteria form filaments in which certain cells are specialized to fix nitrogen. These differentiated cells are called heterocysts. Differentiation is also seen in *Caulobacter* and *Hyphomicrobium.*, both of which have cell cycles in which two cell types alternate: motile, with flagella, and non-motile, without flagella.

While eukaryotes show much greater cellular differentiation and structural diversity than prokaryotes, the converse is true of metabolism. Eukaryotes are very stereotyped metabolically. Prokaryotes are diverse biochemically and possess many pathways not found in eukaryotes, e.g. nitrogen fixation, methane production and anaerobic respiration.

6.3. STRUCTURE OF BACTERIA

Since bacteria contain no internal organelles except ribosomes and the bacterial chromosome, the structure of the bacterial cell is largely the structure of its surrounding layer - the cell envelope, together with the appendages (flagella and pili) which project outwards from this layer. There are two major forms of cell envelope structure, gram-positive and gram-negative. Originally these two classes of bacteria were recognized by a staining reaction. However, this difference in staining is due to fundamental differences in envelope structure and chemical composition.

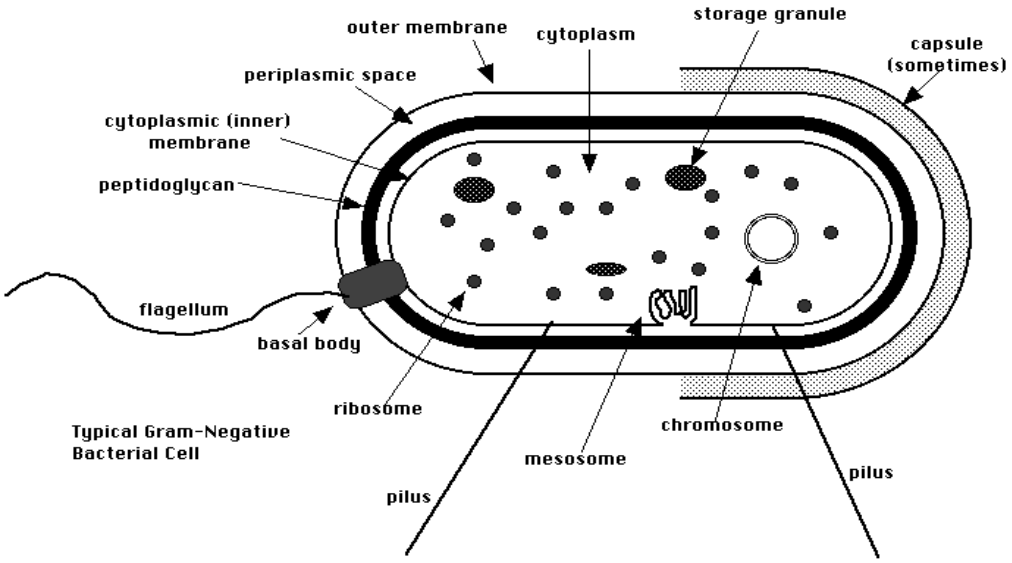


Fig. 1 GENERAL STRUCTURE OF BACTERIA

6.4. CELL WALL

Gram-positive envelope: Thick cell wall with multiple peptidoglycan layers. No outer membrane, periplasmic space or lipopolysaccharide. They possess long chain teichoic acids intertwined among the peptidoglycan (up to 90% peptidoglycan).
Gram-negative envelope: Single thin peptidoglycan layer, but no teichoic acids. Possess an outer membrane containing lipopolysaccharide and a periplasmic space between the outer and inner membranes.

6.4.1 CELL WALL AND GRAM-NEGATIVE CELL ENVELOPE

The Gram stain broadly differentiates bacteria into Gram-positive and Gram-negative groups; a few organisms are consistently Gram-variable. Gram-positive and Gram-negative organisms differ drastically in the organization of the structures outside the plasma membrane but below the capsule: in Gram-negative organisms these structures constitute the cell envelope, whereas in Gram-positive organisms they are called cell wall.

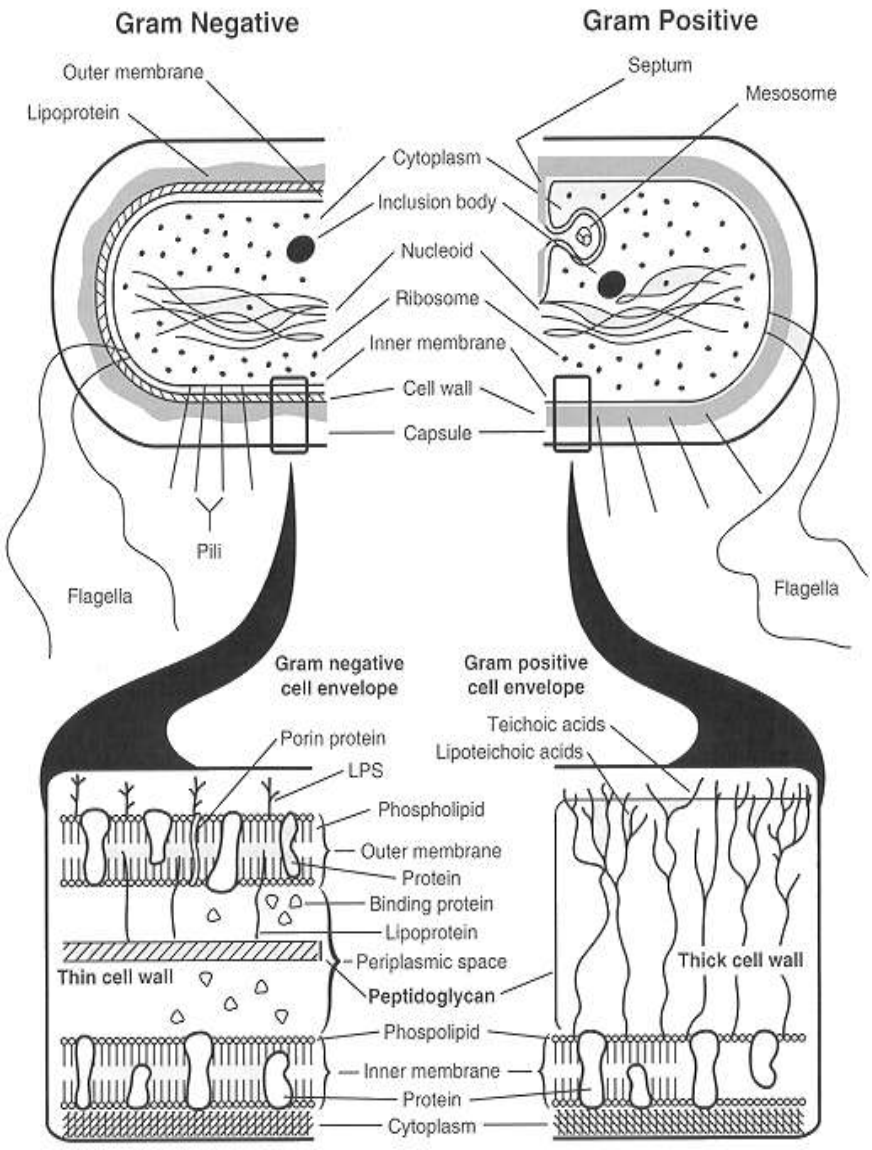


Fig.2 COMPARISON OF THE THICK CELL WALL OF GRAM-POSITIVE BACTERIA WITH THE COMPARATIVELY THIN CELL WALL OF GRAM-NEGATIVE BACTERIA.

Note the complexity of the Gram-negative cell envelope (outer membrane, its hydrophobic lipoprotein anchor; periplasmic space).

Most Gram-positive bacteria have a relatively thick (about 20 to 80 nm), continuous cell wall (often called the sacculus), which is composed largely of peptidoglycan (also known as mucopeptide or murein). In thick cell walls, other cell wall polymers (such as the teichoic acids, polysaccharides, and peptidoglycolipids) are covalently attached to the peptidoglycan. In contrast, the peptidoglycan layer in Gram-negative bacteria is thin (about 5 to 10 nm thick); in *E.coli*, the peptidoglycan is probably only a monolayer thick. Outside the peptidoglycan layer in the Gram-negative envelope is an outer membrane structure (about 7.5 to 10 nm thick). In most Gram-negative bacteria, this membrane structure is anchored

noncovalently to lipoprotein molecules (Braun's lipoprotein), which, in turn, are covalently linked to the peptidoglycan. The lipopolysaccharides of the Gram-negative cell envelope form part of the outer leaflet of the outer membrane structure.

The organization and overall dimensions of the outer membrane of the Gram-negative cell envelope are similar to those of the plasma membrane (about 7.5 nm thick). Moreover, in Gram-negative bacteria such as *E.coli*, the outer and inner membranes adhere to each other at several hundred sites (Bayer patches); these sites can break up the continuity of the peptidoglycan layer. Table 2 summarizes the major classes of chemical constituents in the walls and envelopes of Gram-positive and Gram-negative bacteria.

Chemical component	Examples
Gram positive cell walls	
Peptidoglycan	All species
Polysaccharides	*Streptococcus* group A,B,C substances
Techoic acid	
Ribitol	*S.aureus, B.subtilis, Lactobacillus sp.*
Glycerol	*S.epidermis, Lactobacillus sp.*
Teichuronic acids	*B.licheniformis, M.lysodeikticus*
Peptidoglycolipids	*Corynebacterium sp., Mycobacterium sp., Nocardia sp.*
Glycolipids	*Corynebacterium sp., Mycobacterium sp., Nocardia sp.*
Gram negative envelopes	
LPS	All species
Lipoprotein	*E.coli,*and many enteric bacteria, *Pseudomonas aeruginosa*
Porins	*E.coli, S.typhimurium*
Phospholipids and proteins	All species
Peptidoglycan	Almost all species

TABLE.2. MAJOR CLASSES OF CHEMICAL COMPONENTS IN BACTERIAL WALLS AND ENVELOPES

The basic differences in surface structures of Gram-positive and Gram-negative bacteria explain the results of Gram staining. Both Gram-positive and Gram-negative bacteria take up the same amounts of crystal violet (CV) and iodine (I). The CV-I complex, however, is trapped inside the Gram-positive cell by the dehydration and reduced porosity of the thick cell wall as a result of the differential washing step with 95 percent ethanol or other solvent mixture. In contrast, the thin peptidoglycan layer and probable discontinuities at the membrane adhesion sites do not impede solvent extraction of the CV-I complex from the Gram-negative cell. The above mechanism of the Gram stain based on the structural differences between the two groups has been confirmed by sophisticated methods of electron microscopy. The sequence of steps in the Gram stain differentiation is illustrated diagrammatically in Figure 3. Moreover, mechanical disruption of the cell wall of Gram-positive organisms or its enzymatic removal with lysozyme results in complete extraction of the CV-I complex and conversion to a Gram-negative reaction. Therefore, autolytic wall-degrading enzymes that cause cell wall breakage may account for Gram-negative or variable

reactions in cultures of Gram-positive organisms (such as *Staphylococcus aureus, Clostridium perfringens, Corynebacterium diphtheriae,* and some Bacillus spp).

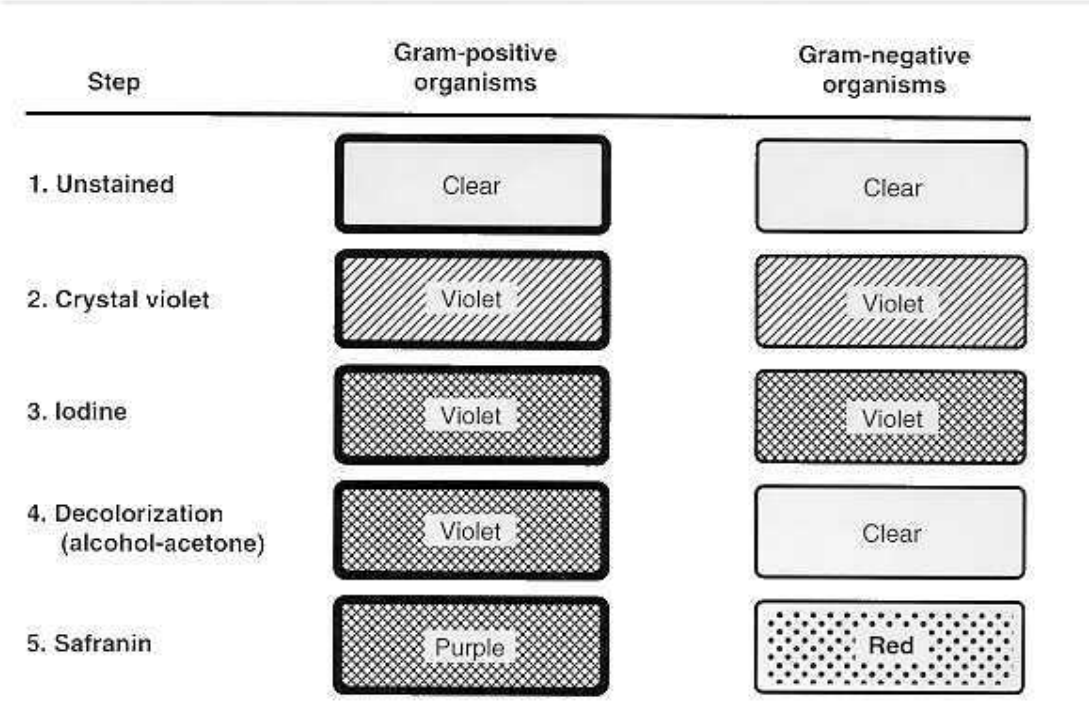


Fig. 3 GENERAL SEQUENCE OF STEPS IN THE GRAM STAIN PROCEDURE AND THE RESULTANT STAINING OF GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

6.5. PEPTIDOGLYCAN

Peptidoglycan (Also called mucopeptide or murein): Long chains of a polysaccharide consisting of alternate residues of N-acetyl-glucosamine (NAG) and N-acetyl-muramic acid (NAM) are crosslinked by short chains of amino acids. The amino acids side chains are attached to the NAM residues and some of the amino acids are in the D-configuration (i.e. opposite to that found in proteins). In *E. coli* and most gram-negative bacteria there is a single molecular layer of peptidoglycan. In *Staphylococcus aureus* and most gram-positives there are many layers of peptidoglycan forming a thick wall. In gram-positives some of the teichoic acid is covalently attached to the peptidoglycan. In gram-negatives the peptidoglycan is covalently attached to lipoprotein molecules which project into the outer membrane.

Unique features of almost all prokaryotic cells (except for Halobacterium halobium and mycoplasmas) are cell wall peptidoglycan and the specific enzymes involved in its biosynthesis. These enzymes are target sites for inhibition of peptidoglycan synthesis by specific antibiotics. The primary chemical structures of peptidoglycans of both Gram-positive and Gram-negative bacteria have been established; they consist of a glycan backbone of repeating groups of β1, 4-linked disaccharides of β 1,4-N-acetylmuramyl-N-acetylglucosamine. Tetrapeptides of L-alanine-D-isoglutamic acid-L-lysine (or diaminopimelic acid)-n-alanine are linked through the carboxyl group by amide linkage of

muramic acid residues of the glycan chains; the D-alanine residues are directly cross-linked to the e-amino group of lysine or diaminopimelic acid on a neighboring tetrapeptide, or they are linked by a peptide bridge. In *S. aureus* peptidoglycan, a glycine pentapeptide bridge links the two adjacent peptide structures. The extent of direct or peptide-bridge cross-linking varies from one peptidoglycan to another. The staphylococcal peptidoglycan is highly cross-linked, whereas that of *E coli* is much less so, and has a more open peptidoglycan mesh. The diamino acid providing the e-amino group for cross-linking is lysine or diaminopimelic acid, the latter being uniformly present in Gram-negative peptidoglycans. The structure of the peptidoglycan is illustrated in Figure 4. A peptidoglycan with a chemical structure substantially different from that of all eubacteria has been discovered in certain archaebacteria. Instead of muramic acid, this peptidoglycan contains talosaminuronic acid and lacks the D-amino acids found in the eubacterial peptidoglycans. Interestingly, organisms containing this wall polymer (referred to as pseudomurein) are insensitive to penicillin, an inhibitor of the transpeptidases involved in peptidoglycan biosynthesis in eubacteria.

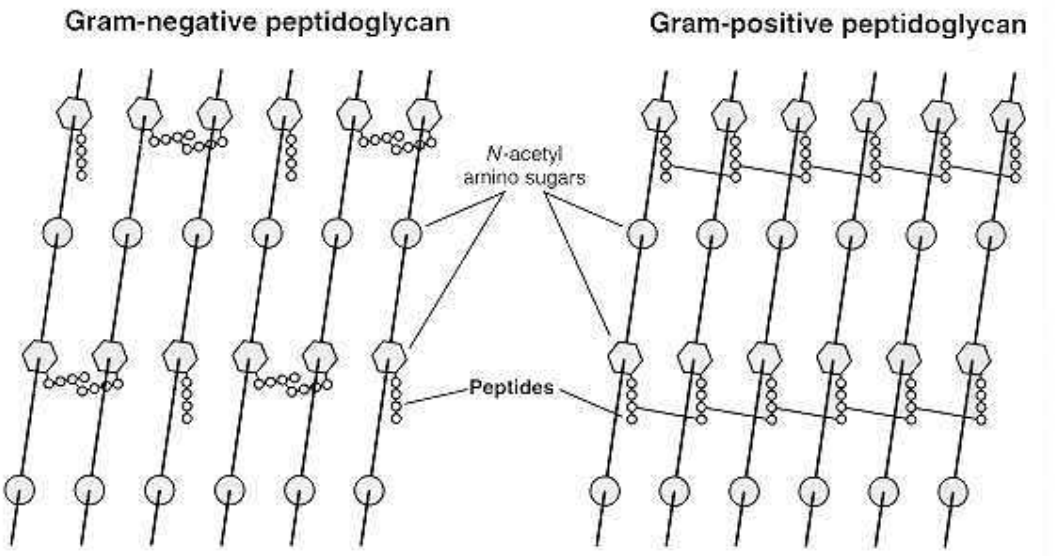


Fig.4 DIAGRAMMATIC REPRESENTATION OF PEPTIDOGLYCAN STRUCTURES

With adjacent glycan strands cross-linked directly from the carboxyterminal D-alanine to the e-amino group of an adjacent tetrapeptide or through a peptide cross bridge ,N-acetylmuramic acid; N-acetylglucosamine.

The ß-1,4 glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine is specifically cleaved by the bacteriolytic enzyme lysozyme. Widely distributed in nature, this enzyme is present in human tissues and secretions and can cause complete digestion of the peptidoglycan walls of sensitive organisms. When lysozyme is allowed to digest the cell wall of Gram-positive bacteria suspended in an osmotic stabilizer (such as sucrose), protoplasts are formed. These protoplasts are able to survive and continue to grow on suitable media in the wall-less state. Gram-negative bacteria treated similarly produce spheroplasts, which retain much of the outer membrane structure. The dependence of bacterial shape on the

peptidoglycan is shown by the transformation of rod-shaped bacteria to spherical protoplasts (spheroplasts) after enzymatic breakdown of the peptidoglycan. The mechanical protection afforded by the wall peptidoglycan layer is evident in the osmotic fragility of both protoplasts and spheroplasts. There are two groups of bacteria that lack the protective cell wall peptidoglycan structure, the Mycoplasma species, one of which causes typical pneumonia and some genitourinary tract infections and the L-forms, which originate from Gram-positive or Gram-negative bacteria and are so designated because of their discovery and description at the Lister Institute, London. The mycoplasmas and L-forms are all Gram-negative and insensitive to penicillin and are bounded by a surface membrane structure. L-forms arising "spontaneously" in cultures or isolated from infections are structurally related to protoplasts and spheroplasts; all three forms (protoplasts, spheroplasts, and L-forms) revert infrequently and only under special conditions.

6.6. TEICHOIC ACIDS

Teichoic acids are only found in gram-positive bacteria. They are polymers in which glycerol or ribitol residues alternate with phosphate groups. The glycerol or ribitol residues may carry amino acid and/or sugar substituents. Some teichoic acids are attached to the peptidoglycan and extend throughout the cell wall layer. Other teichoic acids are covalently attached to lipids in the cytoplasmic membrane - so called "lipoteichoic acids". They are immunogenic but their function is unclear.

Wall teichoic acids are found only in certain Gram-positive bacteria (such as staphylococci, streptococci, lactobacilli, and Bacillus spp); so far, they have not been found in gram- negative organisms. Teichoic acids are polyol phosphate polymers, with either ribitol or glycerol linked by phosphodiester bonds; their structures are illustrated in Figure 5. Substituent groups on the polyol chains can include D-alanine (ester linked), N-acetylglucosamine, N-acetylgalactosamine, and glucose; the substituent is characteristic for the teichoic acid from a particular bacterial species and can act as a specific antigenic determinant. Teichoic acids are covalently linked to the peptidoglycan. These highly negatively charged polymers of the bacterial wall can serve as a caution-sequestering mechanism.

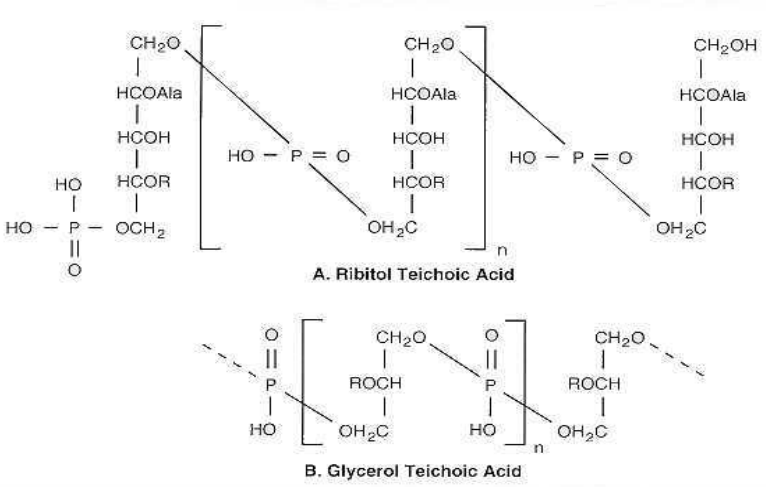


Fig.5 STRUCTURES OF CELL WALL TEICHOIC ACIDS.

(A) Ribitol teichoic acid with repeating units of 1,5-phosphodiester linkages of D-ribitol and D-alanyl ester on position 2 and glycosyl substituents (R) on position 4. The glycosyl groups may abe N-acetylglucosaminyl (a or b) as in S aureus or a-glucosyl as in B subtilis W23. (B) Glycerol teichoic acid with 1,3-phosphodiester linkages of glycerol repeating units (1,2-linkages in some species). In the glycerol teichoic acid structure shown, the polymer may be unsubstituted (R - H) or substituted (R - D-alanyl or glycosyl).

6.6.1 ACCESSORY WALL POLYMERS

In addition to the principal cell wall polymers, the walls of certain Gram-positive bacteria possess polysaccharide molecules linked to the peptidoglycan. For example, the C polysaccharide of streptococci confers group specificity. Acidic polysaccharides attached to the peptidoglycan are called teichuronic acids. Mycobacteria have peptidoglycolipids, glycolipids, and waxes associated with the cell wall.

6.7. LIPOPOLYSACCHARIDES

Lipopolysaccharide is found only in the outer half of the OM. LPS contains a hydrophobic region, Lipid A buried in the membrane and then two polysaccharide regions which project outwards - the core and O-antigen. LPS is sometimes called endotoxin because of its toxic effects on mammalian cells. The toxicity is due to the Lipid A portion of the molecule which consists of two glucosamine residues with 7 long chain fatty acids covalently attached. The core region contains 7 and 8 carbon sugars as well as hexoses and hexosamines. It is often substituted with phosphate and phosphoethanolamine side chains. On the distal end of the core is attached the O-antigen, a chain made of many repeating oligosaccharide units each usually consisting of 3 or 4 sugars. There is great variety in the structure and composition of the O-antigen between different strains of bacteria. *E. coli*, for example, has over 150 possible types of O-antigen, many of which contain rare sugars, e.g. colitose, abequose. The O-antigen is highly immunogenic and is used in the antibody typing of *Salmonella* and other Enterobacteria.

A characteristic feature of Gram-negative bacteria is possession of various types of complex macromolecular lipopolysaccharide (LPS). So far, only one Gram-positive organism, Listeria monocytogenes, has been found to contain an authentic LPS. The LPS of this bacterium and those of all Gram-negative species are also called endotoxins, thereby distinguishing these cell-bound, heat-stable toxins from heat-labile, protein exotoxins secreted into culture media. Endotoxins possess an array of powerful biologic activities and play an important role in the pathogenesis of many Gram-negative bacterial infections. In addition to causing endotoxic shock, LPS is pyrogenic, can activate macrophages and complement, is mitogenic for B lymphocytes, induces interferon production, causes tissue necrosis and tumor regression, and has adjuvant properties. The endotoxic properties of LPS reside largely in the lipid A components. Usually, the LPS molecules have three regions: the lipid A structure required for insertion in the outer leaflet of the outer membrane bilayer; a covalently attached core composed of 2-keto-3deoxyoctonic acid (KDO), heptose, ethanolamine, N-acetylglucosamine, glucose, and galactose; and polysaccharide chains linked to the core. The polysaccharide chains constitute the O-antigens of the Gram-negative bacteria, and the individual monosaccharide constituents confer serologic specificity on these components. Figure 6 depicts the structure of LPS. Although it has been known that lipid A is composed of b1,6-linked D-glucosamine disaccharide substituted with phosphomonester groups at positions 4' and 1, uncertainties have existed about the attachment positions of the

six fatty acid acyl and KDO groups on the disaccharide. The demonstration of the structure of lipid A of LPS of a heptoseless mutant of Salmonella typhimurium has established that amide-linked hydroxymyristoyl and lauroxymyristoyl groups are attached to the nitrogen of the 2- and 2'-carbons, respectively, and that hydroxymyristoyl and myristoxymyristoyl groups are attached to the oxygen of the 3- and 3'-carbons of the disaccharide, respectively. Therefore, only position 6' is left for attachment of KDO units.

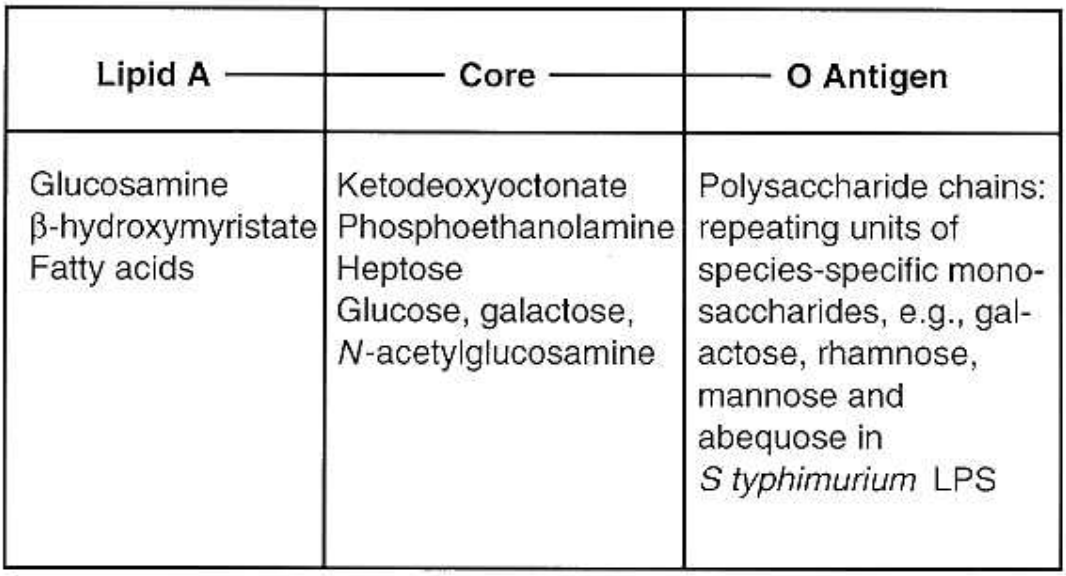


FIG. 6 THE THREE MAJOR, COVALENTLY LINKED REGIONS THAT FORM THE TYPICAL LPS

LPS and phospholipids help confer asymmetry to the outer membrane of the Gram-negative bacteria, with the hydrophilic polysaccharide chains outermost. Each LPS is held in the outer membrane by relatively weak cohesive forces (ionic and hydrophobic interactions) and can be dissociated from the cell surface with surface-active agents.

As in peptidoglycan biosynthesis, LPS molecules are assembled at the plasma or inner membrane. These newly formed molecules are initially inserted into the outer-inner membrane adhesion sites.

6.8. OUTER MEMBRANE OF GRAM-NEGATIVE BACTERIA

Outer Membrane (OM): Found in gram-negatives only. Provides an outer diffusion barrier to molecules greater than 700 to 1500 molecular weight (exact value depends on species). Protects cells against antibiotics, toxic metals, and other noxious chemicals. Consists of protein, lipid, and a unique molecule found only in the OM of gram-negative bacteria - the lipopolysaccharide (LPS).

Outer Membrane Proteins (OMP's) are of three major classes:

I. Structural Proteins. The lipoprotein is covalently bound to the peptidoglycan and serves to bind the OM to the rest of the cell wall. OmpA protein is a structural protein and is also the receptor for the sex pilus during conjugation.

II. The porins are the major proteins of the OM. There are two or three different porins which form pores through the OM. These pores admit molecules up to a size limit (700 to 1500 M.W.) which is characteristic of the bacterial species. Specialised porins exist e.g for phosphate or maltose transport which are induced only under appropriate conditions.

The porins, lipoprotein and OmpA are the most numerous proteins in the bacterial cell along with ribosomal proteins, translation factors, and acyl carrier protein.

III. Specific receptor proteins are found in the OM for certain nutrients which are too large to get through the porins. Many of these are also used as receptors by bacteriophages. Other phages may use LPS or porins as their receptors.

In thin sections, the outer membranes of Gram-negative bacteria appear broadly similar to the plasma or inner membranes; however, they differ from the inner membranes and walls of Gram-positive bacteria in numerous respects. The lipid A of LPS is inserted with phospholipids to create the outer leaflet of the bilayer structure; the lipid portion of the lipoprotein and phospholipid form the inner leaflet of the outer membrane bilayer of most Gram-negative bacteria.

In addition to these components, the outer membrane possesses several major outer membrane proteins; the most abundant is called porin. The assembled subunits of porin form a channel that limits the passage of hydrophilic molecules across the outer membrane barrier to those having molecular weights that are usually less than 600 to 700. Evidence also suggests that hydrophobic pathways exist across the outer membrane and are partly responsible for the differential penetration and effectiveness of certain b-lactam antibiotics (ampicillin, cephalosporins) that are active against various Gram-negative bacteria. Although the outer membranes act as a permeability barrier or molecular sieve, they do not appear to possess energy-transducing systems to drive active transport. Several outer membrane proteins, however, are involved in the specific uptake of metabolites (maltose, vitamin B12, nucleosides) and iron from the medium. Thus, outer membranes of the Gram-negative bacteria provide a selective barrier to external molecules and thereby prevent the loss of metabolite-binding proteins and hydrolytic enzymes (nucleases, alkaline phosphatase) found in the periplasmic space. The periplasmic space is the region between the outer surface of the inner (plasma) membrane and the inner surface of the outer membrane. Thus, Gram-negative bacteria have a cellular compartment that has no equivalent in Gram-positive organisms. In addition to the hydrolytic enzymes, the periplasmic space holds binding proteins (proteins that specifically bind sugars, amino acids, and inorganic ions) involved in membrane transport and chemotactic receptor activities. Moreover, plasmid-encoded b-lactamases and aminoglycoside-modifying enzymes (phosphorylation or adenylation) in the periplasmic space produce antibiotic resistance by degrading or modifying an antibiotic in transit to its target sites on the membrane (penicillin-binding proteins) or on the ribosomes (aminoglycosides). These periplasmic proteins can be released by subjecting the cells to osmotic shock and after treatment with the chelating agent ethylenediaminetetraacetic acid.

Periplasmic Space: Between the OM and inner membrane (IM) of gram-negative cells. This space contains various degradative enzymes eg: 5'-nucleotidase, alkaline phosphatase. It also

contains binding proteins involved in the uptake of amino acids, sugars, etc. When gram-negative cells contain plasmids specifying antibiotic resistance the enzymes which break down antibiotics are located in the periplasmic space, e.g. beta-lactamase. In *E. coli* about 20-40% of the cell volume is in the periplasmic space. The osmotic pressure of the periplasmic space cannot be maintained with simple sugars or inorganic ions as these are too small to be retained by the OM. Instead the periplasmic space contains the membrane-derived oligosaccharides (MDO).

Inner (Cytoplasmic) Membrane (IM): A typical biological membrane comprising about one third lipid and two thirds protein. The lipid bilayer is penetrated by hydrophobic proteins - "intrinsic" proteins. In addition the surface of the lipid layer is covered by 2 to 3 layers of "extrinsic" protein molecules. The lipid bilayer behaves as a 2-dimensional liquid allowing membrane proteins to drift laterally. The IM controls entry into the cytoplasm and acts as an electrical insulator for the electron transport chain. The IM contains synthetic enzymes for components of all layers of the cell envelope together with proteins involved in secretion and chemosensing.

6.9. CAPSULE

Layer found loosely attached to the outside of many bacteria. Not essential and varies with species and growth conditions. It usually consists of simple or complex carbohydrates or, more rarely protein eg: *Acetobacter* - simple polysaccharide, cellulose; *E. coli* - complex polysaccharides made of amino sugars and sugar acids; *Acinetobacter* - regular array of protein subunits. Capsules are protective and keep both bacteriophages and macrophages at a distance. They also protect cells against desiccation.

Some bacteria are enclosed within a capsule. This protects the bacterium, even within phagocytes, helping to prevent the cell from being killed. Encapsulated bacteria grow as 'smooth' colonies, whereas colonies of bacteria that have lost their capsules appear rough. Rough colonies do not generally cause disease. Encapsulated bacteria do not succumb to intracellular killing as easily as bacteria that lack capsules. Strains of *Streptococcus pnuemoniae* that lack capsules do not cause disease. All the bacteria that cause meningitis are encapsulated. Suspending bacteria in India ink is an easy way of demonstrating capsules. Ink particles cannot penetrate the capsular material and encapsulated cells appear to have a halo around them. This is the **Quellung** reaction.

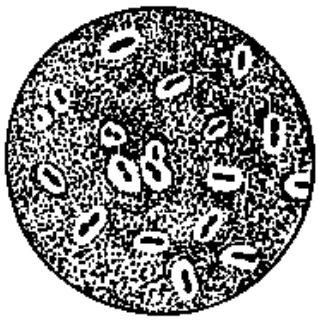


Fig. 7 THE QUELLUNG REACTION

In the 'Quellung' reaction, bacterial cells are resuspended in antiserum that carries antibodies raised against the capsule. This causes the capsule to swell, and this can be easily visualised by suspension in India Ink. The ink particles cannot penetrate the capsule, which appears as a halo around the bacterial cells.

Some bacteria form capsules, which constitute the outermost layer of the bacterial cell and surround it with a relatively thick layer of viscous gel. Capsules may be up to 10 µm thick. Some organisms lack a well-defined capsule but have loose, amorphous slime layers external to the cell wall or cell envelope. The hemolytic *Streptococcus mutans*, the primary organism found in dental plaque is able to synthesis a large extracellular mucoid glucans from sucrose. Not all bacterial species produce capsules; however, the capsules of encapsulated pathogens are often important determinants of virulence. Encapsulated species are found among both Gram-positive and Gram-negative bacteria. In both groups, most capsules are composed of highmolecular-weight viscous polysaccharides that are retained as a thick gel outside the cell wall or envelope. The capsule of *Bacillus anthracis* (the causal agent of anthrax) is unusual in that it is composed of a g-glutamyl polypeptide. Table 3 presents the various capsular substances formed by a selection of Gram-positive and Gram-negative bacteria. A plasma membrane stage is involved in the biosynthesis and assembly of the capsular substances, which are extruded or secreted through the outer wall or envelope structures. Mutational loss of enzymes involved in the biosynthesis of the capsular polysaccharides can result in the smooth-to-rough variation seen in the *Pneumococci*.

The capsule is not essential for viability. Viability is not affected when capsular polysaccharides are removed enzymatically from the cell surface. The exact functions of capsules are not fully understood, but they do confer resistance to phagocytosis and hence provide the bacterial cell with protection against host defenses to invasion.

Genus and species	Capsular substances
Gram positive bacteria	
S.pneumoniae	Polysaccharides, glucose, glucoronic acid, other types, various sugars and amino sugars
Streptococcus species	Polysaccharides, others containing amino sugars, uronic acids
B.anthracis	Gamma Glutamyl polypeptide
Gram negative bacteria	
H.influenzae	Polyribosephosphate
Klebsiella sp.	Polysaccharides, sugars such as hexose, fucose, uronic acids
N.meningitidis	Polysaccharides, sialic acid polymers

TABLE 2.3. NATURE OF CAPSULAR SUBSTANCES FORMED BY VARIOUS BACTERIA

Some bacteria produce slime to help them to stick to surfaces. Slime is produced by several types of pathogenic microbes, and is usually made up from polysaccharides. The slime produced by *Streptococcus mutans* enables it to stick to the surface of teeth, where it helps to form plaque, leading eventually to dental caries. 'Coagulase-negative' staphylococci

live on the skin, and some strains produce a slime that enables them to stick to plastics. These bacteria cause infections associated with implanted plastic medical devices.

6.10. PILUS (plural pili)

Sex pili and common pili are both composed of protein monomers arranged helically. Sex pili are involved in binding male and female cells together for subsequent transfer of DNA during mating and are only produced by male cells. Only one or two sex pili per cell are made. Common pili (also called fimbriae; singular = fimbria) are made by both sexes and appear in higher numbers per cell. They are involved in adhesion to suitable surfaces or in floating - bacteria lacking common pili clump together and sediment.

The terms pili and fimbriae are usually used interchangeably to describe the thin, hairlike appendages on the surface of many Gram-negative bacteria and proteins of pili are referred to as pilins. Pili are more rigid in appearance than flagella. In some organisms, such as Shigella species and *E coli*, pili are distributed profusely over the cell surface, with as many as 200 per cell. As is easily recognized in strains of *E coli*, pili can come in two types: short, abundant common pili, and a small number (one to six) of very long pili known as sex pili. Sex pili can be distinguished by their ability to bind male-specific bacteriophages (the sex pilus acts as a specific receptor for these bacteriophages). The sex pili attach male to female bacteria during conjugation.

Pili in many enteric bacteria confer adhesive properties on the bacterial cells, enabling them to adhere to various epithelial surfaces, to red blood cells (causing hemagglutination), and to surfaces of yeast and fungal cells. These adhesive properties of piliated cells play an important role in bacterial colonization of epithelial surfaces and are therefore referred to as colonization factors. The common pili found on *E coli* exhibit sugar specificity analogous to that of phytohemagglutinins and lectins, in that adhesion and hemagglutinating capacities of the organism are inhibited specifically by mannose. Organisms possessing this type of hemagglutination are called mannose-sensitive organisms. Other piliated organisms, such as gonococci, are adhesive and hemagglutinating, but are insensitive to the inhibitory effects of mannose. Extensive antigenic variations in pilins of gonococci are well known.

6.11. FLAGELLUM (plural flagella)

Prokaryotic flagella are quite different from those of eukaryotes. Bacterial flagella consist of a single filament made of helically arranged protein subunits. The basal structure in the IM acts as a rotor and the whole flagellar shaft rotates. Bacterial flagella are powered directly by the proton motive force, whereas eukaryotic flagella use ATP.

Structurally, bacterial flagella are long (3 to 12 μm), filamentous surface appendages about 12 to 30 nm in diameter. The protein subunits of a flagellum are assembled to form a cylindrical structure with a hollow core. A flagellum consists of three parts: (1) the long filament, which lies external to the cell surface; (2) the hook structure at the end of the filament; and (3) the basal body, to which the hook is anchored and which imparts motion to the flagellum. The basal body traverses the outer wall and membrane structures. It consists of a rod and one or two pairs of discs. The thrust that propels the bacterial cell is provided by counterclockwise rotation of the basal body, which causes the helically twisted filament to whirl. The movement of the basal body is driven by a proton motive force rather than by ATP directly. The ability of bacteria to swim by means of the propeller-like action of the flagella provides them with the mechanical means to perform chemotaxis (movement in response to attractant and repellent substances in the environment). Response to chemical stimuli involves a sophisticated sensory system of receptors that are located in the cell surface and/or

periplasm and that transmit information to methyl-accepting chemotaxis proteins that control the flagellar motor. Genetic studies have revealed the existence of mutants with altered biochemical pathways for flagellar motility and chemotaxis.

Chemically, flagella are constructed of a class of proteins called flagellins. The hook and basal-body structures consist of numerous proteins. Mutations affecting any of these gene products may result in loss or impairment of motility. Flagellins are immunogenic and constitute a group of protein antigens called the H antigens, which are characteristic of a given species, strain, or variant of an organism. The species specificity of the flagellins reflects differences in the primary structures of the proteins. Antigenic changes of the flagella known as the phase variation of H1 and H2 occurs in *Salmonella typhimurium*.

The number and distribution of flagella on the bacterial surface are characteristic for a given species and hence are useful in identifying and classifying bacteria. Figure 8 illustrates typical arrangements of flagella on or around the bacterial surface. For example, *V cholerae* has a single flagellum at one pole of the cell (i.e., it is monotrichous), whereas *Proteus vulgaris and E coli* have many flagella distributed over the entire cell surface (i.e., they are peritrichous). The flagella of a peritrichous bacterium must aggregate as a posterior bundle to propel the cell in a forward direction.

Bacterial cells may carry a single flagellum, and are thus described as monotrichous. If the single flagellum is at one end of a rod-shaped cell it is known as a polar flagellum. If the bacterium carries a single tuft of flagella, it is said to be lophotrichous (*lophos* - Greek for a crest). When the tuft appears at both ends of the cell, the bacterium is amphitrichous (*amphi* - Greek for 'at each end'). Bacteria that are covered all over in flagella are said to be peritrichous (*peri* - around).

Flagella can be sheared from the cell surface without affecting the viability of the cell. The cell then becomes temporarily nonmotile. In time it synthesizes new flagella and regains motility. The protein synthesis inhibitor chloramphenicol, however, blocks regeneration of flagella.

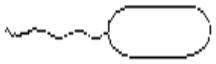
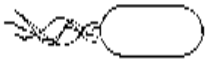
Monotrichous	***Vibrio cholera***	
Lopotrichous	***Bartonella bacilliformis***	
Amphitrichous	***Spirillum serpens***	
Peritrichous	***Escherichia coli***	

Fig.8 ARRANGEMENTS OF FLAGELLA ON BACTERIAL CELLS

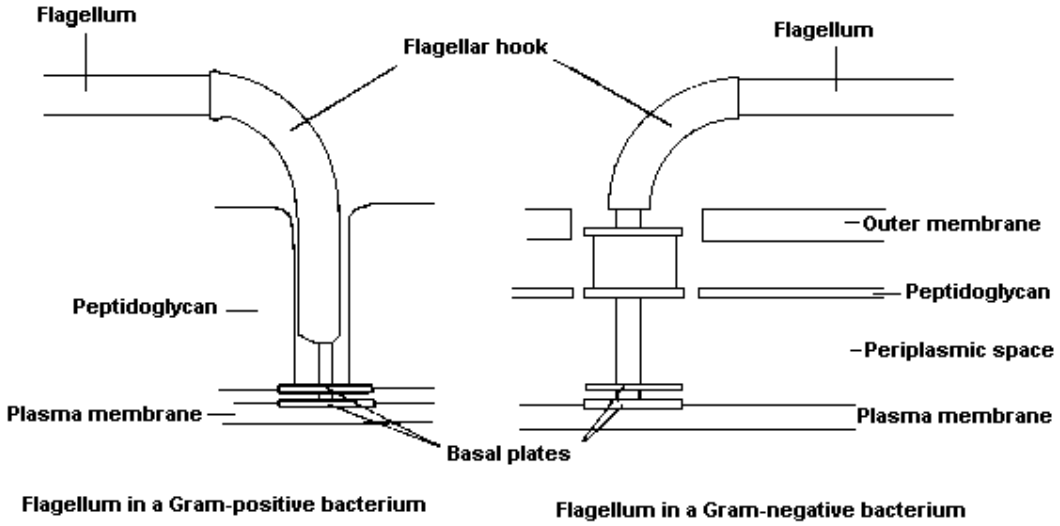


Fig.9 INSERTION OF FLAGELLA INTO BACTERIAL CELLS OF DIFFERENT TYPES

INTRACELLULAR COMPONENTS

6.12. PLASMA (CYTOPLASMIC) MEMBRANES

Bacterial plasma membranes, the functional equivalents of eukaryotic plasma membranes, are referred to variously as cytoplasmic, protoplast, or (in Gram-negative organisms) inner membranes. Similar in overall dimensions and appearance in thin sections to biomembranes from eukaryotic cells, they are composed primarily of proteins and lipids (principally phospholipids). Protein-to-lipid ratios of bacterial plasma membranes are approximately 3: 1, close to those for mitochondrial membranes. Unlike eukaryotic cell membranes, the bacterial membrane (except for Mycoplasma species and certain methylotrophic bacteria) has no sterols, and bacteria lack the enzymes required for sterol biosynthesis.

Although their composition is similar to that of inner membranes of Gram-negative species, cytoplasmic membranes from Gram-positive bacteria possess a class of macromolecules not present in the Gram-negative membranes. Many Gram-positive bacterial membranes contain membrane-bound lipoteichoic acid, and species lacking this component (such as *Micrococcus* and *Sarcina* spp) contain an analogous membrane-bound succinylated lipomannan. Lipoteichoic acids are structurally similar to the cell wall glycerol teichoic acids in that they have basal polyglycerol phosphodiester 1-3 linked chains. These chains terminate with the phosphomonoester end of the polymer, which is linked covalently to either a glycolipid or a phosphatidyl glycolipid moiety. Thus, a hydrophobic tail is provided for anchoring in the membrane lipid layers. As in the cell wall glycerol teichoic acid, the lipoteichoic acids can have glycosidic and D-alanyl ester substituents on the C-2 position of the glycerol.

Both membrane-bound lipoteichoic acid and membrane-bound succinylated lipomannan can be detected as antigens on the cell surface, and the glycerol-phosphate and succinylated mannan chains appear to extend through the cell wall structure. This class of

polymer has not yet been found in the cytoplasmic membranes of Gram-negative organisms. In both instances, the lipoteichoic acids and the lipomannans are negatively charged components and can sequester positively charged substances. They have been implicated in adhesion to host cells, but their functions remain to be elucidated.

Multiple functions are performed by the plasma membranes of both Gram-positive and Gram-negative bacteria. Plasma membranes are the site of active transport, respiratory chain components, energy-transducing systems, the H+-ATPase of the proton pump, and membrane stages in the biosynthesis of phospholipids, peptidoglycan, LPS, and capsular polysaccharides. In essence, the bacterial cytoplasmic membrane is a multifunction structure that combines the mitochondrial transport and biosynthetic functions that are usually compartmentalized in discrete membranous organelles in eukaryotic cells. The plasma membrane is also the anchoring site for DNA and provides the cell with a mechanism (as yet unknown) for separation of sister chromosomes.

6.13. BACTERIAL SPORES

A few species of bacteria have the ability to produce highly resistant structures known as endospores (or simply spores). These resist a range of hazardous environments, and protect against heat, radiation, and desiccation. Endospores form within (hence *endo*-) special vegetative cells known as sporangia (singular *sporangium*). Diseases caused by sporing bacteria include botulism (*Clostridium botulinum*), gas gangrene (*Clostridium perfringens*), tetanus (*Clostridium tetani*) and acute food poisoning (*Clostridium perfringens*, again) All these bacteria are 'anaerobic'. The aerobic sporing bacteria can also cause disease. Anthrax is caused by *Bacillus anthracis. Bacillus cereus* causes two types of food poisoning.

Endospores are highly heat-resistant, dehydrated resting cells formed intracellularly in members of the genera Bacillus and Clostridium. Sporulation, the process of forming endospores, is an unusual property of certain bacteria. The series of biochemical and morphologic changes that occur during sporulation represent true differentiation within the cycle of the bacterial cell. The process, which usually begins in the stationary phase of the vegetative cell cycle, is initiated by depletion of nutrients (usually readily utilizable sources of carbon or nitrogen, or both). The cell then undergoes a highly complex, well-defined sequence of morphologic and biochemical events that ultimately lead to the formation of mature endospores. As many as seven distinct stages have been recognized by morphologic and biochemical studies of sporulating Bacillus species: stage 0, vegetative cells with two chromosomes at the end of exponential growth; stage I, formation of axial chromatin filament and excretion of exoenzymes, including proteases; stage II, forespore septum formation and segregation of nuclear material into two compartments; stage III, spore protoplast formation and elevation of tricarboxylic acid and glyoxylate cycle enzyme levels; stage IV, cortex formation and refractile appearance of spore; stage V, spore coat protein formation; stage VI, spore maturation, modification of cortical peptidoglycan, uptake of dipicolinic acid (a unique endospore product) and calcium, and development of resistance to heat and organic solvents; and stage VII, final maturation and liberation of endospores from mother cells (in some species).

When newly formed, endospores appear as round, highly refractile cells within the vegetative cell wall, or sporangium. Some strains produce autolysins that digest the walls and liberate free endospores. The spore protoplast, or core, contains a complete nucleus, ribosomes, and energy generating components that are enclosed within a modified

cytoplasmic membrane. The peptidoglycan spore wall surrounds the spore membrane; on germination, this wall becomes the vegetative cell wall. Surrounding the spore wall is a thick cortex that contains an unusual type of peptidoglycan, which is rapidly released on germination. A spore coat of keratinlike protein encases the spore contained within a membrane (the exosporium). During maturation, the spore protoplast dehydrates and the spore becomes refractile and resistant to heat, radiation, pressure, desiccation, and chemicals; these properties correlate with the cortical peptidoglycan and the presence of large amounts of calcium dipicolinate.

Recent evidence indicated that the spores of *Bacillus spharicus* were revived which had been preserved in amber for more than 25 million years. Their claims need to be reevaluated. The thin section of the spore shows the ruptured, thick spore coat and the cortex surrounding the spore protoplast with the germinal cell wall that becomes the vegetative wall on outgrowth.

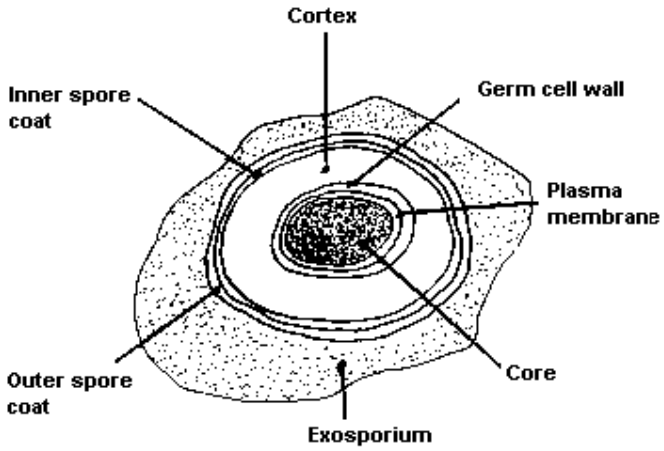


Fig.10 A BACTERIAL SPORE

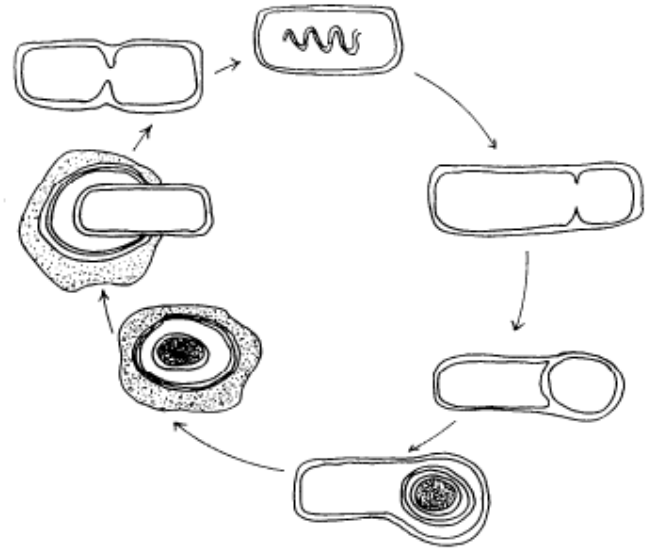


Fig.11 THE CYCLE OF SPORE FORMATION AND GERMINATION

6.14. INCLUSION BODIES AND VOLUTIN GRANULES

Not surrounded by membranes, on the contrary of *Eukarya* inclusion bodies. Concentrated deposits of certain substances are detectable in the cytoplasm of some bacteria. Volutin granules, also known as metachromatic granules, are composed of polyphosphate. They stain an intense reddish purple colour with dilute methylene blue and can be observed by light microscopy they appear as round, dark areas. Volutin serves as a reserve source of phosphate. Another polymer often found in aerobic bacteria, especially under high carbon, low nitrogen culture conditions, is a chloroform soluble, lipid like material, poly – β – hydroxybutyrate (PHB), which can serve as a reserve carbon and energy source. PHB granules can be stained with lipid soluble dyes such as Nile blue. By electron microscopy they appear as clear round areas. Polysaccharide granules, ie glycogen, can be stained brown with iodine. By electron microscopy they appear as dark granules. Another type of inclusion is represented by the intracellular globules of elemental sulfur that may accumulate in certain bacteria growing in environments rich in hydrogen sulfide.

Some bacteria that live in aquatic habitats form gas vacuoles that provide buoyancy. By light microscopy these are bright, refractile bodies; by electron microscopy they are seen to have a regular shape: hollow, rigid cylinders with more or less conical ends and having a striated protein boundary. This boundary is impermeable to water, but the various dissolved gases in the culture medium can penetrate it to fill the cavity. The identifying feature of gas vacuoles is that they can be made to collapse under pressure and thereby lose their refractility.

6.15. MESOSOME

A localized infold in the plasma membrane (more abundant in Gram +ve). This is the site for chemiosmosis in nitrifying *Bacteria*, chromatophores in purple sulfur *Bacteria*, cylindrical vesicle in green *Bacteria* and multilayered membrane thylakoids in *Cyanobacteria*. They bind the chromosome and, like the latter, duplicate themselves during binary fission, when an accessory septal mesosome appears.

Thin sections of Gram-positive bacteria reveal the presence of vesicular or tubular-vesicular membrane structures called mesosomes, which are apparently formed by an invagination of the plasma membrane. These structures are much more prominent in Gram-positive than in Gram-negative organisms. At one time, the mesosomal vesicles were thought to be equivalent to bacterial mitochondria; however, many other membrane functions have also been attributed to the mesosomes. At present, there is no satisfactory evidence to suggest that they have a unique biochemical or physiologic function. Indeed, electron-microscopic studies have suggested that the mesosomes, as usually seen in thin sections, may arise from membrane perturbation and fixation artifacts. No general agreement exists about this theory, however, and some evidence indicates that mesosomes may be related to events in the cell division cycle.

6.16. THE NUCLEOID

Prokaryotic and eukaryotic cells were initially distinguished on the basis of structure: the prokaryotic nucleoid the equivalent of the eukaryotic nucleus is structurally simpler than the true eukaryotic nucleus, which has a complex mitotic apparatus and surrounding nuclear

membrane. The bacterial nucleoid, which contains the DNA fibrils, lacks a limiting membrane. Under the light microscope, the nucleoid of the bacterial cell can be visualized with the aid of Feulgen staining, which stains DNA. Gentle lysis can be used to isolate the nucleoid of most bacterial cells. The DNA is then seen to be a single, continuous, "giant" circular molecule with a molecular weight of approximately 3 X 109. The unfolded nuclear DNA would be about 1 mm long (compared with an average length of 1 to 2 μm for bacterial cells). The bacterial nucleoid, then, is a structure containing a single chromosome. The number of copies of this chromosome in a cell depends on the stage of the cell cycle (chromosome replication, cell enlargement, chromosome segregation, etc). Although the mechanism of segregation of the two sister chromosomes following replication is not fully understood, all of the models proposed require that the chromosome be permanently attached to the cell membrane throughout the various stages of the cell cycle.

Bacterial chromatin does not contain basic histone proteins, but low-molecular-weight polyamines and magnesium ions may fulfill a function similar to that of eukaryotic histones. Despite the differences between prokaryotic and eukaryotic DNA, prokaryotic DNA from cells infected with bacteriophage, when visualized by electron microscopy, has a beaded, condensed appearance not unlike that of eukaryotic chromatin.

6.17. MITOCHONDRIA

FUNCTION
Site of: cellular respiration - redox reactions oxidation of CH_2O --> CO_2
Gas exchange in cell - CO_2 is released & O_2 is reduced
Krebs cycle - oxidizes PYRUVATE ---> CO_2 + H_2O
Respiratory ETC chain & Oxidative Phosphorylation, which makes ATP
Role of: site of conversion of covalent bond energy of food molecules --> into ATP
It couples redox transfer of e- & H^+ protons to ATP-synthase --> ATP
STRUCTURE
Double membrane bound organelle. Outer membrane - contains transport protein porin (passes molecules up to 5K). Inner membrane - very selectively permeable (i.e., impermeant to most molecules). Peri-mitochondrial space - (in between) area where H^+ accumulate. Cristae - inner membranes that hold the respiratory assemblies of ETC.
Mitoplasm-"matrix" aqueous compartment-DNA, ribosomes, KC, etc.
Elongate cylinders to oblate speroids.3-5 μm long by 0.5-1.0 um dia, "shape-shifters", mobile.
Number: 20 to 1,000 per cell; greater number in more active cells can make-up as much as 20% of cell's volume.
Contents: its own DNA - 16,569 nucleotide pairs: about 37 genes, its own ribosomes (prokaryotic size) & protein synthesizing ability enzymes for cellular respiration.

6.18. PLASTIDS OR CHLOROPLASTS

Develops in the light from proplastids, as a plastid. Site of autotrophic metabolism = PHTS, O_2 evolution, CO_2 reduction
SHAPE: oblate spheroid = shape variable (stellate, reticulate)
SIZE: 2-3 μm dia by 5-10 um long & number: 15/20 - 100's/cell
CONTENTS: aqueous stroma (CHLOROPLASM) holds within itself.
1) Internal membrane system made of thylakoid membranes, grana Stacks and intergranal membranes.

2) 70s ribosomes (bacterial size)
3) Lipid droplets
4) Naked DNA pieces (highly supercoiled & repetitive)
5) Starch granules & pyrenoids
6) Enzymes of CO_2 fixation (reduction)

Endosymbionts by Lynn Margulis – 1981

"Mitochondria & Chloroplasts are derived from prokaryotes, which were once free living, but joined into a symbiotic relationship with eukaryotic aerobes during cellular evolution"

Some of the Evidence: many of today's single celled eukaryotes live in oxygen poor places (gut), lack mitochondria, & function anaerobically. *Pelomyxa palustris* is eukaryotic amoeba,that lacks mitochondria, yet holds aerobic bacteria within its cytoplasm (in a symbiotic relationship). Chloroplasts share a common molecular ancestry [DNA sequences are similar] with the cyanobacteria (the 1st photosynthetic prokaryotes).

Number striking similarities of Bacteria & Mitochondria/Chloroplasts:
Both organelles are double membrane bound.
Both are semiautonomous. Derived from themselves that is by divisional fission (i.e., replicate independently from their cell hosts).
Both have their own DNA (a circular molecule like DNA of prokaryotes) & protein biosynthetic systems (can make some of own proteins).
DNA sequence homology, each has similar DNA sequences. Mitochondria DNA related to aerobic bacterial DNA, chloroplast DNA related to cyanobacterial DNA.

6.19. RIBOSOMES

Ribosome is a sub cell ribonucleo-protein particle (RNP) a complex of RNA & Proteins. Site of cellular protein synthesis. Spheroid shape - 17 to 23 nm dia. Found in 3 different places in cells. Free in cytoplasm, as individual subunits or dimers, membrane bound on outer surface of Endoplasmic Reticulum membranes, attached to mRNA molecule in a polysome [or polyribosome].

Unlike animal or plant cells, there is no endoplasmic reticulum to which ribosomes are bound; some ribosomes are free in the cytoplasm, and other especially those involved in the synthesis of proteins to be transported out of the cell, are associated with the inner surface of the cytoplasmic membrane. When the ribosomes of prokaryotes undergo sedimentation in a centrifuge, they have a sedimentation coefficient of 70 Svedberg units (70S) and are composed of two subunits, a 50S and 30S subunit. This is in contrast to the ribosomes of eukaryotic organisms, which have a sedimentation coefficient of 80S and are composed of a 60S and 40S subunit.

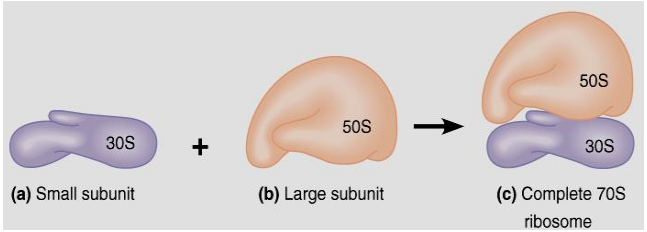


Fig. 12 STRUCTURE OF RIBOSOME AND ITS SUBUNITS

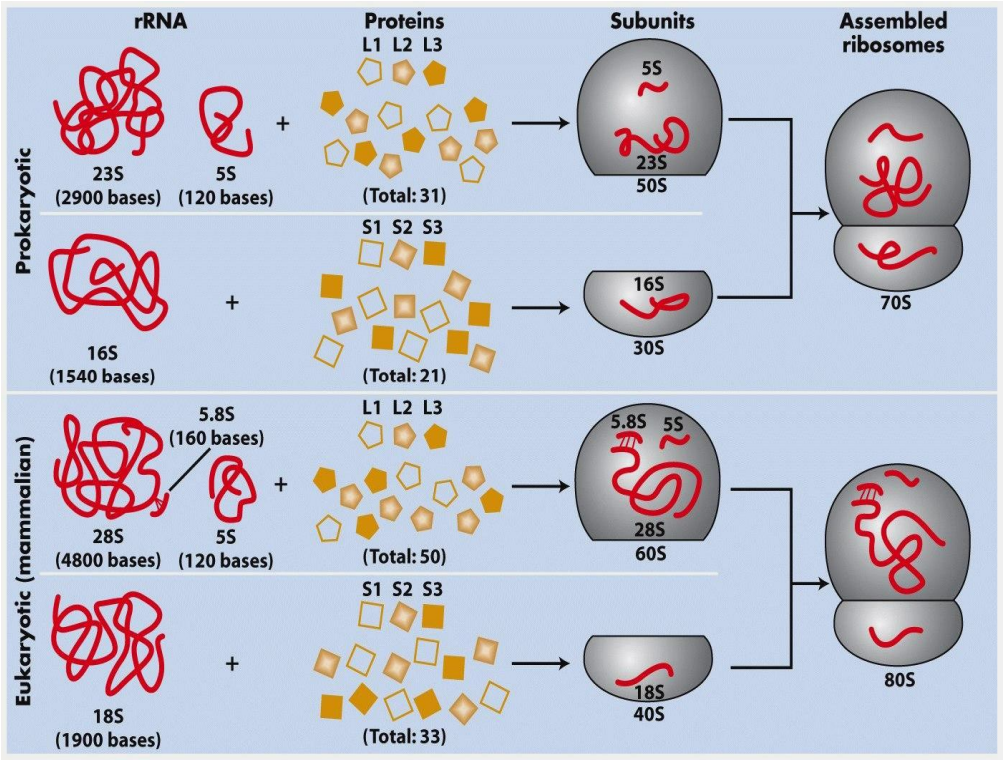


Fig. 13 RIBOSOME SUBUNITS OF PROKARYOTE AND EUKARYOTE

6.20. LET US SUM UP

- Bacteria are classified as Gram positive and Gram negative based on their cell wall.
- Bacteria have complex cellular organization with various external appendages.
- External appendages are pili, flagella and capsule structures.
- Inner cytoplasm has volutin granules the energy storage organelle of the bacteria.

6.21. LESSON END ACTIVITIES

1. Explain the importance of extracellular appendages present in bacteria.
2. Write short note on the cell wall of Gram positive bacteria.
3. What are the modifications that a microorganism undergo during stress and explain about them?
4. What are capsules and how they are useful to bacteria?
5. Explain the outer membrane of Gram negative organisms.
6. Explain the cell wall nature of Gram positive organisms.

6.22. POINTS FOR DISCUSSION

1. Write the difference between Gram positive and Gram negative and explain the cell wall composition with diagram.
2. Explain spore formation process.
3. Explain about teichoic acid, peptidoglycan and lipopolysaccharides.

6.23 REFERENCES

1. Microbiology by Pelczar, Reid and Chan, McGraw Hill Book Company.
2. Microbiology, Fundamental and Applications by R.A. Atlas, McMillan Publishers.
3. General Microbiology by Powar and Daginawala, Himalaya Publishing House.
4. Microbial genetics by David friefelder

LESSON – 7

MICROBIAL METABOLISMS AND ENERGETICS

Contents

7.0. AIMS AND OBJECTIVES

The chapter deals with the microbial metabolism and its energetics.

7.1. INTRODUCTION

The multiplicity of processes performed by all biological systems can be traced, directly or indirectly, to certain chemical reactions. The term metabolism denotes all the organized chemical activities performed by a cell, which compromise two general type- energy production and energy utilization.

7.2. PRINCIPLES OF ENERGETICS

Most cells obtain energy by carrying out chemical reactions which liberate energy. In the course of any chemical reaction, energy available for the performance of useful work is either released or absorbed. The amount of energy liberated or taken up during the course of a reaction is referred to as the free energy change (ΔG) of the reaction. It is expressed in terms of calories. If the ΔG of a chemical reaction has negative value, the reaction releases energy and is termed as exergonic reaction. If the ΔG has positive value, the reaction requires energy and termed as endergonic reaction. Concentration of reactants affects the value of ΔG for a chemical reaction. The concentration of all reactants at 1M in the steady state; referred to as standard concentration.

Under conditions of standard concentration, the free energy change (ΔG) of a reaction is referred to by a special term, $\Delta G°$. In other words, $\Delta G°$ is the amount of free energy released or absorbed when one mole of the reactant is converted to one mole of product at 25°C and one atmosphere of pressure, and under (hypothetical) conditions, where all reactants and products are maintained at 1M concentration.

The $\Delta G°$ or standard free energy change is related to the equilibrium constant K_{eq}, of a chemical reaction by the equation

$$\Delta G^\circ = -RT \ln K_{eq}$$

Where R – Gas constant
T – Absolute temperature
If ΔG° is negative value - K_{eq} is > 1and formation of product is forward.
If ΔG° is positive value - K_{eq} is < 1 and chemical reaction tends to proceed in the reverse direction.

It is also possible to obtain energy from chemical reaction in the form of electric potential. Conversely, it is possible to use electric potential energy to drive a chemical reaction. Electric energy is generated when oxidations occur by the removal of electrons. When the electrons fall through a potential difference or drop, energy is produced. The relationship between an oxidation- reduction potential difference and the standard free energy change is

$$\Delta G^\circ = - nFE^\circ$$

Where n – number of moles of electrons transferred in the reaction.
F – Faraday's constant (23,061 cals/V per equivalent)
E° - Standard oxidation – reduction potential difference

In order for life to continue, it is essential that the energy released from exergonic reactions be used to drive endergonic reactions, and living organisms have developed characteristic ways of coupling exergonic reactions with endergonic reactions. The basic principle behind is there be a common reactant.

The common reactants of greatest use to the cell are those capable of transferring large amounts of free energy, called high energy transfer compounds. A variety of such compounds exists in cells, and although such compounds may possess no more total energy than other compounds, the energy is distributed in such a way within molecule that one portion opposes another, resulting in considerable molecular strain.

S.No	Compound	ΔG°', kcal/mol
1	Adenosine triphosphate (ATP)	-7.3
2	Guanosine triphosphate (GTP)	-7.3
3	Uridine triphosphate (UTP)	-7.3
4	Cytidine triphosphate (CTP)	-7.3
5	Acetyl phosphate	-10.1
6	1,3-Diphosphoglyceric acid	-11.8
7	Phosphoenolpyruvic acid(PEP)	-14.8

TABLE 3.1. SOME OF HIGH ENERGY TRANSFER COMPOUNDS FOUND IN CELLS WITH THEIR STANDARD FREE ENERGY CHANGES UPON HYDROLYSIS

7.3. OXIDATION – REDUCTION REACTIONS

Oxidation is the loss of electrons; reduction is the gain of electrons. Frequently, oxidation reactions are dehydrogenations (reactions involving the loss of hydrogen atoms); since a hydrogen atom consists of a proton plus an electron, a compound which loses a hydrogen atom has essentially lost an electron and therefore has been oxidized. An oxidizing agent (oxidant) will absorb electrons and will therefore become reduced. Eg: Fumaric acid is an oxidizing agent; it absorbs hydrogen atoms (which contain electrons) and becomes

reduced to succinic acid. A reducing agent (reductant) donates electrons, becoming oxidized in the process. One can notice that the reverse of each oxidation reaction is a reduction and the reverse of a reduction reaction is an oxidation. Eg: succinic acid conversion to fumaric acid. Pair of substances which involves in such type of reaction is referred to as oxidation-reduction (O/R) system.

One O/R system may tend to absorb electrons from another O/R system; i.e; the first system will oxidize the second. On the other hand, the tendency of the first system to absorb electrons may be so low that the second system may oxidize the first. This power (the tendency to absorb electrons) is expressed by the standard oxidation – reduction potential or the electromotive potential (E'_o) of an O/R system, which is measured electrically under standardized conditions of comparison (electron donor and its conjugate at 1M concentration, 25°C, and pH 7) and expressed in volts. The more positive the E'_o, the greater the oxidizing ability of the system. Such relationships are very important in understanding the orderly sequence in which biological oxidations occur.

When one O/R system oxidizes another, energy is released. It is important to know the values of E'_o for each system, because the $\Delta G°'$of the overall reaction is directly proportional to the difference in E'_o values. If the voltage difference is large, an amount of free energy sufficient to drive the synthesis of ATP may be liberated.

In respiration, an oxidizable substrate is the primary electron donor. In aerobic respiration the terminal electron acceptor is oxygen; in anaerobic respiration the final electron acceptor is a compound like fumarate, NO_3^-, SO_4^{2-}, or CO_3^{2-}. In fermentation, an organic compound is the final electron acceptor; an oxidizable substrate is the electron donor. In photosynthesis carried out by bacteria, bacteriochlorophylls serve as both electron donors and acceptors. In photosynthesis by green algae, plants and cyanobacteria, water serves as a primary electron donor and $NADP^+$ as a terminal electron acceptor. The paths through which these electrons flow in the various processes are called electron transport chains.

Electron transport chains are sequences of oxidation-reduction reactions that occur in cells. These reactions are mediated by a number of electron carriers and electron carrier enzymes. As the electrons flow through the chains, much of their free energy is conserved in the form of ATP; this process is called oxidative phosphorylation.

The multicomponent electron-transport chains are always associated with membranes. In eukaryotes, they are in mitochondrial or chloroplast membranes; in prokaryotes, they are in the cytoplasmic membrane.

7.4. THE RESPIRATORY CHAIN

A respiratory chain is an electron-transport chain. When a pair of electrons or hydrogen atoms from an oxidizable substrate is coupled with the reduction of an ultimate electron acceptor, such as oxygen, there is a large free-energy change. The flow of electrons through the transport chain allows a stepwise release of this energy, some of which is conserved in the form of ATP at several steps in the chain. At these specific steps the difference in E'_o values is great enough to permit sufficient energy to be liberated for oxidative phosphorylation to occur.

A respiratory chain consists of enzymes having prosthetic groups or coenzymes. These can be regarded as the working parts of the enzymes, and in the case of the respiratory chain each is in fact an O/R system. The oxidized form of each prosthetic group or coenzyme has an absorption spectrum different from that of the reduced form.

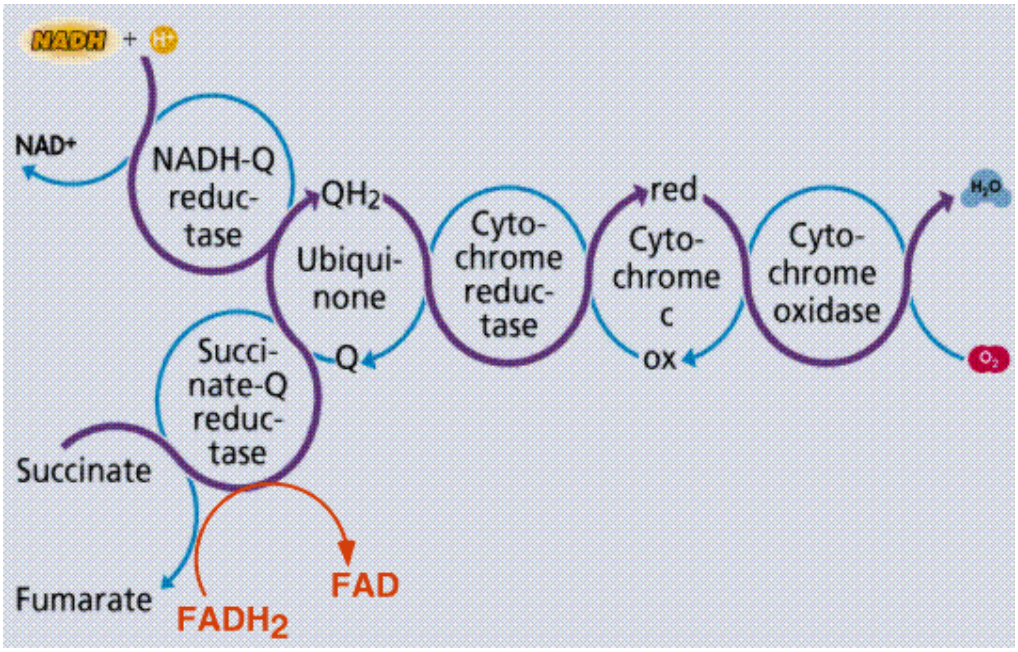


Fig.1. THE RESPIRATORY CHAIN

NAD and NADP: certain enzymes which remove electrons and hydrogen ions from reduced substrates (dehydrogenases) have NAD^+ or $NADP^+$ as their coenzyme. The vitamin niacin forms part of the structure of NAD and NADP and is a precursor in their biosynthesis.

FAD and FMN: another class of dehydrogenases known as flavoproteins exists and contains either FAD or FMN as prosthetic groups. One of the basic parts of their coenzyme structure is the vitamin riboflavin.

Coenzyme Q: it is also called ubiquinone because it is a quinone and is present in all cells. It is a fat-soluble coenzyme. It functions as an acceptor of reducing power from the flavin linked dehydrogenases.

CYTOCHROMES: another major class of oxidative enzymes in the respiratory chain is the cytochromes. The prosthetic group of a cytochrome is a derivative of heme and contains a single iron atom, which is responsible for the oxidative or reductive properties of the enzyme. On the basis of differences in absorption spectra, cytochromes can be divided into three main categories: cytochromes a, cytochromes b and cytochromes c. each of these groups has different function in the respiratory chain and can be further subdivided on the basis of minor differences in absorption spectra, Eg cytochromes c and c_1 or cytochromes a and a_3. The cytochromes act sequentially to transport electrons from coenzyme Q to O_2. Cytochromes a and a_3 together called cytochrome oxidases. Both of them also contain copper. But only cytochrome a_3 can react directly with oxygen.

7.5. ENERGETICS OF METABOLISM

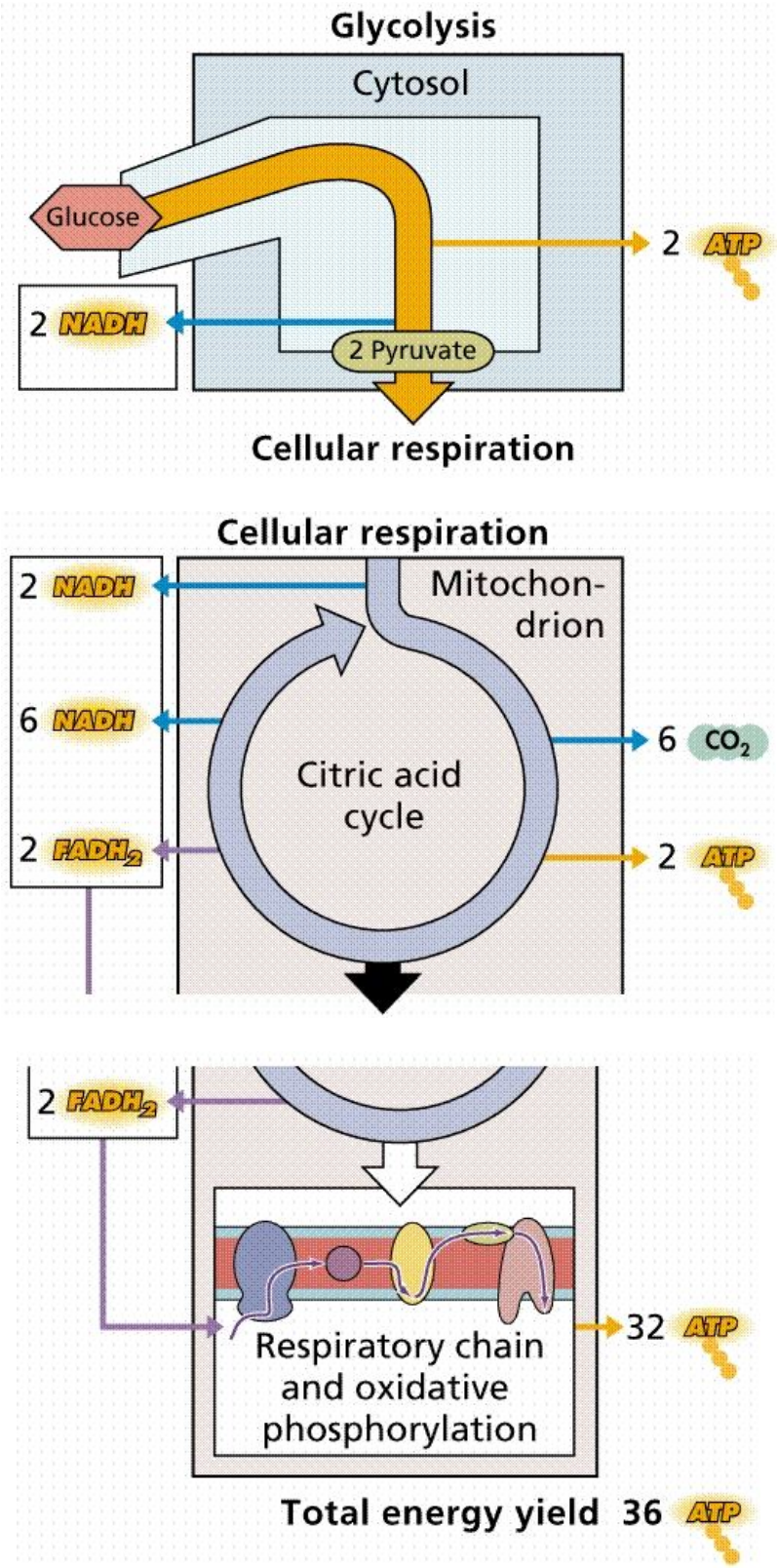


Fig.2. OVERVIEW OF ENERGETIC OF METABOLISM

7.6. LET US SUM UP

- The $\Delta G°$ or standard free energy change is related to the equilibrium constant K_{eq}, of a chemical reaction by the equation $\Delta \mathbf{G°} = \mathbf{-RT \ln K_{eq}}$
- The relationship between an oxidation- reduction potential difference and the standard free energy change is $\Delta \mathbf{G°} = \mathbf{- nFE°}$
- Oxidation is the loss of electrons; reduction is the gain of electrons.
- Electron transport chains are sequences of oxidation-reduction reactions that occur in cells.
- Oxidative phosphorylation - the electrons flow through the chains, much of their free energy is conserved in the form of ATP.
- A respiratory chain consists of enzymes having prosthetic groups or coenzymes.

7.7. LESSON END ACTIVITIES

What is energetics?
What is the equilibrium constant?
What are oxidation and reduction potentials?

Derive the relationship between oxidation and reduction potentials.
Explain about standard free energy change.
Derive the equation $\Delta G° = -RT \ln K_{eq}$

7.8. POINTS FOR DISCUSSION

Explain oxidative phosphorylation
Describe the respiratory chain in microorganisms.
What are the enzymes involved in respiratory chain?
Explain electron transport chain in microorganisms.

7.9 REFERENCES

1. Microbiology by Pelczar, Reid and Chan, McGraw Hill Book Company.
2. Microbiology, Fundamental and Applications by R.A. Atlas, McMillan Publishers.
3. General Microbiology by Powar and Daginawala, Himalaya Publishing House.
4. Microbial genetics by David friefelder

LESSON 8

AEROBIC AND ANAEROBIC GROWTH

Contents

8.0. AIMS AND OBJECTIVES

The chapter deals with the various aerobic and anaerobic processes through which the organisms obtain and utilize the energy for their growth.

8.1. INTRODUCTION

The term metabolism denotes all the organized chemical activities performed by a cell, which comprise two general types, energy production and energy utilization. Energy is the ability to do work, and the work of a bacterial cell is extensive and varied. Energy is utilized for the construction of the physical parts of the cell also it is required for synthesis of enzymes, nucleic acids, polysaccharides and other chemical components. It is also required for repair of damage and mere maintenance of the status quo and for the growth and

multiplication. It is required for the accumulation of certain nutrients in high concentration in the cell. A bacterial cell can be pictured as a dynamo of tremendous energy production.

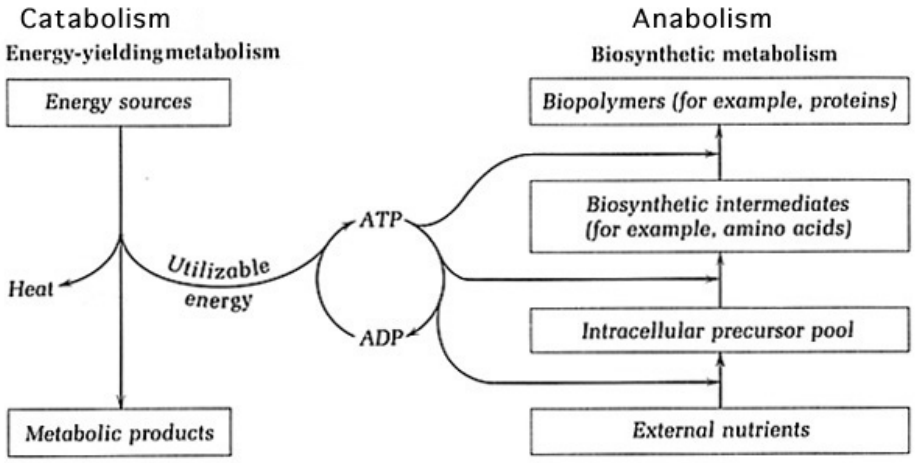


Fig 1. THE RELATIONSHIP BETWEEN CATABOLISM AND ANABOLISM IN A CELL

During catabolism, energy is changed from one form to another, and keeping with the laws of thermodynamics, such energy transformations are never completely efficient, i.e., some energy is lost in the form of heat. The efficiency of a catabolic sequence of reactions is the amount of energy made available to the cell (for anabolism) divided by the total amount of energy released during the reactions.

Metabolism has an energy-generating component, called catabolism, and an energy-consuming, biosynthetic component, called anabolism. Catabolic reactions or pathways produce energy as ATP, which can be utilized in anabolic reactions to build cell material from nutrients in the environment.

Anabolic pathways utilize ATP to provide energy for the synthesis of the monomeric compounds that are required for the manufacture of the small molecules needed in cells, i.e., carbohydrates, lipids, amino acids, nucleotides, vitamins, etc.

8.2. ATP

During catabolism, useful energy is temporarily conserved in the "high energy bond" of ATP - adenosine triphosphate. No matter what form of energy a cell uses as its primary source, the energy is ultimately transformed and conserved as ATP. ATP is the universal currency of energy exchange in biological systems. When energy is required during anabolism, it may be spent as the high energy bond of ATP which has a value of about 8 kcal per mole. Hence, the conversion of ADP to ATP requires 8 kcal of energy, and the hydrolysis of ATP to ADP releases 8 kcal.

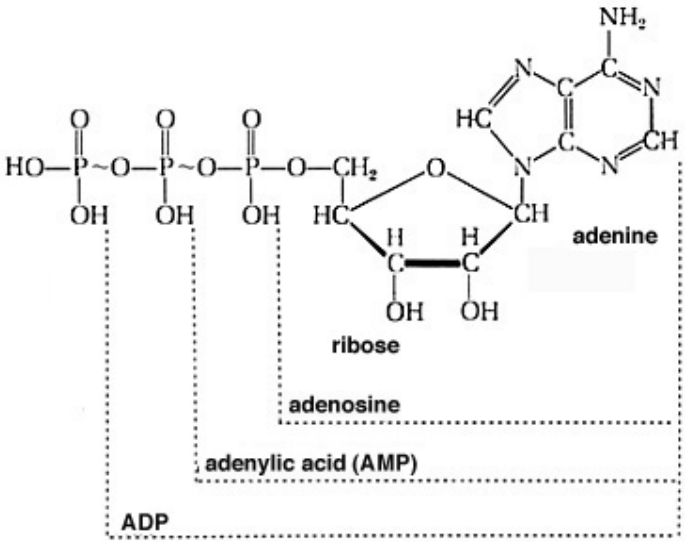


Fig.2. THE STRUCTURE OF ATP

ATP is derived from the nucleotide adenosine monophosphate (AMP) or adenylic acid, to which two additional phosphate groups are attached through pyrophosphate bonds (~P). These two bonds are energy rich in the sense that their hydrolysis yields a great deal more energy than a corresponding covalent bond. ATP acts as a coenzyme in energetic coupling reactions wherein one or both of the terminal phosphate groups is removed from the ATP molecule with the bond energy being used to transfer part of the ATP molecule to another molecule to activate its role in metabolism. For example, Glucose + ATP -----> Glucose-P + ADP or Amino Acid + ATP ----->AMP-Amino Acid + PPi.

Because of the central role of ATP in energy-generating metabolism, expect to see its involvement as a coenzyme in most energy-producing processes in cells.

8.3. NAD

Another coenzyme commonly involved in metabolism, derived from the vitamin niacin, is the pyridine nucleotide, NAD (Nicotinamide Adenine Dinucleotide). The basis for chemical transformations of energy usually involves oxidation/reduction reactions. For a biochemical to become oxidized, electrons must be removed by an oxidizing agent. The oxidizing agent is an electron acceptor that becomes reduced in the reaction. During the reaction, the oxidizing agent is converted to a reducing agent that can add its electrons to another chemical, thereby reducing it, and reoxidizing itself. The molecule that usually functions as the electron carrier in these types of coupled oxidation-reduction reactions in biological systems is NAD and its phosphorylated derivative, NADP. NAD or NADP can become alternately oxidized or reduced by the loss or gain of two electrons. The oxidized form of NAD is symbolized NAD; the reduced form is symbolized as $NADH_2$.

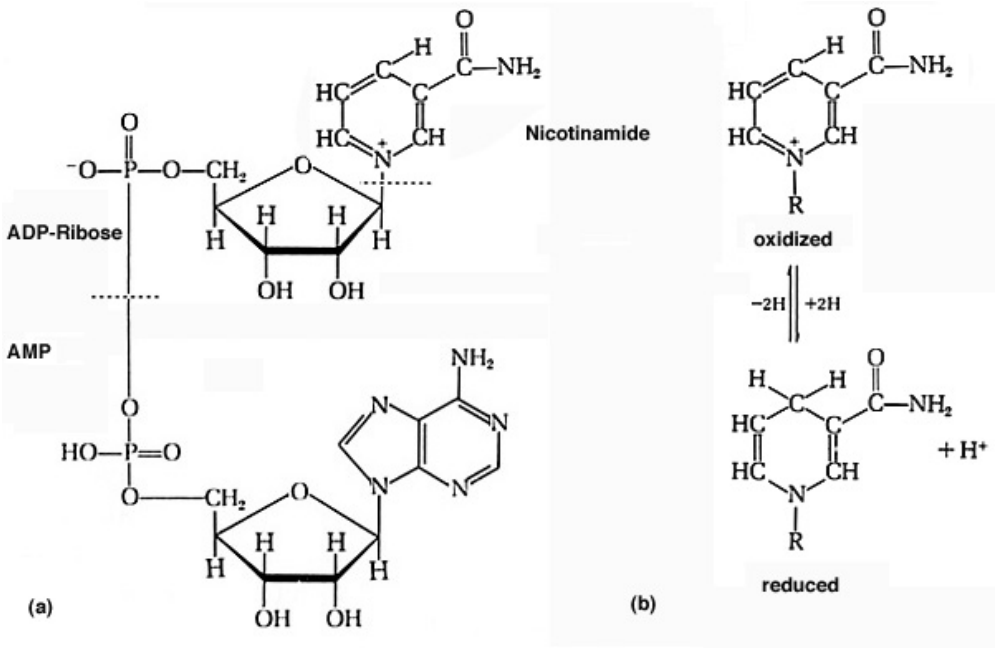


Fig.3. THE STRUCTURE OF NAD

(a) Nicotinamide Adenine Dinucleotide is composed of two nucleotide molecules: Adenosine monophosphate (adenine plus ribose-phosphate) and nicotinamide ribotide (nicotinamide plus ribose-phosphate). NADP has an identical structure except that it contains an additional phosphate group attached to one of the ribose residues. (b) The oxidized and reduced forms of the nicotinamide moiety of NAD. Nicotinamide is the active part of the molecule where the reversible oxidation and reduction takes place. The oxidized form of NAD has one hydrogen atom less than the reduced form and, in addition, has a positive charge on the nitrogen atom which allows it to accept a second electron upon reduction. Thus the correct way to symbolize the reaction is $NAD^+ + 2H$----->$NADH + H^+$. However, for convenience we will hereafter use the symbols NAD and $NADH_2$.

8.4. ATP SYNTHESIS

The objective of a catabolic pathway is to make ATP that is to transform either chemical energy or electromagnetic (light) energy into the chemical energy contained within the high-energy bonds of ATP. Cells fundamentally can produce ATP in two ways: substrate level phosphorylation and electron transport phosphorylation.

8.4.1. SUBSTRATE LEVEL PHOSPHORYLATION (SLP)

It is the simplest, oldest and least-evolved way to make ATP. In a substrate level phosphorylation, ATP is made during the conversion of an organic molecule from one form to another. Energy released during the conversion is partially conserved during the synthesis of the high energy bond of ATP. SLP occurs during fermentations and respiration (the TCA cycle), and even during some lithotrophic transformations of inorganic substrates.

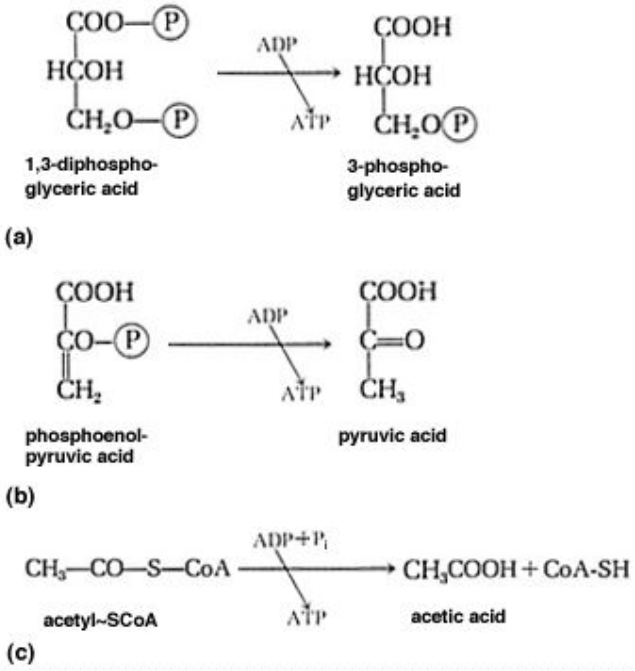


Fig.4. THREE EXAMPLES OF SUBSTRATE LEVEL PHOSPHORYLATION

(a) and (b) are the two substrate level phosphorylations that occur during the Embden Meyerhof pathway, but they occur in all other fermentation pathways which have an Embden-Meyerhof component. (c) is a substrate level phosphorylation found in *Clostridium* and *Bifidobacterium.* These are two anaerobic (fermentative) bacteria that learned how to make one more ATP from glycolysis beyond the formation of pyruvate.

8.4.2. ELECTRON TRANSPORT PHOSPHORYLATION (ETP)

Electron Transport Phosphorylation (ETP) is a much more complicated affair that evolved long after SLP. Electron Transport Phosphorylation takes place during respiration, photosynthesis, lithotrophy and possibly other types of bacterial metabolism. ETP requires that electrons removed from substrates be dumped into an electron transport system (EST) contained within a membrane. The electrons are transferred through the EST to some final electron acceptor in the membrane (like O_2 in aerobic respiration), while their traverse through the ETS results in the extrusion of protons and the establishment of a proton motive force (PMF) across the membrane. An essential component of the membrane for synthesis of ATP is a membrane-bound ATPase (ATP synthetase) enzyme. The ATPase enzyme transports protons, thereby utilizing the PMF (protons) during the synthesis of ATP. The idea in electron transport phosphorylation is to drive electrons through an ETS in the membrane, establish a PMF, and use the PMF to synthesize ATP. Obviously, ETP take a lot more "gear" than SLP, in the form of membranes, electron transport systems, ATPase enzymes, etc.

A familiar example of energy-producing and energy-consuming functions of the bacterial membrane, related to the establishment and use of PMF and the production of ATP, is given in the following drawing of the plasma membrane of *Escherichia coli.*

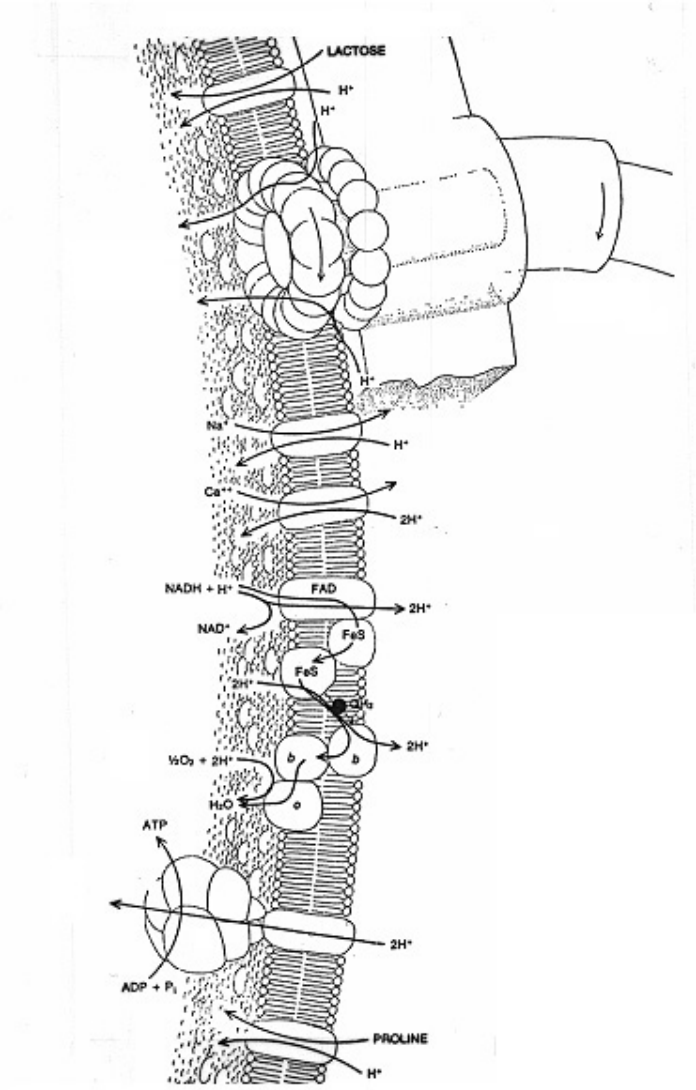


Fig.5. THE PLASMA MEMBRANE OF *Escherichia coli*

The membrane in cross-section reveals various transport systems, the flagellar motor apparatus (S and M rings), the respiratory electron transport system, and the membrane-bound ATPase enzyme. Reduced NADH + H^+ feeds pairs of electrons into the ETS. The ETS is the sequence of electron carriers in the membrane [FAD --> FeS --> QH2 (Quinone) --> (cytochromes) b --> b --> o] that ultimately reduces O_2 to H_2O during respiration. At certain points in the electron transport process, the electrons pass "coupling sites" and this results in the translocation of protons from the inside to the outside of the membrane, thus establishing the proton motive force (PMF) on the membrane. The PMF is used in three ways by the bacterium to do work or conserve energy: active transport (e.g. lactose and proline symport; calcium and sodium antiport); motility (rotation of the bacterial flagellum), and ATP synthesis (via the ATPase enzyme during the process of oxidative phosphorylation or electron transport phosphorylation).

8.5. ENERGY PRODUCTION BY ANAEROBIC PROCESSES

Heterotrophic bacteria can use a variety of organic compounds as energy sources. These compounds include carbohydrates, organic and fatty acids, and amino acids. For many microorganisms the preferred compounds are carbohydrates, especially the 6-carbon sugar glucose.

In the bacteria there exist three major pathways of glycolysis (the dissimilation of sugars): the classic Embden-Meyerhof pathway, which is also used by most eukaryotes, including yeast (*Saccharomyces*): the heterolactic pathway used by lactic acid bacteria, and the Entner-Doudoroff pathway used by vibrios and pseudomonads, including *Zymomonas*. Although the latter two pathways have some interesting applications in the manufacture of dairy products and alcoholic beverages, they will not be discussed further in this section.

8.5.1. GLYCOLYSIS

The most common pathway of glucose catabolism is the Embden-Meyerhof pathway of glycolysis (splitting of sugar). This process occurs widely in microorganisms and other higher organisms. It does not require the presence of oxygen and therefore can occur in both aerobic and anaerobic cells. In aerobic cells, glucose initially gets fermented to intermediate product pyruvate and by respiration in presence of oxygen pyruvate gets converted to carbon dioxide and water. For each molecule of glucose metabolized, two molecules of ATP are used up and four molecules of ATP are formed. Therefore for each molecule of glucose metabolized by glycolysis, there is a net yield of two ATP molecules.

This is the pathway of glycolysis most familiar to biochemists and eukaryotic biologists, as well as to brewers, bread makers and cheese makers. The pathway is operated by *Saccharomyces* to produce ethanol and CO_2. The pathway is used by the lactic acid bacteria to produce lactic acid, and it is used by many other bacteria to produce a variety of fatty acids, alcohols and gases. Some end products of Embden-Meyerhof fermentations are essential components of foods and beverages, and some are useful fuels and industrial solvents. Diagnostic microbiologists use bacterial fermentation profiles (e.g. testing an organism's ability to ferment certain sugars, or examining an organism's array of end products) in order to identify them, down to the genus level.

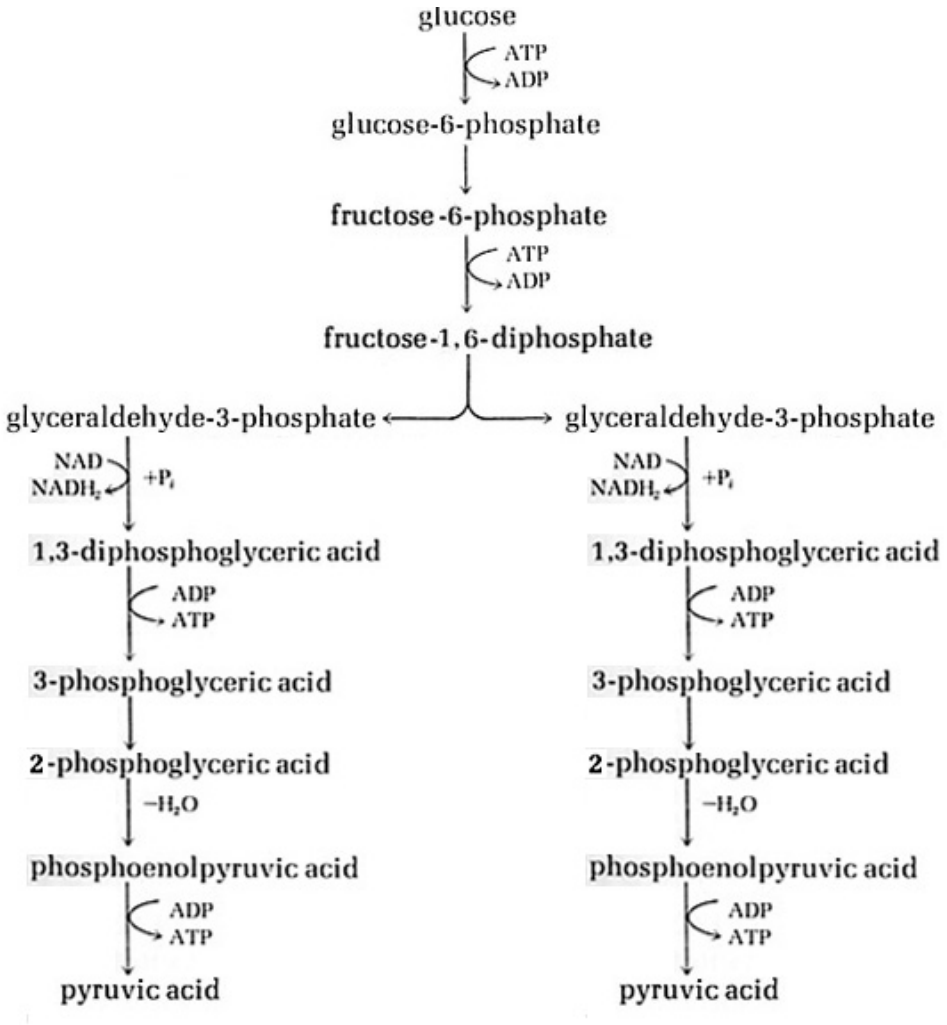


Fig.6. THE EMBDEN MEYERHOF PATHWAY FOR GLUCOSE DISSIMILATION

The overall reaction is the oxidation of glucose to 2 pyruvic acid. The two branches of the pathway after the cleavage are identical.

The first three steps of the pathway prime (phosphorylated) and rearrange the hexes for cleavage into 2 triodes (glyceraldehyde phosphate). Fructose 1,6-diphosphate aldolase is the key (cleavage) enzyme in the E-M pathway. Each triose molecule is oxidized and phosphorylated followed by two substrate level phosphorylations that yield 4 ATP during the drive to pyruvate.

Lactic acid bacteria reduce the pyruvate to lactic acid; yeast reduces the pyruvate to alcohol (ethanol) and CO_2.

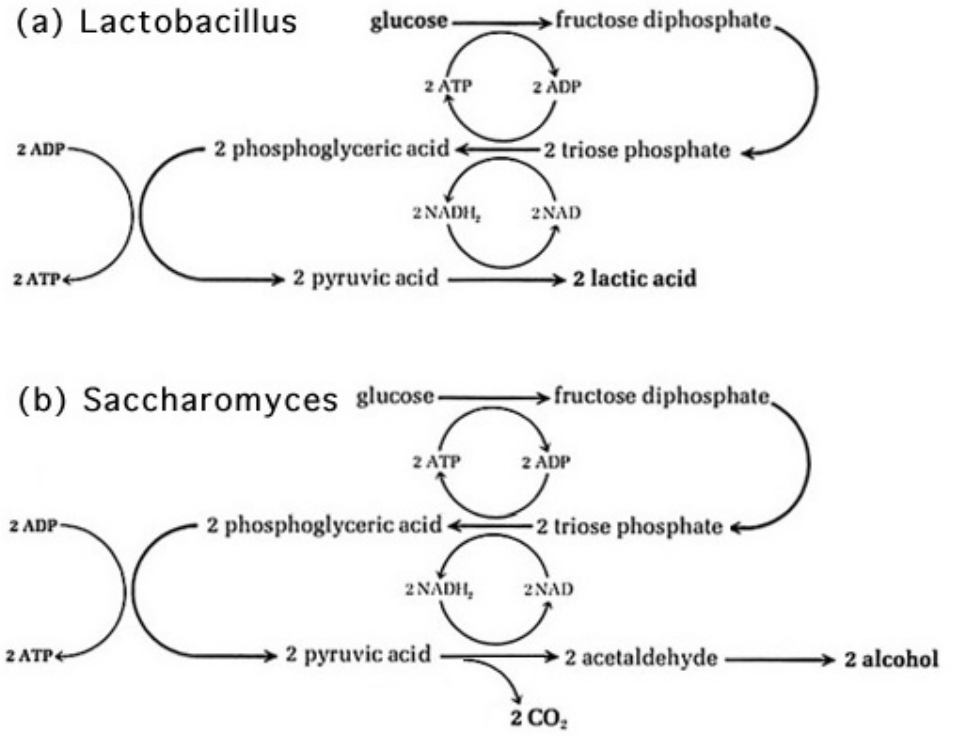


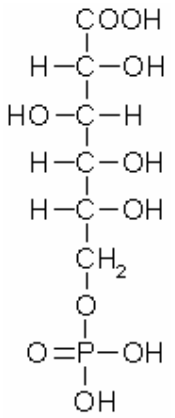
Fig.7.(a) The Embden Meyerhof pathway of lactic acid fermentation in lactic acid bacteria (Lactobacillus) and (b) the Embden Meyerhof pathway of alcohol fermentation in yeast (Saccharomyces).

The pathways yield two moles of end products and two moles of ATP per mole of glucose fermented. The steps in the breakdown of glucose to pyruvate are identical. The difference between the pathways is the manner of reducing pyruvic acid, thereby giving rise to different end products.

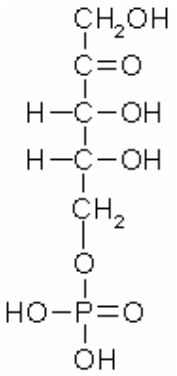
8.5.2. PENTOSE PHOSPHATE PATHWAY

The pathway, unlike glycolysis, is a catabolic reaction pathway. Since it involves some reactions of the glycolytic pathway, it has been viewed as a shunt of glycolysis and hence called as hexose monophosphate shunt (phosphogluconate pathway). Glucose can be oxidized by the HMP shunt with the liberation of electron pairs, which may enter the respiratory chain. This cycle is not generally considered a major energy-yielding pathway in most microorganisms. It provides reducing power in the form of NADPH + H^+, which is required in many biosynthetic reactions of the cell, and it provides pentose phosphates for use in nucleotide synthesis. Although it can produce energy for the cell as an alternate pathway for the oxidation of glucose, it is also a mechanism for obtaining energy from 5-carbon sugars. Specifically, six molecules of glucose 6 phosphate are oxidized to six molecules each of ribulose – 5 – phosphate and carbon dioxide; five molecules of glucose - 6- phosphate are then regenerated from six molecules of ribulose – 5 – phosphate. It is more probable that the pentose phosphate pathway feeds into glycolytic pathway by means of fructose – 6 – phosphate and glyceraldehydes – 3 – phosphate.

Glycolysis is not the only sugar-manipulating pathway in cells. DNA and RNA are both reliant on the production of the 5-carbon sugar ribose, and this is a central chemical in another oxidative pathway called the pentose phosphate pathway (PPP). The pathway generates a variety of sugars (including ribose), and unlike glycolysis, also produces NADPH, which is required by most cells for biosynthetic reactions. The pathway can be fiddled with by enzyme regulation to produce different ratios of NADPH to ribose.



6-phosphogluconate



Ribulose-5-phosphate

There are four essential reactions in the PPP:

1. **Oxidation**: the initial step of PPP oxidises glucose to 6-phosphogluconate, with concomitant production of NADPH.
2. **Decarboxylation**: 6-phosphogluconate is then decarboxylated to ribulose-5-phosphate, producing another molecule of NADPH, and releasing CO_2.
3. **Epimerisation**: ribulose-5-phosphate is an epimer of ribose-5-phosphate and may be converted to it by a simple isomerisation proceeding by an enediol intermediate. This reaction is the same as that catalysing conversion of DHAP and GAP, and glucose- and fructose-6-phosphate in glycolysis, and is catalysed by a similar enzyme.
4. **Transfer (transketolase and transaldolase reactions)**: most cells need far more NADPH than ribose. Consequently, the rest of the PPP consists of a variety of reactions which transfer 2-carbon (transketolase) and 3-carbon (transaldolase) units from sugar to sugar, generating a plethora of trioses, tetroses, pentoses, and even septuloses (epimerisation is also involved in this sequence). These reactions serve to regenerate fructose-6-phosphate and GAP, which may either be fed into glycolysis, or used to regenerate glucose-6-phosphate by gluconeogenesis.

Depending on the relative importance's of NADPH and ribose synthesis, the PPP can be tuned to:

- Generate masses of ribose (dividing cells need this for DNA synthesis):
  5 Glucose-6-P + ATP → 6 Ribose-5-P + ADP
- Generate NADPH and ribose in balanced quantities:
  Glucose-6-P + 2 NADP + H_2O → Ribose-5-P + 2 NADPH + CO_2
- Generate masses of NADPH (adipose tissue needs this for the synthesis of fatty acids) and simultaneously oxidize glucose completely:
  Glucose-6-P + 12 NADP + 7 H_2O → 12 NADPH + 6 CO_2 + P_i
- Any stoichiometry in between these extremes.

The PPP is very important in plants, where is comprises part of the Calvin cycle, which regenerates ribulose-5-phosphate from its carboxylation products. The PPP is often overlooked in biology, but it is of at least as much importance as glycolysis, and may well have evolved before it.

8.5.3. THE ENTNER-DOUDOROFF PATHWAY

Another pathway of glucose metabolism where glucose is phosphorylated to glucose-6-phosphate. It is found in both aerobic and anaerobic prokaryotes but not in eukaryotes. It is fairly widespread, particularly among Gram negative bacteria.

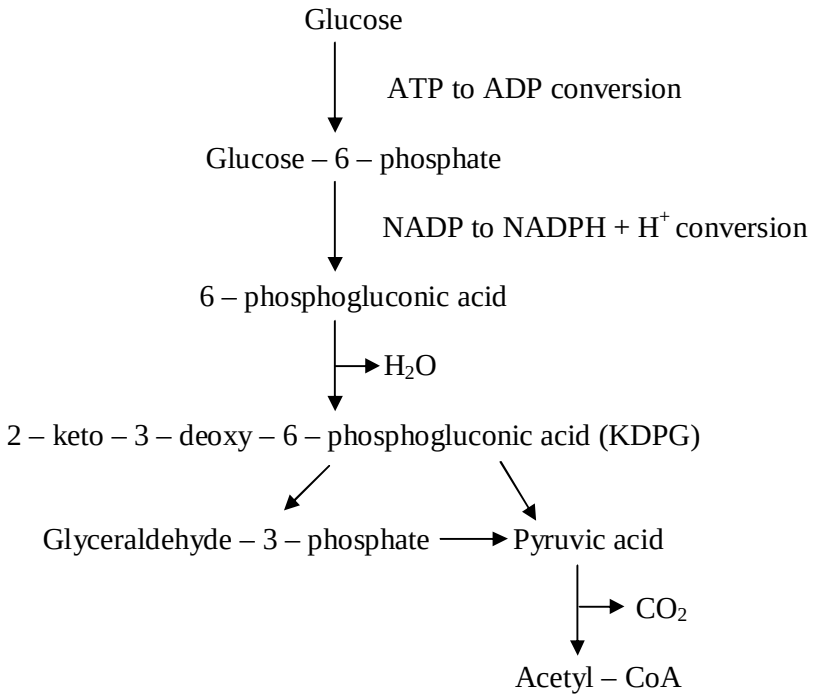


Fig.8. THE ENTNER – DOUDOROFF PATHWAY OF GLUCOSE

8.6. HETEROTROPHIC TYPES OF METABOLISM

Heterotrophy (i.e., chemoheterotrophy) is the use of an organic compound as a source of carbon and energy. It is the complete metabolism package. The cell oxidizes organic molecules in order to produce energy (catabolism) and then uses the energy to synthesize cellular material from these the organic molecules (anabolism). We animals are familiar with heterotrophic metabolism. Fungi and protozoa are all heterotrophs; many bacteria, but just a few archaea, are heterotrophs, Heterotrophic fungi and bacteria are the masters of decomposition and biodegradation in the environment. Heterotrophic metabolism is driven mainly by two metabolic processes: fermentations and respirations.

8.6.1. FERMENTATION

Anaerobes also produce energy by reactions called fermentations, which use organic compounds as electron donors and acceptors. Facultative anaerobic bacteria and obligately anaerobic bacteria employ many different kinds of fermentations to produce energy. The

lactic fermentation is a typical example. *Streptococcus lactis*, bacterium responsible for the normal souring of raw milk, dissimilates glucose to lactic acid, which accumulates in the medium as the sole fermentation product. By glycolysis, one molecule of glucose is converted to two molecules of pyruvic acid with concomitant production of two NADH + H^+. The pyruvic acid (2molecules) is converted to lactic acid (2molecules) and NAD (2molecules) using 2 molecules of NADH + H^+.

In other carbohydrate fermentations, the initial stages of glucose dissimilation frequently, but not always, follow the scheme of glycolysis. Differences in carbohydrate fermentations usually occur in the ways the resulting pyruvic acid is used. Thus pyruvic acid is hub of carbohydrate fermentations. Some anaerobes do not have functional glycolytic system. They may have carbohydrate fermentation pathways that use the pentose phosphate pathway and the Entner – Doudoroff pathway. Fermentations of noncarbohydrate substrates, such as amino acids involve highly specific pathways.

Fermentation is an ancient mode of metabolism, and it must have evolved with the appearance of organic material on the planet. Fermentation is metabolism in which energy is derived from the partial oxidation of an organic compound using organic intermediates as electron donors and electron acceptors. No outside electron acceptors are involved; no membrane or electron transport system is required; all ATP is produced by substrate level phosphorylation.

By definition, fermentation may be as simple as two steps illustrated in the following model. Indeed, some amino acid fermentations by the clostridia are this simple. But the pathways of fermentation are a bit more complex, usually involving several preliminary steps to prime the energy source for oxidation and substrate level phosphorylations.

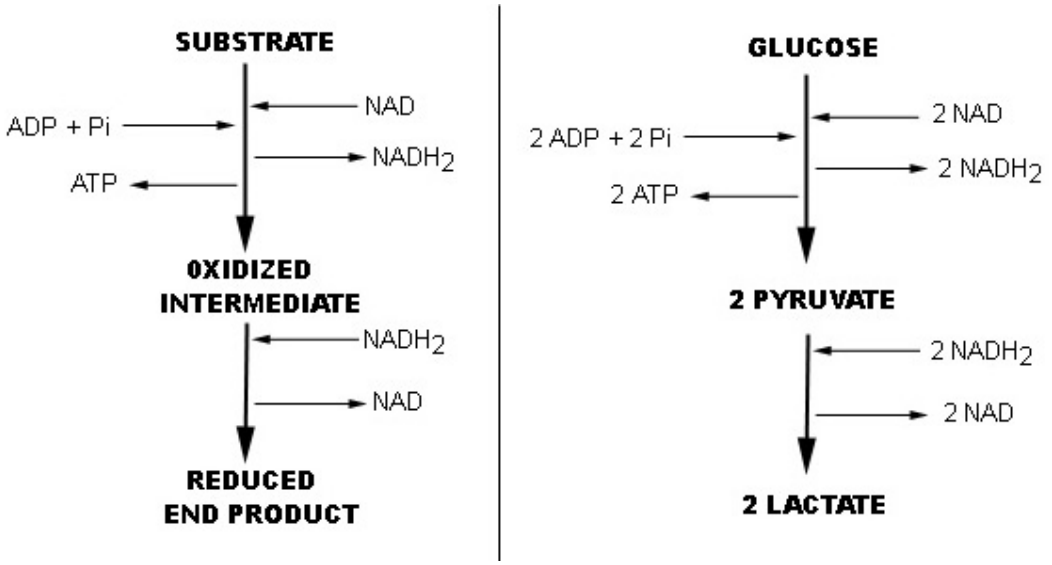


Fig.9. Model fermentation

The substrate is oxidized to an organic intermediate; the usual oxidizing agent is NAD. Some of the energy released by the oxidation is conserved during the synthesis of ATP by the process of substrate level phosphorylation. Finally, the oxidized intermediate is reduced to end products. Note that $NADH_2$ is the reducing agent, thereby balancing its redox

ability to drive the energy-producing reactions. R. In lactic fermentation by *Lactobacillus*, the substrate (glucose) is oxidized to pyruvate, and pyruvate becomes reduced to lactic acid. Redox balance is maintained by coupling oxidations to reductions within the pathway. For example, in lactic acid fermentation via the EmbdenMeyerhof pathway, the oxidation of glyceraldehyde phosphate to phosphoglyceric acid is coupled to the reduction of pyruvic acid to lactic acid.

In biochemistry, for the sake of convenience, fermentation pathways start with glucose. This is because it is the simplest molecule, requiring the fewest enzymatic (catalytic) steps, to enter into a pathway of glycolysis and central metabolism.

Besides lactic acid, Embden-Meyerhof fermentations in bacteria can lead to a wide array of end products depending on the pathways taken in the reductive steps after the formation of pyruvic acid. Usually, these bacterial fermentations are distinguished by their end products into the following groups.

1. Homolactic Fermentation. Lactic acid is the sole end product. Pathway of the homolactic acid bacteria (*Lactobacillus* and most streptococci). The bacteria are used to ferment milk and milk products in the manufacture of yogurt, buttermilk, sour cream, cottage cheese, cheddar cheese, and most fermented dairy products.

2. Mixed Acid Fermentations. Mainly the pathway of the *Enterobacteriaceae*. End products are a mixture of lactic acid, acetic acid, formic acid, succinate and ethanol, with the possibility of gas formation (CO_2 and H_2) if the bacterium possesses the enzyme formate dehydrogenase, which cleaves formate to the gases.

2a. Butanediol Fermentations. Forms mixed acids and gases as above, but, in addition, 2,3 butanediol from the condensation of 2 pyruvate. The use of the pathway decreases acid formation (butanediol is neutral) and causes the formation of a distinctive intermediate, acetoin. Water microbiologists have specific tests to detect low acid and acetoin in order to distinguish non fecal enteric bacteria (butanediol formers, such as *Klebsiella* and *Enterobacter*) from fecal enterics (mixed acid fermenters, such as *E. coli, Salmonella* and *Shigella*).

3. Butyric acid fermentations, as well as the butanol-acetone fermentation (below), are run by the clostridia, the masters of fermentation. In addition to butyric acid, the clostridia form acetic acid, CO_2 and H_2 from the fermentation of sugars. Small amounts of ethanol and isopropanol may also be formed.

3a. Butanol-acetone fermentation. Butanol and acetone were discovered as the main end products of fermentation by *Clostridium acetobutylicum* during the World War I. This discovery solved a critical problem of explosives manufacture (acetone is required in the manufacture gunpowder) and is said to have affected the outcome of the War. Acetone was distilled from the fermentation liquor of *Clostridium acetobutylicum,* which worked out pretty good if you were on our side, because organic chemists hadn't figured out how to synthesize it chemically. You can't run a war without gunpowder, at least you couldn't in those days.

4. Propionic acid fermentation. This is an unusual fermentation carried out by the propionic acid bacteria which include corynebacteria, *Propionibacterium* and *Bifidobacterium.*

Although sugars can be fermented straight through to propionate, propionic acid bacteria will ferment lactate (the end product of lactic acid fermentation) to acetic acid, CO_2 and propionic acid. The formation of propionate is a complex and indirect process involving 5 or 6 reactions. Overall, 3 moles of lactate are converted to 2 moles of propionate + 1 mole of acetate + 1 mole of CO_2, and 1 mole of ATP is squeezed out in the process. The propionic acid bacteria are used in the manufacture of Swiss cheese, which is distinguished by the distinct flavor of propionate and acetate, and holes caused by entrapment of CO_2.

The Embden-Meyerhof pathway for glucose dissimilation, as well as the TCA cycle, is two pathways that are at the center of metabolism in nearly all organisms. Not only do these pathways dissimilate organic compounds and provide energy, they also provide the precursors for biosynthesis of macromolecules that make up living systems. These are sometimes called amphibolic pathways since the have both an anabolic and a catabolic function.

8.6.2. ENERGY PRODUCTION BY AEROBIC PROCESSES OR RESPIRATIONS

Compared to fermentation as a means of oxidizing organic compounds, respiration is a lot more complicated. Respirations result in the complete oxidation of the substrate by an outside electron acceptor. In addition to a pathway of glycolysis, four essential structural or metabolic components are needed: Tricarboxylic acid cycle, membrane and associated electron transport system, outside electron acceptor, trans membranous ATPase system.

1. The **Tricarboxylic acid (TCA) cycle** (also known as the citric acid cycle or the Kreb's cycle): when an organic compound is utilized as a substrate, the TCA cycle is used for the complete oxidation of the substrate. The end product that always results from the complete oxidation of an organic compound is CO_2. TCA cycle is a sequence of reactions that generate energy in the form of ATP and reduced coenzyme molecules ($NADH_2$ and $FADH_2$). It also performs other functions. Many intermediates in the cycle are precursors in the biosynthesis of amino acids, purines, pyrimidines. Eg, oxaloacetic acid and alpha ketoglutaric acid are amino acids precursors. Thus TCA cycle is an amphibolic cycle, which means that it functions not only in catabolic but also in anabolic reactions.

2. A **membrane and an associated electron transport system (ETS)**. The ETS is a **sequence of electron carriers in the plasma membrane** that transports electrons taken from the substrate through the chain of carriers to a final electron acceptor. The electrons enter the ETS at a very low redox potential (E'_o) and exit at a relatively high redox potential. This drop in potential releases energy that can be harvested by the cells in the process of ATP synthesis by the mechanisms of **electron transport phosphorylation**. The operation of the ETS establishes a proton motive force (PMF) due to the formation of a proton gradient across the membrane.

3. An **outside electron acceptor** ("outside", meaning it is not internal to the pathway, as is pyruvate in fermentation). For **aerobic respiration** the electron acceptor is O_2, of course. Molecular oxygen is reduced to H_20 in the last step of the electron transport system. But in the bacterial processes of **anaerobic respiration**, the final electron acceptors may be SO_4 or S or NO_3 or NO_2 or certain other inorganic compounds, or even an organic compound, such as fumarate.

4. A trans membranous **ATPase enzyme** (ATP synthetase). This enzyme utilizes the proton motive force established on the membrane (by the operation of the ETS) to synthesize ATP in the process of **electron transport phosphorylation**. It is believed that the trans membranous Fo subunit is a proton transport system that transports $2H^+$ to the F1 subunit (the actual ATPase) on the inside of the membrane. The 2 protons are required and consumed during the synthesis of ATP from ADP plus Pi. See Figure 6 -the membrane of *E. coli.* The reaction catalyzed by the ATPase enzyme is ADP + Pi + 2 H^+ <----------> ATP. (It is important to appreciate the reversibility of this reaction in order to account for how a fermentative bacterium, without an ETS, could establish a necessary PMF on the membrane for transport or flagellar rotation. If such an organism has a trans membranous ATPase, it could produce ATP by SLP, and subsequently the ATPase could hydrolyze the ATP, thereby releasing protons to the outside of the membrane.)

Figure 10 of aerobic respiration integrates these metabolic processes into a scheme that represents the overall process of respiratory metabolism. A substrate such as glucose is completely oxidized to CO_2 by the combined pathways of glycolysis and the TCA cycle. Electrons removed from the glucose by NAD are fed into the ETS in the membrane. As the electrons traverse the ETS, a PMF becomes established across the membrane. The electrons eventually reduce an outside electron acceptor, O_2, and reduce it to H_20. The PMF on the membrane is used by the ATPase enzyme to synthesize ATP by a process referred to as "oxidative phosphorylation".

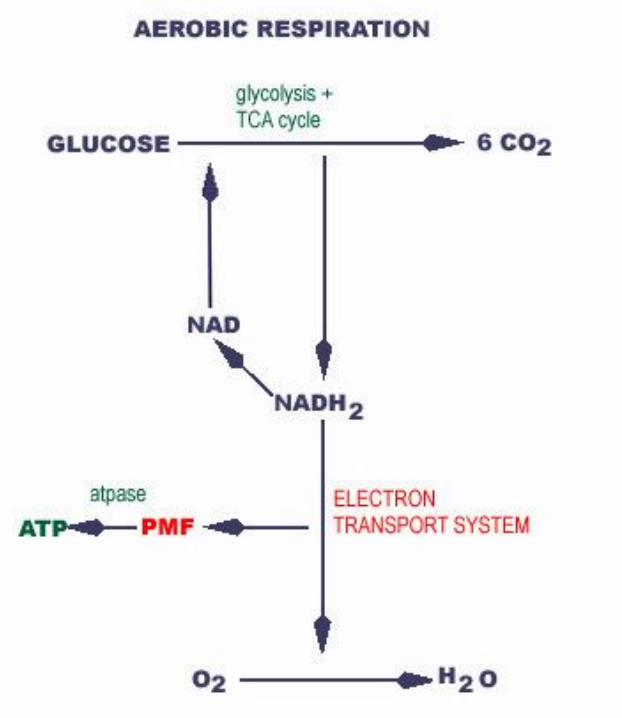


Fig.10. MODEL OF AEROBIC RESPIRATION

The overall reaction for the aerobic respiration of glucose is

Glucose + 6 O_2 ----------> 6 CO_2 + 6 H_2O

In a heterotrophic respiration, glucose is dissimilated in a pathway of glycolysis to the intermediate, pyruvate, and it the pyruvate that is moved into the TCA cycle, eventually becoming oxidized to 3 CO_2. Since 2 pyruvate are formed from one glucose, the cycle must turn twice for every molecule of glucose oxidized to 6 CO_2. The TCA cycle (including the steps leading into it) accounts for the complete oxidation of the substrate and it provide 10 pairs of electrons (from glucose) for transit through the ETS. For every pair of electrons put into the ETS, 2 or 3 ATP may be produced, so a huge amount of ATP is produced in a respiration, compared to fermentation.

The TCA cycle is an important amphibolic pathway, several intermediates of the cycle may be withdrawn for anabolic (biosynthetic) pathways).

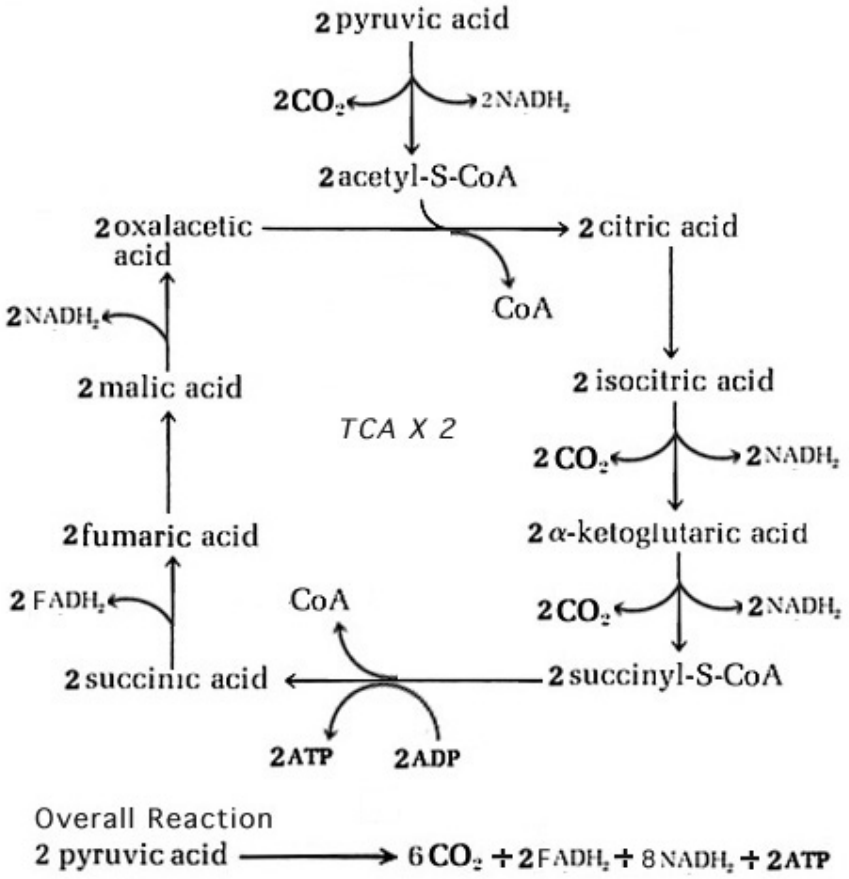


Fig.11. The tricarboxylic acid (TCA) or Kreb's cycle.

Also called the citric acid cycle because citric acid is one of the first intermediates formed during the cycle. When an organic compound is utilized during respiration it is invariably oxidized via the TCA cycle. Combined with the pathway(s) of glycolysis (e.g. Embden-Meyerhof) TCA is central to the metabolism of all heterotrophic respiratory organisms.

8.7. CATABOLISM OF LIPIDS

Glucose is the single most important source of energy for most cells. However, for many microorganisms, other substances, such as lipids and proteins, may be used as alternate sources of energy. The breakdown of lipids or fats begins with the cleavage of triglycerides by the addition of water to form glycerol and fatty acids by means of enzymes called lipases. Glycerol as a component of fats can be converted into an intermediate of the glycolytic pathway (dihydroxyacetone phosphate). The dihydroxyacetone phosphate formed would be broken down by the mechanisms. Fatty acids are oxidized by the successive removal of 2-carbon fragments in the form of acetyl – CoA, a process known as β-oxidation. The acetyl – CoA formed can then enter the TCA cycle, and the hydrogen atoms and their electrons enter the respiratory transport chain, leading to oxidative phosphorylation. There is more energy yield per gram of fat than per gram of carbohydrate. However, relatively few microbial species are effective in breaking down lipids of either simple or complex types, partly because of the limited solubility of lipids.

8.8. CATABOLISM OF PROTEINS

Many heterotrophic microorganisms can degrade exogenous proteins, using the products as carbon and nitrogen energy sources. Since protein molecules are too large to pass into the cell, bacteria secrete exoenzymes called proteases that hydrolyze exogenous proteins to peptides, which are then transported into the cell cytoplasm. Bacteria produce peptidases that breakdown peptides to the individual amino acids, which are then broken down according to the specific amino acid and the species or strain of bacteria breaking it down. Where amino acids are broken down, the carbon skeletons of the amino acids undergo oxidation to compounds that may enter the TCA cycle for further oxidation. Entry into the TCA cycle can be via acetyl CoA, alpha ketoglutaric acid, succinic acid, fumaric acid, or oxaloacetic acid.

8.9. RESPIRATION WITHOUT OXYGEN IN SOME BACTERIA OR ANAEROBIC RESPIRATION

Some bacteria which are ordinarily aerobic can grow anaerobically if nitrate is present. Eg: *Aquaspirillum itersonii*, an aquatic bacterium, is dependent on oxygen unless potassium nitrate is added to the medium. In such cases nitrate essentially substitutes for oxygen as the final electron acceptor in the respiratory chain. The pathways for the dissimilation of the carbon and energy sources are identical with those in aerobic respiration, and electron transport occurs via a respiratory chain similar to that in aerobic cells. Oxygen is replaced as the terminal electron acceptor by nitrate. However, in some strict anaerobes, other compounds, such as carbon dioxide, or ions, such as sulphate ion, can be the terminal electron acceptors.

Respiration in some prokaryotes is possible using electron acceptors other than oxygen (O_2). This type of respiration in the absence of oxygen is referred to as anaerobic respiration. Although anaerobic respiration is more complicated than the foregoing statement, in its simplest form it represents the substitution or use of some compound other than O_2 as a final electron acceptor in the electron transport chain. Electron acceptors used by prokaryotes for respiration or methanogenesis (an analogous type of energy generation in archaea) are described in the table1.

Electron acceptor	Reduced end product	Name of process	Organism
O_2	H_2O	aerobic respiration	*Escherichia, Streptomyces*
NO_3	NO_2, NH_3 or N_2	anaerobic respiration: denitrification	*Bacillus, Pseudomonas*
SO_4	S or H_2S	anaerobic respiration: sulfate reduction	*Desulfovibrio*
fumarate	succinate	anaerobic respiration: using an organic e- acceptor	*Escherichia*
CO_2	CH_4	methanogenesis	*Methanococcus*

TABLE.1. ELECTRON ACCEPTORS FOR RESPIRATION AND METHANOGENESIS IN PROCARYOTES

Biological methanogenesis is the source of methane (natural gas) on the planet. Methane is preserved as a fossil fuel (until we use it all up) because it is produced and stored under anaerobic conditions, and oxygen is needed to oxidize the CH_4 molecule. Methanogenesis is not really a form of anaerobic respiration, but it is a type of energy-generating metabolism that requires an outside electron acceptor in the form of CO_2.

Denitrification is an important process in agriculture because it removes NO_3 from the soil. NO_3 is a major source of nitrogen fertilizer in agriculture. Almost one-third the cost of some types of agriculture is in nitrate fertilizers the use of nitrate as a respiratory electron acceptor is usually an alternative to the use of oxygen. Therefore, soil bacteria such as *Pseudomonas* and *Bacillus* will use O_2 as an electron acceptor if it is available, and disregard NO_3. This is the rationale in maintaining well-aerated soils by the agricultural practices of plowing and tilling. *E. coli* will utilize NO_3 (as well as fumarate) as a respiratory electron acceptor and so it may be able to continue to respire in the anaerobic intestinal habitat.

Sulfate reduction is not an alternative to the use of O_2 as an electron acceptor. It is an obligatory process that occurs only under anaerobic conditions. Methanogens and sulfate reducers may share habitat, especially in the anaerobic sediments of eutrophic lakes such as Lake Mendota, where they crank out methane and hydrogen sulfide at a surprising rate.

Anaerobic respiring bacteria and methanogens play an essential role in the biological cycles of carbon, nitrogen and sulfur. In general, they convert oxidized forms of the elements to a more reduced state. The lithotrophic prokaryotes metabolize the reduced forms of nitrogen and sulfur to a more oxidized state in order to produce energy. The methanotrophic bacteria, which uniquely posses the enzyme methane monooxygenase, can oxidize methane as a source of energy. Among all these groups of prokaryotes there is a minicycle of the elements in a model ecosystem.

8.10. LITHOTROPHIC TYPES OF METABOLISM

Lithotrophy is the use of an inorganic compound as a source of energy. Most lithotrophic bacteria are aerobic respirers that produce energy in the same manner as all aerobic respiring organisms: they remove electrons from a substrate and put them through an electron transport system that will produce ATP by electron transport phosphorylation.

Lithotrophs just happen to get those electrons from an inorganic, rather than an organic, compound.

Some lithotrophs are facultative lithotrophs, meaning they are able to use organic compounds, as well, as sources of energy. Other lithotrophs do not use organic compounds as sources of energy; in fact, they won't transport organic compounds. CO_2 is the sole source of carbon for the methanogens and the nitrifying bacteria and a few other species scattered about in other groups.

Most lithotrophs get their carbon from CO_2 and are thus autotrophs and are properly referred to as lithoautotrophs or chemoautotrophs. The lithotrophs are a very diverse group of prokaryotes, united only by their ability to oxidize an inorganic compound as an energy source.

Lithotrophy runs through the Bacteria and the Archaea. If one considers methanogen oxidation of H_2 a form of lithotrophy, then probably most of the Archaea are lithotrophs.

Lithotrophs are usually organized into "physiological groups" based on their inorganic substrate for energy production and growth.

Physiological group	Energy source	Oxidized end product	Organism
Hydrogen bacteria	H_2	H_2O	*Alcaligenes, Pseudomonas*
Methanogens	H_2	H_2O	*Methanobacterium*
Carboxydobacteria	CO	CO_2	*Rhodospirillum, Azotobacter*
Nitrifying bacteria*	NH_3	NO_2	*Nitrosomonas*
Nitrifying bacteria*	NO_2	NO_3	*Nitrobacter*
Sulfur oxidizers	H_2S or S	SO_4	*Thiobacillus, Sulfolobus*
Iron bacteria	Fe^{++}	Fe^{+++}	*Gallionella, Thiobacillus*

* The overall process of nitrification, conversion of NH_3 to NO_3, requires a consortium of microorganisms.

TABLE 2. PHYSIOLOGICAL GROUPS OF LITHOTROPHS

The hydrogen bacteria oxidize H_2 (hydrogen gas) as an energy source. The hydrogen bacteria are facultative lithotrophs as evidenced by the pseudomonads that fortuitously possess a hydrogenase enzyme that will oxidize H_2 and put the electrons into their respiratory ETS. They will use H_2 if they find it in their environment even though they are typically heterotrophic. Indeed, most hydrogen bacteria are nutritionally versatile in their ability to use a wide range of carbon and energy sources. The bacterial electron transport system.

The methanogens used to be considered a major group of hydrogen bacteria - until it was discovered that they are Archaea. The methanogens are able to oxidize H_2 as a sole source of energy while transferring the electrons from H_2 to CO_2 in its reduction to methane. Metabolism of the methanogens is absolutely unique, yet methanogens represent the most prevalent and diverse group of Archaea. Methanogens use H_2 and CO_2 to produce cell material and methane. They have unique enzymes and electron transport processes. Their

type of energy generating metabolism is never seen in the Bacteria, and their mechanism of autotrophic CO_2 fixation is very rare, except in methanogens.

The carboxydobacteria are able to oxidize CO (carbon monoxide) to CO_2, using an enzyme CODH (carbon monoxide dehydrogenase). The carboxydobacteria are not obligate CO users, i.e., some are also hydrogen bacteria, and some are phototrophic bacteria. Interestingly, the enzyme CODH used by the carboxydobacteria to oxidize CO to CO_2, is used by the methanogens for the reverse reaction - the reduction of CO_2 to CO - in their unique pathway of CO_2 fixation.

The nitrifying bacteria are represented by two genera, *Nitrosomonas* and *Nitrobacter*. Together these bacteria can accomplish the oxidation of NH_3 to NO_3, known as the process of nitrification. No single organism can carry out the whole oxidative process. *Nitrosomonas* oxidizes ammonia to NO_2 and *Nitrobacter* oxidizes NO_2 to NO_3. Most of the nitrifying bacteria are obligate lithoautotrophs, the exception being a few strains of *Nitrobacter* that will utilize acetate. CO_2 fixation utilizes RUBP carboxylase and the Calvin Cycle. Nitrifying bacteria grow in environments rich in ammonia, where extensive protein decomposition is taking place. Nitrification in soil and aquatic habitats is an essential part of the nitrogen cycle.

Lithotrophic sulfur oxidizers include both Bacteria (e.g. *Thiobacillus*) and Archaea (e.g. *Sulfolobus*). Sulfur oxidizers oxidize H_2S (sulfide) or S (elemental sulfur) as a source of energy. Similarly, the purple and green sulfur bacteria oxidize H_2S or S as an electron donor for photosynthesis, and use the electrons for CO_2 fixation (the dark reaction of photosynthesis). Obligate autotrophy, which is nearly universal among the nitrifiers, is variable among the sulfur oxidizers. Lithoautotrophic sulfur oxidizers are found in environments rich in H_2S, such as volcanic hot springs and fumaroles, and deep-sea thermal vents. Some are found as symbionts and endosymbionts of higher organisms. Since they can generate energy from an inorganic compound and fix CO_2 as autotrophs, they may play a fundamental role in primary production in environments that lack sunlight. As a result of their lithotrophic oxidations, these organisms produce sulfuric acid (SO_4), and therefore tend to acidify their own environments. Some of the sulfur oxidizers are acidophiles that will grow at a pH of 1 or less. Some are hyperthermophiles that grow at temperatures of 115 degrees C.

Iron bacteria oxidize Fe^{++} (ferrous iron) to Fe^{+++} (ferric iron). At least two bacteria probably oxidize Fe^{++} as a source of energy and/or electrons and are capable of lithoautotrophic growth: the stalked bacterium *Gallionella*, which forms flocculant rust-colored colonies attached to objects in nature, and *Thiobacillus ferrooxidans*, which is also a sulfur-oxidizing lithotroph.

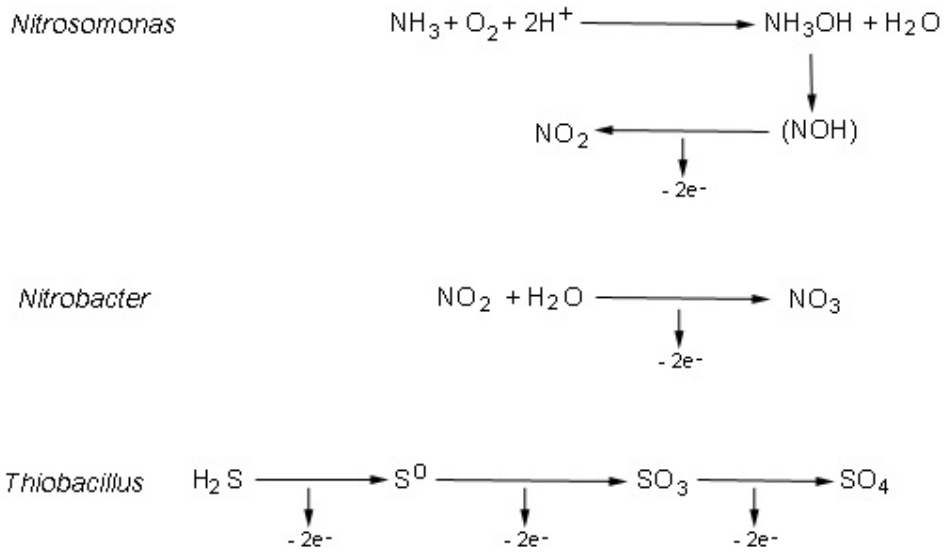


Fig.12. Lithotrophic oxidations.

These reactions produce energy for metabolism in the nitrifying and sulfur oxidizing bacteria.

8.11. HETEROTROPHIC CO_2 FIXATION

This phenomenon (unrelated to autotrophic CO_2 fixation) is important because it provides a mechanism for synthesis of compounds of the TCA cycle from the products of carbohydrate metabolism. Two types of CO_2 fixing reactions occur in heterotrophic bacteria. First type is essentially irreversible and occurs in many bacteria.

Eg:

$$\text{Phosphoenolpyruvate (PEP)} + CO_2 \xrightarrow{\text{PEP carboxylase, acetyl CoA}} \text{oxaloacetate} + Pi$$

A variation of this reaction requires a nucleoside diphosphate

$$PEP + ADP + CO_2 \xrightleftharpoons{\text{PEP carboxylase, } Mg^{2+}} \text{oxaloacetate} + ATP$$

Second type requires the vitamin biotin for activity.

$$ATP + \text{pyruvate} + CO_2 \xrightleftharpoons{\text{Pyruvate carboxylase, biotin, } Mg^{2+}\text{, acetyl CoA}} \text{oxaloacetate} + ADP + Pi$$

8.12. GLYOXYLATE CYCLE

The glyoxylate cycle is used by some microorganisms when acetate is the sole carbon source or during oxidation of primary substrates (such as higher fatty acids) that are cleaved to acetyl CoA without the intermediate formation of pyruvic acid. This pathway does not occur in higher organisms because they are never forced to feed on 2-carbon molecules alone. The specific enzymes of the glyoxylate cycle are isocitrate lyase and malate synthase. Enzymes which carry out replenishment reactions are known as anaplerotic enzymes; their function is to maintain the pool of essential intermediates for biosynthesis.

8.13. ENERGY PRODUCTION BY PHOTOSYNTHESIS

Plants, algae and cyanobacteria are photoautotrophs. They use light as their source of energy and carbon dioxide as their sole source of carbon. In order for carbon dioxide to be useful for metabolism, it must first be reduced to carbohydrate. This process by which light is used to convert carbon dioxide to carbohydrate is called photosynthesis. It has two important requirements – a large amount of energy in the form of ATP, and a large quantity of a chemical reductant, water.

General equation for bacterial photosynthesis in the presence of light and bacteriochlorophyll:

$$2H_2A + CO_2 \longrightarrow (CH_2O)_x + 2A + H_2O$$

Here H_2A represents the chemical reductant, such as the inorganic compounds H_2, H_2S, or $H_2S_2O_3$, or the organic compounds lactate or succinate. If H_2A in the equation stands for H_2S, then A would stand for S.

Phototrophy is the use of light as a source of energy for growth, more specifically the conversion of light energy into chemical energy in the form of ATP. Procaryotes that can convert light energy into chemical energy include the photosynthetic cyanobacteria, the purple and green bacteria, and the "halobacteria" (actually archaea). The cyanobacteria conduct plant photosynthesis, called oxygenic photosynthesis; the purple and green bacteria conduct bacterial photosynthesis or anoxygenic photosynthesis; the extreme halophilic archaea use a type of nonphotosynthetic photophosphorylation mediated by a pigment, bacteriorhodopsin, to transform light energy into ATP.

Photosynthesis is the conversion of light energy into chemical energy that can be used in the formation of cellular material from CO_2. Photosynthesis is a type of metabolism separable into a catabolic and anabolic component. The catabolic component of photosynthesis is the light reaction, wherein light energy is transformed into electrical energy, then chemical energy. The anabolic component involves the fixation of CO_2 and its use as a carbon source for growth, usually called the dark reaction. In photosynthetic procaryotes there are two types of photosynthesis and two types of CO_2 fixation.

The Light Reactions depend upon the presence of chlorophyll, the primary light-harvesting pigment in the membrane of photosynthetic organisms. Absorption of a quantum of light by a chlorophyll molecule causes the displacement of an electron at the reaction center. The displaced electron is an energy source that is moved through a membrane photosynthetic electron transport system, being successively passed from an iron-sulfur protein (X) to a quinone to a cytochrome and back to chlorophyll. As the electron is transported, a proton motive force is established on the membrane, and ATP is synthesized by an ATPase enzyme. This manner of converting light energy into chemical energy is called cyclic photophosphorylation.

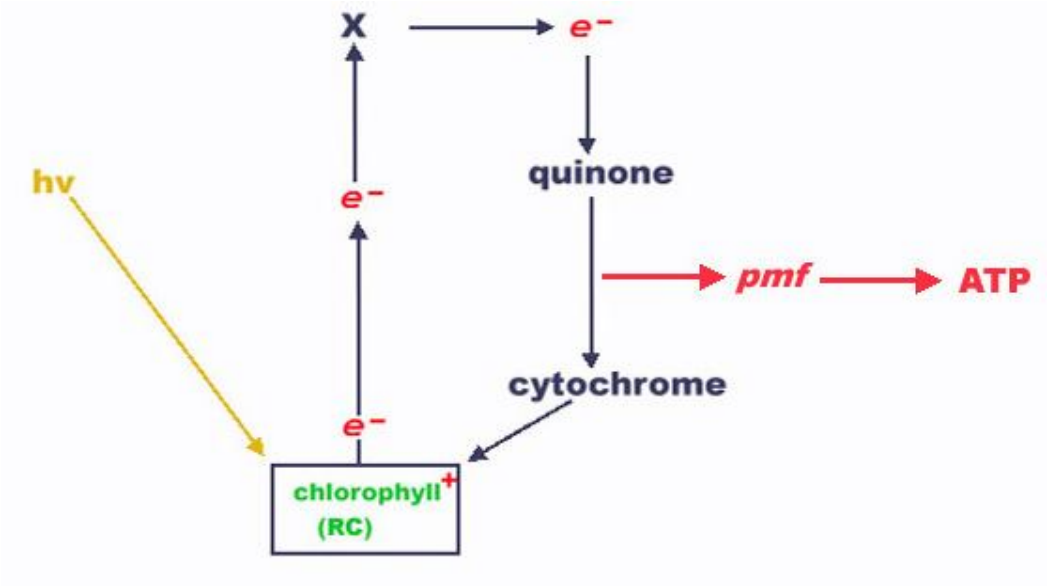


Fig.13. PHOTOSYSTEM I: CYCLICAL ELECTRON FLOW COUPLED TO PHOTOPHOSPHORYLATION

The functional components of the photochemical system are light harvesting pigments, a membrane electron transport system, and an ATPase enzyme. The photosynthetic electron transport system of is fundamentally similar to a respiratory ETS, except that there is a low redox electron acceptor (e.g. ferredoxin) at the top (low redox end) of the electron transport chain, that is first reduced by the electron displaced from chlorophyll.

There are several types of pigments distributed among various phototrophic organisms. Chlorophyll is the primary light-harvesting pigment in all photosynthetic organisms. Chlorophyll is a tetrapyrrole which contains magnesium at the center of the porphyrin ring. It contains a long hydrophobic side chain that associates with the photosynthetic membrane. Cyanobacteria have chlorophyll a, the same as plants and algae. The chlorophylls of the purple and green bacteria, called bacteriochlorophylls are chemically different than chlorophyll a in their substituent side chains. This is reflected in their light absorption spectra. Chlorophyll a absorbs light in two regions of the spectrum, one around 450nm and the other between 650 -750nm; bacterial chlorophylls absorb from 800-1000nm in the far red region of the spectrum.

The chlorophylls are partially responsible for light harvesting at the photochemical reaction center. The energy of a photon of light is absorbed by a special chlorophyll molecule at the reaction center, which becomes instantaneously oxidized by a nearby electron acceptor of low redox potential. The energy present in a photon of light is conserved as a separation of electrical charge which can be used to generate a proton gradient for ATP synthesis.

Carotenoids are always associated with the photosynthetic apparatus. They function as secondary light-harvesting pigments, absorbing light in the blue-green spectral region between 400-550 nm. Carotenoids transfer energy to chlorophyll, at near 100 percent efficiency, from wave lengths of light that are missed by chlorophyll. In addition, carotenoids have an indispensable function to protect the photosynthetic apparatus from photooxidative damage. Carotenoids have long hydrocarbon side chains in a conjugated double bond system.

Carotenoids "quench" the powerful oxygen radical, singlet oxygen, which is invariably produced in reactions between chlorophyll and O_2 (molecular oxygen). Some nonphotosynthetic bacterial pathogens, i.e., *Staphylococcus aureus*, produce carotenoids that protect the cells from lethal oxidations by singlet oxygen in phagocytes.

Phycobiliproteins are the major light harvesting pigments of the cyanobacteria. They also occur in some groups of algae. They may be red or blue, absorbing light in the middle of the spectrum between 550 and 650nm. Phycobiliproteins consist of proteins that contain covalently-bound linear tetrapyrroles (phycobilins). They are contained in granules called phycobilisomes that are closely associated with the photosynthetic apparatus. Being closely linked to chlorophyll they can efficiently transfer light energy to chlorophyll at the reaction center.

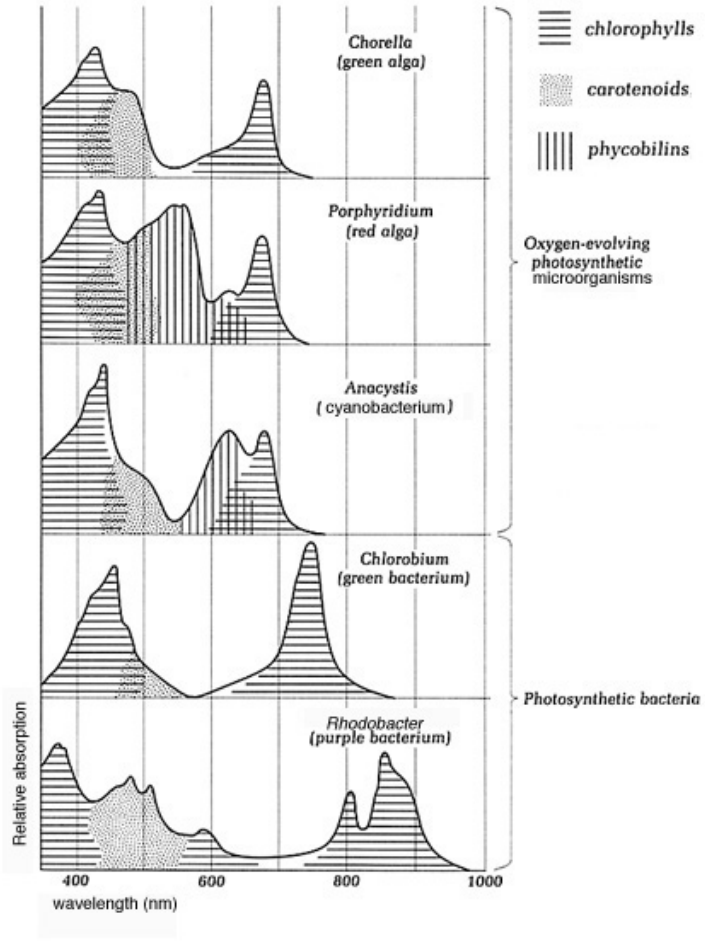


Fig.14. THE DISTRIBUTION OF PHOTOSYNTHETIC PIGMENTS AMONG PHOTOSYNTHETIC MICROORGANISMS

All phototrophic bacteria are capable of performing cyclic. This universal mechanism of cyclic photophosphorylation is referred to as Photosystem I. Bacterial photosynthesis uses only Photosystem I (PSI), but the more evolved cyanobacteria, as well as algae and plants, have an additional light-harvesting system called Photosystem II (PSII). Photosystem II is

used to reduce Photosystem I when electrons are withdrawn from PSI for CO_2 fixation. PSII transfers electrons from H_2O and produces O_2.

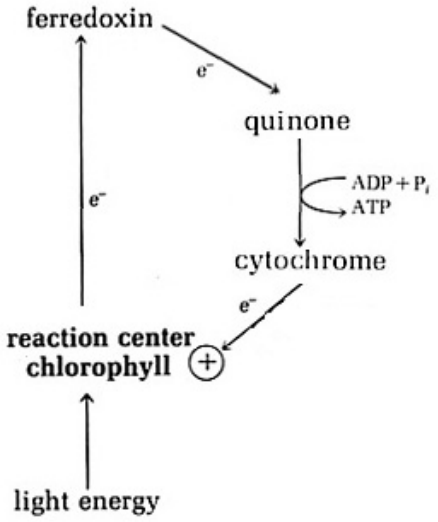


Fig.15. The cyclical flow of electrons during bacterial (anoxygenic) photosynthesis.

A cluster of carotenoid and chlorophyll molecules at the Reaction Center harvests a quantum of light. A bacterial chlorophyll molecule becomes instantaneously oxidized by the loss of an electron. The light energy is used to boost the electron to a low redox intermediate, ferredoxin, (or some other iron sulfur protein) which can enter electrons into the photosynthetic electron transport system in the membrane. As the electrons traverse the ETS a proton motive force is established that is used to make ATP in the process of photophosphorylation. The last cytochrome in the ETS returns the electron to chlorophyll. Since light energy causes the electrons to turn in a cycle while ATP is synthesized, the process is called cyclic photophosphorylation. Bacterial photosynthesis uses only Photosystem I for the conversion of light energy into chemical energy.

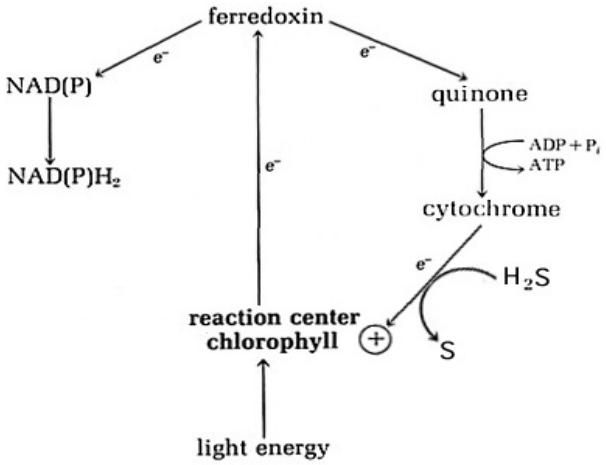


Fig.16. The normally cyclical flow of electrons during bacterial photosynthesis

Must be opened up in order to obtain electrons for CO_2 fixation. In the case of the purple sulfur bacteria, they use H_2S as a source of electrons. The oxidation of H_2S is coupled to PSI. Light energy boosts an electron, derived from H_2S, to the level of ferredoxin, which reduces NADP to provide electrons for autotrophic CO_2 fixation.

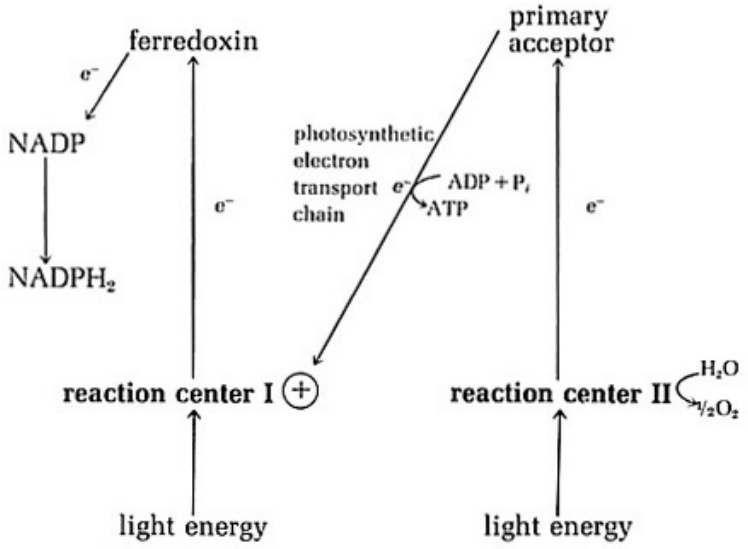


Fig.17. Electron flow in plant (oxygenic) photosynthesis.

Photosystem I and the mechanisms of cyclic photophosphorylation operate in plants, algae and cyanobacteria, as they do in bacterial photosynthesis. In plant photosynthesis, chlorophyll a is the major chlorophyll species at the reaction center and the exact nature of the primary electron acceptors (X or ferredoxin) and the components of the ETS are different than bacterial photosynthesis. But the fundamental mechanism of cyclic photophosphorylation is the same. However, when electrons must be withdrawn from photosystem I (ferredoxin--e^--->NADP in upper left), those electrons are replenished by the operation of Photosystem II. In the Reaction Center of PSII, a reaction between light, chlorophyll and H_2O removes electrons from H_2O (leading to the formation of O_2) and transfers them to a component of the photosynthetic ETS (primary electron acceptor). The electrons are then transferred through a chain of electron carriers consisting of cytochromes and quinones until they reach chlorophyll in PSI. The resulting drop in redox potential allows for the synthesis of ATP in a process called noncyclic photophosphorylation. The operation of photosystem II is what fundamentally differentiates plant photosynthesis from bacterial photosynthesis. Photosystem II accounts for the source of reductant for CO_2 fixation (provided by H_2O), the production of O_2, and ATP synthesis by noncyclic photophosphorylation.

Most of the phototrophic procaryotes are autotrophs, which mean that they are able to fix CO_2 as a sole source of carbon for growth. Just as the oxidation of organic material yields energy, electrons and CO_2, in order to build up CO_2 to the level of cell material (CH_2O), energy (ATP) and electrons (reducing power) are required. The overall reaction for the fixation of CO_2 in the Calvin cycle is CO_2 + 3ATP + 2$NADPH_2$ ----------> CH_2O + 2ADP + 2Pi + 2NADP. The light reactions operate to produce ATP to provide energy for the dark reactions of CO_2 fixation. The dark reactions also need reductant (electrons). Usually the

provision of electrons is in some way connected to the light reactions. A model for coupling the light and dark reactions of photosynthesis is illustrated below.

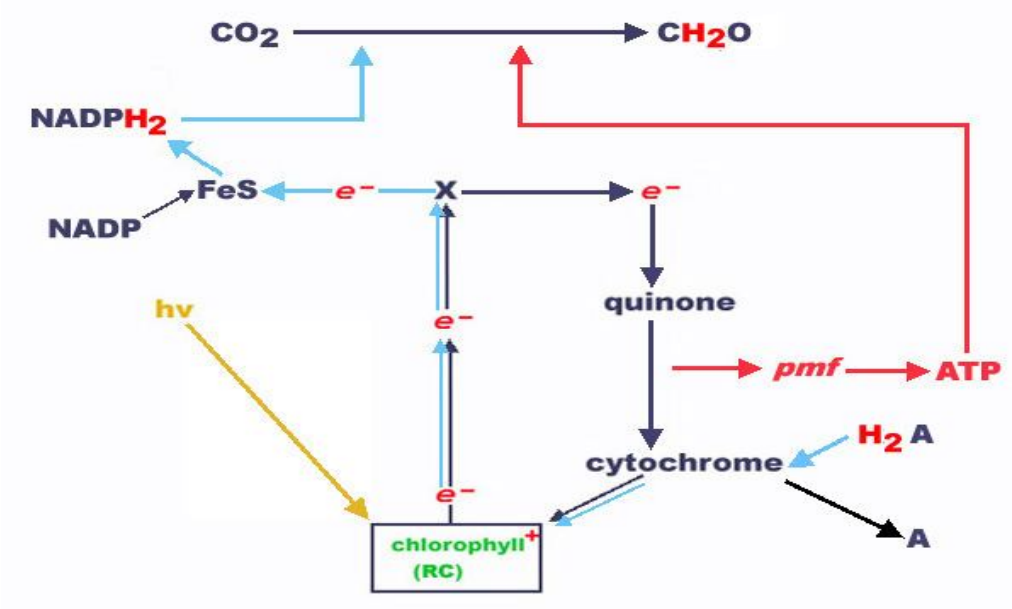


Fig.18. MODEL FOR COUPLING THE LIGHT AND DARK REACTIONS OF PHOTOSYNTHESIS

The differences between plant and bacterial photosynthesis are summarized in Table below. Bacterial photosynthesis is an anoxygenic process. The external electron donor for bacterial photosynthesis is never H_2O, and therefore, purple and green bacteria never produce O_2 during photosynthesis. Furthermore, bacterial photosynthesis is usually inhibited by O_2 and takes place in micro aerophilic and anaerobic environments. Bacterial chlorophylls use light at longer wave lengths not utilized in plant photosynthesis, and therefore they do not have to compete with oxygenic phototrophs for light. Bacteria use only cyclic photophosphorylation (Photosystem I) for ATP synthesis and lack a second photosystem.

Organisms	**Plant photosynthesis**	**Bacterial photosynthesis**
	plants, algae, cyanobacteria	purple and green bacteria
Type of chlorophyll	chlorophyll a absorbs 650-750nm	bacteriochlorophyll absorbs 800-1000nm
Photosystem I (cyclic photophosphorylation)	present	present
Photosystem I (noncyclic photophosphorylation)	present	absent
Produces O_2	yes	no
Photosynthetic electron donor	H_2O	H_2S, other sulfur compounds or certain organic compounds

TABLE.3. DIFFERENCES BETWEEN PLANT AND BACTERIAL PHOTOSYNTHESIS

While photosynthesis is highly-evolved in the procaryotes, it apparently originated in the Bacteria and did not spread or evolve in Archaea. But the Archaea, in keeping with their unique ways, are not without representatives which can conduct a type of light-driven photophosphorylation. The extreme halophiles, archaea that live in natural environments such as the Dead Sea and the Great Salt Lake at very high salt concentration (as high as 25 percent NaCl) adapt to the high-salt environment by the development of "purple membrane", actually patches of light-harvesting pigment in the plasma membrane. The pigment is a type of rhodopsin called bacteriorhodopsin which reacts with light in a way that forms a proton gradient on the membrane allowing the synthesis of ATP. This is the only example in nature of non photosynthetic photophosphorylation. These organisms are heterotrophs that normally respire by aerobic means. The high concentration of NaCl in their environment limits the availability of O_2 for respiration so they are able to supplement their ATP-producing capacity by converting light energy into ATP using bacteriorhodopsin.

8.14. AUTOTROPHIC CO_2 FIXATION

The use of RUBP carboxylase and the Calvin cycle is the most common mechanism for CO_2 fixation among autotrophs. Indeed, RUBP carboxylase is said to be the most abundant enzyme on the planet (nitrogenase, which fixes N_2 is second most abundant). This is the only mechanism of autotrophic CO_2 fixation among eukaryotes, and it is used, as well, by all cyanobacteria and purple bacteria. Lithoautotrophic bacteria also use this pathway. But the green bacteria and the methanogens, as well as a few isolated groups of procaryotes, have alternative mechanisms of autotrophic CO_2 fixation and do not possess RUBP carboxylase.

RUBP carboxylase (ribulose bisphosphate carboxylase) uses ribulose bisphosphate (RUBP) and CO_2 as co-substrates. In a complicated reaction the CO_2 is "fixed" by addition to the RUBP, which is immediately cleaved into two molecules of 3-phosphoglyceric acid (PGA). The fixed CO_2 winds up in the -COO group of one of the PGA molecules. Actually, this is the reaction which initiates the Calvin cycle.

The Calvin cycle is concerned with the conversion of PGA to intermediates in glycolysis that can be used for biosynthesis, and with the regeneration of RUBP, the substrate that drives the cycle. After the initial fixation of CO_2, 2 PGA are reduced and combined to form hexose-phosphate by reactions which are essentially the reverse of the oxidative Embden-Meyerhof pathway. The hexose phosphate is converted to pentose-phosphate, which is phosphorylated to regenerate RUBP. An important function of the Calvin cycle is to provide the organic precursors for the biosynthesis of cell material. Intermediates must be constantly withdrawn from the Calvin cycle in order to make cell material. In this regard, the Calvin cycle is an anabolic pathway. The fixation of CO_2 to the level of glucose ($C_6H_{12}O_6$) requires 18 ATP and 12 $NADPH_2$.

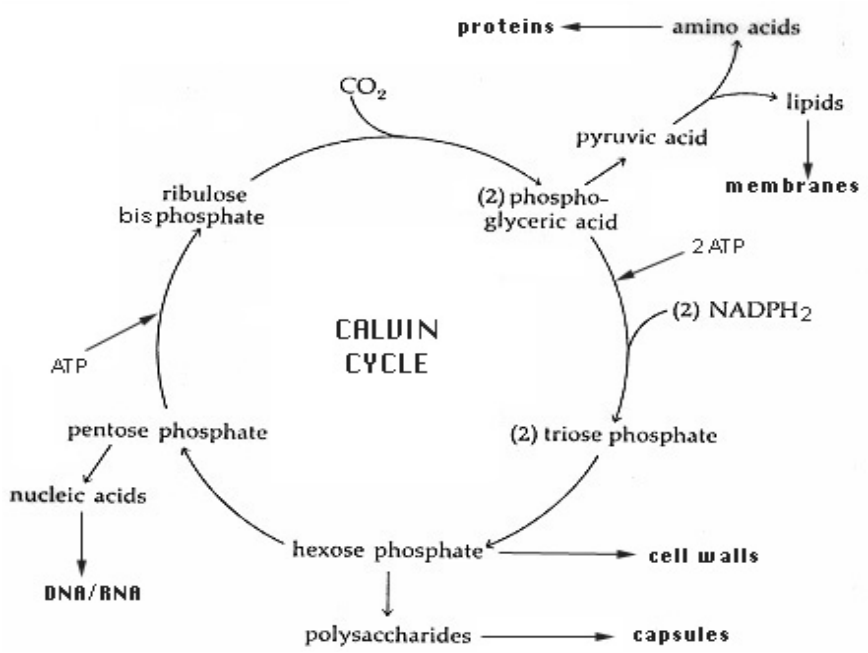


Fig.19. THE CALVIN CYCLE AND ITS RELATIONSHIP TO THE SYNTHESIS OF CELL MATERIALS

The methanogens, a very abundant group of procaryotes, use CO_2 as a source of carbon for growth, and as a final electron acceptor in an energy-producing process that produces methane. If a methanogen is fed labeled CO_2 as a sole form of carbon, 95 percent of the label winds up in methane and 5 percent winds up in cell material. The methanogens fix CO_2 by means of the enzyme CODH (carbon monoxide dehydrogenase) and the Acetyl CoA pathway. Methanogens predominate in anaerobic habitats including the deep sea with its volcanoes, thermal vents and fumaroles, and hence they perform a significant amount of CO_2 fixation on the planet.

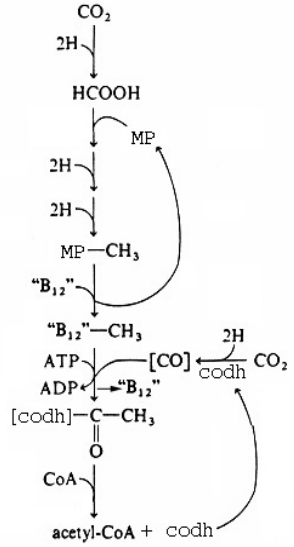


Fig.20. The CODH or acetyl CoA pathway of CO_2 fixation in the methanogens.

The pathway of methanogenesis steadily reduces CO_2 to the methyl (CH_3) level, mediated by the coenzyme methanopterin (MP), related to folic acid. MP-CH_3 may be reduced to methane (not shown) or the MP may be replaced by a vitamin B_{12}-like molecule to enter the pathway of CO_2 fixation. The "B_{12}"-CH_3 is substrate for CO fixation mediated by the CODH. CODH reduces CO_2 to CO and adds the CO to "B12"CH3 to form acetyl-[CODH]. Coenzyme A (CoA) then replaces the CODH, resulting in the formation of Acetyl CoA, which is in the heart of biosynthetic metabolism. The net effect is the reduction of 2 CO_2 to Acetyl CoA.

8.15. CYCLIC AND NONCYCLIC PHOTOPHOSPHORYLATION

Anoxygenic photosynthetic bacteria possess chlorophylls, called bacteriochlorophylls that differ from the chlorophylls of plants in structure and in light absorbing properties. Bacteriochlorophylls absorb light in the infrared region (725 to 1035nm). They are not contained in chloroplasts but are found in extensive membrane systems throughout the bacterial cell.

When a molecule of bacteriochlorophyll absorbs a quantum of light, the energy of the light raises the molecule to an excited state. In this excited state an electron is given off by bacteriochlorophyll. Bacteriochlorophylls thus become positive charge. It then serves as an electron trap or strong oxidizing agent. The energy released in the step between cytochrome b and cytochrome f is used for photophosphorylation – generation of ATP from ADP and inorganic phosphate. The reduction of $NADP^+$ in photosynthetic bacteria is accomplished not by photosynthesis but by using reducing power from constituents of the environment, such as H_2S and other inorganic and organic compounds. Such reduced compounds usually abound in the anaerobic environment of photosynthetic bacteria. Important role of plastaquinone is in the transport of protons originating from water. This modified process has been termed oxygenic photophosphorylation.

8.16. BIOSYNTHESIS

The pathways of central metabolism (i.e., glycolysis and the TCA cycle), with a few modifications, always run in one direction or another in all organisms. The reason - these pathways provide the precursors for the biosynthesis of cell material. When a pathway, such as the Embden-Meyerhof pathway or the TCA cycle, functions to provide energy in addition to chemical intermediates for the synthesis of cell material, the pathway is referred to as an amphibolic pathway. Pathways of glycolysis and the TCA cycle are amphibolic pathways because they provide ATP and chemical intermediates to build new cell material.

Biosynthesis or intermediary metabolism is a topic of biochemistry, more so than microbiology. It will not be dealt with in detail here. The fundamental metabolic pathways of biosynthesis are similar in all organisms, in the same way that protein synthesis or DNA structure are similar in all organisms. When biosynthesis proceeds from central metabolism as drawn below, some of the main precursors for synthesis of prokaryotic cell structures and components are as follows.

- Polysaccharide capsules or inclusions are polymers of glucose and other sugars.
- Cell wall peptidoglycan (NAG and NAM) is derived from glucose phosphate.
- Amino acids for the manufacture of proteins have various sources, the most important of which are pyruvic acid, alpha ketoglutaric acid and oxaloacetic acid.

- Nucleotides (DNA and RNA) are synthesized from ribose phosphate. ATP and NAD are part of purine (nucleotide) metabolism.
- Triose-phosphates are precursors of glycerol, and acetyl CoA is a main precursor of lipids for membranes
- Vitamins and coenzymes are synthesized in various pathways that leave central metabolism. Following figure illustrates the example of heme synthesis proceeds from the serine pathway, as well as from succinate in the TCA cycle.

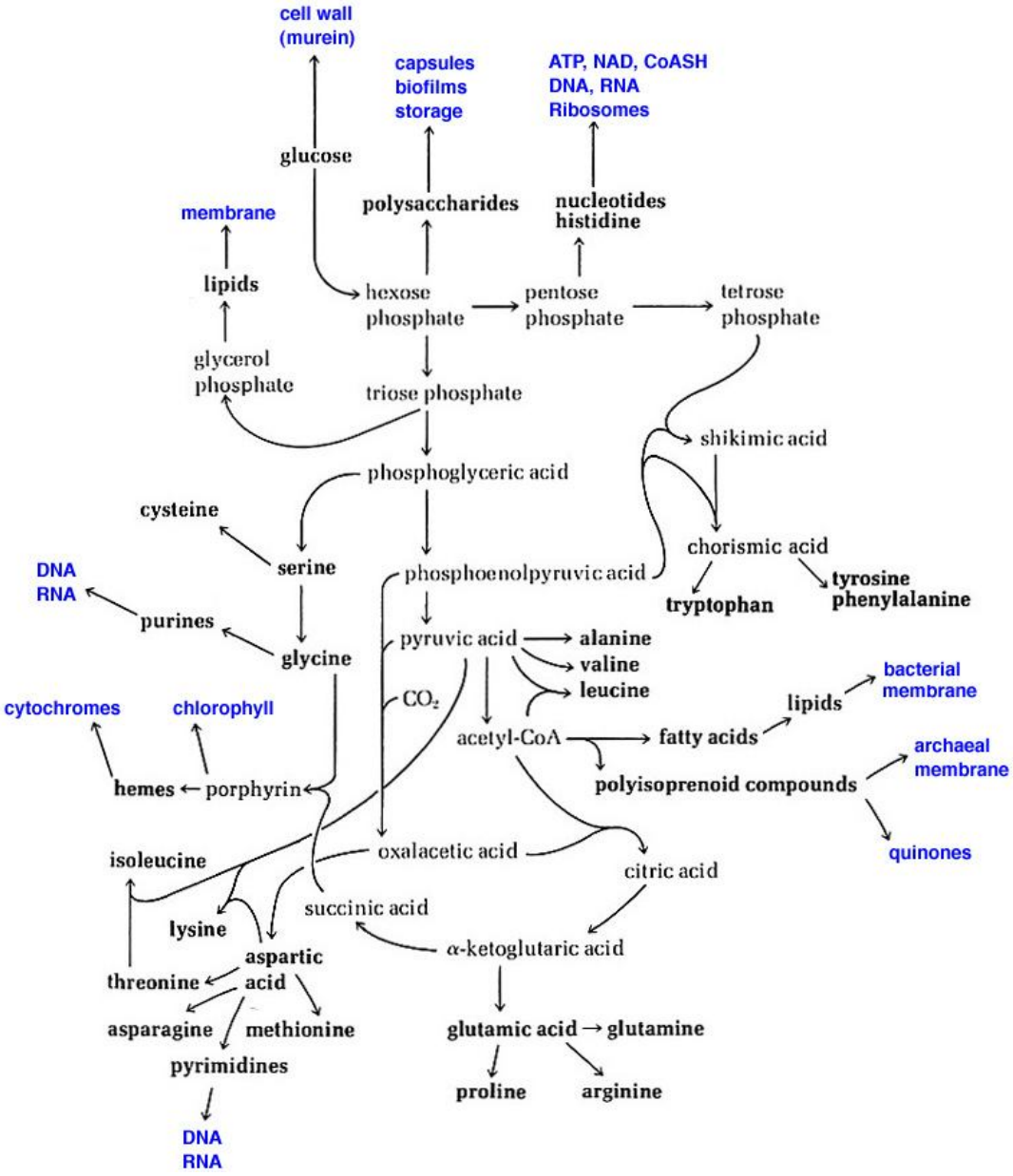


Fig.21. THE MAIN PATHWAYS OF BIOSYNTHESIS IN PROKARYOTIC CELLS

8.17. LET US SUM UP

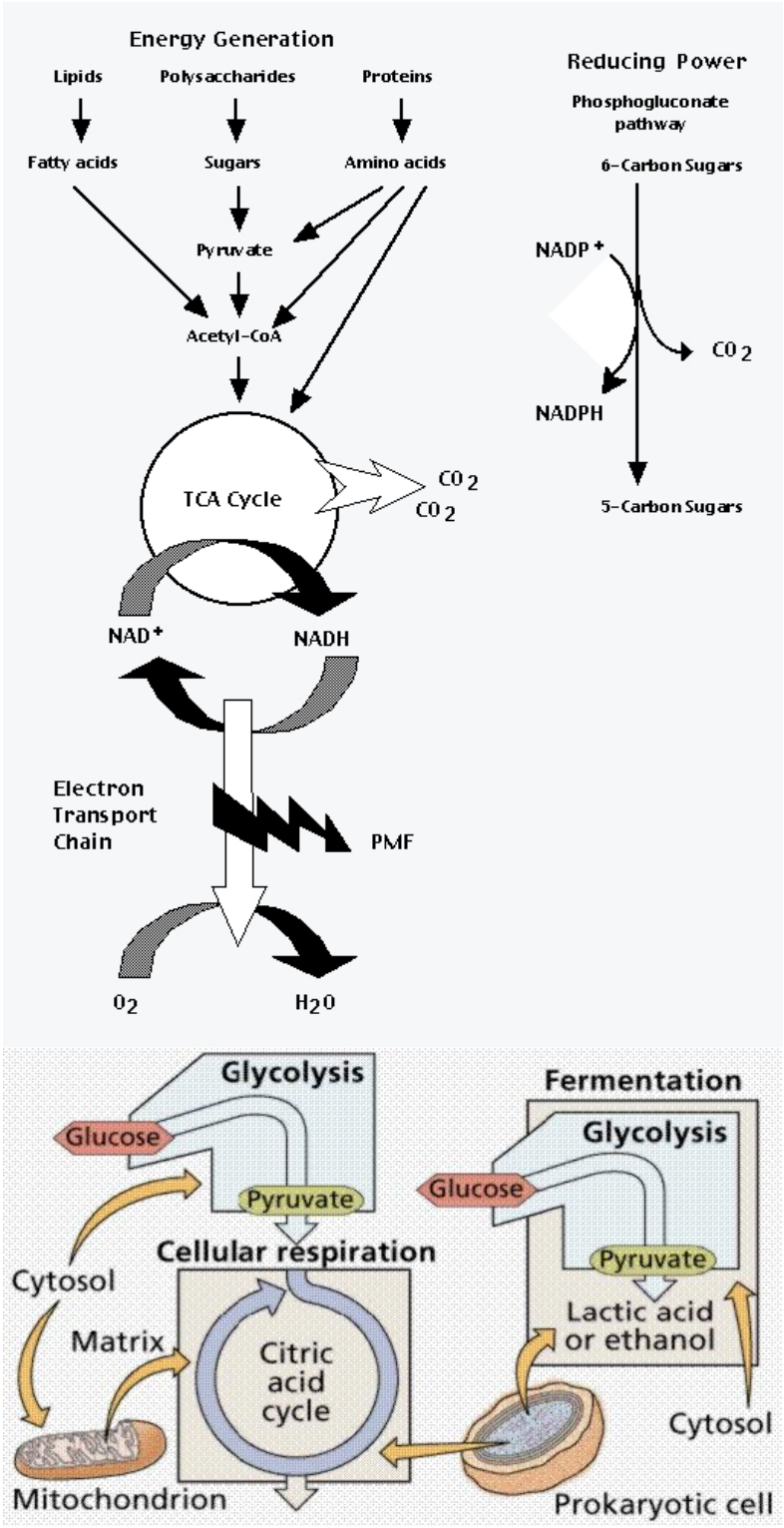


Fig.22.OVERVIEW OF THE METABOLIC PATHWAYS IN A PROKARYOTE

- During catabolism, useful energy is temporarily conserved in the "high energy bond" of ATP - adenosine triphosphate.
- ATP is the universal currency of energy exchange in biological systems.
- In a substrate level phosphorylation, ATP is made during the conversion of an organic molecule from one form to another.
- SLP occurs during fermentations and respiration (the TCA cycle), and even during some lithotrophic transformations of inorganic substrates.
- Electron Transport Phosphorylation takes place during respiration, photosynthesis, lithotrophy and possibly other types of bacterial metabolism.
- The most common pathway of glucose catabolism is the Embden-Meyerhof pathway of glycolysis (splitting of sugar).
- Each molecule of glucose metabolized by glycolysis, there is a net yield of two ATP molecules.
- Lactic acid bacteria reduce the pyruvate to lactic acid; yeast reduces the pyruvate to alcohol (ethanol) and CO_2.
- Phosphogluconate pathway or hexose monophosphate shunt – shunt of glycolysis pathway.
- Essential steps involved in HMP shunt are oxidation, decarboxylation, epimerization and transketolase and transaldolase.
- The Entner-Doudoroff Pathway - glucose metabolism where glucose is phosphorylated to glucose-6-phosphate.
- Anaerobes also produce energy by reactions called fermentations, which use organic compounds as electron donors and acceptors.
- Tricarboxylic acid cycle also called as Kreb’s cycle – complete oxidation of the substrate.
- In some strict anaerobes, other compounds, such as carbon dioxide, or ions, such as sulphate ion, can be the terminal electron acceptors for the anaerobic respiration.
- Lithotrophy is the use of an inorganic compound as a source of energy.
- The glyoxylate cycle is used by some microorganisms when acetate is the sole carbon source or during oxidation of primary substrates (such as higher fatty acids) that are cleaved to acetyl CoA without the intermediate formation of pyruvic acid.
- Organisms use light as their source of energy and carbon dioxide as their sole source of carbon for the growth.
- Phototrophy is the use of light as a source of energy for growth, more specifically the conversion of light energy into chemical energy in the form of ATP.
- Photosynthesis is the conversion of light energy into chemical energy that can be used in the formation of cellular material from CO_2.
- The Light Reactions depend upon the presence of chlorophyll, the primary light-harvesting pigment in the membrane of photosynthetic organisms.
- Chlorophyll is a tetrapyrrole which contains magnesium at the center of the porphyrin ring.
- The Calvin cycle is concerned with the conversion of PGA to intermediates in glycolysis that can be used for biosynthesis, and with the regeneration of RUBP, the substrate that drives the cycle.

8.18. LESSON END ACTIVITIES

Write the glycolysis pathway of prokaryote.
What is substrate level phosphorylation?
Explain heterotrophic CO_2 fixation.

Review the energy production by photosynthesis and analyse the significant features of it.
Write the energetics of metabolism.
Draw the structure of ATP and NADP. Explain its role in metabolism.
Draw the calvin cycle.
Explain the Kreb's cycle.

8.19. POINTS FOR DISCUSSION

Distinguish the aerobic and anaerobic respiration in prokaryote.
Explain about fermentation in microorganisms.
Explain glyoxylate cycle.
Write about photophosphorylation.

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UNIT III MICROBIAL GENOMICS AND REPLICATION

CONTENTS

LESSON - 9

FINE STRUCTURE OF GENE

Contents

9.0 AIMS AND OBJECTIVES

This unit imparts knowledge on the chemical and physical structure of DNA. The chapter also deals with the genomics and the replication process of the microorganisms.

9.1. INTRODUCTION

Various milestones or discoveries in Genetics field are as follows:
1865 – Genes are particulate factors
1903 – Chromosomes are hereditary units
1910 – Genes lie on chromosomes
1913 – Chromosomes contain linear arrays of genes
1927 – Mutations are physical changes in genes
1931 – Recombination is caused by crossing over
1944 – DNA is the genetic material
1945 – A gene codes for a protein
1953 – DNA is a double helix
1958 – DNA replicates semi conservatively
1961 – Genetic code is triplet
1977 – DNA can be sequenced
1997 – Genome can be sequenced

DNA as genetic material has been proved with *Pneumococcus*. It has capsular polysaccharides which is responsible for virulence. There are two types of *Pneumococcus viz.* smooth and rough bacteria. Smooth bacteria – virulent. Rough bacteria – avirulent (without polysaccharide). When heat killed smooth bacteria and live rough bacteria were mixed the smooth bacteria was transformed into virulent.

In yet another experiment with ^{32}P labeled DNA / ^{35}S labeled protein in phage, when the phage infected the bacteria the bacteria was found to have ^{32}P labeled DNA and no ^{35}S labeled protein. This proved that only DNA is transferred.

Deoxyribonucleic acid (**DNA**) is a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms. The main role of DNA molecules is the long-term storage of information and DNA is often compared to a set of blueprints, since it contains the instructions needed to construct other components of cells, such as proteins and RNA molecules. The DNA segments that carry this genetic information are called genes, but other DNA sequences have structural purposes, or are involved in regulating the use of this genetic information.

Chemically, DNA is a long polymer of simple units called nucleotides, with a backbone made of sugars and phosphate groups joined by ester bonds. Attached to each sugar is one of four types of molecules called bases. It is the sequence of these four bases along the backbone that encodes information. This information is read using the genetic code, which specifies the sequence of the amino acids within proteins. The code is read by copying stretches of DNA into the related nucleic acid RNA, in a process called transcription. Most of these RNA molecules are used to synthesize proteins, but others are used directly in structures such as ribososmes and spliceosomes.

Within cells, DNA is organized into structures called chromosomes and the set of chromosomes within a cell make up a genomes. These chromosomes are duplicated before cells divide, in a process called DNA replication. Eukaryotic organisms such as animals, plants and fungi store their DNA inside the cell nucleus, while in prokaryotes such as bacteria it is found in the cell's cytoplasm. Within the chromosomes, chromatin proteins such as histones compact and organize DNA, which helps control its interactions with other proteins and thereby control which genes are transcribed.

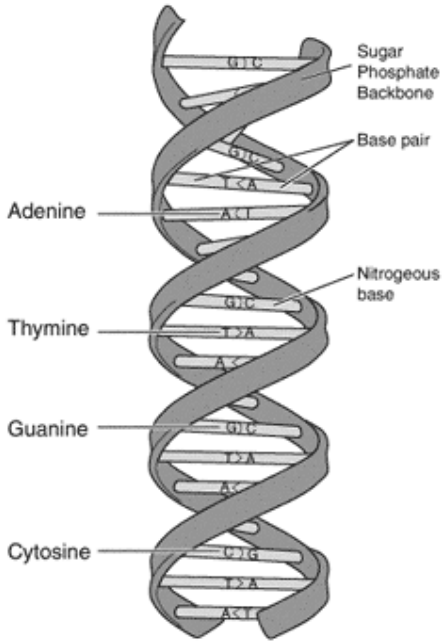

Figure: 1 Structure of Deoxyribonucleic Acid

9.2 FINE STRUCTURE OF GENE

DNA is a long polymer made from repeating units called nucleotides. The DNA chain is 22 to 26 Angstroms wide (2.2 to 2.6 nanometers), and one nucleotide unit is 3.3 Angstroms (0.33 nanometers) long.http://en.wikipedia.org/wiki/DNA - _note-0 Although each individual repeating unit is very small, DNA polymers can be enormous molecules containing millions of nucleotides. For instance, the largest human chromosome, chromosome number 1, is 220 million base pairs long.

In living organisms, DNA does not usually exist as a single molecule, but instead as a tightly-associated pair of molecules. These two long strands entwine like vines, in the shape of a double helix. The nucleotide repeats contain both the segment of the backbone of the molecule, which holds the chain together, and a base, which interacts with the other DNA strand in the helix. In general, a base linked to a sugar is called a nucleoside and a base linked to a sugar and one or more phosphate groups is called a nucleotide. If multiple nucleotides are linked together, as in DNA, this polymer is referred to as a polynucleotide.

9.2.1. CHEMICAL STRUCTURE OF DNA

The backbone of the DNA strand is made from alternating phospphate and sugar residues. The sugar in DNA is 2-deoxyribose, which is a pentose (five carbon) sugar. The sugars are joined together by phosphate groups that form phosphodiester bonds between the third and fifth carbon atoms of adjacent sugar rings. These asymmetric bonds mean a strand of DNA has a direction. In a double helix the direction of the nucleotides in one strand is opposite to their direction in the other strand. This arrangement of DNA strands is called antiparallel. The asymmetric ends of DNA strands are referred to as the 5’ (*five prime*) and 3’ (*three prime*) ends. One of the major differences between DNA and RNA is the sugar, with 2-deoxyribose being replaced by the alternative pentose sugar ribose in RNA.

The DNA double helix is stabilized by hydrogen bonds between the bases attached to the two strands. The four bases found in DNA are adenine (abbreviated A), cytosine (C), guanine (G) and thymine (T). These four bases are shown below and are attached to the sugar/phosphate to form the complete nucleotide, as shown for adenosine monophosphate.

These bases are classified into two types; adenine and guanine are fused five- and six-membered heterocyclic compounds called purines, while cytosine and thymine are six-membered rings called pyrimidines. A fifth pyrimidine base, called uracil (U), usually takes the place of thymine in RNA and differs from thymine by lacking a methyl group on its ring. Uracil is not usually found in DNA, occurring only as a breakdown product of cytosine, but a very rare exception to this rule is a bacterial virus called PBS1 that contains uracil in its DNA. In contrast, following synthesis of certain RNA molecules, a significant number of the uracils are converted to thymines by the enzymatic addition of the missing methyl group. This occurs mostly on structural and enzymatic RNAs like transfer RNAs and ribosomal RNA.

The double helix is a right-handed spiral. As the DNA strands wind around each other, they leave gaps between each set of phosphate backbones, revealing the sides of the bases inside. There are two of these grooves twisting around the surface of the double helix: one groove, the major groove, is 22 Å wide and the other, the minor groove, is 12 Å wide. The narrowness of the minor groove means that the edges of the bases are more accessible in the major groove. As a result, proteins like transcription factors that can bind to specific

sequences in double-stranded DNA usually make contacts to the sides of the bases exposed in the major groove.

9.2.2. PHYSICAL STRUCTURE OF DNA

DNA exists in several possible conformations. The conformations so far identified are: A-DNA, B-DNA, C-DNA, D-DNA, E-DNA, H-DNA, L-DNA, P-DNA,http://en.wikipedia.org/wiki/DNA_-_note-Allemand1998 and Z-DNA. However, only A-DNA, B-DNA, and Z-DNA have been observed in naturally occurring biological systems. Which conformation DNA adopts depends on the sequence of the DNA, the amount and direction of supercoiling, chemical modifications of the bases and also solution conditions, such as the concentration of metal ions and polyamines. Of these three conformations, the "B" form described above is most common under the conditions found in cells. The two alternative double-helical forms of DNA differ in their geometry and dimensions.

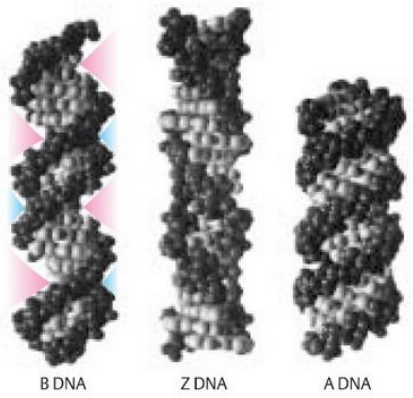

Fig.2. FORMS OF DNA

Properties	B	A	Z
Type of helix	Right	Right	Left
Distance/complete turn	3.4nm	3.2nm	4.5nm
Topology of major groove	Wide, deep	Narrow, deep	Flat
Topology of minor groove	Narrow, shallow	Broad, shallow	Narrow, deep

Table 1 Forms of DNA

The A form is a wider right-handed spiral, with a shallow and wide minor groove and a narrower and deeper major groove. The A form occurs under non-physiological conditions in dehydrated samples of DNA, while in the cell it may be produced in hybrid pairings of DNA and RNA strands, as well as in enzyme-DNA complexes. Segments of DNA where the bases have been chemically-modified by methylation may undergo a larger change in conformation and adopt the Z-form. Here, the strands turn about the helical axis in a left-handed spiral, the opposite of the more common B form. These unusual structures can be recognised by specific Z-DNA binding proteins and may be involved in the regulation of transcription.

Each type of base on one strand forms a bond with just one type of base on the other strand. This is called complementary base pairing. Here, purines form hydrogen bonds to

pyrimidines, with A bonding only to T, and C bonding only to G. This arrangement of two nucleotides binding together across the double helix is called a base pair. In a double helix, the two strands are also held together via forces generated by the hydrophobic effect and pi stacking, which are not influenced by the sequence of the DNA. As hydrogen bonds are not covalent, they can be broken and rejoined relatively easily. The two strands of DNA in a double helix can therefore be pulled apart like a zipper, either by a mechanical force or high temperature. As a result of this complementarity, all the information in the double-stranded sequence of a DNA helix is duplicated on each strand, which is vital in DNA replication. Indeed, this reversible and specific interaction between complementary base pairs is critical for all the functions of DNA in living organisms.

The two types of base pairs form different numbers of hydrogen bonds, AT forming two hydrogen bonds, and GC forming three hydrogen bonds. The GC base pair is therefore stronger than the AT base pair. As a result, it is both the percentage of GC base pairs and the overall length of a DNA double helix that determine the strength of the association between the two strands of DNA. Long DNA helices with a high GC content have stronger-interacting strands, while short helices with high AT content have weaker-interacting strands. Parts of the DNA double helix that need to separate easily, such as the TATAAT Pribnow box in bacterial promoters, tend to have sequences with a high AT content, making the strands easier to pull apart. In the laboratory, the strength of this interaction can be measured by finding the temperature required to break the hydrogen bonds, their melting temperature (also called T_m value). When all the base pairs in a DNA double helix melt, the strands separate and exist in solution as two entirely independent molecules. These single-stranded DNA molecules have no single common shape, but some conformations are more stable than others.

9.3. LET US SUM UP

- Physical properties and structure and different forms of DNA were discussed
- Chemical properties and structure and different forms of DNA were discussed
- G pairs with C using three hydrogen bonds
- A pairs with T using two hydrogen bonds
- DNA structure is double helix stacked on the sugar backbone

http://en.wikipedia.org/wiki/DNA - note-10

9.4. LESSON END ACTIVITIES

1. Write a short note on following
    a. Fine structure of gene
    b. Chemical structure of DNA
    c. Physical structure of DNA

9.5. CHECK YOUR PROGRESS

What are the bases that form DNA structure?
Draw the structure of ribose and deoxyribose sugars.
What are the forms of DNA?

9.6. POINTS FOR DISCUSSION

Explain the forms of DNA.
Write the difference between DNA and RNA.

Explain the chemical properties of DNA.

9.7 REFERENCES

1. Microbiology by Pelczar, Reid and Chan, McGraw Hill Book Company.
2. Microbiology, Fundamental and Applications by R.A. Atlas, McMillan Publishers.
3. General Microbiology by Powar and Daginawala, Himalaya Publishing House.
4. Microbial genetics by David friefelder

LESSON – 10

GENETIC CODE

Contents

10.0 AIMS AND OBJECTIVES

The chapter deals with the scheme of genetic code in general.

10. 1. INTRODUCTION

Relationship between a sequence of DNA and the sequence of the corresponding protein is expressed in the form of genetic code. DNA sequence is read in groups of three nucleotides, each representing one amino acid. Such each trinucleotide sequence is called a codon. Code is read in nonoverlapping triplets (each codon consists of three nucleotides, successive codons are represented by successive trinucleotides) from a fixed starting point (must start at one end).

The **genetic code** is the set of rules by which information encoded in genetic material (DNA or RNA sequences) is translated into proteins (amino acid sequences) by living cells. Specifically, the code defines a mapping between tri-nucleotide sequences called **codons** and amino acids; every triplet of nucleotides in a nucleic acid sequence specifies a single amino acid. Because the vast majority of genes are encoded with exactly the same code, this particular code is often referred to as the canonical or standard genetic code, or simply the genetic code, though in fact there are many variant codes; thus, the canonical genetic code is not universal. For example, in humans, protein synthesis in mitochondria relies on a genetic code that varies from the canonical code. All Codes are degenerate except methionine and tryptophan. All amino acids have more than one codon. Twenty amino acids correspond to 64 triplets. Synonymous codons grouped into families. Eg: GGA, GGU, GGG, GGC – glycine. Code contains punctuation codons such stop and start codons. UAA, UGA, UAG – termination/stop codons. AUG – initiation/start codon (also methionine but removed during notification at amino terminus). Code is not universal. Mitochondria and prokaryotes have different codes.

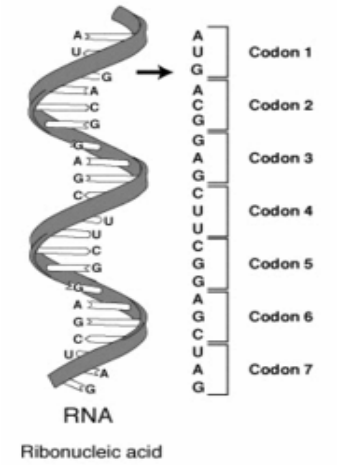

Figure 1: Ribonucleic acid

Some definitions in genetic code are,

- Code letter – nucleotide AUGC, ATGC
- Code word/codon – triplet nucleotide / sequence of nucleotides
- Anti codon – sequence of nucleotides on tRNA that complements the codon – AAA for phenylalanine.
- Genetic code/coding dictionary – table of all code words/codons with specific amino acids.
- Word size – number of letters in a code word. Eg. Three letters in a triplet code
- Non overlapping – only as many amino acids coded as there are code words in end to end sequence.
- Overlapping – more amino acids are coded as sequence.
- Degenerate code – more than one codon for a particular amino acid (UUU, UUC – phenylalanine)
- Ambiguous code – one codon code for more than L amino acid (GGA – glycine, glutamic acid)
- Comma less code – two intermediatary nucleotides between words eg: UUUCCC(non overlapping)
- Reading frame – particular nucleotide sequence – starts at a specific point then partitioned into codons.
- Sense word – codon that specifies an amino acid
- Missense mutation – change in nucleotide sequence – result in different amino acid.
- Nonsense mutation – change in nucleotide sequence – result in no amino acid
- Universality – same code for all organisms. UUU –phenylalanine in bacteria, mouse, man and tobacco.

10.2. INFORMATION TRANSFER VIA GENETIC CODE

The genome of an organism is inscribed in DNA, or in some viruses RNA. The portion of the genome that codes for a protein or RNA is referred to as a gene. In RNA, thymine (**T**) is replaced by uracil (**U**), and the deoxyribose is substituted by ribose. Each protein-coding gene is transcribed into a template molecule of the related polymer RNA, known as messenger RNA or mRNA. This in turn is translated on the ribosome into an aminoacid chain or polypeptide. The process of translation requires transfer RNAs specific

for individual amino acids with the amino acids covalently attached to them, guanosine triphosphate as an energy source, and a number of translation factors. tRNAs have anticodons complementary to the codons in mRNA and can be "charged" covalently with amino acids at their 3' terminal CCA ends. Individual tRNAs are charged with specific amino acids by enzymes known as aminoacyl tRNA synthetases which have high specificity for both their cognate amino acids and tRNAs. The high specificity of these enzymes is a major reason why the fidelity of protein translation is maintained.

There are $4^3 = 64$ different codon combinations possible with a triplet codon of three nucleotides. In reality, all 64 codons of the standard genetic code are assigned for either amino acids or stop signals during translation. If, for example, RNA sequence, UUUAAACCC is considered and the reading frame starts with the first U (by convention, 5' to 3'), there are three codons, namely, UUU, AAA and CCC, each of which specifies one amino acid. This RNA sequence will be translated into an amino acid sequence, three amino acids long. A comparison may be made with computer science, where the codon is the equivalent of a byte, which codes for a single letter in a program (like one amino acid of a protein), and a nucleotide for a bit.

The standard genetic code is shown in the following tables. Table 1 shows what amino acid each of the 64 codons specifies. These are called forward and reverse codon tables, respectively. For example, the codon AAU represents the amino acid asparagine, and UGU and UGC represent cysteine (standard three-letter designations, Asn and Cys respectively).

1st base	2nd base U	2nd base C	2nd base A	2nd base G
U	**UUU (Phe/F) Phenylalanine** **UUC (Phe/F) Phenylalanine** **UUA (Leu/L)Leucine** **UUG (Leu/L)Leucine**	**UCU (Ser/S)Serine** **UCC (Ser/S)Serine** **UCA (Ser/S)Serine** **UCG (Ser/S)Serine**	**UAU (Tyr/Y)Tyrosine** **UAC (Tyr/Y)Tyrosine** **UAA Ochre (*Stop*)** **UAG Amber (*Stop*)**	**UGU (Cys/C) Cysteine** **UGC (Cys/C) Cysteine** **UGA Opal (*Stop*)** **UGG (Trp/W) Tryptophan**
C	**CUU (Leu/L)Leucine** **CUC (Leu/L)Leucine** **CUA (Leu/L)Leucine** **CUG (Leu/L)Leucine**	**CCU (Pro/P)Proline** **CCC (Pro/P)Proline** **CCA (Pro/P)Proline** **CCG (Pro/P)Proline**	**CAU (His/H)Histidine** **CAC (His/H)Histidine** **CAA (Gln/Q)Glutamine** **CAG (Gln/Q)Glutamine**	**CGU (Arg/R) Arginine** **CGC (Arg/R) Arginine** **CGA (Arg/R) Arginine** **CGG (Arg/R) Arginine**
A	**AUU (Ile/I)Isoleucine** **AUC (Ile/I)Isoleucine** **AUA (Ile/I)Isoleucine** **AUG (Met/M) Methionine, *Start*[1]**	**ACU (Thr/T)Threonine** **ACC (Thr/T)Threonine** **ACA (Thr/T)Threonine** **ACG (Thr/T)Threonine**	**AAU (Asn/N)Asparagine** **AAC (Asn/N)Asparagine** **AAA (Lys/K)Lysine** **AAG (Lys/K)Lysine**	**AGU (Ser/S)Serine** **AGC (Ser/S)Serine** **AGA (Arg/R)Arginine** **AGG (Arg/R)Arginine**
G	**GUU (Val/V)Valine** **GUC (Val/V)Valine** **GUA (Val/V)Valine** **GUG (Val/V)Valine**	**GCU (Ala/A)Alanine** **GCC (Ala/A)Alanine** **GCA (Ala/A)Alanine** **GCG (Ala/A)Alanine**	**GAU (Asp/D)Aspartic acid** **GAC (Asp/D)Aspartic acid** **GAA (Glu/E)Glutamic acid** **GAG (Glu/E)Glutamic acid**	**GGU (Gly/G) Glycine** **GGC (Gly/G) Glycine** **GGA (Gly/G) Glycine** **GGG (Gly/G) Glycine**

Table: 1 Details of 64 codons and the amino acid each codon codes for (Direction is 5' to 3')

Codons specify each of the 20 standard amino acids involved in translation is given below,

Ala/A GCU, GCC, GCA, GCG
Leu/L UUA, UUG, CUU, CUC, CUA, CUG
Arg/R CGU, CGC, CGA, CGG, AGA, AGG
Lys/K AAA, AAG
Asn/N AAU, AAC
Met/M AUG
Asp/D GAU, GAC
Phe/F UUU, UUC **Cys/C** UGU, UGC
Pro/P CCU, CCC, CCA, CCG
Gln/Q CAA, CAG
Ser/S UCU, UCC, UCA, UCG, AGU, AGC
Glu/E GAA, GAG
Thr/T ACU, ACC, ACA, ACG
Gly/G GGU, GGC, GGA, GGG
Trp/W UGG
His/H CAU, CAC
Tyr/Y UAU, UAC
Ile/I AUU, AUC, AUA
Val/V GUU, GUC, GUA, GUG
START AUG **STOP** UAG, UGA, UAA

10.3. READING FRAME OF A SEQUENCE

Note that a codon is defined by the initial nucleotide from which translation starts. For example, the string GGGAAACCC, if read from the first position, contains the codons GGG, AAA and CCC; and if read from the second position, it contains the codons GGA and AAC; if read starting from the third position, GAA and ACC. Partial codons have been ignored in this example. Every sequence can thus be read in three reading frames, each of which will produce a different amino acid sequence (in the given example, Gly-Lys-Pro, Gly-Asp, or Glu-Thr, respectively). With double-stranded DNA there are six possible reading frames, three in the forward orientation on one strand and three reverse (on the opposite strand).

The actual frame a protein sequence is translated in is defined by a start codon, usually the first AUG codon in the mRNA sequence. Mutations that disrupt the reading frame by insertions or deletions of a non-multiple of 3 nucleotide bases are known as frame shift mutations. These mutations may impair the function of the resulting protein, if it is formed, and are thus rare in *in vivo* protein-coding sequences. Often such misformed proteins are targeted for proteolytic degradation. In addition, a frame shift mutation is very likely to cause a stop codon to be read which truncates the creation of the protein (example). One reason for the rareness of frame-shifted mutations being inherited is that if the protein being translated is essential for growth under the selective pressures the organism faces, absence of a functional protein may cause lethality before the organism is viable.

10. 4. START/STOP CODONS

Translation starts with a chain initiation codon (start codon). Unlike stop codons, the codon alone is not sufficient to begin the process. Nearby sequences and initiation factors are

also required to start translation. The most common start codon is AUG, which codes for methionine, so most amino acid chains start with methionine.

The three stop codons have been given names: UAG is *amber*, UGA is *opal* (sometimes also called *umber*), and UAA is *ochre*. "Amber" was named by discoverers Richard Epstein and Charles Steinberg after their friend Harris Bernstein, whose last name means "amber" in German. The other two stop codons were named 'ochre" and "opal" in order to keep the "color names" theme. Stop codons are also called termination codons and they signal release of the nascent polypeptide from the ribosome due to binding of release factors in the absence of cognate tRNAs with anticodons complementary to these stop signals.

10. 5. DEGENERACY OF THE GENETIC CODE

The genetic code has redundancy but no ambiguity. For example, although codons GAA and GAG both specify glutamic acid (redundancy), neither of them specifies any other amino acid (no ambiguity). Degenerate codons may differ in their third positions; e.g., both GAA and GAG code for the amino acid glutamic acid. A codon is said to be **fourfold degenerate** if any nucleotide at its third position specifies the same amino acid; it is said to be **twofold degenerate** if only two of four possible nucleotides at its third position specify the same amino acid. In twofold degenerate codons, the equivalent third position nucleotides are always either two purines (A/G) or two pyrimidines (C/T). Only two amino acids are specified by a single codon; one of these is the amino-acid methionine, specified by the codon AUG, which also specifies the start of translation; the other is tryptophan, specified by the codon UGG. The degeneracy of the genetic code is what accounts for the existence of silent mutations.

Degeneracy results because a triplet code designates 20 amino acids and a stop codon. Because there are four bases, triplet codons are required to produce at least 21 different codes. For example, if there were two bases per codon, then only 16 amino acids could be coded for (4^2=16). Because at least 21 codes are required, then 4^3 gives 64 possible codons, meaning that some degeneracy must exist.

These properties of the genetic code make it more fault-tolerant for point mutations. For example, in theory, fourfold degenerate codons can tolerate any point mutation at the third position, although codon usage bias restricts this in practice in many organisms; twofold degenerate codons can tolerate one out of the three possible point mutations at the third position. Since transition mutations (purine to purine or pyrimidine to pyrimidine mutations) are more likely than transversion (purine to pyrimidine or vice-versa) mutations, the equivalence of purines or that of pyrimidines at twofold degenerate sites adds a further fault-tolerance.

A practical consequence of redundancy is that some errors in the genetic code only cause a silent mutation or an error that would not affect the protein because the hydrophilicity or hydrophobicity is maintained by equivalent substitution of amino acids; for example, a codon of NUN (where N = any nucleotide) tends to code for hydrophobic amino acids. Even so, it is a single point mutation that causes a modified hemoglobin molecule in sickle cell diseas. The hydrophilic glutamate (Glu) is substituted by the hydrophobic valine (Val), which reduces the solubility of β-globin. In this case, this mutation causes hemoglobin to form linear polymers linked by the hydrophobic interaction between the valine groups causing sickle-cell deformation of erythrocytes. Sickle-cell disease is generally not caused by a *de novo* mutation. Rather it is selected for in malarial regions (in a way similar to thalassemia),

as heterozygous people have some resistance to the malarial *Plasmodium* parasite (heterozygote advantage).

These variable codes for amino acids are allowed because of modified bases in the first base of the anticodon of the tRNA, and the base-pair formed is called a wobble base pair. The modified bases include inosine and the Non-Watson-Crick U-G base pair

10. 6. VARIATIONS TO THE STANDARD GENETIC CODE

While slight variations on the standard code had been predicted earlier, none were discovered until 1979, when researchers studying human mitochondrial genes discovered they used an alternative code. Many slight variants have been discovered since, including various alternative mitochondrial codes,http://en.wikipedia.org/wiki/Genetic_code - _note-4 as well as small variants such as *Mycoplasma* translating the codon UGA as tryptophan. In bacteria and archaea, GUG and UUG are common start codons. However, in rare cases, certain specific proteins may use alternative initiation (start) codons not normally used by that species.

In certain proteins, non-standard amino acids are substituted for standard stop codons, depending upon associated signal sequences in the messenger RNA: UGA can code for selenocystein and UAG can code for pyrrolysine as discussed in the relevant articles. Selenocysteine is now viewed as the 21st amino acid, and pyrrolysine is viewed as the 22nd. A detailed description of variations in the genetic code can be found at the NCBI web site.

However, all known codes have strong similarities to each other, and the coding mechanism is the same for all organisms: three-base codons, tRNA, and ribosomes, reading the code in the same direction, translating the code three letters at a time into sequences of amino acids.

10.5 LET US SUM UP

- Genetic code the set of rules by which information encoded in genetic material (DNA or RNA sequences) is translated into proteins (amino acid sequences) by living cells have been elaborated
- Genetic code are in the form of three letter named as codons
- Each codon represents an aminoacid
- 64 possible codons are available
- The genetic code has redundancy but no ambiguity

10.6. LESSON END ACTIVITIES

Explain the degeneracy of genetic code
What are the variations to the standard genetic code?

10.7. CHECK YOUR PROGRESS

What are the start and stop codons?
What are the various codons that code for histidine and arginine?

10.8. POINTS FOR DISCUSSION

Discuss in detail about information transfer via genetic code

10.9 REFERENCES

1. Microbiology by Pelczar, Reid and Chan, McGraw Hill Book Company.
2. Microbiology, Fundamental and Applications by R.A. Atlas, McMillan Publishers.
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4. Microbial genetics by David friefelder

LESSON – 11

GENETIC ARRANGEMENT

Contents

11.0. AIMS AND OBJECTIVES

The chapter deals with the gentic organization of organisms.

11.1 INTRODUCTION ON PROKARYOTIC GENOME

Prokaryotic genome is a large circular DNA molecule. It also contains additional small circular / linear DNA molecules called plasmids (with various phenotypic traits). Plasmids are dispensible. Unipartite organism have single genome and a plasmid, whereas multipartite have single genome and large number (17 – 18) linear or circular molecules. There are no introns. In some cases a groups of genes involved in a single biochemical pathway are expressed in conjugation with one another and they are **operon**. Based on size the organisms can be ordered as – eukaryote > bacteria > archaeal. Two important features of prokaryotes are no introns, infrequency of repetitive sequences, however posses some sequence that may be repeated elsewhere. Eg: insertion sequences.

11.2 PHYSICAL STRUCTURE

Most of the prokaryotic genome is of <5Mb size but few are longer. Genome has been localized within the nucleoid. Packaging in a small space is made possible by supercoiling which is facilitated by DNA gyrase and TopoI. In Bacteria HU protein similar to histones in eukaryotes are used for packaging genome into nucleus. Archaea has more histone like proteins for packaging the genome.

Some bacterial genomes are circular and some are linear. Precise status of plasmid with regard to genome is not known. Some can integrate into main genome but others are independent. Most plasmids not essential for the bacterium. Some have very small size plasmid eg: *E.coli*. Some have more or less equal sized DNA molecule (eg: *Vibrio cholerae* 2.96 and 1.07Mb; only 2.96 molecule is essential). Characteristic of plasmid are integron which are a set of genes and other DNA sequences that enable plasmids to capture genes from phages and other plasmids. Some have more complexity like *Borrelia burgdorferi* – 911Kb linear genome with 17/18 linear and circular plasmids. There are five types of plasmids *viz.* virulence (Ti), Resistance (Ampicillin $^{R),}$ killer (colicin), Degradative (Tol) and Fertility (F).

11.3 GENETIC ORGANIZATION

Very less non coding region are present in bacterial genome. Characteristic feature of bacterial genome is operon. **Operon** concept does not hold for Archaean, all genes are not biochemically related. An operon consists of a Structural gene encoding protein or RNA and Regulatory gene encoding for proteins regulating gene expression. Cis acting regulate only of the gene or genes physically connected to it (Eg: operator, promoter, and terminator). Trans acting regulate other genes also (Eg: transcription factors). Control may be negative control and positive control. Negative control suppress protein expression and positive control induce protein expression. Regulatory proteins are trans acting factors that recognize cis acting elements upstream of the gene.

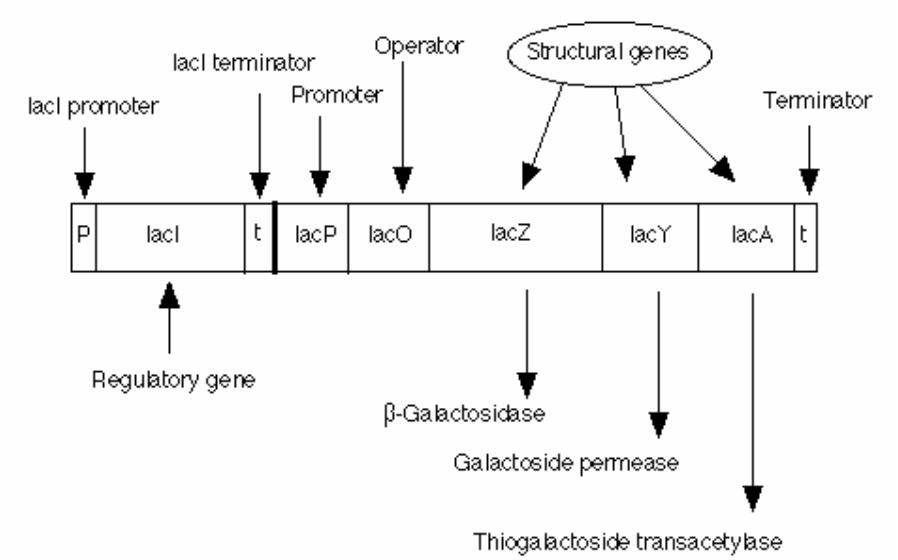

Figure 4: Lac operon model

Lac I – repressor (monomer), forms tetramer binds to operator of lac, blocks transcription, lactose binds to repressor, releases transcription.
Lac Z – β-galactosidase
Lac Y – Permease
Lac A – transacetylase

Transposable elements are DNA molecules that move from one site to another. Movement of DNA elements from one site to another is called transposition. The mobile segments transposons, usually contains easily recognizable genes example, antibiotic resistance. They are designated as Tn followed by a number. Sometimes indicate the phenotype Tn 1 (Amp^R). First discovered transposons do not have any phenotypic character like insertion elements

Insertion sequences are present in the bacterial genome. This is proved by the following experiment. A gene which were highly polar mutated was selected for the study. Mapped first gene of an operon where mutation was seen and downstream proteins were not synthesized. No reversion possible by base analog /frameshift. If plasmid was inserted similar mutation observed and need not be the same site. Mutated gene was larger than original. Hence concluded that a segment of DNA had been inserted.

Termini of each IS elements have inverted repeat sequences of 10-40bp. Insertion can be either left to right/right to left. Different IS elements have different number of bases. This IS elements contain atleast two apparent coding sequences initiated by an AUG and terminated with an in phase stop codon. There are two types of transposons:

- Composite transposons – carrying anterior flanked by two identical / nearly identical copies of an IS element.
- Tn 3 transposon family – three genes
- Transposable phages – Mu and D108

11.4 EUKARYOTIC GENOME

The increase in genome size correlates with the vast expansion of noncoding (i.e., intronic, intergenic, and interspersed repeat sequences) and repeat DNA (e.g., satellite, LINEs, Short interspersed nuclear element (SINEs), DNA (Alu sequence), in red) sequences in more complex muiticellular organisms. This expansion is accompanied by an increase in the number of epigenetic mechanisms (particularly repressive) that regulate the genome. Expansion of the genome also correlates with an increase in size and complexity of transcription units, with the exception of plants. P = Promoter DNA element.

Organisms have a vast array of ways in which their respective genomes are organized. A comparison of the genomic organization of six major model organisms shows size expansion with the increase of complexity of the organism. There is a more than 300-fold difference between the genome sizes of yeast and mammals, but only a modest 4- to 5-fold increase in overall gene number. However, the ratio of coding to noncoding and repetitive sequences is indicative of the complexity of the genome: The largely "open" genomes of unicellular fungi have relatively little noncoding DNA compared with the highly heterochromatic genomes of multicellular organisms.

In particular, mammals have accumulated considerable repetitive elements and noncoding regions, which account for the majority of their DNA sequences (52% non-coding and 44% repetitive DNA). Only 4% of the mammalian genome thus encodes for protein function (including intronic sequences). This massive expansion of repetitive and noncoding sequences in multicellular organisms is most likely due to the incorporation of invasive elements, such as DNA transposons, retrotransposons, and other repetitive elements. The expansion of repetitive elements (such as Alu sequences) has even infiltrated the transcriptional units of the mammalian genome. This results in transcription units that are frequently much larger (30-200 kb), commonly containing multiple promoters and DNA repeats within untranslated introns.

The vast expansion of the genome with noncoding and repetitive DNA in higher eukaryotes implies more extensive epigenetic silencing mechanisms. Studies of the genomic organization is thought to be the future of genomic medicine, which will provide the opportunity for personalized prognoses in clinics.

11.5 CHROMOSOMAL CHARACTERISTICS

Other sequences are used in replication or during interphase with the physical structure of the chromosome.

- Ori, or Origin: Origins of replication.
- MAR: Matrix attachment regions, where the DNA attaches to the nuclear matrix.

Regions of the genome with protein-coding genes include several elements:

- Enhancer regions (Normally up to a few thousand basepairs upstream of transcription)
- Promoter regions (Normally less than a couple of hundred basepairs upstream of transcription) include elements such as the TATA and CAAT boxes, GC elements, etc.

- Exons are the part of the transcript that will eventually be transported to the cytoplasm for translation. When discussing gene with alternate splicing, an exon is a portion of the transcript that could be translated, given the correct splicing conditions. The exons can be divided into three parts
- The coding region is the portion of the mRNA that will eventually be translated.
- Upstream untranslated region (5' UTR can serve several functions, including mRNA transport, and initiation of translation (including, portions of the Kozak sequence). They are never translated into the protein (excepting various mutations).
- The 3' region downstream from the stop codon is separated into two parts:
  - 3' UTR is never translated, but serves to add mRNA stability. It is also the attachment site for the poly-A tail. The poly-A tail is used in the initiation of translation and also seems to have an effect on the long-term stability (aging) of the mRNA.
  - An unnamed region after the poly-A tail, but before the actual site for transcription termination, is spliced off during transcription, and so does not become part of the 3' UTR. Its function, if any, is unknown.

Introns are intervening sequences between the exons that are never translated. Some sequences inside introns function as miRNA, and there are even some cases of small genes residing completely within the intron of a large gene. For some genes (such as the antibody genes), internal control regions are found inside introns. These situations, however, are treated as exceptions.

Many regions of the DNA are transcribed with RNA as the functional form:

- rRNA: Ribosomal RNA are used in the ribosome.
- tRNA: Transfer RNA are used in the translation process by bringing amino acids to the ribosome.
- snRNA: Small nuclear RNA are used in spliceosomes to help the processing of pre-mRNA.
- gRNA: Guide RNA are used in RNA editing.
- miRNA: Micro RNA are small (approximately 24 nucleotides) that are used in gene silencing.
- snoRNA: Small nucleolar RNA are used to help process and construct the ribosome.

Other RNAs are transcribed and not translated, but have undiscovered functions.

11.6 REPETITIVE SEQUENCES

Eukaryote and also human DNA contains large portion of noncoding sequences. As for the coding DNA, the noncoding DNA may be unique or in more identical or similar copies. DNA sequences with high copy numbers are then called repetitive sequences. If the copies of a sequence motif lie adjacent to each other in a block, or an array, we are speaking about tandem repeats, the repetitive sequences dispersed throughout the genome as single units flanked by unique sequence are interspersed repeats.

Satellites are unique sequences that are repeated in tandem in one area. Depending on the length of the repeat, they are classified as either:

- Minisatellite: Short repeats of nucleotides.
- Microsatellite: Very short repeats of nucleotides. Some trinucleotide repeats are found in coding regions (see, Trinucleotide repeat disorder). Most are found in noncoding regions. Their function is unknown, if they have any specific function. They are used as molecular markers and in DNA fingerprinting.

Interspersed sequences are tandem repeats, with sequences that are found interspersed across the genome. They can be classified based on the length of the repeat as SINE and LINE:

- SINE: Short interspersed sequences. The repeats are normally a few hundred base pairs in length. These sequences constitute about 13% of the human genome with the specific Alu sequence accounting for 5%.
- LINE: Long interspersed sequences. The repeats are normally several thousand base pairs in length. These sequences constitute about 21% of the human genome.

Most interspersed repeats originate by a process of transposition, which is "jumping" of a DNA segment to another place of the genome. There are essentially two types of transposable DNA elements, or transposons: DNA transposons and retrotransposons.
http://biol.lf1.cuni.cz/ucebnice/en/repetitive_dna.htm - main

11.7. LET US SUM UP

- Structure of prokaryotic genome which may be include small circular / linear DNA molecules called plasmids have been explained
- Unique features of prokaryotic genome such as repetitive sequences and transposons, are also described
- Operon – characteristic feature of bacterial genome
- Operon comprises of promoter, operator and terminator gene sequences
- DNA molecules that move from one site to another are called as transposable elements
  http://en.wikipedia.org/wiki/DNA - _note-10

11.8. LESSON END ACTIVITIES

What are transposable elements?
What are insertion sequences?
Explain in brief about the chromosomal characteristics
What are the components of lac operon?
Explain the physical structure of genetic arrangement
Explain the gentic organization in brief.

11.9. POINTS FOR DISCUSSION

Give a detailed account on lac operon
Describe the various types DNA Transposons
What are the repetitive sequences?

11.10. REFERENCES

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LESSON – 12

GENETIC SYSTEMS OF MICROORGANISMS

Contents

12.0. AIMS AND OBJECTIVES

The chapter deals with the genetic system of various microorganisms viz., bacteria, virus, yeast and protozoa.

12.1. INTRODUCTION

Inspite the microorganisms have general structure of DNA and RNA material, genetic code and gene arrangements, the genetic system varies based upon the hierarchy of the complexity of the microorganism. These are discussed in general in this chapter.

12.2. GENETIC SYSTEM OF BACTERIA

Species classification based on same morphological structure, does not hold good for bacteria which can interbreed. There are three ways of gene flow.

- Transformation, naked DNA uptake. Prokaryotes living in similar ecological niches exchange genes, increase fitness lateral gene transfer
- Transduction via a bacteriophage, small piece of DNA transferred.
- Conjugation, two bacteria come into physical contact (segment of chromosomal DNA integrated into plasmid called episome transfer).

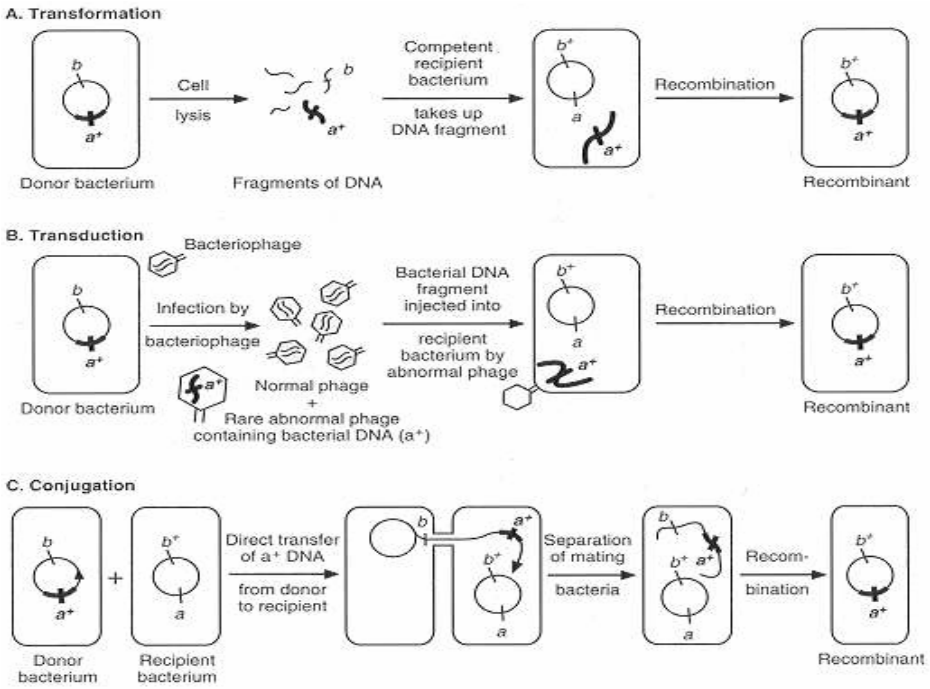

Figure 5: a. Transformation b. Transduction c. Conjugation

12.2.1 BACTERIAL TRANSFORMATION

Bacterial transformation is the process by which bacterial cells take up naked DNA molecules. If the foreign DNA has an origin of replication recognized by the host cell DNA polymerases, the bacteria will replicate the foreign DNA along with their own DNA. When transformation is coupled with antibiotic selection techniques, bacteria can be induced to uptake certain DNA molecules, and those bacteria can be selected for that incorporation. Bacteria which are able to uptake DNA are called "competent" and are made so by treatment with calcium chloride in the early log phase of growth. The bacterial cell membrane is permeable to chloride ions, but is non-permeable to calcium ions. As the chloride ions enter the cell, water molecules accompany the charged particle. This influx of water causes the cells to swell and is necessary for the uptake of DNA. The exact mechanism of this uptake is unknown. It is known, however, that the calcium chloride treatment be followed by heat. When *E. coli* are subjected to 42ºC heat, a set of genes are expressed which aid the bacteria in surviving at such temperatures. This set of genes are called the heat shock genes. The heat shock step is necessary for the uptake of DNA. At temperatures above 42ºC, the bacteria's ability to uptake DNA becomes reduced, and at extreme temperatures the bacteria will die.

Uptake of free DNA molecules from the surrounding medium occurs naturally.
S.pneumococcus: Capsular polysaccharides responsible for virulence

Smooth bacteria – virulent

Rough bacteria – avirulent (without polysaccharide)

Heat killed smooth bacteria + Live rough bacteria ⟶ Transformed ⟶ Virulent

Bacterial strains such as those used by Griffiths and Avery have NATURAL COMPETENCE, i.e. they have the ability to take up DNA from the medium. Natural competence is a genetically programmed physiological state. Natural transformation is distinct from artificial transformation by techniques such as electroporation, protoplast formation, and microprojectiles. In addition, some bacterial strains, such as *E. coli,* can be made ARTIFICIALLY COMPETENT using $CaCl_2$ and heat shock treatment.

The process of transformation in both gram negative and gram positive bacteria is broadly similar. In both cases, DNA fragments must first bind to the exterior of the cell. They are then fragmented which may occur concommitantly with uptake into the periplasm in gram negative bacteria. Finally, one DNA strand is taken up into the cytoplasm while the other is degraded. Recombination appears to be quick in gram negative bacteria but somewhat slower in gram positive bacteria.

Competent **c**ells are the cells that are permeable to DNA. Under certain conditions the uptake is increased various reasons such as change in cell wall material at some stage of growth, induction/activation of receptors on the cells that binds DNA. Only some cells become competent which secrete proteins that activate receptors and convert or produce more competent cells. Calcium chloride treated cells become competent but mechanism is different from natural competency.

DNA uptake may be facilitated by DNA interaction in two ways- reversible binding and irreversible interaction.

Reversible binding – it can be washed off / degraded by DNase.

Irreversible interaction – passage of DNA through cell membrane which requires energy.

Uptake is non specific except in case of *H.influenzae*.

Two experiments were performed.

- Str^s + DNA of Str^r incubated for 15mins and washed free of unincorporated DNA. Then the cells were taken – one part plated give rise to Str^r cells- from other part DNA extracted and used to transform fresh Str^s gave rise to Str^r cells.
- Str^s + DNA of Str^r incubated for two min and washed free of unincorporated DNA. The cells were taken from which one part plated give rise to Str^r cells and from other part DNA was extracted and used to transform fresh Str^s. The DNA did not transform.

These experiments prove that a period during which no transforming DNA can be isolated from potentially transformed cells (about first ten minutes) called eclipse exists. During uptake double strand binds wherein one strand is degraded and only one strand enters the host (Gram positive and Gram negative except *H. influenzae*).

12.2.1.1 MOLECULAR MECHANISM OF TRANSFORMATION

Two possible mechanisms – (a) incoming donor allele replaces the allele of the recipient, (b) donor allele is added to the genome.

An experimental evidence to confirm the molecular mechanism was performed. When x^- strain and DNA from x^+ strain were mixed x^+ strain was formed. The DNA from this transformed cell is mixed with x^- strain. After transformation both x^+ and x^- strains appeared. If (b) mechanism is true then after transformation no x^- strain should be there because all have x^+ and x^- genes. Hence (a) mechanism proved to be true.

It is not known if transformation is a natural phenomenon in all bacteria -- it may be widespread in nature but it can be difficult to observe in the laboratory. As mentioned before, it was discovered first in the gram positive *Streptococcus pneumoniae*. It has since been observed and characterized in the gram negative *Haemophilus influenzae* and in the gram positive *Bacillus subtilis.*

Cells need to be competent to take up DNA from the external milieu. In gram positive bacteria this requires the presence of a DNA-binding protein on the surface of the cell. The presence of this protein is correlated with nutritional shift-down -- i.e. when the cells start to run out of nutrients.

The existence of a ssDNA intermediate within the cell has been inferred from the fact that the cell enters an **ECLIPSE** period after it has been transformed. In theory, once a cell has taken up DNA -- containing a specific marker -- from the medium then it should be

possible to isolate total DNA immediately from the newly transformed cells and then use that DNA in a second transformation -- selecting for the same marker.
However, in naturally transformed *B. subtilis* cells, this is not possible -- no successful transformants will be found. They will only be found if one waits a period of time before isolating total DNA to carry out the second transformation.

During transformation of *B. subtilis*, DNA from the medium is taken up as ssDNA molecules -- but *B. subtilis* cannot be transformed with ssDNA. So, until the ssDNA is converted into dsDNA as a result of recombination with the host chromosome, it will not be possible to obtain any transformants for the selected marker. The eclipse period is the time required to convert ssDNA into a stable dsDNA form.

12.2.1.2 USES OF TRANSFORMATION

- Mapping: based on recombination frequencies between markers. But frequency varies with size hence not reliable. Based on the principle that two markers transform together if they are near enough – but varies with competence and DNA concentration.
- Determine effects of chemical agents on DNA. When a bacterium or an animal is exposed to a particular chemical and mutagenesis occurs, one cannot be sure that the chemical acts directly on DNA. To ensure that pure DNA with a marker is exposed to the chemical and used for transformation. If the marker is inactive it may be sure that the chemical is active on DNA
- Identifying DNA can be done by transforming the isolated DNA and testing the transformants

12.2.2 CONJUGATION

Bacterial conjugation is the transfer of genetic material between bacteria through direct cell-to-cell contact. conjugation is a mechanism of horizontal gene transfer—as are transformation and transduction—although these mechanisms do not involve cell-to-cell contact.This is a mechanism in which plasmid is transferred between donor and recipient cells. F plasmid contains genes that mediate transfer of F to F^- cells. These are genes that regulate plasmid replication. Three transposons (IS2, IS3 and γδ/Tn 1000) are responsible for integration of F into chromosome. When F integrates into chromosomes the cell becomes Hfr donor (Hfr – high frequency) because Hfr cells transfer genes to F^- cell with much frequency than from F^+ cell. Integrated F sequence are always flanked by two copies of one of IS elements in F plasmid. Integration is a reciprocal DNA exchange similar to phage λ integration.

Bacterial conjugation is often incorrectly regarded as the bacterial equivalent of sexual reproduction or mating. It is not actually sexual, as it does not involve the fusing of gametes and the creation of a zygote, nor is there equal exchange of genetic material. It is merely the transfer of genetic information from a donor cell to a recipient. In order to perform conjugation, one of the bacteria, the donor, must play host to a conjugative or mobilizable genetic element, most often a conjugative or mobilizable plasmid or transposon. Most conjugative plasmids have systems ensuring that the recipient cell does not already contain a similar element.

The prototype for conjugative plasmids is the F-plasmid, also called the F-factor. The F-plasmid is an episome (a plasmid that can integrate itself into the bacterial chromosome by genetic recombination) of about 100 kb length. (One kb is one thousand base pairs) It carries its own origin of replication, called oriT. There can only be one copy of the F-plasmid in a given bacterium, either free or integrated (two immediately before cell division). The host

bacterium is called F-positive or F-plus (denoted F+). Strains that lack F plasmids are called F-negative or F-minus (F-).

Among other genetic information, the F-plasmid carries a tra and a trb locus, which together are about 33 kb long and consist of about 40 genes. The tra locus includes the pilin gene and regulatory genes, which together form pili on the cell surface, polymeric proteins that can attach themselves to the surface of F- bacteria and initiate the conjugation. Though there is some debate on the exact mechanism, the pili themselves do not seem to be the structures through which the actual exchange of DNA takes place; rather, some proteins coded in the tra or trb loci seem to open a channel between the bacteria.

When conjugation is initiated, via a mating signal, a relaxase enzyme creates a nick in one plasmid DNA strand at the origin of transfer, or oriT. The relaxase may work alone or in a complex of over a dozen proteins, known collectively as a relaxosome. In the F-plasmid system, the relaxase enzyme is called TraI and the relaxosome consists of TraI, TraY, TraM, and the integrated host factor, IHF. The transferred, or T-strand, is unwound from the duplex plasmid and transferred into the recipient bacterium in a 5'-terminus to 3'-terminus direction. The remaining strand is replicated, either independent of conjugative action (vegetative replication, beginning at the oriV) or in concert with conjugation (conjugative replication similar to the rolling circle replication of lambda phage). Conjugative replication may necessitate a second nick before successful transfer can occur. A recent report claims to have inhibited conjugation with chemicals that mimic an intermediate step of this second nicking event.

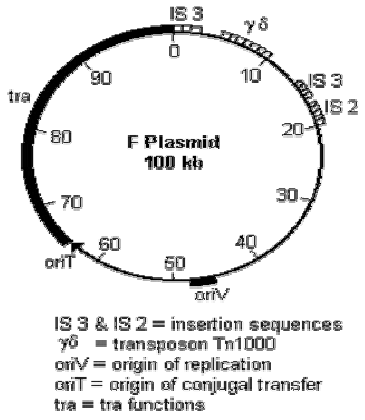

Figure 6 F plasmid

If the F-plasmid becomes integrated into the host genome, donor chromosomal DNA may be transferred along with plasmid DNA. The certain amount of chromosomal DNA that is transferred depends on how long the bacteria remain in contact; for common laboratory strains of E. coli the transfer of the entire bacterial chromosome takes about 100 minutes. The transferred DNA can be integrated into the recipient genome via recombination.

A culture of cells containing non-integrated F plasmids usually contains a few that have accidentally become integrated, and these are responsible for those low-frequency chromosomal gene transfers which do occur in such cultures. Some strains of bacteria with an integrated F-plasmid can be isolated and grown in pure culture. Because such strains transfer chromosomal genes very efficiently, they are called Hfr (high frequency of recombination). The E. coli genome was originally mapped by interrupted mating experiments, in which various Hfr cells in the process of conjugation were sheared from recipients after less than

100 minutes (initially using a Waring blender) and investigating which genes were transferred.

12.2.2.1 CHARACTERISTIC OF INTEGRATION OF F

Two ways by which Hfr cells arise are homologous recombination and replicon fusion. Homologous recombination occurs between two identical IS element (1 in chromosome and 1 in F) and this requires either recombination or transposon gene product. Replicon fusion involves formation of an co integrate mediated by an IS element in F and duplication of a target sequence in the chromosome.

Site where F integrates into the chromosome is not unique. Integration occurs at many sites (20major sites) and frequency of formation of particular Hfr strain varies with IS elements in the site. F can integrate both clockwise and counter clockwise orientation.

12.2.2.2 Hfr TRANSFER

F integrates into chromosome and behaves as if it is a part of chromosome – remains single, circular. F can transfer the entire chromosome of Hfr to F^- cell. Stages of transfer are (i) pairing of donor and recipient, (ii) rolling circle replication donor DNA, (iii) transfer of newly synthesized strand and (iv) conversion of transferred DNA to double stranded. First a portion of F DNA is transferred and chromosomal genes are transferred. Finally the remaining F DNA is transferred.

Difference between F transfer and Hfr transfer

1. 100min for entire chromosome transfer (Hfr) and 2min – F transfer
2. During Hfr transfer the mating pair may break up before entire chromosome is transferred because of long duration.
3. When Hfr and F^- mixed F^- results unless full F DNA is transferred.
4. In Hfr transfer the transfer DNA does not circularize and cannot replicate. The donor DNA recombines and generates recombinants in F^- cells. To recognize recombinants two markers are needed.

$$Hfr\ leu^+\ Str^s \times F^-\ leu^-\ Str^r$$

Leu^+ to select against parental recipient (selected marker)

Str^r to select against parental donor (counter selective marker) – $leu^+\ Str^r$ recombinant – exconjugants.

12.2.2.3 INTERRUPTED MATING AND TIME OF ENTRY MAPPING

The transfer of the Hfr chromosome proceeds at a constant rate from a fixed point determined by the site at which F has been inserted in the Hfr chromosome. Order of transfer will reflect the gene order in the chromosome. Time at which particular genetic loci enter an F^- recipient is related to the positions of loci on the chromosome. By mechanically interrupting transfer during mating at various times mapping of genes can be performed. The time at which a particular gene is transferred can be determined by purposely breaking the mating cells apart at various times and noting the earliest time at which breakage no longer prevents recombination from appearing. This is called the interrupted mating technique. This technique reflects the loci of genes and order of genes by rate of chromosome transfer.

12.2.2.4 FORMATION OF F' PLASMID

Integrated F can excise out of chromosome and may contain genes adjacent to F in the chromosome. This excised sequence form a plasmid called F' plasmid. To prove formation of F' plasmid an exconjugant and a recipient are allowed for short time mating. The resultant

cells if transformed indicates that F' plasmid has transferred because transfer of integrated chromosome takes longer time.

12.2.2.5 RECOMBINATION IN RECIPIENT CELLS

The final stage of bacterial conjugation is the incorporation of a transferred DNA fragment into the recipient chromosome. Linear DNA that enter the cell cannot survive in a cell. DNA is unable to replicate because they lack *ori* / and only circular DNA replicate (like phage DNA) by rolling circle mechanism. DNA may be degraded by cellular nucleases. Hence integration is important. Two exchanges is necessary because linear DNA to be integrated into circular DNA. Only single strand is integrated. Recombination stimulated at specific sequence called chi sequence. High frequency of recombination observed close to the leading end of an Hfr is attributed to the entry of recBCD protein complex at double strand.

Plateau values indicate the efficiency of transfer a marker and probability of recombination. Probability that conjugation will be disrupted before transfer is complete is the major factor for plateau values. Plateau value decrease with time of entry. Since two recombination events is important for integration of marker, recombination frequency is affected by the distance of the marker from the ends of the fragment. **Anomalous plateau value** (low) occurs when the marker is near transfer origin. Later markers also give anomalous value but a high plateau value.

Proteins required for recombination are Rec A and Rec BCD. Polymerization of Rec A on single strand DNA results in the formation nucleoprotein. Synapsis occurs when nucleoprotein interacts with double strand circle but not homologous region hence sequence independent interaction. After the synapis alignment of homologous region occurs and this is called homologous alignment. Post synaptic strand exchange occurs when aligned, but not intertwines and this stage is unstable. RecA promotes displacement of a strand from the double strand molecule and assimilation of new strand. RecA acts as a helicase.

Rec BCD has helicase activity and also nuclease activity (3'-5'strand). When chi is recognised, it nicks DNA just upstream of chi site and D with nuclease activity is released.

In Rec A mutants the recombination decreased by 10^6 fold and in RecBC mutants only 1000 fold. Hence some alternate pathways with RecE and RecF were found to play role in recombination.

When the cells are $RecBCD^-$ and sbc A^+ (suppressor Rec BC) no RecE (nuclease activity similar to D) is produced. Recombination does not occur. Hence cells with $RecBCD^-$ sbc A^- produced recE and recombination occurs. recE and sbc A are located on defective cryptic prophage called Rac. recF gene is inhibited by $sbcB^+$ and therefore, in $recBC^-$ sbc^- strains recombination occurs.

12.2.2.6 CHROMOSOME TRANSFER IN OTHER BACTERIA

Chromosome mobilization involves by chromosome transfer by F' containing strains ie via plasmids. F plasmid transfer create unstable Hfr cell lines. Hence R' plasmids developed. This modified plasmids share a homologous region between plasmid and chromosome.

a) by rDNA technique, a segment of chromosome inserted into the plasmid
b) a transposon inserted into plasmid and plasmid transferred to a cell whose chromosome contain transposon inserted it.

So far all Gram negative have conjugative plasmid. Conjugative plasmids are also present in Gram positive. Instead of pili as in *E.coli* sometimes cell to cell contact can be mediated by diffusible proteins pheromones of recipient cells which cause clumping of number of donor and recipient cells.

12.3 TRANSDUCTION

Bacteriophages are viruses that infect bacteria. In the process of assembling new virus particles, some host DNA may be incorporated in them. The virion head can hold only so much DNA so these viruses

- while still able to infect new host cells
- may be unable to lyze them.

Instead the hitchhiker bacterial gene (or genes) may be inserted into the DNA of the new host, replacing those already there and giving the host an altered phenotype. This phenomenon is called **transduction**. Transduction is the process by which bacterial DNA is moved from one bacterium to another by a virus.

When bacteriophages (viruses that infect bacteria) infect a bacterial cell, their normal mode of reproduction is to harness the DNA replication machinery of the host bacterial cell and make numerous copies of their own DNA or RNA. These copies of bacteriophage DNA or RNA are then packaged into newly synthesized copies of bacteriophage virions.

12.3.1 LYSOGENIC CYCLES

Transduction happens through either the lytic cycle or the lysogenic cycle. If the lysogenic cycle is adopted, the phage chromosome is integrated into the bacterial chromosome, where it can remain dormant for thousands of generations. If the lysogen (eg. UV light) is induced, the phage genome is excised from the bacterial chromosome and initiates the lytic cycle, which culminates in lysis of the cell and the release of phage particles. Two types of lysogenic cycle have been observed in *E.coli* phage λ and *E.coli* phage p1.

- *E.coli* phage λ: linear DNA injected. mRNA synthesis occurs and produce suppressor protein and site specific recombination enzyme. mRNA synthesis is turned off. Recombination between phage DNA and bacterial DNA occurs. Bacterium continues to grow and phage gene also replicate.
- *E.coli* phage p1: no DNA insertion system. Phage becomes a plasmid.

Lysogenic cells are capable of entering either lytic or lysogenic cycle. Bacterium containing a complete set of phage genes called lysogen. Phage DNA in lysogens called a prophage. Important properties of lysogens are resistant to reinjection (immunity) after many generations also. Lysogen can initiate lytic cycle (induction).

Prophage insertion occurs by two region

- BOB region O region – common base sequence.
- POP region where attachment occurs.

Exchange is catalyzed by phage enzyme integrase.

12.3.2 LYTIC CYCLE

The lytic cycle leads to the production of new phage particles which are released by lysis of the host. Adsorption of phage on bacterial surface is by specific receptors. Passage of DNA from the phage through bacterial cell wall is via hypodermic syringe like mechanism which injects the DNA without exposure to surrounding in case of phage with tail, tail less phage. The phage coat breaks open and release nucleic acid into cell wall. Conversion of infected bacterium to a phage producing cell is caused after infection, because bacteria lose ability to replicate (by degradation of host DNA). Production of phage nuclei and proteins occurs. Either synthesize phage specific polymerases or modify the specificity of bacterial polymerases for protein production. There are two stages viz. early proteins and late proteins synthesis. RNA phages should synthesize own polymer.

For the assembly of phage particles structural protein are needed. Catalytic proteins that are not present in phage also participate in assembly. Maturation proteins help in converting DNA into a package able form.

Steps involved are aggregation of phage structural proteins, condensation of nucleic acid and entry into preformed head and attachment of tail to filled head. Release of newly synthesized phage is by the lysis of the cell by 'holin' (disrupts cytoplasmic membrane) and lysozyme (degrade cell wall). Some release progeny continuously by outfolding of cell wall called extrusion.

However, the packaging of bacteriophage DNA is not fool-proof and at some low frequency, small pieces of bacterial DNA, rather than the bacteriophage genome, will be packaged into the bacteriophage genome. At the same time, some phage genes are left behind in the bacterial chromosome.

There are generally two types of mistakes that can lead to this incorporation of bacterial DNA into the viral DNA, leading to two modes of recombination. Two types are generalized and specialized.

- Generalized: produce particles with only bacterial DNA – bacterial DNA fragment got from any part.
- Specialized: produce aprticles with both phage and bacterial DNA in a single DNA molecule – bacterial genes got from particular region of the bacterial chromosome.

12.3.3 GENERALIZED TRANSDUCTION

If bacteriophages undertake the lytic cycle of infection upon entering a bacteria, the virus will take control of the cell's machinery for use in replicating its own viral DNA. If by chance bacterial chromosomal DNA (instead of viral DNA) is inserted into the viral capsid used to contain the viral DNA, while this lytic pathway is proceeding, the mistake will lead to generalized transduction. The new virus capsule now loaded with part bacterial DNA is no longer infectious, but will still attempt to infect another bacterial cell.

When the new DNA is inserted into this recipient cell it can fall to one of three fates:

1. The DNA will be absorbed by the cell and be recycled for spare parts.
2. If the DNA was originally a plasmid, it will re-circularize inside the new cell and become a plasmid again.
3. If the new DNA matches with a homologous region of the recipient cell's chromosome, it will exchange DNA material similar to the actions in conjugation.

This type of recombination is random and the amount recombined depends on the size of the virus being used. *E.coli* phage p1 and *S.typhimurium* phage p22 are the viruses that cause transduction. p22 is most studied.

p22 infects *S. typhimurium* by binding to O antigen part of lipopolysaccharide on outer membrane. After infection recircularizes by binding of cohesive ends. Initially undergoes θ replication then rolling circle replication. Long concatamers of double strand DNA are produced. Nuclease cuts the DNA at pac site and DNA is packed into the head.

In *S. typhimurium* 'pseudo pac' sites – phage nuclease cuts 'pseudo pac' site – packs into head – phage contains only bacterial DNA. It can transfer any region chromosomal DNA and hence called generalized transduction. After injection of bacterial DNA by phage into another host it transduces host. Recipient should be rec^+ and absence of rec^+ no integration can occur. Hence no continuous replication occurs and they form – only tiny micro colonies called abortive transductants.

Proof for the presence of only bacterial DNA has been observed when bacteria was grown in ^{15}N medium. p22 DNA was labeled with ^{14}N labeled DNA. Infected the bacteria by p22 and allowed to grow. Phage progeny centrifuged in $CsCl_2$ and I DNA isolated by density

based separation. Viable phage had only ^{15}N labeled DNA. This proved that the phage had only bacterial DNA. Transduction can be quantified by EOP and MOI units.

- EOP – efficiency of plating – fraction of phage particles that can form a plaque.
- MOI – multiplicity of infection – average number of adsorbed phage per bacterium.

Cotransduction ie. simultaneous transduction of two gene. Mapping by co transduction is possible. Closer the genes are to each other greater the probability they will be co transduced. Frequency of co transduction is inversely proportional to distance between the two genes.

12.3.4 SPECIALIZED TRANSDUCTION

The second type of mistake is called specialized transduction. If a virus removes itself from the chromosome incorrectly, it can leave part of the viral DNA in the chromosome. Some of the bacterial DNA can be packaged into the virion. Mistakes in this process of viral DNA going from the lysogenic to the lytic cycle lead to specialized transduction. There are three possible results from specialized transduction:

1. DNA can be absorbed and recycled for spare parts.
2. The bacterial DNA can match up with a homologous DNA in the recipient cell and exchange it. The recipient cell now has DNA from both itself and the other bacterial cell.
3. DNA can insert itself into the genome of the recipient cell as if still acting like a virus resulting in a double copy of the bacterial genes.

This type of recombination is not random and only small portions of genes are recombined. Example of specialized transduction is λ phages in *Escherichia coli.*

12.3.5 SPECIALIZED TRANSDUCTION BY NON LYSOGEN

When Gal^- cell is infected with mutant λ and mixed with lysate of Gal^+ lysogenic for λ, lysogeny attained will contain almost entire normal λ phage. When plated on galactose only Gal^+ grows. Based on this observation there are two types of transduction.

- Type I: consists of nonlysogenic Gal^+. All colonies are Gal^+ if again resuspended. As they have arisen as a result of two cross overs they replace gal^- gene with gal^+ and contain one gal^+ gene – haploids.
- Type II: λd gal^+. About 1% is Gal^- and lost prophage. As they have arisen as a result of single cross over both Gal^- and Gal^+ are present which are heterogenotes/partial diploids. They are incapable of tail producing and are unstable.

12.3.6 SPECIALIZED TRANSDUCTION BY LYSOGEN

This produce a heterogenate which occurs at high frequency by single cross over. But are highly unstable than type II and gal^- production is greater.

High frequency – transducing lysates (HFT)

Dilysogen is formed by sequential insertion of two phage - $\lambda dgal^-\lambda^+$ - useful λ^+ substitutes the lacking gene in λdgal – yield lysates half of whose phage are transducing particle – called HFT; lysate from single lysogen LFT.

Specialized transducing phage as cloning vehicle

Proved by φ80 specialized transducing phage to clone supF gene from *E.coli*. used as hybridization probes. lacDNA in φ80 as probe, Lac operon induced – mRNA produced radiolabeled – hybridization – determine amount of radioactive mRNA that are hybridized. At present specific cloning vectors are being used.

12.4 PHAGE I (LYTIC PHAGES) BIOLOGY

Viruses are that grow in bacterial cells and are obligate bacterial parasite. It can persist alone but cannot replicate. Minimal function of virus are protection of its nucleic acid from environment and chemicals, delivery of its nucleic acid to the bacterium, conversion of an infected bacterium into phage producing system, release of progeny phage from an infected bacterium.

Structure of the virus may be either Icosahedral tail less or Icosahedral with tail or filamentous. They contain single nucleic acid either single or double strand linear or circular DNA/single strand linear RNA. Exception is φ6 which has three linear double strand RNA.

12.4.1 GENETICS OF λ PHAGE

Phage contains a linear double strand DNA with 48514 base pairs. At 5' end extends 12 bases beyond 3' endonuclease called cohesive ends which is complimentary to one another. They circularize and *E.coli* DNA ligase seals. Out of 46 λ genes 14 are nonessential for lytic and 7 are nonessential for lytic and lysogenic. Important feature of the genome is clustering of related genes.

oL and oR – operator regions

pL and pR – promoter regions

Transcription starts from pL and pR and terminates at tL1 and tR1. L1 codes only for N and the gene product is important for positive regulation of transcription of O and P genes. N binds with *nutL* and *nutR* and RNA polymerase interacts with N which can then ignore tL1 and tR1 and form longer transcripts L2 and R2 ie. Anti termination occurs. O and P region are important for DNA replication. *red* is essential for recombination. O and P genes produce proteins important for DNA replication. These are not continuously required because it is catalytic protein.

Cro genes produce cro protein and when sufficient amount is produced suppresses O_L and turn off mRNA synthesis of all leftward genes. After some time after inhibiting O_L and $P_{L,}$ it inhibits O_R also and blocks O and P synthesis. By that time enough O and P are produced for DNA replication.

Throughout this time R4 is synthesized from pR_2 and terminating at tR4. R4 is leader for late mRNA. Once R3 has been made, Q gene product is expressed and binds to *qut* and prevents tR4 transcription. This allow synthesis of late mRNA. R4 is then extended to form R5 transcript. Late mRNA then encodes for head, tail assembly and lysis proteins.

λ **phage assembly**: Earliest precursor of head are scaffolded prehead. By proteolytic processing groE (host protein) prehead is formed which is used for packaging of concatenated linear DNA. Nu1 A bind near left *cos* site of tandem polymers of linear DNA and becomes attached to specific area of prehead. Protein F1 wound DNA into the prehead and when head filled, D1 protein attaches to outside of Capsid locking the head in place. The left and right *cos* sites are brought close together at the entrance and ter function of A protein cleaves in a staggered fashion and heads are filled. Tail is attached and mature virus is formed.

12.4.2 GENETICS OF *E.coli* PHAGE T_4

There are 4 classes based on structure and life cycle. T even phages are attractive subject because it is easily grown and many mutants can easily be got. The DNA molecules contain an unusual base.

The four classes are

- T1
- T2,T4,T6
- T3,T7
- T5

DNA of **phage T4** has a molecular weight of 10million with 1, 66,000bp and its length is 55µm. They contain adenine, thymine, guanine and 5 – hydroxymethylcytosine (HMC) which is further modified by glycolysation. Other even phages differ in the sugar group. The sugar moieties protect from variety of DNases. They are terminally reductant ie. a sequence of bases repeated at both ends of the molecule.

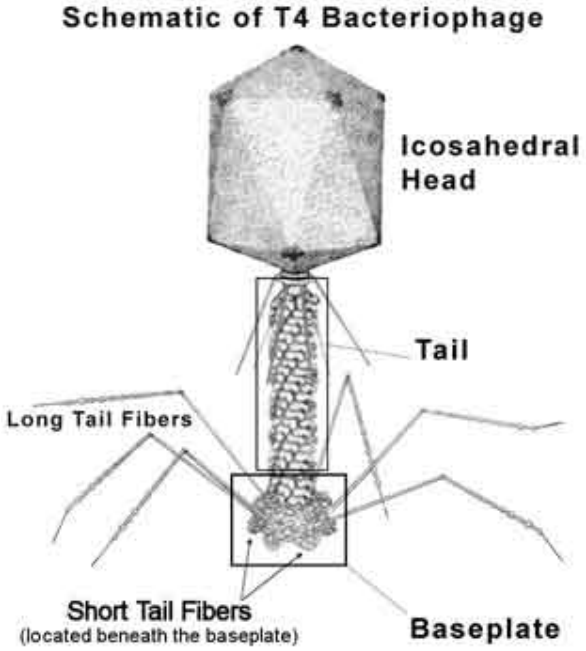

Figure. 7 T4 Bacteriophage

Genetic organization

135 genes are identified which accounts to only 90% and 15-20 genes yet to be found. Two classes of genes are 82 metabolic genes which are only important (DNA synthesis, trans, lysis) and 53 genes responsible for particle assembly.

Steps of life cycle

Phage absorption to bacterial cell wall takes place and DNA is injected into host. The synthesis of host DNA, RNA and proteins is turned off .The synthesis of first phage mRNA begins and degradation of bacterial DNA takes place. When synthesis of late mRNA begins completed head and tails appear. After complete phage particle is assembled lysis of bacteria occurs.

After infection host RNA polymerase is modified and they do not recognize host promoters. The phage mRNA encodes DNases and degrade host DNA. Turning off synthesis of host DNA, RNA and proteins precedes synthesis of nucleases.

Replication of T4 : Unique features of T4 DNA replication are,

- Sources of nucleotide are degraded host DNA which are degraded by 2 endonucleases of genes *denA* and *denB*. They are active only on cytoplasm containing DNA. dNMP are converted to dNTP by *E.coli* enzymes and are provided for replication. 5 phage coded enzymes also supply dNTPs by denovo synthesis.
- Synthesis of HMC: 5-hydroxymethyl cytosine *E.coli* does not posses such enzyme. 2 phage enzyme convert dCMP to dHDP.

dCMP ⟶ dHMP (by T4 hydroxyl methylase)
dHMP ⟶ dHDP (by HMP kinase)
dHDP ⟶ dHTP (by phosphate kinase)

- Prevention of incorporation of cytosine into T4 DNAis important. Cytosine containing DNA gets degraded and cannot be used as template for transcription in late life cycle.Phage produces dCTPase and degrades dCDP and dCTP. Occasionally some cytosine appears in progeny – degraded by DNase (only single strand which has cytosine) – repair mechanism synthesis the strand.
- Glycosylation of T4 DNA: HMC-DNA may be degraded by *E.coli* nucleases – hence glycosylated by two phage enzymes α-glycosyl transferase and β-glycosyl transferase. Transfer glucose occurs from UDPG to HMC.

Assembly: Production of phage particles occurs after assembly of head-tail and packaging of DNA. Assembly of head and tail are separate events and independent processes. Long strand of DNA is packed into head and packaging begins with attachment of end of DNA molecule to a protein contained in phage head. Condensation protein/molecule induces folding. Cuts is made after enough DNA enters the head which is called headful mechanism.

12.4.3 GENETICS OF *E.coli* PHAGE T7

This is midsized phage with molecular weight 26×10^6. It has equal amount of ATGC and no unusual bases. It has terminal redundancy and not permuted. It has very short tail.

Organisation of genes: Among 55 known genes only 34 gene products are known and for 27 genes functions are known. Leaders and spacers sequences are only a few nucleotides long for economic utilization. Genes are clustered according to function and are arranged to provide continuous order according to the time of their function.

Growth cycle: First adsorption of phage onto the bacteria occurs. Initiation of slow injection of phage DNA into host occurs which takes about 10 min for complete injection. This is a significant feature of T7. Initiation of synthesis of phage mRNA takes place. Turning off host transcription begins. Initiation of synthesis of structural proteins occurs after completion of injection. Phage assembly occurs after complete injection. Host cell lysis occurs and progeny is released.

Transcription occurs in two stages.Rarly (uses *E.coli* RNA polymerase) and late (used phage RNA polymerase). Three classes of transcripts – I, II and III – from same strand r strand – synthesis in right ward direction. Three promoters for each transcript.

For Transcript I promoter I is used. Synthesis begins two min after infection and transcription is by bacterial RNA polymerase. Stops at termination site (gene1.3) and cleaved by *E.coli* RNase III to 5 mRNAs. Transcript I proteins mainly are protein kinase (phosphorylate bacterial polymerase – inactivates) and RNA polymerase (takes over transcription work).

Transcript II is first synthesized earlier than III since class II enters before the entry of class III. This starts upstream of termination site of class I and phage RNA polymerase ignores termination. The transcript terminates at next termination site (next to gene 10) (only 90% terminated 10% continuous synthesizing). Transcript II proteins are second inhibitor of *E.coli* RNA polymerase and completely shuts down *E.coli* DNA and Transcript I synthesis. Proteins involved in DNA replication are also synthesised. Structural proteins and Amidases that inhibits overall transcription are also produced. Lysozyme like enzyme causes lysis of host cell.

Eventhough DNA replication is completed and structural proteins formed quickly, there is no assembling because maturation and proteins synthesis is delayed by delay entry. 15 min after infection, transcript II synthesis shuts off and strong promoter pIII alone remains active.

12.4.4 GENETICS OF *E.coli* PHAGE φX174

This contains one circular single strand DNA with 5386 nucleotides. DNA is enclosed in icosahedral head composed of three coat proteins and one integral protein. It has eleven genes and amino acids in proteins exceed the coding capacity. This proves that overlapping genes are found. The phage gets adsorbed to *E.coli* by a spike protein. Unlike T phages the spike protein H enters bacteria along with DNA. DNA is not injected into bacteria and release the phage DNA on the surface. **Gene function**:

A – Rf replication, viral strand synthesis.
A* - Turning of host DNA synthesis
B – Formation of Capsid
C – Formation of phage unit sized DNA
D – Formation of Capsid
E – Lysis of bacteria
F – Major coat protein
G – Major spike port
H – Minor spike port; adsorption to host
J – Core protein – entry of progeny into phage particles
K – Unknown

Replication: Single strand of DNA in phage is (+) strand complimentary to this is (–) strand. Steps:

- Conversion of single strand DNA to closed circular double strand DNA called replicative form I (RFI) – depends on host enzymes.
- Protein n' binds to single strand DNA and acquires a bound ATP and then primase joins preprimosome to form primosome – protein n' used ATP energy to move primosome along the DNA until the priming site – DNA B protein alters the DNA strand and enable primase to initiate synthesis of RNA primer – replication then proceeds.
- Synthesis of many copies of RF I – (-) strand transcribed to produce protein A – protein A cause single strand break in (+) strand (between 4305 and 4306 – replication origin) and remains linked to 5' phosphor termini – forms RFII.
- *E.coli* proteins (synthesis prior to infection) causes (+) strand to be displaced from RFII by looped rolling circle replication – displaced (+) strand cleaved from looped rolling circle – recircularized – used as a template for next synthesis.
- Synthesis of (+) strand for encapsulation – no switching occurs from RFI synthesis to packaging – but packaging system captures + strand before replication – capture is delayed till enough RFI is formed.

Transcription: The genome has three promoters which are activated simultaneously. Except for lysozyme there is no regulation for synthesis. All transcripts from are from (-) strand and hence no transcripts till (-) strand is synthesized. After synthesis RFI (Relicative fork I) synthesis continues and lysozyme activity is delayed because of slow translation. Lysis occurs after 30 min of infection and 500 phage released.

Packaging requires seven proteins. 4 proteins form the particle itself. Head protein F and spike protein G form pentamers in the presence of gene B protein and form an aggregate 12S particle. Gene H protein added to complete spike and gene D protein forms a frame on which a prohead is built from 12S (prohead – has B, D, F, G, H) – gene C protein bound to 5' end of (+) strand directs the 5' end into prohead. Gene J protein initially binds to DNA outside the prohead and organizes the DNA inside the head and consolidates the structure. B protein is released during single strand DNA synthesis. A protein cuts displaced (+) strand and circularizes. D protein is then removed

12.5 BACTERIAL PLASMIDS

Plasmid is a DNA molecule separate from the chromosomal DNA and capable of autonomous replication. It is typically circular and double-stranded. It usually occurs in bacteria, and is sometimes found in eukaryotic organisms (e.g., the *2-micrometre-ring* in *Saccharomyces cerevisiae*).The size of plasmids varies from 1 to over 400 kilobase pairs (kbp). There may be one copy, for large plasmids, to hundreds of copies of the same plasmid in a single cell, or even thousands of copies, for certain artificial plasmids selected for high copy number (such as the pUC series of plasmids). Plasmids can be part of the mobilome, since they are often associated with conjugation, a mechanism of horizontal gene transfer Plasmids often contain genes or gene cassettes that confer a selective advantage to the bacterium harboring them, such as the ability to make the bacterium antibiotic resistant.

Every plasmid contains at least one DNA sequence that serves as an *origin of replication*, or *ori* (a starting point for DNA replication), which enables the plasmid DNA to be duplicated independently from the chromosomal DNA. The plasmids of most bacteria are circular, like the plasmid depicted in Figure8, but linear plasmids are also known, which superficially resemble the chromosomes of most eukaryotes.

12.5.1 ANTIBIOTIC RESISTANCE

http://en.wikipedia.org/wiki/Image:Example_plasmid.png

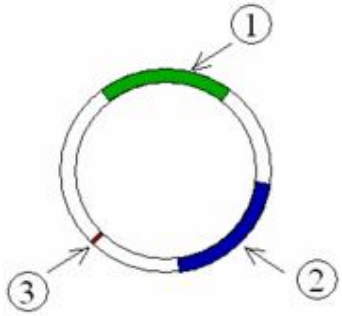

Figure 8 http://en.wikipedia.org/wiki/Image:Example_plasmid.png**Illustration of a plasmid with antibiotic resistances. 1 & 2 Genes that code for resistance. 3 Ori.**

12.5.2

http://en.wikipedia.org/wiki/Image:Example_plasmid.png**EPISOME**http://en.wikipedia.org/wiki/Image:Example_plasmid.png

An **episome** is a plasmid that can integrate itself into the chromosomal DNA of the host organism. For this reason, it can stay intact for a long time, be duplicated with every cell division of the host, and become a basic part of its genetic makeup. This term is no longer commonly used for plasmids, since it is now clear that a region of homology with the chromosome such as a transposon makes a plasmid into an episome. In mammalian systems, the term episome refers to a circular DNA (such as a viral genome) that is maintained by noncovalent tethering to the host cell chromosome.

12.5.3 GROUPING PLASMIDS

One way of grouping plasmids is by their ability to transfer to other bacteria. *Conjugative* plasmids contain so-called *tra-genes*, which perform the complex process of *conjugation*, the sexual transfer of plasmids to another bacterium. *Non-conjugative* plasmids are incapable of initiating conjugation, hence they can only be transferred with the assistance of conjugative plasmids, by 'accident'. An intermediate class of plasmids are *mobilizable*, and carry only a subset of the genes required for transfer. They can 'parasitise' a conjugative plasmid,

transferring at high frequency only in its presence. Plasmids are now being used to manipulate DNA and may possibly be a tool for curing many diseases.
It is possible for plasmids of different types to coexist in a single cell. Seven different plasmids have been found in *E. coli*. But *related* plasmids are often incompatible, in the sense that only one of them survives in the cell line, due to the regulation of vital plasmid functions. Therefore, plasmids can be assigned into *compatibility groups*.
Another way to classify plasmids is by function. There are five main classes:

- *Fertility-F-plasmids*, which contain tra-genes. They are capable of conjugation.
- *Resistance-(R)plasmids*, which contain genes that can build a resistance against antibiotics or poisons. Historically known as R-factors, before the nature of plasmids was understood.
- *Col-plasmids*, which contain genes that *code for* (determine the production of) bacteriocins, proteins that can kill other bacteria.
- *Degradative plasmids*, which enable the digestion of unusual substances, e.g., toluene or salicylic acid.
- *Virulence plasmids*, which turn the bacterium into a pathogen.

Plasmids can belong to more than one of these functional groups.
Plasmids that exist only as one or a few copies in each bacterium are, upon cell division, in danger of being lost in one of the segregating bacteria. Such single-copy plasmids have systems which attempt to actively distribute a copy to both daughter cells.Some plasmids include an *addiction system* or "postsegregational killing system (PSK)", such as the hok/sok (host killing/suppressor of killing) system of plasmid R1 in Escherichia coli. They produce both a long-lived poison and a short-lived antidote. Daughter cells that retain a copy of the plasmid survive, while a daughter cell that fails to inherit the plasmid dies or suffers a reduced growth-rate because of the lingering poison from the parent cell.

12.5.4 APPLICATIONS

Plasmids serve as important tools in genetics and biochemistry labs, where they are commonly used to multiply (make many copies of) or *express* particular genes. Many plasmids are commercially available for such uses.The gene to be replicated is inserted into copies of a plasmid which contains genes that make cells resistant to particular antibiotics. Next, the plasmids are inserted into bacteria by a process called *transformation*. Then, the bacteria are exposed to the particular antibiotics. Only bacteria which take up copies of the plasmid survive the antibiotic, since the plasmid makes them resistant. In particular, the protecting genes are expressed (used to make a protein) and the expressed protein breaks down the antibiotics. In this way the antibiotics act as a filter to select only the modified bacteria. Now these bacteria can be grown in large amounts, harvested and lysed (often using the alkaline lysis method) to isolate the plasmid of interest.Another major use of plasmids is to make large amounts of proteins. In this case, researchers grow bacteria containing a plasmid harboring the gene of interest. Just as the bacteria produces proteins to confer its antibiotic resistance, it can also be induced to produce large amounts of proteins from the inserted gene. This is a cheap and easy way of mass-producing a gene or the protein it then codes for, for example, insulin or even antibiotics.

12.6 DNA TRANSPOSONS

DNA transposons are regarded to be inactive in the human genome due to accumulation of mutations during vertebrate phylogenesis, so we can find only their ancient remnants or "fossils". However, the active transposon derived from the human fossil elements can be engineered with the information gathered from human and other vertebrate genomes.

One example is the Sleeping Beauty transposon, which is a promising component of next generation gene therapy, due to its more specific integration site (than observed e. g. for retroviruses). How a typical DNA transposon functions? The core of the transposable element codes for an enzyme transposase. This enzyme binds to the ends of the element. The ends of the transposon are formed by inverted repeats, which can therefore exchange DNA strands and stabilize the stem-loop structure necessary for transposase action. Transposase then cuts the transposon out and ligates the resulting free chromosomal DNA ends. [Nearly identical mechanism is employed during maturation of immunoglobulin (V-D-J recombination) and TCR (T-cell receptor) genes for excision of the intervening sequences. Interestingly, the enzyme that catalyse this reaction (made from two components RAG1 and RAG2) indeed probably evolved from a transposase.] The free complex transposon-transposase binds to a specific sequence motif elsewhere in the genome, transposase cleaves the host DNA and ligates the transposon into the new place. Thus, the transposon moves by a cut-and-paste mechanism and the copy number remains stable.

12.6.1 RETROTRANSPOSONS

Retrotransposons are most important transposable elements in the human genome. First, they are much more abundant, directly forming at least 45% of the human genome (the estimations vary, but most researchers believe, that it must be even more, since ancient retrotransposons that have been inactivated, have diverged by mutation to the point where they are unidentifiable).Second, retrotransposons are still active in the human genome.

For jumping they require cellular RNA polymerases (II or III) by which they are transcribed into RNA, while the original DNA copy is maintained at the same location. The RNA copy is reverse-transcribed into DNA, and the DNA is inserted into the genome at a new location. Thus, these elements expand in number by a duplication (copy-and-paste) mechanism. As described for the L1 retrotransposon, process of retrotransposition is prone to various mistakes, so the new copies of a retrotransposon would be largely inactivated, because of truncation or point mutation. Because most of the transposon copies are inactive, the further expansion of the retrotransposon family is governed by the few active full-length elements. However, even if all the active elements were lost later during evolution, the genome might be literarly overrun with the fossil members of the sequence family.

Retrotransposons can be further classified as autonomous and nonautonomous. Autonomous retrotransposons are coding for proteins necessary for their transposition, although they are also dependent on host RNA polymerases and DNA repair enzymes for successful jumping. Nonautonomous retrotransposons do not code for any protein and must hijack other transposon´s enzymes to be able of transposition.

Endogenous retroviruses, also called LTR retrotransposons, resemble proviruses of true retroviruses in the composition - they contain long terminal repeats (LTRs), gag, pol, env and prt genes, but at least one of the proteins necessary for assembly of infectious viral particles is mutated or actually missing - env in particular. Thus endogenous retroviruses can move only within cells, otherwise their life cycle is similar to infectious retroviruses, e.g. HIV virus. Although endogenous retroviruses are active in many mammals, including chimpanzee, humans currently contain only fossils (mutated and incapable of transposition), which fill about 8% of the genome. Full-length endogenous retroviruses are typically 7-9 kb long, but as in case of L1 (Fig.9.), many are truncated, especially at the 5´ end. Frequently we can find only standalone LTR, as a result of retroviral insertion and subsequent intrachromosomal recombination between the LTRs or unequal recombination of the homologous chromosomes, leading to deletion of the coding part of the retrovirus.

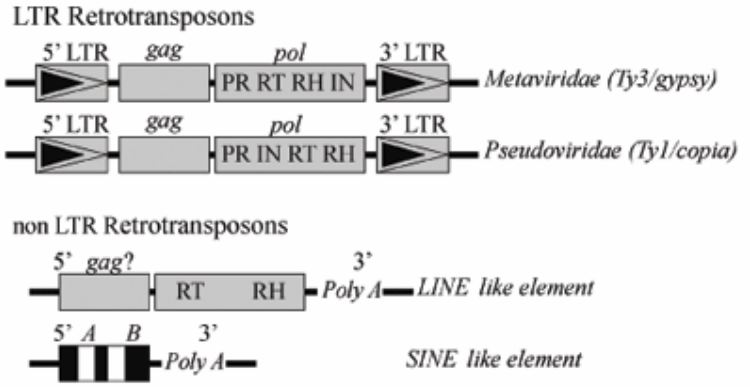

Figure 2 - Schematic representations of the structural features of class I transposable elements (based on Daboussi, 1996 and Havecker *et al.*, 2004). Long terminal direct repeat (LTR) retrotransposons resemble retroviruses in having LTRs flanking an internal domain encoding proteins analogous to the *gag* and *pol* retroviral gene products. The non LTR-retrotransposons lack terminal repeats and carry a poly (A) tail at their 3' ends. Elements with long dispersed nuclear element structures (LINEs) possess two long open reading frames (ORFs), with similarities to *gag* as well as the reverse transcriptase (RT) and RnaseH (RH) genes. Elements with short dispersed nuclear element structures (SINEs) are short elements which contain an internal RNA polymerase III promoter with bipartite structure (boxes A and B) and which rely on RT for mobilization but do not themselves encode the enzyme.

Fig 9: Retrotransposons

LINEs (long interspersed nuclear elements), are **Non LTR autonomous retrotransposons**. They comprise about 21% of the human genome. The active elements belong to the most abundant LINE-1 or L-1 family, which alone comprises 17% of the genome. Of the roughly half milion of L1s in our genome, close to 10,000 are full-length and about 100 are still capable of retrotransposition. Active L1 element is about 6 kb long and contains two open reading frames, ORF1 and ORF2. 5´UTR (untranslated region) functions also as a promoter, 3´UTR contains polyA signal. Function of ORF1 is not clear, it is only known to bind to L1 mRNA, ORF2 contains reverse transcriptase and endonuclease domain and is the enzyme responsible for integration. Life cycle of L1 begins with transcription of L1 DNA by cellular RNA polymerase II and standard maturation into mRNA molecule. The L1 mRNA is transported into cytoplasm and ORF1 translated. Then the translation is reinitiated on an internal ribosomal entry site (IRES) to translate ORF2 (uncanonical and ineffective process in eukaryotes, so only portion of L1 mRNAs get their ORF2 protein). Both proteins immediately bind to the L1 mRNA. This protein-mRNA complex is transported into nucleus. ORF2 cuts chromosomal DNA at the target site (target site is not absolutely specific like it is true for restriction endonucleases, but there is some prefererence for AT rich sequences, cleavage site are approximately TT/AAAA). The DNA cut is unequal (creating sticky ends). Free 3´OH group on one side the cleaved DNA molecule is used by reverse transcriptase of ORF2 to prime the synthesis of the first cDNA strand (target primed reverse transcription). Detailed mechanism of second cDNA strand synthesis is still subject to discussion, but the process ends by stable integration of double stranded L1 DNA on a new place in the genome. Because of staggered DNA break made by transposon endonuclease, the integrated L1 element is flanked by target site duplication 7-20 bp. The reverse transcriptase is often incapable to finish first strand synthesis, resulting in 5´truncation of the newly formed copy. Reverse transcriptase also lacks proofreading (3´to 5´ endonuclease) activity, often introducing mutation into the new copy. Interestingly, L1 mRNA is expressed predominantly

in meiotic and postmeiotic spermatocytes, increasing thus the L1 potencial for copy expansion (copies introduced into germ line can be inherited, as opposed to somatic transposition events).

SINEs (**Nonautonomous retrotransposons**) short interspersed nuclear elements are typically less than 500bp long and have no protein coding potential. The main SINE family in humans is formed by Alu elements (the name is derived by their discovery based on a pair of conserved AluI restriction sites). The greater than 1 million Alu elements in the human genome account for about 11% of its mass.

Alu elements share 282 bp consensus, which is related to, and was presumably derived from the SRP (signal recognition particle) RNA subunit (called 7SL RNA). SRP is a ribonucleo-protein complex that recognizes signal peptide, binds to it and translocates the ribosome-mRNA-nascent peptide complex to endoplasmic reticulum (ER) channel, through which the nascent protein is translocated into the ER lumen or integrated into the membrane. Alus are, like 7SL RNA gene, transcribed by RNA polymerase III. Alu RNA can bind two SRP proteins (9 and 14). Presumably, Alu can thus bind to ribosomes and by its polyA tail it can bind (if the ribosome just happens to translate LINE-1 mRNA) nascent ORF2 protein, and force ORF2 protein to reverse transcribe and integrate its RNA and not the LINE-1 mRNA.

12.6.2 TANDEM REPEATS http://biol.lf1.cuni.cz/ucebnice/en/repetitive_dna.htm - main

Tandem repeats are made of succesive identical or nearly identical (degenerate) repeat units. They vary in length of repeat unit as well as lenght of the whole repeat much, so every classification is not satisfying and must be taken "cum grano salis". The largest repeats, which tend to be composed from large repeat units, are called satellites. The name satellites comes from centrifugation of DNA in density gradients. First, during DNA isolation conventional methods, DNA is subject to shear stress, with resulting DNA fragmentation (note that in vivo one G1 phase chromosome contains 1 DNA molecule). These fragments can be then centrifuged in density gradients so the DNA molecules occupy places in the gradient with the same density as the DNA molecule. Bulk of DNA will form one band. But DNA fragments with significantly different CG/AT content; caused e. g. by large monotonous repeats will form minor "satellite" bands. The denomination of satellite DNA was later broadened to incorporate similarly repetitive sequences that are not forming these satellite bands. Satellite primary repeat units are various, from GGAAT found in satellites 2 and 3 to 171 bp in alpha satellite. But these primary units are often degenerated, conaining certain irregularities. These irregularities can be periodical, forming thus secondary repeat units. Satellite DNA is abundant at centromeres and constitutive heterochromatin. Although human genome is considered completely assembled, the centromere regions and heterochromatin containing satellite sequences are not included, since the sequencing of such regions is from various reasons challenging (absence of restriction sites, difficult sequencing, and almost impossible contig assembly). From the various satellites found at or near the centromere, a family of alpha-satellite repeat (with primary unit 171 bp) probably form functional core of centromeres, as they are important for kinetochore assembly during cell division (some kinetochore proteins bind to the alpha-satellite at centromere, and thus nucleate kinetochore assembly). The function of other satellites is unknown, regarded mostly as junk DNA.

Minisatellites are shorter tandem repeats, in the range of kb, which are enriched in subtelomeric regions of chromosomes. They are often highly polymorphic as to the number of repeat units in a repeat (many alleles in the population) and can be used as genetic markers - VNTR, variable number of tandem repeats. VNTRs are often too large to be amplified by PCR and are therefore typically assayed by Southern blot. Sometimes, certain minisatellites

are hypothesised to have regulatory functions, as e.g. a VNTR in insulin promoter, where different length of the repeat was associated with different types of diabetes. One allele of the insulin VNTR is shown on fig. 7. Telomeres of human chromosomes, formed by several kilobases of the hexamer repeat TTAGGG belong also to the minisatellite range of tandem repeats, although they arise by a specific mechanism - by the enzyme telomerase. Telomerase is composed from a protein subunit with reverse transcriptase activity and an RNA subunit with a sequence complementary to TTAGGG, which serves as a template for the telomere elongation (telomerase protein subunit is related to reverse transcriptase of non-LTR retrotransposons). However, telomeres can elongate even by the passive general mechanism of unequal crossing-over, e.g. in cancer cells. Maybe it should be noted here once again, that the sequence of the human genome comprises the euchromatic regions, bounded proximally, but not including the centromeres and pericentromeric heterochromatin, and distally by telomeres, which are also, together with subtelomeric regions not included.

Microsatellites have repeat units typically 1-5 bp, with repeat length rarely exceeding hundreds of repetitions in order. Most common family of these repeats are 2 bp repeats, from which (CA)n repeats are prevailing. The microsatellites are very common in the genome, highly polymorphic and are very often used as genetic markers. Examples of such genetic markers are in chapter covering linkage.

http://biol.lf1.cuni.cz/ucebnice/en/repetitive_dna.htm - main

12.7. GENETIC SYSTEM OF VIRUS

12.7.1 RNA VIRUS

RNA virus is a virus which belongs to either *Group III*, *Group IV* or *Group V* of the Baltimore classification system of classifying viruses. As such, they possess ribonucleic acid (RNA) as their genetic material and do not replicate using a DNA intermediate. The nucleic acid is usually single-stranded RNA (ssRNA) but can occasionally be double-stranded RNA (dsRNA). Notable human pathogenic RNA viruses include SARS, Influenza and Hepatitis C viruses. Walter Fiers (University of Ghent, Belgium) was the first to establish the complete nucleotide sequence of a gene (1972) and of the viral genome of a virus: Bacteriophage MS2-RNA (1976)

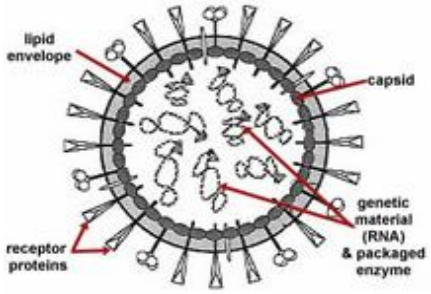

Fig 10: RNA virus

RNA Sense

RNA viruses can be further classified according to the sense or polarity of their RNA into negative-sense and positive-sense RNA viruses. Positive-sense viral RNA is identical to viral mRNA and thus can be immediately translated by the host cell. Negative-sense viral RNA is complementary to mRNA and thus must be converted to positive-sense RNA by an RNA polymerase before translation. As such, purified RNA of a positive-sense virus can directly cause infection though it may be less infectious than the whole virus particle. Purified RNA of a negative-sense virus is not infectious by itself as it needs to be transcribed into positive-sense RNA

Double-stranded RNA viruses

The double-stranded (ds)RNA viruses represent a diverse group of viruses that vary widely in host range (humans, animals, plants**,** fungi, and bacteria**),** genome segment number (one to twelve), and virion organization (T-number, capsid layers, or turrets). Members of this group include the rotaviruses, renowned globally as the commonest cause of gastroenteritis in young children, and bluetongue virus, an economically important pathogen of cattle and sheep. In recent years, remarkable progress has been made in determining, at atomic and subnanometeric levels, the structures of a number of key viral proteins and of the virion capsids of several dsRNA viruses, highlighting the significant parallels in the structure and replicative processes of many of these viruses

Mutation rates

RNA viruses generally have very high mutation rates as they lack DNA polymerases which can find and fix mistakes, and are therefore unable to conduct DNA repair of damaged genetic material. DNA viruses have considerably lower mutation rates due to the proof-reading ability of DNA polymerases within the host cell. Retroviruses integrate a DNA intermediate of their RNA genome into the host genome, and therefore have a higher chance of correcting any mistakes in their genome thanks to the action of proof-reading DNA polymerases belonging to the host cell. Although RNA usually mutates rapidly, recent work found that the SARS virus and related RNA viruses contain a gene that mutates very slowly. The gene in question has a complex three-dimensional structure which is hypothesized to provide a chemical function necessary for viral propagation, perhaps as a ribozyme. If so, most mutations would render it unfit for that purpose and would not propagate

Replication

Animal RNA viruses can be placed into about four different groups depending on their mode of replication.

- Positive-sense viruses have their genome directly utilized as if it were mRNA, producing a single protein which is modified by host and viral proteins to form the various proteins needed for replication. One of these includes RNA replicase, which copies the viral RNA to form a double-stranded replicative form, in turn this directs the formation of new virions.
- Negative-sense viruses must have their genome copied by a RNA polymerase or transcriptase to form positive-sense RNA. This means that the virus must bring along with it the RNA-dependent RNA polymerase enzyme. The positive-sense RNA molecule then acts as viral mRNA, which is translated into proteins by the host ribosomes. The resultant protein goes on to direct the synthesis of new virions, such as capsid proteins and RNA replicase, which is used to produce new negative-sense RNA molecules.
- Double-stranded reoviruses contain up to a dozen different RNA molecules which each code for a mRNA. These all associate with proteins to form a single large complex which is replicated using virally-encoded replicase to form new virions.
- Retroviruses are single-stranded but unlike other single-stranded RNA viruses they use DNA intermediates to replicate. Reverse transcriptase, a viral enzyme that comes from the virus itself after it is uncoated, converts the viral RNA into a complementary strand of DNA, which is copied to produce a double stranded molecule of viral DNA. This DNA goes on to direct the formation of new virions.

12.7.2 RETROVIRUS

All RNA viruses have linear genomes, without significant terminal repeat sequences, and all employ RdRps. These are template-specific, but do not have proofreading ability, and do not make use of RNA primers for replication, as do all DNA polymerases. The RdRps also all specifically recognise different origins of replication at the 3'-termini of both (+) and (-) sense RNAs, whatever the type of genome.

12.7.2.1 ENTRY INTO CELLS

There is a fundamental difference in mechanisms employed to enter host cells between viruses infecting animal cells and viruses infecting plants. This is because animal cells are separated by barriers far less formidable than the thick, rigid and impermeable cell walls consisting of cellulose and pectin that separate plant cells from one another.

12.7.2.2 PLANT VIRUSES

Because plant cell walls are so thick compared to the sizes of the viruses infecting them (>10 m m compared to largely <1 m m), plant viruses have not evolved mechanisms similar to those of bacteriophages for entering their host cells. The only ways that viruses can enter plant cells to cause a primary infection are via:

- A purely mechanical injury that breaches the cell wall and transiently breaches the plasma membrane of underlying cells;
- similar gross injury due to the mouthparts of a herbivorous arthropod, such as a beetle
- injection directly into cells through the piercing mouthparts of sap-sucking insects or nematodes
- carriage into plant tissue on or in association with cells of a fungal parasite
- vertical transmission through infected seed or by vegetative propagation;
- transmission via pollen; and
- Grafting of infected tissue onto healthy tissue.

For example, the ssRNA(-) viruses Tomato spotted wilt virus (TSWV) and Crimean-Congo haemorrhagic fever virus (CCHFV) - both in family Bunyaviridae share a common particle morphology, and infect the cells of their respective arthropod hosts in similar ways: that is, by a specific attachment and a fusion or phagosomal uptake mechanism. CCHFV also infects the the cells of its mammalian hosts similarly (table 3). However, TSWV infects plant cells by injection directly into cells via the piercing mouthparts of its insect vector, the Western flower thrips, and not via membrane interactions. ;;

Once virions are in the cytoplasm, they are generally uncoated to some extent by a variety of processes, including simple dissociation and/or enzyme-mediated partial degradation of the particles, to release the viral genome as a naked RNA or as a nucleoprotein complex.

12.7.2.3 ANIMAL VIRUSES

The initial phase of cell entry starts when attachment proteins on the virion surface attach to specific receptors on the cell surface. Both attachment proteins and receptors are normally glycoproteins; the cellular proteins can be things like transplantation markers (MHC proteins), adhesins, or simply sialyloligosaccharides (sugars attached to glycoproteins) in the case of ortho- and paramyxoviruses. The attachment is normally temperature- and pH-dependent, and is due to the same sorts of molecular structural complementarities - "lock and key" fit - as occur with enzyme-substrate and antibody-antigen binding. There are essentially two different paths that are followed for entry into the cell: these are

- receptor-mediated endocytosis, and
- direct membrane fusion.

The first is perhaps the primary means of viral cell entry, and is simply a subversion of a normal cellular process. Virus particles become attached at multiple sites to cellular receptors, as these consolidate within the plasma membrane. If these complexes migrate to coated pits, they are internalised as clathrin-coated vesicles as part of normal endocytosis. These vesicles quickly fuse with endosomes and then lysosomes, which renders their internal environment considerably more acidic and introduces a host of degradative proteases, lipases, and other enzymes. The pH shift generally triggers conformational changes in the attachment protein complexes, which in the case of enveloped virions, may expose lipophilic "fusion domains" that allow fusion of the viral envelope and the vesicle membrane. This has been show to occur with orthomyxoviruses, for example. In the case of non-enveloped virions, pH-induced conformational changes in the capsid may cause increased hydrophobicity / lipophilicity, which will allow interactions with the vesicle membrane that can cause pore formation. This is known to occur with picornaviruses. In either case, the result is the entry of an RNA-protein complex (=nucleoprotein or nucleocapsid) or of naked RNA into the cytoplasm, which is the most important part of the uncoating process.

Direct membrane fusion as a mode of entering cells is possible only with enveloped viruses, and is common among paramyxoviruses. The viruses require a fusion-promoting protein on their virion surfaces, which, in the presence of consolidated receptor-attachment protein binding, promotes fusion of cell and virion membranes and the release of the nucleoprotein into the cell cytoplasm. This is a pH-independent process, and may occur at the cell surface, or within an endosomal vesicle.

Based on morphology viruses can be classified into:

1. A – TYPE
2. B – TYPE
3. C – TYPE
4. D – TYPE

VIRUS GROUPS	HOSTS
Avian Retro Viruses Subgroups A-E Subgroups F,G	Chicken, Quail, Duck Pheasant
Reticuloendothelial Virus	Chicken And Other Birds, Some Mammals
Murine Leukemia Viruses Ecotropic Xenotropic Amphotropic	 Mouse, rat Mink, Human, and other mammals Most mammals
Mouse Mammary Tumor Virus	Mouse

Table: 3 Morphology classification of animal viruses

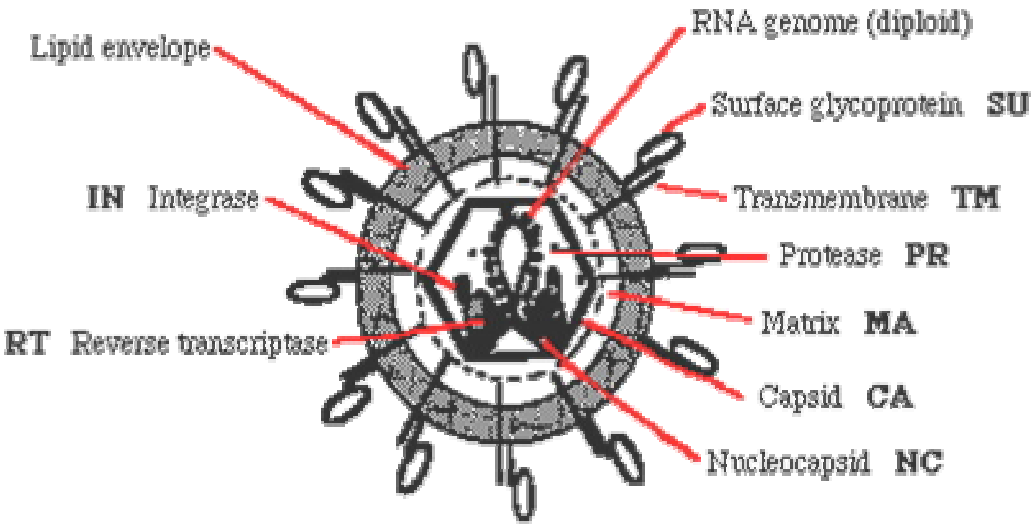

Figure: 11 Retrovirus

Retroviral genome is unique. It has two identical ssRNA.Each is 8.5 to 9.5 Kb size. The 5' end is capped and 3' is polyadenylated. 3 regions called gag, pol, env. Replication of genetic material is through DNA.

12.7.2.4 GENOME EXPRESSION AND REPLICATION

The replication of these viruses is intimately involved with the expression of their genomes: all of the viruses must produce all or most of the components of an RdRp, and often other proteins as well, in order to transcribe full-length complementary RNA molecules from RNA templates. Whereas modes of entry of the viruses split largely along host lines, the exact type of genome of the virus determines the mode(s) of expression and replication. For example,

- ssRNA(+) genomes may be wholly or partially translated upon entry into the cell to produce the RdRp, followed by synthesis of full-length complementary RNA(-) and then of full-length RNA(+), and often also of subgenomic mRNA(s).
- ssRNA(-) genomes must be accompanied into the cell by RdRp for subgenomic mRNAs to be transcribed before translation is possible.
- dsRNA genomes are transcribed conservatively from within virion-derived nucleoprotein complexes, and new genomes are transcribed by newly synthesised RdRp from the mRNAs so liberated.

Additionally, all plant-infecting viruses possess one or more movement-related protein (MP) genes: these are very varied, although there are distinct groups of them, and they appear to derive from host plant genes for chaperonins and plasmodesmata-associated proteins.

ssRNA(+) genomes

All of these viruses have wholly or partially translatable genomes, and as a result are usually infectious as naked RNA. Apart from this common feature, there are few other evolutionary similarities. After partial or complete uncoating upon entry into the cytoplasm, the genomic RNA is recognised by the translation initiation factors and ribosomal subunits and translation of the open reading frame (ORF) nearest the 5' end of the RNA(s) is initiated. If there are still proteins bound to the RNA, which in the case of plant viruses is most likely, this process efficiently strips them off. While all ssRNA(+) genomes have at least one ORF accessible for translation, and those with multicomponent genomes will have more than one,

expression of any with more than one ORF per genome segment will suffer from the limitation that the eukaryotic translation machinery is heavily biased to expressing only the 5'-proximal ORF. Thus, these viruses have evolved two main strategies for expressing their whole genomes. These are:

- expression of the whole of a genome component as a single ORF, which is then proteolytically processed to yield smaller protein products
- expression of 5'-distal ORFs via subgenomic mRNAs

Replication in all cases involves an initial transcription of full-length RNA(-) from an infecting RNA(+) template, and transcription from this of RNA(+), and perhaps also subgenomic mRNA. Replication complexes are usually closely associated with membrane complexes derived from the ER or perhaps nuclear membranes, and free RNA(-) is not found. In some cases it has been shown that coat protein (CP) helps regulate the expression of RNA(+), in that cp- mutants accumulate approximately equal amounts of both senses of RNA, while normal viruses accumulate much more RNA(+), especially as the CP concentration increases late in infection. dsRNA forms of viral genomes and of subgenomic RNAs can be isolated from infected cells for many ssRNA(+) viruses, including most plant viruses, some picorna-like insect viruses, and coronaviruses: this may be how some dsRNA viruses originated.

- **ssRNA(-) viruses**

These viruses seem to be an evolutionarily recent development, as they infect only higher eukaryotes, like arthropods, vertebrates, and higher plants. The viruses infecting plants probably do so as a result of close association of insects and host plants in recent evolutionary times; most of these still also infect an insect vector / alternative host. The group includes the only taxonomic order among RNA(-) viruses: this is the order Mononegavirales, including the families Orthomyxoviridae, Paramyxoviridae, and Filoviridae, all of which have single-component genomes and share a basic genome arrangement and significant sequence similarities (Figure 4). Nearly all of the RNA(-) viruses in Table 1 share a similar major RdRp subunit (L-type protein gene); there are also similarities in their nucleoproteins (N or NP genes).

Replication of all the viruses commences with the transcription by virion-associated RdRp of usually monocistronic mRNAs from genomic RNA(s) in the newly uncoated nucleoprotein complexes. For the segmented genomes of bunya- and orthomyxoviruses, this usually means a single mRNA per segment; for the non-segmented mononegaviruses, this means multiple transcription initiation and termination events on a full-length RNA(-), at intergenic repeated sequences, with transcription apparently usually initiating at the genomic 3'-end with synthesis of a 50-base leader. Transcripts are capped and polyA tailed; the RdRp complex (consisting of L, N / NP and other proteins) adds caps, while tails are apparently added by RdRp stuttering at short polyU repeats at the end of genes. Independent transcription events in paramyxoviruses allow control of level of expression: these viruses transcribe far more mRNAs for structural protein genes at the 3'-end of the genome than for regulatory genes at the 5'-end, possibly due to the progressive failure of the RdRp complex at reinitiating multiple times down the length of the RNA(-). Production of full-length RNA(+) rather than of mRNAs is triggered by binding mainly of newly-synthesised viral N (nucleoprotein) but also of P (RdRp minor subunit) proteins to the 5'-leader sequence, somehow causing the RdRp to ignore all termination and polyadenylation signals. The RNA(+) is then used as template for RNA(-) transcription: this also has a 5'-leader, which is also recognised as an assembly origin by N protein. Thus, concomitant genome or anti-genome synthesis and nucleoprotein assembly occur, with a bias for (-) strand synthesis, possibly due to preferential recognition of the (+) strand 3'-origin.

Commonalities in expression and replication of ssRNA(-) viruses appear to include distinct transcription and replication functions for the RdRp, probably triggered by binding of the virion nucleoprotein (N or NP) subunits. Thus, both RNA(-) and RNA(+) may be found complexed with N proteins in replication complexes. As for ssRNA(+) viruses, glycoproteins (GPs) are generally expressed as are cellular trans- or outer membrane proteins; that is, they have signal sequences that result in translocation into the rough ER during translation, and are subsequently glycosylated according to signals perceived by the cellular machinery.

- **dsRNA viruses**

While it is tempting to speculate that these viruses are the monophyletic survivors of a pre-DNA dsRNA genome era, the truth is that, although there is very wide diversity among dsRNA viruses, at least some of them may descend from ssRNA(+) viruses. Two distinct groups of dsRNA viruses have polymerase affinities with alpha-like and poty-like viruses respectively. Thus, the viruses are certainly polyphyletic in origin, and there is almost certainly a wide variety of mechanisms used for expression and replication. However, many of the viruses have not been well studied, so details are lacking.

Reoviruses are the best-studied dsRNA viruses. Representatives of the family infect plants, animals, and insects, and many infect an insect vector as well as an animal or plant alternate host. The viruses all have a double capsid structure, the outer layer of which is stripped off, partly due to proteolysis, during endocytotic entry. Naked core particles in the cytoplasm are able to transcribe capped and non-polyadenylated genome-segment-length monocistronic mRNAs, via an RdRp activity associated with the insides of the hollow spike structures at 5-fold rotational axes of symmetry. These are extruded into the cytoplasm as they are synthesised, and are translated. Viral products accumulate as viroplasms: associations of viral structural and polymerase proteins and mRNAs result in assembly of immature particles, inside which mRNAs are transcribed to give RNA (-) molecules with which they become base-paired. This is the best-characterised example of conservative replication for any organism. New "core" particles also produce mRNAs, but these appear to be largely uncapped.

12.7.2.5 ASSEMBLY AND EXIT

The processes of assembly of the virions are as varied as their structures; however, there is a logical divide between those with membranes, and those without. The former tend to be considerably more complex than the latter, which may be as simple as a nucleoprotein composed of a single type of protein. There is a commonality between all of the viruses in that their core nucleoproteins assemble either as helices (usually) or as isometric particles. This assembly is usually a simple process, but often very specific, and is driven by increasing concentrations of genomic or pre-genomic RNA and of structural protein. Assembly takes place in the cytoplasm for all except the orthomyxoviruses, which assemble nucleoproteins containing N, PB1, PB2, and PA proteins in the nucleus, from where they are exported to the cytoplasm after association of the complexes with the M1 or matrix protein. The interaction of protein and RNA may be promoted by their sequestration in inclusion bodies or viroplasms, which are often associated with elaborations of internal ER-derived membranes.

For some of the simple naked isometric viruses, specific nucleation of assembly at low CP concentration is followed by complete nucleocapsid assembly as CP concentration increases. For picornaviruses, however, there is a complex assembly process. One model of the process involves assembly of a complete RNA-free provirion. This then undergoes autolytic protein cleavage due to its associating with genomic RNA, which is then encapsidated due to a complicated structural reorganization. In another model, viral RNA is complexed with smaller protein aggregates, which are then further processed. Reoviruses also have a complex assembly process, starting with the mRNA-protein complex, which becomes

a RNAse-sensitive double capsid that does not contain nonstructural (NS) proteins. This synthesises RNA(-) strands, and then undergoes some structural changes to become RNAse-insensitive and to have NS proteins associated with it. Virions may collect in amorphous or paracrystalline arrays inside infected cells: plant viruses especially may accumulate at very high concentrations. Release of such virions may be induced by virus-induced cell lysis, such as is the case with some picornaviruses. However, in most cases release is by cell death followed by membrane degradation.

12.8. GENETIC SYSTEM OF YEAST

12.8.1 GENETICS OF YEAST

S. cerevisiae contains a haploid set of 16 well-characterized chromosomes, ranging in size from 200 to 2,200 kb. The total sequence of chromosomal DNA, constituting 12,052 kb, was released in April, 1996. A total of 6,183 open-reading-frames (ORF) of over 100 amino acids long were reported, and approximately 5,800 of them were predicated to correspond to actual protein-coding genes. A larger number of ORFs were predicted by considering shorter proteins. In contrast to the genomes of multicellular organisms, the yeast genome is highly compact, with genes representing 72% of the total sequence. The average size of yeast genes is 1.45 kb, or 483 codons, with a range from 40 to 4,910 codons. A total of 3.8% of the ORF contain introns. Approximately 30% of the genes already have been characterized experimentally. Of the remaining 70% with unknown function, approximately one half either contain a motif of a characterized class of proteins or correspond to genes encoding proteins that are structurally related to functionally characterized gene products from yeast or from other organisms.

Ribosomal RNA is coded by approximately 120 copies of a single tandem array on chromosome XII. The DNA sequence revealed that yeast contains 262 tRNA genes, of which 80 have introns. In addition, chromosomes contain movable DNA elements, retrotransposons, that vary in number and position in different strains of *S. cerevisiae*, with most laboratory strains having approximately 30.

Other nucleic acid entities also can be considered part of the yeast genome. Mitochondrial DNA encodes components of the mitochondrial translational machinery and approximately 15% of the mitochondrial proteins. γ^o mutants completely lack mitochondrial DNA and are deficient in the respiratory polypeptides synthesized on mitochondrial ribosomes, i.e., cytochrome *b* and subunits of cytochrome oxidase and ATPase complexes. Even though γ^o mutants are respiratory deficient, they are viable and still retain mitochondria, although morphologically abnormal.

The 2-γm circle plasmids, present in most strains of *S. cerevisiae*, apparently function solely for their own replication. Generally *cir*^{o} strains, which lack 2-γm DNA, have no observable phenotype. However, a certain chromosomal mutation, *nib1*, causes a reduction in growth of *cir*$^+$ strains; due to an abnormally high copy number 2-γm DNA

Similarly, almost all *S. cerevisiae* strains contain dsRNA viruses that constitute approximately 0.1% of total nucleic acid. RNA viruses include three families with dsRNA genomes, L-A, L-BC, and M. Two other families of dsRNA, T and W, replicate in yeast but so far have not been shown to be viral. M dsRNA encodes a toxin, and L-A encodes the major coat protein and components required for the viral replication and maintenance of M. The two dsRNA, M and L-A, are packaged separately with the common capsid protein encoded by L-A, resulting in virus-like particles that are transmitted cytoplasmically during vegetative growth and conjugation. L-B and L-C (collectively denoted L-BC), similar to L-A, have a RNA-dependent RNA polymerase and are present in intracellular particles. *KIL*-o mutants, lacking M dsRNA and consequently the killer toxin, are readily induced by growth at elevated temperatures, and chemical and physical agents.

Yeast also contains a 20S circular single-stranded RNA that appears to encode an RNA-dependent RNA polymerase, that acts as an independent replicon, and that is inherited as a non-Mendelian genetic element.

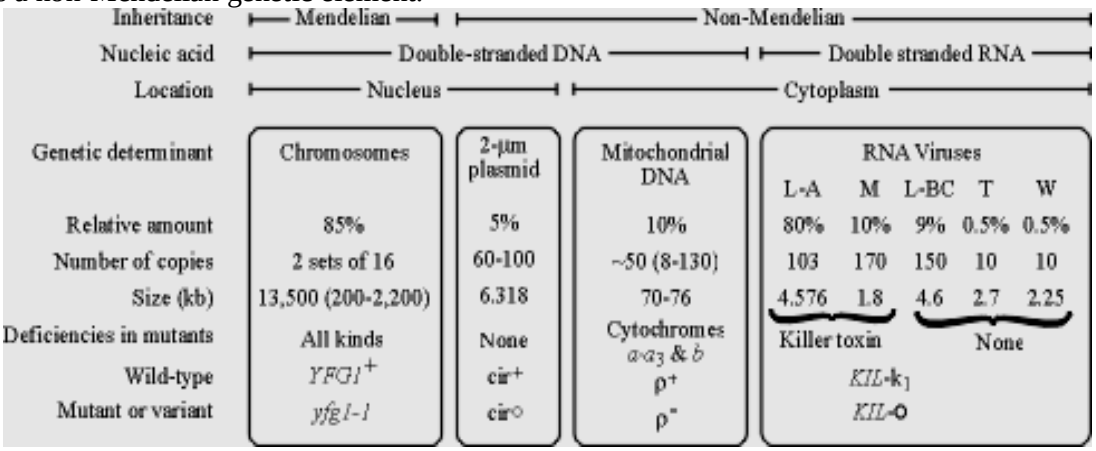

Figure 12: Genetics of Yeast

12.8.1.1 CHROMOSOMAL GENES OF YEAST

The genetic nomenclature for chromosomal genes of the yeast *S. cerevisiae* is now more-or-less universally accepted, using *ARG2* as an example. Whenever possible, each gene, allele, or locus is designated by three italicized letters, e.g., *ARG*, which is usually a describer, followed by a number, e.g., *ARG2*. Unlike most other systems of genetic nomenclature, dominant alleles are denoted by using uppercase italics for all letters of the gene symbol, e.g., *ARG2*, whereas lowercase letters denote the recessive allele, e.g., the auxotrophic marker *arg2*. Wild-type genes are designated with a superscript "plus" (*sup6*$^+$ or *ARG2*$^+$). Alleles are designated by a number separated from the locus number by a hyphen, e.g., *arg2-9*. The symbol γ can denote complete or partial deletions, e.g., *arg2-γ1*. Insertion of genes follow the bacterial nomenclature by using the symbol. For example, *arg2::LEU2* denotes the insertion of the *LEU2* gene at the *ARG2* locus, in which *LEU2* is dominant (and functional), and *arg2* is recessive (and defective).

Phenotypes are sometimes denoted by cognate symbols in roman type and by the superscripts + and -. For example, the independence and requirement for arginine can be denoted by Arg$^+$ and Arg$^-$, respectively. Proteins encoded by *ARG2*, for example, can be denoted Arg2p, or simply Arg2 protein. However, gene symbols are generally used as adjectives for other nouns, for example, *ARG2* mRNA, *ARG2* strains, etc.

Although most alleles can be unambiguously assigned as dominant or recessive by examining the phenotype of the heterozygous diploid crosses, dominant and recessive traits are defined only with pairs, and a single allele can be both dominant and recessive. For example, because the alleles *CYC1*$^+$, *cyc1-717* and *cyc1-γ1* produce, respectively, 100%, 5% and 0% of the gene product, the *cyc1-717* allele can be considered recessive in the *cyc1-717*/*CYC1*$^+$ cross and dominant in the *CYC1-717*/*cyc1γ-1* cross. Thus, sometimes it is less confusing to denote all mutant alleles in lower case letters, especially when considering a series of mutations having a range of activities.

Although superscript letters should be avoided, it is sometimes expedient to distinguish genes conferring resistance and sensitivity by superscript R and S, respectively. For example, the genes controlling resistance to canavanine sulphate (*can1*) and copper sulphate (*CUP1*) and their sensitive alleles could be denoted, respectively, as *can*^{R}*1*, *CUP*^{R}*1*, *CAN*^{S}*1*, and *cup*^{S}*1*.

Wild-type and mutant alleles of the mating-type locus and related loci do not follow the standard rules. The two wild-type alleles of the mating-type locus are designated *MAT*a and

*MAT*γ. The wild-type homothallic alleles at the *HMR* and *HML* loci are denoted, *HMR*a, *HMR*γ, *HML*a and *HML*γ, The mating phenotypes of *MAT*a and *MAT*γ, cells are denoted simply a and γ, respectively. The two letters *HO* denotes the gene encoding the endonuclease required for homothallic switching.

Dominant and recessive suppressors should be denoted, respectively, by three uppercase or three lowercase letters, followed by a locus designation, e.g., *SUP4, SUF1, sup35, suf11*, etc. In some instances UAA ochre suppressors and UAG amber suppressors are further designated, respectively, o and a following the locus. For example, *SUP4*-o refers to suppressors of the *SUP4* locus that insert tyrosine residues at UAA sites; *SUP4*-a refers to suppressors of the same *SUP4* locus that insert tyrosine residues at UAG sites. The corresponding wild-type locus that encodes the normal tyrosine tRNA and that lacks suppressor activity can be referred to as $sup4^{+}$. Intragenic mutations that inactivate suppressors can be denoted, for example, $sup4^{-}$ or *sup4-o-1*. Frameshift suppressors are denoted as *suf* (or *SUF*), whereas metabolic suppressors are denoted with a variety of specialized symbols, such as *ssn* (suppressor of *snf1*), *srn* (suppressor of *rna1-1*), and *suh* (suppressor of *his2-1*)

Capital letters are also used to designate certain DNA segments whose locations have been determined by a combination of recombinant DNA techniques and classical mapping procedures, e.g., *RDN1*, the segment encoding ribosomal RNA.

The general form YCRXXw is now used to designate genes uncovered by systematically sequencing the yeast genome, where Y designates yeast; C (or A, B, *etc*.) designates the chromosome III (or I, II, *etc*.); R (or L) designates the right (or left) arm of the chromosome; XX designates the relative position of the start of the open-reading frame from the centromere; and w (or c) designates the Watson (or Crick) strand. For example, YCR5c denotes *CIT2*, a previously known but unmapped gene situated on the right arm of chromosome III, fifth open reading-frame from the centromere on the Crick strand.

12.8.1.2. MITOCHONDRIAL GENES OF YEAST

Special consideration should be made of the nomenclature describing mutations of mitochondrial components and function that are determined by both nuclear and mitochondrial DNA genes. The growth on media containing nonfermentable substrates (Nfs) as the sole energy and carbon source (such as glycerol or ethanol) is the most convenient operational procedure for testing mitochondrial function. Lack of growth on nonfermentable media (Nfs^{-} mutants), as well as other mitochondrial alterations, can be due to either nuclear or mitochondrial mutations. Nfs^{-} nuclear mutations are generally denote by the symbol *pet*; however, more specific designations have been used instead of *pet* when the gene products were known, such as *cox4*, *hem1*, etc.

The complexity of nomenclatures for mitochondrial DNA genes, is due in part to complexity of the system, polymorphic differences of mitochondrial DNA, complementation between exon and intron mutations, the presence of intron-encoded maturases, diversed phenotypes of mutations within the same gene, and the lack of agreement between various workers. Unfortunately, the nomenclature for most mitochondrial mutations does not follow the rules outline for nuclear mutations. Furthermore, confusion can occur between phenotypic designations, mutant isolation number, allelic designations, loci, and cistrons (complementation groups).

12.8.1.3. YEAST VECTORS

A wide range of vectors is available to meet various requirements for insertion, deletion alteration and expression of genes in yeast. Most plasmids used for yeast studies are shuttle vectors, which contain sequences permitting them to be selected and propagated in *E.*

coli, thus allowing for convenient amplification and subsequent alteration *in vitro*. The most common yeast vectors originated from pBR322 and contain an origin of replication (*ori*), promoting high copy-number maintenance in *E. coli*, and the selectable antibiotic markers, the β-lactamase gene, *bla* (or Amp^R), and sometime to tetracycline-resistance gene, *tet* or (Tet^R), conferring resistance to, respectively, ampicillin and tetracycline.

In addition, all yeast vectors contain markers that allow selection of transformants containing the desired plasmid. The most commonly used yeast markers include *URA3*, *HIS3*, *LEU2*, *TRP1* and *LYS2*, which complement specific auxotrophic mutations in yeast, such as *ura3-52*, *his3-α1*, *leu2-α1*, *trp1-α1* and *lys2-201*. These complementable yeast mutations have been chosen because of their low-reversion rate. Also, the *URA3*, *HIS3*, *LEU2* and *TRP1* yeast markers can complement specific *E. coli* auxotrophic mutations.The *URA3* and *LYS2* yeast genes have an additional advantage because both positive and negative selections are possible, as discussed below (*URA3* and *LYS2*).

Components of common yeast plasmid vectors	YIp	YEp	YRp	YCp
Plasmid				
E. coli genes or segments				
ori, *bla*; *tet*	+	+	+	+
Yeast genes or segments				
URA3; *HIS3*; *LEU2*; *TRP1*; *LYS2*; *etc*.	+	+	+	+
leu2-d	0	+	+	0
2 αm; 2 αm-*ori REP3*;	0	+	0	0
ARS1; *ARS2*; *ARS3*; *etc*.	0	0	+	+
CEN3; *CEN4*; *CEN11*; *etc*.	0	0	0	+
Host (yeast) markers				
ura3-52; *his3-α1*; *leu2-α1*; *trp1-α1*; *lys2-201*; *etc.*	+	+	+	+
Stability	++	+	α	+

Table: 4 yeast plasmid vectors

Although there are numerous kinds of yeast shuttle vectors, those used currently can be broadly classified in either of following three types as summarized in Table: 4 Integrative vectors, YIp; autonomously replicating high copy-number vectors, YEp; or autonomously replicating low copy-number vectors, YCp. Another type of vector, YACs, for cloning large fragments are discussed (Yeast Artificial Chromosomes).

12.8.2 GENETICS OF NEUROSPORA

S.cerevisiaea has with four spores whereas neurospora have asci with eight spores. Two meiotic division followed by one mitotic division results in eight spores arranged in linear order determined by successive meiotic and mitotic divisions.

Fertilization results in zygote (2n). First meiotic division results in two spores (2n) and second meiotic division which results in four spores (n). After mitotic division eight spores (n) are formed.

Genetics of fungi is mainly studied for haploid mapping / tetrad analysis. Provide unique opportunity to analysis total products of meiosis. Yeast has unordered asci. Neurospora has ordered asci.

- **Spore isolation**

Individual ascus was plated on agar. Individual spores were pressed out of ascus. The spores are lined up on the agar. The agar squares are cut, lifted out and placed in individual tubes to grow. Studied are based on phenotypic character. Mutants created to analyze and map chromosomes. Some of the phenotypic characters are,

Yeast – normal pink colony + other markers – mutants many available genes.

Neurospora – filamentous form (wild), fluffy (fl), tuft (tu), dirty (dir), colonial (col4) – mutants. Some sensitivity to sulfa drug (wild) resistant/require for growth (sfo). Some mutant grows in minimal medium (sugar + N_2 source + some organic acids + biotin vitamin). Some requires arg (arg mutant).

- **Genome organization**

Haploid genome has approximately 43Mb chromosomal DNA. Genome sequences has 54% GC content. Individual chromosomes has 4 to 10.3Mb. Little repetitive sequence have found. 2000 different genes have been identified and one active transposon are known. But different inactive DNA sequences represent different transposon families. Except one DNA intermediate element others appear to be retro transposons.

12.8.2.1 TETRAD ANALYSIS

With diploid heterozygous for a gene A there are three features –

- centromeres do not separate following premeiotic DNA replication and hence hold the daughter molecules together.
- Separation occurs after first meiotic division. Chromosome pairing occurs during DNA replication forming four molecule stages.
- Crossing over occurs during four molecule stage before any chromosome separation or cell division.

With two genes A and B, the three features are,

- Reciprocal recombinant genotypes Ab, aB are present in same ascus.
- Both parents present in equal numbers indicating the crossing over occurs after DNA replication.
- Four copies of each of the allele A, a, B, b are present. This reveals that a pair of alleles segregates from each other and the order of segregation.

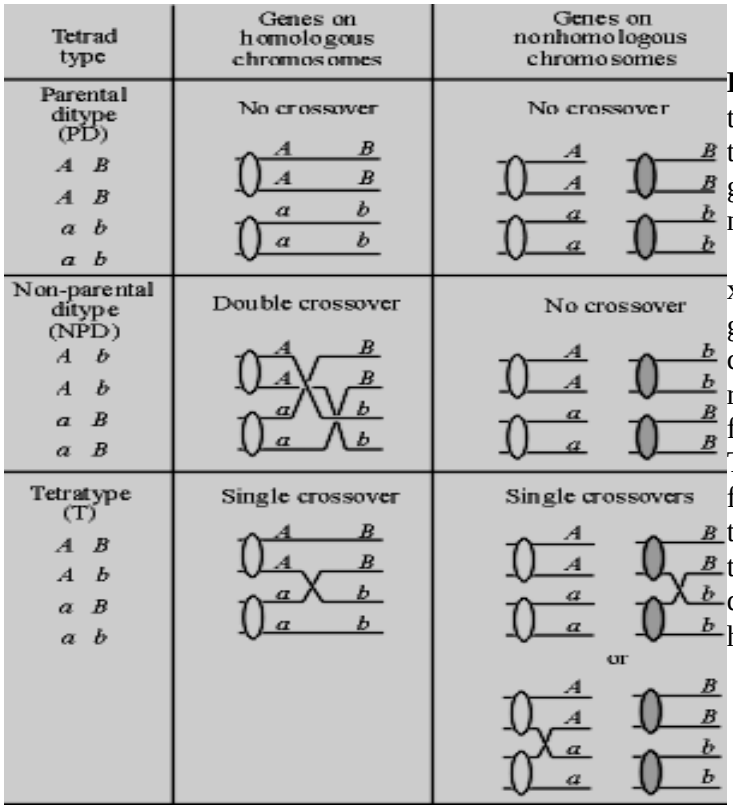

Figure 13 Origin of different tetrad types. Different tetrad types (left) are produced with genes on homologous (center) or nonhomologous (right)

Chromosomes from the cross *AB* x *ab*. When PD > NPD, then the genes are on homologous chromosomes, because of the rarity of NPD, which arise from four strand double crossovers. The tetratype (T) tetrads arise from single crossovers. See the text for the method of converting the %T and %NPD tetrads to map distances when genes are on homologous chromosomes. If

gene are on nonhomologous chromosomes, or if they greatly separated on the same chromosome, then PD = NPD, because of independent assortment, or multiple crossovers. Tetratype tetrads of genes on nonhomologous chromosomes arise by crossovers between either of the genes and their centromere, as shown in the lower right of the figure. The %T can be used to determine centromere distances if it is known for one of the genes

12.9 GENETIC SYSTEM OF PROTOZOA

Protozoa (in Greek *proto* = first and *zoa* = animals) are one-celled eukaryotes (that is, unicellular microbes whose cells have membrane-bound nuclei) that commonly show characteristics usually associated with animals, mobility and heterotrophy. Protozoans were commonly grouped in the kingdom Protista together with the plant-like algae and fungus-like water molds and slime molds. In 21st-century systematics, however, most of the algae are classified in kingdoms such as Plantae and Chromista; and in such cases the remaining life forms are occasionally classified as a kingdom Protozoa. But the name is misleading, since protozoans are neither Animalia nor Metazoa, with the *possible* exception of the enigmatic, moldy MyxozoaProtozoa occupy an important place in the food chains of natural communities where free water is present. Autotrophic flagellates are abundant in the sea and in fresh waters as well as in symbiotic association with animals at various level of organization, including other protozoa. Some groups in particular form important in the diet of a large number of animals.

Protozoa have traditionally been divided on the basis of their means of locomotion, although this is no longer believed to represent genuine relationships

- Flagellates
- Amoeboids
- Sporozoans
    - Apicomplexa
    - Myxozoa
    - Microsporidia
- Ciliates

Medically, ecologically, and evolutionarily speaking, Kinetoplastida (Eukaryota, Excavata, Euglenozoa) is one of the most interesting groups of eukaryotic microorganisms. Kinetoplastids include the trypanosomatid parasites responsible for major human maladies such as sleeping sickness (*Trypanosoma brucei*), Chagas' disease (*Trypanosoma cruzi*), and leishmaniases. Other kinetoplastids are collectively referred to as bodonids. Among these, the best studied are the cryptobiids (assigned, controversially, to *Cryptobia* and *Trypanoplasma*), some of which parasitize commercially important fish. Free-living bodonids such as *Bodo* spp. doubtless play major roles in microbial food webs, consuming bacteria and small eukaryotes

Kinetoplast DNA (kDNA) is the most structurally complex mitochondrial DNA in nature. Unique to the single mitochondrion of unicellular flagellates of the order Kinetoplastida, kDNA is best known as a giant network of thousands of catenated circular DNAs. The kDNA circles are of two types, maxicircles and minicircles. Maxicircles usually range from 20 to 40 kb, depending on the species, and are present in a few dozen identical copies per network. Minicircles, present in several thousand copies per network, are usually nearly identical in size (0.5 to 10 kb, depending on the species) but are heterogeneous in sequence. Maxicircles encode typical mitochondrial gene products (e.g., rRNAs and subunits of respiratory chain complexes) but, remarkably, some of the protein-coding genes are encrypted. To generate functional mRNAs, the cryptic maxicircle transcripts undergo posttranscriptional modification via an intricate RNA editing process that involves insertion and deletion of uridine residues at specific sites in the transcripts.

The genetic information for editing is provided by guide RNAs (gRNAs) that are mostly encoded by minicircles, although a few are encoded by maxicircles. Encoding gRNAs is the only known function of minicircles, and some organisms that edit extensively (such as *Trypanosoma brucei*) possess about 200 different minicircle sequence classes in their network to provide sufficient gRNAs. For reviews on RNA editing. Electron microscopy studies during the 1970s revealed that some members of the early-branching suborder Bodonina, which includes free-living as well as parasitic species, had kDNA that in vivo seemed at odds with the classical network structure. For example, in some species, the kDNA seemed to be dispersed throughout the mitochondrial matrix, uniformly or in multiple foci, rather than being condensed in one region, as it is in species containing a network

1. **Pro-kDNA**. : Electron microscopy of thin sections of *Bodo saltans* (a late-diverging free-living bodonid isolated from a lake) revealed a single bundle-like structure in the mitochondrial matrix that superficially resembles a kDNA disk .As with a kDNA network, the pro-kDNA bundle is situated near the basal body of the flagellum, although there is no information as to whether there are molecular connections between the two. 4',6'-Diamidino-2-phenylindole (DAPI) staining as well as in situ hybridization with a minicircle probe confirmed that this structure contains kDNA (Molecular analysis of pro-kDNA revealed that it is composed not of networks but of individual 1.4-kb minicircles, with only a few very small catenanes. As in kDNA networks, these minicircles are mostly covalently closed and, significantly, are topologically relaxed. It is not known whether they develop gaps, as do kDNA network minicircles, after they have undergone replication. Each minicircle encodes two gRNAs and, like classical kDNA minicircles, contains sequences that cause DNA bending (A tracts phased every 10 bp). The minicircles also contain a short sequence, within a 350-bp conserved region, that resembles the UMS replication origin. The *B. saltans* maxicircle is unusually large (~70 kb), and a 4-kb fragment that has been sequenced contains typical maxicircle genes. However, the gene order and editing patterns differ from those of trypanosomatids . Other bodonids may have pro-kDNA, as electron microscopy of thin sections has revealed kDNA similar to that of *B. saltans* in structure and in location within the mitochondrial matrix. Examples include the free-living *Bodo designis*, *Procryptobia* (*Bodo*) *sorokini*, *Rhynchomonas nasuta*, and *Cephalothamnium cyclopi* . However, there have been no studies on the molecular nature of their kDNAs.
2. **Poly-Kdna :** Inspection of DAPI-stained cells or electron micrographs of the early-branching bodonids *Dimastigella trypaniformis* (a commensal of the intestine of a termite), *Dimastigella mimosa* (a free-living bodonid isolated from a sewage plant), and *Cruzella marina* (a parasite of the intestine of a sea squirt) revealed a kDNA packaging pattern distinct from that of *B. saltans*. Instead of being condensed into a single globular bundle. The kDNA is distributed among various discrete foci throughout the mitochondrial lumen. Molecular studies have shown that poly-kDNA, like pro-kDNA, does not exist in the form of a network. Instead, it consists of monomeric minicircles (1.2 to 2.0 kb, depending on the species), many of which are covalently closed but not supercoiled (one faint band detected by gel electrophoresis of *D. trypaniformis* kDNA migrated as expected for supercoils but could also be a smaller minicircle). Minicircle dimers, but no larger oligomers, were also found, and they were relatively abundant only in *C. marina* (A. Zíková and J. Lukeš, unpublished results). No sequence information is available for poly-kDNA minicircles or maxicircles. Other bodonids apparently have poly-kDNA. Based on Giemsa staining, these include free-living *Rhynchobodo* spp., *Hemistasia phaeocysticola*, and ectoparasitic *Ichthyobodo* (*Costia*) *necatrix* .

3. **Pan-kDNA** : The kDNA of *Cryptobia helicis* (a parasite of the receptaculum seminis of snails) fills most of the mitochondrial matrix like pro-kDNA and poly-kDNA, pan-kDNA does not exist in the form of a network, and almost all of its 4.2-kb minicircles are monomeric. However, one major difference from all the kDNA forms discussed so far is that *C. helicis* minicircles are not relaxed but are supercoiled. Although most minicircles are present as supercoiled monomers, dimers and oligomers are also present. *C. helicis* minicircles contain typical minicircle motifs, including a UMS-related sequence and a bent helix). Maxicircles are ~43 kb, and the two genes partially sequenced so far encode RNAs that are not edited. Pan-kDNA may also occur in free-living *B. caudatus* (as judged from the published data) and *Cryptobia branchialis*, a parasite of fish.
4. **Mega-kDNA** :The most unusual kDNA (from the perspective that the network is the conventional structure) is that of the fish parasite *Trypanoplasma borreli*. This kDNA is distributed fairly uniformly throughout a large region of the mitochondrial matrix. However, molecular studies have indicated that it does not contain minicircles at all. Instead, minicircle-like sequences are tandemly linked into large molecules (possibly circular) of approximately 200 kb. Each minicircle-size unit (1 kb each, cut once by *Sca*I) encodes gRNAs that are unusual in having uridine tails on both 5' and 3' ends. Cloned *Sca*I fragments contain a UMS-related sequence. The gene order and editing patterns of maxicircle genes in this species are significantly different from those of trypanosomatids. In addition to *T. borreli*, light and electron microscopy images suggest that similarly organized mega-kDNA may occur in other species of *Trypanoplasma* and in *Jarrellia*, a parasite of whales.

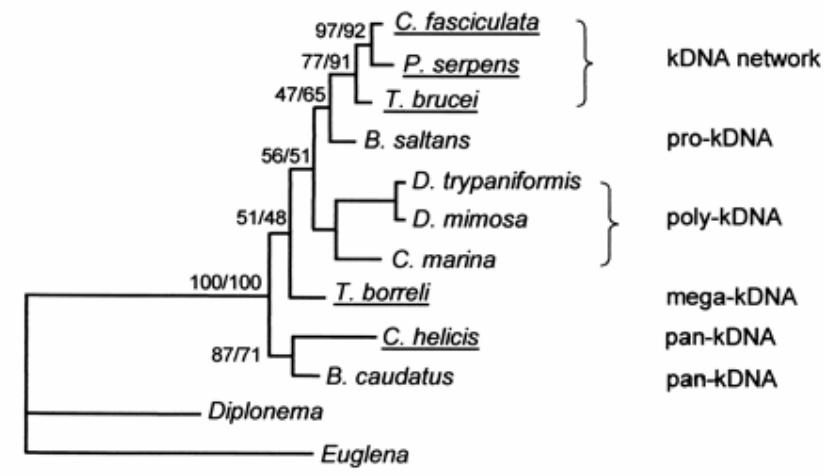

Figure 14: Various kDNA Structure

12.10 EVOLUTION OF KDNA

12.10.1 Phylogeny of the Kinetoplastida

The order Kinetoplastida was originally subdivided into the suborders Bodonina and Trypanosomatina based on morphological characteristics. Subsequently, phylogenetic trees constructed from nuclear rRNA genes confirmed the morphology-based subdivisions and the paraphyletic status of the Bodonina. These trees also established the monophyly as well as the derived character of the Trypanosomatina. These results have been further supported by comparative analyses of the mitochondrial gene order on maxicircle DNA and the RNA editing patterns of these genes and by phylogenetic analysis of the cytochrome oxidase subunit I and II genes. However, the most extended bodonid data set, analyzed with

maximum parsimony and maximum likelihood, has failed to resolve the branching order of the early-diverging bodonid species. Therefore, until more conserved genes can be analyzed, the precise evolution of the early-branching bodonids cannot be definitively traced. However, it is currently believed that *C. helicis* is among the earliest of the bodonids and that *B. saltans* is among the last to diverge.

12.10.1.1 Evolution of kDNA structures.

If one superimposes on the phylogenetic tree the variety of kDNA structures together with their compaction patterns within the mitochondrial matrix, a straightforward and logical pathway for the evolution of kDNA structures can be deduced. We propose that the pan-kDNA of *C. helicis* is the form most similar to the ancestral state. In pan-kDNA, the size, supercoiling, monomeric status, and distribution of minicircles in the mitochondrial matrix resemble those of plasmids. We postulate that the precursor to modern minicircles was derived from a plasmid harbored within the mitochondrion of an ancient flagellate. That *C. helicis* minicircles contain UMS-related and bent DNA sequences is consistent with the hypothesis that they are an ancestor of the minicircles in kDNA network

12.10.2 GENETIC SYSTEM OF MYCOPLASMA

Mycoplasma is a genus of bacteria that lack cell walls. Because they lack cell walls, they are unaffected by most antibiotics such as penicillin that target cell wall synthesis. They can be parasitic or saprophytic. Several species are pathogenic in humans, including *M. pneumoniae* which cause of pneumonia and other respiratory disorders. *M. genitalium*, which is believed to be involved in pelvic inflammatory diseases.

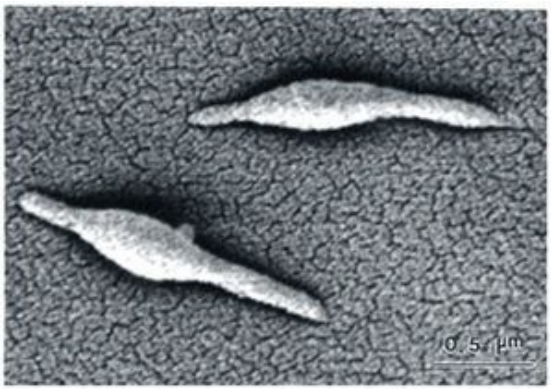

Figure 15: ***Mycoplasma***

Kingdom:Bacteria
Division :Firmicutes
Class :Mollicutes
Order :Mycoplasmatales
Family :Mycoplasmataceae
Genus :Mycoplasma
Species :*M.pneumoniae*
:M.genetalium
etc…

Mycoplasmas are wide spread in nature as parasites, of humans, mammals etc. It usually exhibits a rather strict host & tissue specificity. It is an obligate parasite. The primary habitat of humans and animal mycoplasmas are the mucous surface of respiratory and urinogenital tract. Since mycoplasma cells are bounded by a plastic cell membrane their dominating shape is a sphere. Mollicutes exhibit a variety of morphological entities. These

include pear-shaped cells, flask-shaped cells with terminal tip structures, filaments of various lengths, and helical filaments. The ability to maintain such shapes in the absence of a rigid cell indicates the presence of a cytoskeleton in mycoplasmas. The genomic analysis of *M. pneumoniae* enabled the identification of major proteins building blocks of the cytoskeleton. Some proteins functions in surface exposed adhesion. Proteins such as P1, P30 and other accessory proteins maintain this distribution of adhesion in the membrane. Proteins such as P65 and P200 share characteristic structural features suggesting their function as elements of the cytoskeleton.

Some *Mycoplasma* species are capable of gliding on solid surfaces. The mechanism of this gliding motility is still unknown. The gliding motility genes could not be identified. *M. pneumoniae* is known to be motile and to exhibit chemotactic behaviour.

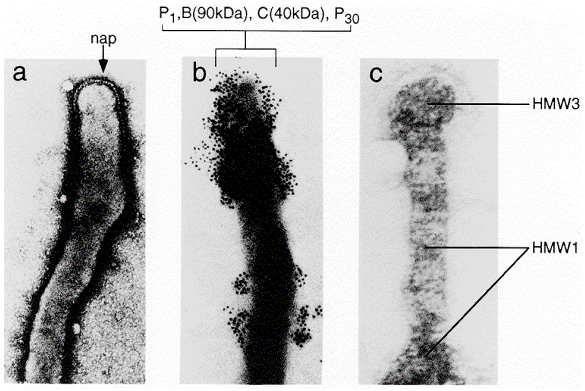

Figure 16: Surface Exposed Protein in *Mycoplasma*

12.10.2.1 CELL DIVISION

The mode of reproduction of mycoplasmas is slightly similar as of other prokaryotes dividing by binary fission. In binary fission cytoplasmic division must be fully synchronized with genome replication. But in mycoplasmas the cytoplasmic division may lag behind genome replication. It results in the formation of multinucleate filaments. Recent genetic data reveals on the genes involved in the cell division process in mycoplasmas.

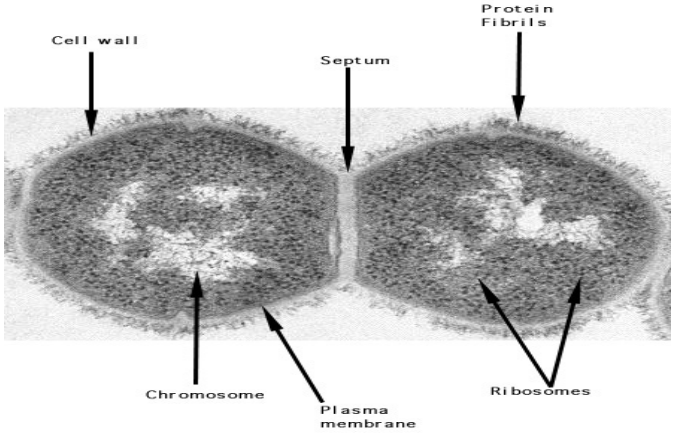

Figure 17: *Mycoplasma* cell division

The genome sizes of *Mycoplasma* species range from 580-1380 kb. Genome sizes are variable even among strains of the same species. One of the reasons for this variability is the frequent occurrence in mollicute genomes of repetitive elements. It consists of segments of protein genes, differing in size and number, or insertion sequence (IS) elements. The

mycoplasma genome has a characteristically low G + C content. The G + C content of mycoplasma genomes is within the range of 24 to 33 mol%. The G + C distribution along the genome is uneven. The average G + C content of the *M.genitalium* genome is 32 mol%. And the average G + C content of the *M.pneumoniae* genome is 40 mol%. Some of the adenine and cytosine residues in mycoplasmal genomes may be methylated.

Very few viruses are known to infect *Mycoplasma* species. The mollicute phage DNA genomes range in size from 4 to 40 kb and may be either circular/linear or single/double stranded. The most recent phage discovered is the lysogenic phage MAV1, infecting *M.arthritidis.* This suggests that MAV1 carries a virulence enhancing factor. The mollicute phages and plasmids serve as cloning and shuttle vectors.

12.10.2.2 ORIGIN OF REPLICATION:

The organization of genes *oriC* site of mollicute genomes and their order (*rnpA-rmpH-dnaAdnaN-gyrB*) are highly conserved. The DNA gyrase subunits A and B (*gyrA* and *gyrB*) has been found at the origin of replication of *M.genetalium & M.pmeumoniae* species. The overall studies concluded that conserved order of genes is not prerequisite for the functional origin of replication.

Sequence data of *M.genetalium* and *M.pneumoniae* indicates these two *Mycoplasma* species carry two DNA polymerase genes. One coding for the larger protein has 3'◊5'exonuclease activity. While the other coding for the protein which resembles Pol C gene from E.coli. This lack 3', 5'exonuclease activity. The mycoplasmal DNA topoisomerases are DNA gyrase and topoisomerase IV.

The % of genes devoted to transcription is higher in the mycoplasma. The genes for the RNA polymerase subunit are basically similar in mycoids and other eubacteria. The genes which encodes for RNA polymerase in M.pneumoniae is *rpo*A, *rpo*B and *rpo* C. This mycoplasma encodes only one *sigma* factor.

The mycoplasmal transcription signals resemble the eubacteria. The mycoplasmal -10region (Pribnow box) and to a lesser extent the -35 region consensus sequences are recognized by the RNA polymerase holoenzyme. SD sequence could be identified upstream of the initiation codon. Termination of transcription is independent of the termination factor Rho. Typical terminator sequences forming a stem-loop structure, followed by a run of U residues, also serve as transcription stops in mollicutes.

The mycoplasmal ribosomes are typically procaryotic in size, shape, and composition. The *M. pneumoniae* ribosomal protein genes are organized in operons. The highly conserved nature of the ribosomal protein genes has contributed to mollicute phylogeny. The rRNA genes are perhaps the best-characterized mollicute genes. The organization of the mollicute rRNA genes generally follows the characteristic eubacterial order, 16S-23S-5S.

The number of tRNA genes in mollicutes is kept to a minimum, with very few gene duplicates. The no of tRNA genes in *M.pneumoniae* is 33 and in *M.capricolum* is 30. The number of anticodons in in *M. pneumoniae* is 32. The mycoplasmal tRNAs contain significantly fewer modified nucleosides.

Most mollicutes have genomes with a very low G + C content due to mutation during their evolution. Under strong A-T pressure is the reassignment in most mollicutes of UGA from a stop codon to a tryptophan codon. The use of UGA as a tryptophan codon indicates the mycoplasmal origin of an element located in chromosome such as IS-like elements.

The no of genes involved in DNA repair is smaller when compared to E.coli. The *M.pneumoniae* codes for only 30 genes for DNA repair. The *ung* gene, coding for uracil-DNA glycosylase removes uracil residues from DNA arising by spontaneous deamination of cytosine residues. The genes that codes for ABC excinucleases combines with pol I, Helicase II and Ligase provides the mechanism for the repair of the UV damage

Gene Transfer: The lack of cell wall in mollicutes facilitates the introduction of exogenous DNA. Increased transformation and transfection efficiencies can be achieved either in the presence of polyethyleneglycol (PEG) or by application of electroporation.

12.11. LET US SUM UP

- The gene flow in prokaryote which include Transformation, Transduction and Conjugation have been elaborated
- Structure of DNA transposons and different types the plasmid are also explained
- Eukaryotic genome structure and its features were also discussed
- Genetic Systems Of Viruses includes types of RNA viruses and retrovirus their multiplication also elaborated
- Genetic systems of fungi - Chromosomal and mitochondrial genes of yeast and Neurospora systems were discussed
- Protozoa Genetic System includes different types of kDNA and their classification have been described

12.12. LESSON END ACTIVITIES

- Write a brief account on various types Bacterial plasmids
- Describe the various types DNA Transposons
- Give a detailed account on retrovirus
- When and how retrovirus will enter the cell
- List out the chromosomal and Mitochondrial Genes in Yeast
- Discuss in detail about tetrad analysis
- Explain the mechanism of parasite identification?

12.13. POINTS TO DISCUSSION

1. List out the promoters involved in phage T7 transcription
2. Describe the following
    - Transformation
    - Transduction
    - Conjugation

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LESSON 13

REPRODUCTION OF MICROORGANISM

Contents

13.0. AIMS AND OBJECTIVES

The chapter deals with the multiplication process of microorganisms in general.

13.1 INTRODUCTION

The organisms utilize the sources available in the environment and replicate the genetic material for their multiplication. This process is discussed in the chapter with bacteria and fungi.

13.2 MULTIPLICATION OF MICROORGANISMS

13.2.1 PHAGE MULTIPLICATION CYCLE

Bacteriophages undergo two types of multiplication cycle. Lytic and lysogenic cycle which have been dealt already in earlier sections.

13.2.2 LYTIC OR VIRULENT PHAGES

Lytic or virulent phages are phages which can only multiply on bacteria and kill the cell by lysis at the end of the life cycle.

a. **Eclipse period** - During the eclipse phase, no infectious phage particles can be found either inside or outside the bacterial cell. The phage nucleic acid takes over the host biosynthetic machinery and phage specified m-RNA's and proteins are made. There is an orderly expression of phage directed macromolecular synthesis, just as one sees in animal virus infections. Early m-RNA's code for early proteins which are needed for phage DNA synthesis and for shutting off host DNA, RNA and protein biosynthesis. In some cases the early proteins actually degrade the host chromosome. After phage DNA is made late m-RNA's and late proteins are made. The late proteins are the structural proteins that comprise the phage as well as the proteins needed for lysis of the bacterial cell.

b. **Intracellular Accumulation Phase** - In this phase the nucleic acid and structural proteins that have been made are assembled and infectious phage particles accumulate within the cell.

c. **Lysis and Release Phase** - After a while the bacteria begin to lyse due to the accumulation of the phage lysis protein and intracellular phage are released into the medium. The number of particles released per infected bacteria may be as high as 1000.

Assay for Lytic Phage can be done by Plaque assay. A plaque is a clear area which results from the lysis of bacteria. Each plaque arises from a single infectious phage. The infectious particle that gives rise to a plaque is called a pfu (plaque forming unit).

13.2.3 LYSOGENIC OR TEMPERATE PHAGE

Lysogenic or temperate phages are those that can either multiply via the lytic cycle or enter a quiescent state in the cell. In this quiescent state most of the phage genes are not transcribed; the phage genome exists in a repressed state. The phage DNA in this repressed state is called a **prophage** because it is not a phage but it has the potential to produce phage. In most cases the phage DNA actually integrates into the host chromosome and is replicated along with the host chromosome and passed on to the daughter cells. The cell harboring a prophage is not adversely affected by the presence of the prophage and the lysogenic state may persist indefinitely. The cell harboring a prophage is termed a **lysogen**.

Circularization of the phage chromosome - Lambda DNA is a double stranded linear molecule with small single stranded regions at the 5' ends. These single stranded ends are complementary (**cohesive ends**) so that they can base pair and produce a circular molecule. In the cell the free ends of the circle can be ligated to form a covalently closed circle.

Site-specific recombination - A recombination event, catalyzed by a phage coded enzyme, occurs between a particular site on the circularized phage DNA and a particular site on the host chromosome. The result is the integration of the phage DNA into the host chromosome.

Repression of the phage genome - A phage coded protein, called a **repressor**, is made which binds to a particular site on the phage DNA, called the **operator**, and shuts off transcription of most phage genes EXCEPT the repressor gene. The result is a stable repressed phage genome which is integrated into the host chromosome. Each temperate phage will only repress its own DNA and not that from other phage, so that repression is very specific (immunity to superinfection with the same phage).

Events Leading to Termination of Lysogeny : Anytime a lysogenic bacterium is exposed to adverse conditions, the lysogenic state can be terminated. This process is called **induction**. Conditions which favor the termination of the lysogenic state include: desiccation, exposure to UV or ionizing radiation, exposure to mutagenic chemicals, etc. Adverse conditions lead to the production of proteases (rec A protein) which destroy the repressor protein. This in turn leads to the expression of the phage genes, reversal of the integration process and lytic multiplication.

13.2.4. LYTIC VS LYSOGENIC CYCLE

The decision for lambda to enter the lytic or lysogenic cycle when it first enters a cell is determined by the concentration of the repressor and another phage protein called **cro** in the cell. The cro protein turns off the synthesis of the repressor and thus prevents the establishment of lysogeny. Environmental conditions that favor the production of cro will lead to the lytic cycle while those that favor the production of the repressor will favor lysogeny.

13.2.5 SIGNIFICANCE OF LYSOGENY

a. **Model for animal virus transformation** - Lysogeny is a model system for virus transformation of animal cells

b. **Lysogenic conversion** - When a cell becomes lysogenized, occasionally extra genes carried by the phage get expressed in the cell. These genes can change the properties of the bacterial cell. This process is called lysogenic or phage conversion. This can be of significance clinically. e.g. Lysogenic phages have been shown to carry genes that can modify the Salmonella O antigen, which is one of the major antigens to which the immune response is directed. Toxin production by Corynebacterium diphtheriae is mediated by a gene carried by a phage. Only those strain that have been converted by lysogeny are pathogenic.

13.2.6. MULTIPLICATION OF BACTERIA

Fortunately bacteria rarely are able to sustain this high rate of multiplication for long because of limitations in nutrient availability and various bacteria feeding organisms (bacteriovores) and bacteria infecting viruses (bacteriophages).

Prokaryotes such as bacteria use a relatively simple form of cell division called binary fission. Typically bacterial chromosomes consist of a single loop of DNA, often called circular DNA. Eukaryotes have a linear DNA molecule. During duplication of the DNA the bacterial chromosome replicates leading to two identical chromosomes attached to separate points of attachment. The cell begins to divide, each cell with an identical chromosome. Bacteria can divide every twenty to thirty minutes. This gives bacteria remarkable powers of multiplication. Consider single bacteria. After 24 hours there would be 2^{48} or 2.81×10^{14} bacteria. This is within the range of the number of eukaryotic cells in the human body.

Binary fission is the process by which all bacteria reproduce. Binary fission results in the separation of a single cell into two identical daughter cells each contain at least one copy of the parental DNA. This process is carried out in a stepwise manner. First the bacterial cells elongate. Then the bacterium replicates its chromosomal DNA. Next the cell envelope pinches inward, eventually meeting. This results in a cross wall being formed and ultimately two distinct cells are present. This is more clearly shown in the figure 18.

Each resulting daughter cell is a clone of the parent cell. Therefore this form of reproduction does not allow for differences in the bacterial genome. However, occasionally a mistake is made as the DNA is copied and one of the new cells may have a slight difference, called a mutation. Bacteria carry out binary fission very efficiently and, when conditions suit them perfectly, can divide once every twenty minutes.

This has obvious implications for dealing with bacterial diseases because since bacteria multiply so rapidly it is critical to treat bacterial diseases swiftly and as completely as possible.

13.2.7. MULTIPLICATION OF FUNGI

Many fungi are successful because they can produce enormous numbers of spores, both by mitosis or meiosis.

13.3. SEXUAL AND ASEXUAL REPRODUCTION IN BACTERIA

Bacteria can reproduce at tremendous speeds. Some bacteria can reproduce as often as once every 20 minutes! However, bacteria have to have certain conditions in which to reproduce. These conditions are not often met, and that is one thing that keeps bacteria from growing out of control.

Bacteria reproduce using two basic methods: **asexual reproduction** and **sexual reproduction**. Asexual reproduction involves only one individual or parent. The offspring generated by asexual reproduction are exact duplicates of the parent. **Binary fission** is the process by which a bacteria splits into two cells. Each cell gets an exact copy of the parent cell's genetic material.

Sexual reproduction involves the joining of two parent cells and the exchanging of genetic materials. In sexual reproduction, the offspring will have a mixture of the parent cells' traits. **Conjugation** is the process by which bacteria join and exchange genetic materials. Once genetic materials are exchanged, each bacteria cell will go through binary fission to produce an offspring with a new genetic makeup.

13.3.1 BINARY FISSION

Bacteria reproduce by a way of asexual reproduction called binary fission. In one bacterium, the single circular chromosome duplicates. Then, the two resulting chromosomes attach to the inside of the plasma membrane. The cell elongates and separates into two strands. Finally, the cell membrane grows inward, the cell wall forms separating two daughter cells each with a chromosome.

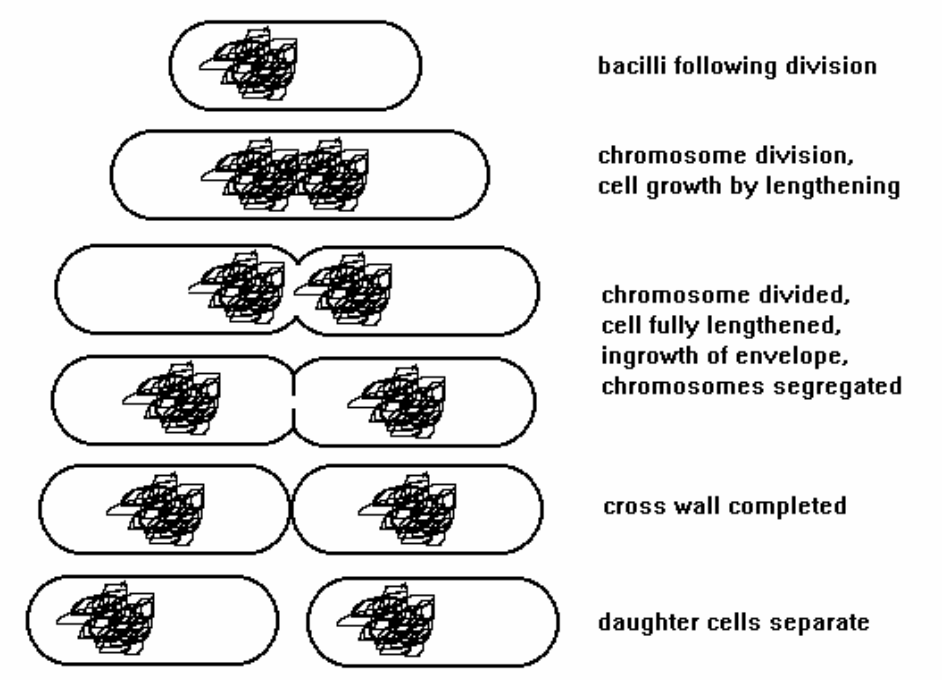


Figure 18 Binary Fission

13.3.2 EXCHANGE OF GENETIC MATERIAL

Bacteria also reproduce with the exchange of DNA. When bacteria exchange DNA, it has a similar effect to sexual reproduction, in that, there is a blending of genes between two organisms. There are three was in which bacteria exchange DNA.

1. *Conjugation* – male cell passes DNA to female cell by means of a conjugation tube (sex pilus).
2. *Transformation* – bacterium takes up DNA released by dead bacteria.
3. *Transduction* – bacteriophages carry DNA from one cell to another.

13.4 SEXUAL AND ASEXUAL REPRODUCTION IN FUNGI.

The ability to reproduce asexually is common to almost all fungi. *S. cerevisiae* can be stably maintained as either heterothallic or homothallic strains. Both heterothallic and homothallic diploid strains sporulate under conditions of nutrient deficiency, and especially in special media, such as potassium acetate medium. During sporulation, the diploid cell undergoes meiosis yielding four progeny haploid cells, which become encapsulated as spores (or ascospores) within a sac-like structure called an ascus (plural asci). The percent sporulation varies with the particular strain, ranging from no or little sporulation to nearly 100%. Many laboratory strains sporulate to over 50%. The majority of asci contains four haploid ascospores, although varying proportions asci with three or less spores are also observed.

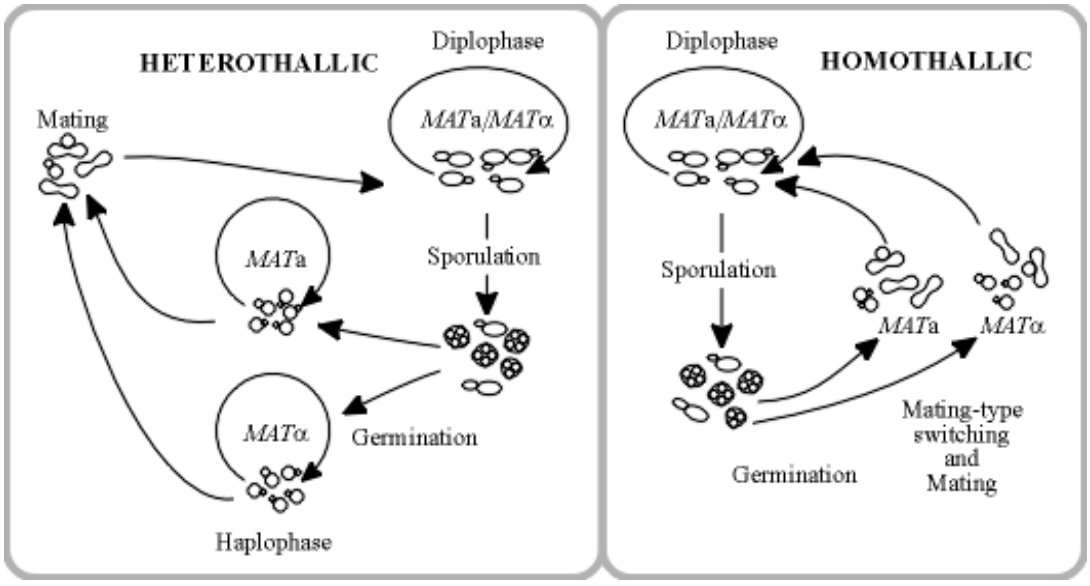


Figure 19 Life cycles of heterothallic and homothallic strains of *S. cerevisiae*.

Heterothallic strains can be stably maintained as diploids and haploids, whereas homothallic strains are stable only as diploids, because the transient haploid cells switch their mating type, and mate.

13.4.1 SEXUAL REPRODUCTION OF FUNGI

Sexual reproduction introduces the possibility of variation into a population, and this is why most fungi have a sexual phase. To achieve sexual reproduction it is necessary to have two mating type haploid nuclei (n + n), or a diploid (2n) nucleus. In the case of the two

haploid nuclei they must fuse to form a diploid first, but once fused the nuclei undergo meiosis, which is the reduction division that potentially brings about variation in the progeny. These events are followed by the formation of spores, which in most cases are resting spores that can withstand adverse conditions.

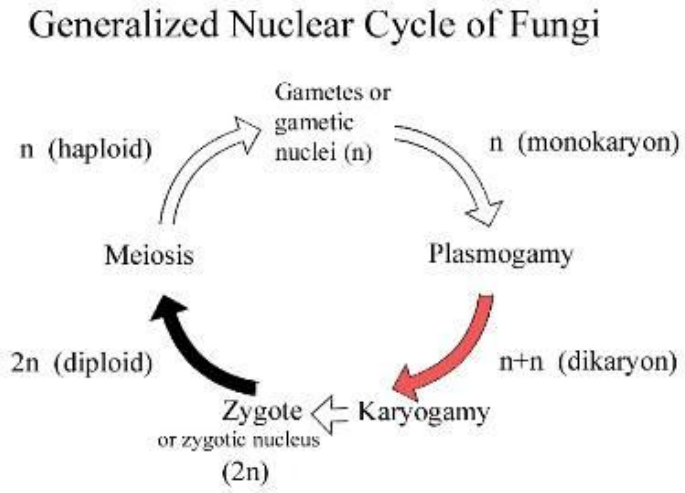


Figure 20 Generalized nuclear cycle of fungi

Some Fungal Anatomy: An Overview	
Antheridia*	Male-like donor of haploid nuclei
Asci	Sac-fungus sexually produced spore-containing structures
Ascocarp	Sac-fungus fruiting body (asci-containing structures)
Ascogonia*	Sac-fungus female-like receiver of haploid nuclei from sac-fungi antheridia
Ascospores	Sac-fungus haploid dispersal stage (spores)
To put the above in more familiar terms: Antheridia (boys) plus **Ascogonia** (girls) leads to the production of **Ascocarp** (~womb/mushroom equivalent) that contain **Asci** (~placenta/basidia equivalent) that contain **Ascospores** that are the *sexually* produced haploid dispersal stage of the sac fungi while the **Conidia** (defined below) are the *asexually* produced haploid dispersal stage of sac fungi	
Basidia	Club-fungus asci equivalent (spore-containing structure)
Basidiocarp	Club-fungus basidia-containing structure
Coenocytic	A fungal hyphae that lacks septa
Conidia*	Sac-fungus asexually produced spores (they don't come from asci)
Dikaryon	A fungus (cell, hyphae, or mycelia) that contains haploid nuclei sourced from different parents
Gametangia*	Gametangia are the sexual organs of fungi and plants (note the common "gamet-" between gamete and gametangia); these are the supplies of haploid nuclei that ultimately will fuse (karyogamy) to form the diploid precursor to

	meiosis
Hyphae	Hyphae are the filamentous cells or linked-together cells that represent the bulk of the bodies of molds and macrofungi (e.g., mushrooms)
Mycelium	Mycelia are tangled masses of hyphae typically found growing within a fungal food source
Septa (septum)	These are crosswalls that separate (distinguish) the cells within hyphae; not all fungi possess crosswalls within all of their hyphae

TABLE 5. FUNGAL ANATOMY: AN OVERVIEW

Sexual reproduction (note: order of terms is relevant):

- Mitosis - Hyphae (ploidy = n)
- Mitosis - Mycelium (ploidy = n)
- Plasmogamy (a process) (= fusion of cytoplasm)
- Dikaryotic stage (ploidy = n + n) (occurs within zygosporangia for Zygomycete, ascogonia for Ascomycete, or hyphae for Basidiomycete)
- Mitosis - Karyogamy (a process) (= fusion of haploid nuclei)
- Diploidy (ploidy = 2n) (occurs within zygosporangia for Zygomycete, ascocarps for Ascomycete, or basidiocarps for Basidiomycete)

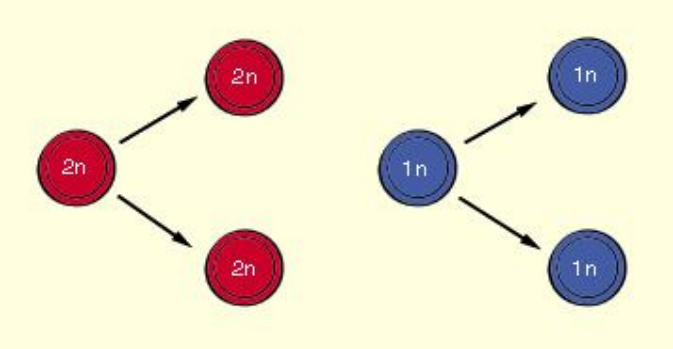


Figure 21 Mitosis

- Meiosis (a process)
- Spore-producing structures (ploidy = n) (= sporangium for Zygomycete, asci for Ascomycete, or basidia for Basidiomycete)
- Spores (ploidy = n) (= spores for Zygomycete, ascospores for Ascomycete, or basidiospores for Basciomycete)
- Germination (a process)
- Mitosis - Hyphae (ploidy = n)
- Mitosis - Mycelium (ploidy = *n*)

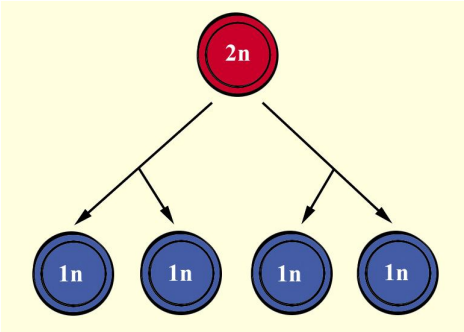


Figure 22 Meiosis

Overview of Fungi Sexual Reproduction
mitosis (m) → Hyphae → (m) → Mycelium →
[Plasmogamy → Dikaryon → (m) → Karyogamy → Diploidy → Meiosis → Spores →]
Germination → (m) → Hyphae → and so on

(1) Ploidy

(a) Fungi typically possess haploid nuclei, except just prior to meiosis

(b) Only following nuclear fusion (karyogamy) are fungi diploid, and mitosis in fungi does not occur in the diploid state

(c) However, many fungi routinely achieve a diploid-like state following cytoplasmic fusion (plasmogamy) that is called a dikaryon state or stage; note that dikaryon is not synonymous with diploid since nuclei remain haploid even if found in same cytoplasm

(2) Plasmogamy

(a) Though fungi nuclei are typically haploid, that doesn't stop haploid nuclei from different fungal parents (e.g., mom and dad equivalents) from being present in the same cytoplasm

(b) The process by which the cytoplasms of two parental fungi fuse is called plasmogamy

(c) Note that plasmogamy may be followed by nuclei fusing, though this does not necessarily occur immediately, and for some fungi the time until nuclear fusion occurs can be greatly extended (days, months, years)

(3) Dikaryon state or stage

(a) The post-plasmogamy condition in which two different haploid nuclei occupy the same cytoplasm is a dikaryon state or stage

(b) Being a dikaryon, since cytoplasms are shared, provides the masking of deleterious alleles of diploidy without the possession of diploid nuclei

(4) Karyogamy

(a) The fusion of haploid nuclei found in dikaryonic fungal cells is called karyogamy

(b) Karyogamy is necessary for the occurrence of meiosis (since haploid nuclei cannot undergo meiosis)

(c) Note that meiotic products are both haploid and therefore are no longer dikaryonic

13.4.2 SEXUAL REPRODUCTION IN THE CHYTRIDIOMYCETES

Sexual reproduction occurs in some members of the chytrids by the production of diploid spores after gametic or somatic fusion of two different mating types. The resulting spore may germinate to produce a diploid vegetative mycelium or it may undergo meiosis to produce a haploid mycelium. The diploid mycelium can also produce resting sporangia in which meiosis occurs, generating haploid zoospores that germinate to produce haploid vegetative mycelium:

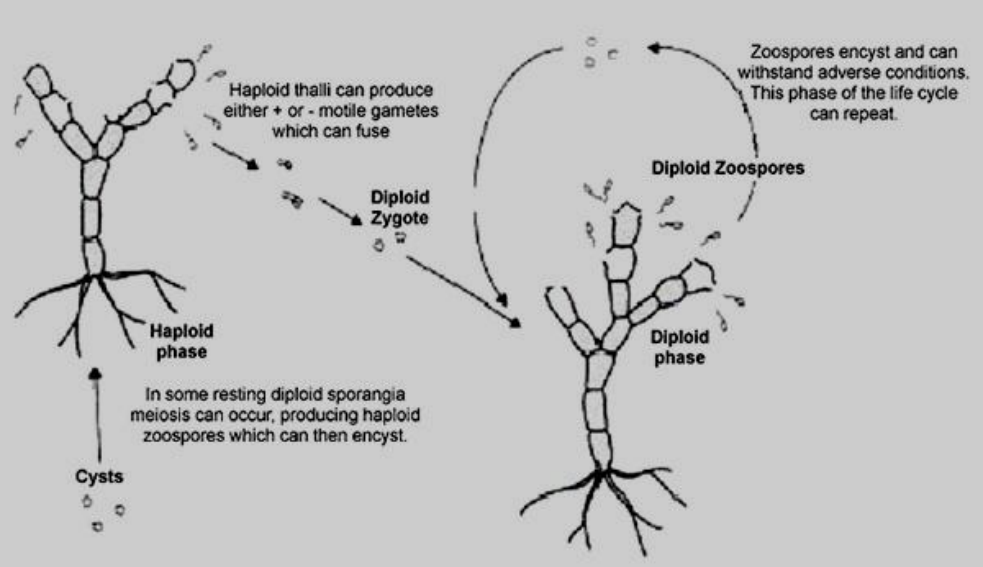


Figure: 23 Sexual Reproduction in the Chytridiomycetes

13.4.3 SEXUAL REPRODUCTION IN THE ZYGOMYCETES

There are two possible nuclear states in the mycelia of this group of fungi. They can have a single type of nucleus in their mycelium, a condition termed homothallism, or they can contain the two mating type nuclei within their mycelium, termed heterothallism. If the fungus is homothallic the first event in the onset of sexual reproduction has to be somatic fusion. This is termed conjugation. To achieve such a mating it is necessary to attract each other and an elaborate sequence of cellular and biochemical events have been established for some of these fungi. This signalling involves the secretion of inducer molecules that are responsible for causing the formation of zygophores, modified hyphal tips, and these then grow towards each other long a gradient of hormone. The exact sequence is shown in Fig24.

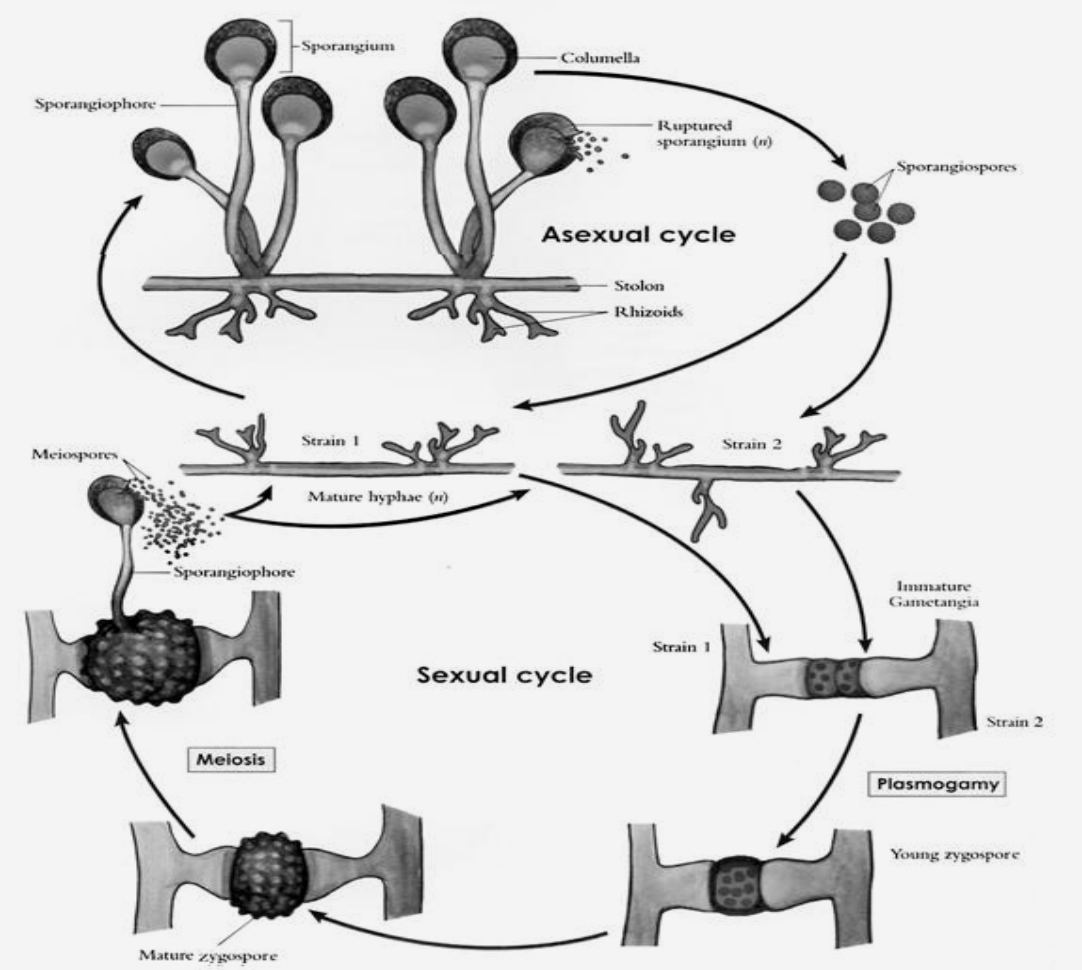


Figure 24 Sexual reproduction in the *Zygomycetes*

Once in contact the two zygophores fuse, and then the nuclei fuse to form the diploid. Meiosis occurs, producing four haploid nuclei, but three may degenerate. The timing of fusion varies from species to species.

13.4.4 SEXUAL REPRODUCTION IN THE ASCOMYCETES

In this group of fungi there are no specialized organs of hyphal fusion, different mating type mycelia merely fuse with each other to form transient dikaryons, mycelia with two mating type nuclei within it. The dikaryotic mycelium can differentiate to from varying amounts of sterile mycelium around what is to become the fertile tissue of the fruit body. In yeasts, a single, diploid yeast will undergo meiosis, producing four haploid progeny cells, but in more complex fungi there are a sequence of cellular and nucleic events that ensure an organized fertile layer.

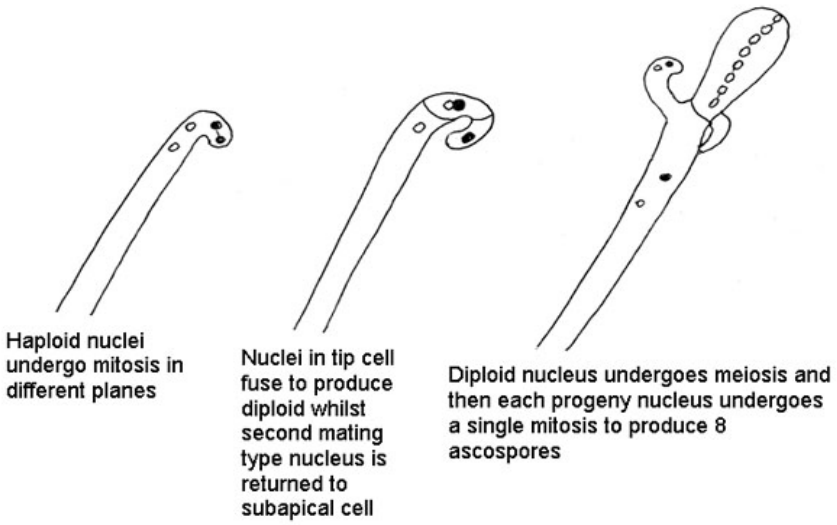


Figure 25 Sexual Reproduction in the Ascomycetes

Spores are delineated around these nuclei in a process called free cell formation, and as most of the cytoplasm is contained around the nucleus and within the spore wall, all that is left outside is cell sap. These modified hyphae are termed Asci, and the spores that are held within them are termed ascospores. The asci are often found packed tightly with other asci, and between a dense layer of supporting sterile tissue. Often the structure is large enough to be seen with the naked eye.

The asci can be aggregated together in various sorts of fruit body which we will see in the practical, including the, cup fungi (*Discomycetes*, apothecial), the flask fungi, (Pyrenomycetes, perithecial), the mildews (*Plectomycetes* cleistothecial) and the fungi with black, crusty stromata (*Loculoascomycetes*, pseudothecial fungi). There are also the yeasts, *Hemiascomycetes*,. Their ascospores are normally formed in loose asci and are not actively discharged. We have not looked at these. When they form ascospores in fruit pulps or liquids they are usually liberated by the disintegration of the ascus wall.

13.4.5 SEXUAL REPRODUCTION IN THE BASIDIOMYCETES

Basidiomycetes are characterised by the most complex and large structures found in the fungi. They are very rarely produce asexual spores. Much of their life cycle is spent as vegetative mycelium, exploiting complex substrates.

A preliminary requisite for the onset of sexual reproduction is the acquisition of two mating types of nuclei by the fusion of compatible mycelium. This creates a dikaryon where single copies of the two mating type nuclei are held within every hyphal compartment for extended periods of time. Maintenance of the dikaryon requires elaborate septum formation during growth and nuclear division.

Onset of sexual spore formation is triggered by environmental conditions and in the larger Basidiomycetes begins with the formation of a fruit body primordium. The primordium expands and differentiates to form the large fruit bodies of mushrooms and toadstools. The mycelium within this structure remains as a dikaryon, diploid formation only occurring within the modified hyphal tip called the basidium. Meiosis occurs within the basidium, and the four products are extruded from the tip of the basidium on sterigma (below). Usually this event occurs across a large area of basidia called a hymenium, or fertile layer. It is usually formed over an extensive sterile layer of tissue like a mushroom gill.

There are three major divisions in the basidiomycetes.

1. *Hymenomycetes*. Basidia are in extensive fertile layer which are susceptible to rain when exposed. Spores are actively discharged from a protected hymenium when ripe. This group includes mushrooms and toadstools, boletes, brackets and coral fungi.
2. *Gasteromycetes*. Hymenia line closed cavities in an initially closed fruit body. Basidiospores are released passively by autolysis of the hymenium, and basidiocarps disintegrate at maturity. This group includes earth balls, puff-balls, stinkhorns and birds nest fungi.
3. *Teliomycetes*. These are the rusts and smuts, neither of which form large, conspicuous, fruit bodies but invade plants and produce characteristic sporulating lesions in plant tissue.

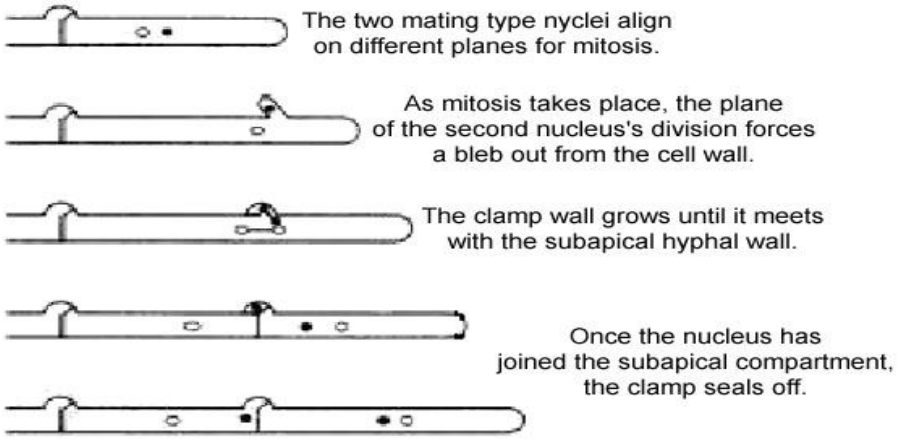


Figure 26 Sexual Reproduction in the basidiomycetes

13.4.6 ASEXUAL REPRODUCTION OF FUNGI

Overview of Fungi Asexual Reproduction
mitosis (m) → Hyphae → (m) → Mycelium → (m) → [Spore-producing structures → (m) → Spores →] Germination → (m) → Hyphae → and so on

Asexual reproduction (note: order of terms is relevant):

Mitosis - Hyphae (ploidy = n)

- Mitosis - Mycelium (ploidy = n)
- Mitosis - Spore-producing structures (ploidy = n)
- Mitosis - Spores (ploidy = n) (= conidia for sac fungi)

Germination (a process)

- Mitosis - Hyphae (ploidy = n)
- Mitosis - Mycelium (ploidy = n)

13.4.7 BUD FORMATION IN YEASTS

In its simplest form asexual reproduction is by budding or binary fission. The onset of the cellular events is accompanied by the nuclear events of mitosis.

The initial events of budding can be seen as the development of a ring of chitin around the point where the bud is about to appear. This reinforces and stabilizes the cell wall. Enzymatic activity and turgor pressure the act to weaken and extrude the cell wall. New cell

wall material is incorporated during this phase. Cell contents are forced into the progeny cell, and as the final phase of mitosis ends a cell plate, the point at which a new cell wall will grow inwards from, forms.

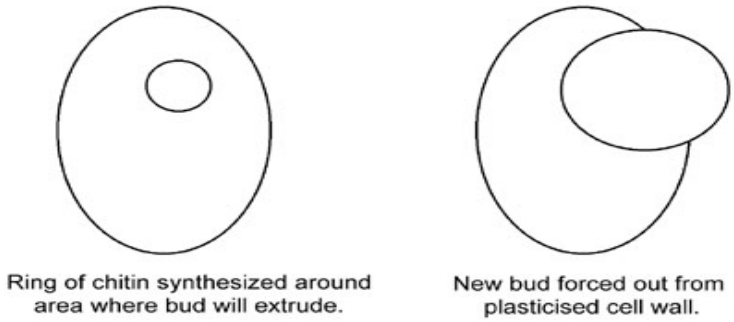


Figure 27 Budding of Yeasts

Separation of the bud from the parent leaves a scar. When chains of yeast cells do not fully separated this can create a pseudo-mycelium.

- **FRAGMENTATION**

  Many fungi can reproduce by fragmentation. Any mycelium that is fragmented or disrupted, provided that the fragment contains the equivalent of the peripheral growth zone, can grow into a new colony. Many fungi are sub-cultured using this hyphal fragment technique. All of this weeks practical plates have been inoculated in this way with a cork bore taken from a colonized donor plate. Cut mycelial tips do not regenerate, but branches can form some distance from the damage point.

- **SPORULATION**

  By far the most important type of asexual reproduction is that of spore formation. Asexual reproduction is extremely important to fungi. It is responsible for the production of large numbers of spores throughout the year. These asexual spores are formed on a phase of the fungal life cycle termed in some texts as the mitosporic, or anamorphic phase. There can be more than one mitosporic state for each species of fungus, and in some cases the mitosporic state of very different species can look very similar. This has contributed to the problems of creating a taxonomy for the fungi that only possess mitosporic states. The sexual stage of the fungus can be termed the teleomorph, and the characteristics of this phase of the life cycle are much more stable and reliable for taxonomic purposes.

  The onset of asexual reproduction is controlled by many different things. Some are environmental, like nutrient levels, CO_2 levels, light levels. Others can fungi have internal time clocks and sporulate anyway in a preset part of the fungal life cycle designed to spread and maximize colonization during one season.

13.4.8 Chytridiomycete asexual reproduction

Chytrids are quite distinct from other fungi as they have extremely simple thalli and motile zoospores. Species within this group are very simple in structure and may only consist of a single cell, perhaps with rhizoids to anchor it on to a substrate.

Asexual reproduction in the chytrids is by the production of motile zoospores, with a single, posterior flagellum, in sporangia. There will be a film of chytrid reproduction available in the practical.

Figure: 28 Holocarpic chytrid

This is a diagram of a holocarpic chytrid, one where the entire thallus consists of only one cell with rhizoids. These are usually parasitic on aquatic plants or fish. The fungus 'feeds' from its substrate via its rhizoids. The entire cell contents will convert to motile zoospores.

13.4.9 *ZYGOMYCETE* ASEXUAL REPRODUCTION

Zygomycete fungal mycelium is coenocytic. At the onset of sporulation large amounts of aerial hyphae are produced. The tips of these aerial hyphae fill with cytoplasmic contents, and the nuclei undergo repeated mitosis. Around each of the nuclei cytoplasm and organelles collect, and by the formation of copious vesicles from the Golgi, each nucleus becomes isolated from the next by a plasma membrane. Within the spaces created by this cytoplasmic cleavage, spore walls begin to form, again by the fusion of Golgi vesicles containing cell wall monomers and enzymes with the spore membrane. A sporangium forms. As these events occur so there is considerable water uptake by the forming sporangium, and as the columella forms the structure comes under considerable turgor pressure. The large sporangia can contain up to 100,000 spores.

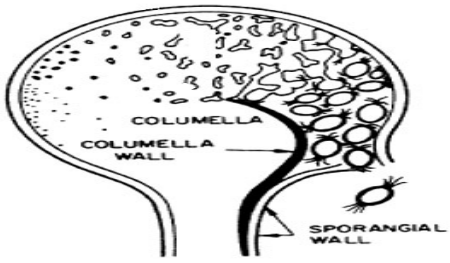


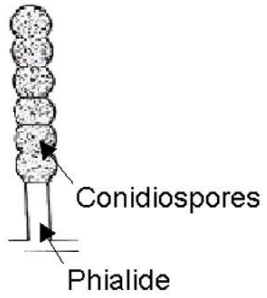
Figure 29 Formation of a sporangium

This diagram is redrawn from Brackers original and shows the development of a sporangium through time. As nuclei undergo repeated mitosis so the Golgi produces membrane bound vesicles filled with spore wall building materials. These coalesce around the nuclei to form a spore. Eventually they are released.

Not all sporangia are as large as this, there are many species with smaller, specialized sporangia, called sporangiola, merosporangia and some are almost conidial, forming single spored sporangia that are only distinguished from the conidium of the higher fungi by the possession of a double spore wall. We will look at some of these structures in the practical.

13.4.10 ASEXUAL REPRODUCTION IN THE ASCOMYCETES (AND DEUTEROMYCETES)

The process of spore formation in most members of the higher fungal groups is again based largely on the formation of aerial mycelium and the differentiation of the hyphal tip. However, unlike the process seen in the *Zygomycetes*, the process here involves something much more like the budding we see in the yeasts. This is termed a blastic process, which involves the blowing out or blebbing of the hyphal tip wall. The blastic process can involve all wall layers, or there can be a new cell wall synthesized which is extruded from within the old wall. As asexual reproduction is sometimes the only form of reproduction seen in some fungi, we have in the past tried to construct elaborate taxonomic schemes based on spore structure and production. However, as I said earlier, these features are notoriously plastic and such schemes have largely been abandoned. The hypha that creates the sporing (conidiating) tip can be very similar to the normal hyphal tip, or it can be differentiated. The commonest differentiation is the formation of a bottle shaped cell called a phialide, from which the spores are produced.



Figuer: 30 Phialide formation in the Ascomycete fungi

Not all of these asexual structures are single hyphae. In some groups the conidiophores (the structures that bear the conidia) are aggregated. In the *Moniliales* all are single with the exception of the aggregations termed coremia or synnema. These produce structures rather like corn-stooks, with many conidia being produced in a mass from the aggregated conidiophores.

Other species of *Ascomycetes* and *Deuteromycetes* form their structures within plant tissue, either as parasite and saprophytes. These fungi have evolved more complex asexual sporing structures, probably influenced by the cultural conditions of plant tissue as a substrate. These structures are called the sporodochium. This is a cushion of conidiophores created from a psuedoparenchymatous stroma in plant tissue. The pycnidium is a globose to flask-shaped parenchymatous structure, lined on its inner wall with conidiophores. The acervulus is a flat saucer shaped bed of conidiophores produced under a plant cuticle, which eventually erupt through the cuticle for dispersal.

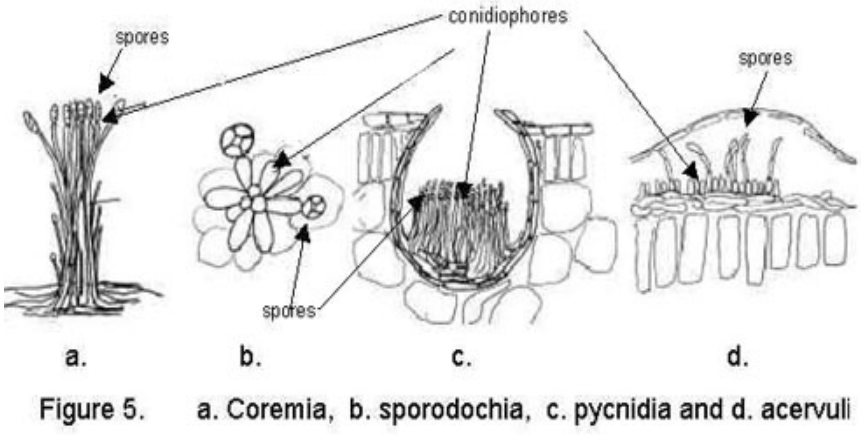


Figure 5. a. Coremia, b. sporodochia, c. pycnidia and d. acervuli

Figure 31 SPORE STRUCTURE

13.5 LET US SUM UP

- Multiplication, Sexual and asexual reproduction of bacteria, bacteriophages, yeast, fungi and Actinomycetes also elaborated.
- Phage multiplication – lytic and lysogenic cycle.
- Conjugation – process by which bacteria join and exchange genetic material.
- Binary fission – process by which a bacteria splits into two cells.
- Transformation – bacterium takes up DNA released by dead bacterium.
- Transduction – bacteriophages carry DNA from one cell to another.

13.6 LESSON END ACTIVITIES

Explain the generalized nuclear cycle of fungi.
Explain sexual reproduction of zygomycetes.
Explain the following: bud formation in yeast, fragmentation, sporulation

What are heterothallic and homothallic strains of yeast?
Describe the asexual reproduction of deuteromycetes.
Describe the phage life cycle.

13.7 POINTS FOR DISCUSSION

1. Describe the various methods for fungal multiplication
2. Write a brief account on
    - Sexual reproduction in the basidiomycetes
    - Sexual reproduction in the ascomycete
    - Sexual reproduction in the chytridiomycetes
    - Asexual reproduction in the ascomycetes

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UNIT IV

MICROBIAL ECOLOGY

CONTENTS

UNIT IV

MICROBIAL ECOLOGY

LESSON 14

SOIL MICROBIOLOGY

Contents

14.0. AIMS AND OBJECTIVES

This unit aims to provide information on the role of microbes in various environments and the physiological adaptation of microbes to the environment.

14.1 INTRODUCTION

Microbial ecology is the relationship of microorganisms with one another and with their environment. It concerns the three major domains of life — Eukaryota, Archaea, and Bacteria — as well as viruses. Microorganisms, by their omnipresence, impact the entire biosphere. They are present in virtually all of our planet's environments, including some of the most extreme, from acidic lakes to the deepest ocean, and from frozen environments to hydrothermal vents.

Microbes, especially bacteria, often engage in symbiotic relationships (either positive or negative) with other organisms, and these relationships affect the ecosystem. One example of these fundamental symbioses is chloroplasts, which allow eukaryotes to conduct photosynthesis. Chloroplasts are considered to be endosymbiotic cyanobacteria, a group of bacteria that are thought to be the origins of aerobic photosynthesis. Some theories state that this invention coincides with a major shift in the early earth's atmosphere, from a reducing

atmosphere to an oxygen-rich atmosphere. Some theories go as far as saying that this shift in the balance of gasses might have triggered a global ice-age known as the Snowball Earth.

They are the backbone of all ecosystems, but even more so in the zones where light cannot approach and thus photosynthesis cannot be the basic means to collect energy. In such zones, chemosynthetic microbes provide energy and carbon to the other organisms.

Other microbes are decomposers, with the ability to recycle nutrients from other organisms' waste poducts. These microbes play a vital role in biogeochemical cycles. The nitrogen cycle, the phosphorus cycle and the carbon cycle all depend on microorganisms in one way or another. For example, nitrogen which makes up 78% of the planet's atmosphere is "indigestible" for most organisms, and the flow of nitrogen into the biosphere depends on a microbial process called fixation.

Due to the high level of horizontal gene transfer among microbial communities, microbial ecology is also of importance to studies of evolution.

14.2. SOIL MICROBIOLOGY

Soil is a complex environment colonized by an immense diversity of microorganisms. Soil microbiology focuses on the soil viruses, bacteria, actinomycetes, fungi, and protozoa, but it has traditionally also included investigations of the soil animals such as the nematodes, mites, and other micro arthropods. These organisms, collectively referred to as the soil biota, function in a belowground ecosystem based on plant roots and litter as food sources. Modern soil microbiology represents an integration of microbiology with the concepts of soil science, chemistry, and ecology to understand the functions of microorganisms in the soil environment.

The surface layers of soil contain the highest numbers and variety of microorganisms, because these layers receive the largest amounts of potential food sources from plants and animals. The soil biota form a belowground system based on the energy and nutrients that they receive from the decomposition of plant and animal tissues. The primary decomposers are the bacteria and fungi.

Microorganisms, especially algae and lichen, are pioneering colonizers of barren rock surfaces. Colonization by these organisms begins the process of soil formation necessary for the growth of higher plants. After plants have been established, decomposition by microorganisms recycles the energy, carbon, and nutrients in dead plant and animal tissues into forms usable by plants. Therefore, microorganisms have a key role in the processing of materials that maintain life on the Earth. The transformations of elements between forms are described conceptually as the elemental cycles.

In the carbon cycle, microorganisms transform plant and animal residues into carbon dioxide and the soil organic matter known as humus. Humus improves the water-holding capacity of soil, supplies plant nutrients, and contributes to soil aggregation. Microorganisms may also directly affect soil aggregation. The extent of soil aggregation determines the workability or tilt of the soil. A soil with good tilt is suitable for plant growth because it is permeable to water, air, and roots.

Soil microorganisms play key roles in the nitrogen cycle. The atmosphere is approximately 80% nitrogen gas (N_2), a form of nitrogen that is available to plants only when it is transformed to ammonia (NH_3) by either soil bacteria (N_2 fixation) or by humans (manufacture of fertilizers). Soil bacteria also mediate denitrification, which returns nitrogen to the atmosphere by transforming NO_3^- to N_2 or nitrous oxide (N_2O) gas. Microorganisms are crucial to the cycling of sulfur, phosphorus, iron, and many micronutrient trace elements.

In addition to the elemental cycles, there are several interactions between plants and microbes which are detrimental or beneficial to plant growth. Some soil microorganisms are pathogenic to plants and cause plant diseases such as root rots and wilts. Many plants form

symbiotic relationships with fungi called *mycorrhizae* (literally fungus-root). *Mycorrhizae* increase the ability of plants to take up nutrients and water. The region of soil surrounding plant roots, the rhizosphere, may contain beneficial microorganisms which protect the plant root from pathogens or supply stimulating growth factors. The interactions between plant roots and soil microorganisms are an area of active research in soil microbiology.

The incredible diversity of soil microorganisms is a vast reserve of potentially useful organisms. Many of the medically important antibiotics are produced by filamentous bacteria known as actinomycetes. The soil is the largest reservoir of these medically important microorganisms.

The numerous natural substances that are used by microorganisms indicate that soil microorganisms have diverse mechanisms for degrading a variety of compounds. Human activity has polluted the environment with a wide variety of synthetic or processed compounds. Many of these hazardous or toxic substances can be degraded by soil microorganisms. This is the basis for the treatment of contaminated soils by bioremediation, the use of microorganisms or microbial processes to detoxify and degrade environmental contaminants. Soil microbiologists study the microorganisms, the metabolic pathways, and the controlling environmental conditions that can be used to eliminate pollutants from the soil environment.

Microbiologists traditionally isolate pure strains of microorganisms by using culture methods. Methods that do not rely on culturing microorganisms include microscopic observation and biochemical or genetic analysis of specific cell constituents. The rates or controlling factors for microbial processes are studied by using methods from chemistry, biology, and ecology. Typically, these studies involve measuring the rate of production and consumption of a compound of interest. The results of these studies are commonly analyzed by using mathematical models. Models allow the information from one system to be generalized for different environmental conditions.

14.3 SOIL AIR

Soil is a three-phase, porous media, composed of solids, liquids, and voids (empty spaces between the solids). The typical amount of total porosity (ratio of void volume to total volume) in a mineral soil ranges from about 40% to 60%. This means approximately 40 to 60% of the volume of a mineral soil is actually empty space between the solid particles (voids).

These voids are filled with air and/or water. The air in the soil is similar in composition to that in the atmosphere with the exception of oxygen, carbon dioxide, and water vapor. In soil air as in the atmosphere, nitrogen gas (dinitrogen) comprises about 78%. In the atmosphere, oxygen comprises about 21% and carbon dioxide comprises about 0.36%. However, in the soil air, oxygen usually is replaced by carbon dioxide, so both range from about 0.4% to 21%.Oxygen is used by plant roots and soil microbes during respiration, and carbon dioxide is released. Thus, in the soil, the oxygen levels are generally less than atmospheric levels and the carbon dioxide levels are generally greater than atmospheric levels.

Some factors that determine the extent of the difference between atmospheric and soil air constituents include depth in the soil profile, soil pore size distribution, and soil water content.

- Depth: Oxygen levels generally decrease with depth in the soil profile due to slow diffusion rates of oxygen from the surface through the soil.
- Pore size distribution: Soils with large pores promote more rapid oxygen diffusion into and through the soil, and carbon dioxide movement out of the soil. Soils with small pores have slower oxygen diffusion into the soil and carbon dioxide diffusion

out of the soil. Sandy soils generally have low total porosity but large individual pores. Clay soils generally have high total porosity but small individual pores.

- Aeration and drainage: Soils with large pores generally have good drainage (less water) and aeration, while soils with small pores generally have poor drainage and aeration. Thus, sands generally have good drainage, while clays have poor drainage and are more likely to become anaerobic (deprived of oxygen) as microbes use oxygen more rapidly than it is replenished through diffusion.
- Soils with more pores filled with water have less space available for air, thus become anaerobic more rapidly than drier soils.
- Water vapor: Soil air has a relative humidity very close to 100%. (Relative humidity is the amount of water vapor actually in the air relative to the amount the air could hold at that temperature.) This is much different than atmospheric air, which may vary in relative humidity between 5% and 100%, sometimes within 24 hours in semi-arid and arid regions. A Boy Scout Survival Kit applies this concept by providing a shovel, piece of plastic, and a cup. Dig a hole in the morning; place the cup in the bottom of the hole. Anchor the plastic with soil around the rim of the hole. Place a rock on the plastic above the cup. During the day, the plastic allows the soil to heat, evaporating water. At night, the plastic allows radiational cooling. As the air cools, water vapor condenses on the plastic and drips into the cup. This provides enough water for subsistence. If cacti or other succulents are available, place some of their vegetation in the hole to enhance the water provision.

14.4 SOIL WATER

It has been demonstrated that soil is not essential for plant growth and indeed plants can be grown hydroponically (in a liquid culture). However, usually plants are grown in the soil and soil properties directly affect the availability of water and nutrients to plants. Soil water affects plant growth directly through its controlling effect on plant water status and indirectly through its effect on aeration, temperature, and nutrient transport, uptake and transformation. The understanding of these properties is helpful in good irrigation design and management.

The soil system is composed of three major components: solid particles (minerals and organic matter), water with various dissolved chemicals, and air. The percentage of these components varies greatly with soil texture and structure. An active root system requires a delicate balance between the three soil components; but the balance between the liquid and gas phases is most critical, since it regulates root activity and plant growth process.

The amount of soil water is usually measured in terms of water content as percentage by volume or mass, or as soil water potential. Water content does not necessarily describe the availability of the water to the plants, nor indicates how the water moves within the soil profile. The only information provided by water content is the relative amount of water in the soil.

Soil water potential, which is defined as the energy required to remove water from the soil, does not directly give the amount of water present in the root zone either. Therefore, soil water content and soil water potential should both be considered when dealing with plant growth and irrigation.

14.5 SOIL MICROORGANISMS

Normal, fertile soils teem with soil microbes. In fact, there may be hundreds of millions to billions of microbes in a single gram [about 4 hundredths of a pound and about the size of a navy bean in volume)(See Table 1)]. The most numerous microbes in soil are the bacteria (unicellular cells lacking a true nucleus) followed in decreasing numerical order by

the actinomycetes (a specialized group of bacteria which contains many members that produce valuable antibiotics), the fungi (singular: fungus) which produce long, slender filaments nicely adapted for exploiting the three-dimensional pore network of the soil, soil algae and cyanobacteria ("blue-green "algae") (photosynthetic microbes which can add small amounts of carbon to soil and which also can be a nuisance in turfgrass golf greens) and soil protozoa (unicellular soil organisms that decompose organic materials as well as consume large numbers of bacteria).

Microbial Group	No./Gram of soil
Bacteria	100,000,000 - 1,000,000,000
Fungi	100,000 - 1,000,000
Algae and Cyanobacteria	1000 - 1,000,000
Protozoa	1000 - 100,000

Table 1. Numbers of Microbes in Soil

Some microorganisms also burrow and channel through soil, which improves soil structure and aggregation, while other microorganisms have the ability to break down resistant organic matter such as lignin, toxins, and pesticides. Microorganisms also have the ability to protect plants from antagonistic pathogens and some can dissolve minerals, making nutrients available to plants.

In addition to the microbes, there are numerous species of soil animals that inhabit soils. These include nematodes (microscopic roundworms which are generally beneficial but some of which are plant parasites of agricultural crops and turfgrasses), microarthropods (mites, springtails, etc.) and larger animals such as the earthworms, burrowing insects, etc. Earthworms are like "Nature's Tillers". They incorporate dead organic matter into soil, ingest it, and excrete the nutrient rich casts on to and in soil. Earthworms improve aeration, water infiltration, drainage, and they enhance nutrient availability and cycling. Dung Beetles and Termites also incorporate dead organic matter into soil. These larger organisms can exert beneficial effects through improved soil structure and improved aeration and drainage due to their channeling activities in the soil.

Soil microbes are important for soil structure also but their effect is more subtle. Soil microbes produce lots of gummy substances (polysaccharides, mucilages, etc.) that help to cement soil aggregates. This cement makes aggregates less likely to crumble when exposed to water. Fungal filaments, called hyphae, also stabilize soil structure because these threadlike structures ramify throughout the soil literally surrounding particles and aggregates like a hairnet. The fungi can be thought of as the "threads" of the soil fabric. It must be stressed that microbes generally exert little influence on changing the actual physical structure of the soil. That's the job of the larger "earthmovers".

Fungi are able to break down resistant materials such as cellulose, gums, and lignin. They dominate in acidic, sandy soils and in fresh organic matter.

Actinomycetes also are able to decompose resistant substances in soil. One type, Frankia, help plants get nutrients needed from the air by breaking triple bonded nitrogen down into ammonium that plants can use. Antibiotics are made from soil Actinomycetes.

Bacteria decompose a wider range of earth material than any other microbe group. Heterotrophs gain their energy and Carbon from other organisms, while Autotrophs synthesize their own energy from light or by chemical oxidation. Some bacteria can fix nitrogen in to forms plants can use.

14.6 BENEFITS OF SOIL MICROBES

In addition to their role in cementing soil aggregates mentioned above, soil microbes are of paramount importance in cycling nutrients such as carbon (C), nitrogen (N), phosphorus (P), and sulfur (S). Not only do they control the forms of these elements [e.g.

specialized soil bacteria convert ammonium N (NH_4^+) to nitrate N (NO_3^-)], they can regulate the quantities of N available to plants. This is especially critical in systems relying on organic fertilizers. It is only through the actions of soil microbes that the nutrients in organic fertilizers are liberated for plants and use by other microbes. Soil microbiologists call this process mineralization [the conversion of organic complexes of the elements to their inorganic forms, e.g., conversion of proteins to carbon dioxide (CO_2) ammonium (NH_4^+) and sulfate (SO_4^-)]. It is perhaps the single-most important function of soil microbes as it recycles nutrients tied up in organic materials back into forms useable by plants and other microbes. In fact, the so-called Principle of Microbial Infallibility (popularized by Dr. Martin Alexander of Cornell University) states that for every naturally occurring organic compound there is a microbe or enzyme system that can degrade it. Note that this applies to naturally occurring compounds. It is obvious that some our persistent pesticides did not conform to this principle and even some naturally occurring compounds are fairly resistant to microbial attack. It is through the process of mineralization that crop residues, grass clippings, leaves, organic wastes, etc., are decomposed and converted to forms useable for plant growth as well as converted to stable soil organic matter called humus. Herein lies another important role for the larger soil animals like earthworms. The large organisms function as grinders in that they reduce the particle size of organic residues making them more accessible and decomposable by the soil microbes. The soil microbial population also further decomposes the waste products of the larger animals. Thus, the activities of different groups of soil organisms are linked in complex "food webs".

One beneficial process carried out exclusively by soil microbes is called nitrogen fixation, the capture of inert N_2 gas (dinitrogen) from the air for incorporation into the bodies of microbial cells. In one well-known form of this process, symbiotic nitrogen fixation, soil bacteria such as *Rhizobium* and *Bradyrhizobium* actually inhabit specialized structures on the roots of leguminous plants (soybeans, cowpeas, beans clovers, etc.) where they fix substantial quantities of nitrogen that becomes available to the host plant. Unfortunately, the root nodule system is not found in the grasses so we cannot rely on it for "free" nitrogen. Nevertheless, free-living (nonsymbiotic) nitrogen-fixing bacteria do associate with roots of grasses where they fix small quantities of nitrogen using carbon compounds (root exudates, sloughed root cells, etc.) produced from the roots as energy sources to drive the energy-expensive nitrogen-fixing enzyme system. Another factor limiting the utility of free-living N_2 fixers is the fact that they will not fix N_2 when exposed to even very low levels of fertilizer nitrogen. Thus in fertile turfgrass soils this process is of limited importance whereas in unfertilized prairie soils the 10 to 25 pounds of N fixed per acre per year is ecologically relevant.

Another benefit of soil microbes is their ability to degrade pest control chemicals and other hazardous materials reaching the soil. Thus through the actions of the soil microflora, pesticides may be degraded or rendered nontoxic lowering their potential to cause environmental problems such as ground and surface water contamination. Of course, there is a "downside" to this microbial capability. In some instances, soil microbes have been shown to degrade soil-applied pesticides so rapidly as to reduce the ability of the chemicals to control the target pests. This phenomenon is known as enhanced degradation and usually results from repeated applications of a chemical to the soil. One way around this problem is to vary the use of pest control chemicals.

Considerable research has been done on applying various microbes as inoculants for various purposes including their use as agents to control plant diseases, to stimulate plant growth (the so-called plant-growth-promoting rhizobacteria; PGPR) and more recently their use in various forms of bioremediation processes. Perhaps the most outstanding example of beneficial use of a soil bacterium is the practice of inoculating legumes with bacteria such as *Rhizobium* and *Bradyrhizobium*. Some crops are nearly self-sufficient in meeting their

nitrogen requirements through this process. The process is so successful because the plant essentially selects the bacterium and builds a habitat, the root nodule, where conditions for nitrogen fixation are optimized.

14.7 ORGANIC MATTER OF SOIL

Soil is composed of minerals and organic matter, as well as living organisms. The minerals are derived from the weathering of "parent material" - bedrock and overlying sub-soil. The organic matter in soil derives from plants and animals. In a forest, for example, leaf litter and woody material falls to the forest floor. This is sometimes called organic material. When it decays to the point it is no longer recognizable it is called soil organic matter. When the organic matter has broken down into a stable humic substances that resist further decomposition it is called humus. Thus soil organic matter comprises all of the organic matter in the soil exclusive of the undecayed material.

Organic matter may refer simply to matter which was once part of a living organism or produced by a living organism. This definition is synonymous with biotic material, and would include a clam's shell and naturally produced urea, while excluding synthetically produced urea. While this definition is useful for modelling nutrient flows, it is not useful in measuring the organic content of soil.

Measurements of organic matter generally measure only organic compounds or carbon, and so is only an approximation of the level of once-living or decomposed matter. Some definitions of organic matter likewise only consider "organic matter" to refer to only the carbon content, or organic compounds, and do not consider the origins or decomposition of the matter. In this sense, not all organic compounds are created by living organisms, and living organisms do not only leave behind organic material. A clam's shell, for example, while biotic, does not contain much organic carbon, so may not be considered organic matter in this sense. Conversely, urea is one of many organic compounds that can be synthesized without any biological activity.

14.7.1 SOIL RHIZOSPHERE

The establishment of large numbers of metabolically active populations of beneficial soil microbes is critical for the success of several environmental remediation and agricultural practices. These microorganisms are successful in getting established in the soil ecosystem due to their high adaptability in a wide variety of environments, their faster growth rate, and their biochemical versatility to metabolize a variety of natural and xenobiotic chemicals. Majority of the microbial population found in the soil is associated with the plant roots, where their numbers can reach up to 10^9 to 10^{12} per gram of soil, leading to a biomass equivalent to 500 kg ha^1. This abundance in vegetated soils is due to the availability of nutrients via plant root exudation, which can stimulate microbial growth in the immediate vicinity of the roots (a region also known as the "rhizosphere"). Hence, the rhizosphere has been promoted as the ideal site to modify microbial populations ("rhizoengineering") to suite various applications in the soil.

The soil zone strongly influenced by plant roots, the rhizosphere, plays an important role in regulating soil organic matter decomposition and nutrient cycling. Processes that are largely controlled or directly influenced by roots are often referred to as rhizosphere processes. These processes may include exudation of soluble compounds, water uptake, nutrient mobilization by roots and microorganisms, rhizosphere-mediated soil organic matter decomposition, and the subsequent release of CO_2 through respiration. Rhizosphere processes are major gateways for nutrients and water. At the global scale, rhizosphere processes utilize approximately 50% of the energy fixed by photosynthesis in terrestrial ecosystems, contribute roughly 50% of the total CO_2 emitted from terrestrial ecosystems, and mediate virtually all

aspects of nutrient cycling. Therefore, plant roots and their rhizosphere interactions are at the center of many ecosystem processes. However, the linkage between rhizosphere processes and soil organic matter decomposition is not well understood. Because of the lack of appropriate methods, rates of soil organic matter decomposition are commonly assessed by incubating soil samples in the absence of vegetation and live roots with an implicit assumption that rhizosphere processes have little impact on the results. Our recent studies have overwhelmingly proved that this implicit assumption is often invalid, because the rate of soil organic matter decomposition can be accelerated by as much as 380% or inhibited by as much as 50% by the presence of live roots.

Two key mechanisms behind the rhizosphere effect on soil organic matter decomposition are: (1) accelerated soil microbial turnover rate, and (2) transpiration-induced drying-rewetting cycles. The role of rhizosphere carbon fluxes in shaping the temperature sensitivity of soil organic matter decomposition is also very much essential. This is related to the current debate about the potential positive feedback mechanism between global warming and the rate of soil organic carbon decomposition. The positive feedback mechanism informs that, if warmer environment accelerates the release of CO_2 from the decomposition of soil organic carbon, the global environment will get even warmer because of the extra CO_2 from soil carbon pool will increase the atmospheric CO_2 concentration and intensifies the greenhouse effect, and this even warmer condition will further lead to more CO_2 release from soil organic carbon pool, therefore forming a viscous circle. Although there are clearly controversies about temperature sensitivity of soil organic matter decomposition among published reports, one issue is widely recognized: the lack of understanding about rhizosphere carbon fluxes and how they may respond to temperature changes is at the heart of the current debate. The rhizosphere effect on soil organic matter decomposition is often large in magnitude and significant in mediating plant-soil interactions.

14.7.2 ORGANIC MATTER DECOMPOSITION

Organic matter may be defined as material that is capable of decay, or the product of decay (humus), or both. Usually the matter will be the remains of recently living organisms, and may also include still-living organisms. Polymers and plastics, although they may be organic compounds, are usually not considered organic material, due to their poor ability to decompose. A clam's shell, while biotic, would not be considered organic matter by this definition because of its inability to decay.

There are some creatures that have the ability to convert naturally occurring compounds and chemicals into food for themselves. They do this by directly eating the naturally occurring compounds, and sometime they secrete enzymes that help them break the compounds down for digesting. These creatures are called 'autotrophs'. The rest are generically referred to as 'heterotrophs', they dine on the autotrophs, or their waste products. These are basically the plants and animals of this microscopic world. On them the rest of life depends, because these creatures form the base of the food pyramid. With them the decomposition of all organic materials begins. And in turn, the renewal of all energy given to us by the sun is continued. Also from the recycling of organic compounds, chemicals, and other materials that is constantly being renewed into new life in a large cycle known as 'The Cycle of Life'.

Saprobic bacteria are heteroptrophs that live on decaying material, such as a dead body. By decomposing organic material for energy, these microorganisms help recycle nutrients like nitrogen and carbon back into the environment. If it were not for these decomposers, the organic carbon in dead and rotting organisms would remain locked underground, effectively stopping the carbon cycle. The carbon dioxide in air would be

quickly depleted, and there would be none left for plants to carry out photosynthesis. Saprobic bacteria are, therefore, one of the most important links in the carbon cycle.

14.7.3 BREAKING DOWN A DEAD BODY

Saprobic bacteria invade every inch of the dead body, and begin eating and digesting its tissues. There are many types of bacteria that live inside the body. These bacteria are the first to begin the process of decomposition after an organism dies. The initial bacteria start physical break-down of muscles. They tear apart the muscle tissue into individual protein strands. More saprobic bacteria soon move in to help break down the organic matter. Because there are so many bacteria in the soil (about 2 billion in 1 gram of fertile garden soil), decomposition occurs more rapidly after a body is buried. Decay that occurs underground or underwater is known as anaerobic decomposition. One specific type of anaerobic decay that is very important to the carbon cycle is biogas production. This process has three steps: hydrolysis, acidification, and methane formation.

- **Hydrolysis -- Splitting a Protein**

Proteins are broken down by an enzyme called protease that is secreted by fermentative bacteria. This enzyme separates proteins (polypeptides) into amino acids (peptides). It accomplishes this depolymerization through a process known as hydrolysis. In hydrolysis, a water molecule is inserted between the two amino acids that are bonded together. This breaks the bond between them by *capping* the free reactive ends with the H and the OH. The protein, therefore, is broken down from long chains into its individual molecules, amino acids.

- **Acidification -- From Serine to Acetic Acid**

In the next step, a new group of bacteria called acetogens take over. These bacteria decompose amino acids into acetic acid, hydrogen gas, nitrogen gas, and carbon dioxide gas. To do this, they need oxygen which they obtain from O_2 dissolved in the body's fluids or bound in the body's structure. While acetogens are anaerobic bacteria, oxygen is not as poisonous to them as to some other anaerobes. The chemical reaction that occurs when acetogens decompose amino acids is:

$$2C_3H_7NO_3 + O_2 \text{ ---> } 2HC_2H_3O_2 + 3H_2 + N_2 + 2CO_2$$

serine (amino acid) + oxygen ---> acetic acid + hydrogen + nitrogen + carbon dioxide

Because acetogens produce acid, the pH of the organic matter falls to about 4 or 5. This low pH is toxic to the next group of bacteria, the methanogens. As the acetogens die out, however, the pH rises and methanogens become more predominant.

- **Biogas Formation -- From Acetic Acid to Methane**

Methanogens are bacteria that produce methane gas. They are very anaerobic (oxygen is toxic to them) and sensitive to pH. These bacteria are also very biologically primitive. Methanogens have, therefore, been a part of the carbon cycle for a long time. They combine the acetic acid made by acetogens with hydrogen gas, and carbon dioxide to produce methane gas, water, and carbon dioxide, according to the following equation:

$$HC_2H_3O_2 + 4H_2 + CO_2 \text{ --> } 2CH_4 + 2H_2O + CO_2$$

acetic acid + hydrogen gas + carbon dioxide --> methane + water + carbon dioxide

Each year, between 531 and 792 million tons (482 - 718 million metric tons) of methane are released into the atmosphere by methanogens. These incredibly busy bacteria,

therefore, recycle a great deal of carbon, making a very significant contribution to the earth's carbon cycle.

14.7.4 BENEFITS OF ORGANIC MATTER

- Nutrient Supply
  Organic matter is a reservoir of nutrients that can be released to the soil. Each percent of organic matter in the soil releases 20 to 30 pounds of nitrogen, 4.5 to 6.6 pounds of P_2O_5, and 2 to 3 pounds of sulfur per year. The nutrient release occurs predominantly in the spring and summer, so summer crops benefit more from organic-matter mineralization than winter crops.
- Water-Holding Capacity
  Organic matter behaves somewhat like a sponge, with the ability to absorb and hold up to 90 percent of its weight in water. A great advantage of the water-holding capacity of organic matter is that the matter will release most of the water that it absorbs to plants. In contrast, clay holds great quantities of water, but much of it is unavailable to plants.
- Soil Structure Aggregation
  Organic matter causes soil to clump and form soil aggregates, which improves soil structure. With better soil structure, permeability (infiltration of water through the soil) improves, in turn improving the soil's ability to take up and hold water.
- Erosion Prevention
  This property of organic matter is not widely known. Data used in the universal soil loss equation indicate that increasing soil organic matter from 1 to 3 percent can reduce erosion 20 to 33 percent because of increased water infiltration and stable soil aggregate formation caused by organic matter.

14.8 FACTORS AFFECTING SOIL MICROBIAL POPULATION

Interestingly, some soil bacteria (the anaerobes) do not even need air to grow and some are "poisoned" by exposure to oxygen. Generally, soil microbes grow best in soils of near neutral pH (7.0) having adequate supplies of inorganic nutrients (N and P, etc.), a balance of air- and water-filled pore space (about 50-60% of water holding capacity) and abundant organic substrates (carbon and energy sources). When any one of these parameter gets too far beyond the normal range some segment of the population will likely be stressed. For example, aerobic (oxygen requiring) bacteria will be at a disadvantage when a soil becomes waterlogged and available O_2 is depleted through respiration of roots, microbes and soil animals. Conversely, anaerobic organisms may predominate leading to unique problems such as the formation of "black layer" caused, at least in part, by the anaerobic sulfate-reducing bacteria. Similarly, if soils become too acidic (down to pH 4 or 5) bacteria and actinomycetes usually decline and fungi assume a more dominant position. Except at cool and warm temperature extremes, the soil microbial population is usually not severely stressed. Most soil microbes grow best at temperatures between 15-30^o Celsius (about 60 to 85^{o}F) and their growth rates increase with increasing temperature up to a point. This is why it is harder to maintain soil organic matter in warm climates. Interestingly, some cold-loving microbes (called cryophiles) can actually grow and cause disease under blankets of snow cover. Such is the case with the so-called snow molds which can damage turfgrasses extensively during winter months. The opposite extreme is found in thermophilic microbes ("heat lovers") that thrive in composts reaching temperatures as high as 65^o C (150^o F). It is the biological heating of composts that actually reduces levels of pathogenic microbes, weed seeds and insects during the composting process.

Without a doubt, the most important limiting factor for microbial growth in soil (assuming moisture is adequate) is the abundance of available organic carbon sources. The vast majority of soil microbes require organic carbon compounds (these are called organotrophs) to oxidize for energy and to build the organic constituents of their cell bodies. Only a few types of soil bacteria get their carbon from CO_2 (autotrophs) and they contribute little to the overall organic matter content of a soil with the possible exception of the cyanobacteria on the surface of closely mown turfs where they may accumulate as dark slippery films. Organic inputs in turfgrass soils come mainly from the grasses themselves in the form of root exudates, lysed root cells, decomposing roots and any clippings returned to the soil. Of course, organic amendments may contribute some useable carbon as well but bear in mind that amendments such as compost, which is essentially microbially decomposed organic materials, do not contain high levels of readily available carbon. Rather, they provide slowly useable substrates and contribute directly to the soil organic matter pool. Also, as a general "rule of thumb" about one third of the organic carbon added to temperate soils remains in the soil as humus and microbial biomass whereas about two thirds of this carbon is returned to the atmosphere as CO_2 through microbial respiration.

Frequently we see statements in the lay literature about chemical fertilizers killing soil microbes or, worse yet, statements indicating these management inputs "sterilize" the soil. It is true that some inputs, e.g., anhydrous ammonia, cause reductions in microbial numbers in the immediate vicinity of the application. After all, ammonia is a toxic gas. However, it quickly equilibrates with the soil solution in the form of ammonium ions and the toxicity subsides. Certain pesticides have been shown to cause similar transient reductions in selected microbial population. But remember, in some cases the microbes simply view these chemicals as food and degrade them fairly quickly.

Organic fertilizers circumvent the criticisms leveled at "synthetic" fertilizers but it should not be forgotten that plants take up nitrogen in the form of ammonium (NH_4^+) or nitrate (NO_3^-) ions regardless of whether it was mineralized from an organic source or applied as in inorganic fertilizer like ammonium nitrate. An advantage of using organics, where practical, is that nutrients are liberated slowly as the microbes mineralize the organic materials. Thus there is low risk for fertilizer burn on plants and less risk for environmental problems due to runoff and leaching. Another potentially negative effect of long-term use of ammonia-based fertilizers is soil acidification due to ammonia oxidation by the nitrifying bacteria. Soil pH can drop below 5.0 after prolonged use of ammonia-based fertilizers and this can cause marked reductions in populations of bacteria and actinomycetes and simultaneous increases in the relative abundance of fungi. Such changes might favor the development of certain fungal plant pathogens. On the other hand, the potato scab disease is reduced by the low pH because the actinomycete which causes it is eliminated. These changes are easily reversed with applications of lime to the soil. Thus we see qualitative changes in the soil populations due to some management inputs but this is a long way from "sterilizing" or "killing" the soil.

With the advent of high-sand golf greens questions have arisen about the need for applying microbes during green construction and thereafter. Sand because of its lack of organic matter supports little microbial growth. However, when mixed with peats, composted rice hulls or other organic amendments it gains the microbial populations associated with those materials. Turfgrasses established from vegetative sprigs also bring their root-associated microbes with them. Once the turfgrass begins growing in the rooting medium of the green, microbes already present will colonize roots and the mechanics of soil organic matter formation will commence. A reasonable practice would be to add a small amount of normal pathogen-free soil to the greens mix as an inoculum. Thus far, there is little scientific evidence indicating the need to inoculate golf greens with selected microorganisms. The

newly constructed green does afford us the possibility of customizing the soil population to some extent. Once we know what we want in these mixes it may be easier to add them "up front" than to add them into an established population already adapted to the prevailing conditions of a particular soil. As our knowledge of soil microbial biodiversity and the factors that control it increases we may find ways of tailoring microbial populations in given environments.

One of the nagging problems of using organisms as inoculants is the tendency for erratic control of pests or failure to observe any benefit from inoculation. Reasons for inconsistencies in response to inoculation can be manifold. There are many reasons why introduced bacteria do not become established when added to the soil in very low numbers. Here we see a number of problems that an introduced microbe must overcome in order to establish itself among the normal population. These include inhibition by toxins, predation by other soil microbes such as the protozoa and a bacterium called *Bdellovibrio*, lysis by viruses called bacteriophages, and a simple inability to compete with the native organisms.

- Microbially produced toxins
- Predatory protozoa
- Lysis by bacteriophage (bacterial viruses)
- Lysis by *Bdellovibrio bacteriovorus*
- Lysis by microbial enzymes
- Inability of introduced microbe to compete

Table2 Some biotic factors responsible for the elimination of introduced microbes

14.9. SOIL MICROBIAL BALANCE

It is well to recall that each soil has an indigenous microbial population that is selected by the prevailing biotic and abiotic factors unique to that soil. Typically it is difficult to add or displace microorganisms to or from a system in such equilibrium. An axiom of microbial ecology often referred to as Beijerinck's Rule (Beijerinck was a Dutch microbiologist who is often considered the "Father" of microbial ecology) states that "Everything (microbes) is everywhere and the milieu (i.e. the environment) selects". Thus each soil is endowed with a stable community of microbes uniquely selected by and adapted to the prevailing physical, chemical, and biological conditions of that soil. Minor perturbations have little effect on this balance.

From the above discussion, one can see that there are many factors, both biotic and abiotic, that can come together to foil our attempts to use beneficial microbes in practical applications. It is because of these inconsistencies that biological alternatives are often met with reluctance by users. There is a greater comfort factor in using a chemical formulation that delivers more consistent results when applied as directed. However, as research progresses and we gain a clearer understanding of the characteristics that make an organisms successful in the soil or rhizosphere environment it is likely that we will see the development of useful microbial products for a number of purposes including increasing plant growth, protecting crops from disease, organisms for use in bioremediation or for enhancing the cleanup of pesticides in rinsates etc. However, one thing will be reasonably certain, those that come to the forefront will be based on sound biological principles and will be backed up by substantial research demonstrating the efficacy of the product in meeting the claims of the manufacturer.

Root-microbe communication is another important process that characterizes the underground zone. Some compounds identified in root exudates that have been shown to play an important role in root-microbe interactions include flavonoids present in the root exudates of legumes that activate *Rhizobium meliloti* genes responsible for the nodulation process. Although the studies are not yet conclusive, these compounds may also be responsible for vesicular-arbuscular mycorrhiza colonization. In contrast, survival of the delicate and physically unprotected root cells under continual attack by pathogenic microorganisms

depends on a continuous "underground chemical warfare" mediated by secretion of phytoalexins, defense proteins, and other as yet unknown chemicals.

The unexplored chemodiversity of root exudates is an obvious place to search for novel biologically active compounds, including antimicrobials. For instance, recently identified rosmarinic acid (RA) in the root exudates of hairy root cultures of sweet basil (*Ocimum basilicum*) elicited by fungal cell wall extracts from *Phytophthora cinnamoni*. Basil roots were also induced to exude RA by fungal in situ challenge with *Pythium ultimum*, and RA demonstrated potent antimicrobial activity against an array of soil-borne microorganisms including *Pseudomonas aeruginosa*. Similar studies by Brigham et al. (1999) with *Lithospermum erythrorhizon* hairy roots reported cell-specific production of pigmented naphthoquinones upon elicitation, and other biological activity against soil-borne bacteria and fungi. Given the observed antimicrobial activity of RA and naphthoquinones, these findings strongly suggest the importance of root exudates in defending the rhizosphere against pathogenic microorganisms.

14.10 LET US SUM UP

- Soil microbiology deals with microbial role in soil fertility by decomposing the dead organic matter into nutrients.
- Blue green algae – photosynthetic microbes that add small amounts of carbon to soil.
- Protozoa – unicellular soil organisms that decompose organic materials as well as consume large number of bacteria.
- Humus – the organic matter broken down into stable humic substances that resist further decomposition.
- Key mechanisms behind rhizosphere effect on soil organic matter decomposition – accelerated soil microbial turnover rate, transcription induced drying rewetting cycles.

14.11 LESSON END ACTIVITIES

Weigh a gram of soil and dilute in water. View under the microscope and try to enumerate the microbial count

Why do they use microbes for sulphur ore mining?

Enumerate the rhizospheric organisms in your garden.

Explain the concepts of soil air and water.

What are the benefits of organic matter?

Explain soil microbial balance.

14.12 POINTS FOR DISCUSSION

What are the benefits of soil microbes?

Explain the organic matter of soil.

Explain organic matter decomposition.

Explain in detail the factors affecting soil microbial population.

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LESSON - 15
AQUATIC AND AEROMICROBIOLOGY

Contents

15.0. AIMS AND OBJECTIVES

The chapter deals with the microbial ecology of the aquatic system.

15.1. INTRODUCTION

Microbial ecology is the relationship of microorganisms with one another and with their environment. It concerns the three major domains of life — Eukaryota, Archaea, and Bacteria — as well as viruses. Microorganisms, by their omnipresence, impact the entire biosphere. They are present in virtually all of our planet's environments, including some of the most extreme, from acidic lakes to the deepest ocean, and from frozen environments to hydrothermal vents.

15.2. AQUATIC MICROBIOLOGY

Microbial communities, including phytoplankton, protozoa, bacteria, archaea, fungi and virus, are by far the most abundant and the most taxonomic and genetically diverse group of organisms in marine pelagic ecosystems. Biological activity, biomass, production and remineralization in these systems are essentially microbial while higher trophic levels (crustaceans, fish, and mammals) play a minor role in quantitative terms.

15.3 MICROBIAL BIOMASS DETERMINATION IN AQUATIC SYSTEM

Attempts can be made to count the number of viable bacteria or **biomass** of various kinds in water (e.g. total coliforms, fecal coliforms, Pseudomonas species, etc.) These tests

are usually performed to count and identify pathogens or to indicate the possible presence of pathogens (e.g. coliform tests). However, they can also be used to count the viable bacteria, fungi, protozoa in water or sediment samples in efforts to understand the ecology and population dynamics of the microbial populations.

Other methods attempt to count the "total" (as opposed to "viable") numbers of bacteria and other microorganisms. These methods (such as the Direct Method of fluorescence staining with acridene orange stain) do not distinguish between living and dead microorganisms in most cases.

Yet other methods use a "surrogate" technique to estimate biomass. They would, for example, use the amount of chlorophyll as a measure of the presence of algal biomass in a water sample.

Direct methods: Membrane filtration of standard volumes onto 8 μM, 2 μM, 0.45 μM and 0.2 μM Millipore or Nucleopore filters followed by direct observation with or without staining. Ultraviolet fluorescence of chlorophyll can be used to detect and count algal cells and fluorescence staining methods (e.g. acridine orange, anilinonaphthalene sulfonic acid dyes, fluorescein isothiocyanate, etc.) can be used for bacteria and fungi. In these cases, a black filter is required. The black filters can be produced from the normal cellulose filters by dyeing the filter with a black dye such as Irgalan black or Dylon 44.

Other methods include the Most Probable Number (MPN) technique, viable counting procedures, and biochemical methods for estimating biomass such as Protein levels, ATP and adenylate charge, lipopolysaccharide (LPS), muramic acid, and chlorophyll. Chlorophyll concentration is particularly important in the aquatic habitat because of the occurrence of photosynthetic algae and bacteria. The chlorophyll can be determined spectrophotometrically or fluorometrically. Chlorophyll a is usually measured but the chlorophylls b and c can be assayed selectively at different wavelengths with a spectrophotometer.

15.4 MICROBIAL ACTIVITY IN AQUATIC SYSTEM

In many cases it is preferable to assay the **microbial activity** rather than the **biomass**. There are innumerable assays for microbial activity, but most rely on some measure of metabolic activity such as respiration, photosynthesis or biochemical pathway or product.
The main categories are:

1. Rate of increase in the number of colonies on media
2. Direct increase in numbers on membrane filters
3. Estimation of rates of multiplication in continuous culture systems
4. Measurement of metabolic activities such as photosynthesis, respiration, substrate utilization or product accumulation

15.4.1 RATE OF INCREASE IN THE NUMBER OF COLONIES ON MEDIA

Has the usual disadvantages associated with all viable counting procedures. It may be useful to follow populations during and after a specific treatment, especially in systems where the population is known or a pure culture is being investigated.

15.4.2 DIRECT INCREASE IN NUMBERS ON MEMBRANE FILTERS

Microorganisms are concentrated on a membrane filter and incubated in contact with natural water at the temperature of the environment. It has been shown that these microorganisms will maintain their growth rates for a short period of time under these conditions.

15.4.3 ESTIMATION OF RATES OF MULTIPLICATION IN CONTINUOUS CULTURE SYSTEMS

The growth rates or generation times of various microorganisms in water systems can be estimated using continuous culture systems. If a chemostat is operated at very slow growth rates, the growth rate of bacteria from natural waters can be estimated using those waters as the medium, even if the dilution rate of the chemostat is higher than their growth rates. This is done by measuring the washout rate of the bacteria and calculating the growth rate from the difference between this (the washout rate) and the dilution rate of the chemostat. Even if only very slow growth is occurring, the bacteria will wash out more slowly than the dilution rate.

15.4.4 MEASUREMENT OF METABOLIC ACTIVITIES

Metabolic activities such as photosynthesis, respiration, substrate utilization or product accumulation may also be used to determine the microbial activity.

A few examples of using metabolic activities of aquatic populations as indicators of activity are given below. Any metabolic activity can be used as a measure of activity it can be measured. Measurement of $^{14}CO_2$, Measurement of uptake of organic substrates, Measurement of CO_2 respired by heterotrophs in aquatic systems and Oxygen uptake measurements are some the methods used to determine the biological activity of microbes in aquatic system.

Many of the methods can be modified so that they only examine parts of the overall population. Any method which uses an added substrate can be modified to use a specific substrate for particular groups of organisms. For example, *Thiobacillus* sp. can be examined by adding 10 mg/L of thiosulphate to the sample and finding the rate of thiosulphate oxidation by iodometric titration methods. Control bottles of sterilized water would be used as the control. Hydrogen and methane oxidizing bacteria could be examined by adding the appropriate substrate.

15.5 AERO MICROBIOLOGY

Of all environments, air is the simplest one and it occurs in a single phase gas. The relative quantities of various gases in air, by volume percentage are nitrogen 78%, oxygen 21 %, argon 0.9%, carbon dioxide 0.03%, hydrogen 0.01 % and other gases in trace amounts. In addition to various gases, dust and condensed vapour may also be found in air.

Various layers can be recognized in the atmosphere upto a height of about 1000 km. The layer nearest to the earth is called as troposphere. In temperate regions, troposphere extends upto about 11 km whereas in tropics up to about 16 km. This troposphere is characterized by a heavy load of microorganisms.

The temperature of the atmosphere varies near the earth's surface. However, there is a steady decrease of about 1 DC per 150 m until the top of the troposphere. Above the troposphere, the temperature starts to increase.

The atmosphere as a habitat is characterised by high light intensities, extreme temperature variations, low amount of organic matter and a scarcity of available water making it a non hospitable environment for microorganisms and generally unsuitable habitat for their growth. Nevertheless, substantial numbers of microbes are found in the lower regions of the atmosphere.

15.5.1 SIGNIFICANCE OF AIR MICROFLORA

Although, when compared with the microorganisms of other environments, air microflora is very low in number, they play a very significant role. This is due to the fact that the air is in contact with almost all animate and inanimate objects. The significance of air flora has been studied since 1799, in which year Lazaro Spallanzani attempted to disprove spontaneous generation. In t 837, Theodore Schwann, in his experiment to support the view

of Spallanzani, introduced fresh heated air into a sterilized meat broth and demonstrated that microbial growth couldn't occur.

This formed the basis of modern-day forced aeration fermentations. It was Pasteur in 1861, which first showed that microorganisms could occur as airborne contaminants. He used special cotton in his air sampler onto which the microorganisms were deposited. He microscopically demonstrated the presence of microorganisms in the cotton. In his famous swan necked flask experiment, he showed that growth could not occur in sterile media unless airborne contamination had occurred.

No microbes are indigenous to the atmosphere rather they represent allochthonous populations transported from aquatic and terrestrial habitats into the atmosphere. Microbes of air within 300-1,000 or more feet of the earth's surface are the organisms of soil that have become attached to fragments of dried leaves, straw or dust particles, being blown away by the wind. Species vary greatly in their sensitivity to a given value of relative humidity, temperature and radiation exposures.

More microbes are found in air over land masses than far at sea. Spores of fungi, especially *Alternaria, Cladosporium, Penicillium* and *Aspergillus* are more numerous than other forms over sea within about 400 miles of land in both polar and tropical air masses at all altitudes up to about 10,000 feet.

Microbes found in air over populated land areas below altitude of 500 feet in clear weather include spores of *Bacillus* and *Clostridium*, ascospores of yeasts, fragments of myceilium and spores of molds and streptomycetaceae, pollen, protozoan cysts, algae, *Micrococcus*, *Corynebacterium* etc.

In the dust and air of schools and hospital wards or the rooms of persons suffering from infectious diseases, microbes such as *Tubercle bacilli, Streptococci, Pneumococci* and *Staphylococci* have been demonstrated.

These respiratory bacteria are dispersed in air in the droplets of saliva and mucus produced by coughing, sneezing, talking and laughing. Viruses of respiratory tract and some enteric tract are also transmitted by dust and air. Pathogens in dust are primarily derived from the objects contaminated with infectious secretions that after drying become infectious dust.

Droplets are usually formed by sneezing, coughing and talking. Each droplet consists of saliva and mucus and each may contain thousands of microbes. It has been estimated that the number of bacteria in a single sneeze may be between 10,000 and 1,00,000. Small droplets in a warm, dry atmosphere are dry before they reach the floor and thus quickly become droplet nuclei.

Many plant pathogens are also transported from one field to another through air and the spread of many fungal diseases of plants can be predicted by measuring the concentration of airborne fungal spores. Human bacterial pathogens which cause important airborne diseases such as diphtheria, meningitis, pneumonia, tuberculosis and whooping cough are described in the chapter "Bacterial Diseases of Man".

15.5.2. AIR MICROFLORA SIGNIFICANCE IN HOSPITALS

Although hospitals are the war fields for combating against diseases, there are certain occasions in which additional new infectious diseases can be acquired during hospitalization. Air within the hospital may act as a reservoir of pathogenic microorganisms which are transmitted by the patients.

Infection acquired during the hospitalization is called nosocomial infections and the pathogens involved are called as nosocomial pathogens. Infections, manifested by the corresponding symptoms, after three days of hospitalization can be regarded as nosocomial infection.

Nosocomial infection may arise in a hospital unit or may be brought in by the staff or patients admitted to the hospital.The common microorganisms associated with hospital infection are *Haemophilus influenzae, Streptococcus pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa,* members of Enterobacteriaceae and respiratory viruses. Development of high antibiotic resistance is a potential problem among nosocomial pathogens. For example, Methicillin Resistant *Staphylococcus aureus* (MRSA) and gentamicin resistant Gram-negative bacilli are of common occurrence. Even antiseptic liquids used would contain bacteria, for example *Pseudomonas,* due to their natural resistance to certain disinfectants and antiseptics and too many antibiotics.

Nosocomial pathogens may cause or spread hospital outbreaks. Nosocomial pneumonia is becoming a serious problem nowadays and a number of pathogens have been associated with it. Frequent agents are *Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa, Enterobacter, Klebsiella, Escherichia coli* and *Haemophilus influenzae*. Other less frequent agents are *enterococci, streptococci* other than *S. pneumoniae, Serratia marcescens, Citrobacter freundii, Acinetobacter sp.* and *Xanthomonas* sp.

In addition *Legionella, Chlamydia pneumoniae* and *Mycobacterium tuberculosis* have also been reported. Nosocomial transmissions of tuberculosis from patients to patients and from patients to health care workers have also been well documented.

There are two main routes of transmission for nosocomial pathogens, contact (either direct or indirect) and airborne spread. Airborne spread is less common than the spread by direct or indirect contact. It occurs by the following mechanisms. The source may be either from persons or from inanimate objects.

In case of spread from persons the droplets from mouth, skin scales from nose, skin exudates and infected lesion transmit diseases such as measles, tuberculosis, pneumonia, staphylococcal sepsis and streptococcal sepsis. Talking, coughing and sneezing produce droplets. Skin scales are shed during wound dressing or bed making.

In case of inanimate sources particles from respiratory equipment and air-conditioning plant may transmit diseases. These include Gram-negative respiratory infection, Legionnaire's disease and fungal infections.

15.5.3 FACTORS AFFECTING AIR MICROFLORA

A number of intrinsic and environmental factors influence the kinds and distribution of the microflora in air. Intrinsic factors include the nature and physiological state of microorganisms and also the state of suspension. Spores are relatively more abundant than the vegetative bacterial cells.

This is mainly due to the dormant nature of spores which enables them to tolerate unfavourable conditions like desiccation, lack of enough nutrients and ultraviolet radiation. Similarly fungal spores are abundant in the air since they are meant for the dispersal of fungi.

The size of the microorganisms is another factor that determines the period of time for which they remain suspended in air. Generally smaller microorganisms are easily liberated into the air and remain there for longer period. Fungal mycelia have a larger size and hence mainly fragments of mycelia will be present in air. The state of suspension plays an important role in the settling of microorganisms in air. Organisms in the free state are slightly heavier than air and settle out slowly in a quiet atmosphere. However, microorganisms suspended in air are only rarely found in the free state.

Usually they are attached to dust particles and saliva. Microorganisms embedded in dust particle settle out rapidly and in a quiet atmosphere they remain airborne only for a short period of time. Droplets which are discharged into the air by coughing or sneezing are also remain suspended in air for a short period of time. When their size decreases by evaporation they remain for a longer period in air.

Environmental factors that affect air microflora include atmospheric temperature, humidity, air current, the height at which the microorganisms are found etc. Temperature and relative humidity are the two important factors that determine the viability of microorganisms in aerosol. Studies with *Serratia marcesens* and *E. coli* show that the airborne survival is closely related to the temperature.

There is a progressive increase in the death rate with an increase in temperature from -18°C to 49°C. Viruses in aerosols show a similar behaviour. Particles of influenza, poliomyelitis and vaccinia viruses survive better at low temperature from 7 to 24°C.The optimum rate of relative humidity (RH) for the survival of most microorganisms is between 40 and 80 percent. Low and high relative humidity cause the death of most microorganisms. Almost all viruses survive better at a RH of 17 to 25 percent.

A notable exception is that of poliomyelitis which survives better at 80 to 81 percent. Survival has been found to be a function of both RH and temperature. At all temperatures, survival is best at the extremes of RH. Irrespective of RH, an increase in temperature leads to decrease in survival time.

Air current influences the time for which either the microorganisms or the particles laden with microorganisms remain suspended in air. In still air the particles tend to settle down. But a gentle air current can keep them in suspension for relatively long periods. Air current is also important in the dispersal of microorganisms as it carries them over a long distance.

Air currents also produce turbulence which causes a vertical distribution of air flora. Global weather patterns also influence the vertical distribution. High altitudes have a limiting effect on the air microflora. High altitudes are characterized by severe conditions like desiccation, ultraviolet radiation and low temperature. Only resistant forms like spores can survive these conditions. Thus high altitudes are characterized by the presence of spores and other resistant forms.

15.5.4 ENUMERATION OF MICROORGANISMS IN AIR

There are several methods, which require special devices, designed for the enumeration of microorganisms in air. The most important ones are solid and liquid impingement devices, filtration, sedimentation, centrifugation, electrostatic precipitation, etc.

However, none of these devices collects and counts all the microorganisms in the air sample tested. Some microbial cells are destroyed and some entirely pass through in all the processes.

Some of the methods are described below.

Impingement in liquids: In this method, the air drawn is through a very small opening or a capillary tube and bubbled through the liquid. The organisms get trapped in the liquid medium. Aliquots of the liquid are then plated to determine its microbial content. Aliquots of the broth are then plated to determine microbial content.

Impingement on solids: In this method, the microorganisms are collected, or impinged directly on the solid surface of agar medium. Colonies develop on the medium where the organism impinges. Several devices are used, of which the settling-plate technique is the simplest. In this method the cover of the Petridish containing an agar medium is removed, and the agar surface is exposed to the air for several minutes. A certain number of colonies develop on incubation of the Petridish.

Each colony represents particle carrying microorganisms. Since the technique does not record the volume of air actually sampled, it gives only a rough estimate. However, it does give information about the kind of microorganisms in a particular area. Techniques wherein a measured volume of air is sampled have also been developed. These are sieve and slit type devices.

A sieve device has a large number of small holes in a metal cover, under which is located a Petridish containing an agar medium. A measured volume of air is drawn, through these small holes. Airborne particles impinge upon the agar surface. The plates are incubated and the colonies counted. In a slit device the air is drawn through a very narrow slit onto a Petridish containing agar medium. The slit is approximately the length of the Petridish. The Petridish is rotated at a particular speed under the slit One complete turn is made during the sampling operation.

Filtration: The membrane filter devices are adaptable to direct collection of microorganisms by filtration of air. The method is similar in principle to that described for water sampling.

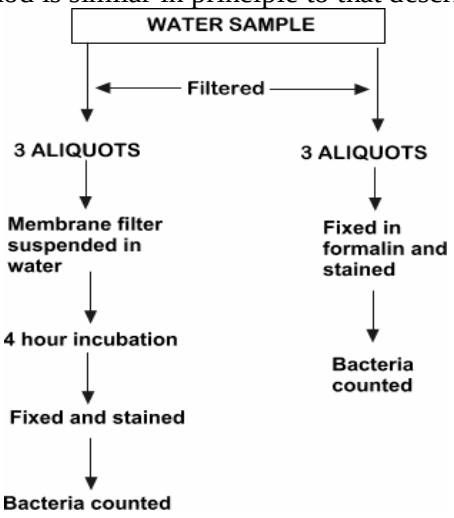

Fig.1. Enumeration of microbes in water

15.5.5 SOURCES OF MICROORGANISMS IN AIR

Although a number of microorganisms are present in air, it doesn't have an indigenous flora. Air is not a natural environment for microorganisms as it doesn't contain enough moisture and nutrients to support their growth and reproduction.

Quite a number of sources have been studied in this connection and almost all of them have been found to be responsible for the air microflora. One of the most common sources of air microflora is the soil.

Soil microorganisms when disturbed by the wind blow, liberated into the air and remain suspended there for a long period of time. Man made actions like digging or ploughing the soil may also release soilborne microbes into the air. Similarly microorganisms found in water may also be released into the air in the form of water droplets or aerosols. Splashing of water by wind action or tidal action may also produce droplets or aerosols.

Air currents may bring the microorganisms from plant or animal surfaces into air. These organisms may be either commensals or plant or animal pathogens. Studies show that plant pathogenic microorganisms are spread over very long distances through air. For example, spores of *Puccinia graminis* travel over a thousand kilometers. However, the transmission of animal diseases is not usually important in outside air.

The main source of airborne microorganisms is human beings. Their surface flora may be shed at times and may be disseminated into the air. Similarly, the commensal as well as pathogenic flora of the upper respiratory tract and the mouth are constantly discharged into the air by activities like coughing, sneezing, talking and laughing.

The microorganisms are discharged out in three different forms which are grouped on the basis of their relative size and moisture content. They are droplets, droplet nuclei and infectious dust. It was Wells, who described the formation of droplet nuclei. This initiated the

studies on the significance of airborne transmission. A brief description of these agents is given below.

Droplets - Droplets are usually formed by sneezing, coughing or talking. Each consists of saliva and mucus. Droplets may also contain hundreds of microorganisms which may be pathogenic if discharged from diseased persons. Pathogens will be mostly of respiratory tract origin. The size of the droplet determines the time period during which they can remain suspended.

Most droplets are relatively large, and they tend to settle rapidly in still air. When inhaled these droplets are trapped on the moist surfaces of the respiratory tract. Thus, the droplets containing pathogenic microorganisms may be a source of infectious disease.

Droplet Nuclei - Small droplets in a warm, dry atmosphere tend to evaporate rapidly and become droplet nuclei. Thus, the residue of solid material left after drying up of a droplet is known as droplet nuclei. These are small, 1-4μm, and light. They can remain suspended in air for hours or days, traveling long distances.

They may serve as a continuing source of infection if the bacteria remain viable when dry. Viability is determined by a set of complex factors including, the atmospheric conditions like humidity, sunlight and temperature, the size of the particles bearing the organisms, and the degree of susceptibility or resistance of the particular microbial species to the new physical environment.

Infectious Dust - Large aerosol droplets settle out rapidly from air on to various surfaces and get dried. Nasal and throat discharges from a patient can also contaminate surfaces and become dry. Disturbance of this dried material by bed making, handling a handkerchief having dried secretions or sweeping floors in the patient's room can generate dust particles which add microorganisms to the circulating air.

Microorganisms can survive for relatively longer periods in dust. This creates a significant hazard, especially in hospital areas. Infective dust can also be produced during laboratory practices like opening the containers of freeze dried cultures or withdrawal of cotton plugs that have dried after being wetted by culture fluids. These pose a threat to the people working in laboratories.

15.5.6 MICROBES FOUND IN AIR

In addition to gases, dust particles and water vapour, air also contains microorganisms. There are vegetative cells and spores of bacteria, fungi and algae, viruses and protozoan cysts. Since air is often exposed to sunlight, it has a higher temperature and less moisture. So, if not protected from desiccation, most of these microbial forms will die.

Air is mainly it transport or dispersal medium for microorganisms. They occur in relatively small numbers in air when compared with soil or water. The micro flora of air can be studied under two headings outdoor and indoor micro flora.

15.6. LET US SUM UP

- Two main routes of transmission of nosocomial pathogens are contact and air borne.
- MRSA – methicillin resistant *Staphylococcus aureus*
- Enumeration of microorganisms – impingement in liquids, impingement on solids, filtration

15.7. LESSON END ACTIVITIES

Prepare nutrient plates and check the sterility of the lab
Enumerate the aerobiology in your area.
What are the factors affecting air microflora?
Explain the significance of air microflora in hospital area.
Explain the measurement of metabolic activities.
Explain the microbial biomass determination in aquatic system.

15.8. POINTS FOR DISCUSSION

Explain in detail the microbial activity in aquatic system.
What are the factors affecting air microflora?
Explain in detail the techniques involved in enumeration of microorganisms in air.

15.9 REFERENCES

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LESSON – 16

INFLUENCES OF FACTORS ON MICROBIAL PHYSIOLOGY

Contents

16.0 AIMS AND OBJECTIVES

The chapter deals with the influences of factors on microbial physiology.

16.1 INTRODUCTION

Climatic factors are very important for the growth of microorganisms. These factors influence the growth of microorganisms. Some of the factors that influence growth are chemical and physical factors.

16.2 INFLUENCE OF CHEMICAL FACTORS ON MICROBIAL GROWTH

Although organisms can be grouped based on their relationship to molecular oxygen, they can also be grouped in relation to their nutritional requirements.

Element	Dryweight(%)	Source	Function
Carbon	50	organic compounds or CO_2	Main constituent of cellular material
Oxygen	20	H_2O, organic compounds, CO_2, and O_2	Constituent of cell material and cell water; O_2 is electron acceptor in aerobic respiration
Nitrogen	14	NH_3, NO_3, organic compounds, N_2	Constituent of amino acids, nucleic acids nucleotides, and coenzymes
Hydrogen	8	H_2O, organic compounds, H_2	Main constituent of organic compounds and cell water
Phosphorus	3	inorganic phosphates (PO_4)	Constituent of nucleic acids, nucleotides, phospholipids, LPS, teichoic acids
Sulfur	1	SO_4, H_2S, S^o, organic sulfur compounds	Constituent of cysteine, methionine, glutathione, several coenzymes
Potassium	1	Potassium salts	Main cellular inorganic cation and cofactor for certain enzymes
Magnesium	0.5	Magnesium salts	Inorganic cellular cation, cofactor for certain enzymatic reactions
Calcium	0.5	Calcium salts	Inorganic cellular cation, cofactor for certain enzymes and a component of endospores
Iron	0.2	Iron salts	Component of cytochromes and certain nonheme iron-proteins and a cofactor for some enzymatic reactions

Table no. 1 **MAJOR ELEMENT, THEIR SOURCES AND FUNCTIONS IN BACTERIAL CELLS**

Broadly two groups are recognized based on the nutritional requirements.

(i) Autotrophs - which can utilize, inorganic nutrients or light as a source of energy, e.g. nitrifying bacteria, algae, etc., and

(ii) Heterotrophs - which require organic compounds as a source of energy, e.g. many bacteria, fungi and protozoa. Although this classification is relatively simple it does not allow us to distinguish organisms based on their energy requirement and the principal carbon source. Currently microorganisms are grouped into four major groups based on the above parameters.

1. **Photoautotrophs -** which use light as an energy source and CO_2 as the carbon source e.g. algae and many photosynthetic bacteria
2. **Photoheterotrophs-** which use light as the energy source and reduced organic compounds as a carbon source. Example is purple non sulphur Bacteria.
3. **Chemoautorophs** - which use inorganic chemicals as a source and CO_2 as a principal carbon source. These organisms obtain their energy by the oxidation of reduced inorganic compounds such as NH_3, NO'_2, H_2, H_2S and Fe^{++}. These organisms are also called chemolithothrophs since these can grow in an inorganic medium in the absence of light.

4. Chemoheterotrophs - which use organic compounds as a source as well as a principal carbon source. The clear distinction between the energy source and the carbon source, which is characteristic of the above three groups is lost since this group can derive both the carbon and energy from a single organic compound. This nutritional classification is arbitrary since some organisms classified as photo. Heterotrophs can also grow in dark as chemoheterotrophs. Thus chemoheterotrophy is an alternate nutritional mode of certain photo heterotrophs and chemoautotrophs.

Type	**Energy Source**	**Carbon Source**	**Examples**
Photolithotrophs	Light	CO_2	Algae, Purple sulphur bacteria, green sulphur bacteria
Photoorganotrophs	Light	Organic Compounds	Purple non sulphur bacteria
Chemolithotrophs	Oxidation of inorganic compounds		Nitrifying bacteria, iron bacteria, H_2 bacteria
Chemoorganotrophs	Oxidation of organic compounds	Organic Compounds	Most bacteria, fungi, protozoa

Table 2 **CLASSIFICATIONS OF HETEROTROPHIC MICROBES**

The main building elements of organisms are carbon, hydrogen, oxygen, nitrogen, sulphur and phosphorus. Growth of an organism involves the conversion of these elements present in an inorganic form to the organic compound that make up the living matter. The energy for this elemental conversion is ultimately derived from solar sources of photosynthesis.

If this were the only process, life would soon cease as the inorganic forms of elements, particularly carbon and nitrogen would be locked into organic matter. In fact, the reverse process mineralisation must also occur and is brought about by the activity of living organisms so that cycles of elements and matter occur. The situation is complicated by the existence in nature of oxidised and reduced states of most of the essential elements; organisms may only be able to use one or other form and further cycles therefore exist between them.

The microbes interconvert these compounds for their own growth and each process is energy consuming. While the different elements are cycled, energy flows through the ecosystem and ultimately lost as heat. Let us consider in some details as how the major elements undergo cyclic changes in nature.

16.2.1 WATER REQUIREMENT

Cells require certain amount of free water to be able to carry out metabolism. **Water activity** ($\mathbf{a_w}$) is used as a quantitative measurement of the availability of water. Microorganisms are separated from their environment by a selectively permeable Plasma Membrane. They are affected by changes in **osmotic concentration** of their surroundings. The availability of water is inversely related to osmotic pressure.

Osmosis is the movement of water into a cell by simple diffusion; occurs from regions of high water concentration to regions of low water concentration.

A **hypotonic solution** is one that has a ***lower*** **solute** concentration than the cell's cytoplasm.If the cell is placed in a hypotonic solution→water enters cell→cell bursts.

A **hypertonic solution** is one that has a ***higher*** **solute** concentration than the cell's cytoplasm.If the cell is placed in a hypertonic solution→water out of cell→ cytoplasm shrink and pulls out of cell (phenomenon called **plasmolysis**).

Osmotolerant organisms can grow in solutions of both high and low water activity. **Halophiles** require environments of low water activity (high osmotic pressure) in order to grow. Ex. *Halobacterium halobium* grows in Dead Sea, Great Salt Lake, evaporating salt flats. Microorganisms growing in a habitat with low water activity (aw) usually maintain a high internal solute concentration in order to retain water.

16.2.2 CARBON REQUIREMENT

Carbon dioxide, either in the atmosphere or in solution in surface waters is the major source of carbon for living organisms. It is converted to organic form by the autotrophs which use it as sole carbon source. The most important in this conversion are the photosynthetic autotrophs (seed plants on land and algae in water) that carry out oxygen producing type of photosynthesis. Photosynthetic and chemosynthetic autotrophs, chiefly bacteria also play role in this conversion.

A small part of the inorganic carbon is also present in the reduced form of methane, in some specific habitat conditions. Methane can be utilised as the sole source of carbon and energy by a special group of aerobic bacteria, methane utilising bacteria which convert it into organic carbon. The total CO_2 in the atmosphere would be completely exhausted with this rate of conversion of inorganic to organic carbon.

However, the reverse process of mineralisation of organic to inorganic carbon by the activity of heterotrophs prevents this exhaustion. The major end product of mineralisation is CO_2, though some methanogenic bacteria also produce CH_4 by anaerobic respiration and fermentation. The conversion of inorganic to organic carbon by plants and by autotrophic microbes is relatively straightforward.

16.2.2.1 ROLE OF MICROBES IN CARBON CYCLE

The carbon cycle is the biogeochemical cycle by which carbon is exchanged between the biosphere, geosphere, hydrosphere, and atmosphere of the Earth.

The cycle is usually thought of as four major reservoirs of carbon interconnected by pathways of exchange. The reservoirs are the atmosphere, the terrestrial biosphere (which usually includes freshwater systems and non-living organic material, such as soil carbon), the oceans (which includes dissolved inorganic carbon and living and non-living marine biota), and the sediments (which includes fossil fuels). The annual movements of carbon, the carbon exchanges between reservoirs, occur because of various chemical, physical, geological, and biological processes. The ocean contains the largest active pool of carbon near the surface of the Earth, but the deep ocean part of this pool does not rapidly exchange with the atmosphere.

The global carbon budget is the balance of the exchanges (incomes and losses) of carbon between the carbon reservoirs or between one specific loop (e.g., atmosphere - biosphere) of the carbon cycle. An examination of the carbon budget of a pool or reservoir can provide information about whether the pool or reservoir is functioning as a source or sink for carbon dioxide.

In the atmosphere Carbon exists primarily as the gas carbon dioxide (CO_2). Although it is a very small part of the atmosphere overall (approximately 0.04% on a molar basis, though rising), it plays an important role in supporting life. Other gases containing carbon in the atmosphere are methane and chlorofluorocarbons (the latter is entirely anthropogenic). The overall atmospheric concentration of these greenhouse gases has been increasing in recent decades, contributing to global warming.

Carbon is taken from the atmosphere in several ways:

- When the sun is shining, plants perform photosynthesis to convert carbon dioxide into carbohydrates, releasing oxygen in the process. This process is most prolific in relatively new forests where tree growth is still rapid.

- At the surface of the oceans towards the poles, seawater becomes cooler and more carbonic acid is formed as CO2 becomes more soluble. This is coupled to the ocean's thermohaline circulation which transports dense surface water into the ocean's interior (see the entry on the solubility pump).
- In upper ocean areas of high biological productivity, organisms convert reduced carbon to tissues, or carbonates to hard body parts such as shells and tests. These are, respectively, oxidized (soft-tissue pump) and redissolved (carbonate pump) at lower average levels of the ocean than those at which they formed, resulting in a downward flow of carbon (see entry on the biological pump).
- The weathering of silicate rock. Carbonic acid reacts with weathered rock to produce bicarbonate ions. The bicarbonate ions produced are carried to the ocean, where they are used to make marine carbonates. Unlike dissolved CO_2 in equilibrium or tissues which decay, weathering does not move the carbon into a reservoir from which it can readily return to the atmosphere.

Carbon can be released back into the atmosphere in many different ways,

- Through the respiration performed by plants and animals. This is an exothermic reaction and it involves the breaking down of glucose (or other organic molecules) into carbon dioxide and water.
- Through the decay of animal and plant matter. Fungi and bacteria break down the carbon compounds in dead animals and plants and convert the carbon to carbon dioxide if oxygen is present, or methane if not.
- Through combustion of organic material which oxidizes the carbon it contains, producing carbon dioxide (and other things, like water vapor). Burning fossil fuels such as coal, petroleum products, and natural gas releases carbon that has been stored in the geosphere for millions of years.
- Production of cement. Carbon dioxide is released when limestone (calcium carbonate) is heated to produce lime (calcium oxide), a component of cement.
- At the surface of the oceans where the water becomes warmer, dissolved carbon dioxide is released back into the atmosphere
- Volcanic eruptions and metamorphism release gases into the atmosphere. These gases include water vapor, carbon dioxide and sulfur dioxide. The carbon dioxide released is roughly equal to the amount removed by silicate weathering; so the two processes, which are the chemical reverse of each other, sum to roughly zero, and do not affect the level of atmospheric carbon dioxide on time scales of less than about 100,000 yr.

In the biosphere around 1,900 gigatons of carbon are present. Carbon is an essential part of life on Earth. It plays an important role in the structure, biochemistry, and nutrition of all living cells.

Autotrophs are organisms that produce their own organic compounds using carbon dioxide from the air or water in which they live. To do this they require an external source of energy. Almost all autotrophs use solar radiation to provide this, and their production process is called photosynthesis. A small number of autotrophs exploit chemical energy sources in a process called chemosynthesis. The most important autotrophs for the carbon cycle are trees in forests on land and phytoplankton in the Earth's oceans. Photosynthesis follows the reaction $6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$

Carbon is transferred within the biosphere as heterotrophs feed on other organisms or their parts (e.g., fruits). This includes the uptake of dead organic material (detritus) by fungi and bacteria for fermentation or decay.

Most carbon leaves the biosphere through respiration. When oxygen is present, aerobic respiration occurs, which releases carbon dioxide into the surrounding air or water, following the reaction $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$. Otherwise, anaerobic respiration

occurs and releases methane into the surrounding environment, which eventually makes its way into the atmosphere or hydrosphere (e.g., as marsh gas or flatulence).

Burning of biomass (e.g. forest fires, wood used for heating, anything else organic) can also transfer substantial amounts of carbon to the atmosphere

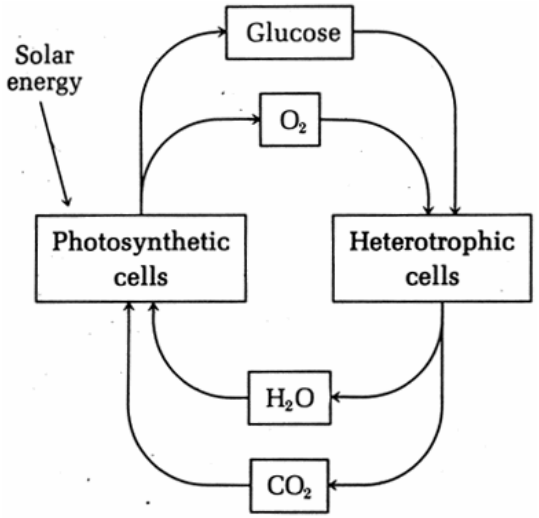


Fig. 1Carbon oxygen cycle

Carbon may also be circulated within the biosphere when dead organic matter (such as peat) becomes incorporated in the geosphere. Animal shells of calcium carbonate, in particular, may eventually become limestone through the process of sedimentation.

Much remains to be learned about the cycling of carbon in the deep ocean. For example, a recent discovery is that larvacean mucus houses (commonly known as "sinkers") are created in such large numbers that they can deliver as much carbon to the deep ocean as has been previously detected by sediment traps. Because of their size and composition, these houses are rarely collected in such traps, so most biogeochemical analyses have erroneously ignored them.

Carbon storage in the biosphere is influenced by a number of processes on different time-scales: while net primary productivity follows a diurnal and seasonal cycle, carbon can be stored up to several hundreds of years in trees and up to thousands of years in soils. Changes in those long term carbon pools (e.g. through de- or afforestation or through temperature-related changes in soil respiration) will thus directly affect global warming.

The seas contain around 36,000 gigatonnes of carbon, mostly in the form of bicarbonate ion. Inorganic carbon, that is carbon compounds with no carbon-carbon or carbon-hydrogen bonds, is important in its reactions within water. This carbon exchange becomes important in controlling pH in the ocean and can also vary as a source or sink for carbon. Carbon is readily exchanged between the atmosphere and ocean. In regions of oceanic upwelling, carbon is released to the atmosphere. Conversely, regions of downwelling transfer carbon (CO_2) from the atmosphere to the ocean. When CO_2 enters the ocean, carbonic acid is formed:

$$CO_2 + H_2O \rightarrow H_2CO_3$$

This reaction has a forward and reverse rate that is it achieves a chemical equilibrium. Another reaction important in controlling oceanic pH levels is the release of hydrogen ions and bicarbonate. This reaction controls large changes in pH:

$$H_2CO_3 \rightarrow H^+ + HCO_3^-$$

Thus carbon is cycled from atmosphere through biosphere to oceans.

16.2.3 NITROGEN REQUIREMENT OF MICROORGANISM

Nitrogen and sulphur axe taken up as NO_3 and SO_4 by most organisms and are subsequently reduced within the cell and utilized in other biosynthetic processes. Some organisms can also use reduced inorganic nitrogen (such as ammonium salts) directly. The nitrogen and sulphur requirements of most organisms can also be met by organic nutrients that contain these two elements in reduced organic combinations such as amino acids. A few organisms can reduce elemental nitrogen to ammonia and this process of nitrogen assimilation is known as biological nitrogen fixation.

In nature **nitrogen** is present in organic form as proteins, and in many inorganic states, the most reduced being NH_3^- and the most oxidised. In atmosphere, there is abundant molecular nitrogen.

There is some nitrogen fixing microbes in nature. Fixation of atmospheric nitrogen requires high energy input. Only prokaryotes are capable of biological nitrogen fixation. The most important group is the rhizobia that form root nodules in leguminous plants. There is some specificity between the bacterial strain and the legume host. Through cross fertilisation the nodulation of specific crops could be increased by inoculation of seeds with appropriate *Rhizobjum* strains. Other associations are also of agricultural value, particularly root nodules in nonlegumes by actinomycetes (*Frankia*) and biological nitrogen fixation by cyanobacteria in paddy fields.

Nitrogen fixation is a rate limiting step of the nitrogen cycle, summarized. There should be an efficient cycling between organic and inorganic forms of nitrogen. Inorganic nitrogen in the form of NH_3 or NO) is converted to organic form mainly by the plants and microorganisms. NO) nitrate is reduced first to NH_3. Microbes differ in their ability to use an inorganic source of nitrogen. The organic nitrogenous compounds are converted to ammonia mainly by the microorganisms.

- **ROLE OF MICROBES IN NITROGEN CYCLE**

All life requires nitrogen-compounds, e.g., proteins and nucleic acids. Air, which is 79% nitrogen gas (N_2), is the major reservoir of nitrogen. But most organisms cannot use nitrogen in this form. Plants must secure their nitrogen in "fixed" form, i.e., incorporated in compounds such as:

- nitrate ions (NO_3^-)
- ammonia (NH_3)
- urea $(NH_2)_2CO$

Animals secure their nitrogen (and all other) compounds from plants (or animals that have fed on plants).

Four processes participate in the cycling of nitrogen through the biosphere.

- nitrogen fixation
- decay
- nitrification
- denitrification

Microorganisms play major roles in all four of these.

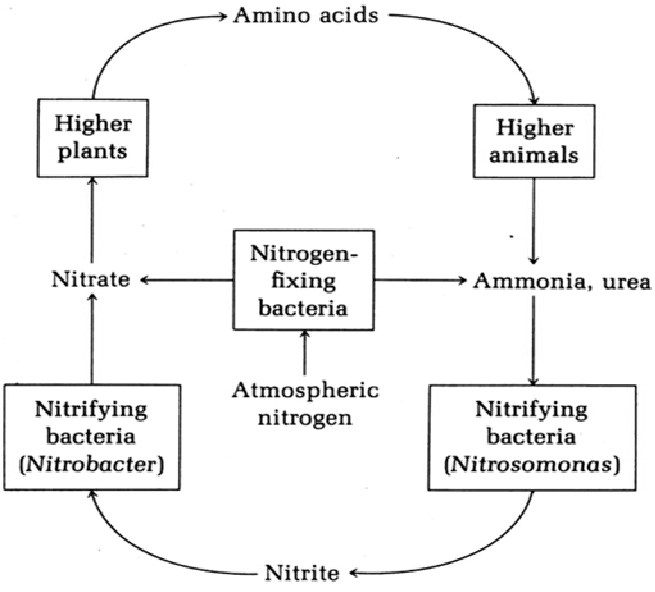


Fig. 2Nitrogen cycle

16.2.4 NITROGEN FIXATION

The nitrogen molecule (N_2) is quite inert. To break it apart so that its atoms can combine with other atoms requires the input of substantial amounts of energy. Three processes are responsible for most of the nitrogen fixation in the biosphere:

- **atmospheric fixation** by lightning
- **biological fixation** by certain microbes — alone or in a symbiotic relationship with some plants and animals
- **industrial fixation**

Atmospheric Fixation: The enormous energy of lightning breaks nitrogen molecules and enables their atoms to combine with oxygen in the air forming nitrogen oxides. These dissolve in rain, forming nitrates that are carried to the earth. Atmospheric nitrogen fixation probably contributes some 5– 8% of the total nitrogen fixed.

Industrial Fixation: Under great pressure, at a temperature of 600°C, and with the use of a catalyst, atmospheric nitrogen and hydrogen (usually derived from natural gas or petroleum) can be combined to form ammonia (NH_3). Ammonia can be used directly as fertilizer, but most of it is further processed to urea and ammonium nitrate (NH_4NO_3).

Biological Fixation: The ability to fix nitrogen is found only in certain bacteria and archaea.

- Some live in a symbiotic relationship with plants of the legume family (e.g., soybeans, alfalfa).
- Some establish symbiotic relationships with plants other than legumes (e.g., alders).
- Some establish symbiotic relationships with animals, e.g., termites and "shipworms" (wood-eating bivalves).
- Some nitrogen-fixing bacteria live free in the soil.
- Nitrogen-fixing *cyanobacteria* are essential to maintaining the fertility of semi-aquatic environments like rice paddies.

Biological nitrogen fixation requires a complex set of enzymes and a huge expenditure of ATP. Although the first stable product of the process is ammonia, this is quickly incorporated into protein and other organic nitrogen compounds.

The proteins made by plants enter and pass through food webs just as carbohydrates do. At each trophic level, their metabolism produces organic nitrogen compounds that return to the environment, chiefly in excretions. The final beneficiaries of these materials are microorganisms of decay. They break down the molecules in excretions and dead organisms into **ammonia**.

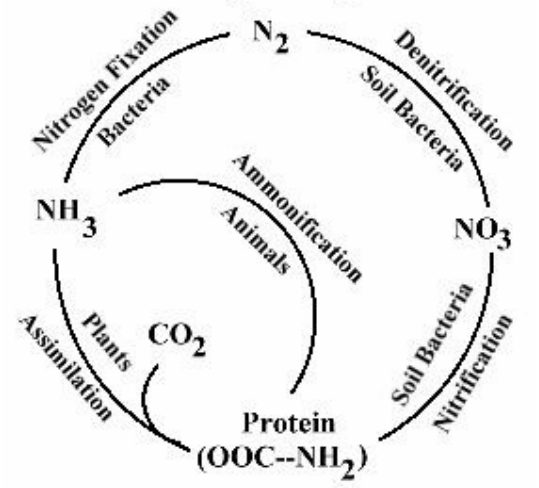


Fig.3 Role of microbes in nitrogen cycle

16.2.5 NITRIFICATION

Ammonia can be taken up directly by plants — usually through their roots. However, most of the ammonia produced by decay is converted into **nitrates**. This is accomplished in two steps:

- Bacteria of the genus ***Nitrosomonas*** oxidize NH_3 to **nitrites** (NO_2^-).
- Bacteria of the genus ***Nitrobacter*** oxidize the nitrites to **nitrates** (NO_3^-).

These two groups of autotrophic bacteria are called nitrifying bacteria. Through their activities (which supply them with all their energy needs), nitrogen is made available to the roots of plants.

Many soils also contain archaeal microbes, assigned to the *Crenarchaeota*, that convert ammonia to nitrites. While more abundant than the nitrifying bacteria, it remains to be seen whether they play as important a role in the nitrogen cycle.

Many legumes, in addition to fixing atmospheric nitrogen, also perform nitrification — converting some of their organic nitrogen to nitrites and nitrates. These reach the soil when they shed their leaves.

16.2.6 DENITRIFICATION

The three processes above remove nitrogen from the atmosphere and pass it through ecosystems. Denitrification reduces nitrates to nitrogen gas, thus replenishing the atmosphere. Once again, bacteria are the agents. They live deep in soil and in aquatic sediments where conditions are anaerobic. They use nitrates as an alternative to oxygen for the final electron acceptor in their respiration. Thus they close the nitrogen cycle.

16.2.7 SULPHUR REQUIREMENT OF MICROBES

Sulphur, like nitrogen is also present in organic and inorganic forms and can also be deficient in soils. Sulphate is the major forms assimilated by microbes and plants. This is reduced in the cell before incorporation into proteins and other organic compounds.

Mineralisation of these organic compounds results in the production of sulphate, or under anaerobic conditions H_2S. H_2S can also be formed anaerobically by dissimilatory reduction of sulphate.

This process is carried out by sulphate reducing bacteria -*Desulphovibrio*, *Desulphomonas* and *Desulpho- maculum*. This process again exhibits the links between different nutrient cycles as sulphate reduction accounts for up to 50% of the carbon mineralised in marine sediments. This process gives rise to the black odor to muds and sediments of estuaries, the color developing from precipitation of ferrous sulphide. Sulphide is believed to be involved in corrosion of mild steel.

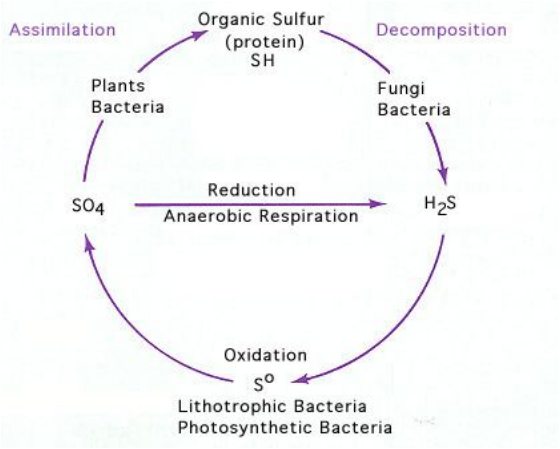


Fig.4 Sulphur cycle

16.2.8 ROLE OF MICROBES IN SULPHUR CYCLE

The environmental sulphur cycle involves many physical, chemical and biological agents. In mineral form sulphur may be present as sulphides (e.g. pyrite, FeS_2, chalcopyrite, FeS.CuS, pyrrhotite, FeS) and/or sulphates (e.g. gypsum, $CaSO_4.2H_2O$, barite, $BaSO_4$). Sulphur in minerals may move through the cycle as a result of the oxidation of sulphides to sulphate and/or the dissolution of sulphates. For example, oxidation of pyrite to sulphuric acid may be immediately followed, in situ, by acid neutralization by calcium carbonate (calcite) to form calcium sulphate (gypsum). The reaction of hydrogen sulphide with dissolved metal ions may precipitate metallic sulphides which are chemically indistinguishable from naturally occurring sulphide minerals.

At some mines, sulphur is added to the cycle as sulphur dioxide in processes such as the Inco/SO_2 process for cyanide destruction in the treatment of tailings. This added sulphur is oxidized to sulphate ion, most of which remains free, but some of which combines with lime, CaO, in the tailings to form gypsum.

Micro-organisms (most frequently bacteria) are often integrally involved in the chemical alteration of minerals. Minerals, or intermediate products of their decomposition, may be directly or indirectly necessary to their metabolism. The dissolution of sulphide minerals under acidic conditions (ARD), the precipitation of minerals under anaerobic conditions, the adsorption of metals by bacteria or algae, and the formation and destruction of organometallic complexes are all examples of indirect micro-organism participation. Where minerals are available as soluble trace elements, serve as specific oxidizing substrates, or are electron donors/acceptors in oxidation-reduction reactions, they may be directly involved in cell metabolic activity.

There are three categories of oxidation-reduction reactions for minerals with micro-organisms:

- Oxidation by autotrophic (cell carbon from carbon dioxide) or mixotrophic (cell carbon from carbon dioxide or organic matter) organisms. Energy derived from the oxidation reaction is utilized in cell synthesis.
- Electron acceptance by minerals (reduction) for heterotrophic (cell carbon from organic matter) and mixotrophic bacteria. Chemical energy is used to create new cell material from an organic substrate.
- Electron donation by minerals (oxidation) for bacterial or algal photosynthesis (reaction is fuelled by photon energy).

Oxidation of sulphur or sulphides for energy production is restricted to the bacterial genus *Thiobacillus,* the genus *Thiomicrospira*, and the genus *Sulfolobus*. These bacteria all produce sulphuric acid (i.e. hydrogen ions, H^+, and sulphate ions, SO_4^{--}) as a metabolic product.

It is these bacteria that are known to accelerate the generation of Acid Rock Drainage (ARD) from pyritic and pyrrhotitic rocks under suitable conditions. The bacteria develop flagella only if they are required for mobility in accessing energy sources.

ARD is the product formed by the atmospheric (i.e. by water, oxygen and carbon dioxide) oxidation of the relatively common iron-sulphur minerals pyrite and pyrrhotite in the presence of (catalysed by) bacteria (*Thiobacillus ferrooxidans*), and any other products generated as a consequence of these oxidation reactions.

An important reaction involving *T. ferrooxidans* is the oxidation of ferrous to ferric iron (Fe^{++} to Fe^{+++})

$$4Fe^{++} + O_2 + 4H^+ = Fe^{+++} + 2H_2O$$

Ferric iron is a powerful oxidizing agent. Even at a Fe^{+++}/Fe^{++} ratio of 1:1,000,000, a Redox potential of greater than +0.4 V is generated which is sufficient for the attack of most base metal sulphides. The general equation for the ferric ion reaction with base metal sulphides is:

$$MS + nFe^{+++} = Mn^+ + S + nFe^{++}$$

Consequently *T. ferrooxidans*, in generating Fe^{+++}, is indirectly responsible for the dissolution of base metal sulphide minerals and the mobilization of metallic cations such as Cu^{++}, Zn^{++}, Pb^{++} and Cd^{++}. Base metal sulphides react only very slowly with sulphuric acid in the absence of ferric iron.

In general, for substantial metal mobilization from base metal sulphides the following conditions must be met:

- Ferric iron for sulphide oxidation
- *T. ferrooxidans* and oxygen for ferrous to ferric oxidation
- pH compatible with *T. ferrooxidans* habitat requirements, typically pH 1.5-3.5

The typical habitat pH of *T. ferrooxidans* of 1.5 to 3.5 is not one that develops spontaneously. It is currently believed that these conditions are produced by a consortium of bacteria acting in succession. Such a succession may include *T. thioparus* at neutral pH, giving way to dominance by *metallogenium* bacteria under mildly acid conditions (pH 3.5 to 4.5), and finally *T. ferrooxidans* dominance at low pH.

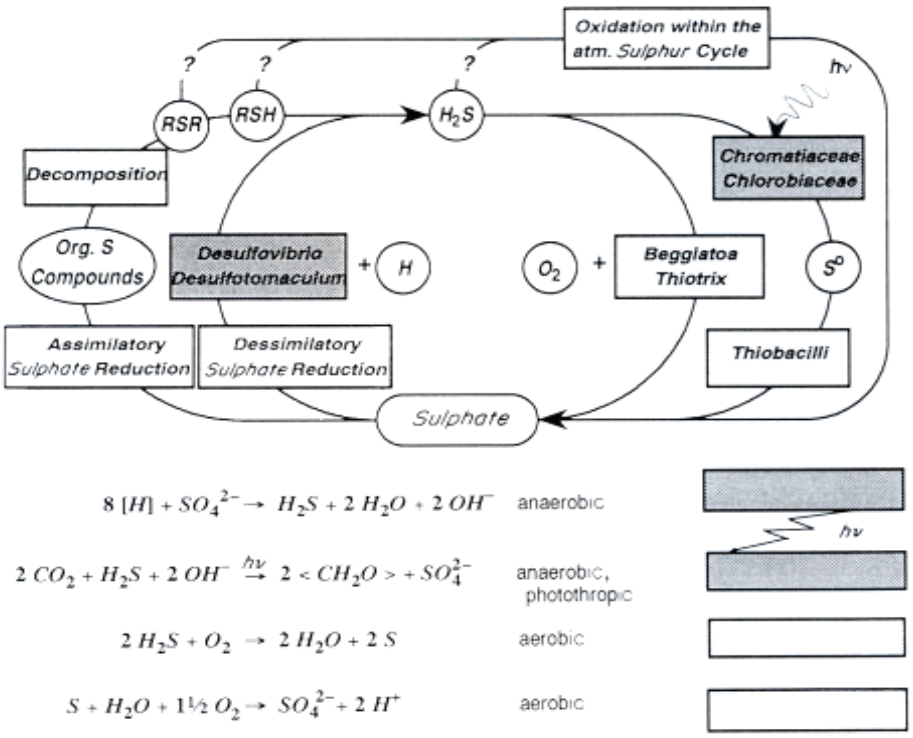


Fig. 5Microbial role in sulphur cycle

- **NATURAL REDUCTION IN THE SULPHUR CYCLE**

The direct reduction of sulphate ions to hydrogen sulphide is effected in nature by specialized, strictly anaerobic bacteria of the genera *Desulfovibrio* and *Desulfotomaculum*.

These sulphate reducing bacteria (SRB) are heterotrophic (cell carbon from organic compounds) organisms that utilize sulphate, thiosulphate, $S_2O_3^{--}$, sulphite, SO_3^{--}, or other reducible sulphur-containing ions as terminal electron acceptors in their respiratory metabolism. In the process these sulphur-containing ions are reduced to hydrogen sulphide.

The bacteria require an organic substrate which is usually a short chain acid such as lactic or pyruvic acid. In nature such substrates are generated by the fermentation activities of other anaerobic bacteria on more complex organic substrates. Thus in natural systems, the specific requirement for a short chain acid by the SRB are met by the availability of a complex organic source and a mixed bacterial system. Lactate is used by the SRB during anaerobic respiration to produce acetate according to the reaction:

$$2CH_3CHOHCOO^- + SO_4^{--} = 2CH_3COO^- + 2HCO_3^- + H_2S$$

This is the major natural process for the conversion (destruction) of sulphate ion. However, the process may be adapted to a controlled engineering process by the use of anaerobic reaction vessels and carbon monoxide, CO, and hydrogen, H_2, or partially oxidized propane or natural gas, as the energy source for the bacteria.

- **OTHER MICRO-ORGANISM REACTIONS IN THE SULPHUR CYCLE**

Sulphate ion is taken up from soil by plants, which incorporate it into protein, and plant protein is consumed by animals that convert plant protein to animal protein. Death of plants and animals allows bacterial decomposition of protein in remains to produce hydrogen sulphide and other products, in processes involving many fungi, actinomycetes and bacteria such as the heterotroph *Proteus vulgaris*.

Some bacteria can function in the transition zone between aerobic and anaerobic environments. Hydrogen sulphide may be oxidized to sulphur by such bacteria which deposit elemental sulphur in their cells while using oxygen as the terminal electron acceptor.

Hydrogen sulphide may also be oxidized to sulphate photosynthetically by the bacteria, *Chromtiacceae* and *Chlorobiaceae.*

16.3. OXYGEN REQUIREMENT OF MICROBES

The response of an organism to O_2 in its environment depends upon the occurrence and distribution of various enzymes which react with O_2 and various oxygen radicals that are invariably generated by cells in the presence of O_2. All cells contain enzymes capable of reacting with O_2. For example, oxidations of flavoproteins by O_2 invariably result in the formation of H_2O_2 (peroxide) as one major product and small quantities of an even more toxic free radical, superoxide or $O_2^{\cdot-}$. Also, chlorophyll and other pigments in cells can react with O_2 in the presence of light and generate singlet oxygen, another radical form of oxygen which is a potent oxidizing agent in biological systems.

In aerobes and aerotolerant anaerobes the potential for lethal accumulation of superoxide is prevented by the enzyme superoxide dismutase. All organisms which can live in the presence of O_2 (whether or not they utilize it in their metabolism) contain superoxide dismutase. Nearly all organisms contain the enzyme catalase, which decomposes H_2O_2. Even though certain aerotolerant bacteria such as the lactic acid bacteria lack catalase, they decompose H_2O_2 by means of peroxidase enzymes which derive electrons from $NADH_2$ to reduce peroxide to H_2O. Obligate anaerobes lack superoxide dismutase and catalase and/or peroxidase, and therefore undergo lethal oxidations by various oxygen radicals when they are exposed to O_2.

All photosynthetic (and some nonphotosynthetic) organisms are protected from lethal oxidations of singlet oxygen by their possession of carotenoid pigments which physically react with the singlet oxygen radical and lower it to its nontoxic "ground" (triplet) state. Carotenoids are said to "quench" singlet oxygen radicals.

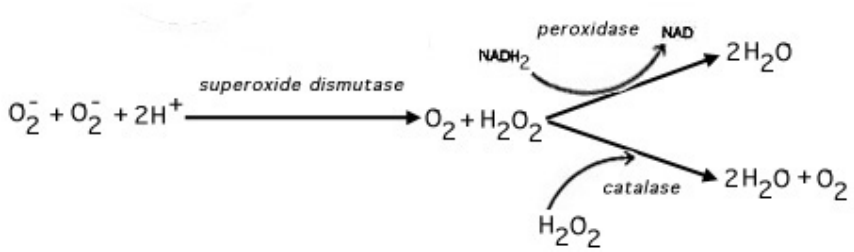


FIG.6 THE ACTION OF SUPEROXIDE DISMUTASE, CATALASE AND PEROXIDASE.

These enzymes detoxify oxygen radicals that are inevitably generated by living systems in the presence of O_2. The distribution of these enzymes in cells determines their ability to exist in the presence of O_2.

16.4 THE INFLUENCE OF PHYSICAL ENVIRONMENTAL FACTORS ON GROWTH

Most microorganisms only grow in fairly moderate environmental conditions, but some, referred to as extremophiles, can grow under harsh conditions that would kill most

other organisms. The kinds of microorganisms found in a given environment and the rates at which they grow can be influenced by a variety of factors, both physical and biochemical. Physical factors include pH, temperature, oxygen concentration, moisture, hydrostatic pressure, osmotic pressure, and radiation.

The physico chemical factors of natural environment determine the rates of microbial growth and the nature and size of the indigenous population.

1. Temperature. Environmental temperature is one of the most important factors affecting the growth rate of microbes. There is a minimum temperature, below which growth does not occur. As we rise above the minimum, rate of growth increases in accordance with the laws governing the effect of temperature on the chemical reactions that make up growth.

These reactions are mostly enzyme catalysed. However, a point is reached the optimum temperature when there is also a very rapid increase the rate of inactivation of heat sensitive cell components, like enzymes, ribosomes, DNA, membranes etc. Above an optimum temperature, this heat denaturation will occur so rapidly that there is a corresponding rapid drop in the rate of growth to give a maximum temperature for growth for that particular microorganism. Most microbes are capable of growth in a temperature range of 20- 3O"C. Most microorganisms have a growth optimum between 20 and 40°C and are called mesophilic. Those inhabiting cold environments such as polar areas can prow at much lower temperatures.

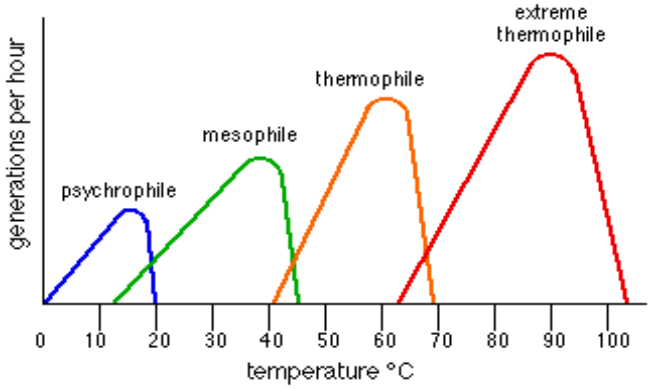


Fig. 7Microbial profile of temperature sensitivity

There is a rich microbial growth on the, surface of glaciers, where they may also cause visible red or green colour. Such microbes are called psychrophilic, that may cause problems in food storage in refrigerators over longer periods. There are also thermophilic that are able to inhabit environments such as compost heaps or hot springs. They can grow at temperatures as high as 80-100°C, when vast majority of live organism die. It appears that microorganisms can grow at any temperature as long as water is in liquid state. Perhaps, by increased stability of most cell components coupled with active repair mechanisms of heat denatured components. Growth should not be confused with survival.

Psychrophiles can grow well at 0°C, have optimal growth at 15°C or lower, and usually will not grow above 20°C.

Mesophiles have growth minima of 15 to 20°C, optima of 20 to 45°C, and maximum of about 45°C or lower.
Thermophiles have growth minima around 45°C, and optima of 55 to 65°C.
Hyperthermophiles have growth minima around 55°C and optima of 80 to 110°C.

Temperature for growth (° C)

Bacterium	Minimum	Optimum	Maximum
Listeria monocytogenes	1	30-37	45
Vibrio marinus	4	15	30
Pseudomonas maltophilia	4	35	41
Thiobacillus novellus	5	25-30	42
Staphylococcus aureus	10	30-37	45
Escherichia coli	10	37	45
Clostridium kluyveri	19	35	37
Streptococcus pyogenes	20	37	40
Streptococcus pneumoniae	25	37	42
Bacillus flavothermus	30	60	72
Thermus aquaticus	40	70-72	79
Methanococcus jannaschii	60	85	90
Sulfolobus acidocaldarius	70	75-85	90
Pyrobacterium brockii	80	102-105	115

Table 3 MINIMUM, MAXIMUM AND OPTIMUM TEMPERATURE FOR GROWTH OF CERTAIN BACTERIA AND ARCHAEA

Genus and species	Optimal growth temp (°C)
Vibrio cholerae	18-37
Photobacterium phosphoreum	20
Rhizobium leguminosarum	20
Streptomyces griseus	25
Rhodobacter sphaeroides	25-30
Pseudomonas fluorescens	25-30
Erwinia amylovora	27-30
Staphylococcus aureus	30-37
Escherichia coli	37
Mycobacterium tuberculosis	37
Pseudomonas aeruginosa	37
Streptococcus pyogenes	37
Treponema pallidum	37
Thermoplasma acidophilum	59
Thermus aquaticus	70
Bacillus caldolyticus	72
Pyrococcus furiosus	100

Table 4 OPTIMUM GROWTH TEMPERATURE OF SOME PROKARYOTES

2. pH. Microbes grow at a wide pH range. Most microbes grow best at pH near neutrality, bacteria usually slightly on the alkaline side and algae and fungi on the acid side. However, some can grow at extreme values of low or high environmental pH. For example, a few bacteria that oxidise inorganic sulphur compounds to H_2SO_4 can grow at pH 0 (i.e. 1M H_2SO_4) other bacteria, as those causing human urinary, tract infections (that hydrolyse urea to produce excess of NH_3 causing a rise in pH) grow at high pH of 11.0.

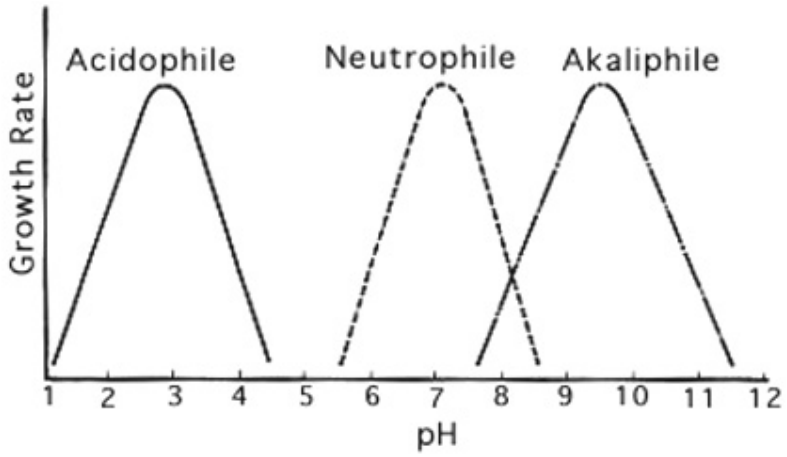
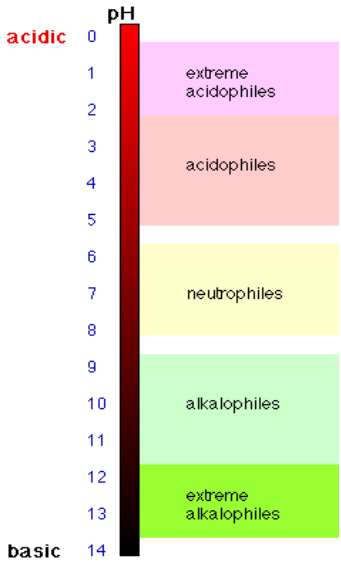


Fig. 8 Growth rate vs pH for three environmental classes of procaryotes

Most free-living bacteria grow over a pH range of about three units. Note the symmetry of the curves below and above the optimum pH for growth.

Microorganisms are classified as:

1. **Acidophiles** grow best between pH 0 and 5.5
2. **Neutrophiles** grow best between pH 5.5 and 8.0
3. **Alkalophiles** grow best between pH 8.5 and 11.5
4. **Extreme alkalophiles** grow best at pH 10.0 or higher



Despite wide variations in habitat pH, the internal pH of most microorganisms is maintained near neutrality either by proton/ion exchange or by internal buffering.

The inability of most bacteria to grow below pH value of 3-4 is utilised in food industry where pickling is a common method of preservation. Acetic acid as vinegar added to food or bacteria themselves due to fermentation may lower the pH.

Organism	**Minimum pH**	**Optimum pH**	**Maximum pH**
Thiobacillus thiooxidans	0.5	2.0-2.8	4.0-6.0
Sulfolobus acidocaldarius	1.0	2.0-3.0	5.0
Bacillus acidocaldarius	2.0	4.0	6.0
Zymomonas lindneri	3.5	5.5-6.0	7.5
Lactobacillus acidophilus	4.0-4.6	5.8-6.6	6.8
Staphylococcus aureus	4.2	7.0-7.5	9.3
Escherichia coli	4.4	6.0-7.0	9.0
Clostridium sporogenes	5.0-5.8	6.0-7.6	8.5-9.0
Erwinia caratovora	5.6	7.1	9.3
Pseudomonas aeruginosa	5.6	6.6-7.0	8.0
Thiobacillus novellus	5.7	7.0	9.0
Streptococcus pneumoniae	6.5	7.8	8.3
Nitrobacter sp	6.6	7.6-8.6	10.0

Table 5 MINIMUM, MAXIMUM AND OPTIMUM PH FOR GROWTH OF CERTAIN PROKARYOTES

3. Oxygen and redox potential. The presence or absence of oxygen divides the organisms into following three main classes. The chief criterion of this grouping is the nature of the energyproducing systems:

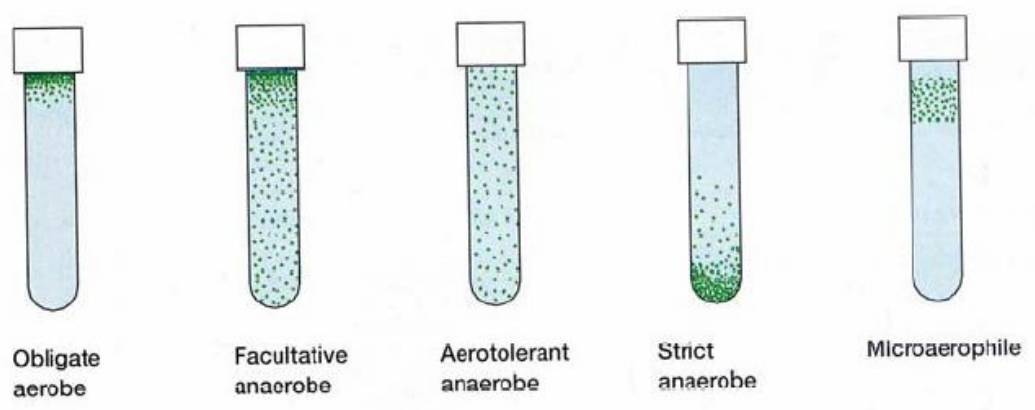
(i)The microbes which require as terminal electron acceptor for oxidation, and if this is the only means of energy production, the organism will be strict aerobe.

(ii) If, in addition, a microbe can obtain energy in the absence, it will be facultative anaerobe. Here growth is usually more abundant in presence of 0_2 than in its absence.

(iii) strict anaerobes have an energy producing system which does not require 0_2, and in addition, they are actually poisoned by oxygen. The aerobic or anaerobic nature of a microbe is related to the normal natural environment of that organism. Thus, methanogenic (methane producing) microbes are strict anaerobes and live in an environment such as the lower gut of animals, swamps, sewage, lake sediments etc. Methanogenic bacteria belong to the genera Methano bacterium, Methanococcus, Methallo, sarcina and Methanospirilum.

Microorganisms differ in their requirements of molecular oxygen (i.e., O_2) as well as other atmospheric gasses (e.g., carbon dioxide).
Obligate aerobes are completely dependent on atmospheric O_2 for growth.
Facultative anaerobes do not require O_2 for growth, but do grow better in its presence
Aerotolerant anaerobes ignore O_2 and grow equally well whether it is present or not.
Obligate (strict) **anaerobes** do not tolerate O_2 and die in its presence.

Microaerophiles are damaged by the normal atmospheric level of O_2 (20%) but require lower levels (2 to 10%) for growth.



On the other hand, methane utilising bacteria are strict aerobes, and must occur in environments where oxygen is available from the air but also supplemented by methane as the result of action of methane producing bacteria in related anaerobic areas. As a result in stratified lake we find, methane producers in the anaerobic sediments, whilst methane utilisers are concentrated at the interface between the aerobic and anaerobic layers where methane is diffusing upwards and oxygen is diffusing downwards. Methane utilisers are the species of Methylosinus, Methylocystis, Methylomonas, Methylobacter and Methylococcus.

Other bacterium, Hyphomicrobium can not utilise CH4 as carbon source, but can utilise other compounds containing one or more methyl groups. These bacteria, whose carbon and energy sources are methane or other compounds having one or more methyl groups, are known as methylotrophs.

4. Osmotic pressure - Most microbes grow within a wide range of environmental osmotic pressure. Their ability to survive O.P. lower than those of cytoplasm is generally related to tough cell wall or to contractile vacuole (water excreting mechanisms). Some microbes are able to tolerate higher levels of O.P. and are known as osmophiles or halophiles. They occur in salt lakes, salt pans and oceans. Most microbes are unable to grow at such high O.P. and this fact is used in preservation of food by salt or sugar.

5. Hydrostatic pressure - The only natural environment with high hydrostatic pressure are the depth of oceans. Here pressure may be as high as 1000 times those on the surface. The microbes present in such depths arc called barophiles.

6. Radiation - Most microbes are killed by high doses of electromagnetic radiations, particularly in the UV range, and by smaller doses of ionising radiation. Visible light is essential for photosynthetic forms.

16.5 WATER AVAILABILITY

Water is the solvent in which the molecules of life are dissolved, and the availability of water is therefore a critical factor that affects the growth of all cells. The availability of water for a cell depends upon its presence in the atmosphere (relative humidity) or its presence in solution or a substance (**water activity**). The water activity (A_w) of pure H_2O is

1.0 (100% water). Water activity is affected by the presence of solutes such as salts or sugars, that are dissolved in the water. The higher the solute concentration of a substance, the lower is the water activity and vice-versa. Microorganisms live over a range of A_w from 1.0 to 0.7. The A_w of human blood is 0.99; seawater = 0.98; maple syrup = 0.90; Great Salt Lake = 0.75. Water activities in agricultural soils range between 0.9 and 1.0.

The only common solute in nature that occurs over a wide concentration range is salt [NaCl], and some microorganisms are named based on their growth response to salt. Microorganisms that require some NaCl for growth are **halophiles**. **Mild halophiles** require 1-6% salt, **moderate halophiles** require 6-15% salt; **extreme halophiles** that require 15-30% NaCl for growth are found among the archaea. Bacteria that are able to grow at moderate salt concentrations, even though they grow best in the absence of NaCl, are called **halotolerant**. Although halophiles are "osmophiles" (and halotolerant organisms are "osmotolerant") the term **osmophiles** is usually reserved for organisms that are able to live in environments high in sugar. Organisms which live in dry environments (made dry by lack of water) are called **xerophiles**.

The concept of lowering water activity in order to prevent bacterial growth is the basis for preservation of foods by drying (in sunlight or by evaporation) or by addition of high concentrations of salt or sugar.

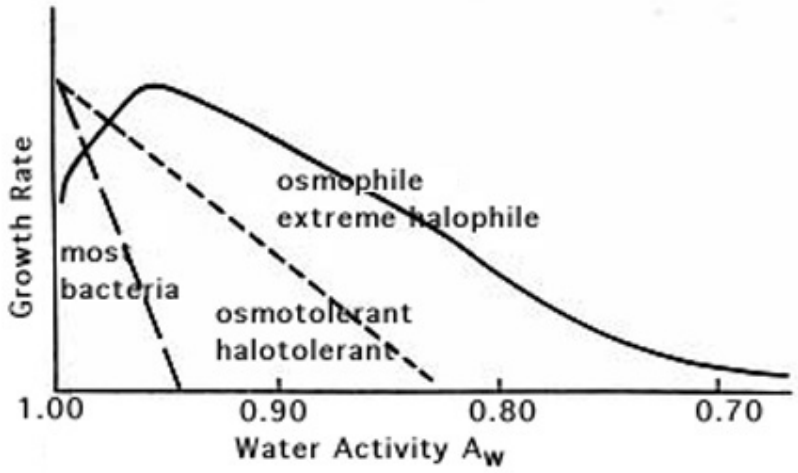


Fig. 9 Growth rate vs osmolarity for different classes of procaryotes

Osmolarity is determined by solute concentration in the environment. Osmolarity is inversely related to water activity (A_w), which is more like a measure of the concentration of water (H_2O) in a solution. Increased solute concentration means increased osmolarity and decreased A_w. From left to right the graph shows the growth rate of a normal (nonhalophile) such as *E. coli* or *Pseudomonas,* the growth rate of a halotolerant bacterium such as *Staphylococcus aureus, and* the growth rate of an extreme halophile such as the archaean *Halococcus. Note* that a true halophile grows best at salt concentrations where most bacteria are inhibited.

Organism	Minimum A_w for growth
Caulobacter	1.00
Spirillum	1.00
Pseudomonas	.91
Salmonella/E. coli	.91
Lactobacillus	.90
Bacillus	.90
Staphylococcus	.85
Halococcus	.75

Table 6 LIMITING WATER ACTIVITIES (A_W) FOR GROWTH OF CERTAIN PROKARYOTES

From the above, two overall groups of principles can be clearly seen.

(1) A microbial species usually has a fairly wide range of environment conditions in which it will grow. This range is generally higher in prokaryotes than eukaryotes, as is in microbes in general when compared with cells of higher plants and animals. The amplitude of this range may reflect either a less sensitive cellular mechanism, or a capacity for controlling the cell wall composition in presence of environmental extremes, in other words, a capacity for homoeostasis. Both possibilities can be considered.

(2) Microbial world as a whole possess an extraordinary capability to occupy extreme environmental niches. Again, this is much marked in prokaryotes. Thus in Dead Sea or near a hot spring there will be exclusively prokaryotes. There is no much competition for available nutrients. Moreover, the simpler structure of prokaryotic cell is more suited for evolutionary adaptation. Eukaryotic microbes are more suited to evolutionary change in direction of multicellular differentiated life form.

There are thus very few places on earth's surface where physico chemical conditions prevent microbial growth. As a group, microbes have tremendous metabolic versatility and are capable of degrading any natural organic material, and also most of the synthetic compounds. They have the potential to sequester nutrients present at very low concentrations. In addition, endospores of bacteria are the most resistant biological structures known and spores of fungi and actinomycetes are the most highly evolved dispersal structures.

16.6. GROWTH IN NATURAL ENVIRONMENTS

Microorganisms will grow in microenvironments until an **environmental or nutritional factor** limit this process. These factors include water, energy, temperature, nutrients, pressure, pH, salinity etc. Limiting factors can change over time and space.

- **Liebig's Law of the Minimum:** The total biomass of an organism will determined by the nutrient present in the lowest concentration relative to the organism' requirements.
- **Shelford's Law of Tolerance:** There are limits to environmental factors below and above which a microbe cannot survive and grow, regardless of nutrient supply.

16.6.1 GROWTH FACTORS

This simplified scheme for use of carbon, either organic carbon or CO_2, ignores the possibility that an organism, whether it is an autotroph or a heterotroph, may require small amounts of certain organic compounds for growth because they are essential substances that the organism is unable to synthesize from available nutrients. Such compounds are called **growth factors**.

Growth factors are required in small amounts by cells because they fulfill specific roles in biosynthesis. The need for a growth factor results from either a blocked or missing metabolic pathway in the cells. Growth factors are organized into three categories.

1. **purines and pyrimidines**: required for synthesis of nucleic acids (DNA and RNA)
2. **amino acids**: required for the synthesis of proteins
3. **vitamins**: needed as coenzymes and functional groups of certain enzymes

Some bacteria (e.g *E. coli*) do not require any growth factors: they can synthesize all essential purines, pyrimidines, amino acids and vitamins, starting with their carbon source, as part of their own intermediary metabolism. Certain other bacteria (e.g. *Lactobacillus*) require purines, pyrimidines, vitamins and several amino acids in order to grow. These compounds must be added in advance to culture media that are used to grow these bacteria. The growth factors are not metabolized directly as sources of carbon or energy, rather they are assimilated by cells to fulfill their specific role in metabolism. Mutant strains of bacteria that require some growth factor not needed by the wild type (parent) strain are referred to as **auxotrophs**. Thus, a strain of *E. coli* that requires the amino acid tryptophan in order to grow would be called a tryptophan auxotroph and would be designated *E. coli* ***trp-*.**

16.6.2 ANTIMICROBIAL COMPOUNDS

Antimicrobial agents are chemicals that kill or inhibit the growth microorganisms. Antimicrobial agents include chemical preservatives and antiseptics, as well as drugs used in the treatment of infectious diseases of plants and animals. Antimicrobial agents may be of natural or synthetic origin, and they may have a static or cidal effect on microorganisms.

16.6.3 TYPES OF ANTIMICROBIAL AGENTS

Antiseptics: microbicidal agents harmless enough to be applied to the skin and mucous membrane; should not be taken internally. Examples include alcohols, mercurials, silver nitrate, iodine solution, alcohols, detergents.

Disinfectants: agents that kill microorganisms, but not necessarily their spores, but are not safe for application to living tissues; they are used on inanimate objects such as tables, floors, utensils, etc. Examples include, hypochlorites, chlorine compounds, lye, copper sulfate, quaternary ammonium compounds, formaldehyde and phenolic compounds.

Common antiseptics and disinfectants and their uses are summarized in Table. Note: disinfectants and antiseptics are distinguished on the basis of whether they are safe for application to mucous membranes. Often, safety depends on the concentration of the compound.

CHEMICAL	ACTION	USES
Ethanol (50-70%)	Denatures proteins and solubilizes lipids	Antiseptic used on skin
Isopropanol (50-70%)	Denatures proteins and solubilizes lipids	Antiseptic used on skin
Formaldehyde (8%)	Reacts with NH_2, SH and COOH groups	Disinfectant, kills endospores
Tincture of Iodine (2% I2 in 70% alcohol)	Inactivates proteins	Antiseptic used on skin Disinfection of drinking water
Chlorine (Cl_2) gas	Forms hypochlorous acid (HClO), a strong oxidizing agent	Disinfect drinking water; general disinfectant
Silver nitrate ($AgNO_3$)	Precipitates proteins	General antiseptic and used in the eyes of newborns
Mercuric chloride	Inactivates proteins by reacting with sulfide groups	Disinfectant, although occasionally used as an antiseptic on skin
Detergents (e.g. quaternary ammonium compounds)	Disrupts cell membranes	Skin antiseptics and disinfectants
Phenolic compounds (e.g. carbolic acid, lysol, hexylresorcinol, hexachlorophene)	Denature proteins and disrupt cell membranes	Antiseptics at low concentrations; disinfectants at high concentrations
Ethylene oxide gas	Alkylating agent	Disinfectant used to sterilize heat-sensitive objects such as rubber and plastics
Ozone	Generates lethal oxygen radicals	Purification of water, sewage

Table 7 COMMON ANTISEPTICS AND DISINFECTANTS

Preservatives: static agents used to inhibit the growth of microorganisms, most often in foods. If eaten they should be nontoxic. Examples are calcium propionate, sodium benzoate, formaldehyde, nitrate and sulfur dioxide.

Some common preservatives added to processed foods:

1. Salt - retards bacterial growth. Not good for blood pressure.
2. Nitrates - can be found in some cheeses, adds flavor, maintains pink color in cured meats and prevents botulism in canned foods. Can cause adverse reactions in children, and potentially carcinogenic.
3. Sulfur Dioxide and Sulfites - are used as preservatives and to prevent browning in alcoholic beverages, fruit juices, soft drinks, dried fruits and vegetables. Sulfites prevent yeast growth and also retard bacterial growth in wine. Sulfites may cause asthma and hyperactivity. They also destroy vitamins.
4. Benzoic Acid and Sodium Benzoate - are used to preserve oyster sauce, fish sauce, ketchup, non-alcoholic beverages, fruit juices, margarine, salads, confections, baked goods, cheeses, jams and pickled products. They have also been found to cause hyperactivity.
   Propionic Acid and Propionates - used in bread, chocolate products, and cheese for lasting freshness.
5. Sorbic Acid and Sorbates - prevent mold formation in cheese and flour confectioneries

PRESERVATIVE	EFFECTIVE CONCENTRATION	USES
Propionic acid and propionates	0.32%	Antifungal agent in breads, cake, Swiss cheeses
Sorbic acid and sorbates	0.2%	Antifungal agent in cheeses, jellies, syrups, cakes
Benzoic acid and benzoates	0.1%	Antifungal agent in margarine, cider, relishes, soft drinks
Sodium diacetate	0.32%	Antifungal agent in breads
Lactic acid	unknown	Antimicrobial agent in cheeses, buttermilk, yogurt and pickled foods
Sulfur dioxide, sulfites	200-300 ppm	Antimicrobial agent in dried fruits, grapes, molasses
Sodium nitrite	200 ppm	Antibacterial agent in cured meats, fish
Sodium chloride	unknown	Prevents microbial spoilage of meats, fish, etc.
Sugar	unknown	Prevents microbial spoilage of preserves, jams, syrups, jellies, etc.
Wood smoke	unknown	Prevents microbial spoilage of meats, fish, etc.

Table 8 COMMON FOOD PRESERVATIVES AND THEIR USES

Chemotherapeutic agents (synthetic antibiotics): antimicrobial agents of synthetic origin useful in the treatment of microbial or viral disease. Examples are sulfonilamides, isoniazid, ethambutol, AZT, nalidixic acid and chloramphenicol. Note that the microbiologist's definition of a chemotherapeutic agent requires that the agent be used for antimicrobial purpose and excludes synthetic agents used for therapy against diseases that are not of microbial origin. Hence, pharmacology distinguishes the microbiologist's chemotherapeutic agent as a "synthetic antibiotic".

Antibiotics: antimicrobial agents produced by microorganisms that kill or inhibit other microorganisms. This is the microbiologist's definition. A more broadened definition of an antibiotic includes any chemical of natural origin (from any type of cell) which has the effect to kill or inhibit the growth of other types cells. Since most clinically-useful antibiotics are produced by microorganisms and are used to kill or inhibit infectious Bacteria, we will follow the classic definition.

Antibiotics are low molecular-weight (non-protein) molecules produced as secondary metabolites, mainly by microorganisms that live in the soil. Most of these microorganisms form some type of a spore or other dormant cell, and there is thought to be some relationship (besides temporal) between antibiotic production and the processes of sporulation. Among the molds, the notable antibiotic producers are *Penicillium* and *Cephalosporium*, which are the main source of the beta-lactam antibiotics (penicillin and its relatives). In the Bacteria, the Actinomycetes, notably *Streptomyces* species, produce a variety of types of antibiotics including the aminoglycosides (e.g. streptomycin), macrolides (e.g. erythromycin), and the

tetracyclines. Endospore-forming *Bacillus* species produce polypeptide antibiotics such as polymyxin and bacitracin. Semisynthetic antibiotics are molecules produced by a microbe that are subsequently modified by an organic chemist to enhance their antimicrobial properties or to render them unique for a pharmaceutical patent.

Chemical class	Examples	Biological source	Spectrum (effective against)	Mode of action
Beta-lactams (penicillins and cephalosporins)	Penicillin G, Cephalothin	*Penicillium notatum* and *Cephalosporium* species	Gram-positive bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Semisynthetic penicillin	Ampicillin, Amoxycillin		Gram-positive and Gram-negative bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Clavulanic Acid	Clavamox is clavulanic acid plus amoxycillin	*Streptomyces clavuligerus*	Gram-positive and Gram-negative bacteria	Suicide inhibitor of beta-lactamases
Monobactams	Aztreonam	*Chromobacter violaceum*	Gram-positive and Gram-negative bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Carboxypenems	Imipenem	*Streptomyces cattleya*	Gram-positive and Gram-negative bacteria	Inhibits steps in cell wall (peptidoglycan) synthesis and murein assembly
Aminoglycosides	Streptomycin	*Streptomyces griseus*	Gram-positive and Gram-negative bacteria	Inhibit translation (protein synthesis)
	Gentamicin	*Micromonospora* species	Gram-positive and Gram-negative bacteria esp. Pseudomonas	Inhibit translation (protein synthesis)
Glycopeptides	Vancomycin	*Streptomyces orientales*	Gram-positive bacteria, esp. Staphylococcus aureus	Inhibits steps in murein (peptidoglycan) biosynthesis and assembly
Lincomycins	Clindamycin	*Streptomyces lincolnensis*	Gram-positive and Gram-negative bacteria esp. anaerobic Bacteroides	Inhibits translation (protein synthesis)

Macrolides	Erythromycin	*Streptomyces erythreus*	Gram-positive bacteria, Gram-negative bacteria Not enterics, Neisseria, Legionella, Mycoplasma	Inhibits translation (protein synthesis)
Polypeptides	Polymyxin	*Bacillus polymyxa*	Gram-negative bacteria	Damages cytoplasmic membranes
	Bacitracin	*Bacillus subtilis*	Gram-positive bacteria	Inhibits steps in murein (peptidoglycan) biosynthesis and assembly
Polyenes	Amphotericin	*Streptomyces nodosus*	Fungi	Inactivate membranes containing sterols
	Nystatin	*Streptomyces noursei*	Fungi (Candida)	Inactivate membranes containing sterols
Rifamycins	Rifampicin	*Streptomyces mediterranei*	Gram-positive and Gram-negative bacteria, Mycobacterium tuberculosis	Inhibits transcription (eubacterial RNA polymerase)
Tetracyclines	Tetracycline	*Streptomyces species*	Gram-positive and Gram-negative bacteria, Rickettsias	Inhibit translation (protein synthesis)
Semisynthetic tetracycline	Doxycycline		Gram-positive and Gram-negative bacteria, Rickettsias Ehrlichia, Borrelia	Inhibit translation (protein synthesis)
Chloramphenicol	*Chloramphenicol*	*Streptomyces venezuelae*	Gram-positive and Gram-negative bacteria	Inhibits translation (protein synthesis)

Table 9 CLASSES OF ANTIBIOTICS AND THEIR PROPERTIES

16.7 METABOLIC INHIBITORS

16.7.1. ANTIMICROBIAL AGENTS USED IN THE TREATMENT OF INFECTIOUS DISEASE

The modern era of antimicrobial chemotherapy began following Fleming's discovery in 1929 of the powerful bactericidal substance penicillin, and Domagk's discovery in 1935 of synthetic chemicals (sulfonamides) with broad antimicrobial activity. In the early 1940's, spurred partially by the need for antibacterial agents in WW II, penicillin was isolated, purified and injected into experimental animals, where it was found to not only cure infections but also to possess incredibly low toxicity for the animals. This fact ushered into being the age of antibiotic chemotherapy and an intense search for similar antimicrobial agents of low toxicity to animals that might prove useful in the treatment of infectious disease. The rapid isolation of streptomycin, chloramphenicol and tetracycline soon followed, and by the 1950's, these and several other antibiotics were in clinical usage.

The most important property of a clinically-useful antimicrobial agent, especially from the patient's point of view, is its selective toxicity, i.e., the agent acts in some way that inhibits or kills bacterial pathogens but has little or no toxic effect on the animal taking the drug This implies that the biochemical processes in the bacteria are in some way different from those in the animal cells, and that the advantage of this difference can be taken in chemotherapy.

Antibiotics may have a cidal (killing) effect or a static (inhibitory) effect on a range of microbes. The range of bacteria or other microorganisms that are affected by a certain antibiotic is expressed as its spectrum of action. Antibiotics effective against procaryotes which kill or inhibit a wide range of Gram-positive and Gram-negative bacteria are said to be broad spectrum. If effective mainly against Gram-positive or Gram-negative bacteria, they are narrow spectrum. If effective against a single organism or disease, they are referred to as limited spectrum.

16.7.2. KINDS OF ANTIMICROBIAL AGENTS AND THEIR PRIMARY MODES OF ACTION

1. **Cell wall synthesis inhibitors:** Cell wall synthesis inhibitors generally inhibit some step in the synthesis of bacterial peptidoglycan. Generally they exert their selective toxicity against eubacteria because human cells lack cell walls.

Beta lactam antibiotics chemically, these antibiotics contain a 4-membered beta lactam ring. They are the products of two groups of fungi, *Penicillium* and *Cephalosporium* molds, and are correspondingly represented by the penicillins and cephalosporins. The beta lactam antibiotics inhibit the last step in peptidoglycan synthesis, the final cross-linking between between peptide side chains, mediated by bacterial carboxypeptidase and transpeptidase enzymes. Beta lactam antibiotics are normally bactericidal and require that cells be actively growing in order to exert their toxicity.

Natural penicillins, such as Penicillin G or Penicillin V, are produced by fermentation of *Penicillium chrysogenum.* They are effective against streptococcus, gonococcus and staphylococcus, except where resistance has developed. They are considered narrow spectrum since they are not effective against Gram-negative rods.

Semisynthetic penicillins first appeared in 1959. A mold produces the main part of the molecule (6-aminopenicillanic acid) which can be modified chemically by the addition of side chains. Many of these compounds have been developed to have distinct benefits or advantages over penicillin G, such as increased spectrum of activity (e.g. effectiveness against Gram-negative rods), resistance to penicillinase or effectiveness when administered orally. Amoxycillin and Ampicillin have broadened spectra against Gram-negatives and are effective orally; Methicillin is penicillinase-resistant.

Clavulanic acid is a chemical sometimes added to a semisynthetic penicillin preparation. Thus, amoxycillin plus clavulanate is clavamox or augmentin. The clavulanate is not an antimicrobial agent. It inhibits beta lactamase enzymes and has given extended life to penicillinase-sensitive beta lactams.

Although nontoxic, penicillins occasionally cause death when administered to persons who are allergic to them. In the U.S. there are 300 - 500 deaths annually due to penicillin allergy. In allergic individuals the beta lactam molecule attaches to a serum protein which initiates an IgE-mediated inflammatory response.

Cephalolsporins are beta lactam antibiotics with a similar mode of action to penicillins that are produced by species of *Cephalosporium.* The have a low toxicity and a somewhat broader spectrum than natural penicillins. They are often used as penicillin substitutes, against Gram-negative bacteria, and in surgical prophylaxis. They are subject to degradation by some bacterial beta-lactamases, but they tend to be resistant to beta-lactamases from *S. aureus*.

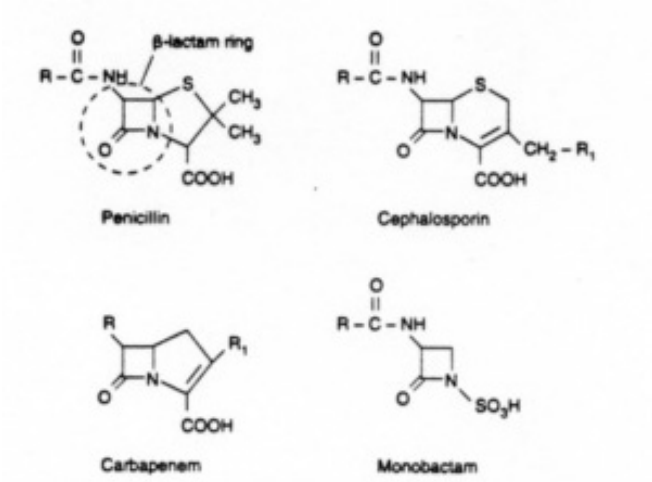


Figure 10 CHEMICAL STRUCTURE OF SOME BETA LACTAM ANTIBIOTICS

Bacitracin is a polypeptide antibiotic produced by *Bacillus* species. It prevents cell wall growth by inhibiting the release of the muropeptide subunits of peptidoglycan from the lipid carrier molecule that carries the subunit to the outside of the membrane. Teichoic acid synthesis, which requires the same carrier, is also inhibited. Bacitracin has a high toxicity which precludes its systemic use. It is present in many topical antibiotic preparations, and since it is not absorbed by the gut, it is given to "sterilize" the bowel prior to surgery.

2. **Cell membrane inhibitors**: disorganize the structure or inhibit the function of bacterial membranes. The integrity of the cytoplasmic and outer membranes is vital to bacteria, and

compounds that disorganize the membranes rapidly kill the cells. However, due to the similarities in phospholipids in bacterial and eucaryotic membranes, this action is rarely specific enough to permit these compounds to be used systemically. The only antibacterial antibiotic of clinical importance that acts by this mechanism is Polymyxin, produced by *Bacillus polymyxa*. Polymyxin is effective mainly against Gram-negative bacteria and is usually limited to topical usage. Polymyxins bind to membrane phospholipids and thereby interfere with membrane function. Polymyxin is occasionally given for urinary tract infections caused by *Pseudomonas* that are gentamicin, carbenicillin and tobramycin resistant. The balance between effectiveness and damage to the kidney and other organs is dangerously close, and the drug should only be given under close supervision in the hospital.

3. **Protein synthesis inhibitors:** Many therapeutically useful antibiotics owe their action to inhibition of some step in the complex process of translation. Their attack is always at one of the events occurring on the ribosome rather than the stage of amino acid activation or attachment to a particular tRNA. Most have an affinity or specificity for 70S (as opposed to 80S) ribosomes, and they achieve their selective toxicity in this manner. The most important antibiotics with this mode of action are the tetracyclines, chloramphenicol, the macrolides (e.g. erythromycin) and the aminoglycosides (e.g. streptomycin).

The aminoglycosides are products of *Streptomyces* species and are represented by streptomycin, kanamycin, tobramycin and gentamicin. These antibiotics exert their activity by binding to bacterial ribosomes and preventing the initiation of protein synthesis. Aminoglycosides have been used against a wide variety of bacterial infections caused by Gram-positive and Gram-negative bacteria. Streptomycin has been used extensively as a primary drug in the treatment of tuberculosis. Gentamicin is active against many strains of Gram-positive and Gram-negative bacteria, including some strains of *Pseudomonas aeruginosa*. Kanamycin is active at low concentrations against many Gram-positive bacteria, including penicillin-resistant staphylococci. Gentamicin and Tobramycin are mainstays for treatment of *Pseudomonas* infections. An unfortunate side effect of aminoglycosides has tended to restrict their usage: prolonged use is known to impair kidney function and damage to the auditory nerves leading to deafness.

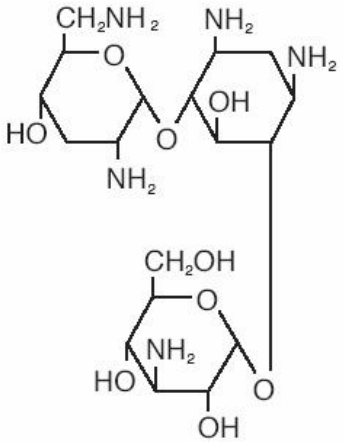


Figure 11 THE CHEMICAL STRUCTURE OF TOBRAMYCIN

The tetracyclines consist of eight related antibiotics which are all natural products of *Streptomyces*, although some can now be produced semisynthetically. Tetracycline, chlortetracycline and doxycycline are the best known. The tetracyclines are broad-spectrum

antibiotics with a wide range of activity against both Gram-positive and Gram-negative bacteria. The tetracyclines act by blocking the binding of aminoacyl tRNA to the A site on the ribosome. Tetracyclines inhibit protein synthesis on isolated 70S or 80S (eucaryotic) ribosomes, and in both cases, their effect is on the small ribosomal subunit. However, most bacteria possess an active transport system for tetracycline that will allow intracellular accumulation of the antibiotic at concentrations 50 times as great as that in the medium. This greatly enhances its antibacterial effectiveness and accounts for its specificity of action, since an effective concentration cannot be accumulated in animal cells. Thus a blood level of tetracycline which is harmless to animal tissues can halt protein synthesis in invading bacteria.

The tetracyclines have a remarkably low toxicity and minimal side effects when taken by animals. The combination of their broad spectrum and low toxicity has led to their overuse and misuse by the medical community and the wide-spread development of resistance has reduced their effectiveness. Nonetheless, tetracyclines still have some important uses, such as in the treatment of Lyme disease.

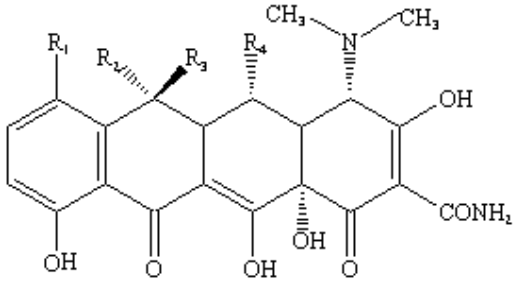


Figure 12 T**HE CHEMICAL STRUCTURE OF TETRACYCLINE**

Chloramphenicol has a broad spectrum of activity that exerts a bacteriostatic effect. It is effective against intracellular parasites such as the rickettsiae. Unfortunately, aplastic anemia, which is dose related, develops in a small proportion (1/50,000) of patients. Chloramphenicol was originally discovered and purified from the fermentation of a *Streptomyces*, but currently it is produced entirely by chemical synthesis. Chloramphenicol inhibits the bacterial enzyme peptidyl transferase thereby preventing the growth of the polypeptide chain during protein synthesis.

Chloramphenicol is entirely selective for 70S ribosomes and does not affect 80S ribosomes. Its unfortunate toxicity towards the small proportion of patients who receive it is in no way related to its effect on bacterial protein synthesis. However, since mitochondria originated from procaryotic cells and have 70S ribosomes, they are subject to inhibition by some of the protein synthesis inhibitors including chloroamphenicol. This likely explains the toxicity of chloramphenicol. The eucaryotic cells most likely to be inhibited by chloramphenicol are those undergoing rapid multiplication, thereby rapidly synthesizing mitochondria. Such cells include the blood forming cells of the bone marrow, the inhibition of which could present as aplastic anemia. Chloramphenicol was once a highly prescribed antibiotic and a number of deaths from anemia occurred before its use was curtailed. Now it is seldom used in human medicine except in life-threatening situations (e.g. typhoid fever).

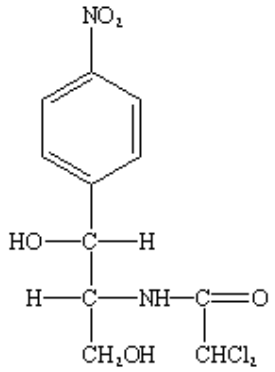


Figure 13 THE CHEMICAL STRUCTURE OF CHLOROAMPHENICOL

The Macrolides is a family of antibiotics whose structures contain large lactone rings linked through glycoside bonds with amino sugars. The most important members of the group are erythromycin and azithromycin. Erythromycin is active against most Gram-positive bacteria, *Neisseria*, *Legionella* and *Haemophilus*, but not against the *Enterobacteriaceae*. Macrolides inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit. Binding inhibits elongation of the protein by peptidyl transferase or prevents translocation of the ribosome or both. Macrolides are bacteriostatic for most bacteria but are cidal for a few Gram-positive bacteria.

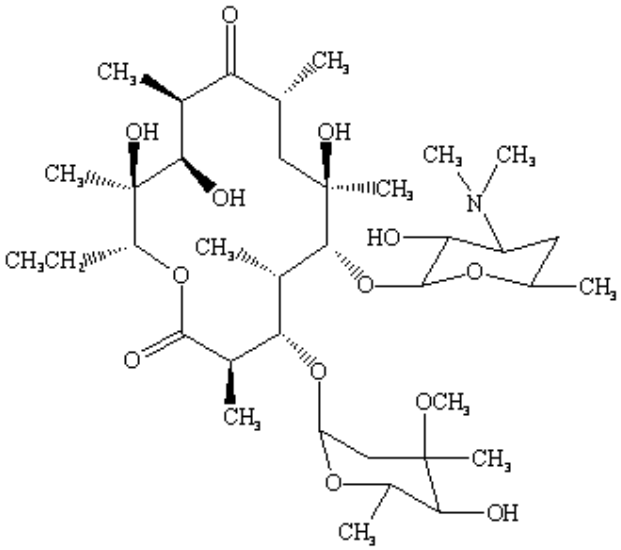


Figure 14 THE CHEMICAL STRUCTURE OF ERYTHROMYCIN

4. **Effects on Nucleic Acids:** Some chemotherapeutic agents affect the synthesis of DNA or RNA, or can bind to DNA or RNA so that their messages cannot be read. Either case, of course, can block the growth of cells. The majorities of these drugs are unselective, however, and affect animal cells and bacterial cells alike and therefore have no therapeutic application. Two classes of nucleic acid synthesis inhibitors which have selective activity against procaryotes and some medical utility are quinolones and rifamycins.

Quinolones are broad-spectrum agents that rapidly kill bacteria and are well absorbed after oral administration. Nalidixic acid and ciprofloxacin belong to this group. They act by inhibiting the activity of bacterial DNA gyrase, preventing the normal functioning of DNA. Bacterial DNA exists in a supercoiled form and the enzyme DNA gyrase, a topoisomerase, is responsible for introducing negative supercoils into the structure. Humans possess DNA gyrase but it is structurally distinct from the bacterial enzyme and remains unaffected by the activity of quinolones. Overuse of these drugs in certain situations is selecting quinolone resistant mutants and these may threaten the long term use of such compounds.

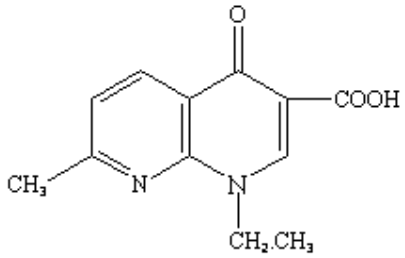


Figure 15 THE CHEMICAL STRUCTURE OF NALIDIXIC ACID

Some quinolones penetrate macrophages and neutrophils better than most antibiotics and are thus useful in treatment of infections caused by intracellular parasites. However, the main use of nalidixic acid is in treatment of lower urinary tract infections (UTI). The compound is unusual in that it is effective against several types of Gram-negative bacteria such as *E. coli*, *Enterobacter aerogenes*, *K. pneumoniae* and species which are common causes of UTI. It is not usually effective against *Pseudomonas aeruginosa*, and Gram-positive bacteria are resistant. However, a fluoroquinolone, Ciprofloxacin (Cipro) was recently recommended as the drug of choice for prophylaxis and treatment of anthrax.

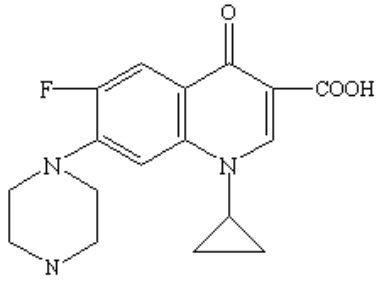


Figure 16 THE CHEMICAL STRUCTURE OF CIPROFLOXACIN

The rifamycins are the products of *Streptomyces*. Rifampicin is a semisynthetic derivative of rifamycin that is active against Gram-positive bacteria (including *Mycobacterium tuberculosis*) and some Gram-negative bacteria. Rifampicin acts quite specifically on eubacterial RNA polymerase and is inactive towards RNA polymerase from animal cells or towards DNA polymerase. The antibiotic binds to the beta subunit of the polymerase and apparently blocks the entry of the first nucleotide which is necessary to activate the polymerase, thereby blocking mRNA synthesis. It has been found to have greater bactericidal effect against *M.tuberculosis* than other anti-tuberculosis drugs, and it has largely replaced isoniazid as one of the front-line drugs used to treat the disease, especially when

isoniazid resistance is indicated. It is effective orally and penetrates well into the cerebrospinal fluid and is therefore useful for treatment of tuberculosis meningitis, as well as meningitis caused by *Neisseria meningitidis.*

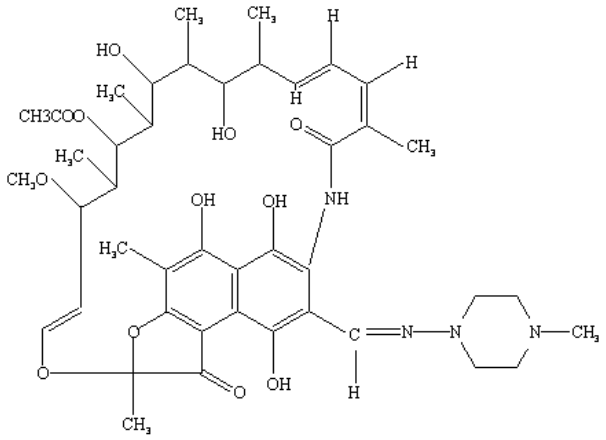


Figure 17 THE CHEMICAL STRUCTURE OF RIFAMPICIN

5. **Competitive Inhibitors**: The competitive inhibitors are mostly all synthetic chemotherapeutic agents. Most are "growth factor analogs", chemicals which are structurally similar to a bacterial growth factor but which do not fulfill its metabolic function in the cell. Some are bacteriostatic and some are bactericidal.

Sulfonamides were introduced as chemotherapeutic agents by Domagk in 1935, who showed that one of these compounds (prontosil) had the effect of curing mice with infections caused by beta-hemolytic streptococci. Chemical modifications of the compound sulfanilamide gave compounds with even higher and broader antibacterial activity. The resulting sulfonamides have broadly similar antibacterial activity, but differ widely in their pharmacological actions. Bacteria which are almost always sensitive to the sulfonamides include *Streptococcus pneumoniae*, beta-hemolytic streptococci and *E. coli.* The sulfonamides have been extremely useful in the treatment of uncomplicated UTI caused by *E. coli*, and in the treatment of meningococcal meningitis (because they cross the blood-brain barrier). The most useful sulfonamides are sulfanilamide, Gantrisin and Trimethoprim.

The sulfonamides are inhibitors of the bacterial enzymes required for the synthesis of tetrahydrofolic acid (THF), the vitamin form of folic acid essential for 1-carbon transfer reactions. Sulfonamides are structurally similar to para aminobenzoic acid (PABA), the substrate for the first enzyme in the THF pathway, and they competitively inhibit that step. Trimethoprim is structurally similar to dihydrofolate (DHF) and competitively inhibits the second step in THF synthesis mediated by the DHF reductase. Animal cells do not synthesize their own folic acid but obtain it in a preformed fashion as a vitamin. Since animals do not make folic acid, they are not affected by these drugs, which achieve their selective toxicity for bacteria on this basis.

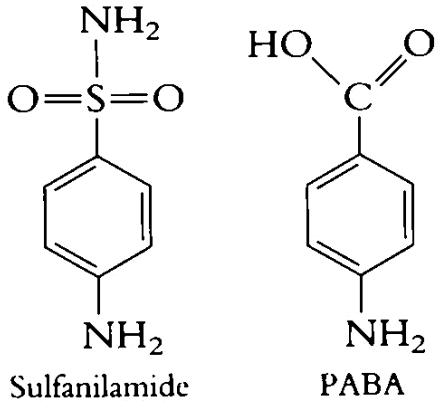


Figure 18 STRUCTURE OF SULFANAMIDE

Sulfanilamide is similar in structure to para-aminobenzoic acid (PABA), an intermediate in the biosynthetic pathway for folic acid. Sulfanilamide can competitively inhibit the enzyme that has PABA as it's normal substrate by competitively occupying the active site of the enzyme.

Three additional synthetic chemotherapeutic agents have been used in the treatment of tuberculosis: isoniazid (INH), para-aminosalicylic acid (PAS), and ethambutol. The usual strategy in the treatment of tuberculosis has been to administer a single antibiotic (historically streptomycin, but now, most commonly, rifampicin is given) in conjunction with INH and ethambutol. Since the tubercle bacillus rapidly develops resistance to the antibiotic, ethambutol and INH are given to prevent outgrowth of a resistant strain. It must also be pointed out that the tubercle bacillus rapidly develops resistance to ethambutol and INH if either drug is used alone. Ethambutol inhibits incorporation of mycolic acids into the mycobacterial cell wall. Isoniazid has been reported to inhibit mycolic acid synthesis in mycobacteria and since it is an analog of pyridoxine (Vitamin B_6) it may inhibit pyridoxine catalyzed reactions as well. Isoniazid is activated by a mycobacterial peroxidase enzyme and destroys several targets in the cell. PAS is an anti-folate. PAS was once a primary anti-tuberculosis drug, but now it is a secondary agent, having been largely replaced by ethambutol.

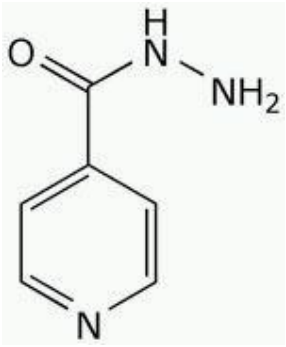


Figure 19 THE CHEMICAL STRUCTURE OF ISONIAZID

16.8 LET US SUM UP

- Water activity (a_w) is used as a quantitative measurement of the availability of water.
- Osmotolerant organisms can grow in solutions of both high and low water activity.
- The total biomass of an organism will determined by the nutrient present in the lowest concentration relative to the organism' requirements.
- There are limits to environmental factors below and above which a microbe cannot survive and grow, regardless of nutrient supply.

16.9 LESSON END ACTIVITIES

What is water activity?
Explain osmotolerant.
Explain about extremophiles.

Explain about Liebig's Law of the Minimum
Explain about Shelford's Law of Tolerance
Write about the nitrogen cycle.

16.10 POINTS FOR DISCUSSION

Describe in detail about growth factors.
Explain about the metabolic inhibitors of antibiotics.
Explain about the action of superoxide dismutase, catalase and peroxidase.
Write about the sulphur cycle.

6.11 REFERENCES

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LESSON 17 RESPONSES AND MODIFICATION OF MICROBES TO ENVIRONMENT

CONTENTS

17.0AIMS AND OBJECTIVES

The chapter deals with the responses and modification of microbes to environment.

17.1 INTRODUCTION

In fungi, unicellular algae, and protozoa, **reproduction** involves a duplication of the nucleus through the asexual process of mitosis and a splitting of the cell in cytokinesis. Reproduction can also occur by a sexual process in which haploid nuclei unite to form a diploid cell having two sets of chromosomes. Various changes then follow to yield a sexually produced offspring. Sexual reproduction has the advantage of mixing chromosomes to obtain genetic variations not possible with asexual reproduction. However, fewer individuals normally result from sexual reproduction than from asexual reproduction. More details on these methods are provided in the chapters on fungi and protozoa.

17.2 GROWTH RESPONSE TO ENVIRONMENT

17.2.1. REPRODUCTION PATTERNS

During their growth cycles, microorganisms undergo reproduction many times, causing the numbers in the population to increase dramatically.

Bacteria reproduce by the asexual process of **binary fission.** In this process, the chromosomal DNA duplicates, after which the bacterial membrane and cell wall grow inward to meet one another and divide the cell in two. The two cells separate and the process are complete.

One of the remarkable attributes of bacteria is the relatively short **generation time**, the time required for a microbial population to double in numbers. The generation time varies among bacteria and often ranges between 30 minutes and three hours. Certain bacteria have very brief generation times. *Escherichia coli*, for example, has a generation time of about 20 minutes when it is dividing under optimal conditions.

17.2.2. THE GROWTH CURVE

The growth of a bacterial population can be expressed in various phases of a **growth curve.** The logarithms of the actual numbers in the population are plotted in the growth curve along the side axis, and the time is plotted at the base. Four phases of growth are recognized in the growth curve.

In the first phase, called the **lag phase**, the population remains at the same number as the bacteria become accustomed to their new environment. Metabolic activity is taking place, and new cells are being produced to offset those that are dying.

In the **logarithmic phase**, or **log phase**, bacterial growth occurs at its optimal level and the population doubles rapidly. This phase is represented by a straight line, and the population is at its metabolic peak. Research experiments are often performed at this time.

During the next phase, the **stationary phase**, the reproduction of bacterial cells is offset by their death, and the population reaches a plateau. The reasons for bacterial death include the accumulation of waste, the lack of nutrients, and the unfavorable environmental conditions that may have developed. If the conditions are not altered, the population will enter its **decline**, or **death phase**. The bacteria die off rapidly, the curve turns downward, and the last cell in the population soon dies.

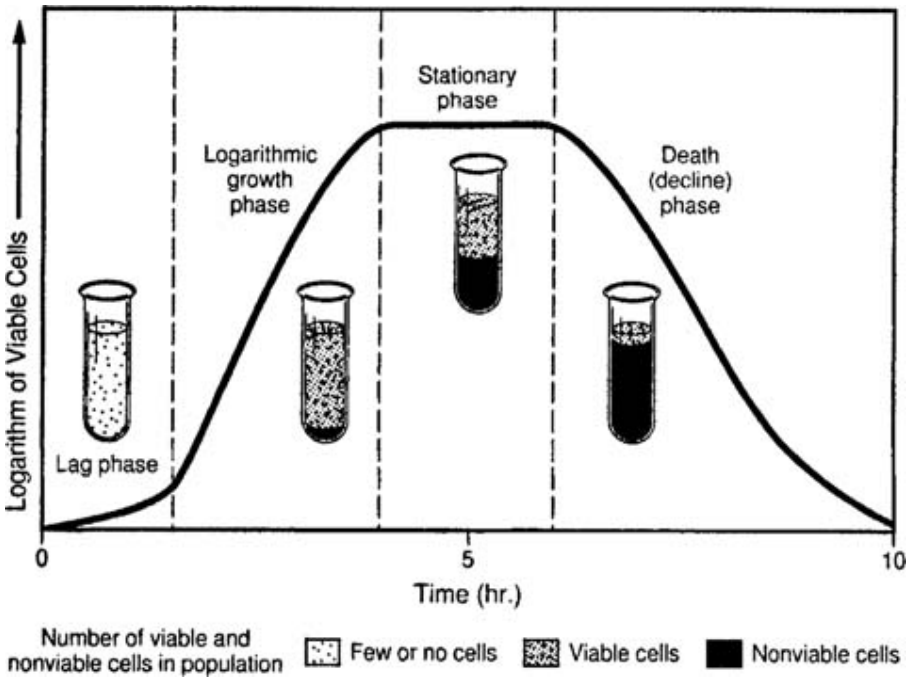

Fig. 11 A GROWTH CURVE OF A BACTERIAL POPULATION SHOWING THE FOUR MAJOR PHASES OF THE CURVE.

17.2.3. BACTERIAL SPORES

A few species of bacteria have the ability to produce highly resistant structures known as endospores (or simply spores). These resist a range of hazardous environments, and protect against heat, radiation, and desiccation. Endospores form within (hence *endo-*) special vegetative cells known as sporangia (singular *sporangium*). Diseases caused by sporing bacteria include botulism (*Clostridium botulinum*), gas gangrene (*Clostridium perfringens*), tetanus (*Clostridium tetani*) and acute food poisoning (*Clostridium perfringens*, again) All these bacteria are 'anaerobic'. The aerobic sporing bacteria can also cause disease. Anthrax is caused by *Bacillus anthracis. Bacillus cereus* causes two types of food poisoning.

Endospores are highly heat-resistant, dehydrated resting cells formed intracellularly in members of the genera Bacillus and Clostridium. Sporulation, the process of forming endospores, is an unusual property of certain bacteria. The series of biochemical and morphologic changes that occur during sporulation represent true differentiation within the cycle of the bacterial cell. The process, which usually begins in the stationary phase of the vegetative cell cycle, is initiated by depletion of nutrients (usually readily utilizable sources of carbon or nitrogen, or both). The cell then undergoes a highly complex, well-defined sequence of morphologic and biochemical events that ultimately lead to the formation of mature endospores. As many as seven distinct stages have been recognized by morphologic and biochemical studies of sporulating Bacillus species: stage 0, vegetative cells with two chromosomes at the end of exponential growth; stage I, formation of axial chromatin filament and excretion of exoenzymes, including proteases; stage II, forespore septum formation and segregation of nuclear material into two compartments; stage III, spore protoplast formation and elevation of tricarboxylic acid and glyoxylate cycle enzyme levels; stage IV, cortex formation and refractile appearance of spore; stage V, spore coat protein formation; stage VI, spore maturation, modification of cortical peptidoglycan, uptake of dipicolinic acid (a unique

endospore product) and calcium, and development of resistance to heat and organic solvents; and stage VII, final maturation and liberation of endospores from mother cells (in some species).

When newly formed, endospores appear as round, highly refractile cells within the vegetative cell wall, or sporangium. Some strains produce autolysins that digest the walls and liberate free endospores. The spore protoplast, or core, contains a complete nucleus, ribosomes, and energy generating components that are enclosed within a modified cytoplasmic membrane. The peptidoglycan spore wall surrounds the spore membrane; on germination, this wall becomes the vegetative cell wall. Surrounding the spore wall is a thick cortex that contains an unusual type of peptidoglycan, which is rapidly released on germination. A spore coat of keratinlike protein encases the spore contained within a membrane (the exosporium). During maturation, the spore protoplast dehydrates and the spore becomes refractile and resistant to heat, radiation, pressure, desiccation, and chemicals; these properties correlate with the cortical peptidoglycan and the presence of large amounts of calcium dipicolinate.

Recent evidence indicated that the spores of *Bacillus spharicus* were revived which had been preserved in amber for more than 25 million years. Their claims need to be reevaluated. The thin section of the spore shows the ruptured, thick spore coat and the cortex surrounding the spore protoplast with the germinal cell wall that becomes the vegetative wall on outgrowth.

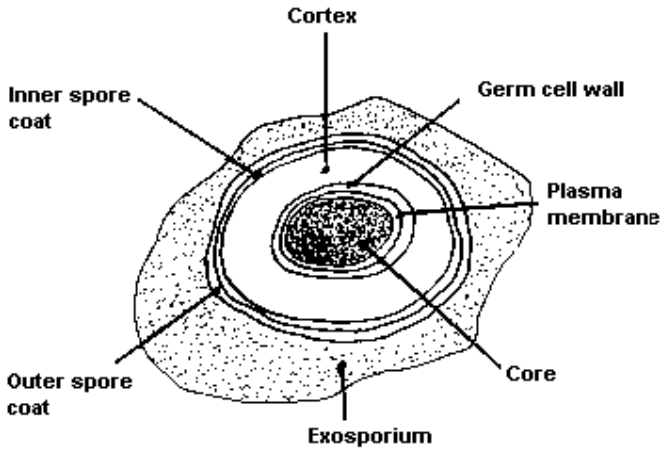

Fig.12 A BACTERIAL SPORE

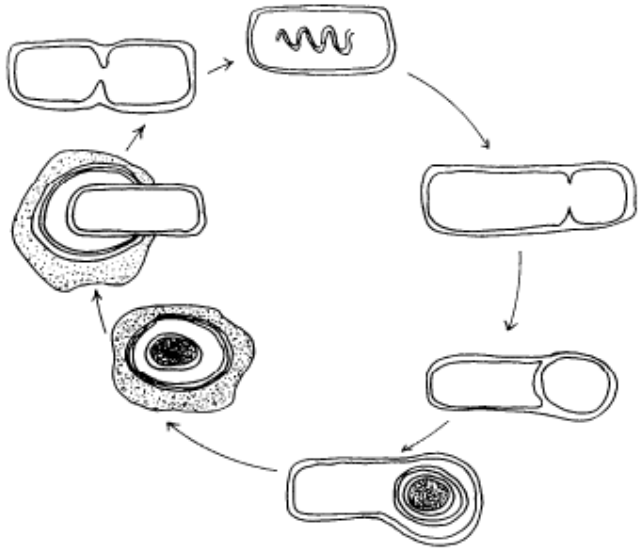

Fig.13 THE CYCLE OF SPORE FORMATION AND GERMINATION

17.2.4. OVERVIEW OF GROWTH RESPONSE OF BACTERIA, YEAST AND FUNGI TO ENVIRONMENT

17.2.4.1. BACTERIAL GROWTH

Bacteria exhibit a wide diversity in metabolic activities, but all have similar cellular structure and reproduction mechanisms. They are classed as *prokaryotic* organisms. The various genera of bacteria are related back to a common ancestral state, however, through evolution, substantial structural and physiological diversity has developed. In Gram-positive cells the polysaccharide murein forms up to 30 molecular layers, while in Gram-negative cells only a single murein layer is present, and lipopolysaccharides and lipoproteins are the main constituents of the cell wall.

Prokaryotes also have no nuclear membrane or other intracellular organelles. Despite this diversity, there are several features common to many bacteria that are important to quantitative microbiology. Bacteria generally reproduce by the process of binary fission, resulting in two daughter cells of equal size. A cell grows by increasing in size, during which time the amount of each new cell component, e.g., protein, RNA, etc., is doubled and the genome is replicated. Cell division is initiated by in growth of the cell wall and eventual formation of a transverse septum. Cell separation proceeds by cleavage of the septum, and two identical daughter cells are formed. There is a difference, however, in how the Gram-positive and Gram-negative bacteria synthesize their cell wall material. Gram-positive bacteria synthesize new cell wall in an equatorial zone along an axis, whereas Gram-negative bacteria synthesize cell wall by intercollation along the whole wall. Incomplete cleavage of the septa will result in chains as is the case for streptococci. Delayed cleavage will result in elongated bacilli structures. The concentration of cell components, i.e., RNA, enzymes, metabolites, etc., in each daughter will be the same as in the parent. This is true, however, only when the cells are growing in an environment that does not necessitate a change in some cell property with time. If, for instance, an environmental change calling for induction or repression of some enzyme occurs between initiation of a cell cycle and cell division, then the daughter cells will have a different level of one or more enzymes than the original parent.

Some bacteria, especially members of the family Bacillaceae and some gliding bacteria, have the ability to form spores to survive in adverse conditions.

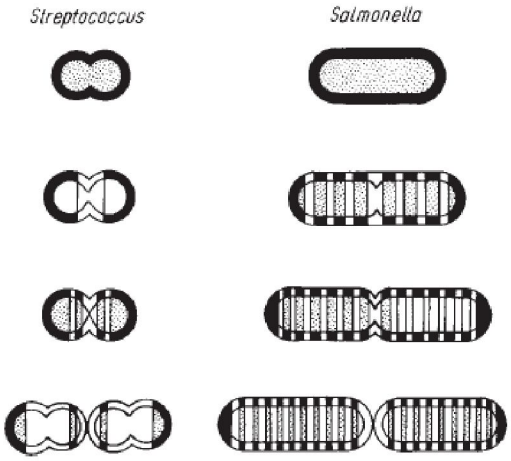

Fig.14 Process of binary fission and cell wall addition of a Gram-positive species of *Streptococcus* and a Gram-negative species of *Salmonella*; dark areas are old cell wall material, light areas represent newly added compounds.

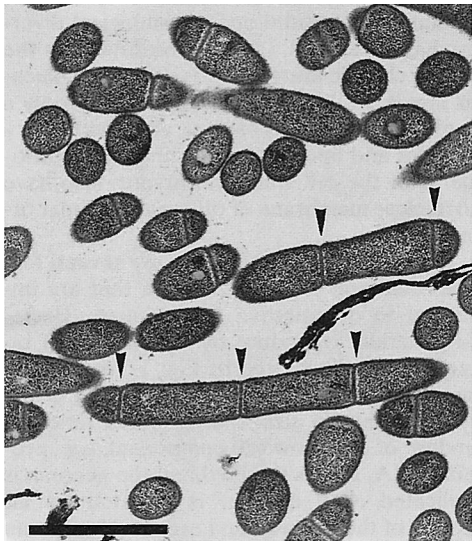

Fig.15 Electron micrograph of an ultrathin section of filamentous gliding Gram-negative *Herpetosiphon auranticus*. Several bacteria are sectioned longitudinally and show complete septa formation (arrow heads), barp2 mm (LÜNSDORF, GBF, 1993).

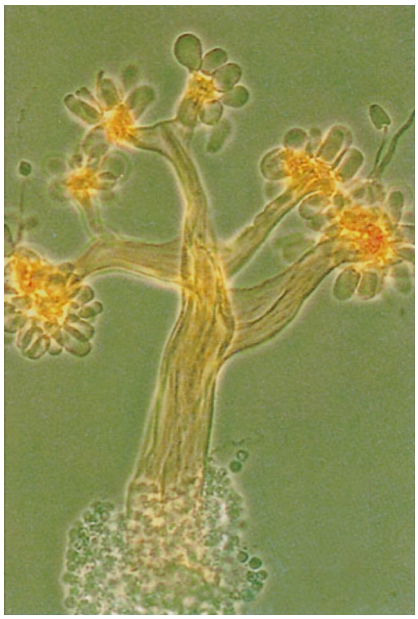

Fig.16 Several fruiting bodies on a sporangiophore and sporangioles of the gliding myxobacterium *Condromyces crocatus* (H. REICHENBACH, GBF).

17.2.4.2. YEAST GROWTH

Yeasts are *eukaryotic* organisms which belong to the fungi. The most prominent members are baker's yeast *Saccharomyces cerevisiae,* brewer's yeast *S. uvarum (carlsbergensis)* and the fodder yeasts *Candida utilis* and *C. tropicalis.* They are fungi that do not form asexual spores or aerial structures and exist as single cells during at least part of their vegetative growth cycle. The most common form of cell division is budding, however, fission following cross-wall formation, mycelial growth by chain elongation, and branching are also observed in some yeast. The yeasts are non-motile and non-photosynthetic. They are either oval or spherical in shape. The size of a yeast cell is dependent on the growth rate; the shorter the doubling time, the larger the cell volume.

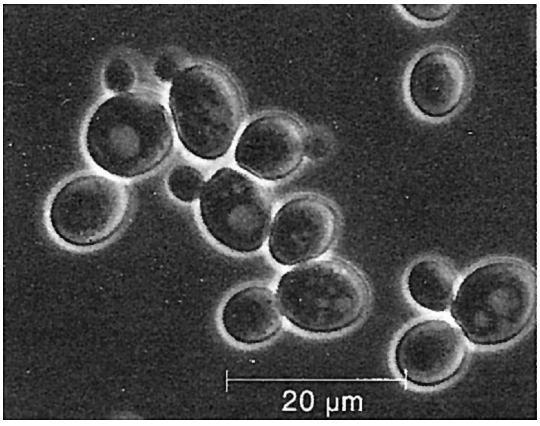

Fig.17 Budding yeasts cells (Saccharomyces cerevisiae) with 1st and 2nd generation daughter cells (NK2, isolated from African palm wine, A. EJIOFOR and M. RHODE, GBF, 1993).

During growth of yeast by budding, there are several distinct events. Initially, the yeast cell undergoes a period of expansion: its volume increases. Shortly after the cell stops expanding, bud emergence occurs. During bud formation, the total volume of the mother plus daughter bud cell is constant, so that bud growth occurs as a consequence of depletion of the mother cell. The bud separates as a single, but smaller cell from the mother. Once separated, the new daughter cell and the original mother cell grow and reach the same size at the same time; thus, the daughter grows faster than the mother. The mother cell will have a bud scar on its surface for each bud that has separated; these can be seen with fluorescent techniques. Unlike bacteria, mother and daughter yeast cells are different. They possess different growth rates, and their cell surface is different. It is possible to count the number of scars and to establish a cell's age in the broth. Thus, there is a distribution of cells having different ages. While yeasts are typically single-celled, their progeny, or daughter cells, will sometimes not separate. When buds do not separate from the mother, the resulting chains of cells are called pseudomycelia.

17.2.4. 3 MYCELIAL GROWTH

Molds, actinomycetes, and some yeasts under aerobic conditions, predominantly grow by the process of hypha chain elongation (e.g., elongation at the tip, also called apical growth) and branching. The hypha, which is divided into individual cells, is a branching tubular structure of 2–10 mm in diameter. The intertwining strands of hyphae are called mycelium. Many fungi can form asexual spores called conidia.

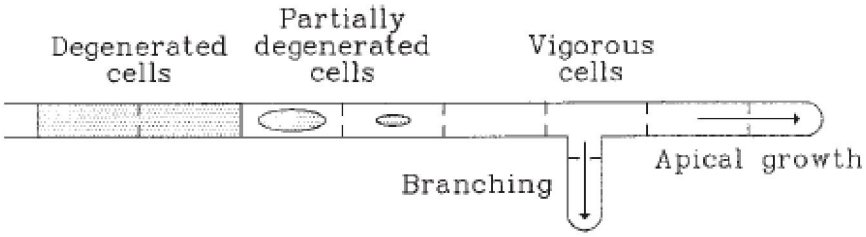

Fig. 18 Schematic diagram of mycelial growth by branching and chain elongation. Shaded areas represent degenerated regions, while unshaded areas are cytoplasmic regions.

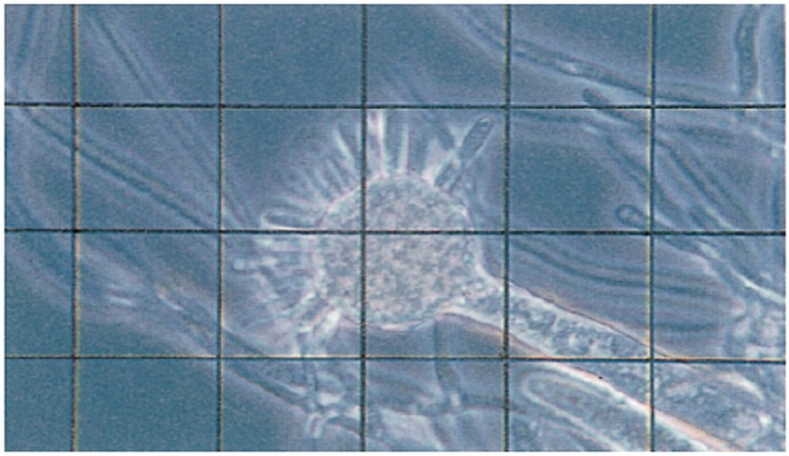

Fig.19 Hyphae and conidial structur with conidiophore, sterigmata, and conidia (spores) of the fungus aspergillus niger. (frinken and heiber, gbf, 1991).

The length of a hyphal chain depends on the growth environment. If left undisturbed, as on an agar surface, the chain can become quite long. In submerged culture, however, there are shear forces that cause hyphae fragmentation. This results in shorter, but more highly branched mycelia. In submerged cultures, the mycelia may exist along with dispersed, diffuse mycelia or may form pellets; the form of growth has an important effect on growth and product formation. Model-based simulations have brought more insight into the process of mycelial growth. Microorganisms respond to their environment. Cells growing within a pellet will "see" a very different environment than those growing in a more diffuse manner. A measurable bulk concentration of nutrient or product exists in the broth. However, the concentration of *nutrient* on the pellet surface is lower due to diffusion through a stagnant liquid boundary layer and cell metabolism. The concentration is reduced further inside in the pellet as a result of metabolism, to such a degree, that, at the center of the pellet, there may be little or no nutrient. This problem is especially important when considering oxygen supply. The *product* concentration, on the other hand, will be higher within the pellet than outside resulting from further diffusional limitation.

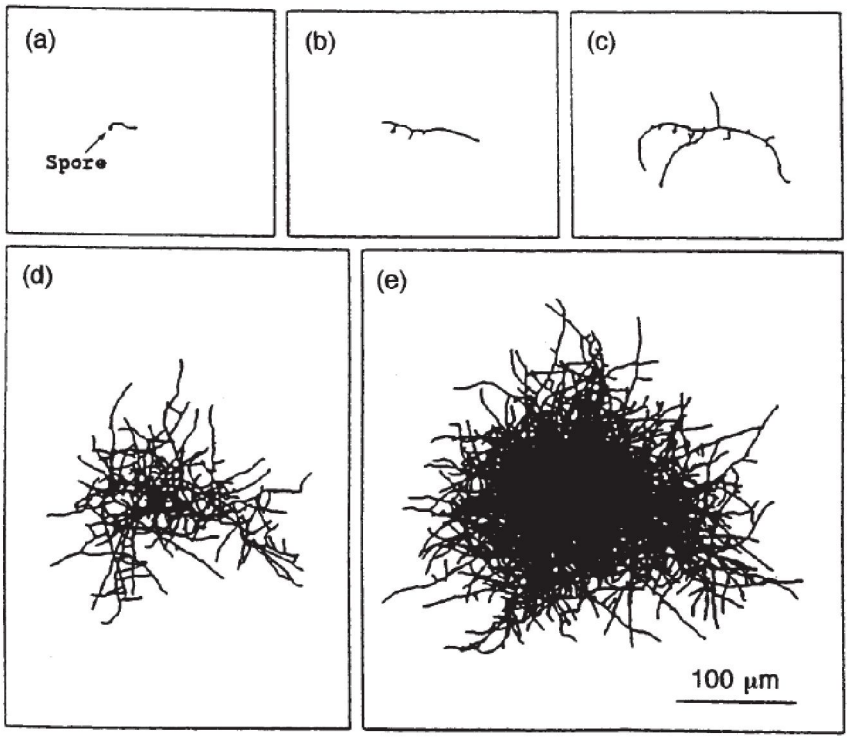

Fig.20 SIMULATED MORPHOLOGICAL DEVELOPMENT OF THE GROWTH OF A MYCELIUM EMERGING FROM A SPORE

This is an important issue only if there is product inhibition. Early in fermentation, the cells in the pellet see a nutrient-rich environment, later the pellets become denser, and growth and metabolism occur predominantly on the periphery. As a restult, cells near the pellet center become starved for one or more nutrients. Mycelial growth leads to an age distribution of cells. Younger cells at the hyphal tip will have different metabolism than cells at or near the origin of growth. This is due to both the aging process inherent in the cell and the differing environments of young and old cells. Ideally, methods of measurement would allow the investigator to distinguish among cells of different ages. However, this is difficult to

achieve, and in most cases, the performance of a mycelial population is *normalized* to the total amount of cell mass present.

17.2.5. GROWTH INHIBITION AND DEATH

17.2.5.1. TYPES OF MICROBIAL GROWTH CONTROL

Inhibition: Microbes or their undesired activities are inhibited by bacteriostatic compounds or techniques
Sterilization: Microbes themselves are killed by bacteriocidal compounds or techniques
Treatment of Inanimate Objects
Decontamination removes or kills most microbes, making an object or surface safe
Disinfection removes most or all pathogens from an object or surface
Sterilization removes or kills all microbes
Treatment of Living Organisms or Tissues
Agents used to treat living organisms or tissues must be nontoxic as well as antimicrobial
Antiseptic compounds can be applied to tissues
Antimicrobial agents can be taken internally
Disinfectants are only applied to inanimate surfaces

17.2.5.2. HEAT

Heat is useful for inanimate objects; macroscopic organisms and their tissues are more sensitive to heat than are microbes
Lethal effects of heat on microbes may be due to:

- Protein denaturation
- Effects on membrane fluidity
- Denaturation of other macromolecules

Kinetics of heat sterilization is first order
Kinetics of Heat Sterilization
Kinetics of thermal death, like those of growth, is first order
First order means that the rate of cell death is directly dependent on the number of cells present
Under a given set of conditions (*e.g.*, temperature) the number of microbes dying per minute is proportional to the number of microbes present
For instance, under a given set of conditions, 90% of the cells present are killed each minute
In this case, if there were initially 1,000,000 cells, after 1 minute 100,000 cells remain (900,000 cells die), and 2 min 10,000 cells remain (990,000 cells have died)

17.2.5.3. DECIMAL REDUCTION TIME

Decimal reduction time is the time required to reduce the number of living microbes by a factor of 10
In the previous example, the decimal reduction time was 1 min
Decimal reduction time can be measured accurately only when large numbers of microbes are present
With smaller numbers of microbes, statistics become important
When 1,000,000 cells are initially present, it is easy to see that 100,000 remain after one DRT
What happens when the initial number of cells is 1?

17.2.5.4. STATISTICS OF THERMAL DEATH

It is more accurate to consider that after 1 DRT, each initial microbe has a 1:10 chance of surviving
With a large population, the number of survivors will average very close to 0.1 of initial
With a small population, it is a matter of chance
Compare to serial dilutions
Take a 1-ml sample
Dilute to 10 ml and take 1 ml (a 1:10 dilution)
Each organism in the original sample has 1:10 chance of being in the selected 1 ml
If there are 10 organisms in the sample, there will on average be 1 organism; in actuality, sometimes there will be 1, sometimes none, sometimes 2 or 3

17.2.5.5. THERMAL DEATH OF A MESOPHILE

This figure shows viable numbers during thermal death (Y axis is percent of initial number of cells remaining viable)
Notice that logarithmic scales never go to zero
As the numbers on the Y-axis reach 1 cell, that means there is on average one cell remaining (about a 30% probability that ≥ 1 cell is present)
Notice that the time required for sterilization depends on how sure you want to be
When average number of cells is 1, there is a 70% chance that the sample is sterile
When average is 0.1, there is approximately 90% chance of sterility

17.2.5.6. THERMAL DEATH TIME

Thermal death time is the amount of time to sterilize a population of a microbe
From what you learned in the previous discussion:
Thermal death time depends on the initial number of organisms
Thermal death time is not a precise number, even when the initial number of organisms is defined, because the death of that last organism is a matter of chance (when only one organism remains, each additional DRT gives a 1:10 chance for survival)

17.2.5.7. VARIATIONS IN DRT

DRT is logarithmically related to temperature
Various organisms may have different sensitivities to temperature
Physiological conditions of the microbe also affect DRT
Effect of Heterogeneous Populations
Imagine what would happen if the population included a mix of species, or cells at various physiological states with various DRT
At first, most susceptible strains would die, reflecting a shorter DRT (more rapid death)
Later, when only more resistant cells remained, DRT would be greater (slower death)
Environmental Factors Affecting DRT
Physiological factors include growth rate and previous incubation temperature
Formation of endospores is a critical physiological factor
Environmental factors (during thermal treatment):

- pH (acidity accelerates death)
- Salt (positive or negative factor)
- Water (sterilization with dry heat requires about 3 h at 180ºC, whereas wet sterilization requires 15 min at 121ºC)

17.3 MODIFICATION TO THE ENVIRONMENT

17.3.1. EUTROPHICATION: THE MICROBIAL RESPONSE TO HIGH NUTRIENT LEVELS

Eutrophication may be defined as the inorganic nutrient enrichment of natural waters, leading to an increased production of algae and macrophytes. Many lakes are naturally eutrophic, and in some cases there is a progressive eutrophication as the lake matures. The term 'eutrophication' is more widely known in relation to human activities, where the artificial introduction of plant nutrients particularly phosphorus and nitrogen) has lead to community changes and a deterioration of water quality in many freshwater systems. This aspect has become increasingly important with increases in human population and more extensive development of agriculture, and eutrophication now ranks with other major anthropogenic effects such as deforestation, global warming, depletion of the ozone layer, and large-scale environmental disturbance in relation to its potentially harmful effect on natural ecosystems.

In terms of aquatic microbiology, eutrophication results in changes in the biomass and taxonomic composition of all groups of microorganisms present in freshwater systems. Some of these effects have already been noted in relation to lake Phytoplankton, river diatom biofilms, planktonic bacteria, and benthic protozoa. Freshwater microorganisms have a central role in the environmental effects of eutrophication, since it is the microbial response which leads to physicochemical changes in water quality and can ultimately disrupt ecological balance and system stability.

17.3.2. THE GROWTH AND IMPACT OF ALGAL BLOOMS

17.3.2.1 ALGAL BLOOMS AND EUTROPHICATION

Algal blooms are simply dense populations of planktonic algae which develop in aquatic systems. They may occur in a wide range of environments, including lakes and rivers, exposed mudflats, and snowpacks – and are part of the normal seasonal development in many ecosystems. In all of these environments, the development of algal blooms can be seen as a balance between the processes of population increase (high growth rate, ability to out-compete other algae) and population loss (effects of grazing, parasitic attack). Increased levels of inorganic nutrients lead to a general increase in primary productivity but may also promote algal blooms at different times of the year. In lentic environments, these blooms include the spring diatom bloom, late-spring blooms of green algae, and summer blooms of dinoflagellates and blue-green algae. Most of these blooms have no adverse effects on the environment, and the increased algal biomass is transferred to other lake biota via the normal food web.

The major problems of eutrophication come with anthropogenic enrichment of the environment and the formation of dense blooms of toxic dinoflagellates (principally marine) and colonial blue-green algae freshwater).

- Toxic dinoflagellates. These organisms are particularly characteristic of marine waters, and are thus largely outside the scope of this volume. They do occur in estuaries, however, and are occasionally seen in major rivers associated with estuaries – so do have some peripheral relevance to freshwater systems. Some of these dinoflagellates are important in the formation of neurotoxins, and are potential hazards in terms of human water contact and food consumption. Toxic dinoflagellates

include *Gambierdiscus toxicus* (CTX toxin), *Gonyaulax Alexandrinum* (STX), *Gymnodinium breve* (brevetoxins), *Diophysis spp.* (Okadaic acid), and *Pfiesteria piscicida* (unknown toxin).

- Colonial blue-green algae. Colonial bluegreens, not dinoflagellates, form the major nuisance- algae of freshwater systems and have the potential to cause deterioration in water quality and adverse environmental effects. In many eutrophic environments and mesotrophic lakes of lesser magnitude, quite dense blue-green blooms occur on an annual basis without any permanent environmental effects. It is only when these algae form very dense accumulations and totally out-compete other algae that their influence becomes severe.

17.3.2.2 COMPETITION WITH OTHER ALGAE

Various hypotheses have been put forward to explain the ability of blue-greens to out-compete other algae, including their optimum growth at high temperature, low-light tolerance, tolerance of low N/P nutrient ratios, depth regulation by buoyancy, resistance to zooplankton grazing, and tolerance of high pH/low CO_2 concentrations. Most of these features probably contribute to the success of blue-greens, without being individually of sole importance. The dominant success of colonial blue-greens probably results from the sum total of all these characteristics, with an overriding requirement for high nutrient input to achieve high biomass levels.

17.3.2.3 OPTIMUM GROWTH AT HIGH TEMPERATURE

Blue-green algae have higher growth optima than do green algae and diatoms. The midsummer increase in abundance of these algae in temperate lakes, and their success in tropical lakes, may be the direct result of their ability to grow well in warm water conditions. Chemostat experiments have suggested that blue-greens isolated from Lake Michigan and Lake Superior (North America) have maximum growth ability at temperatures exceeding 20°C.

Although temperature is important, it is secondary to nutrient requirements. Oligotrophic lakes in the same geographic area as eutrophic lakes, and with the same temperature regime, do not develop bluegreen blooms. A survey of world lakes lead Robarts and Zohary (1987) to conclude that temperature was of subsidiary importance. Tolerance of low-light conditions various physiological studies have suggested that blue-green algae have lower light-energy requirements than green algae and diatoms. Although the ability to grow at low light may seem an unlikely advantage for algae present at the top of the water column in midsummer, the intense self-shading which occurs within turbid bloom populations make this a significant feature. Critical evidence is contradictory, however, with some studies showing that blue-greens are more dominant in turbid conditions, while others show the reverse.

17.3.2.4 ABILITY FOR GROWTH AT LOW N/P RATIOS

The apparent ability of blue-greens to out-grow other algae at low nitrogen/phosphorus (N/P) ratios, has received much attention. General evidence in support of this is not conclusive, and it may be a feature of secondary importance. Analysis of 17 lakes by Smith (1983) concluded that although the blue-green contribution to the phytoplankton could be high in lakes with low TN/TP ratios (<29/1), they only occurred at

low level when ratios were higher. Other studies have been less supportive, and the general importance of low N/P ratios to bluegreens must be queried.

17.3.2.5 DEPTH REGULATION BY BUOYANCY

The ability of vacuolate blue-greens such as Anabaena and Oscillatoria to regulate their depth by buoyancy is probably important both in the early development of algal populations and in the final phase of dominance. In terms of population growth, it allows them to adopt an optimum position within the water column in relation to light and CO_2 availability. Regulation of depth is also important in the diurnal migration of these algae to lower (high-nutrient) parts of the water column, allowing them to continue growth at a time of year when epilimnion nutrient levels have reached a low level. During bloom formation, the rapid flotation of these algae to the lake surface leads to changes in the water chemistry and light regime at the lake surface, depressing the growth of other phytoplankton.

Depth regulation by buoyancy is particularly effective in a static water column and confers an advantage on these organisms in such stable conditions. In lakes where the water column remains stratified but without a high degree of stability, changes in the phytoplankton composition (with the emergence of blue-green dominance) still occur – suggesting that buoyancy is not absolutely essential for bloom formation in these algae.

17.3.2.6 RESISTANCE TO ZOOPLANKTON FEEDING

Filter-feeding zooplankton often appears to feed ineffectively, if at all, on blue-green algae. Blue-green dominance is further promoted by the elimination of competing green algae and diatoms from mixed phytoplankton populations during grazing activities. Even where blue-green algae are ingested, the presence of a thick outer layer of mucilage often allows them to pass through the zooplankter alimentary canal without being digested. The relationship between blue-green algal dominance and zooplankton grazing is an important one. The notion that blue-green algae may become dominant in the short term due to a failure of zooplankton grazing is suggested by several studies. These demonstrate that an abundance of filterfeeding grazers does promote dominance by bluegreens such as Aphanizomenon. In contrast to this short-term effect, the effectiveness of biomanipulation as a control method depends on the long-term activities of zooplankton such as *Daphnia magna* for controlling blue-greens. Lakes which have acquired these zooplankton populations tend to show a shift from blue-green to green algal dominance. The overall relationship between blue-greens and zooplankton grazers at a particular site is thus highly variable, depending on zooplankton biomass, physiological state, species composition, and whether the interaction is being considered short-term or long-term.

17.3.2.7 TOLERANCE OF HIGH pH AND LOW CO_2 CONCENTRATIONS

In conditions of high light intensity, elevated levels of photosynthesis within the epilimnion lead to pronounced CO_2 uptake resulting in strongly alkaline conditions with low CO_2 availability. It has been suggested that blue-green algae (but not other members of the phytoplankton) have the ability to tolerate these extreme environmental conditions – allowing them to continue active growth at a point when other algae are inhibited, thus out-competing other photosynthetic organisms. This concept is supported by the fact that bluegreen dominance occurs in most lakes only when pH is high. Blue-green dominance is absent from lakes where pH does not rise during the summer – including oligotrophic lakes (low algal

biomass, low photosynthesis) and lakes with CO_2 sources other than the atmosphere. The importance of CO_2 and pH to blue-green dominance is also supported by enclosure experiments.

17.3.2.8. AQUATIC MATRIX

In the freshwater environment, water is important both as a major internal constituent of biota and also as the environmental matrix. Because of this dual role, the physical/chemical properties of water are central to the physiology and ecology of freshwater organisms. The properties of water in the liquid state are considered in this section and water in the frozen state.

17.4 INFLUENCE OF THE PHYSICAL PROPERTIES OF WATER ON THE BIOLOGY OF AQUATIC MICROORGANISMS

The physical properties of water exert fundamental and wide-ranging influence on the biology of freshwater microorganisms through their effects on the surrounding aquatic environment. Some of these effects are general to all water bodies, while others are more specific to lotic or lentic systems, respectively.

17.4.1 GENERAL EFFECTS

Temperature characteristics of water are generally important, by modulating the influence of external changes in atmospheric temperature. Low thermal conductivity and high heat capacity modulate diurnal and seasonal changes in temperature. This has wide-ranging implications, including relatively high rates of decomposition (compared with terrestrial systems) by fungi and other saprophytes. Other characteristics of general importance include high surface tension (development of surface neuston communities) and low light absorption/scattering. Deep penetration of the water column and irradiation of sediments promotes photosynthesis and other light-mediated activities in a wide range of environments.

17.4.2 LENTIC ENVIRONMENTS

The physical properties of water are important to lake microorganisms in a number of specific ways including lake stratification, development of ice-blooms, and the establishment of surface biofilm (neuston) microbial communities.

17.4.3. FORMATION OF ICE-BLOOMS

Variations in density with temperature are particularly important in maintaining thermal stratification within standing waters, with temperature normally decreasing with depth in the water column. The density of water, however, reaches a maximum at 3.95°C, resulting in a reverse stratification below ice (temperature increasing with depth) as surface water approaches freezing point. The anomalous temperature – density curve of water is particularly important in ice covered water bodies such as Lake Baikal (Russia), where localized heating below the ice layer in late spring results in the generation of weak convection currents. These are sufficient to maintain microbial communities (particularly diatoms) in suspension below the ice at a time of year when light intensity is increasing, resulting in algal blooms. If the density of water simply decreased with a rise in temperature from 0°C, no downdraft would occur and circulation below the ice would not take place.

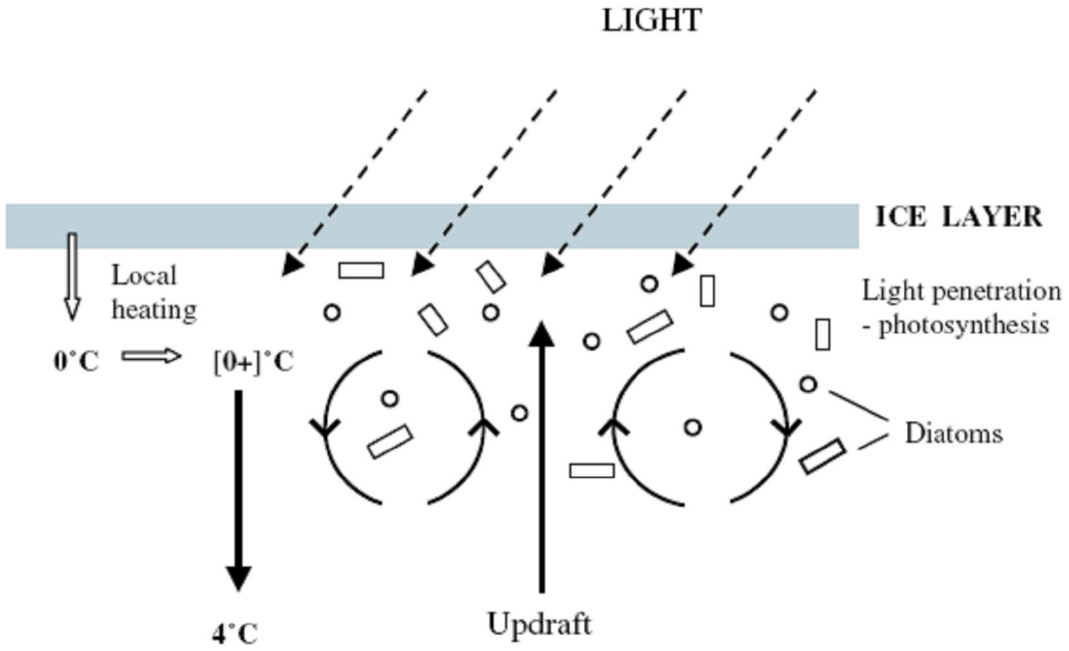

Fig.21 Formation of ice-blooms due to the anomalous temperature-density relationship of water

Under appropriate ice conditions, light penetration leads to turbulent suspension of diatoms (*Aulacoseira baicalensis*) and supports photosynthesis. Local heating raises the temperature of water below the ice (from 0 to 4°C), increasing density and promoting a downdraft. This causes sub-surface water circulation, generating updrafts which carry phytoplankton up into the light zone, where they remain suspended in the turbulent conditions, leading to a diatom bloom below the ice.

17.4..4 SURFACE BIOFILM COMMUNITIES

The high surface tension of water is important in the establishment of a surface biofilm, with an associated community of surface microorganisms. The lowering of surface tension by organic molecules leads to their spontaneous adsorption at the air – water interface, creating a high-nutrient microlayer. This acts as a site for microbial attachment and nutrient supply, leading to the development of dense surface populations of bacteria, algae, and protozoa. These surface microorganisms are part of a larger community of surface biota (neuston), which includes various invertebrates. Many of these, such as the water beetle (Notonecta) and mosquito larvae, exploit the high surface tension of water to attach to the surface.

17.4.5. AQUIFER MICROORGANISMS

Microbial communities in subterranean ground waters exist in various aquatic compartments, from shallow to deep (>200 m) systems, and show considerable physiological diversity. Ground water microorganisms are mainly present within biofilms at the surface of sediment particles, and include both lithotrophic and heterotrophic organisms (Frederickson et al., 1989). Lithotrophic organisms include manganese (Mn IV)- and iron (FeIII)- reducing bacteria in low-oxygen conditions, with sulphate-reducing and methanogenic bacteria

occurring in completely anoxic environments. In the majority of aquifers, the predominant microorganisms are thought to be aerobic or facultatively- aerobic heterotrophs, mostly in the genus Pseudomonas (Kazumi and Capone, 1994). These bacteria may have to experience a range of adverse conditions, including very low nutrient concentrations, and are adapted to grow and survive at extremes of organic carbon availability. Evidence for extreme nutrient limitation of bacteria in aquifers is provided by estimates of inter-related bacterial abundance, nutrient supply, and metabolic activities (Kazumi and Capone, 1994):

- Total bacterial counts in sediments from a range of depths within a pristine (non-contaminated) aquifer have been shown to be directly correlated to total organic carbon levels.
- Values for bacterial abundance and metabolic activity (glucose uptake, total thymidine incorporation per gram dry weight of sediment) in aquifers are generally 10 to 1000-fold lower than soil or sediments of surface aquatic systems.
- The efficiency of glucose uptake is particularly high in aquifer bacteria, with only 10 per cent of added label being recovered as CO_2.

17.4.6. CHEMICAL COMPOSITION OF THE SURFACE MICROLAYER

Most organic chemicals reduce the surface tension of water, releasing free energy at the liquid surface. Because of this, these molecules have a tendency to adsorb to the air–water interface and remain within the surface microlayer – reaching high concentrations.
This surface layer has a thickness of about 0.1–10 mm, depending on the water body, and has a chemical composition quite different from the rest of the water column.

The surface microlayer contains a wide range of both organic and inorganic chemicals.The organic component occurs as both particulate organic material (POM) and dissolved organic material (DOM or DOC). Fatty acids, lipids, and hydrocarbons play an important role at the water surface. These molecules are referred to as ‘dry surfactants’ and are very surface-active. The hydrocarbon assemblage contains poly-aromatic and chlorinated hydrocarbons, including polychlorinated biphenyls (PCBs) and pesticides. PCBs and pesticides can become enriched in surface films from atmospheric deposition, reaching concentrations high enough to cause stress to microorganisms and the reproductive stages of fish and shellfish associated with surface films. Proteins and polysaccharides have been identified in surface films by infrared spectroscopy and other analytical techniques and make up a major part of the organic complement. These ‘wet surfactants’ probably anchor to the water surface via their hydrophobic portions, unfolding as the interface is penetrated. The presence of phenolic and humic material in surface microfilms has been demonstrated by ultraviolet (UV) absorbance. In freshwater systems, these molecules are external (allochthonous) in derivation. These strongly UVabsorbing molecules are susceptible to photooxidation at the water surface, and may act as natural inhibitors of microbial activity.

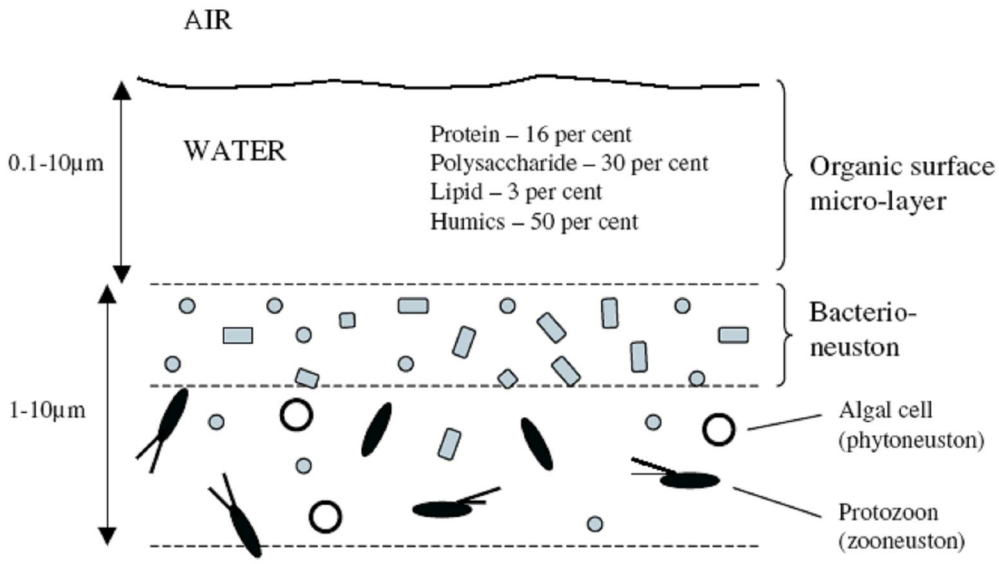

Fig22. Microbial populations associated with the air-water surface biofilm

The bacterioneuston layer varies from single-cell (monolayer) to several-cell (multilayer) thickness. Individual bacterial cells may be free-floating (planktonic) or attached to macromolecules at the edge of the surface microlayer

In addition to dissolved organic material, various inorganic molecules also accumulate in the surface microlayer – including phosphate, ammonia, nitrate, and nitrite ions. These molecules do not lower the surface energy at the air–water interface, so do not adsorb in the same way as organic components. Their presence at the water surface is due to other factors such as excretion and lysis by microfilm organisms, transport in rising air bubbles, and association with organic matter by complexation. The inorganic composition of lake surface biofilms varies with nutrient status, with greater surface film enrichment of phosphate and ammonia nitrogen occurring in eutrophic lakes compared to oligotrophic sites. Accumulation of metals (Cr, Cu, Fe, Pb, Hg, and Zn), present both as particulate and dissolved material, has also been demonstrated in surface films. Although metals appear to have a relatively brief residence time (minutes to hours) in surface films, they are potentially important as toxins for microorganisms and other lake biota.

17.4.7 PHYSICAL PROCESSES AND TRANSFORMATIONS IN THE SURFACE BIOFILM

The surface film is subject to various exchange (input/output) and transformation processes. Input of dry deposition and rain from the atmosphere is counteracted by aerosol formation and evaporation. Aerosol droplets, generated by air turbulence and local heating (insolation), are normally in the range of 2–10 mm diameter, and can transport microorganisms (e.g., bacteria) and chemicals (e.g., algal toxins) away from the water surface. Input of material from the water column is compensated by loss of particulate and soluble matter, mainly by sedimentation and dissolution. Surface biofilms also act as a static barrier to gas exchange, and may influence the temperature at the air–water interface by retarding evaporation or by affecting water movement at the water surface.Various biological

transformations occur within the surface film, including photodegradation and protein denaturation.

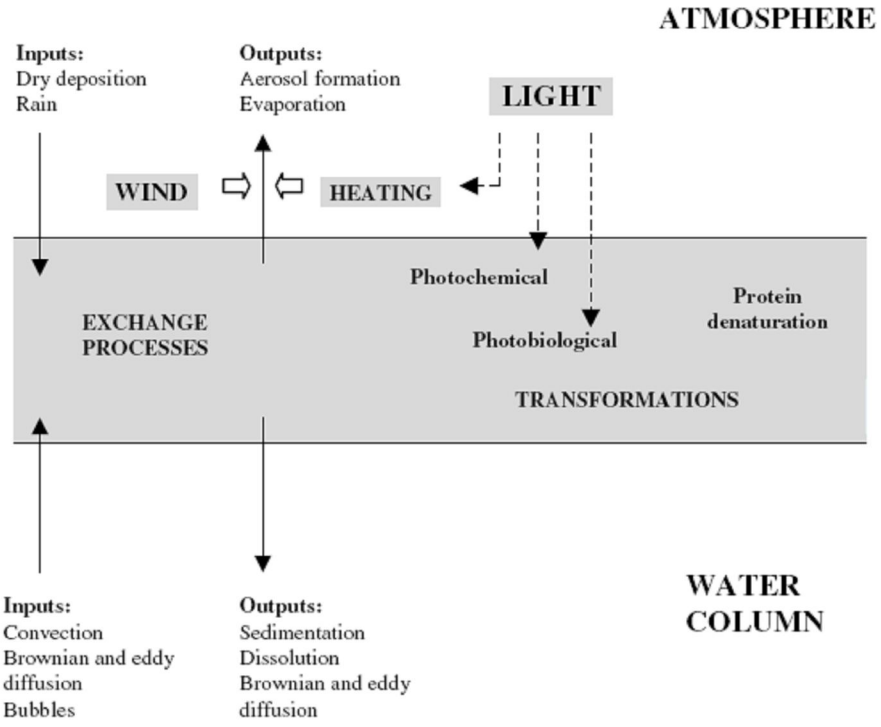

Fig. 23 Physico-chemical exchange and transformation processes in the surface biofilm

Light damage occurs to isolated molecules (photochemical) and whole organisms (photobiological). The surface biofilm is indicated as the central shaded area.

17.4.8 PHOTODEGRADATION

During daylight hours, the air–water interface is in a state of considerable photochemical and photobiological activity. Both visible and UV light have direct effects on chemical and biological (microorganism) components, and also have indirect effects by generating short-lived oxygen radicals in the presence of free oxygen. These radicals are highly reactive and lead to further degradation of organic matter. Aromatic and sulphur-containing components of macromolecules are particularly susceptible to photodegradation by UV absorption. Damage to amino acids and proteins involves a range of processes, including cleavage of N–C bonds to form NH_4, generation of H_2S from cysteine and breakage of glycoside linkages (by hydrolysis) in ribose (but not deoxyribose) nucleotides. Damaging effects of light also have potentially serious effects on surface microorganisms.

17.4.9 PROTEIN DEGRADATION

Studies by Graham and Phillips (1979) on model systems have demonstrated irreversible adsorption and denaturation of proteins at water surfaces. The adsorption process follows a defined sequence of events:

5. diffusion of proteins to the water surface, leading to random collision with the air–water interface

6. a reversible phase of adsorption, in which some proteins are lost (desorbed) while others remain or are recruited – molecular competition is a key aspect of this phase
7. permanent adsorption, with irreversible changes in protein configuration
8. this leads to the formation of a primary protein monolayer, with a thickness of 5–6 nm and an area loading of 2.6– 3.3 mg protein cm^3
9. Continued adsorption, leading to a maximum protein film thickness of >10 nm.

17.4.10 MICROBIAL COMMUNITY AT THE AIR–WATER INTERFACE

Bacteria (bacterioneuston), algae (phytoneuston), and protozoa (zooneuston) all contribute to the surface film community.

17.4.11 BACTERIONEUSTON

High bacterial counts at air–water interfaces have been recorded in numerous reports, suggesting that this is a regular feature of surface biofilms. These organisms probably occur both as single cells and microcolonies, with the bacterial layer in different biofilms ranging from a monolayer to multilayer structure. Some of the bacteria are freely motile (planktonic) while others are attached to the molecular matrix within the surface microlayer. Studies involving model systems suggest that bacterial adhesion to the surface microlayer has similar characteristics to adhesion to solid surfaces, with bacterial surface hydrophobicity and charge strongly influencing surface biofilm adhesion.

The growth and metabolic activity of bacteria within the surface biofilm is influenced by opposing environmental factors. These aspects are promoted by high nutrient levels, but may be limited by unstable temperature conditions, localized accumulation of organic toxins and heavy metals, and intense levels of UV and visible solar radiation. In spite of this environmental stress, studies on nutrient uptake and respiration suggest that a major proportion of the bacterial population is metabolically active and physiologically-adapted to the local conditions. The bacterial population that develops at the water surface is probably initially selected for in terms of ability to adhere to the surface microlayer. After this initial colonization, further growth and survival depend on ability to utilize the organic substrate and to tolerate adverse environmental conditions.

17.4.12 PHYTONEUSTON

Bacteria are generally considered to be the primary colonizers of the water surface film, with algae and protozoa entering the system as secondary populations and extending the biofilm below the zone of attached bacteria. The presence of algae in surface biofilms has been demonstrated by direct observation and by determination of chlorophyll-a. Surface populations of these organisms, however, appear to be highly variable, and the greatest enrichment with phytoneuston appears to occur in discrete patches with high concentrations of associated organic material. These localized accumulations of surface algae increase the thickness and complexity of the biofilm. Algae may enter the surface community in various ways, and may interchange with phytoplankton populations in the water column as part of a diurnal oscillation. As residents of surface films, algae share the same environmental constraints as bacteria – with solar destruction of photosynthetic complexes being additionally important. In spite of these potential limitations, analysis of primary productivity at the water surface suggests higher values than lower down in the main water column.

17.4.13 ZOONEUSTON

Amoebae and ciliate protozoa have been reported at the air–water interface, with a correlation between their population level and the density of bacterioneuston. These secondary colonizers form an important connection between prokaryotes and higher trophic levels, and link with higher organisms that associate with the water surface – including copepods and various larvae.

17.4.14 SNOW MICROBE INTERACTIONS

Interactions between microbes are an important aspect of snow ecosystems and can be identified at three levels – observations on diversity within food webs, correlations between populations of organisms, and direct observations of associations between individual organisms.

Foodwebs : The wide range of biota reported in various studies on snow ecosystems are indicative of complex food webs. In many cases, these food webs are active for only a limited period of time, while free water is available. Population correlations Correlations between population peaks are indicative of a trophic or spatial (e.g., epiphytic) association between biota and have been reported particularly in relation to algal populations. These correlations include the following.

6. Algal and bacterial populations. Thomas (1994) reported a direct correlation between bacterial and algal populations in mountain snow in California, USA. The highest populations of bacteria (3:2 /105 cells ml/1) were found with large populations of algae (4:9 /104 cells ml/1) in red-coloured snow.
7. Algae and filamentous fungi. Both parasitic and non-parasitic fungi show clear populations with algal populations. The latter include snow fungi in the genera Chionaster and Selenotila.
8. Algae and yeasts. It is clear that many fungi occurring in snow environments are present as yeasts. Hoham and Duval (2001) report direct correlations between high yeast and algal populations in areas of mountain snow.

The above correlations of populations of heterotrophic bacteria, filamentous fungi, and yeasts with algae suggests that these organisms are simply growing on dissolved organic carbon (DOC) secreted by algal cells. This indicates that a microbial loop is operating in these ecosystems, much as it does in lake and river environments.

Direct associations: Direct associations between snow biota have been observed at the microscopical level by various authors, including bacterial and fungal associations with algae. (Hoham et al. 1993), for example, reported an association between bacteria and fungi with Chloromonas snow algae from Eastern USA. These organisms were observed adhering to the outer gelatinous coat of resting spores of the alga. Another example is provided by the snow fungus Phacidiun infestans, which grows through melting snowpacks in some parts of the Pacific Northwest of the USA. Snow algae can be seen adhering passively to the fungus, but physical connections between them do not occur as in lichen symbiosis. In these and other instances, observed associations between snow biota are difficult to interpret in functional terms. Spatial association does not necessarily imply symbiosis or any degree of metabolic cooperation between the participants

17.5 BACTERIAL RESISTANCE TO ANTIBIOTICS

Penicillin became generally available for treatment of bacterial infections, especially those caused by staphylococci and streptococci, about 1946. Initially, the antibiotic was

effective against all sorts of infections caused by these two Gram-positive bacteria. Resistance to penicillin in some strains of staphylococci was recognized almost immediately. (Resistance to penicillin today occurs in as many as 80% of all strains of *Staphylococcus aureus*). Surprisingly, *Streptococcus pyogenes* (Group A strep) have not fully developed resistance to penicillin and it remains a reasonable drug of choice for many types of streptococcal infections. Natural penicillins have never been effective against most Gram-negative pathogens (e.g. *Salmonella, Shigella, Bordetella pertussis, Yersinia pestis, Pseudomonas*) with the notable exception of *Neisseria gonorrhoeae*. Gram-negative bacteria are inherently resistant because their vulnerable cell wall is protected by an outer membrane that prevents permeation of the penicillin molecule.

The period of the late 1940s and early 1950s saw the discovery and introduction of streptomycin, chloramphenicol, and tetracycline, and the age of antibiotic chemotherapy came into full being. These antibiotics were effective against the full array of bacterial pathogens including Gram-positive and Gram-negative bacteria, intracellular parasites, and the tuberculosis bacillus. However, by 1953, during a *Shigella* outbreak in Japan, a strain of the dysentery bacillus was isolated which was multiple drug resistant, exhibiting resistance to chloramphenicol, tetracycline, streptomycin, and the sulfanilamides. There was also evidence mounting that bacteria could pass genes for multiple drug resistance between strains and even between species. It was also apparent that *Mycobacterium tuberculosis* was capable of rapid development of resistance to streptomycin which had become a mainstay in tuberculosis therapy.

By the 1960's it became apparent that some bacterial pathogens were developing resistance to antibiotic-after-antibiotic, at a rate faster than new antibiotics could be brought to market. A more conservative approach to the use of antibiotics has not been fully accepted by the medical and agricultural communities, and the problems of emerging multiple-drug resistant pathogens still loom. The most important pathogens to emerge in multiple drug resistant forms so far have been *Mycobacterium tuberculosis* and *Staphylococcus aureus*. An antibiotic sensitivity test performed on an agar plate. The discs are seeded with antibiotics planted on the agar surface. Interpretation of the size of the bacterial "zones of inhibition" relates to the possible use of the antibiotic in a clinical setting. The organism is resistant to the antibiotics planted on the plate at 5 o'clock and 9 o'clock.

Bacterial resistance to an antimicrobial agent may be due to some innate property of the organism or it may due to acquisition of some genetic trait as described below.

Inherent (Natural) Resistance - Bacteria may be inherently resistant to an antibiotic. For example, a streptomycete may have some natural gene that is responsible for resistance to its own antibiotic; or a Gram-negative bacterium has an outer membrane that establishes a permeability barrier against the antibiotic; or an organism lacks a transport system for the antibiotic; or it lacks the target or reaction that is hit by the antibiotic.

Acquired Resistance - Bacteria can develop resistance to antibiotics, e.g. bacterial populations previously-sensitive to antibiotics become resistant. This type of resistance results from changes in the bacterial genome. Acquired resistance is driven by two genetic processes in bacteria: (1) mutation and selection (sometimes referred to as vertical evolution); (2) exchange of genes between strains and species (sometimes called horizontal evolution or horizontal gene transmission).

Vertical evolution is strictly a matter of Darwinian evolution driven by principles of natural selection: a spontaneous mutation in the bacterial chromosome imparts resistance to a member of the bacterial population. In the selective environment of the antibiotic, the wild type (non mutants) is killed and the resistant mutant is allowed to grow and flourish. The mutation rate for most bacterial genes is approximately 10^{-8}. This means that if a bacterial population doubles from 10^8 cells to 2 x 10^8 cells, there is likely to be a mutant present for any given gene. Since bacteria grow to reach population densities far in excess of 10^9 cells, such a mutant could develop from a single generation during 15 minutes of growth.

Horizontal gene transmission (HGT) is the acquisition of genes for resistance from another organism. For example, a streptomycete has a gene for resistance to streptomycin (its own antibiotic), but somehow that gene escapes and gets into *E. coli* or *Shigella*. Or, more likely, some bacterium develops genetic resistance through the process of mutation and selection and then donates these genes to some other bacterium through one of several processes for genetic exchange that exist in bacteria.

Bacteria are able to exchange genes in nature by three processes: conjugation, transduction and transformation. Conjugation involves cell-to-cell contact as DNA crosses a sex pilus from donor to recipient. During transduction, a virus transfers the genes between mating bacteria. In transformation, DNA is acquired directly from the environment, having been released from another cell. Genetic recombination can follow the transfer of DNA from one cell to another leading to the emergence of a new genotype (recombinant). It is common for DNA to be transferred as plasmids between mating bacteria. Since bacteria usually develop their genes for drug resistance on plasmids (called resistance factors [R-factors] or resistance transfer factors [RTFs]), these genetic elements play heavily in the of spread drug resistance to other strains and species during genetic exchange processes.

The combined effects of fast growth rates, high populations of cells, genetic processes of mutation and selection, and the ability to exchange genes, account for the extraordinary rates of adaptation and evolution that can be observed in the bacteria. For these reasons bacterial adaptation (resistance) to the antibiotic environment seems to take place very rapidly in evolutionary time: bacteria evolve fast.

17.5.1 THE MEDICAL PROBLEM OF BACTERIAL DRUG RESISTANCE

Obviously, if a bacterial pathogen is able to develop or acquire resistance to an antibiotic, then that substance becomes useless in the treatment of infectious disease caused by that pathogen (unless the resistance can somehow be overcome with secondary measures). So as pathogens develop resistance, we must find new (different) antibiotics to fill the place of the old ones in treatment regimes. Hence, natural penicillins have become useless against staphylococci and must be replaced by other antibiotics; tetracycline, having been so widely used and misused for decades, has become worthless for many of the infections where it once worked as a "wonder drug".

Not only is there a problem in finding new antibiotics to fight old diseases (because resistant strains of bacteria have emerged), there is a parallel problem to find new antibiotics to fight new diseases. In the past two decades, many "new" bacterial diseases have been discovered (Legionnaire's disease, gastric ulcers, Lyme disease, toxic shock syndrome, "skin-eating" *Streptococci*). We are only now able to examine patterns of susceptibility and resistance to antibiotics among new pathogens that cause these diseases. Broad patterns of

resistance exist in these pathogens, and it seems likely that we will soon need new antibiotics to replace the handful that are effective now against these bacteria, especially as resistance begins to emerge among them in the selective environment antibiotic chemotherapy.

17.6 SUMMARY

3. Soil microbiology deals with microbial role in soil fertility by decomposing the dead organic matter into nutrients.
4. Water microbiology explains the usage of microbes and determination of microbial content in aquatic system
5. Aeromicrobiology elaborates on the microbial population present in air and its sources
6. The impact of nutrients such as C,H,O,N,S and P on microbial physiology is tremendous which have been discussed in detail besides the role of microbes in biogeocycles
7. The growth factors such as amino acids, lipids, carbohydrates, purines, pyrimidines, nucleotides and other factors are having a profound effect on the physiology
8. Microorganisms respond to the environment in changing their reproduction and growth pattern so that they are able to survive and maintain their population in the habitat
9. Microbes also modify their biochemical pathway and physical structure to adapt to the changed environment

17.7 LESSON END ACTIVITIES

- Weigh a gram of soil and dilute in water. View under the microscope and try to enumerate the microbial count
- Why do they use microbes for sulphur ore mining?

3. Add water on the surface of pickle and observe the growth of microbes in a day or two due to dilution of salt content
4. List out the factors that facilitate the growth of microbes in natural environment

17.8 POINTS FOR DISCUSSION

- What are halophilic microbes?
- Explain how the microbes adapt to the environment by changing its reproduction habits.
- Elaborate in detail the chemical changes occurring in microorganisms in response to the environment.

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1. Microbiology by Pelczar, Reid and Chan, McGraw Hill Book Company.
2. Microbiology, Fundamental and Applications by R.A. Atlas, McMillan Publishers.
3. General Microbiology by Powar and Daginawala, Himalaya Publishing House.
4. Microbial genetics by David friefelder

UNIT V - MICROBIAL TECHNIQUES

CONTENTS

LESSON - 18

ISOLATION OF MICROBES

Contents

18.0 AIMS AND OBJECTIVES

Microorganisms play a vital role for the welfare of human. These microbes had to be manipulated for such benefits. Thus the need arises for the study of microbial techniques for well exploitation of microbial population. In this unit, a brief discussion about the various microbial techniques including staining, culture techniques, sterilization methods along with strain improvement techniques.

18.1. INTRODUCTION

The development of microbiology as a scientific discipline dates from Louis Pasteur (1822-95). His studies and interest on fermentation paved way for the evolution of basic principles and techniques in microbiology. In the course of his studies, he introduced techniques of sterilization, varied growth needs of different bacteria. With the advent of microscopy by Antony Van Leeuwenhoek the structural characterization of the microorganism was made possible. Different staining techniques rose for the structure identification of microorganism and were classified into various groups based on their ability to uptake stain.

18.2. ISOLATION OF MICROBES

In natural environments a single kind of bacterium, ie a bacterial species, usually occurs as only one component of a large and complex population containing many other species. To study the characteristics of one species, it must be isolated as pure culture. Once obtained, a pure culture can be maintained or preserved in a culture collection.

18.2.1. SERIAL DILUTION TECHNIQUE

The dilution of a specimen in successive stages. Thus a 1:100 dilution is achieved by combining one part of a 1:10 dilution with nine parts of diluent. Soil harbors a wide variety of microbial life. Most soil organisms are responsible for decomposition and nutrient cycling. With out these micro organisms, life on earth would very quickly come to a halt. Normal soil microbial communities include single–celled bacteria, filamentous bacteria (actinomycetes), fungi, cyanobacteria and algae. To isolate the microbial population from soil, water and air for the beneficial use by man serial dilution technique is used as a preliminary step. Here the heavy counts of microorganisms are reduced or diluted using sterile distil water thus facilitating the isolation of few desirable microorganisms from others by plating the various dilutions on their corresponding media. Usually the dilutions 10^{-2}, 10^{-3} gives rise to fungi; 10^{-4} gives rise to actinomycetes and 10^{-5} and 10^{-6} gives rise to bacteria. Basal medium are used for the enrichment of the growth of microorganisms isolated from soil. The bacterial colonies formed on the medium are calculated by the following formula:

$$\text{CFU \{Colony forming units\}} = \frac{\text{Number of colonies}}{\text{Volume of sample plated} * \text{Dilution factor}}$$

18.2.2. PURE CULTURE TECHNIQUE

Several different methods are used for the isolation of pure cultures of microorganisms. These methods involve separating microorganisms on a solid medium into individual cells that are then allowed to reproduce to form a colony.

18.2.2.1. STREAK PLATE

Several different streaking patterns can be used to achieve separation of individual bacterial cells on the agar surface. In the streak plate technique, a loopful of bacterial cells is streaked across the agar – solidified surface of a nutrient medium. The plates are then incubated under favorable conditions to permit the growth of the microorganisms. The key to this method is that, by streaking, a diluent gradient is established across the face of the plate, so that while confluent growth occurs on part of the plates where the bacterial cells are not sufficiently separated, isolated colonies do develop in another region of the plate. The isolated colonies can then be picked, using sterile loop and restreaked onto a fresh medium to ensure purity.

18.2.2.2. SPREAD PLATE

In the spread plate method a small volume of a suspension of microorganisms is placed on the centre of an agar plate and spread over the surface of the agar by using a sterile glass rod. The glass rod is normally sterilized by dipping in alcohol and flaming to burn off the alcohol. By spreading the suspension over the plate, an even layer of cells is established so that individual microorganisms are separated from the other organisms in suspension and deposited at a discrete location. In order to accomplish this, it is often necessary to dilute the suspension before application to the agar plate prevents overcrowding and the formation of confluent growth rather than the desired development of isolated colonies.

18.2.2.3. POUR PLATE

In the pour plate technique, suspensions of microorganisms are added to melted agar tubes that have been cooled to approximately 42-45°C. the bacteria and agar medium are mixed well and the suspensions are poured into sterile Petri dishes using aseptic technique. The agar is allowed to solidify, trapping the bacteria at separate discrete positions within matrix of the medium. Although the medium holds bacteria in place, it is soft enough to permit growth of bacteria and the formation of discrete isolated colonies both within fluid on the surface of the agar.

18.3. LET US SUM UP

Using serial dilution technique the microorganisms are diluted for efficient separation into pure cultures.

The microbes are isolated into pure culture using quadrant streaking from a consortium of microbes.

Pour plate technique is used to isolate anaerobes.

18.4. LESSON END ACTIVITIES

Take a gram of soil from your garden and try serial dilution technique.

Take 1 ml of water from tank and check for the microbial community present.

Explain the principle of serial dilution technique.

Explain the importance of pure culture techniques.

18.5. POINTS FOR DISCUSSION

Explain the principle and procedure in detail the serial dilution technique.

Explain the importance of spread, streak and pour plate techniques.

18.6 REFERENCES

1. Microbiology by Pelczar, Reid and Chan, McGraw Hill Book Company.
2. Microbiology, Fundamental and Applications by R.A. Atlas, McMillan Publishers.
3. General Microbiology by Powar and Daginawala, Himalaya Publishing House.
4. Microbial genetics by David friefelder

LESSON – 19

CULTURE PRESERVATION TECHNIQUES AND MICROBIAL CULTURE COLLECTION CENTERS

Contents

19.0 AIMS AND OBJECTIVES

The chapter deals with the preservation techniques of the cultured microorganisms.

19.1 INTRODUCTION

Most microbiology labs maintain a large collection of strains, frequently referred to as a stock culture collection. These organisms are needed for laboratory classes and research work. The strains are used for screening of new, potentially effective chemotherapeutic agents; as assay tools for vitamins and amino acids; as agents for the production of vaccines, antisera, antitumor agents, enzymes and organic chemicals; and as reference cultures that are cited in company patents. Several methods have been developed, since not all bacteria respond in a similar manner to a specific method. All methods have ultimate aim of maintaining strains alive and uncontaminated and to prevent nay change in their characteristics.

19.2 METHODS OF MAINTENANCE AND PRESERVATION

19.2.1. PERIODIC TRANSFER TO FRESH MEDIA

Strains can be maintained by periodically preparing a fresh stock culture from the previous stock culture. The culture medium, the storage temperature, and the time interval at which the transfers are made vary with the species and must be ascertained beforehand. The temperature and the type of medium chosen should support a slow rather than a rapid rate of growth so that the time interval between transfers can be as long as possible. Many of the more common Heterotrophs remain viable for several weeks or months on a medium like nutrient agar. The transfer method has the disadvantage of failing to prevent changes in the characteristics of a strain due to the development of variants and mutants.

PRESERVATION IN DISTIL WATER

Over 50 years ago, Castellani introduced the concept that fungi could preserved in distil water. This preservation method was extensively studied on 594 fungal strains, with 62% of the strains growing and maintaining their original morphology. In another study, 76% of yeasts, filamentous fungi and actinomycetes survived storage in distil water for 10 years.

From the margins of well grown fungal cultures, plugs are cut with sterile polypropylene transfer tubes and transferred to cryo vials containing approximately 2 ml of sterile distil water. Alternatively, Wheaton 4 ml glass vials with rubber lined caps may be used. Then the vials are stored at room temperature.

PRESERVATION BY OVERLAYING CULTURES WITH MINERAL OIL

Many bacteria can be successfully preserved by covering the growth on an agar slant with sterile mineral oil. The oil must cover the slant completely; to ensure this, the oil should be about half inch above the tip of the slanted surface. Maintenance of viability under this treatment varies with the species (one month to years). This method of maintenance has the unique advantage that one can remove some of the growth under the oil with a transfer needle, inoculate a fresh medium, and still preserve the original culture. The simplicity of the method makes it attractive, but changes in the characteristics of a strain can still occur.

PRESERVATION BY LYOPHILIZATION (FREEZE – DRYING)

Most bacteria die if cultures are allowed to become dry, although spore and cyst formers can remain viable for many years. However, freeze – drying can satisfactorily preserve many kinds of bacteria that would be killed by ordinary drying. In this process a dense cell suspension is placed in small vials and frozen at -60 to -78°C. The vials are then connected to a high vacuum line. The ice present in the frozen suspension sublimes under the vacuum, ie, evaporates without first going through a liquid water phase. This results in dehydration of the bacteria with a minimum damage to delicate cell structures. The vials are then sealed off under a vacuum and stored in a refrigerator. Many species of bacteria preserved by this method have remained viable and unchanged in their characteristics for more than 30 years. Only minimal storage space is required; hundreds of lyophilized cultures can be stored in a small area. Furthermore, the small vials can be sent conveniently through the mail to other microbiology labs when packaged in special sealed mailing containers. Lyophilized cultures are revived by opening the vials, adding liquid medium, and transferring the rehydrated culture to a suitable growth medium.

STORAGE AT LOW TEMPERATURES

The ready availability of liquid nitrogen has provided the microbiologist with another very useful means for long-term preservation of cultures. In this procedure cells are prepared as a dense suspension in a medium containing a cryoprotective agent such as glycerol or dimethyl sulphoxide, which prevents cell damage due to ice crystal formation during the subsequent steps. The cell suspension is sealed into small ampoules or vials and then frozen at a controlled rate to -150°C. The ampoules or vials are then stored in a liquid nitrogen refrigerator (essentially a large tank having vacuum-insulated walls) either by immersion in the liquid nitrogen (-196°C) or by storage in the gas phase above the liquid nitrogen (-150°C). The liquid nitrogen method has been successful with many species that cannot be

preserved by lyophilization, and most species can remain viable under these conditions for 10 to 30 years or more without undergoing change in their characteristics. This method is quite expensive as at regular intervals the nitrogen refrigerators has to be replaced for the loss due to evaporation.

PRESERVATION USING SILICA GEL

Neurospora has successfully preserved over silica gel. Screw cap tubes, half filled with desiccant activated silica gel (6-12 mesh, grade 40) are oven sterilized. After the tubes have cooled, a skim milk (10%V/V) suspension of conidia or mycelium is dispersed (0.5 ml) into each tube. The tubes are quickly cooled to reduce heat generated as the liquid is absorbed and then vortexed to break up clumps. After being dried at 25°C, they are stored in closed containers with desiccants.

PRESERVATION ON PAPER

Spore forming fungi, actinomycetes, and unicellular bacteria can be preserved by drying the spores on some inert substrates. Fruiting bodies of the Myxobacteria, containing myxospores, may be preserved on pieces of sterile filter paper and stored at room temperature or at 6°C for 5 to 15 years.

Pieces of agar containing fruiting bodies are placed on sterile filter paper in a Petri dish, dried in desiccators under vacuum, and stored at room temperature. Alternatively, vegetative cells are transferred from the growth medium to small pieces of sterile filter paper on water agar and incubated until fruiting bodies develop. After the development, they are allowed to mature for 8 days. The filter papers are then placed into sterile containers, such as screw cap tubes, and dried over silica gel in an evacuated desiccator. After few days the containers are tightly closed and stored.

PRESERVATION ON BEADS

The method was developed by Lederberg, is successful for many bacteria. Porcelain beads (10 – 12 “Fishspine” beads no. 2) are autoclaved in screw cap glass vials (10 ml). Cell suspensions are prepared from 24 to 48 hours culture slants with a 20% (W/V) sucrose solution. The sterile beads are transferred to a sterile Petri dish and inoculated (0.2 – 0.3 ml per bead) with the cell suspension. The beads are returned to the vial with sterile forceps, and the vial is loosely capped and dried in a vacuum desiccator for 72 – 96 hours. A sterile spatula may be used to break the beads apart. Storage is at 25°C in a closed metal cabinet containing Drierite.

PRESERVATION ON SOIL

For many soil borne species, survival in soil is necessary. Survival for up to 20 years in soil has been reported for *Pythium, Fusarium, Verticillium* spp. Survival for >1 year in soil is relatively common for other plant pathogens.

PROTOCOL FOR DRYING ON SOIL

Fungal cultures are grown on agar medium. A spore suspension is prepared and pipetted into tubes of dry, sterile soil (1 ml of suspension per 5 g of soil) and allowed to dry at 25°C the tubes are stored at room temperature.

PROTOCOL FOR PRESERVING ACTINOMYCETES ON SOIL

To 100g of potting soil measured into a 250 ml Erlen meyer flask (twice sterilized for 1 h, twice incubated at 37°C overnight) is added 0.25% dry blood made up of blood fibrin and hemoglobin (10: 1) plus 1% calcium carbonate. The mixture is autoclaved for 1hr, and 20 ml of distil water is added. The flasks are inoculated with a water suspension of the culture (2 ml) and incubated. When mycelial growth is evident, the flasks are covered with parafilm and stored at 5°C. alternatively, screw cap tubes filled to a depth of approximately 4 cm may be used.

19.3 MICROBIAL CULTURE COLLECTION CENTERS

When microbiologists first began to isolate pure cultures, each microbiologist kept a personal collection of those strains having special interest. Subcultures of some strains were often sent to other microbiologists; other subcultures were received and added to the scientist's own collection. Certain strains had taxonomic importance because they formed the basis for descriptions of species and genera. Others had special properties useful for various purposes. However, many important strains became lost or were inadequately maintained. Thus, it became imperative to establish large central collections whose main purpose would be the acquisition, preservation, and distribution of authenticate cultures of living microorganisms.

Many countries have at least one central collection. As example, in France a collection of bacteria is maintained at the Institute Pasteur in Paris; in England the National Collection of Type Cultures is in London; the Federal Republic of Germany maintains the Deutsche Sammlung von Microorganism in Darmstadt; Microbial Type Culture Collection, IMTECH in India and Japan maintains a large collection at the institute for Fermentation in Osaka. Many other such collections exist.

In the United States the major collection is the American Type Culture Collection (ATCC), located in Rockville, Maryland. In 1980 the collection included the following numbers of strains: bacteria, 11500; bacteriophage, 300; fungi and fungal viruses, 13700; protozoa, 720; algae, 130; animal – cell cultures, 500; animala viruses, rickettsiae, and chlamydiae, 1135; and plant viruses, 220. The Quartermaster Research and Development Center, U.S. Army, Natick, Massachusetts, maintains a collection of microbial strains that are associated with deterioration processes. A number of smaller collections of a specialized nature exist, such as the collection of anaerobic bacteria maintained by the Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg.

One of the major functions of a large national collection is the preservation of type strains. The type strain of a species has great taxonomic importance because it is the name bearer strain, or permanent example, of the species. Microbiologists whose propose a new species are expected to deposit the type strain with one or more national collections so that it can be preserved and so that subcultures can be distributed to other workers for study and comparison with other microorganisms.

19.4. LET US SUM UP

Preservation of microbial cultures is very important for the efficient manipulation of the organisms.

Preservation is carried out in various methods to store microbes for decades.

The microbes are preserved for future usage and they are maintained in culture banks or microbial culture collection centres.

19.5. LESSON END ACTIVITIES

Check for the various microbial culture collection centres in India.

Take soil and try for the preservation of microbes in soil method.

List the commonly available microbial culture collection centres.

Explain the preservation of microbes at low temperature.

Explain the preservation using silica gel.

19.6. POINTS FOR DISCUSSION

Explain in detail the preservation by lyophilization.

Explain the preservation of microbes using paper and on beads.

19.7 REFERENCES

1. Microbiology by Pelczar, Reid and Chan, McGraw Hill Book Company.
2. Microbiology, Fundamental and Applications by R.A. Atlas, McMillan Publishers.
3. General Microbiology by Powar and Daginawala, Himalaya Publishing House.
4. Microbial genetics by David friefelder

LESSON 20

STAINING TECHNIQUES

Contents

20.0 AIMS AND OBJECTIVES

The chapter deals with the staining techniques for the morphology study and identification of microorganisms.

20.1 INTRODUCTION

The coloring agents impart a colour to the colorless microorganisms. Due to this coloration the microorganisms become visible, so as to observe it s cell shape, and structures. These stains are composed of a positive and negative ion, one of which is colored and is known as chromophore. The basic dyes are in positive ion while acidic dyes in the negative. Since bacteria are towards negatively charged at pH 7, thus the colored positive ion in a basic dye binds to the negatively charged bacterial cell. Some dyes are classified as acidic dye and others as basic dyes.

20.2 GRAM STAINING

20.2.1 PURPOSE

To become familiar with

15 The chemical and theoretical basis for differential staining procedures.
16 The chemical basis of the gram stain.
17 Performance of the procedure for differentiating between the two principles groups of bacteria: gram – positive and gram – negative.

20.2.2 PRINCIPLE

Differential staining requires the use of at least three chemical reagents that are applied sequentially to a heat fixed smear. The first reagent is called the primary stain. Its function is to impart its color all cells. In order to establish a color contrast the secondary reagent used is the decolorizing agent. Based on the chemical composition of cellular components the decolorizing agent may or may not remove the primary stain from the entire cell or only from certain cell structure's the final reagent the counter stain has a contrasting color to that of the primary stain. Following decolourization, if the primary stain is not washed out, the counter stain cannot be absorbed and the cell or its components will retain the color of the primary stain. If the primary stain is removed, the decolorized cellular components will accept and assume the contrasting color of the counter stain. In this way, cell types or there structures can be distinguished from each other on the basis of the stain that is retained.

The most important differential; stain used in bacteriology is the gram stain named after Dr.Christian Gram it divides bacterial cells into two major groups gram positive and gram negative which makes it, an essential tool for classification and differentiation of micro organisms. The gram stain reaction is based on the difference in the chemical composition of bacterial cell walls. Gram positive cells have a thick peptidoglycan layer, whereas the peptidoglycan layer in gram negative cells is much thinner and surrounded by outer lipid containing layers. Early experiments have shown that if the gram positive cell is denuded of its cell wall by the action of lysozyme or penicillin the gram positive cell will stain gram negative. The gram stain uses four different reagents. Descriptions of these reagents and there mechanisms of action follow.

Primary stain: Crystal violet (Hucker's): The violet stain is used first and stains all cells purple.

Mordant: *Gram's iodine:* This reagent serves as a mordant, a substance that increases the cells' affinity for a stain. It does this by binding to the primary stain, thus forming an insoluble complex. The resultant crystal-violet-iodine complex serves to intensify the color of these stains at this point all cells will appear purple black.

Decolorizing agent: *Ethyl alcohol, 95%:* This reagent serves a dual function as a protein-dehydrating agent and as a lipid solvent. Its action is determined by two factors, the concentration of lipids and the thickness of the peptidoglycan layer in bacterial cell walls. In gram negative cells, the alcohol increases the porosity of the cell wall by dissolving the lipids in the outer layer. Thus the CV-I complex can be more easily removed from the thinner and less highly cross-linked peptidoglycan layer. Therefore, the washing out effect of the alcohol facilitates the release of unbound CV-I complex, leaving the cells colorless or unstained. The much thicker peptidoglycan layer in gram positive cells is responsible for the more stringent retention of the CV-I complex, as the pores are made smaller due to the dehydration effect of the alcohol. Thus the tightly bound primary stain complex is difficult to remove, and the cells remain purple.

Counter stain: *Safranin:* This is the final reagent used to stain red those cells that have been previously decolorized. Since only gram negative cells undergo decolourization, they may now absorb the counter stain. Gram positive cells retain the purple colour of the primary stain.

20.2.3. PRECAUTIONS

The preparation of adequately stained smears requires following precautions:

1. the most critical phase of the procedure is the decolourization step, which is based on the ease with which the CV-I complex is released from the cell. Over decolourization will result in the loss of primary stain, causing gram positive organisms to appear negative.
Under decolourization, however, will not complete remove the CV-I complex, causing Gram negative to appear gram positive.
2. Between applications of the reagents, the slides should be thoroughly washed under running water or water applied with an eye dropper this removes the excess reagent and prepare the slide for the application of the subsequent reagents.
3. The best gram stained preparations are made with fresh cultures i.e., not older than 24 hrs. As the culture age, especially in the case of gram positive cells, the organisms tend to lose their ability to retain the primary stain and may appear to be gram-variable, i.e., some cells will purple while the others will appear red.

20.3 ENDOSPORE STAINING (SCHAEFFER-FULTON METHOD)

20.3.1. PRINCIPLE

Members of the anaerobic genera clostridium and desulfotomaculum and the aerobic genus bacillus are examples of organisms that have the capacity to exist either as metabolically active vegetative cells or as highly resistant, metabolically inactive cell types called spores. When environmental conditions become unfavorable for continuing vegetative cellular activities, particularly with the exhaustion of a nutritional carbon source, these cells have the capacity to undergo sporogenesis and give rise to a new intracellular structure called the endospore, which is surrounded by impervious layers called spore coats. As conditions continue to worsen, the endospore is released from the degenerating vegetative cell and becomes an independent cell called a spore. Because of the chemical composition of spore layers, the spore is resistant to the deleterious effects of excessive heat, freezing, radiation, desiccation, and chemical agents, as well as to the commonly employed micro biological stains, with the return of favorable environmental conditions, the free spore may revert to a metabolically active and less resistant vegetative cell through germination. It should be emphasized that sporogenesis and germination are not means of reproduction but merely mechanisms that ensure cell survival under all environmental conditions.

In practice, the spore stain uses two different reagents.

Primary stain: Malachite green unlike most vegetative cell types that stain by common procedures, the spore, because of its impervious coat, will not accept the primary stain easily. For further penetration, the application of heat is required. After the primary stain is applied and the smear is heated, both the vegetative cell and spore will appear green.

Decolorizing Agent: Water once the spore accepts the malachite green, it cannot be decolorized by tap water; which removes only the excess primary stain. The spore remains green. On the other hand, the stain does not demonstrate a strong affinity for vegetative cell components; the water removes it, and these cells will be colorless.

Counter stain: Safranin: this contrasting red stain is used as the second reagent to color the decolorized vegetative cells, which will absorb the counter stain and appear red. The spores retain the green of the primary stain.

20.4. NEGATIVE STAINING

20.4.1. PURPOSE

Negative staining is performed for the observation of intact microbial structures without disturbing its cellular morphology.

20.4.2. PRINCIPLE

Negative staining requires the use of acid stain such as India ink or nigrosin. The acid stain with its negatively charged chromogen will not penetrate the cells because the negative charge on the surface of the bacteria. Therefore the unstained cells are easily discernible against the colored background. The practical application of negative staining is two fold. First since heat fixation is not required and the cells are not subjected to the distorting effects of chemicals and heat, their natural size and shape can be seen. Second, it is possible to observe bacteria that are difficult to stain, such as some spirilli.

20.5 FLAGELLAR STAINING

It is the part of the bacterial cell meant for locomotion. The flagella can not be seen in the light microscope. A mordant used to build up the diameter of flagella until they become visible microscopically when stained with carbol fuchsin.

20.6 METHYLENE BLUE STAINING

The aqueous or alcoholic solution of a single basic dye is called simple staining for example methylene blue, crystal violet, carbol fuschin and Safranin. These help in studying the cellular shape and structures of microorganisms.

20.7. LET US SUM UP

Grams christain discovered Gram's staining technique.
Gram's staining technique differentiates the microorganism into Gram positive and negative respectively.
Negative staining is done to identify the capsulated organisms.
Flagellar staining differentiates the motile and non motile organisms.

20.8. LESSON END ACTIVITIES

Take curd and identify the microorganisms available using suitable staining technique and report their morphology.
Take *E.coli* culture and check its motility ability using suitable staining technique.
How the microorganisms can be identified?
How the Gram staining should be done. Explain the principle.
Explain the flagellar staining.

20.9. POINTS FOR DISCUSSION

Explain in detail the Gram's staining technique with principle.

Explain the following staining techniques – flagellar, capsule, methylene blue staining.

20.10. REFERENCES

1. Microbiology by Pelczar, Reid and Chan, McGraw Hill Book Company.
2. Microbiology, Fundamental and Applications by R.A. Atlas, McMillan Publishers.
3. General Microbiology by Powar and Daginawala, Himalaya Publishing House.
4. Microbial genetics by David friefelder

LESSON – 21

INOCULUM DEVELOPMENT

Contents

21.0 AIMS AND OBJECTIVES

The chapter discusses about the inoculum development for the industrially important products production.

21.1 INTRODUCTION

It is essential that the culture used to inoculate fermentation satisfies the following criteria:

It must be in a healthy, active state thus minimizing the length of the lag phase in the subsequent fermentation.

It must be available in sufficiently large volumes to provide an inoculum of optimum size.

It must be in a suitable morphological form.

It must be free of contamination.

It must retain its product-forming capabilities.

The process adopted to produce an inoculum meeting these criteria is called inoculum development.

21.2 DEVELOPMENT OF INOCULA FOR YEAST

It is common practice in the British brewing industry to use the yeast from the previous fermentation to inoculate a fresh batch of wort. The brewing terms used to describe this process are crop, referring to the harvested yeast from the previous fermentation, and pitch, meaning to inoculate. The dangers inherent in this practice are the introduction of contaminants and the degeneration of the strain, the most common degenerations being a change in the degree of flocculence and attenuating abilities of yeast. In breweries employing top fermentations these dangers are minimized by collecting yeast to be used for future pitching form middle skimming. During fermentation the yeast cells flocculate and float to the surface, the first cells to do this being the most flocculent and the last cells the least flocculent. As the head of yeast develops, the surface layer is removed and discarded and the underlying cells are harvested and used for subsequent pitching. Therefore, the ‘middle skimmings’ contains cells which have the desired flocculence and which have been protected from contamination by the surface layer of the yeast head. The pitching yeast may be treated to reduce the level of contaminating bacteria and remove protein and dead yeast cells by such treatments as reducing the pH of the slurry to 2.5 to 3, washing with water, washing with ammonium per sulphate and treatment with antibiotics such as polymixin, penicillin and neomycin.

One of the key physiological features of yeast inoculum is the level of sterol in the cells. Sterols are required for membrane synthesis but they are only produced in the presence of oxygen. Thus oxygen is required for sterol synthesis, yet anaerobic conditions are required for ethanol production. This anomaly is resolved traditionally by aerating the wort before inoculation. This oxygen allows sufficient sterol synthesis early in the fermentation to support growth of the cells through out the process that is after the oxygen is exhausted and the process is anaerobic. Boulton et al. developed an alternative approach where the pitching yeast was vigorously aerated prior to inoculation. The yeast was then sterol rich and had no requirement for oxygen during the alcohol fermentation.

21.3 DEVELOPMENT OF INOCULA FOR BACTERIA

The main objective of inoculum development for bacterial fermentations is to produce an active inoculum which will give as short a lag phase as possible in subsequent culture. A long lag phase is disadvantageous because it is not only a waste of time but also medium is consumed in maintaining a viable culture prior to growth. The length of the lag phase is affected by the size of the inoculum and its physiological condition. The inoculum size normally ranges between 3 and 10% of the culture volume. The age of the inoculum is important in the growth of sporulating bacteria, for sporulation is induced at the end of the logarithmic phase.

Keay et al. recommends the use of a 5% inoculum of a logarithmically growing culture of a thermophilic *Bacillus* for the production of proteases.

STOCK CULTURE
Lyophilized with skim milk

↓

MAINTENANCE CULTURE
Agar slope incubated 4 days at 28°C

↓

SEED CULTURE – FIRST STAGE
2 dm^3 flask containing 0.6 dm^3 medium inoculated with culture from one slope; incubated with shaking for 48h at 28°C

↓

SEED CULTURE – SECOND STAGE
40-80 dm^3 fermenter containing 25-50 dm^3 inoculated with 1-1.2% first stage seed culture. Incubated 25-30h at 32°C

↓

PRODUCTION CULTURE
500 dm^3 fermenter 300 dm^3 medium inoculated with 5% second stage seed culture. Incubated at 32° for 140 to 160 h

(The inoculum development for vitamin B_{12} using *Pseudomonas denitrificans)*

DEVELOPMENT OF INOCULA FOR MYCELIAL AND VEGETATIVE FUNGI

In the preparation of inocula for fermentations employing mycelial organisms spore suspension is usually used as seed during an inoculum development programme. The major advantage of a spore inoculum is that it contains far more 'Propagules' than a vegetative culture. Three basic techniques are used to produce high concentration of spores for use as an inoculum.

Sporulation on solidified media: Most fungi and *Streptomycetes* will sporulate on suitable agar media but a large surface area must be employed to produce sufficient spores. Parker described the 'roll-bottle' technique for the production of spores of *Penicillium chrysogenum*.

Sporulation on solid media: Many filamentous organisms will sporulate profusely on the surface of cereal grains from which the spores may be harvested. Substrates such as barley, hard wheat bran, ground maize and rice are suitable for the sporulation of a wide range of fungi. The sporulation is affected by the amount of water added to the cereal before sterilization and the relative humidity of the atmosphere. *Aspergillus ochraces* is sporulated on 'pot' barley or wheat bran.

Sporulation in submerged culture: Many fungi will sporulate in submerged culture. This is more convenient because it is easier to operate aseptically and it may be applied on large scale. The technique was first adopted by Foster et al. for sporulation in *Penicillium notatum* with 2.5% calcium chloride in a defined nitrate-sucrose medium.

Vegetative fungi: An inoculum of vegetative mycelium is used for fungi which do not produce asexual spores. *Gibberella fujikuroi* is such a fungus and is used for the commercial production of Gibberellin. The major problem is the difficulty in obtaining a uniform standard inoculum. The procedure may be improved by fragmenting the mycelium in a homogenizer.

21.5 LET US SUM UP

The brewing terms used to describe this process are crop, referring to the harvested yeast from the previous fermentation, and pitch, meaning to inoculate.

The 'middle skimmings' contains cells which have the desired flocculence and which have been protected from contamination by the surface layer of the yeast head.

The major advantage of a spore inoculum is that it contains far more 'Propagules' than a vegetative culture.

21.6 LESSON END ACTIVITIES

Try to improve the inoculum of bacteria at the laboratory level.

Write about the sporulation on solidified media.

Write about the inoculum development for vegetative fungi.

21.7 POINTS FOR DISCUSSION

Write about the development of inocula for mycelial and vegetative fungi.

Explain about the development of inocula for bacteria.

Write about the development of inocula for yeast.

21.8 REFERENCES

1. Microbiology by Pelczar, Reid and Chan, McGraw Hill Book Company.
2. Microbiology, Fundamental and Applications by R.A. Atlas, McMillan Publishers.
3. General Microbiology by Powar and Daginawala, Himalaya Publishing House.
4. Microbial genetics by David friefelder

LESSON – 22
STERILIZATION METHODS AND CULTIVATION OF MICROBES

CONTENTS

22.0 AIMS AND OBJECTIVES

The chapter deals with the sterilization techniques and culturing of the microorganisms.

22.1 INTRODUCTION

Sterilization implies the complete destruction of all micro organisms including spores. A satisfactory sterilization process is designed to ensure high probability of achieving sterility. Example: A process that kills more than 10^6 spores of a defined, exceptionally high degree of resistance .The methods commonly used in microbiology laboratories are

16. **Dry heat** is sufficient for glass ware, instruments and articles not affected by very high temperature. Water, impermeable oils and waxes can be sterilized by dry heat.
17. **Moist heat** is effective at low temperature and is suitable for culture media, laboratory discards and porous packed materials.
18. **Gaseous chemical** sterilization may be used for heat sensitive articles.
19. **Filter sterilization** is the only means of sterilizing heat labile fluids.
20. **Radiation** is used in the manufacture of sterile packaged products such as disposable plastic syringes and materials being used in clinical microbiology laboratories.
21. **Chemical Disinfection** is the only convenient way for floors, bench tops, wash basins and other laboratory furniture's.

22.2 DRY HEAT

- **Flame sterilization**

Inoculating loops or wires, points of forceps and searing spatulas are held in a bunsen flame till they become red hot, in order to be sterilized. If the loops contain infective proteinaceous material they should be first dipped in chemical disinfectants before flaming to prevent spattering.

Scalpels, needles, mouths of culture tubes, glass slides, cover slips, etc. could be passed a few times through the bunsen flame without allowing them to become red hot.

- **Incineration**

This is an excellent method for rapidly destroying materials such as soiled dressings, animal carcasses, bedding and pathological material. Plastics such as PVC and polythene can be dealt with similarity but polystyrene materials emit clouds of dense black smoke and hence should be autoclaved in appropriate containers.

- **Hot Air Sterilizer {Hot Air Oven}**

This is the most widely used method of sterilization by dry heat. A holding period of 160°C for one hour is used. It is used to sterilize glassware, forceps, scissors, scalpels, all glass syringes, swabs, liquid paraffin, sulphonamides, dusting powder, fats, greases, etc. The oven is usually heated by electricity, with heating elements in the wall of the chamber and it must be fitted with a fan to ensure even distribution of air and elimination of air pockets. Glassware should be perfectly dry before being placed in the oven.

Test tubes, flasks, etc. should be wrapped in kraft paper. For cutting instruments such as those used in ophthalmic surgery, a sterilizing time of two hours at 150°C is recommended. A holding time of one hour at 150°C for oils, glycerol and dusting powder is recommended. The oven must be allowed to cool slowly for about two hours before the door is opened, since the glassware may get cracked by sudden or uneven cooling. Hot air is a bad conductor of heat and its penetrating power is low. Some of the various temperatures and holding time practiced for killing various microorganisms are listed in table 1.

Temperature (°C)	**Holding time (mins)**
160	45
170	18
180	7.5
190	1.5

TABLE 1. VARIOUS TEMPERATURES AND HOLDING TIME IN HOT AIR OVEN

22.3 MOIST HEAT

a) <u>Temperatures below 100°C</u> Pasteurization employs temperature either 63°C for 30minutes (the holder method) or 72°C for 15-20 seconds (the flash process) followed by cooling quickly to 13°C or lower. By these processes all nonsporing pathogens such as *Mycobacteria, Brucellae* and *Salmonellae* are destroyed.

Vaccines of nonsporing bacteria are heat inactivated in special vaccine baths at 60°C for one hour. Serum or body fluids containing coaguable proteins are sterilized at 56°C in water bath for half an hour on several successive days. Some of the various temperatures and holding time practiced for killing various microorganisms are listed in table 2.

Microorganisms	**Temperature (°C)**	**Holding time (mins)**
Staphylococcus aureus	60	30
Streptococcus faecalis	60	60
Clostridium botulinum	120/100	4/330
Poliomyelitis virus	60	30
Hepatitis virus	60	10hr
Bacteriophages	65-80	-
Vegetative forms of bacteria, yeast and	80	5-10

moulds		

TABLE 2. VARIOUS TEMPERATURES AND HOLDING TIME PRACTICED FOR KILLING VARIOUS MICROORGANISMS

b) Temperatures at 100°C **Boiling** kill almost all vegetative bacteria at 90 - 100°C. Some sporing bacteria require considerable periods of boiling. Boiling is not recommended for the sterilization of instruments used for surgical procedures and should be regarded only as a means of disinfection. Nothing short of autoclaving at high pressure can destroy spores and ensure sterilization. Hard water should not be used. Sterilization may be promoted by the addition of 2% sodium bicarbonate to the water. In cases where boiling is considered adequate, the material should be immersed in the water and boiled for a period of 10-30 mins. The lid of the sterilizer should not be opened during the period.

c) Steam at atmospheric pressure (100°C) an atmosphere of free steam is used to sterilize culture media which may decompose if subjected to higher temperatures. A Koch or Arnold steamer is usually used. It is an inexpensive method.

The usual steamer consists of a tinned copper cabinet with the walls suitably lagged. The lid is conical, enabling drainage of condensed steam, and a perforated tray fitted above the water level ensures that the material placed on it is surrounded by steam. One exposure of ninety minutes usually ensures complete sterilization but for media containing sugars or gelatin an exposure of 100°C for 20mins on three successive days is used. This is known as **tyndallization or intermittent sterilization**.

The principle is that the first exposure kills all vegetative bacteria and the spores present, being in a favourable medium, will germinate and be killed on the subsequent occasions. Though this is generally adequate, it may fail in the case of spores of certain anaerobes and thermophiles.

d) Steam under pressure The principle of the autoclave or steam sterilizer is that water boils when its vapour pressure equals that of the surrounding atmosphere. Hence when pressure inside a closed vessel increases, the temperature at which water boil also increases, the temperature at which water boils also increases. Saturated steam has greater penetrative power. When steam comes into contact with a cooler surface it condenses to water gives up its latent heat to that surface (1600ml of steam at 100°C and at atmospheric pressure condenses into one ml of water at 100°C and liberates 518 calories of heat). The large reduction in volume sucks in more steam to the area and the process continues till the temperature of that surface is raised to that of the steam. The condensed water ensures moist conditions for killing the microbes present.

Sterilization is carried out at temperatures between 108°C and 147°C. Materials that could be sterilized are dressings, instruments, laboratory ware, media and pharmaceutical products. Aqueous solutions are done between 108°C and 126°C. Heat is conducted through the walls of the sealed containers until the temperature of the fluid inside is in equilibrium with the steam outside.

Several types are laboratory autoclaves, hospital dressing sterilizers, bowl and instrument sterilizers and rapid cooling sterilizers.

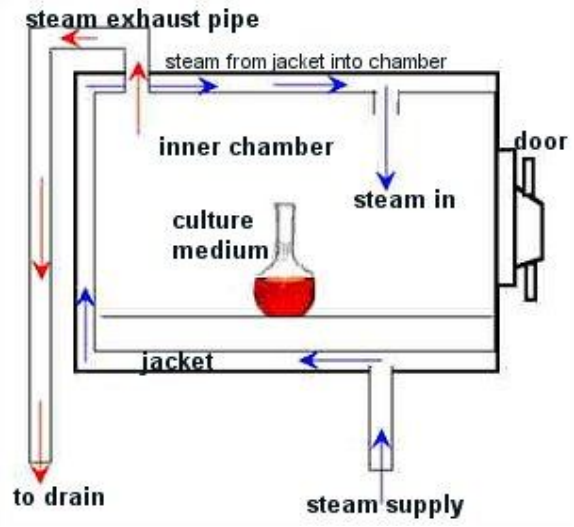

Fig.1 AUTOCLAVE

The laboratory autoclave consists of a vertical or horizontal cylinder of gunmetal or stainless steel, in a supporting sheet-iron case. The lid or door is fastened by screw clamps and made airtight by an asbestos washer. The autoclave has on its lid or upper side a discharge tap for air and steam, a pressure gauge and a safety valve that can be set to blow off at any desired pressure. Heating is done by electricity or gas. Some of the various temperatures and holding time practiced for killing various microorganisms are listed in table 3.

Temperature (°C)	**Holding time (mins)**
121	15
126	10
134	3

TABLE 3. VARIOUS TEMPERATURES AND HOLDING TIME PRACTICED FOR KILLING VARIOUS MICROORGANISMS

The discharge tap is opened slowly and air is allowed to enter the autoclave. If the tap is opened when the pressure inside is high, liquid media will tend to boil violently and spill from their containers and sometimes an explosion may occur. If opened after the pressure inside has fallen below atmospheric pressure, an excessive amount of water would have evaporated and lost from media.

22.4 FILTRATION

Filtration is used to sterilize solutions which are thermo labile and to remove particulate matter from solutions and gases. Eg. Antibiotic solutions, sera, carbohydrate solutions. Filters function by entrapping microorganisms with in the porous structure of the filter matrix. Vacuum or pressure is required to move solutions through the filter. There are four basic types of filters –

1. Candle filters 2. Asbestos filters 3. Sintered glass filters
4. Membrane filters

1. Candle filters are manufactured on different grades of porosity. Used for purification of water for industrial and drinking purposes.
Two types – **Unglazed ceramic filters** can be cleaned with sodium hypochlorite solution after use and withstand scrubbing. Eg. Chamberland and Doulton filters
Diatomaceous earth filters can be cleaned with sodium hypochlorite solution after use and do not withstand scrubbing. Eg. Berkefeld and Mandler filters
2. Asbestos filters are disposable and single use discs. High absorbing capacity and tend to alkalinize filtered solutions and are carcinogenic. Eg. Seitz, carbon, sterimat filters.
3. Sintered glass filters are prepared by heat fusing finely powdered glass particles of graded size. Low absorbance, easy clean, brittle and expensive.
4. Membrane Filters are made from polymeric materials such as cellulose nitrate, cellulose diacetate, poly bicarbonates and poly esters. Used in water purification and analysis, sterilization and sterility testing, preparation of solution for parenteral use. Since it is less absorptive than other filters, they have a faster rate of filtration for any given porosity. Membrane filters are manufactured as discs of 13-293 mm diameter with porosities from 0.015-12 μm. widely used pore size is 0.22 μm. The membrane filters can be sterilized by autoclave.

22.5 RADIATION

Two types of radiation are used for sterilizing purposes: non ionizing and ionizing.
Non ionizing radiation comprises electromagnetic rays with wave lengths longer than visible light and absorbed as heat. Hence considered as a form of hot air sterilization.
Eg: Infra red radiation – rapid mass sterilization of syringes
Ultra violet radiation – disinfecting enclosed areas such as entryways, hospital wards, operation theatres and small virus inoculation rooms and virus laboratories. The efficiency of radiation is dependent on the absorption dose and the choice of radiation dose should be determined by the bio burden and the configuration and composition of the material to be sterilized.

Ionizing radiation has high penetrative power. No appreciable increase in temperature and referred as cold sterilization.
Eg: X-rays, gamma rays and cosmic rays.
These rays are lethal to DNA and other vital cell constituents.
Gamma radiation source is from a radioactive element, usually Co 60. Large commercial plants use gamma radiation for sterilizing most plastics, syringes, swabs, culture plates, catheters, animal feeds, various types of rubber, cardboard, oils, greases, fabrics and metal foils.
Ultrasonic and sonic vibrations show bactericidal property. Microorganisms vary in their sensitivity to them and survivors have been found after such treatment, hence of no practical use.

22.6 CHEMICAL AGENTS

Gaseous chemical – Ethylene oxide sterilizer Medical and surgical articles that cannot with stand even heating at 73°C can be treated with ethylene oxide. This highly lethal gas is an alkylating agent and kills all microorganisms including viruses.
Ethylene oxide is toxic and highly explosive. The gas is mutagenic in a variety of animals. The concentration in the environmental air should not exceed an average of 5 ppm over an 8 hour period. Ethylene oxide should be used in purpose designed pressure vessels capable of

withstand an explosion. It should be operated at sub atmospheric pressure in a spark –free environment.

Mode of action is by alkylating the amino, carboxyl, hydroxyl and sulphydryl groups in protein molecule. Reacts with DNA and RNA.

Used to sterilize heart – lung machines, respirators, sutures, dental equipment.

Formaldehyde is widely employed for fumigation of operation theatres and other rooms. Heat resistant vessels should be used as it generates heat. Formaldehyde gas is generated by adding 150 g of $KMnO_4$ to 280 ml formalin for every 1000 cu ft of room volume.

Betapropiolactone condensation product of ketone and formaldehyde with a boiling point of 163°C. Have low penetrating power and efficient for fumigating than formaldehyde. Has both biocidal and carcinogenic property and has broad spectrum of cidal activity against all microorganisms and viruses.

Disinfectant

A disinfectant is used to eliminate some undesirable organisms in order to prevent their transmission. The disinfectant kills vegetative bacteria, fungi, viruses and occasionally spores by the destruction of proteins, lipids or nucleic acids in the cell or its cytoplasmic membrane.

A disinfectant may have a narrow spectrum of activity against micro organisms. Most Gram positive bacteria are easily killed by disinfectants, how ever, some Gram negative bacteria especially species of *Pseudomonas* are more resistant. Acid – fast bacteria are still more resistant .Action of disinfectant on viruses and spores may be uncertain or absent.

22.7 COMMON DISINFECTANT USED IN LABORATORIES

Phenols: Lister introduced as antiseptic during surgery. The lethal effect of phenols is due to their capacity to cause cell membrane damage, release cell contents and cause lysis. Low concentration precipitates protein and membrane bound oxidases and dehydrogenases.

Eg: phenol (carboxylic acid) – powerful microbicidal.

Lysol and cresol – active against wide range of microorganisms. Toxic to man.

Chlorophenols and chloroxyphenols – less toxic and irritant, less active, inactive against *Pseudomonas*. Various combinations used to control of pyogenic cocci in surgical and neonatal units in hospitals.

Hexachlorophene – toxic

Chlorohexidine (Hibitane) – Non toxic to skin and active against Gram positive organisms. Aqueous solution used in treatment of wound.

- **Hypochlorite**: Hypochlorite is corrosive to metal and textiles and have poor wetting properties. They are readily inactivated by organic materials and cationic detergents. Hypochlorites are corrosive to metal and textiles and have poor wetting properties. They are readily inactivated by organic materials and cationic detergents. Hypochlorites are very active against mycobacterium. The effective concentration for spilt blood and for virology laboratories is 10, 000 parts per million and 1000 ppm for cleaning.
- **Gutturaldehyde**: It is less corrosive than hypochlorite. It is rapid bactericidal and viricidal disinfectant and is effective against mycobacterium and to a lesser extent to spores. The killing of spores may require at least 3 hours exposure. 2% glutaraldehyde is recommended for defined purposes in virology and immunology laboratories including decontamination of centrifuges and automated equipments that can resist the damage by glutaraldehyde.
- **Ethyl alcohol**: Ethanol at 70 %(v\v) in water is highly active against gram positive and gram negative bacteria and acid fast bacilli. However, it is in active against spores. To a lesser extent it is susceptible to in activation by organic matter. It should not be used on heavily solid surface has poor power of penetration. Ethyl alcohol can be removed from the articles by flaming but the temperature reached on the surface is not high enough for

sterilization. Ethanol should be used only on perfectly clean surfaces such as glass slides and the tops of inoculation chambers for the removal of transient bacteria.

Formaldehyde disinfection

For 1000 cubic feet of space, place 500 ml of formaldehyde (40%solution) and 100 ml of water in an electric boiler and switch on the boiler. Leave the room and seal the door. Leave the room filled with formaldehyde and seal for 24 hours.

After 24 hours open the door and window to allow the vapors to dispense and neutralize any residual formaldehyde with ammonia exposing 250 ml ammonia per liter of formalin used.

22.8 CULTIVATION OF MICROORGANISMS

Nutritional and Physical Requirements and Enumeration of Microbial Populations:

As do all other living organisms, microorganisms require certain basic nutrients and physical factors for the sustenance of life. However, their particular requirements vary greatly. Understanding these needs is necessary for successful cultivation of microorganisms in the laboratory.

Nutritional needs: Nutritional needs of microbial cells are supplied in the laboratory through a variety of media. The following list illustrates the nutritional diversity that exists among microbes.

1. Carbon: this is the most essential and central atom common to all cellular structures and functions. Among microbial cells, two carbon-dependent types are noted:
   a. autotrophs: these organisms can be cultivated in a medium consisting solely of inorganic compounds; specifically, they use inorganic carbon in the form of carbon dioxide.
   b. Heterotrophs: these organisms cannot be cultivated in a medium consisting solely of inorganic compounds; they must be supplied with organic nutrients, primarily glucose.

2. Nitrogen: this is also an essential atom in many cellular macromolecules, particularly proteins and nucleic acids. Proteins serve as the structural molecules forming the so-called fabric of the cell and as functional molecules, enzymes that are responsible for the metabolic activities of the cell. Nucleic acids include DNA, the genetic basis of cell life, and RNS, which plays an active role in protein synthesis within the cell. Some microbes use atmospheric nitrogen, others rely on inorganic compounds such as ammonium or nitrate salts, and still others require nitrogen-containing organic compounds such as amino acids.

3. Nonmetallic elements: the major nonmetallic ions used for cellular nutrition are
   - Sulfur: this is integral to some amino acids and is therefore a component of proteins. Sources include organic compounds such as sulfur-containing amino acids, inorganic compounds such as sulfates, and elementary sulfur:
   - Phosphorus: this is necessary for the formation of the nucleic acids DNA and RNA and also for synthesis of the high-energy organic compound adenosine triphosphate, ATP. Phosphorus is supplied in the form of phosphate salts for use by all microbial cells.

18 Metallic elements: Ca^{++}, Zn^{++}, Na^{++}, Cu^{++}, K^{+}, Mn^{++}, Mg^{++}, and Fe^{+2},Fe^{+3} are some of the metallic ions necessary for continued efficient performance of varied cellular activities. Some of these activities are osmoregulation, regulation of enzyme activity, and electron transport during bio oxidation. Remember that these ions are micronutrients and are required in trace concentrations only. Inorganic salts supply these materials.

19 Vitamins: these organic substances contribute to cellular growth and are essential in minute concentrations for cell activities. They are also sources of coenzymes, which are required for the formation of active enzyme systems. Some for normal metabolic activities. Some possess extensive vitamin-synthesizing pathways, whereas others can synthesize only a limited number from other compounds present in the medium.

20 Water: all cells require water in the medium so that the low-molecular-weight nutrients can cross the cell membrane.

21 Energy: active transport, biosynthesis, and biodegradation of macromolecules are the metabolic activities of cellular life. These activities can be sustained only if there is a constant availability of energy within the cell. Two bioenergetic types of microorganisms exist:

i) Phototrophs: these use radiant energy as their sole energy source.

ii) Chemotrophs: these depend on oxidation of chemical compounds as their energy source. Some microbes use organic molecules such as glucose; others utilize inorganic compounds such as H_2O or $NaNO_2$

22 Inducers: the majority of enzymes which are of industrial interest are inducible. Induced enzymes are synthesized only in response to the presence in the environment of an inducer. Inducers are often substrates such as starch or dextrins for amylases, maltose for pullulanase and pectin for pectinases. Some inducers are very potent, such as isovaleronitrile inducing nitralase. Substrate analogues that are not attacked by the enzyme may also serve as enzyme inducers. Most inducers which are included in microbial enzyme media are substrates or substrate analogues, but intermediates and products may sometimes be used as inducers. For example, maltodextrins will induce amylase and fatty acids induce lipase.

One commercial system that has been developed is based on the alcA promoter in *Aspergillus nidulans* to express human interferon α 2. This can be induced by volatile chemicals, such as ethylmethyl ketone, which are added when biomass has increase to an adequate level and the growth medium contains a non-repressing carbon source or low non-repressing levels of glucose. For example yeast mannans for the enzyme α Mannosidase, phenylaceticacid for penicillin acylase and cellulose for cellulase are used as inducers in industry.

23 Precursors: Some components of a fermentation medium help to regulate the production of the product rather than support the growth of the microorganism. Such additives include the precursors. The significance of the different side chains was first appreciated when it was noted that the addition of corn-steep liquor increase the yield of penicillin from 20 units cm^{-3} to 100 units cm^{-3}. Corn-steep liquor was found to contain phenylethylamine which was preferentially incorporated into the penicillin molecule to yield benzyl penicillin (PenicillinG). Phenylaceticacid is still the most widely used precursor in penicillin production. Few examples of precursors are β-iononones for carotenoids, propionate for riboflavin, phenoxyaceticacid for penicillin V, D-Threonine for L-Isoleucine etc.

24 Inhibitors: When certain inhibitors are added to fermentations, more of a specific product may be produced, or a metabolic intermediate which is normally metabolized is accumulated. One of the earliest examples is the microbial production of glycerol. Glycerol production depends on modifying the ethanol fermentation by removing acetaldehyde. The addition of sodium bisulphate to the broth leads to the formation of the acetaldehyde bisulphate addition compound. Since acetaldehyde is no longer available for re-oxidation of $NADH_2$, its place as hydrogen acceptor is taken by dihydroacetone phosphate, produced during glycolysis, the product of this reaction is glycerol-3-phosphate, which is converted to glycerol.

Inhibitors have also been used to affect cell wall structure and increase the permeability for release of metabolites. The best example is the use of penicillin and surfactants in glutamic acid production.

22.9. CULTURE MEDIA

Defined Media – also called Synthetic media, is a type of media that you know exactly how many grams or each ingredient are contained within that media.

Different organisms can be grown optimally on specific defined media. Chemoheterotrophs require nutrients like glucose, amino acids and vitamins which will supply the needed carbon and energy factors.

Those organisms that need to have large numbers of growth factors within their media are called "Fastidious" media. Examples of fastidious organisms are *Neisseria gonorrhea* and *Streptococcus* species.

Complex Media – is one in which the exact chemical amounts are not known. Complex media is probably better than Defined Media, because it has a variety of nutrients as well as growth factors. Examples of common complex media are: MacConkey, TSA and Nutrient Broth. If you add blood to a complex media, it has the ability to grow fastidious organisms well. Once the blood is added, it also becomes an Enriched Media.

Selective Media – selects for a particular type or group of microorganisms. It can allow gram negatives to grow but inhibit gram positives. It can allow gram positives to grow but inhibit gram negatives.

Differential Media – is a media formulation that will express changes in the appearance of the colonies that grow on it or you will see changes in the media it's self by what end products the bacteria have released during the time they were growing on that media.

Anaerobic Media – Obligate anaerobes need a media that will protect them from free oxygen. A Reduced Media is one that contains Thioglycollate, which will bond with free oxygen and remove it from the media. Heat then is used to drive the bonded oxygen from the media before bacteria are inoculated into that media.

Low Oxygen Cultures – are ones that prefer as little oxygen as possible. They prefer a higher percentage of CO_2. These type cultures are needed to mimic the environments of the body tissues or respiratory and intestinal tracts.

Organisms that grow well in high CO_2 atmospheres are called Capnophiles and are also called as "Capneic" organisms.

22.10 LET US SUM UP

Sterilization is an important process in the microbiological study.
Dry heat and moist heat methods are efficient sterilization methods.
Media are important for the culturing of microorganisms.
Organisms that grow well in high CO_2 atmospheres are called capnophiles and are also called as capneic organisms.

22.11 LESSON END ACTIVITIES

Explain the various types of media.
What are extremophiles? Site some examples.
Write about the common disinfectant used in laboratories.

Write about the chemical agents used as sterilization.
Explain the sterilization techniques.
Write the inoculum development of bacteria and yeast.
Give short notes on pure culture isolation technique.

22.12 POINTS FOR DISCUSSION

Write about the nutritional and physical requirements and enumeration of microbial populations.
Write about the dry and moist heat sterilization.
Explain the radiation mode of sterilization.

22.13 REFERENCES

1. Microbiology by Pelczar, Reid and Chan, McGraw Hill Book Company.
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LESSON 23 STRAIN IMPROVEMENT METHODS

CONTENTS

23.0 AIMS AND OBJECTIVES

The chapter deals with the various methods that are involved in the strain improvement.

23.1 INTRODUCTION

Various high-throughput experimental techniques are routinely used for generating large amounts of omics data. In parallel, *in silico* modelling and simulation approaches are being developed for quantitatively analyzing cellular metabolism at the systems level. Thus informative high-throughput analysis and predictive computational modelling or simulation can be combined to generate new knowledge through iterative modification of an in silico model and experimental design. On the basis of such global cellular information we can design cells that have improved metabolic properties for industrial applications.

The indispensable role of biotechnology is increasing in nearly every industry, including the healthcare, pharmaceutical, chemical, food and agricultural industries. Biotechnological production of small-volume high-value drugs, chemicals and bioproducts is well justified economically. However, production of large-volume low-value bioproducts requires the development of lower-cost and higher-yield processes. Towards this goal, improved microorganisms have traditionally been developed through random mutagenesis followed by intelligent screening processes. Rational metabolic and cellular engineering approaches have also been successful in improving strain performance in several cases; however, such attempts were limited to the manipulation of only a handful of genes encoding enzymes and regulatory proteins selected using available information and research experience.

Recent advances in high-throughput experimental techniques supported by bioinformatics have resulted in rapid accumulation of a wide range of omics data at various levels, thus providing a foundation for in-depth understanding of biological processes. Even though our ability to analyze these x-omic data in a truly integrated manner is currently

limited, new targets for strain improvement can be identified from these global data. More recently, several examples of combined analysis of these x-omic data towards the development of improved strains have been reported. Along with these high-throughput experimental techniques, in silico modelling and simulation are providing powerful solutions for deciphering the functions and characteristics of biological systems. These in silico experiments would elevate our capability for understanding and predicting the cellular behaviour of microorganisms under any perturbations (e.g. genetic modifications and/or environmental changes) on a global scale.

Consequently, systems-level engineering of microorganisms can be achieved by integrating high-throughput experiments and *in silico* experiments. The results of genomic, transcriptomic, proteomic, metabolomic and fluxomic studies, the data available in databases, and those predicted by computational modeling and simulation, are considered together within the global context of the metabolic system. This gives rise to new knowledge that can facilitate development of strains that are efficient and productive enough to be suitable for industrial applications.

23.2 HIGH-THROUGHPUT X-OMIC ANALYSES FOR STRAIN IMPROVEMENT

As DNA sequencing has become faster and cheaper the genome sequences of many microorganisms have been completed and many more are in progress. With the complete genome sequences in our hands, post-genomic research (the 'omics' fields) is increasing rapidly. Transcriptomics allows massively parallel analysis of mRNA expression levels using DNA microarrays. Proteomics allows analysis of the protein complement of the cell or its parts by using two-dimensional gel electrophoresis (2DGE) or chromatography coupled with various mass spectrometry methods. Metabolomics enables quantitative profiling of metabolites and metabolic intermediates using chromatography coupled with mass spectrometry or NMR. Fluxomics allows determination of metabolic fluxes based on metabolite balancing and/or isotopomer analysis. In this section, the strategies for strain improvement using these omics studies are described and representative examples are reviewed.

23.3 GENOME ANALYSIS

Comparative analysis of genomes is a relatively simple yet powerful way of identifying the genes that need to be introduced, deleted and/or modified to achieve a desired metabolic phenotype. Genomes of various organisms can be compared, as can wild-type and mutant and/or engineered strains. In one approach, a minimal strain can be designed by deleting unnecessary genes while retaining the essential genes that most effectively use metabolic functions for cell survival and production of specific bioproducts without genomic and metabolic burdens. However, the concept of generating a minimal strain should be taken cautiously. The minimal strain, even after it is successfully developed, can easily become less robust owing to the deletion of many genes, some of which become important under particular culture conditions. Engineering of microorganisms based on comparative genomics has recently been successfully demonstrated. Ohnishi et al. compared the genome sequence of a lysine-overproducing *Corynebacterium* strain with that of the wild-type strain to identify genes with point mutations that might be beneficial for the overproduction of L-lysine. Given that the genome of a particular microorganism tells us what this cell potentially can (and cannot) do, it is the starting point for engineering metabolic pathways. Even though we are currently unable to truly engineer a microorganism at the genome scale we can still benefit

from engineering of local reactions and pathways that can often lead to significantly improved performance of a microorganism. Local targeted engineering based on global information is currently the most appropriate strategy exploiting the benefits of the x-omics revolution. Another important advantage of having complete genome sequences is that genomescale in silico metabolic models can now be developed that can be used to rapidly evaluate the metabolic characteristics, generate hypotheses and suggest possible engineering strategies.

23.4 TRANSCRIPTOME ANALYSIS

Development of high-density DNA microarrays has changed examinations of gene transcription by allowing the simultaneous monitoring of relative mRNA abundance in multiple samples. By comparing transcriptome profiles between different strains or between the samples obtained at different time points and/or under different culture conditions, possible regulatory circuits and potential target genes to be manipulated can be identified. The new information and knowledge generated in this way can be used to engineer the local metabolic pathways for improving the performance of microorganisms. Transcriptome profiles of recombinant *E. coli* producing human insulin-like growth factor I fusion protein (IGF-If) by high cell-density culture (HCDC) were analyzed. Among the w200 genes that were down-regulated after induction, those involved in amino acid and/or nucleotide biosynthetic pathways were selected as the first targets to be manipulated. This was because the expression of these genes is down-regulated during the HCDC of *E. coli*. Amplification of two of these genes, the prsA and glpF genes, encoding the phosphoribosyl pyrophosphate synthetase and glycerol transporter, respectively, allowed a significant increase in IFG-If production (from 1.8 to 4.3 g/L). This demonstrates that the strategy of 'local (targeted) engineering based on global information' allows development of a superior strain by suggesting target genes that would otherwise be difficult to identify.

23.5 PROTEOME ANALYSIS

Considering that most cellular metabolic activities are directly or indirectly mediated by proteins, proteome profiling takes us one step further towards understanding cellular metabolic status. However, it should be noted that not all the protein spots have been identified yet, and therefore information obtainable from the proteome is less than that from transcriptome. Nonetheless, proteome analysis can be a powerful tool when comparative profiling is carried out; one can identify protein spots that show altered intensities under two or more genetically or environmentally different conditions for further analysis and manipulation. For example, the proteome of metabolically engineered *E. coli* XL1-Blue intracellularly accumulating a biodegradable polymer poly(3-hydroxybutyrate) was compared with that of control *E. coli* strain, thus generating new knowledge of the importance of Eda (2-keto-3-deoxy-6-phosphogluconate aldolase) in poly (3- hydroxybutyrate) production by engineered *E. coli*. In another example, the proteomes of recombinant *E. coli* overproducing human leptin were examined.

Interestingly, the expression levels of some enzymes in the serine amino acids biosynthetic pathway decreased significantly, indicating possible limitation of serine family amino acids. This was reasonable as the serine content of leptin is 11.6%, which is much higher than the average serine content of *E. coli* proteins (5.6%). Therefore, one of the down-regulated enzymes, cysteine synthase A (encoded by cysK), was selected for amplification. The co-expression of the cysK gene led to two- and fourfold increase in cell growth and

leptin productivity, respectively. In addition, cysK co-expression could improve production of another serine-rich protein, interleukin-12 *b* chain (serine content of 11.1%), suggesting that this strategy might also be useful for the production of other serine-rich proteins. These examples demonstrate that even the limited information obtained by proteome profiling can successfully lead to designing new strategies for strain improvement.

23.6 METABOLOME AND FLUXOME ANALYSIS

High-throughput quantitative analysis of metabolites has become possible as increasingly sophisticated NMR, gas chromatography mass spectrometry (GC-MS), gas chromatography time-of-flight mass spectrometry (GC-TOF) and liquid chromatography-mass spectrometry (LC-MS) procedures have been developed. Comparative analysis of metabolite profiles under genetic and environmental perturbations makes it possible to analyse the physiological states of cells. In general, the number of metabolites in the cell is far fewer than the number of genes. For example, the number of low molecular mass metabolites in Saccharomyces cerevisiae was estimated to be 560, which is less than one-tenth of the number of genes.

However, there might be many more metabolites that are still unknown to us or difficult to detect. Furthermore, some metabolites that are predicted to exist in genome-wide metabolic reaction networks might be difficult to detect owing to the lack of suitable techniques. The heterogeneous chemistry of different metabolites and availability of only a limited number of chemicals that can be used as standards are making true whole-cell metabolome profiling far from realization. Nonetheless, several good examples of using metabolome profiling for strain improvement can be highligted. One is the integrated analysis of metabolome and transcriptome to improve the yield of lovastatin. Another is the use of metabolome profiling to determine flux distribution in *Corynebacterium glutamicum*. Given that metabolome data can be analyzed together with the fluxome data, metabolome profiling will become an increasingly popular tool in systems biotechnological research. Fluxome analysis – metabolic flux profiles of a cell – takes us one step further towards understanding cellular metabolic status. Because intracellular fluxes are difficult to measure, they are often obtained by computational methods. During the calculation of fluxes, some (although limited amounts of) real experimental data, such as substrate uptake and product excretion rates, are often provided as constraints to make the calculated fluxes more realistic. Isotopomer experiments provide us with additional information on the intracellular fluxes. A frequently used substrate is 13C-labelled glucose, either labeled uniformly or at the specific carbon atom only. As the ^{13}C-labeled substrate is metabolized in the cells, isotopomer distribution can be obtained and used to decipher intracellular flux ratios.

23.7 COMBINED OMICS ANALYSIS

True integration of all x-omic data is still far from reality, but several successful examples of strain improvement by taking combined approaches are available. High cell-density culture (HCDC) is often used to increase the concentration and productivity of a desired product such as recombinant protein. Even though the volumetric productivity (g/LKh) of recombinant protein can be increased by HCDC, it is frequently observed that the specific productivity (g/gDCWKh) decreases as cell density increases. The specific reasons for this phenomenon have been unknown. We recently reported the results of combined transcriptome and proteome analyses during the HCDC of *E. coli*. The most important

finding was that the expression of most of amino acid biosynthesis genes was down-regulated as cell density increased. This finding immediately answers why the specific productivity of recombinant protein is reduced during the HCDC. Therefore, an important metabolic engineering strategy can be suggested for the production of recombinant proteins by monitoring the expression levels of amino acid biosynthesis genes during the HCDC, and particularly before and after induction.

Another integrated analysis of transcriptome and proteome profiles was carried out for *E. coli* W3110 and its L-threonine-overproducing mutant strain. Among the 54 genes showing meaningful differential gene expression profiles, those involved in glyoxylate shunt, the tricarboxylic acid (TCA) cycle and amino acid biosynthesis were significantly up-regulated whereas ribosomal protein genes were down-regulated. In addition, mutation in the thrA and ilvA genes was suggested to have affected overproduction of L-threonine. This combined analysis provided valuable information regarding the regulatory mechanism of L-threonine production and the physiological changes in the mutant strain. Another interesting paper describes the use of combined analysis of transcriptome and metabolome to develop an *Aspergillus* strain overproducing lovastatin, a cholesterol-lowering drug. Improved lovastatin production was initiated by generating a library of strains by expressing the genes thought to be involved in lovastatin synthesis or known to broadly affect secondary metabolite production in the parental strain. These strains were characterized by metabolome and transcriptome profiling, followed by a statistical association analysis to extract potential key parameters affecting the production of lovastatin and (C)-geodin. Using this approach, the target genes were identified and manipulated to improve lovastatin production by 50%. More recently, Kro¨mer et al. performed combined transcriptome, metabolome and fluxome analysis of L-lysine producing *C. glutamicum* at different stages of batch culture. A decrease in glucose uptake rate resulted in the shift of cellular activities from growth to L-lysine production, redirecting the metabolic fluxes from the TCA cycle towards anaplerotic carboxylation and lysine biosynthesis.

During this shift, the intracellular metabolite pools exhibited transient dynamics, including an increase of L-lysine up to 40 mM before its excretion to the medium. The expression levels of most genes involved in L-lysine biosynthesis remained constant whereas the metabolic fluxes showed marked changes, suggesting that metabolic fluxes are strongly regulated at the metabolic level. These are good examples of associating gene expression profile with metabolite formation to enable identification of key genes to be manipulated for improving the strain.

23.8 SYSTEMIC AND INTEGRATIVE STRATEGY FOR DEVELOPING IMPROVED STRAINS

The strategic foundation of systems biotechnology is largely based on the systemic integration of highthroughput x-omic analysis and in silico modelling or simulation. Outlines the conceptual procedure for systems biotechnological research. At the outset, the computational model describing the metabolic system is constructed. This model can be used to analyze and/or predict the system's behaviour for a particular experimental situation under systematic perturbation (e.g. gene deletion or addition and well-designed different culture conditions). The results of this *in silico* study suggest new experimental designs to test the hypothesis generated. The experiments include not only the genetic and metabolic engineering of strains but also high-throughput x-omic experiments to generate more global data. The resultant observations are compared with the prior in silico prediction to validate

the working hypothetical model. In this way, computational model and experimental design are continuously modified in a cyclic manner.

Palsson and colleagues demonstrated the usefulness of constraints-based flux analysis to predict the effects of genetic modifications on cellular metabolic characteristics. The ability of a constraints-based model to describe consequences of genetic modifications was examined by creating *E. coli* mutants and forcing them to undergo adaptive evolution under different growth conditions. The mutant strains evolved to computationally predicted growth phenotypes. This integrated approach of mathematical and experimental work can be applied in designing strains with improved metabolic performance.

In a recent study by Covert et al., a simulation of an *E. coli* genome-scale model was integrated with transcriptional regulatory data and high-throughput growth profiles were obtained using multi-well plates. First, the in silico model of *E. coli* was fine-tuned by incorporating the regulatory circuits using the Boolean rules. The expression of 479 genes is regulated by the products of 104 regulatory genes. Then, the high-throughput growth rate data, gene expression data and the metabolic flux profiles predicted by the in silico model were combined to characterize the metabolic network.

We recently reported the complete genome sequence of *Mannheimia succiniciproducens*, which can produce large amounts of succinic acid, along with other acids. To redesign the metabolic pathways for enhanced succinic acid production it is essential to understand the metabolic characteristics under various conditions. Based on the complete genome sequence, a genome-scale *in silico* metabolic model, composed of 373 reactions and 352 metabolites, was constructed. Metabolic flux analyses were carried out under various conditions, suggesting that CO_2 is important for cell growth as well as the carboxylation of phosphoenolpyruvate to oxaloacetate, which is then converted to succinic acid by the reductive TCA cycle using fumarate as a major electron acceptor. Based on these findings, the strategies for genome-scale metabolic engineering of *M. succiniciproducens* could be suggested. According to Galperin ‘This paper is probably the first example of a new approach to complete genomes, which goes from genome sequence straight to chemical engineering. Such approaches will likely become common in biotechnology of the future’. It is important to note that genome-scale metabolic flux analyses were carried out using the metabolite formation rates from a minimal number of cultivation experiments as additional constraints, thus preventing exhaustive wet experiments while providing realistic simulation results for understanding metabolic characteristics of this relatively unknown bacterium.

23.9 . SUMMARY

Microbes are widely spread in nature.

For the beneficial of human the microorganisms can be manipulated by the recombinant DNA technology.

Microbes can be cultured and handled in *invitro* conditions.

The efficiency of microbes can be improved by strain improvement techniques.

23. 10. LESSON END ACTIVITIES

Explain high through put x-omic analysis for strain improvement.

What is Transcriptome analysis?

Explain proteome analysis

Explain metabolome and fluxome analysis

23.11. POINTS FOR DISCUSSION

Explain strain improvement techniques in detail.
Explain the systemic and integrative strategy for developing improved strains.
Explain combined omics analysis.

23.12. REFERENCES

1. Microbiology by Pelczar, Reid and Chan, McGraw Hill Book Company.
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