MICROBIAL TAXONOMY AND DIVERSITY

Source:
1. Presscot et al
2. Microbiology Principle and exploration By Black
3. Microbiology An Introduction By Tortora et al
4. Textbook of Microbiology By Surender Kumar

Dr Diptendu Sarkar
diptendu81@gmail.com
RKMV
TAXONOMY: THE SCIENCE OF CLASSIFICATION

• From ancient Greek words: *Taxis* meaning Arrangement and *Nomia* meaning Method.

• Taxonomy is the branch of science dealing with **naming, grouping of organisms** on the basis of the **degree of similarity** and **arranging them in an order on the basis of their evolutionary relationship**.

• Therefore in other words, taxonomy is related to **nomenclature, classification** and **phylogeny** of organisms.

• Taxonomy unlike natural sciences such as Botany, Zoology, Physics, Chemistry, etc. is **considered as a synthetic (man made) and multidisciplinary science**.

• It owes its progress on the advancement made in other branches of sciences like morphology, histology, physiology, cell biology, biochemistry, genetics, molecular biology, computational biology etc.

• For classification purposes, organisms are usually organized into **subspecies, species, genera, families and higher orders**.

• For eukaryotes, the definition of species is the ability of similar organisms to **reproduce sexually with the formation of a zygote and to produce fertile offspring**.
Systematics

• Biological systematics is the study of the diversification of living forms, both past and present, and the relationships among living things through time.
• Relationships are visualized as evolutionary trees (synonyms: cladograms, phylogenetic trees, phylogenies).
• Phylogenies have two components: branching order (showing group relationships) and branch length (showing amount of evolution).
• Phylogenetic trees of species and higher taxa are used to study the evolution of traits (e.g., anatomical or molecular characteristics) and the distribution of organisms (biogeography).
• Systematics, in other words, is used to understand the evolutionary history of life on Earth.
Aims of Taxonomy

• Classification of organisms
• Show relationships among organisms
• Way to provide universal identification of an organism
Classification

• It is the orderly arrangement of organisms into groups.
• There is nothing inherently scientific about classification and different groups of scientists may classify the same organism differently.
• For example, clinical microbiologists are interested in the serotype, antimicrobial resistance pattern and toxin production and invasiveness factors in *E.coli*, whereas geneticists are concerned with specific mutations and plasmids.

3 types of Systematics/Taxonomy
• **Evolutionary Systematics**
  Grouping organisms that resemble ancestors
• **Numerical Taxonomy**
  Used mathematical models to group organisms according to overall similarities
• **Phylogenetic Classification System:**
  Groups reflect genetic similarity and evolutionary relatedness
Identification

• It is the **practical use of classification criteria** to distinguish certain organisms from others,
  - to verify the authenticity or
  - utility of a strain or
  - a particular reaction or
  - to isolate and identify the organism that causes a disease.
Nomenclature and Taxonomic Hierarchy

Carolus Linnaeus (1707-78) a Swedish Botanist known as father of taxonomy is credited for establishment of taxonomy as a separate science. He was instrumental in framing the rules for naming the organisms, which he applied uniformly while giving his classification. It was he who popularized the binomial nomenclature that is the modern scientific way of naming organisms.

In binomial nomenclature name of every organism is composed of two parts: first is called generic name representing the taxon – Genus to which it belongs and second is called specific epithet- Species.

- The generic name always starts with capital letter and specific name always with small letter.
- These scientific names are used uniformly regardless of regions/countries or languages, and two different organisms cannot posses same scientific name.
- The names of different organisms used in binomial nomenclature system must be derived from Latin or if names to be used are from different languages they must be treated as Latin.
• The nomenclature of organisms is governed by a set of rules framed by **International Codes of Nomenclature**.

• There are different codes of nomenclature for different groups of organisms for example, naming of bacteria, animals and plants is governed by **International Code for Nomenclature for Bacteria (ICNB)**, **International Code of Zoological Nomenclature (ICZN)** and **International Code of Botanical Nomenclature (ICBN)**, **International Code of Nomenclature for algae, fungi, and plants (ICN)** respectively.

• The scientific name of an organism, when cited in any text, is always mentioned as in italics or underlined font style.

• The name of the author who first gave the correct name as per rules is written in abbreviated form after the specific name and is written in Roman.
**Taxonomical Hierarchy**

- Another aspect of taxonomy is classification, which is the grouping of different organisms on the basis of shared features into different categories called taxa. Different taxa are then arranged in a hierarchical manner starting from lower to higher ranks that is:
  - Species
  - Genus
  - Family
  - Order
  - Class
  - Phylum/Division
  - Kingdom
  - Domain

- This ordered arrangement of various taxa is called **taxonomic hierarchy**.

- In this taxonomic hierarchy each organism is assigned a species name and species of very similar organisms are grouped into a genus. The genera having very similar characteristics are grouped together into a family and similarly several families form an order, several orders into a class and ultimately on the top all similar classes are grouped into a kingdom.

- In recently proposed classifications, a new higher rank- domain/empire has been added on the top of rank, kingdom.
• Earlier taxonomists divided the living organisms on the basis of morphological characters, as there was no any sophisticated tool available to study living world.

• So, whatever information was available it was about visible macro-organisms. Therefore organisms were arranged into two groups- plants and animals based on easily observable (phenotypic) characteristics.

• This system of classification was called two-kingdom classification and it was followed for a very long period in history of biological sciences.
Different characteristics of two kingdoms, Plantae and Animalia.

<table>
<thead>
<tr>
<th>CHARACTER</th>
<th>PLANTAE</th>
<th>ANIMALIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body organization</td>
<td>Simple, Organ systems like excretory, sensory, nervous etc. absent</td>
<td>Well developed and organ systems like excretory, sensory, nervous etc. present</td>
</tr>
<tr>
<td>Mobility</td>
<td>Absent as organs of locomotion are not present</td>
<td>Present due to occurrence of organs of locomotion</td>
</tr>
<tr>
<td>Growth and development</td>
<td>Indefinite</td>
<td>Definite as body grows to certain size and then stop.</td>
</tr>
<tr>
<td>Nutrition</td>
<td>Autotrophic through ether photosynthesis or absorption</td>
<td>Heterotrophic through ingestion</td>
</tr>
</tbody>
</table>
• However, invention of microscope in 16th century made it possible to explore the living world, which was not earlier possible to explore through human-naked-eyes. This exploration revealed an altogether new world of microorganisms sharing features of both plants and animals.

• For example, *Euglena*, a green, *autotrophic*, motile organism having definite shape and size, and obtains food by ingestion process in absence of light. Similarly *Chlamydomonas*, a *photoautotrophic* organism but also has animal-like feature such as motility and definite shape and growth.

• Further, *fungi* although have plant-like features such as immobility, irregular shape and indefinite growth but also posses *heterotrophic* mode of nutrition, a characteristic feature of animals. Therefore such microorganisms could not have appropriate placement in two-kingdom classification.

• In order to classify these microorganisms, *Ernst H. Haeckel* in 1866 proposed a three-kingdom classification in which he added a new kingdom – *Protista*.

• In this new kingdom he included all simple microscopic living organisms such as bacteria, microalgae, protozoa, fungi and sponges.
Further advancement in the techniques of microscopy and their application to biology generated new information that added to the scientific knowledge. Studies of various organisms at cellular and subcellular level in 19th and 20th centuries proved that all organisms are made up of a basic unit called cell, which could be structurally simple (prokaryotic cell) or complex (eukaryotic cell).

All the known organisms posses either prokaryotic or eukaryotic cell organization and therefore they can be accordingly called as ether prokaryotes or eukaryotes, respectively.

Several taxonomists insisted on inclusion of this fact (cell type) in classification of organisms.

In 1956, Lynn Margulis and H. F. Copeland adapted this criterion in their classification and proposed a four-kingdom classification system in which kingdom- Protista was divided into two new kingdoms, Monera containing all prokaryotes and Protoctista containing all simple, microscopic eukaryotic organisms like algae, protozoa and fungi.
Five Kingdom system (Whittakar’s concept)

• In 1969, R. H. Whittaker- an American taxonomist, proposed a five-kingdom classification in which kingdom – Protoctista was split into kingdoms- Protista and Fungi.

• In this classification, cellular organisms have been divided into five kingdoms namely, Monera, Protista, Fungi, Plantae and Animalia.

• This classification is an improvement of earlier proposed four-kingdom classification as in this system a new kingdom- Fungi has been created.

• Whittaker delimited the five kingdoms on the basis of three main criteria viz. cell structure type, degree of cellular organization and mode of nutrition.

• Besides these major characteristics, he has also given importance to characters of ecological role-played and mode of reproduction.

• He also attempted to establish phylogentic relationship amongst various groups of different kingdoms.

• According to him the earliest living forms (progenote) produced prokaryotic organisms or monerans.

• Monera gave rise to protists probably through association of several types of primitive and advanced monerans. Protists in tern gave rise to fungi, plants and animals.
Figure: Whittaker’s five-kingdom classification based on complexity of cell, cellular organization, mode of nutrition and ecological role played.
<table>
<thead>
<tr>
<th>CRITERION</th>
<th>MONERA</th>
<th>PROTISTA</th>
<th>PLANTAE</th>
<th>FUNGI</th>
<th>ANIMALIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell type</td>
<td>Prokaryotic</td>
<td>Eukaryotic</td>
<td>Eukaryotic</td>
<td>Eukaryotic</td>
<td>Eukaryotic</td>
</tr>
<tr>
<td>Cellular organization</td>
<td>Unicellular</td>
<td>Unicellular</td>
<td>Multicellular</td>
<td>Multicellular</td>
<td>Multicellular</td>
</tr>
<tr>
<td>Nutrition mode</td>
<td>Variable-phototrophic/heterotrophic</td>
<td>phototrophic/heterotrophic</td>
<td>Autotrophic (photosynthesis)</td>
<td>Heterotrophic (Absorption)</td>
<td>Heterotrophic (ingestion)</td>
</tr>
<tr>
<td>Reproduction</td>
<td>Asexual</td>
<td>Asexual or sexual without embryo stage</td>
<td>Asexual or sexual with or without embryo stage</td>
<td>Asexual or sexual with spore</td>
<td>Sexual with embryo stage</td>
</tr>
<tr>
<td>Ecological role</td>
<td>Variable</td>
<td>Variable</td>
<td>Producer</td>
<td>Decomposer</td>
<td>Consumer</td>
</tr>
</tbody>
</table>
The Living Being: Five Kingdom system

**1. Kingdom Monera**
- Bacteria and Cyanobacteria
  - Photosynthesis and absorption

**2. Kingdom Protista**
- Microalgae (photoplanktonic forms), Protozoa, Slime moulds
  - Absorption, photosynthesis, ingestion

**3. Kingdom Fungi**
- Microfungi, Macrofungi
  - Absorption

**4. Kingdom Plantae**
- Macroalgae, Bryophytes, Pteridophytes, Gymnosperms, Angiosperms
  - Photosynthesis

**5. Kingdom Animalia**
- Invertebrates, vertebrates
  - Ingestion
KUINGDOM MONERA (ARCHAEBACTERIA, BACTERIA, AND CYANOBACTERIA): CHARACTERISTICS

1. It is the kingdom of all the prokaryotes and includes eubacteria, cyanobacteria (blue-green algae) and archebacteria.

2. The organisms are unicellular, colonial, mycelial and filamentous in form.

3. They lack true nuclei and other membrane bound organelles such as mitochondrion, chloroplast, Golgi bodies, lysosomes etc. and DNA, which is the genetic material and is called nucleoid, is not found associated with histone proteins; cell wall is often present but chemically made up material other than cellulose.

4. Mode of nutrition varies from autotrophy to heterotrophy.

5. Sexual reproduction is absent and asexual reproduction may take place through fission, fragmentation, budding and sporulation.
KINGDOM PROTISTA (PHYTOPLANKTONIC ALGAE, PROTOZOA, AND SLIME MOULDS): CHARACTERISTICS

1. It is a group of organisms differing widely with one another except that they all are simple and minute eukaryotes. It includes microalgae, protozoa and slime moulds.

2. Majority of them are unicellular but some may be colonial in form.

3. They contain true nuclei and membrane bound organelles; cell wall may or may not be present.

4. Nutrition is very diversified and may be autotrophic (via photosynthesis) or heterotrophic (ingestion/absorption).

5. Asexual means of reproduction is common but when organisms reproduce sexually, embryo is not formed.
1. It is the group of mostly multicellular or multinucleate achlorophyllous and spore-producing eukaryotic organisms and includes mildews, moulds, yeasts, morals, truffles, mushrooms, rusts etc.

2. The body of organisms is mycelial in form; cell wall is present and made up of chitin or cellulose.

3. Nutrition is absorptive heterotrophy where organism secretes digestive enzymes into the substrate and then absorbs the digested food.

4. Asexual reproduction is primary mode of reduction and sexual reproduction causes formation of specialized spores.

5. They play the ecological role of decomposer.
KINGDOM PLANTAE (MACROALGAE AND PLANTS): CHARACTERISTICS

1. It includes all coloured multicellular photosynthetic eukaryotic organisms commonly called as plants. The important constituents are macroalgae, bryophytes, pteridophytes, gymnosperms and angiosperms.

2. Plant body is either thalloid (algae and some of bryophytes) or differentiated into root, stem and leaves; nonmotile; Cell wall is present and it is chemically made up of cellulose.

3. Nutrition by: autotrophy (photosynthetic)

4. Both asexual and sexual reproductions occur. An embryo stage is present except in algal group.

5. They play the ecological role of producers.
KINGDOM ANIMALIA (INVEERTEBRATE AND VERTEBRATE ANIMALS): CHARACTERISTICS

1. It is a group of all macroscopic animals derived from zygote and includes sponges, coelentrates, worms, annelids, arthropodes, mollusces, star fishes, fishes, amphibians, reptiles, birds and mammals.

2. Organisms are multicellular with higher degree of body organization where tissue differentiation usually leads to specialized organ formation. Eukaryotic cell is without cell wall and chlorophyll pigments.

3. They exhibit mobility, sensitivity to different stimuli and definite growth.

4. They reproduce primarily by sexual reproduction and embryo stage is usually present.

5. They play ecological role of consumer.
Merits of Five Kingdom System

A. Placement of prokaryotes separately in the kingdom monera is well appreciated as the prokaryotes differ from all other organisms (eukaryotes) in their cellular, physiological, and reproductive organization.

B. Many intermediate or transitional forms occur in unicellular eukaryotes, which had been included both amongst plants and animals. Placement of these unicellular eukaryotes into kingdom Protista has removed this anomaly.

C. Fungi have their own unique physiological, biochemical, and structural characteristics and have never been related to plants. Their placement in the form of a separate kingdom was long overdue.

D. Creation of five kingdom is based on the levels of organization and nutritional strategy which evolved very early and because established in later group that are existing even today.

E. As a result of this system, the animal and plant kingdom appear more homogenous than they were in two-kingdom system

F. Five kingdom system has tried to bring out phytogenetic relationships even amongst the primitive forms hence appear more natural.
Demerits of Five kingdom system

Although this system is considered to be an advanced system but still objections have been raised against this, which are following.

1. It fails to distinguish between archaebacteria and eubacteria.
2. The kingdom- Protista is highly heterogeneous group of organisms, which seems to be having polyphylatic evolution.
3. Placement of algae on the basis of degree of cellular organization into different kingdoms appears to be unrealistic.
4. Red and brown algae placed in kingdom – Plantae are not related to other members.
5. Viruses an important form of life has not been considered in this system of classification.
Three Domain Classification

• Progress done in molecular biology especially in biochemistry and molecular genetics in later decades of 20th century provided new techniques to study and compare organisms.

• The study of chemical structure and sequence of macromolecules such as proteins and nucleic acid can give insights in understanding the functions and evolutionary relationship of different organisms.

• For example, cytochrome c protein, a component of electron transport chain occurring in mitochondria;

  *rbcl* gene, encoding rubisco enzyme present in chloroplast and small subunit ribosomal RNAs (SSUrRNAs) are very useful in such studies.
• Among various probable contender macromolecules, that can help in determining the relationship amongst entire living world, rRNA, fits the requirement the most as this form of ribonucleic acid is (a) found uniformly distributed in all the self-replicating cells as one of the basic components of ribosomes; (b) easy to isolate; (c) structurally stable due to its very low mutation rate, therefore, making it an ideal molecule for detecting the relatedness amongst distant species.

• Carl Woese and George Fox (1977) compared 16s/18s rRNA present in different species, and concluded that rather than two different basic cell types (prokaryotic and eukaryotic cells), as suggested by cytological data, molecularly, there are, in actual, three basic types of cells – one, present in eubacteria
  second, present in archaeabacteria and
  third, present in eukaryotes.
• If organisms are grouped on the basis of three basic cells, the molecular dissimilarity among different cell type based groups appears so prominent, that these groups cannot be considered equivalent to the taxon, kingdom.

• Therefore, Carl Woese et al. introduced a new taxon – domain, above the level of kingdom in their new system of classification, which they proposed in 1990.

• Under this system, life has been divided into three domains, the Bacteria, the Archaea and the Eukarya.
All organisms evolved from cells that formed over 3 billion years ago. The DNA passed on from ancestors is described as conserved. The Domain Eukarya includes the kingdoms Fungi, Plantae, and Animalia. It also includes the protists.

3.5 billion years ago. Living organism from which all currently living organisms descended.

Key Concept

Based on similarities in ribosomal RNA, living organisms are classified into three domains: Bacteria, Archaea, and Eukarya.
## Characteristic features of three domains.

<table>
<thead>
<tr>
<th>CHARACTER</th>
<th>BACTERIA</th>
<th>ARCHAEA</th>
<th>EUKARYA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell type</td>
<td>Prokaryotic</td>
<td>Prokaryotic</td>
<td>Eukaryotic</td>
</tr>
<tr>
<td>Cell wall</td>
<td>Present; contain peptidoglycan</td>
<td>Present; peptidoglycan absent</td>
<td>Present/absent; peptidoglycan absent</td>
</tr>
<tr>
<td>Membrane lipids</td>
<td>Diacyl glycerol, diesters</td>
<td>isoprenoid, glycerol, diethers or diglycerol tetraethers</td>
<td>Glycerol, fattyacyl diesters</td>
</tr>
<tr>
<td>Genetic material</td>
<td>Small circular DNA not associated with histones</td>
<td>Small circular DNA associated with histones like proteins</td>
<td>Large linear DNA associated with histones</td>
</tr>
<tr>
<td>Translation (first amino acid)</td>
<td>Formylmethionine</td>
<td>Methionine</td>
<td>Methionine</td>
</tr>
<tr>
<td>RNA polymerase</td>
<td>One; simple</td>
<td>One; complex</td>
<td>Three; complex</td>
</tr>
<tr>
<td>CHARACTER</td>
<td>BACTERIA</td>
<td>ARCHAEA</td>
<td>EUKARYA</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>---------------------</td>
<td>----------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>tRNA (TΨC arm)</td>
<td>Thymine present</td>
<td>Thymine absent</td>
<td>Thymine present</td>
</tr>
<tr>
<td>Intron</td>
<td>Absent</td>
<td>Present rarely</td>
<td>Present</td>
</tr>
<tr>
<td>Antibiotic sensitivity</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Diphtheria toxin sensitivity</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Reproduction</td>
<td>Spore formation present</td>
<td>Spore formation absent</td>
<td>Spore formation present or absent</td>
</tr>
<tr>
<td>Habit</td>
<td>Variable</td>
<td>Extremophile</td>
<td>Variable</td>
</tr>
</tbody>
</table>
1) Phylogeny of domain Archaea

- Based primarily on rRNA sequence data, domain Archaea is divided into two phyla:

1. Phylum *Crenarchaeota*
   - Originally containing thermophylic and hyperthermophilic sulfur-metabolizing archaea
   - Recently discovered *Crenarchaeota* are inhibited by sulfur & grow at lower temperatures

2. Phylum *Euryarchaeota*
   - Contains primarily methanogenic archaea, halophilic archaea, and thermophilic, sulfur-reducing archaea
2) Phylogeny of domain *Bacteria*

- The 2nd edition of *Bergey’s Manual of Systematic Bacteriology* divides domain *Bacteria* into **23 phyla. Nine** of the more notable phyla are described here.

1. **Phylum Aquiflexa**
   - The earliest “deepest” branch of the *Bacteria*
   - Contains genera *Aquiflex* and *Hydrogenobacter* that can obtain energy from hydrogen via chemolithotrophic pathways

2. **Phylum Cyanobacteria**
   - Oxygenic photosynthetic bacteria
3. **Phylum Chlorobi**
   - The “green sulfur bacteria”
   - Anoxygenic photosynthesis
   - Includes *genus Chlorobium*

4. **Phylum Proteobacteria**
   - The largest group of gram-negative bacteria
   - Extremely complex group, with over 400 genera and 1300 named species
   - All major nutritional types are represented: phototrophy, heterotrophy, and several types of chemolithotrophy
   - Sometimes called the “purple bacteria,” although very few are purple; the term refers to a hypothetical purple photosynthetic bacterium from which the group is believed to have evolved
   - **Divided into 5 classes:** *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria*
Phylum *Proteobacteria* (Cont.....)

- Photosynthetic genera such as *Rhodospirillum* (a purple non-sulfur bacterium) and *Chromatium* (a purple sulfur bacterium)
- Sulfur chemolithotrophs, genera *Thiobacillus* and *Beggiatoa*
- Nitrogen chemolithotrophs (nitrifying bacteria), genera *Nitrobacter* and *Nitrosomonas*
- Other chemolithotrophs, genera *Alcaligenes, Methylobacilllus, Burkholderia*

- The *family Enterobacteriaceae*, the “gram-negative enteric bacteria,” which includes genera *Escherichia, Proteus, Enterobacter, Klebsiella, Salmonella, Shigella, Serratia*, and others
- The *family Pseudomonadaceae*, which includes genus *Pseudomonas* and related genera
- Other medically important *Proteobacteria* include genera *Haemophilus, Vibrio, Campylobacter, Helicobacter, Rickessia, Brucella*
5. Phylum *Firmicutes*
   - “Low G + C gram-positive” bacteria
   - Divided into 3 classes
     - **Class I** – *Clostridia*; includes genera *Clostridium* and *Desulfovibrio maculatum*, and others
     - **Class II** – *Mollicutes*; bacteria in this class cannot make peptidoglycan and lack cell walls; includes genera *Mycoplasma, Ureaplasma*, and others
     - **Class III** – *Bacilli*; includes genera *Bacillus, Lactobacillus, Streptococcus, Lactococcus, Geobacillus, Enterococcus, Listeria, Staphylococcus*, and others

6. Phylum *Actinobacteria*
   - “High G + C gram-positive” bacteria
   - Includes genera *Actinomyces, Streptomyces, Corynebacterium, Micrococcus, Mycobacterium, Propionibacterium*

7. Phylum *Chlamidiae*
   - Small phylum containing the genus *Chlamydia*
8. Phylum *Spirochaetes*

- The spirochaetes characterized by **flexible, helical cells** with a **modified outer membrane** (the outer sheath) and **modified flagella** (axial filaments) located within the outer sheath
- Important pathogenic **genera include Treponema, Borrelia, and Leptospira**

9. Phylum *Bacteroidetes*

- Includes **genera Bacteroides, Flavobacterium, Flexibacter, and Cytophyga; Flexibacter and Cytophyga** are motile by means of “gliding motility”

3) Phylogeny of domain *Eucarya*

The domain *Eucarya* is divided into four kingdoms by most biologists:
- **Kingdom Protista**, including the protozoa and algae
- **Kingdom Fungi**, the fungi (molds, yeast, and fleshy fungi)
- **Kingdom Animalia**, the multicellular animals
- **Kingdom Plantae**, the multicellular plants
Since this is most advance system of classification, it has attempted to address various demerits of five-kingdom classification.

This system recognizes the independent lineages of archaebacteria and bacteria and provides their natural classification.

By introduction of the rank of domain, this system has become natural up to highest level.

Merits of Carl Woese’s system:
<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plantae</td>
<td>Bacteria</td>
</tr>
<tr>
<td>Animalia</td>
<td>Archaea</td>
</tr>
<tr>
<td>Protista</td>
<td>Eukarya</td>
</tr>
<tr>
<td>Monera</td>
<td></td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
</tr>
</tbody>
</table>

- **Three domain classification:** Incorporation of molecular information
- **Five-kingdom classification:** Incorporation of information about cell type, organization and mode of nutrition
- **Four-kingdom classification:** Incorporation of cytological information
- **Three-kingdom classification:** Incorporation of microscopic information about organisms
- **Two-kingdom classification:** Based on morphological information
Definition of “species” in microbiology:

- In microbiology the term species is defined as a collection of strains having similar characteristics. It is the collection of microbial strains, that share many properties, and differ significantly from other groups of strains.

- The original culture of a bacterium based on which the description is derived, forms the type strain, and all other strains that are sufficiently similar to the type strain, together form the species.

- Species are identified by comparison with known “type strains”: well-characterized pure cultures; references for the identification of unknowns.

- The type strain is generally deposited in a type culture collection center.
Type culture collection center

- NCIB: National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland.
- NCTC: National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London, N.W.9., U.K.
- NDRI: National Dairy Research Institute, Karnal 132 001
- American Type Culture Collection (ATCC), Parklawn Drive, Rockville, Maryland, USA.
- Commonwealth Mycological Institute (CMI), England
- Centre de Collections de Types microbents, Switzerland.
- Culture Collection of Algae and Protozoa, Botany School, France
- Microbial Type-Culture Collection, Osaka, Japan.
- USSER Antibiotic Research Institute, Moscow
- Indian Type Culture collection (ITCC), Indian agricultural research institute, new Delhi
- Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India.
<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Culture Collection Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus subsp. aureus</em></td>
<td>ATCC 6538</td>
</tr>
<tr>
<td><em>Bacillus subtilis subsp. spizizenii</em></td>
<td>ATCC 6633</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>ATCC 9027</td>
</tr>
<tr>
<td><em>Clostridium sporogenes</em></td>
<td>ATCC 19404</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>ATCC 10231</td>
</tr>
<tr>
<td><em>Aspergillus brasiliensis</em></td>
<td>ATCC 16404</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>ATCC 8739</td>
</tr>
<tr>
<td><em>Salmonella enterica subsp. enterica serovar typhimurium</em></td>
<td>ATCC 13311</td>
</tr>
</tbody>
</table>
**Strain: Types specimens (Cultures)**

- A strain is defined as a cell population, arising from a single organism as a pure culture.
- Different strains represent genetic variability within a species.
- The most typical strain in a collection is commonly taken as the centrotpe.
- For microorganisms which cannot be cultured, a type can be served by a preserved specimen, a photograph or some other device.
- Sometimes the type are lost and new ones have to be set up to replace them. These newly established types are called neotypes.
- In microbiology, the basic unit of classification is a strain.
- Strain is usually a genetic variant or subtype of a microorganism.
- However, a species may be divided into two or more subspecies based on minor but consistent phenotypic variations within the species, or on genetically determined clusters of strains within the species.
- A genus is a collection of similar species, one of them being the type species. The type species serve as a permanent example of the genus.
- A group of similar genera form a Family.
- A group of similar classes a division and a group of similar divisions a Kingdom, and thus goes the taxonomic hierarchy.
- **Biovars**: Strains that differ in biochemical or physiological differences
- **Morphovars**: Strains that vary in morphology
- **Serovars**: Stains that vary in their antigenic properties
Criteria for the classification of Bacteria

1. Microscopic appearance
2. Colony morphology
3. Cell shape & arrangement
4. Cell wall structure (Gram staining)
5. Special cellular structures
6. Biochemical reactions
7. Growth requirements
8. Serology
9. Phage typing
1. Microscopic appearance

Often does not require sophisticated equipment

Can easily be done anywhere

- Microscopic examination:
  - Cell shape
  - Cell surface
  - Colony color
Microscopic Examination: Cell Shape: Coccus

- **Single Coccus**
- **Diplococcus (paired)**
- **Coccus**
- **Staphylococcus**
- **Streptococcus (chain)**
Arrangements of Cocci

- coccus
- diplococci
- Staphylococci
- streptococci
- sarcina
- tetrad
Microscopic Examination: Cell Shape: Bacillus

- Single Bacillus
- Bacillus
- Single Bacillus (fusiform)
- Streptobacillus (chain)
Microscopic Examination: Cell Shape: Spirillum

Spirillum

Single Spirillum

Multiple Spirillum
Bacterial Morphology

Cocci
- coccus
- diplococci
- diplococci encapsulated
  Pneumococcus
- Staphylococci
  tetrad
  sarcina

Bacilli
- coccobacillus
- bacillus
- diplobacilli
- palisades
- Streptobacilli

Others
- enlarged rod
  Fusobacterium
- Vibrio
- Comma’s form
  Bdellovibrio
- Club Rod
  Corynebacteriaceae
- Helical form
  Helicobacter pylori
- Corkscrew’s form
  Borrelia burgdorferi
- Filamentous
- spirochete
- hypha
- stalk
Types of Flagella

<table>
<thead>
<tr>
<th>Structure</th>
<th>Flagella Type</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monotrichous</td>
<td><em>Vibrio choloreae</em></td>
</tr>
<tr>
<td></td>
<td>Lophotrichous</td>
<td><em>Bartonella bacilliformis</em></td>
</tr>
<tr>
<td></td>
<td>Amphitrichous</td>
<td><em>Spirillum serpens</em></td>
</tr>
<tr>
<td></td>
<td>Peritrichous</td>
<td><em>Escherichia coli</em></td>
</tr>
</tbody>
</table>
Bacterial endospores

Endospores can survive extreme physical and chemical stresses, for e.g., gamma radiation, heat, pressure, etc. The endospore consists of bacterial DNA and part of its cytoplasm surrounded by a very tough outer coating. They are characterized by presence of 5-20% dipicolinic acid. Endospore formation takes place sometimes due to lack of nutrients thus these and survive without nutrients. They are mainly found in soil and water and may survive for long periods of time.
Bacterial Capsules:
It usually consists of polysaccharides, but can be composed of other materials such as polypeptide D-glutamic acid.
2. GRAM STAIN

- distinguishes between Gram + and Gram – bacteria

**Gram positive cell wall**

- Consists of
  - a thick, homogenous sheath of peptidoglycan 20-80 nm thick
  - tightly bound acidic polysaccharides, including teichoic acid and lipoteichoic acid

- Retain crystal violet and stain purple

**Gram negative cell wall**

- Consists of
  - an outer membrane containing lipopolysaccharide (LPS)
  - thin shell of peptidoglycan
  - periplasmic space

- Lose crystal violet and stain pink from safranin counterstain
3. Biochemical Tests

- Each living organism is **unique in its biochemical potentialities**.
- They provide additional information for the identification of the bacterium.
- Clinical microbiology laboratories typically will identify a pathogen in a clinical sample:
  
  - purify the microorganism by plating a single colony of the microorganism on a separate plate, and then perform a series of biochemical studies that will identify the bacterial species.
• **Tests used to identify Gram Positive Bacteria**

1. Catalase Test
2. Mannitol Salt Agar (MSA)
3. Blood Agar Plates (BAP) : Streak-stab technique
4. Taxos P (optochin sensitivity testing)
5. Taxos A (bacitracin sensitivity testing)
6. CAMP Test
7. Bile Esculin Agar
8. Nitrate Broth
9. Spirit Blue agar
10. Starch hydrolysis test
11. Motility Agar
12. Coagulase Test
13. Sulfur Indole Motility Media (SIM)

• **Tests used to identify Gram Negative Bacteria**

1. Oxidase Test
2. Sugar (eg glucose) broth with Durham tubes
3. Methyl Red / Voges-Proskauer (MR/VP)
4. Kliger’s Iron Agar (KIA)
5. Nitrate Broth
6. Motility Agar
7. MacConkey agar
8. Simmon’s Citrate Agar
9. Urease test
Case Study Tests

1. Indole
2. Methyl Red/Voges Proskauer
3. Citrate
4. H2S production in SIM
5. Motility
6. Lactose fermentation
7. Sucrose fermentation
8. Glucose fermentation & gas production
9. Triple Sugar Iron Agar (TSI) test
10. Staphylococcus identification tests
    MSA

Test for enzymes

1. Catalase test
2. Oxidase test
3. Urease test
4. Coagulase test
5. Nitrate reduction
OXIDASE TEST

• Detects the presence of an enzyme “oxidase” produced by certain bacteria which will reduce the dye: tetramethyl-p-phenylene diamine dihydrochloride.
• Positive test is indicated by the development of a purple colour.
• Oxidase positive – *Pseudomonas, Vibrio, Neisseriae*
• Oxidase negative – *Salmonella, Shigella*
TRIPLE SUGAR IRON AGAR (TSI)

• It is a composite media used to study different properties of a bacterium – sugar fermentation, gas production and $H_2S$ production.

• In addition to peptone, yeast extract & agar, it contains 3 sugars – Glucose, Lactose, Sucrose.

• The Iron salt – Ferric citrate indicates $H_2S$ production.

• Phenol red is the indicator.

• It is an orange red medium with a slant and a butt.

• pH of the medium – 7.4
TSI REACTIONS:

Yellow – Acid
Pink – Alkaline

• **Yellow slant / Yellow butt (A/A)** – Lactose fermenters.

• **Pink slant / Yellow butt (K/A)** – Non lactose fermenters.

• **Pink slant / no colour change (K/K)** – Non fermenters

• **Black colour** – H₂S production.

• **Gas bubbles or crack in the medium** – gas production.

• **LF** – *E.coli, Klebsiella*

• **NLF** – *Salmonella, Shigella*

• **H₂S** – *Proteus*
INDOLE TEST

• Used to detect indole production by the organism.
• They produce indole from tryptophan present in peptone water.
• After overnight incubation, a few drops of indole reagent (Kovac’s reagent) is added.
  • Positive indole test – pink ring
  • Negative indole test - yellow ring
• Indole positive – *E.coli*
• Indole negative – *Klebsiella, Salmonella.*
CITRATE UTILIZATION

• Done in **Simmon’s Citrate medium**.

• To detect the **ability of certain bacteria to utilize citrate as the sole source of carbon**.

• Contains **Sodium citrate and bromothymol blue as the indicator**.

• If citrate is utilized, **alkali is produced which turns the medium to blue**.
  • Citrate positive – blue colour
  • Citrate negative – green colour

• Positive – **Klebsiella**

• Negative – **E.coli**
UREASE TEST

• Done in Christensen’s urease medium.
• This test is used to detect organisms that produce urease.
• Urease produced by the organisms split urea into ammonia and CO$_2$.
  • Urease positive – pink colour
  • Urease negative – yellow colour
• Positive – *Proteus, Klebsiella*
• Negative – *E.coli, Salmonella*
DICHTOMOUS KEYS
(DICHOTOMOUS MEANS CUT IN TWO).

• **Dichotomous keys** are widely used for identification.
• In a dichotomous key, identification is based on successive questions, and each question has two possible answers.
• After answering one question, the investigator is directed to another question until an organism is identified.
• Although these keys often have little to do with phylogenetic relationships, they’re invaluable for identification.
• For example, a dichotomous key for bacteria could begin with an easily determined characteristic, such as cell shape, and move on to the ability to ferment a sugar.
The requirements for microbial growth can be divided into two main categories: physical and chemical.

Physical aspects include:
- temperature, pH, and osmotic pressure.

Chemical requirements:
- include sources of carbon, nitrogen, sulfur, phosphorus, oxygen, trace elements, and organic growth factors.

Factors affecting microbial growth:
- Nutrients
- Temperature
- pH
- Oxygen
- Water availability
Nutritional mode

- All organisms
  - Energy source
    - Chemical
      - Chemotrophs
        - Carbon source
          - Organic compounds
            - Chemoheterotrophs
              - Final electron acceptor
                - O₂
                  - All animals: most fungi, protozoa, bacteria
                - Not O₂
                  - Organic compound
                    - Fermentative Streptococcus, for example
                  - Inorganic compound
                    - Electron transport chain Clostridium, for example
          - CO₂
            - Chemoautotrophs
              - Hydrogen-, sulfur-, iron-, nitrogen-, and carbon monoxide-oxidizing bacteria
    - Light
      - Phototrophs
        - Carbon source
          - Organic compounds
            - Photoheterotrophs
              - Use H₂O to reduce CO₂?
                - Yes
                  - Green nonsulfur bacteria, purple nonsulfur bacteria
                  - Oxygenic photosynthesis (plants, algae, cyanobacteria)
                - No
                  - Anoxygenic photosynthetic bacteria (green and purple bacteria)
‘Temperature classes’ of organisms

- **Psychrophile Example**: Escherichia coli
  - Growth rate
  - Temperature, °C

- **Mesophile Example**: Bacillus stearothermophilus
  - Growth rate
  - Temperature, °C

- **Thermophile Example**: Thermococcus celer
  - Growth rate
  - Temperature, °C

- **Hyperthermophile Example**: Pyrodictium brockii
  - Growth rate
  - Temperature, °C
<table>
<thead>
<tr>
<th><strong>TABLE 6.1</strong> The Effect of Oxygen on the Growth of Various Types of Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Effect of Oxygen on Growth</strong></td>
</tr>
<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td>Only aerobic growth; oxygen required.</td>
</tr>
</tbody>
</table>

**Bacterial Growth in Tube of Solid Growth Medium**

1. ![Tube 1](image1)
2. ![Tube 2](image2)
3. ![Tube 3](image3)
4. ![Tube 4](image4)
5. ![Tube 5](image5)

**Explanation of Growth Patterns**

- **Growth occurs only where high concentrations of oxygen have diffused into the medium.**
- **Growth is best where most oxygen is present, but occurs throughout tube.**
- **Growth occurs only where there is no oxygen.**
- **Growth occurs evenly; oxygen has no effect.**
- **Growth occurs only where a low concentration of oxygen has diffused into medium.**

**Explanation of Oxygen’s Effects**

- **Presence of enzymes catalase and superoxide dismutase (SOD) allows toxic forms of oxygen to be neutralized; can use oxygen.**
- **Presence of enzymes catalase and SOD allows toxic forms of oxygen to be neutralized; can use oxygen.**
- **Lacks enzymes to neutralize harmful forms of oxygen; cannot tolerate oxygen.**
- **Presence of one enzyme, SOD, allows harmful forms of oxygen to be partially neutralized; tolerates oxygen.**
- **Produce lethal amounts of toxic forms of oxygen if exposed to normal atmospheric oxygen.**
The activity of microbial enzymes depends on the charge present on the surface of amino acids. Any change in the environmental pH may either enhance or inhibit the enzyme activity.
**low pH**

**Fungi:** - often more acid tolerant than bacteria (opt. pH5)

**Obligate acidophilic bacteria:** *Thiobacillus ferrooxidans*

**Obligate acidophilic Archaea:** *Sulfolobus*, *Thermoplasma*

**Most critical: Biological membrane restrict $H^+$ ions entry**

---

**high pH**

- Few alkaliphiles (pH10-11)
- Bacteria: *Bacillus* spp.
- Archaea
- Often also halophilic
- Sometimes: $H^+$ gradient replaced by $Na^+$ gradient (motility, energy)
- Industrial applications (especially „exoenzymes“):
  - Proteases/lipases for detergents (*Bacillus licheniformis*)
  - pH optima of these enzymes: 9-10
Bacterial growth: Osmosis

### TABLE 5.2 Water activity of several substances

<table>
<thead>
<tr>
<th>Water activity, $a_w$</th>
<th>Material</th>
<th>Examples of organisms growing at stated water activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.000</td>
<td>Pure water</td>
<td>Caulobacter, Spirillum</td>
</tr>
<tr>
<td>0.995</td>
<td>Human blood</td>
<td>Streptococcus, Escherichia</td>
</tr>
<tr>
<td>0.980</td>
<td>Seawater</td>
<td>Pseudomonas, Vibrio</td>
</tr>
<tr>
<td>0.950</td>
<td>Bread</td>
<td>Most gram-positive rods</td>
</tr>
<tr>
<td>0.900</td>
<td>Maple syrup, ham</td>
<td>Gram-positive cocci such as <em>Staphylococcus</em></td>
</tr>
<tr>
<td>0.850</td>
<td>Salami</td>
<td>Saccharomyces rouxii (yeast)</td>
</tr>
<tr>
<td>0.800</td>
<td>Fruit cake, jams</td>
<td>Saccharomyces bailii, <em>Penicillium</em> (fungus)</td>
</tr>
<tr>
<td>0.750</td>
<td>Salt lakes, salted fish</td>
<td>Halobacterium, <em>Halococcus</em></td>
</tr>
<tr>
<td>0.700</td>
<td>Cereals, candy, dried fruit</td>
<td>Xeromyces bisporus and other xerophilic fungi</td>
</tr>
</tbody>
</table>

Soil: water activity = 0.9 – 1.0

In general: bacteria normally have higher osmotic pressure than environment = „positive water balance“

**Osmophiles:** - grow in presence of high sugar concentration  
**Xerophiles** - grow in „dehydrated“ environments
Habitat

Based on habitat

- Halophiles (Saline areas)
- Thermoacidophiles (Hot springs)
- Mechanoens (Marshy areas)
Bacterial growth: Halophiles

Halophiles:
- requirement for Na+
- grow optimally in media with low water activity
- Mild: 1-6 % NaCl
- Moderate: 6-15 % NaCl
- extreme: 15 – 30% NaCl

most other organisms would be dehydrated

Growth rate

Non-halophile Example: Escherichia coli

Halotolerant Example: Staphylococcus aureus

Halophile Example: Vibrio fischeri

Extreme halophile Example: Halobacterium salanarium
Sensitivity to antibiotics

Antibiotic Sensitivity Tests

- **Diffusion**
  - Kirby-Bauer Method
- **Dilution**
  - Stokes Method
  - Tube Dilution
  - Agar Dilution
- **Diffusion & Dilution**
  - E-Test

Qualitative Methods

Quantitative Methods

---

K Hari Krishnan  
Tirunelveli Medical College  
DS/MICRO/RKMV
<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>content (μg/disc)</th>
<th>growth-inhibition zone (mm)</th>
<th>resistance</th>
<th>Interpretation of growth-inhibiting zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sephalexin</td>
<td>10</td>
<td>0</td>
<td>strong</td>
<td>below 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12~13</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>above 14</td>
</tr>
<tr>
<td>flumequine</td>
<td>10</td>
<td>0</td>
<td>strong</td>
<td>below 12</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>13~16</td>
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<td>furazolidone</td>
<td>10</td>
<td>0</td>
<td>strong</td>
<td>below 11</td>
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<td>12~13</td>
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<td></td>
<td></td>
<td></td>
<td>above 14</td>
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<tr>
<td>gentamycin</td>
<td>10</td>
<td>13</td>
<td>moderate</td>
<td>below 12</td>
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<td>13~14</td>
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<td>12~13</td>
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<td></td>
<td>above 14</td>
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<tr>
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<td>30</td>
<td>none</td>
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<td>15~18</td>
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<tr>
<td>tiamulin</td>
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<td>28</td>
<td>none</td>
<td>below 11</td>
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<td>12~13</td>
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<td>below 12</td>
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<td>kanamycin</td>
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<td>9</td>
<td>strong</td>
<td>below 13</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>above 18</td>
</tr>
</tbody>
</table>
Phage typing

- **Phage typing** is a method used for detecting single strains of **bacteria**.

- It is used to trace the source of outbreaks of infections.

- The **viruses** that infect bacteria are called **bacteriophages** ("phages" for short) and some of these can only infect a single strain of bacteria.

- These phages are used to identify different strains of bacteria within a single species.

- A culture of the strain is grown in the agar and dried. A grid is drawn on the base of the **petri dish** to mark out different regions. Inoculation of each square of the grid is done by a different phage. The phage drops are allowed to dry and are incubated: **The susceptible phage regions will show a circular clearing where the bacteria have been lysed**, and this is used in differentiation.
5. Serological Tests

• Use group specific antiserum isolated from the plasma of animals, that have been sensitized to the organism
  • The antiserum contains antibody proteins that react with antigens on the unknown organism.
  • The reaction can be detected by examining agglutination or by using sera labeled with colorimetric or fluorescent labels

Advantages:
  • Highly specific
  • Does not usually require the organism to be isolated into pure culture
  • Can be used to identify organisms that can’t be grown on medium
In a procedure called a *slide agglutination test*, samples of an unknown bacterium are placed in a drop of saline on each of several slides. Then a different known antiserum is added to each sample. The bacteria agglutinate (clump) when mixed with antibodies that were produced in response to that species or strain of bacterium; a positive test is indicated by the presence of agglutination.

(a) Positive test

(b) Negative test
*Salmonella typhi* and *Salmonella paratyphi* A, B and C cause enteric fever (typhoid and paratyphoid) in human.
6. **G + C content (Nucleic Acid Base Composition)**

- 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 base composition.
- DNA contains four purine and pyrimidine bases: adenine (A), guanine (G), cytosine (C), and thymine (T).
- In double-stranded DNA, A pairs with T, and G pairs with C. Thus the \(\frac{(GC)}{(AT)}\) ratio or G + C content, the percent of G + C in DNA, reflects the base sequence and varies with sequence changes as follows:

\[
\text{Mol\% (G+C)} = \frac{G+C}{G+C+A+T} \times 100' 
\]

- The base composition of DNA can be determined in several ways.
- The G +C content often is determined from the melting temperature (Tm) of DNA.
- The Tm is defined as the temperature, at which 50% of double stranded DNA is changed to single-standard DNA.
• The higher the melting temperature, the greater the guanine-cytosine (GC) content of the DNA. In double-stranded DNA, three hydrogen bonds join GC base pairs, and two bonds connect AT base pairs.

• As a result, **DNA with a greater G + C content have more hydrogen bonds, and its strands separate at higher temperatures—that is, it has a higher melting point.**

• DNA melting can be easily followed **spectrophotometrically** because the **absorbance of DNA at 260 nm (UV light) increases during strand separation.**

• When a DNA sample is slowly heated, the absorbance increases as hydrogen bonds are broken and reaches a plateau when all the DNA has become single stranded.

• The midpoint of the rising curve gives the melting temperature, a direct measure of the G + C content.

• **The G + C content of strains within a particular species is constant.**

• **If two organisms differ in their G + C content by more than about 10%, their genomes have quite different base sequences.**

• On the other hand, **it is not safe to assume that organisms with very similar G + C contents also have similar DNA base sequences,** because **two very different base sequences can be constructed 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.**
Since a sequence of single-stranded DNA needs to find its complementary strand to reform a double helix, common sequences renature more rapidly than rare sequences. Indeed, the rate at which a sequence will renature, is proportional to the number of copies of that sequence in the DNA sample.

A sample with a highly-repetitive sequence will renature rapidly, while complex sequences will renature slowly. However, instead of simply measuring the percentage of double-stranded DNA versus time, the amount of renaturation is measured relative to a $C_0t$ value.

The $C_0t$ value is the product of $C_0$ (the initial concentration of DNA), $t$ (time in seconds), and a constant that depends on the concentration of cations in the buffer. Repetitive DNA will renature at low $C_0t$ values, while complex and unique DNA sequences will renature at high $C_0t$ values. The fast renaturation of the repetitive DNA is because of the availability of numerous complementary sequences.
7. Nucleic acid hybridization

- The similarity between genomes can be compared more directly by use of nucleic acid hybridization studies.
- It is a technique, in which single-stranded nucleic acids (DNA or RNA) are allowed to interact, so that, complexes called hybrids are formed, by molecules with similar, complementary sequences.

If a mixture of single-stranded DNA (ssDNA) formed by heating double-stranded (ds) DNA, is cooled and held at a temperature about 25°C below the $T_m$, strands with complementary base sequences will reassociate to form stable dsDNA, whereas non-complementary strands will remain unpaired. Because strands with similar, but not identical, sequences associate to form less temperature stable dsDNA hybrids.

- Incubation of the mixture at 30 to 50°C below the $T_m$, allows hybrids of more diverse ssDNAs to form.
- Incubation at 10 to 15°C below the $T_m$, permits hybrid formation only with almost identical strands.
In one of the more widely used hybridization techniques, nylon filters with bound nonradioactive DNA strands, are incubated at the appropriate temperature with ssDNA fragments, made radioactive with $^{32}$P, $^3$H, or $^{14}$C.

After radioactive fragments are allowed to hybridize with the membrane-bound ss-DNA, the membrane is washed to remove any non-hybridized ssDNA and its radioactivity is measured.

The quantity of radioactivity bound to the filter, reflects the amount of hybridization, and thus the similarity of the DNA sequences.

The degree of similarity or homology is expressed as the percent of experimental DNA radioactivity retained on the filter, compared with the percent of homologous DNA radioactivity bound under the same conditions.

Two strains whose DNAs show at least 70% relatedness under optimal hybridization conditions, and less than a 5% difference in $T_m$, often, but not always, are considered members of the same species.

If DNA molecules are very different in sequence, they will not form a stable, detectable hybrid.

Therefore DNA-DNA hybridization is used to study only closely related microorganisms.
1. Heat to separate strands
2. Combine single strands of DNA
3. Cool to allow renaturation of double-stranded DNA
4. Determine degree of hybridization

Complete hybridization: Organisms identical
Partial hybridization: Organisms related
No hybridization: Organisms unrelated
More distantly related organisms can be compared by carrying out DNA-RNA hybridization experiments, using radioactive ribosomal or transfer RNA.

Distant relationships can be detected because rRNA and tRNA genes represent only a small portion of the total DNA genome, and have not evolved as rapidly as most other microbial genes.

The technique is similar to that employed for DNA-DNA hybridization: membrane bound DNA is incubated with radioactive rRNA, washed, and counted.

An even more accurate measurement of homology is obtained by finding the temperature required to dissociate and remove half the radioactive rRNA from the membrane; the higher this temperature, the stronger the rRNA-DNA complex and the more similar the sequences.
Sequence Conservation Level
- nearly universal
- intermediate
- hypervariable
• **Fluorescent In Situ Hybridization (FISH)**: Fluorescent dye–labeled RNA or DNA probes are used to stain microorganisms in place, or in situ.

• This technique is called **fluorescent in situ hybridization**, or FISH. Cells are treated so the probe enters the cells and reacts with target DNA in the cell (in situ).

• FISH is used to determine the identity, abundance, and relative activity of microorganisms in an environment and can be used to detect bacteria that have not yet been cultured.

• Using FISH, researchers discovered a tiny bacterium called *Pelagibacter* (pel-AJ-e⁻-bak-ter) in the ocean and determined that it’s related to the rickettsias.

• As probes are developed, FISH can be used to detect bacteria in drinking water or bacteria in a patient without the normal 24-hour or longer wait required to culture the bacteria.
8. Nucleic acid sequencing: Ribotyping and Ribosomal RNA Sequencing

- RNAs from small ribosomal subunits (16S and 18S rRNAs from procaryotes and eucaryotes, respectively) have become the molecules of choice for inferring microbial phylogenies and making taxonomic assignments at the genus level. The small subunit rRNAs (SSU rRNAs) are almost ideal for studies of microbial evolution and relatedness because they play the same role in all microorganisms.

- Comparative analysis of 16S rRNA sequences from thousands of organisms has demonstrated the presence of oligonucleotide signature sequences. These are short, conserved nucleotide sequences that are specific for a phylogenetically defined group of organisms. Thus the signature sequences found in Bacteria are rarely or never found in Archaea and vice versa.

- Likewise, the 18S rRNA of eucaryotes also bears signature sequences that are specific to the domain Eucarya.

- Either complete rRNAs or, more often, specific rRNA fragments can be compared.

- The proper alignment of SSU rRNA nucleotide sequences and the application of computer algorithms enable sequence comparison between any number of organisms.

- When comparing rRNA sequences between two microorganisms, their relatedness can be represented by an association coefficient, or $S_{ab}$ value. The higher the $S_{ab}$ values, the more closely the organisms are related to each other.

- If the sequences of the 16S rRNAs of two organisms are identical, the $S_{ab}$ value is 1.0.

- After $S_{ab}$ values have been determined, a computer calculates the relatedness of the organisms and summarizes their relationships in a tree or dendrogram.
9. Genomic Fingerprinting

- A group of techniques called **genomic fingerprinting** can also be used to classify microbes and **help determine phylogenetic relationships**.
- Genomic fingerprinting does not involve nucleotide sequencing.
- Instead, it **employs the capacity of restriction endonucleases** to recognize specific nucleotide sequences.
- Thus the **pattern of DNA fragments generated by endonuclease cleavage** (called restriction fragments) **is a direct representation of nucleotide sequence**.
- The comparison of restriction fragments between species and strains is the basis of **restriction fragment length polymorphism (RFLP)** analysis.
Another assay is based on highly conserved and repetitive DNA sequences present in many copies in the genomes of most gram-negative and some gram-positive bacteria.

There are three families of repetitive sequences: the 154 bp BOX elements, the 124–127 bp enterobacterial repetitive intergenic consensus (ERIC) sequence, and 35–40 bp repetitive extragenic palindromic (REP) sequences.

These sequences are generally found at distinct sites between genes—that is, they are intergenic.

Because they are conserved among genera, oligonucleotide primers can be used to specifically amplify the repetitive sequences by PCR.

Different primers are used for each type of repetitive element, and the results are classified as arising from BOX-PCR, ERIC-PCR, or REP-PCR.

In each case the amplified fragments from many microbial samples can be resolved and visualized on an agarose gel. Each lane of the gel corresponds to a single bacterial isolate, and the pattern created by many samples resembles a UPC bar code.

The “bar code” is then computer analyzed using pattern recognition software as well as software that calculates phylogenetic relationships.

Because DNA fingerprinting enables identification to the level of species, subspecies, and often strain, it is valuable not only in the study of microbial diversity, but in the identification of human, animal, and plant pathogens as well.
10. Amino Acid Sequencing

- There are several ways to compare proteins. The most direct approach is to determine the amino acid sequence of proteins with the same function.
- Because protein sequencing is slow and expensive, more indirect methods of comparing proteins frequently have been employed.
- The electrophoretic mobility of proteins is useful in studying relationships at the species and subspecies levels.
- Antibodies can discriminate between very similar proteins, and immunologic techniques are used to compare proteins from different microorganisms.
- The amino acid sequences of proteins directly reflect mRNA sequences and therefore represent the genes coding for their synthesis.
- The value of a given protein in taxonomic and phylogenetic studies varies.
- The sequences of proteins with dissimilar functions often change at different rates; some sequences change quite rapidly, whereas others are very stable.
- Nevertheless, if the sequences of proteins with the same function are similar, the organisms possessing them may be closely related.
Searching on the web: BLAST at NCBI

Very fast computer dedicated to running BLAST searches.

Many databases that are always up to date (e.g., NR and Human Genome).

Nice simple web interface.

But you still need knowledge about BLAST to use it properly.
Understanding your BLAST output: 1. Graphic display

The display can help you see that some matches do not extend over the entire length of your sequence => useful tool to discover domains.
Understanding your BLAST output: 3. Alignment

A good alignment should not contain too many gaps and should have a few patches of high similarity, rather than isolated identical residues spread here and there.
BLASTting DNA: BLASTN output

- DNA double-stranded molecule => genes may occur on either strand
- plus strand (the query sequence), minus strand (reverse complement)
- If the similarity between query and subject is on the same strand: plus/plus
- If the minus strand of the query sequence is similar to a database sequence: plus/minus with the subject sequence in reverse coordinates (flipped)

Score = 87.7 bits (44), Expect = 2e-15 Identities = 57/60 (95%), Gaps = 1/60 (1%)
Strand = Plus / Plus
Query: 1  ggtgttttagagatcttgtttaacctgttaccaactgttctcoggtttatattgtggag 60

Sbjct: 96694 ggtgttttagagat-tgtttaacctgttaccaactgttctcoggtttatattgtggag 96752

Score = 52.0 bits (26), Expect = 1e-04 Identities = 26/26 (100%)
Strand = Plus / Minus
Query: 18 tgggtttacctgttaccaactgttctc 43

Sbjct: 40758 tgggtttacctgttaccaactgttctc 40783
Flow Cytometry

- Flow cytometry can be used to identify bacteria in a sample without culturing the bacteria.
- In a flow cytometer, a moving fluid containing bacteria is forced through a small opening.
- The simplest method detects the presence of bacteria by detecting the difference in electrical conductivity between cells and the surrounding medium.
- If the fluid passing through the opening is illuminated by a laser, the scattering of light provides information about the cell size, shape, density, and surface, which is analyzed by a computer.
- Fluorescence can be used to detect naturally fluorescent cells, such as *Pseudomonas*, or cells tagged with fluorescent dyes.
- A proposed test that uses flow cytometry to detect *Listeria* in milk could save time because the bacteria wouldn’t need to be cultured for identification.
- Antibodies against *Listeria* can be labeled with a fluorescent dye and added to the milk to be tested.
- The milk is passed through the flow cytometer, which records the fluorescence of the antibody-labeled cells.
## Relative Taxonomic Resolution of Various Molecular Techniques

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
<th>Subspecies</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genome sequencing</td>
<td>16S rRNA sequencing</td>
<td>Genomic fingerprinting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16S rDNA sequencing</td>
<td>Mol % G+C</td>
<td>DNA-DNA hybridization</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA-DNA hybridization</td>
<td>Multilocus sequence typing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multilocus sequence typing</td>
<td>Whole cell protein profiling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole cell protein profiling</td>
<td>Genomic fingerprinting</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Approaches for Assessing Diversity

- **Culture**
- **Nucleic acid extraction**
- **Organism isolation**
- **Molecular characterization**

Microbial community

Phenotype
Molecular Phylogenies

- The evolutionary relationships among organisms are summarized in diagrams called phyllogenetic trees, or simply phylogenies.
- These trees may only show the relationships among the organisms, or they may superimpose the relationships on a time line to indicate how each of the organisms evolved.
- A phylogeny that only shows the relationships is an unrooted tree, where one that show their derivation is a rooted tree.
- In both rooted and unrooted trees, the lineage bifurcate to produce branches.
- The branches at the tips of the tree called terminal branches, lead to the organisms that are under study.
- Each bifurcation in a tree repreasens a common ancestor of the organisms farther out in the tree.
- In molecular analysis of evolutionary relationships, the organisms are represented by DNA or protein sequences.
- Some analysis are based on a single gene or gene product. Other analysis combine data obtained by sequencing different genes or gene products. Sometimes the analyses utilize nongenic DNA sequences to ascertain the relationship among organisms.
Cladograms

- Cladograms are maps, that show evolutionary relationships among organisms (clado- means branch).
- Each branch point on the cladogram is defined by a feature shared by various species on that branch.
- Historically, cladograms for vertebrates were made using fossil evidence; however, rRNA sequences are now being used to confirm fossil-based assumptions. rRNA sequencing is primarily used to make cladograms for microorganisms.
- The small rRNA subunit used has 1500 bases, and computer programs do the calculations.
- The steps of constructing cladogram are as follows:
  1. Two rRNA sequences are aligned
  2. The percentage of similarity between the sequences are calculated
  3. The horizontal branches are drawn in a length proportional to the calculated percent similarity. All species beyond a node (branch point) have similar rRNA sequences, suggesting that they arose from an ancestor at that node.
Building a cladogram.

- Determine the sequence of bases in an rRNA molecule for each organism.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. brevis</td>
<td>AGUCCAGAGC</td>
</tr>
<tr>
<td>L. sanfranciscensis</td>
<td>GUAAAAGAGC</td>
</tr>
<tr>
<td>L. acidophilus</td>
<td>AGCGGAGAGC</td>
</tr>
<tr>
<td>L. plantarum</td>
<td>ACGUUAGAGC</td>
</tr>
</tbody>
</table>

Only a short sequence of bases is shown for this example.

- Calculate the percentage of similarity in the nucleotide bases between pairs of species. For example, there is a 70% similarity between the sequences for *L. brevis* and *L. acidophilus*.

<table>
<thead>
<tr>
<th>Pair</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. Brevis/L. sanfranciscensis</td>
<td>50%</td>
</tr>
<tr>
<td>L. brevis/L. acidophilus</td>
<td>70%</td>
</tr>
<tr>
<td>L. Brevis/L. plantarum</td>
<td>60%</td>
</tr>
<tr>
<td>L. Sanfranciscensis/L. acidophilus</td>
<td>50%</td>
</tr>
<tr>
<td>L. sanfranciscensis/L. plantarum</td>
<td>50%</td>
</tr>
<tr>
<td>L. Plantarum/L. acidophilus</td>
<td>60%</td>
</tr>
</tbody>
</table>
- Construct a cladogram.
- The length of the horizontal lines corresponds to the percent similarity values.
- Each branch point, or node, in the cladogram represents an ancestor common to all species beyond that node.
- Each node is defined by a similarity in rRNA present in all species beyond that branch point.
Numerical taxonomy or taximetrics or phenetics

Peter H. A. Sneath and Robert Sokal have defined numerical taxonomy as “the grouping by numerical methods of taxonomic units into taxa, on the basis of their character states.”

It was Michel Adanson, a French botanist, who for the first time put forward a plan for assigning numerical values to the similarity between organisms, as possible for the classification, and such classifications came to be known as Adansonian classifications.

- Phenetics means as observed in the phenotype.
- Phenetic characters refers to all the observable or measurable characters of an organism.
- The result of phonetic relationship is often summarized with a tree-like network called a phenogram.
- Phenogram is a type of diagram, which is based on the phonetic data.
- Lines called phenon lines, represent lines of % similarity of phonetic features between organisms.
Principle of numerical taxonomy used in bacteriology:

i. Each character of bacteria has is assigned a value of one (1),

ii. Then we simply determine how many characteristics a pair of species share

iii. The higher the percentage shared, the higher their relatedness

iv. Strict statistical criteria can then be applied to determine how related a pair of species should be to occur in the same genus, same family, etc.
1. Collection of data. The bacterial strains have to be chosen and examined for a number of properties or taxonomic characters.

2. The data must be coded and scaled in an appropriate fashion.

3. The similarity or resemblance between the strains is calculated. This yields a table of similarities (similarity matrix) based on the chosen set of characteristics.

4. The similarities are analyzed for taxonomic structure, to yield the groups or clusters that are present, and the strains are arranged into phenons (phonetic groups) which are broadly equated with taxonomic groups (taxa).

5. The properties of the phenons can be tabulated for publication or further study, and the most appropriate characters can be chosen on which to set up identification systems.
Let us consider six **OTUs** (operational taxonomic units): S, T, W, X, Y, and Z to determine the phonetic relationship:

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>T</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>W</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>X</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Y</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Z</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

- From the data we can **calculate simple matching coefficient**, by counting the number of character states for each character in common between two OTUs, and dividing this number by the total number of characters used.
- The coefficient between S and T = \( \frac{\text{character states in common}}{\text{total of characters}} \) = 8/10 = 0.8.
- Such calculations are done all pairs of OTUs, and the resultant figures are depicted next slide in a **data matrix** of **coefficient of association**.
- The higher the coefficient of association, the most closely related are the OTUs.
• Now based on this data matrix of coefficient of association, a phenogram can be constructed.

• In phenogram, the vertical lines indicating groups or clusters of OTUs are based on the coefficients of association.

Data matrix of coefficient of association

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>T</th>
<th>W</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.8</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>0.5</td>
<td>0.7</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>0.7</td>
<td>0.5</td>
<td>0.8</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y</td>
<td>0.4</td>
<td>0.2</td>
<td>0.3</td>
<td>0.5</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Z</td>
<td>0.3</td>
<td>0.1</td>
<td>0.4</td>
<td>0.6</td>
<td>0.9</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Here, S and T as well as W and X are similar at the 0.8 level and Y and Z are similar at 0.9 level.

So, the former two pairs of OTUs are more similar to each other than either pairs is to latter.

The level at which they are connected is based on an average value of the pairs S to W, S to X, T to W and T to X:

\[
\frac{(S \text{ to } W) + (S \text{ to } X) + (T \text{ to } W) + (T \text{ to } X)}{4} = \frac{0.5 + 0.7 + 0.7 + 0.5}{4} = 0.24 / 4 = 0.6
\]

The relationship of Y and Z to the other four OTUs is assessed in the same way and found to be...?
Problems with Numerical Taxonomy

a. The numerical methods are useful in phenetic classifications and not phylogenetic classifications.

b. The proponents of “biological” species concept, may not accept the specific limits bound by these methods.

c. Character selection is the greatest disadvantage in this approach. If characters chosen for comparison are inadequate, the statistical methods may give less satisfactory solution.

d. Different taxonometric procedures may yield different results. A major difficulty is to choose a procedure for the purpose and the number of characters needed in order to obtain satisfactory results.