Chapter
12

Microbial Genetics

Learning Objectives

After studying this chapter the students will be able to,

- *Define gene, genome, genetic code, genotype, phenotype, mutagen, wildtype*
- *Describe transcription and translation*
- *Classify mutations and its types and Understand how mutants are formed*
- *Know the mode of action of physical and chemical mutagens*
- *Identify the purpose of and outline the procedure for Ames test*
- *Compare the gene transfer mechanisms*
- *Know the types of cloning vectors used in genetic engineering*
- *Describe how plasmids and bacteriophages are used to transfer foreign DNA*
- *Explain the role of restriction enzymes in recombinant DNA technology*
- *Know the types of restriction enzymes*
- *Understand agarose gel electrophoresis and PCR techniques*
- *Explain RAPD and RFLP*

Chapter Outline

12.1 Concept of Gene

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The fundamental unit of information in living systems is the **gene**. **Genome** is the set of all genes and genetic signals of a cell. The information contained in genes is converted to molecules that determine the metabolism, structure and form of microorganisms. Gene is expressed through a sequence of events. A gene can be defined biochemically as a segment of DNA (or, in a few cases, RNA) that encodes the information required to produce a functional biological product. The final product is usually a protein. Not all genes are involved in protein synthesis; some code instead for rRNA and tRNA.

The central dogma of molecular biology, comprises the three major processes (Figure 12.1). The first is **replication**, the

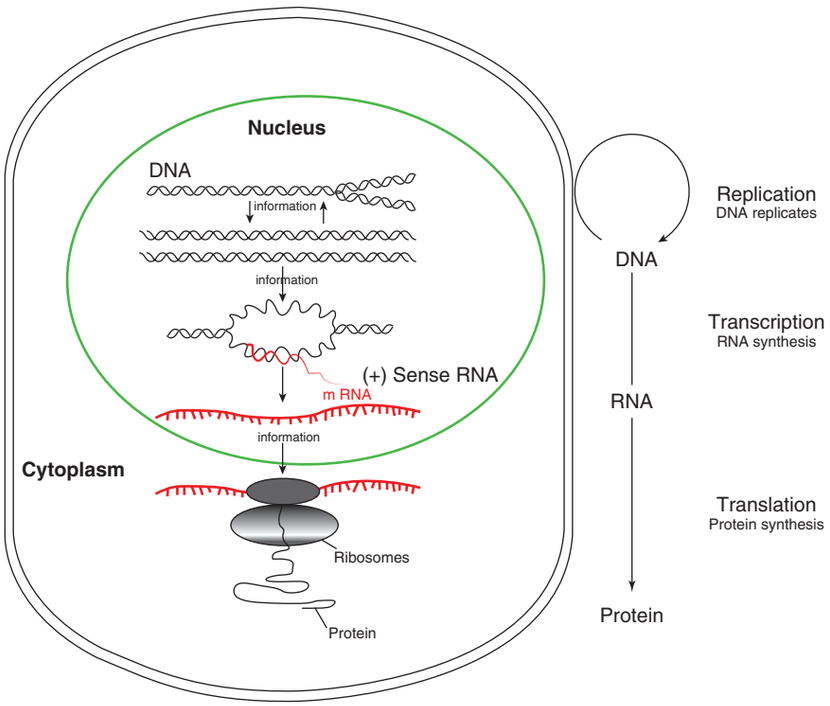

Figure 12.1: Central dogma of molecular biology

copying of parental DNA to form daughter DNA molecules with identical nucleotide sequences. The information contained in the base sequence of DNA is copied into protein molecule through an RNA molecule. The second is **transcription**, production of mRNA from DNA. It is the process by which the segment corresponding to a particular gene is selected and an RNA molecule is synthesized. The third is **translation**, The production of an amino acid sequence from an RNA base sequence. The genetic message encoded in messenger RNA (mRNA) is translated on the ribosomes into a polypeptide with a particular sequence of amino acids. The order of amino acid in a polypeptide chain is determined by DNA base sequence.

12.2 Transcription

An important feature of RNA synthesis is that even though the DNA molecule being copied is double stranded, in any particular region of DNA only one strand serves as a template. The DNA strand copied into RNA molecule is called CODING OR SENSE STRAND.

The synthesis of RNA consists of five discrete stage (Figure 12.2):

1. Promoter recognition: RNA polymerase binds to DNA within a specific base sequence (20–200 bases long) called a promoter. The sequence TATAAT (or a nearly identical sequence) often called a pribnow box or – 10 region is found as part of all prokaryotic promoters.

   The RNA polymerase of the bacterium *E.coli* consists of five protein subunits. Four of the subunits comprise the core enzyme (catalyzes the joining of the nucleoside triphosphates to the RNA) and fifth subunit, the σ subunit (required for promoter binding).
2. Local unwinding of DNA occurs and RNA polymerase forms an open promoter complex.

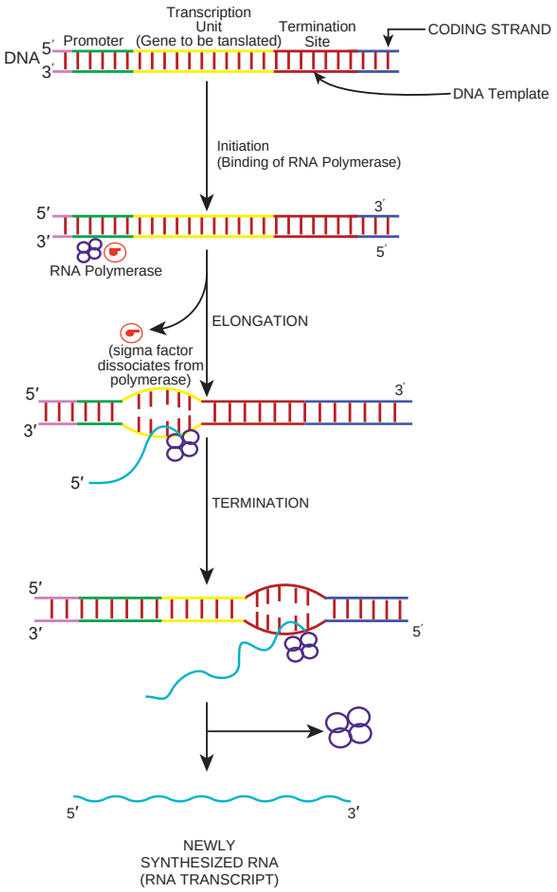

Figure 12.2: Major events in transcription

3. The first nucleoside triphosphate is placed at polymerization start site (near to the initial binding site) and synthesis begins.
4. RNA polymerase then moves along the DNA, adding ribonucleotides, to the growing RNA chain.
5. RNA polymerase reaches chain termination sequence and both the newly synthesized RNA and the polymerase are released. Two kinds of termination events are known those that are self – terminating (dependent on the base sequence only) and those that require the presence of the termination protein Rho.

Initiation of a second round of transcription need not await completion of the first, for the promoter becomes available once RNA polymerase has polymerized 50–60 nucleotides. In bacteria most mRNA molecules are degraded within a few minutes after synthesis. This degradation enables cells to dispense with molecules that are no longer needed.

In prokaryotes mRNA molecules commonly contain information for the amino acid sequences of several different polypeptide chains. In this case, such a molecule is called polycistronic mRNA. Cistron is a term used to mean a base sequence encoding a single polypeptide chain. The genes contained in polycistronic mRNA molecule (Figure 12.3) often encode the different portions of a metabolic pathway. For example, in *E. coli* the ten enzymes needed to synthesize histidine are encoded in one mRNA molecule.

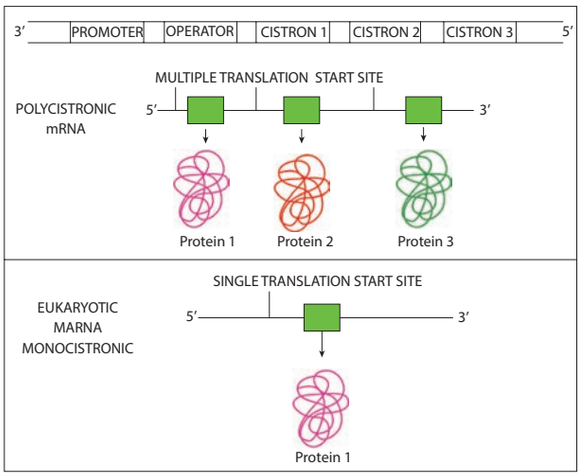

Figure 12.3: Polycistronic mRNA

In prokaryotes the immediate product of transcription (called the primary transcript) is mRNA, in contrast in eukaryotes the primary transcript must be converted to mRNA. This conversion called RNA processing consists of two types of events- modification of termini and excision of untranslated sequences (non-coding sequence or introns) embedded

within coding sequences (exons). Introns excision and the joining of exons to form an mRNA molecule is called RNA splicing. The introns are present in almost all eukaryotic transcripts but are rare in the free – living unicellular eukaryotes such as yeast. Some bacterial genes do contain introns.

Synthesis of rRNA and tRNA

Ribosomal RNA and tRNA are also transcribed from genes. The production of these molecules is not as direct as synthesis of bacterial mRNA. The main difference is that these RNA molecules are excised from large primary transcripts. Highly specific RNA excise rRNA and tRNA from these large transcripts, and other enzymes produce the modified bases in tRNA.

12.3 Genetic Code

A tRNA molecule "reads" the base sequence of mRNA. The language read by the tRNA molecules is called the genetic code, which is a set of relations between sequences of three adjacent bases on an mRNA molecule and particular amino acids. (A RNA base sequence (a set of 3 bases) corresponding to a particular amino acid is called a codon). The genetic code is the set of all codons. Only four bases in DNA serve to specify 20 amino acids in proteins, so some combination of bases is needed for each amino acid. Before the genetic code was elucidated, it was reasoned that if all codons were assumed to have the same number of bases, then each codon would have to contain at least three bases. Codons consisting of pairs of bases would be insufficient because four bases can form only $4^2 = 16$ pairs, and there are 20 amino acid. Triplets of bases would suffice because, these can form $4^3 = 64$ triplets. In fact, the genetic code is a triplet code, and all 64 possible codons carry information of some sort. Several different codons designate the same amino acid. Furthermore, in translating mRNA molecules the codons do not overlap but are used sequentially. The same genetic code is used by almost all biological systems and hence is said to be universal (exceptions are mitochondria and a few unusual microorganisms). The codons are by convention written with the 5′ end at the left. The complete code is shown in Table 12.1.

Features of the Code:

- Sixtyone codons correspond to amino acids. Four codons are signals. These are the three stop codons – UAA, UAG, UGA – and the one start codons, AUG. The start codons (initiation codon) also specifies the amino acid methionine. In rare cases, certain other codon (E.g. GUG) initiate translation. No normal tRNA molecule has an anticodon (a sequence of three bases on tRNA that can base – pair with a codon sequence in the mRNA) complementary to any of the stop codons UAG, UAA or UGA, which is why these codons are stop signals.
- The code is highly redundant i.e. more than one codons code for an amino acid. Only tryptophan and methionine are specified by one codon. The synonymous codons usually differ only in third base (except for serine, leucine and arginine).

First letter	Second letter: U	Second letter: C	Second letter: A	Second letter: G	Third letter
U	UUU, UUC – Phe; UUA, UUG – Leu	UCU, UCC, UCA, UCG – Ser	UAU, UAC – Tyr; **UAA Stop**; **UAG Stop**	UGU, UGC – Cys; **UGA Stop**; UGG Trp	U C A G
C	CUU, CUC, CUA, CUG – Leu	CCU, CCC, CCA, CCG – Pro	CAU, CAC – His; CAA, CAG – Gln	CGU, CGC, CGA, CGG – Arg	U C A G
A	AUU, AUC, AUA – Ile; **AUG Met**	ACU, ACC, ACA, ACG – Thr	AAU, AAC – Asn; AAA, AAG – Lys	AGU, AGC – Ser; AGA, AGG – Arg	U C A G
G	GUU, GUC, GUA, GUG – Val	GCU, GCC, GCA, GCG – Ala	GAU, GAC – Asp; GAA, GAG – Glu	GGU, GGC, GGA, GGG – Gly	U C A G

Table 12.1: Genetic code

The 20 amino acids and their abbreviations	
Amino acid	3-letter abbreviation
Alanine	Ala
Arginine	Arg
Asparagine	Asn
Aspatic acid	Asp
Aspartic acid & Asparagine	Asx
Cysteine	Cys
Glutamine	Gln
Glutamic acid	Glu
Glutaine or Glutamic acid	Glx
Glycine	Gly
Histidine	His
Isoleucine	IIe
Leucine	Leu
Lysine	Lys
Methionine	Met

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1. How many of the 64 codons can be made from the three nucleotides A, U, and C?
2. If codons were four bases long, how many codons would exist in a genetic code?

12.4 Translation

RNA is translated from the 5′ end of the molecule toward the 3′ end. Polypeptides are synthesized from the amino terminus toward the carboxyl terminus, by adding amino acids one by one to the carboxyl end. Not all the base sequences in an mRNA is translated into amino acid sequences of polypeptides. Initiation of polypeptide synthesis may begin hundreds of nucleotides from the 5′ – P terminus of the RNA. The section of untranslated RNA before the region encoding the first polypeptide chain is called a **leader**, which in some cases contains regulatory sequences that influence the rate of protein synthesis. The major events in translation are (Figure 12.4).

1. An mRNA binds to the surface of a protein synthesizing particle, the **Ribosome**.
2. The tRNA – amino acid complexes (made by the aminoacyl tRNA synthetases) bind sequentially, one by one, to the mRNA molecule that is attached to the ribosome.

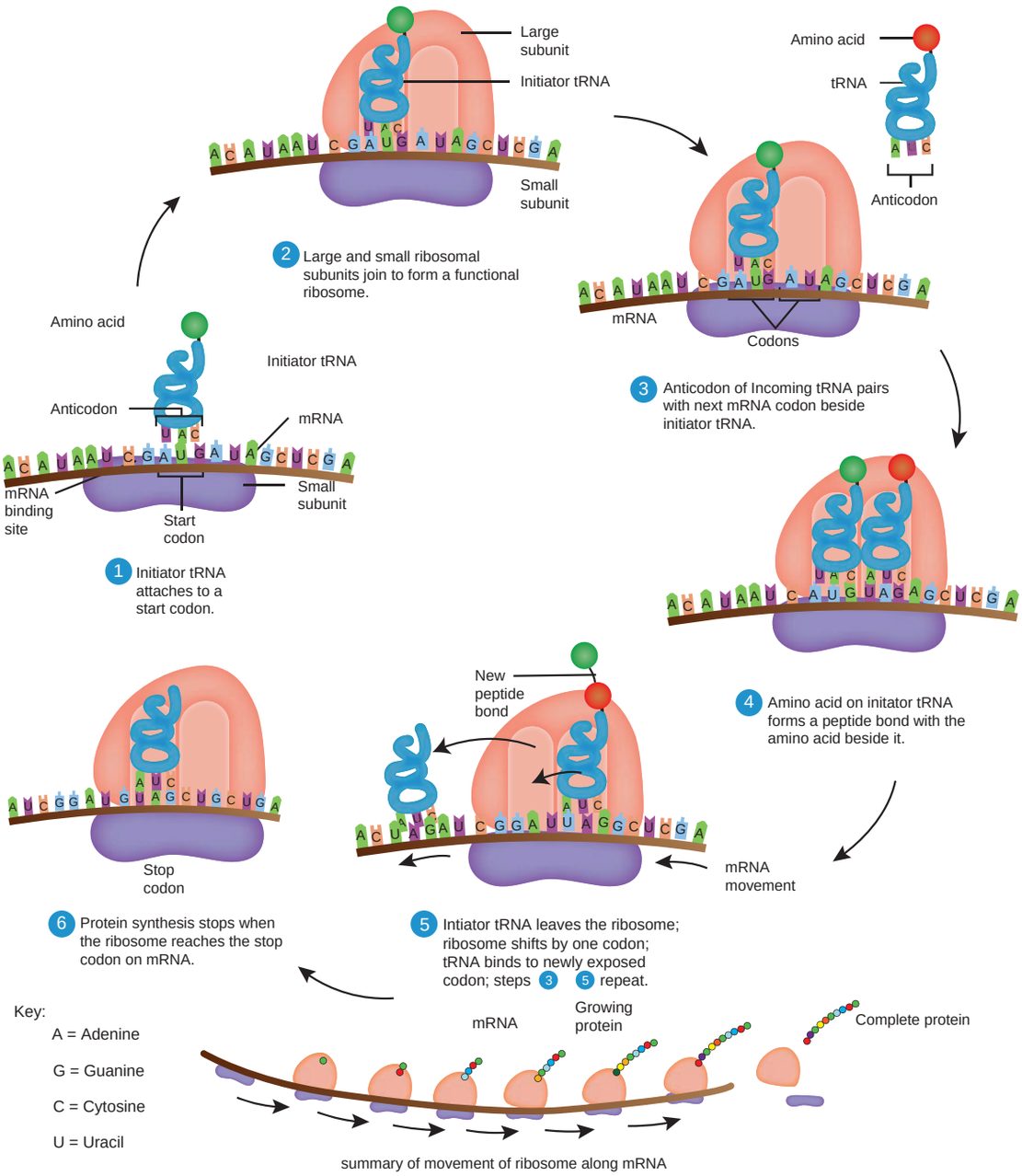

Figure 12.4: Major events in Translation

3. Peptide bonds are made between successively aligned amino acids.
4. Finally the chemical bond between the tRNA and its attached amino acids is broken and the completed protein is removed.

- The 3′ terminal of the tRNA molecule (Figure 12.5) is covalently linked to the amino acid corresponding to the particular mRNA codon
- When an amino acid has become attached to a tRNA molecule, the tRNA is said to be acylated or charged
- An important feature of initiation of polypeptide synthesis in both prokaryotes and eukaryotes is the use of a **specific initiating tRNA molecule**. In prokaryotes this tRNA molecule is acylated with the modified amino acid N – formyl methionine (fMet). This

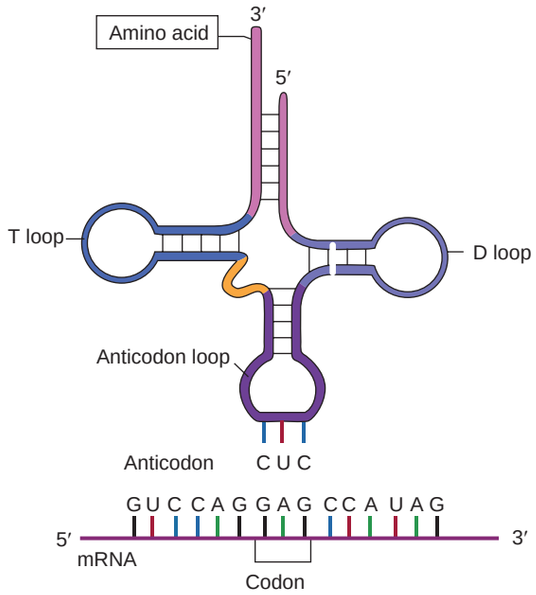

Figure 12.5: tRNA

tRNA is often designated **tRNA^{fMet}**. Both tRNA^{fMet} and tRNA^{Met} recognize the codon AUG, but only tRNA^{fMet} is used for initiation. All prokaryotic proteins while being synthesized have fMet at the amino terminus. However, this amino acid is frequently deformylated or removed later.

- The usual form of translation unit is a polyribosome or polysome wherein an mRNA is covered with ribosomes.

Redundancy and the Wobble Hypothesis

The identity of the third base of a codon appears to be unimportant. (The first base in a codon is at the 5′ end and the third base is at the 3′ end). Wobble refers to the less stringent requirement for base pairing at the third position of the codon than at the first two positions. That is the first two bases must follow Watson and Crick base pairing rule (A with U, or G with C), but the third base pair can be of a different type (for example, G with U). The Wobble hypothesis explains the pattern of redundancy in the code in that certain anticodons (For example, those containing U and G in the first position of the anticodons) can pair with several codons during translation (Figure 12.6).

12.5 Types of Mutations

The base sequence of DNA determines the amino acid sequence of a protein. The chemical and physical properties of each protein are determined by its amino acid sequence, so a single amino acid change is capable of altering the activity of, or even completely inactivating, a protein. **Genotype** refers to the genetic composition

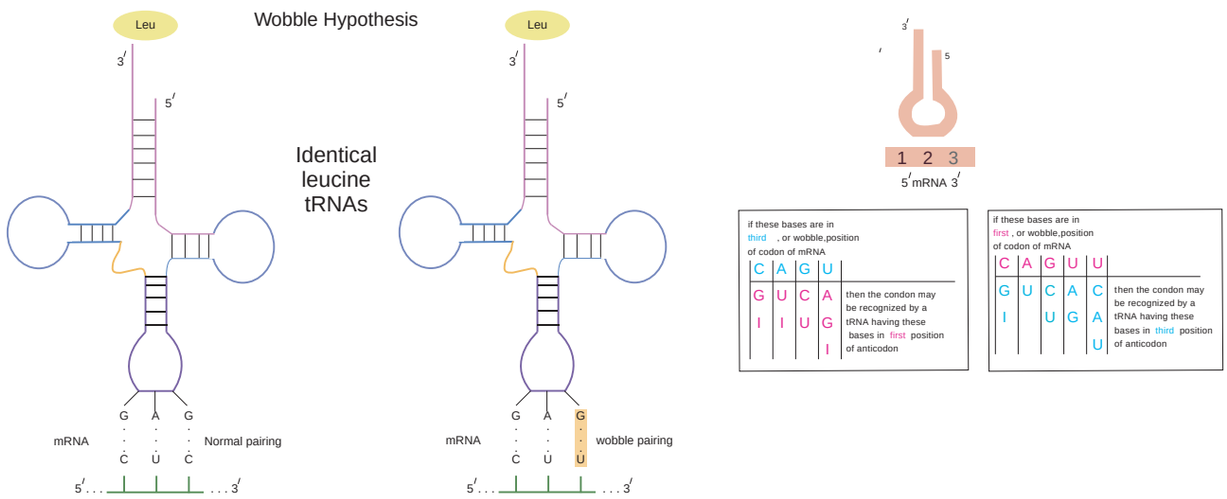

Figure 12.6: Wobble hypothesis

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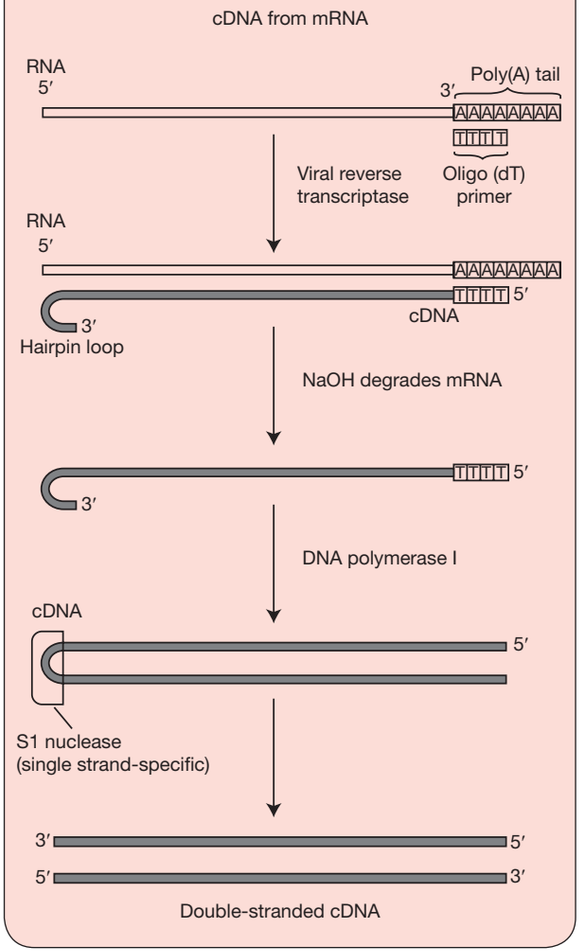
In 1970 Howard Temin and David Baltimore independently discovered the enzyme **reverse transcriptase** that retroviruses use to produce DNA copies of their RNA genome. This enzyme can be used to construct DNA copy, called **complementary DNA** (cDNA), of any RNA as shown in figure below . Thus genes or major portions of the gene can be synthesized from mRNA.



of an organism. **Phenotype** is an observable property of organism. The functional form of a gene is called **Wildtype** because presumably this is the form found in nature.

Mutation is the process by which the sequence of base pairs in a DNA molecule is altered.The alteration can be a single base pair substitution, insertion or deletion.

Mutations can be divided into two general categories:

1. **Base–pair substitution mutation** involves a change in the DNA such that one base pair is replaced by another.
   - A mutation from one purine – pyrimidine base pair to the other purine –pyrimidine base pair is a transition mutation (Figure 12.7a). E.g. AT to GC, CG to TA.
   - A mutation from a purine - pyrimdine base pair to a pyrimidine – purine base pair is a transversion mutation (Figure 12.7b). E.g. AT to TA, CG to GC.
2. **Base pair insertion or deletions** involves the addition or deletion of one base pair. If one or more base pairs are added to or deleted from a protein coding gene, the reading frame of an mRNA can change downstream of the mutation. An addition or deletion of one base pair, for example, shifts the mRNA's downstream reading frame by one base, so that incorrect amino acids are added to the polypeptide chain after the mutation site. This type of mutation, called a frame shift mutation (Figure 12.8) usually results in a nonfunctional protein.

Frame shift mutations:

- May generate new stop codons, resulting in a shortened protein.
- May result in a read through of the normal stop codon, resulting in longer than normal proteins
- Or may result in a complete alteration of the amino acid sequence of a protein.

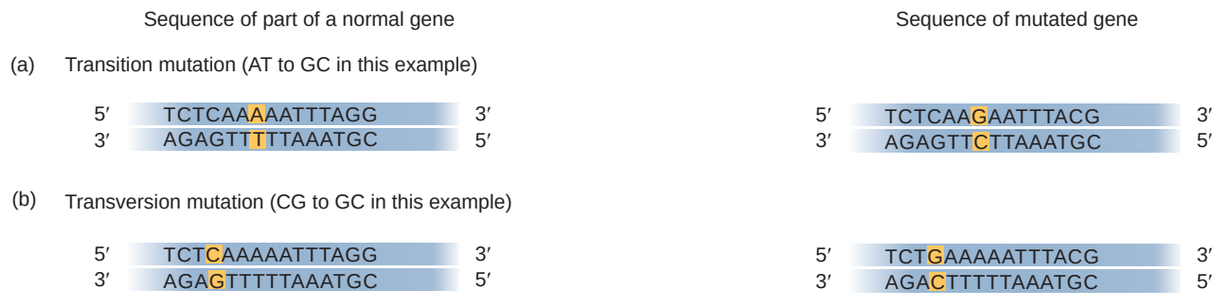


Figure 12.7: **(a)** Transition mutations **(b)** transversion mutations

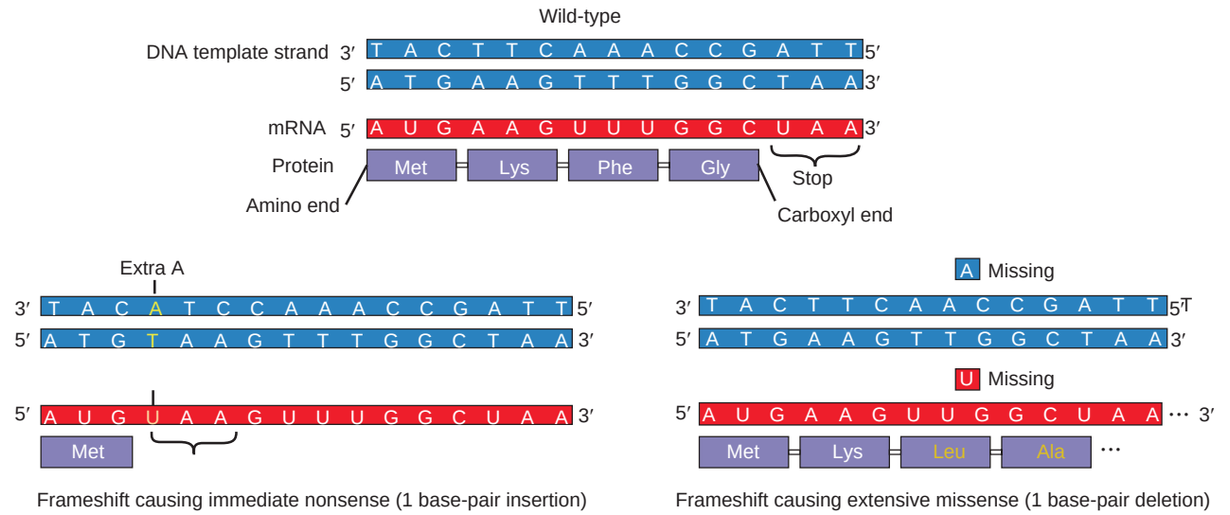


Figure 12.8: Frameshift mutations

Point mutations are single base changes, that do not affect the reading frame, that is, the mutation only makes a single change in a single codon, and everything else is undisturbed.

Mutations can also be defined according to their effects on amino acid sequences in proteins. They are

1. A missense mutation (Figure 12.9a) is a gene mutation in which a base – pair change in the DNA changes a codon in an mRNA so that a different amino acid is inserted into the polypeptide.
2. A neutral mutation (Figure 12.9b) is a subset of missense mutations in which the new codon codes for a different amino acid that is chemically equivalent to the original and therefore does not affect the proteins function. Consequently, the phenotype does not change.
3. A silent mutation (Figure 12.9c) is also a subset of missense mutations that occurs when a base – pair change in a gene alters a codon in the mRNA such that the same amino acid is inserted in the protein. In this case, the protein obviously has a wild type function.
4. A nonsense mutation (Figure 12.9d) is a gene mutation in which a base – pair change in the DNA, changes a codon in an mRNA to a stop (nonsense) codon (UAG, UAA or UGA). Nonsense mutation cause premature chain termination so instead of complete polypeptides, shorter than normal polypeptide fragments (often nonfunctional) are formed.

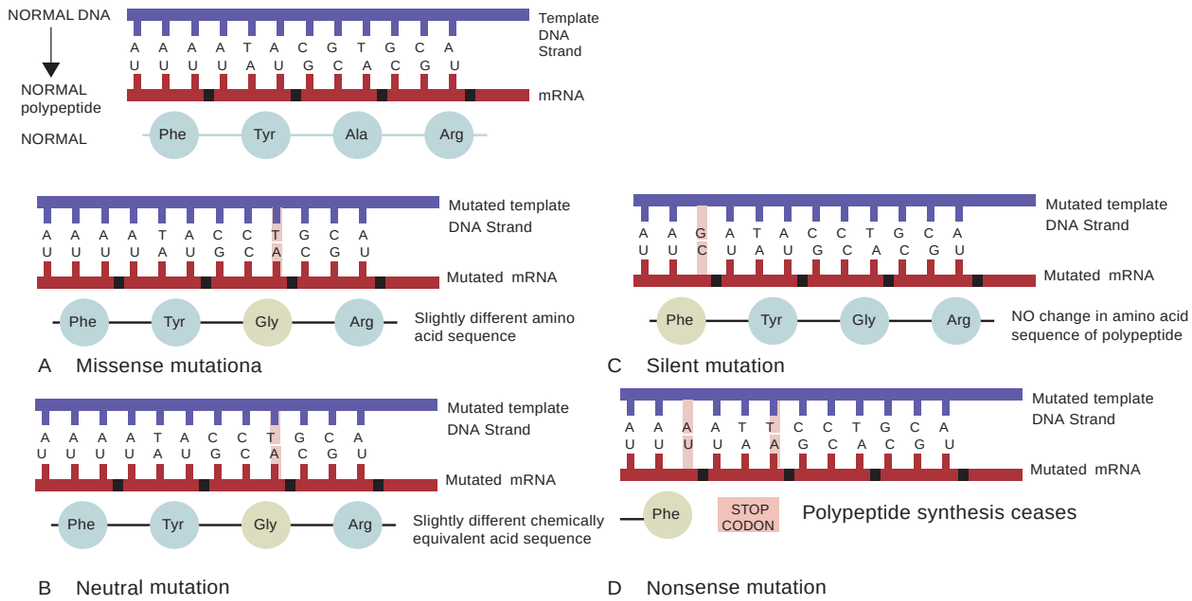


Figure 12.9: **(a)** Missense **(b)** neutral **(c)** silent **(d)** nonsense mutation respectively

Forward mutations change the genotype from wild type to mutant and reverse mutations (or reversions or back mutations) change the genotype from mutant to wild type or to partially wild type. An organism which has reverted is a Revertant. The effects of mutation may be diminished or abolished by a suppressor mutation. Suppressor mutation is a mutation at a different site from that of the original mutation. A suppressor mutation masks or compensates for the effects of the initial mutation, but it does not reverse the original mutation.

12.6 Formation of Mutants

The term mutant refers to an organism in which either the base sequence of DNA or the phenotype has been changed. A mutant is an organism whose genotype differs from that found in nature. The process of formation of mutant organism is called mutagenesis. In nature and in the laboratory, mutations sometimes arise spontaneously, without any help from the experimenter. This is called spontaneous mutagenesis. The two mechanisms that are most important for spontaneous mutagenesis are

1. Errors occurring during replication and
2. Spontaneous alteration of bases.

Mutations can also be induced experimentally by application of mutagens. Mutagens are agents that cause mutations.

Mutagens and their Mode of Action

Physical Mutagens

UV radiation: UV light causes mutations because the purine and pyrimidine bases in DNA absorb light strongly in the ultraviolet range (254 to 260 nm). At this wavelength, UV light induces point mutations primarily by causing photochemical changes in the DNA. One of the effects of UV radiation on DNA is the formation of abnormal chemical bonds between adjacent pyrimidine

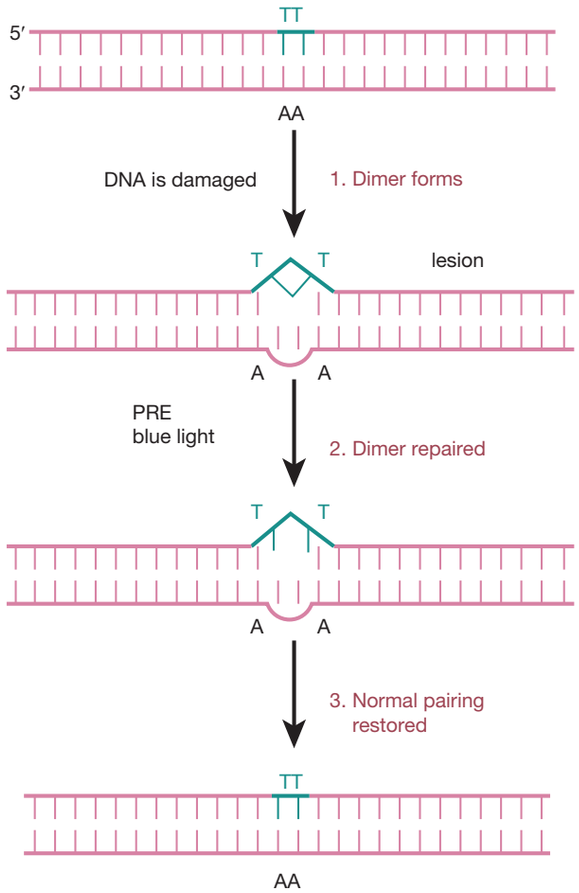


Figure 12.10: UV induced DNA damage

molecules in the same strand, or between pyrimidines on the opposite strands, of the double helix. This bonding is induced mostly between adjacent thymines, forming what are called thymine dimers (Figure 12.10), usually designated TT. This unusual pairing produces a bulge in the DNA strand and disrupts the normal pairing of T's (thymines) with corresponding A's(adenines) on the opposite strand. If UV induced genetic damage is not repaired, mutations or cell death may result.

Chemical Mutagens

Chemical mutagens include both naturally occurring chemicals and synthetic substances. These mutagens can be grouped into different classes on the basis of their mechanism of action. They are

i. **Base analogs** are bases that are similar to the bases normally found in DNA. E.g. 5 – bromouracil (5-BU). TA to CG (Figure 12.11).

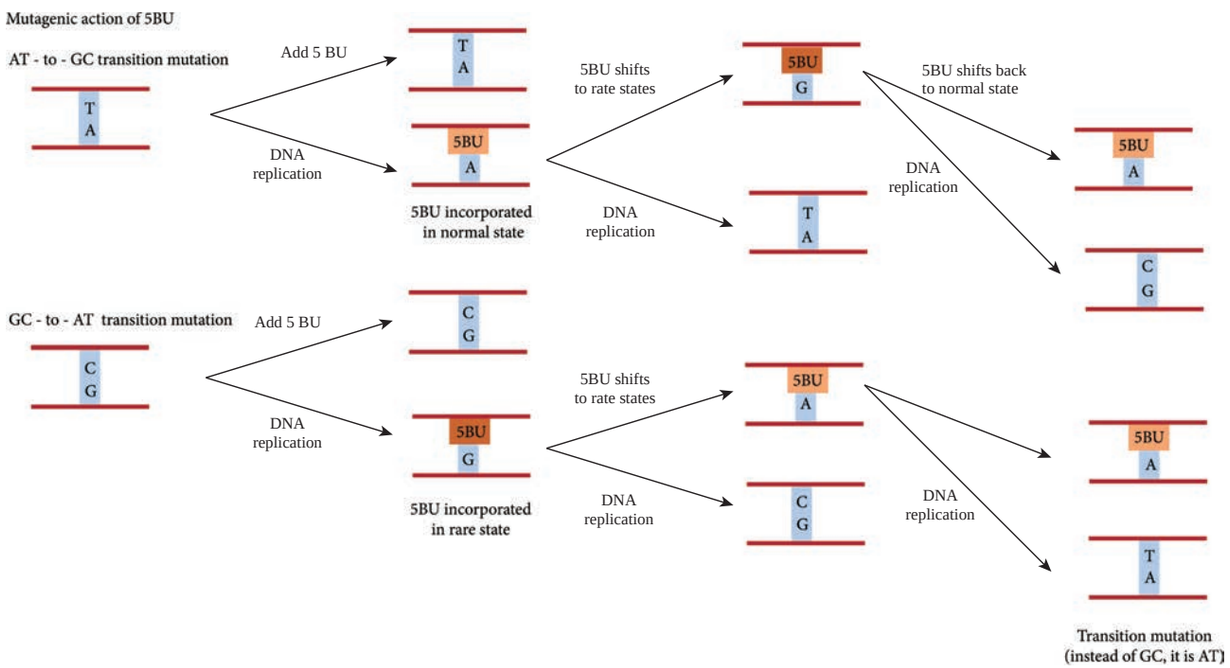


Figure 12.11: Mutagenic effects of 5-BU

Not all base analogs are mutagens. For example, AZT (Azidothymidine), one of the approved drugs given to patients with AIDS, is an analog of thymidine, but it is not a mutagen, because it does not cause base pair changes.

ii. **Base Modifying Agents** are chemical that act as mutagens by modifying the chemical structure and properties of bases. The three types of mutagens that work in this way are

1. A deaminating agent e.g.: Nitrous acid removes amino groups (- NH_2) from the bases guanine, cytosine, and adenine.
2. Hydroxylamine (NH_2 OH) is a hydroxylating mutagen that react specifically with cytosine, modifying it by adding a hydroxyl group (OH) so that it can pair solely with adenine instead of with guanine.
3. Alkylating agents like methymethane sulfonate (MMS) introduces alkyl groups onto the bases at a number of location.

iii. Intercalating agents

Acridine, proflavin, ethidium bromide are a few examples of intercalating agents. These insert (intercalate) themselves between adjacent bases in one or both strands of the DNA double helix. Intercalating agents can cause either additions or deletions.

Original base	Mutagen	Modified base Pairing partner	Predicted transition
Cytosine	Nitrous acid (H_2NO) →	Uracil ═ Adenine	CG TA
Cytosine	Hydroxylamine (NH_2OH) →	Hydroxyl amino cystosine ═ Adenine	CG TA
Guanine	Methylmethane sulfonate(MMS) (alkylating agent) →	O-Methylguanine ═ Thymine	CG TA

Figure 12.12: Action of three base modifying agents.

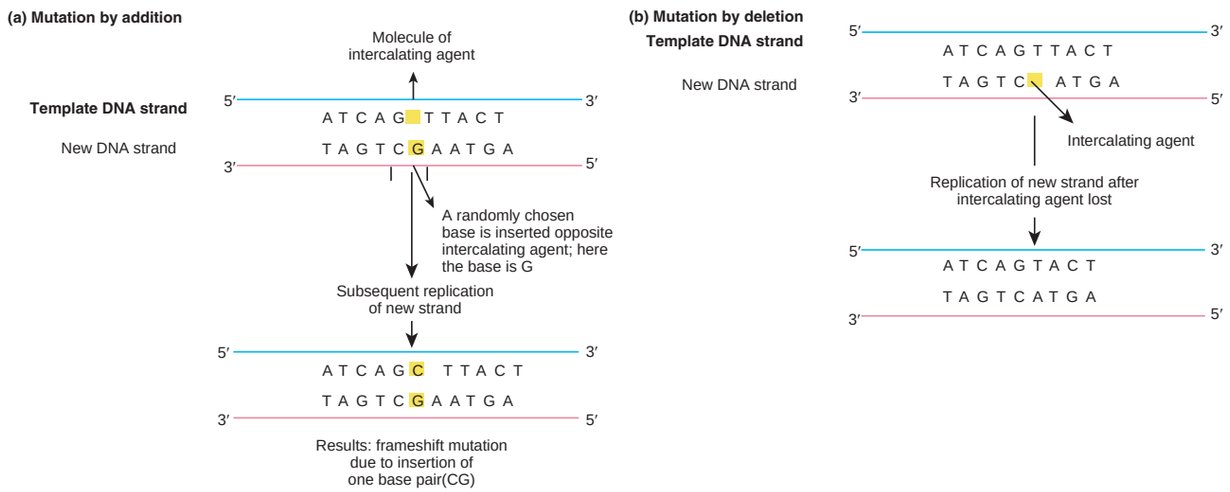


Figure 12.13: Mutations due to intercalating agents

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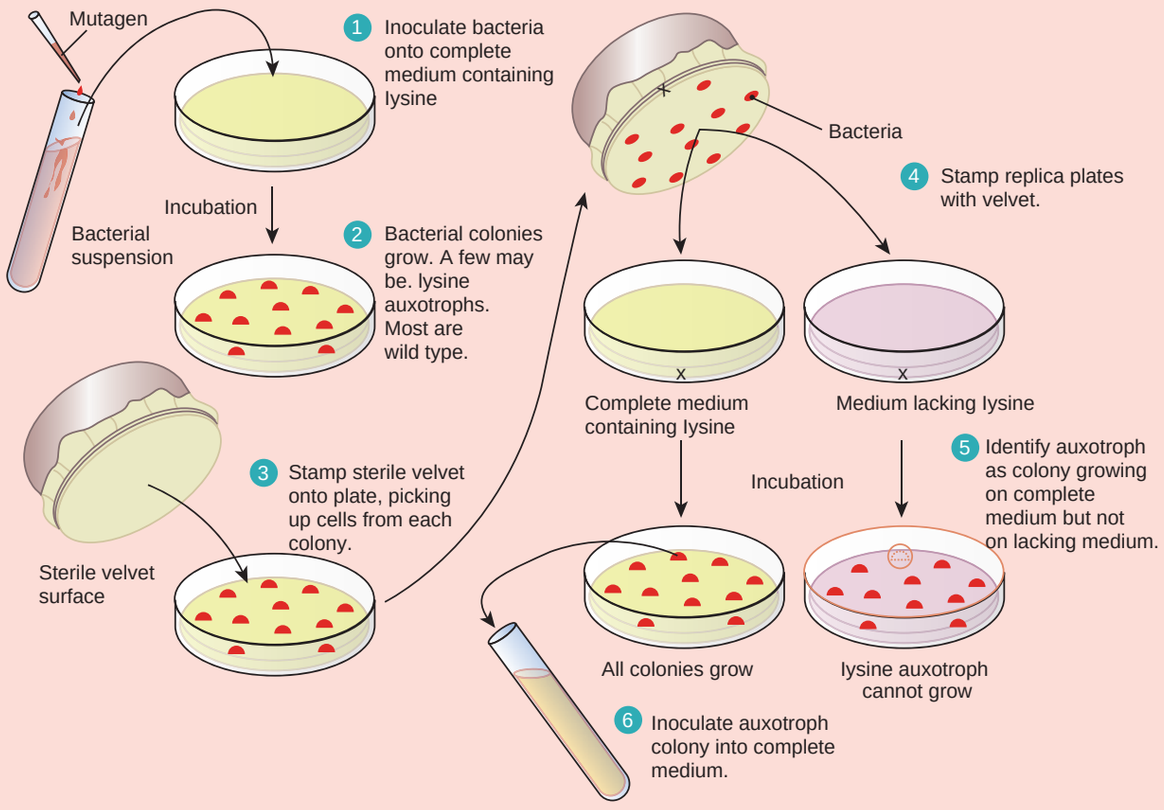
Isolation and detection of Mutants

Once mutations are induced, then, they must be detected if they are to be studied. Selection and screening procedures historically have helped geneticists isolate mutants of interest from a heterogenous mixture in a mutagenized population. When isolating mutants of a particular organism, one must know the normal or wild type characteristics so as to recognize an altered phenotype. A suitable detection system for the mutant phenotype under study also is needed. Detection systems in bacteria and other haploid organisms are straightforward because any new allele should be seen immediately, even if it is recessive mutation. The detection of mutants can be direct and complex. For example, the replica plating technique is used to detect auxotrophic mutants (mutants which are deficient in synthesizing a particular biochemical compound). **Replica plating technique** distinguishes between mutant and wild type strain based on their ability to grow in the absence of a particular biosynthetic end product Figure below. A lysine auxotroph, for instance, will grow on lysine supplemented media but not on a medium lacking an adequate supply of lysine because it cannot synthesize this amino acid.



The Ames Test: A Screen for Potential Carcinogens

Everyday we are exposed to a wide variety of chemicals in our environment, such as drugs, cosmetics, food additives, pesticides, and industrial compounds. Many of these chemicals can have mutagenic effects, including genetic diseases and cancer. Some banned chemical warfare agents (e.g. mustard gas) also are mutagens.

A number of chemicals (subclass of mutagens) induce mutations that result in tumorous or cancerous growth. These chemical agents are called chemical carcinogens. Directly testing the chemicals for their ability to cause tumors in animals is time consuming and expensive. However, the fact that most chemical carcinogens are mutagens led Bruce Ames to develop a simple, inexpensive, indirect assay for mutagens. In general Ames test is an indicator of whether the chemical is a mutagen. The Ames test assays the ability of chemicals to revert mutant strains of the bacterium *Salmonella typhimurium* to wild type. The mutant strain of *S.typhimurium* is auxotrophic to histidine (his^-), that is it requires histidine for its growth and cannot grow in the absence of histidine. The mutant strain is grown in a histidine deficient medium containing the chemical to be tested. A control plate is also set up which does not contain the chemical. After incubation the control plates may have few colonies resulting from spontaneous reversion of the his- strain. Compared to the control plates if there are increased number of colonies on test plate, it indicates that the chemical has reverted the mutant strain back to wild type. This chemical is likely to be a carcinogen. Figure 12.14 shows steps in Ames test.

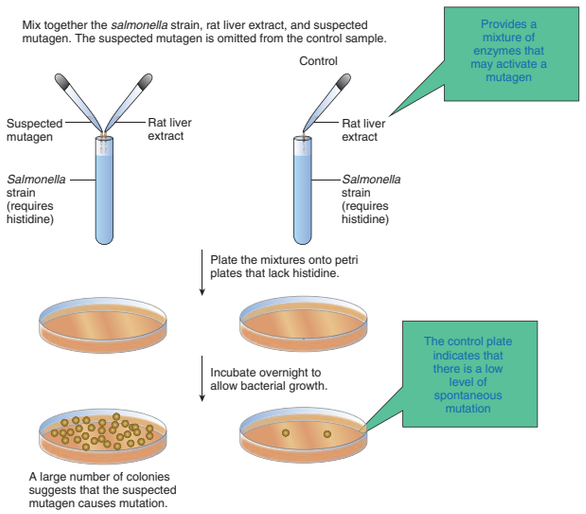


Figure 12.14: Steps in Ames test

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DNA Repair

Both prokaryotes and eukaryotes have a number of repair systems that deal with different kinds of DNA damage. All the systems use enzymes to make correction. Without this repair systems lesions would accumulate and be lethal to the cell or organism. Not all lesions are repaired, and mutations do appear, but at low frequencies. At high doses of mutagens, repair systems are unable to correct all of the damage, and cell death may result. We can group repair systems into different categories on the basis of the way they operate. Some systems correct damaged areas by reversing the damage. This type of repair is called direct correction or direct reversal. Other systems excise the damaged areas and then repair the gap by new DNA synthesis. Some of the DNA repair systems are

- Mismatch repair by DNA polymerase proofreading
- Repair of UV induced pyrimidine dimers- Photo reactivation or Light repair
- Base excision repair
- Nucleotide excision repair

With recombinant DNA technology it is possible to mutate a gene at specific positions in the test tube by SITE SPECIFIC MUTAGENESIS and then introduce the mutated gene back into the cell and investigate the phenotypic changes produced by the mutation in vivo. Such techniques enable geneticists to study, for example, genes with unknown function and specific sequences involved in regulating a gene's expression.

12.7 Transfer of Genetic Material

Normally, genes and the characteristics they code for are passed down from parent to progeny. This is called vertical gene transfer. Bacteria and some lower eukaryotes are unique in that they can pass DNA from one cell of the same generation to another. The exchange of genes between two cells of the same generation is referred to as horizontal gene transfer. Mechanisms like transformation, transduction and conjugation takes place naturally and may bring about genetic variation and genetic recombination. These gene transfer mechanisms are also employed in genetic engineering to introduce desired gene into the cells. Introducing a foreign gene or recombinant DNA into the cells is one of the techniques used in genetic engineering. The success of cloning depends on the efficiency of gene transfer process. The most commonly employed gene transfer methods are transformation, conjugation, transduction, electroporation, lipofection and direct transfer of DNA. The choice of the method depends on the type of host cell (bacteria, fungi, plant, animal). Figure 12.15 shows methods of DNA transfer.

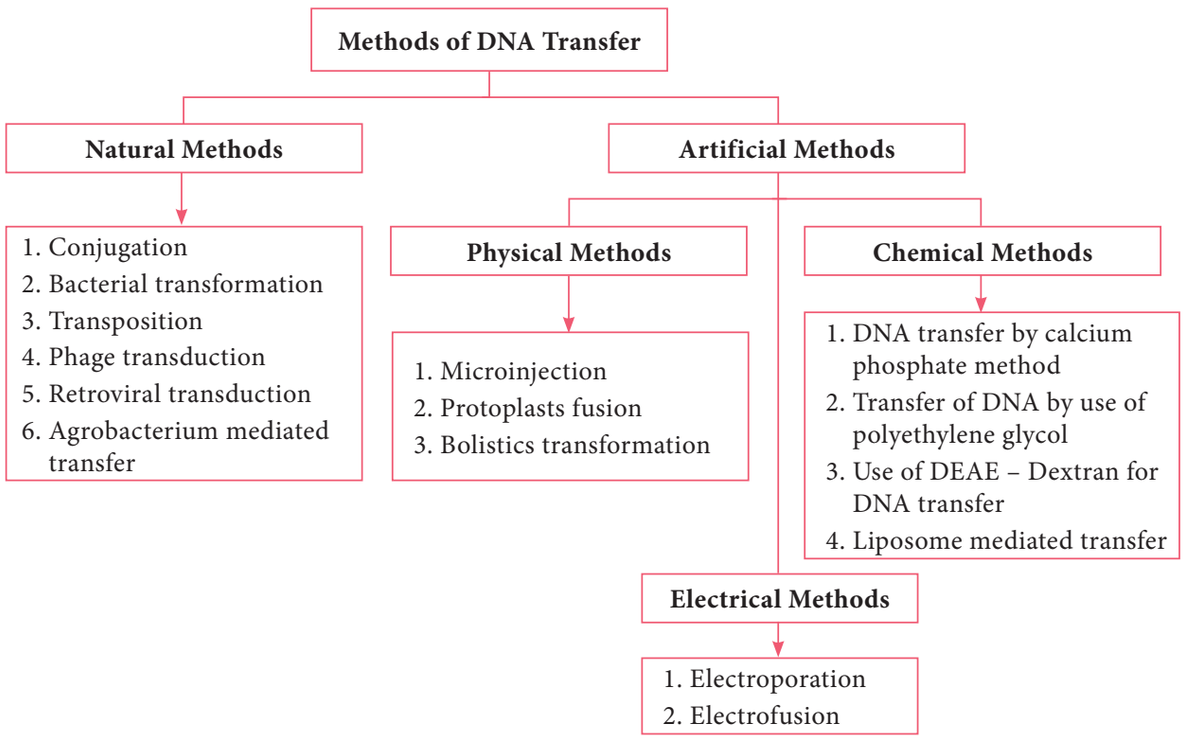


Figure 12.15: Methods of DNA transfer

Note: The term Transfection is used for the transfer of DNA into eukaryotic cells by various physical or chemical means.

12.7.1 Transformation

Transformation is genetic alteration of a cell resulting from the direct uptake, incorporation and expression of exogenous genetic material (exogenous DNA) from its surroundings. Transformation occurs naturally in some species of bacteria, but it can also take place by artificial means in other cells. For transformation to happen, bacteria must be in a state of competence. Competence refers to the state of being able to take up exogenous DNA from the environment. There are two forms of competence: natural and artificial. Transformation works best with DNA from closely-related species. The naturally-competent bacteria carry sets of genes that provide the protein machinery to bring DNA across the cell membrane(s).

There are some differences in the mechanisms of DNA uptake by gram positive and gram negative cells. However, they share some common features that involve related proteins. The DNA first binds to the surface of the competent cells on a DNA receptor, and passes through the cytoplasmic membrane via DNA translocase. Only single stranded DNA may pass through, one strand is therefore degraded by nucleases in the process, and the translocated single-stranded DNA may then be integrated into the bacterial chromosomes. Figure 12.16 shows mechanism of transformation.

Artificial competence can be induced in laboratory by procedures that involve making the cell passively permeable to DNA. Typically, the cells are incubated in a solution containing divalent cations; most commonly, calcium chloride solution under cold condition, which is then exposed to a pulse of heat shock. Electroporation is another method of promoting competence. Using this method, the cells are briefly shocked with an electric field of 10–20 kV/cm which is thought to create holes in the cell membrane through which the plasmid DNA may enter. After the electric shock, the holes are rapidly closed by the cell's membrane-repair mechanisms.

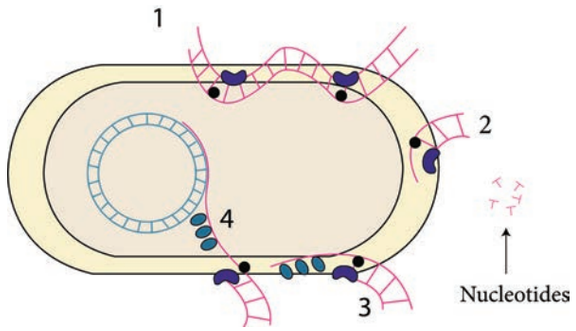


Figure 12.16: Mechanism of transformation **1.** Binding of DNA; **2.** Degradation of one strand; **3.** Entry of ssDNA; **4.** Integration into host chromosome.

12.7.2 Conjugation

The initial evidence for bacterial conjugation, came from an experiment performed by Joshua Lederberg and Edward L Tatum in 1946. Later in 1950, Bernard Davis gave evidence that physical contact of the cells was necessary for conjugation. During conjugation, two live bacteria (a donor and a recipient) come together, join by cytoplasmic bridges (e.g. pilus) and transfer single stranded DNA (from donor to recipient).

Inside the recipient cell, the new DNA may integrate with the chromosome (rather rare) or may remain free (as is the case with plasmids). Conjugation can occur among the cells from different genera of bacteria, while transformation takes place among the cells of a bacterial genus.

A plasmid called the fertility or F factor plays a major role in conjugation. The F factor is about 100 kilobases long and bears genes responsible for cell attachment and plasmid transfer between specific bacterial strains during conjugation. F factor is made up of

a. tra region (tra operon / transfer genes): genes coding the F pilus and DNA transfer,

b. Insertion sequence: genes assisting plasmid integration into host cell chromosome.

Thus, the F factor is an **episome** - a genetic material that can exist outside the bacterial chromosome or be integrated into it.

During $F^+ \times F^-$ mating or conjugation (Figure 12.17a) the F factor replicates by the rolling circle mechanism and a copy moves to the recipient. The channel for DNA transfer could be either the hollow F pilus or a special conjugation bridge formed upon contact. The entering strand is copied to produce double – stranded DNA.

F factor can integrate into the bacterial chromosome at several different locations by recombination between homologous insertion sequences present on both the plasmid and host chromosomes. The integration of F factor into bacterial chromosome results in formation of HFR (High Frequency Recombination) cell. When integrated, the Fplasmid's tra operon is still functional; the plasmid can direct the synthesis of pili, carry out rolling circle replication, and transfer genetic material to an F^- recipient cell. An HFR cell is so called because it exhibits a very high efficiency of chromosomal gene transfer in comparison with F^+ cells. In F^+ cells the independent F factor rarely transfer chromosomal genes hence the recombination frequency is low. Figure 12.17b shows formation of HFR cell. When an HFR cell is mated with F^- cell the F^- recipient does not become F^+ unless the whole chromosome is transferred as explained in Figure 12.17c. The connection usually breaks before this process is finished. Thus, complete F factor usually is not transferred, and the recipient remains F^-.

Because the F plasmid is an episome, it can leave (deintegrate) the bacterial chromosome. Sometimes during this process, the plasmid makes an error in excision and picks up a portion of the chromosomal material to form an F′ plasmid. Figure 12.17d shows formation of F′. During $F'XF^-$ conjugation (Figure 12.17e) the recipient becomes F′ and is a partially diploid since it has two set of the genes carried by the plasmid.

The natural phenomenon of conjugation is now exploited for gene transfer and Recombinant DNA technology. In general, the plasmids lack conjugative functions and therefore, they are not as such capable of transferring DNA to the recipient cells. However, some plasmids with conjugative properties can be prepared and used.

12.7.3 Transduction

Transduction is the transfer of bacterial genes from one bacteria to other by viruses. Example: Bacteriophage (Bacterial viruses). To understand the

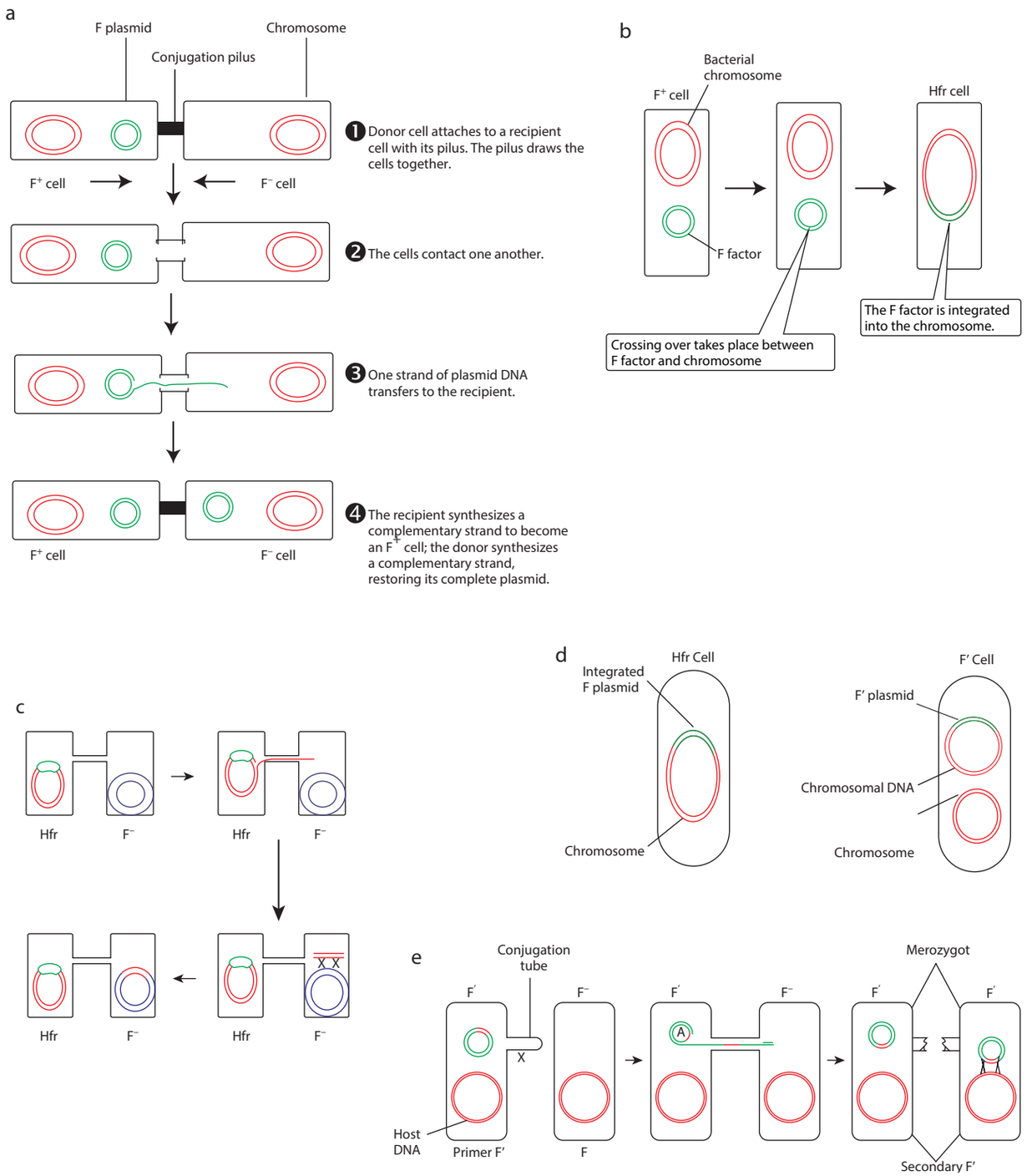


Figure 12.17: Mechanism of conjugation **(a)** $F^+ \times F^-$ **(b)** HFR cell Formation **(c)** HFR $\times$ F^- **(d)** F′ formation **(e)** F′ $\times$ F^-

role of bacteriophage in gene transfer, the lifecycle of bacteriophage is described below briefly.

After infecting the host cell, a bacteriophage (phage for short) often takes control and forces the host to make many copies of the virus. Eventually the host bacterium bursts or lyses and releases new phages. This reproductive cycle is called a lytic cycle because it ends in lysis of the host.

The lytic cycle (Figure 12.18) has four phases.

1. Attachment - Virus particle attaches to a specific receptor site on the bacterial surface.
2. Penetration - the genetic material, which is often double stranded DNA, then enters the cell.
3. Biosynthesis - After adsorption and penetration, the virus chromosome forces the bacterium to make viral components-viral nucleic acids and proteins.
4. Assembly - Phages are assembled from the virus components. Phage nucleic acid is packed within the virus's protein coat.
5. Release - mature viruses are released by cell lysis.

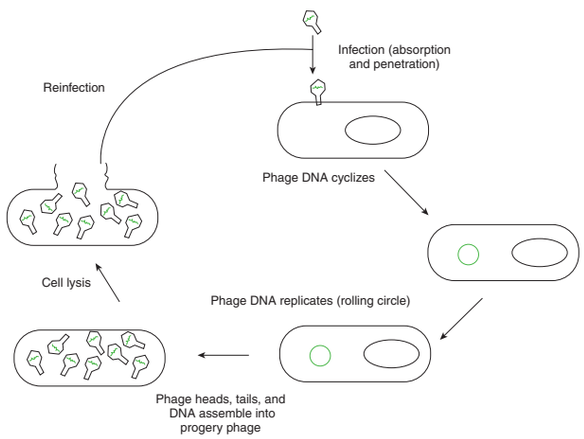


Figure 12.18: Lytic cycle

Bacterial viruses that reproduce using a lytic cycle often are called **virulent bacteriophages** (e.g. T phages) because they destroy the host cell. The genome of many DNA phages such as the lambda phage, after adsorption and penetration do not take control of its host and does not destroy the host. Instead the viral genome remains within the host cell and is reproduced along with the bacterial chromosome. The infected bacteria may multiply for long periods while appearing perfectly normal. Each of these infected bacteria can produce phages and lyses under appropriate environmental conditions. This relationship between phage and its host is called lysogeny (Figure 12.19).

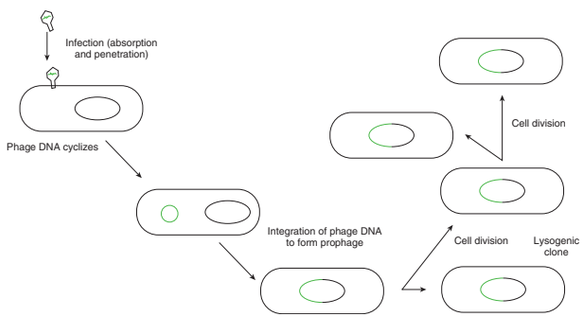


Figure 12.19: Lysogeny

Bacteria that can produce phage particles under some conditions are said to be **lysogens** or **lysogenic bacteria**. Phages which are able to establish lysogeny are called **temperate phages**.

The latent form of virus genome that remains within the host without destroying the host is called the prophage.

The prophage usually is integrated into the bacterial genome. Sometimes phage reproduction is triggered in a lysogenized culture by exposure to UV radiation or other factors. The lysosomes are then destroyed and new phages released – This phenomenon is called induction (Figure 12.20).

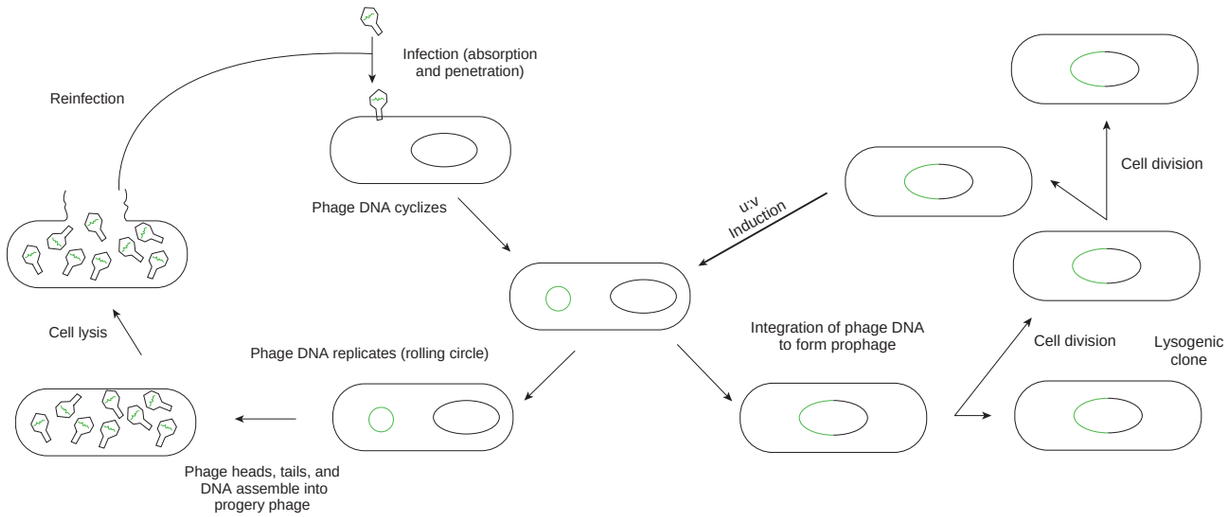


Figure 12.20: Induction of lysogen

Sometimes, bacterial genes are incorporated into a phage capsid because of errors made during the virus life cycle. The virus containing these genes then infects them into another bacterium, resulting in the transfer of genes from one bacterium to the other. Transduction may be the most common mechanism for gene exchange and recombination in bacteria.

There are two very different kinds of transduction.

1. Generalized transduction
2. Specialized transduction

Generalized transduction (Figure 12.21a) occurs during the lytic cycle of virulent and temperate phages. During the assembly stage, when the viral chromosomes are packaged into protein capsids, random fragments of the partially degraded bacterial chromosome also may be packaged by mistake. The resulting virus particles often injects the DNA into another bacterial cell but does not initiate a lytic cycle. Thus in generalized transduction any part of the bacterial chromosome can be transferred. Once the DNA has been injected it may integrate into the recipient cell's chromosome to preserve the transferred genes. About 70 to 90% of the transferred DNA is not integrated but is often able to survive and express itself. However, if the transferred DNA is degraded gene transfer is unsuccessful.

Specialized Transduction (Figure 12.21b) is also called restricted transduction in which only specific portions of the bacterial genome is carried by the phage. When a prophage is induced to leave the host chromosome, exicision is sometimes carried out improperly. The resulting phage genome contains portions of the bacterial chromosome next to the integration site. When this phage infects another bacterium, it transfers the bacterial genes from the donor bacterium along with phage DNA. Here only the bacterial genes that are close to the site of prophage are transferred. So, this transduction is called specialized.

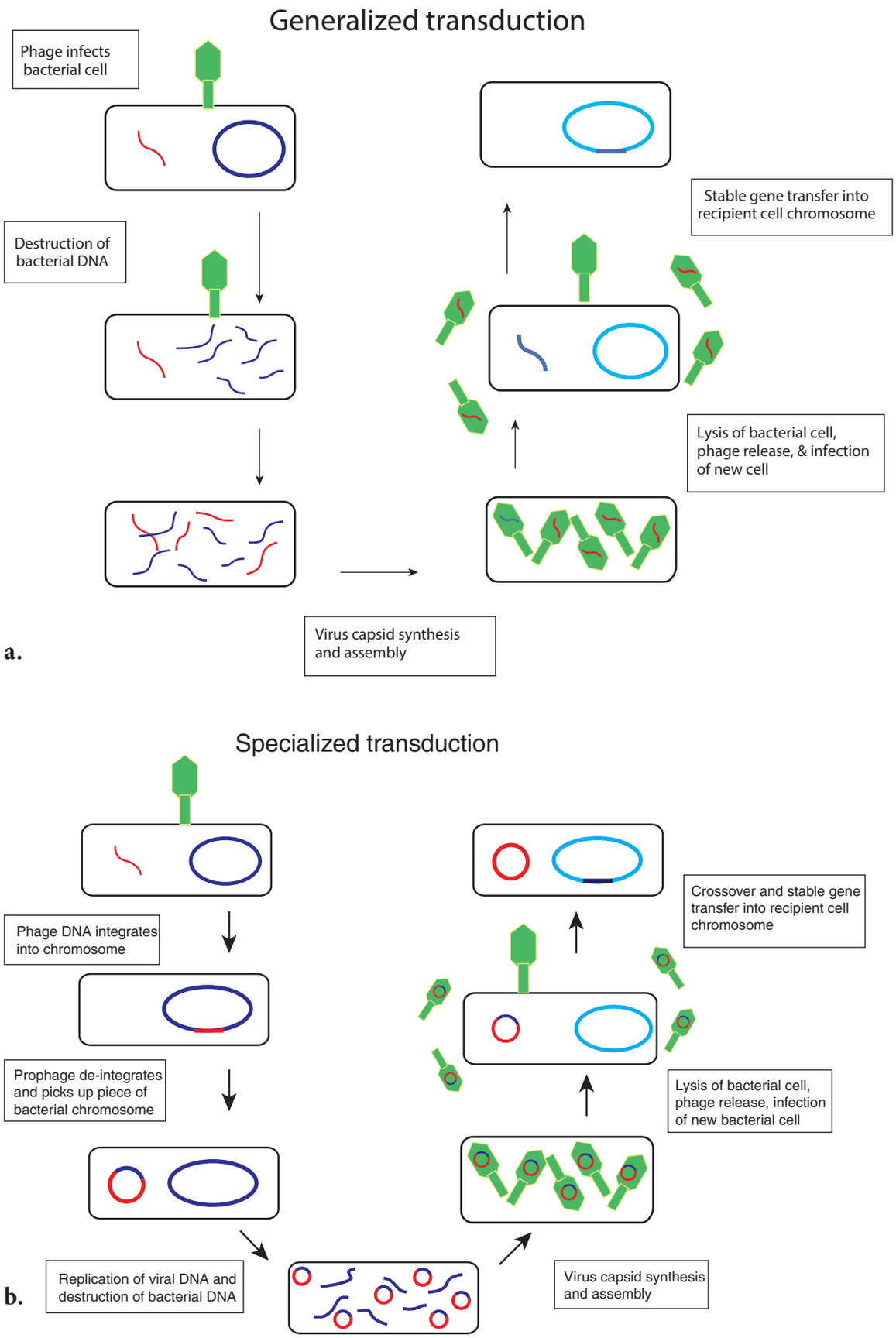


Figure 12.21: **(a)** Generalized Transduction **(b)** Specialized Transduction

Infobits

The chromosomes of bacteria, viruses, and eukaryotic cells contain pieces of DNA that move around the genome. Such movement is called transposition. DNA segments that carry the genes required for this process and consequently move about chromosomes are transposable elements or transposons. Transposons are also called jumping genes because they can jump from one DNA to another, resulting in mutation of the cell. They were first discovered in 1951 by Barbara McClintock whose significant discovery was ignored by scientific community for many years. She was awarded the Nobel Prize in 1983.

12.8 Recombinant DNA Technology

One of the practical applications of microbial genetics and the technology arising from it is the recombinant DNA technology. The deliberate modification of an organism's genetic information by directly changing its nucleic acid genome is called genetic engineering and is accomplished by a collection of methods known as recombinant DNA technology. Recombinant DNA technology opens up totally new areas of research and applied biology. Thus, it is an essential part of biotechnology, which is now experiencing a stage of exceptionally rapid growth and development. In general sense, **recombination** is the process in which one or more nucleic acids molecules are rearranged or combined to produce a new nucleotide sequence. Usually genetic material from two parents is combined to produce a recombinant chromosome with a new, different genotype. Recombination results in a new arrangement of genes or parts of genes and normally is accompanied by a phenotypic change.

There are many diverse and complex techniques involved in gene manipulation. However, the basic principles of recombinant DNA technology are reasonably simple, and broadly involve the following stages (Figure 12.22).

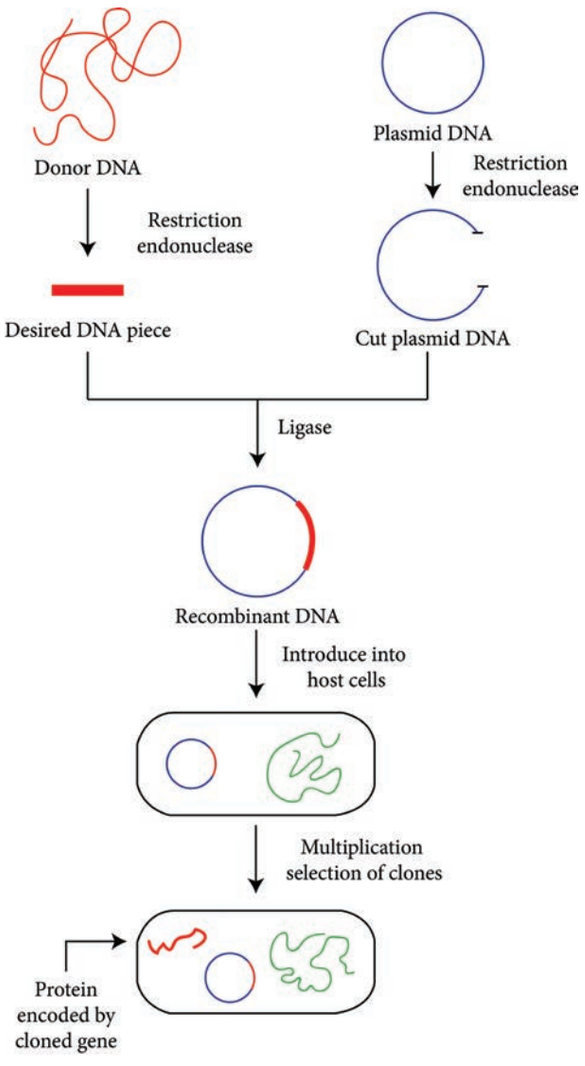


Figure 12.22: Basic principles of recombinant DNA technology

1. Isolation of DNA from the source (Donor)
2. Generation of DNA fragments and selection of the desired piece of DNA
3. Insertion of the selected DNA into a cloning vector (Example: a plasmid) to

create a recombinant DNA or chimeric DNA.

4. Introduction of the recombinant vectors into host cells (Example: bacteria)
5. Multiplication and selection of clones containing the recombinant molecules
6. Expression of the gene to produce the desired product.

Cloning in the molecular biology sense (as opposed to cloning whole organisms) is the making of many copies of a segment of DNA, such as a gene. Cloning makes it possible to generate large amounts of pure DNA, such as genes, which can then be manipulated in various ways, including mapping, sequencing, mutating and transforming cells. An overview of cloning strategies in recombinant DNA technology is shown in Figure 12.23.

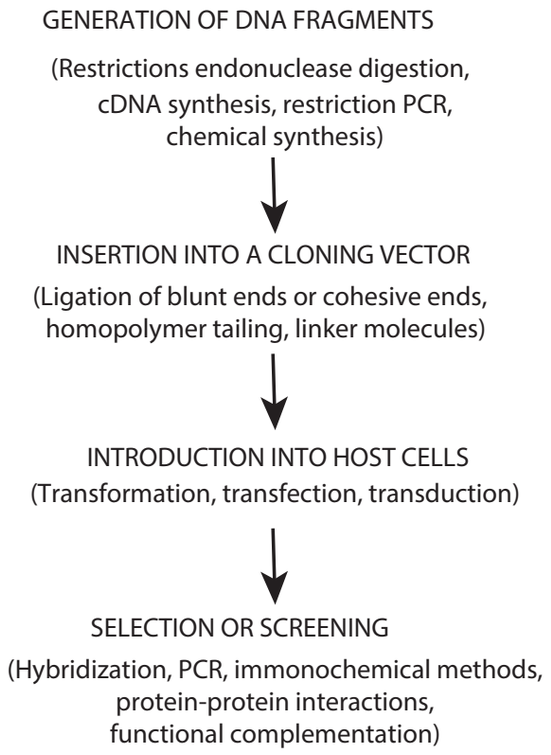


Figure 12.23: An overview of cloning strategies in recombinant DNA technology

12.9 Vectors, Types and Characteristics

Vectors are the DNA molecules, which carry a foreign DNA fragment to be cloned. They are cloning vehicles, examples of which are Plasmids, Bacteriophages, cosmids, phagemids and artificial chromosomes. The vector types differ in the molecular properties they have and in the maximum size of DNA that can be cloned into each.

Characteristics of an ideal vector.

1. Should be small in size
2. Should contain one or more restriction site
3. Should be self replicating
4. Should contain an origin of replication sequence (ori)
5. Should possess genetic markers (to detect the presence of vectors in recipient cells)

Plasmid Cloning Vectors

Bacterial plasmids are extra chromosomal elements that replicate autonomously in cells. Their DNA is circular and double stranded and carries sequences required for plasmid replication (ori sequence) and for the plasmid's other functions. (Note: A few bacteria contain linear plasmids. Example: *Streptomyces species, Borellia burgdorferi*). The size of plasmids varies from 1to 500 kb. Plasmids were the first cloning vectors. DNA fragments of about 570kb are efficiently cloned in plasmid cloning vectors. Plasmids are the easiest to work with. They are easy to isolate and purify, and they can be reintroduced into a bacteria by transformation. Naturally occurring plasmid vectors rarely possess all the characteristics of an ideal vector. Hence plasmid cloning vectors are

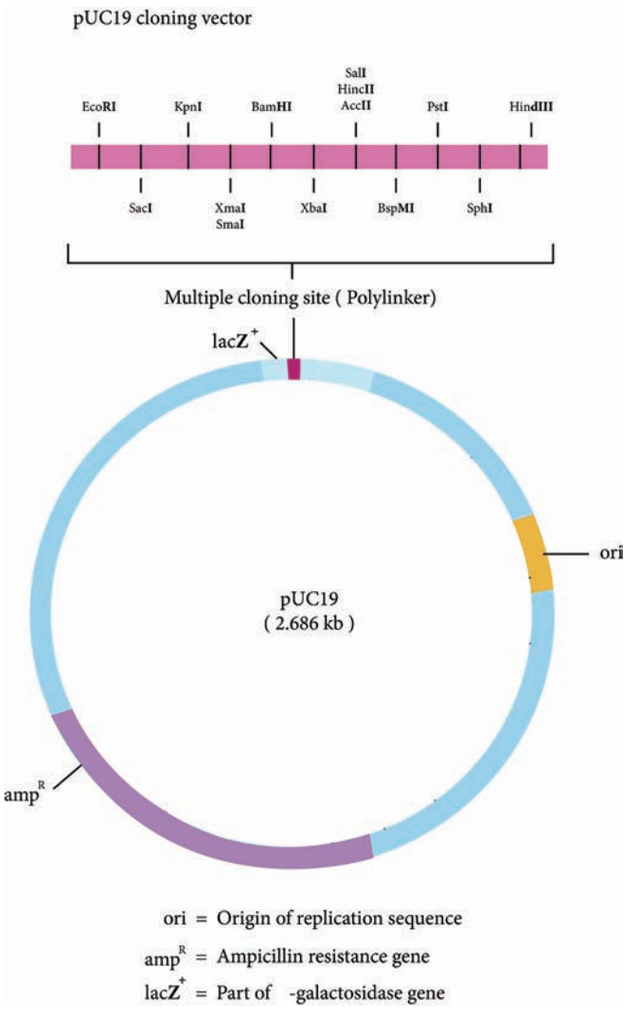
derivatives of natural plasmids and are "engineered" to have features useful for cloning DNA.

Examples of plasmid cloning vectors : pBR 322 (plasmid discovered by Bolivar and Rodriguez 322) and pUC 19 (plasmid from University of California). Herbert Boyer and Stanley Cohen in 1973 showed it was possible to transplant DNA segments from a frog into a strain of *Escherichia coli* using pSC101, a genetically modified plasmid, as the vector. The work laid the foundation for the birth of Genetech, the first company dedicated to commercialization of recombinant DNA.

Figure 12.24a and 12.24b shows genetic maps of plasmid cloning vectors PUC19 and PBR322 respectively.

Plasmid cloning vector PUC 19 has 2,686 –bp and has following features:

1. It has a high copy number; so many copies of a cloned piece of DNA can be generated readily.
2. It has amp R (ampicillin resistant) selective marker
3. It has a number of unique restriction sites clustered in one region, called a multiple cloning site (MCS) or polylinker
4. The MCS is inserted into part of the E.coli β – galactosidase (lac Z^+) gene. Figure 12.25 illustrates how a piece of DNA can be inserted into a plasmid cloning vector such as pUC19



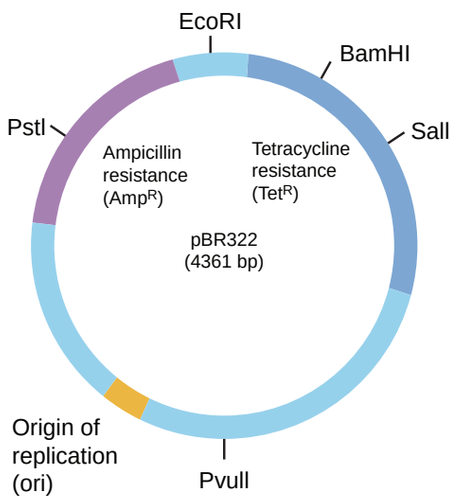


Figure 12.24: (a) PUC19 **(b)** PBR322

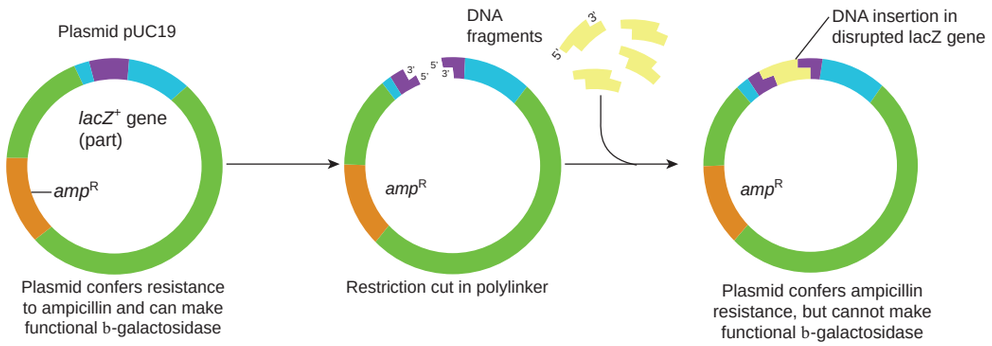


Figure 12.25: Insertion of a piece of DNA into the plasmid cloning vector pUC19 to produce a recombinant DNA molecule

Bacteriophage as Cloning Vectors

They are viruses that replicate within the bacteria. A phage can be employed as vector since a foreign DNA can be spliced into phage DNA, without causing harm to phage genes. The phage will reproduce (replicate the foreign DNA) when it infects bacterial cell. Both single and double stranded phage vectors have been employed in recombinant DNA technology. Derivatives of phage can carry fragments up to about 45 kb in length. Example PI bacteriophage and phage λ.

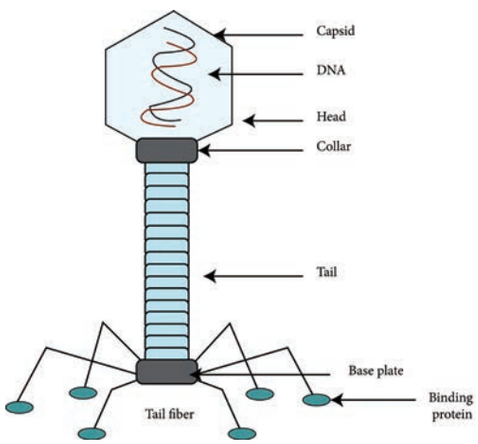


Figure 12.26: Structure of phage λ

The main advantage of using phage vectors is that foreign DNA can be packed into the phage (invitro packaging), the latter in turn can be injected into the host cell very effectively (Note: no transformation is required). Figure 12.28 shows how a λ phage is used for cloning.

Infobits

Terms associated with plasmids:

1. Low copy number plasmids are plasmids that occur low in number in each cell.
2. High copy number are plasmids that occur high in number in each cell.
3. Conjugative plasmids carry a set of transfer genes (tra genes) that facilities bacterial conjugation.
4. Non – conjugative plasmidsare plasmids that do not possess transfer genes.
5. Stringent plasmids are plasmids that are present in a limited number (1–2 per cell).
6. Relaxed plasmids are plasmids that occur in large number in each cell.
7. F plasmids possess genes for their own transfer from one cell to another
8. R plasmids carry genes resistance to antibiotics.

Cosmids: Cosmids are the vectors possessing the characteristics of both plasmid and bacteriophage. The advantage with cosmids is that they carry larger fragments of foreign DNA (35–45 kb) compared to plasmids.

Phagemids: Phagemids are the combination of plasmid and phage and can function as either plasmid or phage. Since they posses functional origins of replication of both plasmid and phage λ they can be propagated (as plasmid or phage) in appropriate E.coli.

Artificial chromosome Vectors: Artificial chromosomes are cloning vectors that can accommodate very large pieces of DNA, producing recombinant DNA molecules resembling small chromosomes. Example: Yeast Artificial Chromosome (YAC), Bacterial Artificial Chromosomes (BACs)

Plasmid shuttle Vectors: The plasmid vectors that are specifically designed to replicate in two or more different host organisms(say in E.coli and yeast) are referred to as shuttle vectors. The origins of replication for two hosts are combined in one plasmid.

Expression vectors: An expression vector is a cloning vector containing the

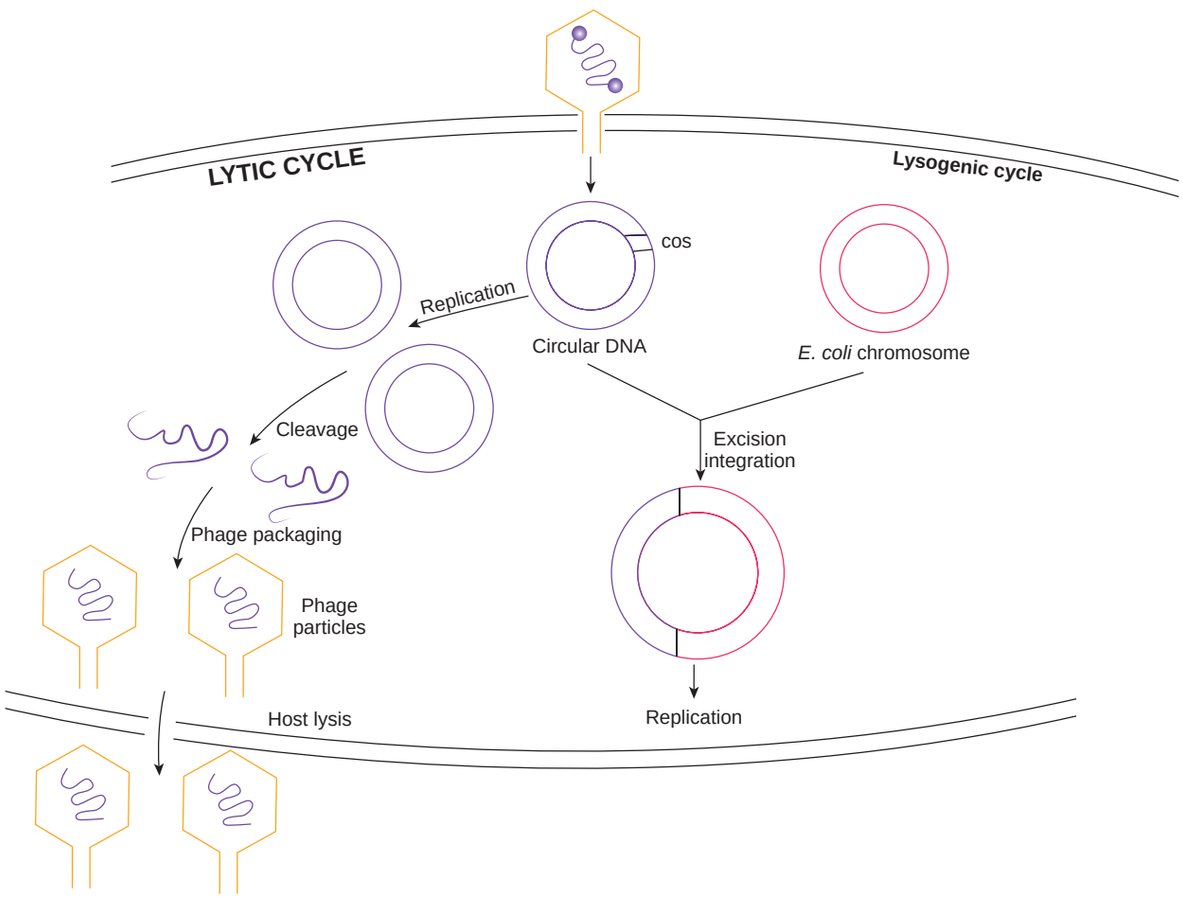


Figure 12.27: Life cycle of phage λ

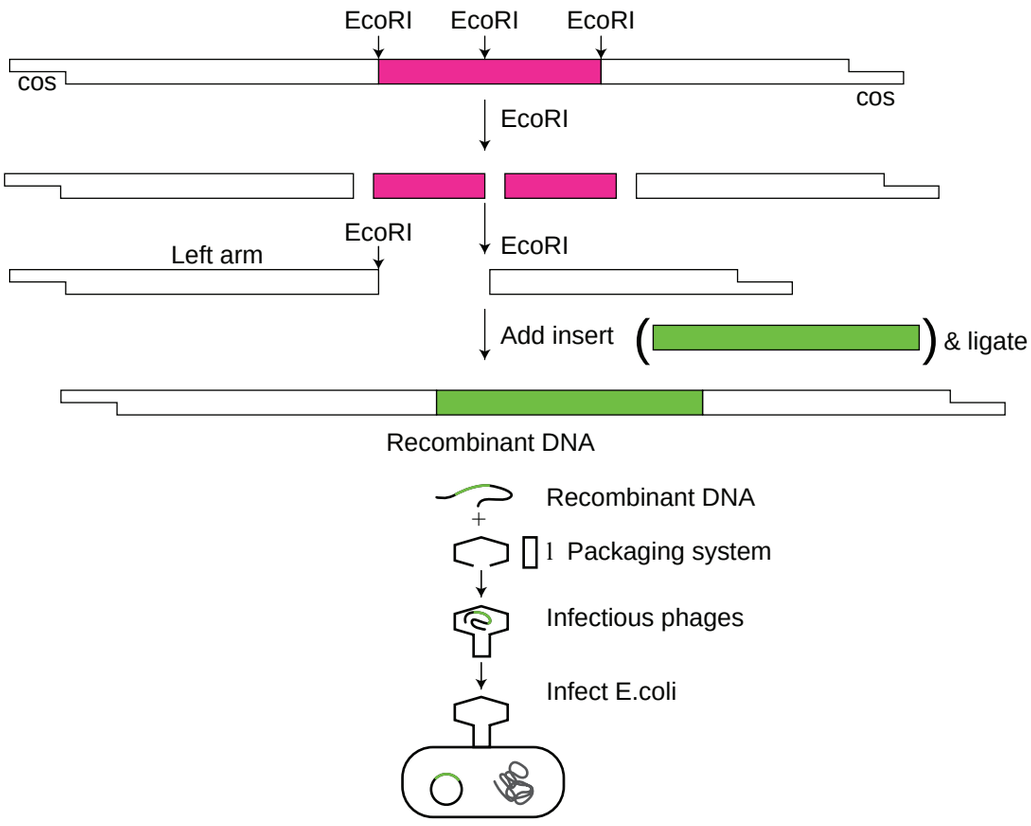


Figure 12.28: Cloning using a λ phage

regulatory sequences (promoter sequence) necessary to allow the transcription and translation of a cloned gene or genes.

12.10 Restriction Enzymes

In 1960s Swiss microbiologist Werner Arber and American microbiologists Hamilthon Othanel Smith and Daniel Nathans discovered restriction enzymes. The discovery, for which the three men shared the 1978 Nobel Prize for Physiology or Medicine. Restriction enzymes or restriction endonucleases are one of the most important groups of enzymes for the manipulation of DNA. It is one of the important molecular tools used by a genetic engineer. These are the bacterial enzymes that recognize a specific base sequence in a DNA molecule (from any source) and make two cuts one in each strand generating 3′ – OH and 5′ – P termini. They were first discovered in E.*coli*. E.*coli* produces the restriction enzyme to cut the viral DNA and protect itself. The host E.coli DNA is protected by its own restriction enzyme since its methylated. Since these enzymes restrict the viral replication the word restriction is added to these enzymes. Hind II was the first discovered restriction endonuclease.

The site where the DNA is cut by a restriction enzyme is called recognition sequence. Restriction endonucleases can specifically recognize DNA with a particular sequence of 4-8 nucleotides and cleave. Each recognition sequence has two fold rotational symmetry i.e. the same nucleotide sequence occurs on both strands of DNA which run in opposite direction. Such sequences are referred to as palindromes, since they read similar in both directions (forwards and backwards). Majority

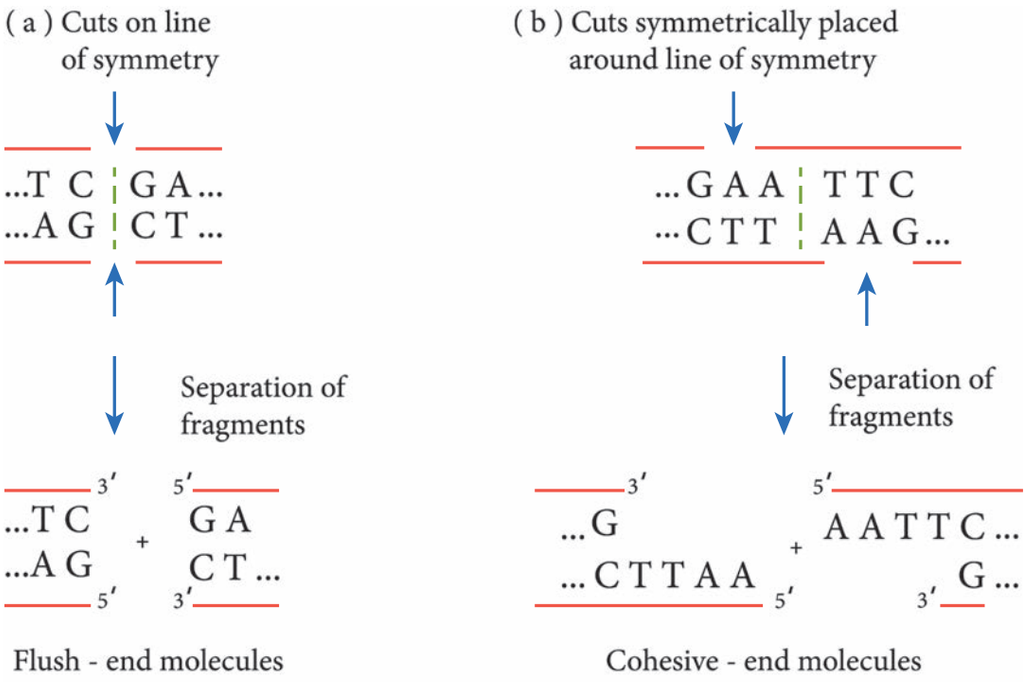


Figure 12.29: Two types of cuts made by restriction enzymes

Type	No of Enzyme and sub units	Cleavage site	Examples	Bacterial source
I	One with 3 sub units for recognition cleavage and methylation	1000 bp from recognition site	EcoK1	*Escherichia coli*
			Cfr A1	*Citrobacter fruendii*
II	Two different enzymes to cleave or modify the recognition sequence	Same as recognition or close to recognition site	Eco R1	*Escherichia coli*
			Alu I	*Arthrobacter luteus*
III	One with 2 subunits	24- 26 bp from recognition site	Hinf III	*Haemophilus influenzae*
			Pst II	*Providencia stuarti*

Table 12.2: Types and features of restriction enzyme

of restriction endonucleases (particularly type II) cut DNA at defined sites within recognition sequence. Type II restriction enzymes make two single – stand breaks) one break in each strand. There are two distinct arrangements of these breaks 1. both breaks at the center of symmetry (generating flush or blunt ends) or 2. breaks that are symmetrically placed around the line of symmetry generating cohesive ends. Figure 12.29 shows two types of cuts made by restriction enzymes. The arrow indicates the cleavage site. The dashed line is the center of symmetry of the sequence (Table 12.2).

Application of Recombinant DNA Technology

a. Production of medically useful proteins such as somatostain, insulin, human growth hormone and interferon. It decreases the dependency on human tissues and solves problem of limited production.

b. Development of synthetic vaccines for instance, vaccines for malaria and rabies a recombinant hepatitis vaccine is already commercially available.

c. Gene therapy

d. Diagnosis of infection diseases.

e. To manufacture industrially important products like enzymes using bacteria, fungi and cultured mammalian cells.

12.11 Techniques in Genetic Engineering

PSHQCW

There are several techniques used in recombinant DNA technology or gene manipulation. The most frequently used methods are agarose gel electrophoresis, isolation and purification of nucleic acids, nucleic acid blotting techniques, DNA sequencing, chemical synthesis of DNA, gene transfer methods, polymerase chain reaction, construction of gene library, radiolabeling of nucleic acids etc, few of them are discussed here.

12.11.1 Agarose Gel Electrophoresis

Electrophoresis refers to the movement of charged molecules in an electric field. The negatively charged molecules move towards the positive electrode while the positively charged molecules migrate towards the negative electrode. Gel electrophoresis is a routinely used analytical technique for the separation and purification of specific DNA fragments. The gel is composed of either polyacrylamide or agarose. Polyacrylamide gel electrophoresis (PAGE) is used for the separation of smaller DNA fragments while agarose electrophoresis is convenient for the separation of DNA fragments ranging in size from 100 base pairs to 20 kilobase pairs.

A genomic library is a collection of clones that contains at least one copy of every DNA sequence in an organism's genome. Like libraries with books, genomic libraries are a great source of information; in this case, the information is about the genome. Specific sequences in cDNA libraries and genomic libraries can be identified via a number of approaches, including the use of specific antibodies, cDNA probes and oligonucleotide probes

Human artificial chromosome (HAC)-based vectors offer a promising system for delivery and expression of full-length human genes of any size into human cells, and a tool for determining human chromosome function. It does not have the problem of limited cloning capacity of other vectors, and it also avoids possible insertional mutagenesis caused by integration into host chromosomes by viral vector.

Gel electrophoresis can also be used for the separation of RNA molecules. A diagramatic view of the agarose gel electrophoresis unit is shown in Figure 12.30a.

Steps

1. Gel is set with wells on one end.
2. The gel is placed in an electrophoresis apparatus and covered with buffer solution.
3. The DNA samples along with tracer dye are placed in the wells of gel.

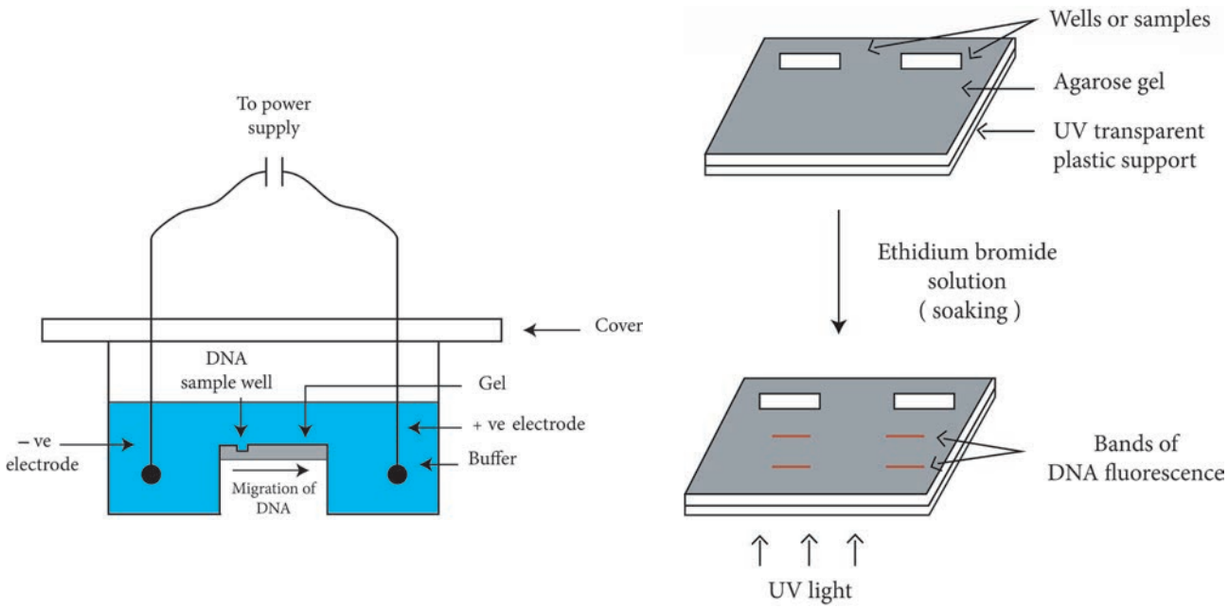


Figure 12.30: **(a)** agarose gel electrophoresis unit **(b)** DNA electrophoresis gel

4. Power supply is switched on and gel is run till the tracer dye reaches the end of the gel.

As the DNA is negatively charged, DNA fragments move through the gel towards the positive electrode. The rate of migration of DNA is dependent on the size and shape. In general, smaller linear fragments move faster than the larger ones. Hence, gel electrophoresis can be conveniently used for the separation of a mixture of DNA fragments, based on their size. The bands of the DNA can be detected by soaking the gel in ethidium bromide solution (Ethidium bromide can also be added to molten agarose prior to setting the gel). When activated by ultraviolet radiation, DNA base pairs in association with ethidium bromide, emit orange fluorescence. And in this way the DNA fragments separated in agarose electrophoresis can be identified (Figure 12.30b).

PAGE is composed of chains of acryl amide monomers crosslinked with methylene bisacryalmide units. The pore size of the gel is dependent on the total concentration of monomers and the cross links. PAGE is used for the separation of single stranded DNA molecules that differ in length by just one nucleotide. Agarose gels cannot be used for this purpose. This is because polyacrylamide gels have smaller pore sizes than agarose gels and allow precise separation of DNA molecule from 10–1500 bp.

HOTS

1. Explain how gel electrophoresis can be used to determine the size of a PCR product.

12.11.2 Polymerase Chain Reaction (PCR)

The PCR technique has already proven exceptionally valuable in many areas of molecular biology, medicine, and biotechnology. PCR technique has great practical importance and impact

on biotechnology. Between 1983 and 1985 American biochemist Kary Mullis developed PCR technique that made it possible to synthesize large quantities of a DNA fragment without cloning it. Mullis received the 1993 Nobel Prize for Chemistry for his invention. PCR is a cell free amplification technique.

Figure 12.31 outlines how PCR technique works. To amplify (make large quantities) a particular DNA sequence by PCR a reaction mixture (often 100µl or less in volume) containing the following are required.

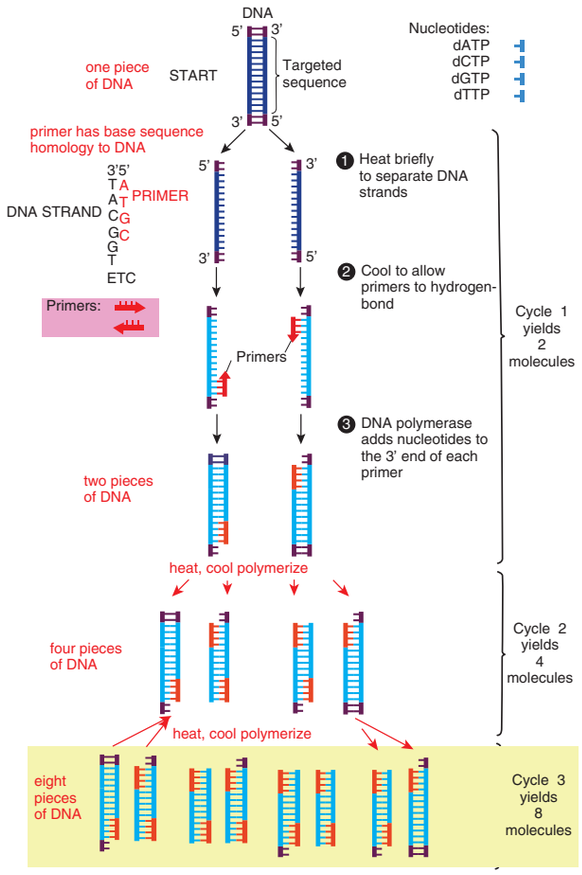


Figure 12.31: Steps in PCR

1. **Target DNA**
2. **Two primers**–These are synthetic oligonucleotides, usually about 20 nucleotides long. These are fragments with sequences identical to those flanking the targeted sequence.
3. **Thermostable DNA polymerase**– Two popular enzymes employed in the PCR technique are Taq polymerase from the thermophilic bacterium *Thermus aquaticus* and the vent polymerase from *Theromococcus litoralis*. These polymerases employed in PCR technique are able to function at high temperatures.
4. **Four deoxyribonucleoside triphosphates** (dNTPs)- dCTP, dATP, dGTP, dTTP

Infobits

Cloned genes and other DNA sequences often are analyzed to determine the arrangement and specific locations of restriction sites. The analytical process involves cleavage of the DNA with restriction enzymes, followed by separation of the resulting DNA fragments by agarose gel electrophoresis. The sizes of the DNA fragments are calculated, enabling restriction maps to be constructed. The many DNA fragments produced by cleaving genomic DNA show a wide range of sizes, resulting in a continuous smear of DNA fragments in the gel. In this case, specific gene fragments can be visualized only by transferring them to membrane filter by southern blotting, hybridizing a specific labelled probe with the DNA fragments, and detecting the hybrids. A similar procedure, Northern blotting is used to analyze the sizes and quantities of RNAs isolated from cell.

Taq polymerase lacks proof reading exonuclease (3′–5′) activity which might contribute to errors in the products of PCR. Some other thermostable DNA polymerases with proof reading activity have been identified. Example: Tma DNA polymerase from *Thermotoga maritana*.

Steps in PCR

1. **Denaturation:** The target DNA containing the sequence to be amplified is heat denatured to separate its complementary strands at temperature 94 °C–95 °C.
2. **Annealing:** The temperature is lowered to 37 °C–55 °C so that the primers can hydrogen bond or anneal to the DNA on both sides of the target sequence. Because the primes are present in excess the targeted DNA strands normally anneal to the primers rather than to each other.
3. **Extension:** Heat resistant DNA polymerase extends the primers and synthesizes copies of the target DNA sequence using the deoxyribonucleoside triphosphate's at 70 °C–75 °C.

The three – step cycle (Figure 12.32) is repeated to obtain copies of target DNA in large numbers. At the end of one cylcle, the targeted sequences on both strands have been copied. When the three – step cycle is repeated, the four strands from the first cycle are copied to produce eight fragments. The third cycle yields 16 products. Theoretically, 20 cycles will produce about one million copies of the target DNA sequence. Each cycle of PCR takes about 3 – 5 minutes.

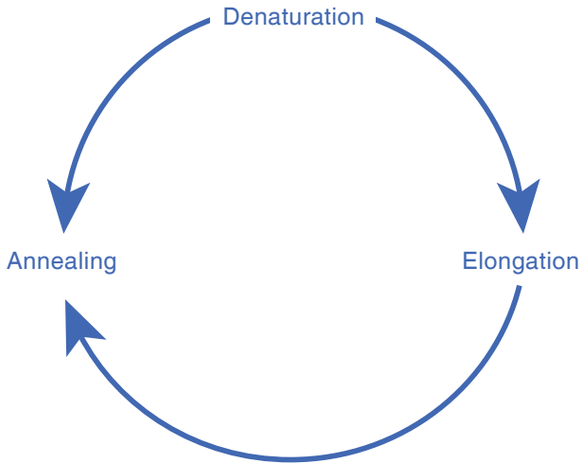


Figure 12.32: Three steps PCR cycle

The PCR technique has now been automated and is carried out by a specially designed machine (Figure 12.33) PCR machines are now fully automated and microprocessor controlled. They can process up to 96 samples at a time. PCR machines can carry out 25 cycles and amplify DNA 10^5 times in as little as 57 minutes.

The PCR has many applications in research and in commercial arena, including generating specific DNA segments for cloning or sequencing, amplifying DNA to detect specific genetic defects, and amplifying DNA for fingerprinting in crime scene investigation.

PCR technology is improving continually. Various forms of PCR are available. RNA too can be efficiently used in PCR procedures. Cellular RNAs and RNA viruses may be studied even when the RNA is present in very small amounts (as few as 100 copies can be transcribed and amplified). Quantitative PCR is quite valuable in virology and gene impression studies. PCR is

modified as per the specific demands of the situation. Thus there are many variations in the original PCR Examples nested PCR, inverse PCR, reverse transcription PCR, time quantitative PCR, RAPD, RFLP, AFLP.

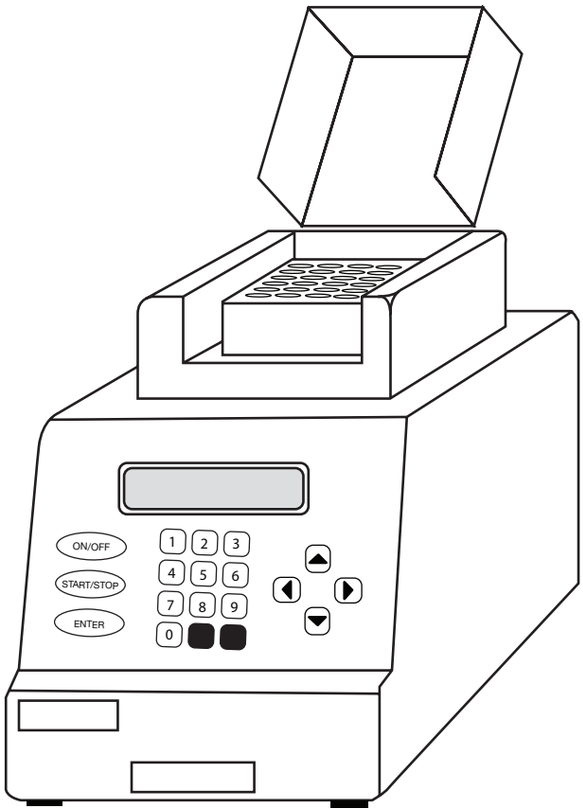


Figure 12.33: PCR machine

HOTS

Both PCR and Cloning allow for the production of many copies of a DNA sequence. What are the advantages of using PCR instead of cloning to amplify a DNA template?

What advantages are there to using a DNA polymerase for PCR that has proofreading activity?

Infobits

In 1970s American molecular biologists Allan M. Maxam and Walter Gilbert and English biochemist Frederick Sanger developed some of the first techniques for DNA sequencing. Gilbert and Sanger shared the 1980 Nobel Prize for Chemistry for their work. Dideoxy procedure is one of the procedure used to sequence DNA.

Summary

The fundamental unit of information in living systems is the gene. Genome is the set of all genes and genetic signals of a cell. Gene is expressed through a sequence of events. The central dogma of molecular biology, comprises the three major processes replication, transcription and translation. The genetic message encoded in messenger RNA (mRNA) is translated on the ribosomes into a polypeptide with a particular sequence of amino acids. An RNA base sequence (a set of 3 bases) corresponding to a particular amino acid is called a codon. The genetic code is the set of all codons. The genetic code is a triplet code, and all 64 possible codons carry information of some sort. The code is highly redundant. Polypeptides are synthesized from the amino terminus toward the carboxyl terminus, by adding amino acids one by one to the carboxyl end. An important feature of intiation of polypeptide synthesis in both prokaryotes and eukaryotes is the use of a specific initiating tRNA molecule.

Mutation is the process by which the sequence of base pairs in a DNA molecule is altered. Mutations can be divided into

base pair substitution mutation and base pair insertion or deletions.

Frame shift mutation usually results in a nonfunctional protein. An addition or deletion of one base pair, for example, shifts the mRNA's downstream reading frame by one base, so that incorrect amino acids are added to the polypeptide chain after the mutation site. Point mutations are single base changes, that do not affect the reading frame, that is, the mutation only makes a single change in a single codon,

Mutations can also be defined according to their effects on amino acid sequences in proteins. They are missense mutation, silent mutation, nonsense mutation. Forward mutations change the genotype from wild type to mutant and reverse mutations (or reversions or back mutations) change the genotype from mutant to wild type or to partially wild type.

The term mutant refers to an organism in which either the base sequence of DNA or the phenotype has been changed. The

Infobits

DNA Fingerprinting Or DNA Profiling:

DNA fingerprinting is the present day genetic detective in the practice of modern medical forensics. The underlying principles of DNA fingerprinting are briefly described.

The structure of each person's genome is unique. The only exception being monozygotic identical twins (twins developed from a single fertilized ovum). The unique nature of genome structure provides a good opportunity for the specific identification of an individual. The DNA fingerprint is an analysis of the nitrogenous base sequence in the DNA of an individual.

The original DNA fingerprinting technique was developed by Alec Jaffreys in 1985. Although the DNA fingerprinting is commonly used, a more general term DNA profiling is preferred. This is due to the fact that a wide range of tests can be carried out by DNA sequencing with improved technology.

Applications of DNA fingerprinting:

The amount of DNA required for DNA fingerprint is remarkably small. The minute quantities of DNA from blood strains, body fluids, hair fiber or skin fragments are enough. Polymerase chain reaction is used to amplify this DNA for use in fingerprinting. DNA profiling has wide range of applications – most of them related to medical forensics. Some important ones are listed below.

- Identification of criminals, rapists, thieves etc.
- Settlement of paternity disputes.
- Use in immigration test cases and disputes.

In general, the fingerprinting technique is carried out by collecting the DNA from a suspect (or a person in a paternity of immigration dispute) and matching it with that of a reference sample (from the victim of a crime, or a close relative in a civil case).

process of formation of mutant organism is called mutagenesis. Ames test is an indicator of whether the chemical is a mutagen. The Ames test assays the ability of chemicals to revert mutant strains of the bacterium Salmonella typhimurium to wild type. The most commonly employed gene transfer methods are transformation, conjugation, transduction, electroporation, lipofection and direct transfer of DNA. Transformation is genetic alteration of a cell resulting from the direct uptake, incorporation and expression of exogenous genetic material (exogenous DNA) from its surroundings. Competence refers to the state of being able to take up exogenous DNA from the environment. During conjugation, two live bacteria (a donor and a recipient) come together, join by cytoplasmic bridges (e.g. pilus) and transfer single stranded DNA (from donor to recipient). Transduction is the transfer of bacterial genes from one bacteria to other by viruses, e.g. Bacteriophage (Bacterial viruses). **Recombination** is the process in which one or more nucleic acids molecules are rearranged or combined to produce a new nucleotide sequence. Cloning in the molecular biology sense (as opposed to cloning whole organisms) is the making of many copies of a segment of DNA, such as a gene. Cloning makes it possible to generate large amounts of pure DNA, such as genes, which can then be manipulated in various ways

Vectors are the DNA molecules, which carry a foreign DNA fragment to be cloned. They are cloning vehicles, examples of which are Plasmids, Bacteriophages, cosmids, phagemids and artificial chromosomes. Bacterial plasmids are extra chromosomal elements that replicate autonomously in cells. They are viruses that replicate within the bacteria. Cosmids are the vectors possessing the characteristics of both plasmid and bacteriophage. Phagemids are the combination of plasmid and phage, and can function as either plasmid or phage. The plasmid vectors that are specifically designed to replicate in two or more different host organisms (say in E. *coli* and yeast) are referred to as shuttle vectors. An expression vector is a cloning vector containing the regulatory sequences (promoter sequence) necessary to allow the transcription and translation of a cloned gene or genes. Restriction enzymes are the bacterial enzymes that recognize a specific base sequence in a DNA molecule (from any source) and make two cuts one in each strand generating 3′ – OH and 5′ – P termini.

There are several techniques used in recombinant DNA technology or gene manipulation. The most frequently used methods are agarose gel electrophoresis, isolation and purification of nucleic acids, nucleic acid blotting techniques, DNA sequencing, chemical synthesis of DNA, gene transfer methods, polymerase chain reaction, construction of gene library, radiolabeling of nucleic acids etc. Gel electrophoresis is a routinely used analytical technique for the separation and purification of specific DNA fragments. PCR is a cell free amplification technique. The three – step cycle is repeated to obtain copies of target DNA in large numbers.

The DNA markers are highly useful for genetic mapping of genomes. RFLPS (Restriction Fragment Length Polymorphisms), VNTRs (mini satellites or Variable Number Tandem Repeats),

STRs (Microsatellites or Simple Random Repeats), SNPs (Single Nucleotide Polymorphisms) are types of DNA sequences (stretch of DNA) which can be used as markers. These markers are used in disease diagnosis and DNA fingerprinting.

Evaluation

Multiple choice questions

1. Which of the following properties is essential for the function of a tRNA molecule?

   a. Recognition of a codon
   b. Recognition of an anticodon
   c. Ability to distinguish one amino acid from another
   d. Recognition of DNA molecule

2. Which chain termination codon could be formed by a single base change from UCG, UGG and UAU?
   a. UAA b. UAG
   c. UGA d. AUG

3. Which of the following base-pair changes are transitions?
   a. AT → TA b. AT → GC
   c. Both a and b d. GC → AT

4. UV light usually causes mutations by a mechanism involving
   a. One-strand breakage in DNA
   b. Deletion of DNA segments
   c. Induction of thymine dimers and their persistence
   d. Inversion of DNA segments

5. The form of genetic information used directly in protein synthesis is
   a. DNA b. mRNA
   c. rRNA d. tRNA

6. _____ display one anticodon each
   a. eukaryotic mRNAs
   b. transfer RNAs
   c. ribosomal RNAs
   d. mRNAs

7. _____ contains exons and introns.
   a. Eukaryotic mRNAs b. rRNA
   c. tRNAs d. primers

8. The symbol lac^+ refer to
   a. genotype b. phenotype
   c. both a & b d. none

9. _____ sequence terminates protein synthesis
   a. UAA b. UAG
   c. UGA d. All the above

10. The principal start codon corresponds to which amino acid?
   a. Valine
   b. arginine
   c. Methionine
   d. Isoleucine

11. Number of nucleoprotein subunit in a prokaryotic ribosome
   a. 2 b. 4
   c. 5 d. 6

12. A deletion occurs that eliminates a single amino acid in a protein. How many base pairs were deleted?
   a. 1 b. 2
   c. 3 d. 4

13. During conjugation plasmids undergo
   a. Theta replication

b. rolling circle replication

c. sigma replication

d. gamma replication

14. If a plasmid is mobilizable but nonconjugative, what function does it lack?

a. Antibiotic resistance

b. Fertility

c. Colicinogenic

d. Restriction sequences

15. The uptake of naked DNA from the surrounding is known as

a. Transduction

b. Conjugation

c. Transformation

d. Lysis

16. The F plasmid makes a cell

a. Donor b. Recipient

c. Resistant d. None

17. Which of the following is more efficient in transferring chromosomal DNA

a. F^+cell b. F^-cell

c. Hfr cell d. R^+cell

18. Which of the following statement is true

a. Protein is the only gene product

b. A functional gene product is protein or might also be one of several classes of RNA molecules

c. Carbohydrate is the only gene product

d. Lipids are the only gene product

19. DNA is transcribed into

a. mRNA b. tRNA

c. sRNA d. hnRNA

20. Which of the following is found as part of all prokaryotic promoters

a. Pribnow box

b. Shine dalgarno sequence

c. AUG sequence

d. UAG sequence

Answer the following

1. What is the direction of synthesis of RNA?
2. Define coding strand.
3. What parts of a mRNA molecule not translated? Ans. Leader & Introns
4. How many codons could be contained in a four-letter code? Ans 4^4=256
5. What is the principal start codon and to what amino acid does it correspond?
6. Restriction endonucleases are naturally found in bacteria. What purpose do they serve?
7. There are many varieties of cloning vectors that are used to propagate cloned DNA. One type of cloning vector used in E.coli is a plasmid vector. What features does a plasmid vector have that makes it useful for constructing and cloning recombinant DNA molecules?
8. What is shuttle vector and why is it used?
9. What information and materials are needed to amplify a segment of DNA using PCR?
10. In most PCR reactions, a DNA polymerase that can withstand short periods of very high (near boiling) temperatures is used. why?

11. The sequence of nucleotides in an mRNA is 5′-AUG-ACCCAUU-CAUUGGUCUCGUUAG-3′. Assuming that ribosomes could translate this mRNA, how many amino acids long would you expect the resulting polypeptide chain to be?
12. The N-terminus of a protein has the sequence Met-His-Arg-Lys-Val-His-Cys-Gly. A molecular Biologist wants to synthesize a DNA chain that can encode this portion of the protein. How many DNA sequences can encode this polypeptide?
13. Explain the process by which an infected bacterium releases progeny phage.
14. Define coding strand.
15. Distinguish a missense and a nonsense mutation.
16. By what mechanism does 5-bromouracil induce mutations?
17. Define the term conjugative.
18. How does an Hfr cell differ from F^+ cell?
19. How are F′ plasmids produced?
20. Define a lysogen.
21. Restriction enzymes generate two types of termini. What are they?
22. Explain cosmids and the advantages resulting from the use of a cosmid?
23. Explain the use of bacteriophage in cloning DNA fragment.
24. What are expression vectors?
25. Diagramatically describe the plasmid cloning vector PUC19.
26. How is the natural phenomenon of conjugation used to transfer foreign gene?
27. List the stages involved in Recombinant DNA technology.
28. Discuss RAPD and RFLP.

MICROBIOLOGY

PRACTICAL MANUAL

Std XII Microbiology Practical Manual

Higher Secondary – Second Year Practical Examination

Microbiology	
Marking Scheme	
Allotment of Marks	
Internal Assessment	05 marks
External Assesment	15 marks
Total	**20 marks**
Internal Assessment (Practicals) Marks Break Up	
1. Record Note Book	03 marks
2. Skill of performing Experiments	02 marks
Total	**05 marks**
External Assessment Mark Break Up	
1. Major Practical	09 marks
2. Spotters	06 marks
Total	**15 marks**
I. Major Practical (Any one out of 5 questions) 9×1 = 9 marks	
• Aim	01 mark
• Principle	02 marks
• Procedure	03 marks
• Diagram	01 marks
• Observation	01 marks
• Results	01 marks
Total	**09 marks**
II. Spotters (Any three – one from each category) 2×3 = 6 marks	
• Identification	½ marks
• Two salient points	1 mark
• Diagram	½ mark
Total	**02 marks × 3 spotters = 6marks**

Key for Practical Examination

I. Major Practical (Any one) 9×1 = 9 marks

1. Determine the gram nature of microorganism present in the given sample (curd/idly batter/yeast)
2. Identify whether the given fungus is *Aspergillus* or *Mucor* or *Rhizopus* based on its microscopic characteristics.
3. Determine the blood group of the given blood sample.
4. Carry out blood staining using field's stain and observe the erythrocytes and leucocytes.
5. Identify whether the given culture is catalase positive.

II. Spotters

A. Specimen	2 marks
B. Slide	2 marks
C. Spotter	2 marks

1. Gram's staining of curd/idly batter/yeast

Aim: To determine the gram nature of microorganism present in the given sample(curd/ idly batter/yeast) by Gram's staining technique.

Theory and Principle:

Based on gram staining reaction bacteria can be divided into two large groups called gram positive and gram negative. Gram positive bacteria have thicker peptidoglycan compared to the gram negative bacteria. The lipid content of the cell wall of gram negative bacteria is higher than that of gram positive bacteria. Both gram positive and gram negative bacteria take up the primary stain crystal violet and stain red. When decolorized the porosity and permeability of the gram negative bacteria increases due to which the crystal violet iodine complex is given out by the gram negative bacteria. Further the gram negative bacteria takes up the counterstain safranin and stains red. Hence when bacteria are observed under microscope after gram staining the gram positive cells appear violet and gram negative cells appear red.

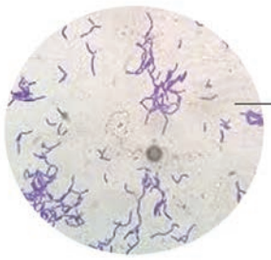
Requirements:

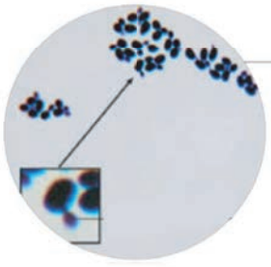
- Clean grease free slide
- Nichrome loop
- Given culture
- Crystal violet
- Grams iodine
- Decolorizer(Acetone Alcohol)
- Safranin
- D/W

Procedure:

1. Take a loopful of the given culture and place on the slide.
2. Prepare a smear and heat fix it.
3. Cover the smear with Crystal Violet for one minute.
4. Wash gently
5. Add Grams iodine for one minute
6. Decolorise with acetone alcohol
7. Wash the slide immediately
8. Cover the smear with safranin for a minute
9. Wash and Air dry.
10. Observe the slide under high power and oil immersion objectives.
11. Record your observations.

Diagram: (Any one Diagram)





Observation Table: (any one shape and stain)

Sr. no	Morphology	Arrangement	Colour of Cytoplasm	Colour of Background	Inference
1.	Rod (bacilli)	Singles, chains	Violet	colourless	Gram positive
2.	Oval yeast cells	Singles, budded	Violet	colourless	Gram positive

Results: Gram staining of the given culture revealed gram positive violet colored rod-shaped bacteria in chains.

2. Identification of the fungus (Aspergillus/Mucor/Rhizopus) by wet mount using LPCB.

Aim: To identify whether the given fungus is *Aspergillus* or *Mucor* or *Rhizopus* based on microscopic characteristics by wet mount method using lactophenol cotton blue stain.

Theory and Principle :

Filamentous fungi are reliably identified by their characteristics microscopic morphology such as shape, size and arrangement of spores and hyphae. Fungi are eukaryotic and range from unicellular yeast to multicellular molds. They reproduce by producing spores.

Common fungi are Aspergillus, Mucor and Rhizopus. They are filamentous and collectively form mycelium. The morphology of the hyphae and spores can be identified using a simple wet mount technique using lactophenol cotton blue stain.

The organism suspended in the stain are killed due to the presence of phenol. Lactic acid preserves fungal structures and cotton blue stains the fungal cell wall.

Fungi	Characteristics of Hyphae	Spores borne in
Aspergillus sp.	Septate	Conidiophore bear conidia
Mucor and Rhizopus sp.	Aseptate	Sporangiosphore bear sporangium containing sporangiospore.

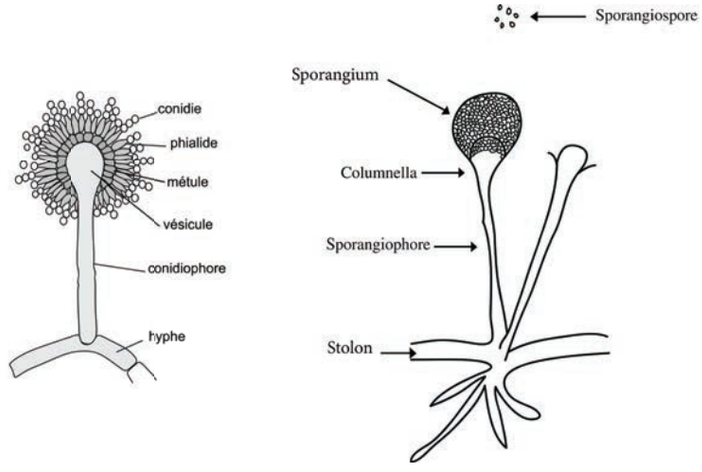
Requirements :

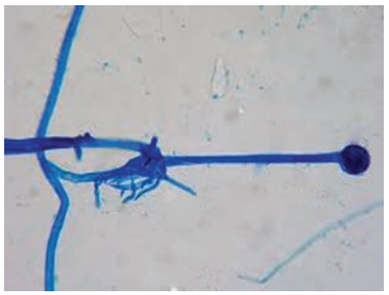
- Clean grease free slide
- Coverslip
- Forcep
- Teasing needle
- Distilled water
- Lactophenol Cotton Blue

Procedure:

1. Take a clean slide.
2. Place a drop of water on the slide.
3. With the help of forceps transfer the fungal mycelium.
4. Tease it with needle to separate the filaments (hyphae).
5. Add a drop of lactophenolcotton blue.
6. Gently place a coverslip avoiding air bubble formation.
7. Observe under low power and high power objective lens.
8. Read the observations and interpret.

Diagram:





Observation;

Filamentous hyphae bearing sporangia were observed.

Results:

Wet mount using lactophenol cotton blue was carried to identify the fungus sample. Hyphae with sporangium bearing sporangiospores were observed. It is likely to be of mucor species.

3. Blood Grouping

Aim: To determine the blood group of the blood sample by the slide agglutination test.

Theory and Principle:

Blood grouping is an essential requirement before blood is transfused from one person to another.It is also useful in settling paternity disputes and medicolegal problems.

Red blood cells contain blood group antigens. Antibodies to the blood group antigens are present in the blood plasma.The antigens are generally determined and are responsible for blood types. When RBCs of a person are mixed with corresponding antiserum, agglutination occurs due to antigen-antibody reactions.

Materials Required

- Blood sample (anticoagulated)
- Sterile cotton
- Sterile lancet
- Clean dry grease free slides or white tile
- Toothpicks
- Marker pen
- Commercially available Anti A sera, Anti B sera and Anti D sera

Procedure

1. Prick the finger under aseptic conditions
2. Place a drop of blood on the slide on each side marked as A, B and D.
3. Add a drop of antiserum A , B and D on A, B and D side respectively.
4. Mix with toothpick using separate toothpicks for each mixture.
5. Wait for 2 mins and observe for clumping reaction if any confirm it by observing under microscope.
6. Interpret the results and report.

Interpretation

If agglutination on A side the blood group is A

If agglutination seen on B side the blood group is B

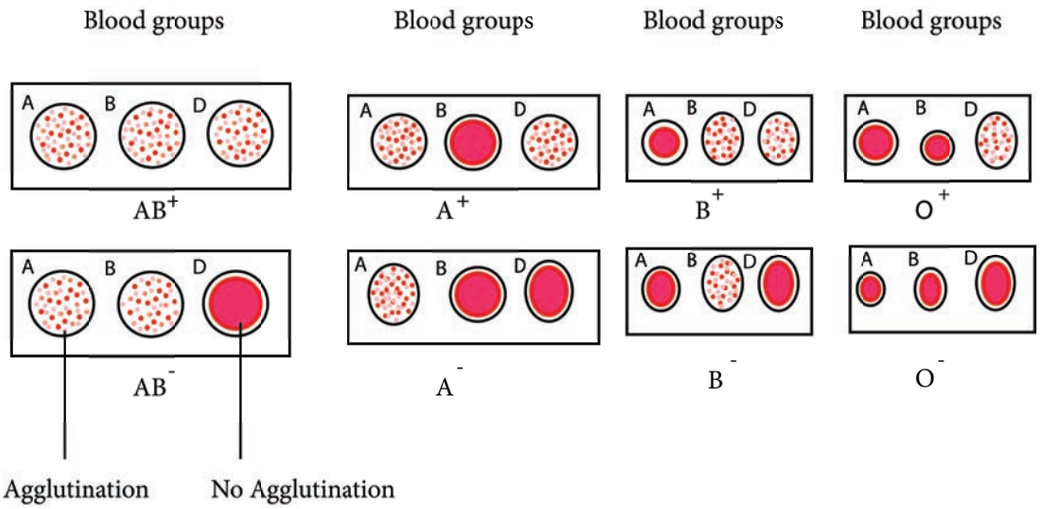
If Agglutination on both A and B side the blood group is AB

If No agglutination on A and B side the blood group is O

If agglutination is seen on D side the blood group is Rh(D) positive

If No agglutination on D side the blood group is Rh(D) negative.

Diagram: (any one depending on the results)



Observation: (will vary with the type of blood group an example is given below)

Agglutination is seen on A, B and D side

Result: The blood group of the blood sample was determined by slide agglutination test and was found to be AB Rh positive.

4. Blood Staining

AIM

To make a blood smear ,stain it using Field's stain and observe the erythrocytes and leucocytes.

Theory and Principle:

Blood smears are used to determine leukocyte differentials, to evaluate erythrocyte, platelet and leukocyte morphology, and, if necessary, to estimate platelet and leukocyte counts. It is also used for diagnosis of parasites like plasmodium in the blood.

Field's Stain is a romanowsky stain, used for rapid processing of blood specimens and is used to stain thick and thin films. It consists of two differential stain.**Field stain A**

which is methylene blue and Azure dissolved in a phosphate buffer solution.It is the basic component of the stain and **Field stain B** made up of Eosin Y in a buffer solution which is the acidic component of the stain.These basic and acidic dyes induce several colours when applied to cells.The fixator, methanol, does not allow any additional changes to the slide. The basic component of peripheral white blood cell(cytoplasm) is stained with acid dye and the acid component that is nucleic acid of the nucleus takes on the basic dye and is stained blue to violet. The neutral components of the cells are stained by both dyes(Field's stain A and B solution).

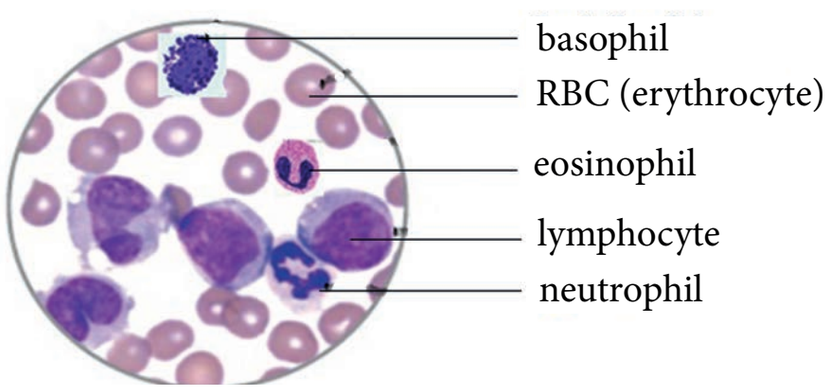
Requirements

- Cotton
- Spirit
- Blood sample
- Clean grease free slides
- Methanol fixative
- Field's stain A and Field's stain B.

Procedure

1. Finger Prick under aseptic condition.
2. Place a small drop of blood, on one side about 1-2 cm from one end of a slide.
3. Without delay place another slide at an angle of 45° to make contact with the drop.
4. Spread it over an area of about 2 cm^2(The film should be distributed so thinly that it appears transparent.
5. After air drying the thin blood film,immerse or fix the smear in methanol for 1 minutes.
6. Flood or dip the slide in Field's Stain A for 2-3 seconds.
7. Wash it with distilled water,
8. Flood or dip the slide in Field's Stain B for 2-3 seconds and wash with distilled water.
9. Now air dry the smear and observe under microscope.

Diagram



Observation

TYPE OF CELL	COLOUR OF CYTOPLASM	COLOUR OF NUCLEUS	COLOUR OF GRANULES
RBC	pink	-	-
WBCs(leucocytes)			
Neutrophil	pink	blue	lilac
Eosinophil	pink	blue	orange
Basophil	pink	blue	Dark blue black
lymphocyte	blue	violet	-

Results

The blood smear was stained using field's stain and erythrocytes and leucocytes were observed under microscope.

5. Test for Catalase

Aim

To test whether the given culture is catalase positive by the catalase test

Theory And Principle

Catalase test demonstrates the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide (H_2O_2). It is used to differentiate those bacteria that produces an enzyme catalase, such as *staphylococci*, from non-catalase producing bacteria such as *streptococci*.

The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. The culture should not be more than 24 hours old.

Requirements

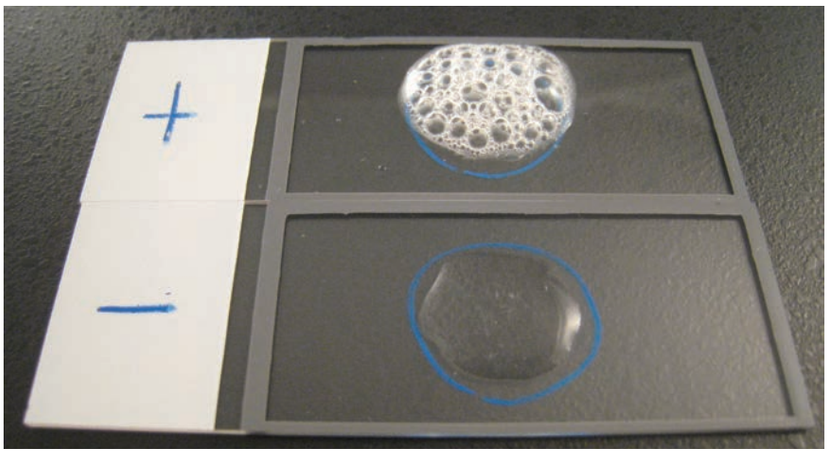
- Slides
- Nichrome loop or toothpick
- 24hour old culture
- 3%hydrogen peroxide
- Dropper

Procedure

Slide Method

1. Use a loop or sterile wooden stick to transfer a small amount of colony growth in the surface of a clean, dry glass slide.
2. Place a drop of 3% H_2O_2 in the glass slide.
3. Observe for the evolution of oxygen bubbles.

Diagram



$2H_2O_2 \xrightarrow[\text{catalase}]{} 2H_2O + O_2$ (gas bubbles)

Observation (any one to be reported depending on the culture)

Positive: Copious bubbles produced, active bubbling

Examples: *Staphylococci, E. coli, Enterobacter, Klebsiella, Shigella, Yersinia, Pseudomonas.*

Negative: No or very few bubbles produced.

Examples: *Streptococcus and Enterococcus* sps.

Result

The given culture was found to be catalase positive as determined by the catalase slide test.

6. Widal Test (Slide Test)

Aim

To carry out the widal test for the given blood sample and to determine the presence of antibodies against salmonella antigens.

Theory And Principle

Widal test is a serological test which is used for the diagnosis of enteric fever or typhoid fever. Typhoid or enteric fever is caused by a gram negative bacteria *Salmonella enterica* (*Salmonella* Typhi or *Salmonella* Paratyphi). *Salmonella* possess O antigen on their cell wall and H antigen on their flagella. On infection, these antigen stimulates the body to produce specific antibodies which are released in the blood. The Widal test is used to detect these specific antibodies in the serum sample of patients suffering from typhoid using antigen-antibody interactions. These specific antibodies can be detected in the patient's serum after 6 days of infection (fever).

Salmonella Typhi possesses O antigen on the cell wall and H antigen on flagella. *Salmonella* Paratyphi A and *S.* Paratyphi B also possess O antigen on their cell wall and but have AH and BH antigen on their flagella respectively.

Widal test is an agglutination test in which specific typhoid fever antibodies are detected by mixing the patient's serum with killed bacterial suspension of Salmonella carrying specific O, H, AH and BH antigens and observed for clumping ie. Antigen-antibody reaction. The main principle of Widal test is that if homologous antibody is present in patient's serum, it will react with respective antigen in the suspension and gives visible clumping on the test slide.

Requirements

Fresh serum

The complete kit containing five vials containing stained Salmonella antigen

- *S.* Typhi ⟶ O antigen
- *S.* Tyhhi ⟶ H antigen
- *S.* Paratyphi ⟶ AH antigen
- *S.* Paratyphi ⟶ BH antigen

Widal positive control

Widal test card or slide

v) Applicator stick

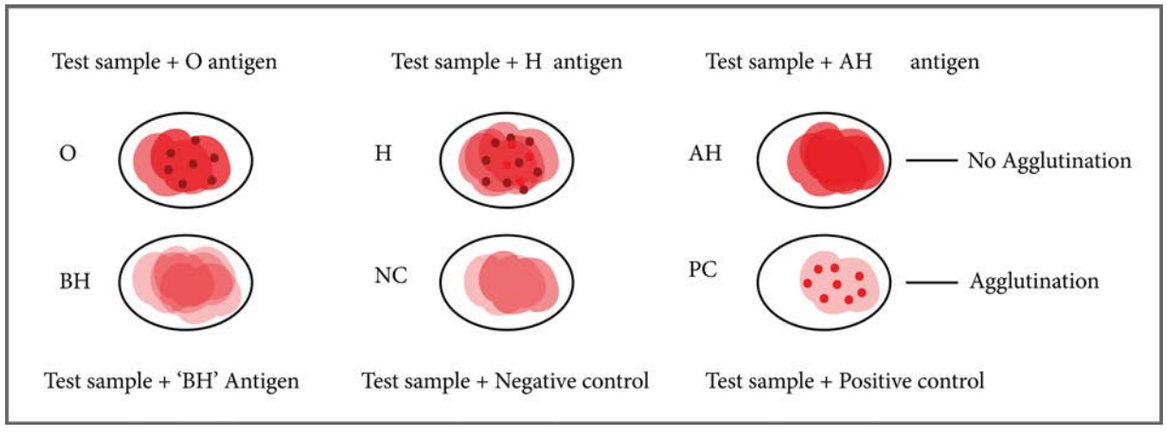
Procedure

- Widal test can be done in two ways-one is rapid test on slide and another is tube test in which result may be obtained after one night of incubation.

Rapid slide test:

1. Clean the glass slide or test card supplied in the kit well and make it dry.
2. Label the circles (1, 2, 3, 4, 5 and 6) in the test card as O, H, AH, BH, Negative control and Positive control
3. Place a drop of undiluted test serum in each of the four labelled circle (1, 2, 3 and 4) ie O, H, AH and BH and place a drop of Negative control serum in circle 5 and Positive control in circle 6.
4. Place a drop of antigen O, H, AH and BH in circle 1, 2, 3, and 4 respectively and no antigen in circle 5 and O/H antigen in circle 6.
5. Mix the content of each circle with a separate wooden applicator stick and spread to fill the whole area of the individual circle.
6. Rock the test card for a minute and observe for agglutination.

Diagram



Observation

Agglutination was observed in O and H side within a minute which indicates the presence of antibodies in the serum sample against Salmonella typhi antigens.

Proceed for quantitative slide test or tube test for the quantitative estimation of the titre of the antibody.

Result

Qualtative widal test was carried out using rapid slide agglutination method. Antibodies against O and H antigens of Salmonella typhi were detected in the serum.

7. Demonstration of rhizobium from root nodules and its isolation

Aim:

To demonstrate the presence of rhizobium in root nodules by gram staining and isolate them on a nutrient medium.

Theory and Principle:

Leguminous plants like cowpea, red gram , black gram contain root nodules formed by rhizobium.Rhizobium in the soil enter into the roots of leguminous plant and form nodules and establish symbiotic association. Bacteria derive nutrients from the plants. The rhizobacteria fix nitrogen which is beneficial to the plant. Rhizobium is a symbiotic N_2 fixer found to occur as bacteroids in the root nodules of leguminous plants. They can be easily isolated and cultured in vitro.

Based on gram staining reaction bacteria can be divided into two large groups called gram positive and gram negative. Gram positive bacteria have thicker peptidoglycan compared to the gram negative bacteria. The lipid content of the cell wall of gram negative bacteria is higher than that of gram positive bacteria. Both gram positive and gram negative bacteria take up the primary stain crystal violet and stain red. When decolorized the porosity and permeability of the gram negative bacteria increases due to which the crystal violet iodine complex is given out by the gram negative bacteria. Further the gram negative bacteria takes up the counterstain safranin and stains red. Hence when bacteria are observed under microscope after gram staining the gram positive cells appear violet and gram negative cells appear red. **Rhizobia are Gram- negative rods which are motile with bi-polar, sub-polar and peritrichous flagella**.

Rhizobium grows well on Yeast Extract Mannitol Agar (YEMA). Congo red added to the medium differentiates rhizobia that stand out as white, translucent, glistening elevated, small colonies with entire margin, in contrast to the red stained colonies of Agrobacterium and other bacteria.

Requirements:

1. Root nodules (pink) of any leguminous plant
2. **Congo red, Yeast Extract, Mannitol Agar (pH 6.8 – 7.0):**

Mannitol	10.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Yeast extract	1.0 g
$CaCO_3$	3.0 g
Agar	25.0 g

Congo red (1% aqueous)

2.5 ml (1.0 g in 100 ml)

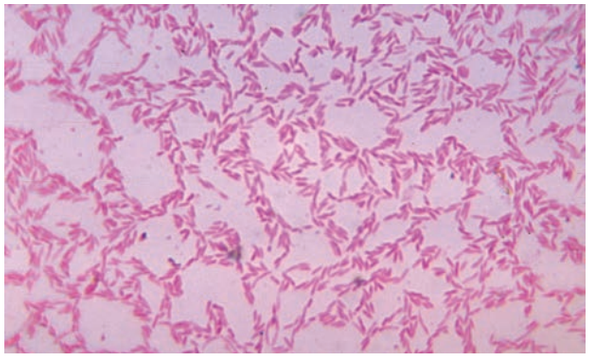
Distilled water 1000.0 ml

3. Inoculation loop
4. Bunsen burner/laminar clean air flow hood.
5. Slides and glass rod.
6. Petri plates with YEMACR medium.
7. Sterile distilled water.
8. 95% alcohol and 0.1% $HgCl_2$.

Procedure:

1. Wash the root system under a slow stream of running tap water, taking care to see that the nodules are intact.
2. Select pink nodules and remove them
3. Wash and keep the nodules in 95% ethanol for a minute, wash and transfer them to 0.1% $HgCl_2$.
4. Remove after five minutes and wash the nodules about four to five times with sterile distilled water.
5. Place the nodule on a sterile slide in a drop of sterile distilled water and crush it either with a sterile glass rod or a flat tipped forceps.
6. Remove a loopful of this cloudy suspension and streak inoculate on YEMACR plates and label.
7. Incubate in dark at 28°-30°C for 2-3 days and observe the colonies.
8. Make a smear of the remaining crushed material and gram stain and observe the gram negative bacilli. Even samples from the colonies can be gram stained.

Diagram:



Observation

Gram's stain

Organism	Morphology	Arrangement	Colour of cytoplasm	Colour of Background	Inference
Rhizobium from root nodule.	Rod (bacilli)	Singles	red	colourless	Gram negative

Colony characteristics of rhizobium on YEMA after incubation for 2-3 days at room temperature

Size – 2-4 mm

Shape- circular

Colour – White

Margin - entire

Elevation – convex, raised

Opacity - semitranslucent

Texture – creamy

Consistency – mucilaginous

Gram nature – gram negative

Motility – actively motile

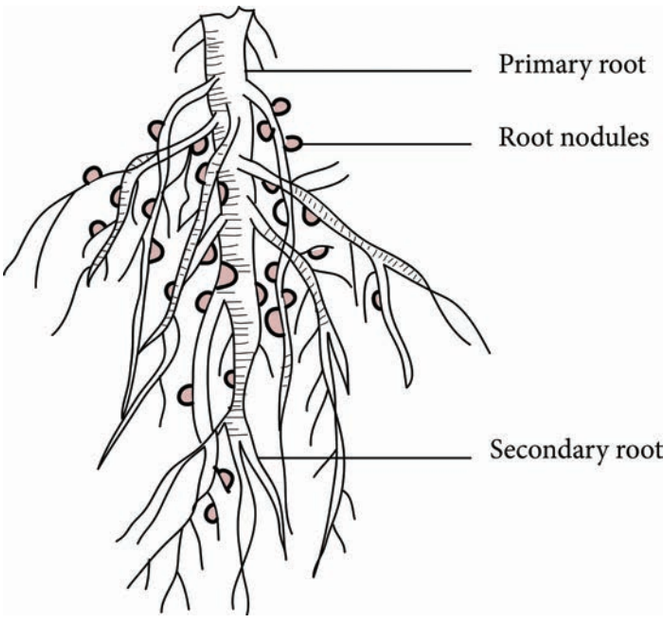
Results: Gram staining of the root nodule exudate revealed the presence of gram negative rods.

The colony characteristics of rhizobia were studied after isolation on YEMA medium. White, creamy, mucoid colonies were obtained.

Spotters

II A) Specimen

1. Root nodules of leguminous plant

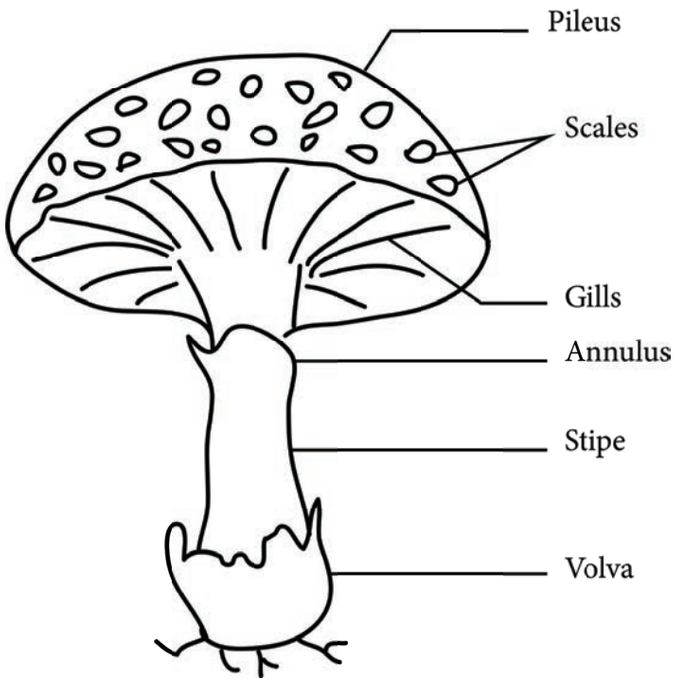


- Leguminous plants like cowpea, red gram contain root nodules formed by rhizobium.
- Rhizobium in the soil enter into the roots of leguminous plant and form nodules and establish symbiotic association.
- Bacteria derive nutrients from the plants.
- The rhizobacteria fix nitrogen which is beneficial to the plant.

2. Tikka leaf spot of groundnut plant

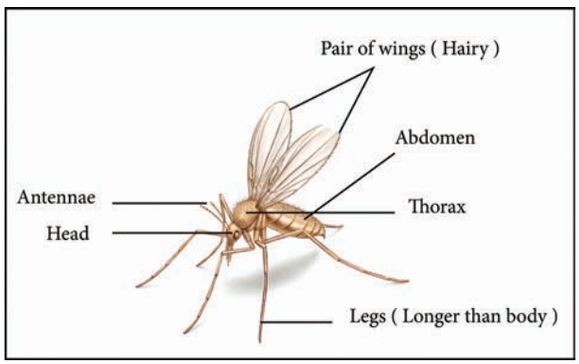
- Tikka leaf spot disease is a kind of fungal disease seen in groundnut leaf.
- This disease is caused by Cercaspora personata.
- Brown spots surrounded by a yellow halo appear on the upper surface of the leaf.
- The fungal spores can be demonstrated if the leaf is processed and observed under microscope.

3. Mushroom



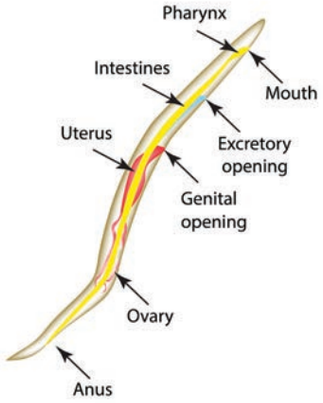
- Mushroom is a saprophytic fungus.
- Primary mycelium grows from basidiospores.
- It has high protein content and edible mushrooms are used as food.
- Example: Agaricus species and Pleurotus species.

4. Sand fly



- Bite of an infected sandfly transmits leishmania donovani infection.
- Female sandfly during a blood meal ingest free as well as intracellular amastigotes in the blood.
- In the midgut these are transformed to flagellated promastigote.

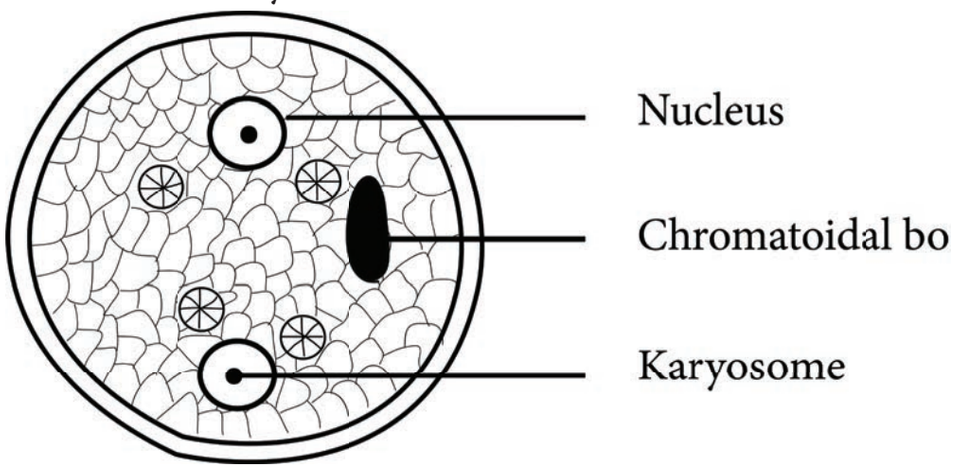
5. Ascaris



- The adult worm of ascaris lives in the small intestine of humans
- They are large cylindrical worms with tapering ends, the anterior end being thinner than the posterior end
- The adult male worm is smaller than female worms.

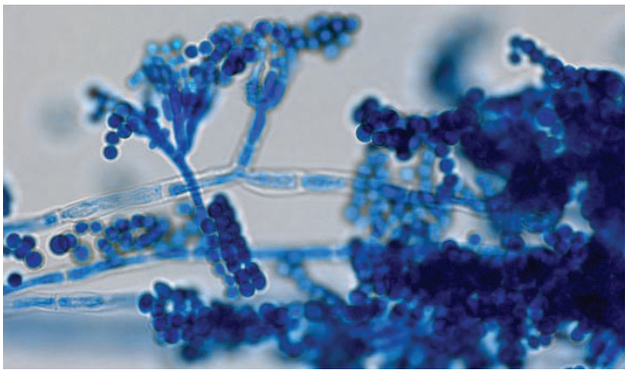
IIB) Slide

6. Cyst of Entamoeba histolytica



- Cyst is one of the three forms of entamoeba histolytica
- A mature cyst is a quadrinucleate spherical body.
- Mature cysts are passed in the stool of infected person
- Direct examination of wet mount of stool for cysts is diagnostic of intestinal amoebiasis

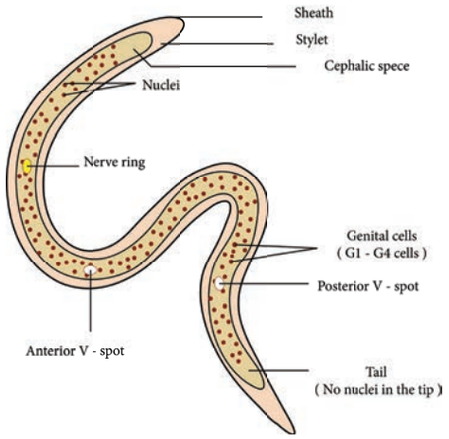
7. Penicillium species



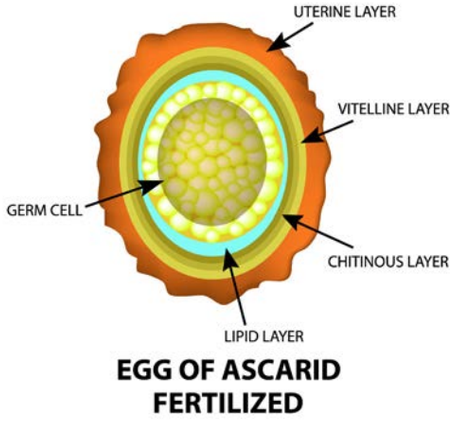
- Colony of penicillium are initially white and fluffy and later produce pigmented spores and turn into shades of green or blue green
- Hyphae are hyaline and septate
- Condiophores are long, give rise to branching phialids
- Phialids branch and give the appearance of brush or penicillins
- They produce sterigmata bearing chain of conidia (spores) which are oval or spherical and measure 1-2micrometer.

8. Microfilariae

- Filariasis is caused by nematodes (roundworms) like *Wuchereria bancrofti* that inhabit the lymphatics and subcutaneous tissues.
- The female worms release the first stage larvae called microfilariae, which are detected in the peripheral blood.
- Identification of microfilariae by microscopic examination is the most practical diagnostic procedure.
- The blood sample can be a thick smear, stained with Giemsa.
- The larva measures about 290microns in length and 6-7micron in breath.

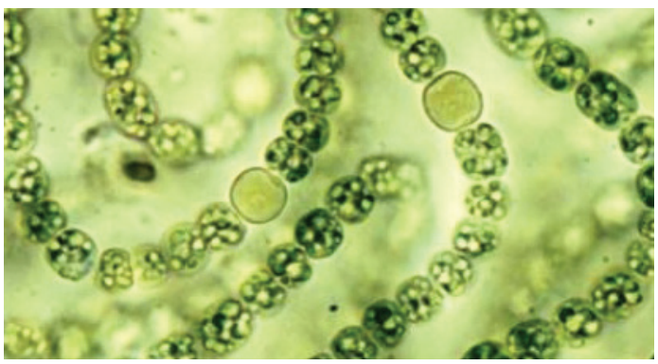


9. Egg of Ascaris lumbricoides



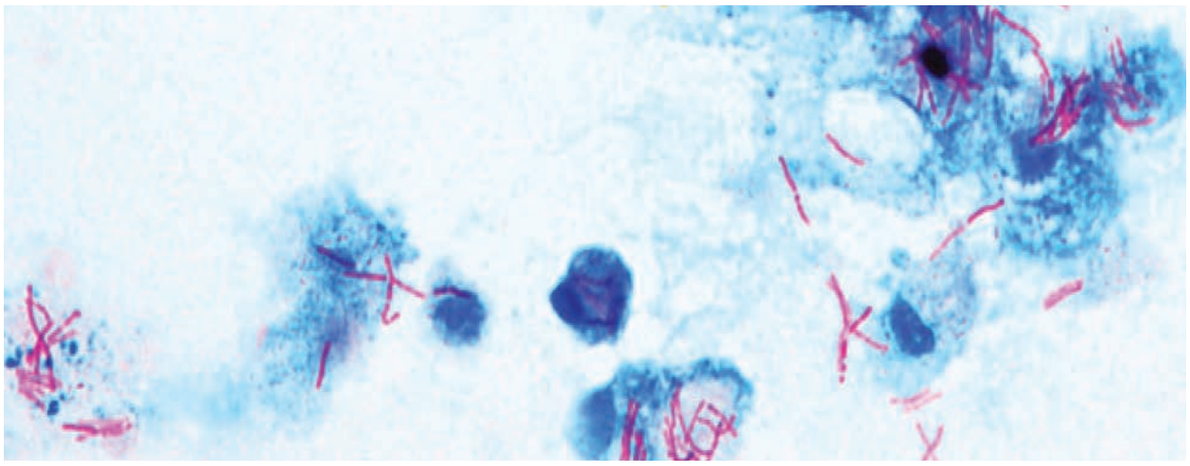
- These are passed in stool of the infected host.
- Brownish due to bile pigment.
- Fertilised eggs are rounded and have a thick shell (chitinous).
- Unfertilised eggs are elongated and larger than fertile eggs.
- When ingested through water or contaminated food by human it causes Ascariasis.
- Microscopic identification of eggs in the stool is the most common method for diagnosing intestinal ascariasis.

10. Heterocysts of Nostoc



- Heterocysts are specialized structures having thick cell wall formed in some filamentous blue green algae like Nostoc, Anabena.
- They may be terminal or found in between the vegetative cells attached to it by means of pores.
- They are sites of atmospheric nitrogen fixation.
- They serve as a store house of food material.

11. Acid fast bacilli

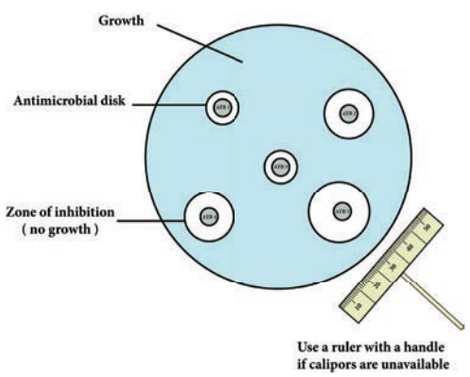


- Acid fast bacilli contains mycolic acid in their cell walls hence do not get stained easily, however once stained cannot be decolourised easily.
- Special method like Ziehl- Neelson's Carbol fuchsin is used to stain acid fast bacilli.
- The acid- fast bacilli are stained red in colour while the non acid fast cells appear blue when counterstained with methylene blue.
- Mycobacterium tuberculosis is and acid fast bacilli.

IIC) Spotter

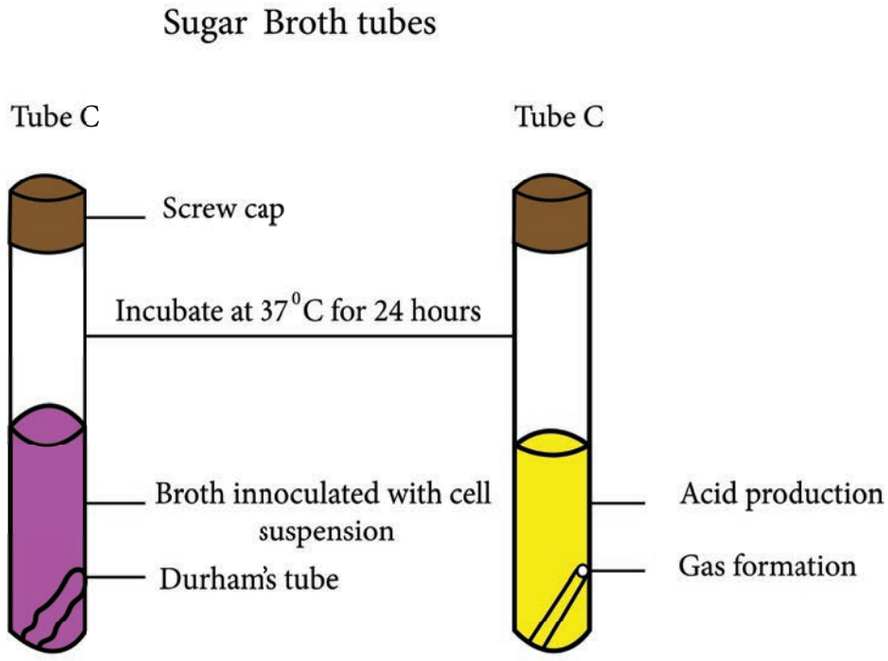
12. Antibiotic sensitivity plate set up by Kirby Bauer technique

- Kirby Bauer technique is used to determine the susceptibility of the organism to various antimicrobial agents.
- Standard suspensions of rapidly growing test bacterium is inoculated on the surface of muller hinton agar plates.
- Antibiotic discs are pressed on the surface of the seeded plates.
- The zone of inhibition or the zone of growth determines the degree of susceptibility of the organism towards antibiotic.



13. Sugar fermentation tube showing acid and gas production

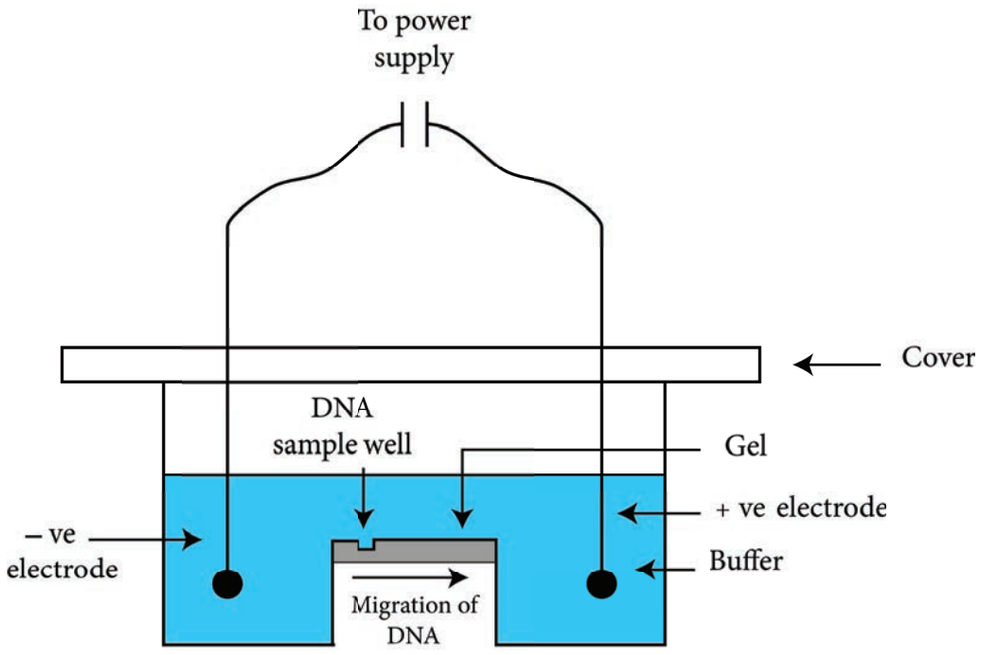
- Carbohydrate broth with bromocresol purple as indicator is used for testing the ability of pure bacterial culture to ferment a specific sugar like lactose, xylose, mannitol and other sugars.
- Acid production is indicated by colour change of the indicator from purple to yellow
- Gas production is indicated by an air bubble in the durham's tube.
- Escherichia coli ferments lactose producing acid and gas.



In the above image Test tube C was innoculated with cell suspension and incubated at 37 C for 24 hours and after innoculation the purple colour of broth was changed to yellow colour with gas bubble formation in durham's tube.

14. Agarose gel electrophoresis apparatus.

- Electrophoresis refers to the movement of charged molecules in an electric field.
- The negatively charged molecules move towards the positive electrode while the positively charged molecules migrate towards the negative electrode.
- Gel electrophoresis is a routinely used analytical technique for the separation and purification of specific DNA fragments.
- As the DNA is negatively charged, DNA fragments move through the gel towards the positive electrode. The rate of migration of DNA is dependent on the size and shape.



15. Spoiled food

- Spoilage is a process in which food deteriorates such that its quality of edibility is reduced.
- Food poisoning may result on eating contaminated or spoiled food.
- Foods spoil due to attacks from enzymes, oxidation and microorganisms.
- These include bacteria, mold, yeast, moisture, temperature and chemical reaction.

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