TOOLS OF RECOMBINANT DNA TECHNOLOGY

Essential components necessary for genetic engineering encompass.

1. Enzymes for DNA manipulation
   (i) Restriction enzymes
   (ii) Polymerase enzymes
   (iii) Ligase enzymes
2. Vectors
3. Host organisms

1) Enzymes

A diverse array of specialized enzymes finds application in genetic engineering processes.

(i) Restriction Enzymes (RE)

In 1963, researchers isolated two enzymes responsible for inhibiting the growth of bacteriophage in E. coli. One of these enzymes, methylase, added methyl groups to DNA, while the other enzyme was identified as a restriction endonuclease, which cut DNA molecules. These restriction enzymes act as molecular scissors, precisely cleaving genes (DNA) into defined fragments.

These fragments have various applications:

(i) Determining the arrangement of genes on chromosomes.
(ii) Analyzing the chemical composition of genes and DNA regions that regulate gene functions.
(iii) Creating novel combinations of genes.

Five years later, the first restriction endonuclease, Hind II, was isolated and characterized. Hind II was found to cut DNA molecules at specific points by recognizing a distinct sequence of six base pairs, known as the recognition sequence for Hind II.

The sequence provided,

5' GT (Pyrimidine: T or C) (Purine: A or G) AC3'
3'CA (Purine: A or G) (Pyrimidine: T or C) TG 5'

Illustrates a specific pattern in DNA where certain base pairs are paired in a specific order.

Apart from Hind II, there are over 900 other restriction enzymes that have been discovered, originating from more than 230 bacterial strains. Each of these enzymes recognizes distinct recognition sequences, contributing to the diversity of restriction enzyme functionality.

Note: Restriction enzymes are sourced solely from prokaryotic organisms, serving as their inherent defense mechanism against bacteriophage infections.

The naming convention for these enzymes typically involves the initial letter representing the genus name, followed by the next two letters representing the species of the prokaryotic cell from which they were extracted.

For instance, EcoRI originates from Escherichia coli strain RY13. In EcoRI, the 'R' denotes the strain's designation "Rough". The Roman numeral accompanying the names denotes the sequence in which the enzymes were isolated from that bacterial strain.

Restriction enzymes are categorized within a broader class of enzymes known as nucleases.

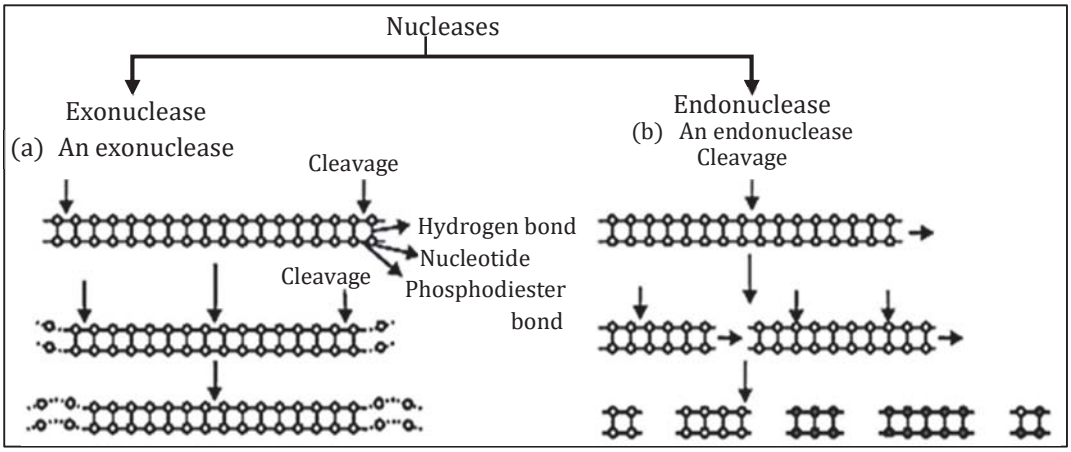

Figure: Depiction of reactions facilitated by two distinct types of nucleases.
(a) Exonuclease: This enzyme eliminates nucleotides from the end of a DNA molecule.
(b) Endonuclease: This enzyme cleaves internal phosphodiester bonds within the DNA molecule.

Every restriction enzyme identifies a particular palindromic nucleotide sequence within DNA. A palindrome refers to a word, phrase, number, or sequence of units that reads the same forward and backward.

A DNA palindrome refers to a sequence of base pairs that maintains its identical reading orientation on both strands. Typically ranging from 4 to 8 nucleotides in length, these sequences exhibit symmetry when read in either the 5' → 3' or the 3' → 5' direction.

For instance, the following sequences display this characteristic symmetry.

5’ — GAATTC — 3’
3’ — CTTAAG — 5’

Certain type II restriction enzymes have distinctive characteristics including their origin, recognition sequence, and cleavage site.

S.No.	Restriction Enzyme	Source	Recognition sequence and site of Cleavage
1.	Eco RI	Escherichia coli RY 13	↓ 5'-G-A-A-T-T-C-3' 3'-C-T-T-A-A-G-5’ ↑
2.	Hin d II	Haemophilus influenzae Rd	↓ 5'G-T-C-G-A-C-3' 3’-C-A-G-C-T-G-5' ↑
3.	Hin d III	Haemophilus influenzae Rd	↓ 5'-A-A-G-C-T-T3' 3'-T-T-C-G-A-A-5' ↑
4.	Bam H I	Bacillus amyloliquefaciens H	↓ 5'-G-G-A-T-C-C-5 3-C-C-T-A-G-G-5' ↑
5.	Sal I	Streptomyces albus	↓ 5-G-T-C-G-A-C-3 3-C-A-G-C-T-G-5 ↑

6.	Sma I	Serratia marcescens	↓ 5'-C-C-C-G-G-G-3' 3'-G-G-G-C-C-C-5' ↑
7.	Alu I	Arthrobacter luteus	↓ 5'-A-G-C-T-3' 3'-T-C-G-A-5' ↑
8.	Eco R II	Escherichia Coli R245	↓ 5'-C-C-T-G-G-3' 3'-G-G-A-C-C-5' ↑
9.	Hae III	Haemophilus aegyptius	↓ 5-G-G-C-C-3' 3-C-C-G-G-5' ↑
10.	Sca I	Streptomyces caespitosus	↓ 5-A-G-T-A-C-T-3' 3-T-C-A-T-G-A-5' ↑

Working of Restriction Enzymes

Every restriction endonuclease operates by scrutinizing the length of a DNA sequence. Upon encountering its specific recognition sequence, the enzyme binds to the DNA and cleaves each of the two strands of the double helix at precise locations along their sugar-phosphate backbones.

Certain restriction enzymes, such as EcoRI, cleave the DNA strand slightly offset from the center of the palindrome sites, but still between the same two bases on opposite strands. As a result, single-stranded segments are left at the ends of the DNA molecule. These stretches, referred to as sticky ends, cohesive ends, or staggered ends, are illustrated in the figure below. They earn this designation because they are capable of forming hydrogen bonds with their complementary cut counterparts. The adhesive nature of these ends facilitates the subsequent action of the enzyme DNA ligase.

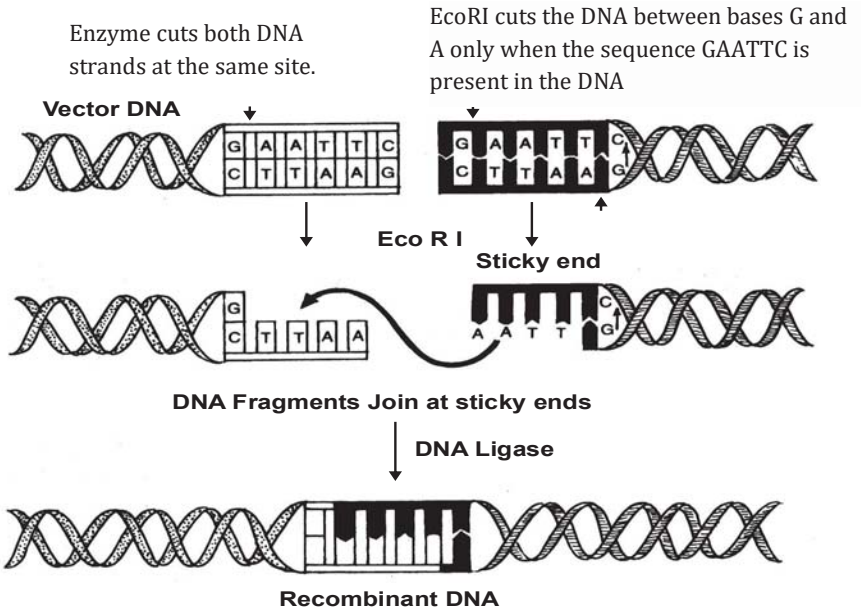

Fig:- Formation of recombinant DNA by action of restriction endonuclease enzyme- Eco RI

Certain restriction enzymes cleave the DNA strand directly at the center of the palindrome sequence. These resulting ends are referred to as blunt ends or flush ends.

For example, the restriction enzyme Sma I

```
        ↓
5' – C C C G G G - 3'
3' – G G G C C C – 5'
        ↑
5' – C C C  G G G - 3'
3' – G G G  C C C - 5'
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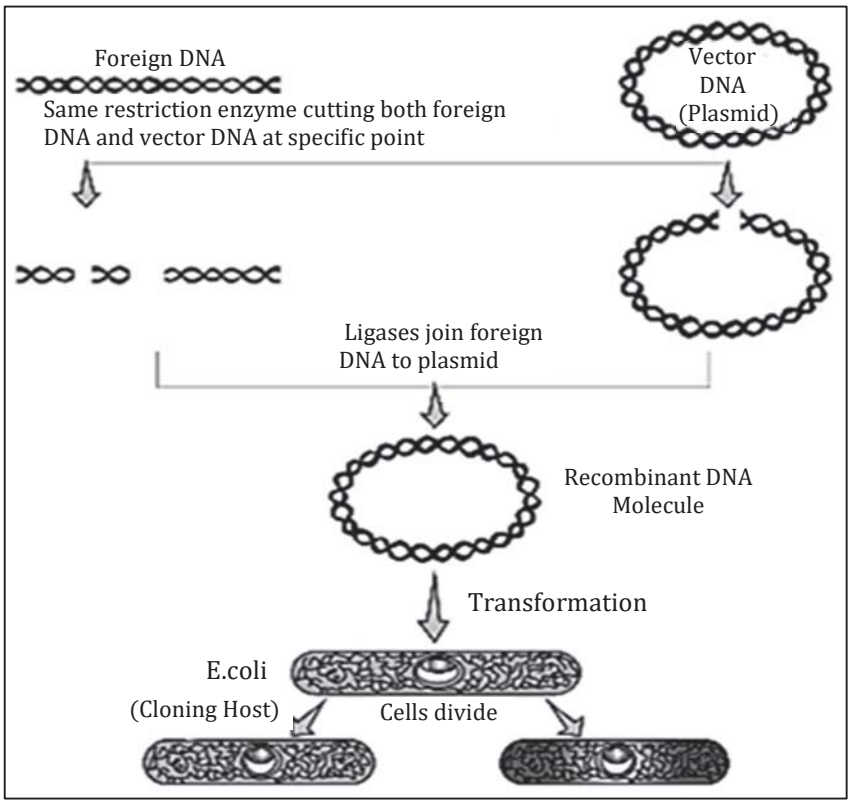
Restriction endonucleases play a pivotal role in genetic engineering by facilitating the formation of recombinant DNA molecules, which consist of DNA segments from diverse sources or genomes. When the same restriction enzyme is used to cut DNA, the resulting fragments exhibit similar sticky ends. These sticky ends can be subsequently ligated together, end-to-end, using DNA ligases.



DNA Ligases (Molecular glue)

This enzyme facilitates the formation of phosphodiester bonds between neighboring nucleotides, thereby covalently joining two separate fragments of double-stranded DNA. It harnesses cellular energy for this process. The most frequently employed ligase in recombinant DNA technology is T_4 DNA ligase, derived from bacteriophage T_4.

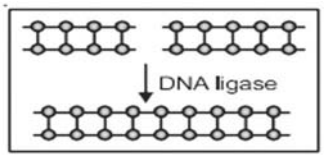


Fig. : Joining two molecules

DNA Polymerases

These enzymes facilitate the creation of a fresh DNA strand that matches an already existing DNA template. This synthesis proceeds in the direction from 5' to 3'.

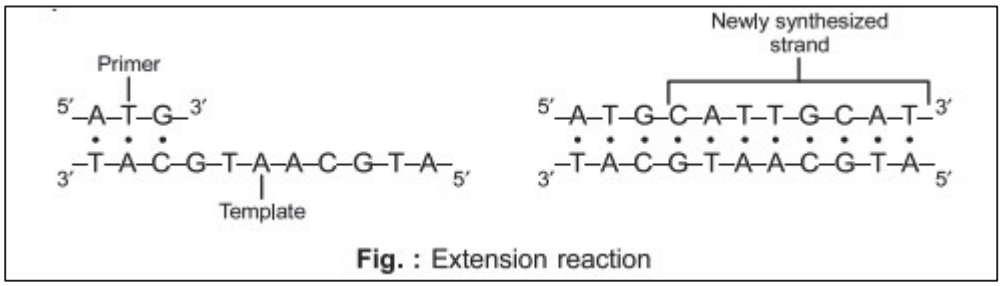


Fig. : Extension reaction

Example: What is the length of the recognition sequence for the initial restriction enzyme that was isolated?

Solution: The recognition sequence consists of six base pairs.

Example: In which organism's plasmid was the antibiotic resistance gene, as isolated by Cohen and Boyer, inserted?

Solution: The antibiotic resistance gene was inserted into a plasmid native to Salmonella typhimurium.

Cloning Vectors

A vector serves as a carrier or vehicle that transports a foreign segment of DNA into the host organism. These vectors are engineered to facilitate the following functions:

(i) Facilitating the easy attachment of foreign DNA.

(ii) Enabling the differentiation between recombinants and non-recombinants.

The essential characteristics of a vector include:

(i) Origin of replication (ori)

This sequence marks the starting point for DNA replication, allowing any DNA segment linked to it to replicate within the host cells. When a DNA segment is transferred to a different organism, it usually cannot replicate in the progeny cells of the host organism. However, if it integrates into the recipient's genome, it may replicate and be inherited along with the host DNA. This integration occurs because the foreign DNA becomes part of a chromosome that possesses replication capabilities. Chromosomes contain a specific DNA sequence known as the 'origin of replication.' Therefore, linking the foreign DNA with the origin of replication allows it to replicate and multiply within the host organism. This process can also be referred to as cloning or generating multiple identical copies of a template DNA. Additionally, this sequence regulates the copy number of the linked DNA. To obtain numerous copies of the target DNA, it should be cloned in a vector with an origin that supports a high copy number.

(ii) Selection Marker

This element aids in the identification and removal of non-transformed cells while selectively promoting the growth of transformed ones. Typically, genes conferring resistance to antibiotics like ampicillin, chloramphenicol, tetracycline, or kanamycin are utilized as beneficial selectable markers for E. coli. Regular E. coli cells lack resistance to these antibiotics. Additionally, the lac Z gene, responsible for encoding β-galactosidase enzyme, can serve as a basis for selection. This enzyme acts upon its substrate to produce a blue-colored product, making the substrate chromogenic, i.e., capable of producing color.

(iii) Cloning Sites

For integrating foreign DNA, the vector should ideally possess minimal, preferably singular, recognition sites for commonly employed restriction enzymes. Having multiple recognition sites within the vector would yield numerous fragments, complicating the process of gene cloning. The gene of interest is inserted at a restriction enzyme site located within any of the antibiotic resistance genes or selectable marker genes. Consequently, the recombinant vector forfeits antibiotic resistance due to the insertion of foreign DNA into one selectable marker. However, it can still be distinguished from non-recombinant ones due to the presence of intact antibiotic resistance for another selectable marker. A cluster of unique restriction enzyme sites closely situated in a plasmid constitutes a region termed the multiple cloning site (MCS).

(iv) Vector Size

It is advisable for the vector to be small since larger molecules are prone to degradation during purification processes.

Examples of commonly utilized vectors in Recombinant DNA Technology (RDT) include:

(A) Plasmids

These are additional chromosomal, circular, non-essential, double-stranded, autonomous, self-replicating DNA fragments found in bacterial and some yeast cells. They may confer properties such as antibiotic resistance in bacteria or virulence in organisms like Agrobacterium tumefaciens, where the Ti plasmid is involved.

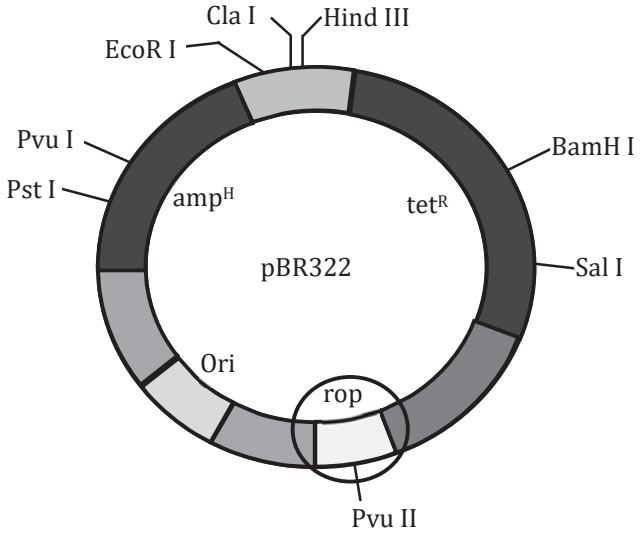


Fig.: The E. coli cloning vector pBR322 is depicted, highlighting specific restriction sites (Hind III, EcoR I, BamH I, Sal I, Pvu II, Pst I, Cla I), as well as the origin of replication (ori) and the antibiotic resistance genes (amp and tet).

Rop codes for the proteins involved in the replication of the plasmid Here is a detailed rephrased explanation of the name pBR322:

The name pBR322 is broken down as follows:

"p" denotes that it is a plasmid.

"BR" stands for Boliver and Rodriguez, indicating the researchers who were involved in its development.

"322" serves to differentiate this particular plasmid from others created within the same laboratory, providing a unique identifier for this specific plasmid.

Table: Contrasting Characteristics of Plasmid DNA and Chromosomal DNA

Characteristic	Plasmid DNA	Chromosomal DNA
Structure	Double-stranded	Double-stranded
Shape	Circular	Linear or Circular
Protein Association	Naked, devoid of histone protein	Coated with histone protein
Essential Genes	Does not carry essential genes for cell function	Carries essential genes necessary for cell function
Replication	Can replicate independently of the main genome	Replicates along with the genome
Presence of Introns	Introns are absent	Both exons and introns are present

Characteristics of pBR322

1. Size: 4.3 kilobases (kb)
2. Dual Antibiotic Resistance Genes: This plasmid harbors two sets of antibiotic resistance genes, necessitating a two-step selection process.
3. Moderately High Copy Number.

Selection via Antibiotic Resistance: The incorporation of foreign DNA occurs at a restriction site within one of the two antibiotic resistance genes.

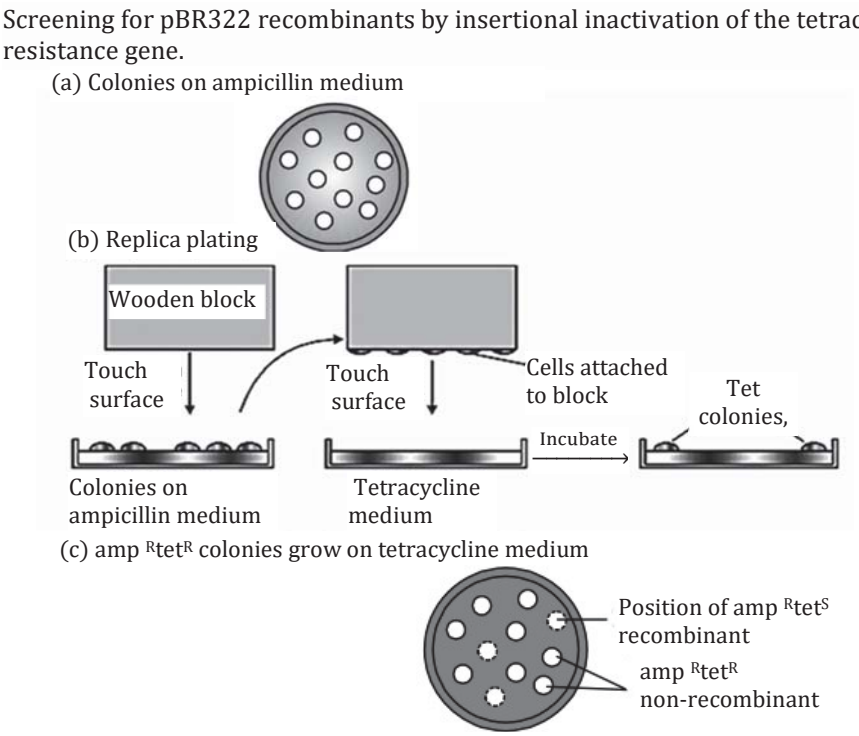


Fig.: (a) Cells are plated onto ampicillin agar: all the transformants produce colonies.
(b) The colonies are replica plated onto tetracycline medium.
(c) The colonies that grow on tetracycline medium are amptet and therefore non-recombinants. Recombinants (amp $^{R}tet^{R}$) do not grow, but their position on the ampicillin plate is now known.

For instance, foreign DNA can be ligated at the BamH I site of the tetracycline resistance gene within the pBR322 vector. Consequently, recombinant plasmids lose their resistance to tetracycline following the insertion of foreign DNA. However, they can still be distinguished from non-recombinant plasmids by culturing the transformed cells on a medium containing tetracycline. Those transformants able to grow on ampicillin-containing medium are subsequently transferred to a medium containing

tetracycline. Recombinant plasmids will proliferate on ampicillin-containing medium but not on tetracycline-containing medium. Conversely, non-recombinant plasmids will exhibit growth on the medium containing both antibiotics. In this scenario, one antibiotic resistance gene aids in the selection of transformants, while the other antibiotic resistance gene becomes "inactivated" due to the insertion of foreign DNA, facilitating the selection of recombinants.

Drawback of pBR322: The process of selecting recombinants via antibiotic inactivation presents a challenge as it necessitates the simultaneous plating of cells on two separate plates containing different antibiotics.

Addressing the limitation of pBR322 can be achieved by employing an alternative plasmid such as pUC8.

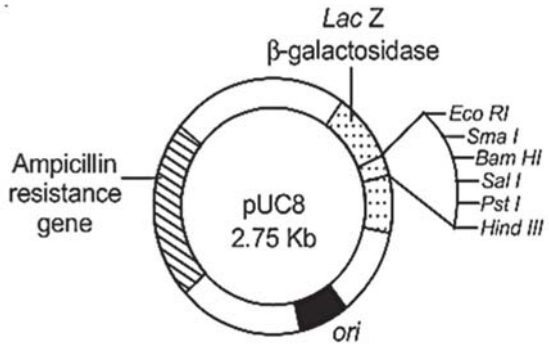


Fig. : Structure of pUC8

Advantages

1) Enhanced Copy Number: Achieving a higher copy number ranging from 500 to 700.
2) Streamlined Identification of Recombinant Cells: Identification of recombinant cells is simplified into a single step by plating cells onto agar medium containing ampicillin and X-gal, a chromogenic substrate for the ß-galactosidase enzyme encoded by lac Z.

Blue/White Selection

The pUC8 plasmid offers an alternative selectable marker, distinguishing between recombinant and non-recombinant cells based on their capacity to generate color in the presence of a chromogenic substrate. In this process, foreign DNA is integrated within the coding sequence of the enzyme ß-galactosidase, leading to the inactivation of the gene responsible for its synthesis, termed insertional inactivation. Colonies exhibit a blue coloration when the plasmid in the bacteria lacks an insert, owing to the presence of the chromogenic substrate. However, the presence of an insert results in the insertional inactivation of ß-galactosidase, causing the colonies to remain colorless, thereby identifying them as recombinant colonies.

(B) Bacteriophage

A bacteriophage is a type of virus that specifically infects bacterial cells, capable of replicating within these cells independently of the host's chromosomal DNA regulation. Due to their abundance within bacterial cells, bacteriophages exhibit a remarkably high copy number of their own genome.

By linking an external DNA segment with a bacteriophage, it becomes feasible to amplify its quantity to match the copy number of the bacteriophage. Various bacteriophages serve as cloning vectors, with the Lambda (λ) and M13 phage vectors being among the most frequently utilized:

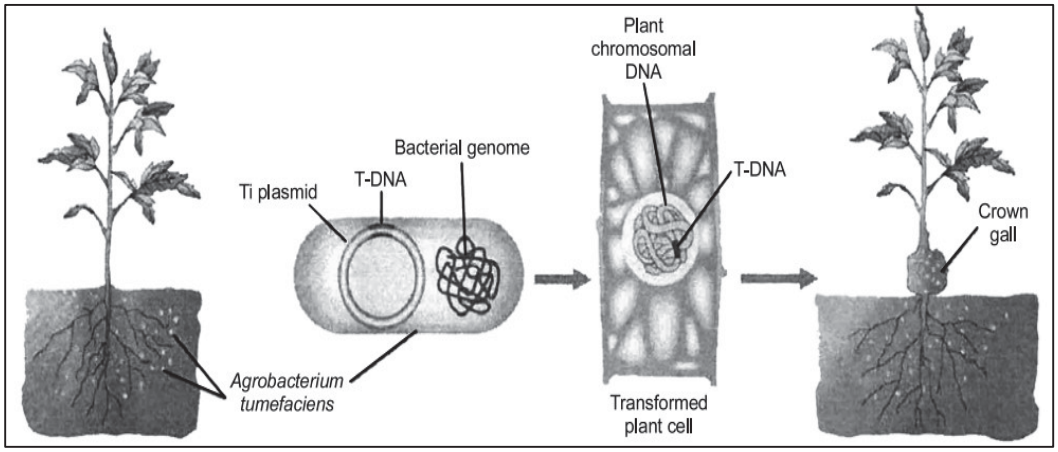
(i) Lambda (λ) Phage Vector: This vector facilitates the cloning of DNA fragments of up to 23 kilobases (kb) in length, where one kilobase represents a sequence of 1000 nucleotides.
(ii) M13 Phage Vector: An example of a filamentous phage that infects Escherichia coli (E. coli). Foreign DNA can be inserted into M13 without disrupting any essential genes.

(C) Vectors for Gene Cloning in Plants

Ti Plasmid of Agrobacterium tumefaciens: Agrobacterium tumefaciens, a pathogenic bacterium affecting several dicot plants, possesses the capability to deliver a DNA fragment called "T-DNA" into normal plant cells, inducing the formation of tumors and directing these cells to produce specific chemicals required by the pathogen. This bacterium typically invades plants at wound sites, transforming them and nearby cells to generate tumors known as crown gall.

Upon encountering a damaged plant cell, the bacterium transfers a T-DNA fragment from its plasmid into the host cell, where it integrates into the plant cell's chromosome at a random position. The Ti plasmid, responsible for inducing tumor formation (hence "Ti"), has undergone modifications to serve as a cloning vector. Although it no longer poses a pathogenic threat to plants, it retains its ability to deliver genes of interest into a variety of plant species.

Disarming the Ti plasmid involves replacing the harmful T-DNA originally present in A. tumefaciens with the desired gene of interest, rendering it safe for use in genetic engineering applications.



(D) Viruses Utilized for Gene Cloning in Animals: Retroviruses

Within the realm of animal virology, retroviruses possess the ability to transform normal cells into cancerous ones. However, these retroviruses have been rendered non-pathogenic and repurposed to serve as vehicles for delivering desirable genes into animal cells.

Consequently, once a gene or DNA fragment has been incorporated into a suitable vector, it is transferred into a bacterial, plant, or animal host, where it undergoes replication.

(E) Shuttle Vectors

These vectors possess the unique capability to replicate in both eukaryotic cells and Escherichia coli (E. coli). Essentially, they contain two distinct origins of replication and selectable marker genes—one tailored for eukaryotic cells and the other for E. coli. Examples include the YEp (Yeast Episomal Plasmid) and Modified Ti plasmid.

Example: To expedite selection, the gene of interest is inserted at a specific position within plasmid pBR322.
Solution: It is inserted within the "MCS" region situated within the antibiotic resistance genes.

Example: In the scenario where the gene of interest is inserted at the Pvu I site in pBR322, what antibiotic resistance would the recombinant bacteria display?
Solution: Tetracycline resistance.

Example: Enumerate two fundamental characteristics of vectors.
Solution: 1. Facilitates facile linking of foreign DNA.
2. Enables straightforward discrimination between recombinant and non-recombinant entities for selection purposes.

Competent Host

A competent host plays a pivotal role in Recombinant DNA Technology by facilitating the efficient insertion of a cloning vector into its cells, thereby yielding the desired outcomes sought by scientists.

Understanding the concept of a competent host requires grasping the hydrophilic nature of DNA. Given that DNA is composed of a bilipid layer, any substance insoluble in lipids cannot traverse the cell membrane. Therefore, to introduce DNA into a specific host, it is essential to render the host cell competent. There are several methods employed to prepare such competent hosts.

Once prepared, the competent hosts can receive recombinant DNA by incubating the cells with the desired DNA at low temperatures (typically on ice). Subsequently, the cells are subjected to a brief heat shock at around 42 degrees Celsius, followed by another cooling step on ice. This process allows the recombinant DNA to be effectively incorporated into the host cells.