Unit 1

Basic principles – mechanism of natural gene transfer by Agrobacterium, generation of foreign DNA molecules, restriction enzymes, their types and target sites, cutting and joining DNA molecule, linkers, adaptors, homopolymers, enzymes used in genetic engineering, cloning vehicles and their properties, natural plasmids, *in-vitro* vectors, cosmids and T-DNA based hybrid vectors

Introduction

Recombinant DNA technology is also called as genetic engineering technique used to transfer DNA isolated from one organism to other to produce a new characteristics. Paul Berg, Herbert W. Boyer, and Stanley N. Cohen invented recombinant DNA technology to artificially introduce into the DNA into the genome of another organism and then replicated and expressed by that other organism was largely. Paul Berg developed the first recombinant DNA molecules that combined DNA from SV40 virus and lambda phage. Later in 1973, Herbert Boyer and Stanley Cohen develop recombinant DNA technology, showing that genetically engineered DNA molecules may be cloned in foreign cells.

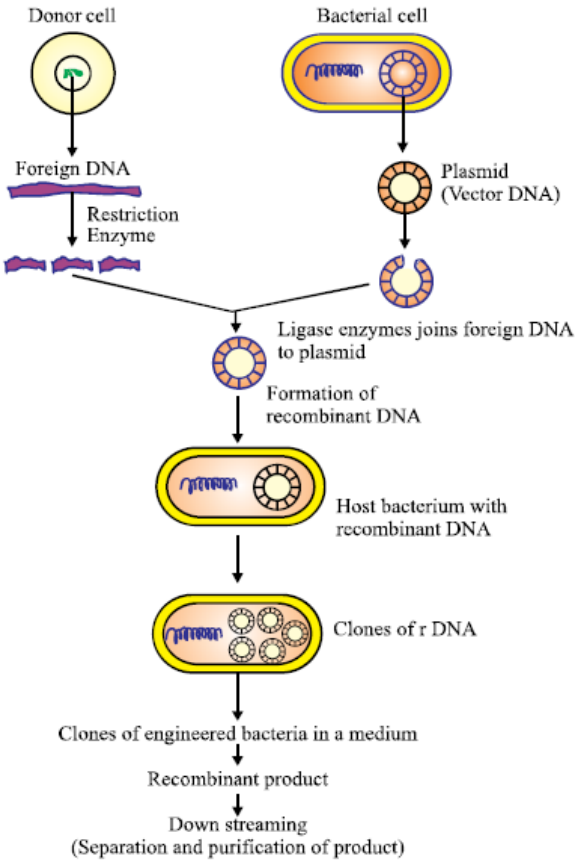


Image.ref. https://www.sarthaks.com/?qa=blob&qa_blobid=13290088062392600704

Agrobacterium mediated gene transfer in plants

Agrobacterium is a gram negative bacterium found in soil has ability to transfer genetic material to cause crown gall disease, tumor in plants, it also affects fungi, large algae and ascomycetes. Agrobacterium contains tumor inducing plasmid with virulence genes, T-DNA, origin of replication, opine catabolism sites.

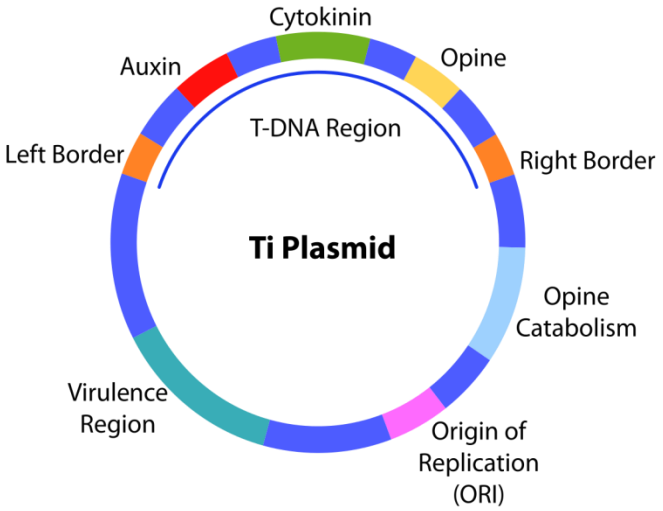
Agrobacterium recognition phenolic compound acetosyringone secreted by the plant wound site initiates the tumor induction. "Activated" *Agrobacterium* transfers a particular gene segment called transfer DNA (T-DNA) from the Ti plasmid. After T-DNA integrated into the chromosomal DNA in the nucleus of the host plant, genes for opine synthesis and tumor-inducing factors on the T-DNA are transcribed in the infected cells. This expression of the foreign gene in the host plant results in neoplastic growth of the tumors, providing increased synthesis and secretion of opine for bacterial consumption. Opine is the condensation of an amino acid with a keto acid or sugar and is a major carbon and nitrogen source for *Agrobacterium* growth. Different *A. tumefaciens* strains produce different opine phenotypes of crown gall tumors because a particular opine expressed in the tumor is used for particular bacterial growth. Most common *Agrobacterium* strains produce an octopine or a nopaline form of opines. Octopine and nopaline are derivatives of arginine. Agropine is discovered in octopine-type tumors, and it is derived from glutamate.

The second condition that plays an important role in gene transfer from *Agrobacterium* to the plant cell is the virulence (*vir*) region that is outside of the T-DNA and close to the left border with a nearly 25 kb length. Previous studies have shown that *vir* region contains six main genes (*virA*, v*irB*, *virC*, *virD*, *virE*, and *virG*) . *VirA* codes for a receptor that detects and correlates with phenolic compounds leaking out of damaged plant cells, and as a consequence, *virG* is stimulated. Stimulated *VirG* takes charge of the transcription operator task for itself and the other *vir* genes. *virC* enables to separate from the borders, while *virD* gene provides the regeneration of the T-DNA strand; *virB* and *virE* genes facilitate the move of the T-DNA from the bacterium to the plant cell.

Gene	Product	Function
ocs	Octopine synthase	Opine synthesis
nos	Nopaline synthase	Opine synthesis
trns1 (iaaH, auxA)	Tryptophan-2-mono-oxygenase	Auxin synthesis
trns2 (iaaM, auxB)	Indoleacetamide hydrolase	Auxin synthesis
trnr (ipt, cyt)	Isopentyltransferase	Cytokinin synthesis
trnL	Unknown	Unknown, mutations affect tumor size
frs	Fructopine synthase	Opine synthesis
mas	Mannopine synthase	Opine synthesis
ags	Agropine synthase	Opine synthesis

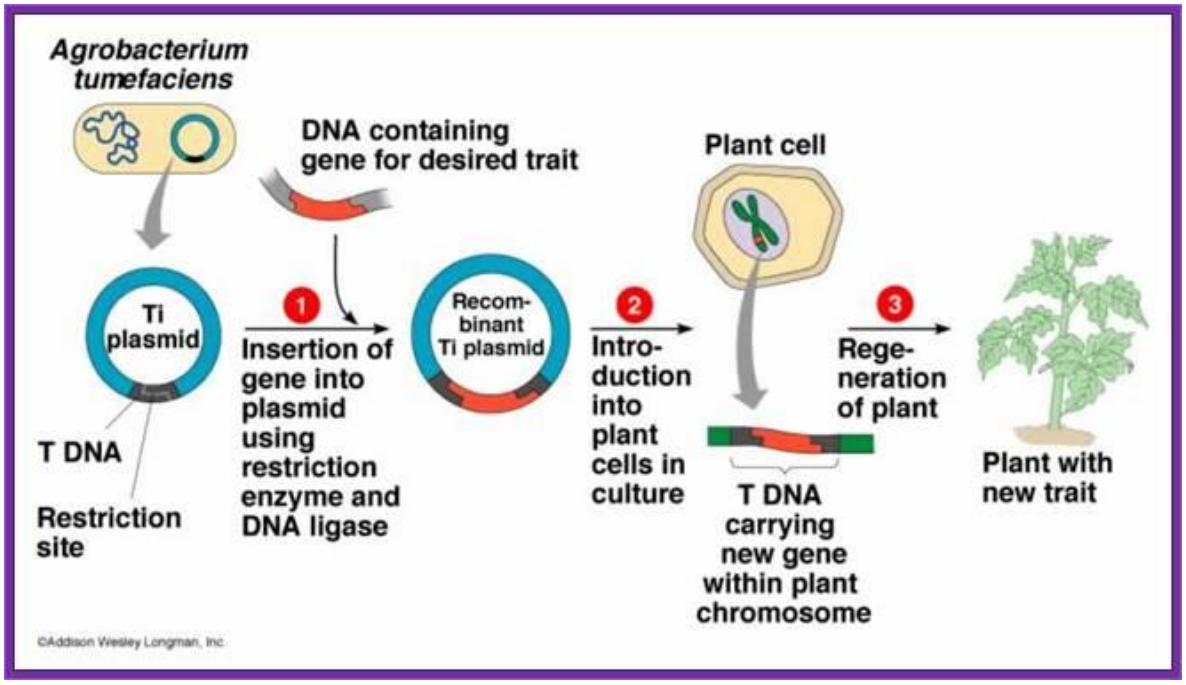
Function of T-DNA genes

Image.ref.https://slideplayer.com/slide/17850277/106/images/6/Table+%3A+Functions+of+various+T DNA+genes.jpg



Ti plasmid

Fig.ref.https://upload.wikimedia.org/wikipedia/commons/thumb/d/d1/Ti_plasmid.svg/1200px-Ti_plasmid.svg.png

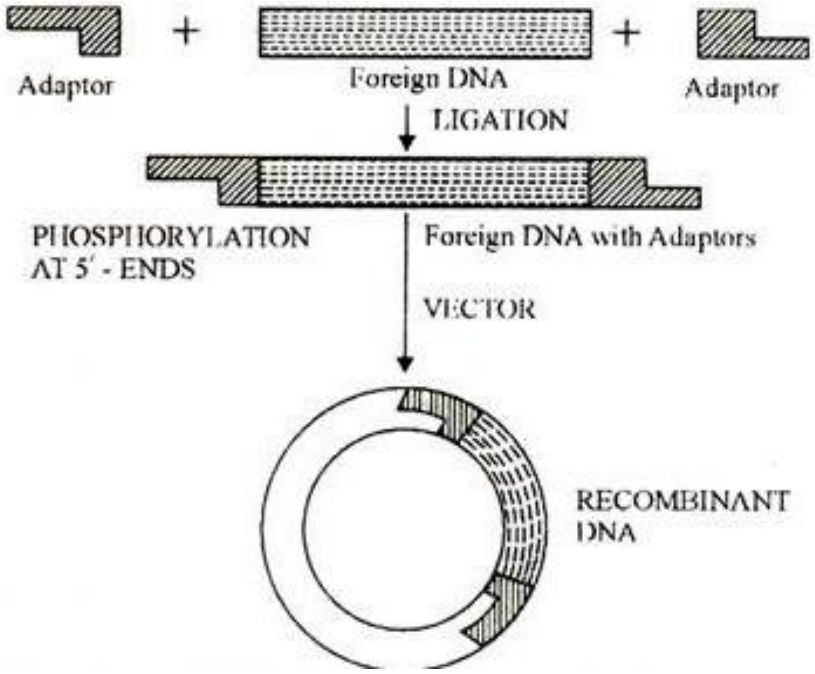


Agrobacterium mediated gene transfer

Image ref. https://mol-biol4masters.masters.grkraj.org/html/Genetic_Engineering4D-Transformation-Plant_Cells_files/image001.jpg

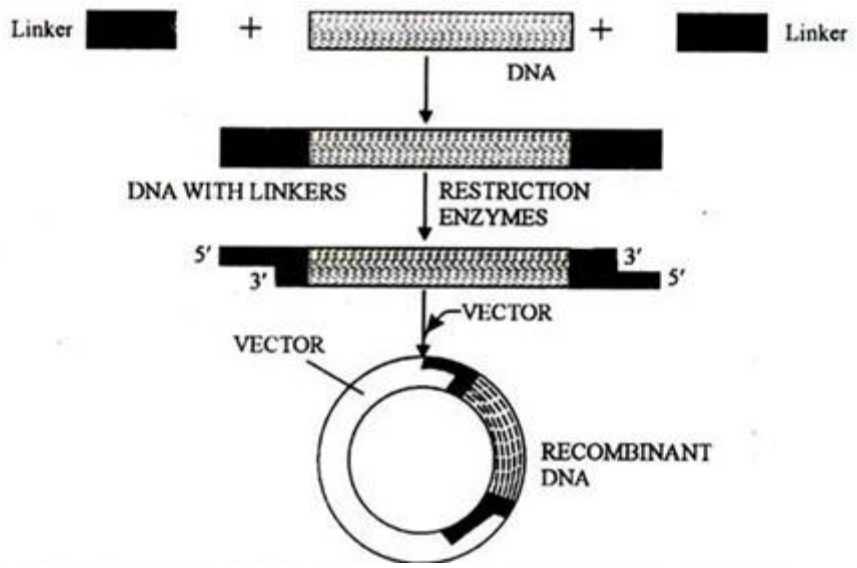
Linkers and Adaptors

Linkers and adaptors are short, chemically synthesized, double stranded DNA sequences sequence helps in the modifications of ends of DNA fragments when treated with restriction enzymes with the help of ligase to join the DNA fragments. Linkers have one or more restriction endonuclease sites can be ligated to the blunt ends of foreign DNA or vector DNA, EcoRI-linker is a common example of frequently used linkers adaptors have one or both sticky ends. The foreign DNA is ligated with adaptor on both ends. This new molecule, so formed, is then phosphorylated at the 5′-terminal. Finally foreign DNA modified with adaptors is integrated into the vector DNA to form the recombinant DNA molecule. Different types of linkers and adaptors are used for different purposes they can create cohesive ends of DNA fragments by treatment with a specific restriction endonuclease. The sticky end of individual adaptor molecule base pair with each other to form dimer so the new DNA molecule still blunt ended this problem is overcome by chemical structure ends of the adaptor molecule.



Function of adaptors in r-DNA technology

Image.ref. https://www.biologydiscussion.com/wp-content/uploads/2016/01/clip_image012-4.jpg



Function of linkers in r-DNA technology

Image.ref. https://www.biologydiscussion.com/wp-content/uploads/2016/01/clip_image010_thumb-5.jpg

Enzymes used in genetic engineering

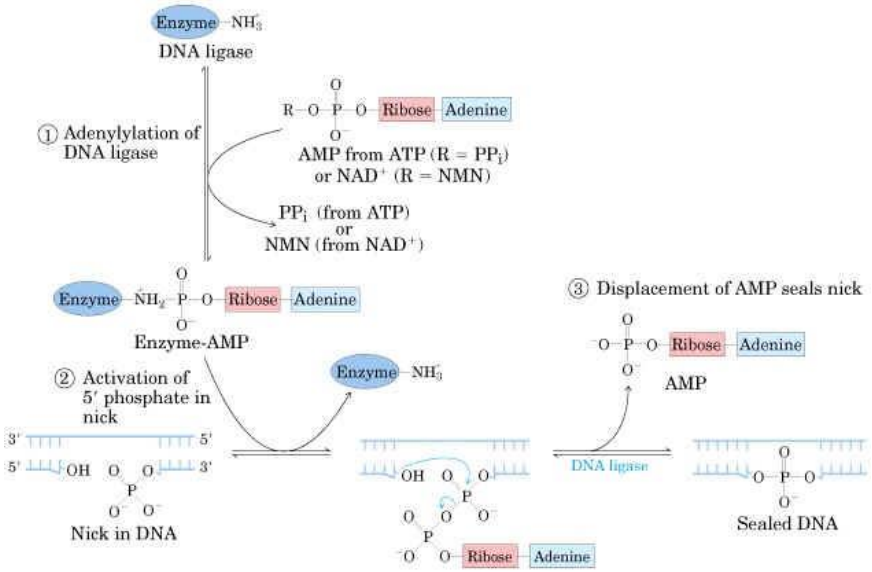
DNA ligase

The DNA ligases are responsible for joining of two DNA segments during replication, repair and recombination. By the formation of alpha phosphodiester bond between free OH group at the 3' end of other DNA strand and phosphate group at 5' end of the other. It is an energy dependent process in bacteria NAD^+ supplies the energy whereas in eukaryotes ATP play the role.

Mechanism of DNA ligase

a) The adenosyl group of the ATP or NAD+ is transferred to the enzyme ligase to form covalent enzyme-AMP complex in which the AMP is linked to the epsilon amino group of lysine residue of the enzyme through posphamide bond.
b) The enzyme then transfers the adenyl group to the 5' phosphoryl terminus of the nick to form a pyrophosphate grouping. In effect, AMP is attached to the 5'-phosphoryl terminus.
c) The final step is a nucleophilic attack of the 3'-OH group on the 5' activated phosphorus atom. A phosphodiester bond is formed and AMP is released. This reaction is driven by the hydrolysis of the pyrophosphate group. Thus two high-

energy phosphate bonds are spent in forming a phosphodiester bridge in the DNA backbone.



Pictorial view mechanism of DNA ligase

Image.ref.https://mol-biol4masters.masters.grkraj.org/html/Prokaryotic_DNA_Replication6-Ligases_files/image005.jpg

Terminal deoxynucleotidyl transferase

Terminal deoxynucleotidyl transferase a template independent DNA polymerase catalyses the addition of nucleotides to the 3' terminus of a DNA molecule. Cobalt is a necessary cofactor, however the enzyme catalyzes reaction upon Mg and Mn administration *in-vitro*. It is used for the formation of homopolymers tailing and cohesive ends. It can also add nucleotides to blunt or recessed 3' ends.

Alkaline phosphatase

Alkaline phosphatase was Used for the removal of single phosphate groups from 5´-ends of linear vectors to prevent re-circularization during cloning or to dephosphorylate DNA. Alkaline phosphatase obtained from various sources, including E-coli and calf intestinal tissue.

T4 polynucleotide kinase

T4 polynucleotide kinase was obtained from E-coli infected with T4, T4 Polynucleotide Kinase catalyses the transfer of the terminal phosphate of ATP to 5′-hydroxyl termini of polynucleotide's such as DNA and RNA and it is reverse mechanism of alkaline phosphatase.

DNA polymerase

DNA polymerase - I (Kornberg enzyme)

Its Molecular weight is 109kd and the concentration is around 400 molecules per cell. When treated with proteases like Trypsin, it gets cleaved into two fragments possessing different function. The large fragment called klenow fragment posses both polymerase and 3'-->5' exonuclease activity. The small fragment possesses only 5'-->3' exonuclease activity.

DNA Polymerase-II

Structure and function are not completely elucidated. It has polymerase activity along with proof reading function, it has 5'-->3' polymerase activity and 3'-->5' exonuclease activity.

DNA Polymerase-III

A primary enzyme involved in DNA replication Holoenzyme of DNA pol-III consists of ten subunits with high polymerization rate about 1000 nucleotide per second. Core enzyme of DNA poly-III contains three subunit alpha, epsilon, theta. Of three subunits alpha subunit posses polymerizing activity and epsilon posses proof reading activity. These are the two function of the enzyme. It has 5'-->3' polymerase activity and 3'-->5' exonuclease activity .

Restriction endonuclease

Restriction enzyme is also called as restriction endonuclease which cuts the DNA at specific sites, protein produced by bacteria while infected with bacteriophage or some viruses. When a phage infects a bacterium, it inserts its DNA into the bacterial cell so that it might be replicated. The restriction enzyme prevents replication of the phage DNA by cutting it into many pieces, thus eliminating infecting organisms DNA they can differentiate their own DNA from foreign DNA by methylating enzyme to methylate its own DNA by activation of two complex systems. Restriction enzymes can be isolated from bacterial cells and used in the laboratory to manipulate fragments of DNA, such as those that are indispensible tools of recombinant DNA technology.

Each restriction enzyme recognizes a short, nucleotide bases called as palindromic sequence, these regions are called recognition sequences, or recognition sites, and are randomly distributed throughout the DNA. There are three types of restriction enzymes are recognized, type I, II and III differs in structure, cleavage site, specificity, and cofactors

Type-I Restriction Endonucleases

These are bifunctional enzymes with both endonucleases and methylases activity with three different subunits, they require Mg^{++} ions, ATP and s-adenosyl methionine for their

functioning. These enzymes have the recognition sequences of about 15 bp length and cleave the DNA about 1000 bp away from the 5′ end of the sequence located within the recognition site.

Type-II Restriction Endonucleases

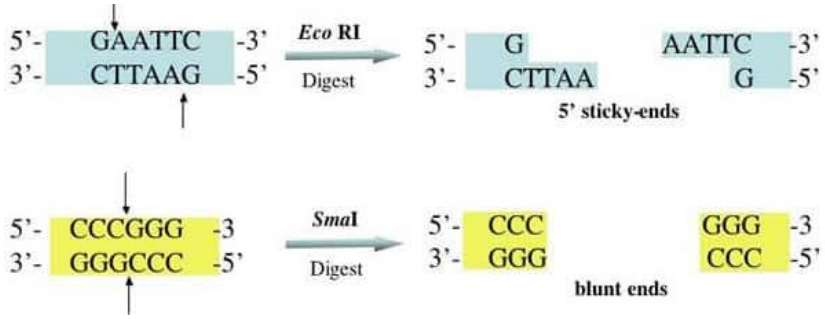
These are unifunctional enzyme with separate endonucleases and methylases activity, with two identical subunits, require Mg^{++} ions for their functioning and more stable . It is the most important endonucleases in r-DNA technology. These enzymes cleavage in both the strands of DNA, at or near the restriction site. Type-II Restriction Endonuclease enzymes are in the form of palindromic sequences the first half of one strand of DNA is the mirror image of the second half of other strand of that DNA double helix, examples of Type-II Restriction endonucleases include, EcoRI, PvuII, AluI, HaeIII etc

Type-III Restriction Endonucleases

These are bifunctional enzymes with both endonucleases and methylases activity with two different subunits, they require Mg^{++} ions, ATP for their functioning. These enzymes have the recognition sequences of about 24-26 bp length and cleave the DNA from the 3′ end of the sequence located within the recognition site. These are not used for gene cloning. They are the intermediate enzymes between Type-I and Type-II restriction endonuclease.

Blunt end and sticky end

Many restriction endonucleases cleaves the strands of DNA to form blunt end and sticky end or cohesive ends. Restriction enzymes such as PvuII, HaeIII, AluI are the examples of restriction endonucleases producing blunt ends. Blunt ends may also be referred to as flush ends breakdown of only phosphodiester bonds at the same sites. In sticky end the two strands of DNA are cut at two different points results into the generation of protruding ends.



Pictorial view of blunt end and sticky end

Image ref. https://lightcat-files.s3.amazonaws.com/problem_images/f6d23daf26d20771-1588696272324.jpg

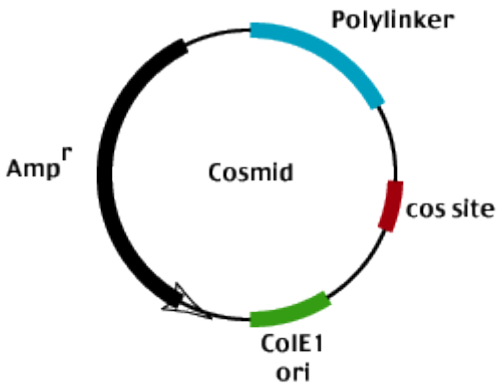
Name of the enzyme	Source	Recognition site and cleavage site	Nature of cut ends
Eco R1	*E. coli* RY13	5'–G|AATTC–3' 3'–CTTAA|G–5'	Sticky
Hind III	*Haemophilus influenzae* Rd	5'–A|AGCTT–3' 3'–TTCGA|A–5'	Stlcky
Bam HI	*Bacillus amyloliquifaciens* H	5'–G|GATCC–3' 3'–CCTAG|G–5'	Sticky
Sal I	*Streptomyces albus* G	5'–G|TCGAC–3' 3'–CAGCT|G–5'	Sticky
Bal I	*Brevibacterium albidum*	5'–TGG|CCA–3' 3'–ACC|GGT–5'	Blunt
Hae III	*Haemophilus aegiptius*	5'–GG|CC–3' 3'–CC|GG–5'	Blunt
Sma I	*Serratia marcescens*	5'–CCC|GGG–3' 3'–GGG|CCC–5'	Blunt

Some examples of restriction enzymes and there recognition sites

Image.ref. https://studiousguy.com/wp-content/uploads/2018/09/Restriction-Enzymes.jpg

Cosmids

Cosmids vectors are hybrid of lambda and plasmids, they can replicate likje plasmid and package like phage, containing one or two cohesive (cos) sites in the same orientation, an origin of replication (ColE1), and a selectable marker (amp^R). Cosmid can carry a DNA inserts about three times as large as those carried by lambda itself. The development of cosmid vector was based on the observation that about 200 bp DNA sequencing in the lambda genome called as cos site is required for lambda DNA packaging into phage particle during lytic infections. Cleavage at cos sites by the lambda terminase protein during phage packaging produces a 12 nucleotides cohesive end and the termini of the linear lambda genome.

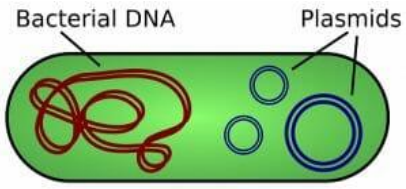


Pictorial view of cosmid

Image.https://faculty.tru.ca/dnelson/courses/biol335/335notes/3recdna/2-vectors/lambda%20vectors/recDNA5c.html

Plasmids

Plasmids are small, circular, self replicative chromosomal DNA found in bacteria, and they can also be found in archaea and multicellular organisms. Which creates beneficial to their host organisms and they have separate genes from their hosts, they are not considered to be independent life



Chromosomal DNA in red and plasmids in blue.

Image.ref. https://upload.wikimedia.org/wikipedia/commons/thumb/c/cf/Plasmid_%28english%29.svg/1200px-Plasmid_%28english%29.svg.png

Functions of Plasmids

Plasmids are very small, self replicative, contain a few genes with a specific function. Multiple plasmids can coexist in the same cell, each with different functions. Plasmids have many different functions. They may contain genes that enhance the survival of an organism, either by killing other organisms or by defending the host cell by producing toxins. Some plasmids facilitate the process of replication in bacteria.

Types of Plasmids

Conjugative and Non-Conjugative

Bacteria reproduce by sexual conjugation, which is the transfer of genetic material from one bacterial cell to another, either through direct contact or a bridge between the two cells. Some plasmids contain genes called transfer genes that facilitate the beginning of conjugation. Non-conjugative plasmids cannot start the conjugation process, and they can only be transferred through sexual conjugation with the help of conjugative plasmids.

Incompatibility

Bacteria with two different plasmid co-occur if they are compatible with each other in other words one plasmid does not affect the expression of other plasmid. An incompatible plasmid will be expelled from the bacterial cell one plasmid affects the other plasmid.

Types of Plasmids

There are five major types of plasmids fertility F-plasmids, resistance plasmids, virulence plasmids, degradative plasmids, and Col plasmids.

Fertility F-plasmids

Fertility plasmids, also known as F-plasmids, contain transfer genes that allow genes to be transferred from one bacteria to another through conjugation. Bacteria that have the F-plasmid are known as F positive (F^+), and bacteria without it are F negative (F^-). When an F^+ bacterium conjugates with an F^- bacterium, two F^+ bacterium result. There can only be one F-plasmid in each bacterium.

Resistance Plasmids

Resistance plasmid or R plasmids contain genes that help a bacterial to create resistance against environmental factors such as poisons or antibiotics. Some resistance plasmids can transfer themselves through conjugation. When this happens, a strain of bacteria can become resistant to antibiotics. The bacteria may even become resistant to these antibiotics within five years. According to NPR, overuse of antibiotics to treat other infections, like urinary tract infections, may lead to the proliferation of drug-resistant strains.

Virulence Plasmids

virulence plasmid turns the bacterium into a pathogen that causes infection to humans, bacteria that cause disease can be easily spread and replicated among affected individuals. Some bacteria has several virulence plasmids. *E. coli* is found naturally in the human gut and in other animals, but certain strains of *E. coli* can cause severe diarrhea and vomiting. *Salmonella enterica* is another bacterium that contains virulence plasmids**.**

Degradative Plasmids

Degradative plasmids a special type of plasmid having genes for special enzymes that break down specific compounds that are not commonly found in nature, such as camphor, xylene, toluene, and salicylic acid. Degradative plasmids are conjugative.

Col Plasmids

Col plasmids have the ability to produce protein colicins that make bacteriocins which kill other bacteria and thus defend the host bacterium Bacteriocins are found in many types of bacteria including *E. coli*, which gets them from the plasmid ColE1.

Reference

1. Gene cloning - an introduction by TA Brown, Chapman and Hall.
2. Life science, fundamental and practice II by Usha meena and Pranav kumar