Genetic Engineering

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Genetic engineering primarily *involves* the *manipulation* of *genetic material (DNA)* to achieve the desired goal in a pre-determined way. Some *other terms* are also in common use to describe genetic engineering.

- *Gene manipulation*
- *Recombinant DNA (rDNA) technology*
- *Gene cloning (molecular cloning)*
- *Genetic modifications*
- *New genetics.*

BRIEF HISTORY OF RECOMBINANT DNA TECHNOLOGY

The present day DNA technology has its roots in the experiments performed by *Boyer and Cohen in* 1973. In their experiments, they successfully recombined two plasmids (pSC 101 and pSC 102) and cloned the new plasmid in *E. coli.* Plasmid pSC 101 possesses a gene resistant to antibiotic tetracycline while plasmid pSC 102 contains a gene resistant to another antibiotic kanamycin. The newly developed recombined plasmid when incorporated into the bacteria exhibited resistance to both the antibiotics-tetracycline and kanamycin.

The second set of experiments of Boyer and Cohen were more organized. A gene encoding a protein (required to form rRNA) was isolated from the cells of African clawed frog *Xenophs laevis,* by use of a restriction endonuclease enzyme *(ECoRI).* The same enzyme was used to cut open plasmid pSC 101 DNA. Frog DNA fragments and plasmid DNA fragments were mixed, and pairing occurred between the complementary base pairs. By the addition of the enzyme DNA ligase, a recombined plasmid DNA was developed. These new plasmids, when introduced into *E. coli,* and grown on a nutrient medium resulted in the production of an extra protein (i.e. the frog protein). Thus, the genes of a frog could be successfully transplanted, and expressed in *E. coli.* This made the real beginning of modern rDNA technology and laid foundations for the present day molecular biotechnology.

Some biotechnologists who admire Boyer - Cohen experiments divide the subject into two chronological categories.

1. BBC-biotechnology **Before Boyer and Cohen.**
2. ABC-biotechnology **After Boyer and Cohen.**

Some recent developments include:

- Creating mouse models of human cancers.
- Creating porcine models to study the progression of heart diseases.
- Knockout mice (mice with a certain gene missing from their genome) to study protein function and its consequences on metabolism and development.
- Anti-sense mRNA technology.
- Inserting foreign genes into crops to increase yield (genetically modified foods).
- Inserting recombinant DNA into farm animals to create pharmaceutically relevant peptide therapeutics (like insulin and growth factor).

MOLECULAR TOOLS OF GENETIC ENGINEERING

An engineer is a person who designs, constructs (e.g. bridges, canals, railways) and manipulates according to a set plan. The term genetic engineer may be appropriate for an individual who is involved in genetic manipulations. The ***genetic engineer's tool kit*** or molecular tools namely the enzymes most commonly used in recombinant DNA experiments are briefly described.

RESTRICTION ENDONUCLEASES

Restriction endonucleases are one of the most important groups of enzymes for the manipulation of DNA. These are the bacterial enzymes that can cut/split DNA (from any source) at specific sites. They were first discovered in *E. coli* restricting the replication of bacteriophages, by cutting the viral DNA (The host *E. coli* DNA is protected from cleavage by addition of methyl groups). Thus, the enzymes that restrict the viral replication are known as ***restriction enzymes*** or restriction endonucleases.

Hundreds of restriction endonucleases have been isolated from bacteria, and some of them are commercially available. The progress and growth of biotechnology is unimaginable without the availability of restriction enzymes.

Nomenclature

Restriction endonucleases are named by a standard procedure, with particular reference to the bacteria from which they are isolated. The first letter (in italics) of the enzymes indicates the genus name, followed by the first two letters (also in italics) of the species, then comes the strain of the organism and finally a Roman numeral indicating the order of discovery. A couple of examples are given below.

EcoRI is from *Escherichia (E) coli (co),* strain Ry13 *(R)* and first endonuclease (I) to be discovered.

Hindlll is from *Haemophilus* (H) influenzae *(in),* strain Rd *(d)* and the third endonucleases *(III)* to be discovered.

Types of endonucleases

At least 4 different types of restriction endonucleases are known-type 1 (e.g *Ecok12),* type II (*e.g. EcoR1),* type III (*e.g. EcoPI)* and type IIs. Their characteristic features are given in *Table 1.* Among these, type II restriction endonucleases are most commonly used in gene cloning.

TABLE 1: Characteristics of different types of restriction endonucleases

Type	***Salient features***
I	A single enzyme with 3 subunits for recognition, cleavage and methylation. It can cleave up to 1000 bp from recognition site.
II	Two different enzymes either to cleave or modify the recognition sequence. Cleavage site is the same or close to recognition site.
III	A single enzyme with 2 subunits for recognition and cleavage. Cleavage site is 24-26 bp from recognition site.
IIs	Two different enzymes, cleavage site is up to 20 bp from recognition site.

Recognition sequences

Recognition sequence is the *site where the DNA is cut* by a restriction endonuclease. 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 *(Table 2).* Such sequences are referred to as palindromes, since they read similar in both directions (forwards and backwards).

Cleavage patterns

Majority of restriction endonucleases (particularly type II) cut DNA at defined sites within recognition sequence. A selected list of enzymes, recognition sequences, and their products formed is given in *Table 2.*

The cut DNA fragments by restriction endonucleases may have mostly *sticky ends* (cohesive ends) or *blunt ends,* as given in Table 2. DNA fragments with sticky ends are particularly *useful*

for recombinant DNA experiments. This is because the single-stranded sticky DNA ends can easily pair with any other DNA fragment having complementary sticky ends.

Table 2: Some restriction enzymes with sources, recognition sequences and the products.

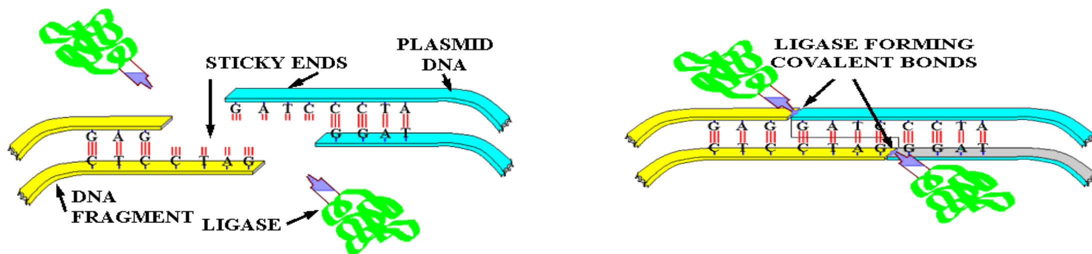
Some restriction enzymes

Enzyme	Source organism	Restriction recognition site in double-stranded DNA	Structure of the cleaved products
(a)			
EcoRI	*Escherichia coli*	5′ —G—A—A—T—T—C— —C—T—T—A—A—G— 5′	—G 5′ A—A—T—T—C— —C—T—T—A—A 5′ G— 5′ overhang
PstI	*Providencia stuartii*	5′ —C—T—G—C—A—G— —G—A—C—G—T—C— 5′	—C—T—G—C—A 3′ G— —G 3′ A—C—G—T—C— 3′ overhang
SmaI	*Serratia marcescens*	5′ —C—C—C—G—G—G— —G—G—G—C—C—C— 5′	—C—C—C G—G—G— —G—G—G C—C—C— Blunt ends
(b)			
HaeIII	*Haemophilus aegyptius*	5′ —G—G—C—C— —C—C—G—G— 5′	—G—G 5′ C—C— —C—C 5′ G—G— Blunt ends
HpaII	*Haemophilus parainfluenzae*	5′ —C—C—G—G— —G—G—C—C— 5′	—C C—G—G— —G—G—C 5′ C— 5′ overhang

DNA LIGASES

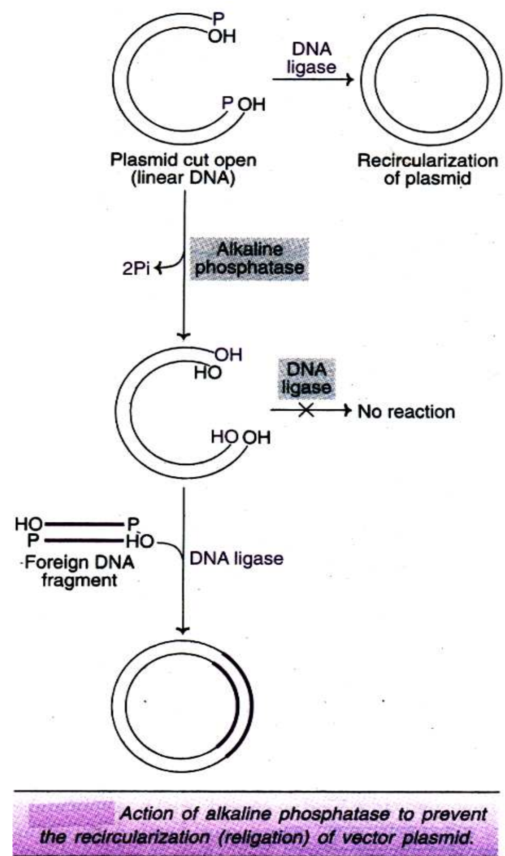
The cut DNA fragments are covalently joined together by DNA ligases. These enzymes were originally isolated from viruses. They also occur in *E. coli* and eukaryotic cells. DNA ligases actively participated in cellular DNA repair process. The action of DNA ligases is absolutely required to permanently hold DNA pieces. This is so since the hydrogen bonds formed between the complementary bases (of DNA strands) are not strong enough to hold the strands together. DNA ligase joins (seals) the DNA fragments by forming a phosphodiester bond between the phosphate group of 5' -carbon of one deoxyribose with the hydroxyl group 3' -carbon of another deoxyribose.

Fig 1: Action of DNA ligase in the formation of phosphodiester bond



ALKALINE PHOSPHATASE

Alkaline phosphatase is an enzyme involved in the removal of phosphate groups. This enzyme is useful to prevent the unwanted ligation of DNA molecules which is a frequent problem encountered in cloning experiments. When the linear vector plasmid DNA is treated with alkaline phosphatase, the 5'-terminal phosphate is removed. This prevents both recircularization and plasmid DNA dimer formation. It is now possible to insert the foreign DNA through the participation of DNA ligase.



Action of alkaline phosphatase to prevent the recircularization (religation) of vector plasmid.

NUCLEASES

Nucleases are the enzymes that break the phosphodiester bonds (that hold nucleotides together) of DNA. *Endonucleases* act on the internal phosphodiester bonds while *exonucleases* degrade DNA from the terminal ends. Restriction endonucleases, described already, are good examples of endonucleases. Some other examples of endo and exonucleases are listed.

Endonucleases

S1 Nuclease

S1 *nuclease is an enzyme that selectively cuts and degrades single-stranded portions of DNA.* It is a glycoprotein consisting of 82% protein and 18% carbohydrate units. The molecular weight of the enzyme is 38,000 daltons. This enzyme breaks the phosphodiester bond between two nucleotides in single stranded portion of DNA and then degrades single stranded extensions. It does not degrade double-stranded portions of DNA and RNAs.

Uses:

1. SI nuclease is used to degrade the hairpin loop formed while making a duplex DNA from complementary DNA strand (cDNA).
2. It is used to remove unwanted tail sequences from DNA fragments to make blunt ends.
3. It is used to remove the extra adenine base from DNAs prepared by polymerase chain reaction.
4. It can also be used to determine the degree of complementary base pairing between DNA strands during hybridization

Exonucleases

- Exonuclease III cuts DNA and generates molecules with protruding 5'-ends.
- Nuclease Bal 31 is a fast acting 3'-exonuclease. Its action is usually coupled with slow acting endonucleases.

Besides the DNA cutting enzymes, there are RNA specific nucleases, which are referred to as *ribonucleases (RNases).*

POLYMERASES

The group of enzymes that catalyze the synthesis of nucleic acid molecules are collectively referred to as polymerases. It is customary to use the name of the nucleic acid template on which the polymerase acts *(Fig.* 6.68). The three important polymerases are given below.

- *DNA-dependent DNA polymerase* that copies DNA from DNA.
- *RNA-dependent DNA polymerase* (reverse transcriptase) that synthesizes DNA from RNA.
- *DNA-dependent RNA polymerase* that produces RNA from DNA.

Polynucleotide Kinase

Polynucleotide kinase transfers a phosphate from ATP to *5'OH* group of dephosphorylated DNA or RNA. It consists of four identical sub-units and its molecular weight is 34,000 daltons.

Uses:

1. Polynucleotide kinase is used to rephosphorylate the 5' end of dephosphorylated vector DNA in rDNA. Then only DNA ligase can seal the nick between the vector DNA and target DNA (desired DNA).

2. It is used to transfer radioactive P32 from ATP to dephosphorylated *5'* end of DNA or RNA for labelling. The labelling technique is used.

i) To make hybridization probes.

ii) To make diagnostic kits.

iii) To analyse the base sequence of DNA.

iv) To construct restriction maps.

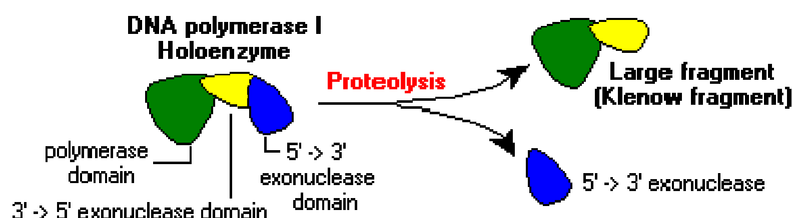
Terminal nucleotidyl transferase: *Terminal nucleotidyl transferase* is an enzyme that adds mononucleotide triphosphates to 3' -OH group of DNA fragment. While adding the nucleotide, a pyrophosphate is released free. This enzyme adds nucleotides without the aid of a template strand. It is made up of two non-identical sub-units and the molecular weight is 32,000 daltons.

Uses:

1. Terminal transferase is used to make homopolymer cohesive tails at 3' end of ON A fragments. Thus it is of much use injoining blunt ended DNA fragments while constructing DNA.

2. It is used to make radioactive DNA probes.

Klenow fragment: The 5' -> 3' exonuclease activity of *E. coli's DNA Polymerase* makes it unsuitable for many applications. However, this pesky enzymatic activity can readily be removed from the holoenzyme. Exposure of DNA polymerase I to the protease subtilisin cleaves the molecule into a small fragment, which retains the 5' > 3' exonuclease activity, and a large piece called Klenow fragment. The large or Klenow fragment of DNA polymerase I has DNA polymerase and 3' -> 5' exonuclease activities, and is widely used in molecular biology.



***Taq* DNA polymerase:** *Taq* polymerase is a heat stable enzyme used in the polymerase chain reaction (PCR) to amplify segments of DNA in the lab. It was discovered in the heat-loving bacterium *Thermus aquaticus*, and without it, we couldn't amplify DNA.

Ribonuclease: Enzymes that break down RNA.

RNase H activity: RNase H is a ribonuclease that degrades the RNA from RNA-DNA hybrids, such as are formed during reverse transcription of an RNA template. This enzyme functions as both an endonuclease and exonuclease in hydrolyzing its target.

Reverse transcriptase: Reverse transcriptase is a common name for an enzyme that functions as a RNA-dependent DNA polymerase.

DNA polymerase activity: In the retroviral life cycle, reverse transcriptase copies only RNA, but, as used in the laboratory, it will transcribe both single-stranded RNA and single-stranded DNA templates with essentially equivalent efficiency. In both cases, an RNA or DNA primer is required to initiate synthesis.

HOST CELLS - THE FACTORIES OF CLONING

The hosts are the *living systems or cells* in which the carrier of recombinant DNA molecule or *vector can be propagated.* There are different types of host cells-prokaryotic (bacteria) and eukaryotic (fungi, animals and plants). Some examples of host cells used in genetic engineering are given in following *Table.*

Group	Examples
Prokaryotic	
Bacteria	*Escherichia coli*
	Bacillus subtilis
	Streptomyces sp
Eurkaryotic	
Fungi	*Saccharomyces cerevisiae*
	Aspergillus nidulans
Animals	Insect cells
	Oocytes
	Mammalian cells
	Whole organisms
Plants	Protoplasts
	Intact cells
	Whole plants

Host cells, besides effectively incorporating the vector's genetic material, must be conveniently cultivated in the laboratory to collect the products. In general, *'microorganisms* are *preferred* as host cells, since they *multiply faster* compared to cells of higher organism (plants or animals).

Prokaryotic Hosts

Escherichia coli

The bacterium, *Escherichia coli* was the first organism used in the DNA technology experiments and continues to be *the host* of *choice* by many workers. Undoubtedly, *E. coli,* the simplest Gram negative bacterium (a common bacterium of human and animal intestine), has played a key role in the development of present day biotechnology. Under suitable environment, *E. coli* can double in number every 20 minutes. Thus, as the bacteria multiply, their plasmids (along with foreign DNA) also multiply to produce millions of copies, referred to as colony or in short *clone.* The term clone is broadly used to a mass of cells, organisms or genes that are produced by multiplication of a single cell, organism or gene.

Limitations of *E. coli*: There are certain limitations in using *E. coli* as a host. These include- causation of diarrhea by some strains, formation of endotoxins that are toxic, and a low export ability of proteins from the cell. Another *major drawback* is that *E. coli* (or even other prokaryotic organisms) *cannot perform post-translational modifications.*

Bacillus subtilis

Bacillus subtilis is a rod shaped non-pathogenic bacterium. It has been used as a host in industry for the production of enzymes, antibiotics, insecticides etc. Some workers consider *B .subtilis* as an *alternative to E. coli.*

EUKARYOTIC HOSTS

Eukaryotic organisms are preferred to produce human proteins since these hosts with complex structure (with distinct organelles) are more suitable to synthesize complex proteins. The *most commonly used* eukaryotic organism is the *yeast, Saccharomyces cerevisiae.* It is a non-pathogenic organism routinely used in brewing and baking industry. Certain fungi have also been used in gene cloning experiments.

Mammalian cells

Despite the practical difficulties to work with and high cost factor, mammalian cells (such as mouse cells) are also employed as hosts. The advantage is that certain complex proteins which

cannot be synthesized by bacteria can be produced by mammalian cells e.g. tissue plasminogen activator. This is mainly because the mammalian cells *possess the machinery to modify the protein to its final form* (post-translational modifications).

It may be noted here that the gene manipulation experiments in higher animals and plants are usually carried out to alter the genetic makeup of the organism to create transgenic animals and transgenic plants, rather than to isolate genes for producing specific proteins.

VECTORS - THE CLONING VEHICLES

Vectors are the DNA molecules, which can *carry* a *foreign DNA fragment to be cloned.* They are self- replicating in an appropriate host cell. The most important vectors are plasmids, bacteriophages, cosmids and phasmids.

Characteristics of an ideal vector

An ideal vector should be small in size, with a single restriction endonuclease site, an origin of replication and 1-2 genetic markers (to identify recipient cells carrying vectors). Naturally occurring plasmids rarely possess all these characteristics.

PLASMIDS

Plasmids are extra chromosomal, double- stranded, circular, self-replicating DNA molecules. Almost all the bacteria *have* plasmids containing a low copy number (1-4 per cell) or a high copy number (10-100 per cell). The size of the plasmids varies from 1 to 500 kb. Usually, plasmids contribute to about 0.5 to 5.0% of the total DNA of bacteria **(Note:** A few bacteria contain linear plasmids e.g. *Streptomyces* sp, *Borella burgdorferi).*

Plasmids as Cloning Vectors

A. Chang and *N. Cohen* (1973) first proved the use of plasmids as gene cloning vectors. They isolated plasmids from two different strains of bacteria and fused them using restriction enzyme and DNA ligase. The fused DNA (chimeric plasmid) was then introduced into *E.coli* cells and its expression was studied. They found out that genes of both the plasmids express their traits in the *E.coli.*

Biotechnologists have adopted the following method for gene cloning:

1. A DNA segment is isolated from the genomic DNA using a restriction enzyme.
2. A suitable plasmid vector is isolated from a strain of bacterium.
3. The plasmid is cut with the same restriction enzyme used to isolate the desired DNA.

4. The source DNA is inserted into the cleaved plasmid DNA using DNA ligase. As a result *chimeric DNA (rDNA)* is formed.
5. The rDNA is then introduced into a host bacterium that has to be manipulated. Here plasmid carries the inserted desired DNA to bacterium and being safe in it. So plasmid is known as a *gene cloning vector* or *cloning vehicle.*

Characteristics of Ideal Plasmid Vectors

The ideal cloning vectors must have the following characteristic features-

1.Size: The plasmid must be small in size. The small size is helpful for easy uptake of chimeric DNA by host cells and for the isolation of plasmid without damage.

2.Copy number: The plasmid must be present in multiple copies.

3.Genetic markers: The plasmid must have one or a few genetic markers. These markers help us for the selection of organism that has recombinant DNA.

4.Origin of Replication: The plasmid must have its own origin of replication <nd regulatory genes for self-replication.

5.Unique Restriction Sites: The plasmid must have unique restriction sites for common restriction enzymes in use.

6.Insertional Inactivation: The plasmid must have unique sites for restriction enzymes in marker genes. This will help us for the selection of recombinants by insertional inactivation method.

7.Pathogenicity: The plasmid should not have any pathogenic property.

Types of plasmids

There are many ways of grouping plasmids. They are categorized as ***conjugative*** if they carry a set of transfer genes *(tra* genes) that facilitates bacterial conjugation, and ***non-conjugative,*** if they do not possess such genes.

Another classification is based on the copy number. ***Stringent plasmids*** are present in a limited number (1-2 per cell) while ***relaxed*** *plasmids* occur in large number in each cell.

F-plasmids possess genes for their own transfer from one cell to another, while ***R-plasmids*** carry genes resistance to antibiotics.

In general, the conjugative plasmids are large, show stringent control of DNA replication, and are present in low numbers. On the other hand, non- conjugative plasmids are small, show relaxed control of DNA replication, and are present in high numbers.

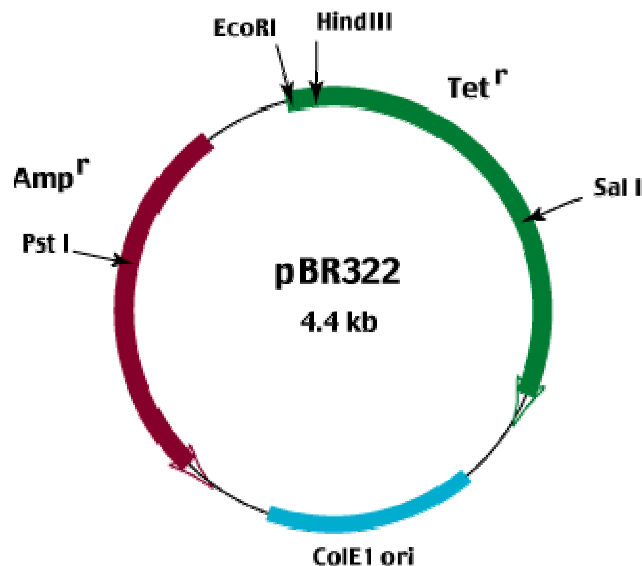
Nomenclature of plasmids

It is a common practice to designate plasmid by a lower case *p,* followed by the first letter(s) of researcher(s) names and the numerical number given by the workers. Thus, ***pBR322*** is a plasmid discovered by *Bolivar* and Rodriguez who designated it as **322.** Some plasmids are given names of the places where they are discovered e.g. ***pUC*** is plasmid from University of California.

pBR322 - the most common plasmid vector

pBR322 of *E. coli* is the most popular and widely used plasmid *vector,* and is appropriately regarded as the parent or grandparent of several other *vectors.*

pBR322 has a DNA sequence of 4,361 bp. It carries genes resistance for ampicillin (Amp') and tetracycline (Tel^{r}) that *serve* as markers for the identification of clones carrying plasmids. The plasmid has unique recognition sites for the action of restriction endonucleases such as *EcoRI, HindI/I, BamHI, Sail* and *Pstll* (***Fig***).



Other plasmid cloning vectors

The other plasmids employed as cloning *vectors* include pUC19 (2,686 bp, with ampicillin resistance gene), and derivatives of pBR322- pBR325, pBR328 and pBR329.

BACTERIOPHAGES

Bacteriophages or simply *phages* are the *viruses* that *replicate within the bacteria.* In case of certain phages, their DNA gets incorporated into the bacterial chromosome and remains - there permanently. Phage *vectors* can accept short fragments of foreign DNA into their genomes. The

advantage with phages is that they *can take up larger DNA segments than plasmids.* Hence phage *vectors* are preferred for working with genomes of human cells.

Bacteriophage λ

Bacteriophage lambda (or simply ***phage λ),*** a virus of *E. coli,* has been most thoroughly studied and developed as a *vector.* In order to understand how bacteriophage functions as a *vector,* it is desirable to know its structure and life cycle. Phage A consists of a head and a tail (both being *proteins)* and its *shape* is *comparable* to a miniature hypodermic syringe. The DNA, located in the head, is a linear molecule of about 50 kb. At each end of the DNA, there are single-stranded extensions of 12 base length each, which have cohesive (cos) ends. On attachment with tail to *E. coli,* phage A injects its DNA into the cell. Inside *E. coli,* the phage linear DNA cyclizes and gets ligated through cos ends to form a circular DNA. The phage DNA has two fates-lytic cycle and lysogenic cycle.

Lytic cycle: The circular DNA replicates and it also directs the synthesis of many proteins necessary for the head, tail etc. of the phage. The circular DNA is then cleaved (to form cos ends) and packed into the head of the phage. About 100 phage particles are produced within 20 minutes after the entry of phage into *E. coli.* The host cell is then subjected to lysis and the phages are released. Each progeny phage particle can infect a bacterial cell, and produce several hundreds of phages. It is estimated that by repeating the lytic cycle four times, a single phage can cause the death of more than one billion bacterial cells. *If* a *foreign DNA is spliced into phage DNA, without causing harm to phage genes, the phage will reproduce (replicate the foreign DNA) when it infects bacterial cell.* This has been exploited in phage vector employed cloning techniques.

Lysogenic cycle: In this case, the phage DNA (instead of independently replicating) becomes integrated into the *E. coli* chromosome and replicates along with the host genome. No phage particles are synthesized in this pathway.

Use of phage λ as a vector

Only about 50% of phage A DNA is necessary for its multiplication and other functions. Thus, as much as 50% (*i.e.* up to 25kb) of the phage DNA can be replaced by a donor DNA for use in cloning experiments. However, several restriction sites are present on phage A which is not by itself a suitable vector. The A-based phage vectors are modifications of the natural phage with much reduced number of restriction sites. Some of them are discussed hereunder.

Insertion vectors: They have just one unique cleavage site, which can be cleaved, and a foreign DNA ligated in. It is essential that sufficient DNA (about 25%) has to be deleted from the vector to make space for the foreign DNA (about 18kb).

Replacement vectors: These vectors have a pair of restriction sites to remove the non-essential DNA *(stuffer DNA)* that will be replaced by a foreign DNA. Replacement vectors can accommodate up to 24kb, and propagate them.

Many phage vector derivations (insertion/replacement) have been produced by researchers for use in recombinant DNA technology.

The main advantage of using phage vectors is that the foreign DNA can be packed into the phage *(in vitro* packaging), the latter in turn can be injected into the host cell very effectively (Note No transformation is required).

Phage M_{13} vectors

Phage M_{13} (bacteriophage M_{13}) is a single-stranded DNA phage of *E. coli.* Inside the host cell, M_{13} synthesizes the complementary strand to form a double-stranded DNA (replicative form DNA; *RF DNA).* For use as a vector, RF DNA is isolated and a foreign DNA can be inserted on it. This is then returned to the host cell as a plasmid. Single- stranded DNAs are recovered from the phage particles. Phage M13 is useful for sequencing DNA through Sanger's method.

COSMIDS

Cosmids are the vectors possessing the characteristics of both plasmid and bacteriophage λ. *Cosmids* can be constructed by adding a fragment of phage A DNA including *CDS* site, to *plasmids.* A foreign DNA (about 40 kb) can be inserted into cosmid DNA. The recombinant DNA so formed can be packed as phages and injected into *E. coli (Fig. 10).* Once inside the host cell, cosmids behave just like plasmids and replicate. The advantage with cosmids is that they can carry larger fragments of foreign DNA compared to plasmids.

Phasid vectors

Phasids are the combination of plasmid and phage and can function as either one (*i.e.* as plasmid or phage). Phasids possess functional origins of replication of both plasmid and phage λ, and therefore can be propagated (as plasmid or phage) in appropriate *E .coli.* The vectors phasids may be used in many ways in cloning experiments.

ARTIFICIAL CHROMOSOME VECTORS

Human artificial chromosome (HAC)

Developed in 1997 (by H. Willard), human artificial chromosome is a ***synthetically produced vector DNA, possessing the characteristics of human chromosome.*** HAC may be considered as a self-replicating micro chromosome with a size ranging from 1/10th to 1/5th of a human chromosome. The advantage with HAC is that it can carry human genes that are too long. Further, HAC can carry genes to be introduced into the cells in gene therapy.

Yeast artificial chromosomes (YACs) Introduced in 1987 (by M. Olson), yeast artificial chromosome (YAC) is a synthetic DNA that can accept large fragments of foreign DNA (particularly human D A). It is thus possible to clone large D A pieces by using YAC. YACs are the most sophisticated yeast vectors, and represent the ***largest capacity vectors*** available. They possess centromeric and telomeric regions, and therefore the recombinant DNA can be maintained like a yeast chromosome.

Bacterial artificial chromosomes (BACs)

The construction of BACs is based on one F-plasmid which is larger than the other plasmids used as cloning vectors. BACs can accept DNA inserts of around 300 kb. The advantage with bacterial artificial chromosome is that the instability problems of YACs can be avoided. In fact, a major part of the *sequencing* ***of human genome*** has been accomplished by using a library of BAC recombinant.

SHUTTLE VECTORS

The plasmid vectors that are specifically designed to ***replicate in two different hosts*** (say in *E. coli* and *Streptomyces* sp.) are referred to as shuttle vectors. The origins of replication for two hosts are combined in one plasmid. Therefore, any foreign D A fragment introduced into the vector can be expressed in either host. Further, shuttle vectors can be grown in one host and then shifted to another host (hence the name shuttle). A good number of eukaryotic vectors are shuttle vectors.

CHOICE OF A VECTOR

Among the several factors, the size of the foreign DNA is very important in the choice of vectors. The efficiency of this process in often crucial for determining the success of cloning.

METHODS OF GENE TRANSFER

Introducing a foreign DNA (i.e. the gene) into the cells is an important task in biotechnology. The efficiency of this process is often crucial for determining the success of cloning. The most

commonly employed gene transfer methods, namely transformation, conjugation, electroporation and lipofection, etc., are generally used. However, partical bombardment and electroporation techniques are described here.

PARTICLE BOMBARDMENT (BIOLISTICS)

Particle (or ***microprojectile) bombardment*** is the most effective method for gene transfer, and creation of transgenic plants. This method is versatile due to the fact that it can be successfully used for the DNA transfer in mammalian cells and microorganisms.

The microprojectile bombardment method was initially named as biolistics by its inventor Sanford *(1988).* ***Biolistics*** is a combination of *biological* and ***ballistics.*** There are other names for this technique ***particle gun, gene gun, bioblaster.***

A diagrammatic representation of micro- projectile bombardment system for the transfer of genes in plants is depicted in ***Fig.***

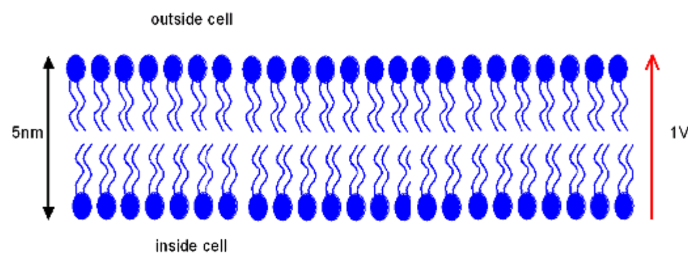
Microcarriers (microprojectiles), the tungsten or gold particles coated with DNA, are carried by macrocarriers (rnacroprojectiles). These macrocarriers are inserted into the apparatus and pushed downward by rupturing the disc. The stopping plate does not permit the movement of macrocarrier while the microcarriers (with DNA) are propelled at a high speed into the plant material. Here the DNA segments are released which enter the plant cells and integrate with the genome.

Electroporation: Electroporation is based on the principle that high *voltage* electric pulses can induce cell plasma membranes to fuse. Thus, electroporation is a technique involving *electric field-mediated membrane permeabilization.* Electric shocks can also induce cellular uptake of exogenous DNA (believed to be *via* the pores formed by electric pulses) from the suspending solution. Electroporation is a simple and rapid technique for introducing genes into the cells from various organisms (microorganisms, plants and animals).

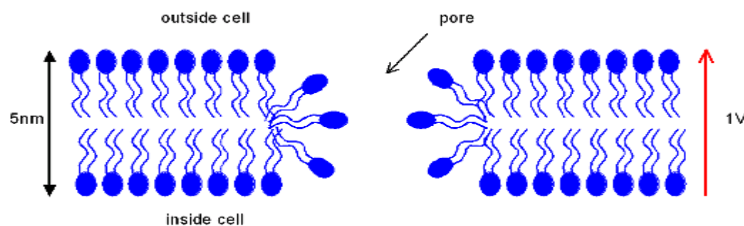
The basic technique of electroporation for transferring genes into mammalian cells is depicted in *Fig.* 6.11. The cells are placed in a solution containing DNA and subjected to electrical shocks to cause holes in the membranes. The foreign DNA fragments enter through the holes into the cytoplasm and then to nucleus.

Electroporation is an *effective* way to transform *E. coli* cells containing plasmids with insert DNAs longer than 100 kb. The transformation efficiency is around 10^9 transformants per microgram of DNA for small plasmids (about 3kb) and about 10^6 for large plasmids (about 130 kb).

Normal Cell Membrane

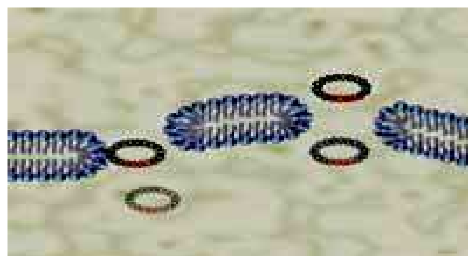


Disrupted Cell Membrane



Normal Cell Membrane

Through the pores the plasmids enters in to the cell



GENE CLONING STRATEGIES

A clone refers to **a *group* of** organisms, cells, ***molecules*** or other objects, ***arising from* a *single individual.*** Clone and colony are almost synonymous.

Recombinant DNA technology: concept and techniques.

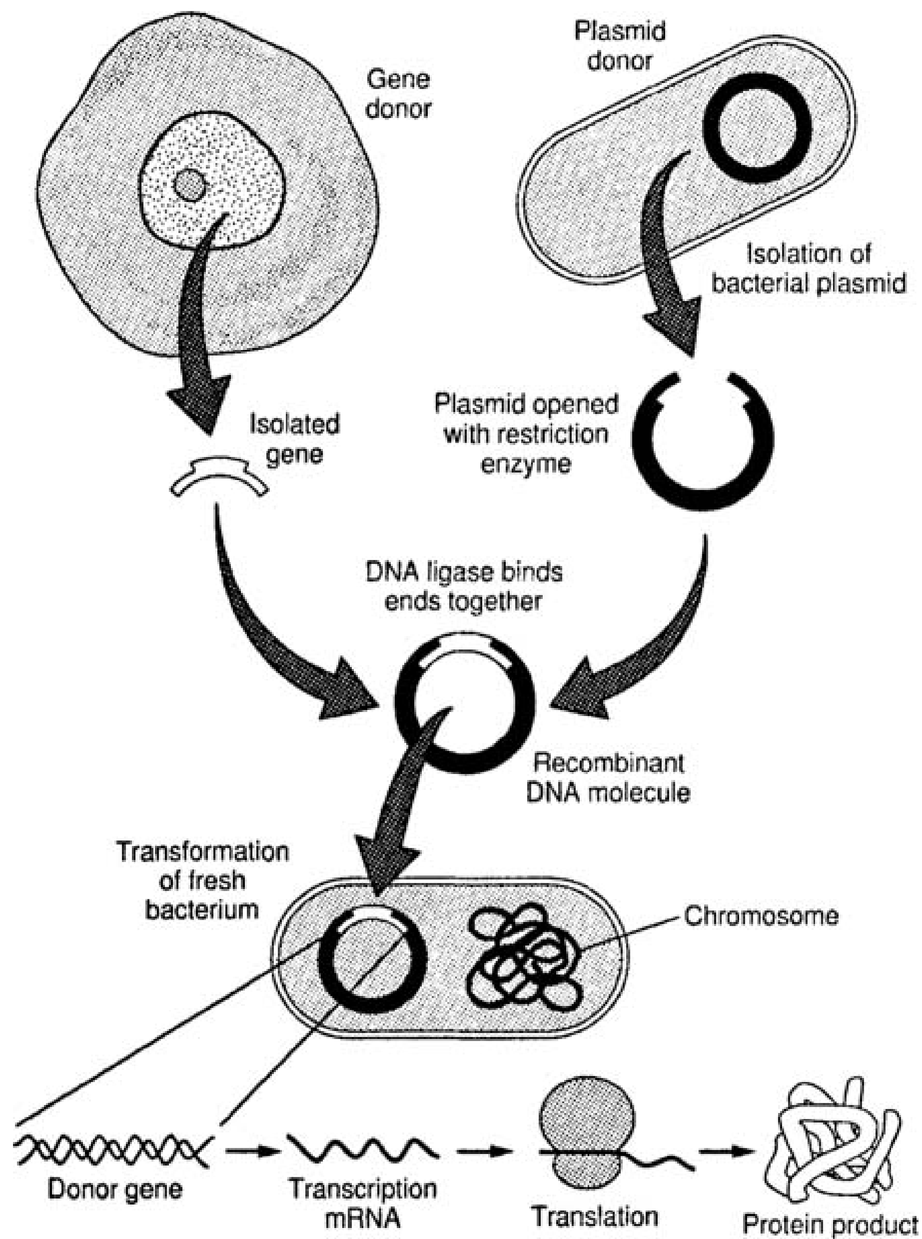
Gene cloning strategies in relation to recombinant DNA technology broadly involve the following aspects

Methodology/steps in Genetic Engineering

1. Preparation/Isolation of desired genes.
    a. Restriction digestion of genomic DNA & Separated by electrophoresis.
    b. Reverse Transcription.
    c. DNA synthesiser / gene machine.
2. Isolation of DNA vector.
3. Construction of recombinant DNA.
4. Introduction of recombinant DNA into the host cell.
5. Screening & Selection of recombinants.
6. Expression of Cloned genes.

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.

1. Generation of DNA fragments and selection of the desired piece of DNA (e.g. a human gene).
2. Insertion of the selected DNA into a cloning vector (e.g. a plasmid) to create a ***recombinant DNA* or *chimeric DNA*** (Chimera is a monster in Greek mythology that has a I ion's head, a goat's body and a serpent's tail. This may be comparable to Narasimha in Indian mythology).
3. Introduction of the recombinant vectors into host cells (e.g. bacteria).
4. Multiplication and selection of clones containing the recombinant molecules.
5. Expression of the gene to produce the desired product.



Applications in sericulture

- Pest Resistance Mulberry varieties (other than insect pests; as silkworm is also an insect).
- Disease Resistance Mulberry Varieties.
- Herbicide Resistance Mulberry Varieties.
- Nitrogen Fixing Mulberry varieties.
- High Leaf Yielding Mulberry Varieties.
- Improved Quality of Mulberry Leaf.

- New strains of silkworm with improved yield and quality.
- New strains of silkworm with more resistance/tolerance against disease causing pathogens *i.e.*, Protozoan, Viral, Fungal & Bacterial. As well as Pests *i.e.*, Uzi fly.
- Production of beneficial proteins for human benefits *eg*. Insulin, thrombolytic enzymes, growth hormone, *etc*., instead of silk.

Acknowledgements

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