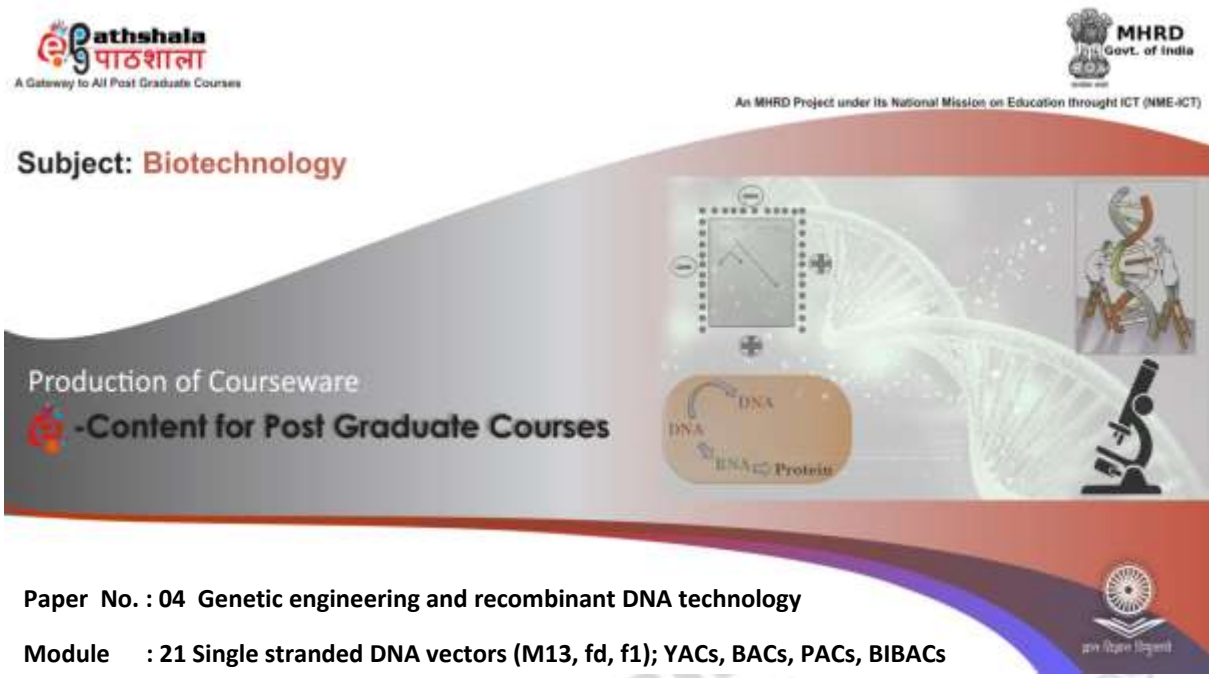

Paper No. : 04 Genetic engineering and recombinant DNA technology

Module : 21 Single stranded DNA vectors (M13, fd, f1); YACs, BACs, PACs, BIBACs

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Description of Module	
Subject Name	Biotechnology
Paper Name	Genetic Engineering and Recombinant DNA Technology
Module Name/Title	Single stranded DNA vectors (M13, fd, f1); YACs, BACs, PACs, BIBACs
Module Id	21
Pre-requisites	
Objectives	Single stranded DNA vectors (M13, f1, fd), Yeast Artificial Chromosomes (YACs), Bacterial Artificial Chromosomes (BACs), P1 derived Artificial Chromosomes (PACs), Binary Bacterial Artificial Chromosomes (BIBACs), Summary
Keywords	Bacteriophage M13, Bacteriophage f1, Bacteriophage fd, YACs, BACs, PACs, BIBACs

A. Single stranded DNA vectors (M13, fd, f1); YACs, BACs, PACs, BIBACs

Cloning vectors have been utilized in recombinant DNA technology not only for replication fucntions, but now a days these are a wonderful tool for various kinds of expression studies, sequencing and mutagenesis related applications. The basic requirements like origin of replication, a partition function, suitable selectable markers for easy and fast identification of clones without any necessity of expression. Besides the ease in transformations, the best advantage of cloning in to a phage vectors like, M13 is that the DNA ones packaged in to phage particles, remain unaffected by any environmental influence. Phage DNA are also modified to have a fragment responsible for expression of a selectable marker for quick detection of lysogens. But all these phage based cloning vectors have their limitation to have a particular size of DNA fragments. While the development of artificial chromosomes opened the door for cloning of large DNA fragment without any hurdle. The advent of various artificial chromosomes helped a lot in whole genome sequencing projects and cloning of long genes having large size intronic regions. The following table represents idea about various cloning vectors having origin of replications, size of DNA can be accommodated and their mode of delivery in to the host cells with their conformational structures.

Vector	Host	Insert Range (kb)	Introduction on to host cell	Origin of Replication	Vector structure
M13	E. coli	1-4	Transduction	f1	Circular virus
λ- Phage	E. coli	20-30	Transduction	f1	Linear virus
PACs	E. coli	100-300	Electroporation	P1	Linear dsDNA
BACs	E. coli	300-350	Electroporation	OriS, repE	Circular plasmid
YACs	Yeast	200-2000	Transformation	ARS	Linear chromosome

Ff class Bacteriophages

The Ff class bacteriophages like M13, fd, and f1 have been widely used in molecular cloning and various derived vectors are extensively used for recombinant DNA technology. These filamentous, F-specific coli phages have important characters that enable them to be used a cloning vectors. The most important feature is that they donot kill their hosts and second is that they allow the packaging themselves into phage particles although they are having

greater size than phage unint length. Another important feature is that they replicate as double stranded, super coiled DNA but are packaged in to viron as single stranded circular molecule. Consequently it allows to obtain sigle stranded circular DNA in pure form for futher processing. This makes these vectors conveniently utilized in sequencing purposes and in oligo nucleotide- directed mutagenesis experiments.

In simple words it can be summarized as-

- Infect only negative bacteria bearing the F episome
- They don't kill their host.
- They allow the packaging themselves into phage particles although they are having greater size than phage unint length.
- Replicate as such in double-stranded condition where as packaged in to viron in circular, single-stranded molecule.
- Target and infect only those bacteria which have F-episome.

Bacteriophage fd

- Bacteriophage fd, a filamentous bacteriophage infects specifically *Escherichia coli* strains.
- Bacteriophage fd have various structural as well as genetic resemblance with Enterobacteria phage M13.
- Bacteriophage fd modified to display immunogenic peptides are widely utilized in case of vaccines related studies.

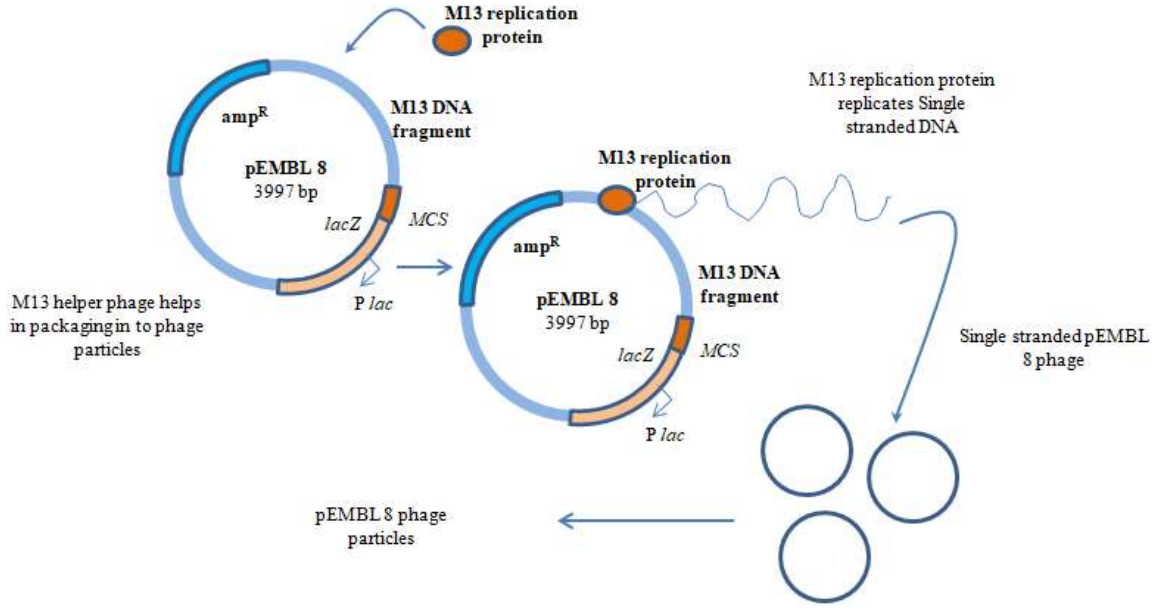
Bacteriophage f1

Bacteriophage f1, filamentous bacteriophage, structurally categorized into class I phages having great similarities with Ff class phages like M13 and fd. Bacteriophage F1 is a rod shaped single stranded DNA phage having a molecular mass of 1.6 x 107 Dalton and 11.3% of total weight of viron. The size of viron range between 850 nm long and 4.3 to 6.3 nm wide. Thousands of major coat protein molecules are arranged into fishcale like alpha helix making a sheath like arrangement. At one end of the filament major coat proteins make a sheath cap and at other end which is blunt due to presence of 3 to 5 copies of each VII and IX proteins. The extended bead like structure is formred by 5 copies of each III and VI proteins.

Approximate size of f1 phage genome is 6407 bases. The genome comprise of total 11 genes, while two genes are overlapping. Five genes encode viron related proteins while three are responsible for synthesis and rest genes are used for assembly. For the nomenclature of genes, these are written in Roman numerals from I to XI, and the sequence of these genes comes as II(X), V, VII, IX, VIII, III, VI, I(XI), IV, intergenic region (IG or IGR) having some intergenic regions (IG or IGR) Also. The intergenic regien sequences are responsible for termination, cleavage of replication and the binding of gene II and IHF.

Bacteriophage M13 and its importance in molecular cloning

M13 was first isolated from wastewater from the city Munich by Hofschneider in 1963. M13 is a unique bacteriophage having a single stranded DNA packed in a two layer filamentous capsid, madeup of only 3 proteins. M13 have a smaller single stranded circular genome of 6407 nucleotides sequenced by Sanger in 1982. Very few number of genes which are necessary for integration and initiation of replications are present in it genome. M13 bacteriophage infects only F type of bacterial host cells, and inters in to bacterial cytoplasm through F-pilli. M13 do not lyse the host cells, it just slowed the bacterial machinery by 1/3 for synthesis and packing of its genome. The single stranded DNA helps in sequencing and site directed mutagenasis.



Bacteriophage M13 phage is filamentous phage that infects E. coli via F-pilus. The genome is a single stranded circular DNA of size ~6.4kb surrounded by a proteinaceous coat. The DNA strand present in phage is called plus (+) strand. After entering to E. coli host, it converts into double stranded DNA molecule called replicative form (RF) by utilizing bacterial machinery. M13 phage as cloning vector can be obtained in both single stranded as well as double stranded form. Replicative form double stranded vector are modified and replicated inside E. coli host similar to a plasmid vector. Single stranded vectors can be isolated by collecting M13 phage.

Phagemid vectors

Phagemid vectors are plasmids having a small segment of a filamentous phage M-13, fd, or F1 phage capable to carry up to 10 kb passenger DNA. Examples: pEMBL series of plasmids

pBluescript family plasmids

The M13 origin of replication enables the packaging of the plasmid in to the capsid of a M-13 phage when we co-infected by M13 helper phage. Phagemids generally encode no or one kind of coat protein. The structural and functional proteins responsible to completion of the life cycle of phagemid are encoded by helper phage and generally transcribed by the host. In M13 bacteriophage, DNA is replicated by the rolling circle mechanism. In this mechanism, one strand is nicked and the free 3'OH is extended by DNA polymerase. The 3' end on the circle is extended while the growing point rolls around the circle template. The 5'end is displaced and forms a tail of single stranded DNA. The single stranded tail is converted into double stranded DNA by synthesis involving RNA primers.

By this way Phagemid has certain advantages:

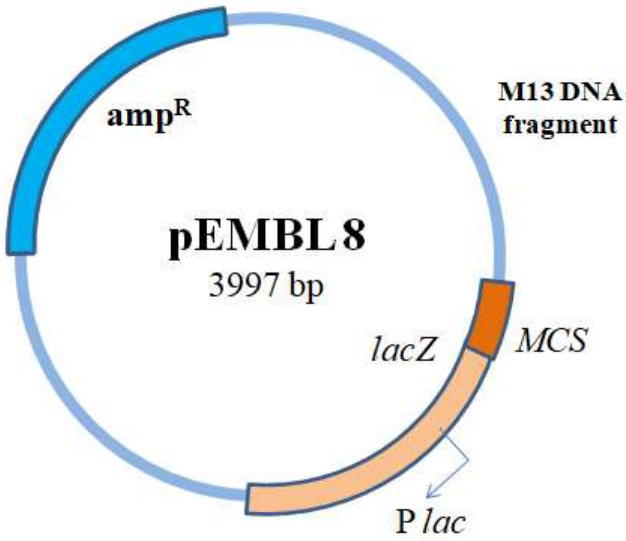
1. The carrying capacity of phagemid is higher than phage vectors.
2. Phagemid has higher efficiency in transformation than phage vectors.
3. Phagemids are genetically more stable than recombinant phage vectors.
4. Phagemids can be exploited to generate single stranded DNA template for sequencing purposes.

5. Single stranded phagemid vectors inside the phage can be targeted for site-directed mutagenesis.
6. Single stranded vectors can be used to generate hybridization probes for mRNA or cDNA.

One of the first hybrid phagmid vectors was pEMBL constructed in 1983. They are characterized by the presence of –

1) The *bla* gene, is responsible of selectable marker as ampicillin resistance.
2) Alpha-peptide is a short fragment of beta-galactosidase (lacZ) genes and containing MCS
3) The intragenic (IG) region of phage F1.

These vectors have been used successfully for DNA sequencing with the dideoxy method, and can be used for other purposes for which M13 derivatives are used. However, the pEMBL plasmids have the advantage of being smaller than M13 vectors, and the purification of DNA is simpler. In addition, long inserts have a higher stability in pEMBL plasmids than M13 vectors.



Within bacteriophage such as M-13 the replication process is complex. Phage DNA molecule generally carry several genes essential for the replication including genes for components and phage coat protein and phage specific DNA replicative enzymes. Alteration in any of genes will impair or destroys the replicative ability. So there is less freedom to modify phage DNA molecule. For the in vitro packaging of phage particles of phagemid vector like pEMBL8.

The pEMBL8 was made by transferring 1300 bp fragment of M13 in to pUC8. This piece of M13 in pEMBL8 contains signal sequences recognized by the enzyme that converts the normal double stranded M13 molecule in to single stranded DNA before secretion of new

phage particles. The signal sequence is still remains functional even though detached from rest of the genome of M13. When a normal M13 is used as helper phage, provides necessary replicative enzymes and phage coat proteins.

High-cloning capacity vectors

The high capacity cloning vectors are generally used for the construction of genomic libraries. These include cosmids, bacterial artificial chromosomes (BACs), P1-derived artificial chromosomes (PACs) and yeast artificial chromosomes (YACs). They are designed to handle longer DNA inserts, much larger than for λ replacement vectors. So they require lower number of recombinants to be screened for identification of a particular gene of interest. Artificial chromosomes are DNA molecules assembled in vitro from defined constituents that can function like natural chromosomes.

Types of artificial chromosomes:

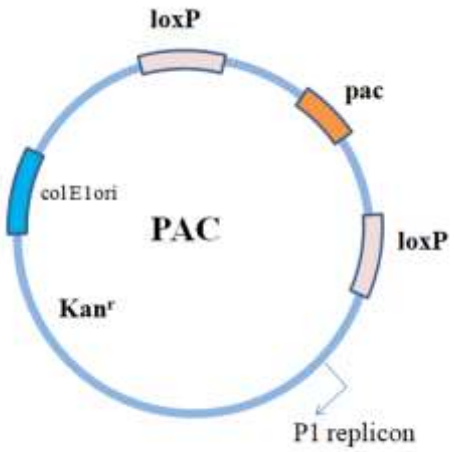
1) PACs: P1-derived artificial chromosomes
2) BACs: Bacterial artificial chromosomes
3) BIBACs: Binary Bacterial artificial chromosomes
4) YACs: Yeast artificial chromosomes
5) HACs: Human artificial chromosomes
6) MACs: Mammalian artificial chromosomes

Bacteriophage P1

The bacteriophage P1 is a temperate phage that infects E. coli. In its lysogenic cycle the P1 genome remains as a plasmid in the bacterium. One of its important feature is that, it hijacks the host machinery and integrates in to host genome. The viron P1 has icosahedral head and a tail with six fibres which help it to anchor the host cell wall. The P1 phage have a comparatively large genome approx 93 kb, as a linear double stranded DNA molecule. After insertion in to host it get circularized and replicates as plasmid. The phage P1 has two ori, OriR responsible for lysogenic cycle, where as OriL replicates it during lytic cycle. It can carry a foreign DNA up to 100 kb and able to replicate it in to the host cytoplasm.

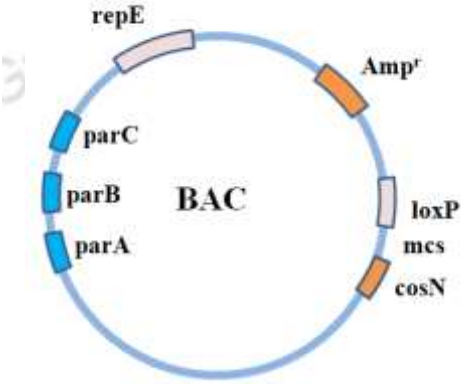
P1 derived Artificial Chromosome (PAC)

1) These are *E. coli* compatible vectors.
2) PAC vectors have low copy number.
3) The maintenance is stable for foreign DNA.
4) Can accommodate up to 150 kbp foreign DNA.



The Bacterial Artificial Chromosome vectors are modified by adding some important features of P1 bacteriophage to creat chimeraless, stable cloning vectors system for genome mapping having a cloning capacity of about 60 to 150 kbps of foreign DNA fragments. The P1 derived Artificial Chromosomes are introduced in to host cells usually though electroporation.

Bacterial Artificial Chromosome (BAC)



Bacterial Artificial Chromosomes were prepared during human genome project for preparation of large insert libraries having a cloning capacity of about 300 kbps. During the human genome project BACs got popularized due their high cloning capacity and easy maintenance. Usually BACs are maintained as single copy plasmids in per host cells and stable as such over hundreds of generations. For partitioning basically three genes are responsible, namely parA, parB and parC. The BACs can be maintained only in bacterial host deficient in homologous recombination (means it should have recA- gene)The total size of a BAC is about 7.4 kb and is introduced through electroporation in to the host cells. A single copy F-plasmid origin of replication (ori) is found in BAC.

The F (fertility) plasmid is relatively large and vectors derived from it have a higher capacity than normal plasmid vectors. F-plasmid has F (fertility) factor which controls the replication and maintain low copy number. Also conjugation can take place between F+ bacteria (male) and F- bacteria (female) to transfer F-plasmid via pilus. Common gene components of a bacterial artificial chromosome are:

1. oriS, repE – F for plasmid replication and regulation of copy number.
2. parA and parB for maintaining low copy number and avoiding two F plasmids in a single cell during cell division.
3. A selectable marker for antibiotic resistance; some BACs also have lacZ at the cloning site for blue/white selection.
4. T7 and Sp6 phage promoters for transcription of inserted genes.

Bacterial Artificial Chromosomes are Bacterial cloning vector (derived from F plasmid) that can accommodate up to 350 kb (most commonly 120–150 kb) DNA sequences, and has a considerably lower error rate than the still larger capacity yeast artificial chromosome (YAC). BACs usually exist in a single copy per cell. Random BACs are selected at random from a genomic library and are then shotgun-sequenced. Most BAC vectors lack selectable markers suitable for mammalian cell selection but can be retrofitted by employing the Cre/loxP site specific recombination system.

Binary Bacterial Artificial Chromosome (BIBAC)

BIBACs are binary expression vectors developed basically to propogate in Agrobacterium as well as in *E. coli*. These vectors are the combination of two technologies *i.e.,* Bacterial artificial chromosome and binary expression systems. These vectors have a capacity to carry about 160 kbps DNA in to the host cell. The BIBACs eliminated unnecessary gene verification steps by many subcloning activities and are able to transfer genes directoly to the plant cells. The advent of BIBACs reduced both time and cost of tedious plant transformation expresiment from transfer of unrelated genes into plant cells. With the aid of BIBACs it is now possible to check expression of genes in a pathway or the whole biochemical pathways itself. These vectors can express a series of genes or as entire biochemical pathway in a plant cell.

A unique BamHI cloning site is present in BIBAC, this single BamHI site makes it suitable for the construction of genomic DNA libraries. Two pair of NotI and SfiI restriction endonucleases are also found as flanking the BamHI site. Virulence helper plasmids are used with BIBACs to enhance its acceptability for large DNA fragments in plants.

Yeast Artificial Chromosome (YAC)

First described in 1983 by Murray and Szostak, a yeast artificial chromosome has sequences to exist inside E. coli as a circular plasmid and contains sequences to maintain as linear nuclear chromosome in yeast. The number of clones in a genomic library can be greatly reduced. YAC vectors have following elements:

1. coli origin of replication
2. Yeast origin of replication
3. Elements of eukaryotic yeast chromosome (centromere and telomere region)
4. Selection markers for both the hosts (Bacterial as well as Yeast)
5. Maintained as linear DNA-like chromosome.
6. Introduced into the yeast cells by electroporation.

YAC is a vector used to clone DNA fragments larger than 100 kb and up to 3,000 kb. YACs are useful for the physical mapping of complex genomes and for the cloning of large genes.

Yeast artificial chromosomes are created placing a centromere(CEN), telomeres (TEL), and an autonomous replicating sequence (ARS) element together to replicate autonomously and conserve itself in yeast cells. ARS are supposed to fuction as initiation point for DNA replication in yeasts. A circular plasmid is restriction digested to creat a lenear YAC vector. These YAC are cleaved in cented to creat two arms and the foreign DNA are ligated between these two arms forming a single linear molecule. TRP1 and URA3 genes are included in the YAC vector to provide a selection system for identifying transformed yeast cells that include YAC by complementing recessive alleles trp1 and ura3 in yeast host cell.

B. Summary

The single stranded bacteriophages Ff, Fd, F1 and M-13 vectors provide beautiful cloning system for getting single stranded DNA molecules. The phagemid Vectors fills the gap between high capacity cloning vectors and sequencing vectors. While the bacterial artificial chromosomes, binary bacterial artificial chromosomes , P1 derived artificial chromosomes and yeast artificial chromosomes have different advance functions like construction of genomic libraries, expression studies, plant transformations etc.

Bibliography:

Cooke H. 2001. Mammalian artificial chromosomes as vectors: progress and prospects; Cloning Stem Cells, 3(4): 243-249.

Dente L., Cesareni G., Cortese R. 1983. pEMBL: a new family of single stranded plasmids; Nuc. Acid Res. 11 (6) 1645-1655.

Hall B.G. 2004. Predicting the evolution of antibiotic resistance genes. Nat Rev Microb 2 (5): 430–435.

http://bioinfo2010.wordpress.com/2009/07/08/vector-bacteriophage-lambda-and-m13-7th-april/

Kim et al . 1992. Stable propagation of cosmid-sized human DNA inserts in an F-factor based vector . Nucleic Acids Res. 20 (5): 1083–1085.

Kim et al. 1995. Construction and utility of a human chromosome 22-specific Fosmid library; Genetic Analysis: Biomol Eng 12 (2): 81–84.

Qi H., Lu H., Qiu H.J., Petrenko V. et al. 2012. Phagemid Vectors for Phage Display: Properties, Characteristics and Construction; J.Mol. Bio (417), 129-143.

Shizuya H. 1992. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. Proc Natl Acad Sci USA, 89(18): 8794–8797.

Tursun B, Cochella L, Carrera I, Hobert O. 2009. A Toolkit and Robust Pipeline for the Generation of Fosmid-Based Reporter Genes in C. elegans . PLoS ONE 4(3): e4625.