BHARATHIDASAN UNIVERSITY
Tiruchirappalli- 620024
Tamil Nadu India

Programme: M.Sc., Biotechnology (Environment)

Course Title : Genetic Engineering
Course Code: CC 07

Unit-II
Cloning Vectors

Dr T Sivasudha
Professor
Department of Environmental Biotechnology

Vectors are self- replicating DNA molecules . that serves as the carrier for the transfer or insertion of foreign gene(s) into another cell, where it can be replicated and/or expressed.

Vector is a latin word derived from '*vehere'* means carrier
Examples
Plasmid
Phage
Hybrid vector
Artificial Chromosomes.

Types of Vectors

Cloning vector

A cloning vector is a genome that can accept the target DNA and increase the number of copies through its own autonomous replication.

These vectors are only useful for storing a genetic sequence.

Expression Vector

A vector that is used for expressing a gene contained within the cloned DNA. For a gene to give rise to a protein product, an expression vector must be used that contains the necessary elements for a host cell to transcribe and translate the gene.

Vector Element	Description
Origin of Replication (ORI)	DNA sequence which allows initiation of replication within a plasmid by recruiting transcriptional machinery proteins
Antibiotic Resistance Gene	Allows for selection of plasmid-containing bacteria.
Multiple Cloning Site (MCS)	Short segment of DNA which contains several restriction sites allowing for the easy insertion of DNA. In expression plasmids, the MCS is often downstream from a promoter.
Insert	Gene, promoter or other DNA fragment cloned into the MCS for further study.
Promoter Region	Drives transcription of the target gene. Vital component for expression vectors: determines which cell types the gene is expressed in and amount of recombinant protein obtained.
Selectable Marker	The antibiotic resistance gene allows for selection in bacteria. However, many plasmids also have selectable markers for use in other cell types.
Primer Binding Site	A short single-stranded DNA sequence used as an initiation point for PCR amplification or sequencing. Primers can be exploited for sequence verification of plasmids.

Characteristics of Cloning Vectors

Multiple cloning site

The ability to self-replicate

A selectable marker gene selectable characteristic so that transformed cells may be recognized from untransformed cells

Multiple, restriction enzyme recognition sites so that DNA fragments can be cloned into the vector relatively easily

Reporter gene allows screening and identifies the correct recombinant among the recombinants after cloning is performed.

Major types of cloning Vectors

Plasmid. Circular extrachromosomal DNA that autonomously replicates inside the bacterial cell. Plasmids generally have a high copy number, such as pUC19 which has a copy number of 500-700 copies per cell.

Phage. Linear DNA molecules derived of bacteriophage lambda. Can be replaced with foreign DNA without disrupting its life cycle.

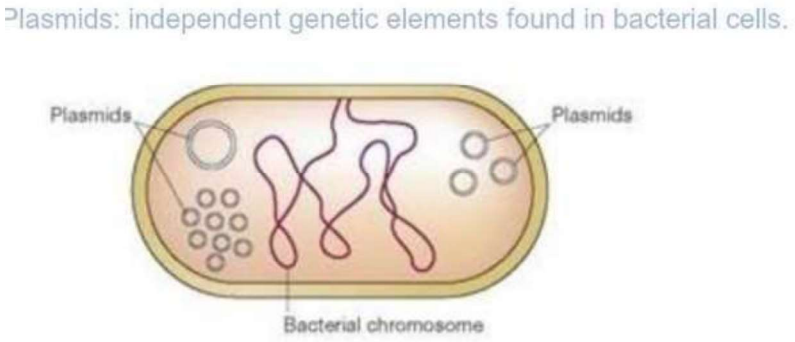
Cosmids. Another circular extrachromosomal DNA molecule that combines features of plasmids and phage.

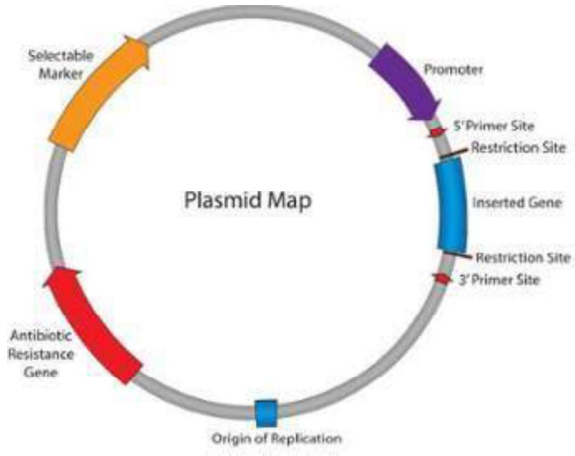
Bacterial Artificial Chromosomes. Based on bacterial mini-F plasmids.

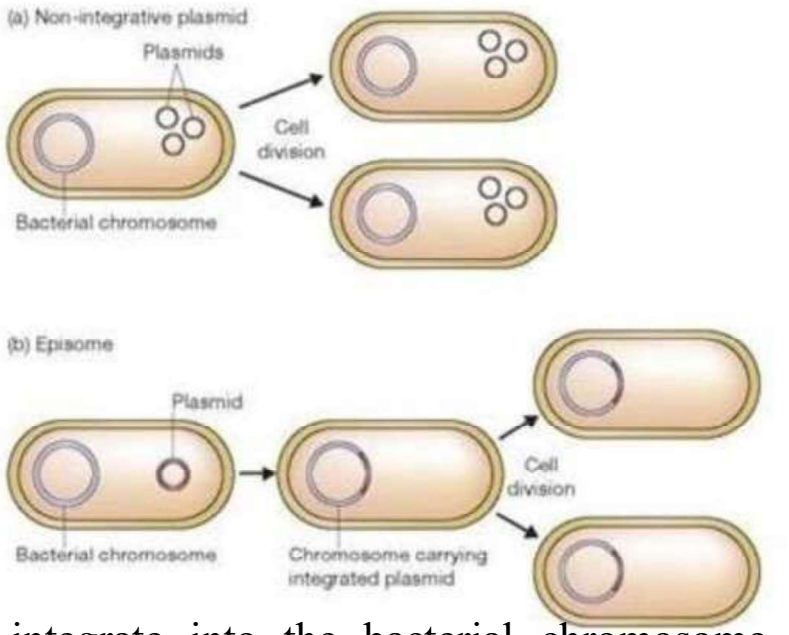
Yeast Artificial Chromosomes. This is an artificial chromosome that contains telomeres (disposable buffers at the ends of chromosomes which are cut off during cell division) with origins of replication, a yeast centromere (part of a chromosome that links sister chromatids or a dyad), and a selectable marker for identification in yeast cells.

Plasmids

- Plasmid vectors are used to clone DNA ranging in size from several base pairs to several thousands of base pairs (100bp - 10kb).
- The term plasmid was first introduced by the American molecular biologist Joshua Lederberg in 1952.





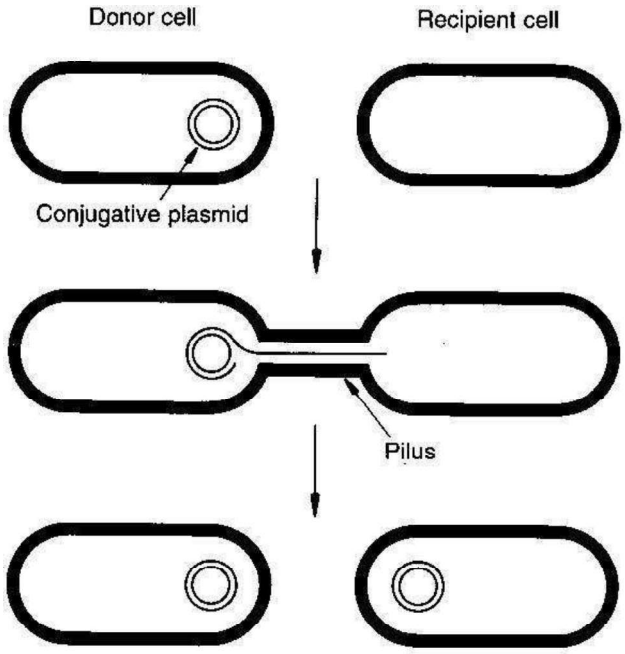


Some plasmids may integrate into the bacterial chromosome. They are called ***episome or integrative plasmids***. At this stage they replicate along with the bacterial chromosome.

The plasmids in this way are classified into 2 types

Stringent & Relaxed plasmid

- Some plasmids, especially the larger ones, are stringent and have a low copy number of perhaps just one or two per cell. stringent plasmids encode protein factors that are necessary for their own replication,
- Relaxed plasmids, are present in multiple copies of 50 or more per cell. relaxed plasmids replicate using host derived proteins



- One way of grouping plasmids is by their ability to transfer to other bacteria. Conjugative plasmids contain tra genes, which perform the complex process of conjugation, the transfer of plasmids to another bacterium.
- Non-conjugative plasmids are incapable of initiating conjugation, hence they can be transferred only with the assistance of conjugative plasmids.

Another way to classify plasmids is by function. There are five main classes:

•**Fertility F-plasmids**: which contain tra genes. They are capable of conjugation and result in the expression of sex pilli.

•**Resistance plasmids**: which contain genes that provide resistance against antibiotics or poisons. They were historically known as R-factors.

•**Col plasmids**: which contain genes that code for bacteriocins, proteins that can kill other bacteria.

•**Degradative plasmids**: which contains genes for the digestion of unusual substances, e.g. toluene and salicylic acid.

•**Virulence plasmids**: which turn the bacterium into a pathogen.

History , pBR series

- First vetors to develop was pBR322
- “P” indicates plasmid
- “BR” identifies the laboratory in which vector was originally constructed.
- (BR stands for Bolivar and Rodriguez, the two researchers who develop pBR322)
- “322” distinguishes the plasmid from others developed in the same laboratory.

Cloning Vectors based on *E. coli* Plasmids

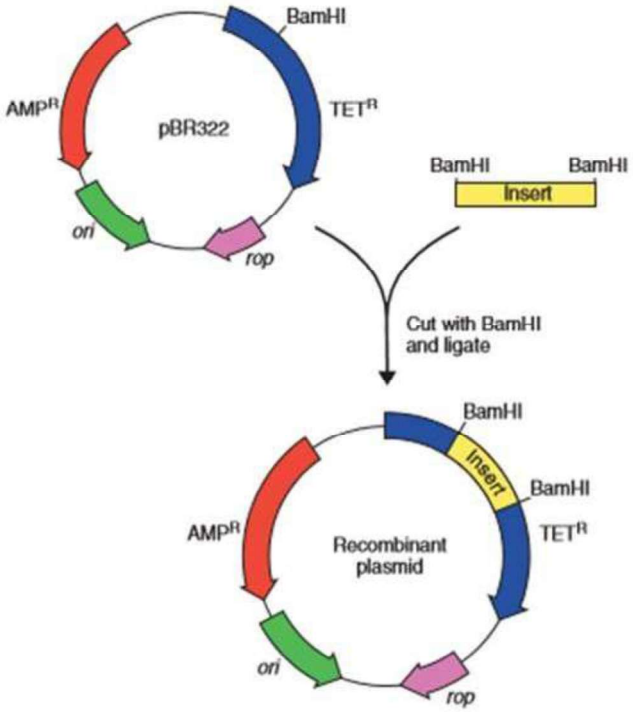
pBR322

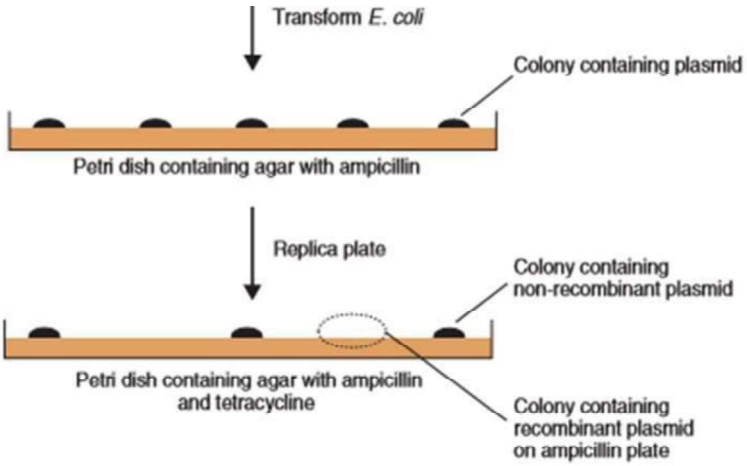
- The plasmid pBR322 was the first widely used plasmid vector. It is a small plasmid (4363 bp). pBR322 contains the following components.

- Origin of replication: carries the Col EI replication origin and rop gene to ensure reasonably high plasmid copy number (15–20 copies per cell)

- Antibiotic resistance genes. as selectable markers. The ampicillin resistance gene (termed bla or, more commonly, AMPr), and the tetracycline resistance gene (termed tet or TETr)

- Cloning sites. The plasmid carries a number of unique restriction enzyme recognition sites. Some of these are located in one or other of the antibiotic resistance genes. For example, sites for PstI, PvuI and SacI are found within AMPR, and sites for BamHI and HindIII are located within TETR


BamHI
AMP^R
TET^R
pBR322
ori
rop
BamHI
BamHI
Insert
Cut with BamHI
and ligate
BamHI
Insert
BamHI
AMP^R
TET^R
Recombinant
plasmid
ori
rop


Transform E. coli
Colony containing plasmid
Petri dish containing agar with ampicillin
Replica plate
Colony containing
non-recombinant plasmid
Petri dish containing agar with ampicillin
and tetracycline
Colony containing
recombinant plasmid
on ampicillin plate

- The antibiotic resistance genes in pBR322 allow for the direct selection of recombinants in a process called insertional inactivation. For example, if we want to clone a DNA fragment into the BamHI site of pBR322

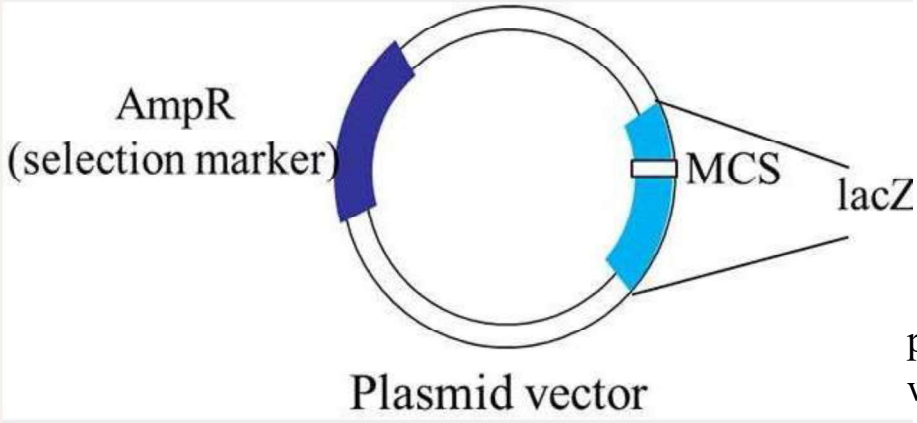
- The insert DNA will interrupt the gene responsible for tetracycline resistance, but the gene for ampicillin resistance will not be altered.

- Transformed cells are first grown on bacterial plates containing ampicillin to kill all the cells that do not contain a plasmid.

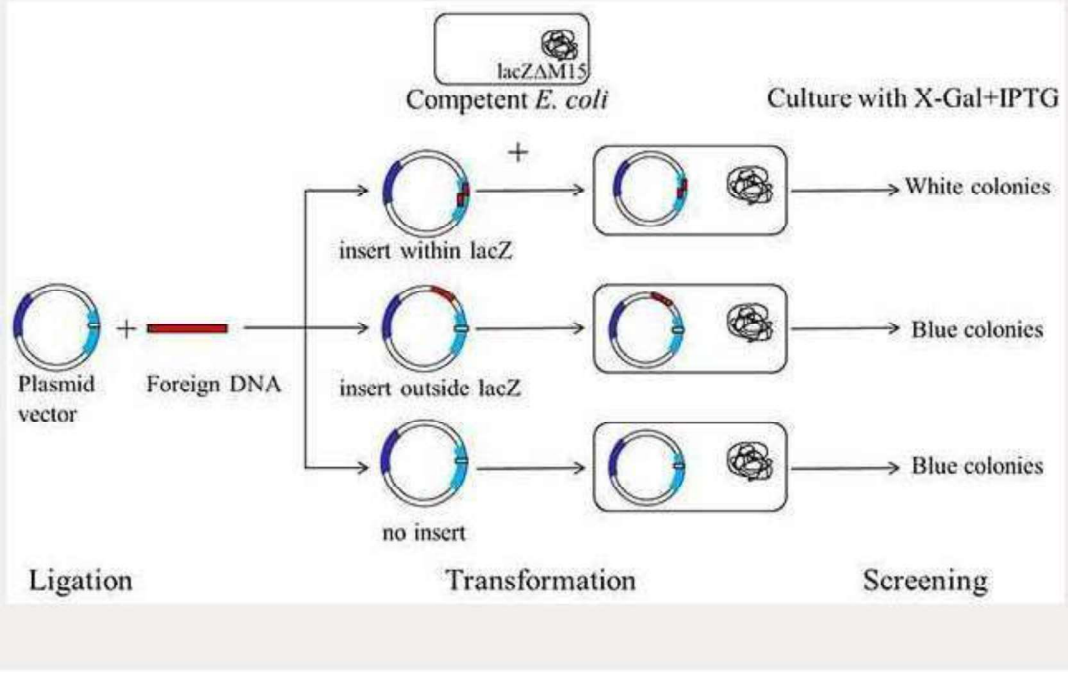
- Those cells that grow on ampicillin are then replica plated onto medium containing both ampicillin and tetracycline.

- Those cells that grow in the presence of the ampicillin, but die under tetracycline selection, contain plasmids that have foreign DNA inserts

- In other words, the insertion of a foreign DNA fragment into an antibiotic resistance gene inactivates the gene product and leads to antibiotic sensitivity



A schematic representation of a typical plasmid vector that can be used for blue-white screening.



2: *A schematic representation of a typical blue-white screening procedure.*

- Blue-white screening is a rapid and efficient technique for the identification of recombinant bacteria. It relies on the activity of β-galactosidase, an enzyme occurring in *E. coli*, which cleaves lactose into glucose and galactose.

- The presence of lactose in the surrounding environment triggers the lacZ operon in *E. coli*. The operon activity results in the production of β-galactoisdase enzyme that metabolizes the lactose. Most plasmid vectors carry a short segment of lacZ gene that contains coding information for the first 146 amino acids of β-galactosisdase. The host *E. coli* strains used are competent cells containing lacZΔM15 deletion mutation. When the plasmid vector is taken up by such cells, due to α-complementation process, a functional β-galatosidase enzyme is produced.

- The plasmid vectors used in cloning are manipulated in such a way that this α-complementation process serves as a marker for recombination. A multiple cloning site (MCS) is present within the lacZ sequence in the plasmid vector. This sequence can be nicked by restriction enzymes to insert the foreign DNA. When a plasmid vector containing foreign DNA is taken up by the host *E. coli*, the α-complementation does not occur, therefore, a functional β-galactosidase enzyme is not produced. If the foreign DNA is not inserted into the vector or if it is inserted at a location other than MCS, the lacZ gene in the plasmid vector complements the lacZ deletion mutation in the host *E. coli* producing a functional enzyme.

- For screening the clones containing recombinant DNA, a chromogenic substrate known as X-gal is added to the agar plate. If β-galactosidase is produced, X-gal is hydrolyzed to form 5-bromo-4-chloro-indoxyl, which spontaneously dimerizes to produce an insoluble blue pigment called 5,5’-dibromo-4,4’-dichloro-indigo. The colonies formed by non-recombinant cells, therefore appear blue in color while the recombinant ones appear white. The desired recombinant colonies can be easily picked and cultured.

- Isopropyl β-D-1-thiogalactopyranoside (IPTG) is used along with X-gal for blue-white screening. IPTG is a non-metabolizable analog of galactose that induces the expression of lacZ gene. It should be noted that IPTG is not a substrate for β-galactosidase but only an inducer. For visual screening purposes, chromogenic substrate like X-gal is required.

Advantages and Disadvantages of using Plasmids

- pBR322 was a breakthrough for molecular biology as the first widely used plasmid for molecular cloning, but the double screening procedure required to identify recombinant clones was both time consuming and error prone.

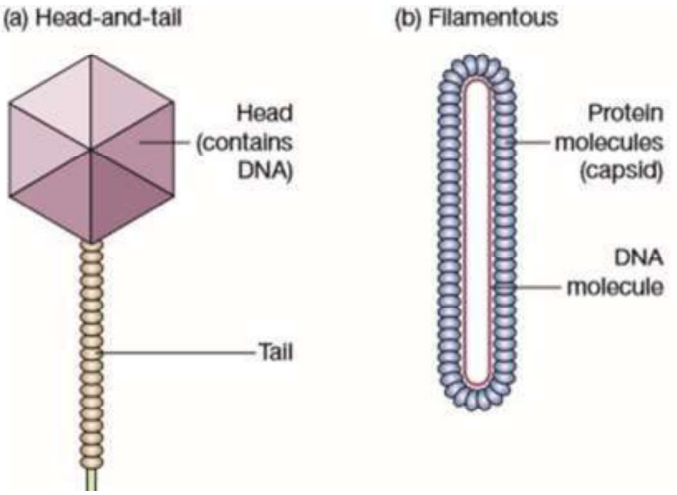
- The major cloning advantage of pUC plasmids over pBR322 is that foreign DNA fragments can be cloned into a variety of restriction enzyme sites and recombinants rapidly screened

- The efficiency at which the plasmid is transferred to a bacterial cell is very low.
- The capacity of plasmids to carry large fragments of foreign DNA is limited. Most plasmids become unstable if their overall size exceeds about 15 kbp.

Bacteriophages

Bacteriophages, or phages as they are commonly known, are viruses that specifically infect bacteria.

Like all viruses, phages are very simple in structure, consisting merely of a DNA (or occasionally ribonucleic acid (RNA)) molecule carrying a number of genes, including several genes for replication of the phage, and its components protein coat and phage specific replicative enzymes. Alteration in any of the gene can impair replicative ability of resultant molecule.

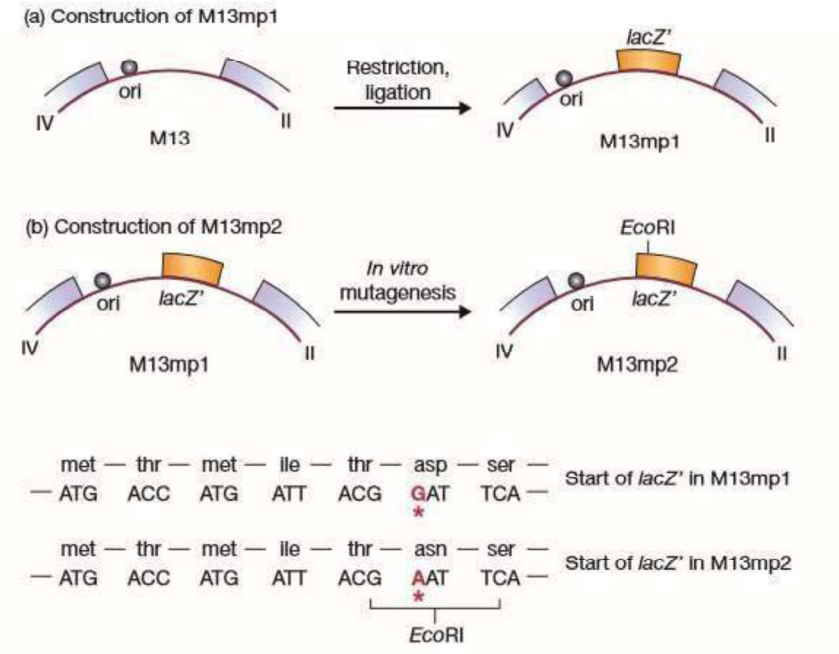


M13—a filamentous phage

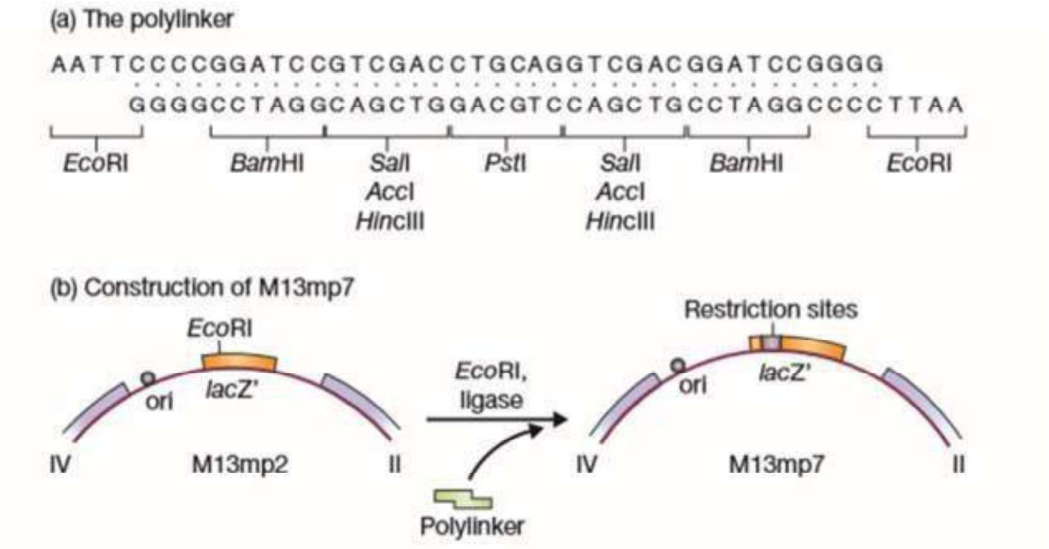
The normal M13 genome is 6.4 kb in length, but most of this is taken up by ten closely packed genes. Each essential for the replication of the phage.

There is only a single 507-nucleotide intergenic sequence into which new DNA could be inserted without disrupting one of these genes, and this region includes the replication origin which must itself remain intact. Clearly there is only limited scope for modifying the M13 genome.

- The first step in construction of an M13 cloning vector was to introduce the *lacZ'* gene into the intergenic sequence. This gave rise to M13mp1, which forms blue plaques on X-gal agar (Figure a). M13mp1 does not possess any unique restriction sites in the *lacZ'* gene. It does, however, contain the hexanucleotide GGATTC near the start of the gene. A single nucleotide change would make this GAATTC, which is an *Eco*RI site. This alteration was carried out using *in vitro* mutagenesis, resulting in M13mp2 (Figure b)

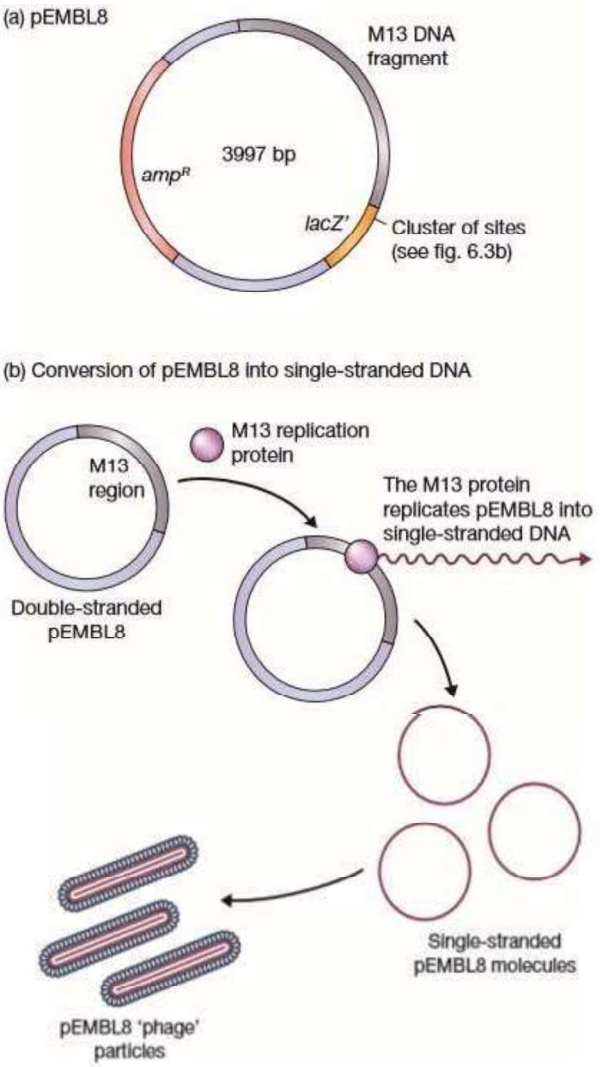


- The next step in the development of M13 vectors was to introduce additional restriction sites into the *lacZ'* gene. This was achieved by synthesizing in the test tube a short oligonucleotide, called a **polylinker**, which consists of a series of restriction sites and has *Eco*RI sticky ends (Figure a). This polylinker was inserted into the *Eco*RI site of M13mp2, to give M13mp7 (Figure b), a more complex vector with four possible cloning sites (*Eco*RI, *Bam*HI, *Sal*I, and *Pst*I). The polylinker is designed so that it does not totally disrupt the *lacZ'* gene: a reading frame is maintained throughout the polylinker, and a functional, though altered, b-galactosidase enzyme is still produced.



Hybrid plasmid–M13 vectors

- Although M13 vectors are very useful for the production of single-stranded versions of cloned genes, they do suffer from one disadvantage.
- There is a limit to the size of DNA fragment that can be cloned with an M13 vector, with 1500 bp generally being looked on as the maximum capacity, though fragments up to 3 kb have occasionally been cloned.
- To get around this problem a number of hybrid vectors ("**phagemids**") have been developed by combining a part of the M13 genome with plasmid DNA.
- An example is provided by pEMBL8, which was made by transferring into pUC8 a 1300 bp fragment of the M13 genome. (Figure a)
- This piece of M13 DNA contains the signal sequence recognized by the enzymes that convert the normal double-stranded M13 molecule into single-stranded DNA before secretion of new phage particles.


(a) pEMBL8
M13 DNA fragment
3997 bp
ampR
lacZ'
Cluster of sites (see fig. 6.3b)
(b) Conversion of pEMBL8 into single-stranded DNA
M13 region
M13 replication protein
The M13 protein replicates pEMBL8 into single-stranded DNA
Double-stranded pEMBL8
Single-stranded pEMBL8 molecules
pEMBL8 'phage' particles

- This signal sequence is still functional even though detached from the rest of the M13 genome, so pEMBL8 molecules are also converted into single-stranded DNA and secreted as defective phage particles (Figure b).

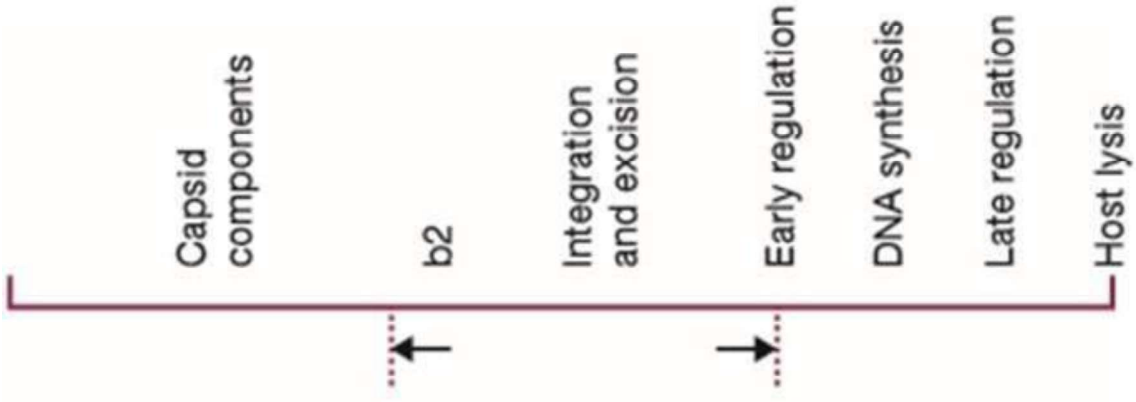
- All that is necessary is that the E. coli cells used as hosts for a pEMBL8 cloning experiment are subsequently infected with normal M13 to act as a helper phage, providing the necessary replicative enzymes and phage coat proteins.

- pEMBL8, being derived from pUC8, has the polylinker cloning sites within the lacZ′ gene, so recombinant plaques can be identified in the standard way on agar containing X-gal.

Segments of the λ genome can be deleted without impairing viability

- The λ genetic map, showing the position of the main non-essential region that can be deleted without affecting the ability of the phage to follow the lytic infection cycle

- Removal of non-essential region decreases the size of the resulting λ molecule by up to 15 kb. This means that as much as 18 kb of new DNA can now be added.



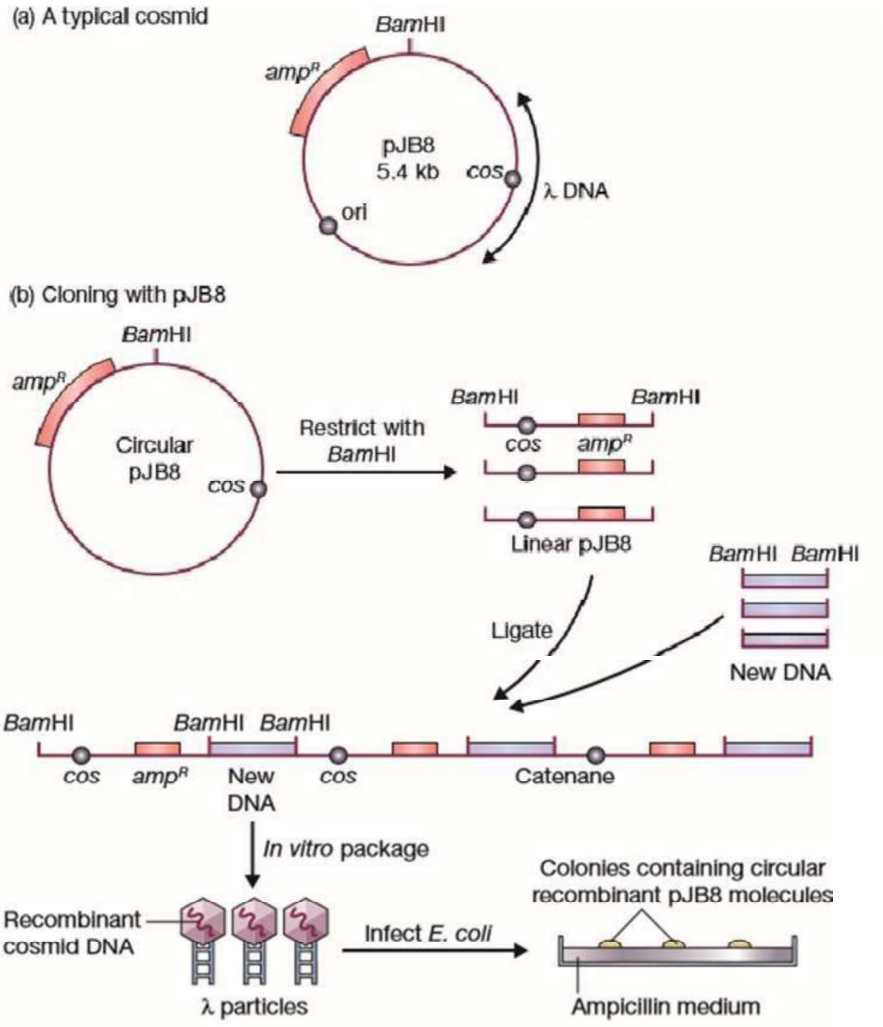
Cosmid

Cosmids are hybrids between λ phage DNA molecule and a bacterial plasmid.

A cosmid is basically a plasmid that carries a cos site (Figure a). It also needs a selectable marker, such as the ampicillin resistance gene, and a plasmid origin of replication, as cosmids lack all the λ genes and so do not produce plaques. Instead colonies are formed on selective media, just as with a plasmid vector.

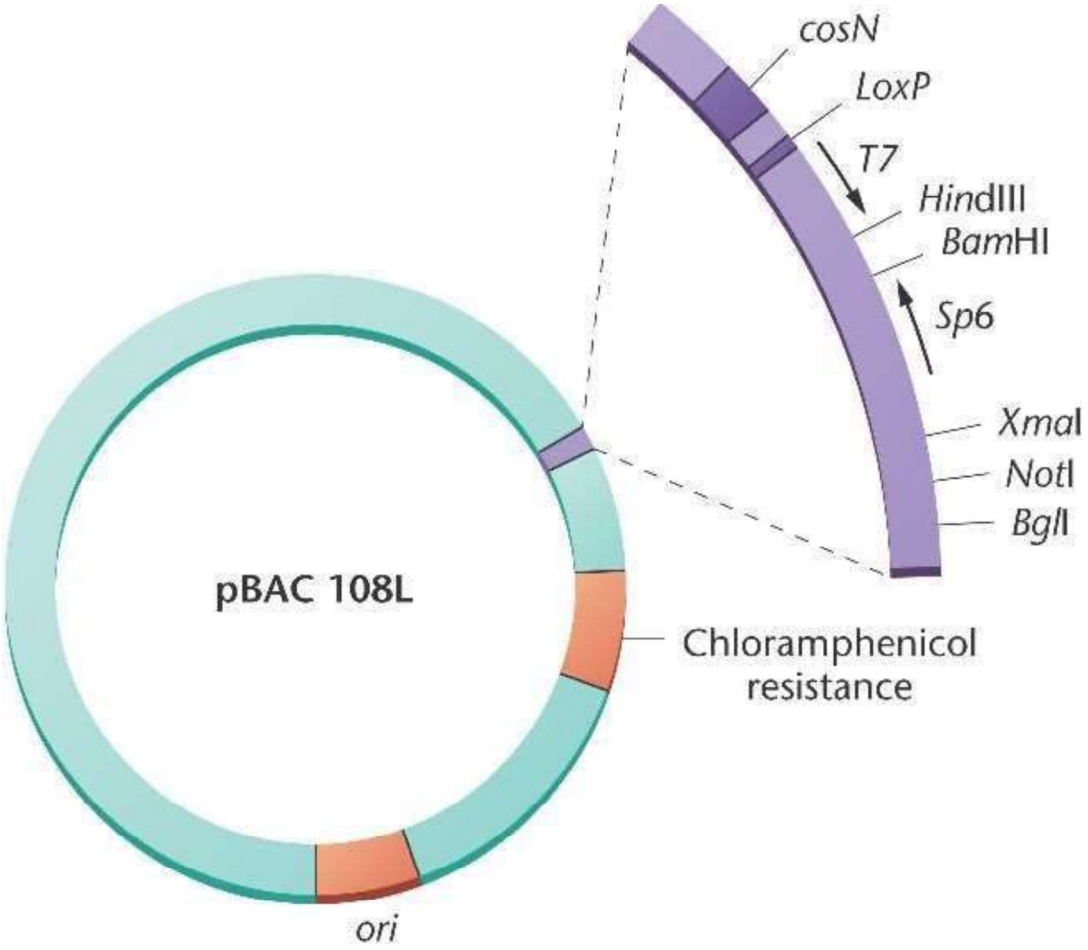
Cosmids also possess a unique restriction enzyme recognition site into which DNA fragments can be ligated. After the packaging reaction has occurred, the newly formed λ particles are used to infect E. coli cells. The DNA is injected into the bacterium like normal λ DNA and circularizes through complementation of the cos ends.

Infected cells are plated onto a selective medium and antibiotic-resistant colonies are grown.


(a) A typical cosmid
BamHI
ampR
pJB8
5.4 kb
cos
λ DNA
ori
(b) Cloning with pJB8
BamHI
ampR
Circular
pJB8
cos
Restrict with
BamHI
BamHI
BamHI
cos
ampR
Linear pJB8
BamHI BamHI
New DNA
Ligate
BamHI
BamHI BamHI
cos
ampR
New
DNA
cos
Catenane
In vitro package
Recombinant
cosmid DNA
λ particles
Infect E. coli
Colonies containing circular
recombinant pJB8 molecules
Ampicillin medium

BACs

- A bacterial artificial chromosome is an engineered large DNA molecule used to clone DNA segment in a bacterial cell.
- Based on well known natural F1 plasmid (allows conjugation between two bacterial cells).
- MCS present for insertion of foreign DNA.
- 150-300 kb can be inserted.
- Copy no is 2 per cell.
- Used in analysis of large genome.
- First BACs vector is pBAC108L.


cosN
LoxP
T7
HindIII
BamHI
Sp6
XmaI
NotI
BglI
pBAC 108L
Chloramphenicol resistance
ori

Cloning genomic DNA into a BAC

1. Genomic DNA is isolated from a desired source and used restriction enzymes to cleave the target DNA into fragments.

2. The BAC is digested by restriction enzymes in the cloning sites ***Hin*dIII and *Bam*HI**.

3. Those two elements recombine by the DNA ligase and attach into a host bacterium.

4. As the bacterial cells grow and divide, they amplify the BAC DNA, which can then be isolated and used in sequencing DNA.

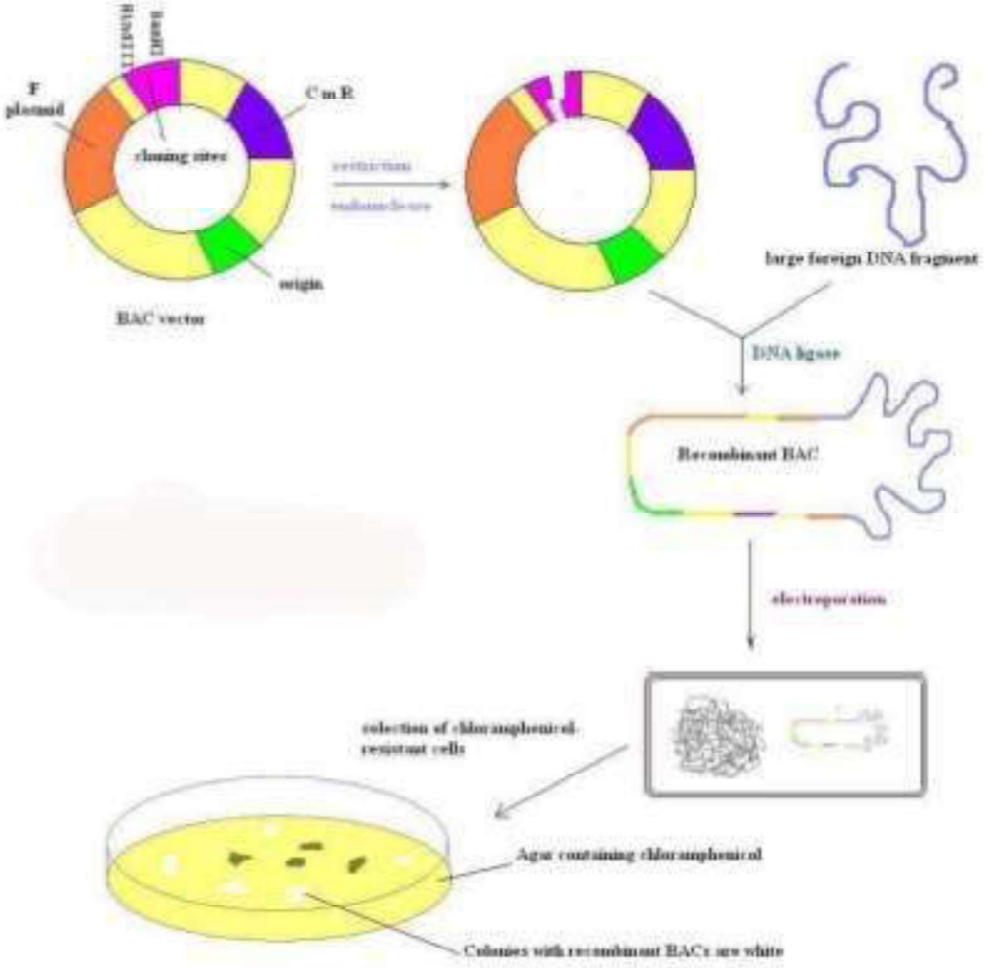


Fig: BAC as a Cloning vector

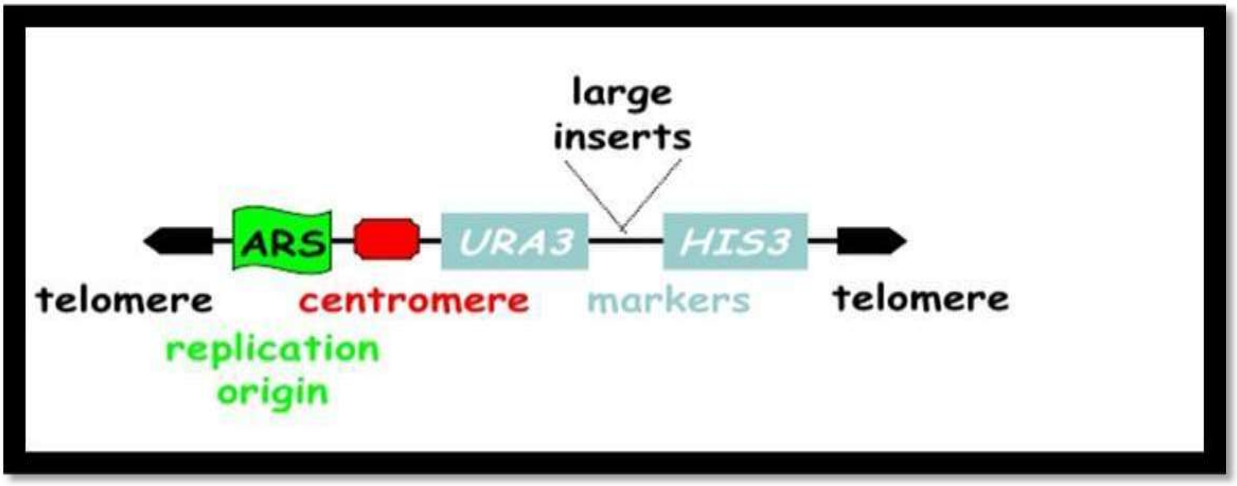
Advantages:

- Useful for cloning up to 200 kb, but can be handled like
- regular bacterial plasmid vectors.
- Useful for sequencing large stretches of chromosomal DNA; frequently used in genome sequencing projects.
- More stable
- Easier to work with than the YACs.

- **Disadvantage:**
- Difficult to transform bacteria.

YACs Vector

- Capable of carrying inserts of 200-2000 kbp in yeast

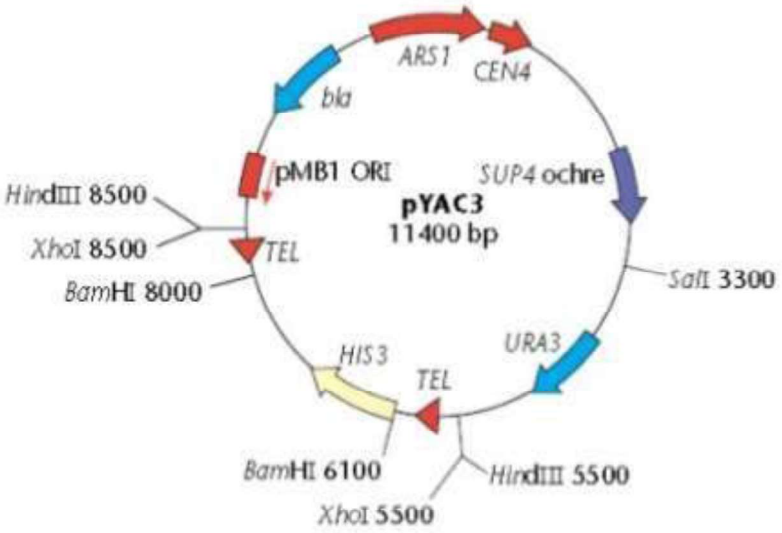


Yeast artificial chromosome(YAC) vectors allow the cloning, within yeast cells.

- ***A yeast centromere (CEN4)*:** The yeast centromere is specified by a 125 bp DNA segment.
- ***Yeast autonomously replicating sequence (ARS)*:** Yeast *ARS* elements are essentially origins of replication
- ***Yeast telomeres (TEL)*:** Telomeres are the specific sequences (5 - TGTGGGTGTGGTG-3) that are present at the ends of chromosomes in multiple copies and are necessary for replication and chromosome maintenance.

- YAC contains the telomere, centromere, and origin of replication elements. If these elements are spliced into DNA in the proper location and orientation, then a yeast cell will replicate the artificial chromosome along with the other, natural chromosomes. The target DNA is flanked by the telomere regions that mark the ends of the chromosome, and is interspersed with the centromere region that is vital for replication. Finally, the start site for the copying process is present. In essence, the yeast is fooled into accepted genetic material that mimics a chromosome.

YACs are plasmid shuttle vectors capable of replicating and being selected in common bacterial hosts such as *Escherichia coli*, as well as in the budding yeast *Saccharomyces cerevisiae*.



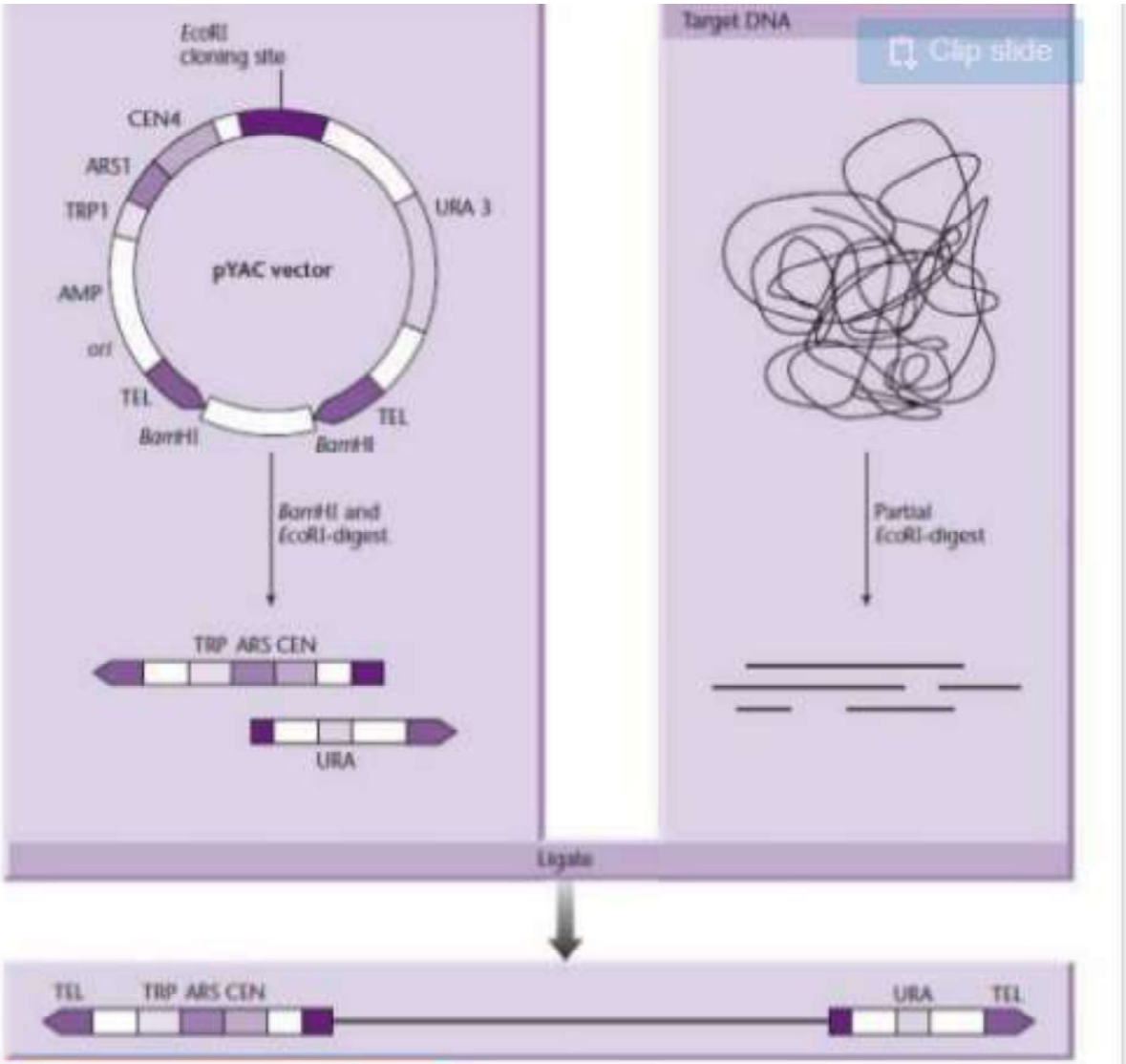


Fig. 11.3
Construction of a yeast artificial chromosome containing large pieces of cloned DNA. Key regions of the pYAC vector are as follows: TEL, yeast telomeres; ARS1, autonomously replicating sequence; CEN4, centromere from chromosome 4; *URA3* and *TRP1*, yeast marker genes; Amp, ampicillin-resistance determinant of pBR322; *ori*, origin of replication of pBR322.

Advantages:

- Very useful in mapping the human genome because they could accommodate hundreds of thousands of kilobases each.

•YACs containing a megabase or more are known as “megaYACs.”

Disadvantages:

- They are inefficient (not many clones are obtained per microgram of DNA)
- Hard to isolate from yeast cells unstable