PLASMIDS

Plasmids are circular, self-replicating double stranded DNA present in most of the bacteria, but can be found in a few archaea and eukaryotic organisms. They serve as a back-up of helpful genes for their host organism, such as providing antibiotic resistance. Plasmids transfer their genetic material to other bacteria. The genetic materials transfer through mainly three mechanisms: transformation, transduction, and conjugation.

VECTORS

A vector (eg. plasmid) is any vehicle, often a virus or a plasmid that is used to ferry a desired DNA sequence into a host cell as part of a molecular cloning procedure.

There are many types of cloning vectors, but the most commonly used ones are genetically engineered plasmids. Cloning is generally first performed using Escherichia coli, and cloning vectors in E. coli include plasmids, bacteriophages (such as phage λ), cosmids, and bacterial artificial chromosomes (BACs).

Yeast artificial chromosome (YAC)

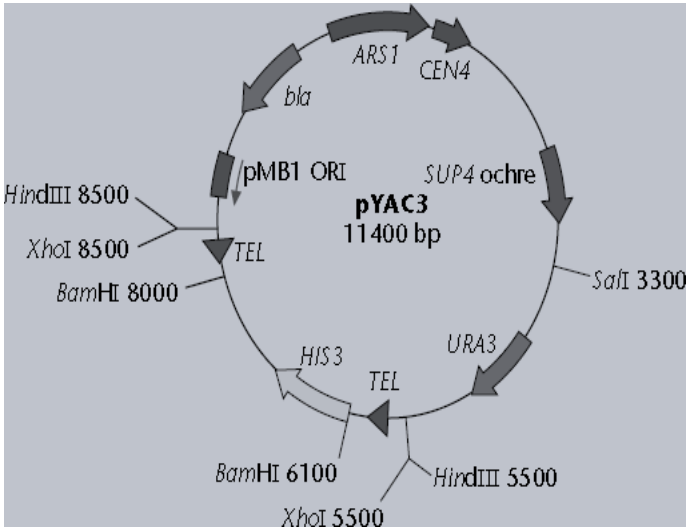
Yeast artificial chromosome (YAC) is a human-engineered DNA molecule used to clone DNA sequences in yeast cells. Segments of an organism's DNA, up to one million base pairs in length, can be inserted into YACs.

Yeast artificial chromosomes (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. They are of relatively small size (approximately 12 kb) and of circular form when they are amplified or manipulated in *E. coli*, but are rendered linear and of very large size, i.e. several hundreds of kilobases (kb), when introduced as cloning vectors in yeast.

The YACs, with their inserted DNA, are then taken up by yeast cells. As the yeast cells grow and divide, they amplify the YAC DNA, which can be isolated and used for DNA mapping and sequencing.

The latter process involves cleavage at strategically located sites by two restriction enzymes, which break them in two linear DNAarms. These are subsequently ligated with the appropriate DNA insert before transformation into recipient yeast cells.

In this linear form, these specialized vectors contain all three cis-acting structural elements essential for behaving like natural yeast chromosomes: an autonomously replicating sequence (ARS) necessary for replication; a centromere (CEN) for segregation at cell division; and two telomeres (TEL) for maintenance.



YACs have several advantages over other large cap

acity vectors: these include accommodation of DNA segments thousands of kilobases in size and stable maintenance of cloned eukaryotic DNA due to the compatibility with the yeast replication machinery.

Construction of Yeast Artificial Chromosomes

After plasmid DNA purification, two distinct digestions are performed: the first with BamHI that cuts twice adjacent to the two telomeric DNA sequences flanking the HIS3 gene, which therefore is excised from the plasmid and lost. This first digestion generates a long linear fragment carrying telomeric sequences at each end. The second digestion consists of the opening of

thecloning site within the SUP4 gene. As a result of this second digestion, two linear fragments are produced as left and right arms of the future linear YAC.

The selective markers are thus separated: TRP1 adjacent to ARS1 and CEN4 on the left arm and URA3 on the right arm. Large DNA fragments with ends compatible to the cloning site, obtained from the desired genome source by digestion with an appropriate restriction endonuclease, are ligated with phosphatase-treated YAC arms, to create a single yeast-transforming DNA molecule.

Primary transformants can be selected for complementation of the ura3 mutation in the host, and successively for complementation of the host *trp1* mutation, thereby ensuring the presence of both chromosomal arms. Transformant colonies containing the exogenous DNA insert within the SUP4 gene are detected by their red colour, due to the inactivation of the suppressor activity and the consequent accumulation of a red metabolic precursor in *ade* host cells.

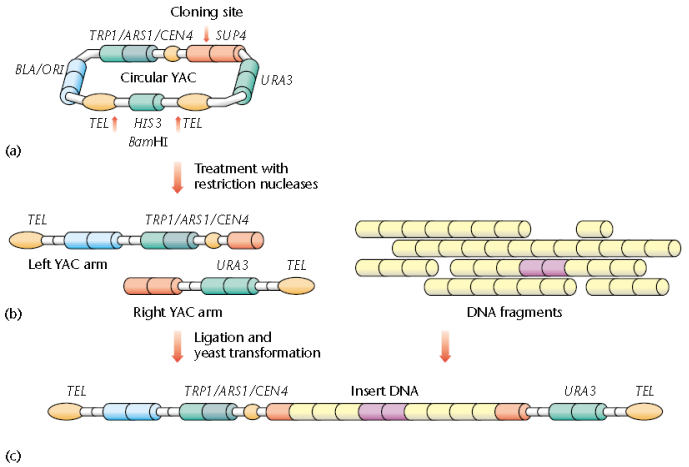


Fig: Construction of the yeast artificial chromosome. (a) A circular YAC vector able to replicate in *Escherichia coli* due to the presence of bacterial ori and bla gene (blue cylinder) and propagated in yeast cells as a linear molecule containing all necessary chromosomal elements: yeast centromere CEN4 (orange circle), autonomous replication sequence ARS1 (dark green cylinder) and two telomeric sequences TEL (orange ellipse) functional in yeast after linearization with the BamHI restriction endonuclease. The yeast SUP4 gene (red cylinder) contains a cloning site used as a colour marker for selection of YACs containing exogenous insert DNA. (b) DNA fragments with ends compatible to the YAC cloning site are prepared from source DNA. After double digestion of the YAC vector, the markers used to select for transformants are separated on two chromosomal arms: TRP1 on the left and URA3 on the right arm (light green cylinders).

Biological Features of YACs

Large DNA (>100 kb) is ligated between two arms. Each arm ends with a yeast telomere, so that the product can be stabilized in the yeast cell. Interestingly, larger YACs are more stable than shorter ones, which favors cloning of large stretches of DNA.

One arm contains an autonomous replication sequence (ARS), a centromere (CEN) and a selectable marker (*trp1*). The other arm contains a second selectable marker (*ura3*)

Insertion of DNA into the cloning site inactivates a mutant expressed in the vector DNA and red yeast colonies appear.

Transformants are identified as those red colonies which grow in a yeast cell that is mutant for *trp1* and *ura3*. This ensures that the cell has received an artificial chromosome with both telomeres (because of complementation of the two mutants) and the artificial chromosome contains insert DNA (because the cell is red).

Construction of YACs by Homologous Recombination

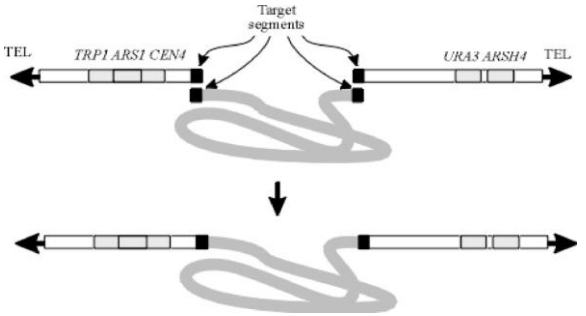


Figure: Recombinational targeted cloning with YAC vectors. A yeast strain is transformed with a mixture of the two YAC vector arms and large fragments of DNA.Recombination *in vivo* results in the formation of a specific YAC clone. The two YAC vector arms are derived from linearized plasmids that contain targeting segments that are homologous to the termini of the DNA segment that is to be cloned.

The potential to use YAC cloning technology has been enhanced by the ability to use homologous recombination for manipulating exogenous DNA in the yeast host. In recombinationally-targeted YAC cloning, YACs are assembled *in vivo*, by recombination, and not by ligation *in vitro*. Recombination takes place between a target segment DNA, and the YAC vector that contains sequences homologous to these targets. Transformation of the two YAC vectors arms and the exogenous segment, flanked by the target segments, followed by recombination, results in the formation of the desired stable YACs.

Applications of YACs

Withholding of DNA inserts thousands of kilobases in size and steady preservation of cloned eukaryotic DNA due to the compatibility with the yeast replication mechanisms. Yeast artificial chromosomes correspond to the top tools for the learning of eukaryotic genomes and for mobilisation of huge genetic elements among bacteria and eukaryotes

Appropriately modified, YACs can be utilized in many different organisms, for cloning or genome analysis. Chromosomal translocation (chromosome abnormality that occurs due to re arrangement of parts among non homologous chromosomes) can be studied by means of disposable YACs that do not contain genetic information necessary for cell function.

Another major application of YACs is in the study of regulation of gene expression by cis-acting, controlling DNA elements, that are present either upstream or downstream of large eukaryotic genes, after the transfer of these YACs from yeast to mammalian cells.

Artificial chromosome vectors have made easier the mapping and sequencing of complex genomes at a progressively more rapid rate. Conventional bacterial cloning agents have remained significant for the learning of rather short clones, but for the cloning of very huge DNA pieces artificial chromosomes have totally replaced prior bacterial systems, counting the lambda phage-based cosmid vector.

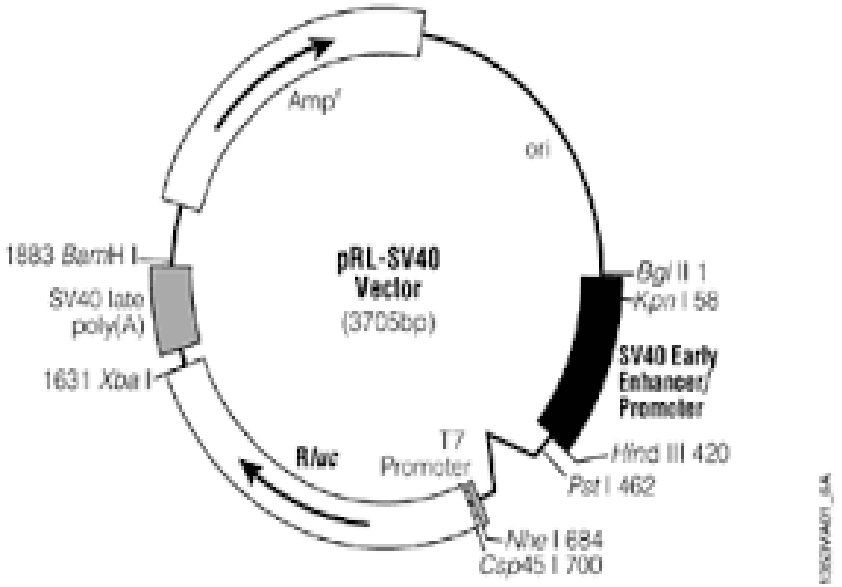
Simian Virus 40

Simian Virus 40 (SV40) is a nonenveloped, double-stranded DNA virus with a 5.2 kb circular genome. SV40 vectors have broad specificity, can be produced in high titers, and have high transduction efficiency (Strayer, 2000).

In addition, the ability of these vectors to infect nondividing cells makes them attractive for LDGT. However, the SV40 is a small virus, and therefore, the capacity of SV40 to accommodate exogenous DNA is limited to 4.7 kb, which could potentially be a major drawback. In mouse livers recombinant SV40 vectors (rSV40) were shown to transduce efficiently in vivo, and the transgene expression was found to be at comparable levels both in the regenerated and preoperative livers rSV40 may persist in vivo despite extensive cell division (Strayer et al., 2002).

In addition, for liver targeting, they can be injected into the tail-veins of mice. Using this approach rSV40 vectors were shown to reach the liver and express the luciferase transgene within a few days (Arad et al., 2005).

Collectively, these results indicate that rSV40 vectors are highly suitable for LDGT.



Some of the specific features of an SV40 vector are as follows

Capacity of the transgene is limited to 5 Kb.

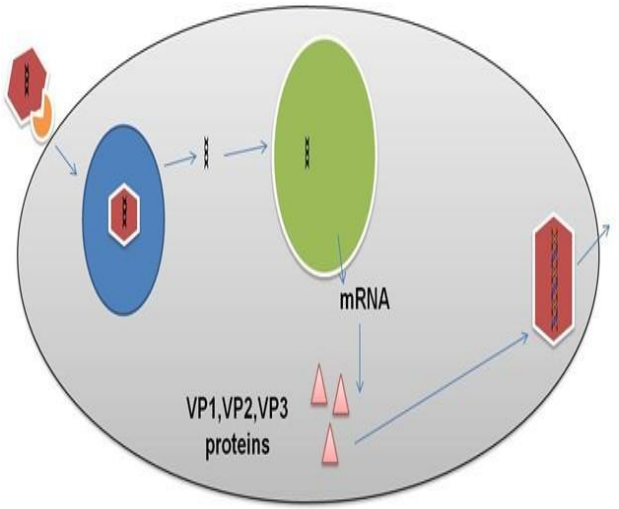
Both resting as well as dividing cells are equally transduced by SV40 based vectors.

High level of transduction efficiency.

Long lasting transgene expression. It has been reported that the transgene expression once established will remain lifelong.

Neutralizing antibody against recombinant

Replication of SV40 in the host cell



#The late proteins of SV40 are synthesized from the opposite strand.

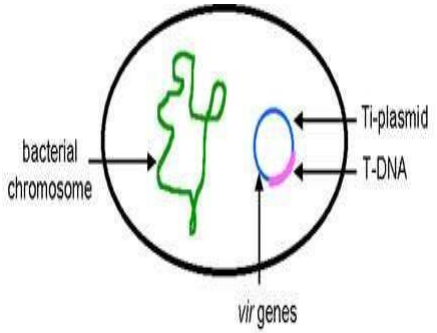
#Out of four structural proteins VP1 is the most abundant one.

#The minimum viral specific sequence required to assemble the virion consists of origin of replication and encapsidation sequences they were overlaps with the early promoter region in the genome.

#The transcription of the genome and the transgene is governed by pol III promoter.

Agrobacterium tumefaciens

- vir genes- essential for the excision and transportof the T-DNA to the wounded plant cell.
- Cytokinin-plant hormone for cell plant divisionand tumorous growth
- Auxin-another plant hormone (inducing stem andleaf elongation, inducing parthenocarpy and preventing aging)
- Opines- a class of chemicals that serve as a source of nitrogen for *A. tumefaciens*, but not for most other organisms.
- Acetylsyringone - produced by wounded plant cells (phenolic compound).
  -plays a further role in the infection process, because at higher concentrations it activates the virulence genes (**Vir** genes) on the T_i plasmid
- rod-shaped, Gram-negative soil bacterium, causes **crown gall** disease, especially members of the rose family such as apple, pear, peach, cherry, almond, raspberry and roses.
- Tumor formation is the result of the transfer, integration and expression of genes on a specific segment of A.Tumefaciens plasmid DNA called
  the T-DNA. *
- Tumor-inducing plasmid



This disease is one of the most widely known, because of its remarkable biology.

Basically, the bacterium transfers part of its DNA to the plant, and this DNA integrates into the plant's genome, causing the production of tumours and associated changes in plant metabolism.

Agrobacteria are biological vectors for introduction of genes into plants.

- Agrobacteria attach to plant cell surfaces at wound sites.
- The plant releases wound signal compounds, such as acetosyringone.
- The signal binds to virA on the *Agrobacterium*

membrane.

- VirA with signal bound activates *virG*.
- The T-DNA is transferred to the plant cell, where it integrates in nuclear DNA.
- T-DNA codes for proteins that produce hormones and opines. Hormones encourage growth of the transformed plant tissue. Opines feed bacteria a carbon and nitrogen source.
- The T-DNA is transferred to the plant cell, where it integrates in nuclear DNA.
- T-DNA codes for proteins that produce hormones and opines. Hormones encourage growth of the transformed plant tissue. Opines feed bacteria a carbon and nitrogen source.

Application

*Insect resistance

*Disease resistance

*Abiotic stresses

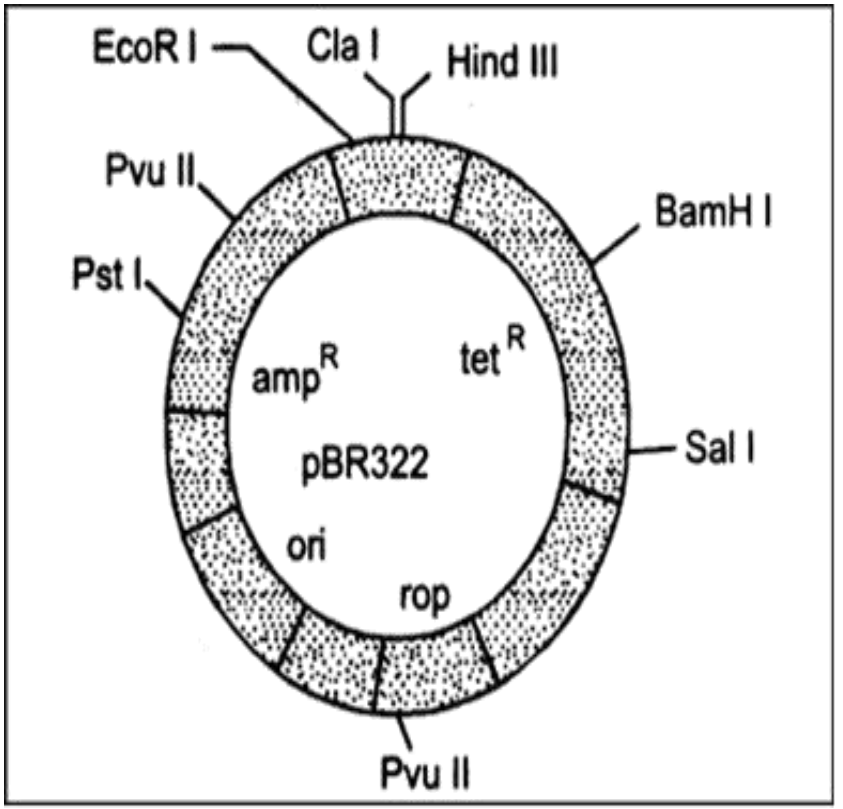
*Herbicide Tolerance

Environmental and biosafety aspects

*Environmental release and biosafety have been subjects of debate regarding transgenic plants, especially for food crop like rice.

*Demand for marker-free transgenic plants is high.

pBR322


EcoR I
Cla I
Hind III
Pvu II
BamH I
Pst I
amp R
tet R
pBR322
Sal I
ori
rop
Pvu II

The main characteristics of pBR322 are:

- **Restriction sites:** BamH I, Hind III, Sal I, Pvu I, Pvu II, Pst I, EcoR I, Cla I
- **Selectable marker:** antibiotic resistance genes for ampicillin (amp^R) and tetracycline (tet^R)
- **ORI:** the origin of replication
- **ROP:** It codes for proteins, which are involved in the process of replication of plasmid

Different antibiotic resistance genes act as a restriction site and to ligate foreign DNA and for the selection of transformants. The gene, where the foreign DNA is inserted becomes inactive.

Alternative selectable marker: Mostly these have an ability to produce some colour after reacting with a chromogenic substance. The alternative markers are used for the ease of differentiating recombinants from non-recombinants, e.g. gene coding for β-galactosidase.

When a foreign gene is inserted between the gene coding for β-galactosidase**,** the recombinant cell does not produce the enzyme β-galactosidase due to inactivation of the gene. In the presence of a chromogenic substrate, non-recombinants form blue colour colonies and recombinants form colourless colonies.

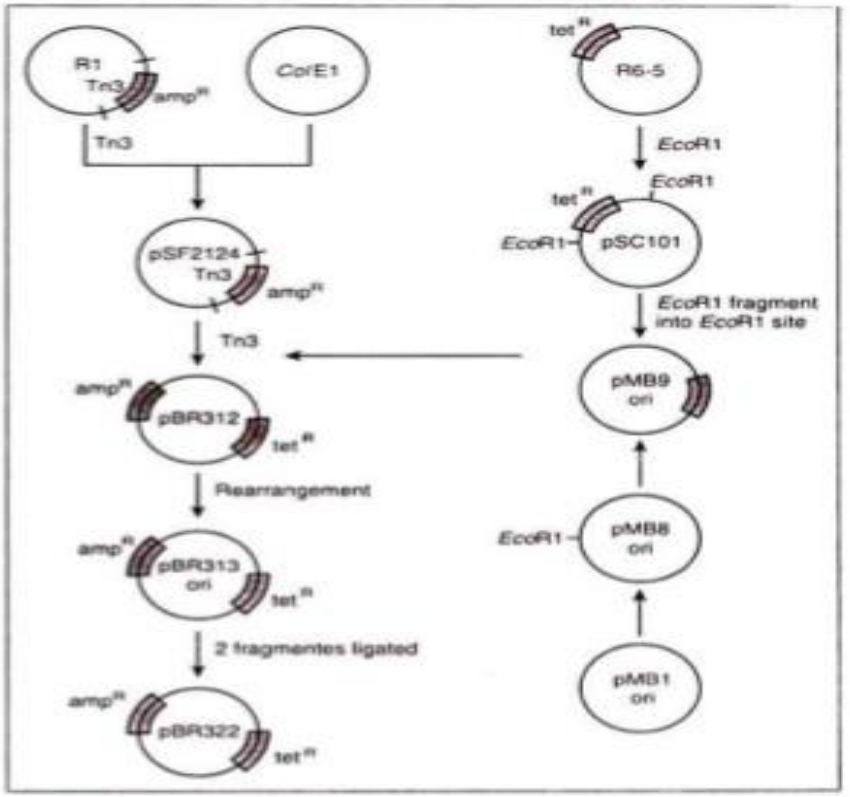


Fig. 4.15: The Pedigree of pBR322

Recombinant selection with pBR322 is done by **insertional inactivation of an antibiotic resistance gene**.

For example, A recombinant pBR322 molecule, one that carries an extra piece of DNA in the BamHI site is no longer able to confer tetracycline resistance on its host, as one of the necessary genes is now disrupted by the inserted DNA. Cells containing this recombinant pBR322 molecule are still resistant to ampicillin, but sensitive to tetracycline (amp^R tef^s).

Uses of pBR322:

It is widely used as a cloning vector. In addi-tion to this, it has been widely used as a model

1. Small size (~ 4.4 kb) enables easy purifi-cation and manipulation.

2. Two selectable markers (amp and tet) al-low easy selection of recombinant DNA.

3. It can be amplified up to 1000-3000 copies per cell when protein synthesis is blocked by the application of chloramphenicol.

Disadvantages of pBR322:

1. It has very high mobility i. e; it can move to another cell in the presence of a conjugative plasmid like F-factor. Due to this, the vector may get lost in a population of mixed host cells.

2. There is a limitation in the size of the gene of interest that it can accommodate.

3. Not a very high copy number is present as is expected from a good vector.

4. The screening process time- consuming and laborious.