Vectors

Cloning Vector

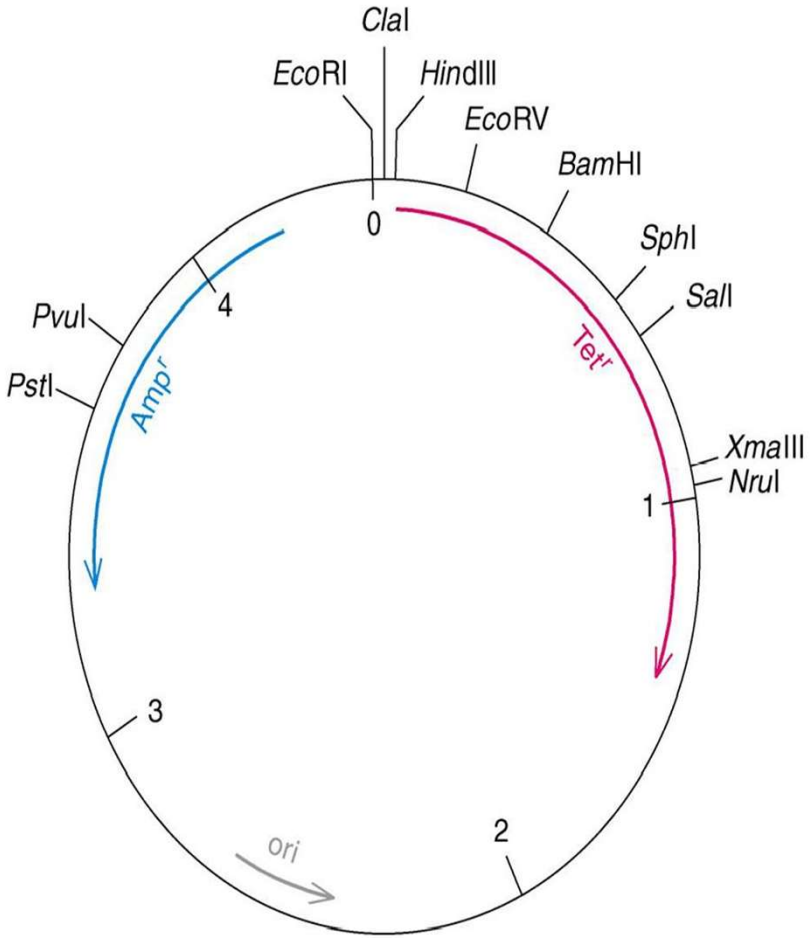
- It is the central component of a gene cloning process.
- A small piece of DNA into which a foreign DNA fragment can be inserted.
- The insertion of the fragment is carried out by treating the vector and the foreign DNA with a restriction enzyme that creates the same overhang, then ligating the fragments together.

Characteristics of a cloning vector

- **Ori (Origin of replication) is a specific sequence of nucleotide from where replication starts**
- **It should have selectable marker gene**
- **It should have restriction sites: a synthetic multiple cloning site (MCS) can be inserted into the vector**
- **Replicate inside the host cell to form multiple copies of the recombinant DNA molecule.**
- **Less than 10kb in size.**

Contd….

- **Origin of Replication: Allow the vector as well as the foreign DNA to amplify in the host cell**
- **Selectable Marker: Antibiotic resistance genes- Allow the host to grow on selective media; Can selectively amplify this specific vector in the host cell**
- **Multiple Cloning Sites: Allow insertion of foreign DNA**



Types of Cloning Vectors

- They allow the exogenous DNA to be inserted, stored, and manipulated mainly at DNA level.
- Types

1. Plasmid vectors
2. Bacteriophage vectors
3. Cosmids
4. Phagemids
5. Fosmids
6. BACs & YACs

Plasmid Vector

- **Plasmid vectors are double-stranded, extra-chromosomal DNA molecules, circular, self-replicating.**
- ***Advantages:***
  - **Small, easy to handle**
  - **Easy purification**
  - **Straightforward selection strategies**
  - **Useful for cloning small DNA fragments (< 10kbp)**

- ***Disadvantages:***
  - **Less useful for cloning large DNA fragments (> 10kbp)**

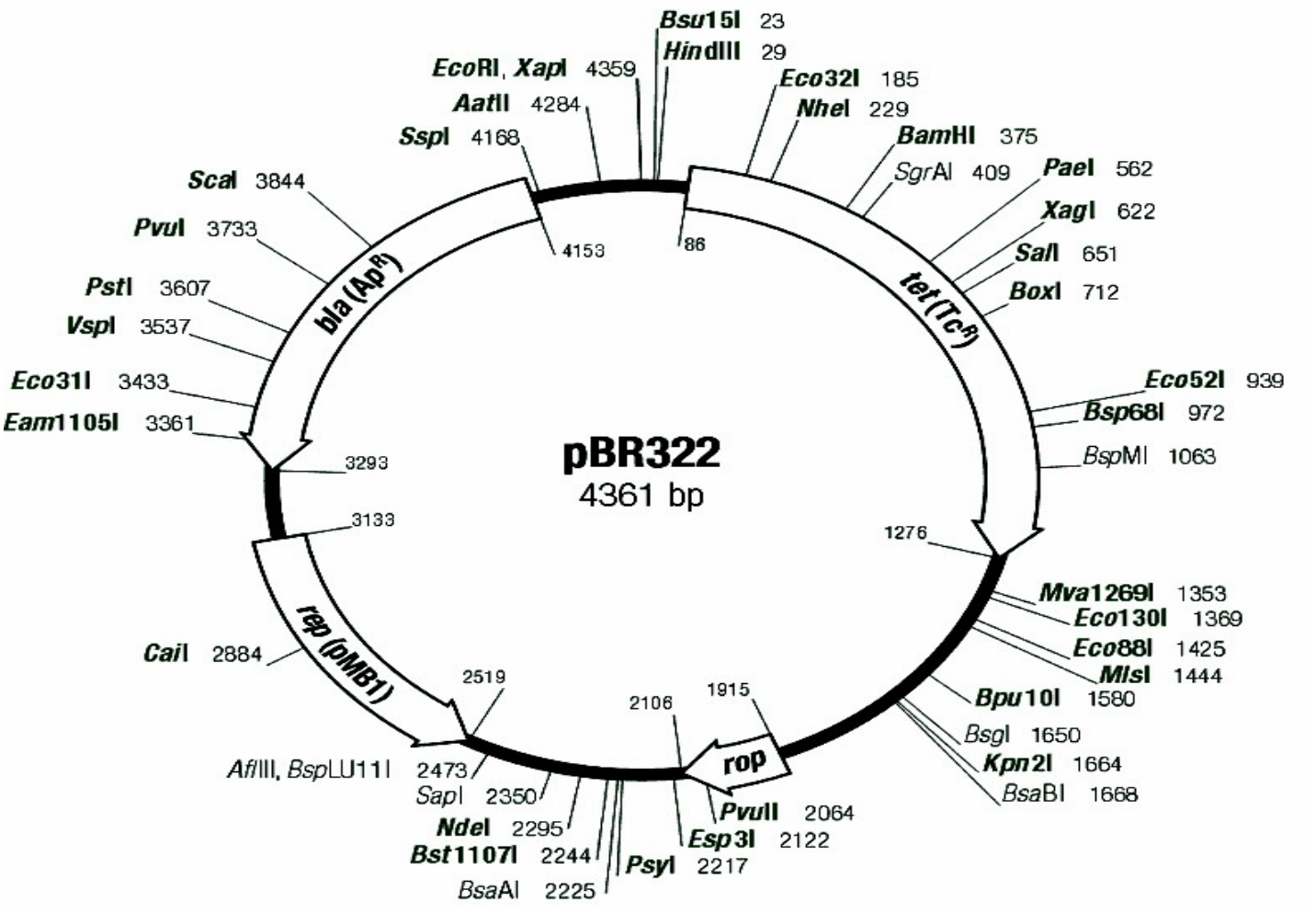
A plasmid vector for cloning

1. Contains an origin of replication, allowing for replication independent of host's genome.
2. Contains Selective markers: Selection of cells containing a plasmid
   - antibiotic resistance
   - blue-white screening
3. Contains a multiple cloning site (MCS)
4. Easy to be isolated from the host cell.
5. Plasmids range in size from 1.0kb to 250kb

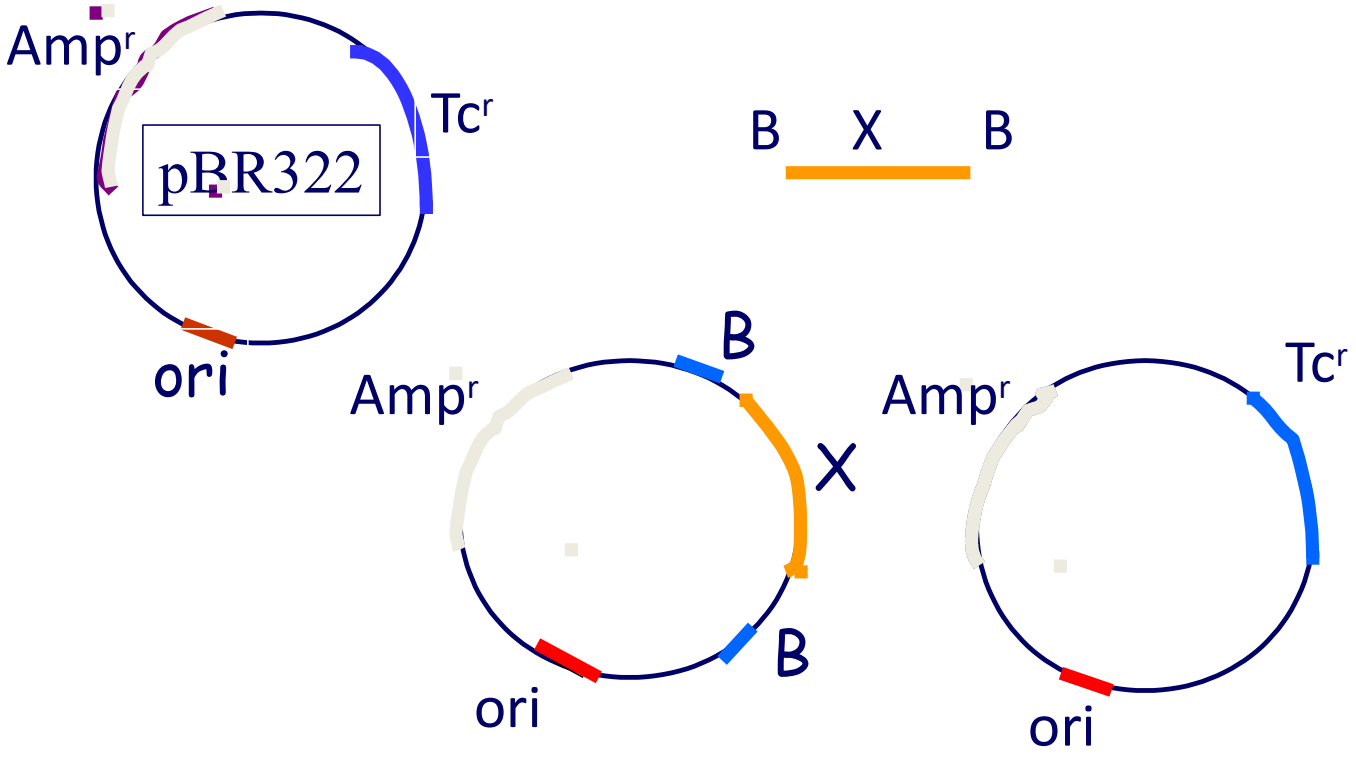
pBR322

- It was one of the first vectors to be developed in 1977.
- The 'p' indicates that it is plasmid, 'BR' indicates Bolivar and Rodriguez
- '322' distinguishes it from the other plasmids produced in the same laboratory e.g. pBR325, pBR327, pBR328.
- It is 4363bp in size i.e. less than 10kb
- It carries two sets of antibiotic resistance genes i.e. either ampicillin or tetracycline can be used as a selectable marker.
- Each of the marker genes carries unique restriction sites and insertion of DNA into these sites inactivates the specific marker site. e.g. insertion of new DNA with Pst1, Puv1, Ppa1 or Sca1 inactivates the amp^R gene.
- It has a high copy number. They are about 15 molecules present in transformed cells but it can be increased to 1000 to 3000 by plasmid amplification in the presence of protein synthesis inhibitor i.e. chloramphenicol.
- The vector comprises DNA derived from three different naturally occurring plasmids: the amp^R gene is from R1 plasmid, tet^R from R6-5 plasmid and the ori gene from pMB1 plasmid.

Features of pBR322

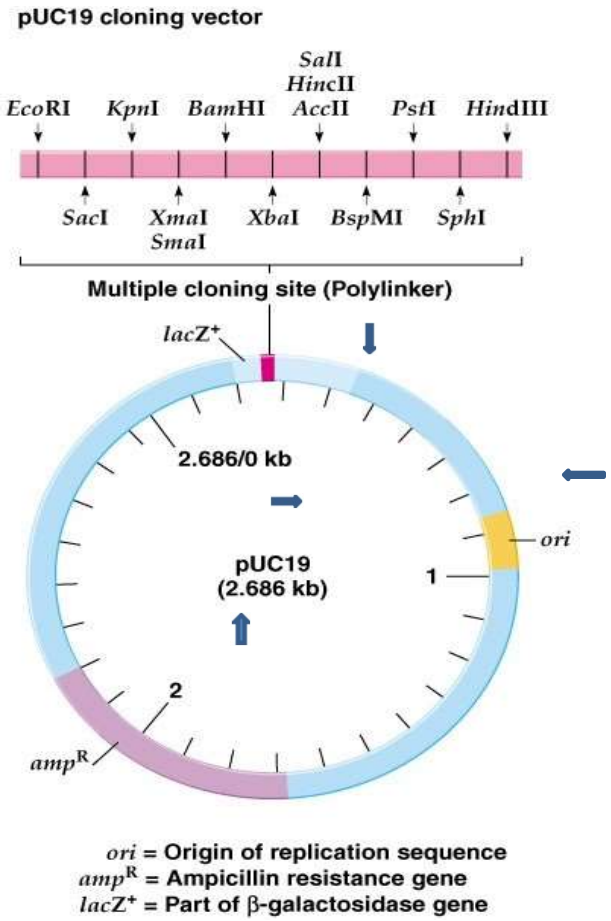


Screening by insertional inactivation of a resistance gene



Ampicillin resistant?	*yes*	*yes*
Tetracycline resistant?	*No*	*yes*

FROM pBR322 to pUC



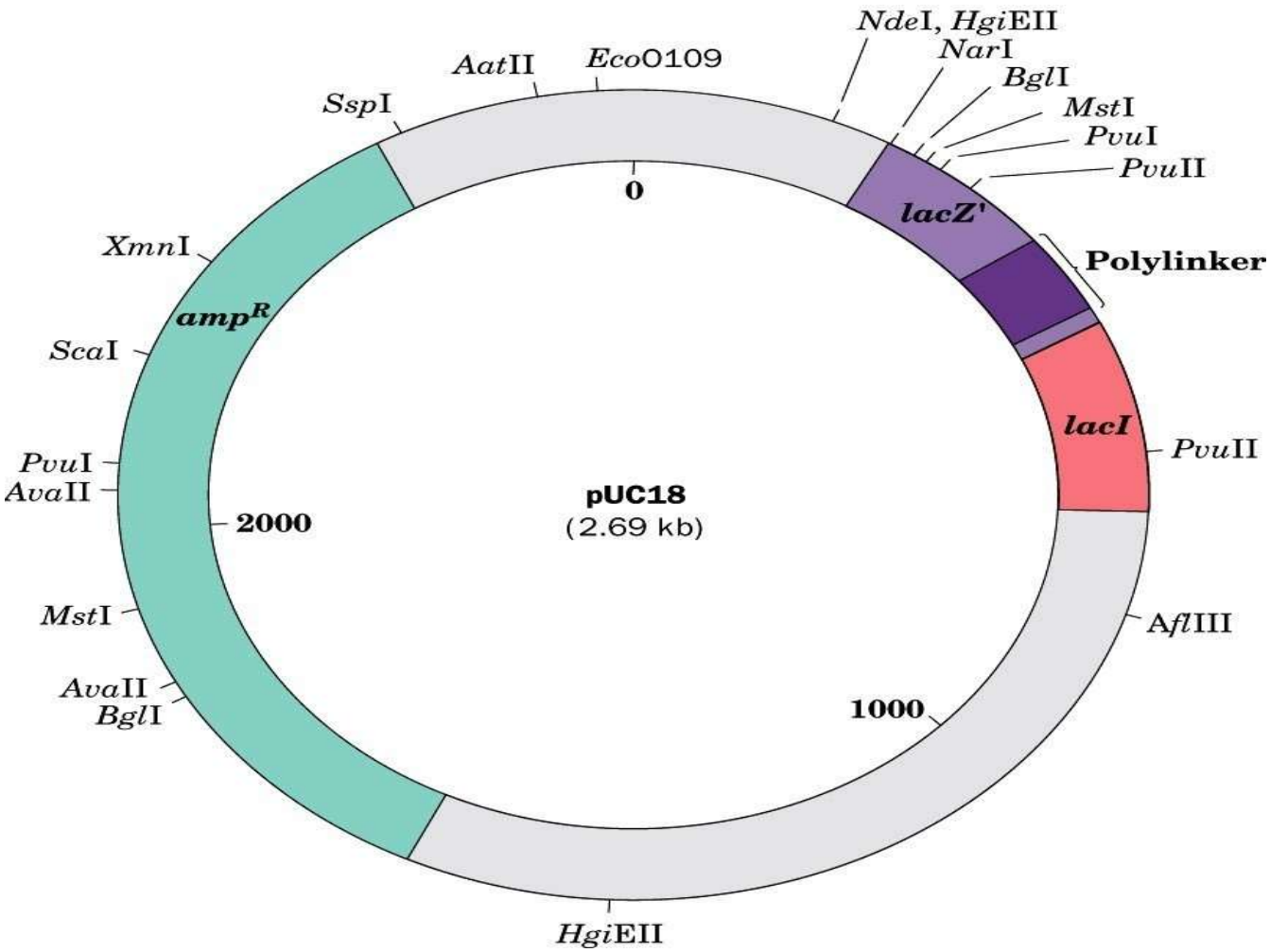
- pBR322 requires double screening
- pBR322 has limited number of restriction site

For these reasons pUC (on the left) was engineered

pUC8- Lac selection plasmid

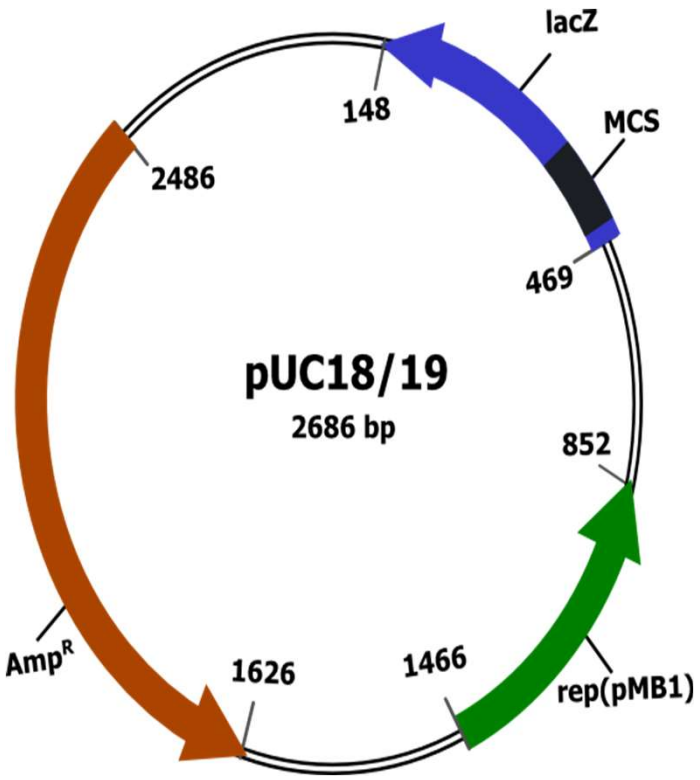
- It is 2750bp in size and is one of the most popular *E. coli* cloning vectors.
- Derived from pBR322 in which only the ori and the amp^R genes remain.
- The nucleotide sequence of amp^R gene has been changed so that it no longer contains the unique restriction sites.
- The restriction sites are clustered into the *lac* Z' gene.
- It has a high copy number of 500-700 molecules per cell even before amplification.
- The identification of the recombinant cells can be achieved by a single step process i.e. by plating onto agar medium containing ampicillin and X-gal.

The pUC18 cloning vector



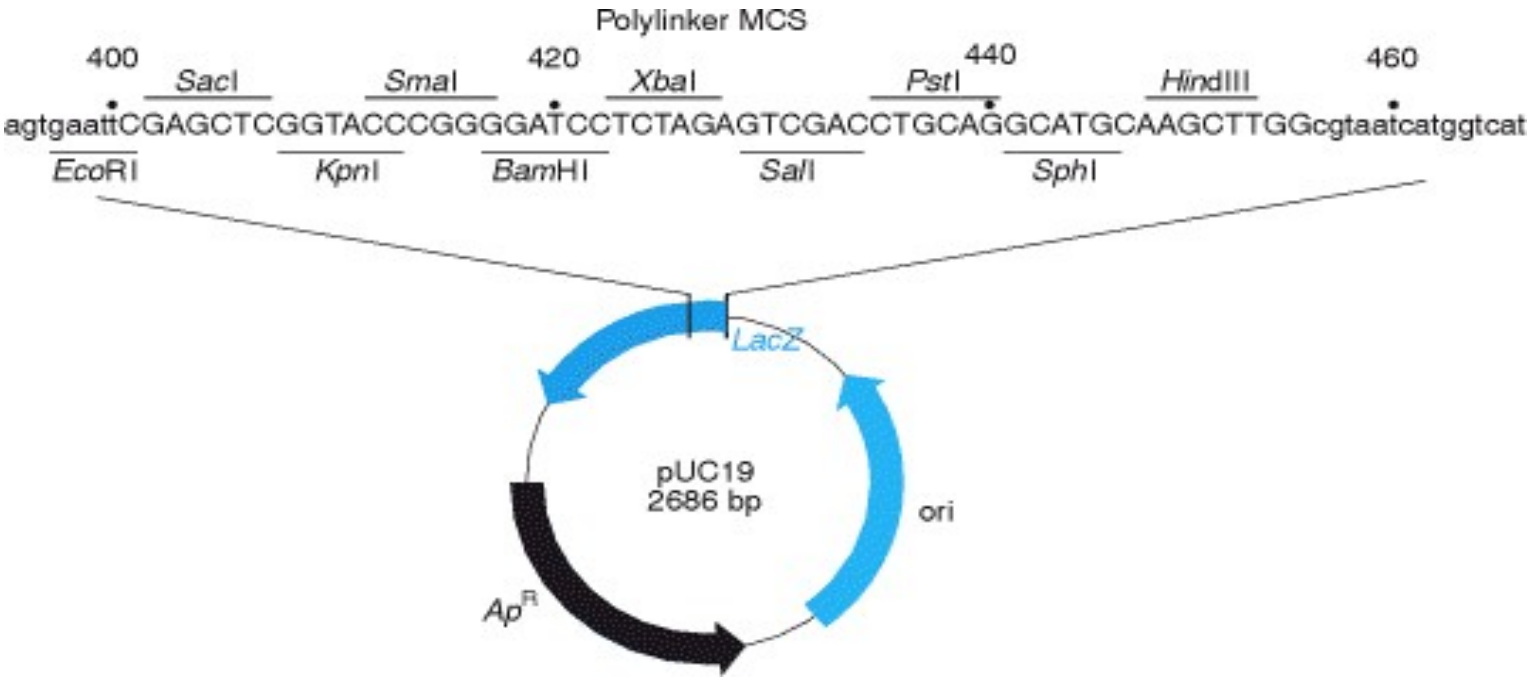
Next Major Advance in Plasmid(ology)

- The inclusion of polylinkers into plasmid vectors
- Polylinker is a tandem array of restriction endonuclease sites in a very short expanse of DNA
- For example, pUC18's polylinker
  - Sites for 13 RE's
  - Region spans the equivalent of 20 amino acids or 60 nucleotides



The Polylinker Advantage

- Unique sites (usually)
- Insert excision facilitated
- Restriction endonuclease mapping and Subcloning made easier
- Directional cloning



Blue-White screening for pUC18

- Colonies with recombinant plasmids are white, and colonies with nonrecombinant plasmids are blue.
- Resistant to ampicillin, has (amp^r gene)
- Contains portion of the lac operon which codes for beta-galactosidase.
- X-gal is a substrate of beta-galactosidase and turns blue in the presence of functional beta-galactosidase is added to the medium.
- Insertion of foreign DNA into the polylinker disrupts the lac operon, beta-galactosidase becomes non-functional and the colonies fail to turn blue, but appear white.

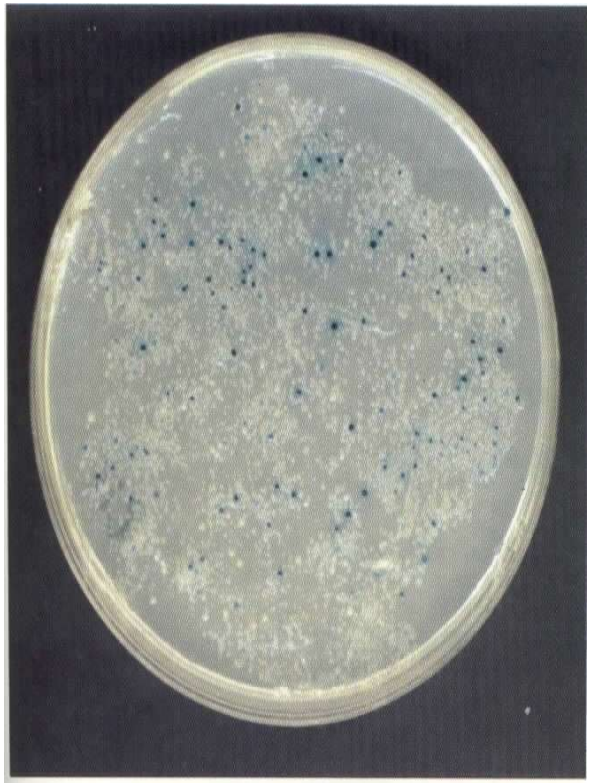


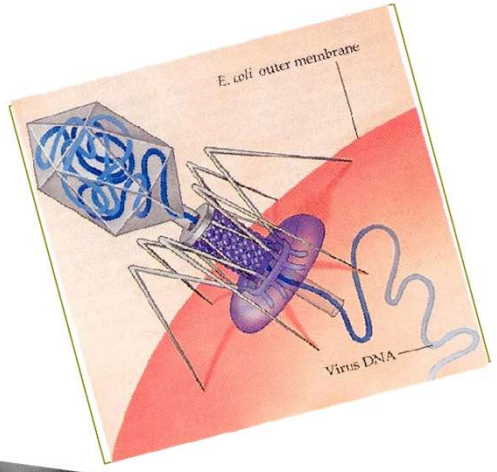
Figure 9.8 Blue-white screening on medium with ampicillin, X-gal, and IPTG. Blue colonies contain nonrecombinant plasmids. White colonies contain recombinant plasmids and can be isolated directly from this plate.

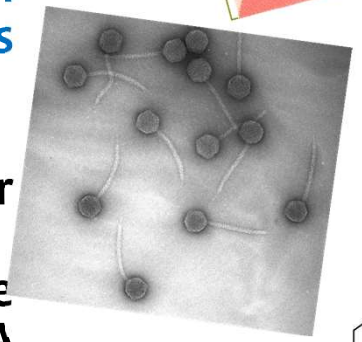
Bacteriophage

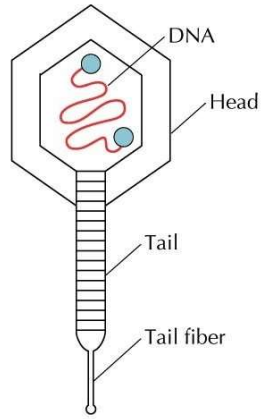
- These are the viruses that specifically infect bacteria and during infection inject the phage DNA into the host cell where it undergoes replication.
- The phages are simple in structure and consist of DNA molecule having several genes for phage replication which is surrounded by a capsid made up of proteins.

λ Phage

- It is 49kb in size and is used as a cloning vector because:
  - The linear double stranded DNA molecule has a stretch of 12 nucleotides at its either ends which act as sticky ends or cohesive ends (cos sites)
  - They can base pair to form a circular DNA molecule which is important for insertion into the bacterial genome.
  - Another role of cos sites is in the formation of large number of λ DNA molecules by rolling circle mechanism of replication

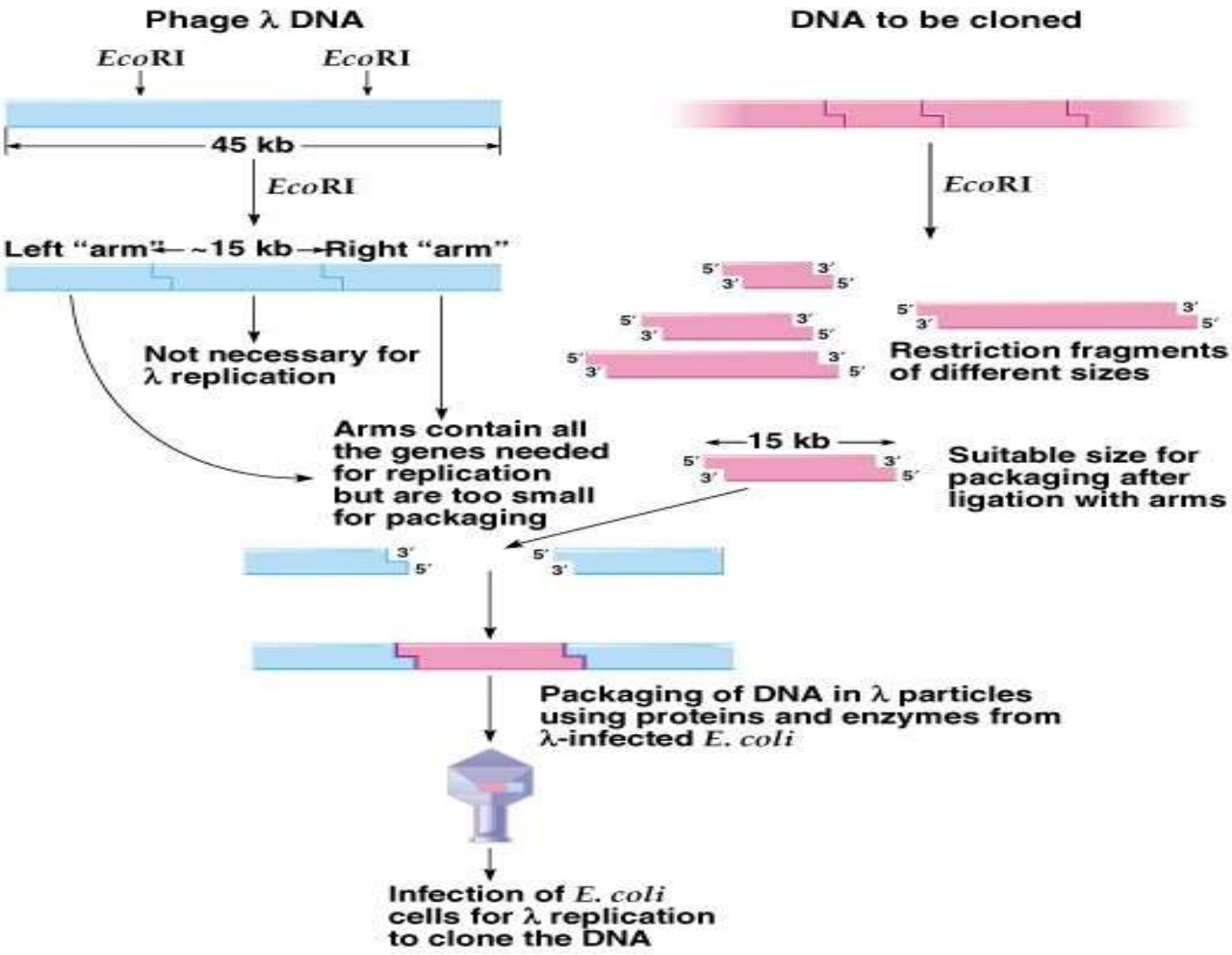






λ Phage Cloning Vector

- It has a large size genome (49kb) and only 3kb new DNA can be inserted because if the size of the molecule is more than 52kb then it can not be packaged into the head of the phage.
- The phage has more than one recognition sequence for almost all the restriction endonucleases. So the use of any restriction enzyme will break the phage DNA into number of small fragments.
- Despite these disadvantages, λ phages are used to clone large DNA (5kb to 25kb) molecules.


Phage λ DNA
EcoRI
EcoRI
45 kb
EcoRI
Left "arm"
~15 kb
Right "arm"
Not necessary for λ replication
Arms contain all the genes needed for replication but are too small for packaging
DNA to be cloned
EcoRI
Restriction fragments of different sizes
15 kb
Suitable size for packaging after ligation with arms
Packaging of DNA in λ particles using proteins and enzymes from λ-infected E. coli
Infection of E. coli cells for λ replication to clone the DNA

Phage λ cloning vectors:

- Engineered version of bacteriophage λ (infects *E. coli*).
- Central region of the λ chromosome (linear) is cut with a restriction enzyme and digested DNA is inserted.
- DNA is packaged in phage heads to form virus particles.
- Phages with both ends of the λ chormosome and a 37-52 kb insert replicate by infecting *E. coli*.
- Phages replicate using *E. coli* and the lytic cycle.
- Produces large quantities of 37-52 kb cloned DNA.
- Like plasmid vectors, large number of restriction sites available; phage λ cloning vectors are useful for larger DNA fragments than pUC19 plasmid vectors.

M13 Phage

- It is 6407 nucleotides in length, circular and consists of a single stranded DNA molecule and is used as a cloning vector because It is less than 10kb in size.

- Single-stranded, circular genome, 6.4 kb
- Infect only F+ bacteria, using pilus F- coded
- Can clone pieces of DNA up to 6X the M13 genome size (36 kb) -- but the larger the DNA, the less stable the clone is…..
- Drawback: foreign DNA can be unstable (slows down host cell growth, so deletions confer a selective advantage)

M13 Phage Cloning Vector

- The M13 genome is 6.4kb in length
- Consists of ten closely packed genes for replication of the phage.
- There is a single 507 nucleotide intergenic sequence (IS) into which new DNA can be inserted
- This region includes the *ori* gene

Useful for

- Sequencing
- Site-directed mutagenesis (later)
- Any other technique that requires single stranded DNA

Bacteriophage vectors

Advantages:

- Useful for cloning large DNA fragments (10 - 23 kb)
- Inherent size selection for large inserts

Disadvantages:

- Less easy to handle

Uses of Bacteriophages:

Lambda phage cloning vectors:

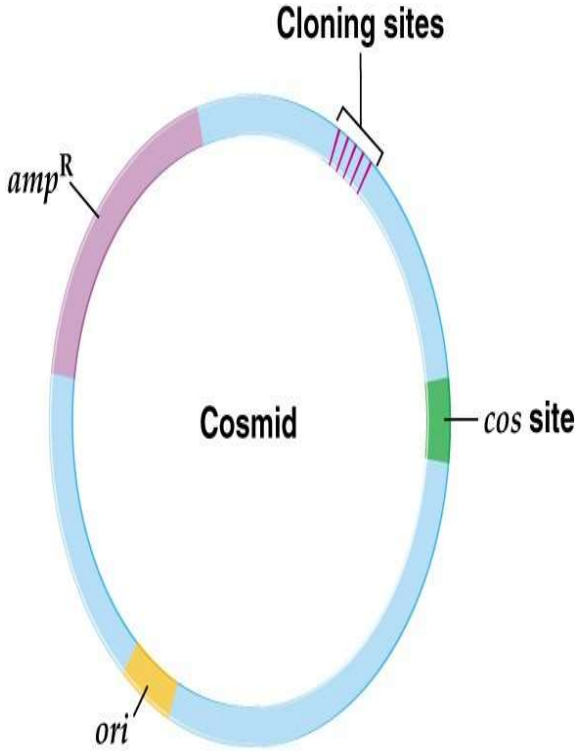
- For gene cloning of large DNA fragments (eukaryotic genes)
- Excellent selection capability (stuffer stuff)
- Clone lots of precisely-sized DNA fragments for library construction

M13 based cloning vectors:

- Single-stranded DNA
- Sequencing
- Site-directed mutagenesis

Cosmids

- Features of both plasmid and lambda phage cloning vectors.
- Circular.
- Do not occur naturally
- Origin (*ori*) sequence for *E. coli*.
- Selectable marker, e.g. amp^R.
- Restriction sites (for cloning).
- Contain Phage λ (lambda) cos sites which permits packaging into λ phage heads and therefore introduction to *E. coli* cells.
- Packaging only occurs with 37-52 kb fragments - selection for large fragments
- Useful for 37-52 kb.
- Packaged DNA is inserted into cells and then replicates as a very large plasmid



Bacterial Artificial Chromosomes (BACs):

Vectors that enable artificial chromosomes to be created and cloned into *E. coli*.

Features:

- Useful for cloning up to 200-300 kb, but can be handled like regular bacterial plasmid vectors.
- Useful for sequencing large stretches of chromosomal DNA; frequently used in genome sequencing projects.

Like other vectors, BACs contain:

- Origin (*ori*) sequence derived from an *E. coli* plasmid called the F factor.
- Multiple cloning sites (restriction sites).
- Selectable markers (antibiotic resistance).

BACs: Bacterial Artificial Chromosomes

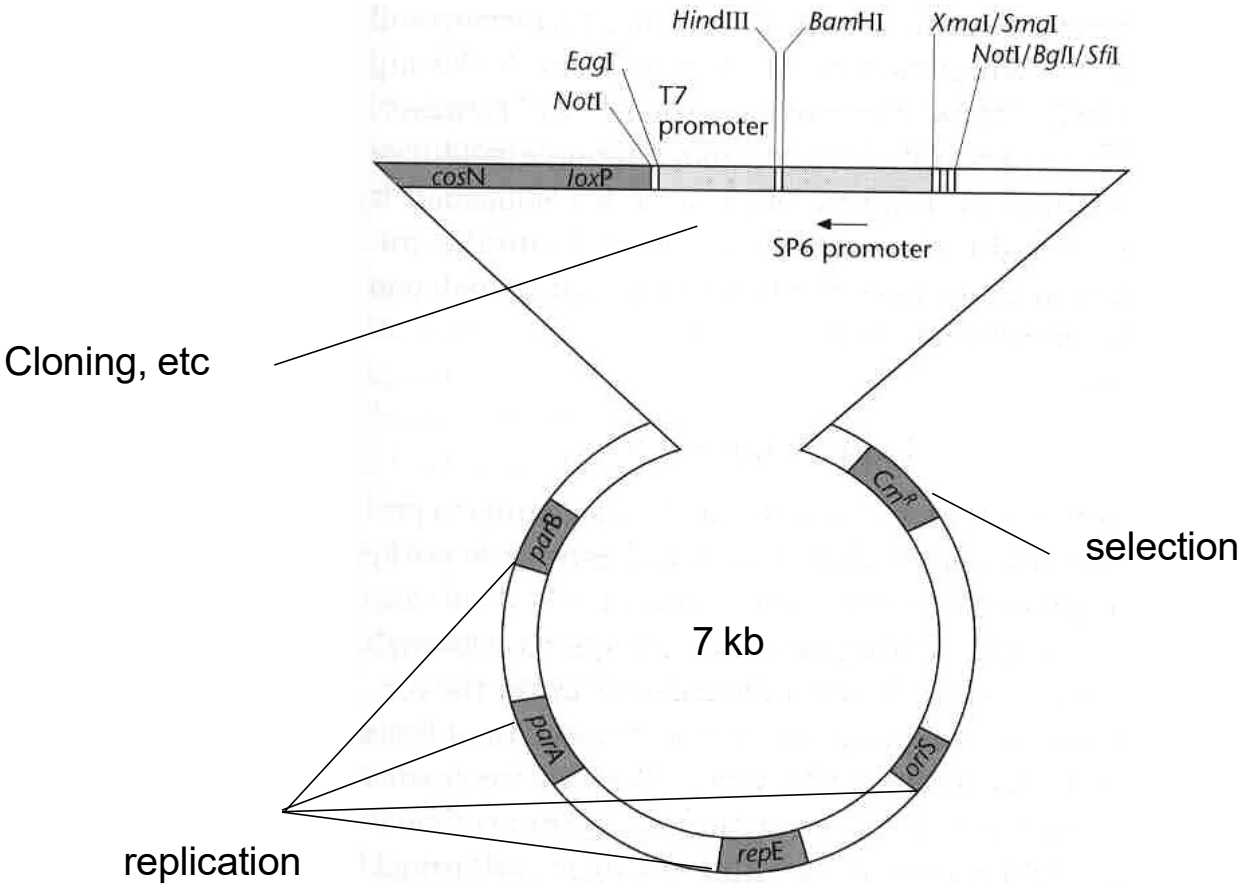
- Based on the F factor of E. coli:
  - 100 kb plasmid, propagates through conjugation
  - low copy number (1-2 copies per cell)
  - 2 genes (parA and parB): accurate partitioning during cell division

- BACs: just have par genes, replication ori, cloning sites, selectable marker

- Can propagate very large pieces of DNA: up to 300 kb

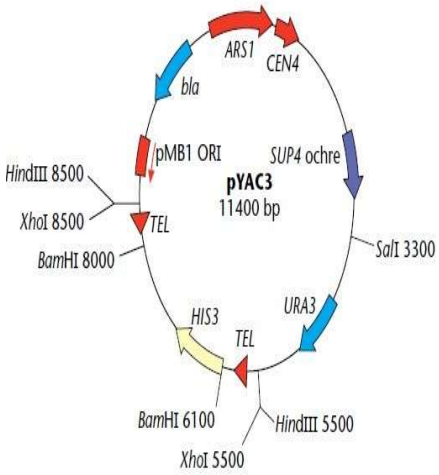
- Relatively easy to manipulate: move into cells by transformation (electroporation)

General BAC vector



YACs: Yeast Artificial Chromosomes

- Vectors that enable artificial chromosomes to be created and cloned into yeast.
- Based on the chromosome of Yeast



Features:

- CEN1, centromere sequence◊segregation
- TEL, telomere sequences◊extremity protection
- ARS1, autonomous replicating sequence◊replication
- Selectable marker (amino acid dependence, etc.) on each arm.
- *Amp*
- ori, origin of replication for propagation in an *E. coli* host.
- Restriction sites (for DNA ligation).
- Useful for cloning very large DNA fragments up to 500
- kb; useful for very large DNA fragments.

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DISADVANTAGES OF YAC

- Very fragile and prone to breakage,
- Unstable, with their foreign DNA inserts often being deleted
- Loss of the entire YAC during mitotic growth
- Difficult to separate the YAC from the other host chromosomes
- The yield of DNA is not high

Shuttle vectors:

1. Capable of replicating in two or more types of hosts.
2. Replicate autonomously, or integrate into the host genome and replicate when the host replicates.
3. Commonly used for transporting genes from one organism to another (i.e., transforming animal and plant cells).

VECTOR pYES2.1

What determines the choice of vector

- insert size

  vector size

  restriction sites

  copy number

  cloning efficiency

  ability to screen for inserts

Application of Recombinant DNA technology

- Recombinant DNA is widely used in biotechnology, medicine and research.
- The most common application of recombinant DNA is in basic research, in which the technology is important to most current work in the biological and biomedical sciences.
- Recombinant DNA is used to identify, map and sequence genes, and to determine their function.
- Recombinant proteins are widely used as reagents in laboratory experiments and to generate antibody probes for examining protein synthesis within cells and organisms.
- Many additional practical applications of recombinant DNA are found in industry, food production, human and veterinary medicine, agriculture, and bioengineering.

Applications of rDNA technology

Manufacture of proteins/hormones Interferon, plasminogen activating factor, blood clotting factors, insulin, growth hormone.

- **AIDS test:** Has become simple & rapid
- **Diagnosis of molecular diseases:** sickle cell anaemia, thalassaemia, familial hypercholesterolaemia, cystic fibrosis
- **Prenatal diagnosis:** DNA from cells collected from amniotic fluid, chorionic villi

Gene Therapy:

- This is achieved by cloning a gene into a vector that will readily be taken up & incorporated into genome of a host cell.

ADA deficiency has been successfully treated

Application in Agriculture:

- Genetically engineered plants are developed to resist draught & diseases. Good quality of food & increased yield of crops is also possible.

- **Industrial Application:**

  Enzymes---use to produce sugars, cheese, detergents.

  Protein products---used as food additives, increases nutritive value, besides imparting flavour.

- **Application in forensic medicine:** The restriction analysis pattern of DNA of one individual will be very specific(DNA fingerprinting),but the pattern will be different from person to person. Helps to identify criminals & to settle disputes of parenthood of children.

- **Transgenesis:** Gene replacement therapy will not pass on to offspring. Therefore genes are transferred into fertilised ovum which will be found in