RECOMBINANT DNA TECHNOLOGY

II M.Sc., MICROBIOLOGY
SEMESTER III

UNIT I (TOOLS AND ENZYMES IN rDNA TECHNOLOGY)

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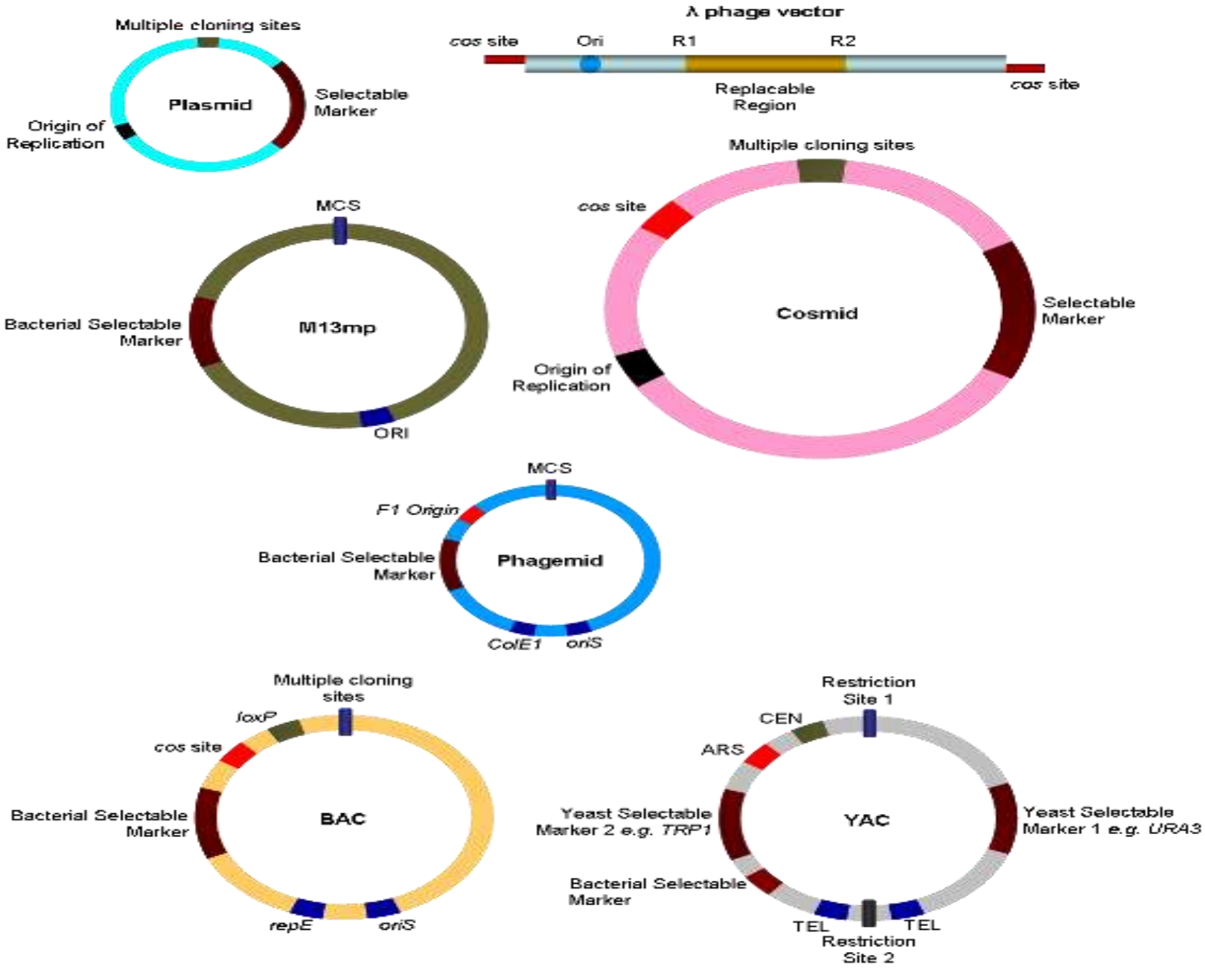


Types of Vectors

CLONING VECTORS

INTRODUCTION

CLONING :

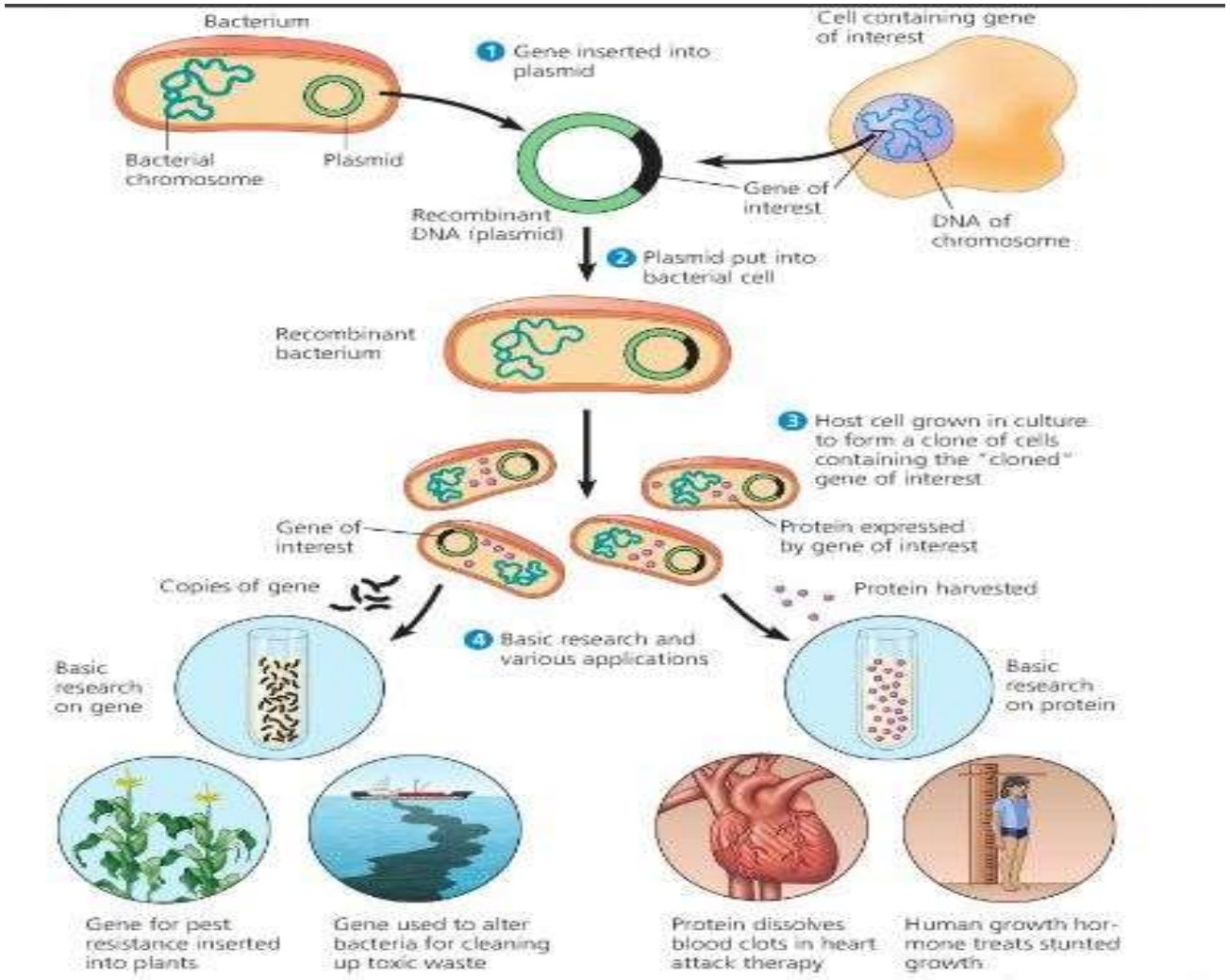
- Cloning is the process of producing similar populations of genetically identical individuals that occurs in nature.
- Cloning refers to processes used to create copies of DNA fragments (molecular cloning), cells (cell cloning), or organisms.

VECTORS

- A cloning vector is a small piece of DNA, taken from a virus, a plasmid, or the cell of a higher organism, that can be stably maintained in an organism, and into which a foreign DNA fragment can be inserted(for cloning purposes).

STEPS INVOLVED IN GENE CLONING

1. Generation of the DNA fragment
2. Construction of the recombinant DNA molecule by joining of DNA fragment with a vector
3. Introduction of the recombinant vector into host cell, multiplication of recombinant DNA molecule along with host cell



Properties and construction of a vector DNA molecule

1. Capability of autonomous replication

- Bacterial and viral genomes contain only one origin of replication while eukaryotes contain multiple origins

2. Small size, low molecular weight

- In small molecules the chances of occurrence of unique sites for restriction enzymes increases
- Efficiency of gene transfer is high with small vector molecules

3. Presence of selectable marker gene(s), reporter genes

- For easy detection of recombinants E.g.: antibiotic resistant genes, *lac* z or resistant to toxins, etc
- Reporter genes(these genes that allow successful clone to be easily identified. Such feature present in cloning vectors is used in blue-white selection.)

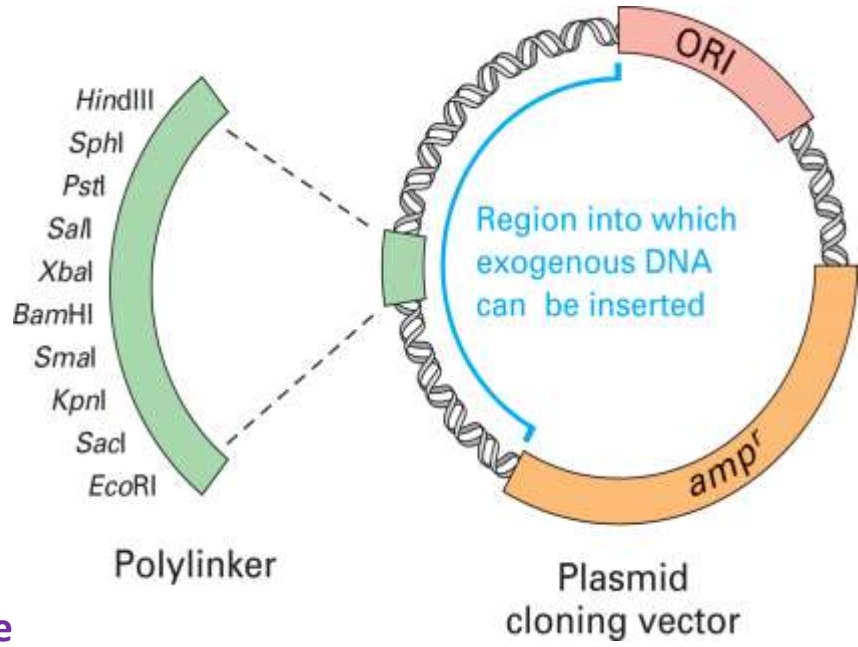
4. Presence of unique restriction enzyme sites or multiple cloning sites for inserting the target DNA

- Position of these restriction sites should be such that the insertion of a segment of DNA in any of these restriction sites bring about a phenotypic change in the characteristic of a vector molecule e.g.: loss of gene expression or loss of resistance to an antibiotic

5. Ease of purification

6. No effect on the replicative ability of vector due to insertion of target DNA

7. Ease of reintroduction into host cell with high efficiency



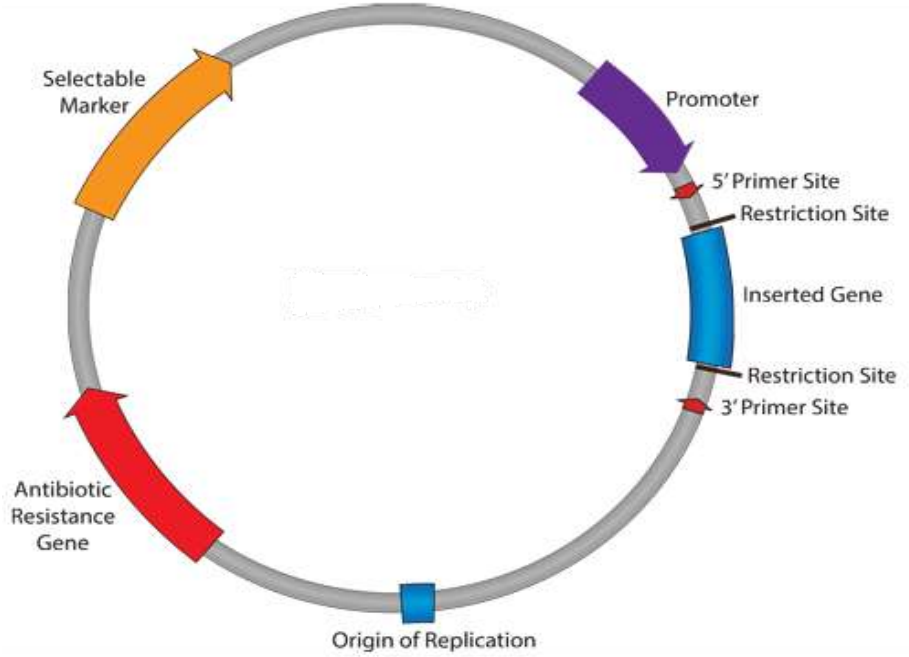
Properties and construction of a vector DNA molecule

- **8. Biological containment**
- Vectors should be biologically contained with no possibility of gene escape
- This can be achieved by non-conjugative and non-mobilized plasmid vectors

9. Presence of operator, promoters and ribosome binding sites

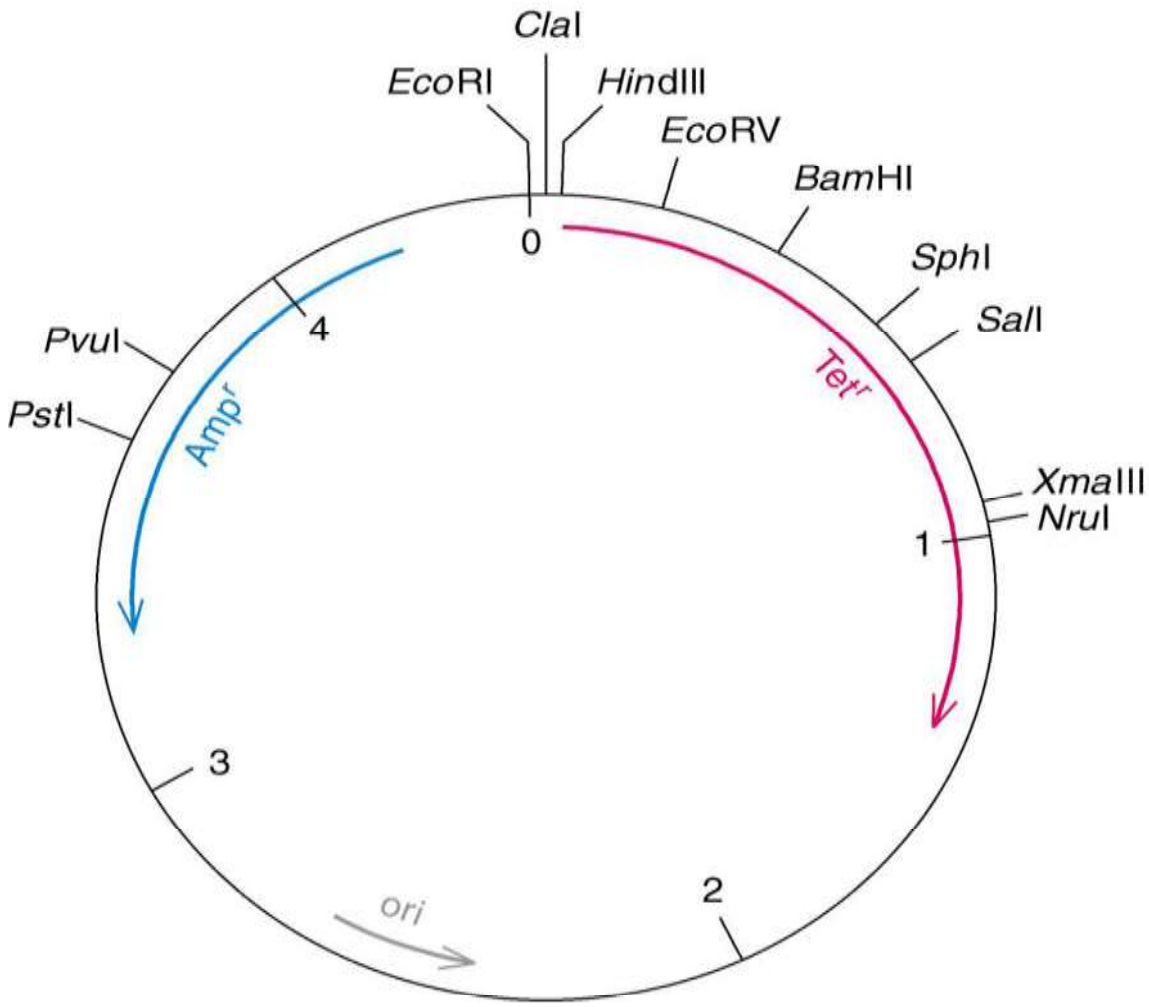
10. Presence of two different origins of replication or broad host range origin of replication

E.g.: shuttle vectors that contain two different origins of replication



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.**



Cloning vectors and expression vectors:

- **Cloning vectors** are used to amplify DNA fragments, usually in *E. coli.*

  They do not need a promotor to express the target sequence.

- **Expressing vectors** require specific promotors(prokaryotic, eukaryotic or RNA promotors depending on what kind of cell you wish to express) the gene product in.

CLONING VECTOR

- A cloning vector is a small piece of DNA, taken from a virus, a plasmid, that can be stably maintained in an organism, and into which a foreign DNA fragment can be inserted for cloning purposes.
- FEATURES -
  - Origin of replication.
  - Cloning site.
  - Selectable marker.
  - Reporter gene.

EXPRESSION VECTOR

- **The expression vector** is usually a plasmid or virus designed for protein expression in cells.
- FEATURES-

Expression vectors must have strong promoter, a strong termination codon, adjustment of the distance between the promoter and the cloned gene, and the insertion of a transcription termination sequence and a portable translation initiation sequence

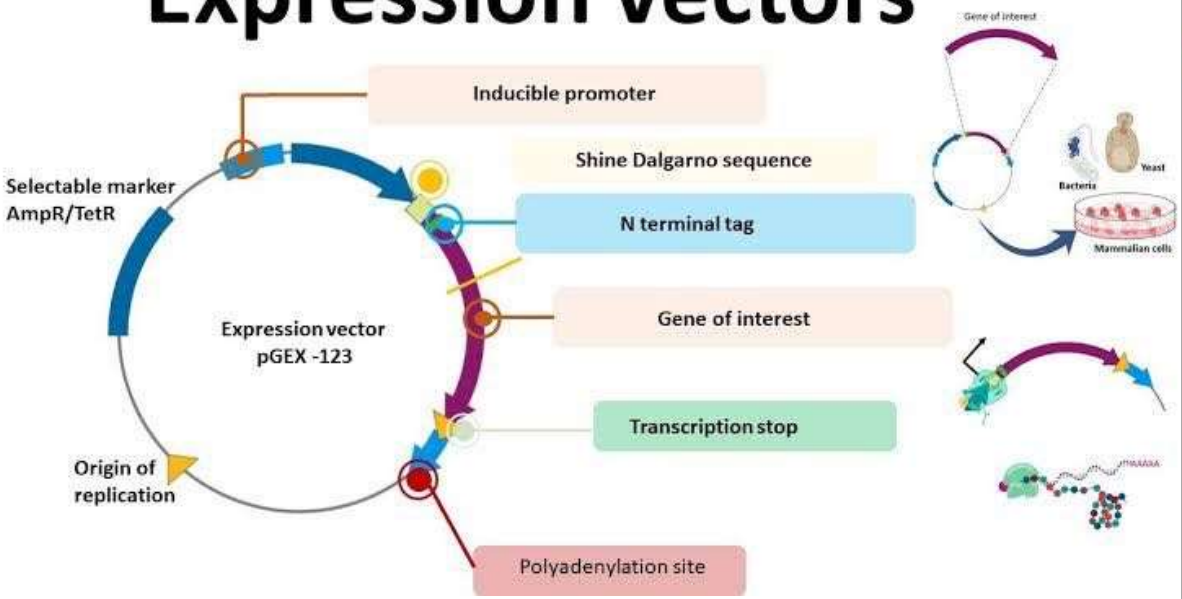
Expression vectors

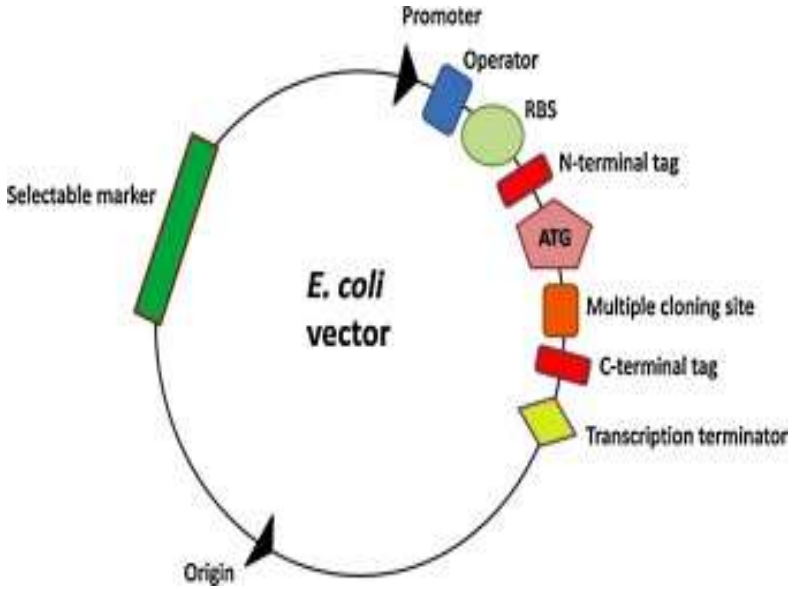
- An expression vector, otherwise known as an expression construct, is generally a plasmid that is used to introduce a specific gene into a target cell.
- Once the expression vector is inside the cell, the protein that is encoded by the gene is produced by the cellular-transcription and translation machinery.
- The plasmid is frequently engineered to contain regulatory sequences that act as enhancer and promoter regions and lead to efficient transcription of the gene carried on the expression vector.
- The goal of a well-designed expression vector is the production of large amounts of stable messenger RNA.
- Expression vectors must have expression signals such as a **strong promoter**, **a strong termination codon**, **adjustment of the distance between the promoter and the cloned gene,** **and** **the insertion of a transcription termination sequence** **and** **a PTIS** (portable translation initiation sequence).

- Expression vectors require not only transcription but translation of the vector's insert, thus requiring more components than simpler transcription-only vectors. Expression vectors require sequences that encode for:
  - Polyadenylation tail: Creates a polyadenylation tail at the end of the transcribed pre-mRNA that protects the mRNA from exonucleases and ensures transcriptional and translational termination: stabilizes mRNA production.
  - Minimal UTR length: UTRs contain specific characteristics that may impede transcription or translation, and thus the shortest UTRs or none at all are encoded for in optimal expression vectors.
  - Kozak sequence: Vectors should encode for a Kozak sequence in the mRNA, which assembles the ribosome for translation of the mRNA.

- After expression of the gene product, the purification of the protein is required; but since the vector is introduced to a host cell, the protein of interest should be purified from the proteins of the host cell. Therefore, to make the purification process easy, the cloned gene should have a tag. This tag could be histidine (His) tag or any other marker peptide.

Expression vectors





Shuttle Vector vs Expression Vector

More Information Online WWW.DIFFERENCEBETWEEN.COM

	Shuttle Vector	Expression Vector
DEFINITION	Shuttle vector is usually a plasmid that is not designed for gene expression studies in cells	Expression vector is usually a plasmid or virus that is designed for gene expression studies in cells
NEED FOR REGULATORY SEQUENCES	Does not need regulatory sequences such as strong promoter, enhancer, inducer and portable translation initiation sequence (PTIS) and strong terminator	Needs regulatory sequences such as strong promoter, enhancer, inducer and portable translation initiation sequence (PTIS) and strong terminator
USES	Used to quickly make multiple copies of the gene and used for in vitro experiment and modifications	Used for precise control of the expression of a recombinant protein used in protein production applications
EXAMPLES	Yeast and Ecoli shuttle vector (pJDB219) and *Bacillus subtilis* and *E.coli* shuttle vectors (pHV14, pEB10, pHP3)	pCI mammalian vector

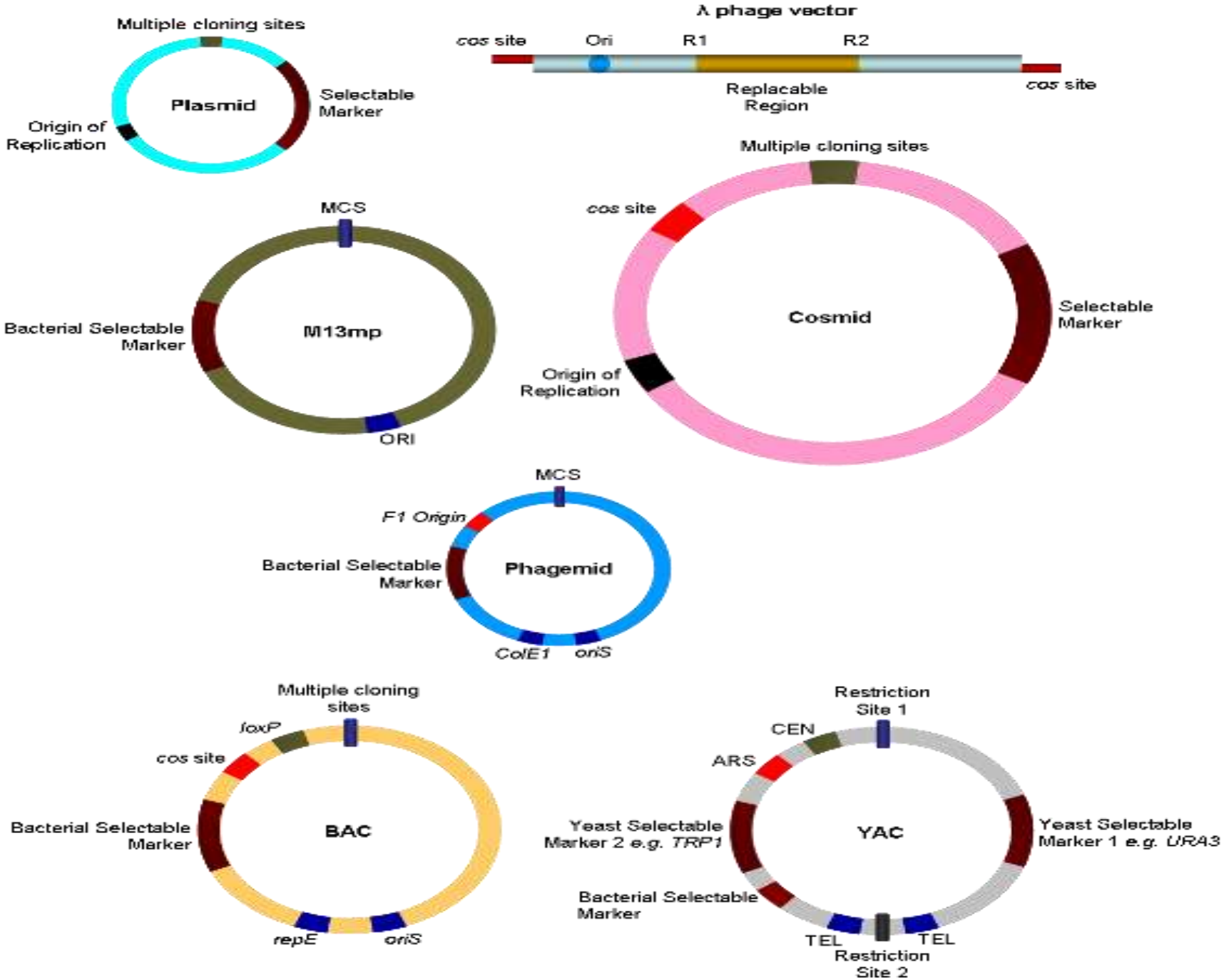
Types of Cloning Vectors

- 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

TYPES OF CLONING VECTORS

Vectors	targeted host
• Plasmid	Bacteria, Streptomyces
• Bacteriophages	Bacteria
• Cosmid	Bacteria
• Yeast Cloning Vectors	Yeasts
• Ti & Ri Plasmids	Transformation of cloned gene in higher plants.



Types of Vectors

Vector	Insert size	Source	Application
Plasmid	≤ 15 kb	Bacteria	Subcloning and downstream manipulation, cDNA cloning and expression assays
Phage	5-20 kb	Bacteriophage λ	Genomic DNA cloning, cDNA cloning and expression library
Cosmid	35-45 kb	Plasmid containing bacteriophage λ cos site	Genomic library construction
BAC (bacterial artificial chromosome)	75-300 kb	Plasmid ocntaining ori from E.coli F- plasmid	Analysis of large genomes
YAC (yeast artificial chromosome)	100-1000 kb (1 Mb)	Saccharomyces cerevisiae centromere, telomere and autonomously replicating sequence	Analysis of large genome, YAC transgenic mice
MAC (mammalian artificial chromosome)	100 kb to > 1 Mb	Mammalian centromere, telomere and origin of replication	Under development for use in animal biotechnology and human gene therapy

Types of Plasmids

- **Cloning Plasmids** - Cloning vectors are simple, often contain only a bacterial resistance gene, origin and MCS.
- **Expression Plasmids** - Used for gene expression. They contain promoter terminator sequences and the inserted gene. An expression vector can also include an enhancer sequence which increases the amount of protein or RNA produced.
- **Gene knock-down Plasmids** - Used for reducing the expression of an endogenous gene. This is frequently accomplished through expression of an shRNA targeting the mRNA of the gene of interest.
- **Reporter Plasmids** - Used for studying the function of genetic elements. They contain a reporter gene (e.g.,, luciferase or GFP) that offers a read-out of the activity of the genetic element.
- **Viral Plasmids** - used in delivery of genetic material into target cells just like a virus . One can use these plasmids to create viral particles, such as lentiviral, retroviral, and adenoviral particles.

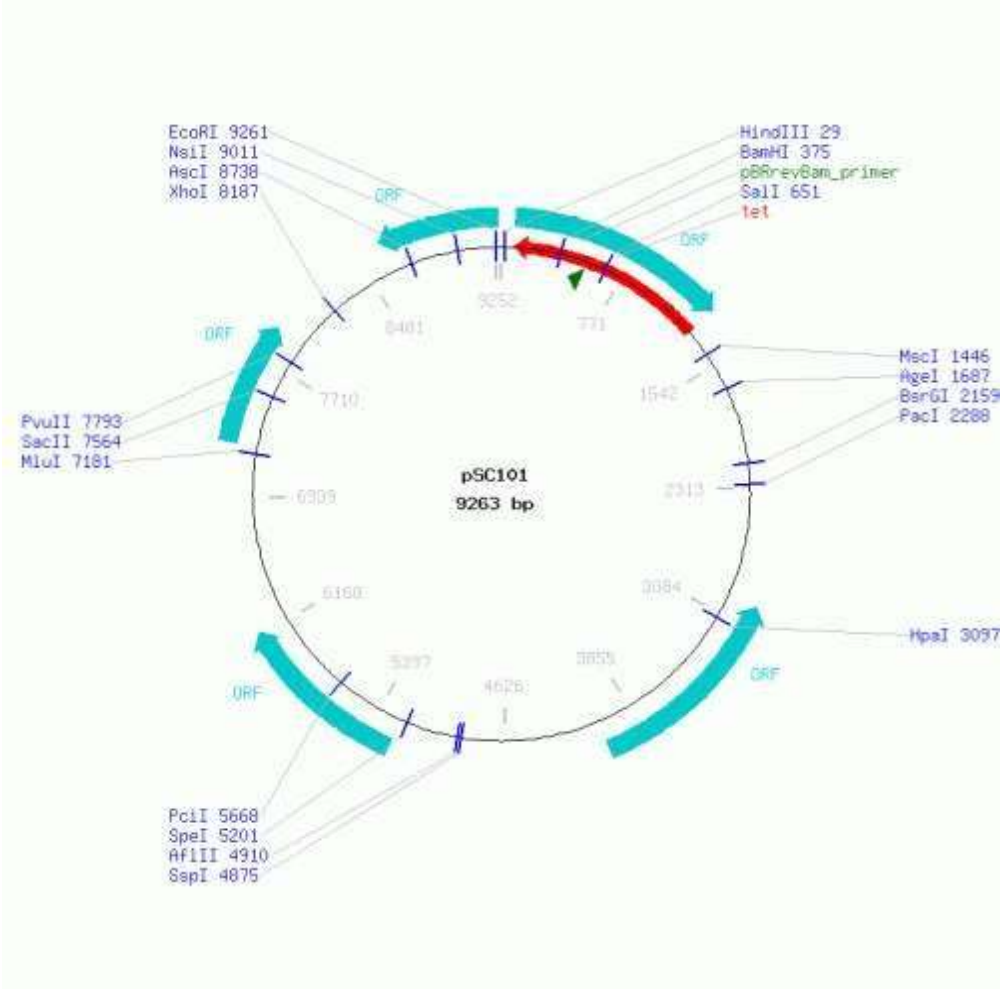
Natural plasmid vectors for *E. coli*

pSC101

- First used for in vitro cloning of eukaryotic DNA
- 9kbp in size
- Low copy number(1-2 copies)
- Has advantage of a single *Eco* RI site at which DNA can be inserted
- Has selectable marker for tetracycline resistance
- Derived from the conjugative plasmid R6-5

Disadvantages are

- Large size
- Stringent replicative control
- Low copy number
- Low insert capability



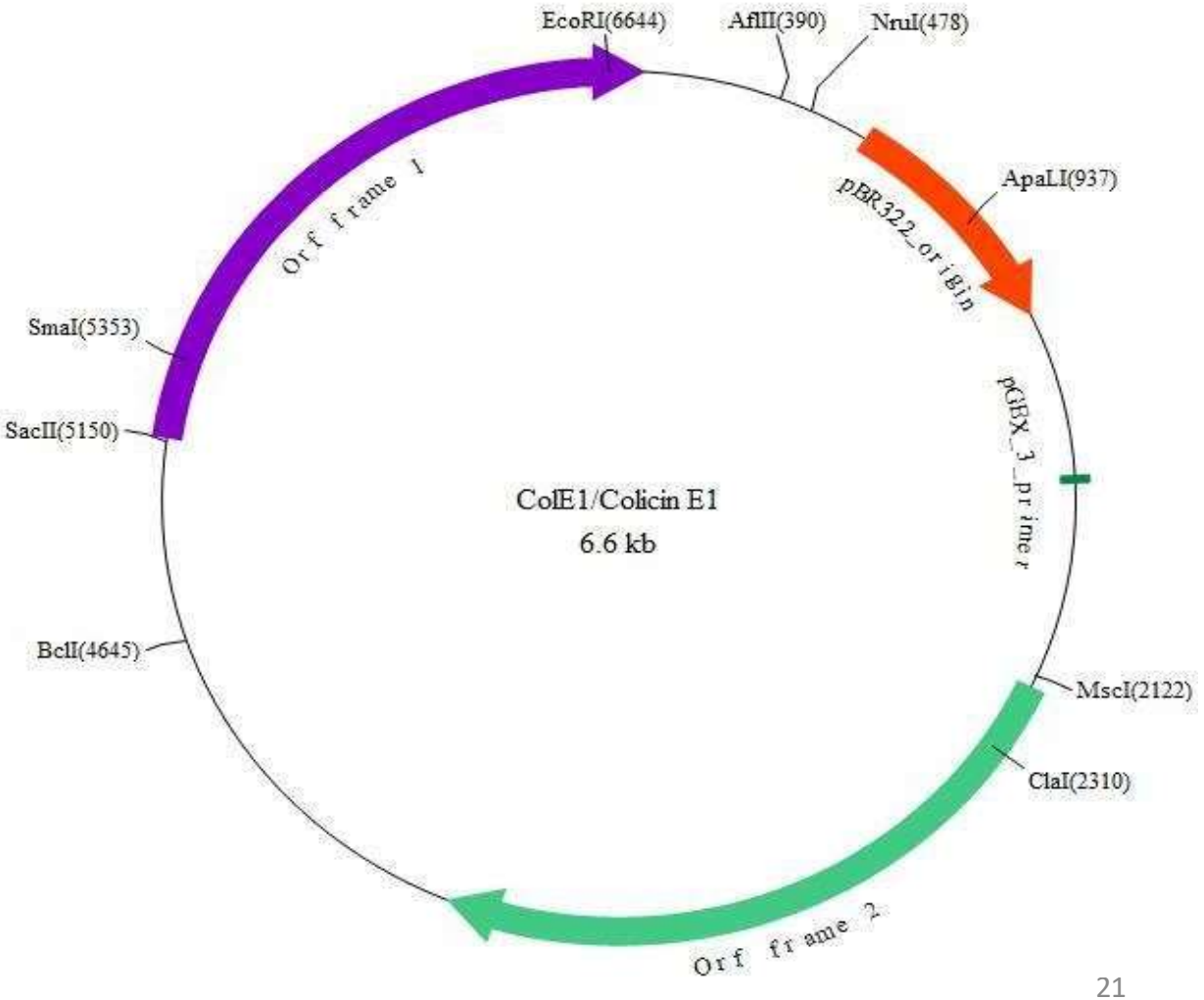
pSF 2124(RSF2124)

Produced by transfer of the ampicillin resistance gene

- Has ability for colicin biosynthesis
- Has high copy number
- Has single sites for Bam H1 and EcoR1
- Not currently used as vector as it does not provide easy selection by insertional inactivation
- Mobilizable plasmid

Col E1 Plasmid

- Small, circular colicingenic plasmid
- Codes for 57kDa protein toxin
- Size is 6,466bp
- Has *cea* gene for colicin production
- *imm* for immunity against colicin
- *kill* for killer or lysis protein
- *mob* for mobilization



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
   - ✓ twin 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 e.g. pUC8 is 2.1 kb and TOL is 117 kb in size.

TABLE 4.3 Replication origins of several *E. coli* plasmid vectors

Plasmid	*ori*	Copy number
pBR322	pMB1	15–20
pUC vectors	pMB1 mutant	100s
pET vectors	pMB1 mutant	100s
pBluescript	pMB1 mutant	100s
pACYC184	p15A	10–12
pSC101	pSC101	5

Plasmid Vector

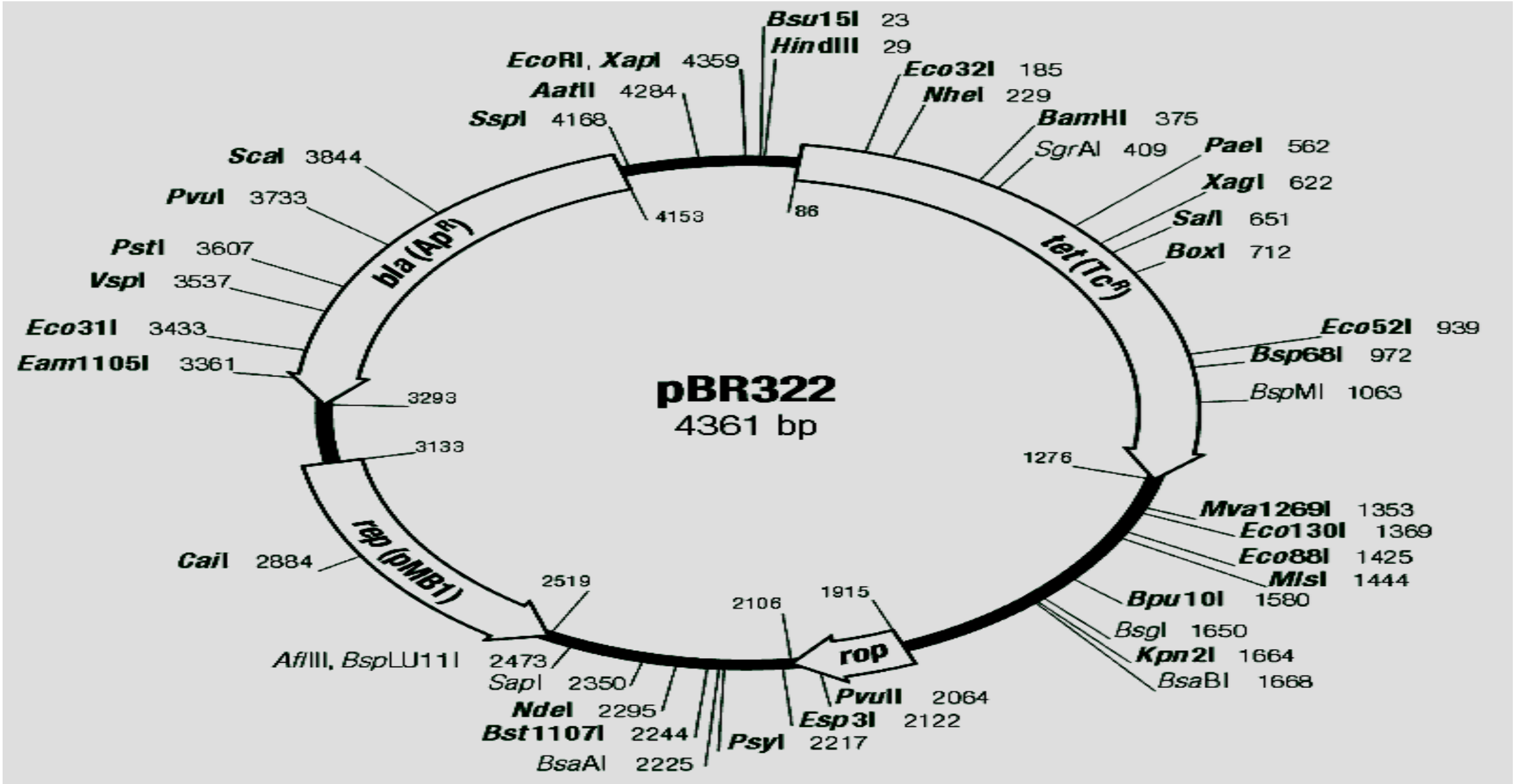
- **Plasmid vectors are double-stranded, extra-chromosomal DNA molecules, circular, self-replicating.**
- ***Advantages:***
  - **Small, easy to handle**
  - **Easy purification**
  - **Straightforward selection strategies**
  - **Useful for cloning small DNA fragments (< 10kbp)**

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

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. There 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.
- 11 unique R. sites within tetR gene and 6 R.sites inside ampR gene, sites where the plasmid can later be cut to insert foreign DNA.

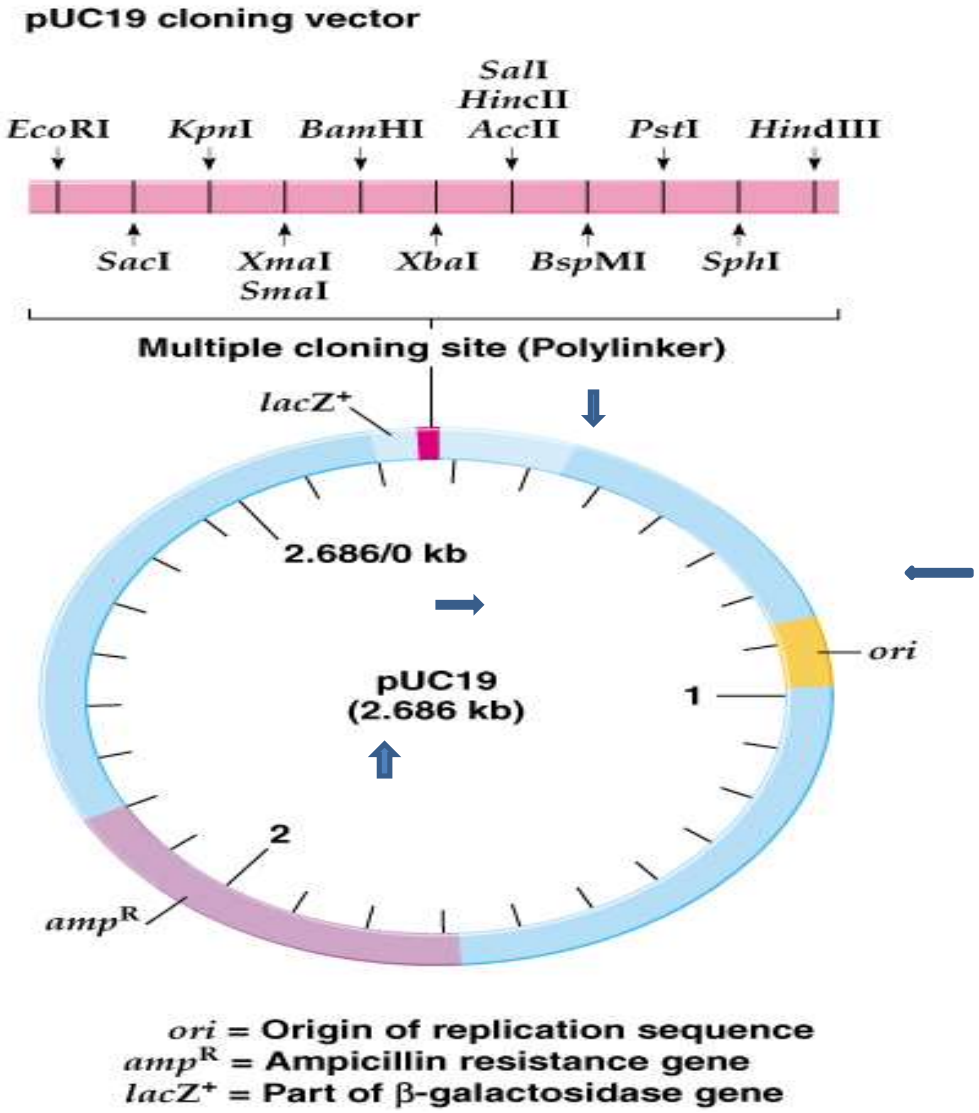
Features of pBR322



pBR327

- **It was produced by removing a 1089bp segment from pBR322.**
- **The amp^R and tet^R genes are intact.**
- **It has high copy number than pBR322 i.e. 30-45 molecules per *E. coli* cell. Thus, more the copies of the cloned genes more will be the effect of the cloned gene on the host cell detectable.**
- **The deletion destroys the conjugative ability of the vector which is important for biological containment.**

FROM pBR322 to pUC



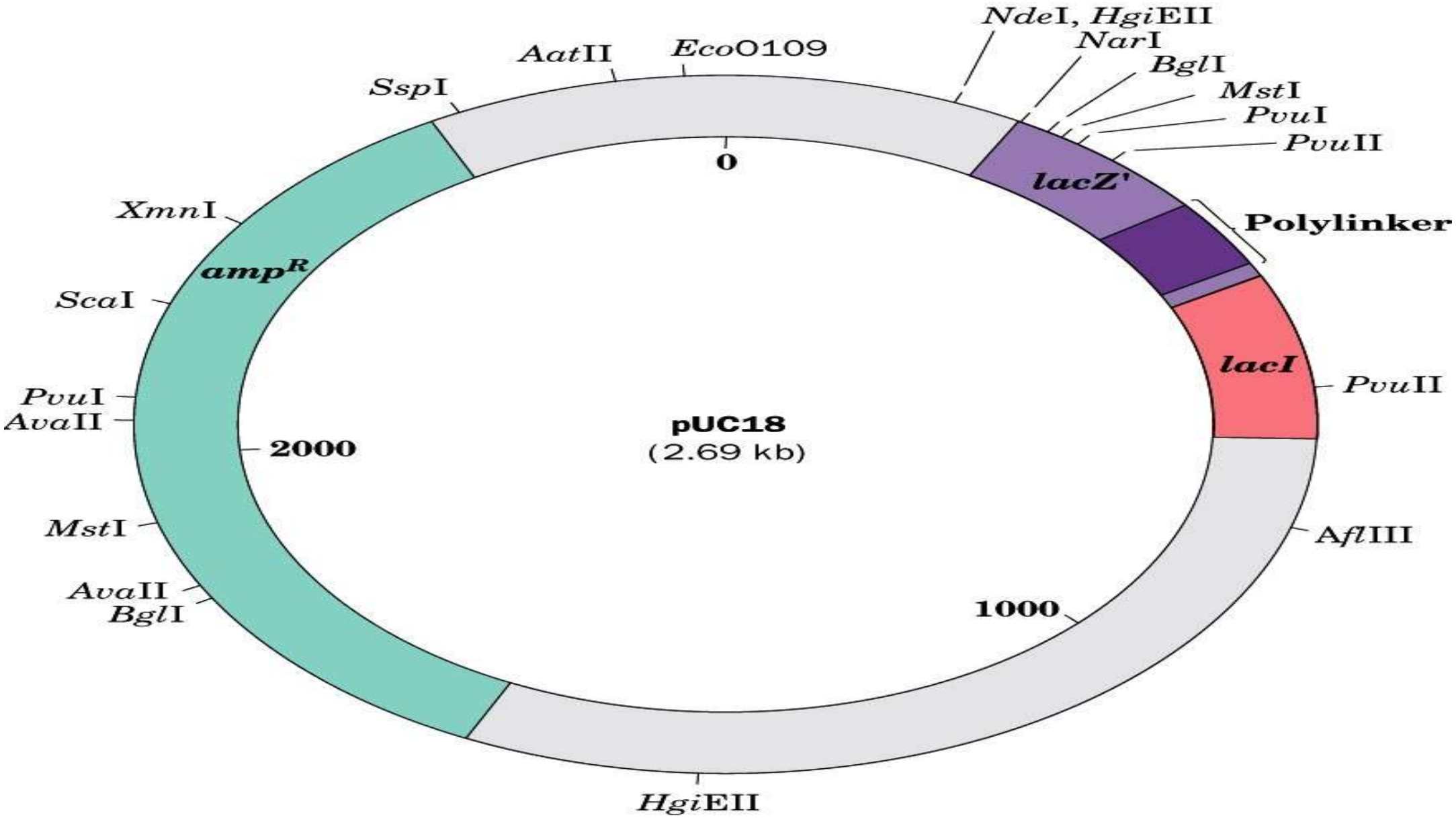
- pBR322 requires double screening
- pBR322 has limited number of restriction site

For these reasons pUC (on the left) was engineered

pUC8- Lac selection plasmid

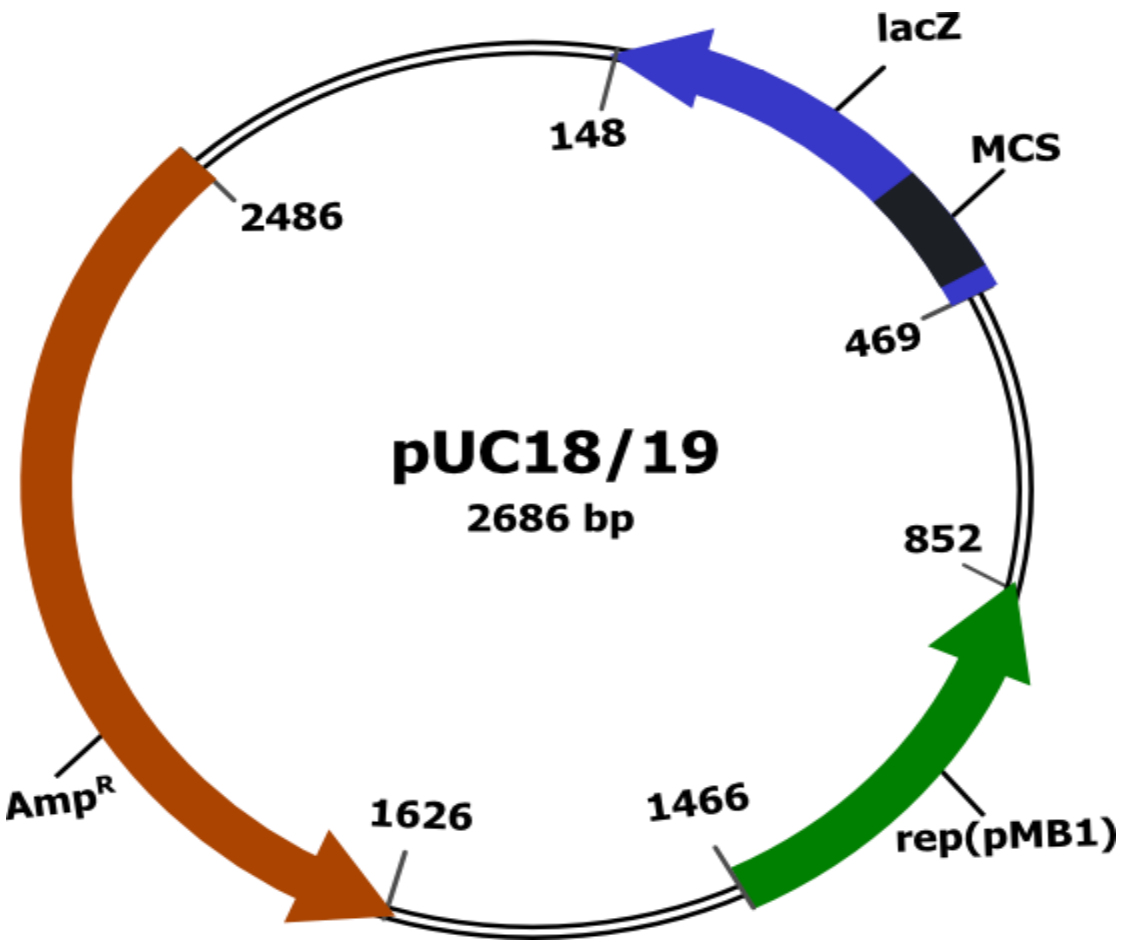
- **It is 2750bp in size and is one of the most popular *E. coli* cloning vectors.**
- **Derived from pBR322 in which only the ori and the amp^R genes remain.**
- **The nucleotide sequence of amp^R gene has been changed so that it no longer contains the unique restriction sites.**
- **The restriction 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 clustering of the restriction sites allows a DNA fragment with two different sticky ends to be cloned without resorting to additional manipulations.**
- **pUC18/pUC19 is a vector having different combinations of restriction sites and provide greater flexibility for the DNA to be cloned.**
- **The restriction site clusters in these vectors are the same as the clusters in M13mp series of vectors. Thus, the DNA cloned in pUC vectors can be transferred directly to its M13mp counterpart enabling the cloned gene to be obtained as a single stranded DNA.**

The pUC18 cloning vector



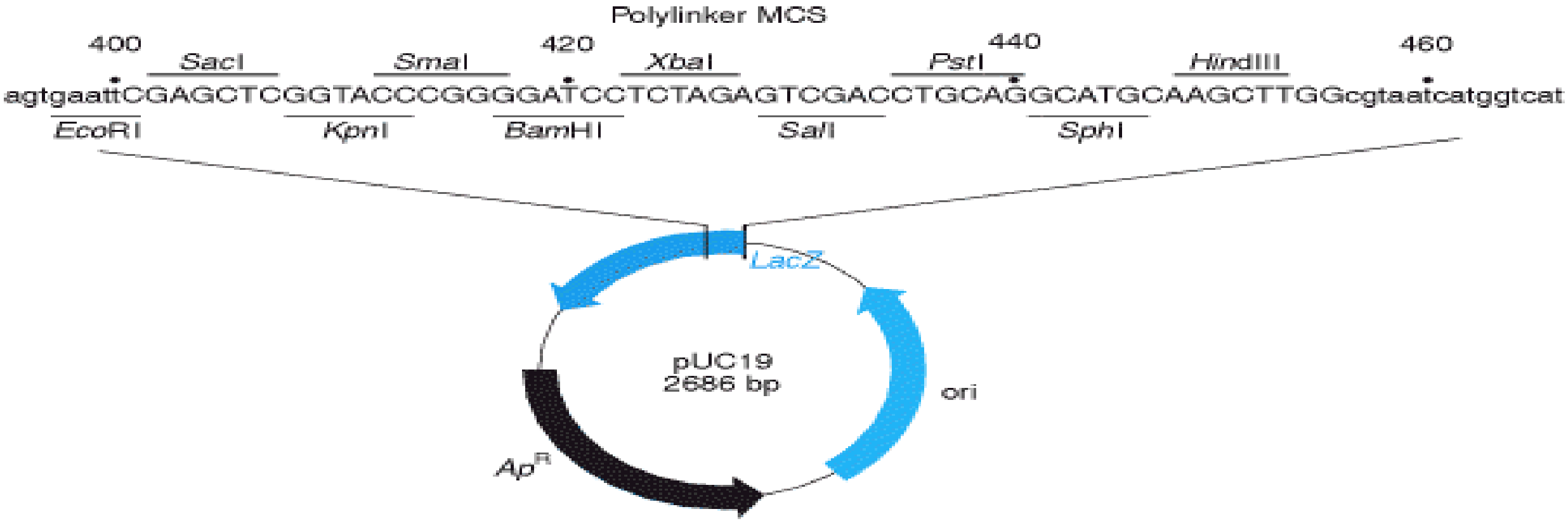
Polylinkers in Plasmid

- 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.

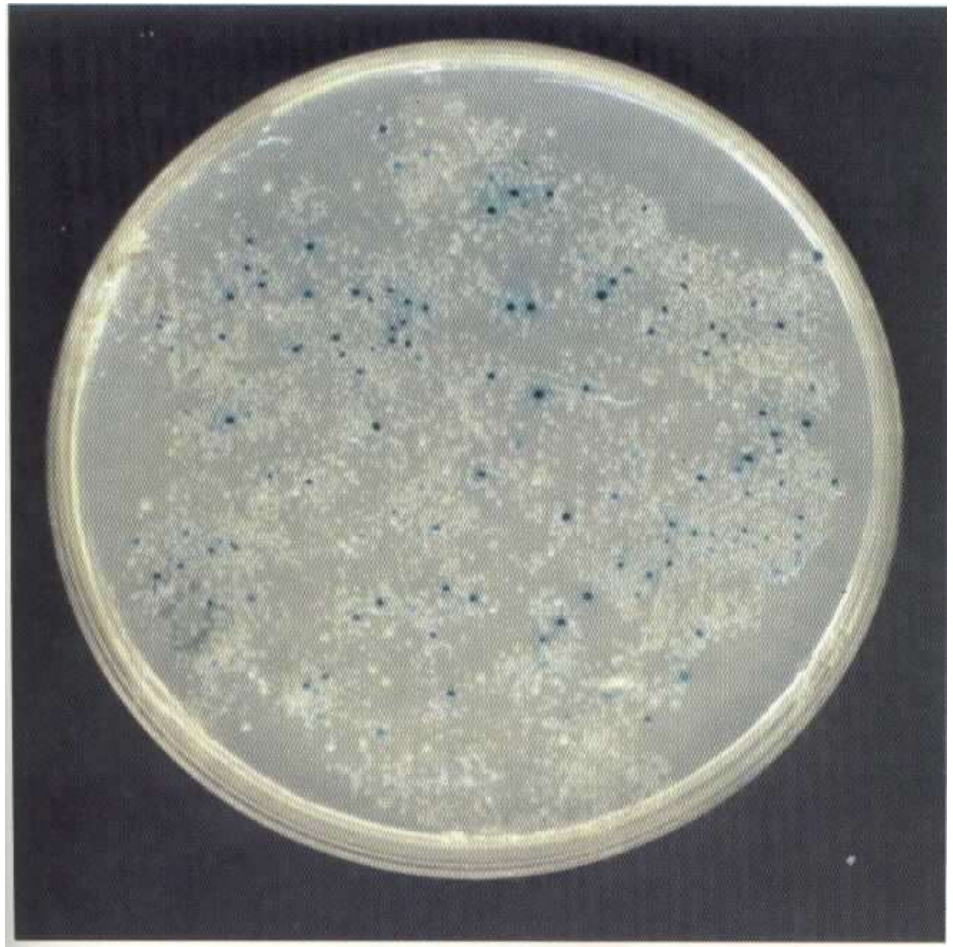


Figure 9.8 Blue-white screening on medium with ampicillin, X-gal, and IPTG. Blue colonies contain nonrecombinant plasmids. White colonies contain recombinant plasmids and can be isolated directly from this plate.

pGEM3Z

- It is very similar to pUC vector and is of same size (2750bp).
- It carries the amp^R and *Lac* Z gene. The cluster of restriction sites is present in the *Lac* Z gene.
- It has two promoter sequences i.e. T7 promoter (RNA polymerase of T7 bacteriophage) and SP6 (RNA polymerase of SP6 phage) promoter sequences that lie on the either side of the cluster of restriction sites.
- These promoters act as the sites for the attachment of RNA polymerase. Thus if a recombinant pGEM3Z is mixed with RNA polymerase in a test tube, transcription occurs and RNA copies of the cloned fragment are synthesized.

APPLICATIONS OF PLASMIDS VECTORS

- **In genetic engineering:** used to make copies of particular genes.
- **Production of large amounts of proteins:** This is a cheap and easy way of mass-producing a gene or the protein it then codes for, for example, insulin or even antibiotics.
- **Molecular studies:** of Plasmids are used in molecular studies of various organisms i.e., in synthetic biology, medicine, ecology.
- **Plasmids in Antibiotic Resistance:** In addition plasmids carry antibiotic resistance genes and their spread in pathogenic bacteria is of great medical importance.
- **Gene therapy:** Plasmid may also be used for gene transfer into human cells as potential treatment in gene therapy so that it may express the protein that is lacking in the cells.

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.

- On the basis of structure
  - Head and Tail phages: e.g. λ phage
  - Filamentous phages: e.g. M13

- On the basis of phage infection cycle

Lytic Phage:

✓ The infection cycle is completed very quickly and the release of new phage particles is associated with the lysis of the host cell

Lysogenic phage:

✓ The phage DNA gets integrated into the bacterial DNA known as PROPHAGE and after many cell divisions released by the lysis of the host cell e.g. λ phage.

✓ Some phages do not form prophages and the new phage particles are continuously assembled and released from the host cell without the lysis of the host cell e.g. M13 phage.

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.
✓The double stranded replicative form of the genome acts like a plasmid.
✓Genes cloned can be obtained in the form of single stranded DNA which is helpful in gene sequencing and *in vitro* mutagenesis.
✓It is easily prepared from the culture of infected *E. coli* cells.

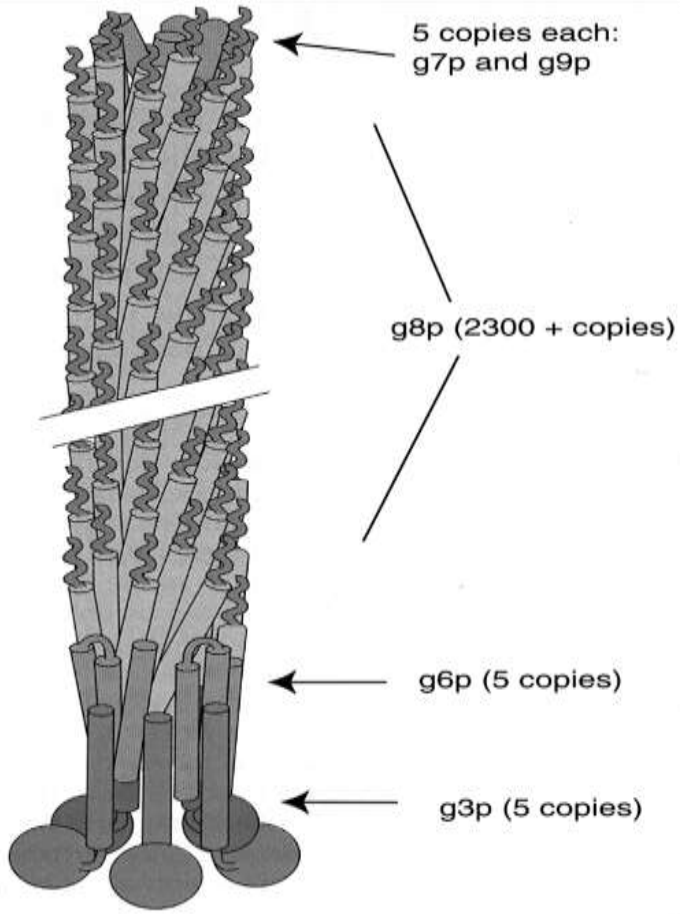
➢Single-stranded, circular genome, 6.4 kb
➢Infect only F+ bacteria, using pilus F- coded
➢Can clone pieces of DNA up to 6X the M13 genome size (36 kb) -- but the larger the DNA, the less stable the clone is…..

- Drawback: foreign DNA can be unstable (slows down host cell growth, so deletions confer a selective advantage)

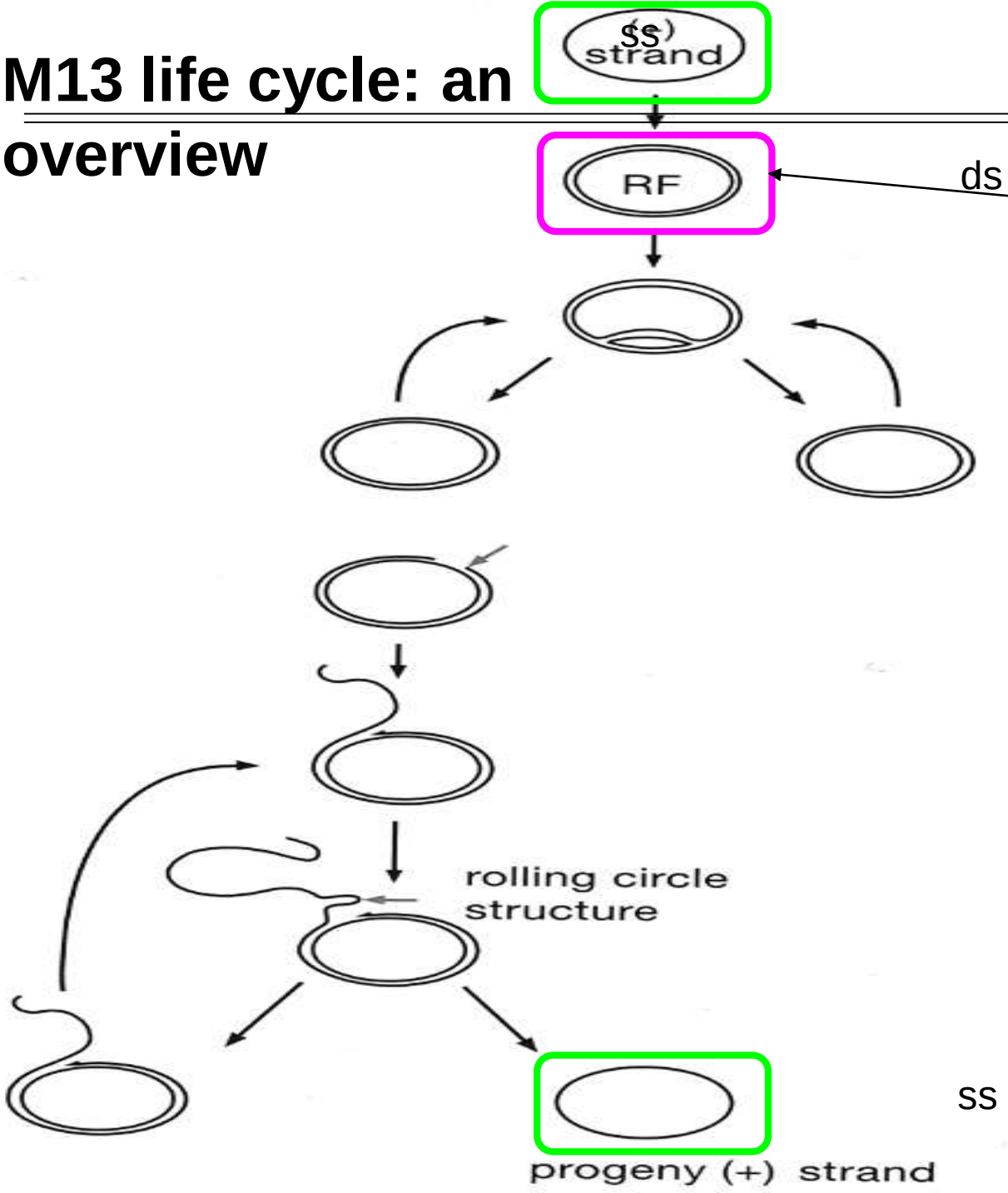
M13 Phage Cloning Vector

- 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 DN



M13 life cycle: an overview



Infecting single-stranded circular viral DNA is converted to double-stranded replicating form (RF) DNA by host-encoded enzymes.

Isolate for cloning

Several rounds of replication occur through θ structures.

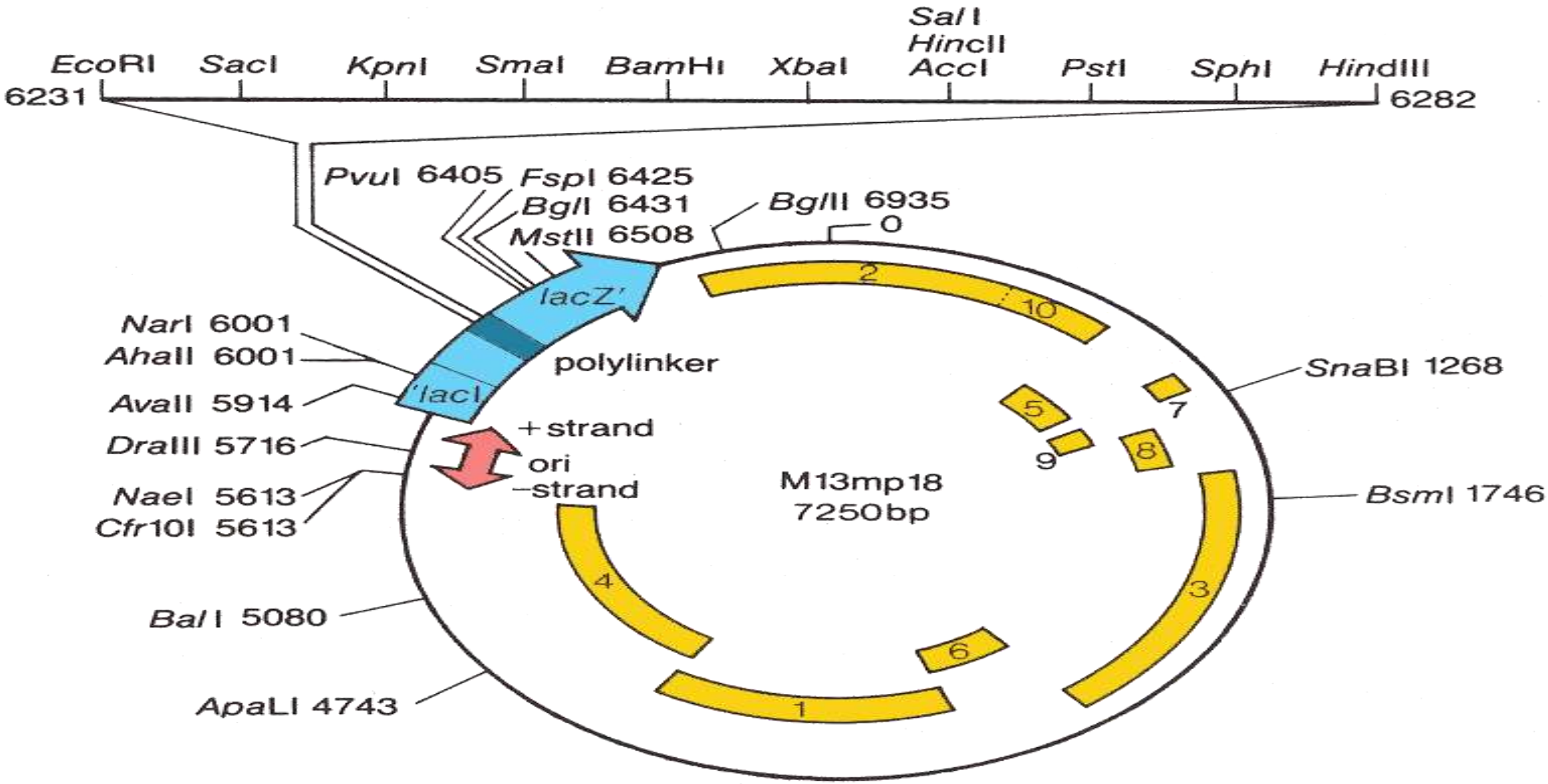
The (–) strand of the RF DNA is transcribed into viral mRNAs.

The viral gene II product introduces a nick at a specific site (red arrow) in the (+) strand of the RF DNA.

Progeny (+) strand is synthesized continuously by movement of the replication machinery around the (–) strand template (rolling circle replication).

The completed progeny (+) strand is cleaved from the rolling circular structure by the viral gene II product (red arrow). The progeny strand then circularizes.

Synthesis of progeny (+) strand continues.


EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
HincII
AccI
PstI
SphI
HindIII
6231
6282
PvuI 6405
FspI 6425
BglI 6431
MstII 6508
BglII 6935
0
lacZ'
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polylinker
NarI 6001
AhaII 6001
AvaII 5914
DraIII 5716
NaeI 5613
Cfr10I 5613
+strand
ori
−strand
M13mp18
7250bp
SnaBI 1268
BsmI 1746
BalI 5080
ApaLI 4743
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M13 Vectors

- M13mp1-The *lac Z* gene was introduced in the IS and it does not have any unique restriction site in the gene.
- M13mp2- This vector was constructed by including the EcoR1 site in the *lac Z* gene. This was done by a single nucleotide change in GGATTC near the start of the *lac Z* gene to GAATTC by *in vitro* mutagenesis. The β-galactosidase enzyme remains functional in the vector.
- M13mp7- It was formed by inserting a polylinker into the EcoR1 site of *lac Z* gene of M13mp2. A polylinker is a short nucleotide sequence that consists of number of restriction sites (EcoR1, BamH1, Sal1 and Pst1) and has a EcoR1 sticky ends. This polylinker does not disrupt the function of *Lac Z* gene.

Advantage of the M13 vectors

- Genome of this phage is a single-stranded DNA, so DNA fragments cloned into this vector can be recovered in single-stranded form.
- Single-stranded DNA can be an aid to site-directed mutagenesis, by which we can introduce specific, premeditated alterations into a gene

M13 doesn't lyse cells, but it does slow them down

"lawn" of *E. coli*

M13 infections form plaques, but they are "turbid"

λ Phage

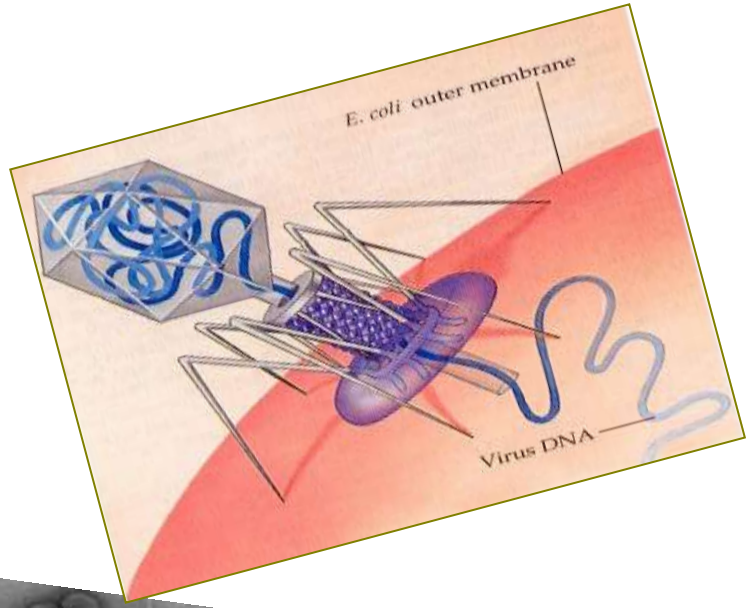
- It is 49kb in size and is used as a cloning vector because:

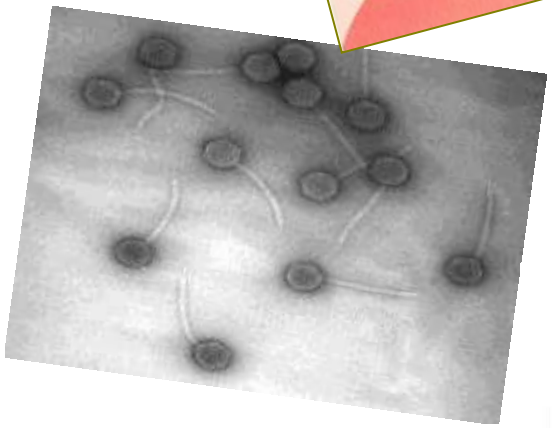
✓ The genes related in terms of function are clustered together in the genome and allows the genes to be switched on and off as a group rather than individually.

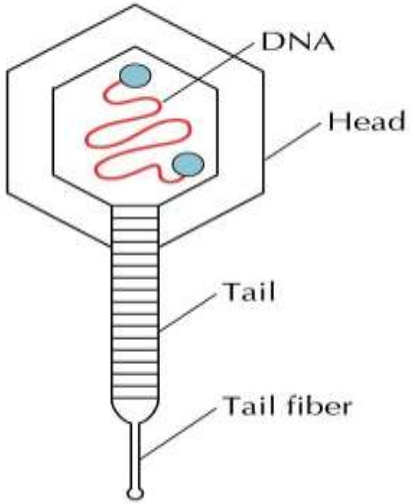
✓ The linear double stranded DNA molecule has a stretch of 12 nucleotides at its either ends which act as sticky ends or cohesive ends (cos sites)

✓ They can base pair to form a circular DNA molecule which is important for insertion into the bacterial genome.

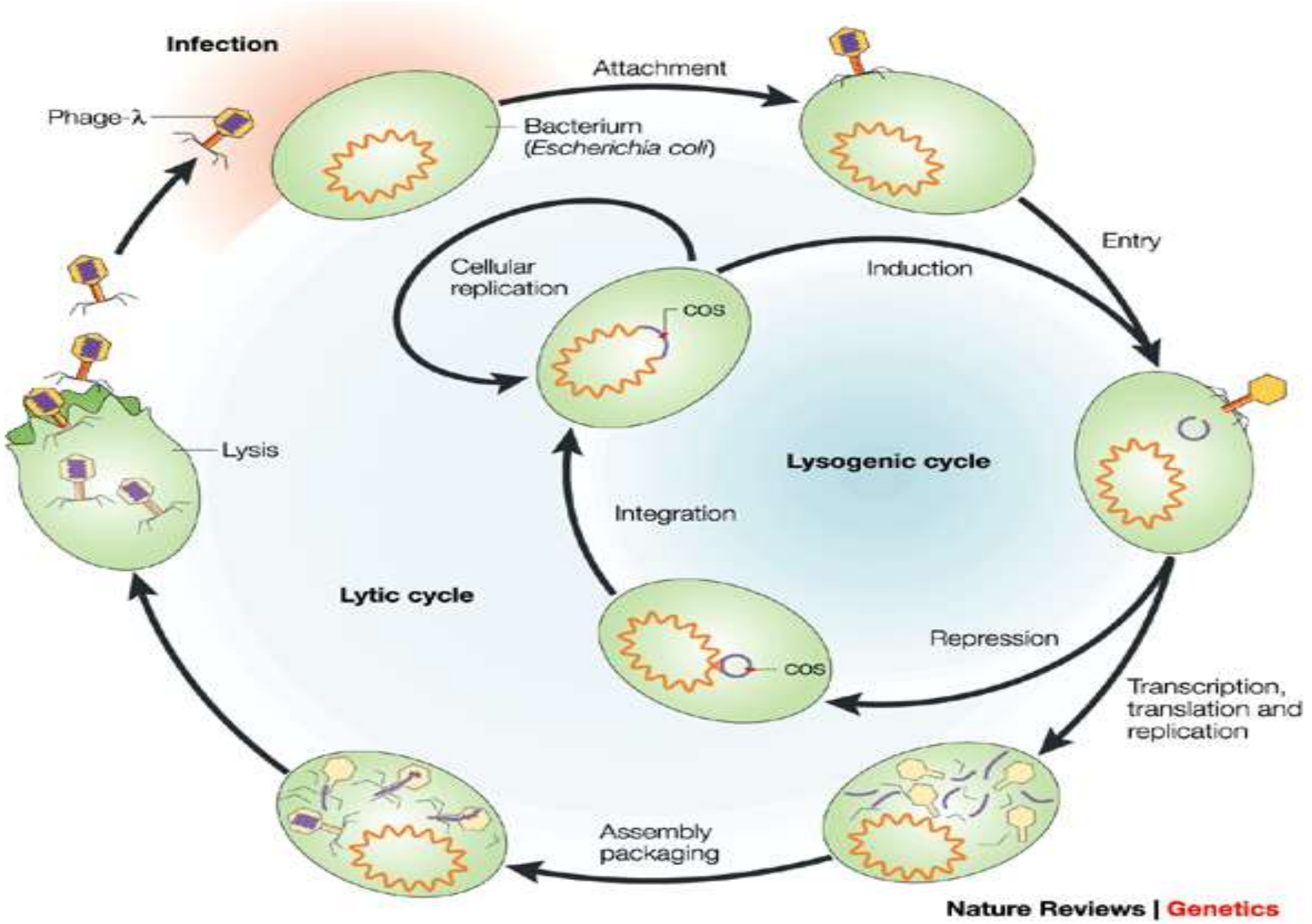
✓ Another role of cos sites is in the formation of large number of λ DNA molecules by rolling circle mechanism of replication (Cancatamers).

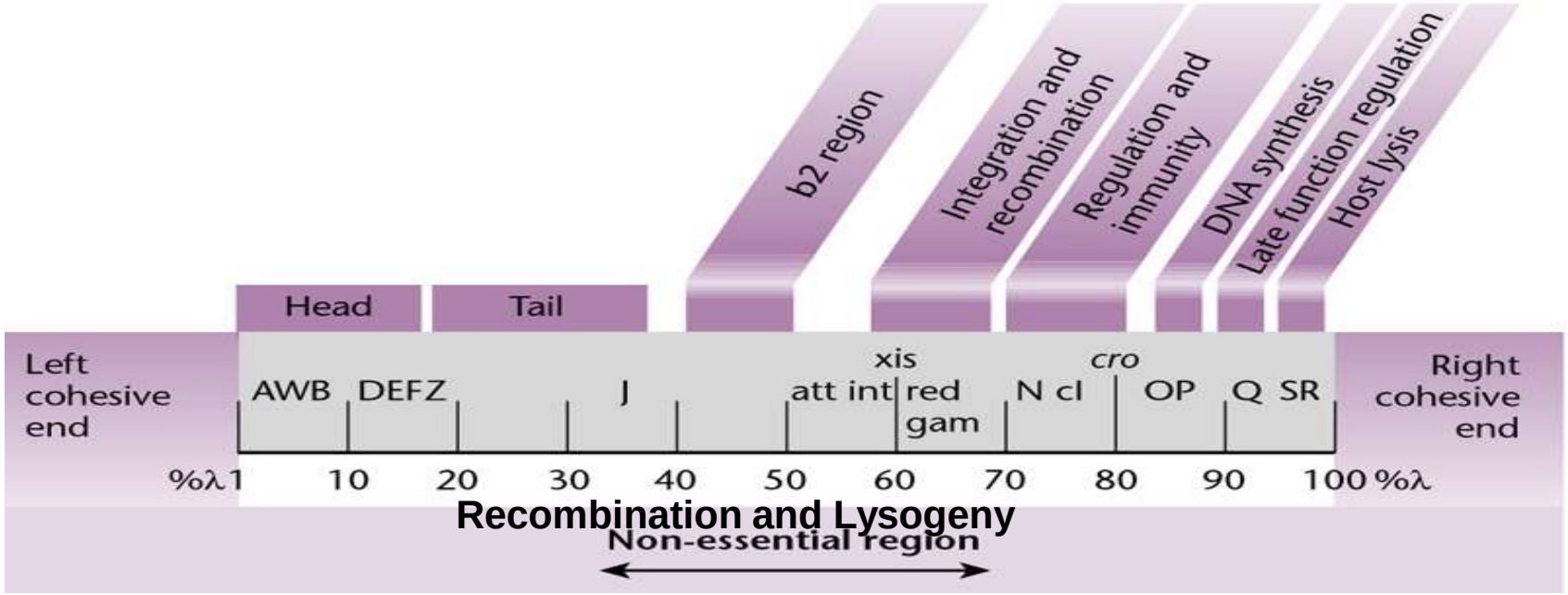


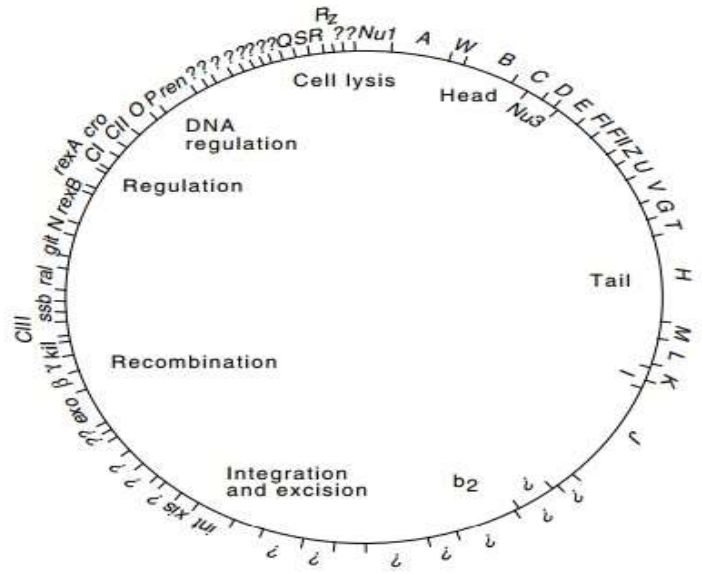




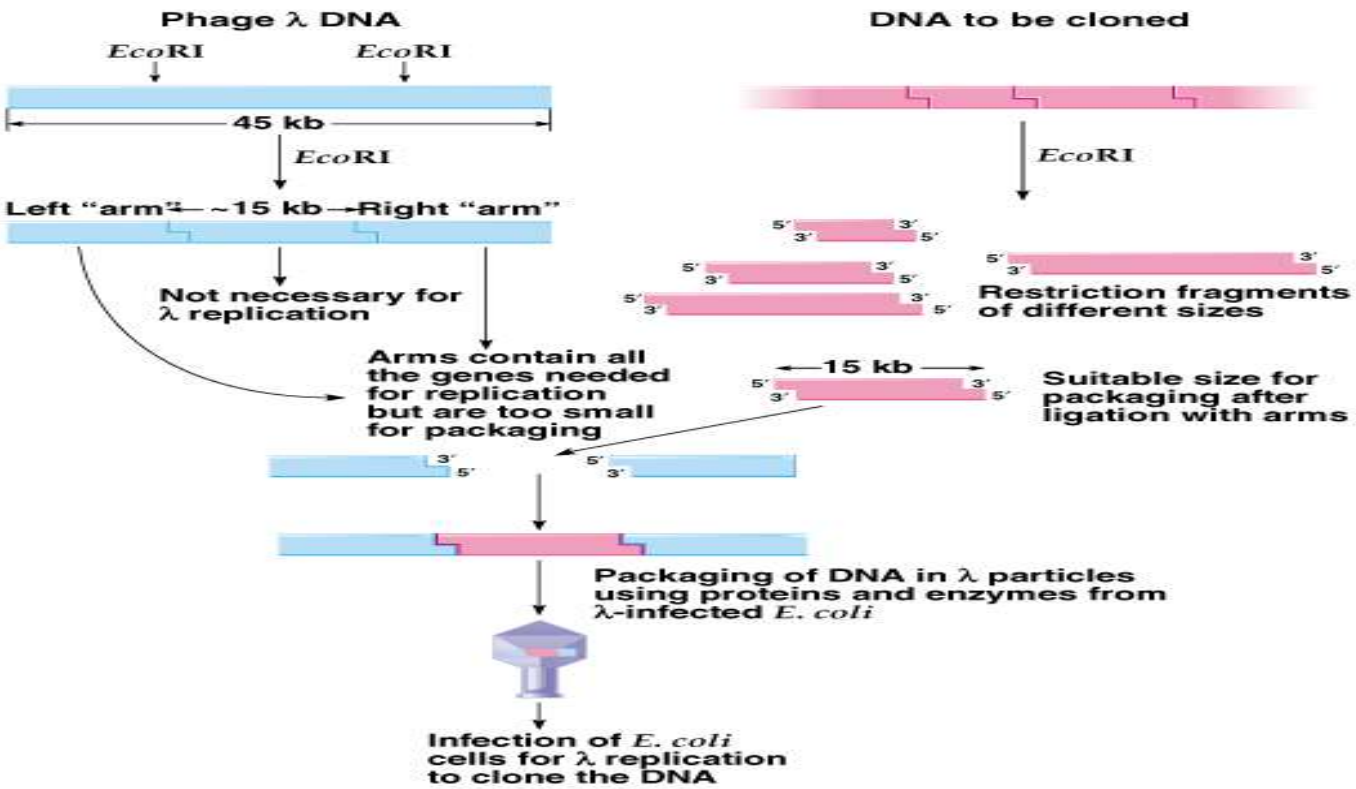
Lytic and Lysogenic cycle

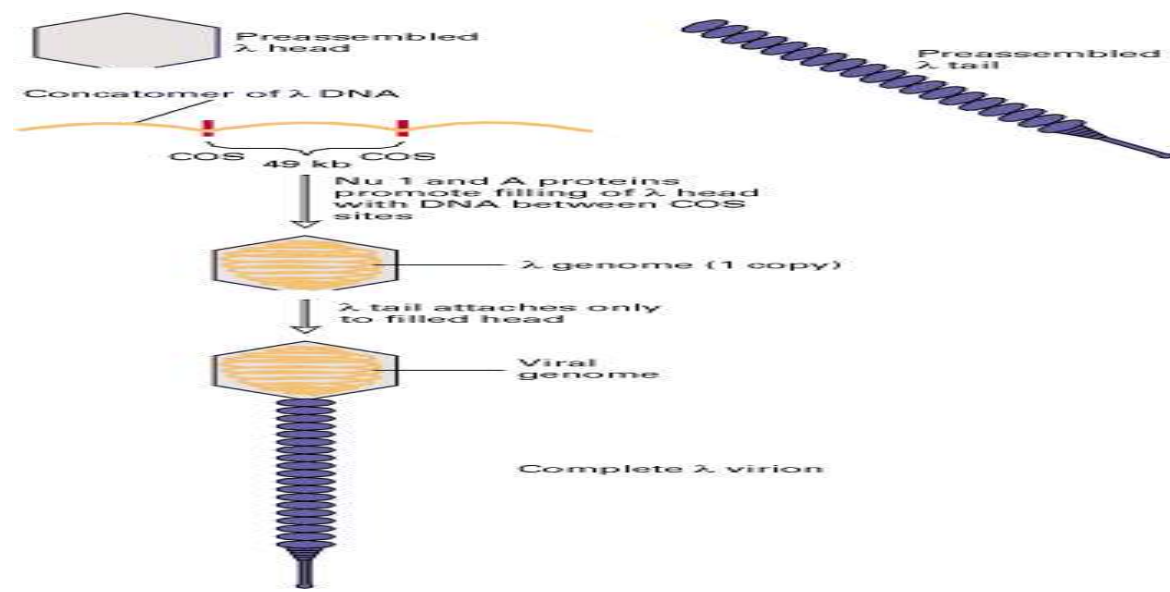


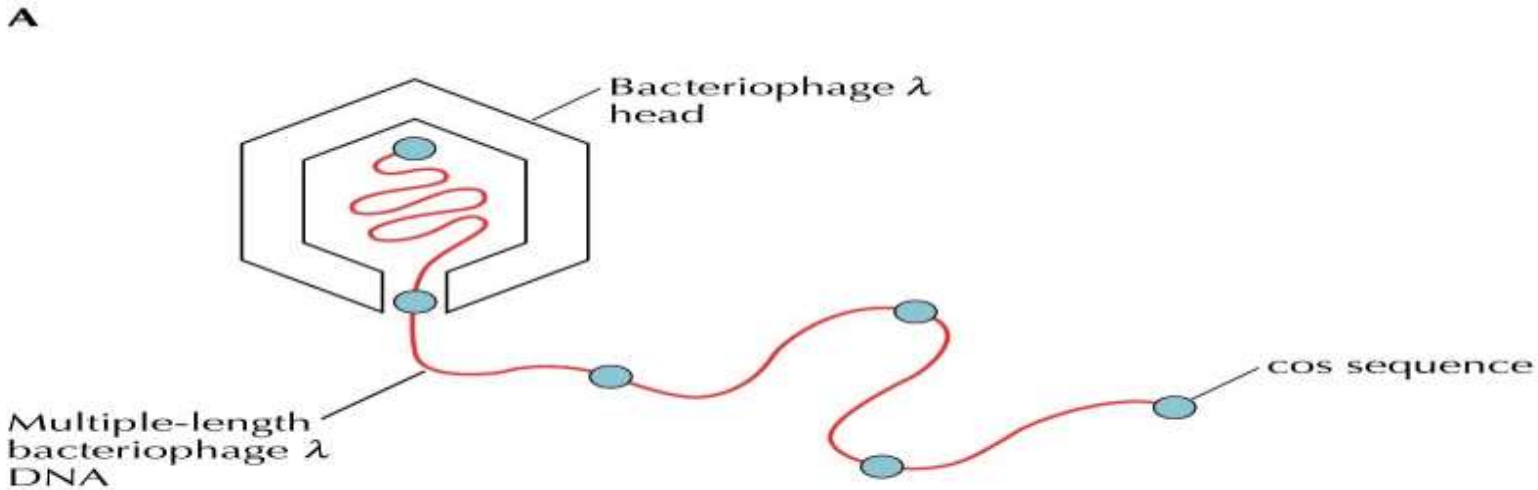


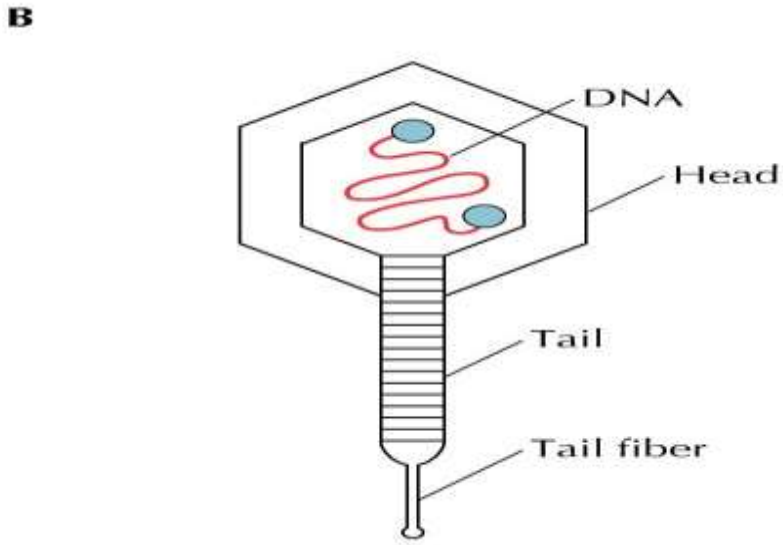


- Cos site: At the ends short (12bp) ss- complementary region “cohesive or sticky” ends--- circulation after infection
- Left Arm: Structural genes for head and tail
- Central Region: genes for lysogenic growth and recombination/insertion of genome into baterial genome
- Right Arm: genes involved in DNA replication and lytic growth
- Only 30 kb is required for lytic growth.
- Thus, one could clone 19 kb of “foreign” DNA.
- Packaging efficiency 78%-105% of the lambda genome.

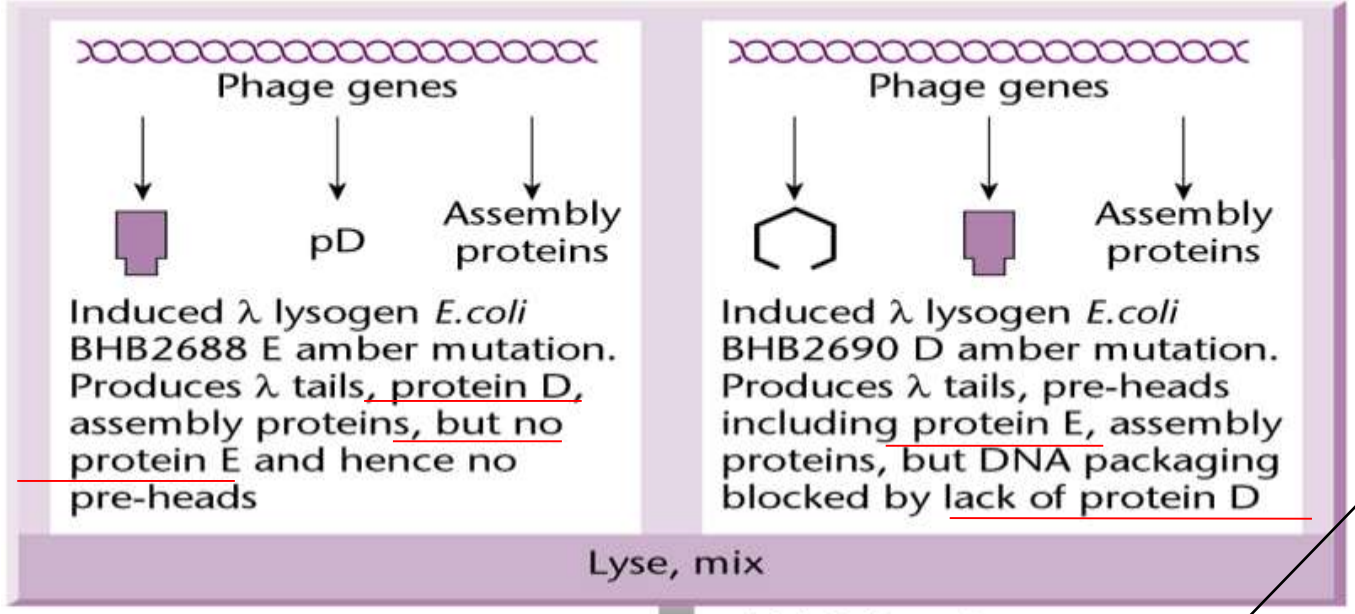

Phage λ DNA
EcoRI
EcoRI
45 kb
EcoRI
Left "arm" ~15 kb Right "arm"
Not necessary for λ replication
Arms contain all the genes needed for replication but are too small for packaging
DNA to be cloned
EcoRI
Restriction fragments of different sizes
15 kb
Suitable size for packaging after ligation with arms
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


Preassembled λ head
Preassembled λ tail
Concatomer of λ DNA
COS
49 kb
COS
Nu 1 and A proteins promote filling of λ head with DNA between COS sites
λ genome (1 copy)
λ tail attaches only to filled head
Viral genome
Complete λ virion

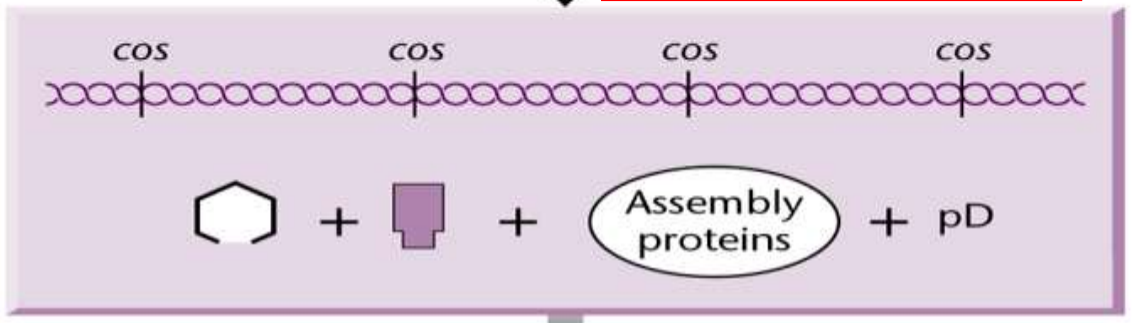

A
Bacteriophage λ head
Multiple-length bacteriophage λ DNA
cos sequence

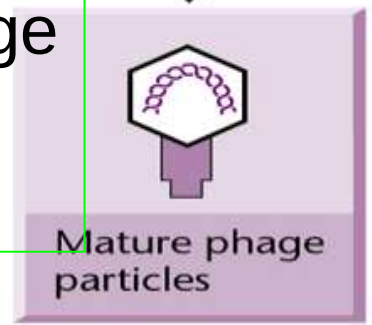

B
DNA
Head
Tail
Tail fiber

How to transfer recombinant lambda into cells?

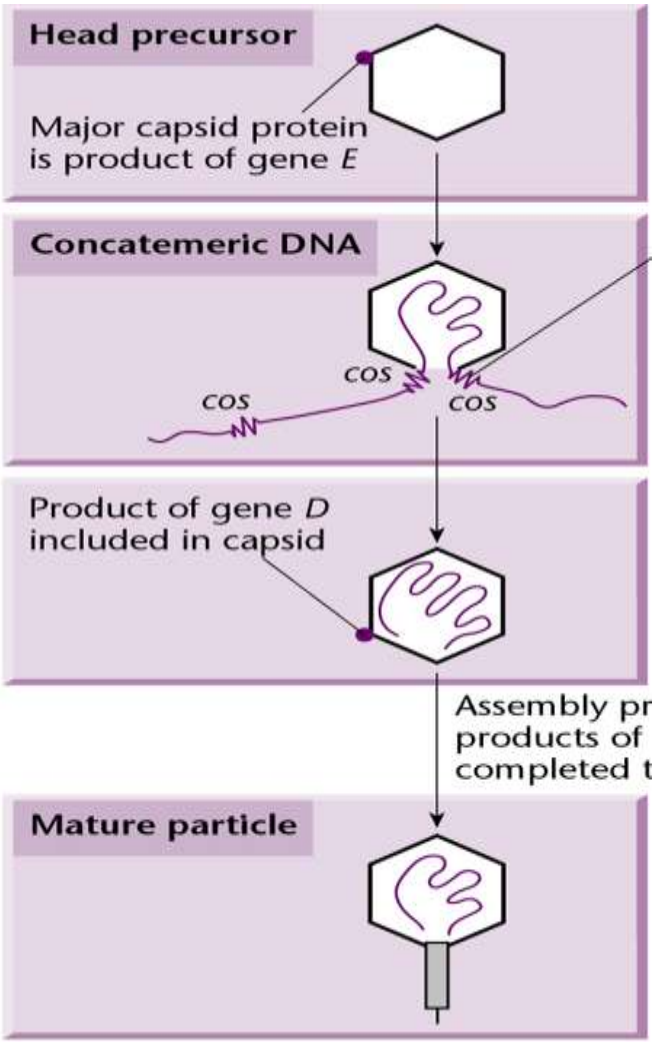


Add ATP and concatemerized λDNA





The packaged phage particles are infectious



Insertion Vectors

- Contain a unique cleavage site whereby foreign DNA with size of 5–11 kb may be inserted.
- Contain single site for cloning DNA inserts
- Designed to be packaged with/without inserted DNA
- Minimum size for packaging – 38 kb so can only clone in 10 kb (or less) inserts
- Used in cloning cDNA in expression vectors
- **λgt10: It can clone up to 8kb of new DNA inserted in the EcoR1 site located in the c1 gene.**
- **λZAP11: In this vector up to 10kb of DNA can be inserted in to any of the 6 restriction sites within the polylinker. This inactivates the lac Z gene of the vector**

Replacement Vectors

- This vector has two recognition sites for the restriction endonuclease used for cloning.
- These sites replace the segment of DNA (Stuffer fragment) from the vector genome by the DNA to be cloned.
- These vectors can carry large pieces of DNA (8–24) kb than insertion vectors.
- *Left arm*:
  - head & tail proteins
- *Right arm*:
  - DNA synthesis
  - regulation
  - host lysis
- *Deleted central region*:
  - integration & excision
  - regulation
- λEMBL4: This vector can be used to insert up to 20kb of DNA.

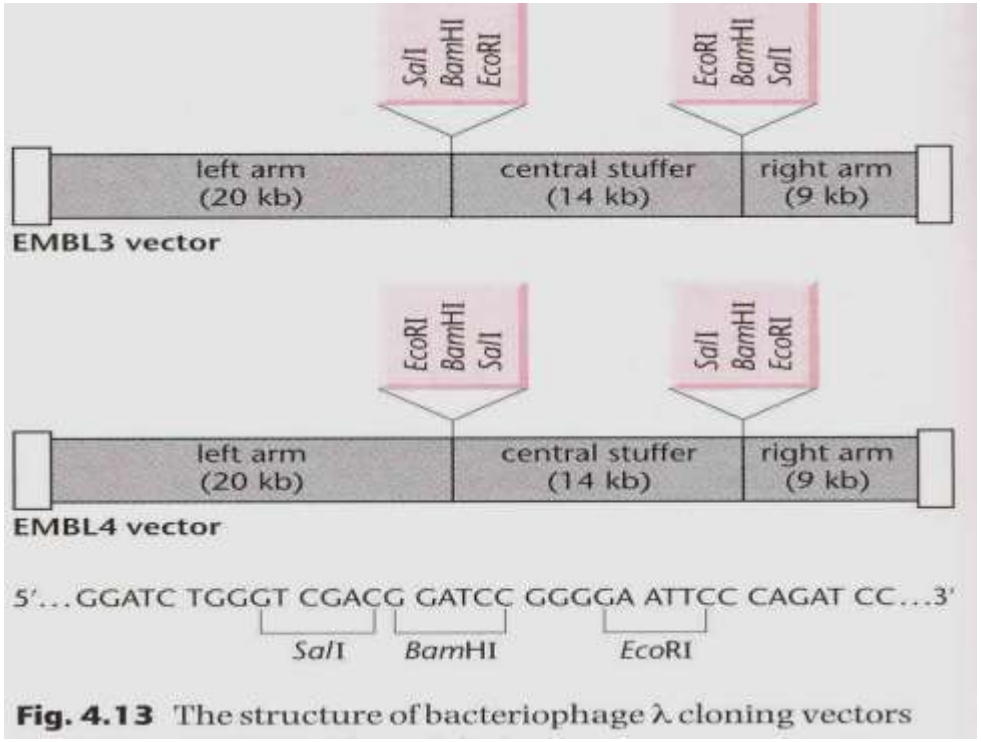


Fig. 4.13 The structure of bacteriophage λ cloning vectors

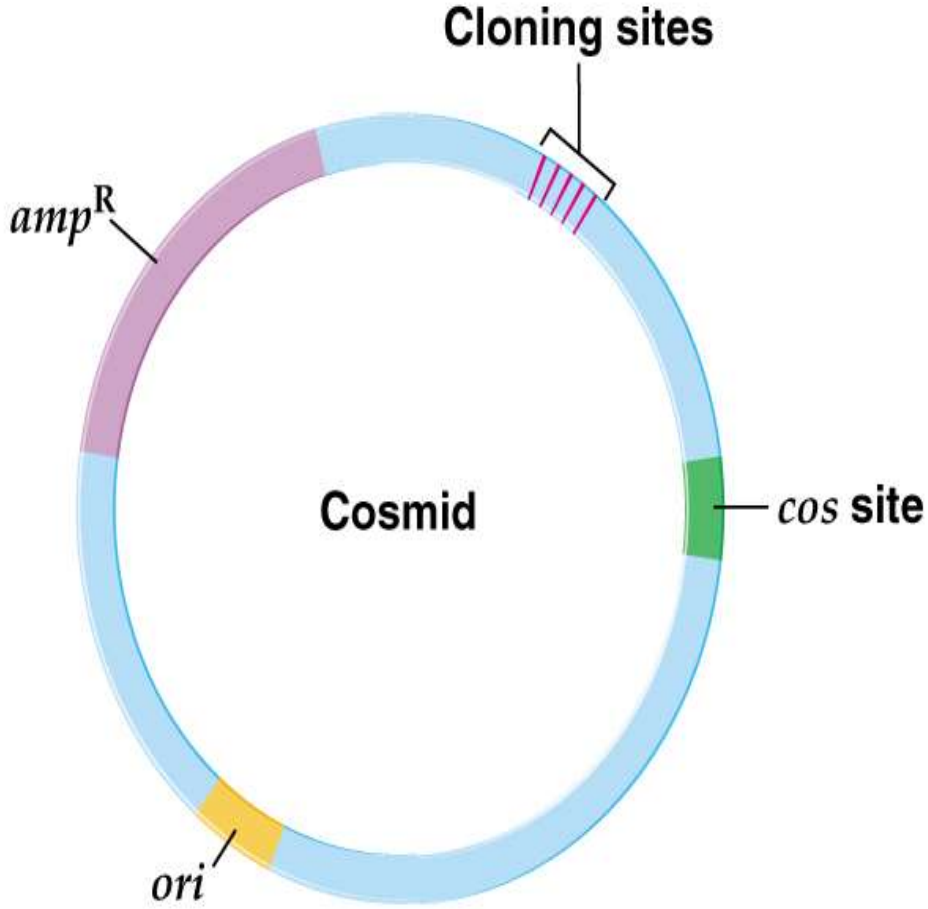
Insertion v/s replacement vectors

- Modified lamda phage genome has single restriction enzyme sites.
- **Small chunk of λ DNA was deleted**
- One of them can used to restriction genome and small size DNA fragment can be inserted without deleting the phage DNA
- The size of the fragment is around **0 to 10kb**
- **Two molecules of vector (left, right arm) and one molecule of DNA Insert.**
- Selection of recombinants depends on cI (λgt10) or lacZ (λZAP11)
- **Insertional Inactivation** of cI gene or LacZ gene will give clear or white palques are recombinants
- Examples: λgt10, λZAP11

- Stuffer fragment is deleted with help two restriction enzymes and same enzymes restriction sites were used to replace **DNA Insert of foreign origin**
- Size of DNA insert is **20 to 23kb**
- **Two molecules of vector (left, right arm) and one molecule of DNA Insert are ligated and that molecule only have right size to pack into the heads of phage**
- Selection of the recombinants **size of the clone, Spi2 phenotype**
- **All phage progeny are Recombinants (100%)**
- Examples: λ EMBL4, λGEM11, λGEM12, Charon

Cosmids

- They were first described by Collins and Hohn in 1978.
- 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

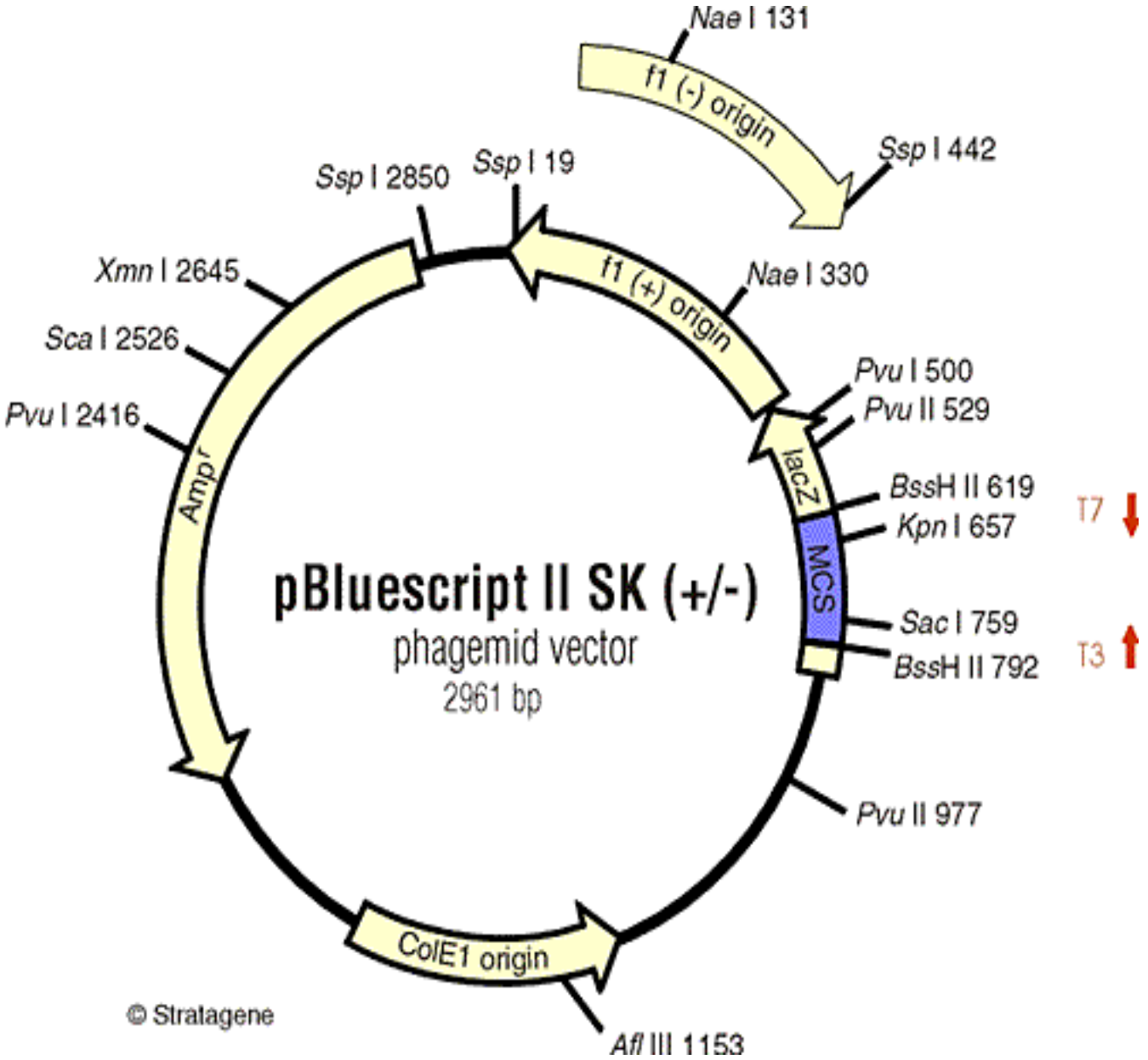


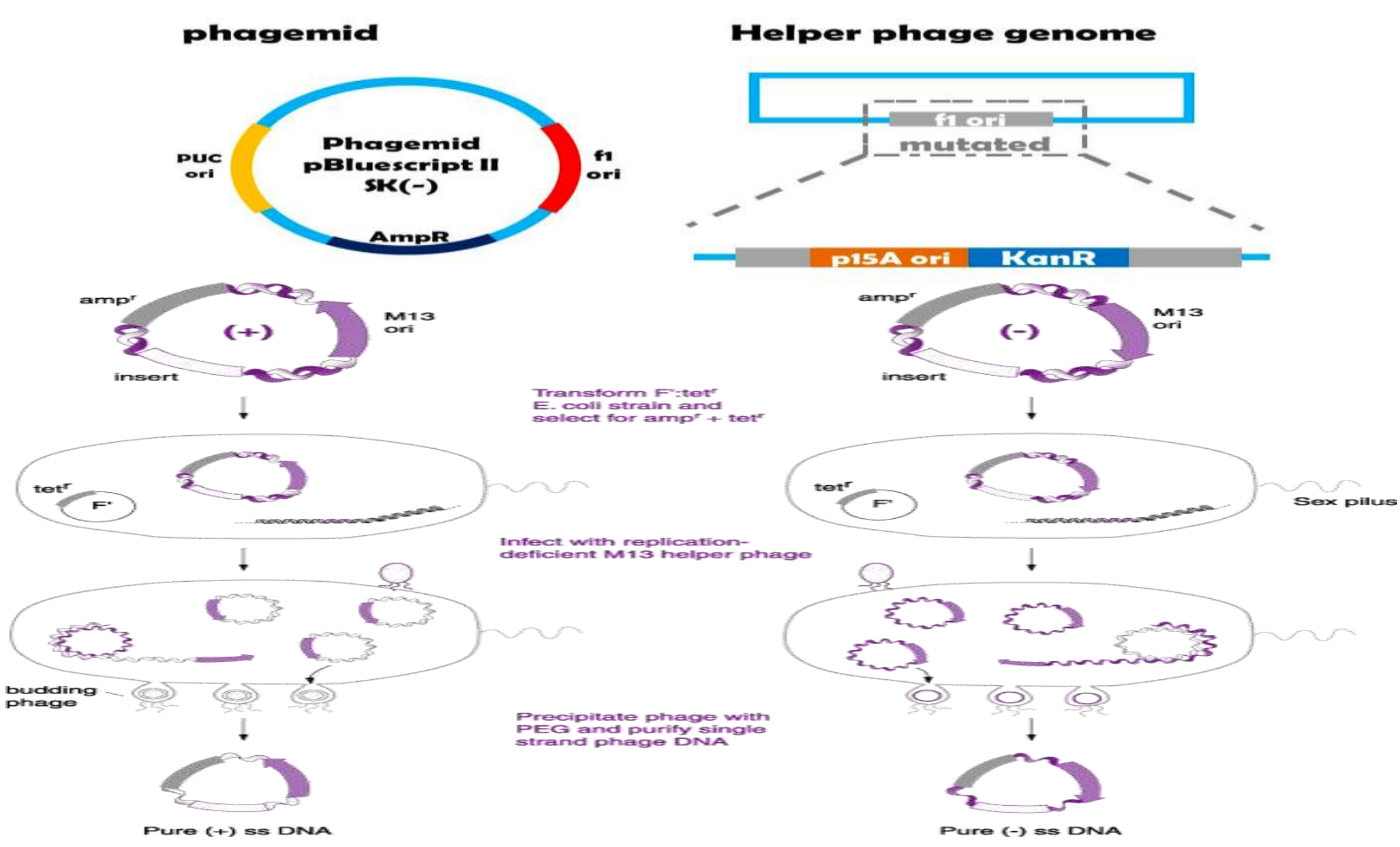
Phagemids/Phasmids: plasmid/F1M13 hybrids

- A phagemid or phasmid is a plasmid that contains an f1 origin of replication from an f1 phage.
- It can be used as a type of cloning vector in combination with filamentous phage M13.
- A phagemid can be replicated as a plasmid, and also be packaged as single stranded DNA in viral particles.
- Phagemids contain an origin of replication (ori) for double stranded replication, as well as an f1 ori to enable single stranded replication and packaging into phage particles.
- In a plasmid, a phagemid can be used to clone DNA fragments and be introduced into a bacterial host by a range of techniques, such as transformation and electroporation.
- However, infection of a bacterial host containing a phagemid with a 'helper' phage, for example VCSM13 or M13K07, provides the necessary viral components to enable single stranded DNA replication and packaging of the phagemid DNA into phage particles.
- The 'helper' phage infects the bacterial host by first attaching to the host cell's pilus and then, after attachment, transporting the phage genome into the cytoplasm of the host cell.
- Inside the cell, the phage genome triggers production of single stranded phagemid DNA in the cytoplasm. This phagemid DNA is then packaged into phage particles.

Phagemids/Phasmids: plasmid/M13 hybrids

- The phage particles containing ssDNA are released from the bacterial host cell into the extracellular environment.
- Filamentous phages retard bacterial growth but, contrasting with the lambda phage and the T7 phage, are not generally lytic.
- Helper phages are usually engineered to package less efficiently (via a defective phage origin of replication)\ than the phagemid so that the resultant phage particles contain predominantly phagemid DNA.
- F1 Filamentous phage infection requires the presence of a pilus so only bacterial hosts containing the F-plasmid or its derivatives can be used to generate phage particles.




phagemid
Helper phage genome
Phagemid
pBluescript II
SK(-)
puc
ori
f1
ori
AmpR
f1 ori
mutated
p15A ori
KanR
amp^r
(+)
M13
ori
insert
amp^r
(-)
M13
ori
insert
Transform F':tet^r
E. coli strain and
select for amp^r + tet^r
tet^r
F'
tet^r
F'
Sex pilus
Infect with replication-
deficient M13 helper phage
budding
phage
Precipitate phage with
PEG and purify single
strand phage DNA
Pure (+) ss DNA
Pure (-) ss DNA

ARTIFICIAL CHROMOSOMEs

- ✓ Linear or Circular.
- ✓ 1 0r 2 copies per cell.
- ➢ Different types –
  - Bacterial Artificial Chromosome (BAC)
  - Yeast Artificial Chromosome (YAC)
  - P1 derived artificial chromosome (PAC)
  - Mammalian Artificial Chromosome (MAC)
  - Human Artificial Chromosome. (HAC)
- YAC – Cloning in yeast
- BAC & PAC – Bacteria
- MAC & HAC – Mammalian & Human cells.

Bacterial Artificial Chromosomes (BACs):

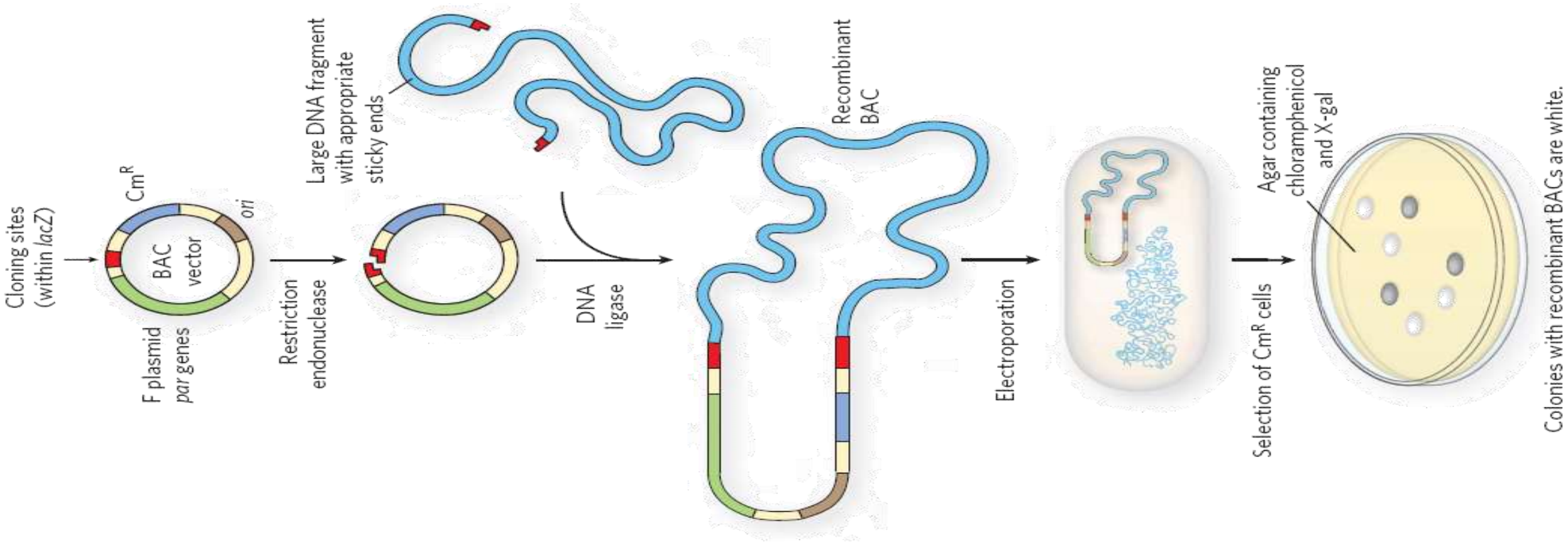
Vectors that enable artificial chromosomes to be created and cloned into *E. coli*.

Features:

- Based on the F factor of E. coli:
  - ✓ 100 kb plasmid, propagates through conjugation
  - ✓ low copy number (1-2 copies per cell)
- 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).
  - ✓ 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)



Bacterial artificial chromosomes (BACs) as cloning vectors. After treatment with an appropriate restriction endonuclease, a BAC and a long fragment of DNA are ligated. The recombinant BAC is transferred into *E. coli* by electroporation, and colonies with recombinant BACs are selected by growth on media containing both the antibiotic chloramphenicol and X-gal, the substrate for –galactosidase that produces a colored product.

YEAST ARTIFICIAL CHROMOSOME

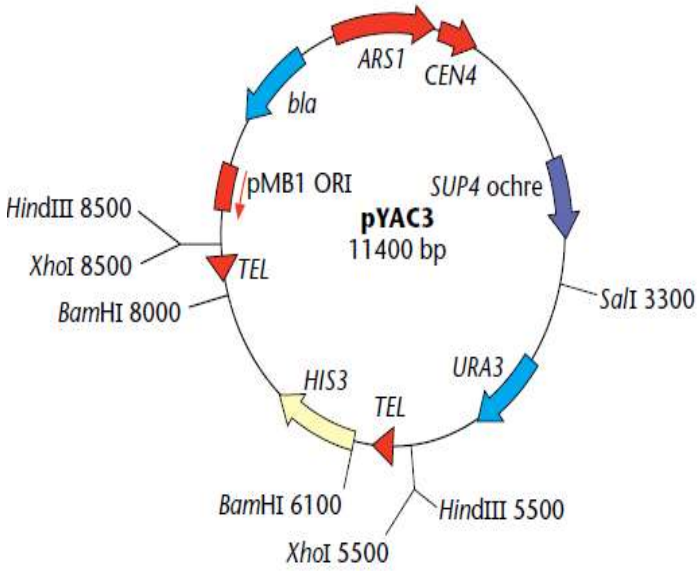
- YAC are genetically engineered chromosomes derived from the DNA of the yeasts.
- Capable of carrying inserts of 100 - 1000 kbp.
- A YAC can be considered as a functional artificial chromosome since it includes three specific DNA sequences:
- **TEL**: Telomere located at each chromosome end, protects the linear DNA from degradation by nucleases.
- **CEN**: Centromere which is the attachment site for mitotic spindle fibers, "pulls" one copy of each duplicated chromosome into each new daughter cell.
- **ORI**: Replication origin sequences which are specific DNA sequences.

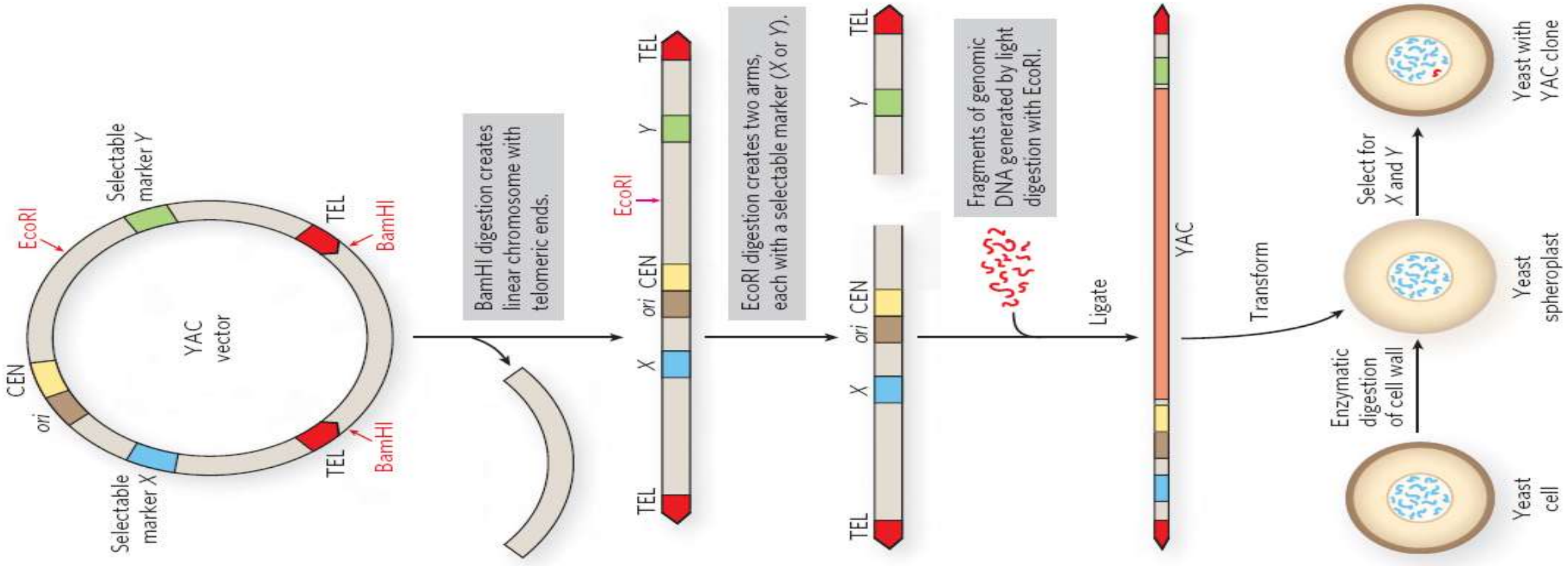
YACs: Yeast Artificial Chromosomes

- 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).
- Acquiring 150kbp it acquires chromosome like features
- *SUP4* gene, a suppressor tRNA gene which overcomes the effect of the *ade-2* ochre mutation and restores wild-type activity, resulting in colorless colonies.
- The host cells are also designed to have recessive *trp1* and *ura3* alleles which can be complemented by the corresponding *TRP1* and *URA3* alleles in the vector, providing a selection system for identifying cells containing the YAC vector.
- Useful for cloning very large DNA fragments up to 500 kb; useful for very **large DNA fragments.**





Construction of a yeast artificial chromosome (YAC). A YAC vector includes an origin of replication (*ori), a centromere (CEN), two telomeres (TEL),* and selectable markers (here designated *X and Y). Two* separate DNA arms are generated by digestion with BamHI and EcoRI, each arm having a telomeric end and one selectable marker. A large DNA fragment, produced by EcoRI digestion, is ligated to the two arms, creating a YAC. The YAC is transferred into yeast cells (which have been prepared by removing the cell wall to form spheroplasts). The transformed cells are selected for *X and Y, and the* surviving cells propagate the DNA insert.

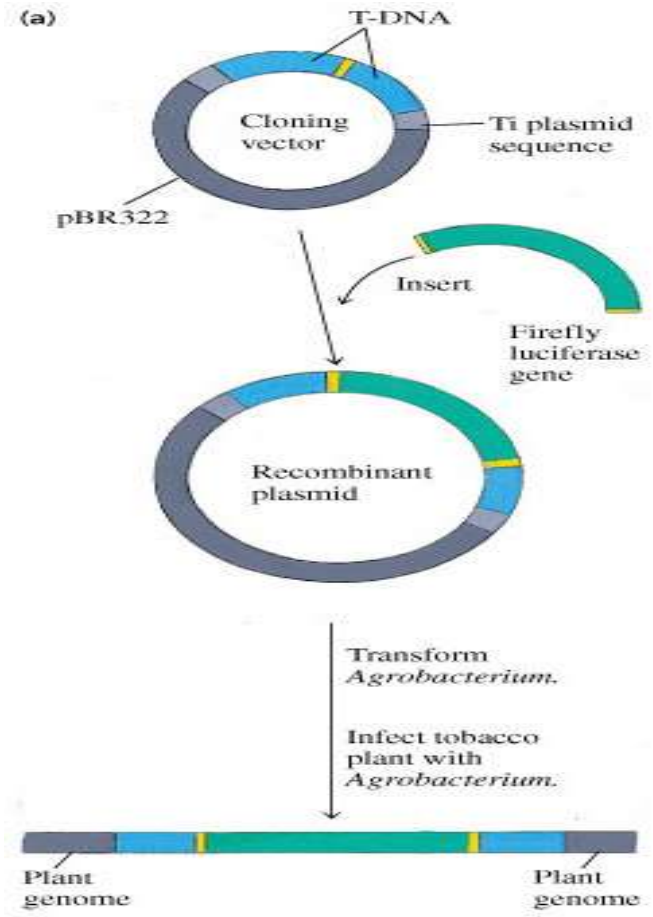
Shuttle vectors:

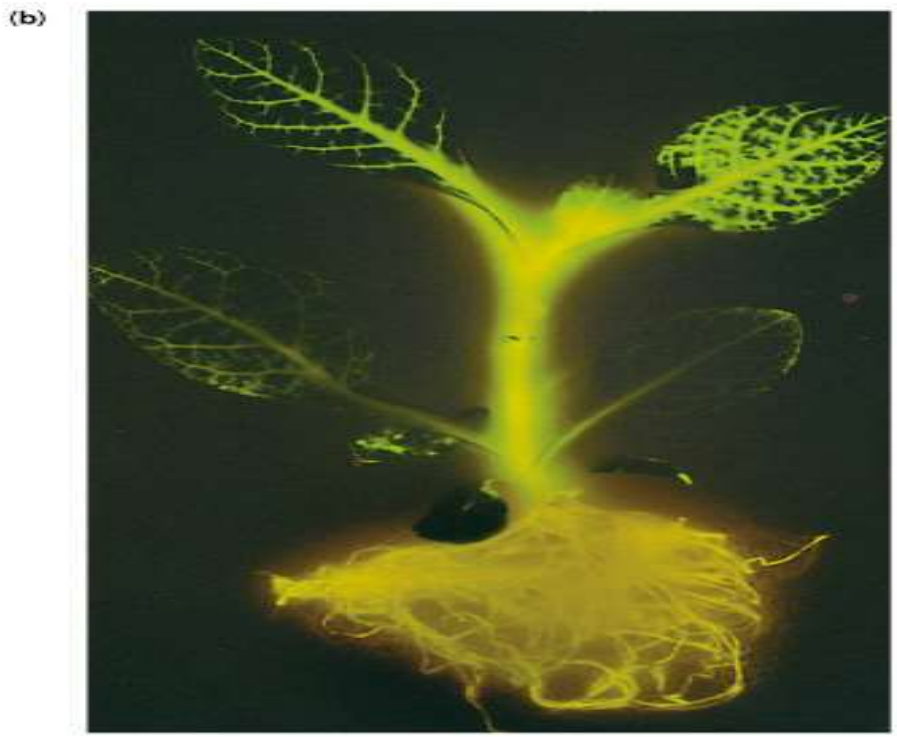
1. Capable of replicating in two or more types of hosts.
2. Replicate autonomously, or integrate into the host genome and replicate when the host replicates.
3. Commonly used for transporting genes from one organism to another (i.e., transforming animal and plant cells).

Example:

*Insert firefly luciferase gene into plasmid and transform *Agrobacterium*.

*Grow *Agrobacterium* in large quantities and infect tobacco plant.





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