Department of BSBE
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BT 207

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Vectors

Requirements for Cloning

- Enzymes
- Cloning Vectors
- Selection methods
- Expression Host
- Methods for introducing DNA into a host cell

V**ector -** a DNA molecule used as a vehicle to artificially carry foreign genetic material into another cell, where it can be replicated and/or expressed.

The four major types of vectors are plasmids, viral vectors, cosmids, and artificial chromosomes.

Of these, the most commonly used vectors are plasmids.

Vectors have an origin of replication, a multiple cloning site, and a selectable marker.

Types of vectors:

1. Cloning
2. Expression
3. Shuttle vectors

Vectors

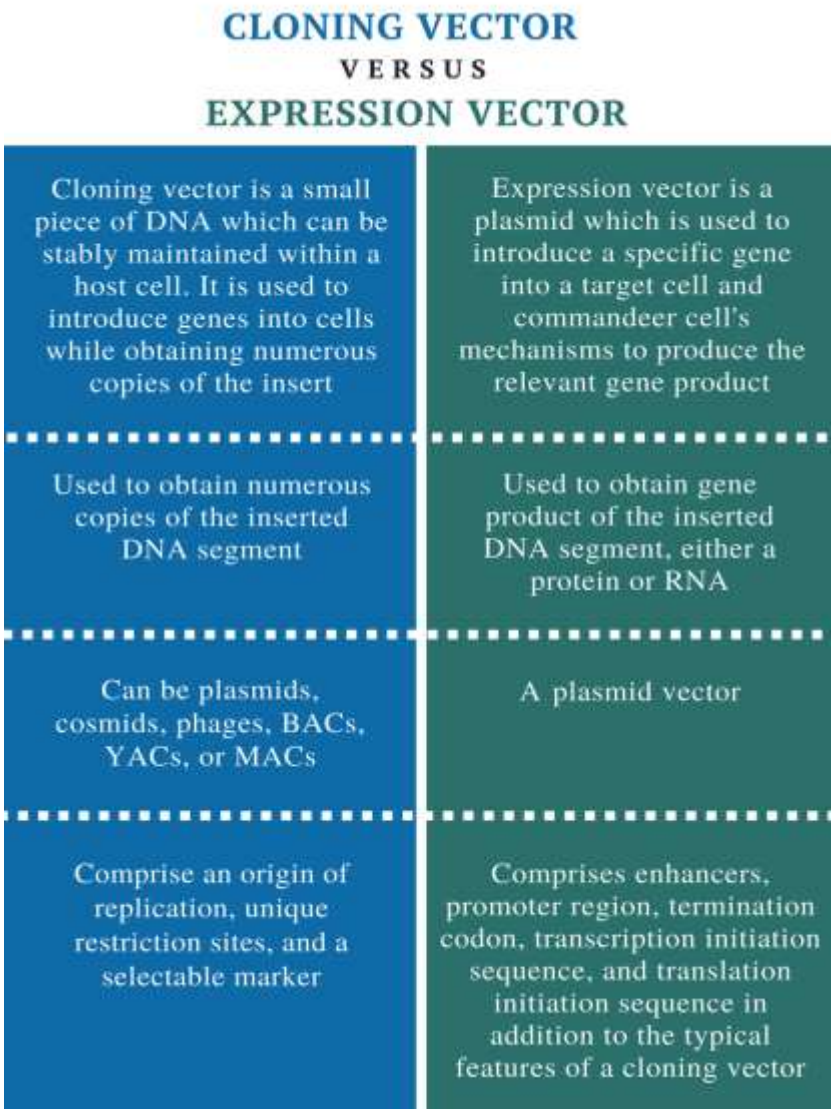
Type:

1. Cloning
2. Expression
3. Shuttle vectors

Difference between cloning and expression vector:

Cloning vector is used to carry foreign DNA segments into any host cell.

Expression vector is a type of plasmid, which contains suitable expression signals to maximize target gene expression.



Cloning vector

- **Cloning vector** - a small piece of DNA that can be stably maintained in an organism, and into which a foreign DNA fragment can be inserted for cloning purposes.
- The vector contains features that allow for the convenient insertion or removal of a DNA fragment to or from the vector.
- Treating the vector and the foreign DNA with a restriction enzyme that cuts the DNA.
- DNA fragments thus generated contain either blunt ends or overhangs known as sticky ends.
- Vector DNA and foreign DNA with compatible ends can then be joined together by molecular ligation.
- There are many types of cloning vectors, but the most commonly used ones are genetically engineered plasmids.
- Others are:
- Bacteriophages (such as phage λ)
- Cosmids
- Fosmids
- Bacterial artificial chromosomes (BACs)
- Yeast artificial chromosomes (YACs)

Should have: Origin of replication, unique restriction site, selectable marker

What determines the choice vector?

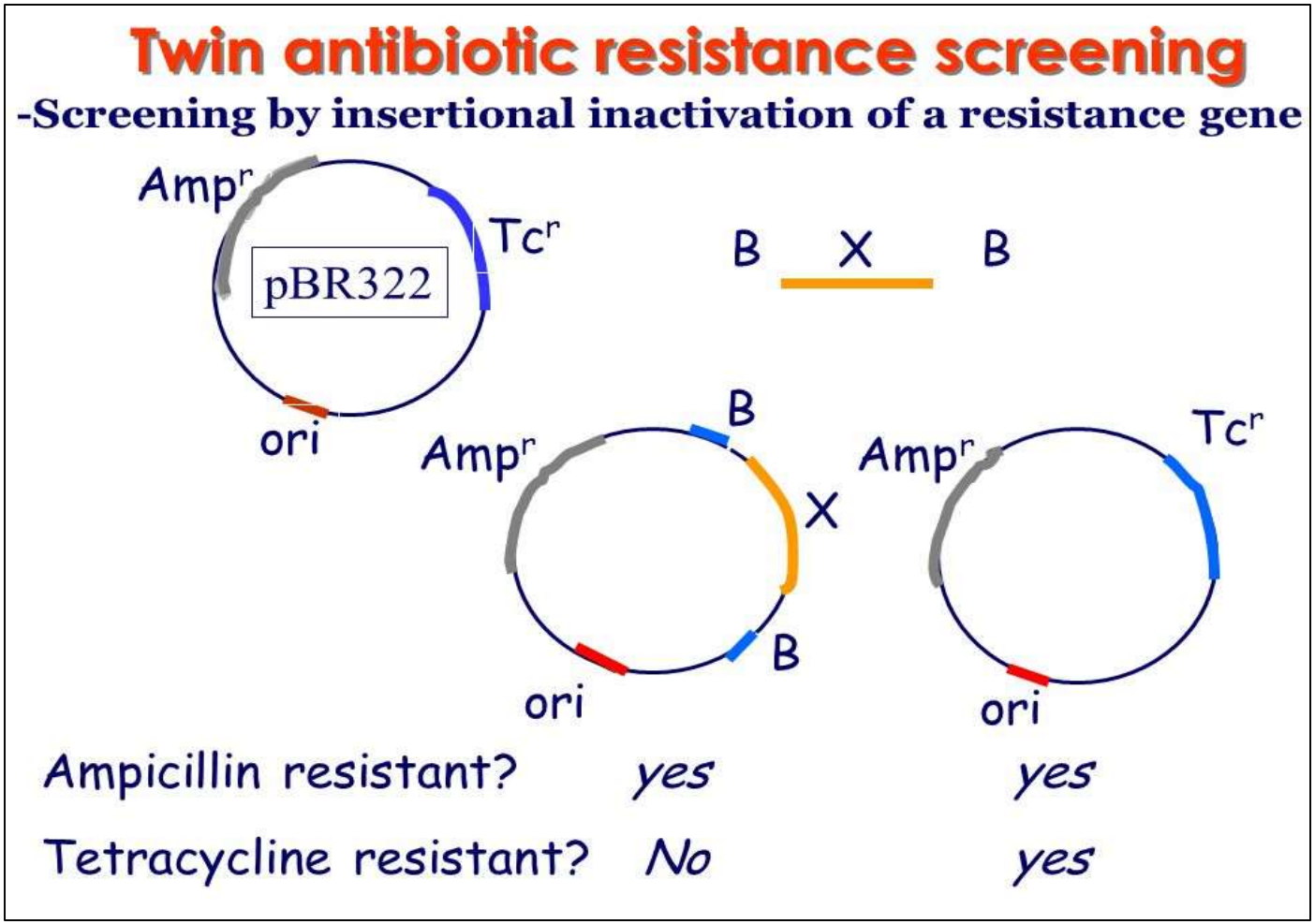
- Insert size
- Vector size
- Restriction sites
- Regulatory sequences (as promoters)
- Copy number
- Ability to screen for inserts
- Cloning efficiency

- **What down-stream experiments do you plan?**

Table 5.1 Maximum DNA insert possible with different cloning vectors. YACs are discussed on p. 159.

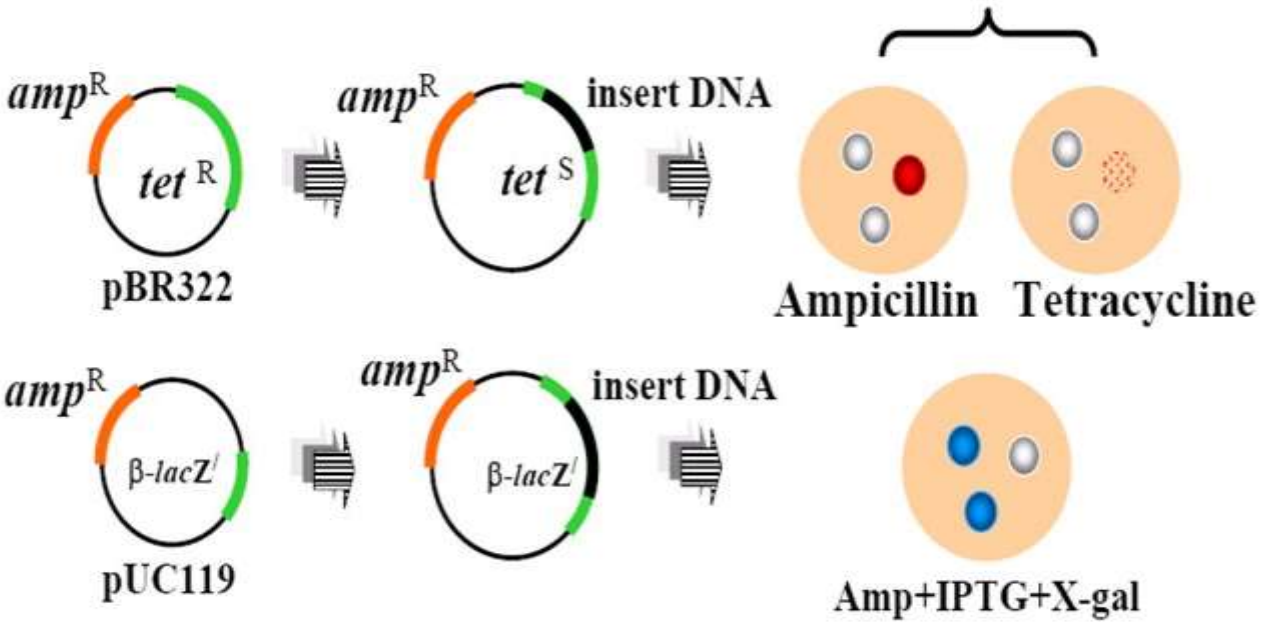
Vector	Host	Insert size
λ phage	*E. coli*	5–25 kb
λ cosmids	*E. coli*	35–45 kb
P1 phage	*E. coli*	70–100 kb
PACs	*E. coli*	100–300 kb
BACs	*E coli*	≤ 300 kb
YACs	*Saccharomyces cerevisiae*	200–2000 kb

SCREENING

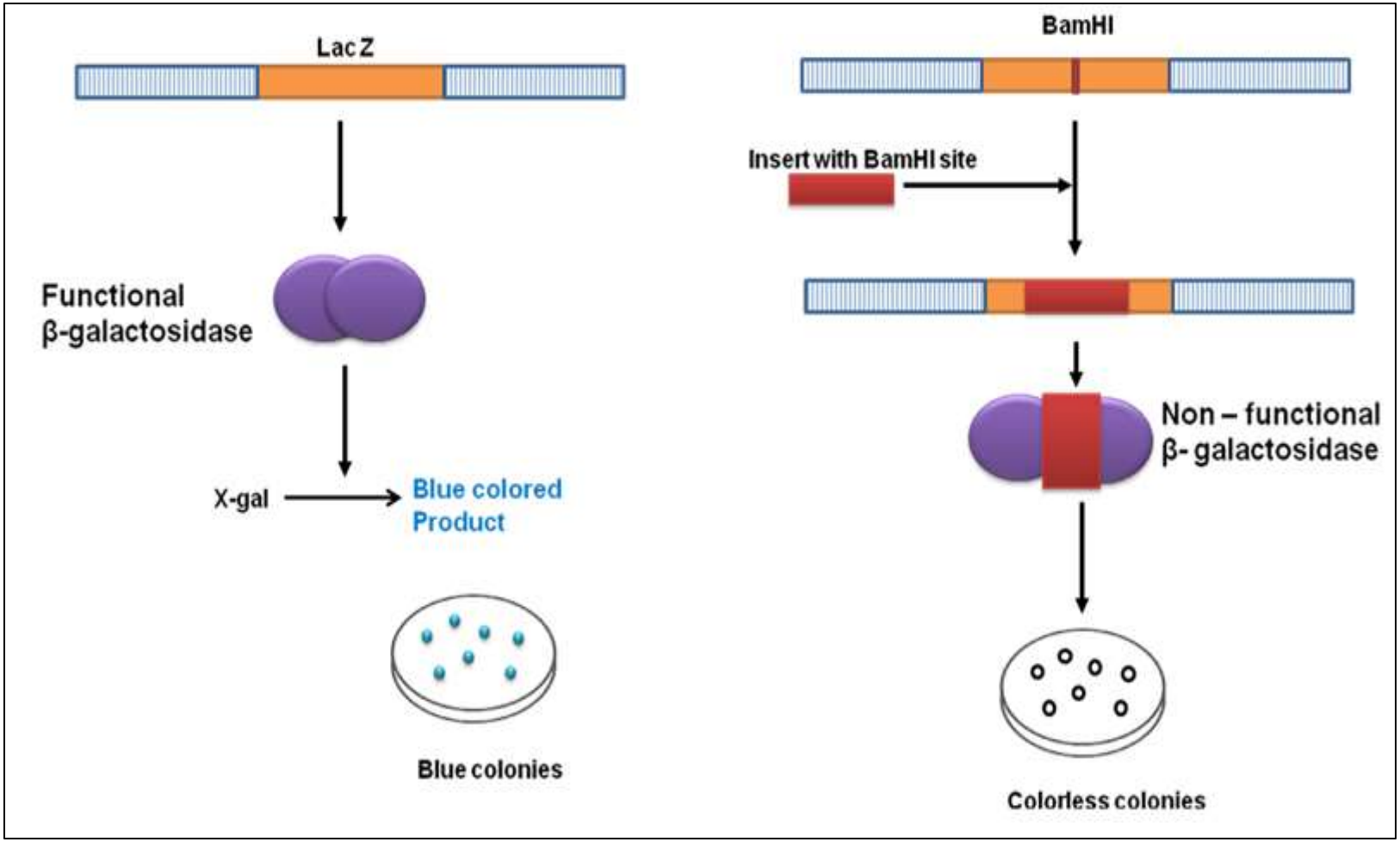

Twin antibiotic resistance screening
-Screening by insertional inactivation of a resistance gene
Amp^r
Tc^r
pBR322
ori
B
X
B
B
Amp^r
X
B
ori
Amp^r
Tc^r
ori
Ampicillin resistant?
yes
yes
Tetracycline resistant?
No
yes

H1-2 Twin antibiotic resistance

A vector with two antibiotic resistance genes can be used to screen for recombinants if the target fragment is inserted into one of the genes, thus insertionally inactivating it.



Blue white screening



Cloning vectors

Allowing the exogenous DNA to be inserted, stored, and manipulated mainly at DNA level.

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

What determines the choice vector?

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- Vector size
- Restriction sites
- Regulatory sequences (as promoters)
- Copy number
- Cloning efficiency
- Ability to screen for inserts

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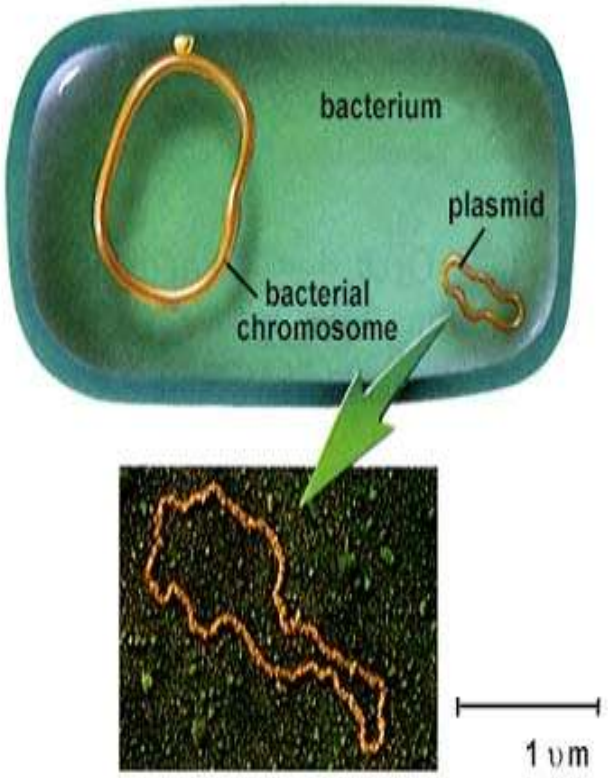
YACs	*Saccharomyces cerevisiae*	200–2000 kb

Plasmid vectors

- Double-stranded, circular, self-replicating, extra-chromosomal DNA molecules.
- Circular DNA molecules present in the cytoplasm of the bacteria
- Capable of autonomous replication
- Can transfer genes from one cell to other
- Act as vectors in genetic engineering.
- Can also be present in Yeasts



Advantages:

Small, easy to handle

Useful for cloning small DNA fragments (< 10kbp)

Disadvantages:

Less useful for cloning large DNA fragments (> 10kbp)

Plasmid vector:

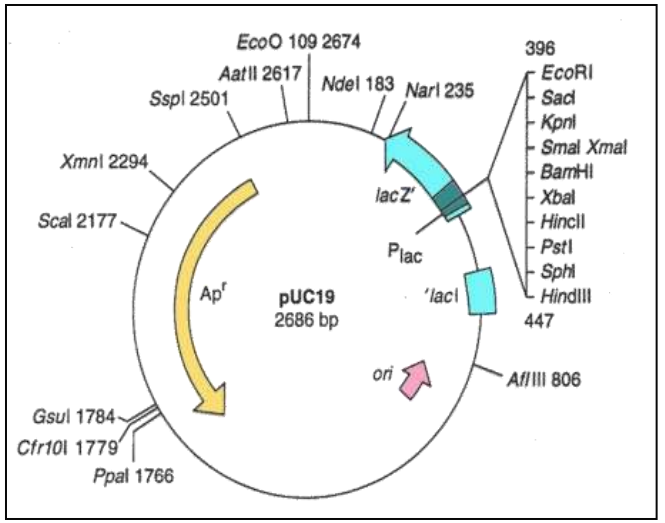
Plasmid vectors may encode genetic information for properties:

1. Resitance to Antibiotics
2. Bacteriocin production
3. Enterotoxin production
4. Enhanced pathogenicity
5. Reduced Sensitivity to mutagens
6. Degrade complex organic molecules

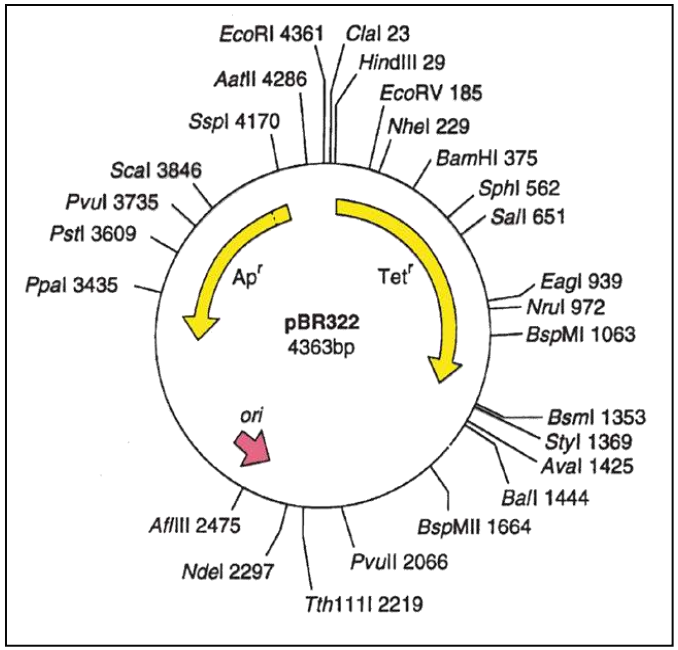
- Contains an origin of replication, allowing for replication independent of host's genome.
- Contains Selective markers: Selection of cells containing a plasmid

  twin antibiotic resistance

  blue-white screening
- Contains a multiple cloning site (MCS)
- Easy to be isolated from the host cell.



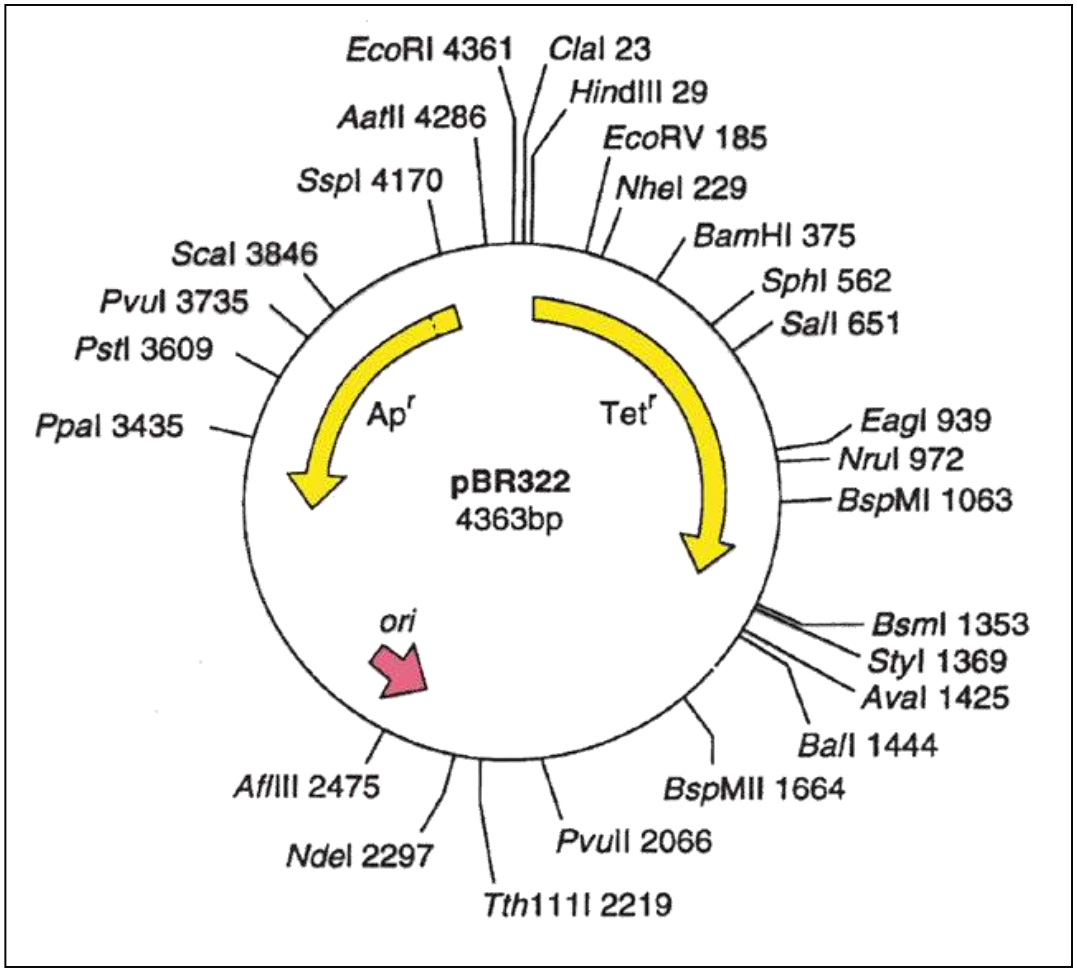
Vector Map



- **pBR322** is a plasmid and was one of the first widely used *E. coli* cloning vectors. Created in 1977 in the laboratory of Herbert Boyer at the University of California, San Francisco.

- Named after Francisco Bolivar Zapat - p stands for "plasmid," and BR for "Bolivar" and "Rodriguez.“

- pBR322 is 4361 base pairs in length and has two antibiotic resistance genes – the gene *bla* encoding the ampicillin resistance (Amp^R) protein, and the gene *tetA* encoding the tetracycline resistance (Tet^R) protein.
- It contains the origin of replication of pMB1, and the *rop* gene, which encodes a restrictor of plasmid copy number.
- The plasmid has unique restriction sites for more than forty restriction enzymes. Eleven of these forty sites lie within the Tet^R gene. There are two sites for restriction enzymes HindIII and ClaI within the promoter of the Tet^R gene.
- There are six key restriction sites inside the Amp^R gene.
- The circular sequence is numbered such that after the unique EcoRI site, the count increases through the Tet^R gene.
- The Amp^R gene is penicillin beta-lactamase.
- Promoters P1 and P3 are for the beta-lactamase gene. P3 is the natural promoter, and P1 is artificially created by the ligation of two different DNA fragments to create pBR322. P2 is in the same region as P1, but it is on the opposite strand and initiates transcription in the direction of the tetracycline resistance gene.

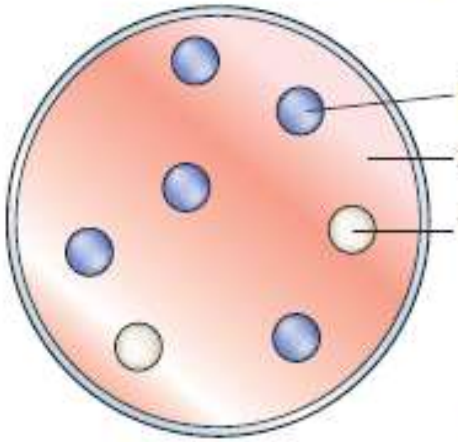

EcoRI 4361
ClaI 23
HindIII 29
AatII 4286
EcoRV 185
SspI 4170
NheI 229
BamHI 375
ScaI 3846
SphI 562
PvuI 3735
SalI 651
PstI 3609
Ap^r
Tet^r
PpaI 3435
EagI 939
NruI 972
pBR322
4363bp
BspMI 1063
ori
BsmI 1353
StyI 1369
AvaI 1425
BalI 1444
AflIII 2475
BspMII 1664
NdeI 2297
PvuII 2066
Tth111I 2219

pBR322

pUC series

- Cloning vector
- The features of pBR322 have been enhanced by the construction of a series of plasmids termed pUC (produced at the University of California).
- There is an antibiotic resistance gene for tetracycline and origin of replication for *E. coli*. There is presence of multiple restriction sites termed the multiple cloning site (MCS).
- It additionally codes for a portion of a polypeptide called β-galactosidase facilitating blue white screening.
- When the pUC plasmid has been used to transform the host cell *E. coli* the gene may be switched on by adding the inducer IPTG (isopropyl-β-D-thiogalactopyranoside).
- Its presence causes the enzyme β-galactosidase to be produced. The functional enzyme is able to hydrolyse a colourless substance called X-gal (5-bromo-4-chloro-3-indolylb-galactopyranoside) into a blue insoluble material (5,50-dibromo-4,40–dichloro indigo).
- However if the gene is disrupted by the insertion of a foreign fragment of DNA, a non-functional enzyme results which is unable to carry out hydrolysis of X-gal.
- This makes the recombinant pUC plasmid to be easily detected as it is white or colourless in the presence of X-gal.
- An intact non-recombinant pUC plasmid will be blue since its gene is fully functional and not disrupted. This elegant system, termed blue/white selection.

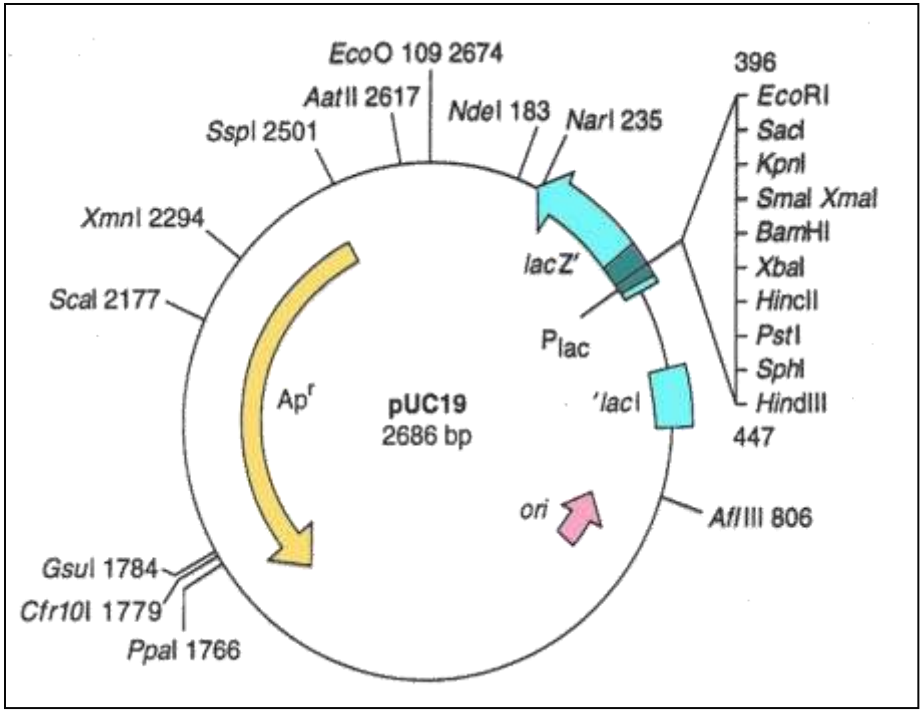
Screening for pUC8 recombinants

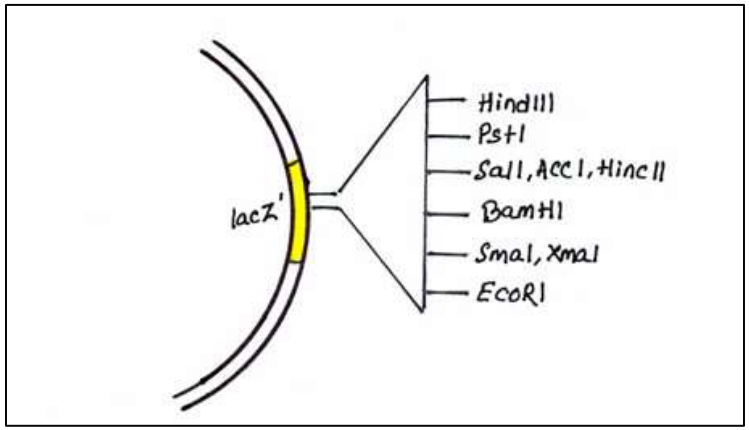


Blue colonies = β-galactosidase synthesized
X-gal → blue product

White colonies = β-galactosidase not synthesized
X-gal → no blue product

Recombinants are screened by plating onto agar containing X-gal and IPTG.


EcoO 109 2674
AatII 2617
NdeI 183
NarI 235
SspI 2501
XmnI 2294
ScaI 2177
GsuI 1784
Cfr10I 1779
PpaI 1766
396
EcoRI
SacI
KpnI
SmaI XmaI
BamHI
XbaI
HincII
PstI
SphI
HindIII
447
lacZ'
Plac
'lacI
Ap^r
pUC19
2686 bp
ori
AflIII 806


lacZ'
HindIII
PstI
SalI, ACCI, HincII
BamHI
SmaI, XmaI
EcoRI

pUC19

- **pUC19** is one of a series of plasmid cloning vectors created by Joachim Messing and co-workers.
- The designation "pUC" is derived from the classical "p" prefix (denoting "plasmid") and the abbreviation for the University of California, where early work on the plasmid series had been conducted.
- It is a circular double stranded DNA and has 2686 base pairs.
- pUC19 – blue white screening.
- pUC18 is similar to pUC19, but the MCS region is reversed. Notably, it has a N-terminal fragment of β-galactosidase (*lacZ*) gene of *E. coli*.
- The multiple cloning site (MCS) region is split into codons 6-7 of the lacZ gene, providing for many restriction endonucleases restriction sites.
- In addition to β-galactosidase, pUC19 also encodes for an ampicillin resistance gene (amp^R), via a β-lactamase enzyme that functions by degrading ampicillin and reducing its toxicity to the host.
- The *ori* site, or origin of replication, is derived from the plasmid pMB1.
- pUC19 is small but has a high copy number. The high copy number is a result of the lack of the *rop* gene and a single point mutation in the ori of pMB1.
- The *lacZ* gene codes for β-galactosidase. The recognition sites for HindIII, SphI, PstI, SalI, XbaI, BamHI, SmaI, KpnI, SacI and EcoRI restric tion enzymes have been derived from the vector M13mp19

pUC vectors carry different combinations of restriction sites and show greater flexibility in the types of DNA fragment that can be cloned.

Clustering of the restriction sites allows a DNA fragment with two different sticky ends to be cloned without involving linker attachment.

DNA cloned into a member of the pUC series can be transferred directly to its M13mp counterpart, because of the same restriction site clusters and it can be analysed by DNA sequencing or in vitro mutagenesis.

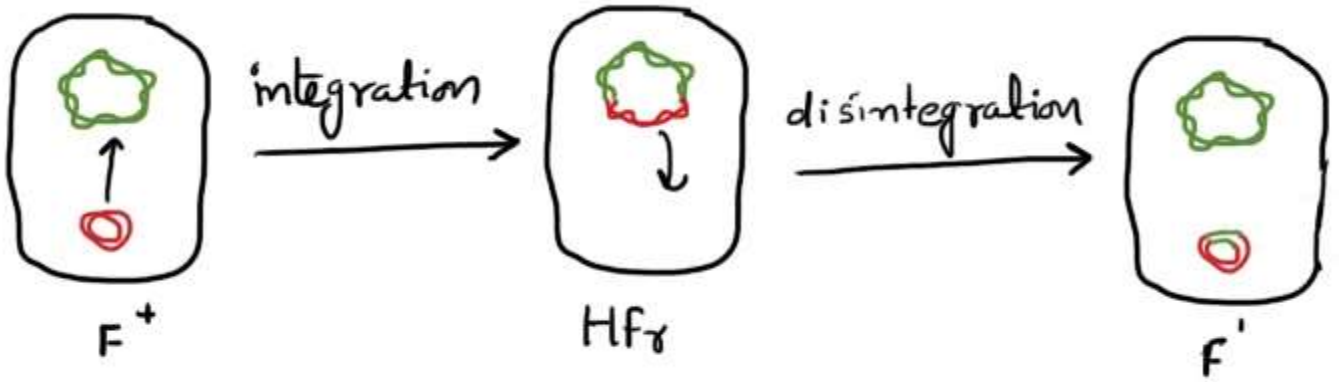
Bacteriophage Lambda phage

Phage Lambda

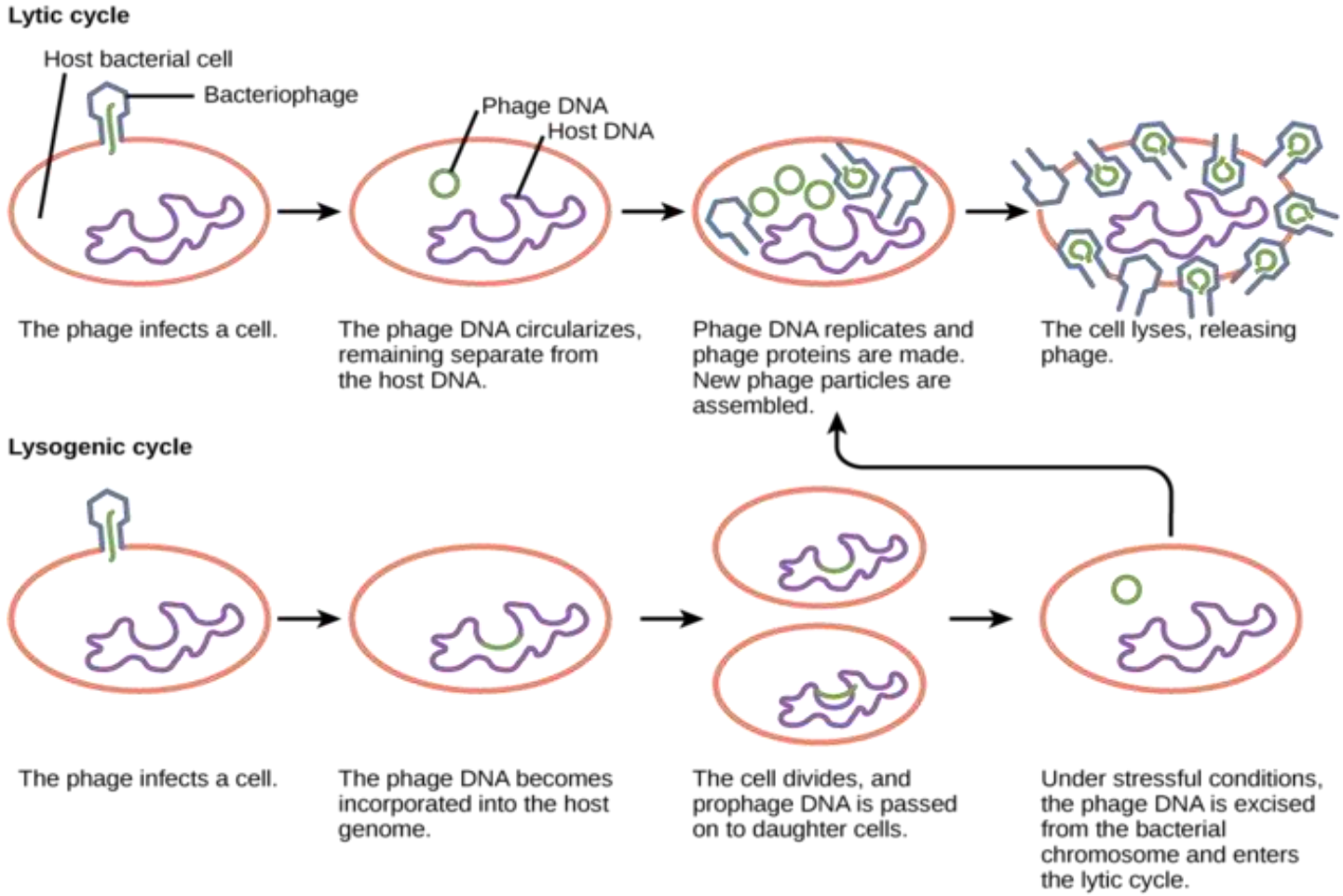
M13

P1 Phage

T4, T7



Strain types

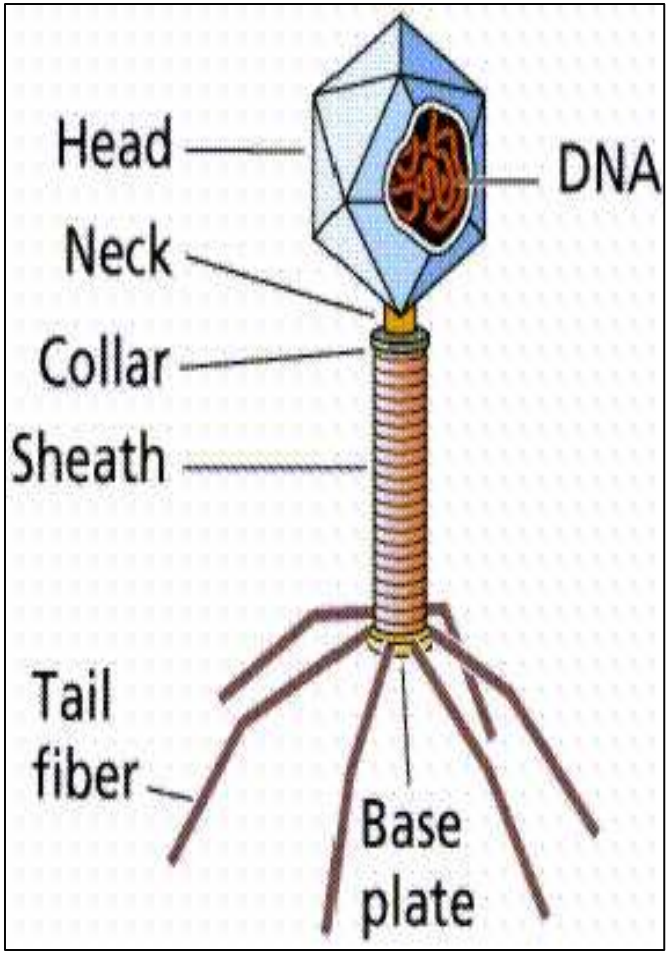


Lytic versus lysogenic cycle: A temperate bacteriophage has both lytic and lysogenic cycles.
In the lytic cycle, the phage replicates and lyses the host cell. In the lysogenic cycle, phage DNA is incorporated into the host genome, where it is passed on to subsequent generations. Environmental stressors such as starvation or exposure to toxic chemicals may cause the prophage to excise and enter the lytic cycle.

Bacteriophage vectors

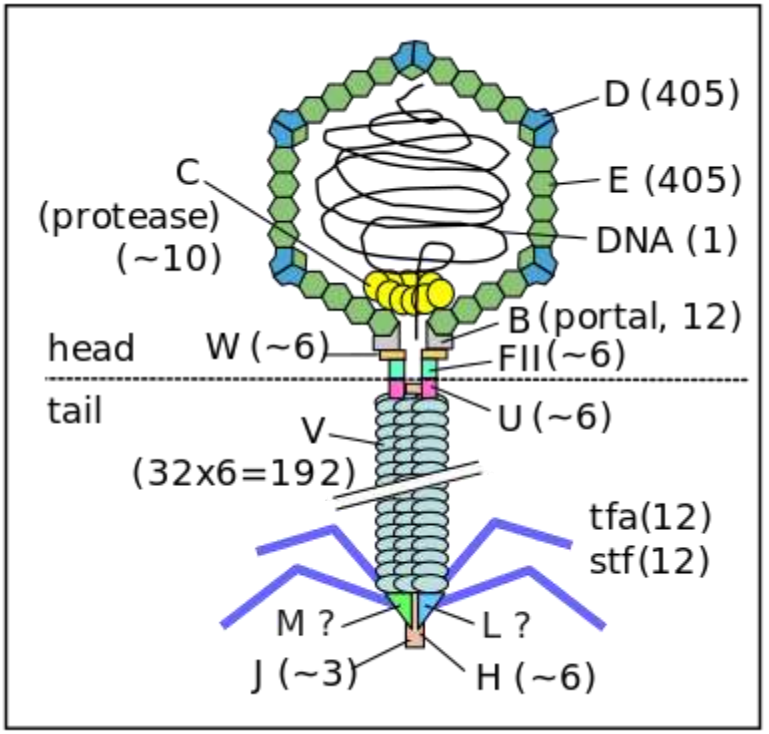
- ***Advantages:***
  - Useful for cloning large DNA fragments
  - 10-23kbp

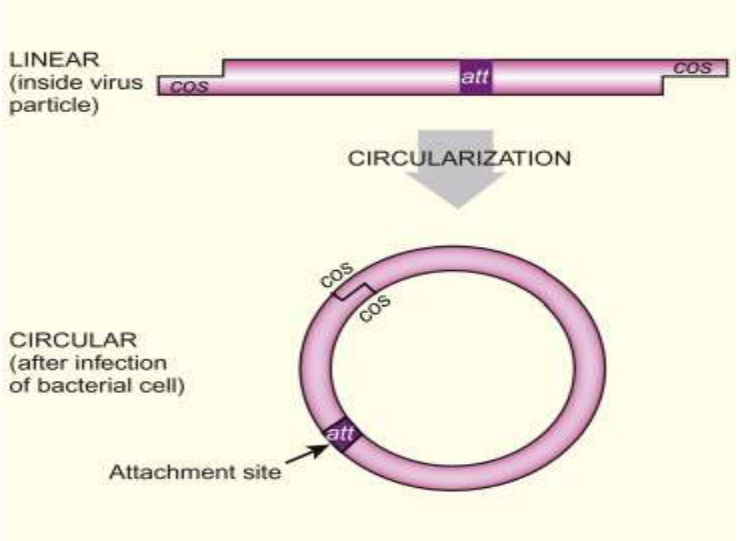
    (May go upto - 50 kbp in some cases)
  - Inherent size selection for large inserts
- ***Disadvantages:***
  - Less easy to handle
  - Ex – Lambda phage, M13, P1



Page Lambda

- ***Enterobacteria phage λ*** (**lambda phage**, **coliphage λ**, - infects the bacterial species *Escherichia coli*.
- The phage particle consists of a head (also known as a capsid), a tail, and tail fibers.
- The head contains the phage's double-stranded linear DNA genome.
- During infection, the phage particle recognizes and binds to its host, *E. coli*, causing DNA in the head of the phage to be ejected through the tail into the cytoplasm of the bacterial cell.





Bacteriophage

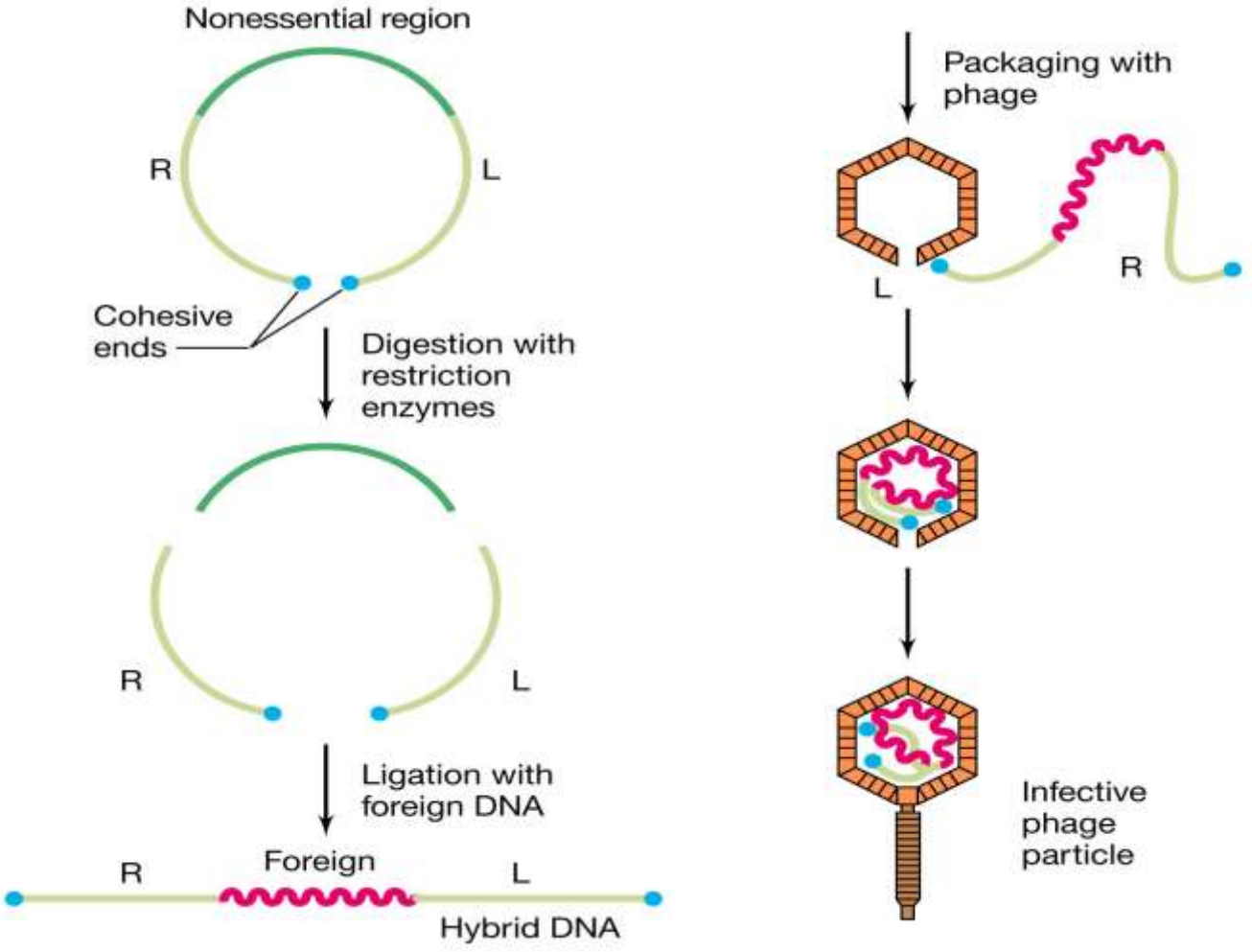
- The bacteriophages used for cloning are the M13 phage and phage λ
- Upper limit - amount of DNA that can be packed into a phage (~ 50 kb)
- Allows foreign DNA to be inserted into phage DNA
- Phage cloning vectors may need to have some non-essential genes deleted, for example the genes for lysogeny since using phage λ as a cloning vector involves only the lytic cycle.
- There are two kinds of λ phage vectors - insertion vector and replacement vector.

1. Insertion vectors contain a unique cleavage site whereby foreign DNA with size of 5–11 kb may be inserted.
2. In replacement vectors, the cleavage sites flank a region containing genes not essential for the lytic cycle, and this region may be deleted and replaced by the DNA insert in the cloning process, and a larger sized DNA of 8–24 kb may be inserted.

- There is a lower size limit for DNA that can be packed into a phage, and vector DNA that is too small cannot be properly packaged into the phage.

- This property can be used for selection - vector without insert may be too small, therefore only vectors with insert may be selected for propagation.

Bacteriophage lambda as a cloning vector



transduction: transfer of host genes from one cell to another by a virus

(Fig. 10.44, p. 311, Madigan et al.)

Substitution Vectors/Replacement vectors

λ-vectors

- *Left arm*:
  - head & tail proteins
- *Right arm*:
  - DNA synthesis
  - regulation
  - host lysis
- *Deleted central region*:
  - integration & excision
  - regulation

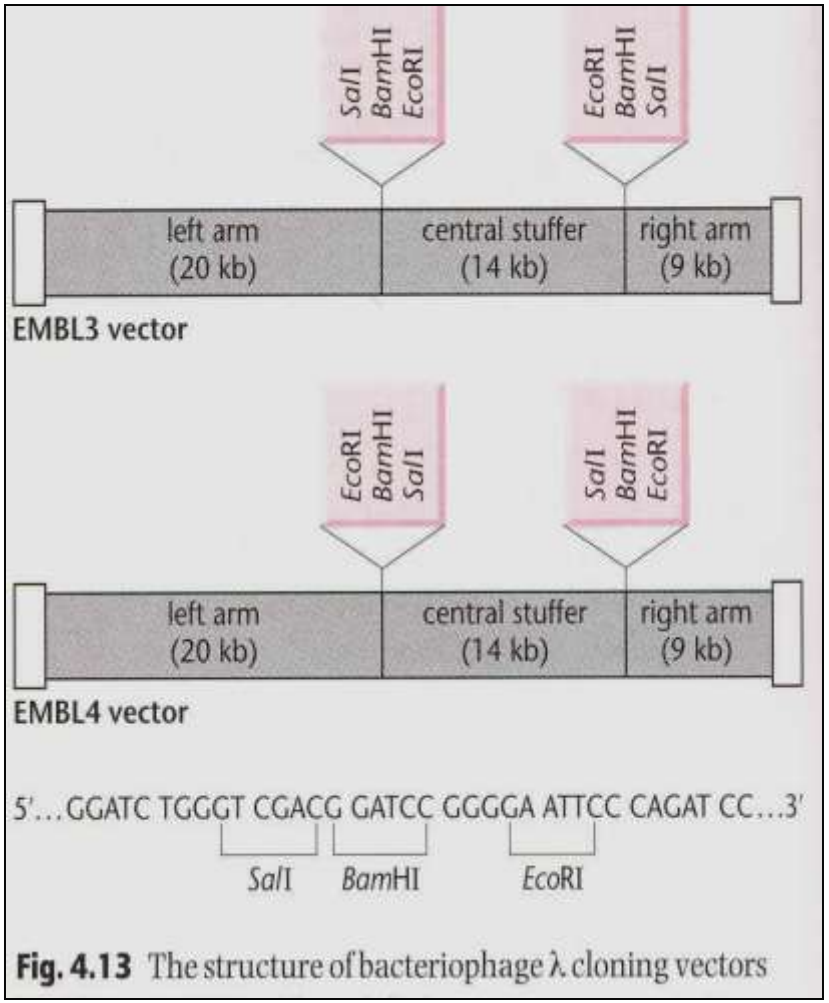


Fig. 4.13 The structure of bacteriophage λ cloning vectors

LAMBDA REPLACEMENT VECTORS

Replacement vectors

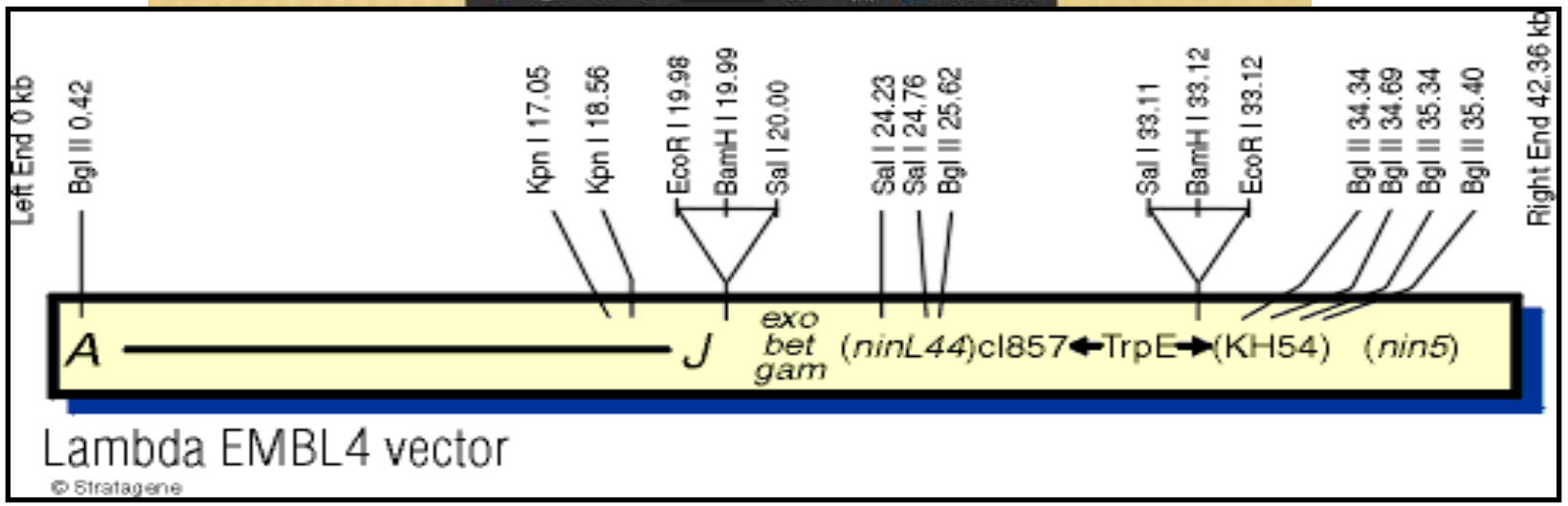
- A replacement vector has two recognition sites for the restriction endonuclease used

for cloning. These sites flank a segment of DNA that is replaced by the DNA to be cloned,.

- Often the replaceable fragment (or **"stuffer fragment"** in cloning jargon) carries additional restriction sites that can be used to cut it up into small pieces, so that its own re-insertion during a cloning experiment is very unlikely.
- Replacement vectors are generally designed to carry larger pieces of DNA than insertion vectors can handle.

An example of a replacement vectors is:

- **LAMBDA EMBL4** can carry up to 20 kb of inserted DNA by replacing a segment flanked by pairs of *Eco*RI, *Bam*HI, and *Sal*I sites. Any of these three restriction endonucleases can be used to remove the stuffer fragment, so DNA fragments with a variety of sticky ends can be cloned. Recombinant selection with eEMBL4 can be on th ype.



1.Inserting genes in replicative circular forms

2.Cloning involves replacing the central nonessential “stuffer fragment” by ligating insert DNA between the two arms

3. Introducing recombinant DNA molecule into a bacterial cell
Two methods: a)Transfection b)In vitro packaging

Transfection – Equivalent to transformation but Phage DNA with insert in circular form is inserted into competent cell, instead of plasmid

Transformation – Introducing plasmid into bacterial competent cell

Packaging the recombinant DNA into phage particles in vitro and infecting *E. coli* cells with the recombinant phage particles.

Bacteriophage M13

Bacteriophage

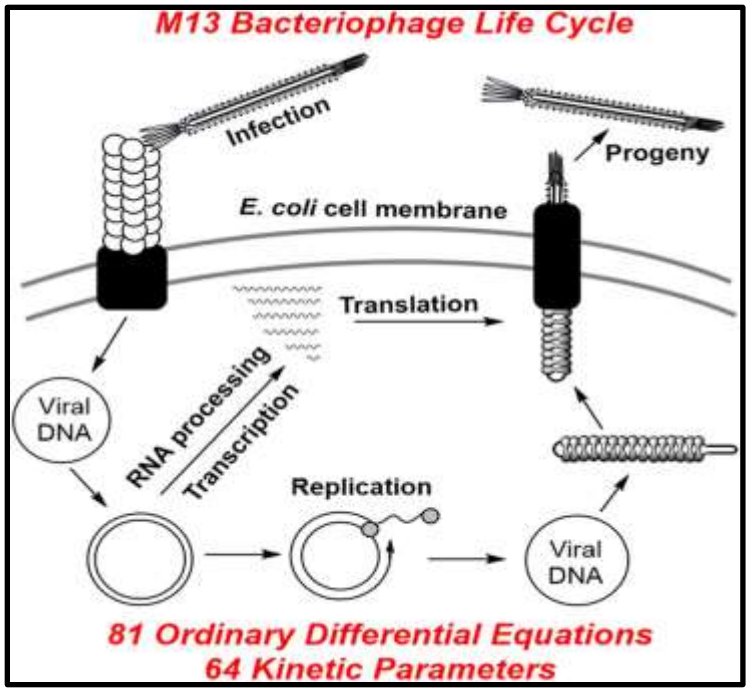
Viruses that infect and replicate only in bacterial cells

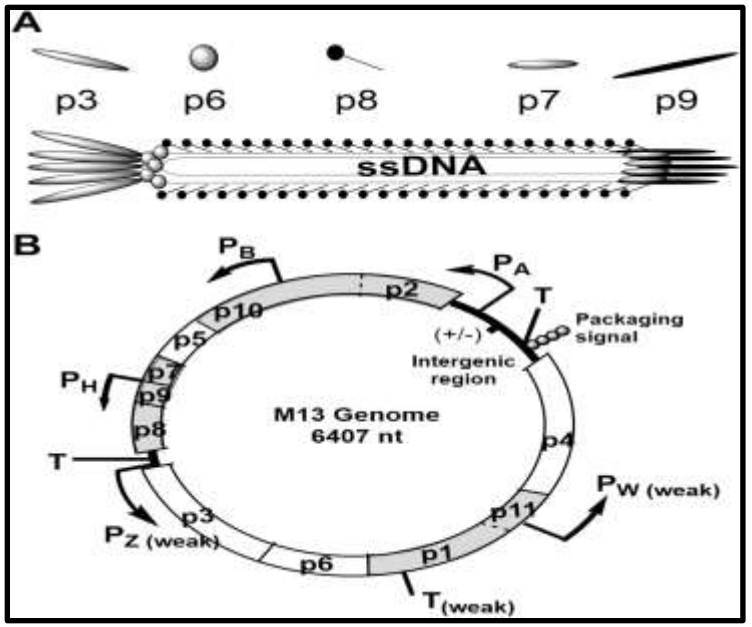
Bacteriphage M13 - **M13** is one of the Ff phages (fd and f1 are others), a member of the family filamentous bacteriophage (inovirus).

Ff phages are composed of circular single-stranded DNA (ssDNA)

m13 phage is 6407 nucleotides long and is encapsulated in approximately 2700 copies of the major coat protein p8

capped with about 5 copies each of four different minor coat proteins (p3 and p6 at one end and p7 and p9 at the other end)





Bacteriophage M13 – filamentous bacteriophage
circular DNA genome (6407 bp long)
– packaged inside rod-shaped protein capsid
M13 phage particles bind to F pilus and only infects F^+, Hfr, F' cells
Single-stranded DNA genome enters cell designated as "+" strand
"+" strand repaired to double-stranded **replicative form (RF)**

The double-stranded, closed-circular, replicative form (RF) of M13 DNA is present in high copy numbers in infected cells, and its physical characteristics are essentially identical to those of closed-circular plasmid DNAs.

M13 cloning vectors

RF can be digested with restriction endonucleases

inserts can be cloned in – like plasmid

"+" strands from phage particles

– convenient source of single-stranded DNA

– used for sequencing and site-directed mutagenesis

different sized DNA molecules packaged as phage particle

– phage with inserts > 2 kb replicated slower

– produce different size phage particles

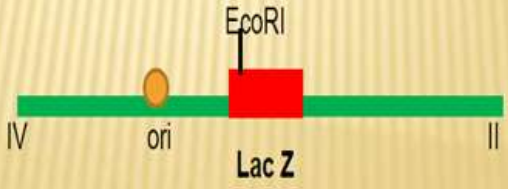
CONSTRUCTION M13 AS PHAGE VECTOR

1. The first step in construction of an M13 cloning vector was to introduce the *lacZ'* gene into the intergenic sequence.
2. This gave rise to M13mp1, which forms blue plaques on X-gal agar.
3. M13mp1 does not possess any unique restriction sites in the *lacZ'* gene.

M13MP1 VECTOR

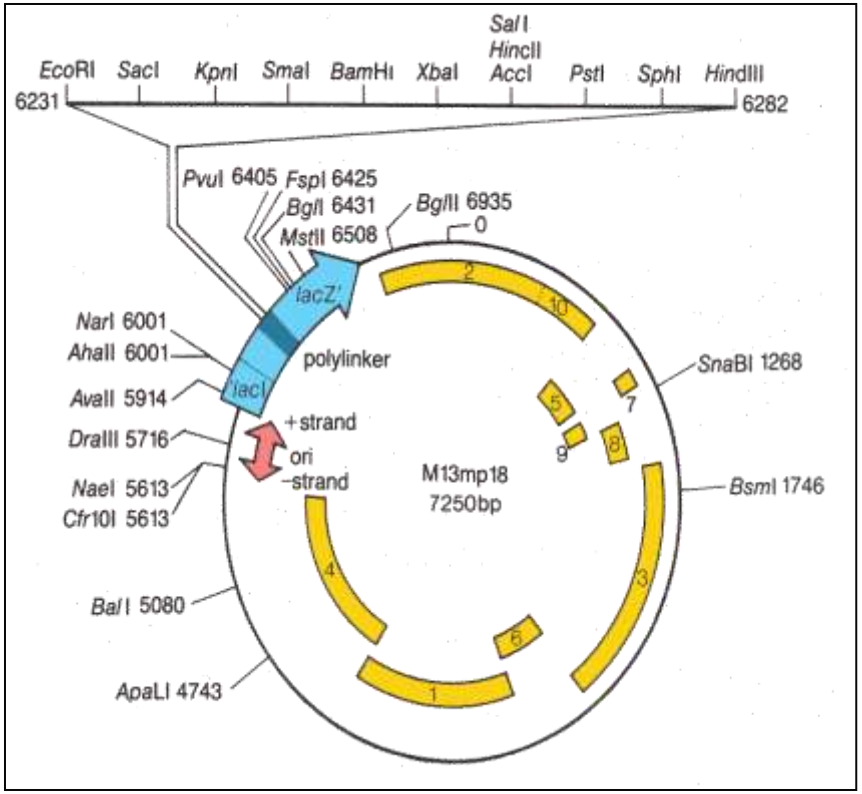
M13 MP 2 VECTOR

- It contains 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.



M13MP7 VECTOR

- A **polylinker**, which consists of a series of restriction sites and has *Eco*RI sticky ends.
- This polylinker was inserted into the *Eco*RI site of M13mp2, to give M13mp7 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. Although it is altered, b-galactosidase enzyme is still produced.

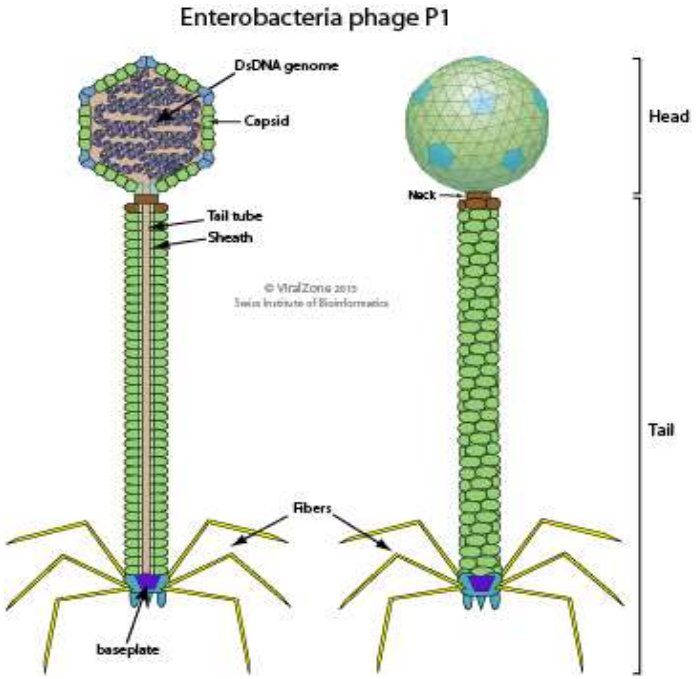


P1 phage - **P1** is a temperate bacteriophage that infects *Escherichia coli* and some other bacteria.

Genetic material – Double stranded DNA

Temperate phage - ability of some bacteriophages to display a lysogenic life cycle.

When undergoing a **lysogenic cycle** the phage genome **exists as a plasmid** in the bacterium.



P1 PHAGE

- **P1** is a temperate bacteriophage (phage) that infects *Escherichia coli* and a some other bacteria.
- When undergoing a lysogenic cycle the phage genome exists as a plasmid in the bacterium. Unlike other phages it integrate into the host DNA.
- P1 has an icosahedral "head" containing the DNA attached to a contractile tail with six tail fibers.

GENOME OF P1 PHAGE

- The genome of the P1 phage is moderately large, around 93Kbp in length. In the viral particle it is in the form of a linear double stranded DNA molecule. Once inserted into the host it circularizes and replicates as a plasmid.
- The genome is especially rich in Chi sequences recognized by the bacterial recombinase **RecBCD.**
- The genome contains two origins of replication, oriR which replicates it during the lysogenic cycle and oriL which replicates it during the lytic stage.

P1 PHAGE AS VECTOR

- The development of a bacteriophage P1 cloning system capable of accepting DNA fragments as large as 100 kilobase pairs (kbp).
- Phage particles has two P1 loxP recombination sites to cyclize the packaged DNA once it has been injected into a strain of *Escherichia coli* containing the P1 Cre recombinase, a kanamycin resistant gene to select bacterial clones containing the cyclized DNA.
- P1 plasmid replicon to stably maintain that DNA in *E. coli* at one copy per cell chromosome, and a lac promoter-regulated P1 lytic replicon to amplify the DNA before it is reisolated.

P1 PHAGE VECTOR SYSTEM

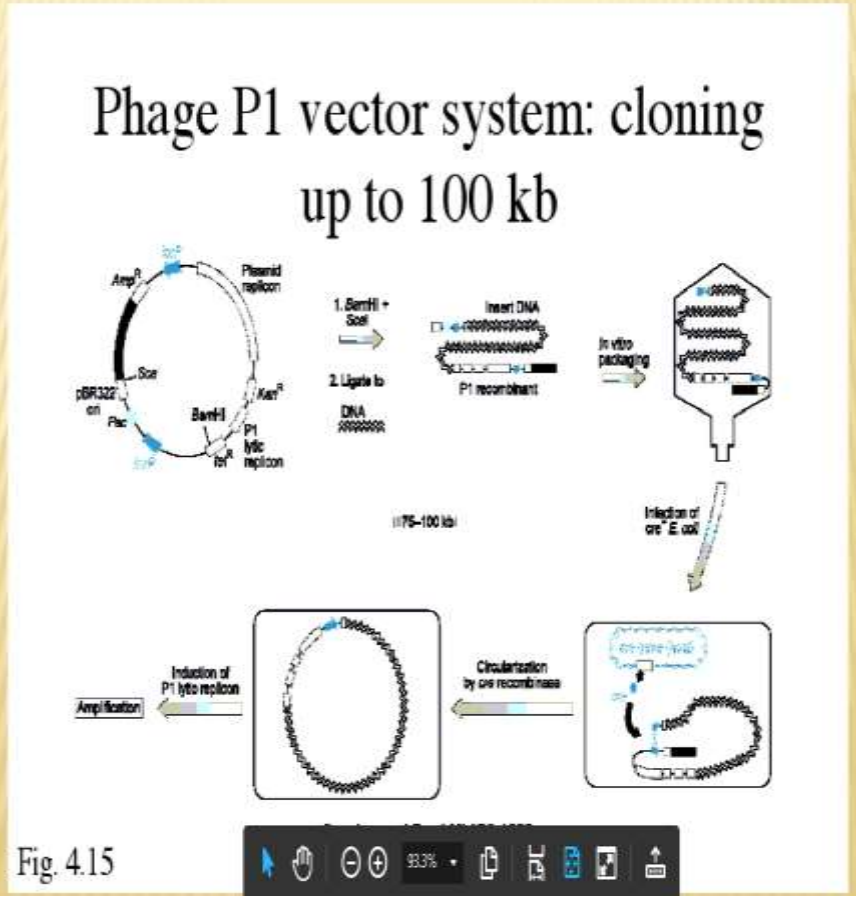
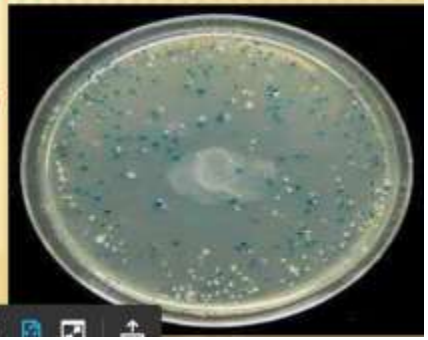


Fig. 4.15

SELECTION OF RECOMBINANTS

- The vector is then inserted into a competent host cell viable for transformation, which are then grown in the presence of X-gal.
- Cells transformed with vectors containing recombinants will produce white colonies; non-recombinant plasmids (i.e. only the vector) grow into blue colonies.



Questions