VECTORS

What are vectors?

Vectors are the DNA molecules that have ability to replicate in an appropriate host cell and into which the DNA insert is integrated for cloning.

Eg: Plasmids, Phages, Viruses etc.

Types of cloning vectors

1. plasmids (about 20kb inserts)
2. Bacteriophage 30-50kb inserts
3. Cosmids (35-50kb insert)
4. BACs

- Use fertility F plasmid
- 75-300kb inserts possible
- developed during the human genome project

5. YACs

- Mimics yeast chromosome
- Contains all regions for replication (yeast ori and centromere)
- 100-1000kb inserts possible.
- developed during the human genome project

Vectors

- Autonomous replication
- Less than 10kb in size
- Easy to isolate and purify
- Ability to integrate
- Unique target sites eg: MCS
- Vector sequence to be devoid of methylase-Resistance
- Suitable marker genes

Most of the natural vectors do not have all the required functions so useful vectors have been constructed

Plasmid Vectors

PLASMIDS:

Autonomously Replicating double stranded Covalently Closed Circular DNA

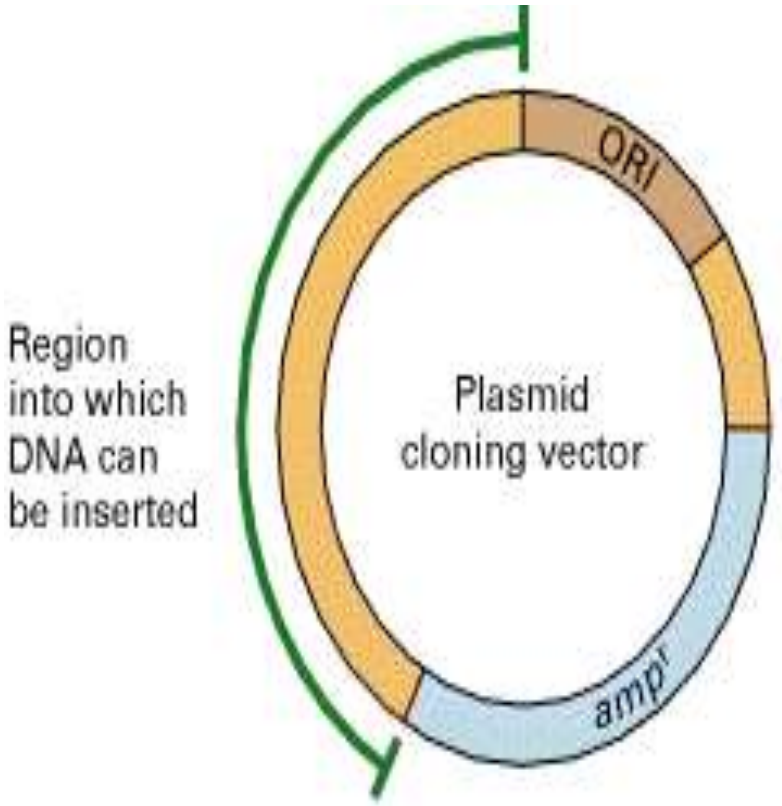
Types of plasmids

Vectors: 2 types

- Cloning vectors
  - Propogation of the DNA in the host: REPLICATION
- Expression vectors
  - Production of specified proteins: OPERON Regulation

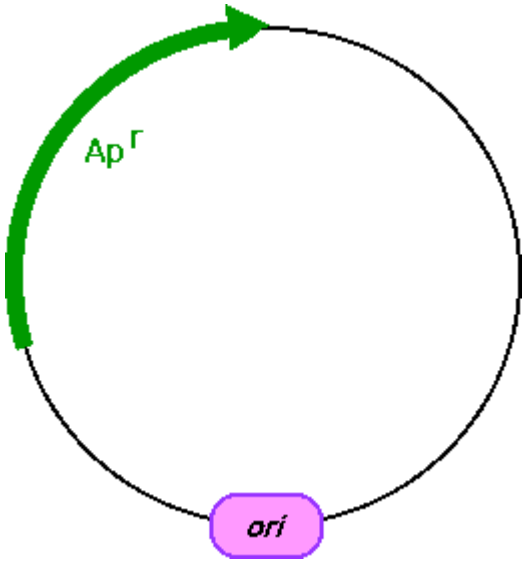
PLASMID VECTORS

- **Plasmid vectors are ≈1.2–3kb and contain:**
- **replication origin (ORI) sequence**
- **a gene that permits selection,**
- **Here the selective gene is *amp*r; it encodes the enzyme b-lactamase, which inactivates ampicillin.**
- **Exogenous DNA can be inserted into the bracketed region .**



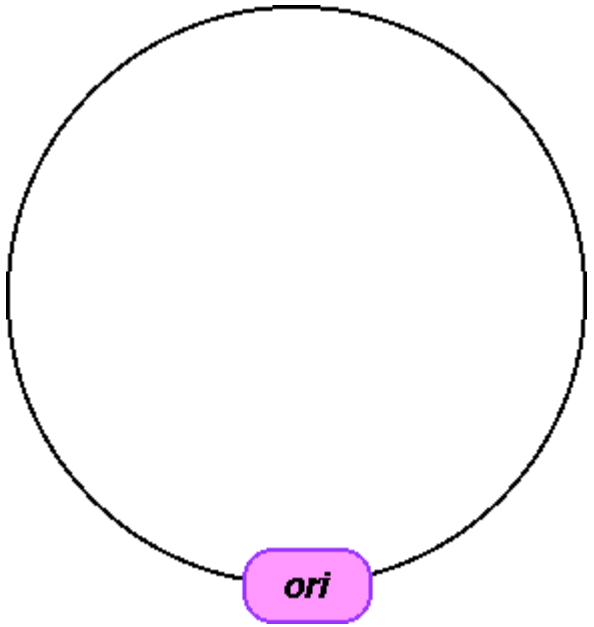
SELECTIVE MARKER

- **Selective marker** is required for maintenance of plasmid in the cell.
- Because of the presence of the selective marker the plasmid becomes useful for the cell.
- **Under the selective conditions**, only cells that contain plasmids with selectable marker can survive
- Genes that confer resistance to various antibiotics are used.
- Genes that make cells resistant to ampicillin, neomycin, or chloramphenicol are used



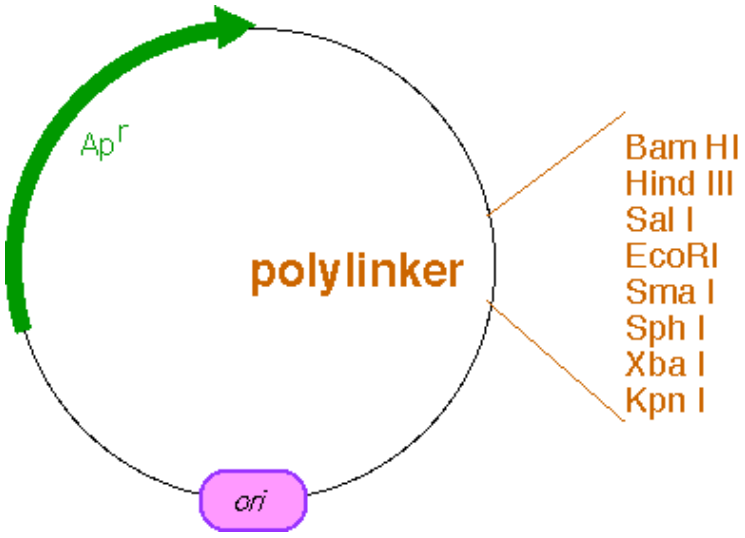
ORIGIN OF REPLICATION

- **Origin of replication** is a DNA segment recognized by the cellular DNA-replication enzymes.
- Without replication origin, DNA cannot be replicated in the cell.



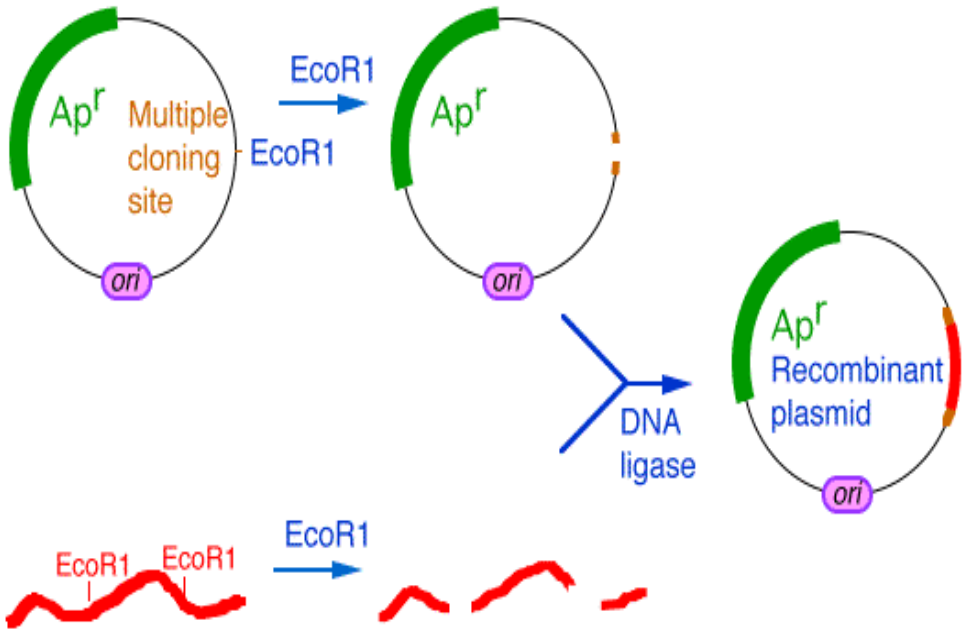
MULTIPLE CLONING SITE

- Many cloning vectors contain a **multiple cloning site** or **polylinker**: a DNA segment with several unique sites for restriction endonucleases located next to each other
- Restriction sites of the polylinker are not present anywhere else in the plasmid.
- Cutting plasmids with one of the restriction enzymes that recognize a site in the polylinker does not disrupt any of the essential features of the vector



MULTIPLE CLONING SITE

- **Gene to be cloned can be introduced into the cloning vector at one of the restriction sites present in the polylinker**



Properties of Plasmids:

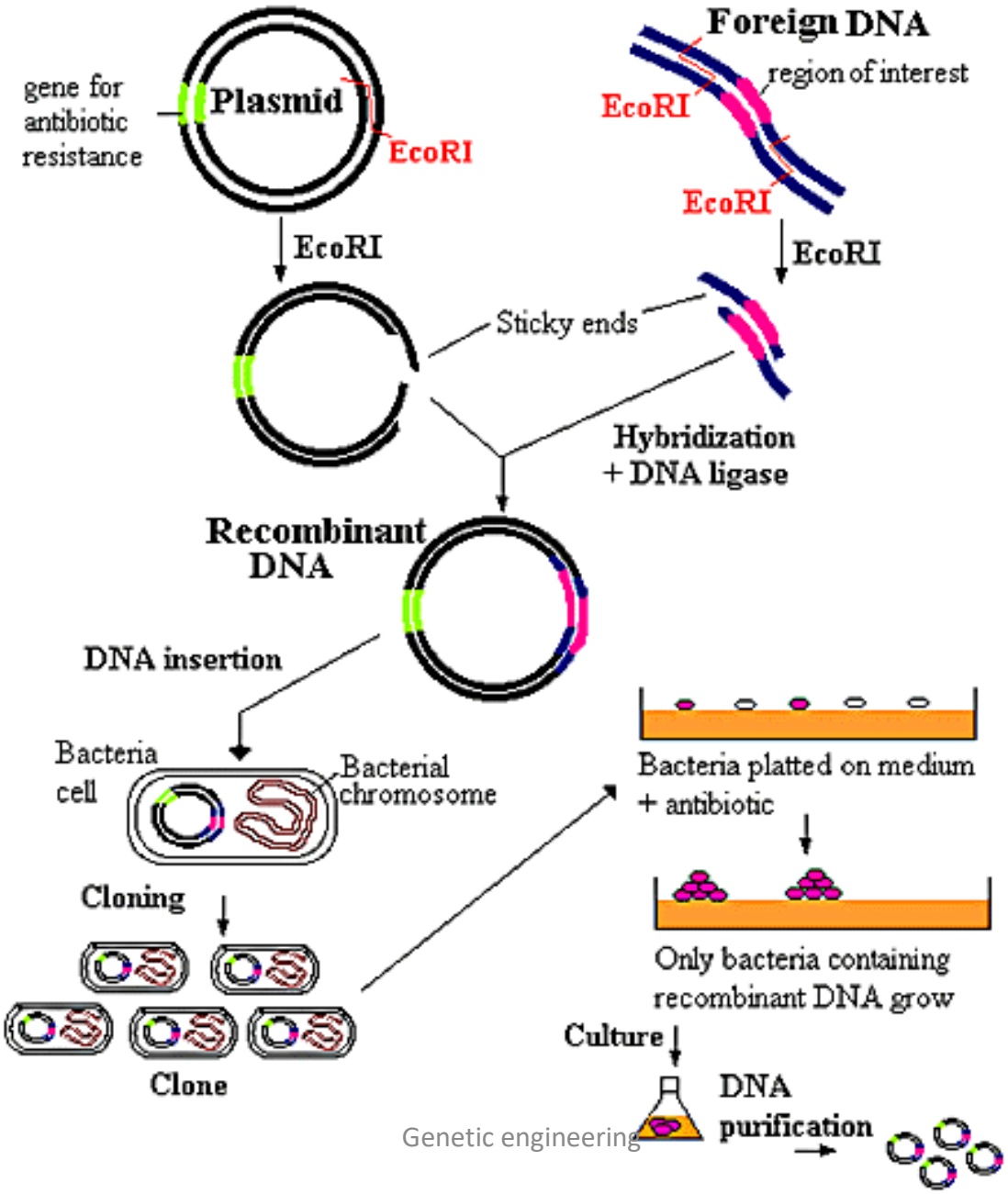
- Replicon
- Not required for survival of the host: EXCEPTIONS!
- Size: 1-250kb
- Have variable marker genes
- Easy to integrate, isolate and purify.

Plasmid incompatibility????

Types of plasmids:

1. F-Plasmids: Fertility factor
2. R-Plasmids: Resistance to antibiotics
3. Col plasmids: production of bacteriocins
4. Virulence plasmids: pathogenicity
5. Degradative plasmids: ability to degrade effluents *etc.*

Cloning using plasmid vector

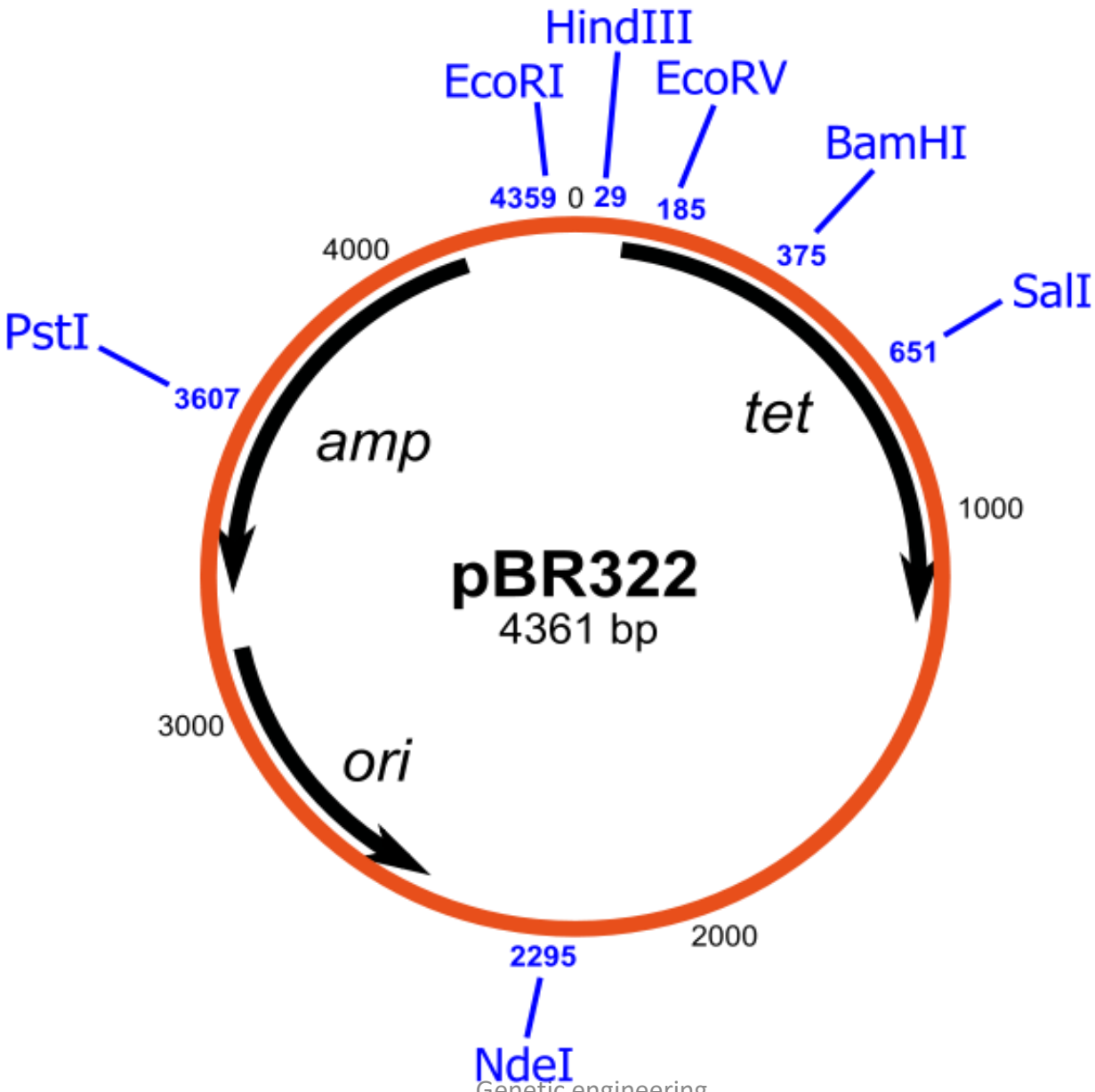


Plasmids as ideal vector:

- Can be readily isolated from cells
- Possesses a single restriction site for 1/ more RE
- Easy selection and screening possible.
- small

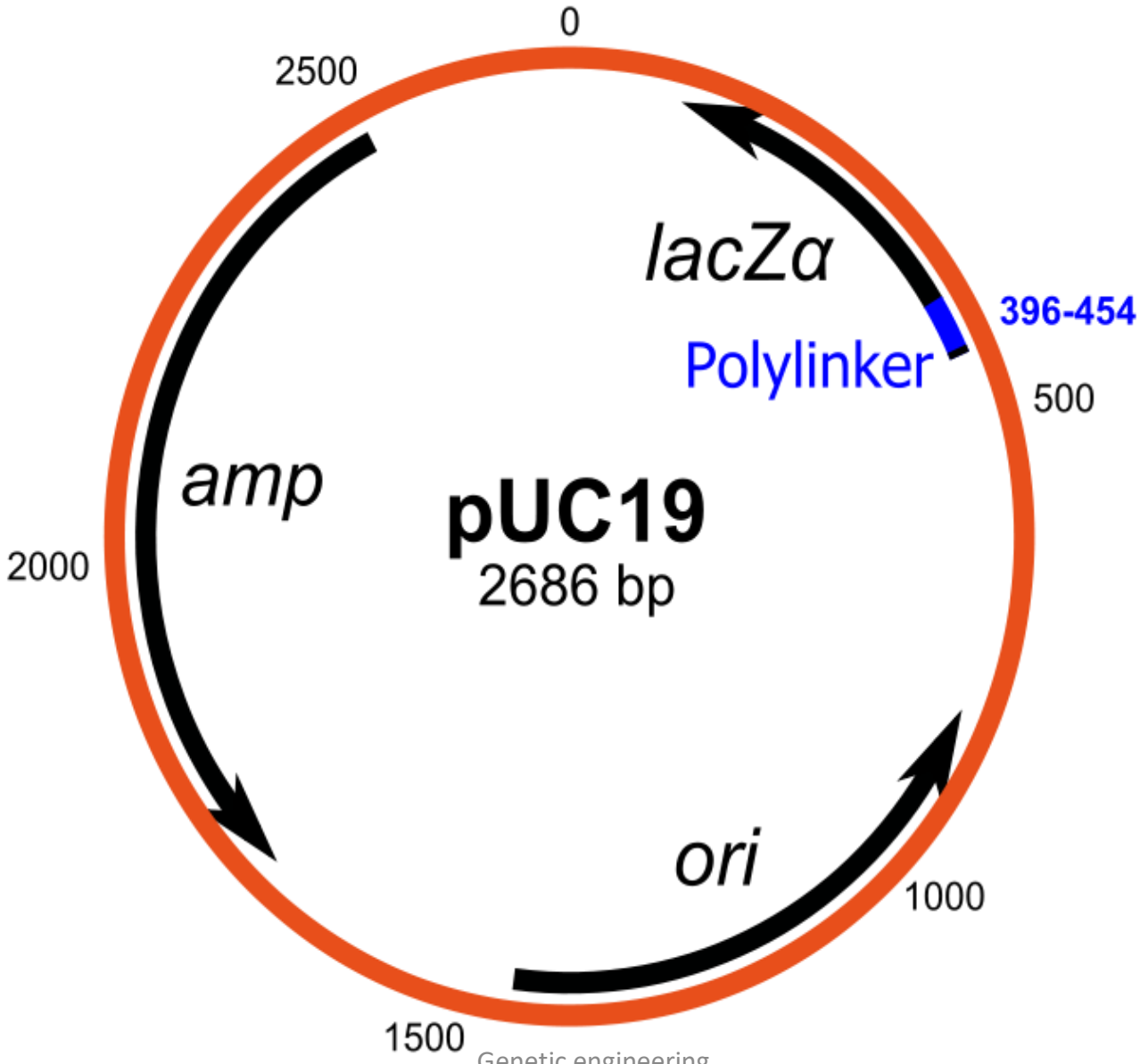
1. pBR 322

- First artificial cloning vector (1977) from E. coli plasmid ColE1 by Boliver and Rodriguez
- 4.362Kb size
- Copy no: 15-20 per cell. (If required the copy no can be amplified in chloramphenicol containing media)
- Has genes conferring resistance to antibiotics: Amp, tetr etc
- Has about 20 sites for unique R like EcoRIV, BamHI, SalI *etc*. in the tet^r
- Construct of pBR322:
  - ***Replicon:***plasmid pMB1
  - ***amp^R*** gene: plasmid RSF2124
  - ***tet^R*** gene: plasmid pSC101


HindIII
EcoRI
EcoRV
BamHI
4359
0
29
185
375
4000
SalI
651
PstI
3607
amp
tet
1000
pBR322
4361 bp
3000
ori
2000
2295
NdeI

2. pUC vectorseries

- 1983: Messings and coworkers-plasmid at University of California.
- Size: 2686bp
- Has Multiple Cloning Site (MCS)
- Exhibits Insertional Inactivation phenomenon
- Examples of the series: pUC 8/9/12/13/18/19 *etc.*
- Insert: less than 10kb
- Construct:
  - Ori (E. col ColE1)
  - Amp^r gene
  - Lac Z' gene


0
500
1000
1500
2000
2500
lacZα
396-454
Polylinker
amp
pUC19
2686 bp
ori

Selection by Blue white colony:

Cells containing pUC19 are grown in IPTG (Isopropylthiogalactoside-Inducer to *lac* operon) containg media.

↓

Lac Z' gene produces hybrid β-Galactosidase.

↓

X-Gal (5-bromo-4-chlorinolyl- β galactosidase) is a chromogenic substrate which if hydrolyzed gives blue color formation.

↓

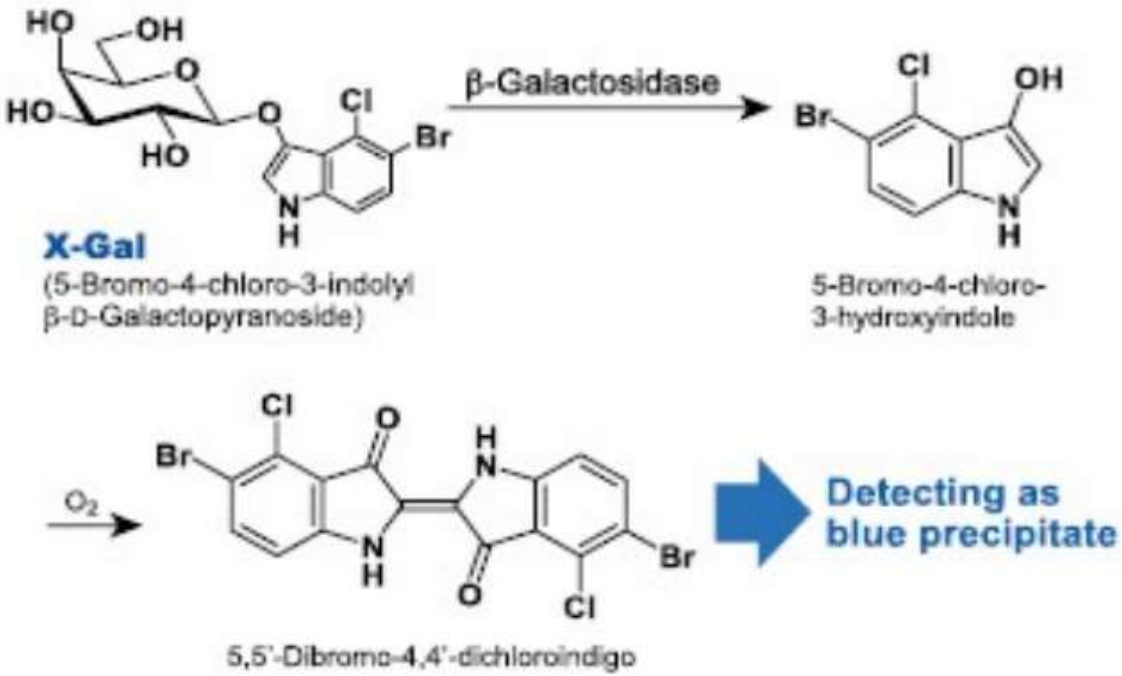
If gene inserted in the lacZ' gene, no enzyme will be formed, hence no hydrolysis of chromogen **(insertional inactivation)**

↓

Colorless colonies: contain the recombinant plasmid

Blue colonies: Not the recombinant plasmids.

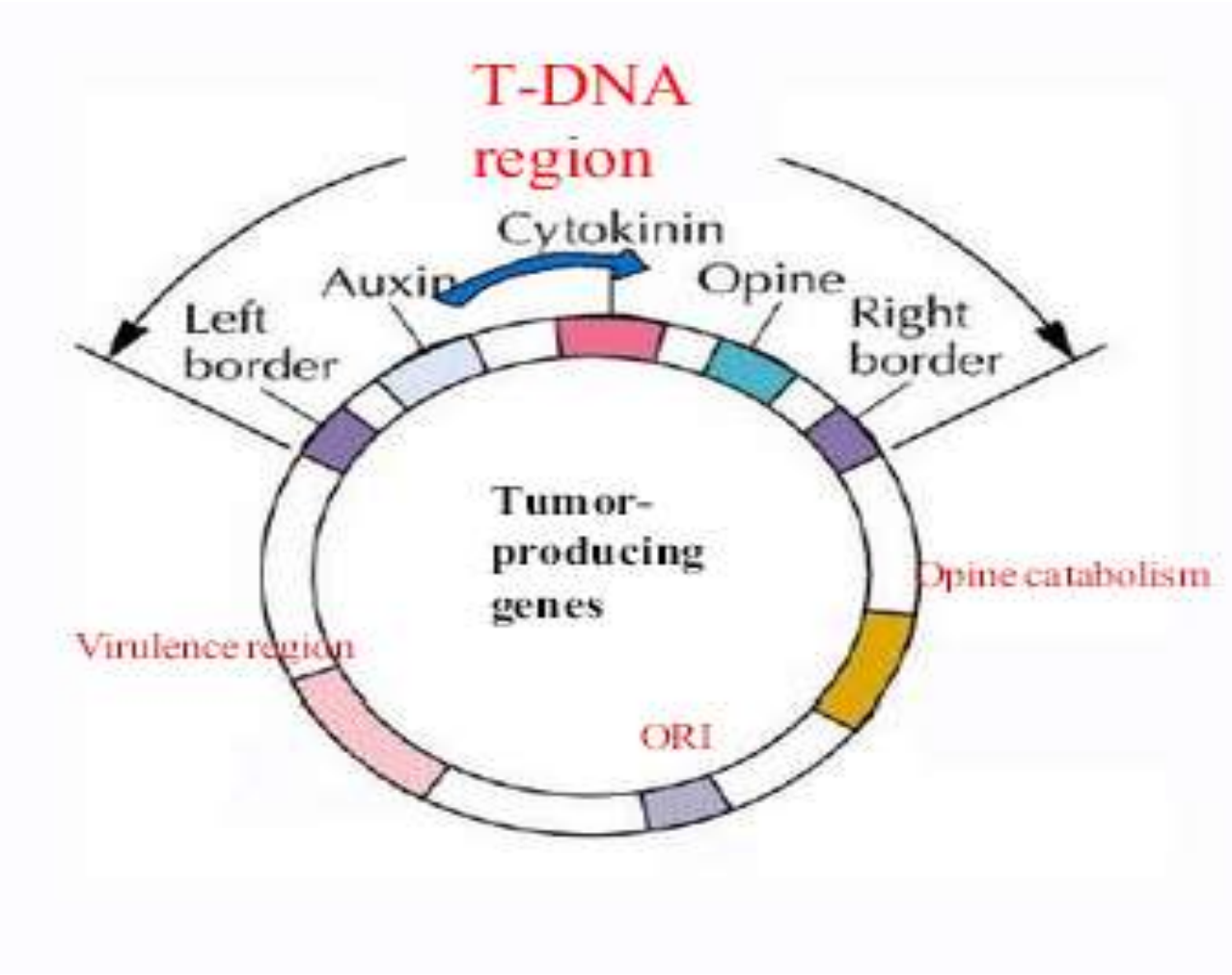
Insertional inactivation is a technique used in rDT where a plasmid pBR322 is used to disable expression of a gene



X-Gal hydrolysis with β-galactosidase.

3. Ti-plasmid vector : Plants

- *Agrobacterium tumefaciens* causes Crown Gall disease.
- It is a soil bacterium that infects only dicotyledonous plants. At the crown region and induces tumor formation.
- pTi (Tumor inducing) plasmid: 180-250kb in size
- Ti plasmid is flanked by direct repeating sequences (25bp)on the left and right side.
- Contains
  - T–DNA: (23-25kb) used for insertion.
  - *Tra* genes : transfer from one cell to another
  - Onc genes for oncogenecity
  - Ori: fro replication
  - Inc: incompatibility


T-DNA
region
Cytokinin
Auxin
Opine
Left
border
Right
border
Tumor-
producing
genes
Opine catabolism
Virulence region
ORI

Vir Gene	Function
Vir A, Vir G	Sense phenolic compounds from wounded plant cells and induce expression of other virulence genes
VirD2	Endo nuclease; cuts T-DNA at right border to initiate T-strand synthesis
Vir D1	Topiosomerase; Helps Vir D2 to recognise and cleave within the 25bp border sequence
Vir D2	Covalently attaches to the 5I end of the T-strand, thus forming the T-DNA Complex. Also guides the T-DNA complex through the nuclear pores
Vir C	Binds to the 'overdrive' region to promote high efficiency T-strand Synthesis
Vir E2	Binds to T-strand protecting it from nuclease attack, and intercalates with lipids to form channels in the plant membranes through which the T-complex passes
Vir E1	Acts as a chaperone which stabilises Vir E2 in the *Agro bacterium*
Vir B & Vir D4	Assemble into a secretion system which spans the inner and outer bacterial membranes. Required for Export of the T-complex and Vir E2 into the plant cell

Mechanism of infection by Ti plasmid

Plant gets wounded (grazing, natural calamities *etc*.)

Plant releases phenoilc compounds like acetosynringone etc.

Autoinduction of *vir* genes of Ti plasmid

The enzyme produced makes a nick at right end of T DNA

Hence the Gene of interest is easily transferred to the plant cell

Ri Plasmids

Bacteriophage vectors

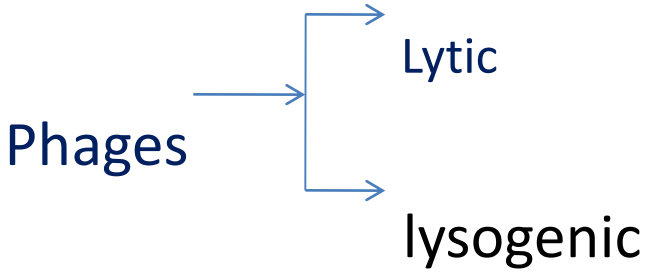
- Viruses that infect bacterial cells are called phages
- Phage genome has lot of non-essential region (req. for cell lysis etc.)

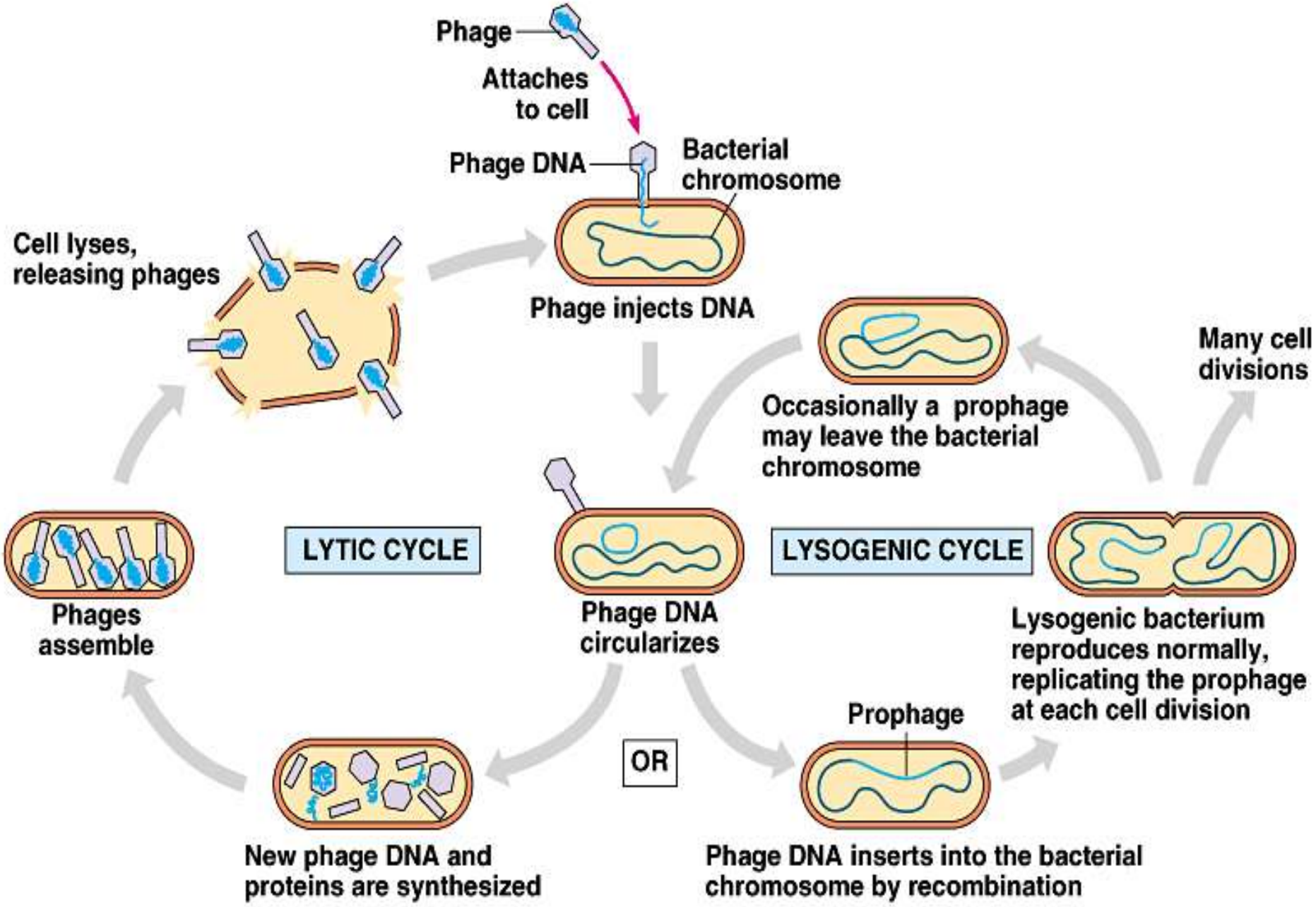
2 classes of vectors:

- 1. Insertion vectors (18-20kb insert)
- 2. Replacement vectors

Foreign DNA of up to **25kb** can be Inserted into phage vectors.

Phage λ and M13 are the most commonly used viral cloning vectors.

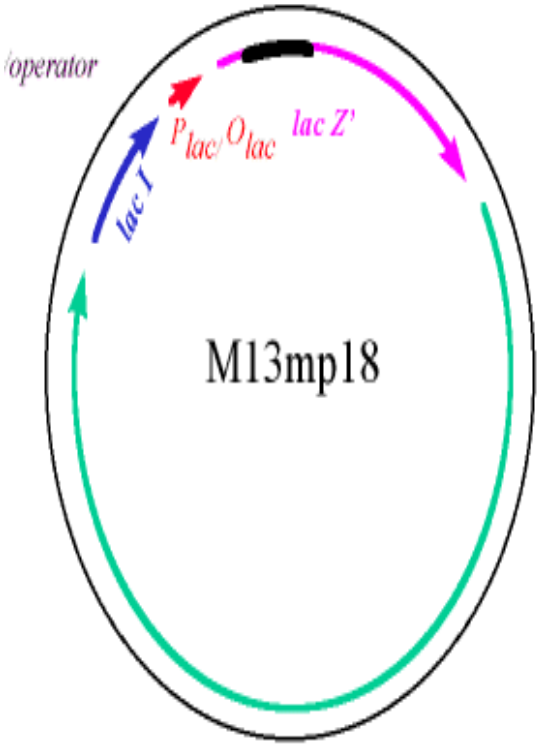




M13-Phage

- Filamentous phage of *E. coli* containing sex pilli
- Attaches at the receptors specified by F Plasmid of sex pilli followed by transfection and delivery into cells through the lumen of pilli.
- Genome: ss circular DNA, 6407bp in size
- Ten genes back to back-Replication.
- Only 507bp stretch available for insertion

Called Intergenic Seq.(IS).



- Advantages:
  1. Has MCS for insertion.
  2. Easy selection by blue white colony method.

Phage cloning vectors:

Advantages:

- DNA can be packed in vitro into the phages and transduced into *E. coli* with higher efficiency
- Foreign DNA upto 25kb can be inserted into the phage vectors.
- Screening and storage of recombinant DNA is easier.

PHAGEMIDS:

Phagemid vectors:

These are plasmids that contain ori for ssDNA containing phage

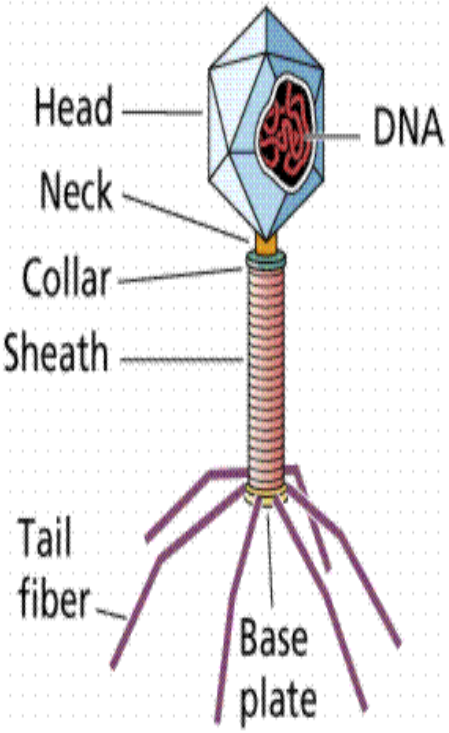
- Important in DNA sequencing and site directed mutagenesis experiments.

  Eg: pBluescript IIK S +/-

λ-Phage

- Contains proteinaceous head with a long tail.
- Its genome is made of 50 genes –about 48.514kb
- Has 12Ntd long projections at both the ends :

COS sites which helps in circularization and ligation.



Cosmids:

These are hybrid vectors derived from plasmids containing cos sites of phageλ.

(Cosmid: Cos site + plasmid)

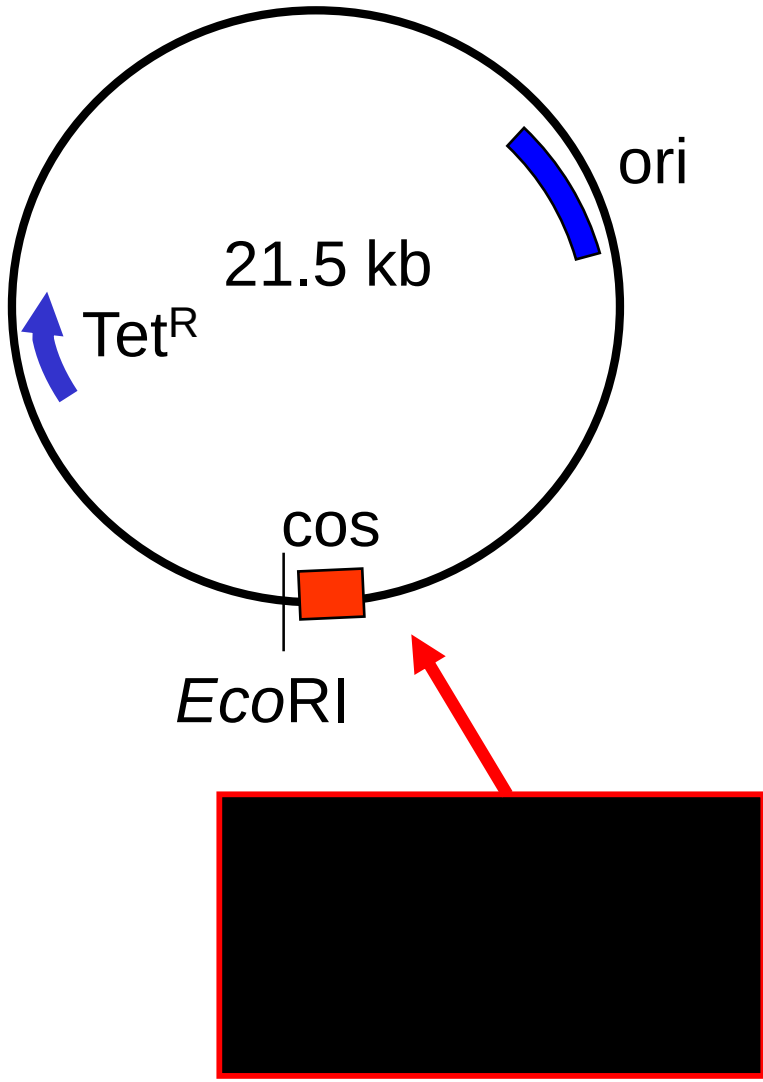
- First developed by Collins and Hohn (1978) from phage λ and *E. coli* Col E1

Characteristics:

1. Has *ori* sequence for autonomous replication.
2. Small size
3. A marker gene encoding antibiotic resistance
4. Special cleavage site for insertion of foreign DNA

Cosmids

- Hybrid vectors: plasmids that contain bacteriophage lambda cos sites
- DNA (~ 33-48 kb) cloned into restriction site, the cosmid packaged into viral particles and these phages used to infect *E.coli*
- Cosmid can replicate in bacterial cell, so infected cells grow into normal colonies
- Insert DNA limited by the amount of DNA that can fit into phage capsule
- Somewhat unstable, difficult to maintain



BACs

1. Use fertility F plasmid
2. 75-300kb inserts possible
3. developed during the human genome project.
4. Large low copy number plasmids (have ori and selectable marker)
5. Can be electroporated into E. coli
6. Useful for sequencing genomes, because insert size 100 - 300kb

YACs

1. Mimics yeast chromosome
2. Contains all regions for replication (yeast ori and centromere)
3. 100-1000kb inserts possible.
4. developed during the human genome project.
5. Can be grown in E.coli and Yeast
6. Miniature chromosome (contains ori, selectable markers, two telomeres, and a centromere
7. Can accept 200 kb -1000 kb; useful for sequencing

Shuttle vectors:

Vectors that can exist both in prokaryotes as well as eukaryotes are called as shuttle vectors

- Diff between prokaryotes and eukaryotes Introns and Exons
- Eg. Yeast Episomal Plasmid YEp

Characteristics:

- Contain 2 'ori' sequences: ori^{E} and ori^{Euk}
- Vector must be stable, non pathogenic and non stress inducing
- Must replicate in many hosts (bacteria, yeast & plants)
- Vector should be small in size to accommodate DNA inserts.
- Easy to select and screen

EXPRESSION VECTORS

- Produces large amounts of a specific protein
- Permits studies of the structure and function of proteins
- Can be useful when proteins are rare cellular components or difficult to isolate

Expression vectors:

- Expression of cloned genes is carried out by inserting the promoter sequence and terminator sequence.
- The cloning vectors which contain these signals for protein synthesis are called expression vectors.
- Example: pSOMI containing promoter operator for production of chain of somatostatin

RETROVIRAL VECTORS

- Retroviral vectors are used to introduce new or altered genes into the genomes of human and animal cells.
- Retroviruses are RNA viruses.
- The viral RNA is converted into DNA by the viral reverse transcriptase and then is efficiently integrated into the host genome
- Any foreign or mutated host gene introduced into the retroviral genome will be integrated into the host chromosome and can reside there practically indefinitely.
- Retroviral vectors are widely used to study oncogenes and other human genes.

Common problems with bacterial expression systems

- Low expression levels:
  - change promoter
  - change plasmid
  - change cell type
  - add rare tRNAs for rare codons on second plasmid
- Severe protein degradation:
  - use proteasome inhibitors and other protease inhibitors
  - try induction at lower temperature
- Missing post-translational modification: co-express with kinases etc.
- Glycosylation will not be carried out:
  - use yeast or mammalian expression system
- Misfolded protein (inclusion bodies):
  - co-express with GroEL, a chaperone
  - try refolding buffers

REPORTER GENE VECTORS

- **A gene that encodes a protein whose activity can be easily assayed in a cell in which it is not normally expressed**
- **These genes are linked to regulatory sequences whose function is being tested**
- **Changes in transcriptional activity from the regulatory sequences are detected by changes in the level of reporter gene expression**

SHUTTLE VECTORS

- **Shuttle vectors can replicate in two different organisms, e.g. bacteria and yeast, or mammalian cells and bacteria.**
- **They have the appropriate origins of replication.**
- **Hence one can clone a gene in bacteria, maybe modify it or mutate it in bacteria, and test its function by introducing it into yeast or animal cells.**

The Major Limitation of Cloning in Plasmids

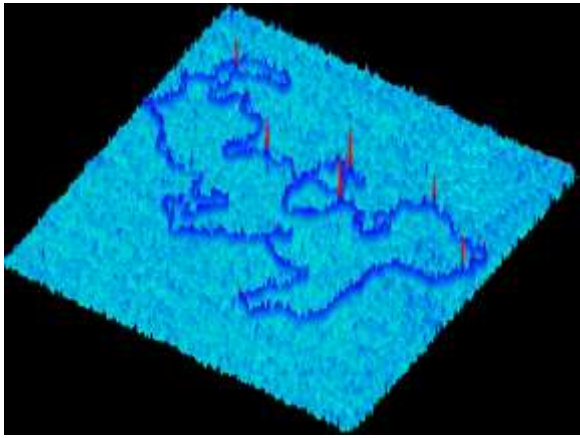
- **Upper limit for clone DNA size is 12 kb**
- **Requires the preparation of “competent” host cells**
- **Inefficient for generating genomic libraries as overlapping regions needed to place in proper sequence**
- **Preference for smaller clones to be transformed**
- **If it is an expression vector there are often limitations regarding eukaryotic protein expression**

Phage Cloning Vectors

- Fragments up to 23 kb can be may be accommodated by a phage vector
- Lambda is most common phage
- 60% of the genome is needed for lytic pathway.
- Segments of the Lambda DNA is removed and a stuffer fragment is put in.
- The stuffer fragment keeps the vector at a correct size and carries marker genes that are removed when foreign DNA is inserted into the vector.
- Example: Charon 4A Lambda
- When Charon 4A Lambda is intact, beta-galactosidase reacts with X-gal and the colonies turn blue.
- When the DNA segment replaces the stuffer region, the lac5 gene is missing, which codes for beta-galactosidase, no beta-galactosidase is formed, and the colonies are white.

Cosmid Cloning Vectors

- Fragments from 30 to 46 kb can be accommodated by a cosmid vector.
- Cosmids combine essential elements of a plasmid and Lambda systems.
- Cosmids are extracted from bacteria and mixed with restriction endonucleases.
- Cleaved cosmids are mixed with foreign DNA that has been cleaved with the same endonuclease.
- Recombinant cosmids are packaged into lambda caspids
- Recombinant cosmid is injected into the bacterial cell where the rcosmid arranges into a circle and replicates as a plasmid. It can be maintained and recovered just as plasmids.



Shown above is a 50,000 base-pair long DNA molecule bound with six EcoRI molecules, and imaged using the atomic force microscope. This image clearly indicates the six EcoRI "sites" and allows an accurate restriction enzyme map of the cosmid to be generated.

http://homer.ornl.gov/cbps/afmimaging.htm

Bacterial Artificial Chromosomes(BACs) and Yeast Artificial Chromosomes(YACs)

- BACs can hold up to 300 kbs.
- The F factor of E.coli is capable of handling large segments of DNA.
- Recombinant BACs are introduced into E.coli by electroportation (a brief high-voltage current). Once in the cell, the rBAC replicates like an F factor.
- Example: pBAC108L
- Has a set of regulatory genes, OriS, and repE which control F-factor replication, and parA and parB which limit the number of copies to one or two.
- A chloramphenicol resistance gene, and a cloning segment.

- YACs can hold up to 500 kbs.
- YACs are designed to replicate as plasmids in bacteria when no foreign DNA is present. Once a fragment is inserted, YACs are transferred to cells, they then replicate as eukaryotic chromosomes.
- YACs contain: a yeast centromere, two yeast telomeres, a bacterial origin of replication, and bacterial selectable markers.
- YAC plasmid→Yeast chromosome
- DNA is inserted to a unique restriction site, and cleaves the plasmid with another restriction endonuclease that removes a fragment of DNA and causes the YAC to become linear. Once in the cell, the rYAC replicates as a chromosome, also replicating the foreign DNA.

Bacterial artificial chromosomes (BAC):

The F factor plasmid has the ability to continue to function even when integrated into a complete bacterial chromosome.

Highly modified F plasmids have been generated that are capable of cloning very large inserts of up to 300,000 base pairs.

One feature is the incorporation of cut sites for restriction endonucleases with eight base cut sites.

Such endonucleases cut DNA less frequently and thus generate larger fragments for cloning.

Bacterial artificial chromosomes are sometimes introduced into their host cells by electroporation, which consists of a brief treatment with high voltage electric current that momentarily disrupts the cell membranes and facilitates entry of large DNA molecules.

Once in the cell, the BAC replicate like F plasmids.

Yeast Artificial Chromosomes (YAC):

a yeast artificial chromosome (YAC) contains

a yeast origin of replication a centromere,

a telomere at each end

a large inserted DNA sequence of up to about 500 kb

Prior to insertion of the foreign DNA, the essential components of the YAC are maintained in bacterial cells as circular plasmids.