PLASMID CLONING VECTORS

Sources:

1. Biotechnology By B.D. Singh,
2. Gene cloning and DNA analysis By TA Brown,
3. Advance and Applied Biotechnology By Marian Patrie,
4. Molecular Biotechnology By Glick *et al* and
5. Principle of Gene Manipulation By sandy b Primrose *et al.*

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Introduction:

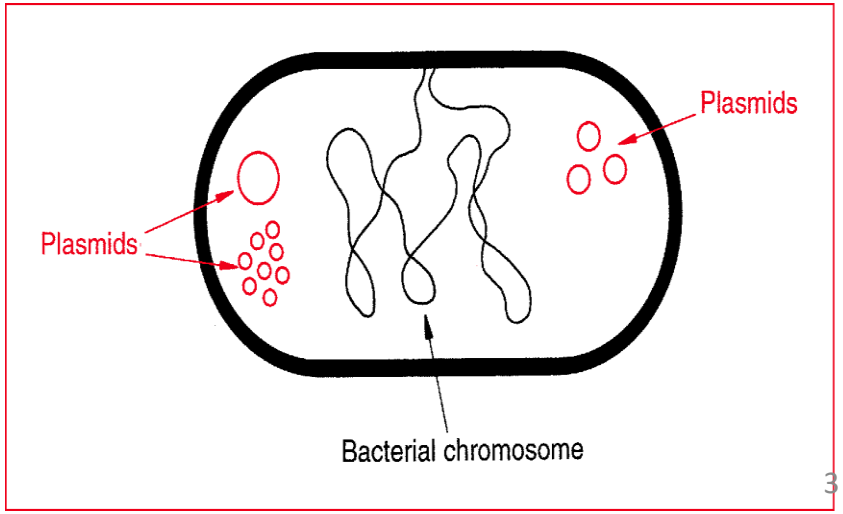
- **DNA molecule used for carrying an exogenous DNA into a host organism and facilitates stable integration and replicate autonomously in an appropriate host cell is termed as Vector.**
- Molecular cloning involves series of sequential steps which includes restriction digestion of DNA fragments both target DNA and vector, ligation of the target DNA with the vector and introduction into a host organism for multiplication. Then the fragments resulted after digestion with restriction enzymes are ligated to other DNA molecules that serve as vectors.

In general, vectors should have following characteristics:

- **Capable of replicating inside the host.**
- **Have compatible restriction site for insertion of DNA molecule (insert).**
- **Capable of autonomous replication inside the host (*ori* site).**
- **Smaller in size and able to incorporate larger insert size.**
- **Have a selectable marker for screening of recombinant organism.**

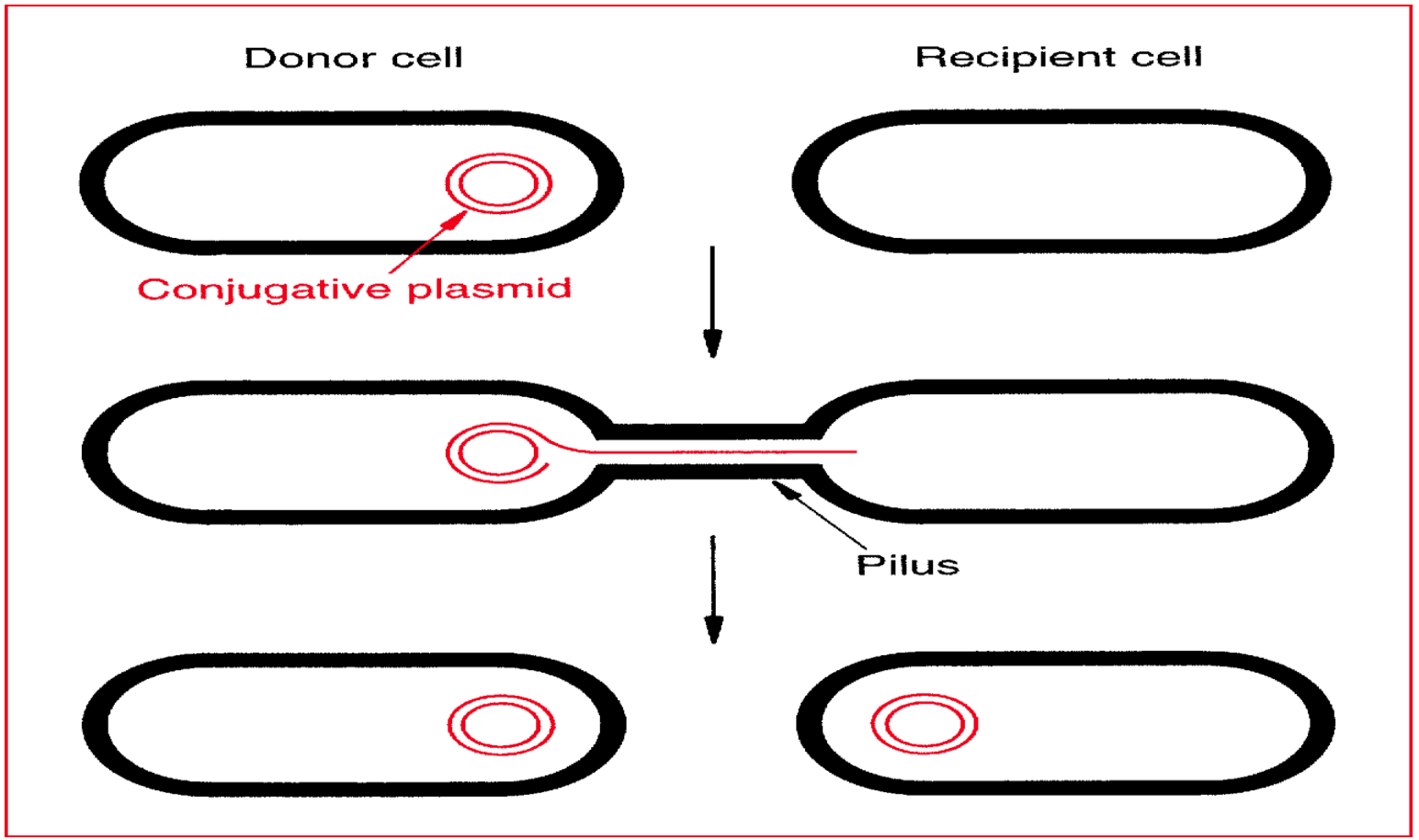
Plasmids:

- **Plasmids are naturally occurring extra chromosomal double-stranded circular DNA molecules which can autonomously replicate inside bacterial cells.**
- Plasmids range in size from about **1.0 kb to over 250 kb**.
- **Plasmids encode only few proteins required for their own replication (replication proteins) and these proteins encoding genes are located very close to the *ori*.**
- All the other proteins required for replication, e.g. **DNA polymerases, DNA ligase, helicase, etc., are provided by the host cell.** Thus, only a small region surrounding the *ori* site is required for replication.
- Other parts of the plasmid can be deleted and foreign sequences can be added to the plasmid without compromising replication.



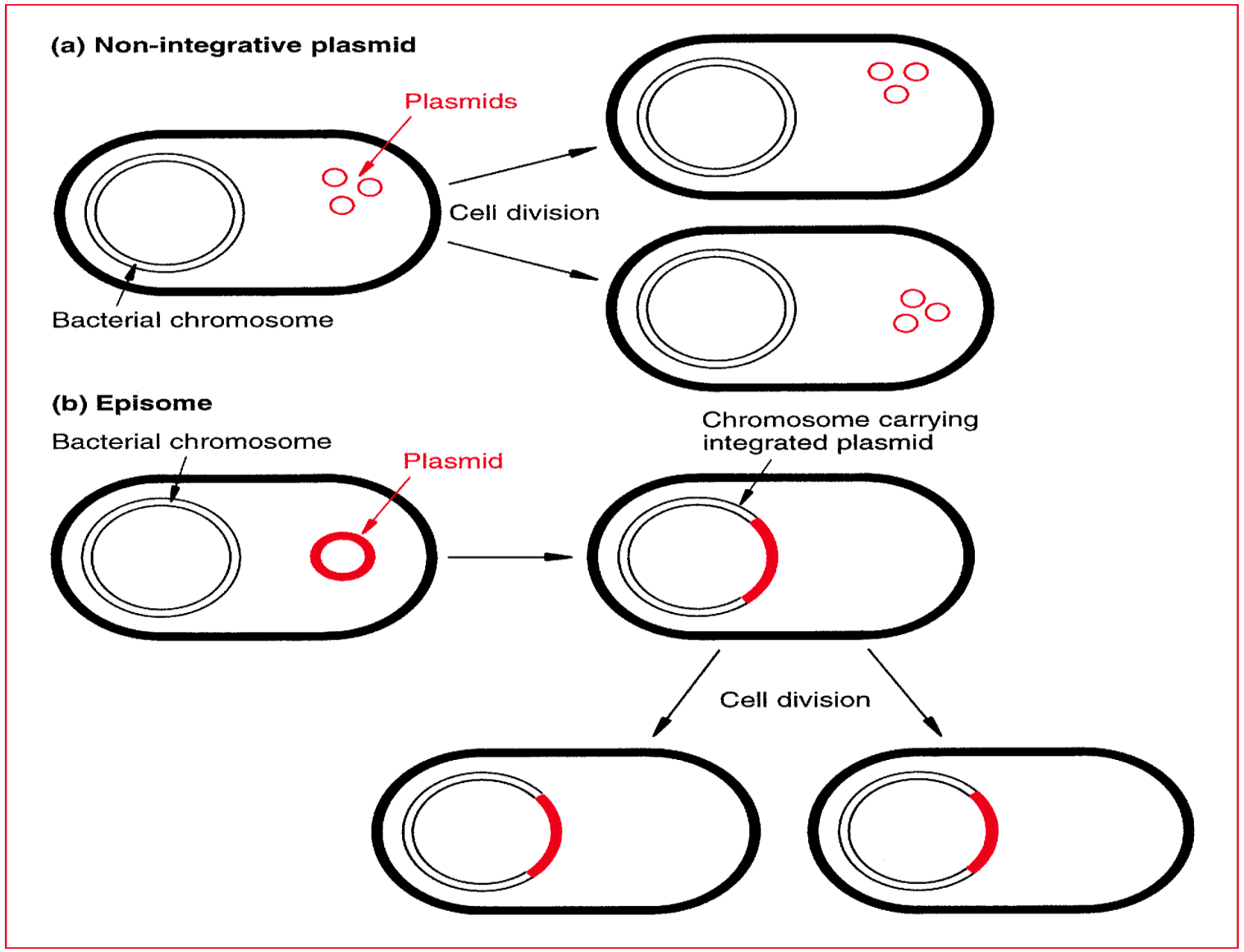
• **The host range of a plasmid is determined by its *ori* region**. Plasmids whose *ori* region is derived from plasmid Col E1 have a restricted host range. They only **replicate in enteric bacteria**, such as *E. coli, Salmonella,* etc. Plasmids of the RP4 type will **replicate in most gram negative bacteria, to which they are readily transmitted by conjugation.** Plasmids like RSF1010 are not conjugative but can be transformed into a wide range of gram -ve bacteria. Plasmids isolated from *Staphylococcus aureus* have a broad host range and can replicate in many other gram-positive bacteria.

- Some of the phenotypes which the naturally occurring plasmids confer on their host cells:
  - **Antibiotic resistance**
  - **Antibiotic production**
  - **Degradation of aromatic compounds**
  - **Hemolysis production**
  - **Sugar fermentation**
  - **Enterotoxin production**
  - **Heavy metal resistance**
  - **Bacteriocin production**
  - **Induction of plant tumors**
  - **Hydrogen sulphide production**



Plasmid transfer by conjugation between bacterial cells. The donor and recipient cells attach to each other by a pilus, a hollow appendage present on the surface of the donor cell. A copy of the plasmid is then passed to the recipient cell. Transfer is thought to occur through the pilus, but this has not been proven and transfer by some other means (e.g. directly across the bacterial cell walls) remains a possibility.

- **Most plasmids exist as double-stranded circular DNA molecules. However, the inter-conversion of super coiled, relaxed covalently closed circular DNA and open circular DNA is possible.**
- Not all plasmids exist as circular molecules. Linear plasmids have been found in a variety of bacteria, e.g. *Streptomyces sp.* and *Borrelia burgdorferi.*
- However, few types of plasmids are also able to replicate by integrating into bacterial chromosomal DNA; these are known as **integrative plasmids or episomes.** They are found mainly in prokaryotes but some eukaryotes are also found to harbour them. In prokaryotes they are found in *Escherichia coli, Pseudomonas* species, *Agrobacterium* species etc. In eukaryotes they are mainly found in *Saccharomyces cerevisiae.*



Replication strategies for
(a) a non-integrative plasmid, and
(b) an episome.

Plasmid classification

The plasmids are divided into 6 major classes as described below depending on the phenotype:

i) **Resistance or R plasmids** carry genes which give resistance to the bacteria from one or more chemical agents, such as antibacterial agents. R plasmids are very important in clinical microbiology as they can have profound consequences in the treatment of bacterial infections. Eg: RP4 plasmid, which is commonly found in *Pseudomonas* and in many other bacteria.

ii) **Fertility or F plasmids** are conjugative plasmid found in F+ bacterium with higher frequency of conjugation. F plasmid carries transfer gene (*tra)* and has the ability to form Conjugation Bridge (F pilus) with F- bacterium. Eg: F plasmid of *E. coli*.

iii) **Col plasmids** have genes that code for colicins, proteins that kill other bacteria. Eg: ColE1 of *E. coli*.

iv) **Degradative plasmids** allow the host bacterium to metabolize unusual molecules such as toluene and salicylic acid. Eg TOL of *Pseudomonas putida*.ca

v) **Virulence plasmids** confer pathogenicity on the host bacterium. Eg: Ti plasmids of *Agrobacterium tumefaciens*, which induce crown gall disease on dicotyledonous plants.

vi) **Cryptic Plasmids** do not have any apparent effect on the phenotype of the cell harboring them. They just code for enzymes required for their replication and maintenance in the host cell.

Based on the origin or source of plasmids, they have been divided into two major classes: such as natural and artificial.

i) **Natural plasmids**: They occur naturally in prokaryotes or eukaryotes. Example*:* ColE1.

ii) **Artificial plasmids**: They are constructed *in-vitro* by re-combining selected segments of two or more other plasmids (natural or artificial). Example: pBR322.

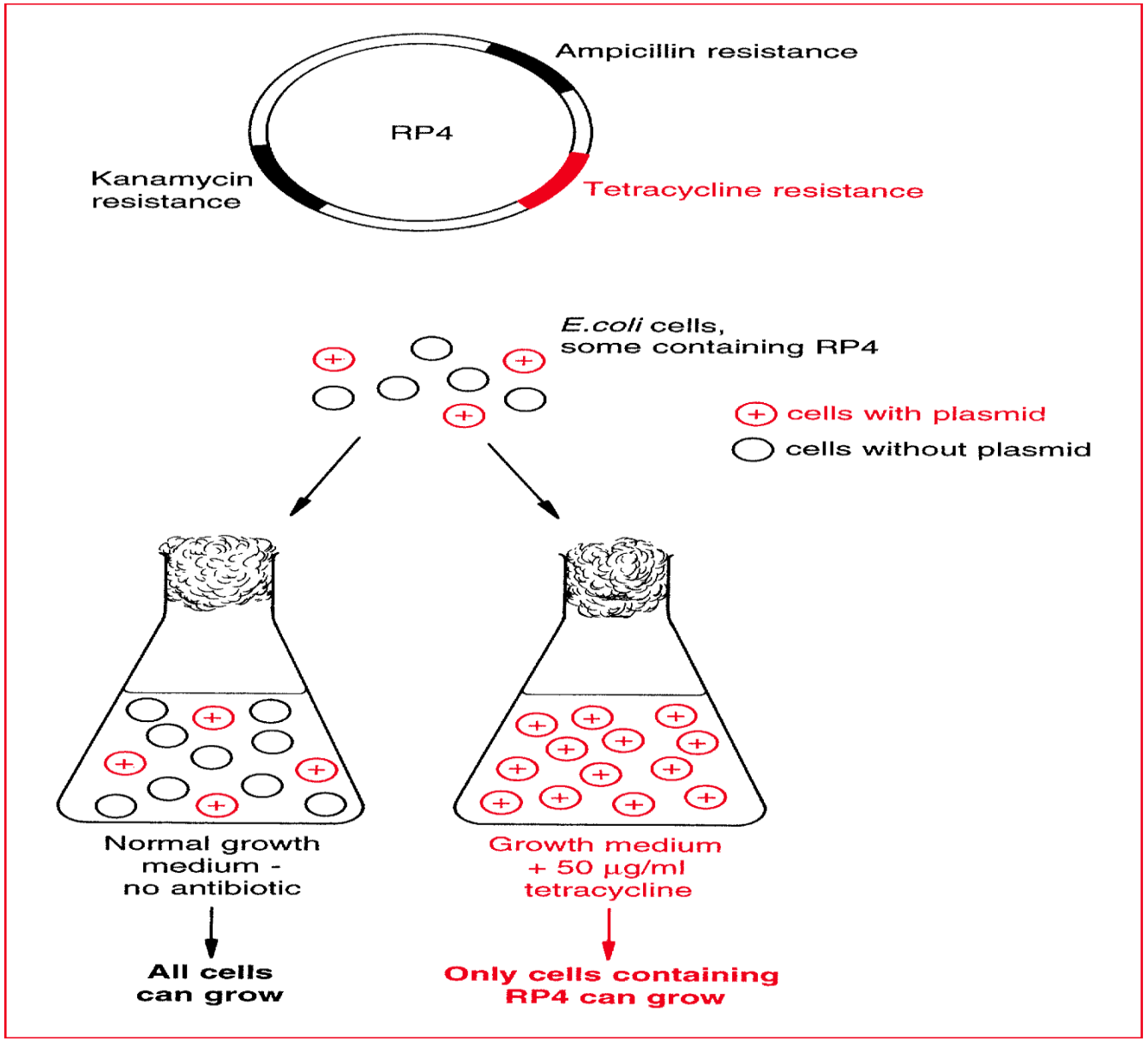


Figure : The use of antibiotic resistance as a selectable marker for a plasmid. RP4 (top) carries genes for resistance to ampicillin, tetracycline and kanamycin.
Only those *E. coli* cells that contain RP4 (or a related plasmid) are able to survive and grow in a medium that contains toxic amounts of one or more of these antibiotics.

Sizes of representative plasmids.

	SIZE		
PLASMID	**NUCLEOTIDE LENGTH (kb)**	**MOLECULAR MASS (MDa)**	**ORGANISM**
pUC8	2.1	1.8	*E. Coli*
ColE1	6.4	4.2	*E. Coli*
RP4	54	36	*Pseudomonas* and others
F	95	63	*E. Coli*
TOL	117	78	*Pseudomonas putida*
pTiAch5	213	142	*Agrobacterium tumefaciens*

Natural Plasmids .

Plasmid	Size (kb)	Origin	Host range	Antibiotic resistance	Additional marker genes showing insertional inactivation
RSF1010	8.6	*E.coli* (strain K-12)	Broad host range	Streptomycin and sulfonamides	None
ColE1	6.6	*E.coli*	Narrow host range	None	Immunity to colicin E1
R100	94.2	*E.coli*	*E.coli* K-12, *Shigella flexneri* 2b	Streptomycin, chloramphenycol, tetracycline	Mercuric (ion) reductase, putative ethidium bromide (EtBr) resistant protein.

Characteristics of ideal plasmid vectors

1. **Size:** plasmid must be small in size. The small is helpful for easy uptake of cDNA by host cells and for the isolation of plasmid without damage. **Ideal vector should be less than or equal to 10kb.** The small size is essential for easy introduction in cell by transformation, transduction and electroporation.
2. **Copy number:** the plasmid must be present in **multiple copies**.
3. **Genetic markers:** plasmid must have **one or few genetic markers**. These markers help us for the selection of organism that has recombinant DNA
4. **Origin of replication:** the plasmid must have **its own orogin of replication and regulatory genes for the self-replication.**
5. **Unique restriction sites:** the plasmid must have unique restriction sites common restriction enzymes in use.
6. **Multiple cloning sites:** This property permits the insertion of gene of interest and plasmid re-circularization.
7. **Insertional inactivation:** the plasmid must have unique sites for restriction enzymes in marker genes. This will help us for the selection of recombination by insertional inactivation method.
8. **Pathogenicity:** the plasmid should not have any pathogenic property.
9. **Should not be transferred by conjugation:** This property of vector molecule prevents recombinant DNA to escape to natural population of bacteria.
10. **Selectable make gene:** Vector molecules should have some detectable traits. These traits enable the transformed cells to be identified among the non-transformed ones. eg. antibiotic resistance gene.

NOTE :

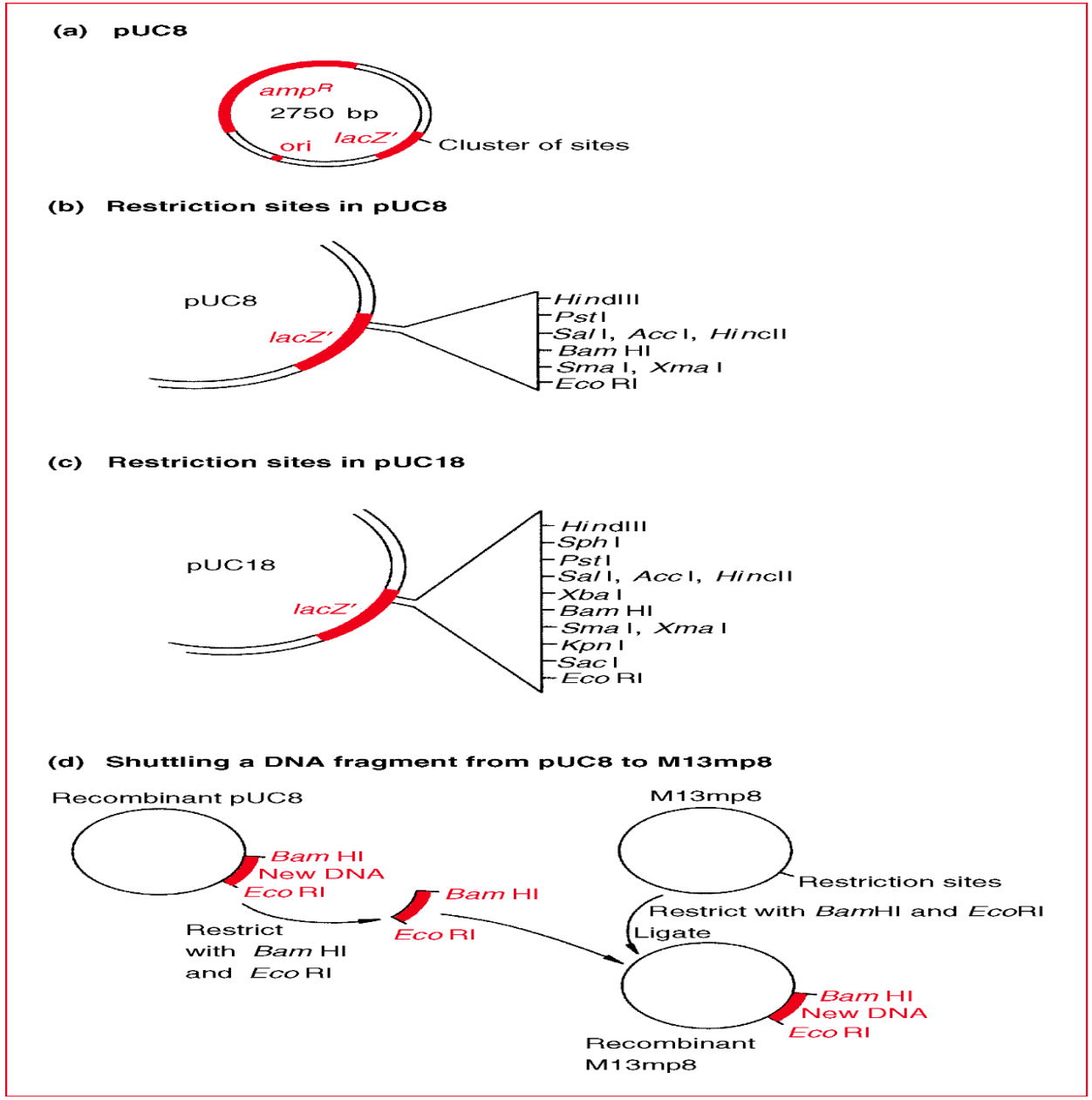
- It should be kept in mind that the **DNA molecule used as vectors have coevolved with their natural host species, and hence are adopted to function well in them and in their closely related species.**
- Therefore, the choice of vector depends on host species into which DNA insert or gene is to be cloned.
- In addition, most naturally occurring vectors do not have all required functions; therefore, useful vectors have been created by joining together segments performing species functions (called modules) from two or more natural entities.

Multiple cloning sites (MCS)

- **MCS is a synthetic DNA segment that has a cluster of unique sites for restriction enzymes**. It is inserted into a gene cloning vector with a view to increasing the number of gene cloning sites.
- The size of **MCS usually from 60 bp to 84 bp.** The number and arrangement of restriction sites varies from MCS to MCS in different vectors. As MCS is a cluster of many restriction sites, it is also known as **polylinker or polylinker sequence**.

USES:

1. MCS are used to increase gene cloning sites in vector DNAs.
2. As they have unique sites for many restriction enzymes, DNA segments with different types of cut-ends can be inserted into the vector.
3. Restriction enzymes of choice can be used to insert a gene into the vector.



Properties of good host

A good host has the following features:

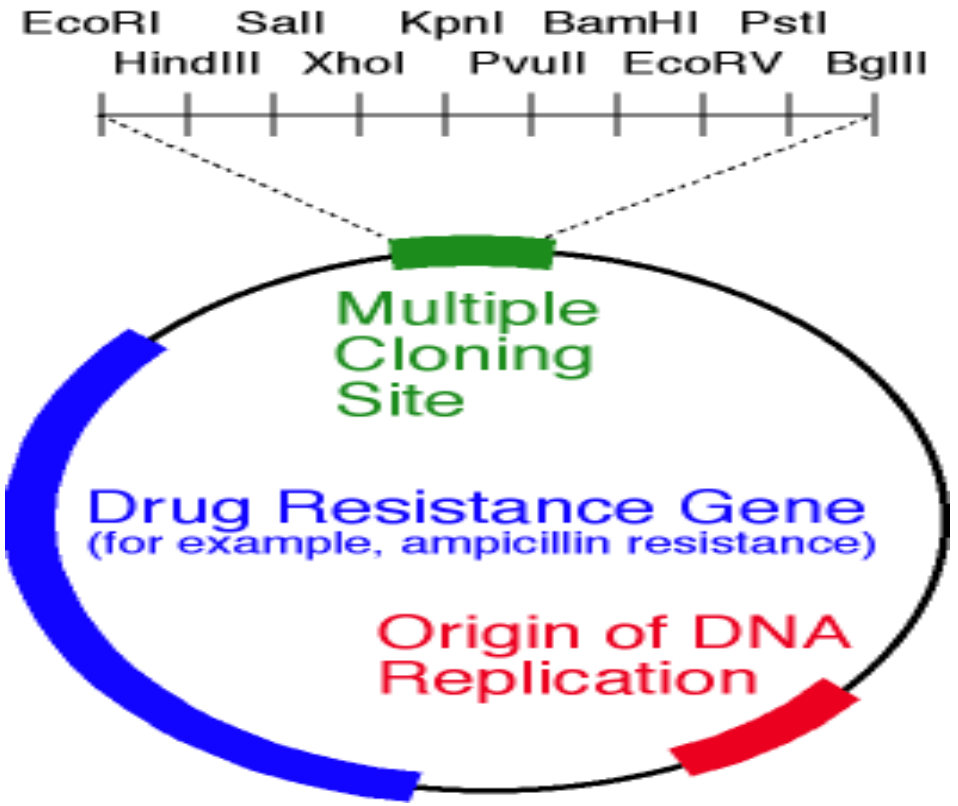
1. **Be easy to transform**
2. **Support the replication of recombinant DNA**
3. **Be free from elements that interfere with replication of recombinant DNA**
4. **Lack active restriction enzymes, e.g., E.coli K12 substrain HB 101.**
5. **Should not have methylases, since, these enzymes would methylate the replicated recombinant DNA which, as a result, would become resistant to useful restriction enzymes.**
6. **Be deficient in normal recombinant function, so, that, the DNA insert is not altered by recombination events.**

ARTIFICIAL PLASMIDS:

- **Naturally occurring plasmids has several limitations**; for example, **some are stringent and not relaxed (pSC101), some has poor marker genes (ColE1), and some are too large (RSF2124).** To overcome the limitations of natural vectors, artificial plasmid are designed by combining different elements from diverse sources.
- Artificial plasmids vectors are **classified into two broad types based on their use:**
    1. **Cloning vector**
    2. **Expression vector**
- Apart from the following, there is another class of vectors known as **shuttle vector. Shuttle vectors can be propagated in two or more different host species (both in prokaryotes and eukaryotes). Hence, inserted DNA can be manipulated and replicated in two different cellular systems.**
- **Cloning vectors are designed for efficient transfer of foreign DNA into the host. Expression vectors have efficient machinery for cloning and expression of foreign gene in the host system.**
- Selection of a vector depends upon various criteria decided by the experimental goal.

Cloning Vector:

A cloning vector is defined as a vector used for replication of a cloned DNA fragment in a host cell. These vectors are frequently engineered to contain “ori” – origin of replication sites particular to the host organism. Examples of commonly used cloning vectors are: pUC18, pUC19, pBluescript vectors.



Types of Cloning Vectors:

- Cloning vectors extensively used in molecular cloning experiments can be considered under following types: **plasmid**, **phage vector** and **cosmid.**
- Different vectors have different insert size and also vary in mode of replication inside the host.
- **Mammalian genes are usually too large (~100 kb), and thus suffer from restrictions in complete inclusion, with the conventional cloning vectors, having limited insert size.**
- Vectors engineered more recently, known as **artificial chromosomes**, **have overcome this problem by mimicking the properties of host cell chromosomes.** They have much larger insert size than other vectors.

Different type of cloning vectors

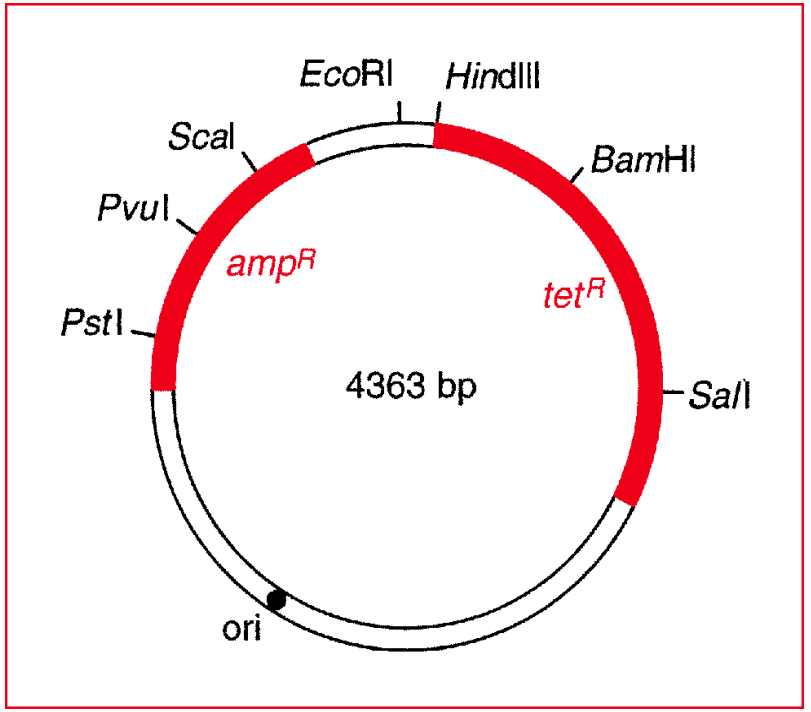
Vector	Insert size	Source	Application
Plasmid	≤ 15 kb	Bacteria	cDNA cloning and expression assays
Phage	5-20 kb	Bacteriophage λ	Genomic DNA cloning, cDNA cloning and expression library
Cosmid	35-45 kb	Plasmid containing a bacteriophage λ *cos* site	Genomic library construction
BAC (bacterial artificial 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

EXAMPLES OF CLONING VECTOR:

pBR322

pBR322 is a widely-used *E. coli* cloning vector. It was created in **1977 in the laboratory of Herbert Boyer at the University of California San Francisco.** The *p* stands for "**plasmid**" and *BR* for "**Bolivar**" and "**Rodriguez**", researchers who constructed it. '322' distinguishes this plasmid from others developed in the same laboratory (there are also plasmids called pBR325, pBR327, pBR328, etc.).

- pBR322 is 4363 base pairs in length.
- pBR322 plasmid has the following elements:
  - ***"rep"*** replicon from plasmid pMB1 which is responsible for replication of the plasmid.
  - ***"rop"*** gene encoding Rop protein, are associated with stability of plasmid and also controls copy number (increase number). The source of "*rop*" gene is pMB1plasmid.
  - ***"tet"*** gene encoding tetracycline resistance derived from pSC101 plasmid.
  - ***"bla"*** gene encoding β lactamase which provide ampicillin resistance (source: transposon Tn3).



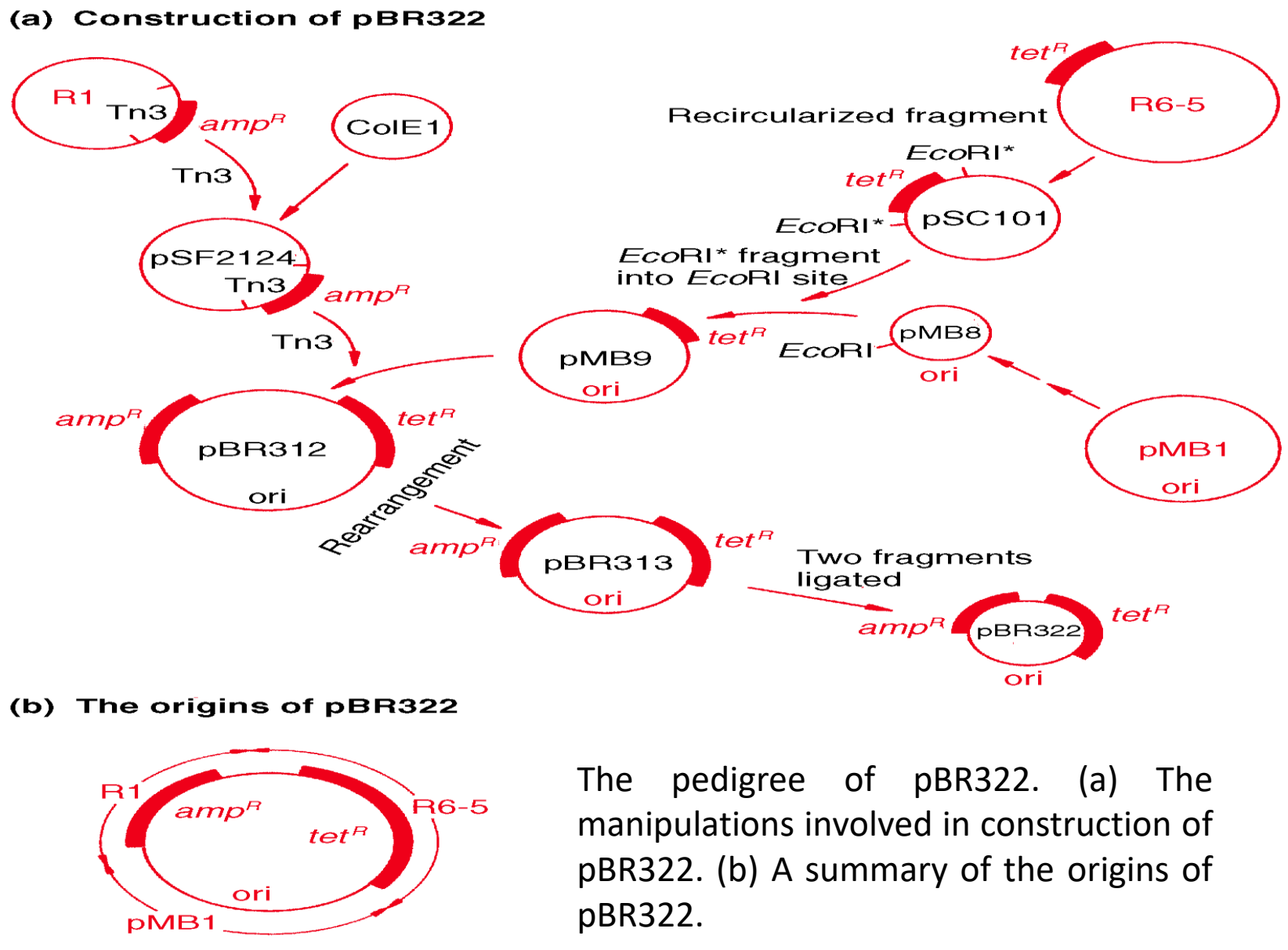
A map of pBR322 showing the positions of the ampicillin resistance (*ampR*) and tetracycline resistance (*tetR*) genes, the origin of replication (ori) and some of the most important restriction sites

Useful features of pBR322

1) **The first useful feature of pBR322 is its size.** pBR322 is 4363 bp, which means that not only can the vector itself be purified with ease, but so too can any recombinant DNA molecules constructed with it. **Even with 6 kb of additional DNA**, a recombinant pBR322 molecule is still of a manageable size.
2) **The second feature of pBR322 is that, it carries two sets of antibiotic resistance genes.** Either ampicillin or tetracycline resistance can be used as a selectable marker for cells containing the plasmid, and **each marker gene contains unique restriction sites that can be used in cloning experiments.**
3) **A third advantage of pBR322 is that it has a reasonably high copy number.** Generally, there are about 15 molecules present in a transformed *E. coli* cell, but this **number can be increased up to between 1000 and 3000 by plasmid amplification in the presence of a protein synthesis inhibitor such as chloramphenicol.** An *E. coli* culture therefore provides a good yield of recombinant pBR322 molecules.

5) It has 528 restriction sites for 66 restriction enzymes. Among them 20 restriction enzymes cut it at unique restriction sites. Tetracycline has 6 unique sites for 6 restriction enzymes. Ampicillin gene has 3 unique restriction site.

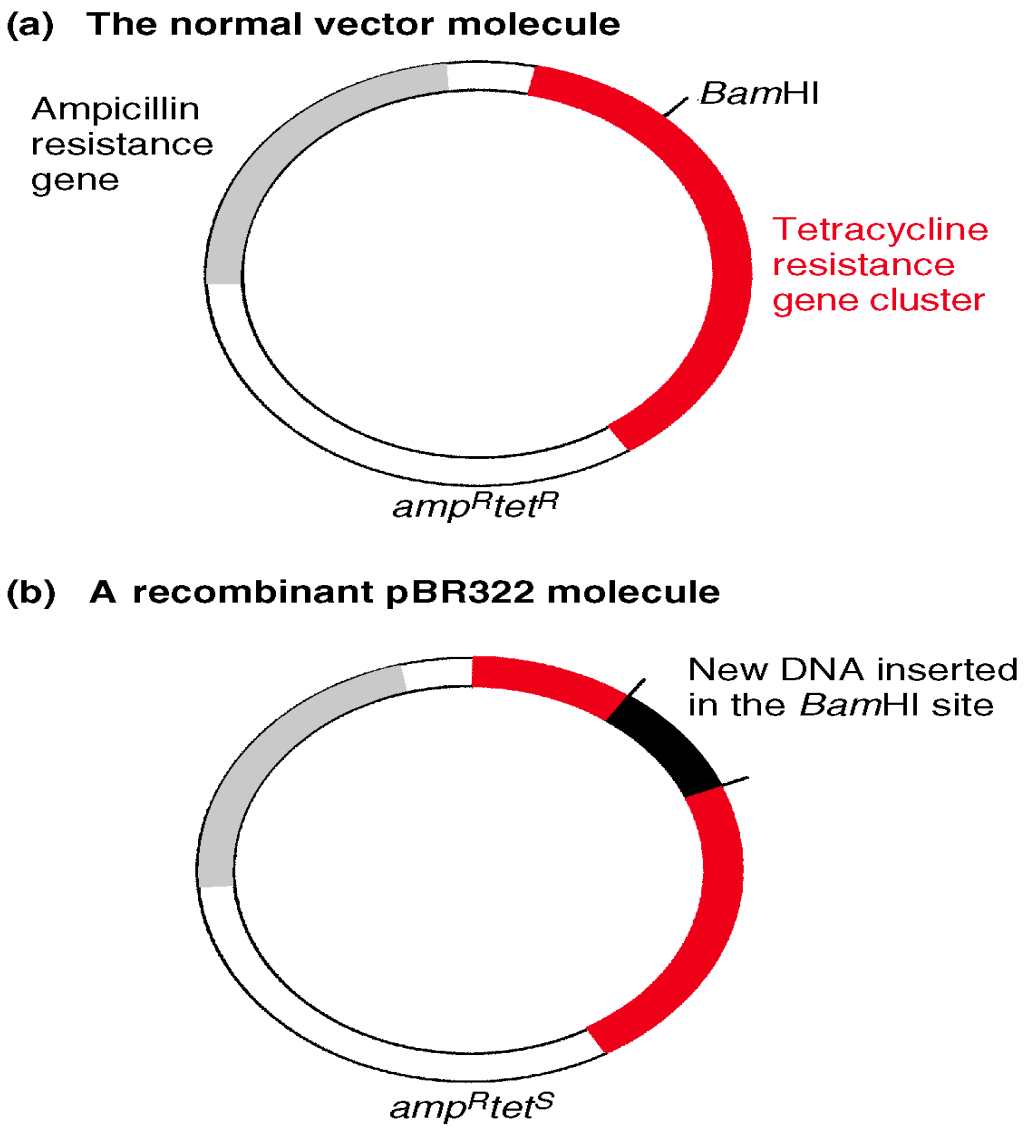
6) The sequences other than Tet and Amp genes, have unique sites for 1 restriction enzymes. There is no restriction inactivation when gene is inserted into any one of these sites.



The pedigree of pBR322. (a) The manipulations involved in construction of pBR322. (b) A summary of the origins of pBR322.

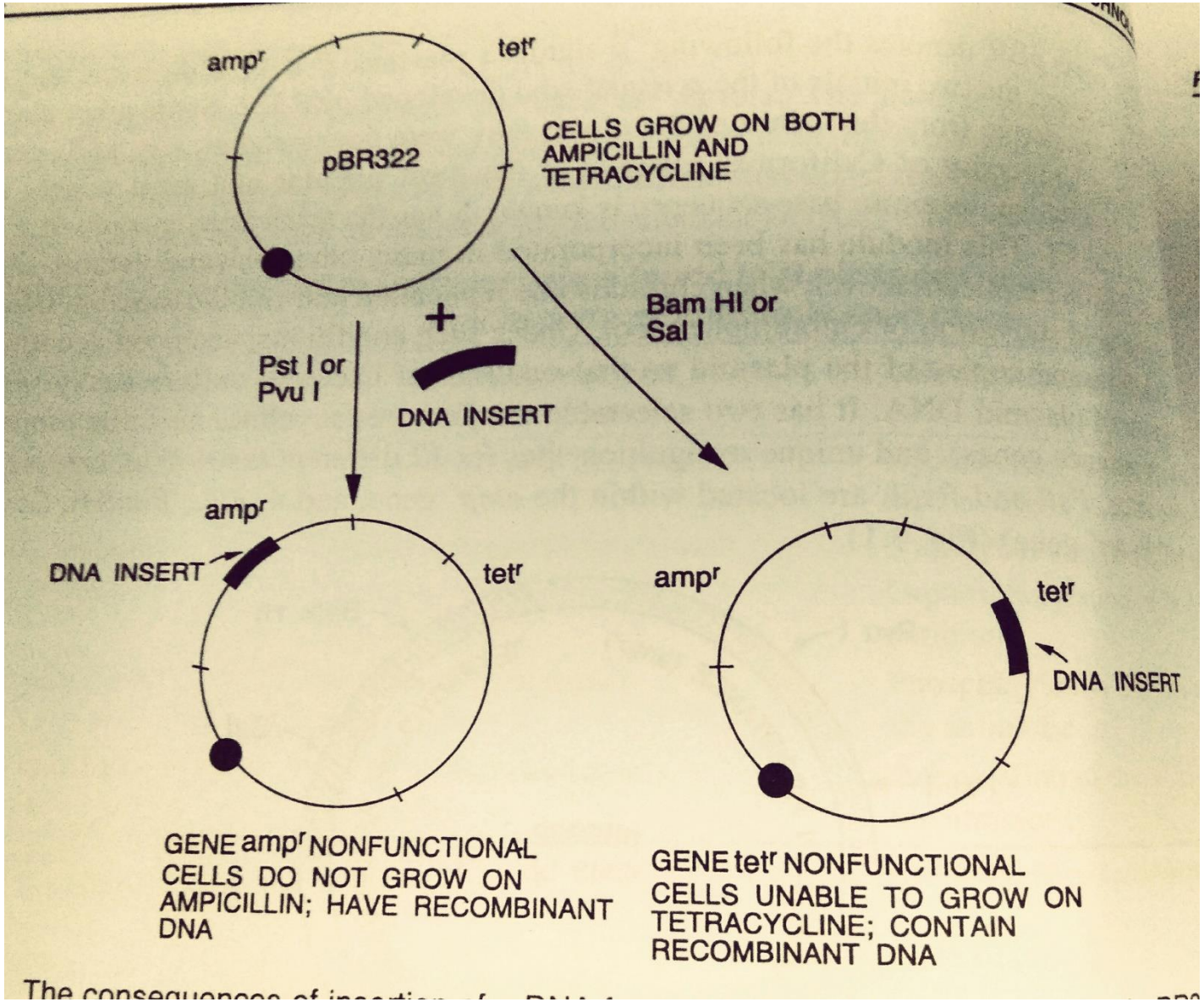
- The amp^R gene originally resided on the **plasmid R1, a typical antibiotic resistance plasmid that occurs in natural populations of *E. coli* .**
- The tet^R gene is derived from R6-5, a second antibiotic resistance plasmid.
- The replication origin of pBR322, which directs multiplication of the vector in host cells, is originally from pMB1, which is closely related to the colicin-producing plasmid ColE1 .

Recombinant selection with pBR322: Insertional inactivation of an antibiotic resistance gene



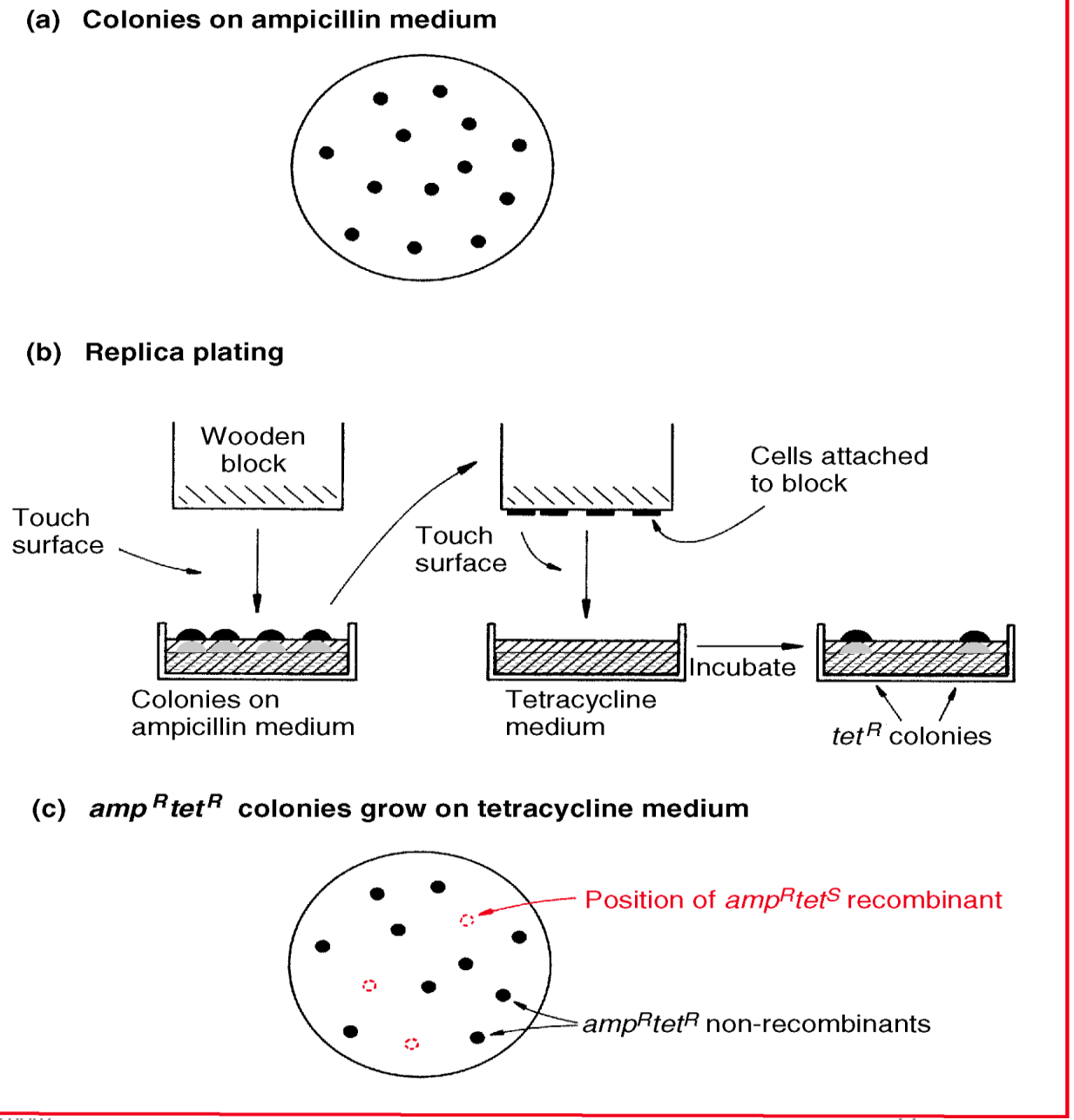
- pBR322 has several unique restriction sites that can be used to open up the vector before the insertion of a new DNA fragment . ***Bam*HI, for example, cuts pBR322 at just one position, within the cluster of genes that code for resistance to tetracycline.**
- A recombinant pBR322 molecule, one that carries an extra piece of DNA in the *Bam*HI site, **is no longer able to confer tetracycline resistance on its host, as one of the necessary genes is now disrupted by the inserted DNA.**
- Cells containing this recombinant pBR322 molecule are still **resistant to ampicillin, but are sensitive to tetracycline (*amp*^{R} *tet*^{S}).**

Screening for pBR322 recombinants is performed in the following way.



- After transformation, the cells are plated onto an **ampicillin medium** and incubated until colonies appear .
- All of these colonies are transformants **(remember, untransformed cells are *amp*^{S} and so do not produce colonies on the selective medium)** but only a few contain recombinant pBR322 molecules; **most will contain the normal, self ligated plasmid.**

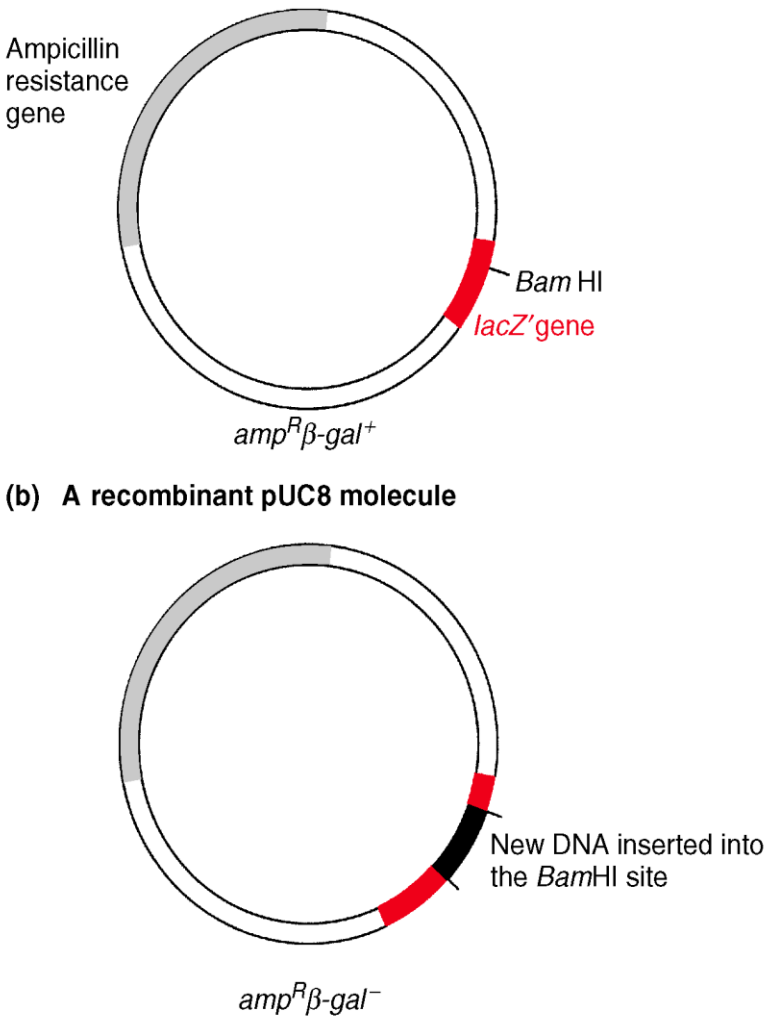
- To identify the recombinants the colonies are **replica plated onto agar medium that contains tetracycline**
- After incubation, some of the original colonies regrow, but others do not .
- Those that do grow, **consist of cells that carry the normal pBR322 with no inserted DNA,** and therefore **a functional tetracycline resistance gene cluster (*amp*^{R}*tet*^{R}).**
- The colonies that **do not grow on tetracycline agar are recombinants (*amp*^{R}*tet*^{S}).**
- Reference back to the original ampicillin agar plate reveals the positions of these colonies, enabling samples to be recovered for further study.

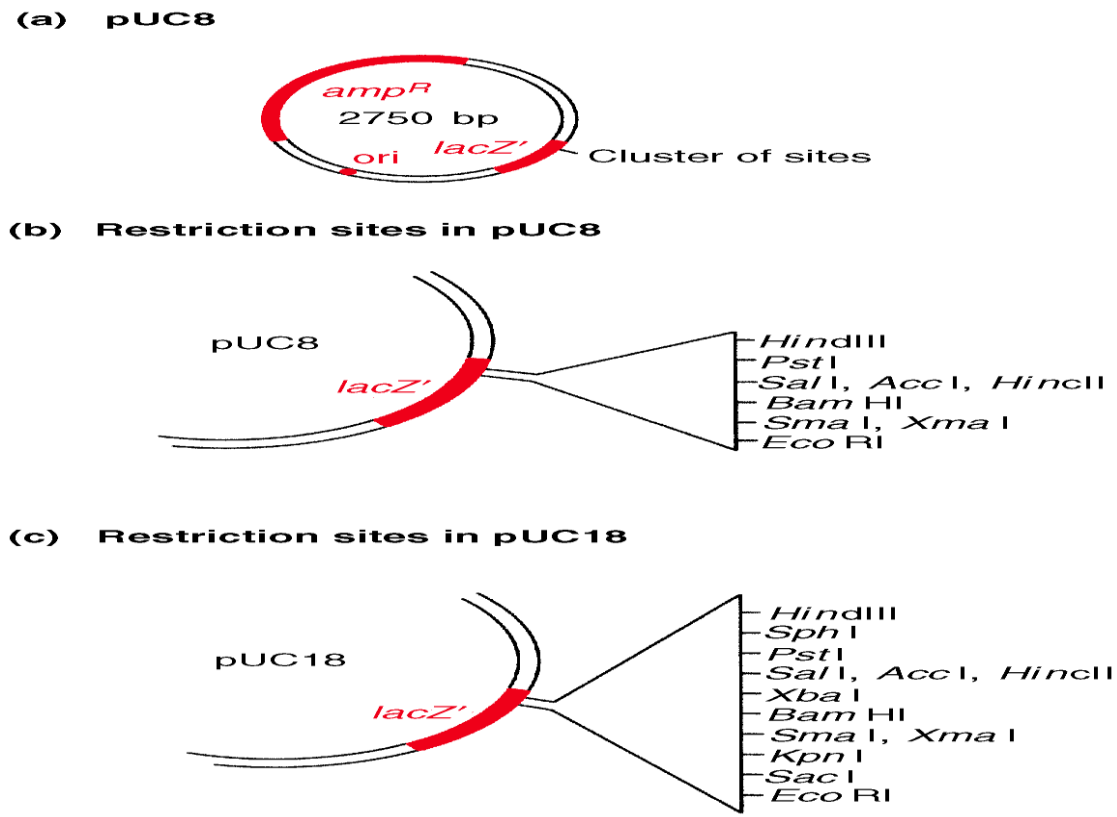


pUC plasmids:

- pUC plasmids are **small, high copy number plasmids of size 2686bp.**
- This series of cloning vectors were developed by **Messing and co-workers in the University of California**. The **p in its name stands for plasmid and UC represents the University of California.**
- **pUC vectors contain a *lacZ* sequence and multiple cloning site (MCS) within *lacZ.*** This helps in use of broad spectrum of restriction endonucleases and permits rapid visual detection of an insert.
- **pUC18 and pUC19 vectors are identical apart from the fact that the MCS is arranged in opposite orientation.**
- **<u>pUC vectors consists of following elements:</u>**
  - pMB1 ***"rep"*** **replicon region derived from plasmid pBR322** with single point mutation (to increase copy number).
  - ***"bla"*** gene encoding **β lactamase which provide ampicillin resistance** which is derived from pBR322. This site is different from pBR322 by two point mutations.
  - ***E.coli*** **lac operon** system.
- **"*rop*" gene is removed from this vector which leads to an increase in copy number.**

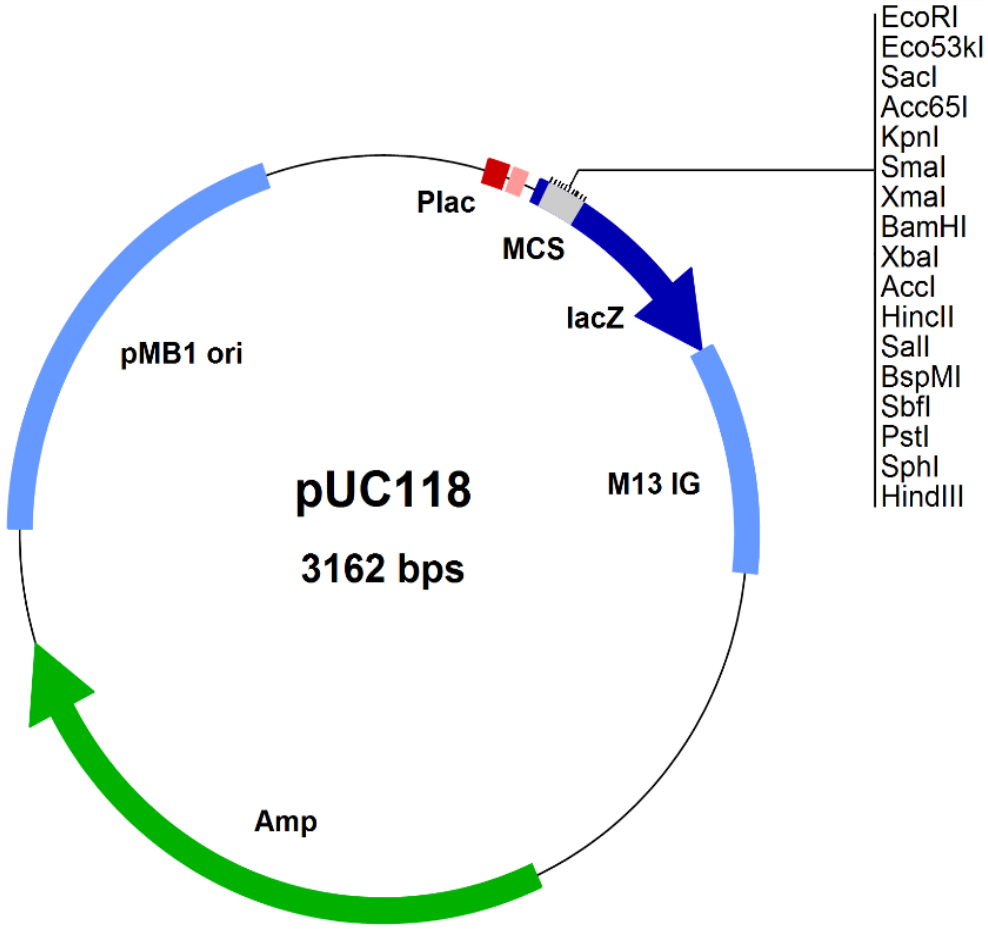
pUC8: A Lac selection plasmid





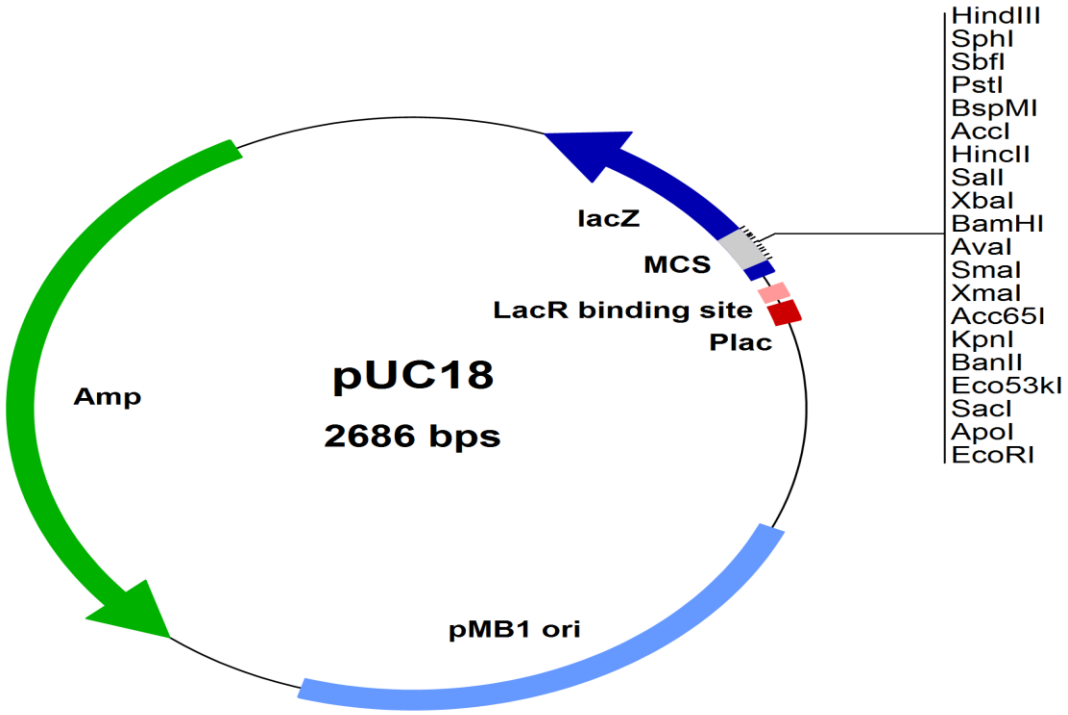
The cloning vector pUC8: (a) the normal vector molecule; (b) a recombinant molecule containing an extra piece of DNA inserted into the *Bam*HI site.

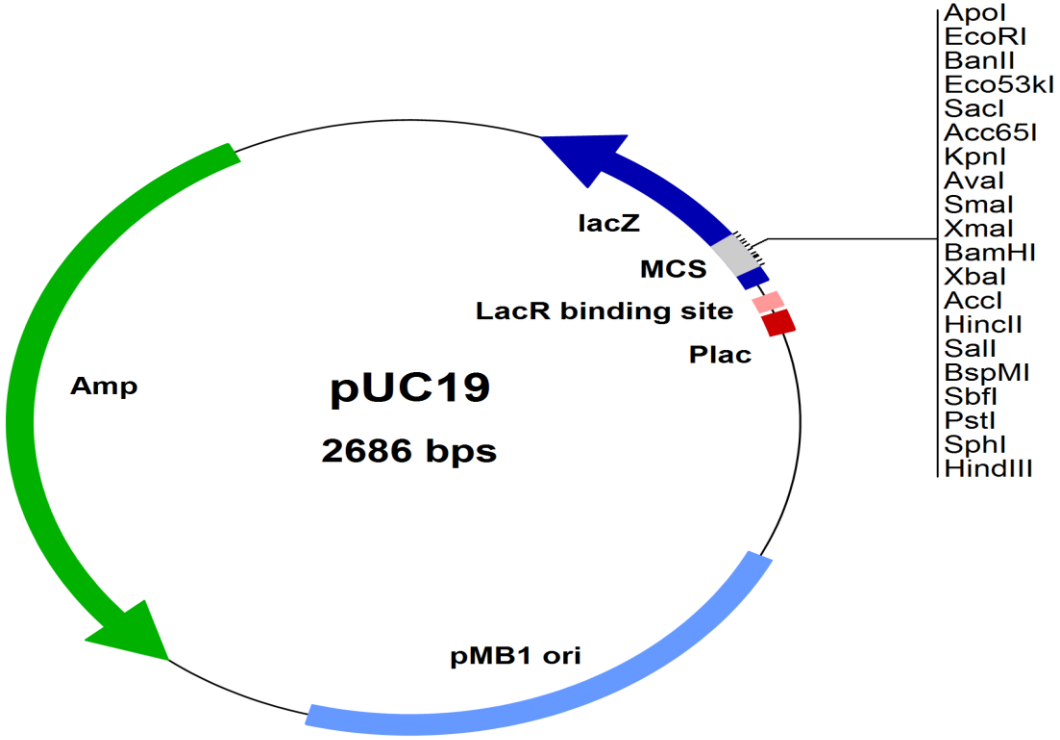
- An MCS is a short DNA sequence consisting of restriction sites for many different restriction endonucleases.
- **The MCS is inserted into the *lacZ* sequence, which encodes the promoter and the α-peptide of β-galactosidase.**
- Insertion of the **MCS into the *lacZ* fragment does not affect the ability of the α-peptide to mediate complementation, while cloning DNA fragments into the MCS does.**
- Therefore, recombinants can be **detected by blue/white screening** on growth medium containing X gal in presence of IPTG as an inducer.



- The native *lacZ* promoter (P*lac*) is situated just upstream of the cloned gene, allowing expression of genes on inserts that are correctly oriented. Most of the nonessential DNA has been removed to provide the ability to clone larger fragments. An ampicillin marker is included for selection of transformants.

- The pUC plasmids were engineered from the pBR322 origin of replication to include the alpha portion of the beta-galactosidase gene (*lacZ'*), complete with its promoter. **The beta portion of *lacZ* was included in the chromosome of the host, so that the host containing the plasmid was Lac+.**





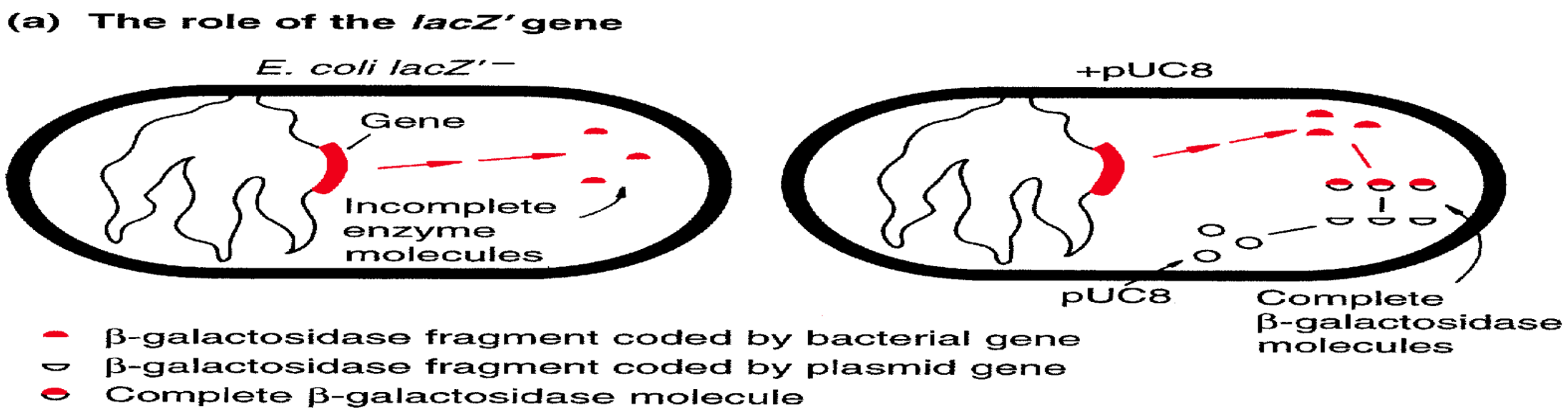
- The restriction enzyme sites **(6 in pUC8/9; 10 in pUC18/19)** are clustered together on an oligonucleotide (called the polylinker, or multiple cloning site), which is in-frame within the first few amino acids of the *lacZ'* reporter gene (thus providing insertional inactivation, Lac+ → Lac-).

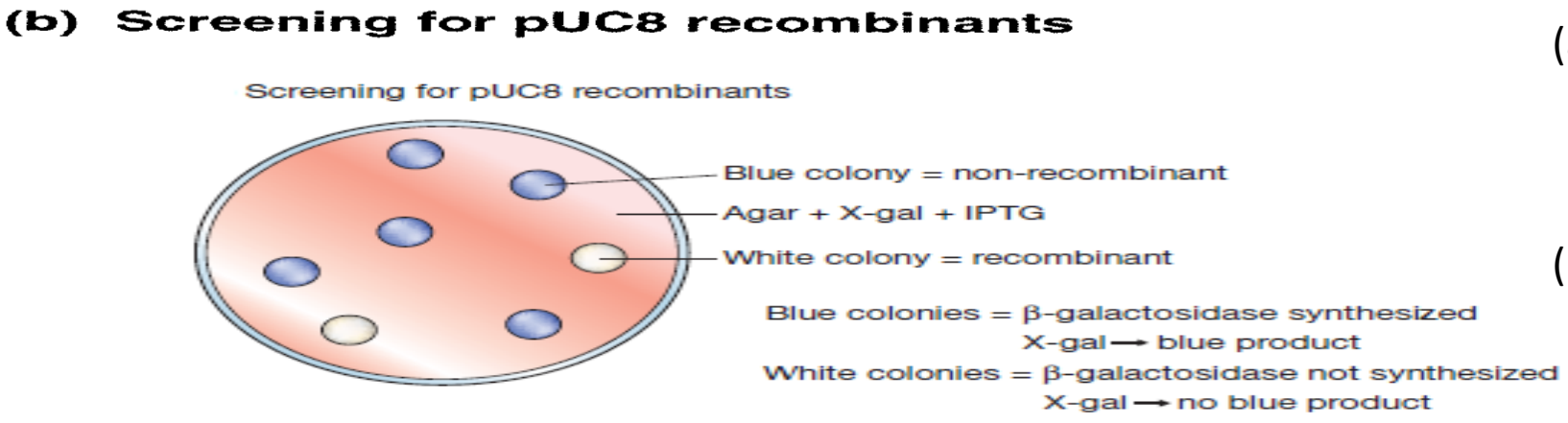
Recombinant selection with pUC vectors: Alpha complementation/ blue-white screening / Insertional inactivation

- The lac-Z gene product (**β-galactosidase**) is a **tetramer**, and **each monomer is made of two parts – lacZ-alpha, and lacZ- omega.**
- **If the alpha fragment was deleted, the omega fragment is non-functional;** however, **alpha fragment functionality can be restored in transformation via plasmid. Hence, then name alpha-complementation.**
- **The *E. coli* enzyme β-galactosidase is a homo-tetramer of the protein product of the *lacZ* gene.**
- **Certain mutations in the 5' region of *lacZ* prevent subunit association.**
- **Because monomers lack enzyme activity, the failure to assemble leads to a Lac^- phenotype.**

- **The activity of the enzyme β –galactosidase is easily monitored by inducing in the growth medium the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β–galactoside (Xgal).**
- This compound is colorless, but on **cleavage, releases a blue indolyl derivative.**
- On solid medium, colonies that are **expressing active β –galactosidase are blue in colour, while those without the activity are white in colour.**
- **This is often referred as blue/white screening.**

The rationale behind insertional inactivation of the *lacZ*$^{\zeta}$ gene carried by pUC8.





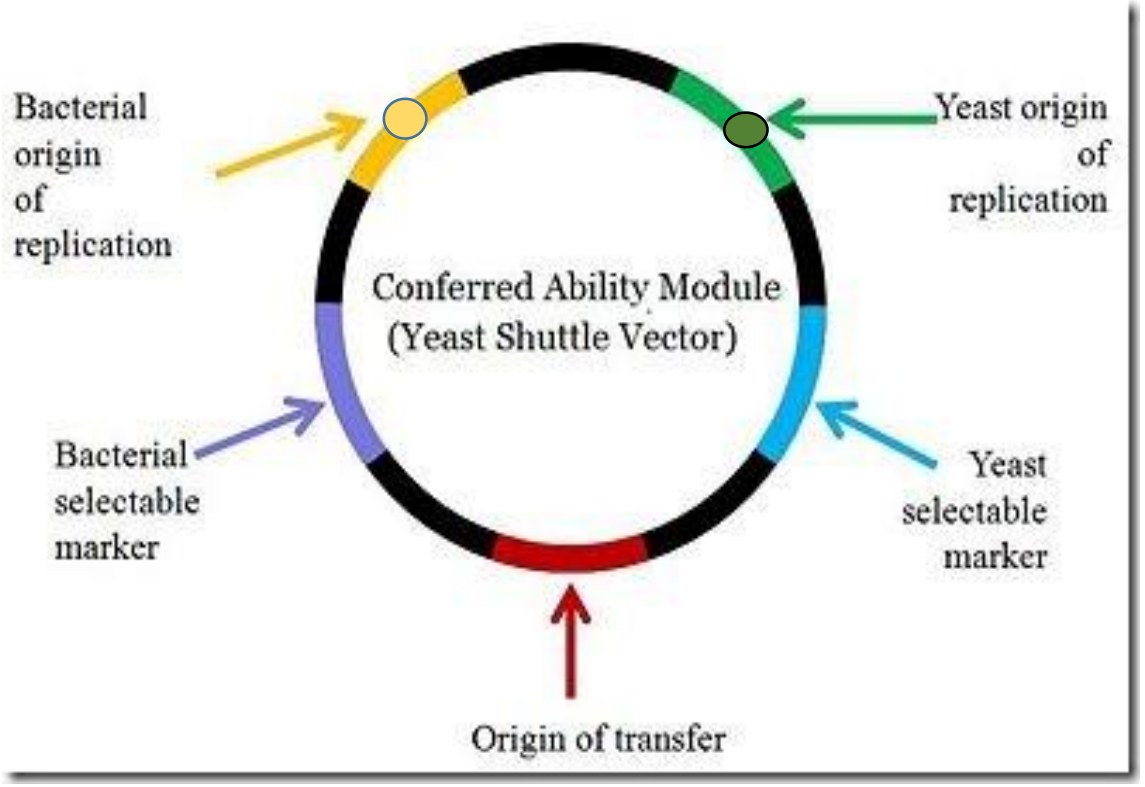
(a) The bacterial and plasmid genes complement each other to produce a functional b-galactosidase molecule.

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

SHUTTLE VECTORS

- **The plasmid cloning vector that can replicate in two different organisms is called shuttle vector. It has two origin of replication, each of which is specific to a host**.
- Since, shuttle vectors replicate in two different hosts, they are known as **bifunctional vectors.**
- A shuttle vector is a vector that **can propagate in two different host species, typically *E. coli and* a eukaryotic host species**. For example, **pMK3/4 has a gram-positive ori for cloning in *Bacillus subtilis*, and a gram-negative ori for cloning in *E. coli*.**

- In this way, genetic engineering may be done in *E. coli* where it is easier and transferred to *B. subtilis* for final expression and excretion.
- **Many eukaryotic expression vectors are shuttle vectors, since they are first produced in *E. coli,* and then introduced into the eukaryotic host.**

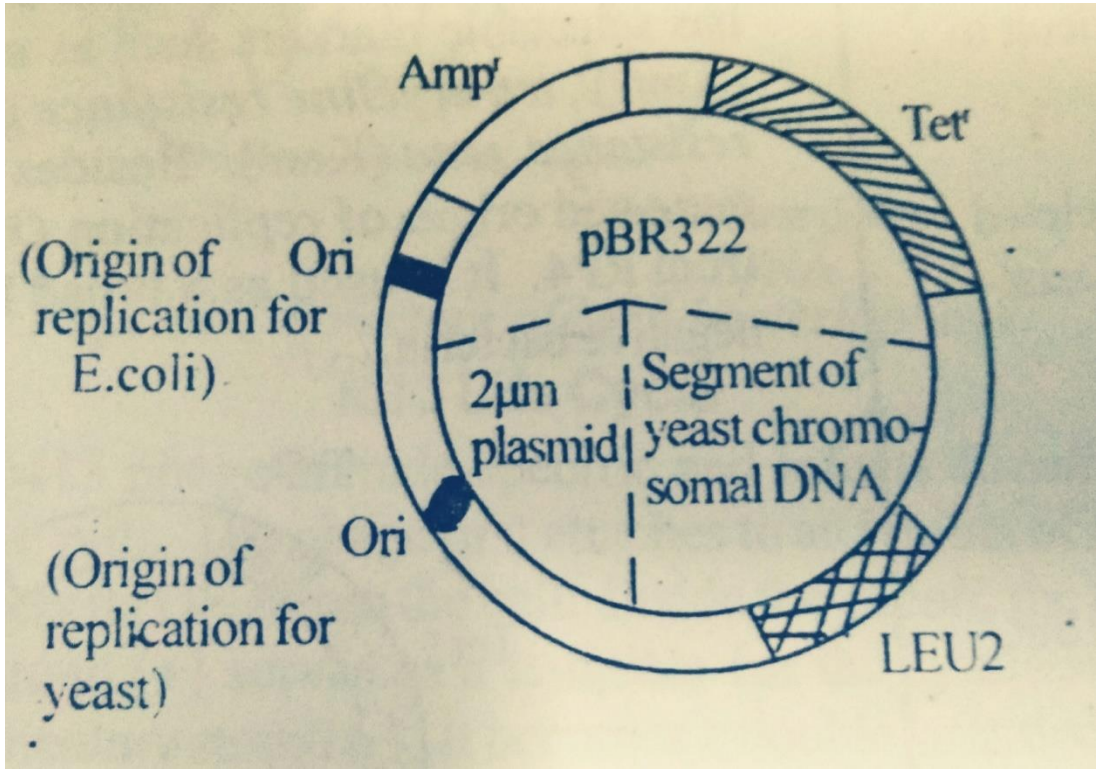


Advantages of shuttle vectors :

Shuttle vectors can be used to shuttle (move to and fro) a gene between two different hosts. After expression studies in the second host, if there is a need for further manipulation, the rDNA can be modified it in the first host.

pJDB 219

- The pJDB219 shuttles an inserted DNA between **E.coli cell and Yeast cell.** It consists of the entire sequence of a pBR322 and a 2ɥm plasmid and a selectable marker gene LEU2 of yeast chromosome.

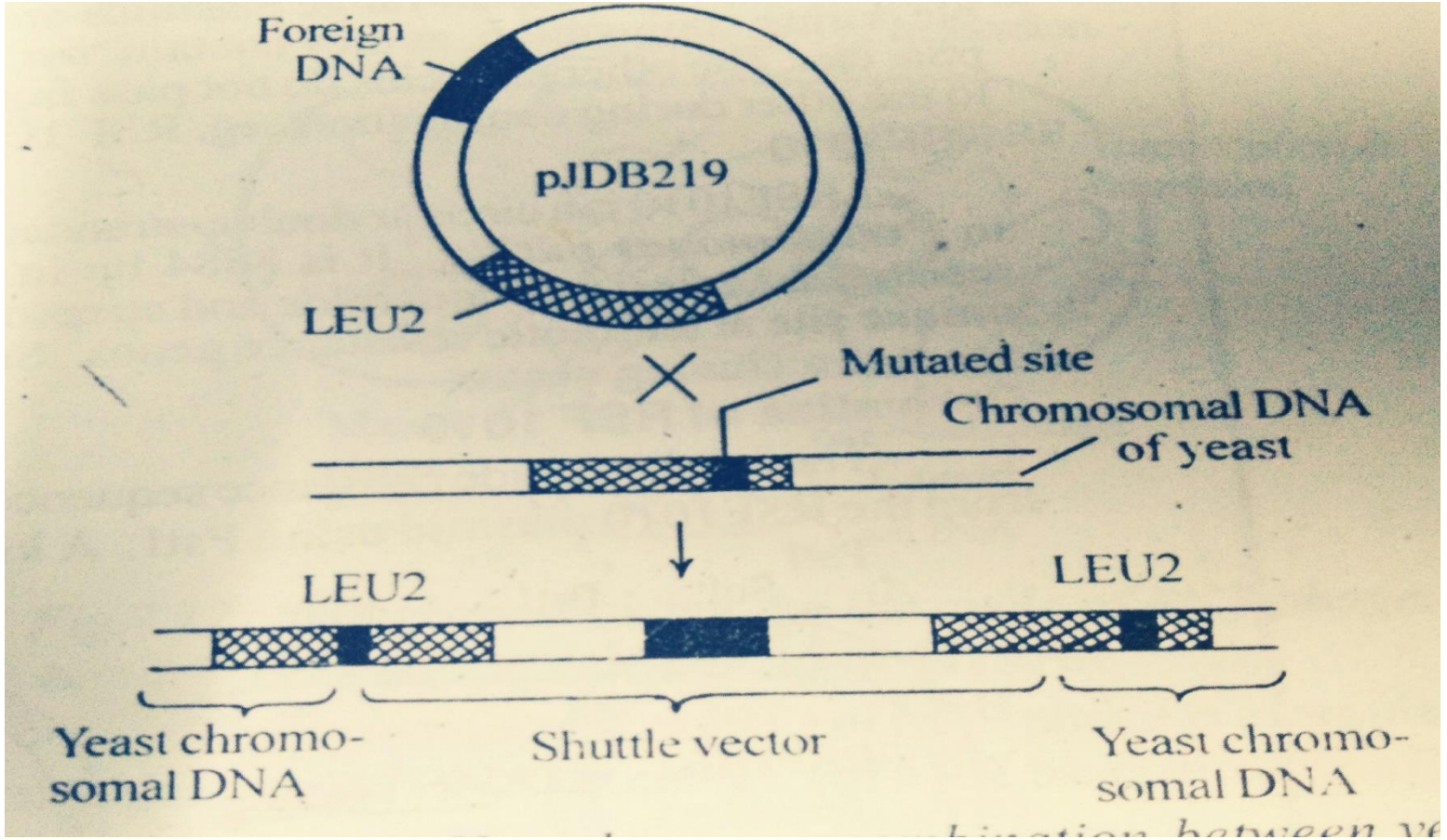


- The **pBR322 derivative encodes for ampicillin resistance (Amp^R) and tetracycline resistance (Tet^R). It has an origin of replication essential for replication in E.coli.**
- **2ɥm plasmid of yeast contributes an origin of replication of plasmid in yeast cells.**
- **The LEU2 gene encodes for isopropyl malate dehydrogenase that converts pyruvic acid into leucine. The leucine can easily be assayed by growing the transformants in a medium lacking leucine.**

- The foreign DNA segment is inserted into pJDB219 to construct an rDNA. The rDNA is introduced into E.coli cells by **bacterial transformation.** The recombinant E.coli cells are selected by insertional inactivation method.

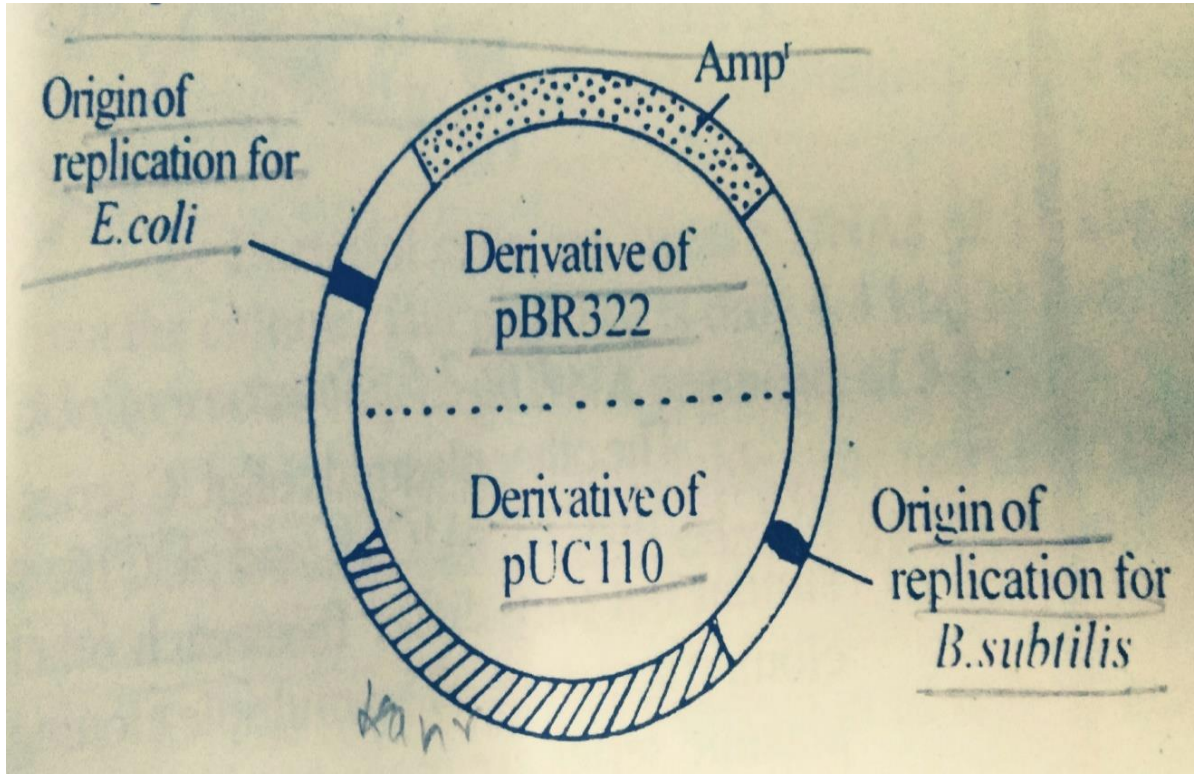
- The **selected E.coli cells are then cultured in a medium containing chloramphenicol to increase the copy number of the plasmid.** Then the plasmids are isolated from E.coli **and introduced into LEU2 mutant yeast cells** by transformation. **The transformed yeasts (*Saccharomyces cerevisiae*) are selected by growing them in a medium lacking leucine.**

- **The LEU2 gene of the plasmid undergoes recombination with LEU2 gene of yeast chromosome.** As a result, the entire plasmid (rDNA) becomes a part of the chromosome.



pEB10

- **pEB10** is a circular, double-stranded plasmid.
- It is **8.9 Kbp in size**.
- It has **two selectable markers- ampicillin resistance gene (Amp^R) and kanamycin resistance gene (Kan^R).**
- The **ampicillin resistance gene determinant is derived from pBR322.**
- **The kanamycin resistance determinant is a derivative of pUB110.**
- **It has an origin of replication for replication of the plasmid in *E,coli.***
- It has yet **another origin of replication that switches on replication in *Bacillus subtilis*.**
- As the pEB10 has two different origins of replication, it **can replicate both in *E,coli* and *B.subtilis.***



- The desired gene is first inserted into pEB10 to construct rDNA.
- The rDNA is introduced in ***E.coli*** cell by bacterial transformation.
- ***E.coli* cells are naturally resistant to chloramphenicol that inhibits protein synthesis. But *B.subtilis* is sensitive to chloramphenicol.**
- **The recombinant *E,coli* culture is treated with chloramphenicol to increase the copy number of pEB10 in the *E.coli.***
- The amplified rDNA is isolated, purified and introduced into *Bacillus subtilis* by transformation.
- The transformants are screened and mass cultured for the expression of the cloned gene.