Cloning Vectors in Bacteria
Plasmids and Phages

Bacterial Strains and Plasmid Vectors

Bacteria as hosts

eg.- *Eschericia coli, Bacillus subtilis*

1. They are easily grown
2. They are cheap to grow
3. They grow fast
4. They are easily manipulated in the laboratory
   a) DNA can be inserted - transformation
   b) DNA can be easily isolated
5. Non-pathogenic
6. Genetically stable
7. Bacteria contain natural plasmids and viruses which are useful vectors for recombinant DNA
8. Equipped with appropriate enzymes to allow replication of the vector

Bacterial Engineered Host Strains

- A few mutations are common to all or most expression strains to accommodate high protein levels including:
- **ompT:** Strains harboring this mutation are deficient in outer membrane protease VII, which reduces proteolysis of the expressed recombinant proteins.

- **lon protease:** Strains where this is completely deleted (designated lon or Δlon) similary reduce proteolysis of the expressed proteins.

  hsdS$_B$ (r$_B^-$ m$_B^-$): These strains have an inactivated native restriction/methylation system. This means the strain can neither restrict nor methylate DNA.

- **Dcm/Dam:** Similarly, strains with this mutation are unable to methylate cytosine/adenine within a particular sequence.
-

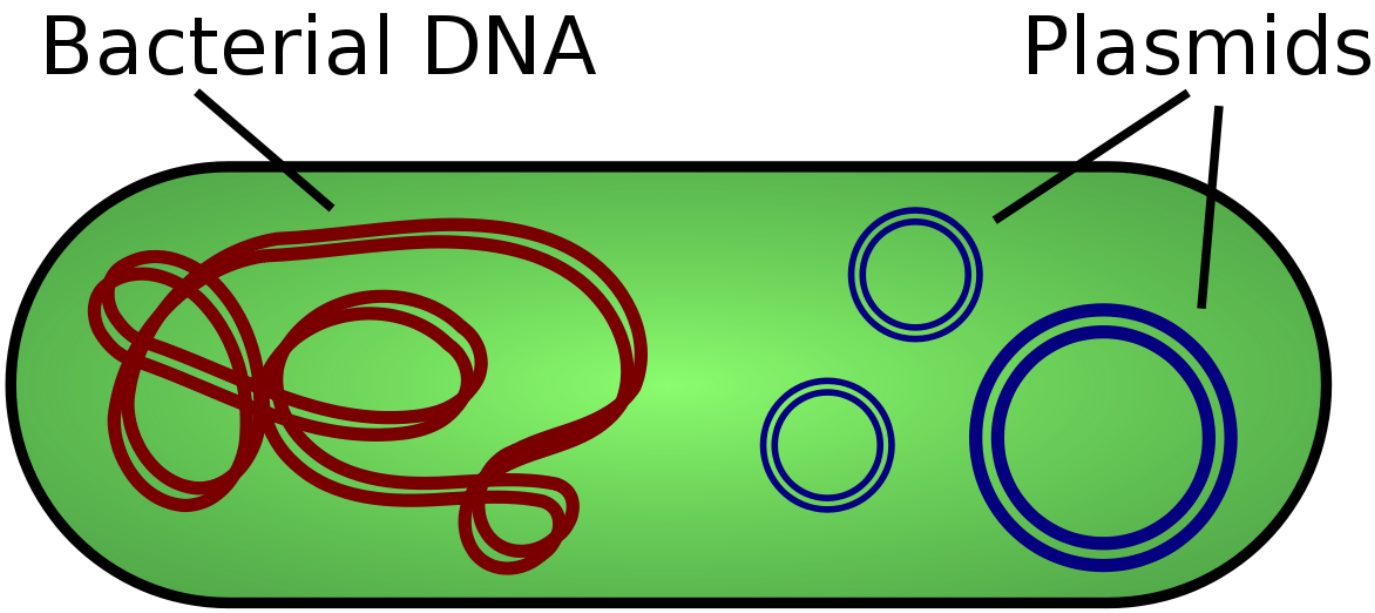
Commonly Used Bacterial Strains

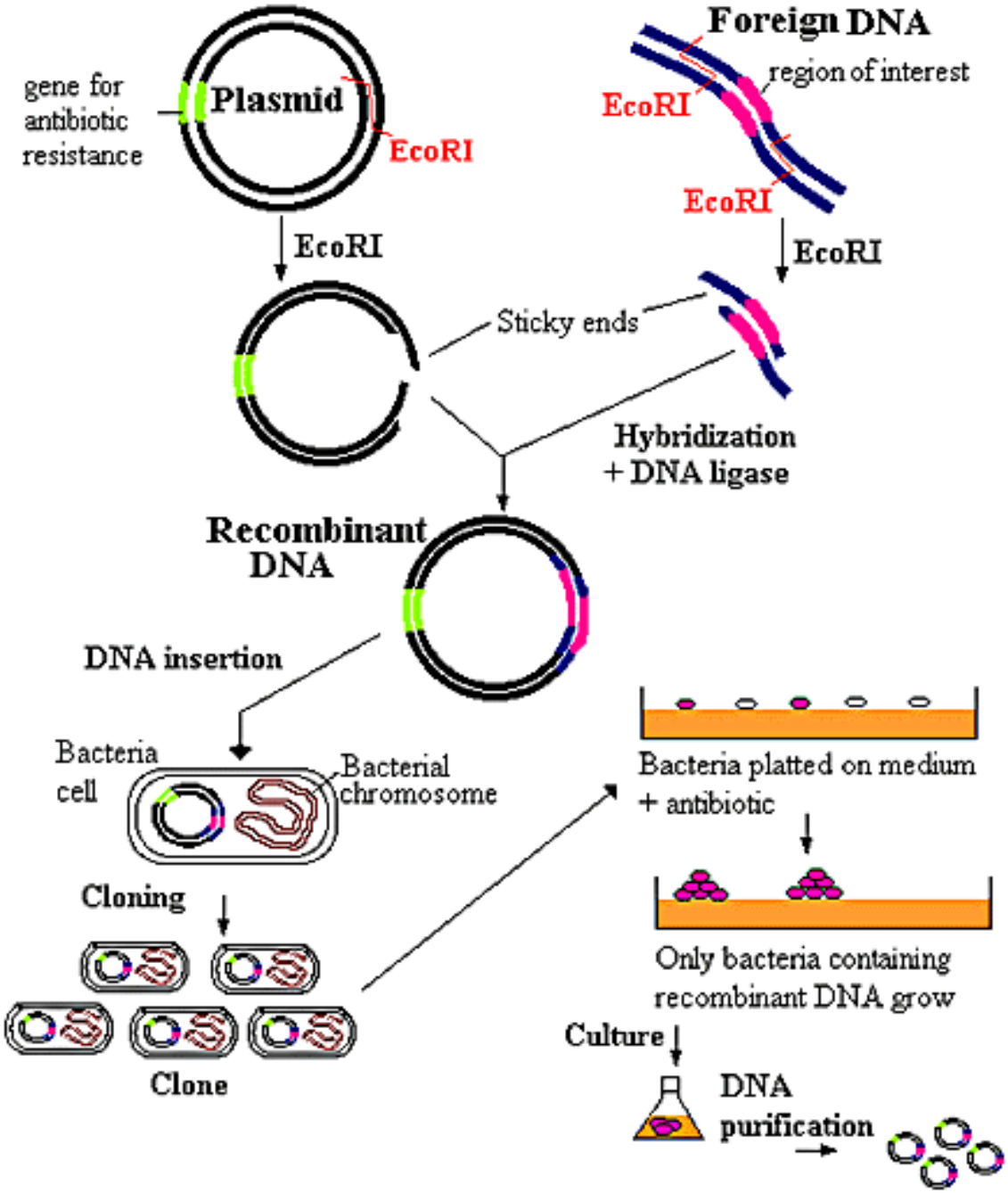
Strain	Resistance	Key Features	Use
BL21 (DE3)		Basic IPTG-inducible strain containing T7 RNAP (DE3)	General protein expression
BL21 (DE3) pLysS*	Chloramphenicol (pLysS)	pLysS expresses T7 lysozyme to reduce basal expression levels; expression vector cannot have p15A origin of replication	Expression of toxic proteins
BL21 (DE3) pLysE*	Chloramphenicol (pLysE)	pLysE has higher T7 lysozyme expression than pLysS; expression vector cannot have p15A origin of replication	Expression of toxic proteins
BL21 star (DE3)		Lacks functional RNaseE which results in longer transcript half-life	General expression; not recommended for toxic proteins
BLR (DE3)	Tetracycline	RecA-deficient; best for plasmids with repetitive sequences.	Expression of unstable proteins

Bacterial Strains

Strain	Resistance	Key Features	Use
Origami2 (DE3)**	Streptomycin and Tetracycline	Contains highly active thioredoxin reductase and glutathione reductase to faciliate proper folding; may increase multimer formation	Expression of insoluble proteins
Rosetta2 (DE3)*	Chloramphenicol (pRARE)	Good for “universal” translation; contains 7 additional tRNAs for rare codons not normally used in *E. coli.* Expression vector cannot have p15A origin of replication	Expression of eukaryotic proteins
Lemo21 (DE3)*	Chloramphenicol (pLemo)	Rhamnose-tunable T7 RNAP expression alleviates inclusion body formation. Expression vector cannot have p15A origin of replication	Expression of toxic, insoluble, or membrane proteins

Plasmids





Cloning into a plasmid

Plasmids- Small circular piece of extrachromosomal DNA

Circular and linear (Streptomyces sp, Borrelia burgdoferi)

Plasmids are classified

1. by their ability to be transferred to other bacteria (+ce of tra genes)

A. **Conjugative**
   The sexual transfer of plasmids to another bacterium through a pilus. those plasmids possess the 25 genes required for transfer
B. **Non-conjugative**
   Non-conjugative plasmids don't initiate conjugaiton.

PLASMID CLASSIFICATION BY FUNCTION

1. **Fertility-(F) plasmids**,
   They are capable of conjugation (they contains the genes for the pili).

2. **Resistance-(R) plasmids,**
   contain gene (s) that can build resistance against one or several antibiotics or poisons.

3. **Col-plasmids,**
   contain genes coding for colicines, proteins that can kill other bacteria
.
4. **Degradative plasmids,**
   able to digest unusual substances, e.g., toluene or salicylic acid.

5. **Virulence plasmids,**
   turn a bacterium into a pathogen.

3. Size and Copy number

High copy number = 10-300 copies / cell → generally Non conjugative, relaxed

Low copy number = 1-4 copies / cell → generally conjugative, HMW, stringent

Plasmid	Size		Organism
	Nucleotide length (kb)	Molecular wt (MDa)	
pBR345	0.7	0.46	*E. coli*
pBR322	4.362	2.9	*E. coli*
ColEl	6.36	4.2	*E. coli*
RP4	54	36	*Pseudomonas* + others
F	95	63	*E. coli*
TOL	117	78	*Pseudomonas putida*
pTiAch5	213	142	*Agrobacterium tumefaciens*

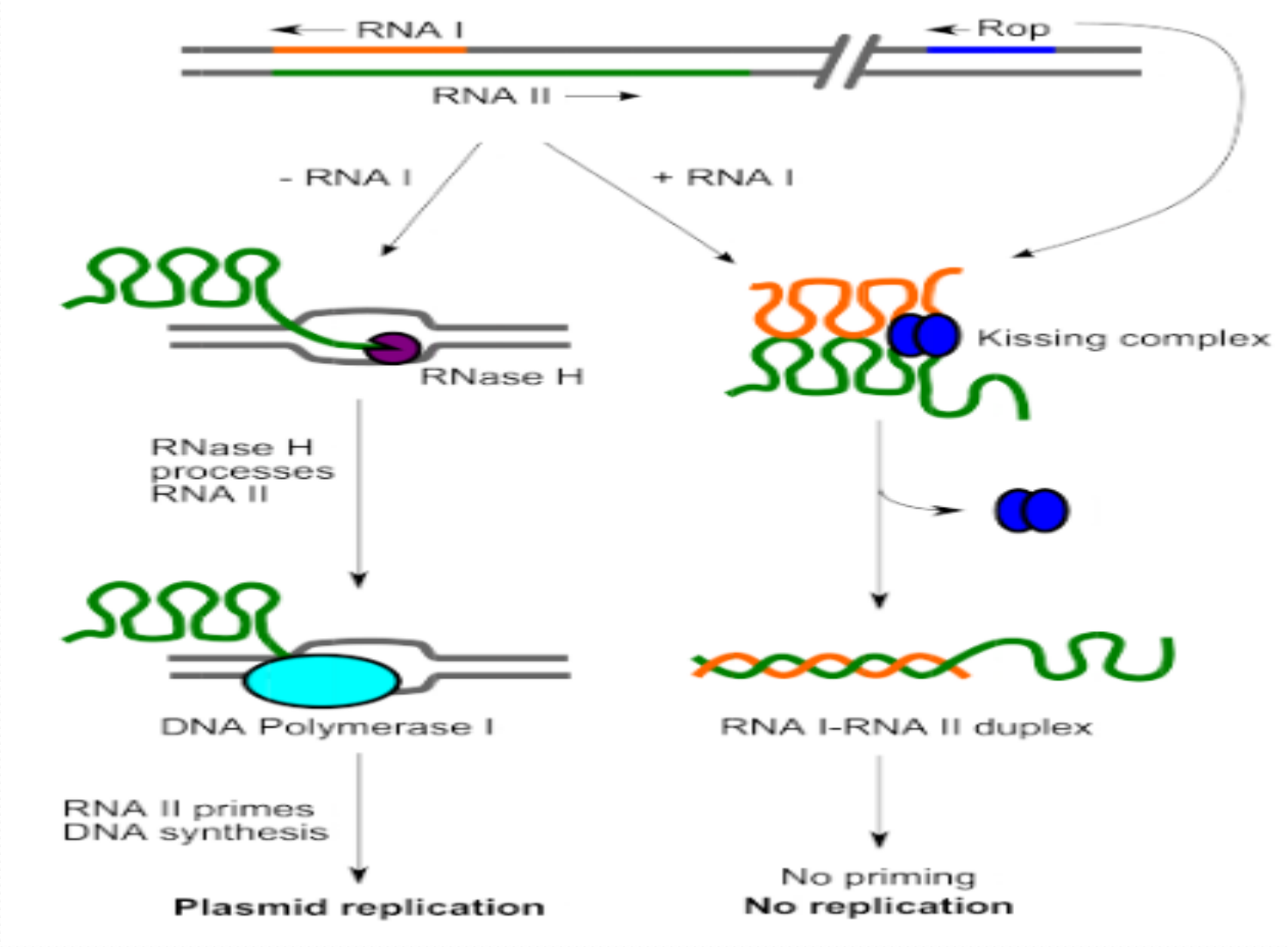
TABLE 4.2 Copy numbers of some plasmids

Plasmid	Approximate copy number
F	1
P1 prophage	1
RK2	4–7 (in *E. coli*)
pBR322	16
pUC18	~30–50
plJ101	40–300

Copy number

- Regulated by initiation of plasmid replication
  - Antisense RNA
  - Proteins binding to repeated sequences called iterons

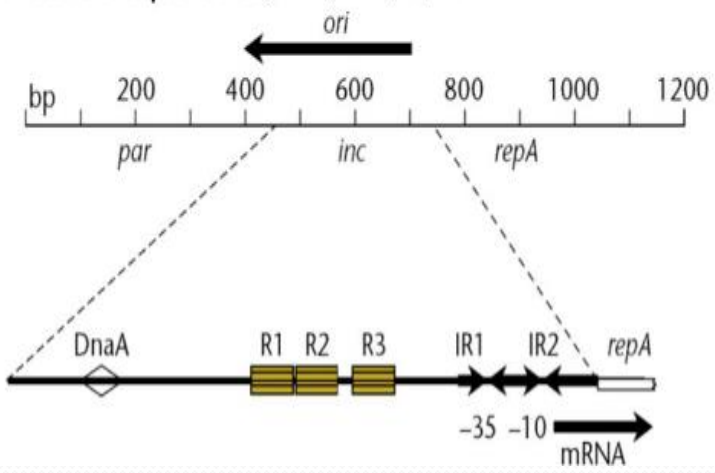
Control of Copy number (Replication Initiation control)

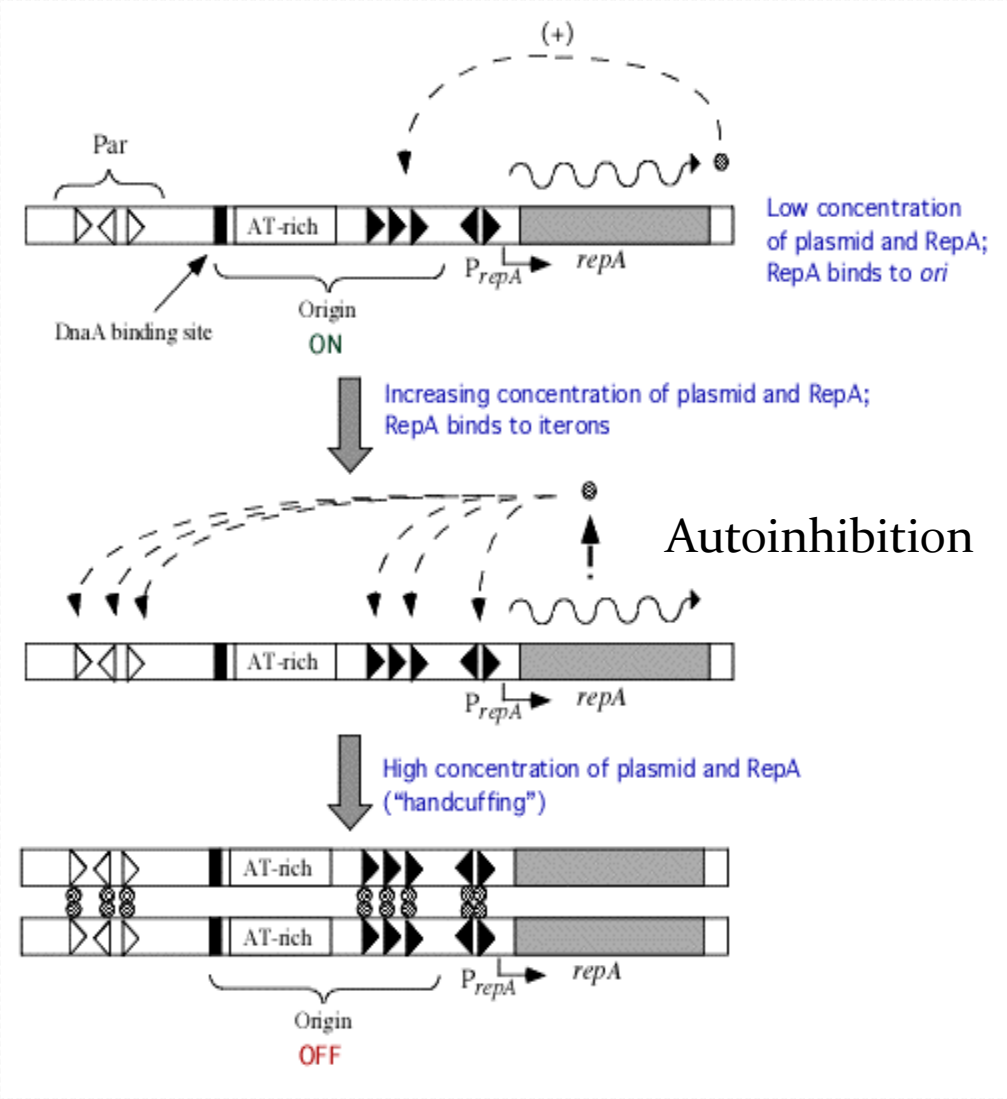


Control of Copy number (Replication Initiation control)

Iteron plasmids

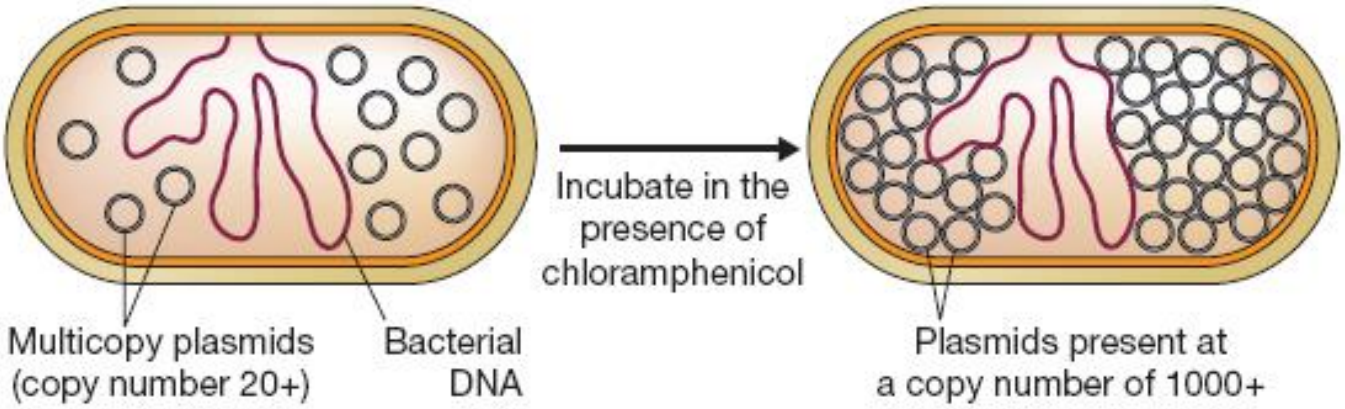
- *ori* contains *repA* gene
  - Sole plasmid-encoded protein required for replication
  - 3 iteron sequences, R1, R2, & R3





Handcuffing plasmids (RepA)

PLASMID AMPLIFICATION



Plasmid replication

- Must be a self-replicating genetic unit
- Plasmid DNA must replicate every time host cell divides or it will be lost
- All self replication plasmids have a *ori*: origin of replication → it determines host and copy number.
- Plasmid segregation is maintained by a *par* locus-a partition locus that ensures each daughter cells gets one plasmid. Not all plasmids have such sequences. Essential for low copy number plasmids.

Stringent

Plasmids are said to be under stringent control of replication when they are dependent on the presence of initiation proteins synthesized by the host cell in order to start their own replication. In general, these types of plasmids tend to be low copy number.

Relaxed

Conversely, plasmids that can initiate DNA replication independently of the host's initiation proteins are said to be under relaxed control, as they only require the host's replication machinery for elongation and termination. These types of plasmids tend to be high copy number.

INCOMPATIBILITY GROUPS:

Plasmid incompatibility refers to the inability of two **plasmids** to coexist stably over a number of generations in the same bacterial cell line. Generally, closely related **plasmids** tend to be **incompatible**, while distantly related **plasmids** tend to be compatible.

How do you know if your plasmids are incompatible?

Scientists have developed a system of bacterial incompatibility groups based on similarity of replication and partitioning systems where the plasmids belonging to the same group are incompatible.

Using plasmid incompatibility for therapeutic advantage-

Although it may seem like plasmid incompatibility may be standing in your way to bacterial cloning glory, scientists have applied these concepts to deal with the more sinister plasmids of the microbiological world. (Want to know more about these plasmid types? Scientists have designed small, high copy incompatible plasmids that lead to asymmetrical plasmid loss of the usually large, low copy virulence plasmids. This strategy has been successful in displacing, or "curing" the virulence plasmids from the bacterial pathogens *Yersinia pestis*, *Agrobacterium tumefaciens*, and *Bacillus anthracis* (Lui et al., 2012, Ni et al., 2008, Uraji et al., 2002). Scientists are beginning to use plasmid incompatibility to combat antimicrobial resistance. Using novel plasmids based partly on plasmid incompatibility with antibiotic-resistance plasmids, scientists have expelled antibiotic resistant plasmids from *Enterobacteriaceae* family members within the mouse gut (Kamruzzaman et al., 2017).

- **Plasmid Host Range- determined by its 'ori' region**

- Narrow host range plasmids:
  - most of the naturally occurring plasmids have a restricted host range.
  - E.g. ColE1 and its derivatives grow only in *E. coli* and related organisms such as *Salmonella*.

- Broad host range plasmids:

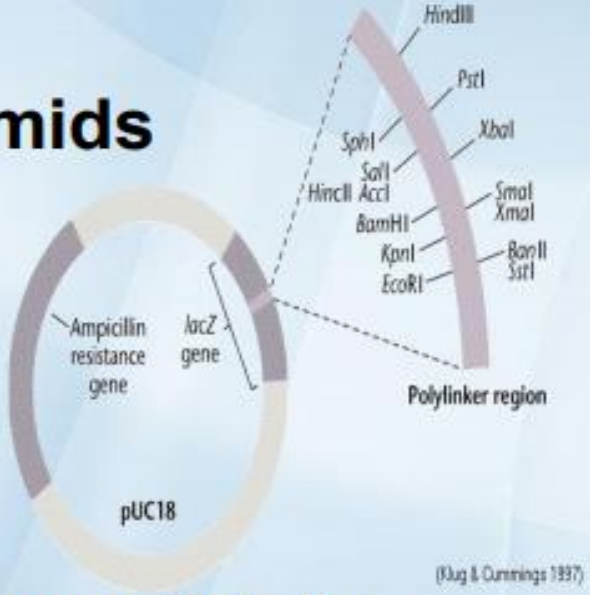
- Possess less specific *ori* region such that it can function in a number of bacterial species.
- Most of them require very few of the host-encoded proteins
- Generally plasmids of the incompatibility group P, Q, and W have a broad host range; also called as **promiscuous plasmids** e.g. RP4, RSF1010, RK2 etc.
- Can replicate in a no. of bacteria such as *E. coli, Agrobacterium, Pseudomonas, Rhizobium* etc.

- Conjugative/non-conjugative
- Plasmid Size
- Plasmid Copy Number
- Plasmid Host Range
- Plasmid Compatibility

PLASMID VECTORS

Plasmid vectors are used to clone DNA ranging in size from several base pairs to several thousands of base pairs (100bp -10kb).

Features of many modern Plasmids



(Klug & Cummings 1997)

- Small size
- Origin of replication
- Multiple cloning site (MCS)
- Selectable marker genes
- Some are expression vectors and have sequences that allow RNA polymerase to transcribe genes
- DNA sequencing primers

Markers (Antibiotic resistance)

Ampicillin (beta-lactam class) inhibits synthesis of bacterial cell wall
amp resistance depends upon production of an enzyme beta-lactamase, that catalyzes beta-lactam ring degradation in the periplasmic space.

Tetracycline binds to 30S subunit of the ribosome to prevent ribosome translocation
tet resistance produces a protein that prevents tetracycline from entering the cell.

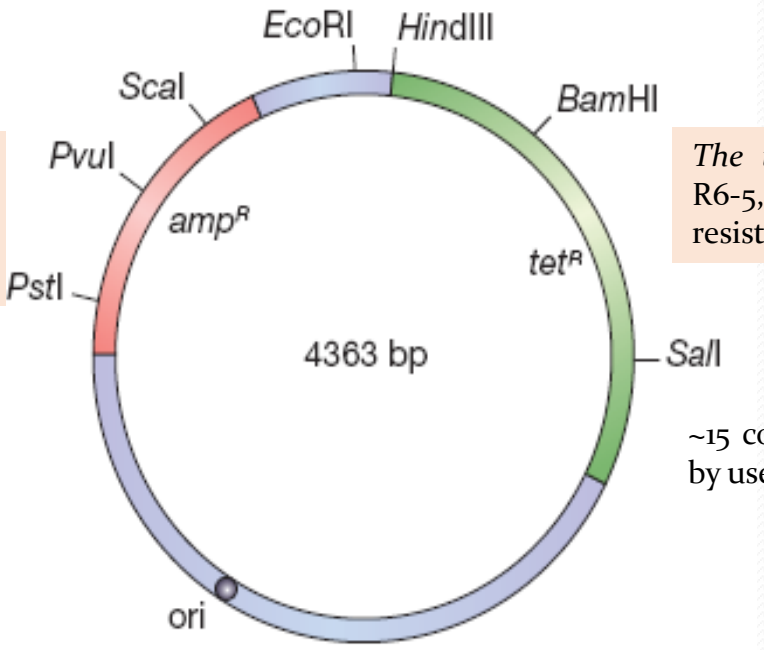
Chloramphenicol binds to the 50S subunit of the ribosome to prevent protein synthesis
cat (chloramphenicol resistance) produces an enzyme system component that converts chloramphenicol to a form that cannot bind the ribosome.

Kanamycin (and the closely related neomycin) are aminoglycosides that bind sub-components of the ribosome and prevent protein synthesis.
kan (and neo) resistance depend upon the synthesis of an aminoglycoside phosphotransferase located in the periplasmic space that inhibits their transport into the cell.

pBR322- One of the earliest plasmid cloning vecto

pBR322 comprises DNA derived from three different naturally occurring plasmids.

The *ampR gene originally resided on the plasmid R1, a typical antibiotic* resistance plasmid that occurs in natural populations of *E. coli*



The tetR gene is derived from R6-5, a second antibiotic-resistant plasmid.

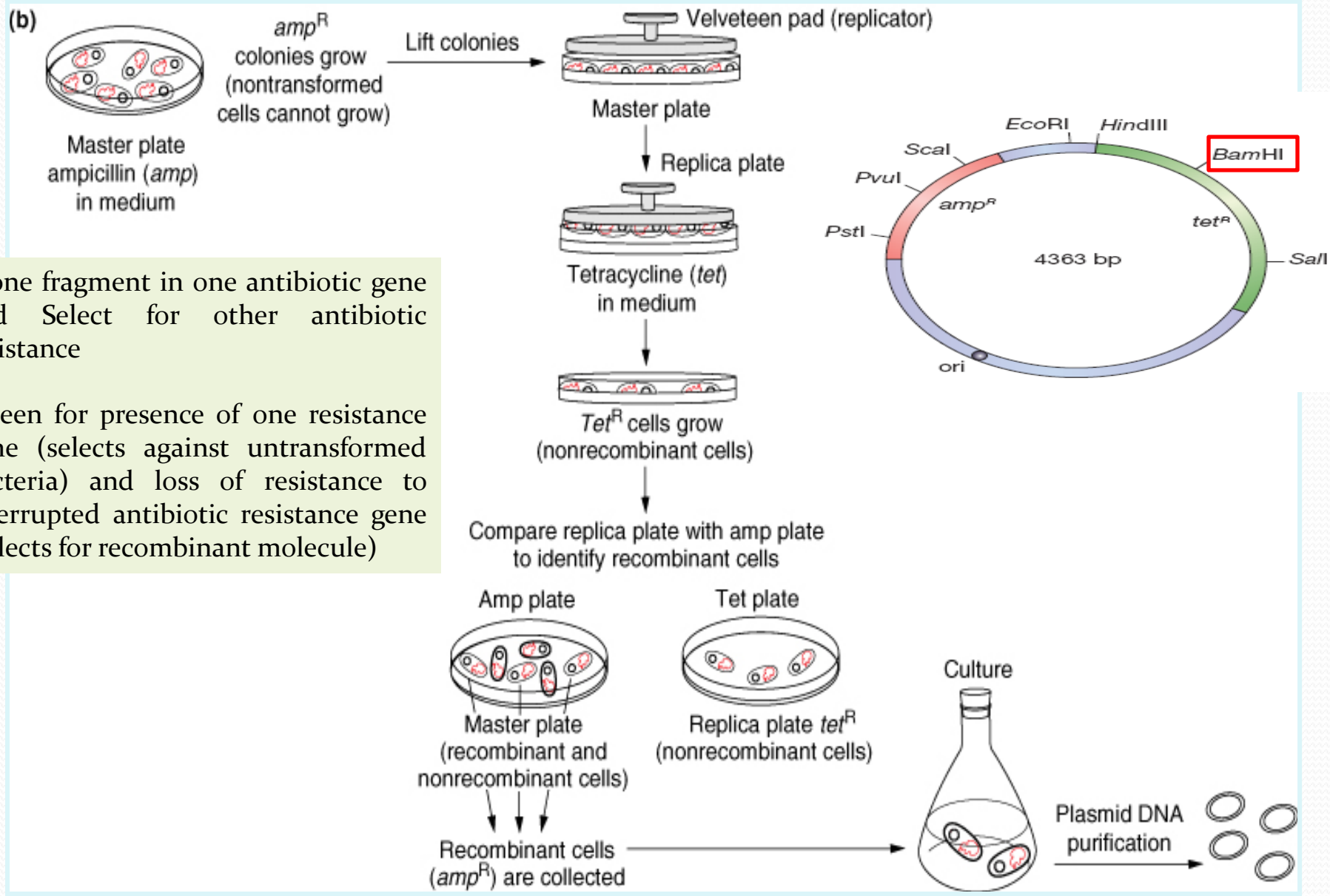
~15 copies/cell (may be increased to ~1000 by use of chloramphenicol)

The *ori* is originally from pMB1, which is closely related to the colicin-producing plasmid ColE1.

The name "pBR322" conforms with the standard rules for vector nomenclature:
"**p**" - plasmid.
"**BR**"- BR stands for Bolivar and Rodriguez, the two researchers who developed pBR322.
"**322**" distinguishes this plasmid from others developed in the same laboratory (there are also plasmids called pBR325, pBR327, pBR328,

Screening bacteria by replica plating

Eg. The cloning is performed in Bam HI Site



Clone fragment in one antibiotic gene and Select for other antibiotic resistance

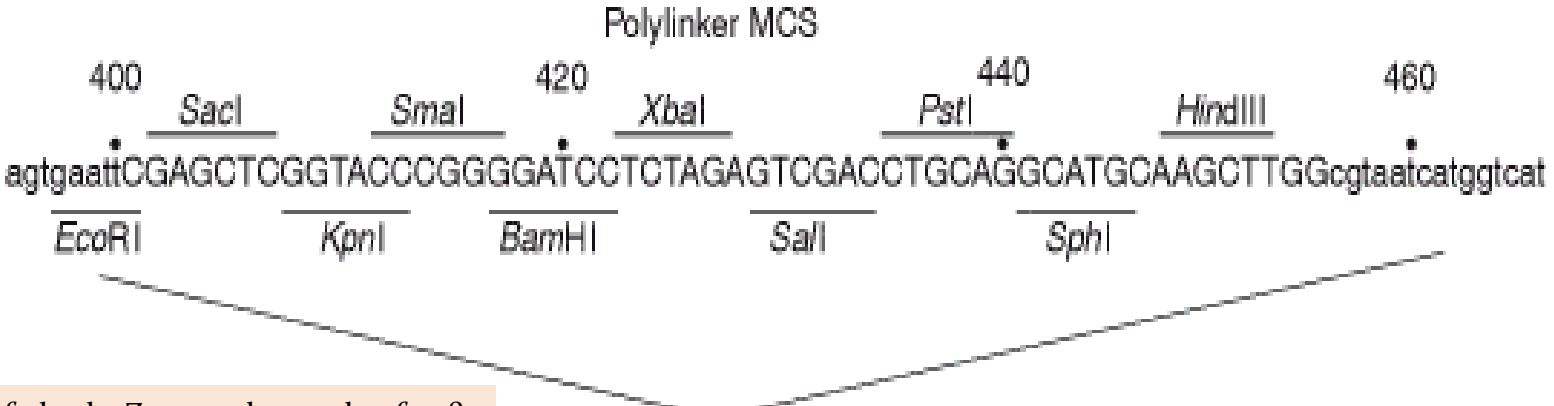
Screen for presence of one resistance gene (selects against untransformed bacteria) and loss of resistance to interrupted antibiotic resistance gene (selects for recombinant molecule)

pBR327

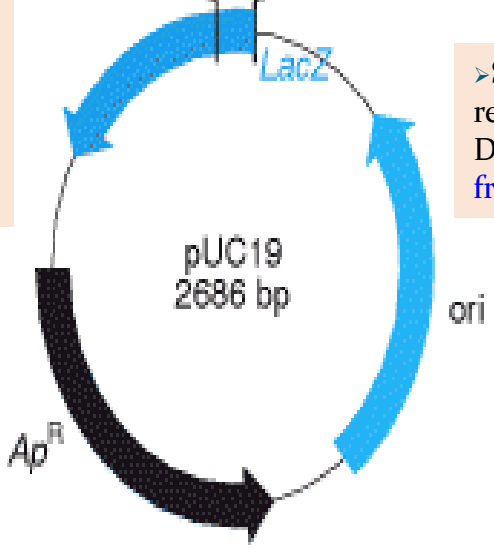
- It was made by deleting a 1089 segment from pBR322
- The Amp and Tet resistance genes were intact
- Higher copy number ~30-45/E coli cell
- The deletion also led to the loss of conjugation ability of the plasmid vector- (Important for Biological Containment)

pUC vector series (pUC18/19)- Advanced Vectors

- Inclusion of Polylinker (also called multiple cloning site) of ~ 60 bases which has sites for multiple restriction endonucleases



Have a region of the lacZ gene that codes for β-galactosidase. This region also contains a polylinker. Thus, insertion of a foreign DNA into any of the restriction sites results in an altered non-functional enzyme. Thus, facilitates Blue-White Screening

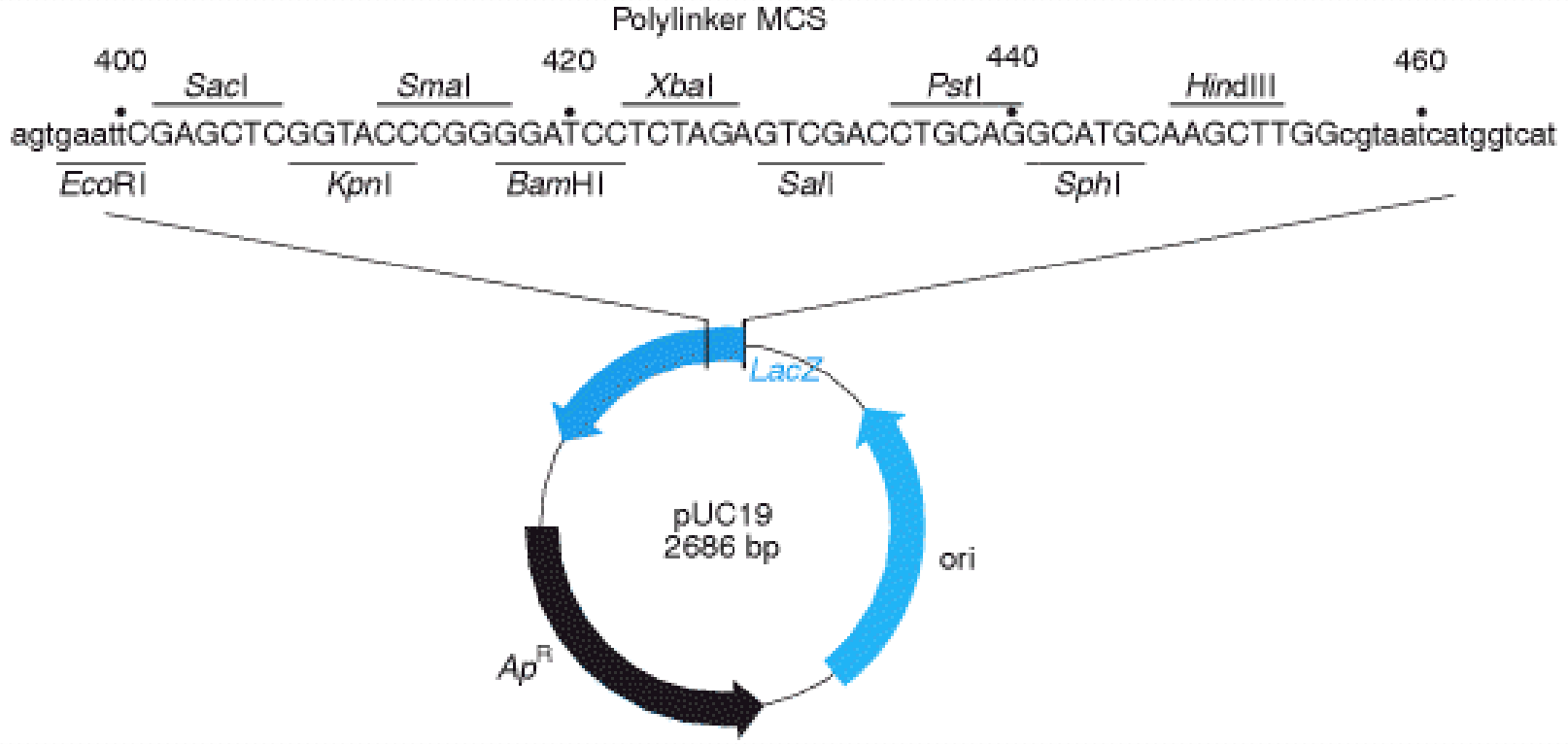


- Shorter than pBR322 (due to removal of most of non-essential DNA) and thus can clone larger fragments

The name "**pUC**" conforms with the standard rules for vector nomenclature:
"**p**" - plasmid.
"**UC**"- stands for University of California where early work on the vector was conducted

The polylinker advantage

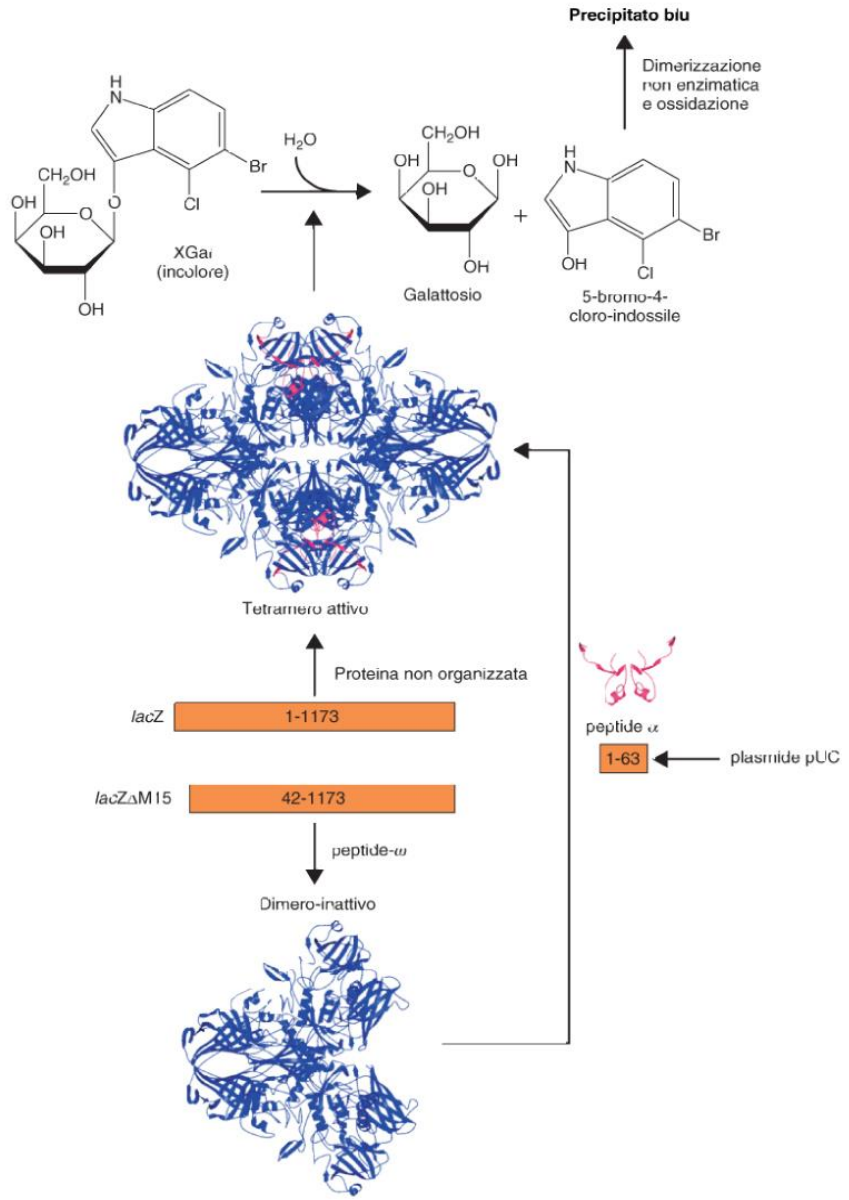
- Unique sites (usually)
- Insert excision facilitated
- Restriction endonuclease mapping and Subcloning made easier
- Directional cloning



Blue White Screening-Alpha complementation:

LacZ→ Beta galactosidase
(Homotetramer)
1021aa

- Bacteria carry mutant allele (LacZΔM15) lacking N-terminal domain → inactive protein
- Alpha peptide carried by plasmid
- Exploits X-Gal (5-bromo-4-cloro-3-indolil-Betagalattoside), a chromogenic substrate analog to galactose
- MCS inserted into LacZ alpha peptide →



Another Major Advance: Blue-White Screening

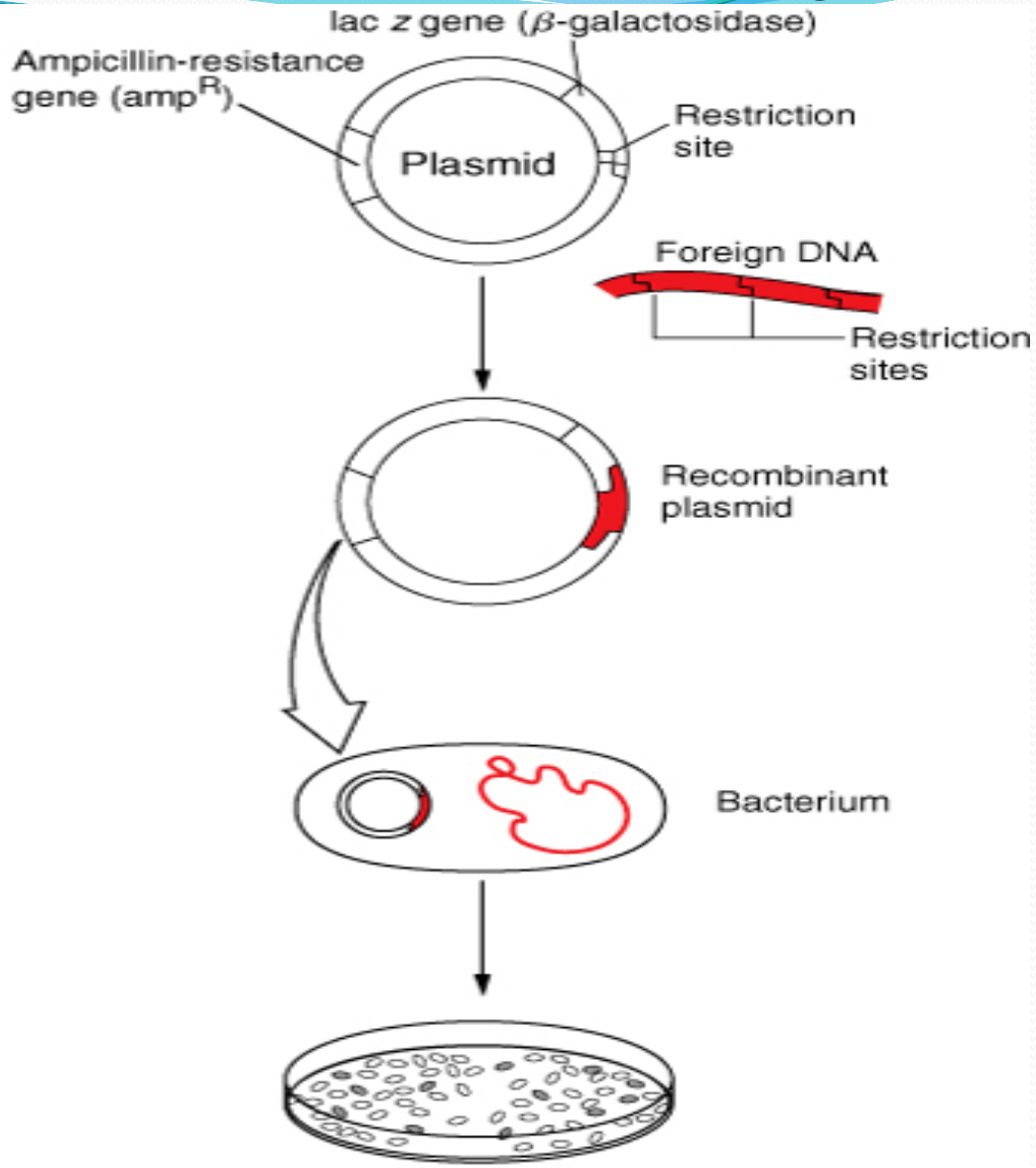
1) Plasmid DNA and foreign DNA are both cut with the same restriction enzyme.

2) Foreign DNA is inserted into the plasmid, where it inactivates the lac z gene.

3) The recombinant plasmid is introduced into a bacterium, which becomes ampicillin-resistant.

4) All treated bacteria are spread on a nutrient agar plate containing ampicillin and β-galactosidase substrate and incubated.

5) White colonies that appear must contain foreign DNA. Blue (gray in this illustration) colonies do not contain foreign DNA.



The **blue colonies contain** "self" religated **plasmids** that **do** not have DNA inserts interrupting the lac Z gene.

White colonies consist of bacteria that carry **plasmids** that have DNA insert fragments that interrupt the lac Z gene.

Beware False Positives:

a) Blue-white screening only indicates the presence of AN insert, not necessarily YOUR insert.

b) A small sequence of foreign DNA may be inserted into MCS and change the reading frame of lacZ gene. This results in **false positive white** colonies.

pUC8

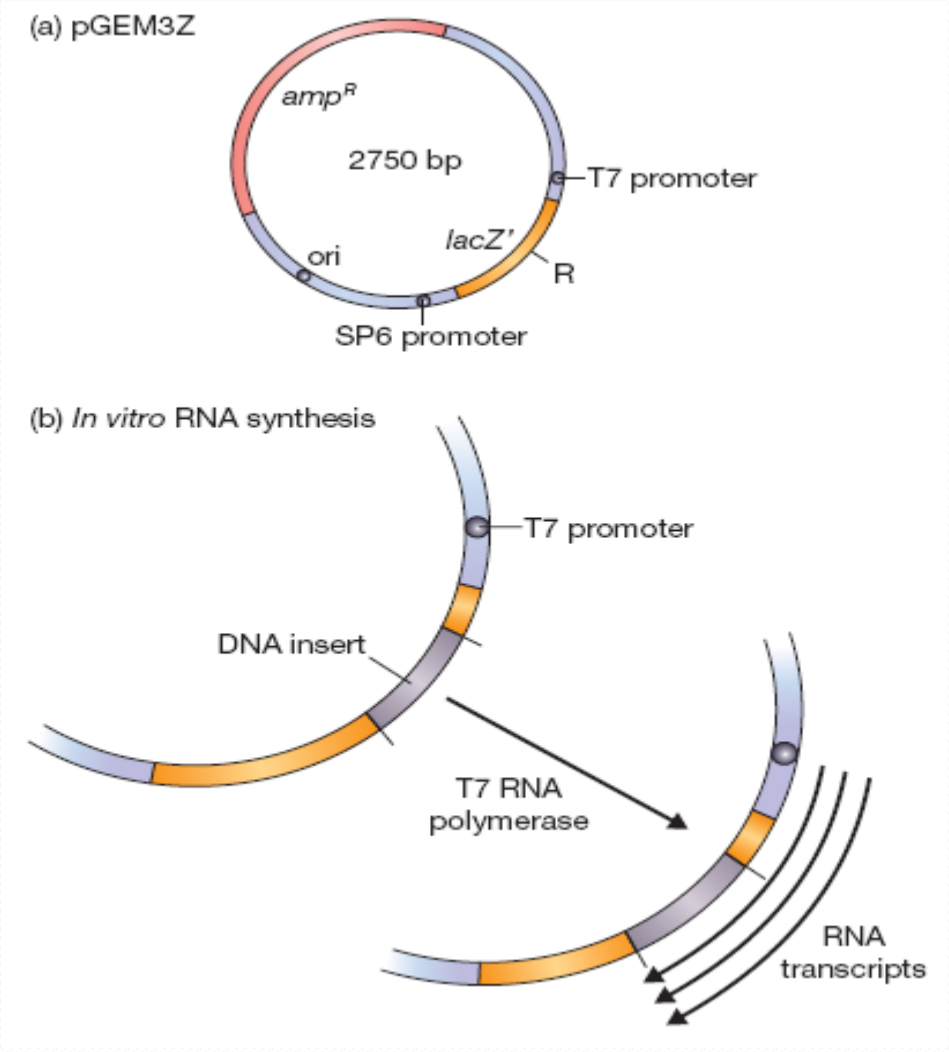
500-700 copies/cell
(due to chance mutation in the ori)

Identification of recombinants (blue white screening)

Multiple Cloning Site (MCS)

Subcloning in M13 mp series vector easy

pGEM3Z—Introduction of promoter for in vitro transcription of cloned DNA



Identification of recombinants (blue white screening)

MCS

2 promoters for in vitro transcription or probe preparation

(phage promoters-T7 and SP6)