Introduction:

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Employment opportunity

Basic gene cloning: plasmids and transformation

- Plasmid biology
- How to add DNA to a plasmid
- Transformation of *E. coli*

The key role of *E. coli* and an RNA expression plasmid in mRNA vaccine production (Pfizer/BioNTech, specifically)

https://www.nytimes.com/interactive/2021/health/pfizer-coronavirus-vaccine.html?action=click&module=Spotlight&pgtype=Homepage

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Readings:

1) *22 MC4 Plasmids*. History of plasmid development, plasmid replicons, care of E. coli and plasmids, specialized plasmids, transformation, alpha complementation.

2) *25 MC4 Antibiotics*. Summary of several antibiotics used for plasmid maintenance, and how they work

3) 24 MC4 Cloning in Plasmids. Practical aspects of cloning and transformation, plasmid dephosphorylation. Discussion of recombinational and ligation independent cloning.

4) Gibson assembly 2009. Report demonstrating easy DNA assembly using overlapping sequences.

5) IVA (in vitro assembly) 2016. Cloning without ligation.

6) Link: www.addgene.org, a nonprofit plasmid repository

Cloning: isolation and propagation of a specific piece of DNA

Cloning vector: a movable DNA element that is modified to contain another piece of DNA

General features:

- Easy to isolate and purify
- Can be manipulated in the test tube
- Can be moved into a new cell
- Selectable: easy to propagate and test for presence or absence

Types of cloning vectors:

- Plasmid
- Virus
- Artificial chromosome
- transposon

Bacterial plasmid: Non-chromosomal DNA molecule

- Small, circular, supercoiled
- Replicates independently of the bacterial chromosome
- Copy number in cell is maintained through an origin of replication (replicon)
- Selectable through a gene that is beneficial for the host bacterium, eg. antibiotic resistance

A useful plasmid

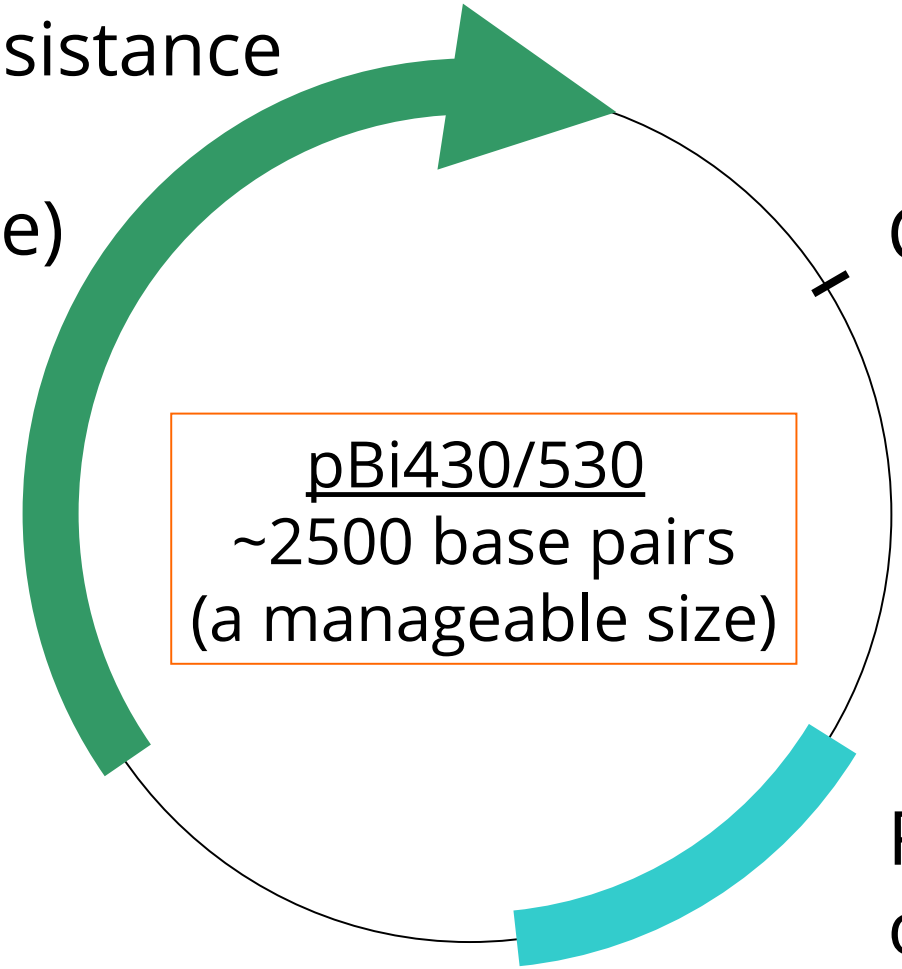
1. Is relatively small
   -- Gives higher copy #, stability, and transforming efficiency
   -- Can accept larger pieces of DNA
   -- Easier to handle (less susceptible to breakage)

2. Has a selectable marker (e.g. antibiotic resistance)

3. Has single sites for many restriction enzymes

Plasmid: basic parts list

gene that encodes antibiotic resistance (for plasmid maintenance)



Cloning site

Replicon/origin of replication (for plasmid maintenance)

Replicon: how the plasmid replicates

- A DNA sequence and associated factors
  - origin of replication, ori: a site on the DNA
  - protein and RNA factors that manage replication

- The replicon helps define the “ copy numbe r”, the number of plasmid copies per cell

A few common plasmid replicons

PLASMID	REPLICON	COPY #
pBR322	pMB1	15-20
pUC19	Modified form of pMB1 (RNA II mutation)	500-700
pACYC	p15A	18-22
pSC101	pSC101	~5

Plasmid copy number is an important consideration

- High copy number plasmids
  - More copies/cell = easy to make a lot of DNA in a plasmid prep
  - Used for routine manipulation of small (<15 kb) recombinant DNAs

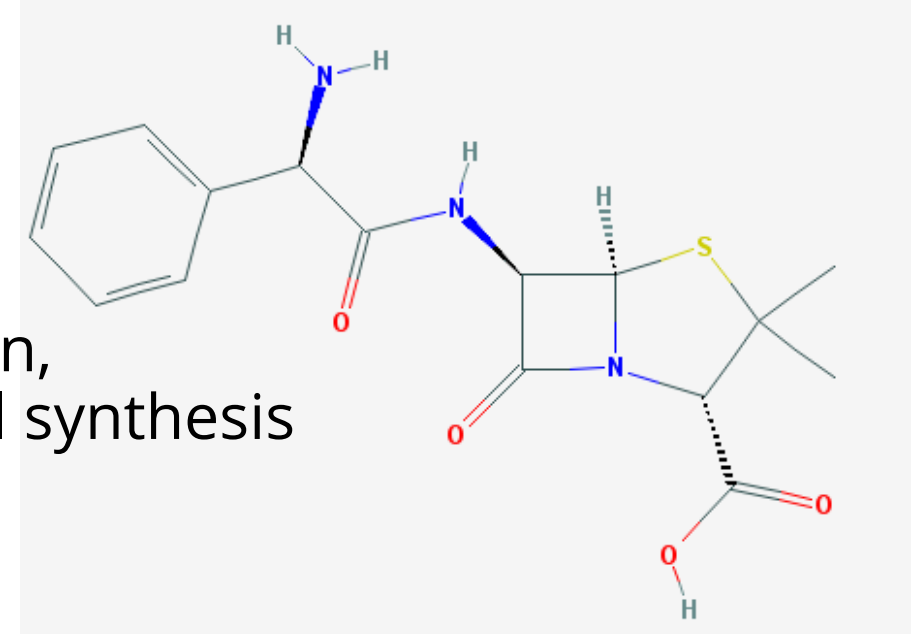
- Low copy number plasmids
  - For genes that are lethal or unstable in high copy number plasmids
  - Bacterial Artificial Chromosomes (BACs) that can propagate large (>100 kb) recombinant DNAs. Lots of DNA is a drag on cell physiology

How to make sure a plasmid is maintained

- Plasmids contain selectable markers: genes carried by the plasmid that confer functions required for host survival

- Selection: only those cells with the plasmid will survive
  - Allows transformation (a rare event) to be easily detected
  - Cells cannot lose the plasmid, even if it causes a selective disadvantage (eg. slow growth or toxic gene product)

Antibiotic resistance genes



- Antibiotic
  - ampicillin (related to penicillin, carbenicillin) inhibits cell wall synthesis

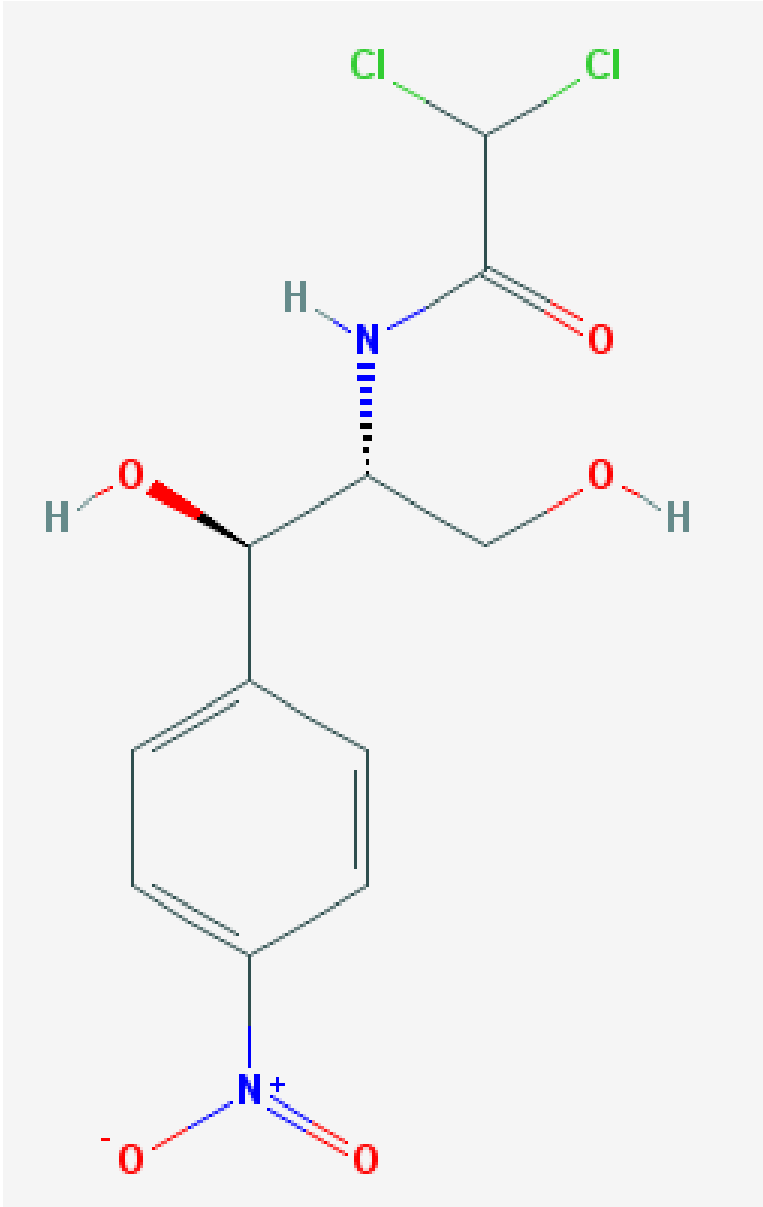
- Resistance gene
  - beta lactamase (bla) breaks down ampicillin, so cells carrying this gene are called amp^r

Keep in mind

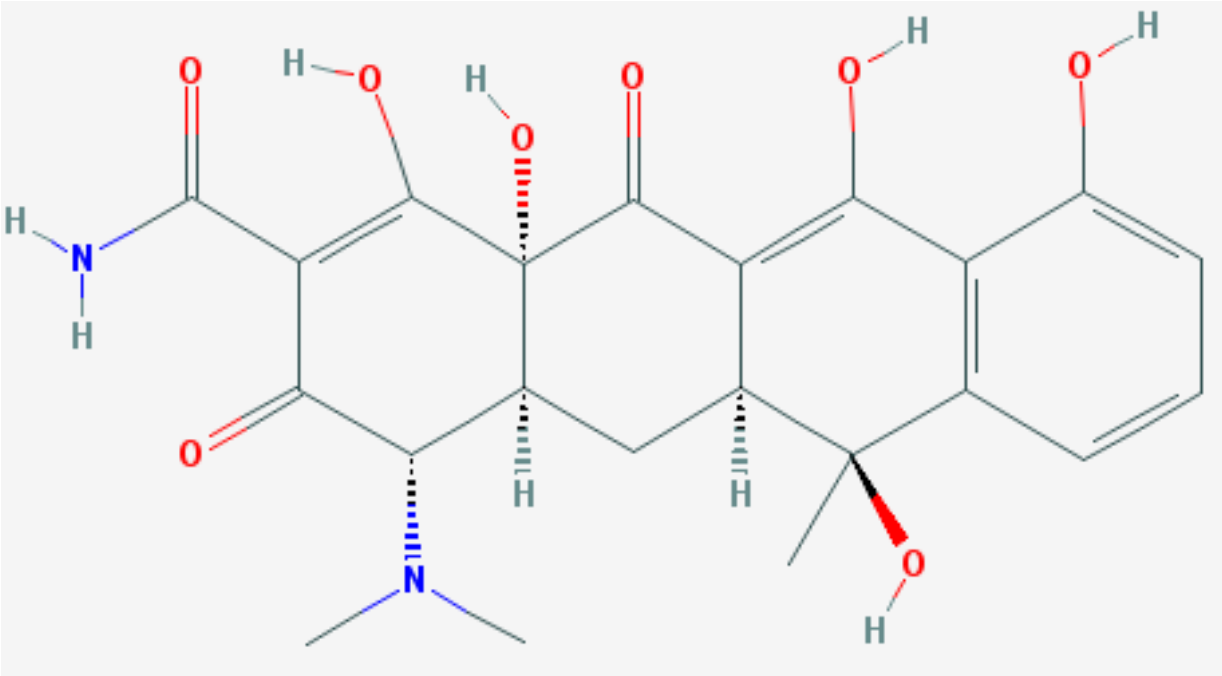
- beta-lactamase is secreted into the medium where it breaks down the antibiotic and depletes it
- ampicillin sensitive cells can grow, defeating the selection

Antibiotic resistance genes

- Antibiotic
  - chloramphenicol (cm) inhibits translation (peptidyl transferase activity of the ribosome)
- Resistance gene
  - chloramphenicol acetyl transferase (CAT) inactivates chloramphenicol



Antibiotic resistance genes

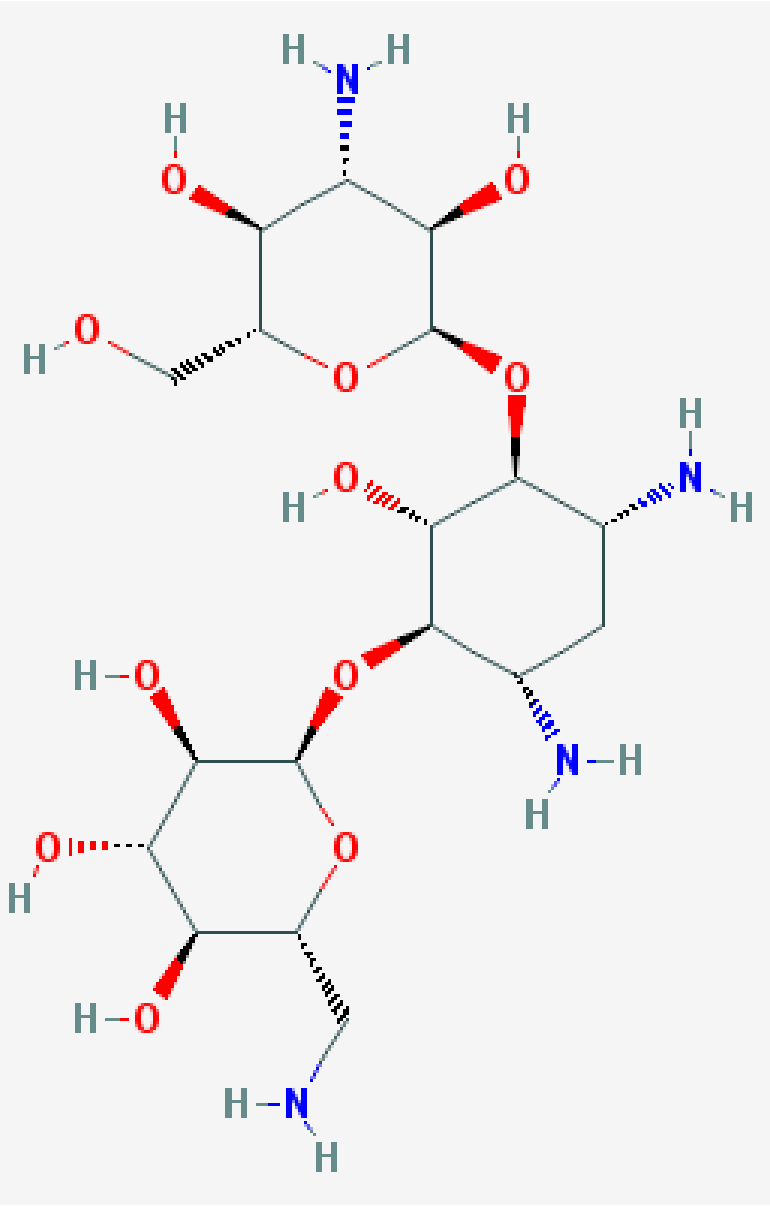


- Antibiotic
  - tetracycline inhibits translation (interacts with ribosome 30S subunit)

- Resistance gene
  - Tet A (C) protein confers resistance to by moving this antibiotic out of the cell

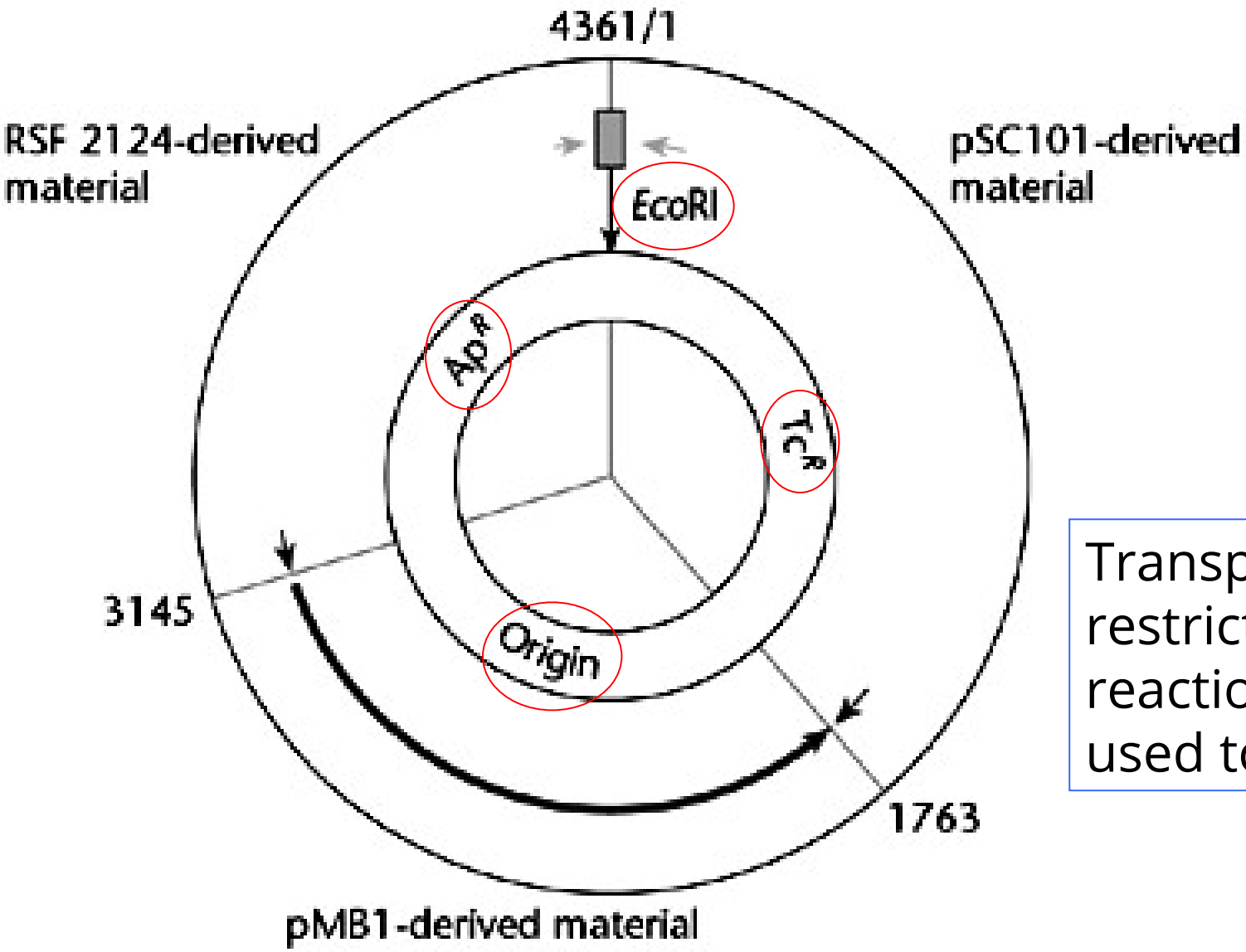
Antibiotic resistance genes

- Antibiotic
  - kanamycin: aminoglycoside antibiotic, inhibits translation (interacts with ribosome 30S subunit)
  - others of this class include G418, neomycin

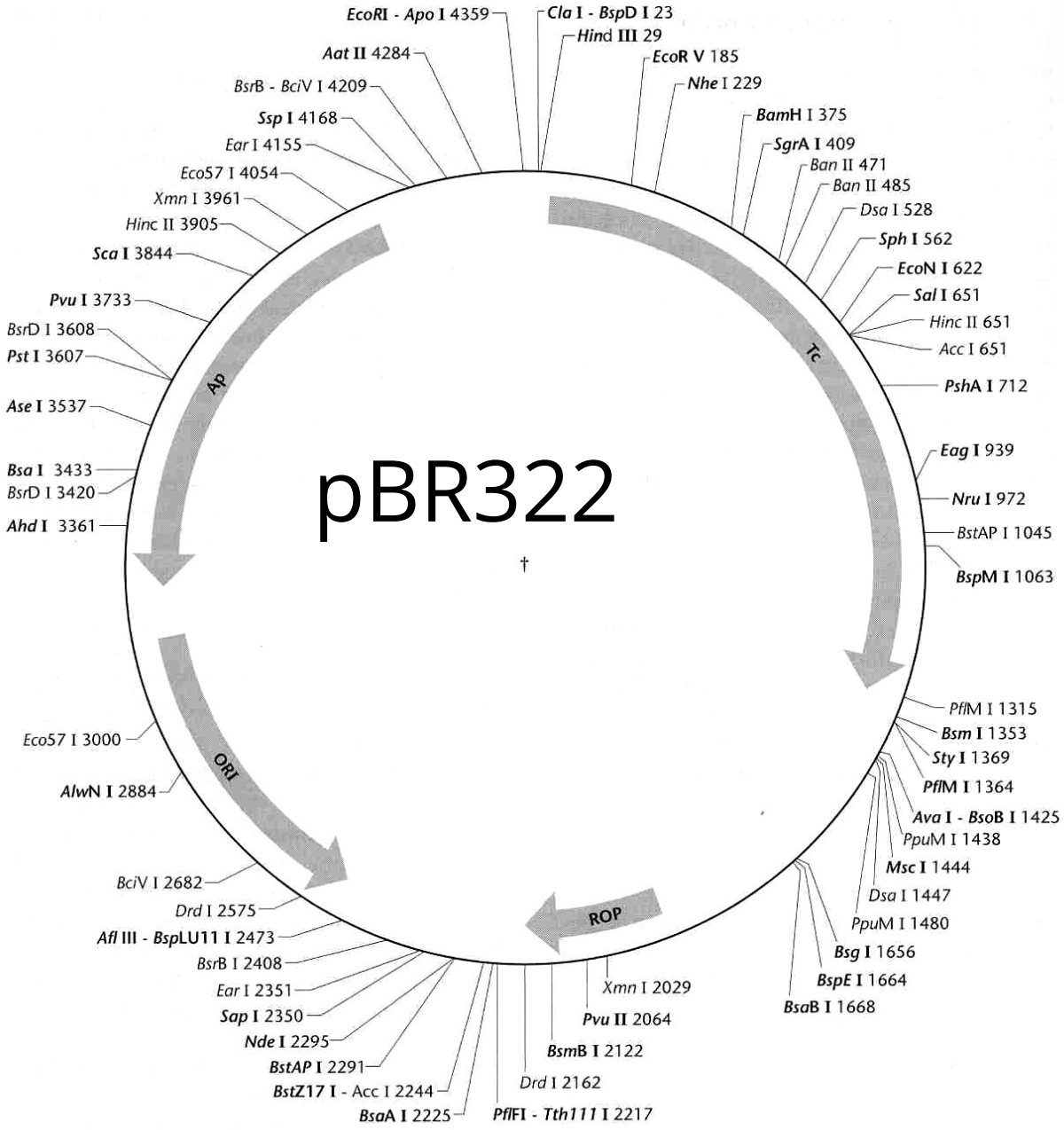


- Resistance gene
  - bacterial aminophosphotransferase inactivates kanamycin by phosphorylation

pBR322: the first widely used plasmid



Transposition and restriction/ligation reactions were used to make it



Using pBR322

Clone into sites in the Tc^r gene,

Plate on ampicillin, then 'replica plate' on tetracycline.

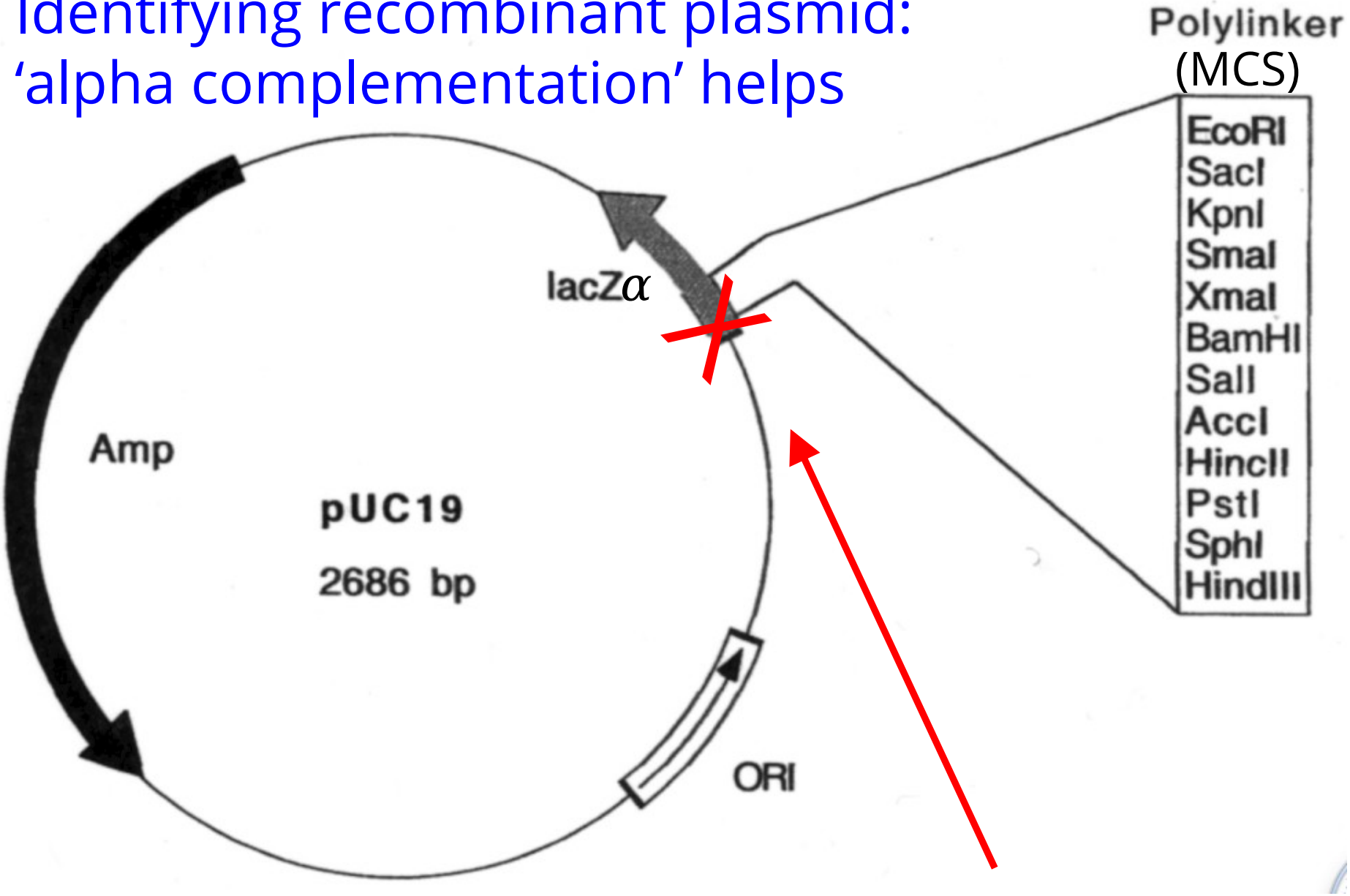
Recombinants are amp resistant, tet sensitive

But: pBR322 has **low copy number**, **large size**

pUC plasmids: improved cloning vectors

- Reduced size (about 2000 bp), easier for cells to handle
- Multiple cloning site (MCS, also called “ poly-linke r”): unique sites for lots of different restriction enzymes
- Very high copy number (mutation in RNA II)
- A “ blue-white” screening tool for recombinants (“ alpha complementation” is disrupted by foreign DNA in the MCS)

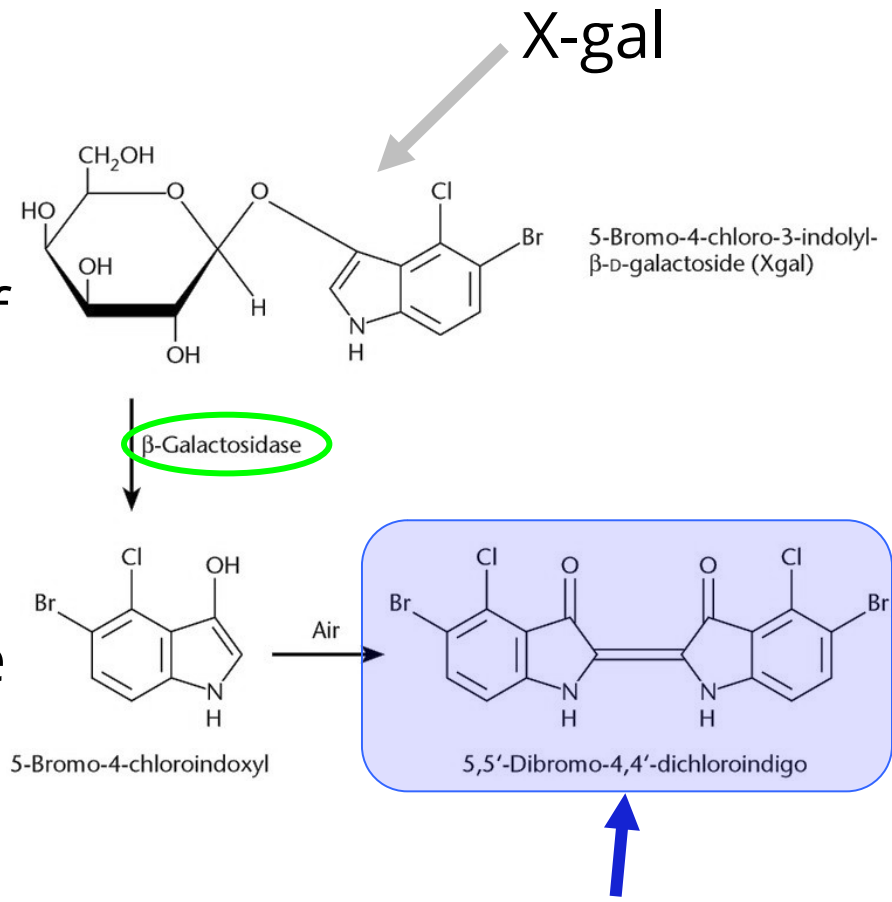
Identifying recombinant plasmid: ‘alpha complementation’ helps



DNA cloned in the MCS interrupts the lacZ gene alpha fragment

Alpha complementation

- Plasmid has the N-terminus of beta galactosidase (alpha fragment)
- Host *E. coli* has the C-terminus of beta galactosidase (omega fragment)
- Both the N- and C-terminal fragments are needed for enzyme activity

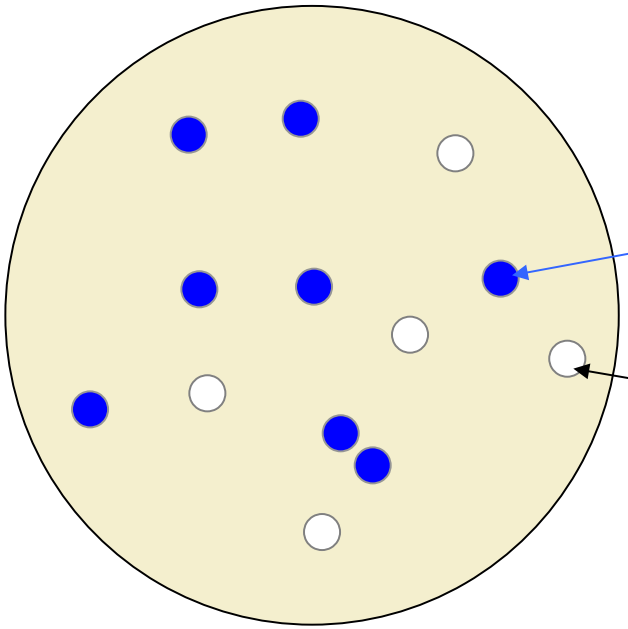


- if beta galactosidase is present, X-gal is cleaved, producing a bright blue product

Bright blue

Alpha complementation on a petri plate

- Plasmid has alpha fragment of beta galactosidase with an MCS, bacterial host has omega fragment
- Clone DNA into the MCS, no alpha fragment
- No alpha fragment, no functional B-galactosidase
- No B-gal, no blue color
- white colonies



Colony with no DNA in MCS. Don't want.

Colony with cloned DNA in MCS. Want.

pUC19 transformation plate

Many specialized plasmids are available

- For expression of specific genes
  - Plasmids are engineered to include“ tags” on expressed protein, to assist in purification or tracking
- For cloning of unstable or toxic genes
  - Low copy number
- For cloning gigantic pieces of DNA
  - Low or single copy number

Want a new plasmid? Addgene.org, a plasmid repository

- https://www.addgene.org/

Keeping track of your plasmid sequence and structure

- A plasmid editor (ApE)
  - Save plasmid sequence
  - Include annotations to the plasmid
  - http://biologylabs.utah.edu/jorgensen/wayned/ape/

How to create a recombinant plasmid (general protocol)

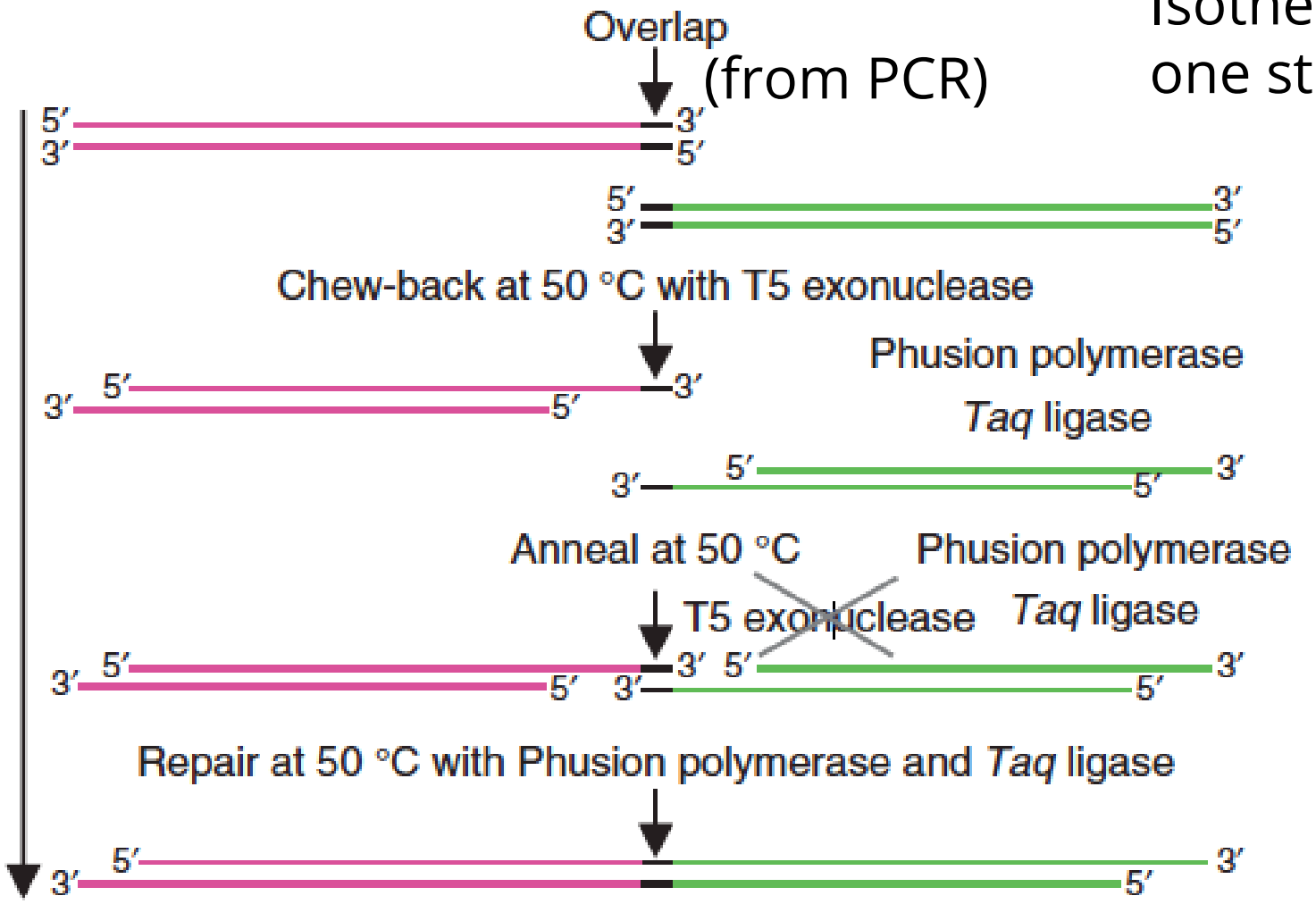
1) Prepare the plasmid vector
   a) Make the plasmid linear (PCR, or restriction enzyme)
   b) Purify, quantify the DNA

2) Prepare the DNA to be cloned
   a) Obtain the DNA
      - PCR
      - Small pieces of genomic DNA
   b) Treat the ends of the DNA to make them clonable
      - Primers with modified 5’ ends
      - Add sequence to 3’ ends with terminal transferase
      - Ligate adaptor sequences to ends

3) Combine the plasmid and clone DNA
   a) Standard cloning: ligase
   b) Gibson Assembly: Exonuclease, DNA pol, ligase
   c) In vivo assembly (IVA): the cell assembles pieces with overlapping ends

Gibson DNA assembly: make synthetic genes, pathways, or *entire genomes*.

Isothermal:
one step at 50°C



1) 5′→3′ exo (T5 exo is heat-labile)

2) Annealing

3) Repair (Phusion DNA pol and Taq ligase are heat stable)

Gibson et al. (2009) Nature Methods **6**, p. 343

Gibson assembly example

Cloning vector: plasmid pUC19

Cloned DNA: Pf1831 (*Pyrococcus furiosus* histone protein)

```
ATGGGAGAATTGCCAATTGCCCCAGTTGACAGGCTTATAAGAAAGGCAGG
TGCTGAAAGAGTTAGCGAGCAAGCAGCCAAAGTCCTAGCAGAGTACCTCG
AAGAGTACGCTATTGAGGTCGCAAAGAAGGCAGTAGAGTTCGCAAGGCAC
GCAGGTAGAAAGACTGTTAAGGTTGAAGACATTAAGCTCGCAATTAAGAG
CTGA
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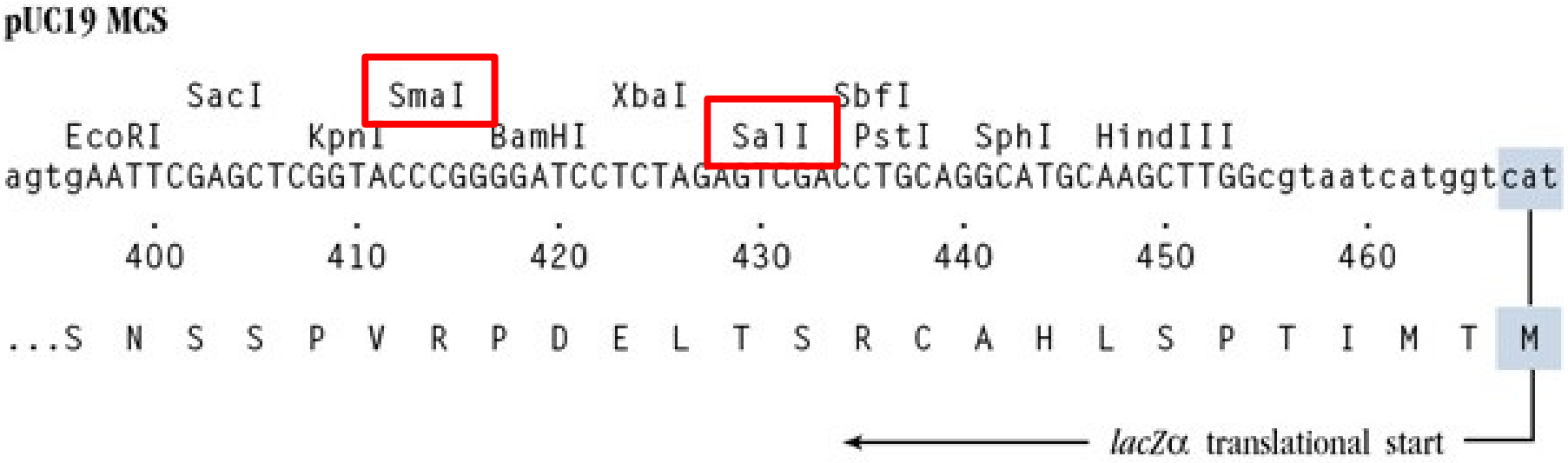


Cloning strategy: http://nebuilder.neb.com/

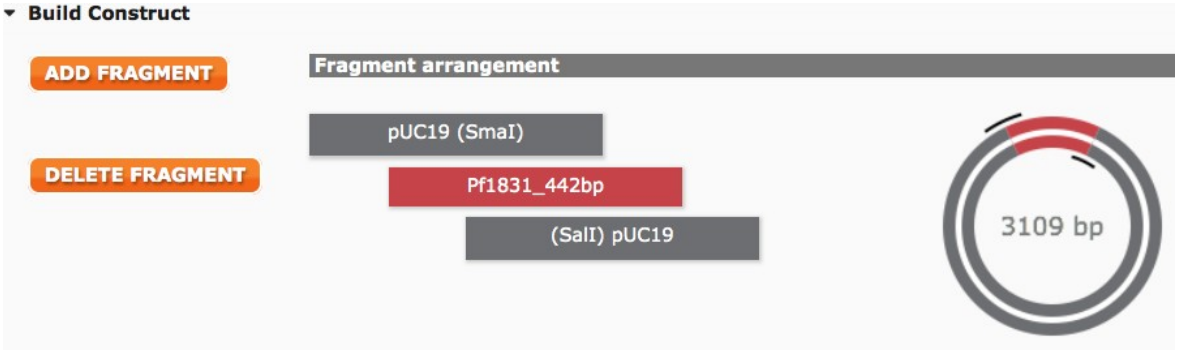
Parameters for cloning by Gibson assembly:

The final product has to be circular

Need at least a 15 bp overlap at the cloning junctions (this is accomplished by extra sequences at the 5’ end of the PCR primers)

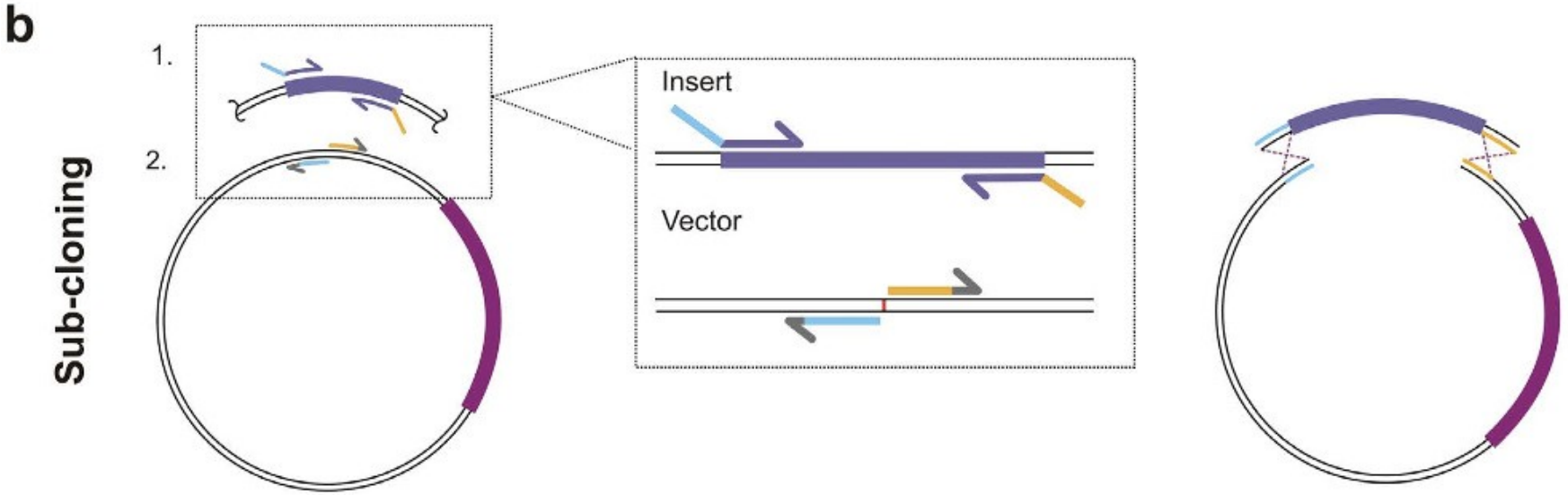
Picking primers: can be done manually, or with an automated online tool

Overlaps	Oligo (Uppercase = gene-specific primer)	Anneals	F/R	3' Tm	3' Ta *
pUC19	tcgagctcggtacccGGCAATCTATTTGGAAATC	Pf1831	Fwd	56.0°C	**56.0°C**
pUC19	ttgcatgcctgcaggGATATACTTTAATTTCGGGAGG	Pf1831	Rev	57.2°C	**56.0°C**



“IVA” cloning: In Vivo Assembly

- *E. coli* has a recombination machinery that only requires short regions of homology (15-20 base pairs)
- Transform cells with DNA with overlaps, the recombination machinery will put them together automatically, and with reasonably high efficiency



You do

Cell does

Moving DNA:
transformation of *E.coli* with plasmid DNA

Cells acquire DNA by

- Chemical competence
- Electroporation
- Natural competence (in some lucky cases)

E.coli strain: must be antibiotic sensitive, and should not have restriction enzyme systems

Following transformation, the cells are cultured on a petri plate

Making chemically competent *E. coli*

- Grow cells to “ mid-log” phase (A_{600} of 0.4) spin to get cell pellet
- Suspend cells in $CaCl_2$ (100 mM), pellet again
- Suspend in small volume of $CaCl_2$/glycerol
- Freeze cells (-80°C) or go straight to transformation protocol

Transformation of chemically competent cells

DNA binds to cells →

- Mix DNA and competent cells, on ice for 30 min.

DNA uptake by cells →

- Heat shock (42°C) for 1.5 minutes
- Add growth media, 37°C for 1 hour

Cells recover →

- Plate on growth medium plus selection (antibiotic) for the plasmid

Selection occurs →

Efficiency: ~10^5 – 10^8 antibiotic resistant colonies/microgram plasmid DNA

Transformation by electroporation

- Grow cells to A_{600} of 0.4
- Centrifuge and resuspend in water + 10% glycerol (do this 4 times to reduce conductivity)
- Place cells with DNA in electrode-containing cuvette, deliver electrical pulse
- If there is arcing (sparks) transformation efficiency will be poor (uneven transfer of charge). To avoid this make sure the ion concentration is very low (less than 10 mM salt)
- > 10^9 transformants/microgram DNA (ideally)

Setting up a transformation – treatments and controls

1. *No plasmid* (negative control, nothing should grow on this plate)
2. *Supercoiled plasmid* of a known concentration (to determine efficiency of competent cells)
3. *Plasmid DNA* backbone: without clone DNA (background transformants)
4. *Plasmid DNA with clone DNA* (desired products)

Ideal results from a transformation

1) No DNA--No colonies

2) 2 nanograms (2×10^{-3} micrograms) supercoiled plasmid DNA--500 colonies (efficiency of cells: 2.5×10^{5} transformants per microgram DNA)

3) Plasmid alone--small number of colonies

4) Plasmid + insert—lots more colonies than #3

Identify recombinant plasmids

- Screen colonies/plasmids for cloned DNA by PCR
- Alpha complementation: white colonies represent presence of insert DNA blocking functional beta galactosidase

Confirm clones by sequencing

When cloning a piece of DNA consider:

1) Choice of plasmid: cloning sites? antibiotic? replicon?

2) Adding DNA to plasmid: how will the reaction be set up to give the desired product?

3) Moving DNA by transformation: what strain of *E. coli* will you transform into? Which method for transformation?

4) Screening for successful ligation products (recombinant plasmid DNA): how will the recombinant plasmids be identified?

Basic gene cloning: plasmids and transformation, cutting and pasting

- Plasmid biology
- Adding DNA to a plasmid
- Transformation of *E. coli*

Vectors for *E.coli* part II

I. Bacteriophage (bacterial viruses): lambda and M13

II. Moving and storing large DNA molecules: PACs, and BACs

Readings:

1) *28 MC4 Lambda and M13*. Short introduction to these historically important molecular biology tools, also two M13 protocols.

2)33 MC4 High capacity vectors. Summary of vector types, with an emphasis on bacterial artificial chromosomes (BACs)

3) *29 MC4 Cre/Lox*. Discussion of the cre-lox system for forcing site-specific recombination

Bacteriophages:
viral vectors for molecular cloning

I. M13: a filamentous phage
   -- Life cycle
   -- genome structure

II. Lambda: a "head and tail" phage
   -- Life cycle
   -- Basic cloning in lambda

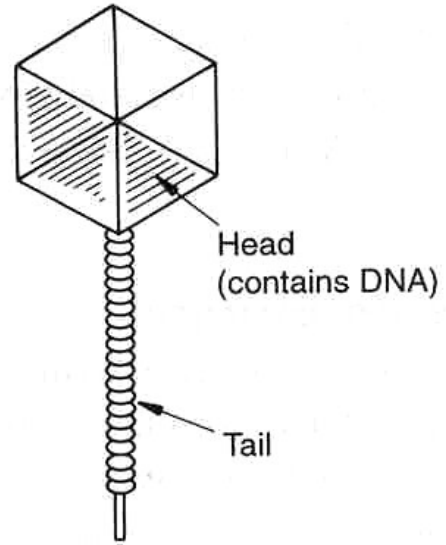
Bacteriophages

Viruses that infect bacteria

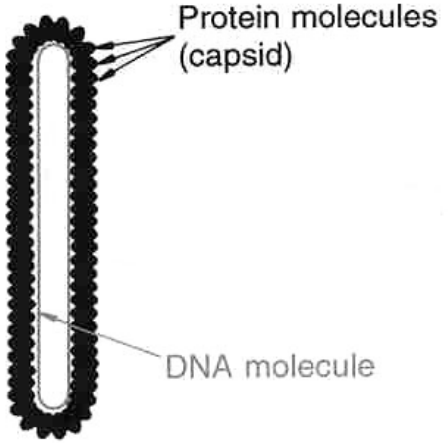
Morphologies:

a) Head and tail

b) Filamentous



(a) Head-and-tail



(b) Filamentous

- Nucleic acid molecule
  - Carrying genes for infection and replication
  - Surrounded by a protective protein coat (capsid)

- Infection (instead of transformation):
  - Phage attaches to outside of bacterium, injects DNA
  - Phage DNA is replicated, capsid proteins are made
  - Phage assembled and released

Use of bacteriophages in cloning:

M13 -- single-stranded DNA genome

- Then:
  - Sequencing
  - Site-directed mutagenesis
- Now:
  - Phage display of foreign peptides

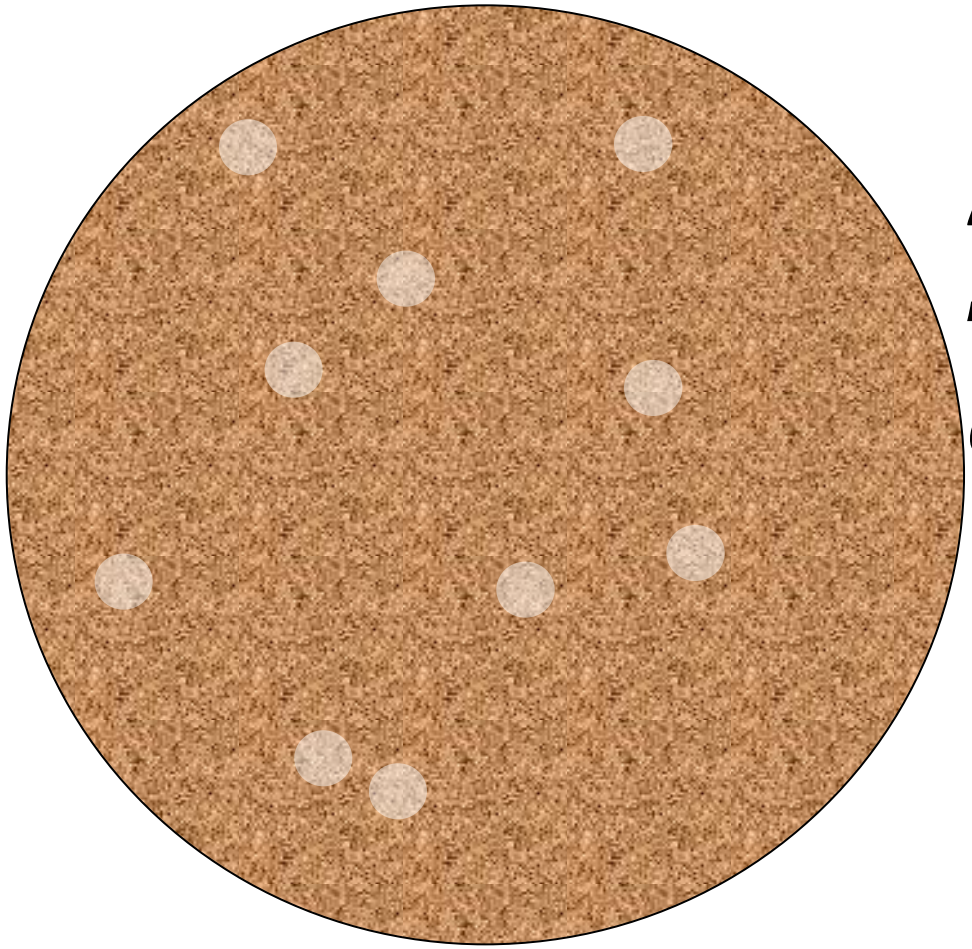
Lambda -- large-ish DNA fragments (25 kilobases)

- For gene cloning (large eukaryotic genes)
- Good selection schemes for recombinants
- Clone lots of precisely-sized DNA fragments for library construction

M13: a filamentous bacteriophage

- Single-stranded, circular genome, 6.4 kb
- Can carry foreign DNA up to 6X the M13 genome size (36 kb) -- the larger the DNA, the less stable the clone
- Drawback: foreign DNA can be unstable (slow host cell growth – deletions confer a selective advantage)

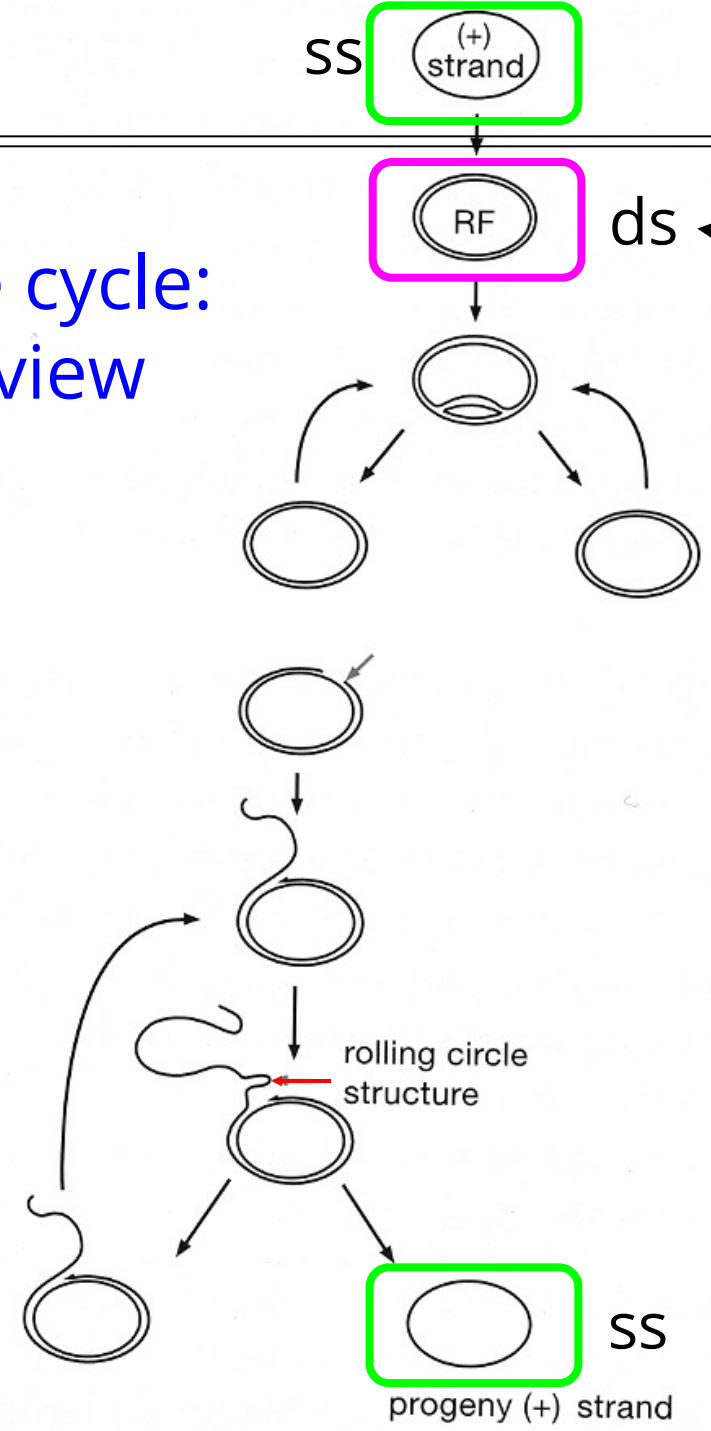
M13 doesn’ t lyse cells, but it slows them down



“ lawn ” of *E. coli*

M13 infections form ‘turbid’ plaques

M13 life cycle: an overview



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

Isolate for cloning

Several rounds of replication occur through θ structures.

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

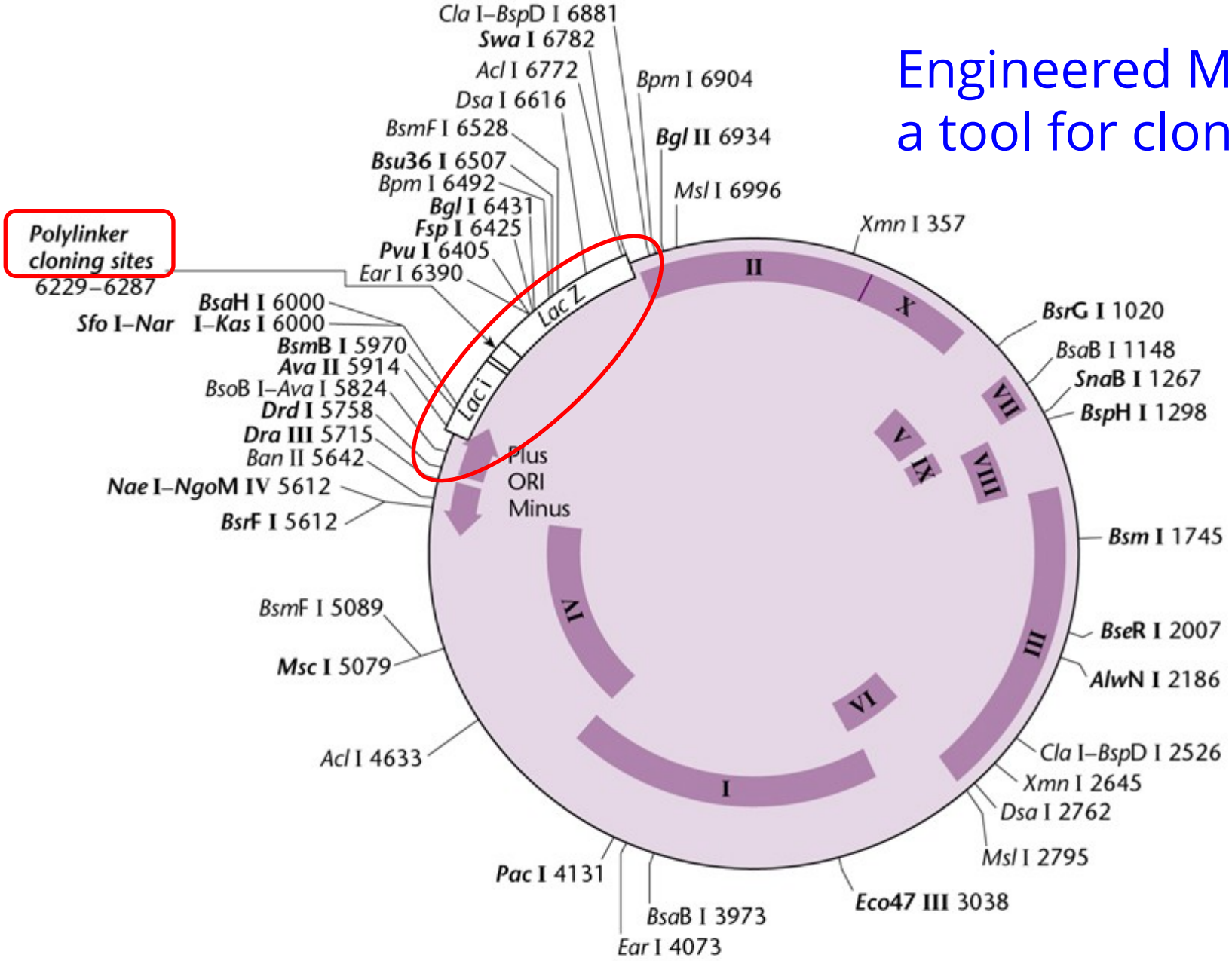
The viral gene II product introduces a nick at a specific site (red arrow) in the (+) strand of the RF DNA.

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

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

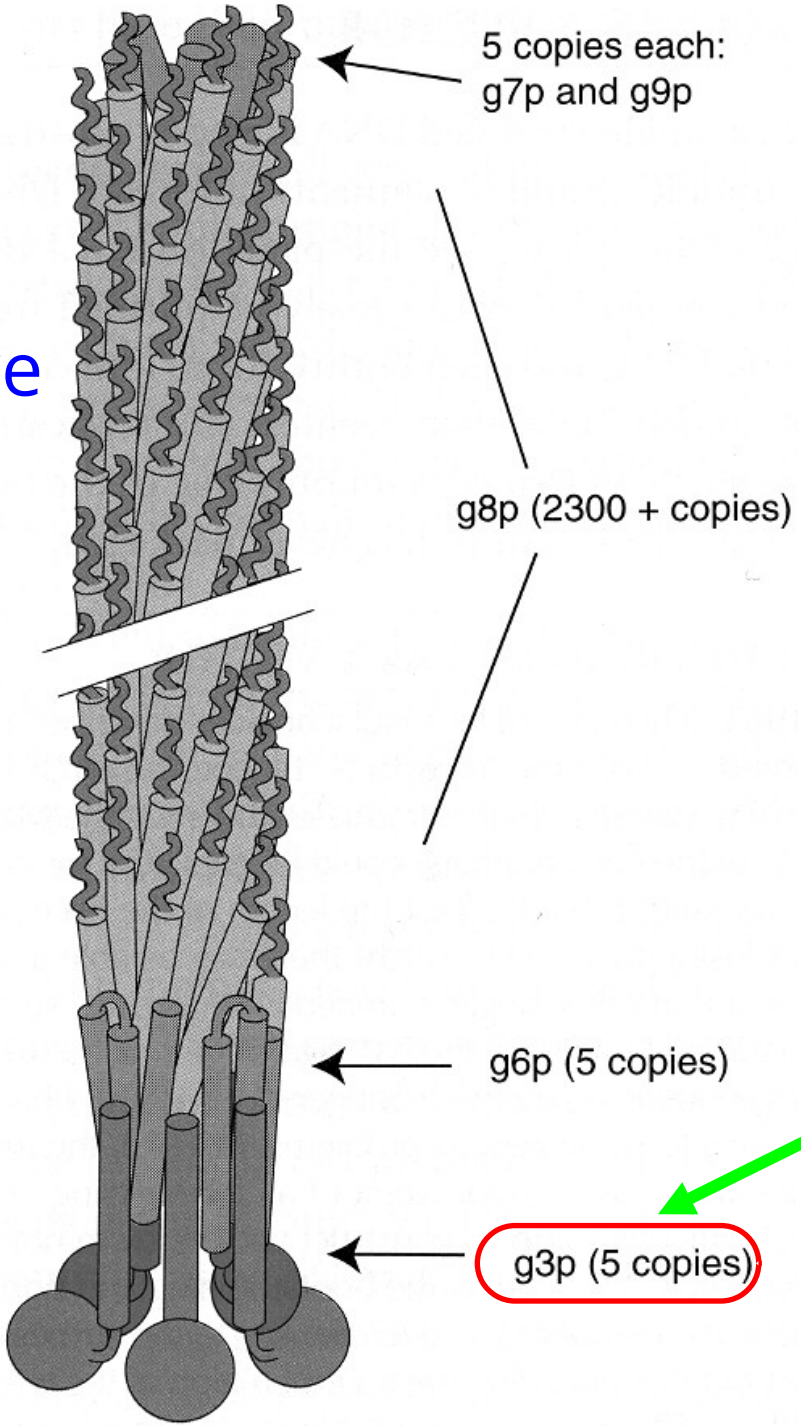
Synthesis of progeny (+) strand continues.

Engineered M13: a tool for cloning



alpha complementation (like pUC plasmids)

M13 phage particle structure



Variable length, determined by DNA size

Used in ‘ phage display’ techniques

Bacteriophage lambda

- “ head and tail” phage, well-studied model
- Large, linear genome--48.5 kb
  - Central region of genome (“ stuffer”) is dispensable for infectious growth--it can be engineered out
- Two infectious phases:
  - Lytic: replicating phase (killing/lysing cell)
  - Lysogenic: latent phase (integrated, waiting for opportunity)

- Can hold 5-25 kb DNA fragments

Lambda as a cloning vector

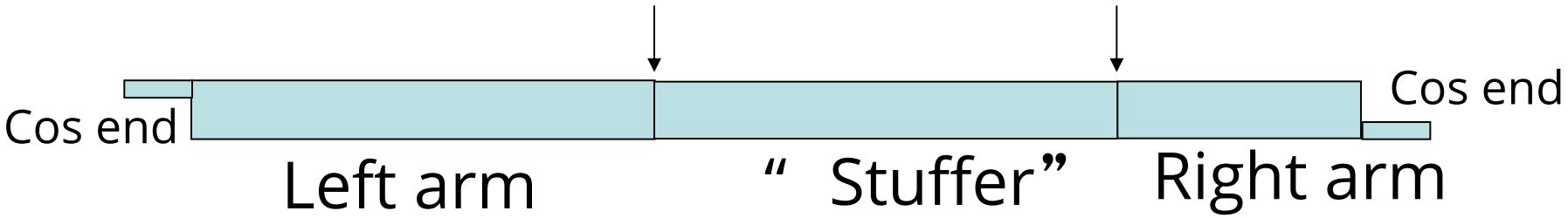
Phage capsid:

- Size of head is invariant
- Capsid must be filled
  - Too little DNA and it's not infectious
  - Too much DNA and the genome won't fit

a)Insertional vectors: can only increase genome size by 5% (about 3 to 5 kb)

b)Replacement vectors: remove " stuffer" , can clone larger pieces of DNA, 8 to 24 kb (sufficient for many eukaryotic genes)

Cloning in lambda phage – the genome view



1) Cut out stuffer, save right and left arms

2) Ligate with foreign DNA

3) “ Package” ligation mixture into phage heads

4) Plate mixture on *E. coli*, individual plaques represent recombinant clones

Selection for *recombinant* lambda

There is a minimal size of DNA that can be packaged in lambda phage heads

- If the stuffer is removed the ligated “ arms” cannot be packaged (too small)
- Presence of recombinant DNA makes the phage large enough, so the recombinants are selected

Cloning large DNA fragments

1) Make genomic *libraries*: the larger the DNA fragment, the fewer you need to make a complete *library* (representing the entire genome in fragments)

2) Clone DNA large enough to contain an entire eukaryotic gene

To get a functional gene, you need the whole gene and its regulatory regions

- Average human gene (exons and introns): 27 kilobases
- Regulatory regions may add another 10 to 100 kilobases upstream/downstream of gene

Vectors for large recombinant DNA fragments

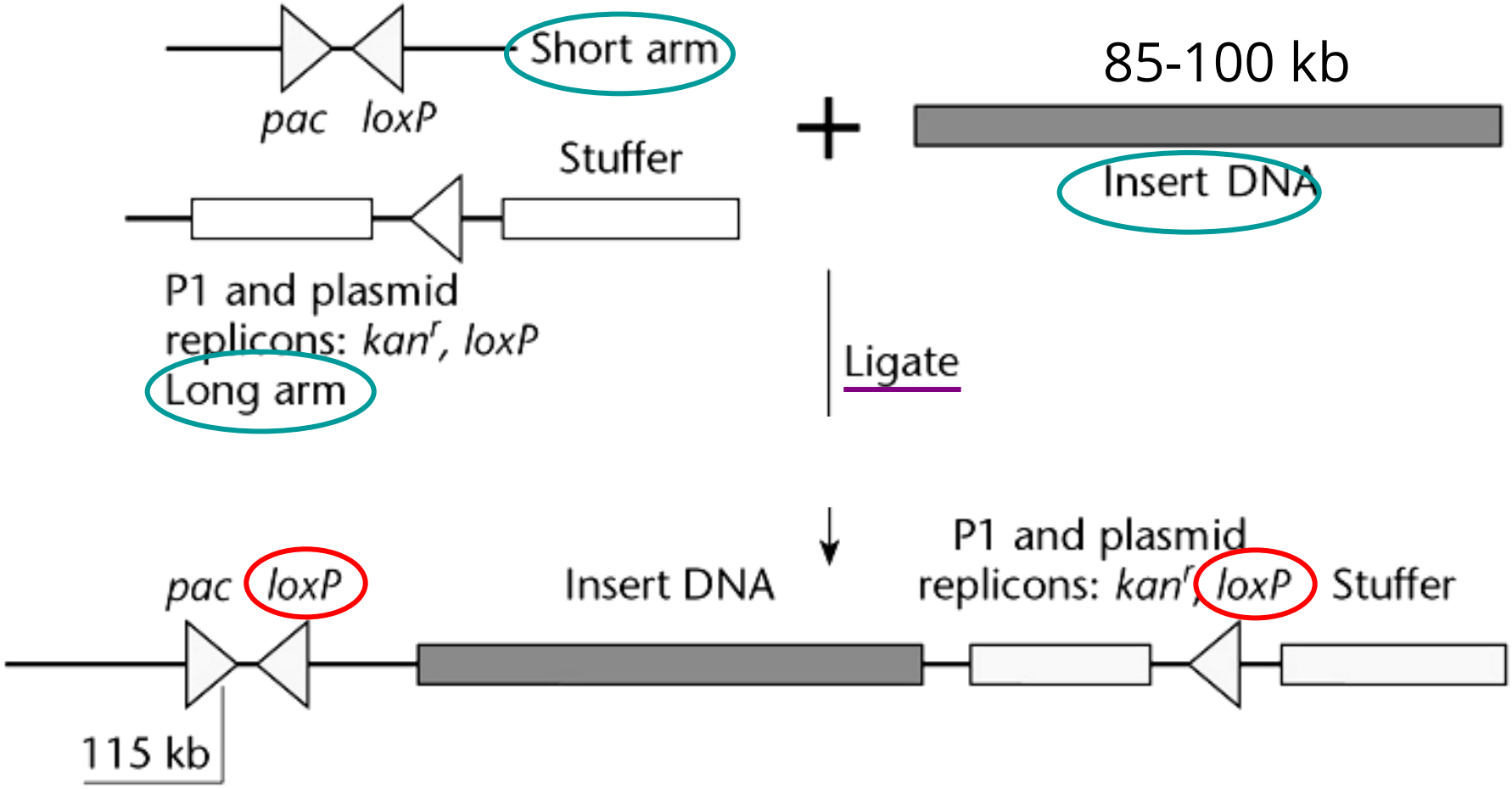
- Bacteriophage P1 plasmid: 70-100 kb
- YAC: 250-400 kb (or higher)
- PAC: 130-150 kb
- BAC: 120-300 kb (up to 700 kb is possible)

TABLE 1. High-capacity vectors for genomic cloning

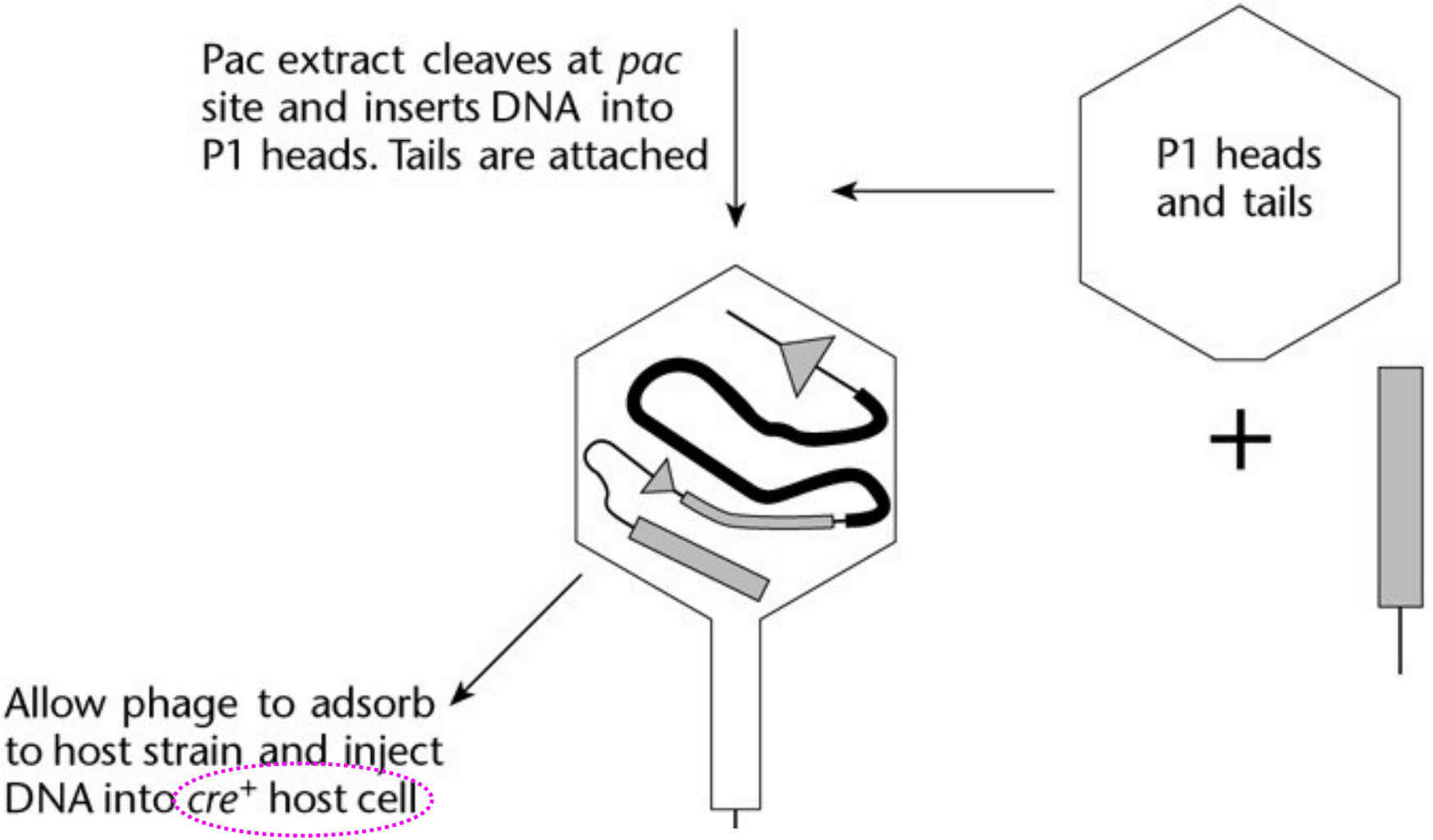
Vector	Capacity (kb)	Replicon	Host	Copy number	Recovery of cloned DNA
P1	70–100	P1	*Escherichia coli*	1 (amplifiable)	Alkaline extraction
YAC	250–400	ARS	Yeast	1	Pulse-field gels
PAC	130–150	P1	*E. coli*	1	Alkaline extraction
BAC	120–300	F	*E. coli*	1	Alkaline extraction

BACs are most commonly used

Phage P1 vectors:
clone large DNA fragments (85-100 kb)

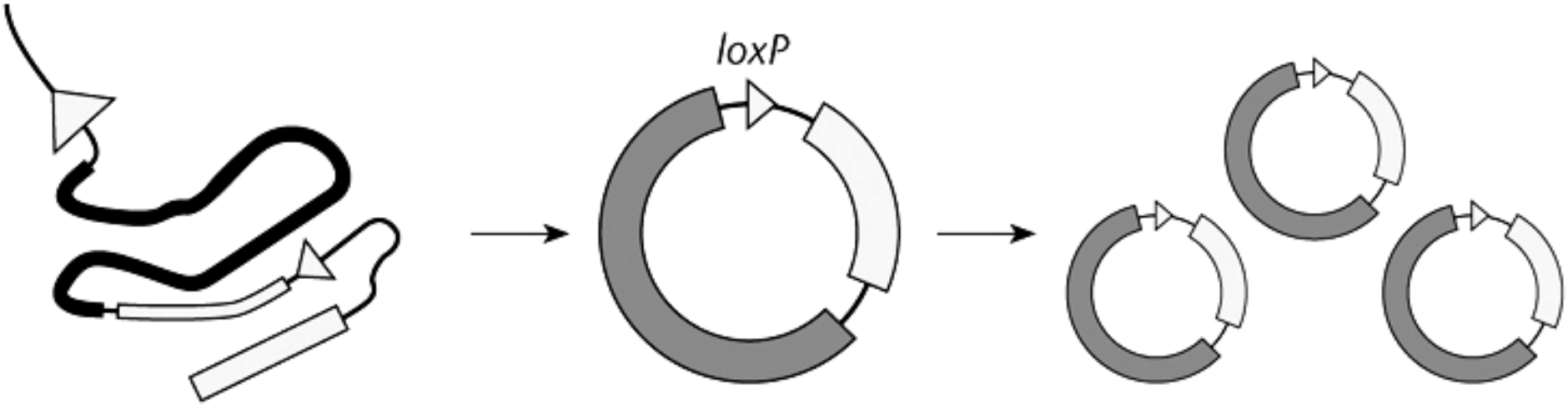


Phage P1 vectors



Efficiency of packaging is typically low: thus it is not good for making large genomic libraries

Phage P1 vectors

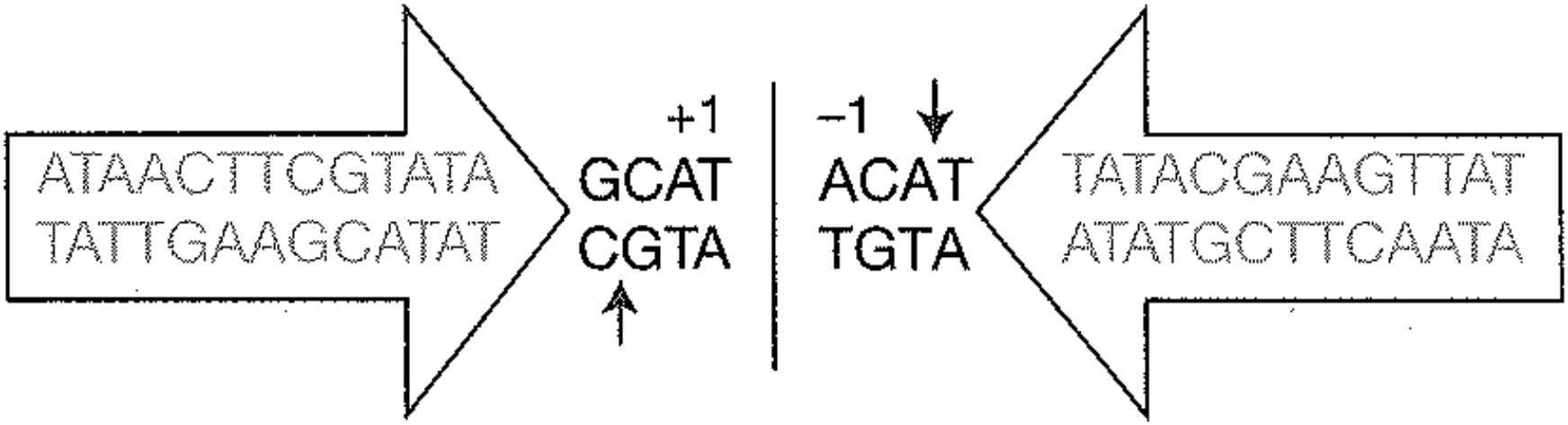


Cre recombinase protein circularizes injected DNA at the *loxP* sites. DNA replicates using plasmid replicon. Plasmid copy number is increased by induction of P1 lytic replicon.

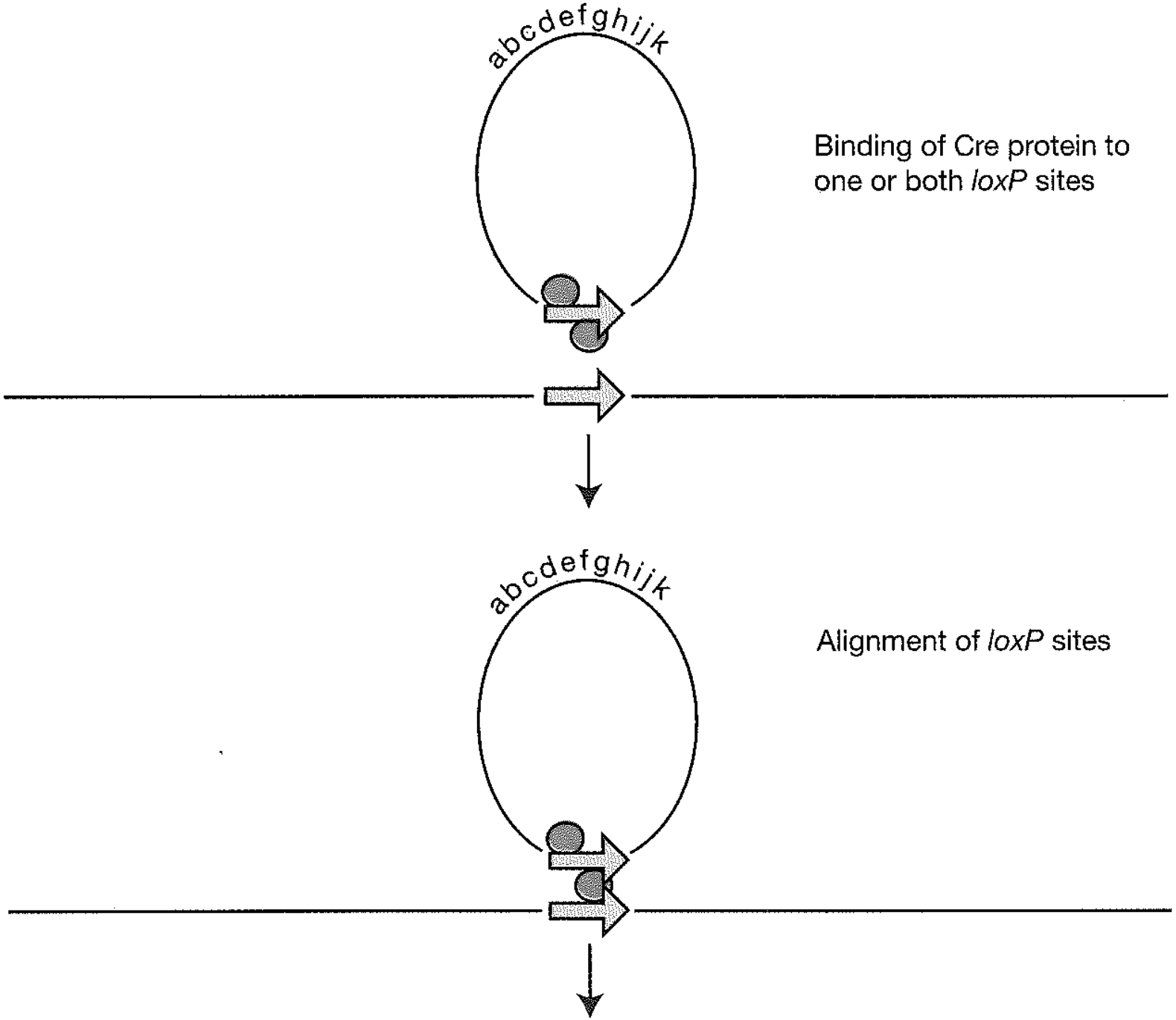
PACs: like P1 vectors but the DNA is not packaged (transfer by electroporation)

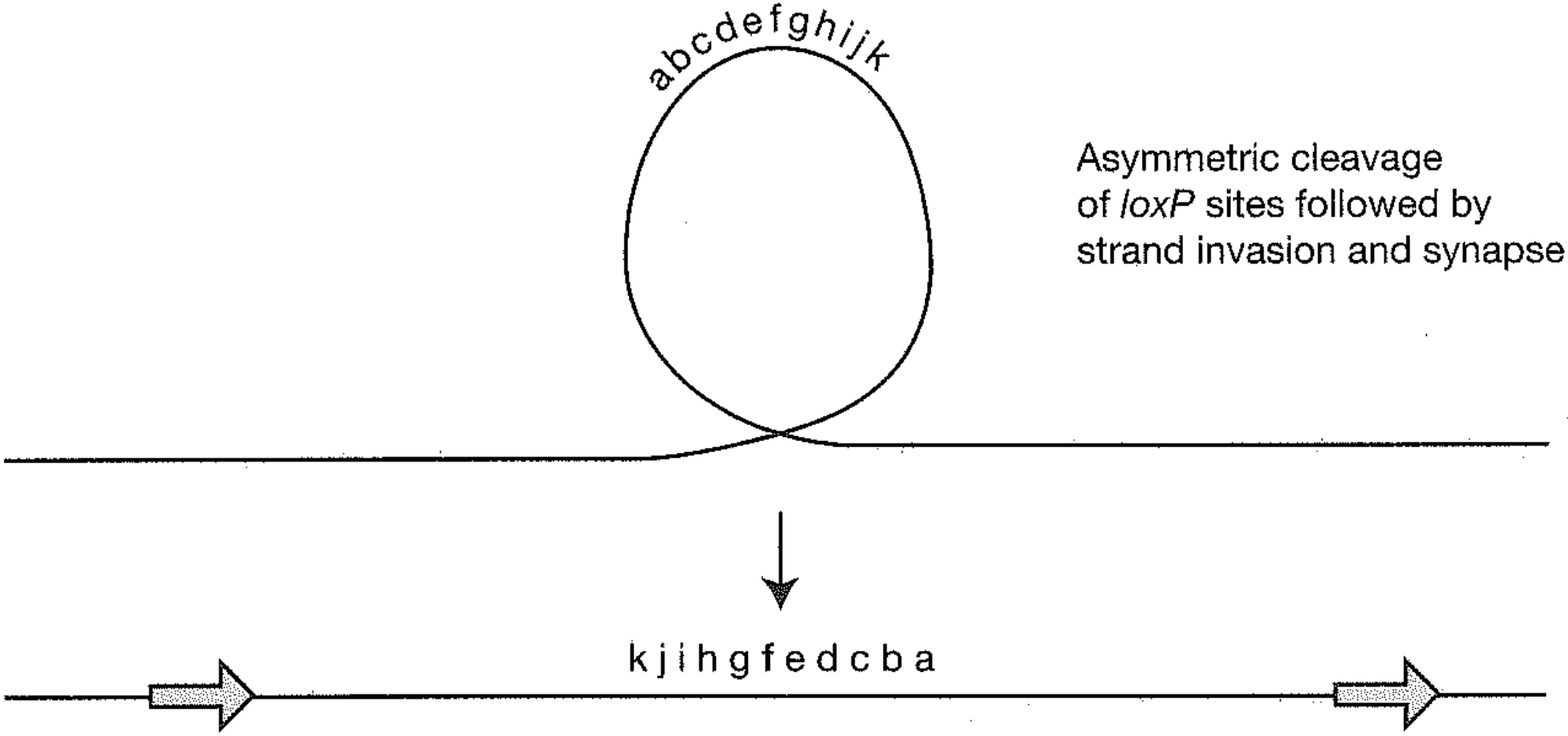
Cre and Lox:
a site-specific recombination system

The loxP site



Two 13 bp inverted repeats separated by an asymmetric linker


abcdefghijk
Binding of Cre protein to one or both *loxP* sites
abcdefghijk
Alignment of *loxP* sites


abcdefghijk
Asymmetric cleavage
of loxP sites followed by
strand invasion and synapse
kjihgfedcba

BAC: Bacterial Artificial Chromosome

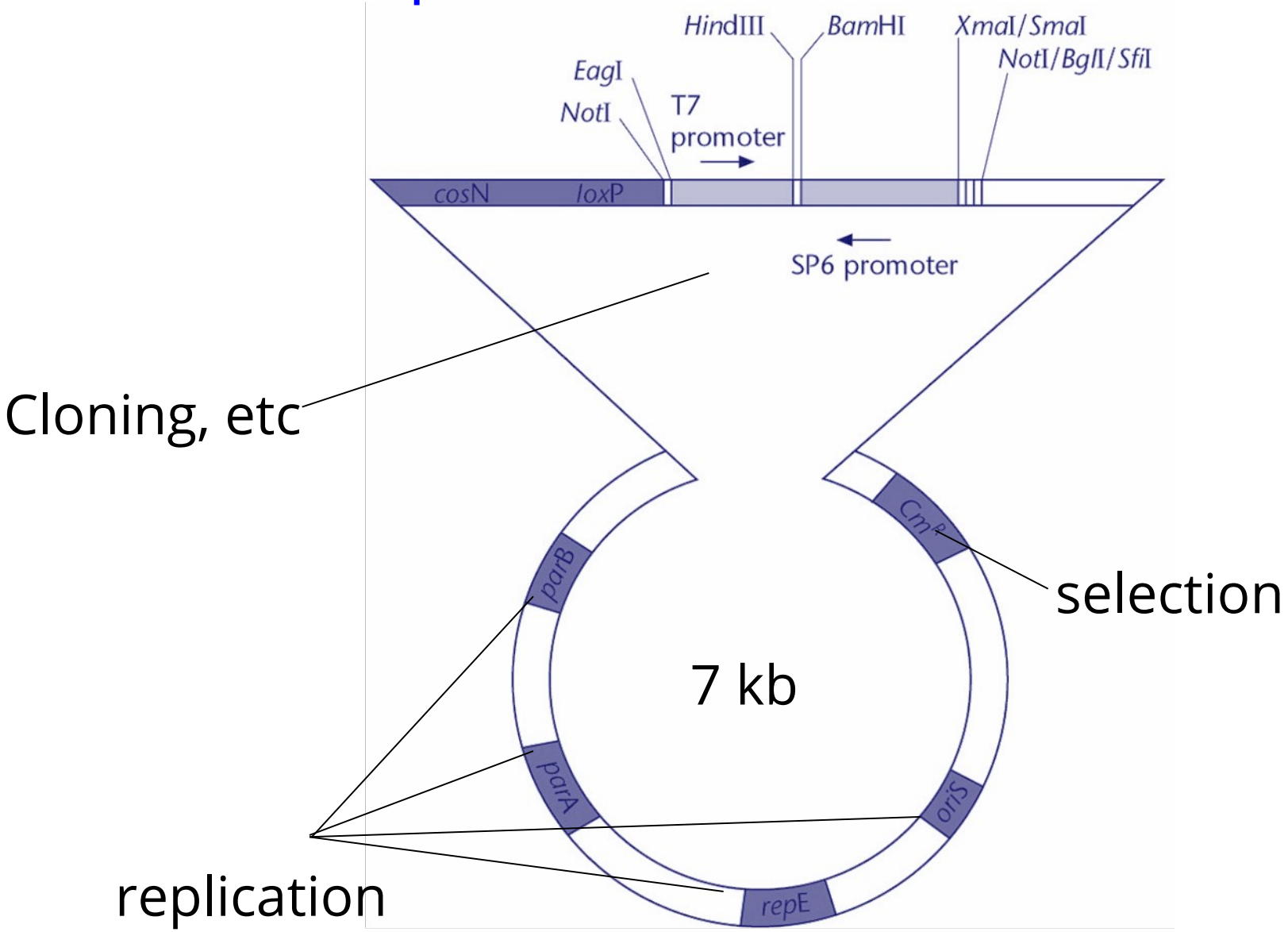
- F factor of E. coli:
  --100 kb plasmid, propagates through conjugation
  --low copy number (1-2 copies per cell)
  --2 genes (parA and parB): accurate partitioning during cell division

- BACs: engineered to have par genes, replication ori, cloning sites, selectable marker

- Holds very large pieces of DNA: up to 300 kb

- Fairly easy to manipulate: move into cells by transformation (electroporation)

BAC vector map



Vectors for *E.coli* part II

I. Bacteriophage lambda and M13

II. Moving and storing large DNA molecules: PACs and BACs