Lecture 2: How to Clone a Gene

Cloning

- Cloning utilizes specialized DNA technology to produce multiple, exact copies of a single gene or other segment of DNA to obtain enough material for further study.
- The resulting cloned (copied) collections of DNA molecules are called clone libraries.
- A second type of cloning exploits the natural process of cell division to make many copies of an entire cell.
- The genetic makeup of these cloned cells, called a cell line, is identical to the original cell.
- A third type of cloning produces complete, genetically identical animals, such as the famous Scottish sheep, Dolly, and plants such as rice.
- Often times, making subclones will be necessary, which involves breaking a cloned fragment into smaller fragments for further cloning.

Cloning a gene involves making a recombinant DNA molecule.

- A recombinant DNA molecule is one that combines DNA from two sources to create new genetic material, which is able to replicate and express under certain conditions.
- Stanley Cohen and Herbert Boyer created the first recombinant DNA organism (1973) using recombinant DNA techniques pioneered several years earlier by Paul Berg.
- An example of a recombinant is the human gene for insulin which was cloned into a carrier DNA (vector).
- The resulting circular DNA molecule was placed in bacteria to produce insulin in large quantities for treating diabetics.
- Such bacteria are recombinant organisms.
- It is possible to create genetically modified organisms because of the universal nature of the genetic code.

Basic Components of Recombinant DNA Technology

- Restriction endonucleases – enzymes that cleave DNA into pieces
- Insert – target DNA fragment or gene
- Vector – an engineered DNA molecule used to transfer and propagate various insert DNAs
- DNA ligase – enzyme that joins two separate DNA molecules together at the ends
- Host – surrogate cell harboring recombinant DNA for amplification and possible expression
- Techniques to deliver recombinant DNA into the host

Nucleases

- **Endonuclease – nucleases that cleave phosphoester bonds within a nucleic acid chain**
  - **They may be specific for RNA or for single-stranded or double-stranded DNA.**
- **Exonuclease – nucleases that cleave phosphoester bonds one at a time from the end of a polynucleotide chain**
  - **They may be specific for either the 5′ or 3′ end of DNA or RNA.**

Restriction Endonucleases

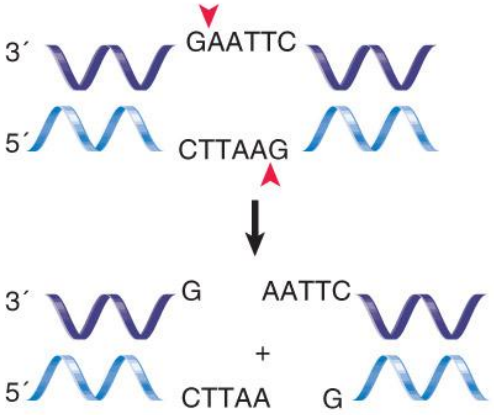
- **Werner Arber, Hamilton Smith and Daniel Nathans discovered that enzymes (endonucleases) isolated from bacteria cut foreign DNA at precise locations.**
- **There are three types of restriction endonucleases: I, II, and III.**
- **Type I cleaves 1000 bp away from the recognition site.**
- **Type II cleaves DNA at defined positions close to or within their recognition sequences and produces discrete restriction fragments and distinct gel banding patterns. Type II enzymes are used in the laboratory for routine DNA analysis and gene cloning.**
- **Type III cleaves 24-26 bp away from the site.**

Type II Restriction Endonucleases

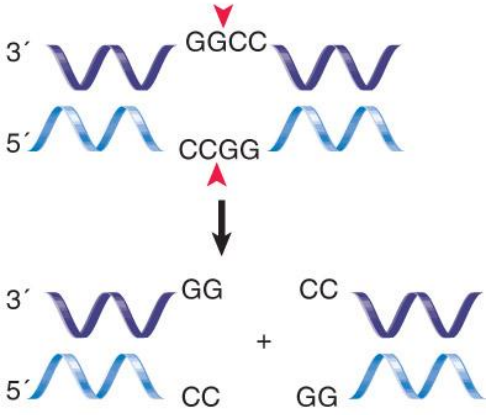
- More than 3000 type II restriction endonucleases have been discovered.
- They recognize short, usually palindromic, sequences of 4–8 bp and, in the presence of Mg^{2+}, cleave the DNA within or in close proximity to the recognition sequence.
- The precise mechanism of cleavage has not yet been established for any restriction enzyme—the main uncertainty concerns the number of Mg^{2+} ions directly involved in cleavage.
- Cleavage in the two strands usually occurs in a concerted fashion and leads to inversion of configuration at the phosphorus group.
- The products of the reaction are DNA fragments with a 3′-OH and a 5′-phosphate.

Restriction Endonucleases

Staggered cut

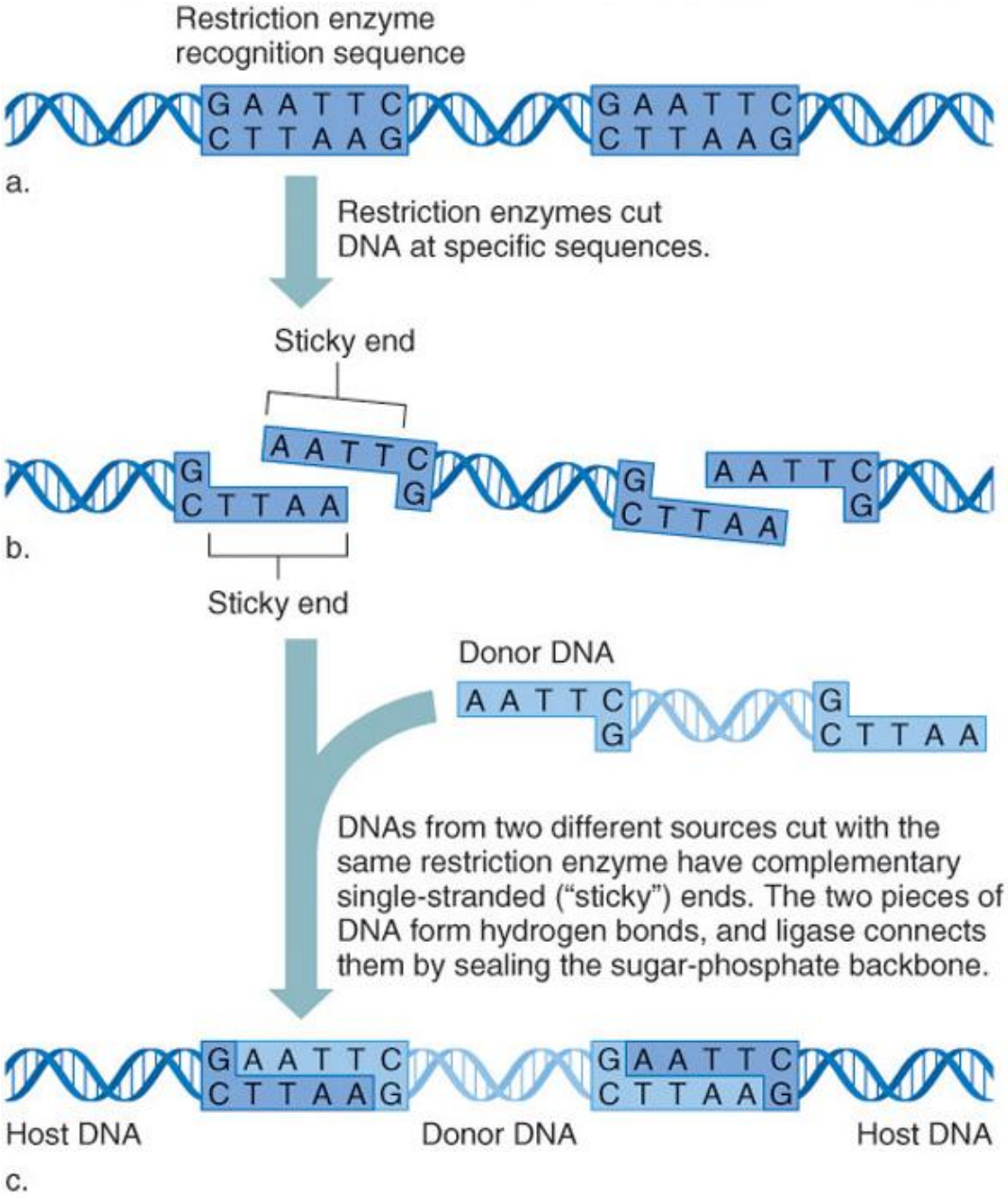


Blunt cut

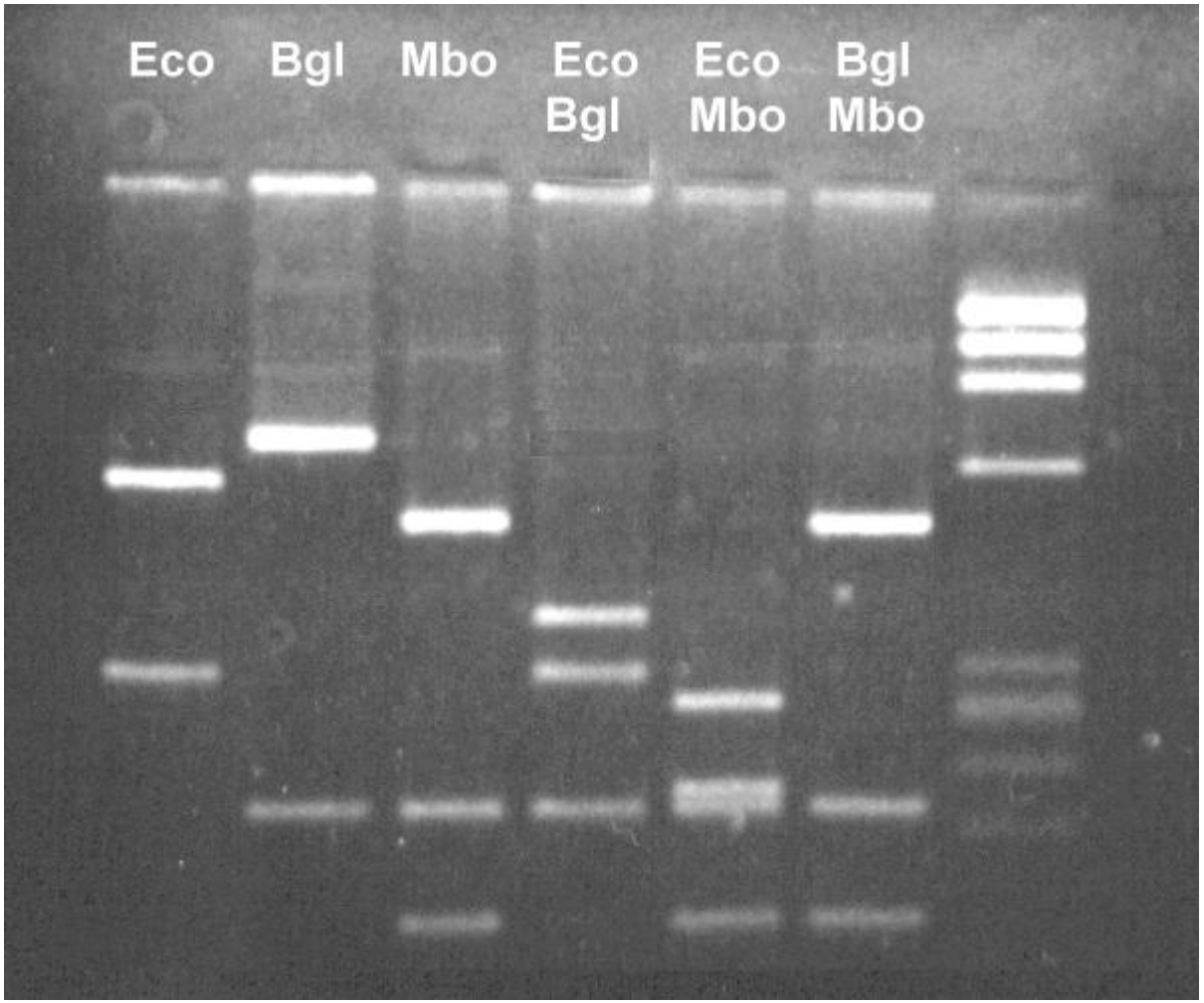


Restriction endonucleases can be used to cleave DNA into defined fragments.

Creating Recombinant DNA Molecules

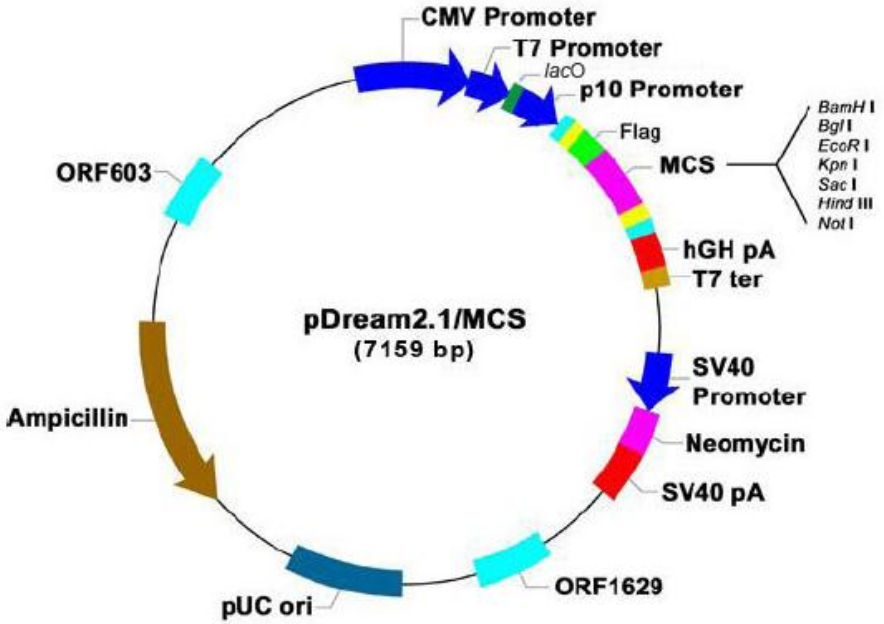


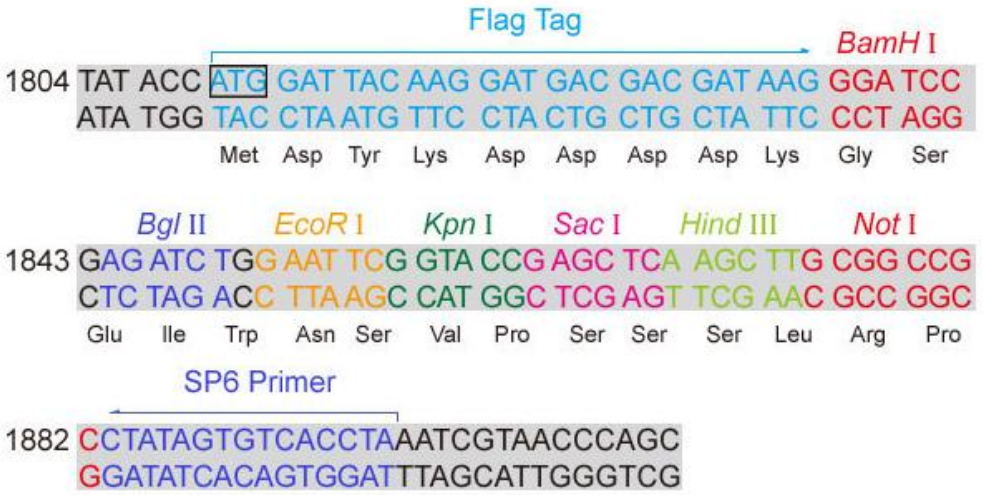
Gel Electrophoresis of DNA Cut With Different Restriction Enzymes



Restriction Map of Recombinant Plasmid Vector Created by GenScript

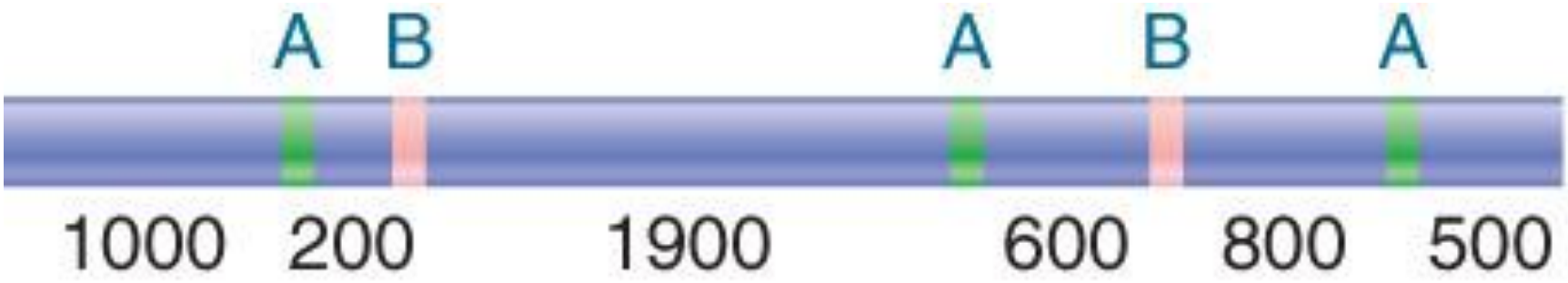
- **GenScript pDream2.1/MCS is an excellent expression vector.**
- **There are seven restriction enzyme sites in MCS.**
- **A gene cloned into MCS can be expressed in any one of the three major protein expression systems: bacteria, Insect cells and mammalian cells.**





Restriction Map

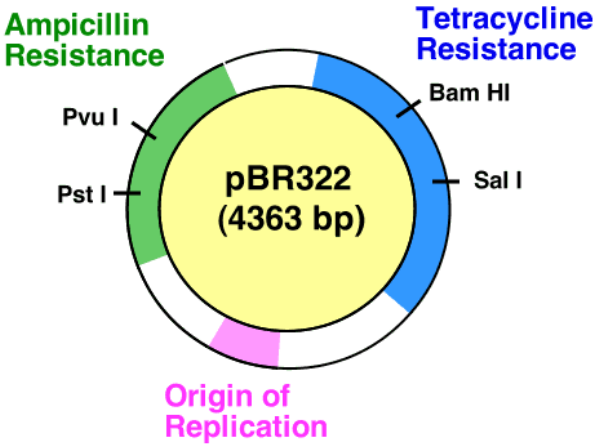
A map can be generated by using the overlaps between the fragments generated by different restriction enzymes.

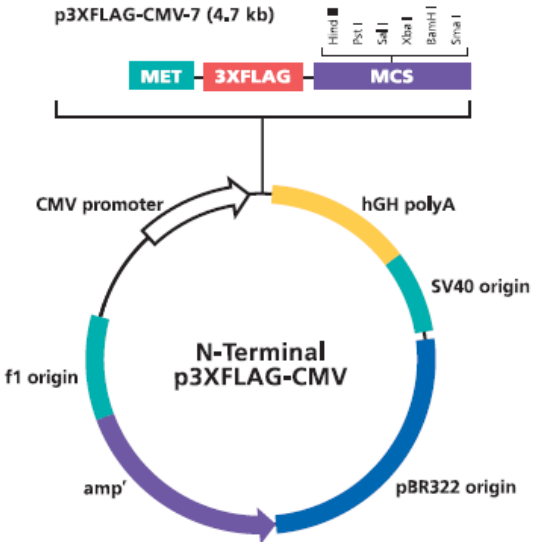


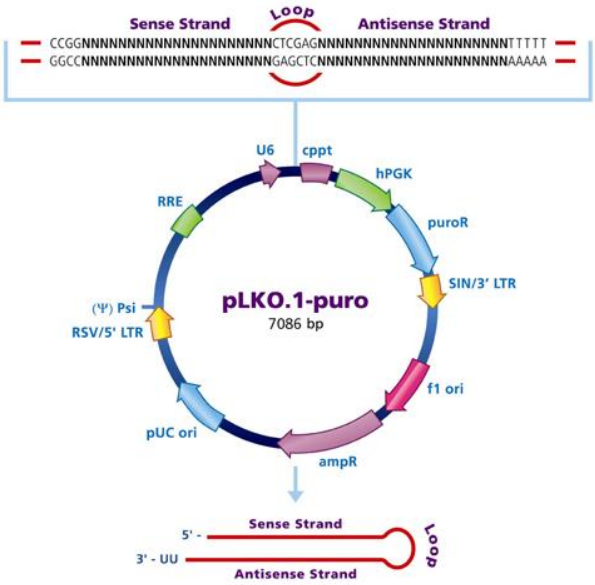
A restriction map is a linear sequence of sites separated by defined distances on DNA.

Vectors

- Vectors are DNA molecules that can be moved into and replicated in an organism independent of the host.
- They are shuttles for moving and copying another DNA fragment or gene.
- Vectors are classified by:
  - ✓ the organisms which replicate the vector
  - ✓ the size of DNA that can be inserted
- A vector contains an origin of replication (ori), selective marker(s) and multiple cloning site (MCS).







Common Vectors

Vector	Host	Size of insert accepted (kb)
Plasmid	*E. coli*	<15
Cosmid (plasmid/λ site)	*E. coli*	<50
Fosmid (F plasmid ORI + λ *cos* site)	*E. coli*	<50
Bacteriophage	*E. coli*	<100
P1(contains deletion in λ)	*E. coli*	<125
Bacterial artificial chromosome (BAC)	*E. coli*	100-500
Yeast artificial chromosome (YAC)	Yeast	250-1,000
P1-derived Artificial Chromosome (PAC)	*E. coli*	70-100
Ti plasmid	Plant	varies
Viral vector (retrovirus, lentivirus, adenovirus, adeno-associated virus)	Mammalian cells	varies
Engineered nanoparticles	Mammalian cells	varies

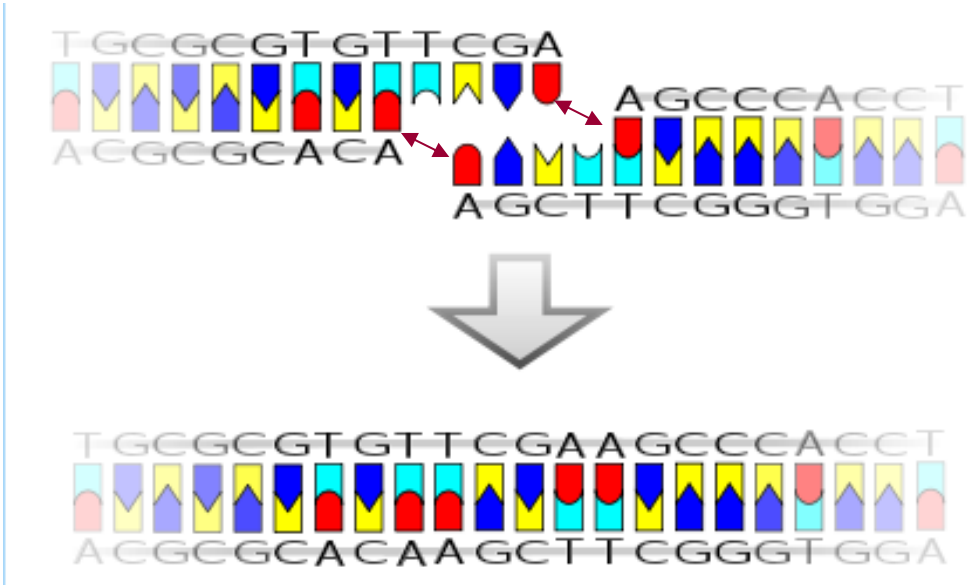
Plasmid – Circular Extrachromosomal DNA

Cloning vectors can be specialized for different purposes.

- Cloning a fragment of DNA requires specially engineered vectors.
- Shuttle vectors can be propagated in more than one type of host cell.
- Expression vectors contain promoters that allow transcription of any cloned gene.
- They generally contain a multiple cloning site (MCS)—a sequence of DNA containing a series of tandem restriction endonuclease sites.

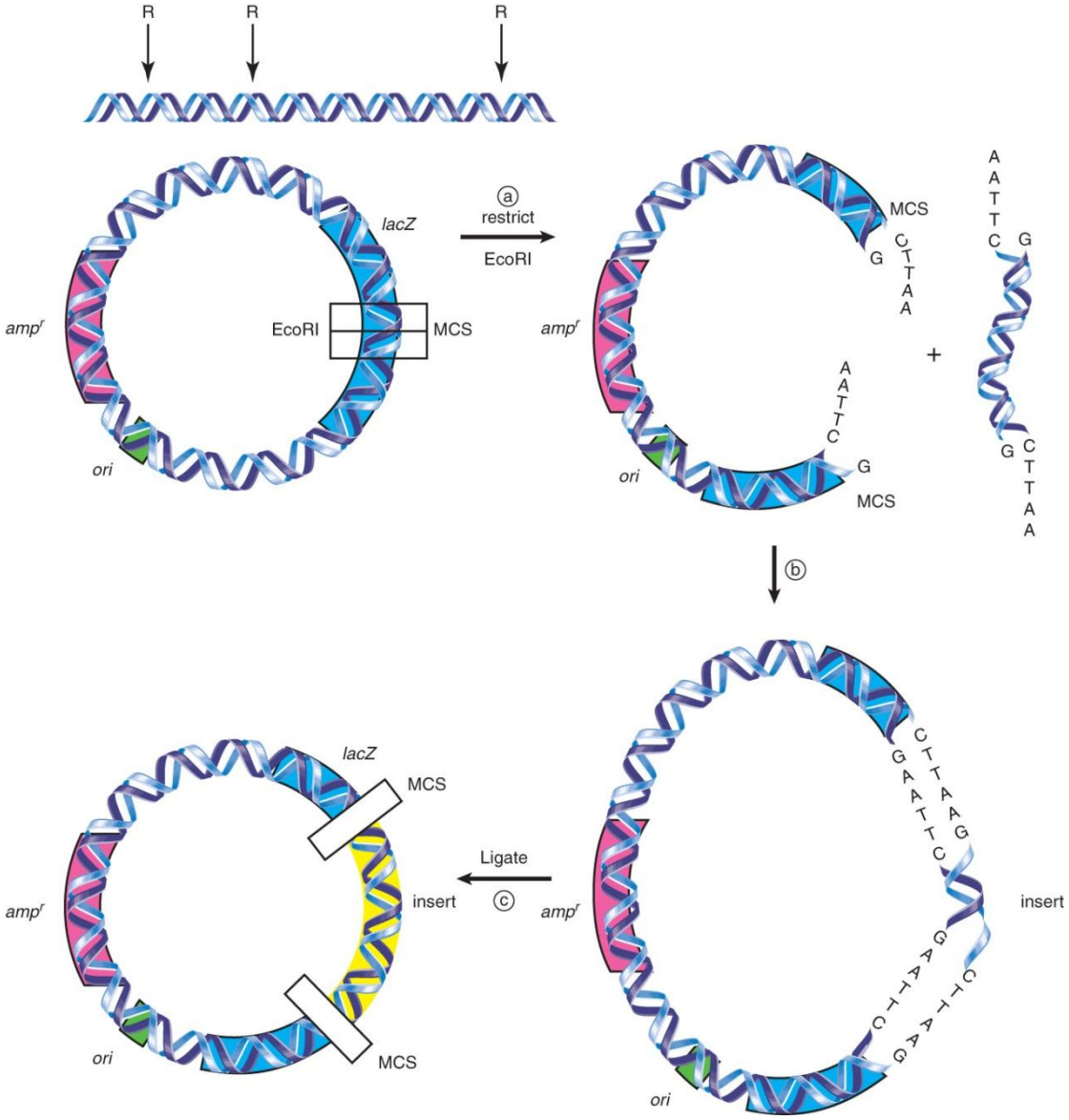
DNA Ligase

DNA ligase is an enzyme, identified by Martin Gellert in 1967, that joins two DNA molecules by forming two covalent phosphodiester bonds between 3' hydroxyl ends of one nucleotide ("acceptor") with the 5' phosphate end of another ("donor").

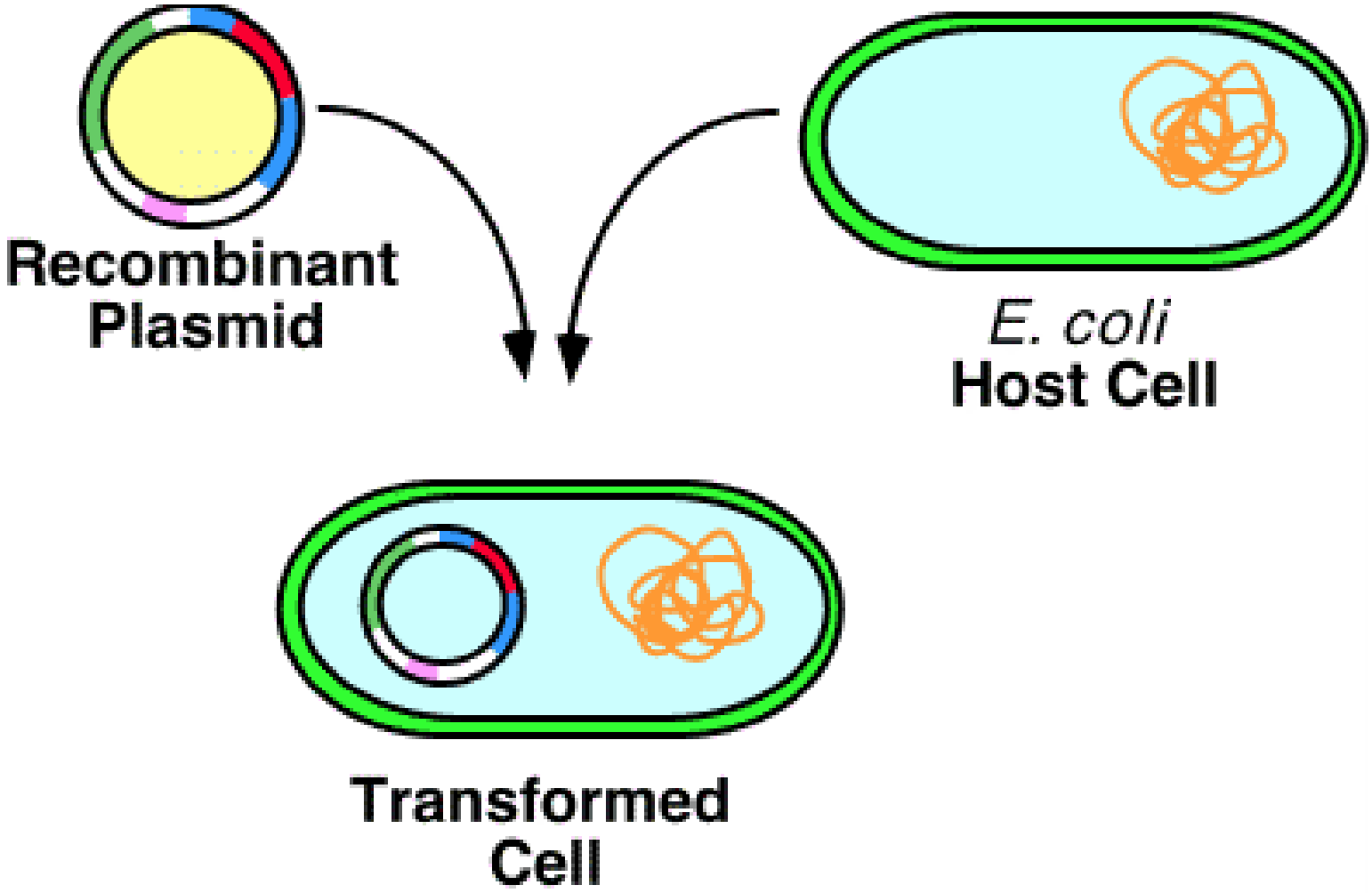


Cloning: plasmid transformation

- **A plasmid contains three key sites: *ori*, antibiotic-resistant gene and *lacZ* with an MCS, together with the insert DNA to be cloned and an appropriate restriction enzyme.**
- **Restricted insert fragment and vector will be combined and ligated together.**
- **The final pool of this DNA can be transformed into *E. coli*.**



Transformation – acquisition of new genetic material by incorporation of added exogenous, nonviral DNA



Selecting Recombinant Molecules

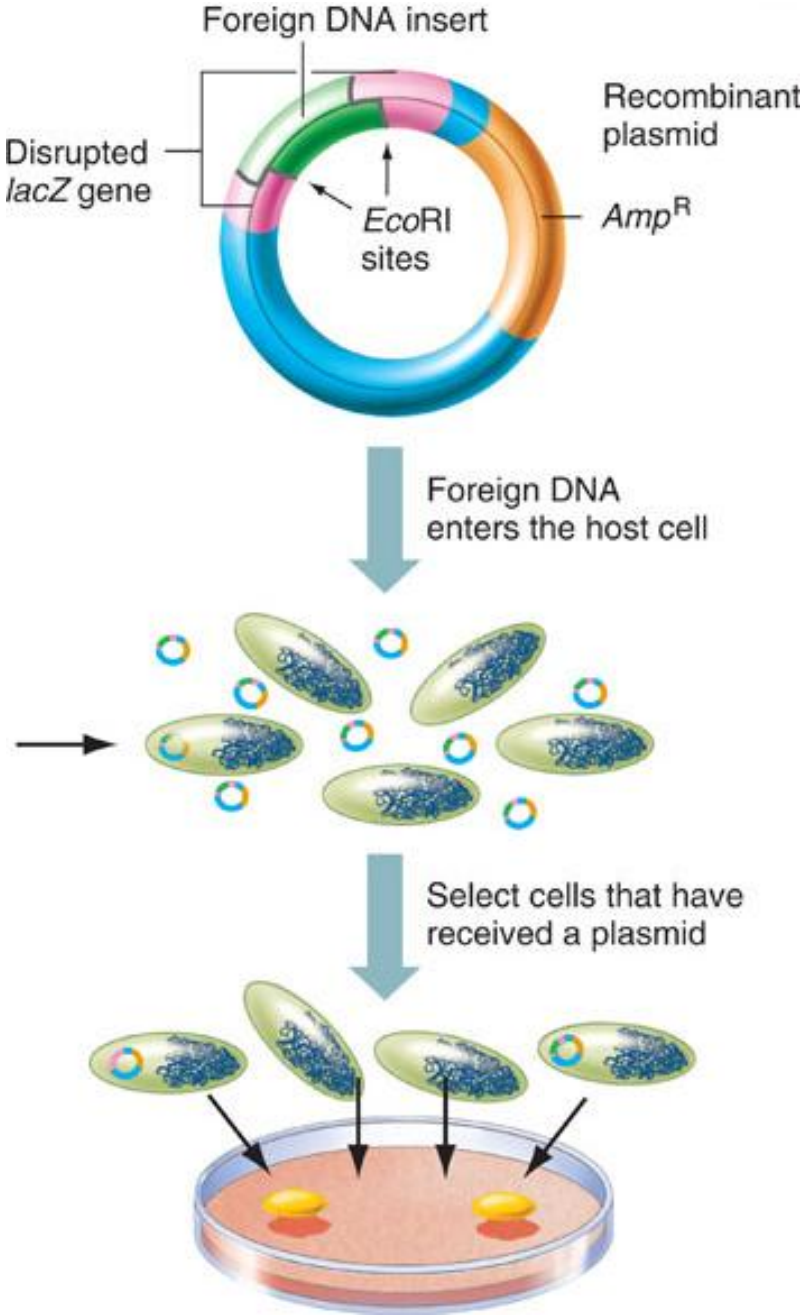
Three types of cells can result from attempts to introduce a DNA molecule into a bacterial cell:

- **Cells without vector**
- **Cells with vector but no inserted DNA**
- **Cells with vector and inserted DNA**

Selecting for Cells with Vector

- **Vectors are commonly engineered to carry antibiotic-resistance genes.**
- **Host bacteria die in the presence of the antibiotic.**
- **Bacteria harboring the vector survive.**
- **Growing cells on media with antibiotics ensures that all growing cells must carry the vector.**

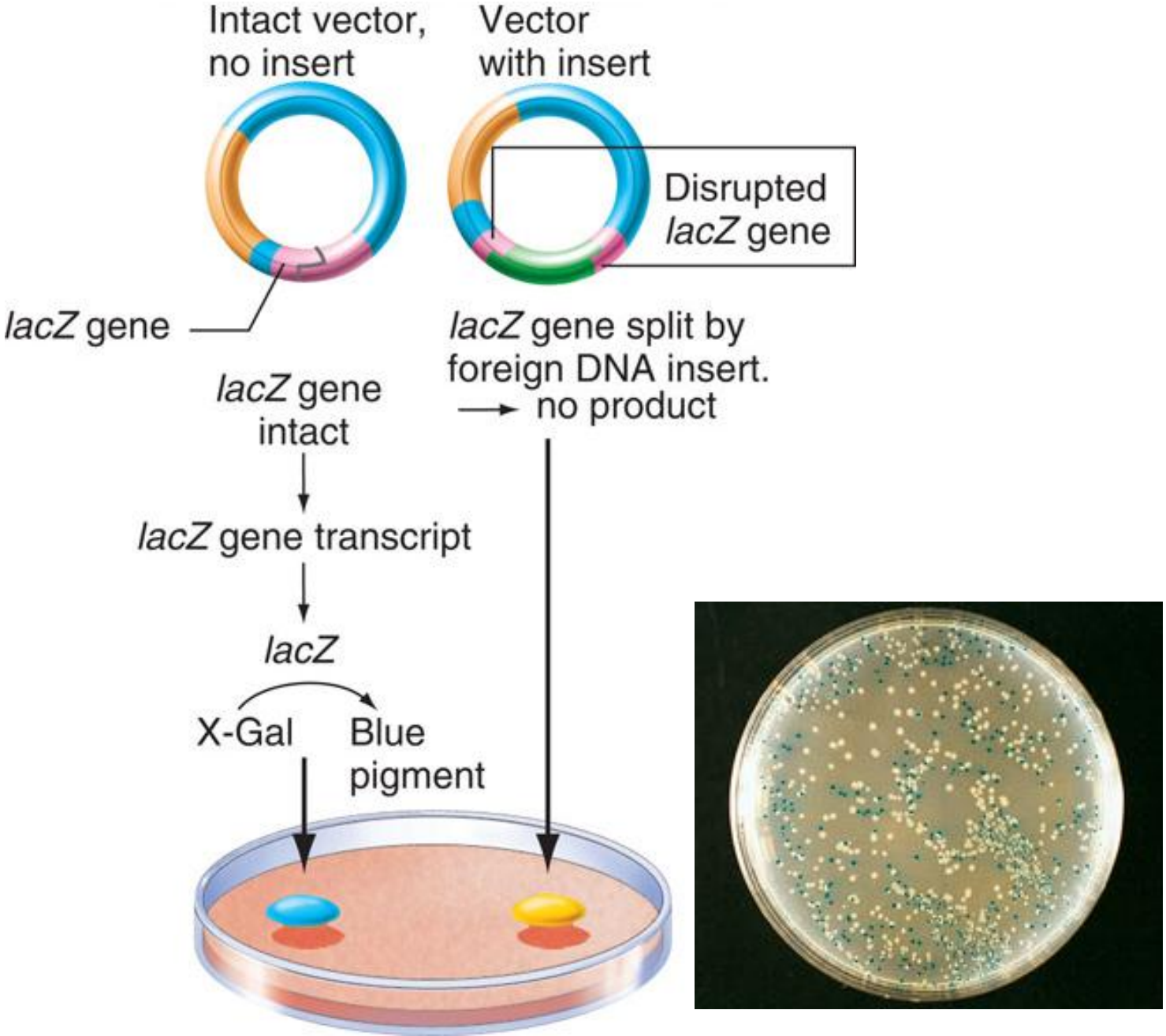
Selecting for Cells with Vector



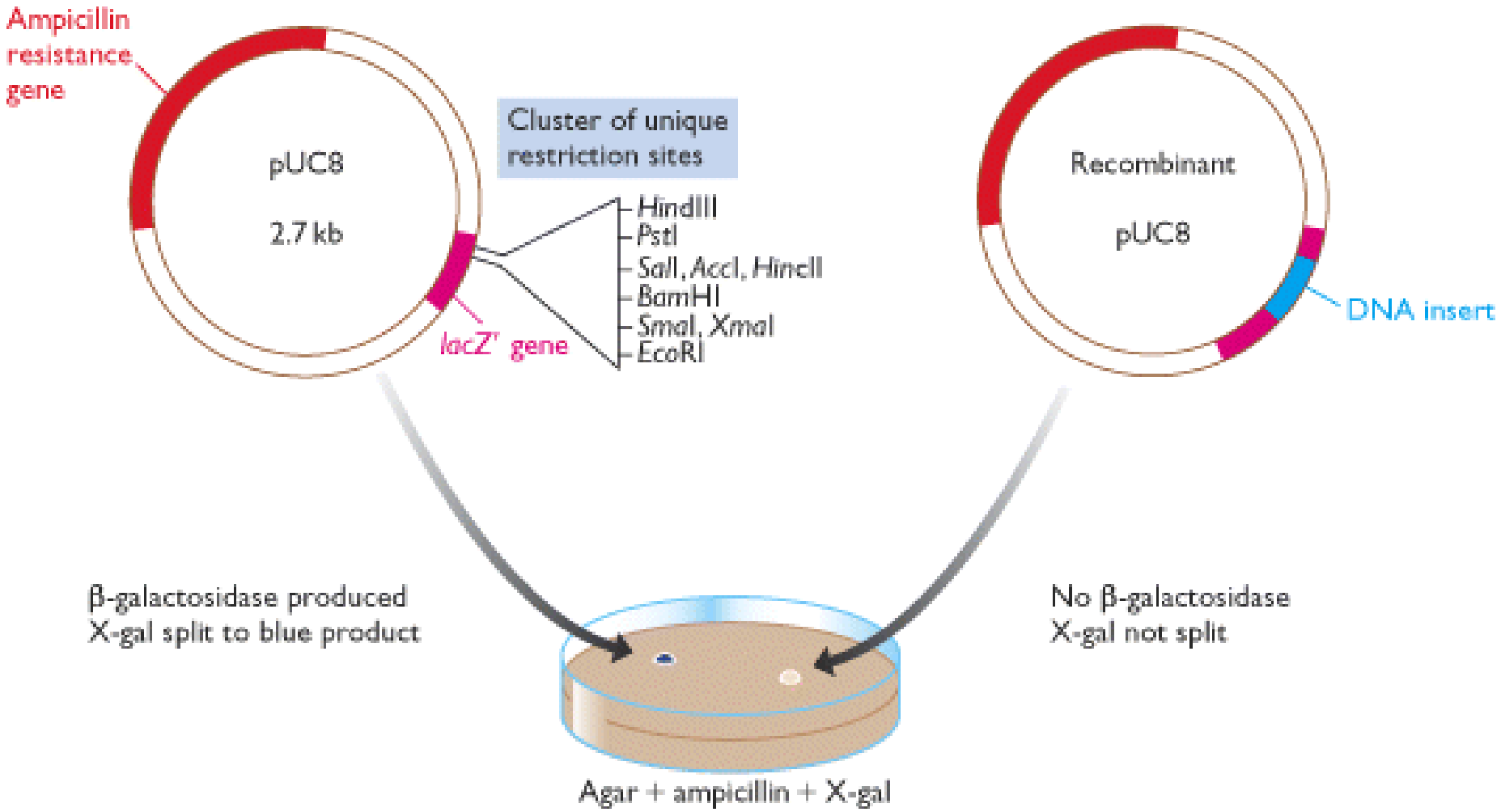
Selecting for Cells with Inserted DNA

- **The site of insertion of the DNA of interest can be within a gene on the vector.**
- **Insertion of a DNA fragment will disrupt the vector gene.**
- **The vector gene *lacZ* produces an enzyme which allows the bacteria to turn blue in the presence of certain media.**
- **Insertions in the *lacZ* gene prevent *lacZ* enzyme production and the bacteria are white.**
- **Bacteria with vector that are white carry a DNA inserted in the lacZ gene. (Rare mutations in the lacZ gene also will render white bacteria.)**

Selecting for Cells with Inserted DNA



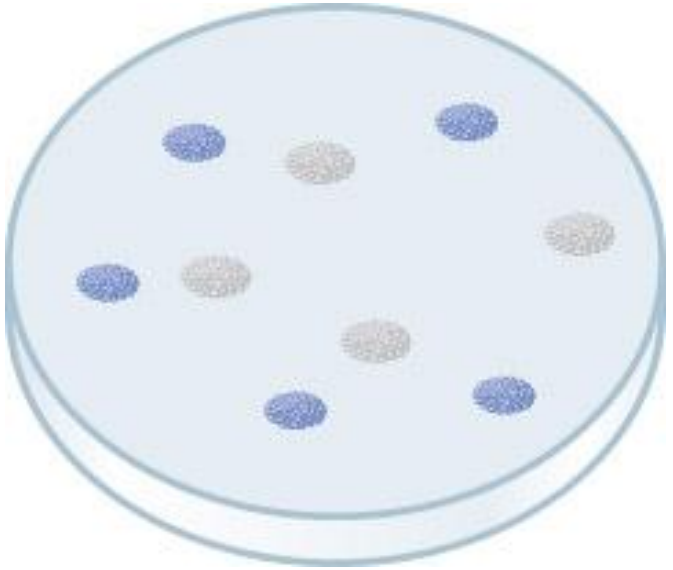
Recombinant Selection with pUC8



Blue-white Colony Selection

Blue/white selection allows the identification of bacteria that contain the vector plasmid and vector plasmids that contain an insert.

E. coli **colonies on agar plates with ampicillin, IPTG, and the color indicator X-gal**



Blue/white Colony Screening Assay, Distinguishing Recombinant Colonies (white) Among Non-recombinant Ones (blue)

- **X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) is an inert chromogenic substrate for beta-galactosidase, an enzyme that promotes lactose utilization.**

- **Beta-galactosidase hydrolyzes X-Gal into a colorless galactose and 4-chloro-3-brom-indigo which forms an intense blue precipitate. Induction of the *lacZ* gene with IPTG leads to the hydrolysis of X-Gal and to the development of blue colonies.**

- **The IPTG is a highly stable synthetic analog of lactose. It inactivates the *lac* repressor and induces synthesis of beta-galactosidase, an enzyme that promotes lactose utilization.**

Delivery of Recombinant DNA Molecules

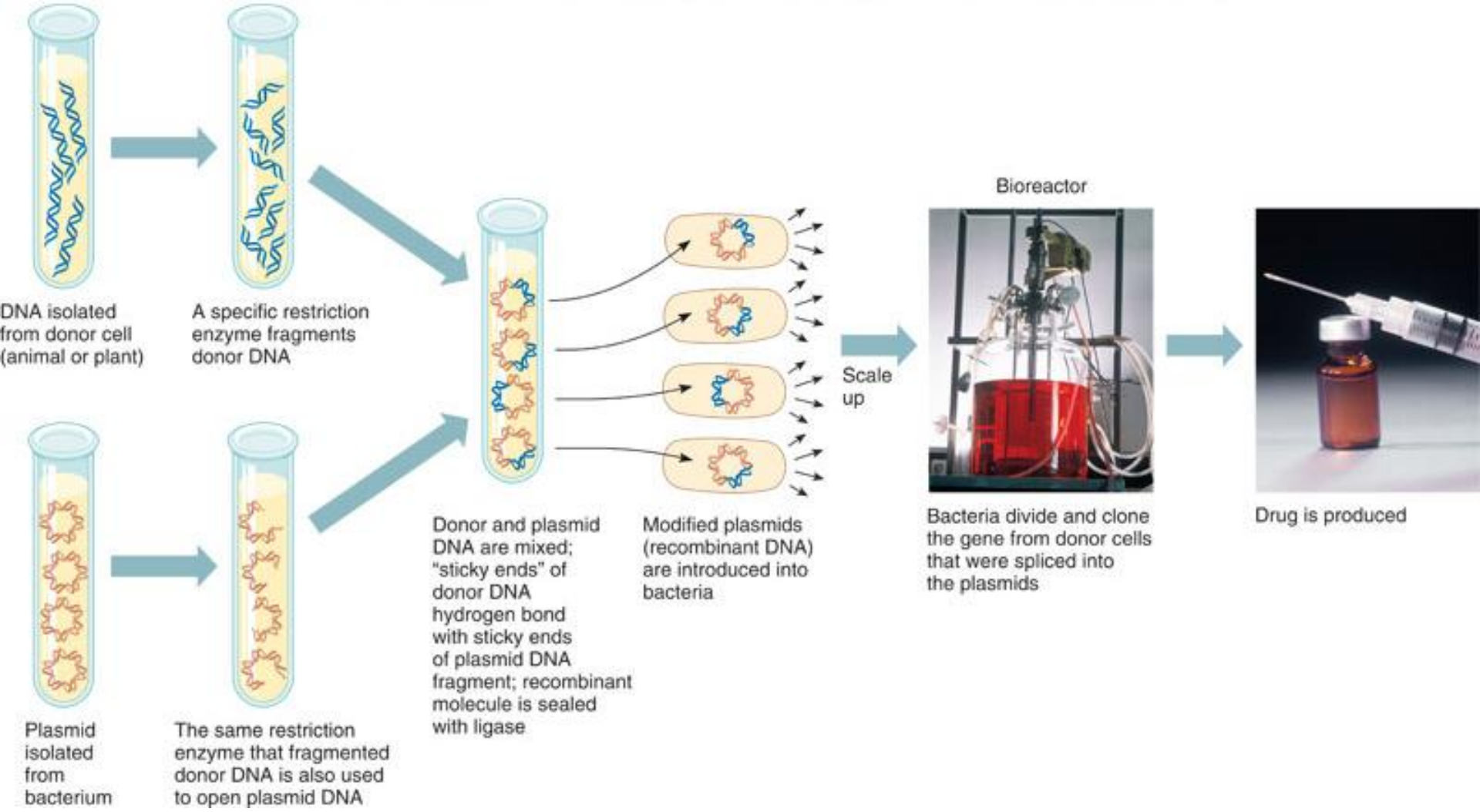
Introduction methods focus on getting recombinant DNA molecule (vector and insert) across the membrane of the host cell:

- ✓ **Electroporation – electricity opens a temporary hole**
- ✓ **Microinjection – microscopic needle injects DNA**
- ✓ **Liposomes – fatty bubbles move across membranes**
- ✓ **Particle bombardment – DNA coated "bullets" are shot into cell**
- ✓ **Chemicals – salts open a temporary hole**
- ✓ **Viruses – DNA or RNA viruses infect cell**
- ✓ ***Ti* plasmid – tumor-inducing genes are removed, gene of interest inserted and modified plasmid introduced into plant protoplasts**

Applications of Recombinant DNA Technology

- **Large-scale production of proteins by genetically engineered bacteria.**
  - ✓ **Examples: insulin, growth hormone, interferon's and blood clotting factors (VIII and IX).**
- **Vaccine development**
- **Gene therapy for genetic diseases**
- **Food production**
- **Stem cell research: iPS (induced pluripotent stem cells)**

Recombinant DNA Technology



Commercial Fermenters