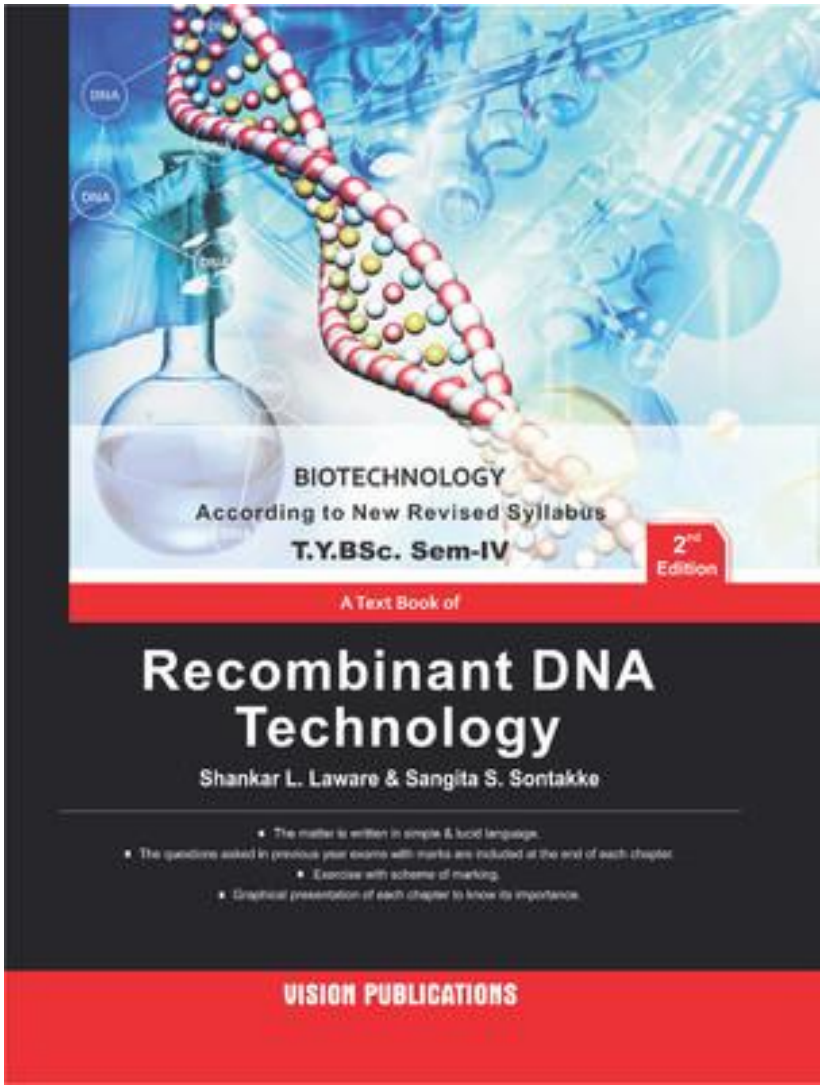
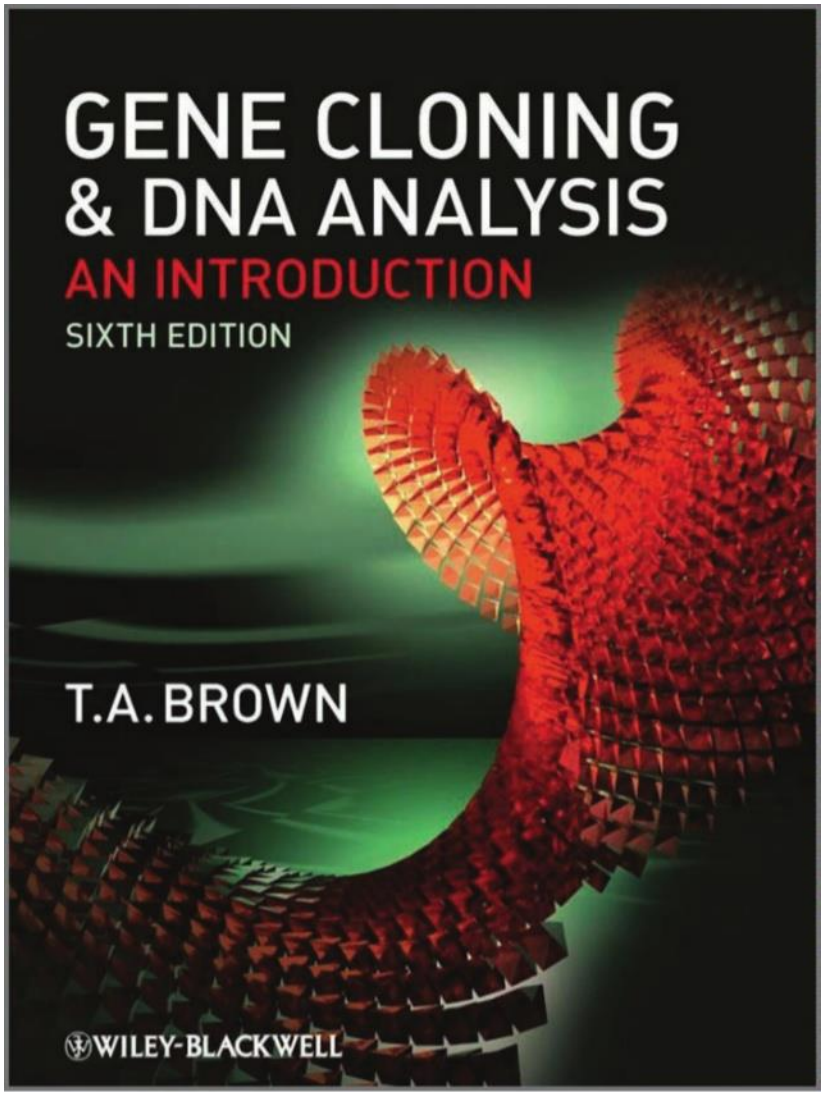
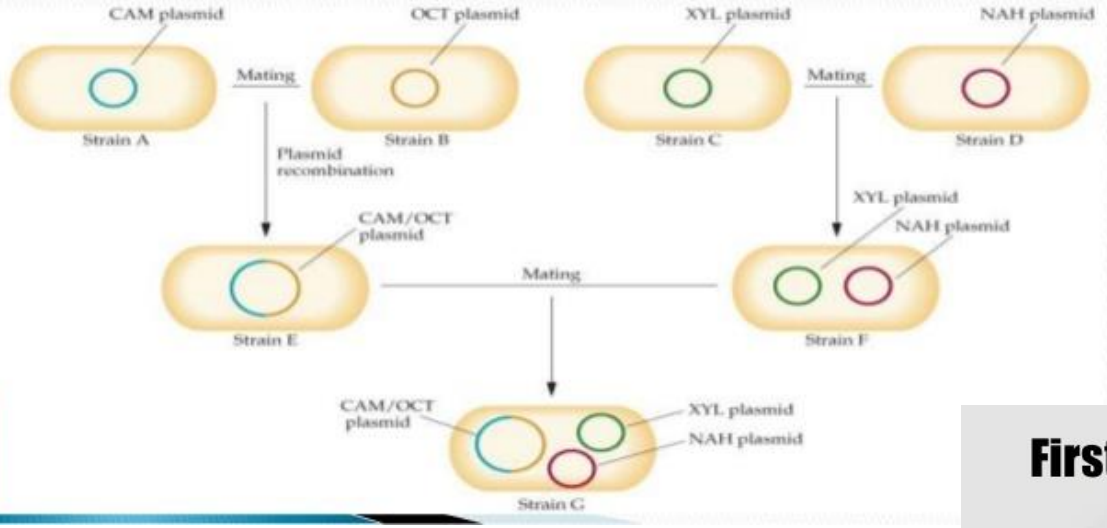

BIOTECHNOLOGY
According to New Revised Syllabus
T.Y.BSc. Sem-IV
2nd Edition
A Text Book of
Recombinant DNA Technology
Shankar L. Laware & Sangita S. Sontakke
VISION PUBLICATIONS


GENE CLONING
& DNA ANALYSIS
AN INTRODUCTION
SIXTH EDITION
T.A. BROWN
WILEY-BLACKWELL

How the idea evolves ??
Why to study this topic ??
What's the importance ??
Do they have any commercial / industrial importance

- **Prof. Ananda Mohan Chakraborty** et al. (1980) developed and patented a "**superbug**" that degraded petroleum (camphor, octane, xylene, and naphthalene) by plasmid transfers.
- transformed *Pseudomonas putida* with plasmids derived from four different bacteria involved in hydrocarbon degradation.



Understanding the concept and importance

First Patent on a Genetically Modified Microrganisms

First patent to Ananda Mohan Chakrabarty for a genetically modified Pseudomonas bacterium that would eat up oil spills.

US Patent No. 4259444

United States Patent [19]
Chakrabarty
[11] 4,259,444
[45] Mar. 31, 1981

[54] MICROORGANISMS HAVING MULTIPLE COMPATIBLE DEGRADATIVE ENERGY-GENERATING PLASMIDS AND PREPARATION THEREOF

[75] Inventor: Ananda M. Chakrabarty, Lathan, N.Y.

[73] Assignee: General Electric Company, Schenectady, N.Y.

[21] Appl. No.: 360,563

[22] Filed: Jan. 7, 1972

[51] Int. Cl.² C12N 15/00
[52] U.S. Cl. 435/172; 435/253; 435/284; 435/281; 435/820; 435/875; 435/877
[58] Field of Search ..... 195/28 R, 1, 3 H, 3 R, 95/96, 78, 79, 112; 435/172, 253, 264, 820, 281, 875, 877

[56] References Cited
PUBLICATIONS

Annual Review of Microbiology vol. 26 Annual Review Inc. 1972 pp. 362-368.
Journal of Bacteriology vol. 106 pp. 468-478 (1971).
Bacteriological Reviews vol. 33 pp. 210-263 (1969).

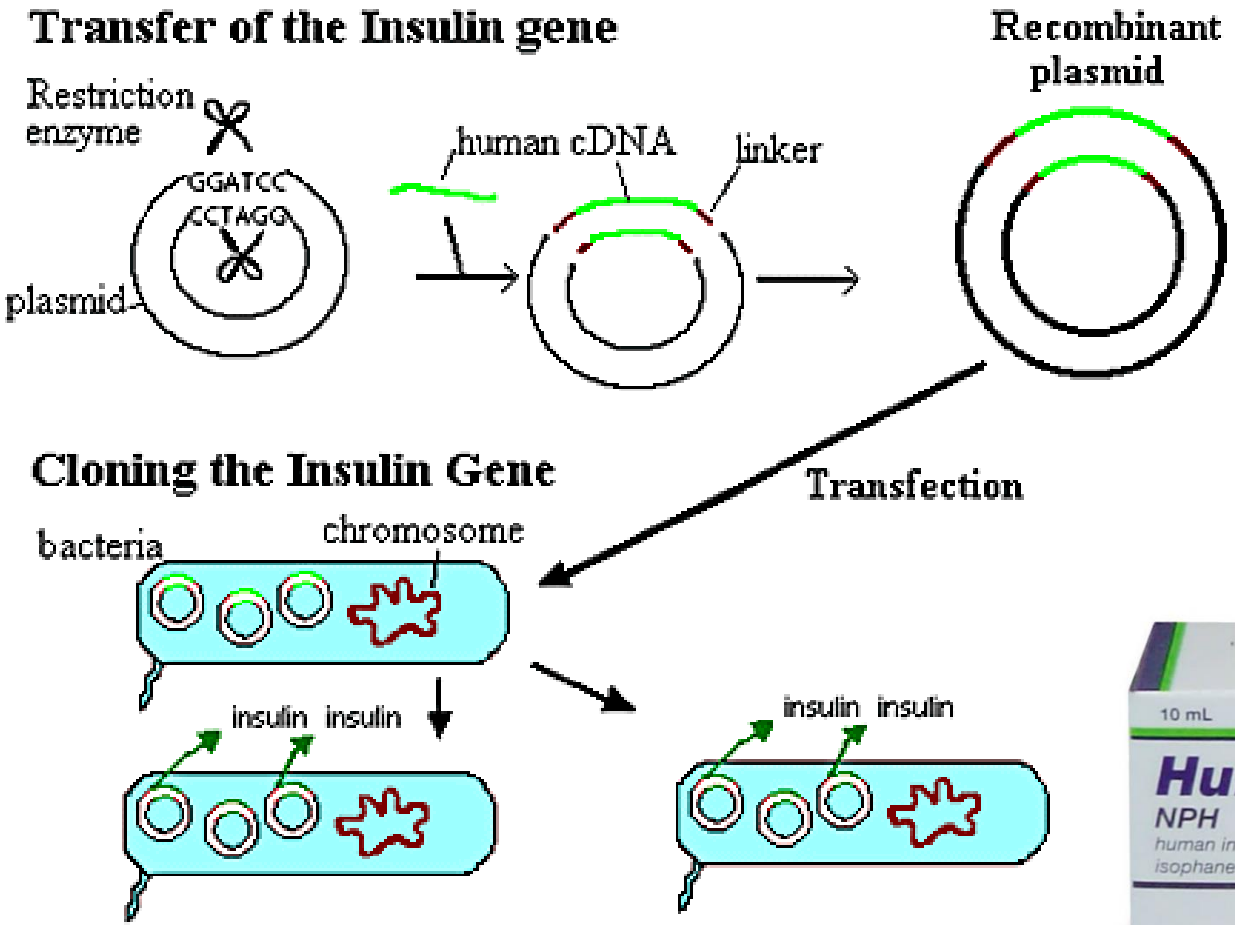
Primary Examiner—R. B. Penland
Attorney, Agent, or Firm—Leo I. MaLossi; James C. Davis, Jr.

[57] ABSTRACT

Unique microorganisms have been developed by the application of genetic engineering techniques. These microorganisms contain at least two stable (compatible) energy-generating plasmids, these plasmids specifying separate degradative pathways. The techniques for preparing such multi-plasmid strains from bacteria of the genus Pseudomonas are described. Living cultures of two strains of Pseudomonas (*P. aeruginosa* [NRRL B-5472] and *P. putida* [NRRL B-5473]) have been deposited with the United States Department of Agriculture, Agricultural Research Service, Northern Marketing and Nutrient Research Division, Peoria, Ill. The *P. aeruginosa* NRRL B-5472 was derived from *Pseudomonas aeruginosa* strain 1c by the genetic transfer thereto, and containment therein, of camphor, octane, salicylate and naphthalene degradative pathways in the form of plasmids. The *P. putida* NRRL B-5473 was derived from *Pseudomonas putida* strain PpG1 by genetic transfer thereto, and containment therein, of camphor, salicylate and naphthalene degradative pathways and drug resistance factor RP-1, all in the form of plasmids.

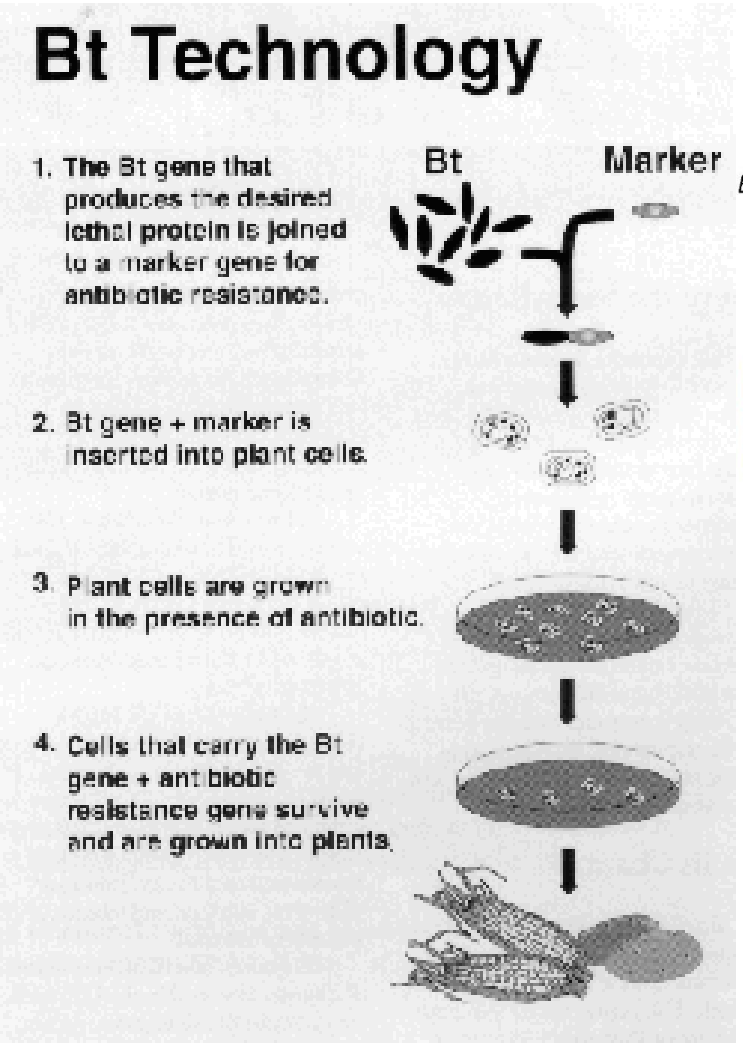
18 Claims, 2 Drawing Figures

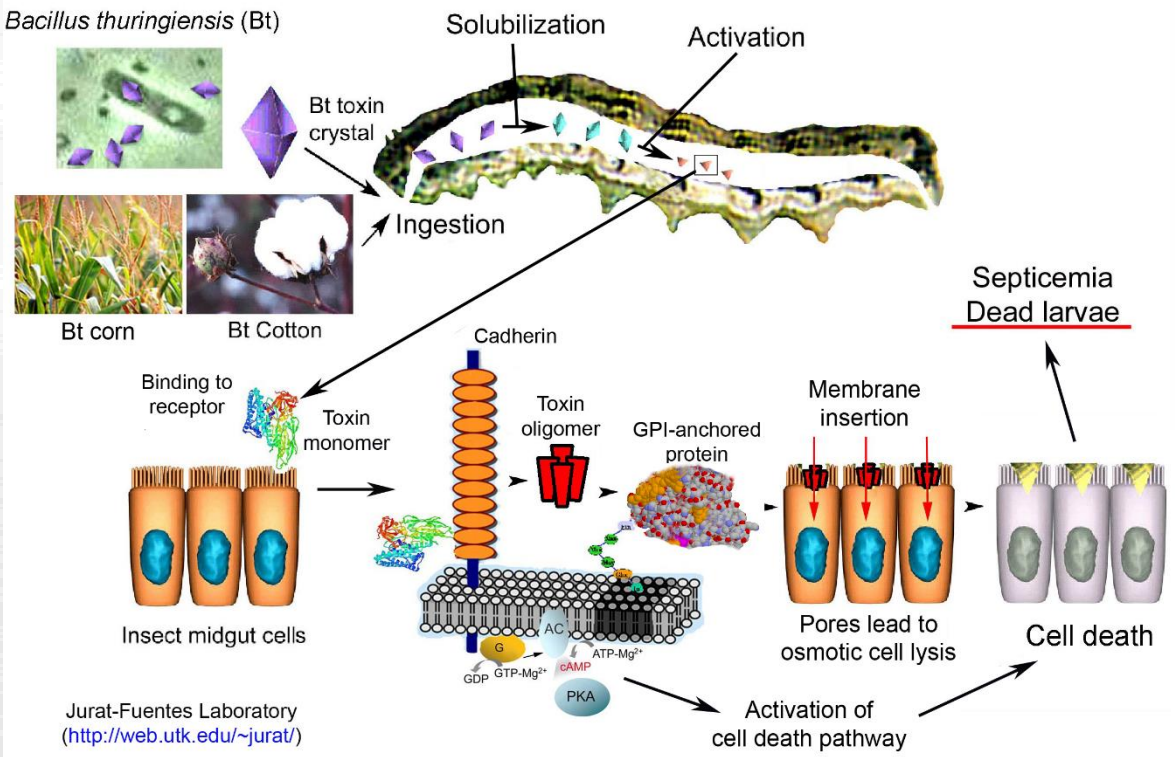
Do they have any commercial / industrial importance ??



Transfer and cloning of the Insulin gene

Do they have any commercial / industrial importance ??





Contents : Basics and techniques envolved

- Introduction to Recombinant DNA Technology and DNA Cloning
- What Makes a Good Vector?
- How Do You Identify and Clone a Gene of Interest?
- Laboratory Techniques and Applications of Recombinant DNA Technology

Introduction

- 1970s: Gene cloning became a reality
  - **Clone** – a molecule, cell, or organism that was produced from another single entity
- Made possible by the discovery of:
  - **Restriction Enzymes** – DNA cutting enzymes (molecular scissors)
  - **Plasmid DNA Vectors** – circular form of self-replicating DNA
    - Can be manipulated to carry and clone other pieces of DNA

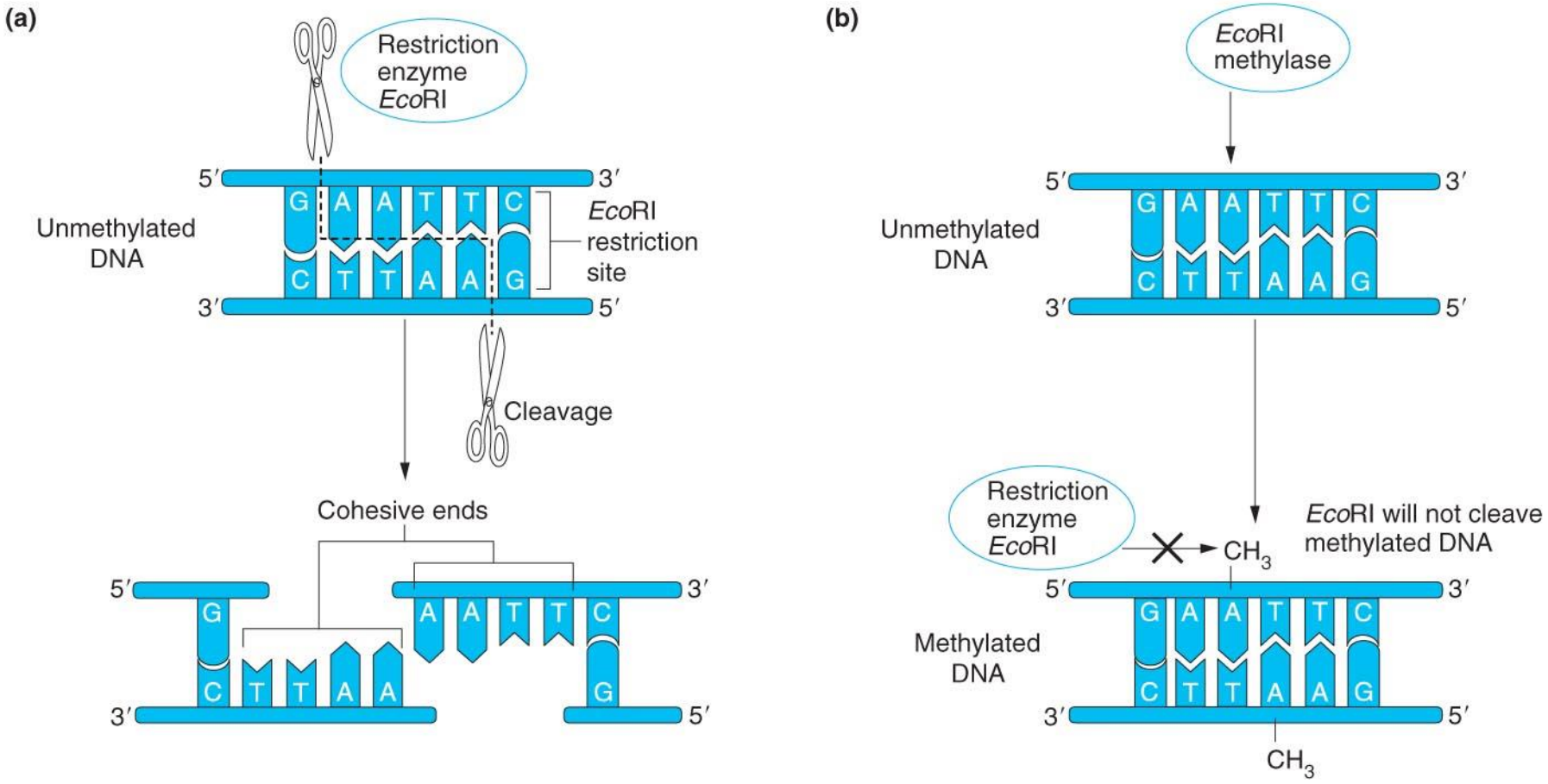
Restriction enzyme

- Restriction Enzymes

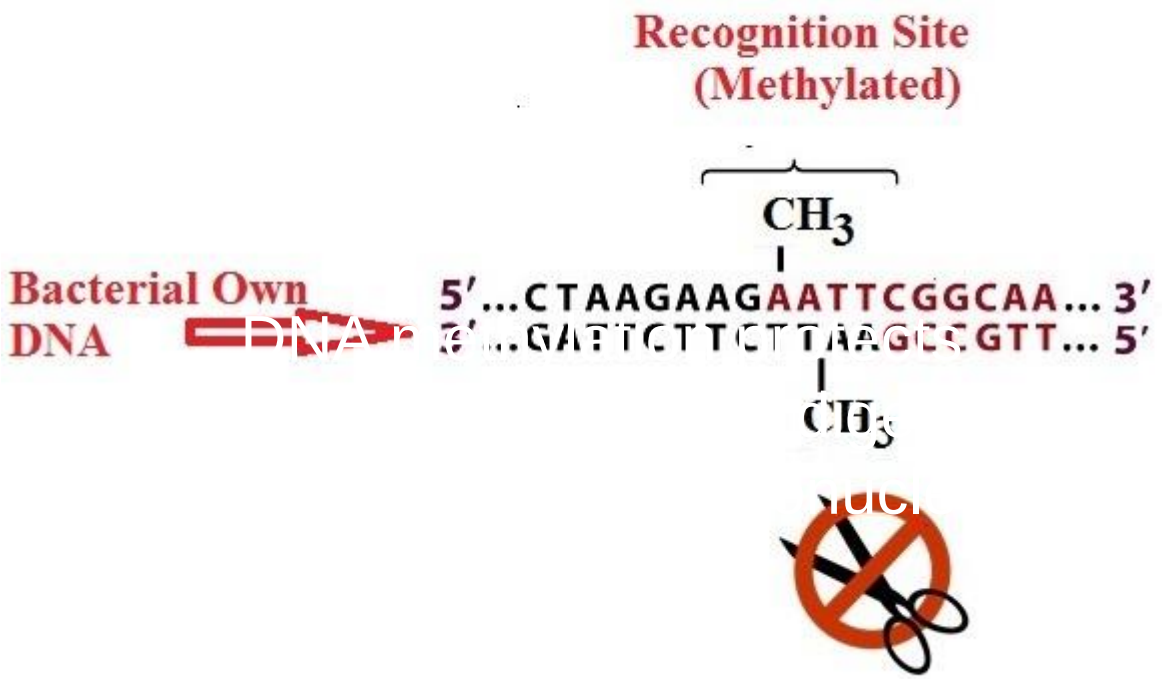
Primarily found in bacteria (**they use these for defense**)

- Cut DNA by cleaving the phosphodiester bond that joins adjacent nucleotides in a DNA strand
- Bind to, recognize, and cut DNA within specific sequences of bases called a **restriction site**
  - Each restriction site is a **palindrome** – reads same forward and backward on opposite strands of DNA
- There are 4 or 6 bp cutters because they recognize restriction sites with a sequence of 4 or 6 nucleotides

Restriction enzyme



- **Why don't restriction enzymes digest bacteria DNA?**



DNA methylation protects bacterial DNA from digestion from their own endonucleases

Restriction enzyme

Digestion

- Blunt ends
  - Cut both strands of DNA at same location
- Sticky/cohesive ends
  - Produce staggered cuts; single stranded “sticky” ends
  - Any DNA cut with the same enzyme will have ends with the same sequence
    - Can combine DNA from different sources and seal cuts with enzyme ligase

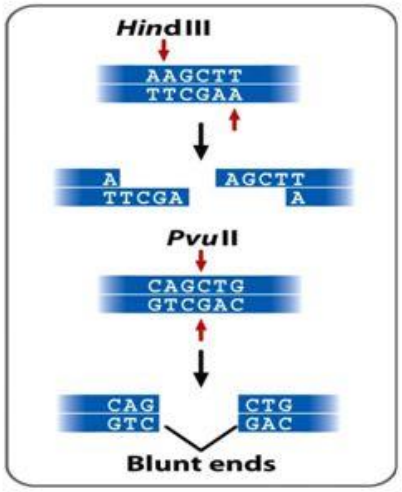


Figure 19-2a
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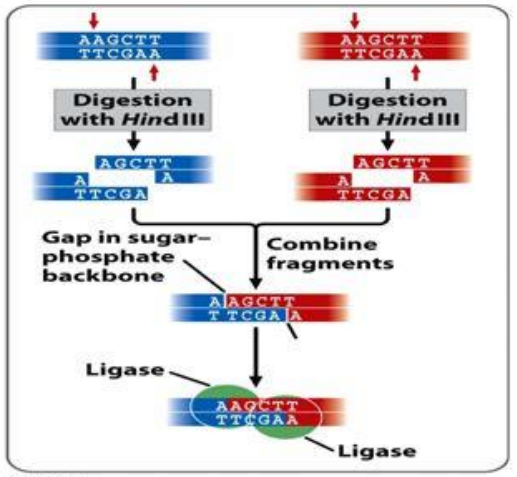

Figure 19-2b
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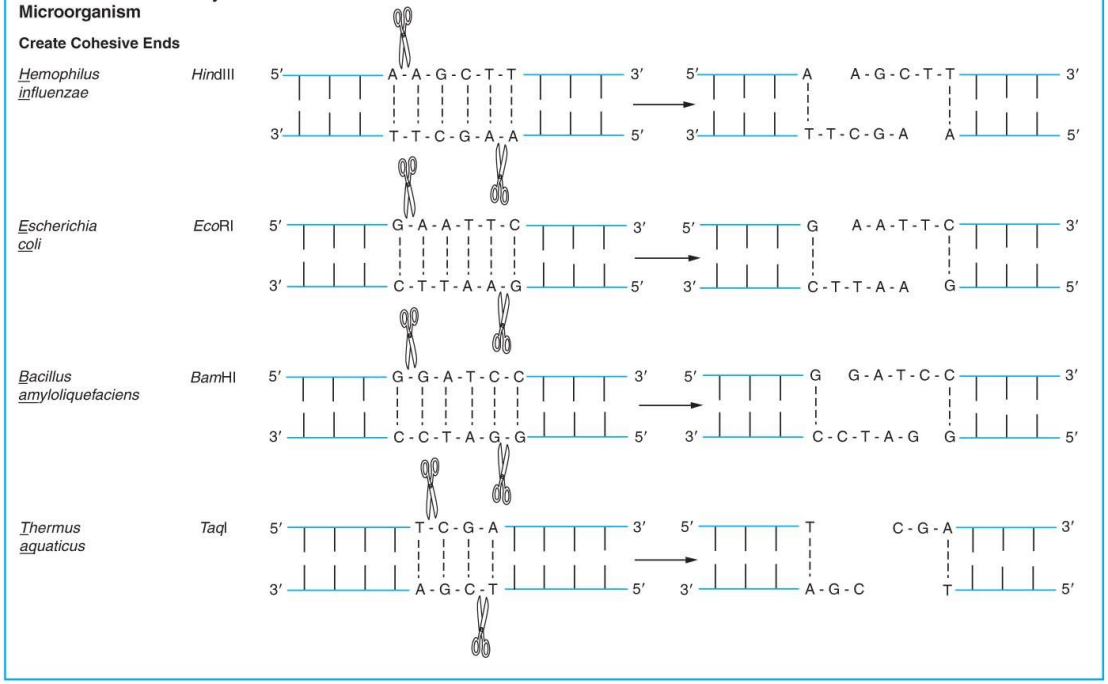
Restriction enzyme

TABLE 3.1 COMMON RESTRICTION ENZYMES

Source Microorganism	Enzyme	Restriction Site
Create Cohesive Ends		
Hemophilus influenzae	*Hin*dIII	5′ A-A-G-C-T-T 3′ / 3′ T-T-C-G-A-A 5′
Escherichia coli	*Eco*RI	5′ G-A-A-T-T-C 3′ / 3′ C-T-T-A-A-G 5′
Bacillus amyloliquefaciens	*Bam*HI	5′ G-G-A-T-C-C 3′ / 3′ C-C-T-A-G-G 5′
Thermus aquaticus	*Taq*I	5′ T-C-G-A 3′ / 3′ A-G-C-T 5′



- **Would EcoRI cut the following sequence? Work in groups to explain your answer.**
- **5'CTCGAGTTCGAG3'**
- **3'GAGCTCAAGCTC5'**

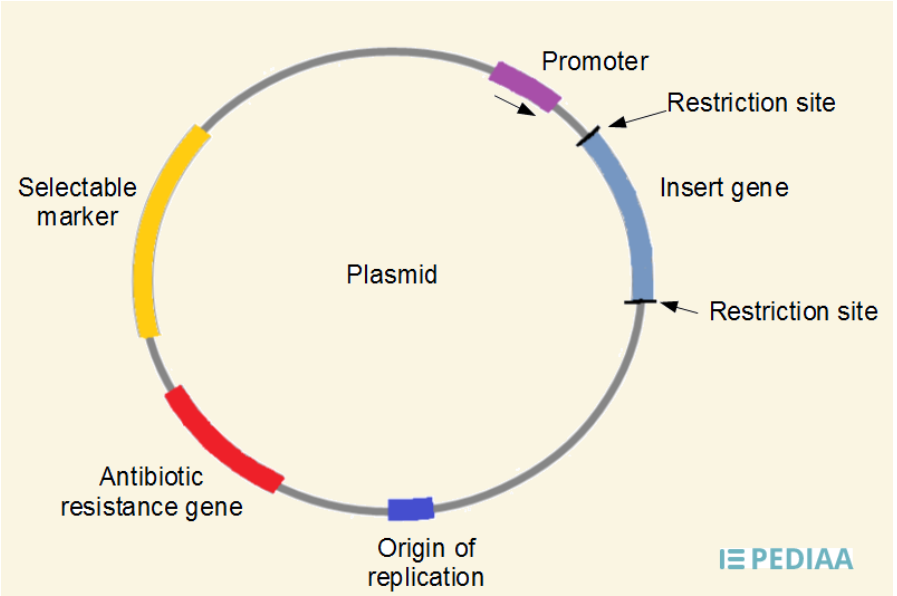
Restriction enzyme

- Advantage of enzymes that produce sticky ends
  - Preferred for cloning because DNA fragments with sticky ends can be easily joined together because they base pair with each other by forming weak hydrogen bonds

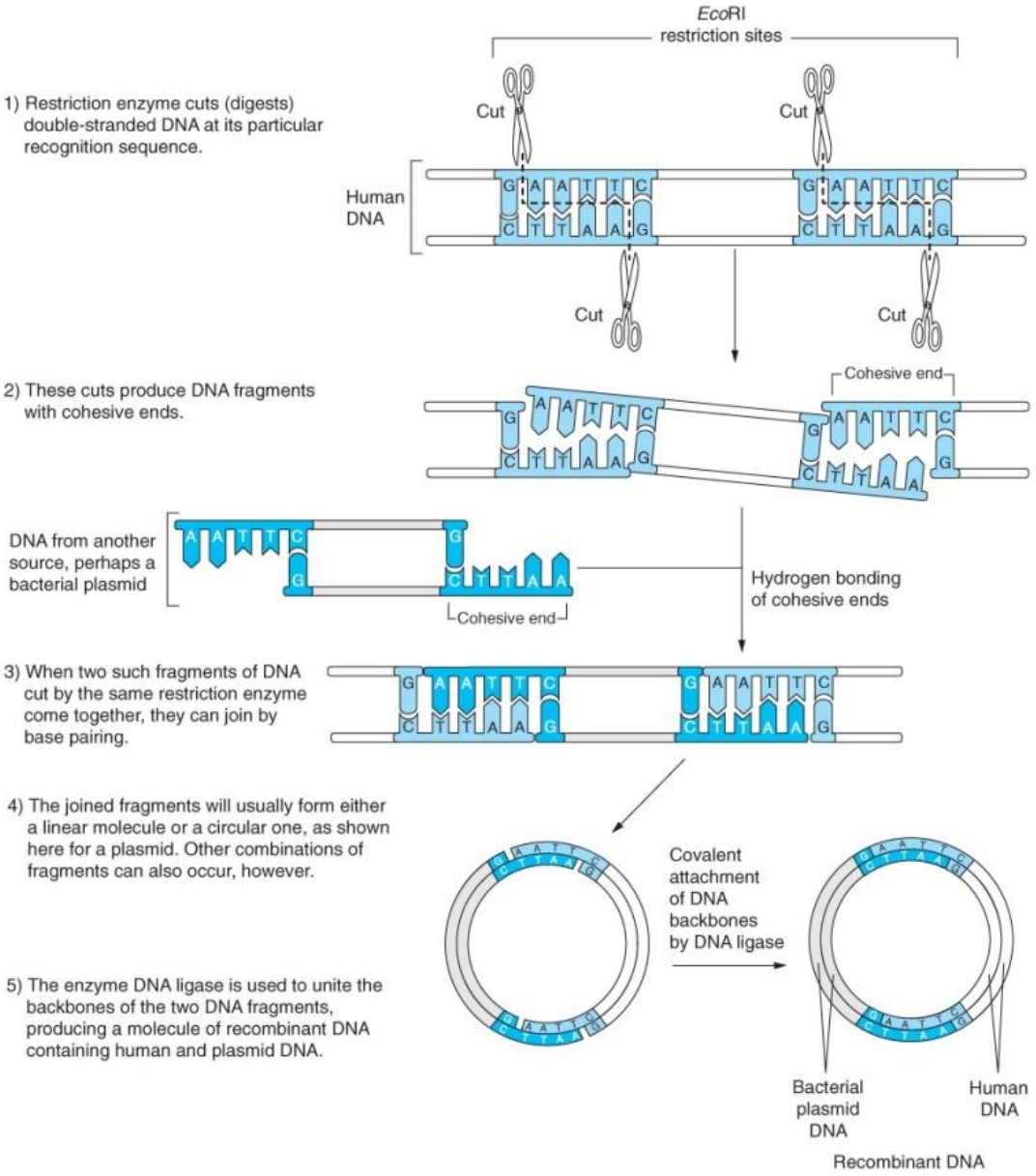
Derivation of the EcoRI name		
Abbreviation	**Meaning**	**Description**
E	*Escherichia*	genus
co	*coli*	specific species
R	RY13	strain
I	First identified	order of identification in the bacterium

The Plasmid

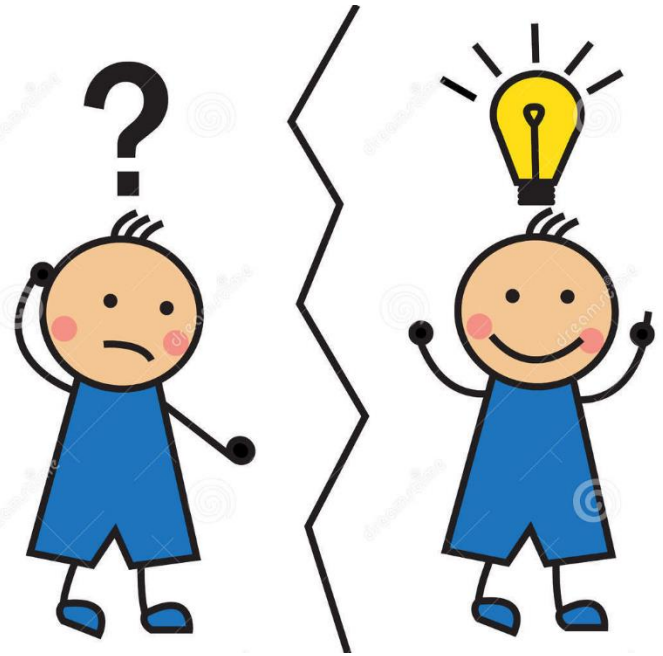
- Plasmid DNA – small circular pieces of DNA found primarily in bacteria
- Are considered **extrachromosomal DNA** because they are in the cytoplasm in addition to the bacteria chromosome
- Are small approximately 1 to 4 kb
- Can replicate independently of chromosome
- Can be used as **vectors** – pieces of DNA that can accept, carry, and replicate other pieces of DNA



Creating recombinant DNA



What should be the next step logically ??

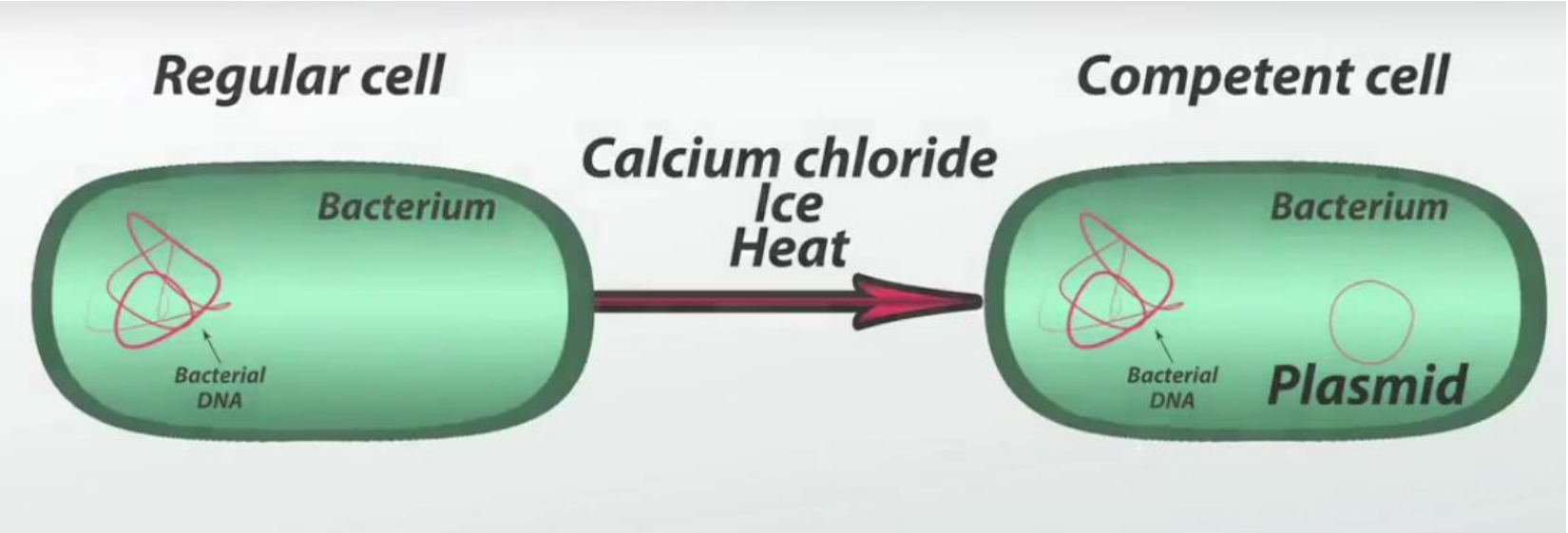


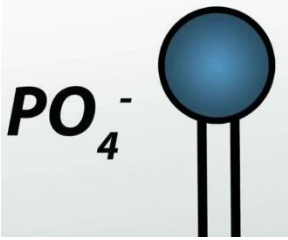
Put it in the bacterial system. Development of competent cells.

- **Transformation of Bacterial Cells**
  - very inefficient process
  - A process for inserting foreign DNA into bacteria
    - Treat bacterial cells with calcium chloride
    - Add plasmid DNA to cells chilled on ice
    - Heat the cell and DNA mixture
    - Plasmid DNA enters bacterial cells and is replicated and express their genes
  - *Electroporation*
  - Apply brief pulse of high voltage electricity to create tiny holes in the bacteria cell wall that allow the DNA construct to enter.

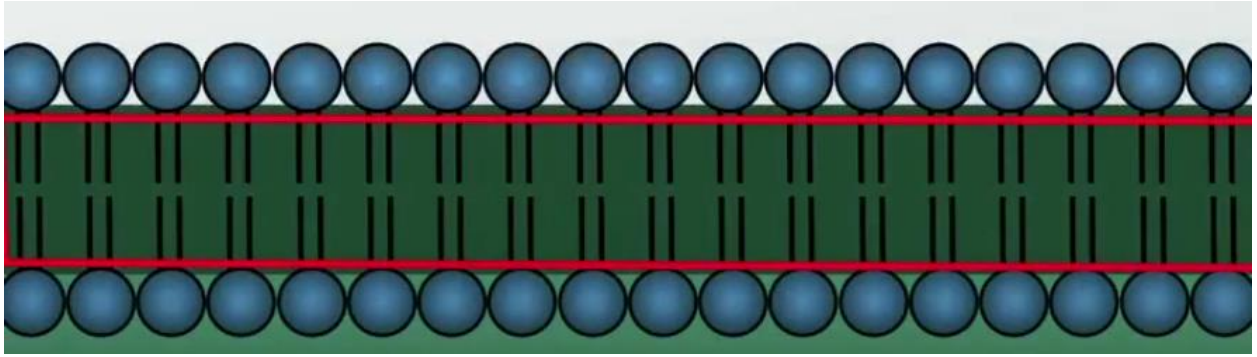
	Step in Experiments	Outcome
I	Mouse injected with SIII strain	----------
II	Mouse injected with RII strain	----------
III	Mouse injected with Heat Killed SIII strain	----------
IV	Mouse injected with Heat Killed SIII & living R II strain	----------
V	Mouse injected with Heat Killed SIII+ living RII strain + DNase enzyme	----------
VI	Mouse injected with Heat Killed SIII+ living RII strain + Protease enzyme	----------

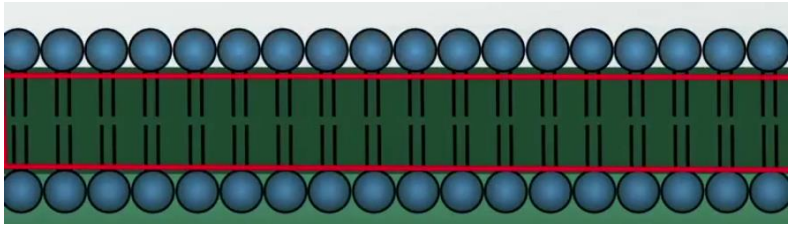
It should take-up the engineered construct



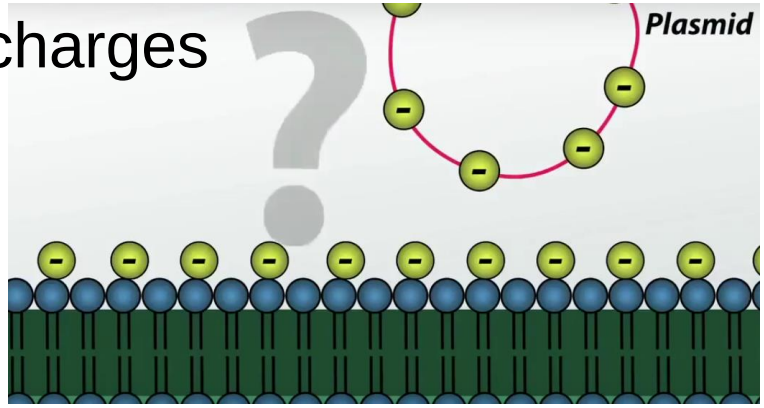


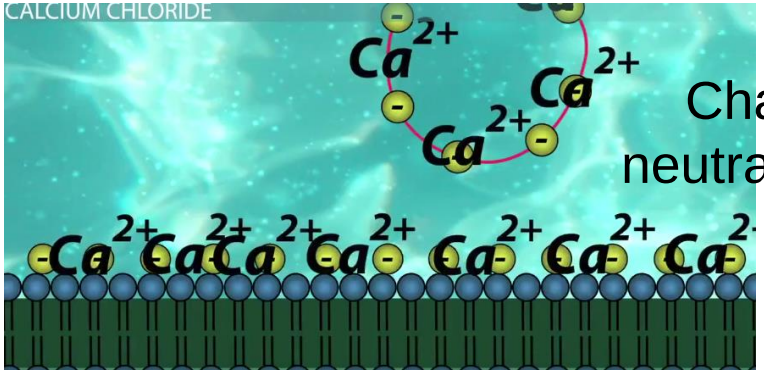
Hydrophobic tail inside and hydrophilic head outside



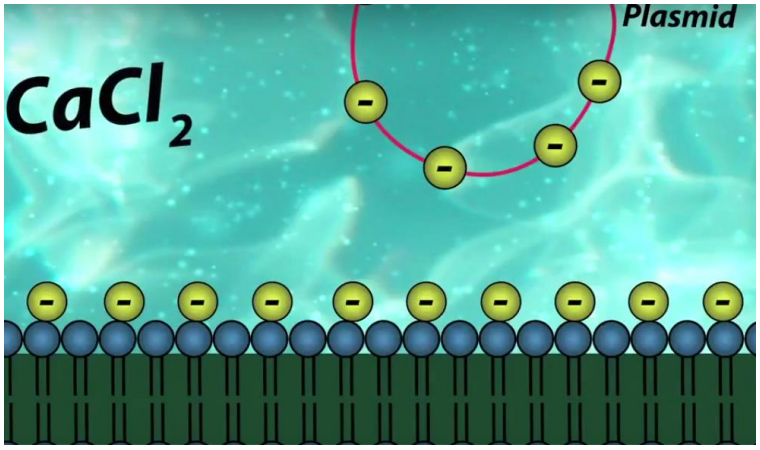


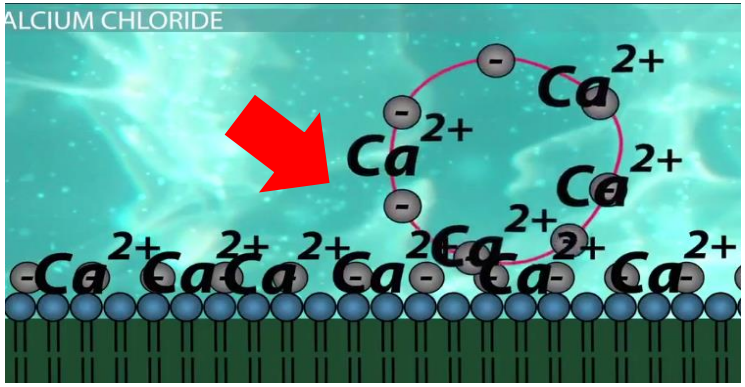
Like charges





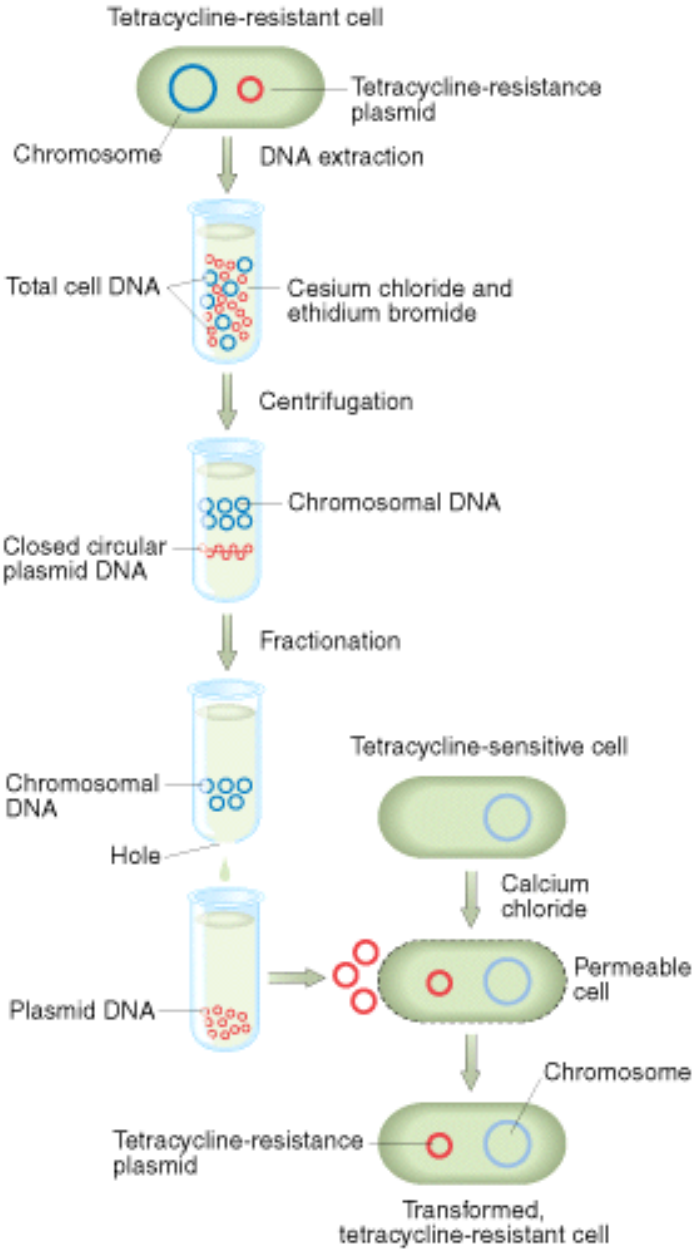
Charge neutralization



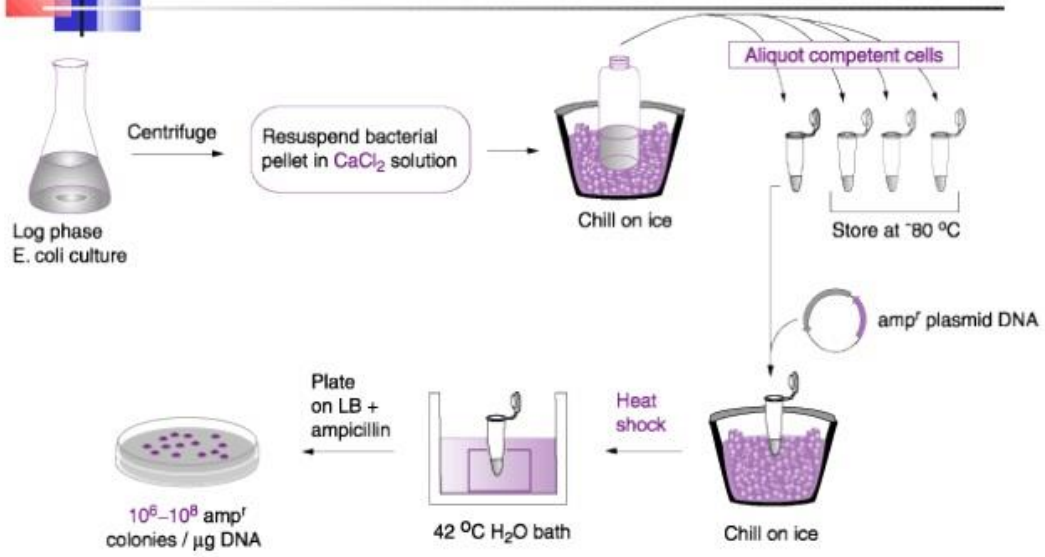


HEAT SHOCK

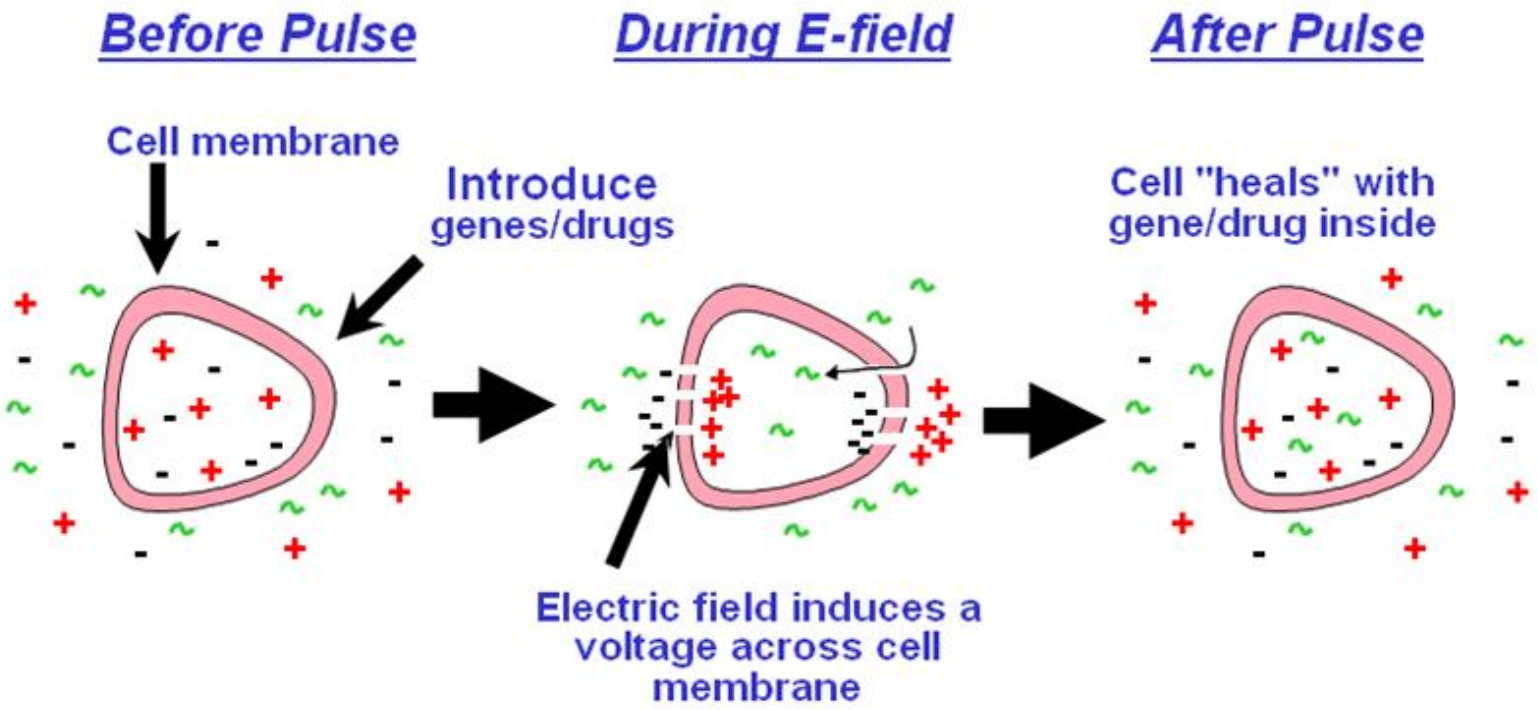
Getting the chimera near the cell wall is just a half story



CHEMICAL TRANSFORMATION WITH CALCIUM CHLORIDE



Electroporation

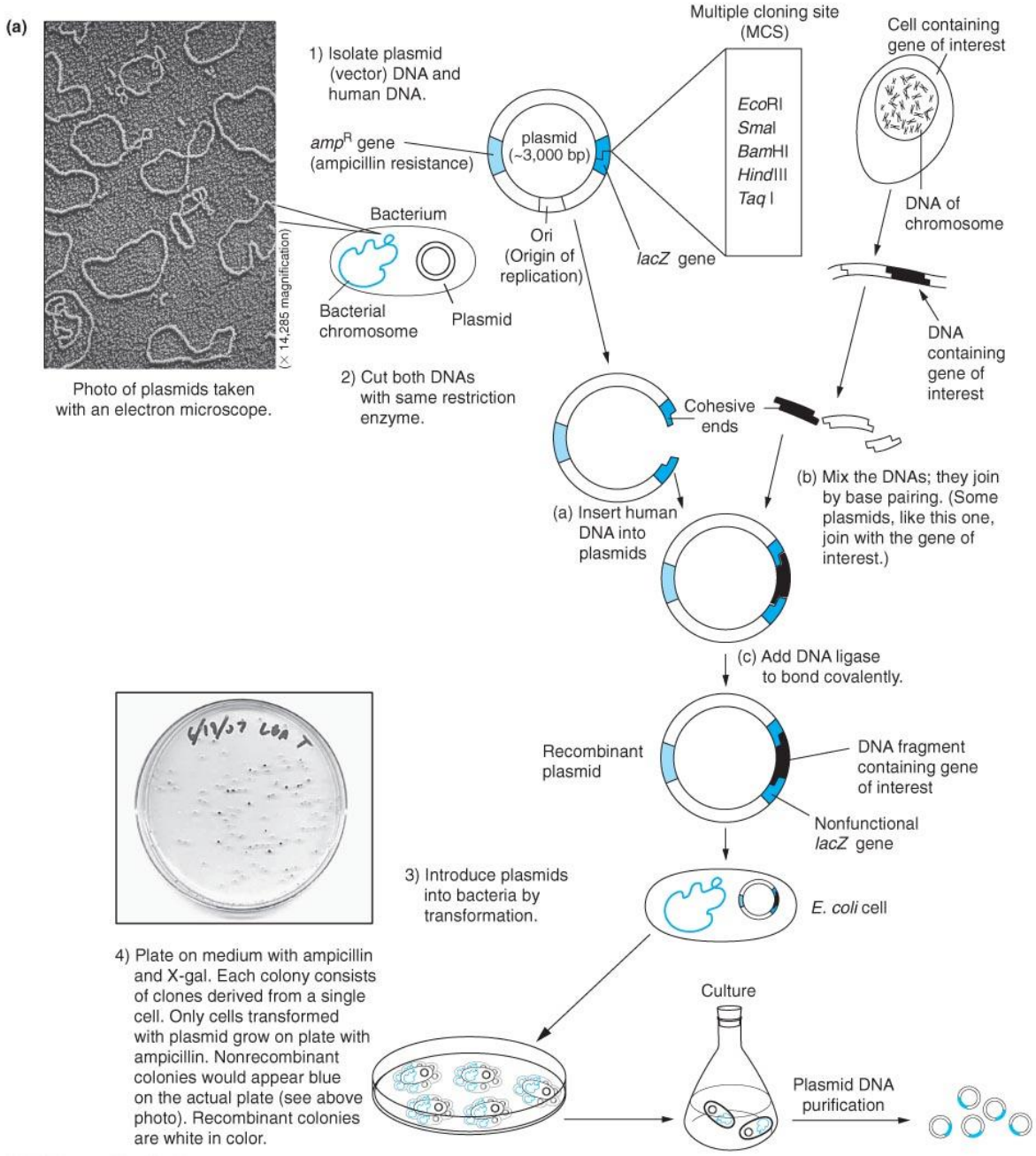


- **Selection of recombinant bacteria after transformation**

  - **Selection** is a process designed to facilitate the identification of recombinant bacteria while preventing the growth of non-transformed bacteria and bacteria that contain plasmid without foreign DNA

  1. Antibiotic selection – plate transformed cells on plates containing different antibiotics to identify recombinant bacteria and non-transformed bacteria
     - **Does not select for plasmid containing foreign DNA vs. recircularized plasmid (What is this ?????)**

- Selection of recombinant bacteria after transformation (Another way)
  2. Blue-white selection
  - DNA is cloned into the restriction site in the lacZ gene
  - When it is interrupted by an inserted gene, the lacZ gene cannot produce functional Beta gal
  - When Xgal (artificial lactose) is added to the plate, if functional lacZ is present = blue colony. Formation of blue color metabolite.
  - Non-functional lacZ = **white colony = clone =** genetically identical bacterial cells each containing copies of recombinant plasmid

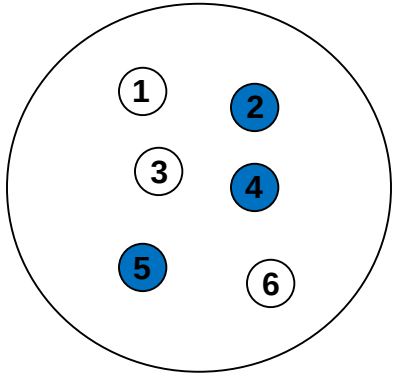

(a)
(× 14,285 magnification)
Photo of plasmids taken with an electron microscope.
1) Isolate plasmid (vector) DNA and human DNA.
ampR gene (ampicillin resistance)
plasmid (~3,000 bp)
Multiple cloning site (MCS)
EcoRI
SmaI
BamHI
HindIII
Taq I
Ori (Origin of replication)
lacZ gene
Bacterium
Bacterial chromosome
Plasmid
Cell containing gene of interest
DNA of chromosome
DNA containing gene of interest
2) Cut both DNAs with same restriction enzyme.
Cohesive ends
(a) Insert human DNA into plasmids
(b) Mix the DNAs; they join by base pairing. (Some plasmids, like this one, join with the gene of interest.)
(c) Add DNA ligase to bond covalently.
Recombinant plasmid
DNA fragment containing gene of interest
Nonfunctional lacZ gene
3) Introduce plasmids into bacteria by transformation.
E. coli cell
4) Plate on medium with ampicillin and X-gal. Each colony consists of clones derived from a single cell. Only cells transformed with plasmid grow on plate with ampicillin. Nonrecombinant colonies would appear blue on the actual plate (see above photo). Recombinant colonies are white in color.
Culture
Plasmid DNA purification

- Assume you used a plasmid that contains the lacz gene in the restriction enzyme site. The plasmid has an antibiotic resistance gene. Following transformation, you grow up the cells on an agar plate containing the antibiotic. Here are your results.

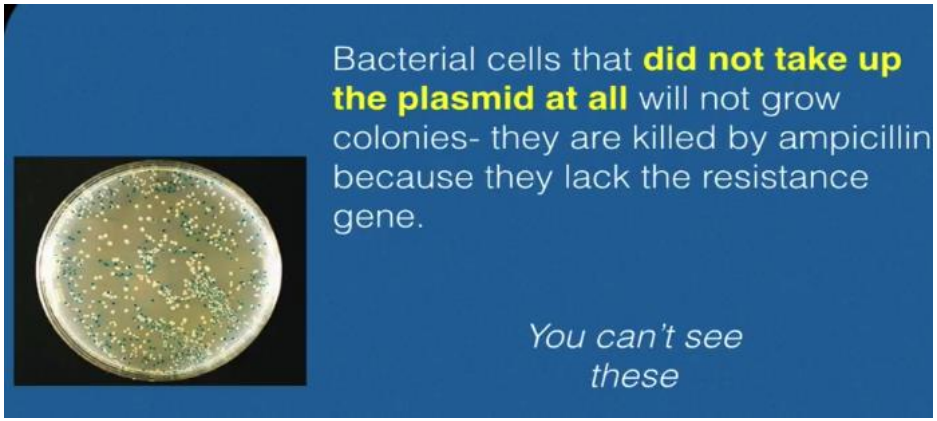
What is this ?????

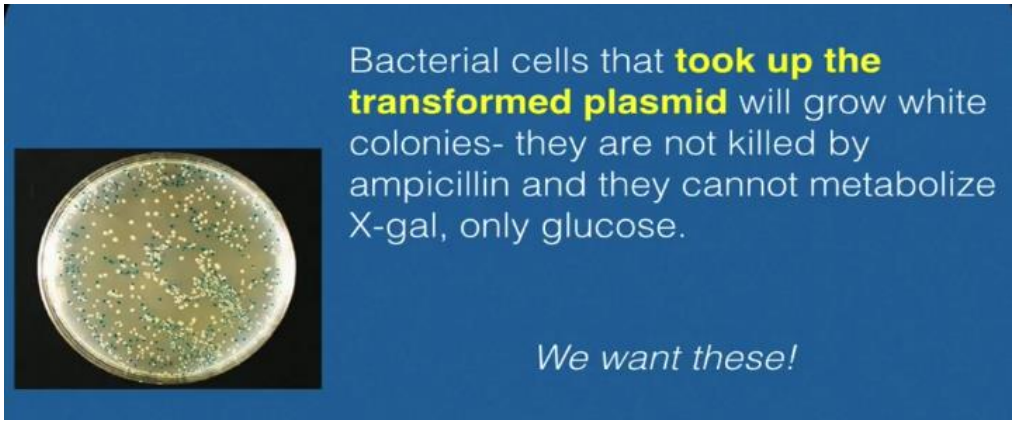
&

Why is this ?????



The possible situation after transformation





Final purification of the bacteria containing the gene of interest

- **Practical Features of DNA Cloning Vectors**
  - Size – small enough to be separated from chromosomal DNA of host plasmid
  - Origin of replication (ori) – site for DNA replication that allow plasmids to replicate independently from host chromosome
    - Copy number: number of plasmids in the cell (normally small but plasmids have high copy numbers)
  - Multiple cloning site (MCS) – recognition sites for several restriction enzymes in which insert is cloned into
  - Selectable marker genes – allow to select for transformed colonies
  - RNA polymerase promoter sequences – used for transcription *in vitro* and *in vivo*
  - DNA sequencing primers

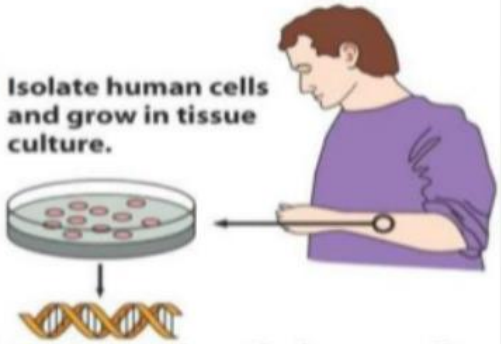
TABLE 3.2 A COMPARISON OF DNA VECTORS AND THEIR APPLICATIONS

Vector Type	Maximum Insert Size (kb)	Applications	Limitations
Bacterial plasmid vectors (circular)	~ 6–12	DNA cloning, protein expression, subcloning, direct sequencing of insert DNA	Restricted insert size; limited expression of proteins; copy number problems; replication restricted to bacteria
Bacteriophage vectors (linear)	~ 25	cDNA, genomic and expression libraries	Packaging limits DNA insert size; host replication problems
Cosmid (circular)	~ 35	cDNA and genomic libraries, cloning large DNA fragments	Phage packaging restrictions; not ideal for protein expression; cannot be replicated in mammalian cells
Bacterial artificial chromosome (BAC, circular)	~ 300	Genomic libraries, cloning large DNA fragments	Replication restricted to bacteria; cannot be used for protein expression
Yeast artificial chromosome (YAC, circular)	200–2,000	Genomic libraries, cloning large DNA fragments	Must be grown in yeast; cannot be used in bacteria
Ti vector (circular)	Varies depending on type of Ti vector used	Gene transfer in plants	Limited to use in plant cells only; number of restriction sites randomly distributed; large size of vector not easily manipulated

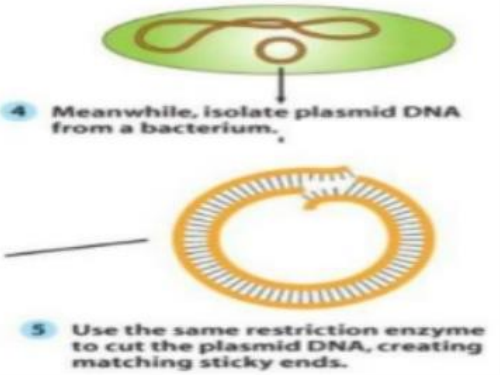
Important

- Types of Vectors
  - Bacterial plasmid vectors – can clone inserts that are smaller than 7 kb; some express eukaryotic proteins from genes poorly
  - Bacteriophage vectors
  - Cosmid vectors
  - Expression vectors
  - Bacterial Artificial Chromosomes (BAC)
  - Yeast Artificial Chromosomes (YAC)
  - Ti vectors

1 Isolate human cells and grow in tissue culture.

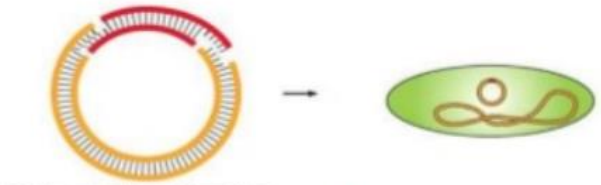


2 Isolate DNA from the human cells.



4 Meanwhile, isolate plasmid DNA from a bacterium.

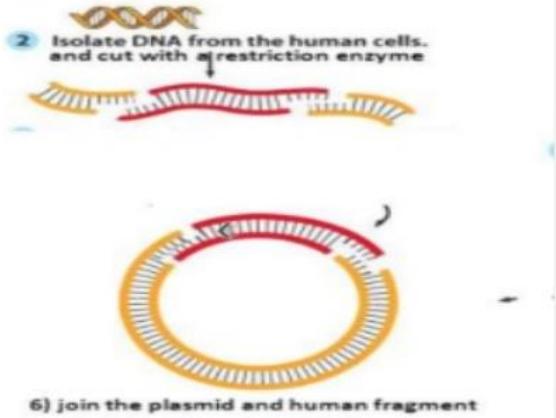
5 Use the same restriction enzyme to cut the plasmid DNA, creating matching sticky ends.



Mix the recombinant plasmid with bacteria.

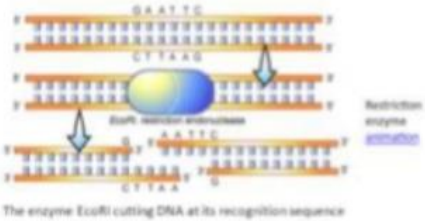
7 Allow new bacteria to incorporate the recombinant plasmid into the bacterial cell, then screen bacteria to find the ones that have incorporated the human gene for insulin.

Screening bacterial cells to learn which contain the human insulin gene is the hard part.



6) join the plasmid and human fragment

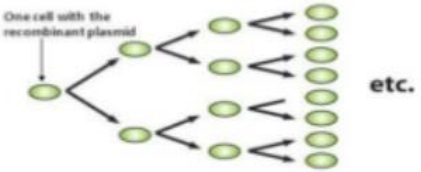
Restriction Enzymes are Enzymes That Cut DNA Only at Particular Sequences



The enzyme EcoRI cutting DNA at its recognition sequence

Different restriction enzymes have different recognition sequences.

This makes it possible to create a wide variety of different gene fragments.



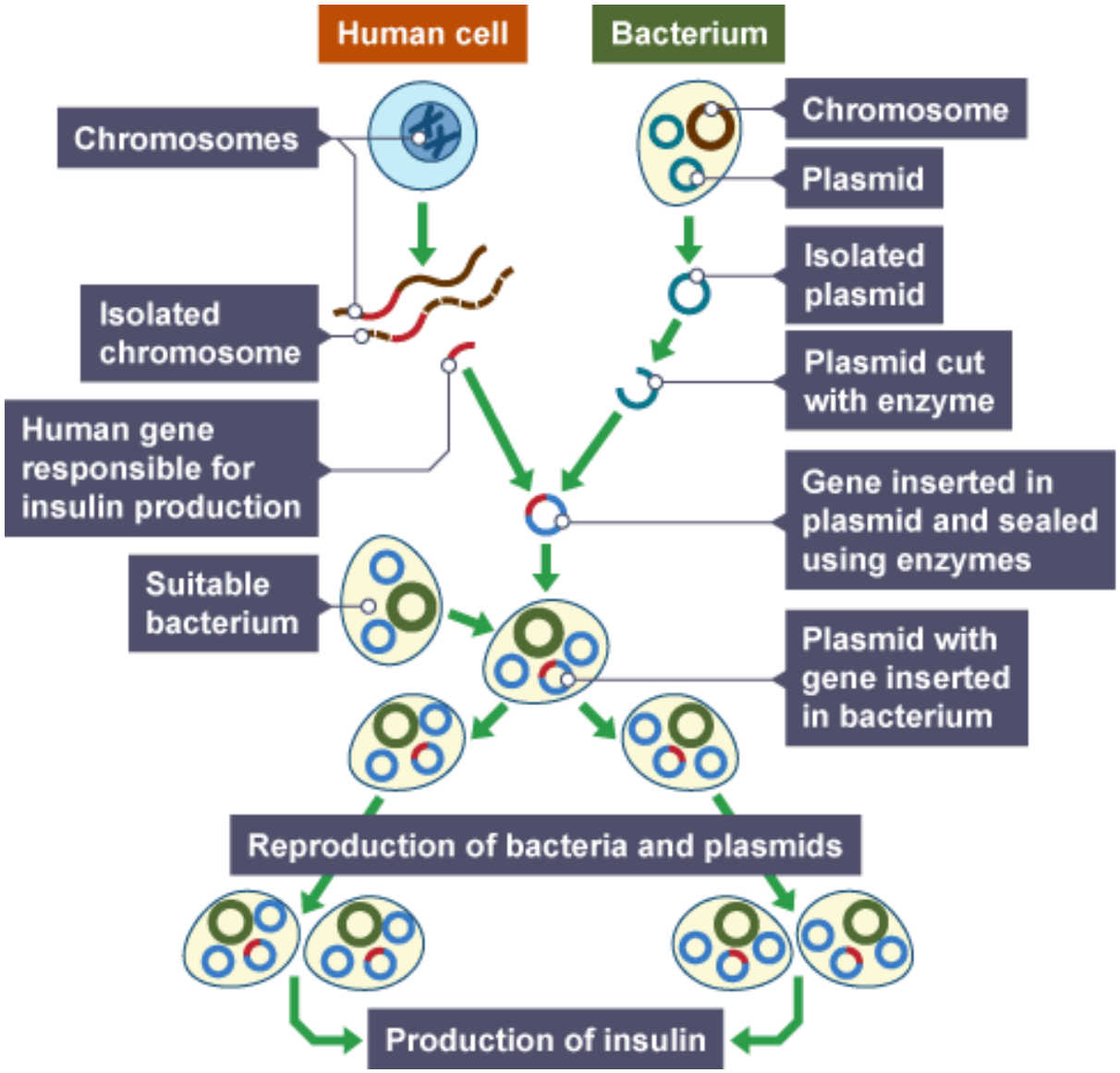
8 Grow trillions of new insulin-producing bacteria.

A fermentor used to grow recombinant bacteria.

This is the step when gene cloning takes place.

The single recombinant plasmid replicates within a cell.

Then the single cell with many recombinant plasmids produces trillions of like cells with recombinant plasmid – and the human insulin gene.


Human cell
Bacterium
Chromosomes
Chromosome
Plasmid
Isolated chromosome
Isolated plasmid
Plasmid cut with enzyme
Human gene responsible for insulin production
Gene inserted in plasmid and sealed using enzymes
Suitable bacterium
Plasmid with gene inserted in bacterium
Reproduction of bacteria and plasmids
Production of insulin

Industrial engineering steps followed in product development