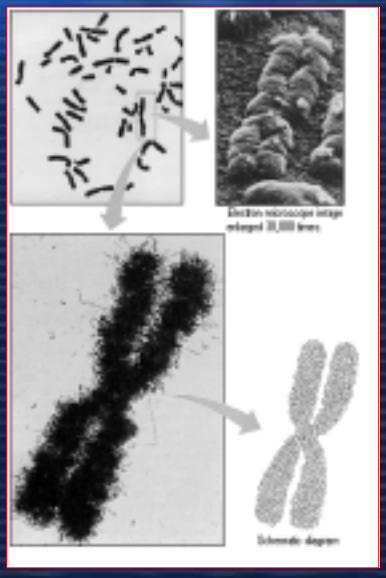
Reference

- T.A. Brown, Gene Cloning, Chapman and Hall
- S.B. Primrose, Molecular Biotechnology, Blackwell

Why Gene Cloning is Important?

- A century ago, Gregor Mendel :
  - Basic assumption (each heritable property of an organism) is controlled by a factor (gene).
- In 1900, Mandel's law → the birth of genetics.
- what these genes are and exactly how they work

The Early Development of Genetics

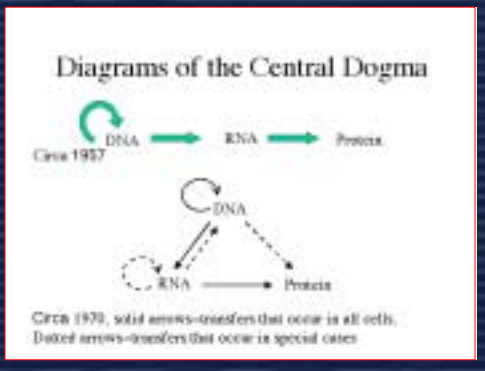


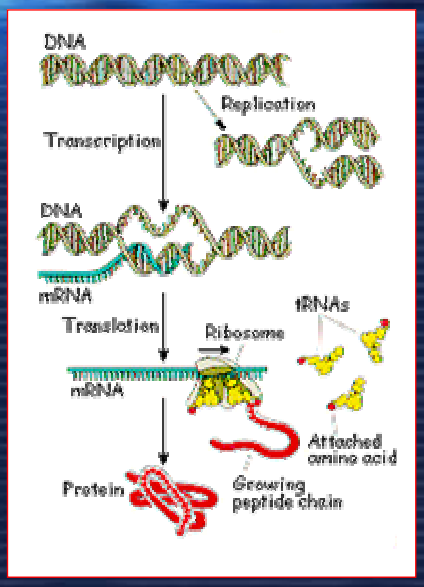
- In 1903, Sutton, W
  - Proposed that genes reside on chromosomes

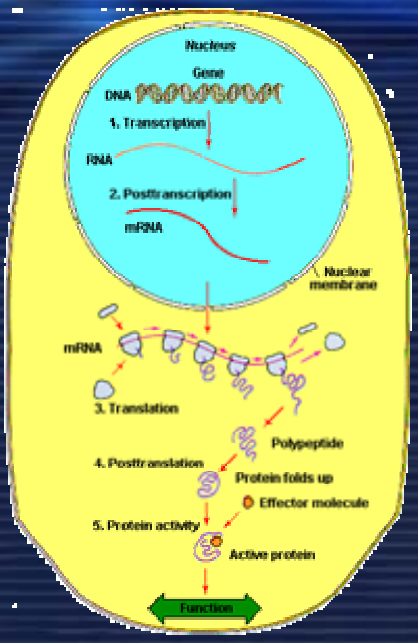
- In 1910, Morgan, TH
  - Experimental backing on that --> development of the techniques for gene mapping (To establish the structure or structural details or location)
  - By 1922, a comprehensive analysis of the relative positions of over 2000 genes on the four chromosomes of the fruit fly.

    (*Drosophilia melanogaster*)
- In 1944, Avery, MacLeod and McCarty
- In 1952, Hershey and Chase
  - Experimental results were shown that DNA is the genetic material.
  - Conventional idea : genes were made of protein

- In 1952-1966, Delbruck, Chargaff, Crick and Monod
  - The structure of DNA was elucidated.
  - The genetic code was cracked.
  - The process of transcription and translation were described.







The Advent of Gene Cloning

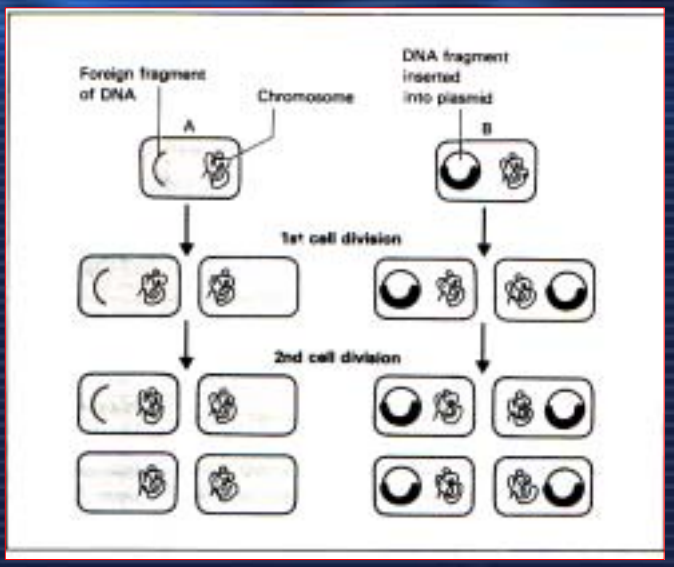
- In the late 1960's ; The experimental techniques were not sophisticated.
- In 1971 ~ 1973 ; A new experimental techniques were developed.
  - Recombinant DNA technology or Genetic engineering based on the process of gene cloning
  - This led to rapid and efficient DNA sequencing techniques that enabled the structures of individual genes to be determined.
- In the 1990s ; started with massive genome sequencing projects including the human project. This led to procedures for studying the regulation of individual genes.

- **Genome : A complete single set of the genetic material of a cell or of an organism.**

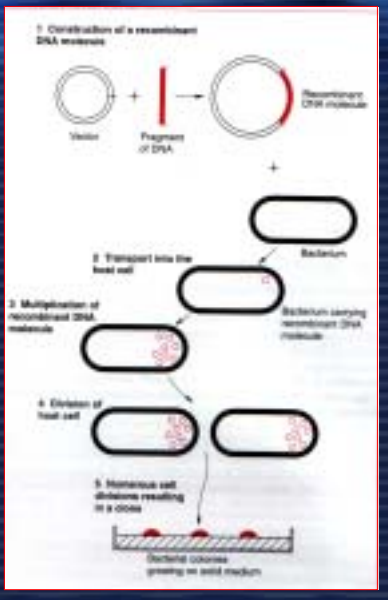
- From the techniques, modern biotechnology is appeared for the production of proteins and other compounds needed in medicine and industrial processes.

Introduction to Gene Cloning

- Let's suppose that we wish to construct a bacterium that produces human insulin.
- It might be thought that all that is required is to introduce the human insulin gene into its new host.
- Case A : The enzyme DNA polymerase, which makes copies of the DNA, does not initiate the process at random but at selected sites known as origins of replication.
  - Replication: The process whereby a new daughter DNA molecule is synthesized from a parent DNA molecule.
- Case B : Recombinant DNA technology - Replication of the insulin gene in its new host by inserting the gene into a cloning vector. A cloning vector is simply a DNA molecule possessing an origin of replication and which can replicate in the host cell of choice.



Basic Steps of Gene Cloning



Basic Steps of Gene Cloning

1) A fragment of DNA , containing the gene to be cloned, is inserted into a circular DNA molecule (vector) → "Recombinant DNA molecule" or "Chimera"
2) The vector acts as a vehicle that transports the gene into a host cell (usually, bacterium) → possibly other types of living cell.
3) Within the host cell the vector multiplies, producing numerous identical copies not only of itself but also of the gene that it carries.

Basic Steps of Gene Cloning

4) When the host cell divides, copies of the recombinant DNA molecule are passed to the progeny and further vector replication takes place.
5) After a large number of cell divisions, a colony, or clone, of identical host cells is produced. Each cell in the clone contains one or more copies of the recombinant DNA molecule. → The gene is cloned.

Cloning vehicles & Clone

- Cloning vehicles
  - DNA molecule must be capable of entering a host cell and replicating to produce multiple copies of itself.
- Clone
  - A group of cells derived from a single parental cell by asexual reproduction.

Two naturally occurring types of DNA molecule

- Plasmids
  - A small circles of DNA found in bacteria and some other organisms.
  - Plasmids can replicate independently of the host cell chromosome.
- Virus chromosomes
  - The chromosomes of bacterophages (viruses that specifically infect bacteria)
  - During infection, the bacteriophage DNA molecule is injected into the host cell where it undergoes replication.

Basic Skills for Handling DNA

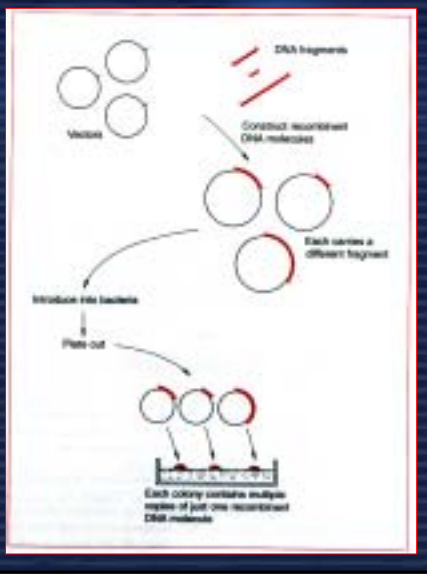
1. Preparation of pure samples of DNA (cloning vehicle and the gene)
1. Cutting DNA molecules (cut a specific point) ; purified restriction endonucleases
2. Analysis of DNA fragment sizes
3. Joining DNA molecules together ; purified ligases
4. Introduction of DNA into host cells
5. Identification of cells that contain recombinant DNA molecules

The Diversity of Cloning Vectors

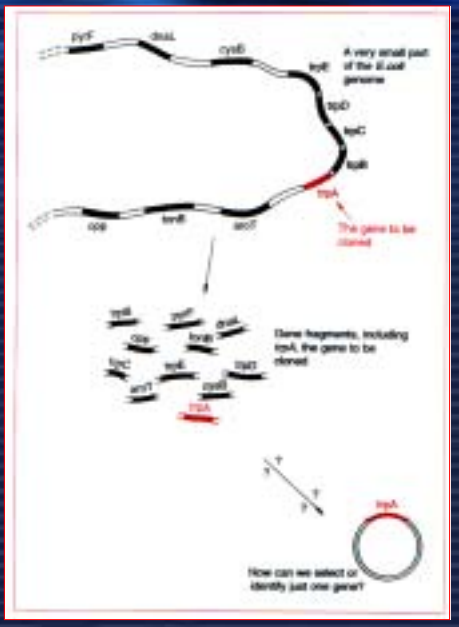
- A wide variety of different cloning vectors are available. All are derived from naturally occuring plasmids or viruses. Those could be modified in various ways.

Why Gene Cloning is so Important?

- Gene isolation by cloning
  - Cloning can provide a pure sample of an individual gene.

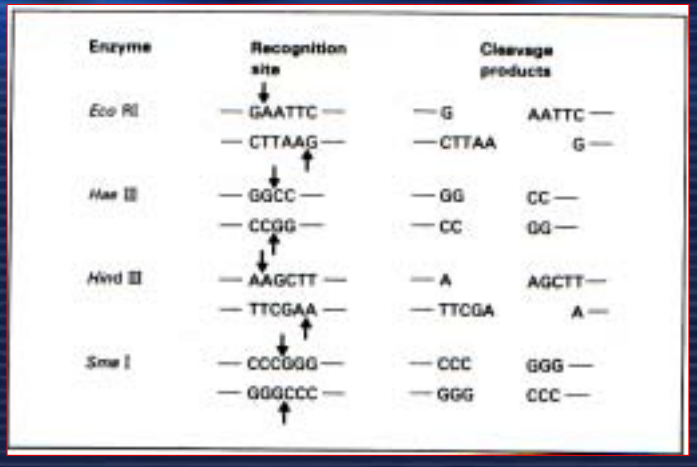


- The Problem of selection of just one gene.
- Only cells containing the desired recombinant DNA molecule can devide and the clone of interest is automatically selected.



Restriction Endonucleases.

- In order to insert foreign DNA into a plasmid, use is made of special enzymes known as restriction endonucleases.
- Restriction endonucleases
  - These enzymes cut large DNA molecules into shorter fragments by cleavage at specific nucleotide sequences called regognition sites.
  - These enzymes are highly specific deoxyribonucleases (DNases).

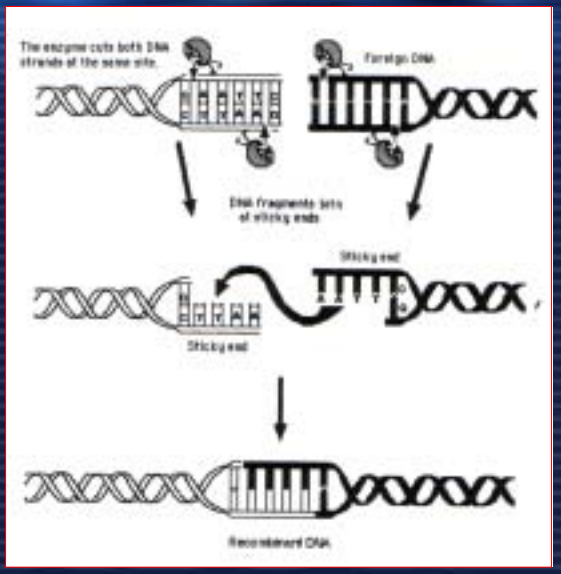


The recognition sites for some common restriction endonucleases.

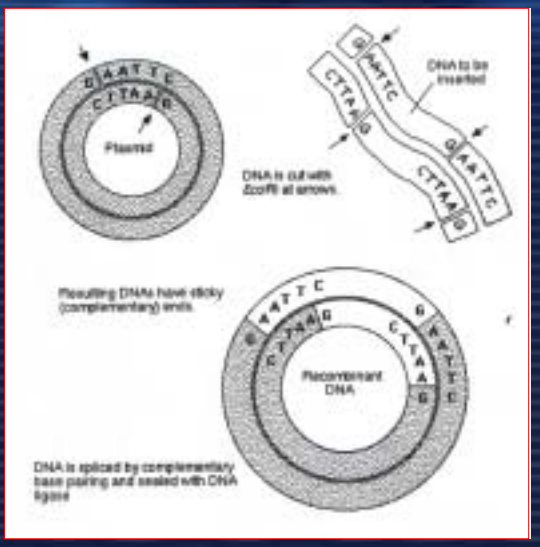
Examples of Restriction Enzymes

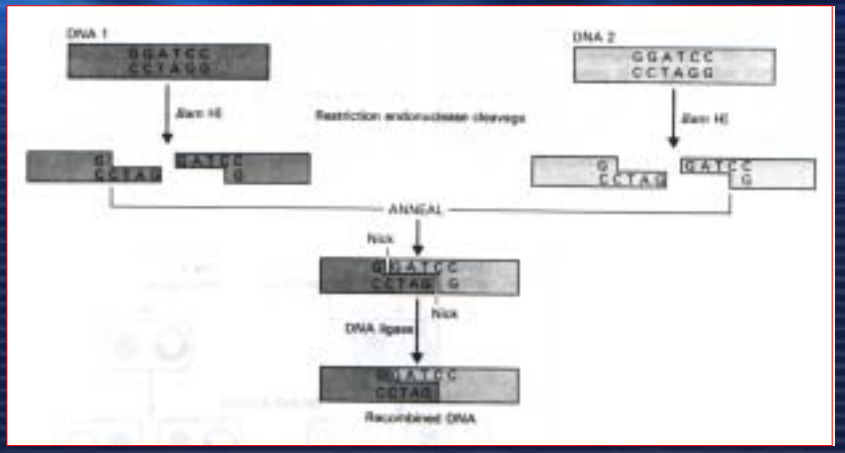
Enzyme	Organism from which derived	Target sequence (cut at •) 5' --->3'
Ava I	Anabaena variabilis	C• C/T C G A/G G
Bam HI	Bacillus amyloliquefaciens	G• G A T C C
Bgl II	Bacillus globigii	A• G A T C T
Eco RI	Escherichia coli RY 13	G• A A T T C
Eco RII	Escherichia coli R245	• C C A/T G G
Hae III	Haemophilus aegyptius	G G • C C
Hha I	Haemophilus haemolyticus	G C G • C
Hind III	Haemophilus influenzae Rd	A• A G C T T
Hpa I	Haemophilus parainfluenzae	G T T • A A C
Kpn I	Klebsiella pneumoniae	G G T A C • C
Mbo I	Moraxella bovis	•G A T C
Mbo I	Moraxella bovis	•G A T C
Pst I	Providencia stuartii	C T G C A • G
Sma I	Serratia marcescens	C C C • G G G
Sstl	Streptomyces stanford	G A G C T • C
Sal I	Streptomyces albus G	G • T C G A C
Taq I	Thermophilus aquaticus	T • C G A
Xma I	Xanthamonas malvacearum	C • C C G G G

Restriction Enzyme Action of EcoRI



Insertion of a DNA Sample into a plasmid



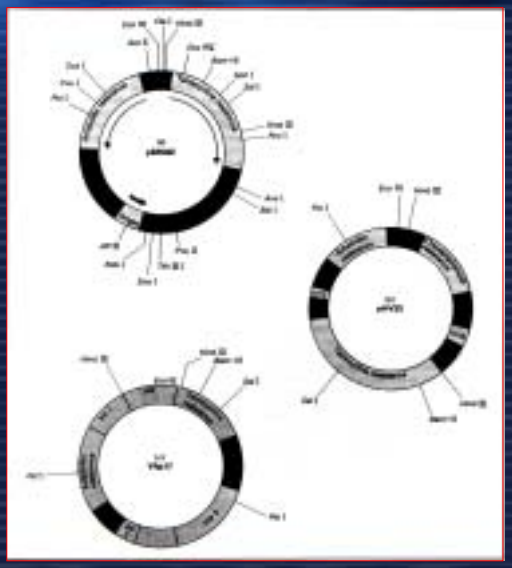


Fragments of the foreign DNA are inserted into plasmid vectors cut open with the same enzyme or one which produces a match end.

Sticky end & Blunt end

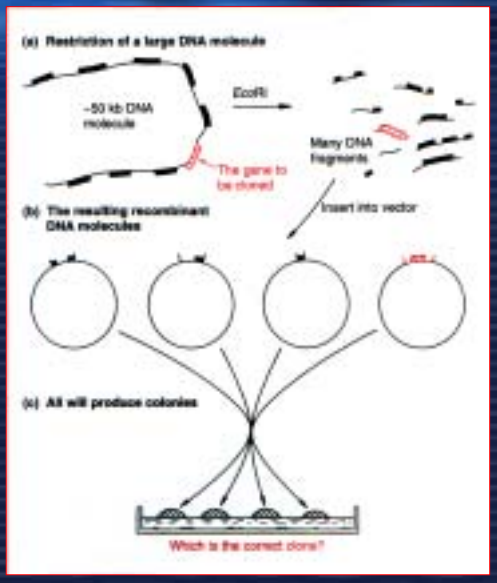
- Sticky end : Some enzymes cut the two helices a few base pairs apart, generating two fragments with single-strand protrusions called sticky end.
- Blunt end (Flush end) : Some enzymes make a simple double-stranded cut in the middle of the recognition sequence.

Three plasmid vectors used for gene cloning



a. The structure of pBR322, a vector used with E. coli.
b. The structure of pHV33, a vector which can replicate in both E. coli and B. subtilis.
c. The structure of YRp17, a vector which can replicate in both E. coli and yeast.

The Problem of Selection

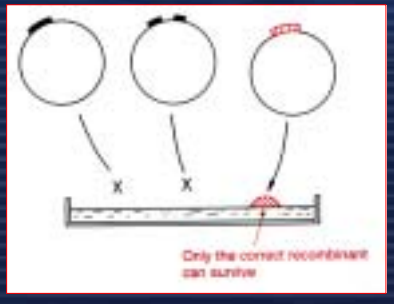


a. Even the simplest organisms, such as *E. coli*, contain several thousand genes, and a restriction digest of total cell DNA will produce not only the fragment carrying the desired gene, but also many other fragments carrying all the other genes.

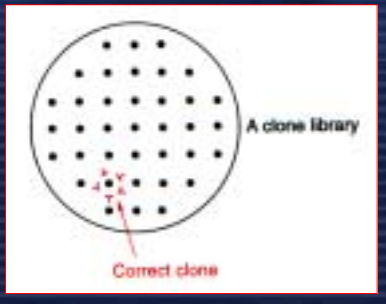
b. During the ligation reaction there is of course no selection for an individual fragment: numerous different recombinant DNA molecules are produced, all containing different pieces of DNA.

c. A variety of recombinant clones are obtained after transformation and plating out. After the correct one must be identified.

Two Basic Strategies for obtaining the Correct Clone

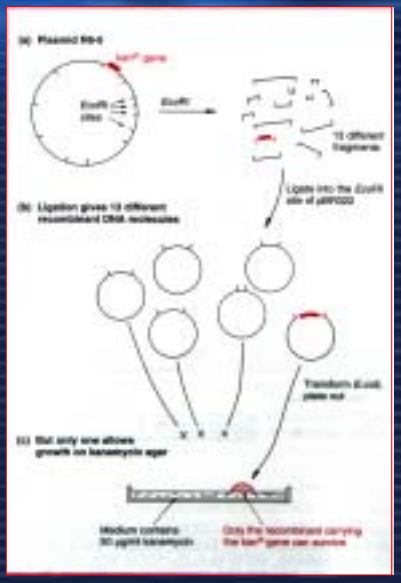


Direct selection



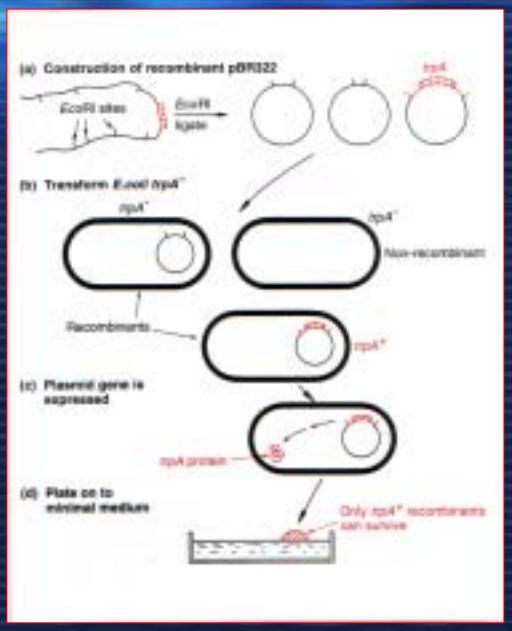
Clone identification

Direct Selection



- Plating-out method : only the correct recombinant can survive.
- The simplest example of direct selection occurs when the desired gene specifies resistance to an antibiotic (kanamycin).

- An experiment to clone the gene for kanamycin resistance from plasmid R 6-5 to pBR322
  a. This plasmid carries genes for resistances to several antibiotics. The kanamycin resistance gene lies within one of the 13 *Eco*RI fragments.
  b. To clone this gene the EcoRI fragments of R6-5 would be inserted into the *Eco*RI site of a vector such as pBR322.
  c. In this case, kanamycin resistance gene can be used as the selectable marker. Transformants are plated on to kanamycin agar, on which the only cells able to survive and produce colonies are those recombinants that contain the cloned kanamycin resistance gene.



- An experiment to clone the gene *trp*A from *E. coli.*
- This gene codes for the enzyme tryptophan synthase, which is involved in biosynthesis of the essential amino acid tryptophan.
- *trp*A- : a mutant strain of *E. coli* that has a non-functional *trp*A gene and is able to survive only if tryptophan is added to the

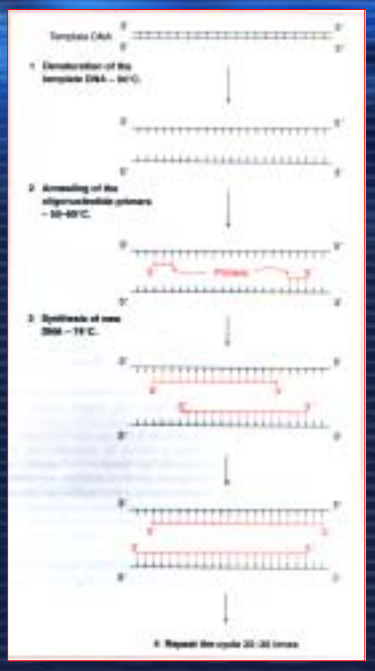
a. E. coli mutant can be used to clone the correct version of the *trp*A gene. Digestion with a restriction endonuclease, followed by ligation into a vector, produces numerous recombinant DNA molecules, one of which may, with luck, carry an intact copy of the *trp*A gene(functional gene).
b. The ligation mixture is used to transform the auxotrophic *E. coli* trpA- cells.
c. The vast majority of the resulting transformants will be auxotrophic, but a few have the plasmid-borne copy of the correct *trg*ene. These recombinants are non-auxotrophic; they no longer require tryptophan as the cloned gene is able to direct production of tryptophan synthase.
d. Direct selection is performed by plating transformants onto minimal medium, which has no tryptophan. Auxotrophs cannot grow on minimal medium, so the only colonies to appear are recombinants that contain the cloned *trp*A gene.

Identification of the Clone From a Gene Library

- Analysis of the individual clones to identify the correct one.
- Problems of marker rescue;
  - Many bacterial mutants are not auxotrophs.
  - Foreign genes (from animals and plants) sometimes do not function in the bacterial cell.

The Polymerase Chain Reaction (PCR)

- **In 1985, Kary Mullis invented the polymerase chain reaction (PCR)**
- **What is PCR?**
  - **PCR is carried out in a single test tube simply by mixing DNA with a set of reagents and placing the tube in a thermal cycler, a piece of equipment that enables the mixture to be incubated at a series of temperatures that are varied in a preprogrammed manner.**

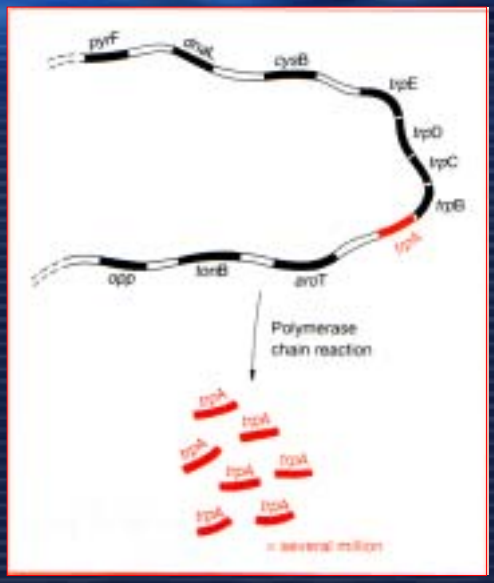


The basic steps in the PCR

Basic Steps in PCR

1) The mixture is heated to 94˚C, at which temperature the hydrogen bonds that hold together the two strands of the double-stranded DNA molecule are broken, causing the molecule to **denature**.
2) The mixture is cooled down to 50 - 60˚C. the two strands of each molecule could join back together at this temperature, but most do not because the mixture contains a large excess of short DNA molecules, called **oligonucleotides** or **primers**, which **anneal** to the DNA molecules at specific positions.
3) The temperature is raised to 74˚C. This is the optimum working temperature for the ***Taq* DNA polymerase** that is present in the mixture. This enzyme attaches to one end of each primer and synthesizes new strands of DNA, complementary to the template DNA molecules, during this step of the PCR.
4) The temperature is increased back to 94˚C. The double-stranded DNA molecules, each of which consists of one strand of the original molecule and one new strand of DNA, denature into single strands. By repeating the cycle 25 times the double-straned molecule that we began with is converted into over 50million new double-stranded molecules.

Gene Isolation by PCR



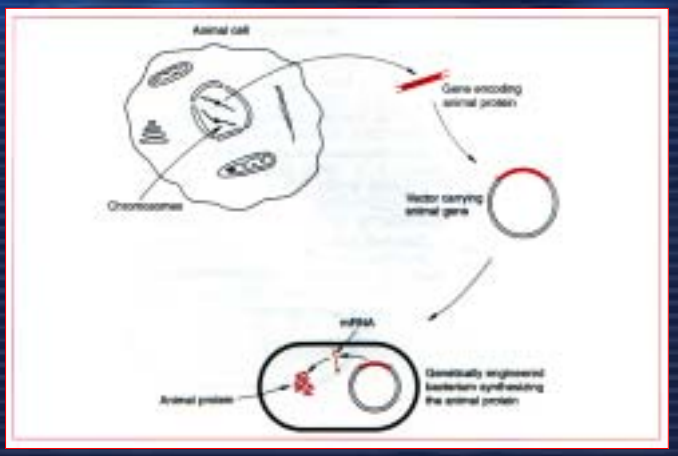
The PCR can also be used to obtain a pure sample of a gene. If the primers anneal either side of the gene of interest, many copies of that gene will be synthesized. A PCR experiment can be completed in a few hours, whereas it takes weeks if not months to obtain a gene by cloning.

Why then is Gene Cloning Still Used?

1) **It is easy to synthesize a primer with a preddetermined sequence, but if the sequences of the annealing sites are unknown then the appropriate primers cannot be made. In this case, PCR cannot be used to isolate genes and that has to be done by cloning.**
2) **There is a limit to the length of DNA sequence that can be copied by PCR. Five kilobases(kb) can be copied fairly easily, and segments to 40kb can be dealt with using specialized techniques, but this is shorter than the lengths of many genes, especially those of humans and other vertebrates. Cloning must be used for long gene.**

- **Even if the sequence of a gene is not known, it may still be possible to determine the appropriate sequences for a pair of primers, based on what is known about the sequence of the equivalent gene in a different organism.**

The Impact of Cloning on Research and Biotechnology

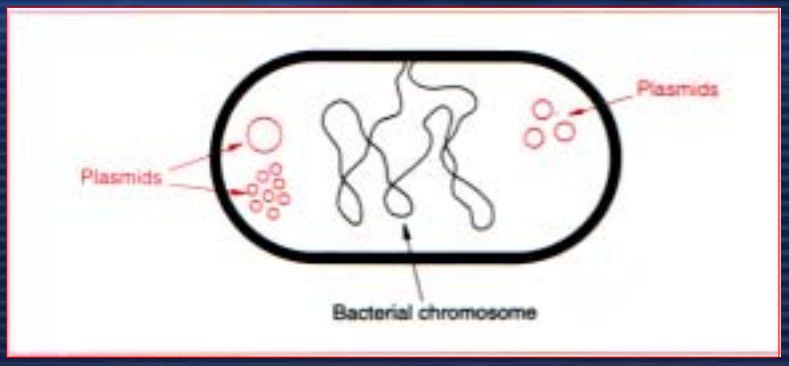


Gene cloning, PCR, the recombinant techniques provide a way in which mammalian proteins can be produced in bacterial cells.

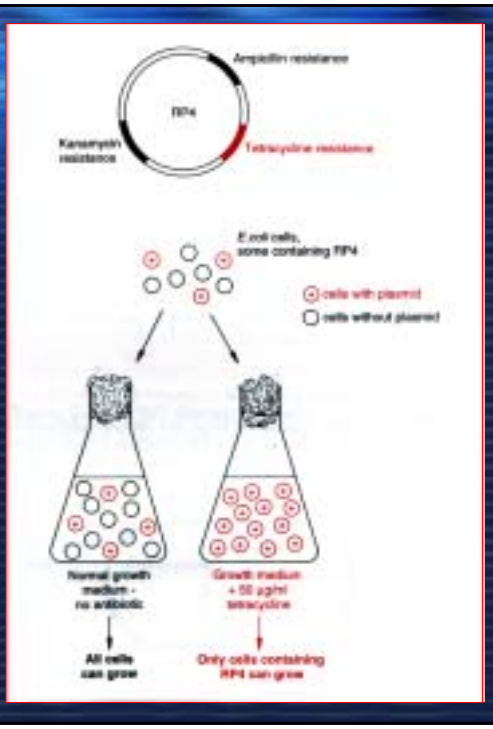
Vehicles : Plasmids and Bacteriophages

- Numerous copies of the recombinant DNA molecule can be produced and passed to the daughter cells.
- A cloning vehicle needs to be relatively small, ideally less than 10kb in size; large molecules tend to break down during purification and also more difficult to manipulate.
- The most important type : plasmids and bacteriophage chromosomes.

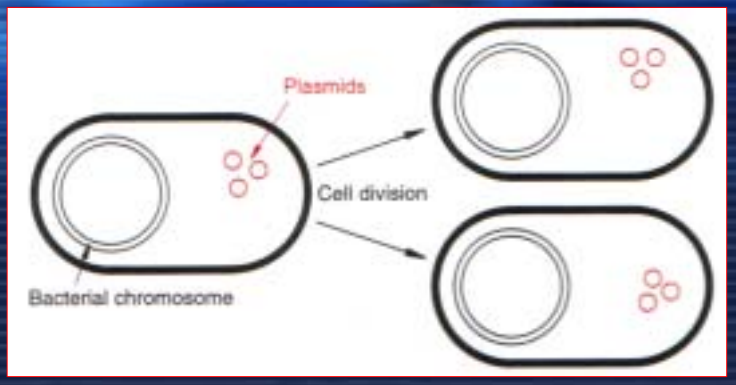
Basic Features of Plasmids



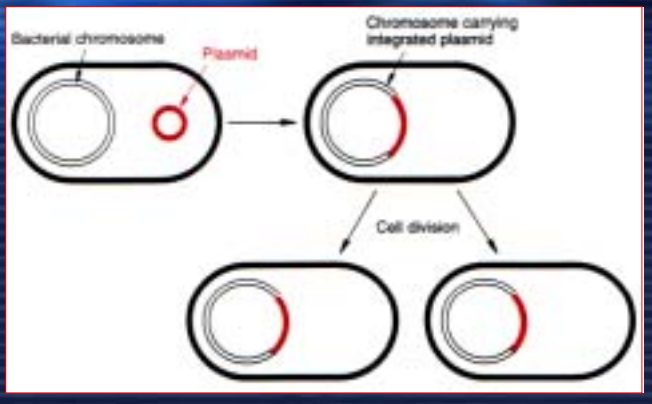
- Plasmids : a circular molecules of DNA that lead to an independent existence in the bacterial cell.



- It carries antibiotic resistance genes. In the Lab., it was used as a selectable marker to ensure that bacteria in a culture contain a particular plasmid.



- All plasmids posses at least one DNA sequence that can act as an origin of replication ; multiply independently of the main bacterial chromosome (non-integrative plasmid)
- The smaller plasmids make use of the host cell's own DNA replicative enzymes in order to make copies of themselves. The larger plasmids carry genes that code for special enzymes that are specific for plasmid replication.



- A few types of plasmid are also able to replicate by inserting themselves the bacterial chromosome (Integrative plasmids : episomes)
- At some stage these plasmids exist as independent elements.

Size and Copy Number

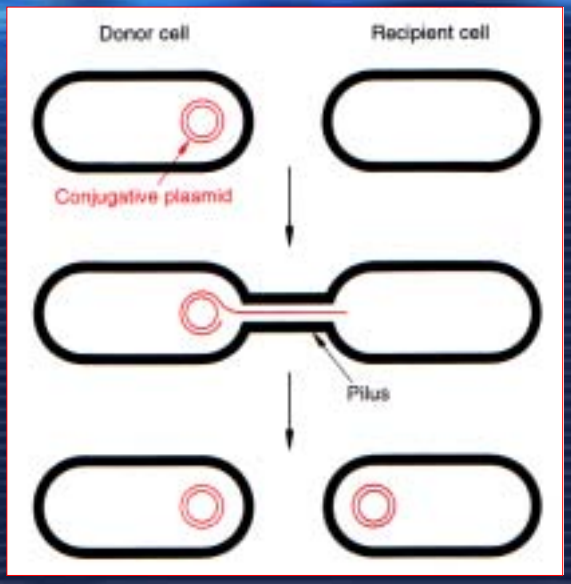
- Plasmid range : 1 ~ 250kb

Plasmid	Size		Organism
	Nucleotide length (kb)	Molecular wt (MDa)	
pUC8	2.1	1.8	*E. coli*
ColEI	6.4	4.2	*E. coli*
RP4	54	36	*Pseudomonas* + others
F	95	63	*E. coli*
TOL	117	78	*Pseudomonas putida*
pTiAch5	213	142	*Agrobacterium tumefaciens*

- Large plasmids may be adapted for cloning under some circumstances.
- Copy number : the number of molecules of an individual plasmid that are normally found in a single bacterial cell.

Conjugation and Compatibility

- Conjugative plasmids
  - The ability to promote sexual conjugation between bacterial cells.
  - Conjugation and plasmid transfer are controlled by a set of transfer or *tra* genes.
- Non-conjugative plasmids
  - Absence of *tra* genes
  - Under some circumstances, it may be cotransferred along with a conjugative plasmid when both are present in the same cell.



Plasmid transfer by Conjugation between bacterial cells.

Plasmid Classification

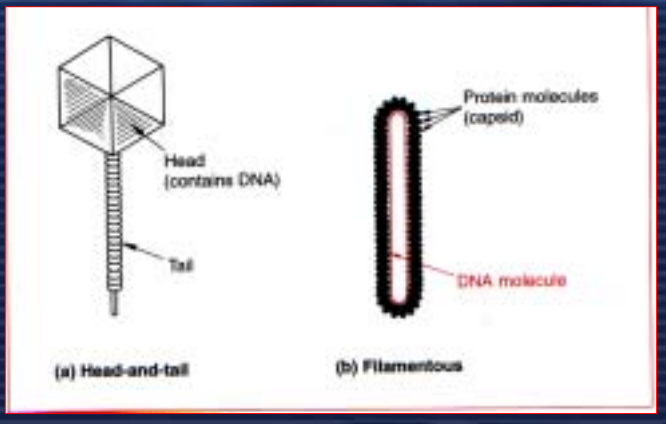
- It's based on the main characteristic coded by the plasmid genes.

1. Fertility or "F" plasmids
   - carry only *tra* genes
   - promote conjugal transfer of plasmids
   - Ex) F plasmid of *E. coli*
2. Resistance or "R" plasmids
   - carry genes conferring on the host bacterium resistance to one or more
   - antibacterial agents.
   - Ex) RP4 in Pseudomonas
3. Col plasmids
   - codes for colicins(proteins that kill other bacteria)
   - Ex) ColE1 of *E. coli*
4. Degradative plasmids
   - allows the host bacterium to metabolize unusual molecules such as toluene and salicylic acid
   - Ex) TOL of *Pseudomonas putida*
5. Virulence plasmids
   - confer pathogenicity on the host bacterium
   - Ex) Ti plasmids of *Agrobacterium tumefaciens*

Eukaryotic Plasmid

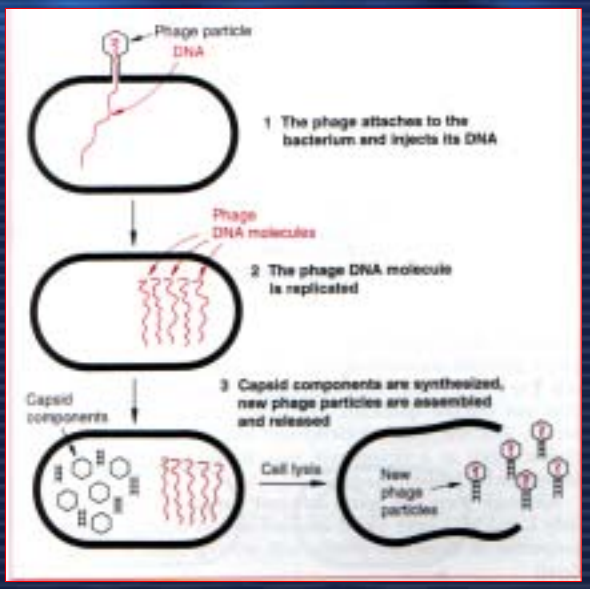
- *Saccharomyces cerevisiae*
  → 2 µm circle
- However, It is suspected that many higher organisms simply do not harbour plasmids within their cells.

Basic Features of Bacteriophages



Viruses that specifically infect bacteria

The General Pattern of Infection



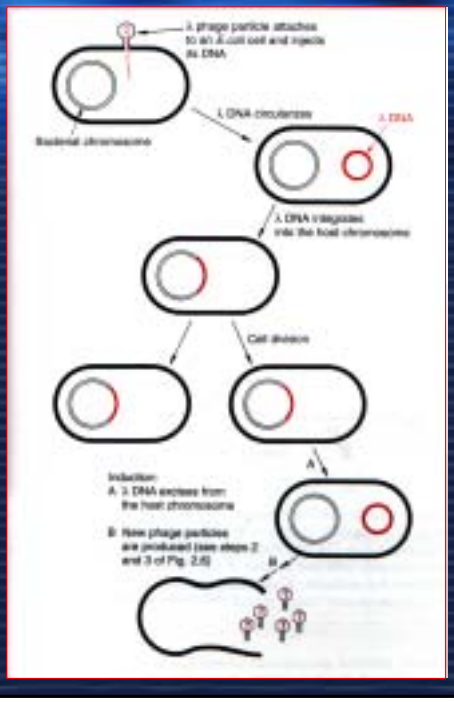
- The general pattern of infection is a three-step process.

1. The phage particle attaches to the outside of the bacterium and injects its DNA chromosome in to the cell.
2. The phage DNA molecule is replicated, usually by specific phage enzymes coded by genes on the phage chromosome.
3. Other phage genes direct synthesis of the protein components of the capsid, and new phage particles are assembled and released from the bacterium.

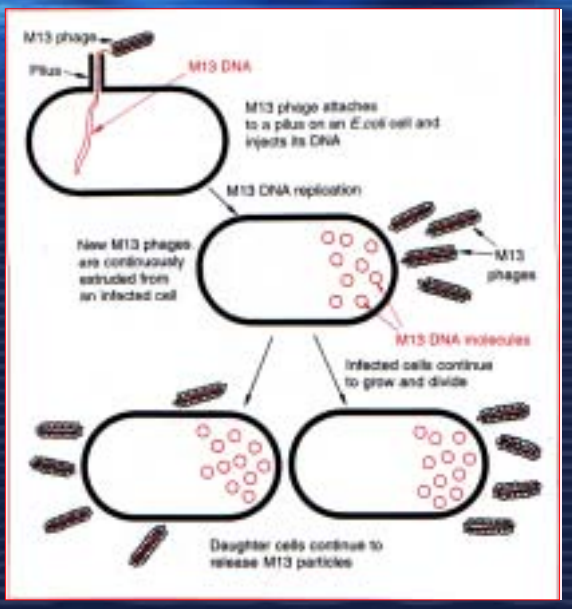
- Lytic cycle
  - The entire infection cycle takes less than 20 min. DNA replication is immediately followed by synthesis of capsid proteins, the phage DNA molecule is never maintained in a stable condition in the host cell.

Lysogenic Phages

- is characterized by retention of the phage DNA molecule in the host bacterium, possibly for many thousands of cell divisions.
- Prophage
  - The integrated form of the phage DNA (the phage DNA is inserted into the bacterial genome, similar to episomal insertion)
- Lysogen
  - A baterium (carries a prophage) usually physiologically indistinguishable from an uninfected cell.
- Prophage is eventually released from the host genome and the phage reverts to the lytic mode and lyses the cell.



The lysogenic infection cycle of bacteriophage λ



The infection cycle of bacteriophage M13

The M13 DNA is not integrated into the bacterial genome. Cell lysis never occurs.