RECOMBINANT DNA TECHNOLOGY

BASIC CONCEPTS

By:

DR. ANNIKA SINGH

DEPARTMENT OF BIOTECHNOLOGY

INSTITUTE OF BIOSCIENCE AND

BIOTECHNOLOGY

BASICS CONCEPTS

Engineering is the use of scientific principles to design and build something (machines, structures, and other items, tunnels,, vehicles, and buildings etc.)

The term *engineering* is derived from the Latin *ingenium*, i.e. "cleverness" and *ingeniare*, meaning "to contrive, devise"

Technology is the sum of techniques, skills, methods, and processes used in the production of goods or services or in the accomplishment of objectives, such as scientific investigation.

The simplest form of technology is the development and use of basic tools.

Genetic engineering, also called **genetic modification** or **genetic manipulation:** It is a set of technologies used to change the genetic makeup of cells, including the transfer of genes within and across species boundaries to produce improved or novel organism.

BASICS CONCEPTS

What Is A Gene?

Gene is a unit of hereditary information -:Gregor Mendel in 1866.

DNA is the genetic material and encoded the information required to produce a single enzyme : Oswald Avery and colleagues In 1944

Many genes encode proteins and functional RNA molecules.

A gene can be defined as the region of DNA (or RNA in case of virus) that controls a discrete hereditary characteristic, usually corresponding to a single protein or RNA. This includes the entire functional unit, along with coding (exons) and noncoding sequences (introns and regulatory sequences).

Major role of DNA within the cell:

- It functions as information repository including instructions for biosynthesis of different biomolecules of the cells.
- Pass on the information to the next generation.

BASICS CONCEPTS

What is genome?

Genome is the complete set of genetic information of a cell or an organism. In diploid organisms, it refers to the haploid set of chromosomes present in a cell.

What is recombinant DNA?

Recombinant DNA is any artificially created DNA molecule which brings together DNA sequences that are not usually found together in nature.

What is Cloning: The propagation of recombinant DNA inside a particular host cell so that many copies of the same sequence are produced is known as *cloning*.

Until the mid-1980s, all cloning was cell-based (rDNAhad to be introduced into a suitable host cell for amplification e.g. *E. coli*).

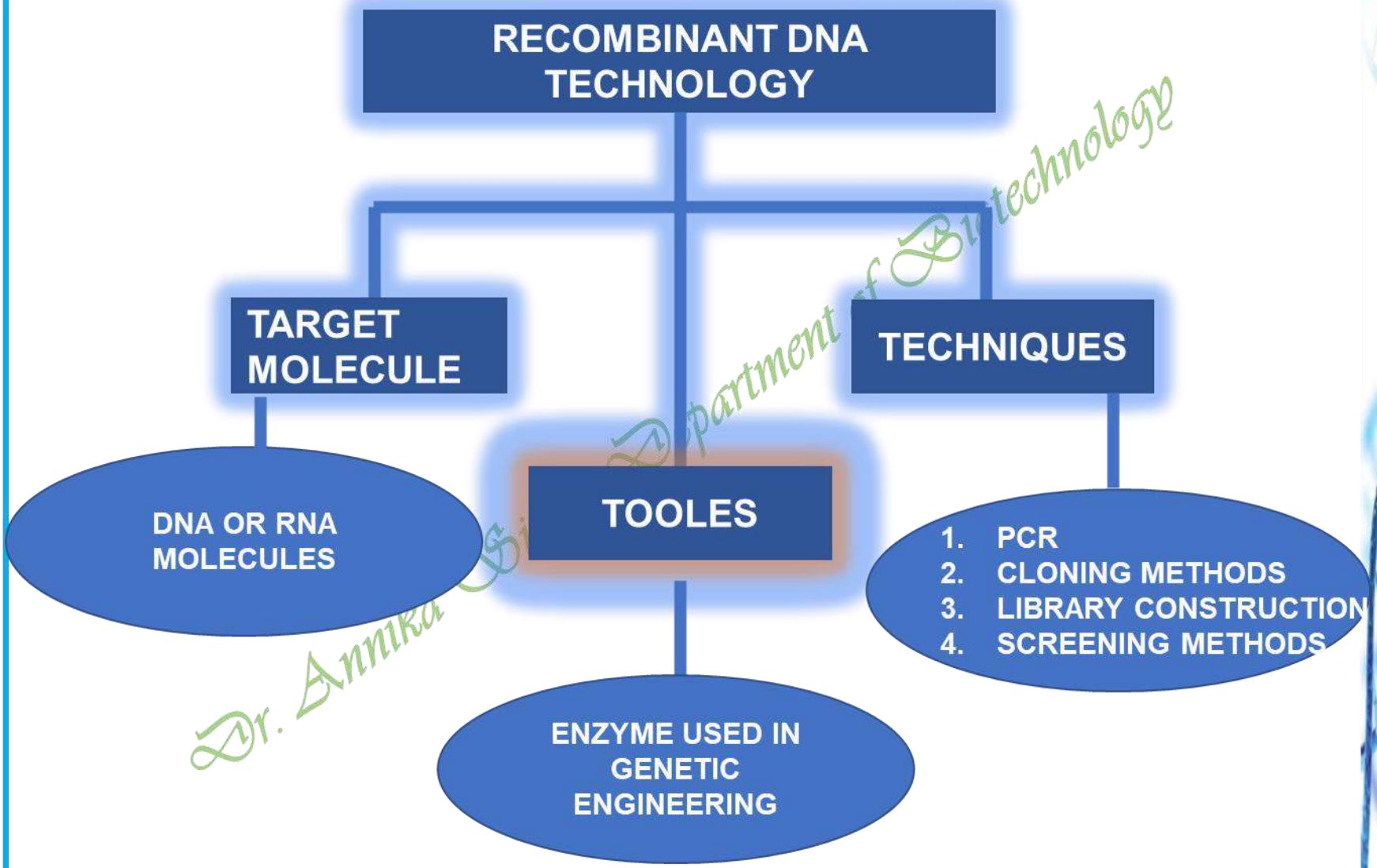
*Polymerase chain reaction (PCR,*1983*)* allowed DNA sequences to be amplified *in vitro* using pure enzymes.

BASICS CONCEPTS

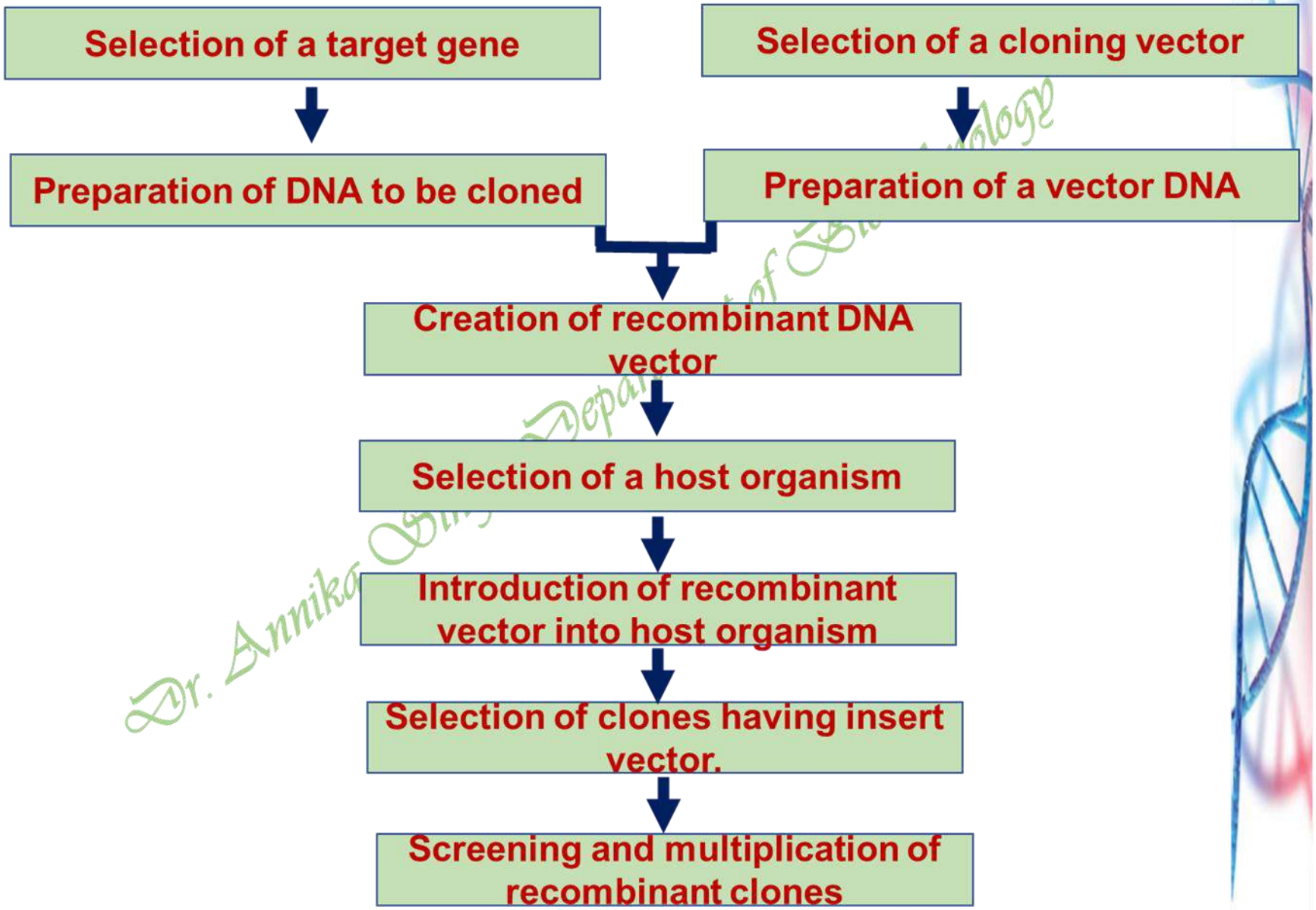
What is gene cloning?

The production of exact copies (*clones*) of a particular gene or DNA sequence using genetic engineering techniques.

- In 1972, Paul Berg created the first recombinant DNA molecules by combining DNA from the monkey virus SV40 with that of the lambda virus.
- In 1973 Herbert Boyer and Stanley Cohen created the first transgenic organism by inserting antibiotic resistance genes into the plasmid of an *Escherichia coli* bacterium.
- In 1974 world's first transgenic animal was created by Rudolf Jaenisch
- He developed transgenic mouse by introducing foreign DNA into its embryo,



MAJOR STEPS OF MOLECULAR CLONING



BASIC STEPS IN GENE CLONING

RECOMBINANT DNA

***Recombinant DNA* is any artificially created DNA molecule which brings togethe DNA sequences that are not usually found together in nature.**

GENE CLONING

The propagation of recombinant DNA inside a particular host cell so that many copies o the same sequence are produced is known as *cloning*.

VECTOR

In molecular cloning, a **vector** is a DNA molecule used as a vehicle to artificially carr foreign genetic material into another cell, where it can be replicated and/or expressed

A vector containing foreign DNA is termed recombinant DNA.

The four major types of vectors are

- Plasmids
- Viral Vectors
- Cosmids
- Artificial Chromosomes

BASIC STEPS IN GENE CLONING

Basic Steps In Gene Cloning

1. Construction of Recombinant DNA
2. Transport in to the Host
3. Multiplication of rDNA
4. Division of host cell
5. Production of a Clone

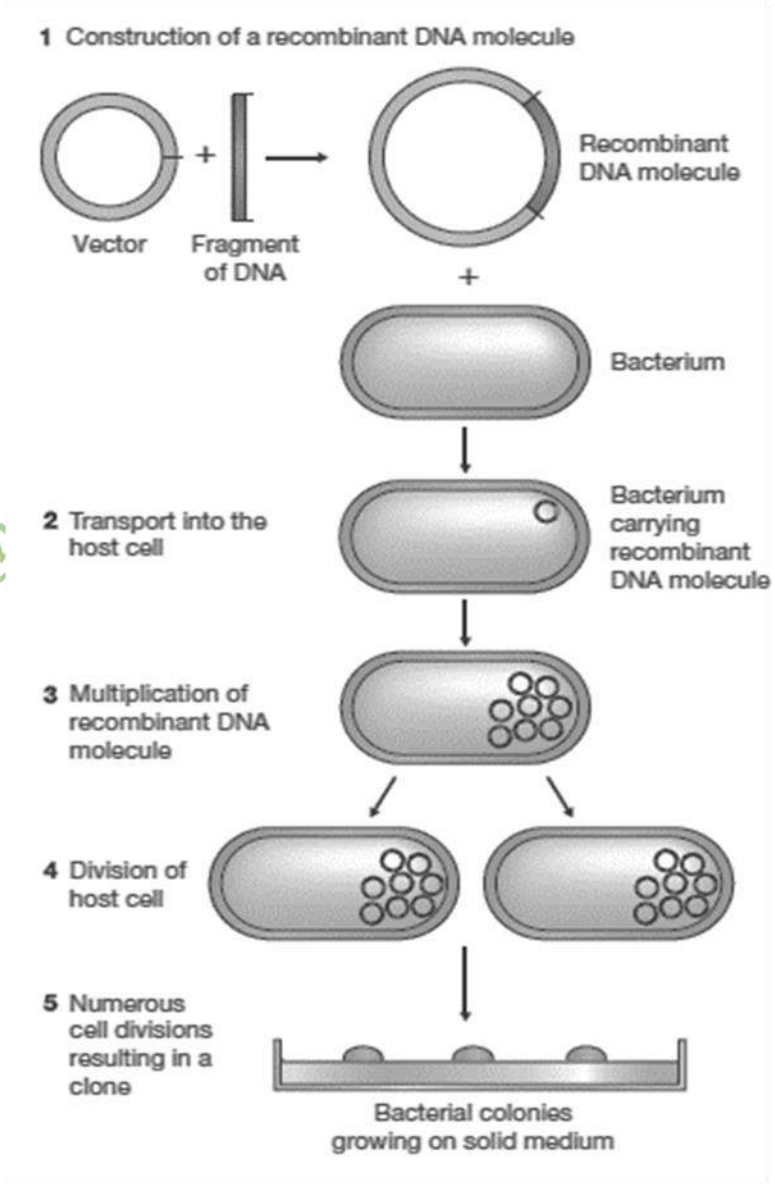


*Figure adapted from Gene cloning **and DNA Analysis:** An Introduction by **T.A. BROWN**

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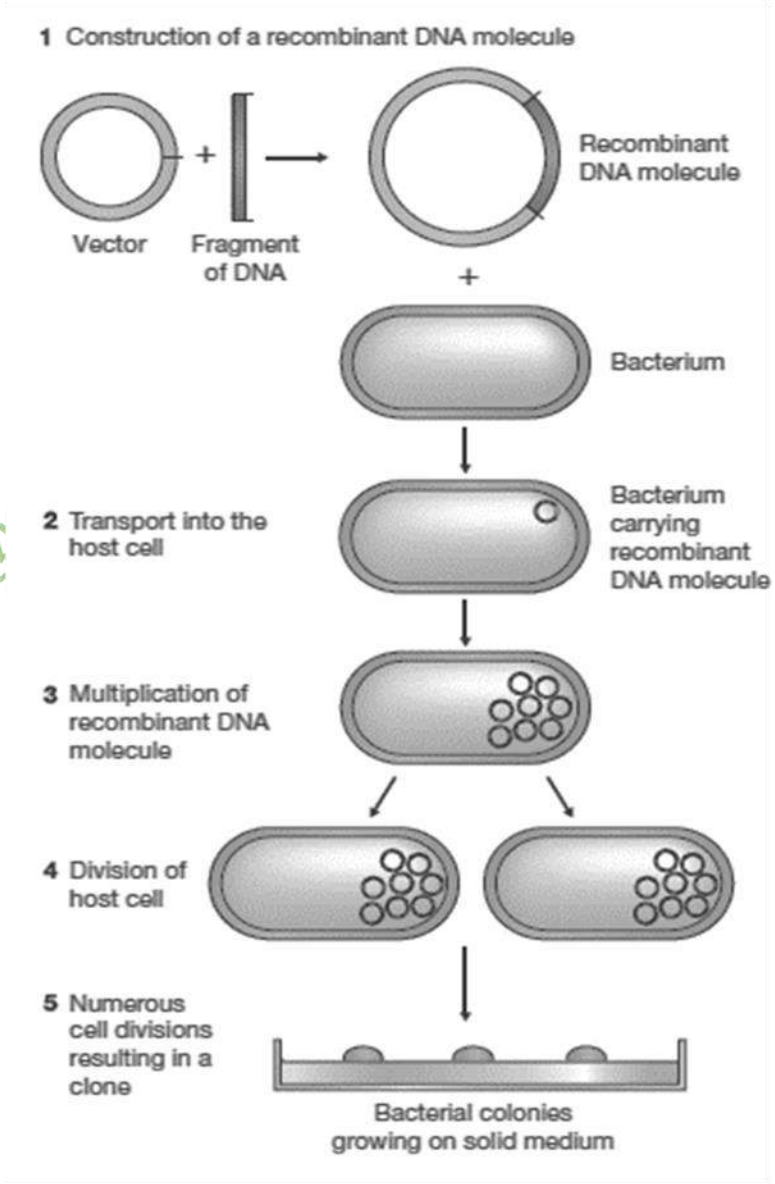

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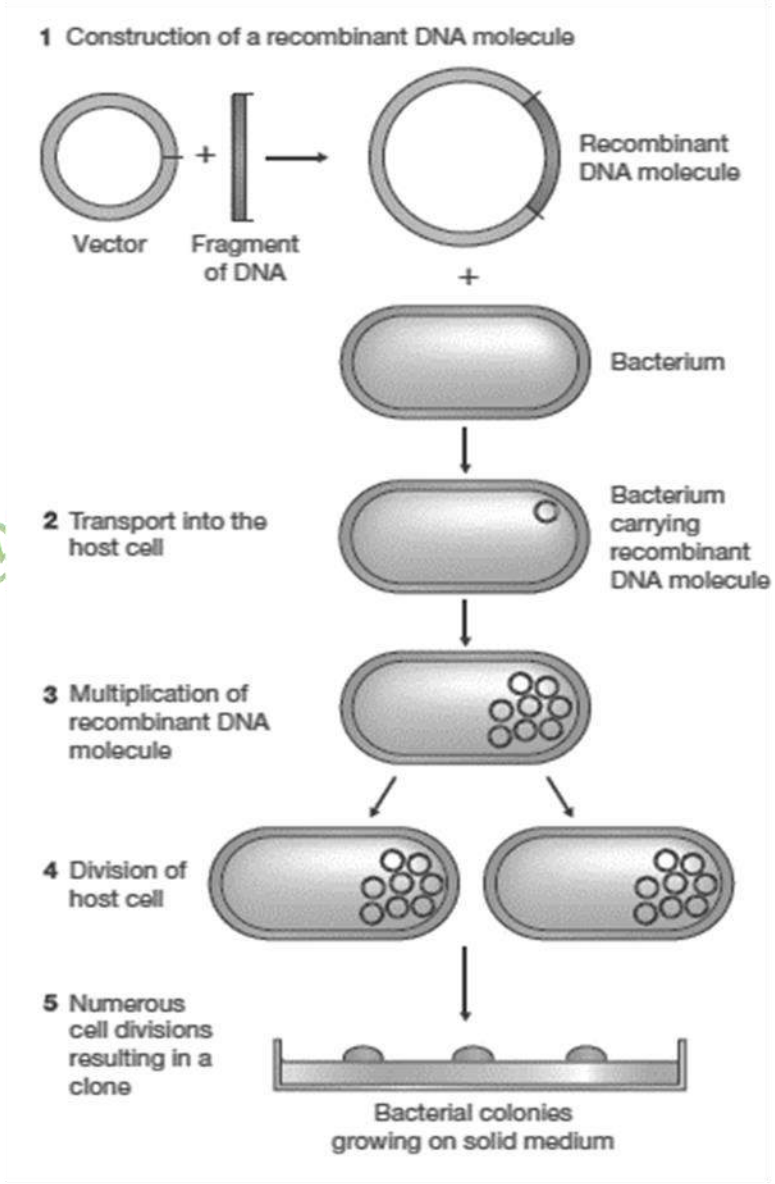

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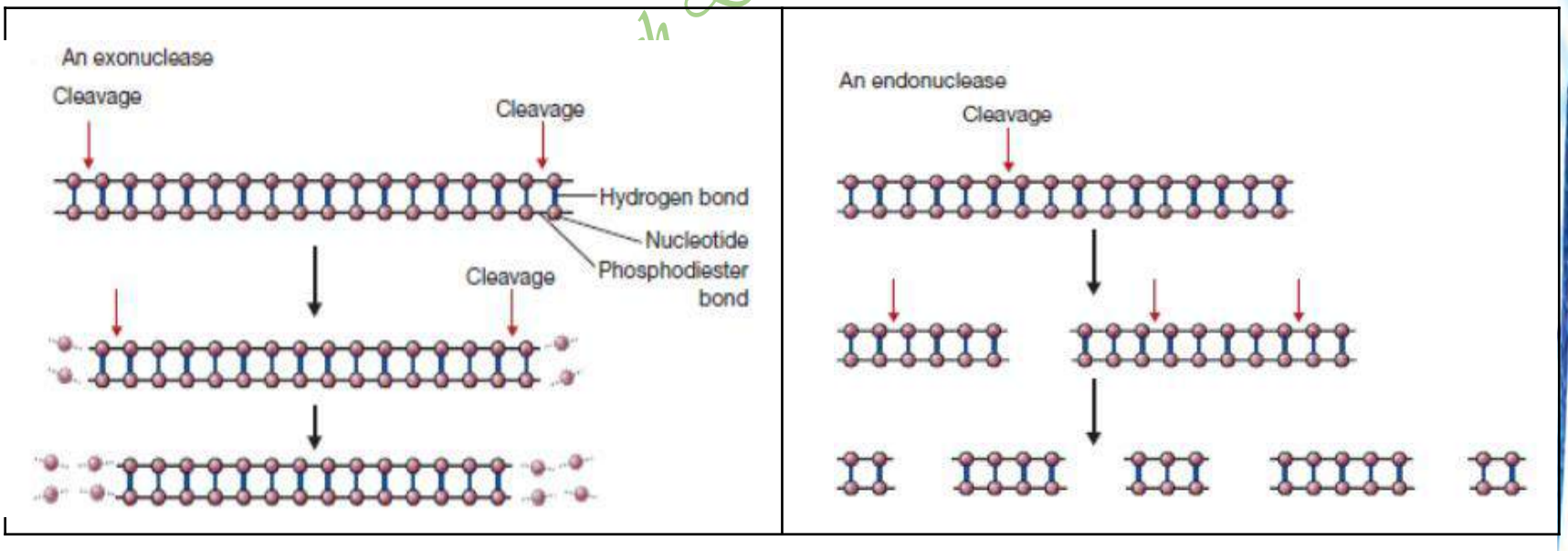
ENZYMES USED IN GENETIC ENGINEERING

NUCLEASES

Nucleases degrade DNA molecules by breaking the phosphodiester bonds that link one nucleotide to the next in a DNA strand. There are two different kinds of nuclease

1. **Exonucleases** remove nucleotides one at a time from the end of a DNA molecule

1. **Endonucleases** are able to break internal phosphodiester bonds within a DNA molecule.



ENZYMES USED IN GENETIC ENGINEERING

Exonucleases

Exonucleases are classified on the basis of types of strands hydrolysed

Bal31 removes nucleotides from both strands of a double-stranded molecule.

E. coli exonuclease III degrade just one strand of a double-stranded molecule, leaving single-stranded DNA as the product

ENDONUCLEASE

- **Endonucleases** are classified further on the basis of types of strands hydrolysed.
- S1 endonuclease (from the fungus *Aspergillus oryzae*) only cleaves single strands
- Deoxyribonuclease I (DNase I), which is prepared from cow pancreas, cuts both single- and double-stranded molecules.
- DNase I is non-specific in that it attacks DNA at any internal phosphodiester bond
- **Restriction endonucleases** cleave double-stranded DNA only at a limited number of specific recognition sites

ENZYMES USED IN GENETIC ENGINEERING

Endonuclease

The reactions catalysed by different **Endonuclease**.

(a) S1 nuclease, which cleaves only single-stranded DNA, including single-stranded nicks in mainly double-stranded molecules.

(b) DNase I, which cleaves both single- and double-stranded DNA.

(c) A restriction endonuclease, which cleaves double-stranded DNA, but only at a limited number of sites.

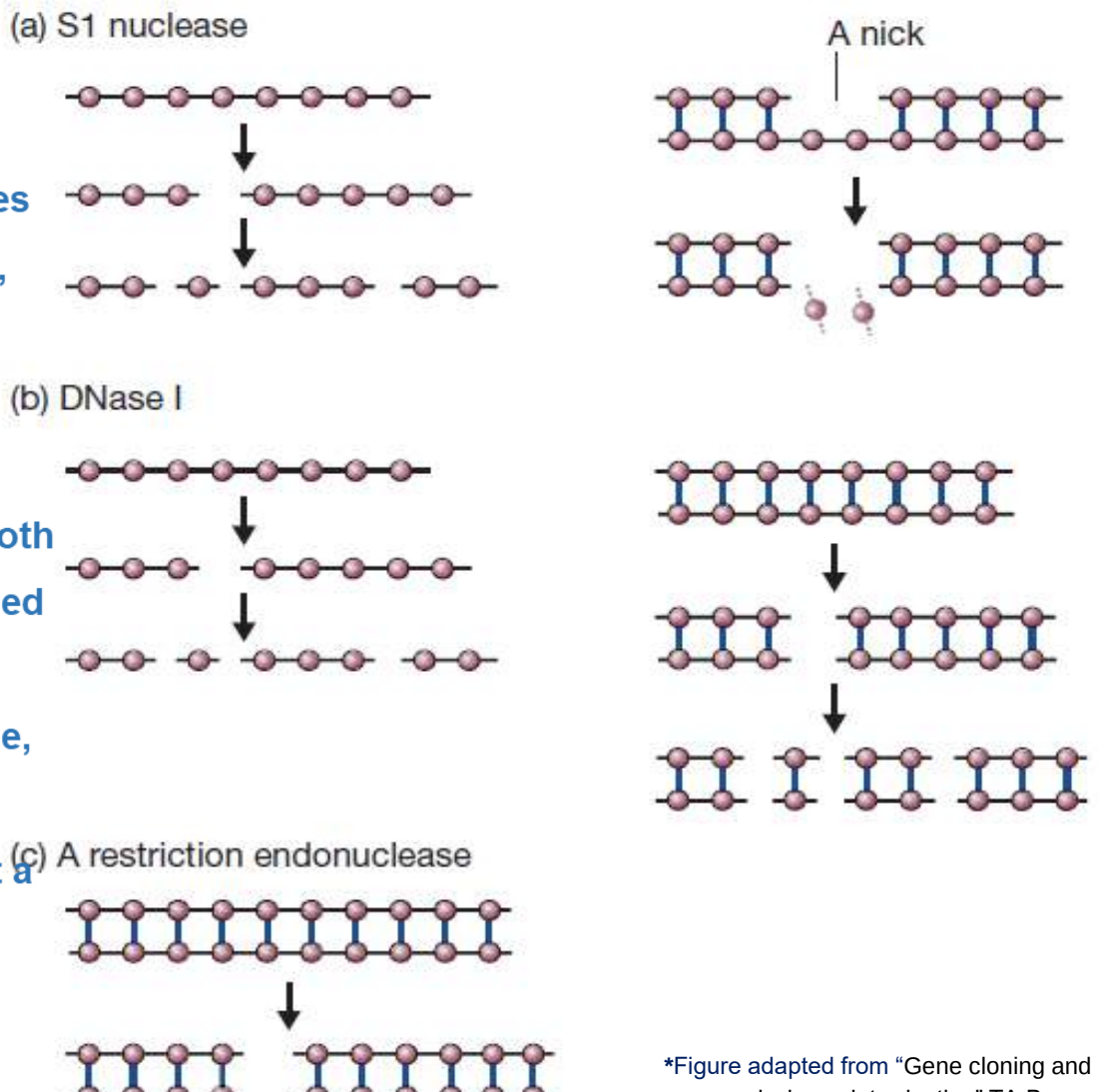


*Figure adapted from "Gene cloning and DNA analysis: an introduction" TA Brown

RESTRICTION ENZYMES

- Restriction enzymes are exclusively produced by bacteria in order to protect themselves against viral infection by degrading viral nucleic acid, hence restricting the growth of a particular bacteriophage.
- Restriction endonucleases were discovered in early 1950s, when some strains of bacteria were shown to be immune to bacteriophage infection, a phenomenon referred to as **host-controlled restriction**.
- Discovery of Restriction enzymes, which led to Nobel Prizes for W. Arber, H. Smith, and D. Nathans in 1978, was one of the key breakthroughs in the development of genetic engineering.
- The bacterium's own DNA, the destruction of which would of course be lethal, is protected from attack because it carries additional methyl groups that block the degradative enzyme action
- These degradative enzymes are termed **restriction endonucleases** and are synthesized by many, perhaps all, species of bacteria.
- To date, almost 4000 different enzymes have been isolated

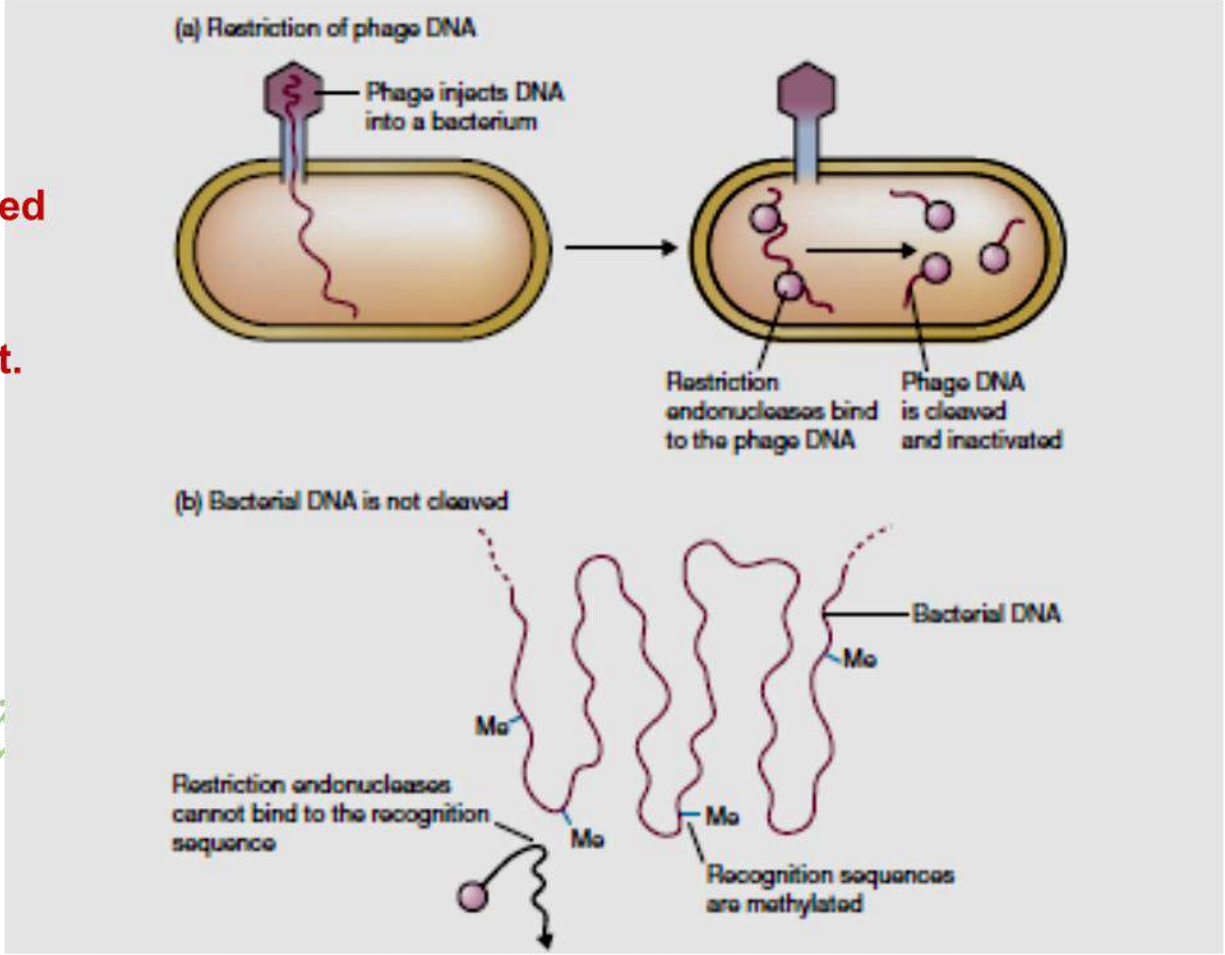
RESTRICTION ENDONUCLEASES

The function of a restriction endonuclease in a bacterial cell.

(a) Phage DNA is cleaved

But

(b) Bacterial DNA is not.

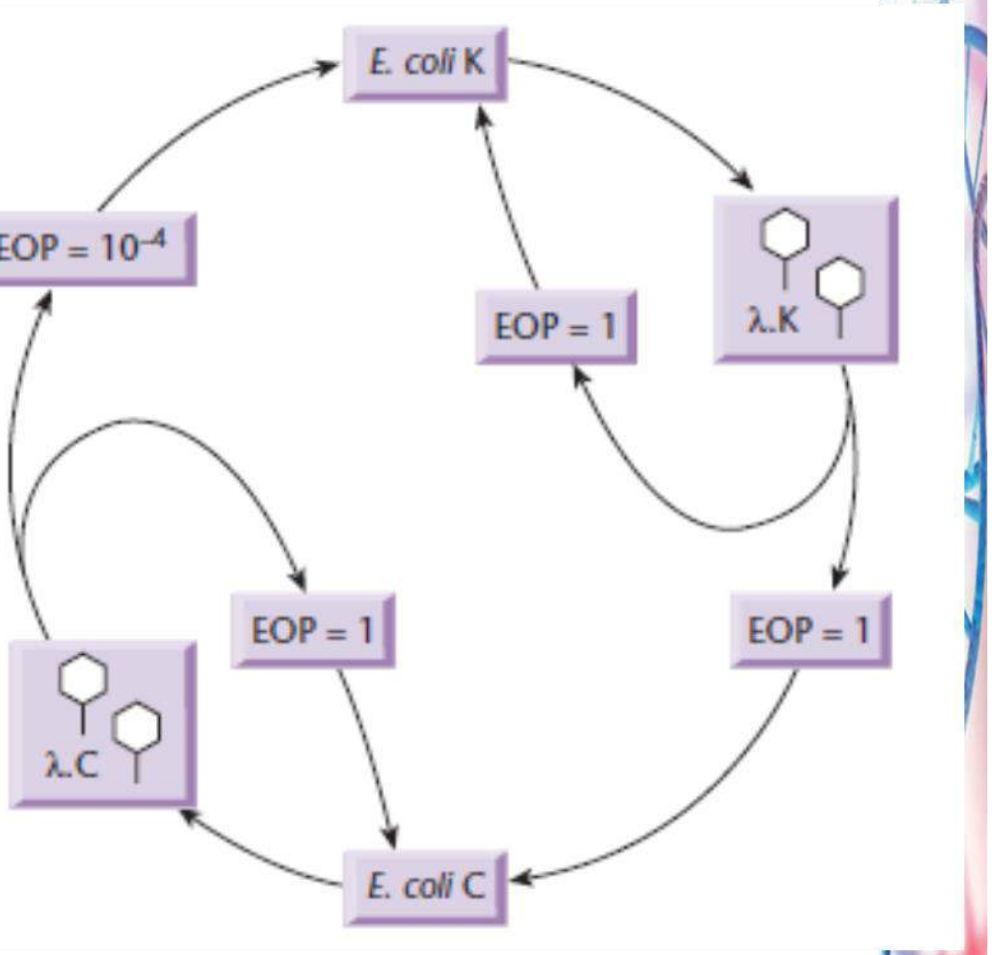


- Purified restriction endonucleases allow the molecular biologist to cut DNA molecules in the precise, reproducible manner required for gene cloning.

HOST-CONTROLLED RESTRICTION AND MODIFICATION

phage λ in *E. coli* strain K, analyzed by efficiency of plating (EOP).

- When two different strains of E coli were infected with λ-phage, it was found that strain K was resistant in comparison to strain C.
- When strain K was re-infected with λ-phage that was isolated from the E coli K (λ K) plate efficiency of plating increases i.e. strain had shown sensitivity against λ-phage K, however λ-phage C show same pattern.
- RM system specific to λ-phage was active in strain K



RECOGNITION SEQUENCES

- Restriction enzymes recognize a specific sequence of nucleotides and produce a double-stranded cut in the DNA.
- The recognition sequences can also be classified by the number of bases in its recognition site, usually between 4 and 8 bases, and the number of bases in the sequence will determine how often the site will appear by chance in any given genome, e.g., a 4-base pair sequence would theoretically occur once every 4^4 or 256bp, 6 bases, 4^6 or 4,096bp, and 8 bases would be 4^8 or 65,536bp.
- Many of them are palindromic, meaning the base sequence reads the same backwards and forwards. Examples are:

R-M SYSTEM

- There are four major different kinds of R-M system are known:
- **Type I enzymes** (EC 3.1.21.3) cleave at sites distant from a recognition site; require both ATP and S-adenosyl-L-methionine to function; multifunctional protein with both restriction digestion and methylase (EC 2.1.1.72) activities.
- **Type II enzymes** (EC 3.1.21.4) cleave within or at short specific distances from a recognition site; most require magnesium; single function (restriction digestion) enzymes independent of methylase.
- **Type III enzymes** (EC 3.1.21.5) cleave at sites a short distance from a recognition site; require ATP (but do not hydrolyse it); S-adenosyl-L-methionine stimulates the reaction but is not required; exist as part of a complex with a modification methylase
- **Type IV enzymes** target modified DNA, e.g. methylated, hydroxy methylated and glucosyl-hydroxy methylated DNA

R-M SYSTEM

TABLE: COMPARITIVE PROPERTIES OF MAJOR RESTRICTION ENDONUCLEASES

Property	Type I RE	Type II RE	Type III RE
Abundance	Less common than Type II	Most common	Rare
Recognition site	Cut both strands at a non-specific location > 1000 bp away from recognition site Single	Cut both strands at a specific, usually palindromic recognition site (4–8 bp)	Cleavage of one strand, only 24-26 bp downstream of the 3' recognition site
Restriction and modification	Single multi functional enzyme	Separate nuclease and methylase	Separate enzymes sharing a common subunit
Cofactors	ATP, Mg2+, SAM	Mg2+	Mg2+ (SAM)
Nuclease subunit structure	Heterotrimer	Homodimer	Heterodimer
DNA cleavage requirements	Two recognition sites in any orientation	Single recognition site	Two recognition sites in a head-to-head orientation
DNA translocation	Yes	No	No
Site of methylation	At recognition site	At recognition site	At recognition site
Enzymatic turnover	No	Yes	Yes

Which type of restriction enzyme is usefull for genetic engineering?

- Most of the useful R-M systems are of type II. They have a number of advantages over type I and III systems.
- First, restriction and modification are mediated by separate enzymes so it is possible to cleave DNA in the absence of modification.
- Secondly, the restriction activities do not require cofactors such as ATP or *S*-adenosylmethionine, making them easier to use.
- Most important of all, type II enzymes recognize a defined, usually symmetrical, sequence *and cut within it*.
- Many of them also make a staggered break in the DNA and the usefulness of this will become apparent.
- type IIs systems have similar cofactors and macromolecular structure to those of type II systems, but it restrict at a distance from the recognition site limits their usefulness

NOMENCLATURE OF RESTRICTION ENDONUCLEASES

- more than 3500 different Type II restriction enzymes have been characterized.
- Each enzyme is named after the bacterium from which it was isolated, using a naming system based on bacterial genus, species and strain
- A suitable nomenclature system for restriction endonucleases was proposed by Smith and Nathans (1973). The key features are:
- The species name of the host organism is identified by the first letter of the genus name and the first two letters of the specific epithet to generate a three-letter abbreviation. This abbreviation is always written in italics. e.g. *Eco*
- Where a particular strain has been the source then this is identified. e.g. *EcoR*
- When a particular host strain has several different R-M systems, these are identified by roman numerals. e.g. *EcoRI*

NOMENCLATURE OF RESTRICTION ENDONUCLEASES

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

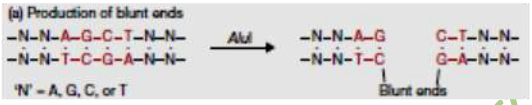
- Homing endonucleases are named in a similar fashion except that intron-encoded endonucleases are given the prefix “I-” (e.g. I-*Ceu*I)
- Intein endonucleases have the prefix “PI-” (e.g. PI-*Psp*I).
- Where it is necessary to distinguish between the restriction and methylating activities, they are given the prefixes “R” and “M”, respectively, e.g. R.*Sma*I and M.*Sma*I.

NOMENCLATURE OF RESTRICTION ENDONUCLEASES

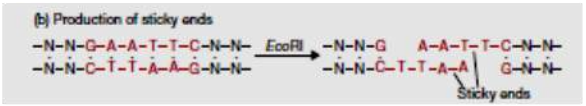
Enzyme	Source	Recognition sequence	Cut
EcoRI	Escherichia coli	5'GAATTC3'CTTAAG	5'---G AATTC---3'3'---CTTAA G---5'
BamHI	Bacillus amyloliquefaciens	5'GGATCC3'CCTAGG	5'---G GATCC---3'3'---CCTAG G---5'
HindIII	Haemophilus influenzae	5'AAGCTT3'TTCGAA	5'---A AGCTT---3'3'---TTCGA A---5'
TaqI	Thermus aquaticus	5'TCGA3'AGCT	5'---T CGA---3'3'---AGC T---5'
SmaI	Serratia marcescens	5'CCCGGG3'GGGCCC	5'---CCC GGG---3'3'---GGG CCC---5'

BLUNT ENDS AND STICKY ENDS

- **Blunt end or flush end** Many restriction endonucleases make a simple double-stranded cut in the middle of the recognition sequence e.g. *Pvu*II and *Alu*I areblunt end cutters.



- **Sticky ends or cohesive ends** some restriction endonucleases cut DNA in such a way that, the two DNA strands are not cut at exactly the same position but instead the cleavage is staggered, usually by two or four nucleotides, so that the resulting DNA fragments have short single-stranded overhangs at each end.



- These are called sticky ends or cohesive ends, as base pairing between them can stick the DNA molecule back together again.

RESTRICTION ENDONUCLEASES

ENZYME	ORGANISM	RECOGNITION SEQUENCE[a]	BLUNT OR STICKY END
EcoRI	*Escherichia coli*	GAATTC	Sticky
BamHI	*Bacillus amyloliquefaciens*	GGATCC	Sticky
BglI	*Bacillus globigii*	AGATCT	Sticky
PvuI	*Proteus vulgaris*	CGATCG	Sticky
PvuII	*Proteus vulgaris*	CAGCTG	Blunt
HindIII	*Haemophilus influenzae* R_d	AAGCTT	Sticky
HinfI	*Haemophilus influenzae* R_f	GANTC	Sticky
Sau3A	*Staphylococcus aureus*	GATC	Sticky
AluI	*Arthrobacter luteus*	AGCT	Blunt
TaqI	*Thermus aquaticus*	TCGA	Sticky
HaeIII	*Haemophilus aegyptius*	GGCC	Blunt
NotI	*Nocardia otitidis-caviarum*	GCGGCCGC	Sticky
SfiI	*Streptomyces fimbriatus*	GGCCNNNNNGGCC	Sticky

adapted from "Gene cloning and DNA analysis: an introduction" TA Brown

RESTRICTION ENDONUCLEASES

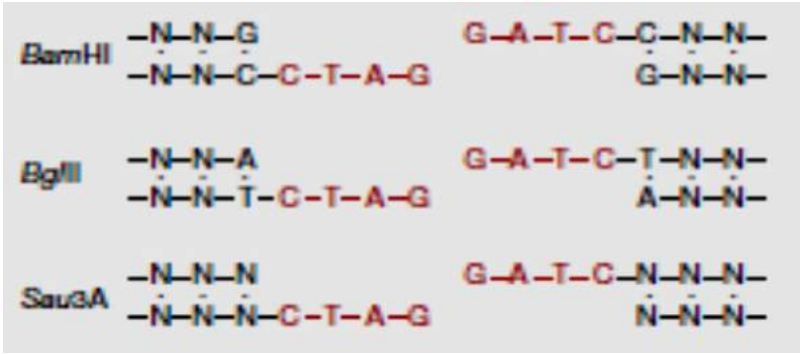
ISOSCHIZOMERS

- Restriction enzymes which are specific to the same recognition sequence and cut site. For example, SphI (CGTAC/G) and BbuI (CGTAC/G)
- The first enzyme discovered which recognizes a given sequence is known as the prototype; all subsequently identified enzymes that recognize that sequence are isoschizomers.
- Isoschizomers are isolated from different strains of bacteria and therefore may
- Some isoschizomers allows identification of methylation state of the restriction site while isolating it from a bacterial strain.
- For example, the restriction enzymes HpaII and MspI are isoschizomers, as they both recognize the sequence 5'-CCGG-3' when it is unmethylated. But when the second C of the sequence is methylated, only MspI can recognize it while HpaII cannot.

RESTRICTION ENDONUCLEASES

- **NEOSCHIZOMERS**: are restriction enzymes that recognize the same nucleotide sequence as their prototype but cleave at a different site.
- For example, SmaI (CCC/GGG) and XmaI (C/CCGGG)

  Kpn1 (GGTAC/C) and Acc651 (G/GTACC)
- **ISOCAUDOMERS:** are pairs of restriction enzymes that have slightly different recognition sequences, but upon cleavage of DNA, generate identical overhanging termini sequences.
- These sequences can be ligated to one another, but then form an asymmetrical sequence that cannot be cleaved by a restriction enzyme.
- Examples of isocaudomers are:



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