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Tiruchirappalli
Tamil Nadu - India

Programme : M.Sc Biotechnology
Course Title : Genetic Engineering
Course code :22BTCC6

Unit -1
Tools of Genetic Engineering

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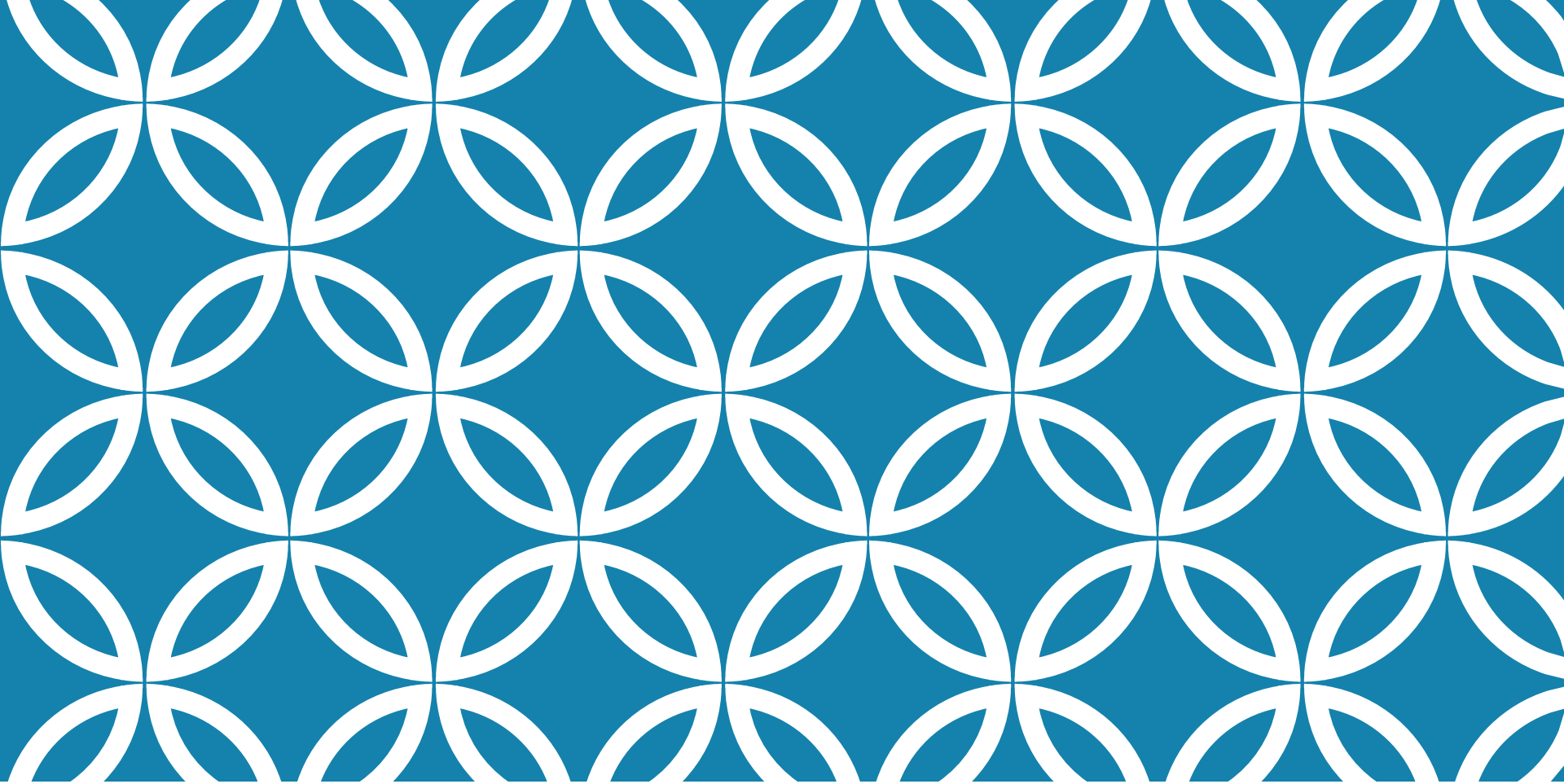
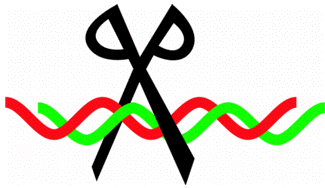


RESTRICTION ENZYMES

HOST CONTROLLED RESTRICTION AND MODIFICATION



Viruses infect the host cells by injecting their DNA into the cells. This viral DNA hijacks the host cell's machinery for reproduction of viral progeny, resulting in the host cell's death.

To overcome the viral infection, many bacteria and archaea have evolved several mechanisms. A major protective mechanism involves the use of restriction enzymes to degrade the invading viral DNA by cleaving it at specific restriction sites.

Restriction enzymes prevents infection by effectively destroying the foreign DNA introduced by an infectious agent (such as a bacteriophage).

The **Host-Controlled Restriction-Modification System** (HCRMS) is a bacterial mechanism used for defending against foreign DNA, such as that from viruses (bacteriophages) or plasmids.

This system consists of two primary components: **restriction enzymes** and **modification enzymes**. These enzymes work together to provide a protective mechanism, ensuring that bacterial cells can distinguish between self and non-self DNA, preventing the integration of harmful or foreign genetic material.

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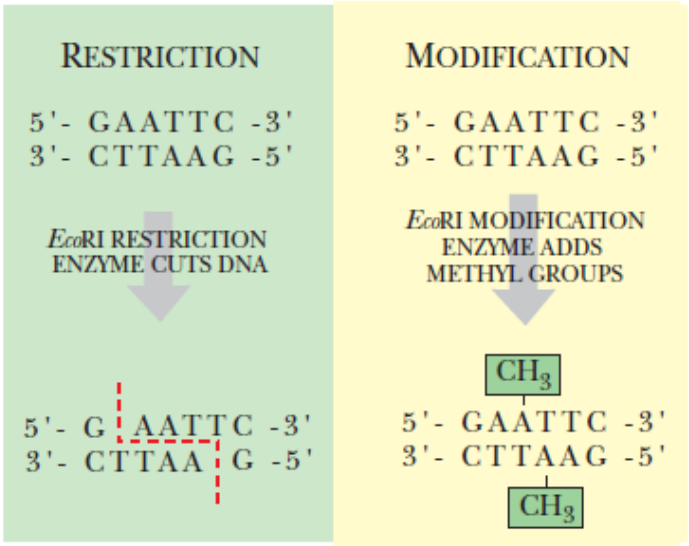


Image courtesy :Molecular Biology by Clark et al

HOST CONTROLLED RESTRICTION AND MODIFICATION

Approximately one-quarter of known bacteria possess Restriction Modification systems and of those about one-half have more than one type of system.

As the sequences recognized by the restriction enzymes are very short, the bacterium itself will almost certainly contain some within its genome.

The host cell protects its own DNA from being cleaved by employing other enzymes called methylases, which methylate adenine or cytosine bases within host recognition sequences.

Bacterial defense against viral infection by restriction-modification complexes

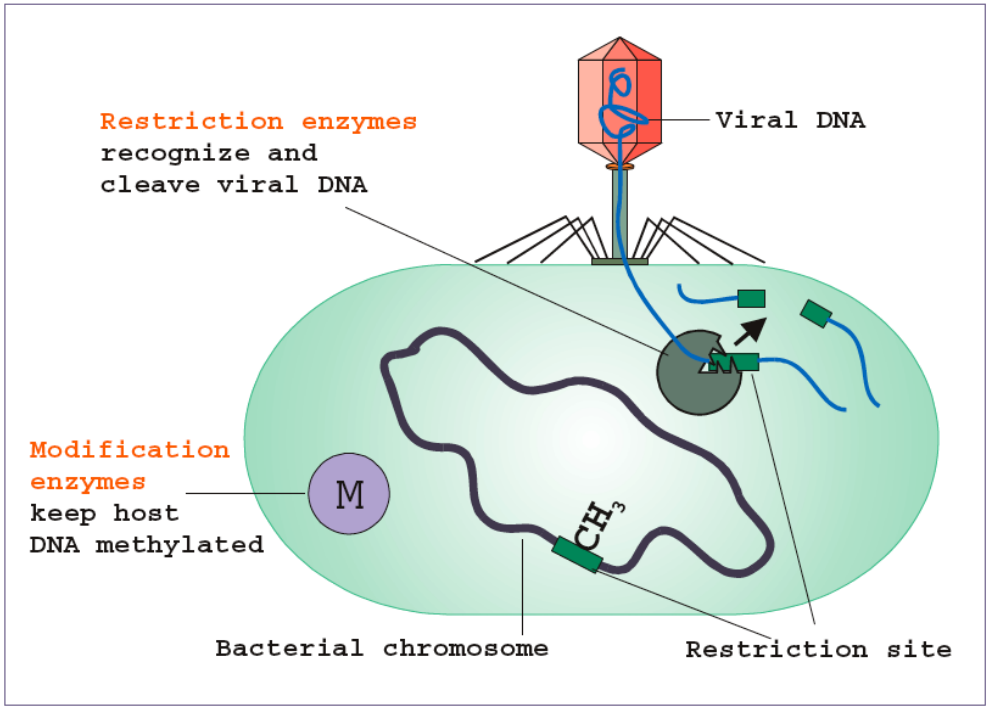


Image source : RLA college

HOST CONTROLLED RESTRICTION AND MODIFICATION

For each of the restriction enzyme, the host cell produces a corresponding methylase that methylates and protects the host DNA from degradation. These enzymes make up the restriction-modification (R-M) systems.

The restriction enzymes catalyze the hydrolysis of the bond between the 3’-oxygen atom and the phosphorus atom in the phosphodiester backbone of DNA. The enzymes require Mg^{2+} or other divalent ions for their activity.

Werner Arber, Daniel Nathans, and Hamilton O. Smith were awarded the Nobel Prize for Physiology or Medicine in 1978 for their discovery and characterization of restriction enzymes, which led to the development of recombinant DNA technology.

RESTRICTION ENDONUCLEASES

Restriction - Because for the way they work, they restrict virus to only one host bacterial strain. They are also restricted to acting on only specific DNA sequences

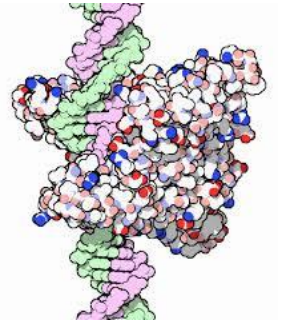
Endonuclease - They cut nucleic acids in the middle not just the ends.

Restriction enzymes, also known as R**estriction endonucleases**, are a class of enzymes that cut DNA at specific sequences. These enzymes play a crucial role in the defense mechanisms of bacteria against viral infections (phages).

Restriction enzymes are classified as endonucleases. Their biochemical activity is the hydrolysis ("digestion") of the phosphodiester backbone at specific sites in a DNA sequence. By "specific" we mean that an enzyme will only digest a DNA molecule after locating a particular sequence.

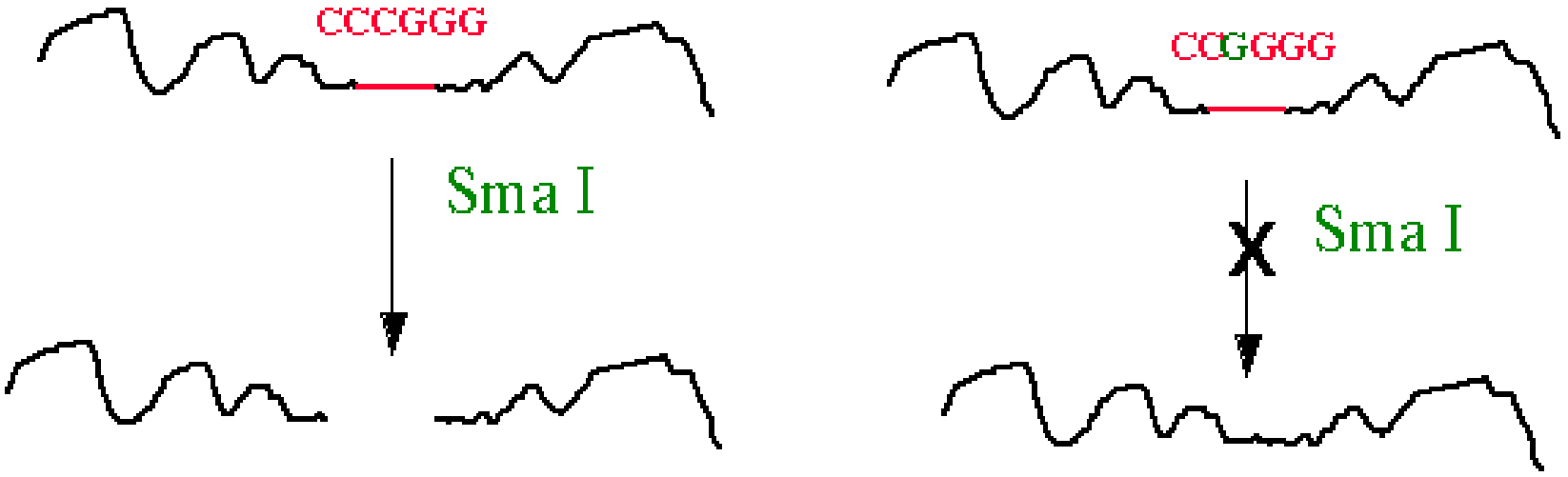
Over 3000 restriction enzymes have been studied in detail, and more than 600 of these are available commercially

RESTRICTION ENDONUCLEASES GENERAL CHARACTERISTICS



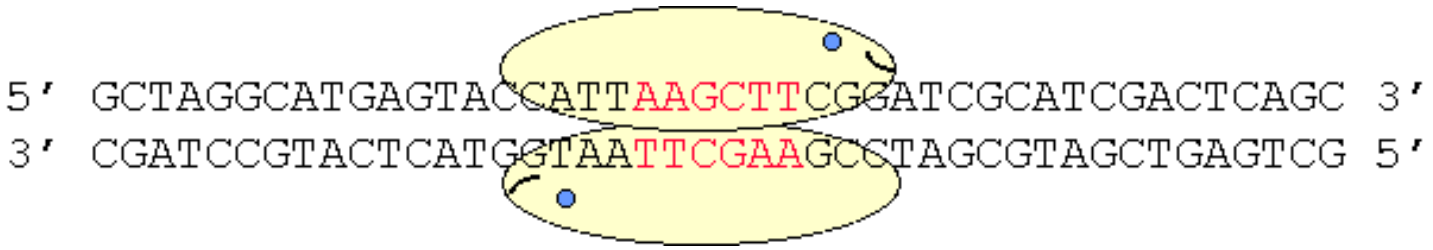
Restriction enzymes recognize specific nucleotide sequences in DNA and cut both strands of the DNA molecule. Different restriction enzymes recognize different sequences in DNA. The recognition sequence can be 4, 5, 6, 7 or 8 nucleotides long.

Restriction enzymes cut DNA ONLY if the exact recognition sequence is present:



RESTRICTION ENDONUCLEASES

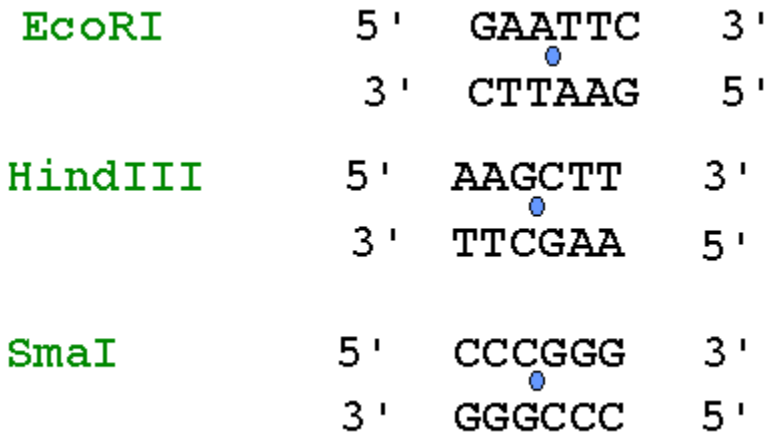
RESTRICTION ENZYMES often act as dimers; each restriction enzyme subunit recognizes THE SAME (5'->3') nucleotide sequence in complementary DNA strands:



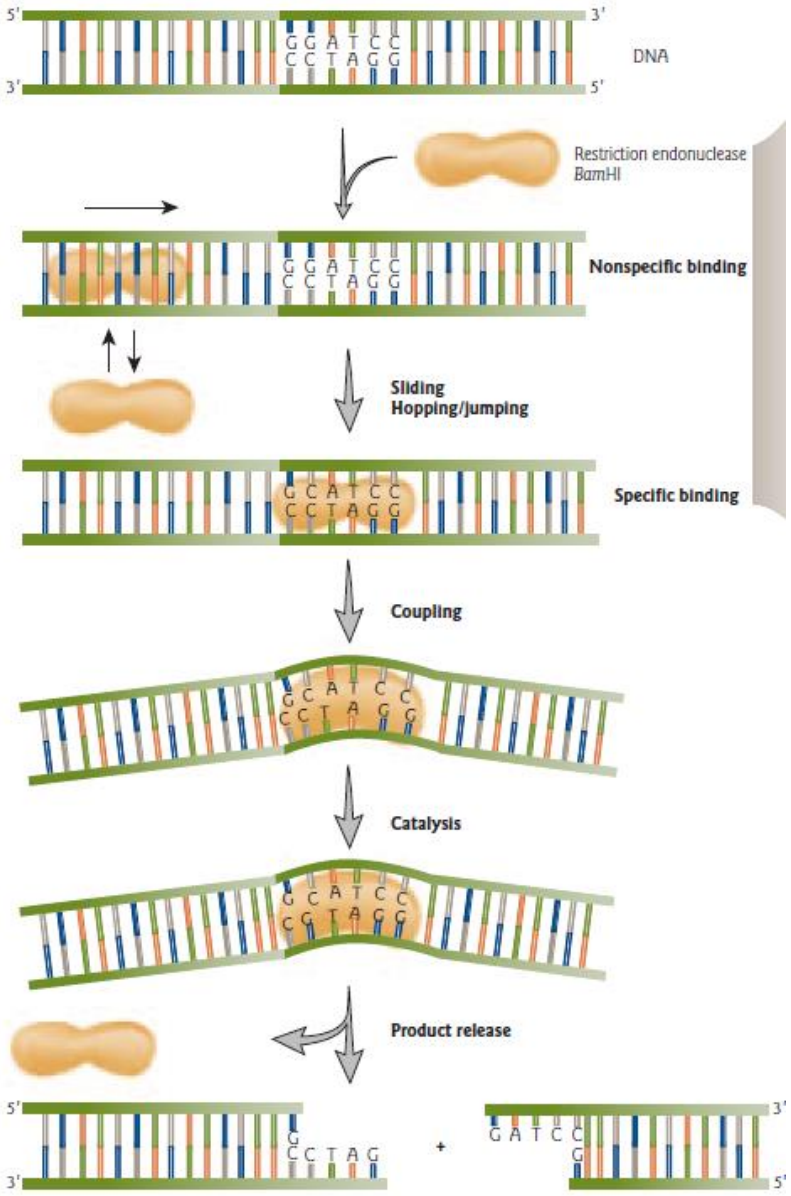
The sequence recognized by the restriction enzyme to cut the DNA is called RESTRICTION SITE, or RECOGNITION SITE.

The recognition site consistsof4-8basepairs.

As a result, restriction enzymes usually recognize palindromic sequences:

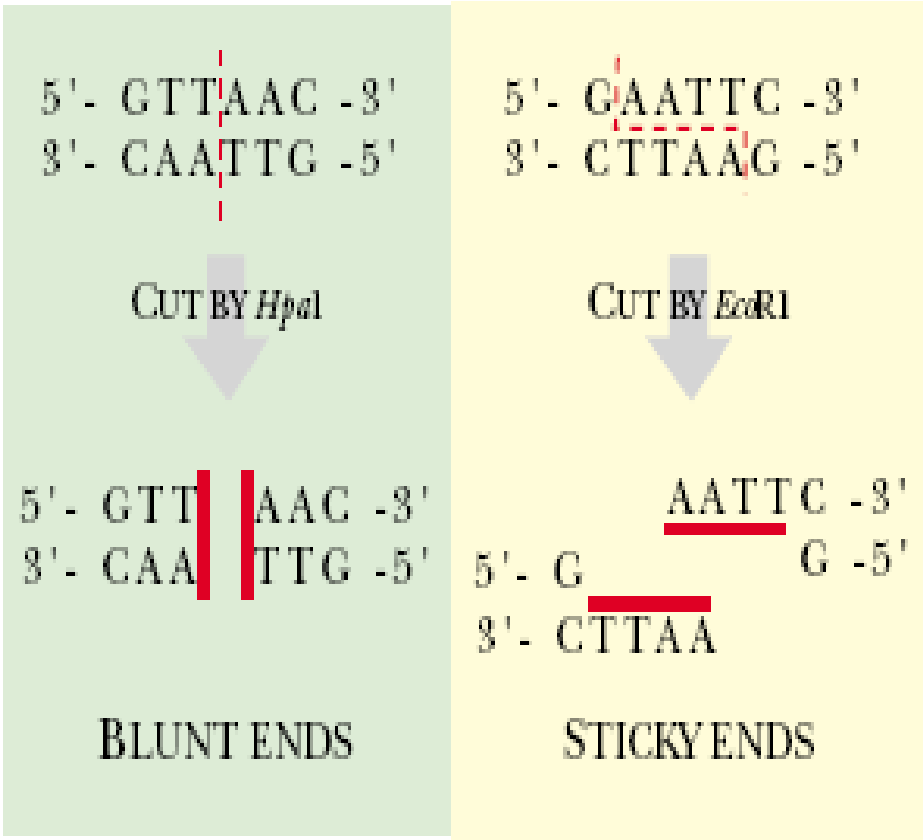


RESTRICTION ENDONUCLEASES ACTION



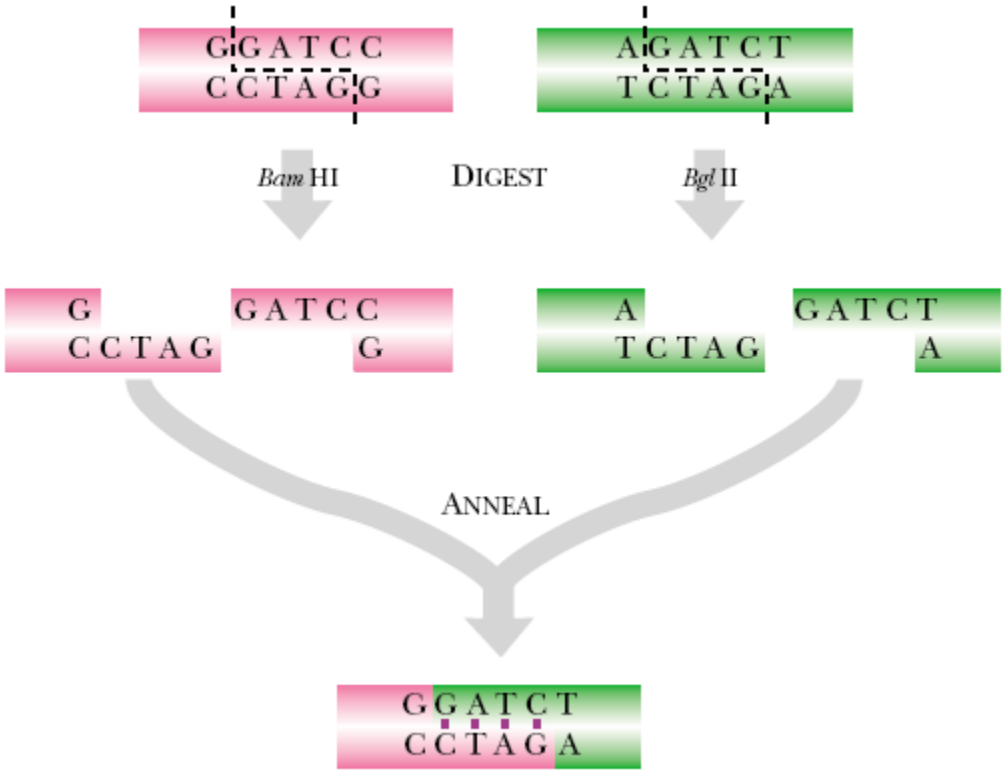
TWO DIFFERENT WAYS OF CUTTING

Restriction enzymes produces either blunt end or sticky ends (overhang)



If two different pieces of DNA are cut with the same restriction enzyme or enzymes that generate the same overhang, the same sticky ends are generated

However, if two sticky ends made by two different enzymes are ligated together, a hybrid site is formed that cannot be cut by either enzyme



RESTRICTION ENZYME NOMENCLATURE

Since their discovery in the 1970s, more than 100 different restriction enzymes have been identified in different bacteria.

Each enzyme is named after the bacterium from which it was isolated using a naming system based on bacterial genus, species and strain.

Enzyme	Enzyme source	Recognition sequence
*Sma*I	*Serratia marcescens*, 1st enzyme	CCCGGG
*Hae*III	*Haemophilus aegyptius*, 3rd enzyme	GGCC
*Hin*dII	*Haemophilus influenzae*, strain d, 2nd enzyme	GTPyPuAC
*Hin*dIII	*Haemophilus influenzae*, strain d, 3rd enzyme	AAGCTT
*Bam*HI	*Bacillus amyloliquefaciens*, strain H, 1st enzyme	GGATCC

NOMENCLATURE

The species name of the host organism is identified by the first letter of the genus name

The first two letters of the specific epithet to generate a three letter abbreviation

This abbreviation is always written in italics.

When a particular host strain has several different R-M systems, these are identified by roman numerals

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

TYPES OF RESTRICTION ENZYMES

There are six classes of restriction endonucleases: types I, II,III, IV,V and VI.

All types of enzymes recognize specific short DNA sequences and carry out the endonucleolytic cleavage of DNA to give specific double-stranded fragments with terminal 5'-phosphates.

They differ in their recognition sequence, subunit composition, cleavage position, and cofactor requirements.

Type I: These enzymes both recognize and cut DNA at distant sites from their recognition sequences, requiring ATP and S-adenosylmethionine for activity.

Type II: The most widely used in molecular biology, these enzymes recognize specific palindromic sequences and cut DNA within or near the recognition site.

Type III: These also require ATP but cut DNA at sites that are a fixed distance from the recognition sequence, much like Type I enzymes.

Type IV: These recognize and cut modified DNA, such as methylated or glucosylated DNA.

Type V: These enzymes (such as CRISPR-associated nucleases) use RNA guides to direct DNA cleavage.

TYPE I RESTRICTION ENZYMES

Type I restriction endonucleases are components of prokaryotic DNA restriction-modification mechanisms that protects the organism against invading foreign DNA.

These enzymes are widespread in eubacteria and archaea. In enteric bacteria they have been subdivide into four families: types IA, IB, IC and ID.

Type I restriction enzymes have distinct characteristics that differentiate them from other types including their molecular structure, recognition sequence, and cutting behaviour.

TYPE I RESTRICTION ENZYMES

General Characteristics of Type I Restriction Enzymes

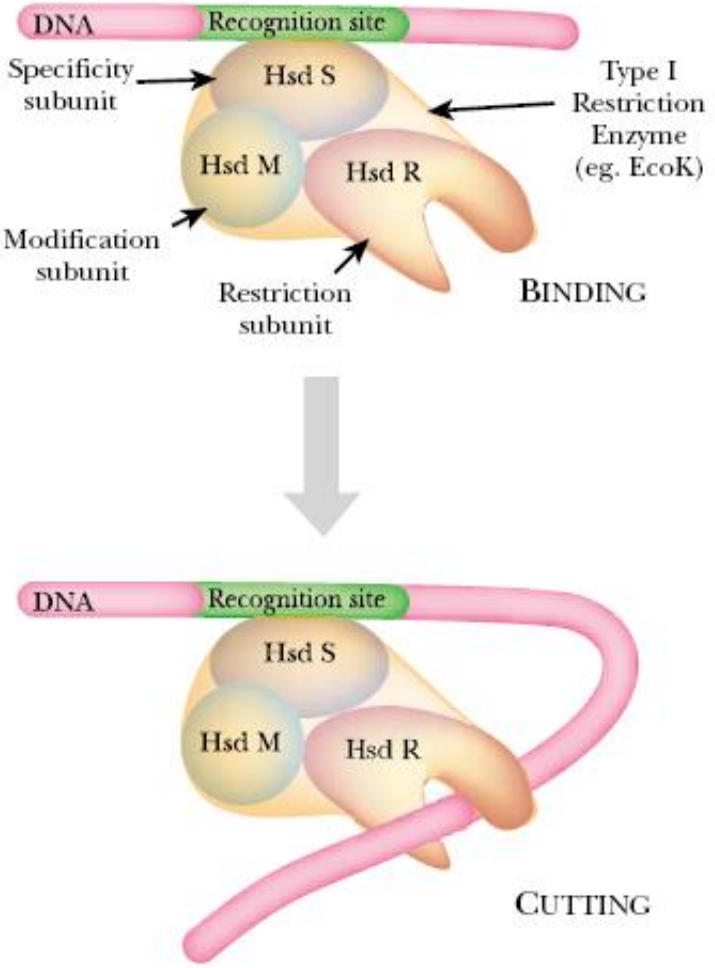
1.Structure:

Type I restriction enzymes are **multi-subunit complexes**. They consist of three distinct protein subunits:

Restriction (R) subunit: Responsible for recognizing the target DNA sequence and cleaving the DNA.

Modification (M) subunit: Responsible for methylating the host's DNA to protect it from cleavage by the restriction subunit. This is crucial for the enzyme's ability to distinguish between self and foreign DNA.

DNA-binding (B) subunit: Involved in binding the DNA molecule.



TYPE I RESTRICTION ENZYMES

2. Recognition and Cleavage:

- Type I enzymes recognize specific DNA sequences, usually **6-8 base pairs** in length, similar to other restriction enzymes.
- These enzymes cleave DNA at sites that are **far from the recognition sequence**, rather than at the recognition site itself (which is characteristic of Type II restriction enzymes).
- The **cleavage mechanism** is unusual because Type I restriction enzymes cut the DNA at random locations that are a distance away from the recognition sequence. This distance is typically about **1,000 to 5,000 base pairs**.
- The cleavage result is the formation of **non-specific fragments**, unlike Type II enzymes, which generate **specific DNA fragments** at or near the recognition site.

3.ATP Requirement:

- Type I restriction enzymes require **ATP** to function, both for the methylation process and for the cleavage of the DNA.
- ATP hydrolysis is involved in the enzymatic activity of the R subunit and is necessary for DNA translocation and cleavage.

TYPE I RESTRICTION ENZYMES

4.Methylation Activity:

- The modification subunit of Type I enzymes is responsible for adding a methyl group to the host's DNA to protect it from restriction. This methylation occurs at **specific bases** within the recognition sequence.
- As a result, the host DNA remains uncut, while foreign DNA (such as from a phage) that lacks the methylation pattern is cleaved.

5.Example Enzymes:

- A well-known example of a Type I restriction enzyme is the **EcoKI** enzyme from *Escherichia coli*. EcoKI is a complex enzyme that consists of three subunits and recognizes the sequence **5'-AACGTT-3'**. It cuts the DNA approximately 1,000-3,000 base pairs away from the recognition sequence.

TYPE I RESTRICTION ENZYMES

Mechanism of Action

1. **DNA Recognition**: The enzyme first binds to its specific DNA recognition site. The recognition sequence is typically a palindromic sequence, though Type I enzymes recognize specific sequences that are a bit more complex.

2. **DNA Methylation**: After the enzyme recognizes the target sequence, the modification (M) subunit adds a methyl group to the DNA, protecting the host's DNA from being cleaved by the restriction enzyme.

3. **Cleavage**: The restriction (R) subunit performs the actual cleavage of the foreign DNA. However, instead of cutting the DNA directly at the recognition sequence, the enzyme cuts the DNA at **random sites** away from the recognition sequence, usually in a somewhat **random manner** along the DNA strand.

4. **Endonuclease Activity**: Once the DNA is cleaved, the resulting fragments are of uneven lengths and can be of varying complexity.

TYPE I RESTRICTION ENZYMES

Classification of Type I Restriction Enzymes

Type I restriction enzymes are classified into **three subgroups** based on the specific features of their subunits:

1. **Type IA**: These enzymes are found in many bacteria and typically recognize palindromic sequences. They require a co-factor for activity and also exhibit methylase activity.

2. **Type IB**: These enzymes are less common but share similar characteristics with IA enzymes. They exhibit a similar mode of DNA cleavage and methylation.

3. **Type IC**: These are less characterized than IA and IB enzymes, but they share structural and functional similarities.

TYPE I RESTRICTION ENZYMES

Importance and Applications

1. **Bacterial Defense**: Type I restriction enzymes are an essential part of bacterial defense against invading viruses, particularly bacteriophages. By cleaving foreign DNA, these enzymes prevent the virus from replicating within the bacterium.

2. **Molecular Biology**:
   - Type I enzymes are generally less utilized in molecular biology techniques because they cleave DNA at a distance from the recognition site, which results in large, non-specific fragments.
   - They are, however, important for understanding the mechanisms of restriction-modification systems and DNA-protein interactions.

3. **Evolutionary Significance**: These enzymes are considered part of the evolutionary arms race between bacteria and phages. The methylation activity ensures that the host's DNA is protected, while the restriction activity is directed at foreign DNA, ensuring the bacterium's survival.

TYPE-II RESTRICTION ENZYME

Type II restriction enzymes are among the most widely studied and utilized class of restriction enzymes in molecular biology. These enzymes play a crucial role in bacterial defense mechanisms by recognizing and cleaving specific DNA sequences. They are widely used in various laboratory applications such as cloning, gene editing, and molecular mapping.

1. Definition and Function

Type II restriction enzymes (also known as Type II restriction endonucleases) are proteins that cut DNA at specific sites. They are part of the bacterial immune system, which helps protect against viral DNA (such as bacteriophages). These enzymes recognize a particular short sequence of DNA (often 4-8 base pairs long), known as the **recognition site** or **restriction site**, and cleave the DNA within or near this sequence.

Unlike other types of restriction enzymes (e.g., Type I and Type III), **Type II enzymes** have the following distinguishing features:

- **Specificity**: They recognize palindromic DNA sequences (sequences that read the same forwards and backwards on complementary strands).
- **Cleavage**: They cut the DNA in a predictable manner, typically at or near the recognition site.
- **Independence**: They do not require ATP or other cofactors for their activity, unlike Type I enzymes, which require ATP for both restriction and modification activities.

TYPE-II RESTRICTION ENZYME

2. Mechanism of Action

Type II restriction enzymes typically cut double-stranded DNA in two ways:

- **Blunt Ends**: Some Type II enzymes cut straight through both strands at the same position, resulting in blunt ends (no overhangs). These are generally less efficient for ligation.
- **Sticky (or Cohesive) Ends**: Many Type II enzymes make staggered cuts in the DNA, leaving overhanging single-stranded regions on either side of the cut. These sticky ends are highly valuable for recombinant DNA technology because they can pair with complementary sticky ends from other DNA fragments.

Example of cleavage mechanism:

- **EcoRI**: This enzyme recognizes the sequence GAATTC and cuts between the G and the A on each strand, producing sticky ends with a 5' overhang.

TYPE-II RESTRICTION ENZYME

Structure and Mechanism of Action

The catalytic mechanism of Type II restriction enzymes involves the recognition of specific DNA sequences by the enzyme's structure. The enzyme binds to the DNA, and a magnesium ion (Mg^{2+}) typically aids in the cutting of the phosphodiester bond between nucleotides.

- **Recognition Domain**: This is the part of the enzyme that binds to the DNA sequence.
- **Catalytic Domain**: This part is responsible for cutting the DNA strand.

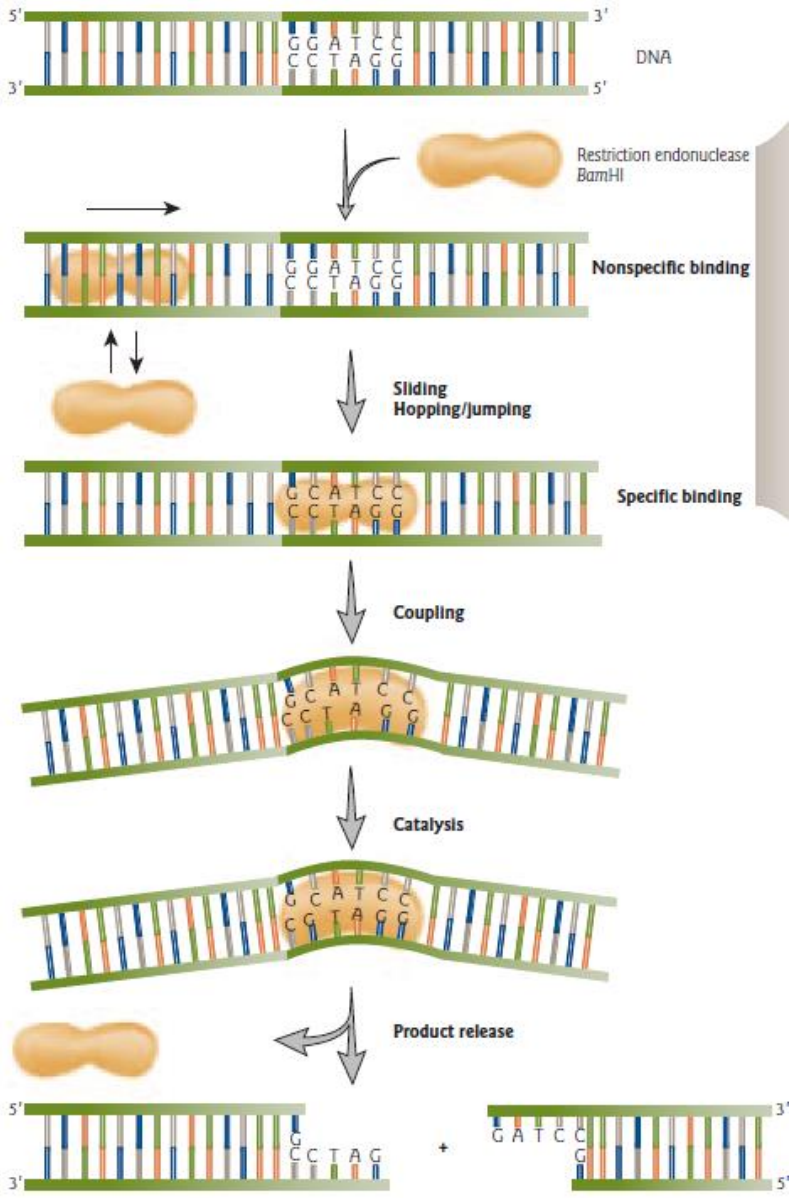
Once the recognition sequence is bound, the enzyme forms a complex with the DNA, positioning the catalytic domain to cleave the phosphodiester bond at the precise location.

TYPE-II RESTRICTION ENZYME

Mechanism of Action at the Molecular Level

The action of Type II restriction enzymes typically involves two steps:

1. **Recognition**: The enzyme specifically recognizes the target DNA sequence through its DNA-binding domain.
2. **Cleavage**: The enzyme then cleaves the DNA at the phosphodiester backbone, either creating blunt ends or sticky ends, depending on the enzyme.

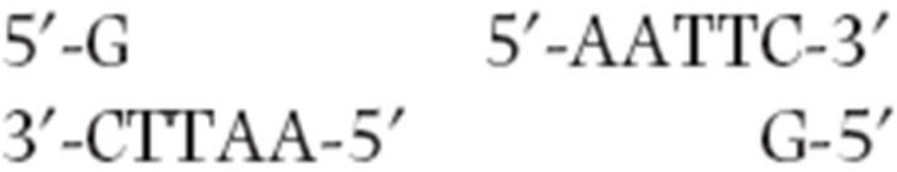


TYPE-II RESTRICTION ENZYME

Mechanism of Action at the Molecular Level

Most of the type II restriction endonucleases recognize and cleave DNA within particular sequences of four to eight nucleotides which have a twofold axis of *rotational symmetry.*

Such sequences are often referred to as *palindromes because of their similarity* to words that read the same backwards as forwards



TYPE-II RESTRICTION ENZYME

Notable Type II Restriction Enzymes

Some of the most widely used Type II restriction enzymes include:

- **EcoRI**: Recognizes the sequence **GAATTC** and cuts between the G and the A.
- **HindIII**: Recognizes the sequence **AAGCTT** and cuts between the A and the A.
- **BamHI**: Recognizes the sequence **GGATCC** and cuts between the G and the A.
- **SalI**: Recognizes the sequence **GTCGAC** and cuts between the G and the T.
- **XbaI**: Recognizes the sequence **TCTAGA** and cuts between the T and the C.

These enzymes have become indispensable tools for molecular cloning, restriction fragment length polymorphism (RFLP) analysis, and recombinant DNA technology.

Table 3.3 Some restriction endonucleases and their recognition sites.

Enzyme	Recognition sequence
4-Base cutters	
*Mbo*I, *Dpn*I, *Sau*3AI	/GATC
*Msp*I, *Hpa*II	C/CGG
*Alu*I	AG/CT
*Hae*III	GG/CC
*Taq*I	ACGT/
6-Base cutters	
*Bgl*II	A/GATCT
ClaI	AT/CGAT
*Pvu*II	CAG/CTG
*Pvu*I	CGAT/CG
*Kpn*I	GGTAC/C
8-Base cutters	
*Not*I	GC/GGCCGC
*Sbf*I	CCTGCA/GG

TYPE-II RESTRICTION ENZYME

Applications in Molecular Biology

Type II restriction enzymes are essential tools in molecular biology and biotechnology. Their applications include:

- **DNA Cloning**: By cutting DNA at specific sequences, Type II enzymes help generate compatible sticky ends for ligating DNA fragments together. This is a fundamental step in creating recombinant DNA molecules.
- **Gel Electrophoresis**: After cutting DNA into fragments, the resulting pieces are separated by size using agarose gel electrophoresis. This allows for the analysis of DNA fragment length distribution.
- **Restriction Mapping**: Type II restriction enzymes can be used to create a map of a DNA molecule by determining the positions of the cutting sites. This technique is important in the sequencing of genomes or identifying the arrangement of genes.
- **Gene Editing**: These enzymes are often used in combination with other techniques like CRISPR/Cas9 for precise genome editing.
- **Diagnostic Techniques**: Restriction enzymes are utilized in PCR-based techniques and Southern blotting to detect specific DNA sequences.

ADVANTAGE OF TYPE II

restriction and modification are mediated by separate enzymes so it is possible to cleave DNA in the absence of modification

The restriction activities do not require cofactors such as ATP or S-*adenosylmethionine, making them easier* to use.

Type II enzymes recognize a defined, usually symmetrical, sequence *and cut within it*.

Many of them also make a staggered break in the DNA

TYPE III RESTRICTION ENZYMES

Type III restriction enzymes are one of the six recognized types of restriction endonucleases that play an essential role in the immune defense of bacteria. They are part of the bacterial system to protect against foreign DNA, like plasmids or bacteriophages, by cleaving DNA at specific recognition sites. Type III restriction enzymes are distinct in their mode of action and their structural and functional properties compared to other types of restriction enzymes.

TYPE III RESTRICTION ENZYMES

Structure

Type III restriction enzymes are heterodimeric proteins, meaning they are composed of two different subunits that function together. These enzymes consist of:

- **One restriction (R) subunit:** This subunit is responsible for recognizing the specific DNA sequence.
- **One modification (M) subunit:** This subunit is responsible for adding a methyl group to the DNA, protecting it from cleavage by the restriction subunit.

The restriction subunit typically recognizes a specific 6-8 base pair sequence within the DNA, and the modification subunit ensures that the bacterial genome is protected by methylating the same sequence.

TYPE III RESTRICTION ENZYMES

Recognition Sequence

Type III restriction enzymes recognize asymmetric sequences of 4 to 6 base pairs, which are often palindromic (i.e., the sequence reads the same forward and backward). However, they typically cleave DNA at a defined position **away** from the recognition site, usually 20 to 30 bases downstream.

For example, the restriction enzyme **EcoP15I**, a well-known Type III enzyme, recognizes the palindromic sequence:

However, it cleaves the DNA 26-27 bases away from the recognition site.

TYPE III RESTRICTION ENZYMES

Mode of Action

Type III restriction enzymes exhibit a distinctive mechanism of action compared to other restriction enzyme types. The key steps include:

- **DNA Binding**: The enzyme binds to the DNA at the recognition site.
- **DNA Methylation**: The modification subunit methylates the recognition site to protect the host DNA.
- **Cleavage**: The restriction subunit introduces a double-strand break in the DNA at a fixed distance from the recognition sequence. However, this cutting does not occur directly at the recognition sequence, as in Type II enzymes, but is spaced by a few nucleotides.
- **ATP-Dependent**: The cleavage reaction is ATP-dependent, and these enzymes require ATP hydrolysis to function effectively. The enzyme typically requires energy to perform both methylation and cleavage activities.

TYPE III RESTRICTION ENZYMES

Cleavage Site

Unlike Type II restriction enzymes, which cut within or near the recognition site, Type III enzymes cleave DNA at a position 20-30 base pairs **downstream** from the recognition site. This cleavage occurs in both strands of the DNA, producing a double-strand break.

Protective Role and DNA Modification

The modification subunit is key in preventing the enzyme from cutting the bacterial host's own DNA. It adds a methyl group to the recognition sequence, which protects the DNA from cleavage. Without this modification, the enzyme would also cleave the host genome at the recognition sequence, resulting in potential self-damage.

This mechanism is part of a broader **restriction-modification system** that provides bacterial cells with protection against invading foreign DNA, such as from viruses (bacteriophages) or plasmids. The bacteria defend themselves by recognizing and cutting foreign DNA while safeguarding their own genome via methylation.

TYPE III RESTRICTION ENZYMES

Example of Type III Restriction Enzyme

- **EcoP15I**: This is a classic example of a Type III restriction enzyme. It recognizes the sequence 5'-AACGTT-3' and cleaves DNA approximately 26-27 bases downstream of this sequence.
- **MspI**: Another example, which works similarly by recognizing a specific sequence and cutting at a defined distance.

TYPE III RESTRICTION ENZYMES

Applications

While Type III restriction enzymes are less commonly used in molecular biology research than their Type II counterparts, they still offer some valuable applications:

- **Genome Mapping**: Type III enzymes can be used for mapping the genome of organisms due to their ability to cut DNA at specific distances from a known recognition sequence.
- **Synthetic Biology**: These enzymes have potential in genetic engineering, where precise control of DNA cleavage at specific locations is required.
- **Restriction Enzyme Analysis**: Type III enzymes are useful for distinguishing between DNA sequences by recognizing specific nucleotide patterns.

TYPE III RESTRICTION ENZYMES

Differences Between Type III and Other Types of Restriction Enzymes

Type III restriction enzymes differ significantly from other types of restriction enzymes in the following ways:

- **Recognition Site**: Type III enzymes generally recognize asymmetric sites (4–6 bp), while Type II enzymes recognize symmetric palindromic sequences.
- **Cleavage Position**: Type III enzymes cleave DNA 20–30 bases downstream of their recognition sequence, unlike Type II enzymes, which cleave directly at or near the recognition site.
- **Requirement for ATP**: Type III enzymes require ATP for both the methylation and cleavage processes, unlike Type II enzymes, which are ATP-independent.
- **DNA Modification**: Type III enzymes also include a modification subunit, which methylates the DNA to protect the host genome from cleavage.

TYPE IV RESTRICTION ENZYMES

Type IV restriction enzymes are distinct from the more widely known Type II enzymes due to their unique properties and functions.

1. General Characteristics of Type IV Restriction Enzymes

Type IV restriction enzymes are relatively less studied compared to other types, but they exhibit unique features that distinguish them from their counterparts:

- **Targeting Modified DNA:** Type IV enzymes primarily recognize and cleave **modified DNA**, particularly **methylated** DNA. This is in contrast to other types, like Type II enzymes, which recognize and cut **unmodified, palindromic DNA sequences**.
- **Recognition of DNA Modifications:** These enzymes usually target **methylated** or **hydroxymethylated** bases within a DNA sequence. This methylation is often a result of bacterial self-protection mechanisms against foreign DNA, such as phages or plasmids.
- **Modification-dependent:** Type IV enzymes are sometimes grouped under the classification of **"modification-dependent" restriction enzymes** because their action is often dependent on a DNA modification status (like methylation).

TYPE IV RESTRICTION ENZYMES

2. Function and Mechanism of Action

The role of Type IV restriction enzymes can be described as follows:

- **Defense Mechanism:** These enzymes act as a defense mechanism for bacteria, similar to how other restriction enzymes work. They protect bacterial genomes from foreign DNA, such as viral DNA, by recognizing modified bases on the incoming foreign DNA and cutting it.

- **Cleavage Site:** Unlike Type II restriction enzymes, which typically cut at specific, defined recognition sequences, Type IV enzymes often cut the DNA **near or within** the modified bases. This suggests that their action is closely linked to the methylation status of the DNA rather than a rigid recognition of a specific sequence.

- **Non-Palindromic Recognition:** Type IV enzymes often do not require palindromic sequences, which are characteristic of Type II restriction enzymes. Instead, they may recognize modifications like **methyl groups** added to adenine (m6A) or cytosine (m5C), commonly seen in the bacterial genome as a defense against foreign DNA.

TYPE IV RESTRICTION ENZYMES

3. Structural Features

Type IV restriction enzymes have distinctive structural features, which include:

- **Multicomponent Systems:** These enzymes are often part of **multi-protein complexes**. They may require additional factors or cofactors to facilitate their action, unlike simpler enzymes like Type II.
- **DNA Modification and Restriction Complexes:** Some Type IV enzymes belong to complexes that include both a modification enzyme (which methylates the DNA) and a restriction enzyme (which cleaves the DNA). This system ensures that the bacterial DNA is protected from self-cleavage while still being able to cut foreign, unmethylated DNA.

TYPE IV RESTRICTION ENZYMES

4. Example of Type IV Restriction Enzymes

One well-characterized example of Type IV restriction enzymes is **McrBC** (Methylcytosine restriction endonuclease B). Here's a closer look at this enzyme:

- **McrBC System:** The McrBC enzyme complex cleaves DNA that is methylated at the **cytosine** residue (m5C). It recognizes DNA containing methylcytosine modifications and cuts the DNA near those sites. This system is particularly important for defending against viral DNA that has been methylated at specific cytosine residues, as viral genomes can often acquire modifications that differ from the host's natural modification pattern.

- **Other Examples:** The Mrr system (methylation-dependent restriction) and the McrA and McrA-B systems are other examples of bacterial defense systems involving Type IV restriction enzymes.

TYPE IV RESTRICTION ENZYMES

5. Biological Significance and Applications

Type IV restriction enzymes have significant biological roles, especially in the context of **immune defense**:

- **Prokaryotic Immune System:** These enzymes are part of the bacterial **immune system**, providing protection from foreign genetic material, especially from bacteriophages or plasmids that have been modified to escape recognition by other types of restriction enzymes.

- **Applications in Molecular Biology:** While Type IV enzymes are less commonly used in molecular biology compared to Type II enzymes, their ability to cleave modified DNA can be harnessed for specific tasks that require the targeting of methylated DNA. They have potential applications in gene editing and genome analysis where modifications need to be specifically recognized or edited.

TYPE IV RESTRICTION ENZYMES

6. Challenges in Studying Type IV Enzymes

Due to their specific requirement for DNA modifications, studying Type IV enzymes can be more challenging than studying other types. Some of the difficulties include:

- **Limited Recognition Sequences:** Since these enzymes rely on methylation rather than sequence recognition, it is more difficult to predict exactly where cleavage will occur in a given DNA molecule.
- **Lack of Broad Recognition:** Because Type IV enzymes often target specific methylation patterns rather than sequences, their activity can be more difficult to control in laboratory settings.

TYPE V RESTRICTION ENZYMES

Type V restriction enzymes are a class of endonucleases that are part of the broader family of restriction-modification systems. Unlike Type II restriction enzymes, which recognize specific DNA sequences and cleave within or near these sites, Type V restriction enzymes use a distinctive mechanism and function. These enzymes are less well-known than the classic Type II enzymes, but they have unique properties that make them important in molecular biology and genetic research.

1. Definition and Classification

Type V restriction enzymes are part of a classification system established to categorize restriction enzymes based on their sequence specificity, reaction mechanism, and co-factor requirements. The classification is made by the **REBASE (Restriction Enzyme Database)**.

- **Type I** and **Type II** enzymes are the most commonly known, where Type II is the classic enzyme that recognizes specific palindromic DNA sequences and cuts within or near them.
- **Type V** enzymes, however, are classified by their unique mechanisms of action and the types of DNA cleavage they mediate.

TYPE V RESTRICTION ENZYMES

2. Mechanism of Action

Type V restriction enzymes are characterized by **CRISPR-associated (Cas) proteins**, which originally evolved as part of the bacterial immune system for targeting and cutting foreign DNA. These enzymes function differently from traditional restriction enzymes in that they do not cleave the DNA at their recognition site immediately. Instead, they typically require **guide RNA** (gRNA) and a **CRISPR-associated (Cas) protein** to mediate cleavage.

Key aspects of their mechanism:

- **Recognition and Cleavage:** Type V enzymes use a **guide RNA** to locate a specific target sequence within the DNA. Upon binding, the associated Cas protein induces DNA cleavage. The cleavage occurs in a **DNA sequence-specific manner** but can involve cutting DNA strands **away from** the recognition sequence, leading to a **double-strand break**.

CRISPR-Cas System Link: Many Type V enzymes are related to the CRISPR/Cas9 system, which has become widely used in gene editing technologies. The Cas proteins involved in Type V enzymes, such as **C2c1**, **C2c2**, and **Csm/Csn systems**, facilitate DNA cleavage in a programmable

TYPE V RESTRICTION ENZYMES

3. Types of Type V Enzymes

Several classes of Type V restriction enzymes exist, mainly based on the different CRISPR-associated proteins involved. They are:

- **C2c1 Family (Type V-A):** These enzymes recognize and cut specific DNA sequences in a targeted manner using a CRISPR guide RNA and Cas protein. They are similar in function to CRISPR-Cas9 but differ in their mechanisms of action, including differences in the target DNA recognition and cleavage site.
- **C2c2 Family (Type V-B):** This is another family of Type V restriction enzymes that have gained attention due to their ability to target specific DNA sequences. C2c2 uses single-stranded RNA as a guide for DNA recognition and cleavage.
- **Csm/Csn Family (Type V-C):** In these systems, multiple CRISPR-associated proteins work in concert to bind and cleave specific DNA sequences. They can also be programmed to target specific sites in a DNA sequence for precise editing.

TYPE V RESTRICTION ENZYMES

4. Functional Significance and Applications

Type V restriction enzymes, particularly those from the CRISPR-Cas family, are important for their ability to be programmed to target nearly any sequence of DNA. This has led to several practical applications, especially in the fields of genetic engineering and molecular diagnostics.

Applications:

- **Genome Editing:** The CRISPR-Cas system, which includes Type V enzymes, is widely used for gene editing, allowing researchers to introduce specific mutations or insert new genetic material into a host organism's genome.
- **Synthetic Biology:** The programmability of Type V restriction enzymes enables their use in designing synthetic genetic circuits, manipulating microbial systems, and creating engineered organisms with novel traits.
- **Diagnostics:** CRISPR-based diagnostic tools, like **SHERLOCK** and **DETECTR**, utilize Type V enzymes to detect specific DNA or RNA sequences, enabling rapid, sensitive disease diagnosis (e.g., for pathogens such as SARS-CoV-2).
- **Protection Against Viruses:** Type V enzymes, as part of the bacterial immune system, provide defence mechanisms against bacteriophages and other foreign genetic material. This characteristic is fundamental to understanding bacterial evolution and defence strategies.

TYPE V RESTRICTION ENZYMES

5. Comparison with Other Restriction Enzymes

While Type II restriction enzymes, like **EcoRI** and **BamHI**, are widely used for cloning, cutting DNA at specific sequences, and studying genetic material, Type V restriction enzymes are more complex and provide more flexible, programmable DNA targeting.

- **Type II Enzymes:** Typically cut DNA within or near their recognition sequences. These enzymes are simpler and have a long history of use in molecular biology.
- **Type V Enzymes:** In contrast, these enzymes (related to CRISPR systems) are more complex and can be programmed for very specific DNA targeting. They enable more sophisticated applications in genome editing and molecular diagnostics.

TYPE V RESTRICTION ENZYMES

6. Limitations and Challenges

Despite their promising applications, Type V restriction enzymes also face challenges:

- **Complexity:** The need for guide RNAs and precise control over the CRISPR-associated proteins can complicate experimental design and increase the difficulty of their use.
- **Off-Target Effects:** Although CRISPR/Cas systems are highly specific, off-target effects where DNA is cleaved at unintended locations remain a potential concern in genome editing.

VARIATIONS ON CUTTING AND JOINING DNA MOLECULES

In order to join two fragments of DNA together, it is not essential that they are produced by the same restriction endonuclease.

Many different restriction endonucleases produce compatible cohesive ends.

For example, *AgeI (A/CCGGT) and AvaI C/CCGGT)* produce molecules with identical 5′ overhangs and so can be ligated together.

ISOSCHIZOMERS AND NEOSCHIZOMERS

Recognition site and cleavage specificity

Another important way to classify and compare restriction enzymes is **isoschizomers** and **neoschizomers**.

- **Isoschizomers** are restriction enzymes that have the same recognition sequence *and* the same specificity. For instance, AgeI and BshT1 recognize and cleave 5′-A↓CCGGT-3′ in the same pattern. Nevertheless, a set of isoschizomers may differ in site preferences, reaction conditions, methylation sensitivity, and star activity.
- **Neoschizomers** recognize the same nucleotide sequence but cleave DNA at different positions. Examples of neoschizomers are SmaI (5′-CCC↓GGG-3′) and XmaI (5′-C↓CCGGG-3′), which both recognize 5′-CCCGGG-3′ but cleave them differently and thus generate different types of ends (in this case, blunt ends for SmaI and 5′ protruding ends for XmaI).