University of Jijel
Faculty of Nature and Life Sciences
Departement of Applied Microbiology and Food Sciences

Genetic engenneering Applied in microbial biotechnology

Master (2nd year)
applied Microbiology

Pr. Mohamed Sifour
University of Jijel

Content

- Overview : the basic concepts of molecular biology, the tools of molecular biology, genetic expression.
- Genetic manipulation of microorganisms used in industry: mutation, cloning, etc. Screening, selection and analysis of recombinant strains
- Stability of recombinant strains
- The application of genetically modified strains in industry
- Production of recombinant proteins for therapeutic use: insulin, growth hormone, vaccines, etc.

Definition

Genetic engineering

Genetic engineering is the manipulation of an organism's genes using biotechnology such as recombinant DNA technology.

Genetic engineering is a set of molecular biology techniques that isolate specific genes, reconstruct them, and then reinsert them into cells or organisms. A technique that involves removing, modifying, or adding genes to a DNA molecule [of an organism] in order to change the information it contains.

The concept of genetic engineering, which involves the artificial manipulation, modification, and recombination of DNA or other nucleic acid molecules to alter organisms, has garnered significant interest over the past few decades.

Genetic engineering plays a crucial role in the rational and precise advancement of microbial biotechnology. Certain microbial components have proven invaluable in the creation of genetic tools, such as CRISPR-Cas systems and thermostable DNA polymerase enzymes. These tools are essential for the development of genetic engineering strategies. For instance, the discovery of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas components in bacteria has revolutionized genome editing. These breakthroughs can enhance the biotechnological capabilities of specific microorganisms, such as improving antibiotic production efficiency.

These two disciplines are interdependent and often challenging to differentiate. Together, they have transformed both the industrial sector and the field of medicine.

Microbial biotechnology

There are many definitions of biotechnology. One of the broadest is the one given at the United Nations Conference on Biological Diversity (also called the Earth Summit) at the meeting held in Rio de Janeiro, Brazil in 1992. That conference defined biotechnology as

"any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use."

Many examples readily come to mind of living things being used to make or modify processes for specific use. Some of these include the use of microorganisms to make the antibiotic, penicillin or the dairy product, yoghurt; the use of microorganisms to produce amino acids or enzymes are also examples of biotechnology.

For millennia, microorganisms have contributed to our daily lives by providing essentials like bread, beer, and wine. In recent times, the technological application of microorganisms—known as microbial biotechnology—has become a critical factor in producing vital natural bioactive compounds.

Microbial biotechnology, the technological application of microorganisms, has been instrumental in producing significant natural bioactive products. These include antibiotics, antifungals, anticancer drugs, antiparasitics, antivirals, immunosuppressants, toxoid vaccines, and therapeutic enzymes. However, microbial biotechnology extends beyond alcohol fermentation, antibiotic synthesis, and molecular biology breakthroughs. It is a dynamic field where continuous exploration leads to fresh insights and discoveries.

Microbial biotechnology focuses on industries and services using microorganisms or their **products** (microorganisms of interest used in manufacturing, transformation or degradation processes).

These applications are numerous:
- production of metabolites of microbial origin;
- transformation of raw materials into fermented foods,
- treatment of wastewater and effluents,
- waste recovery,
- degradation of pollutants;
…

Gene Cloning: Tools & Techniques

Genetic manipulation of organisms:

1- Conventional approach (Breeding)
2- Recombinant DNA (rDNA) Technology

Conventional Breeding

Techniques in conventional breeding
1- Mutagenesis in bacteria:
e.g Induction of mutation in bacteria to increase the yield of antibiotics

2- Hybridization in plants and animals:
e.g. Hybridization between two plants one of them is virus-resistant and the other gives high quality crop but it is prone to viral infections.
e.g Hybridization between cows with high yield of milk with others with high meat production

General steps in conventional breeding.

1. Determine the breeding objective (s)
e.g. protein production, disease resistance, yield increase, improved quality.
2.Assemble genetic variability.
-e.g. to increase the protein content of a plant, there must exist a plant somewhere with high protein content so it can be used in the breeding program.
3. Recombine the variation
-In animals, the conventional way of producing hybrid is to mate selected parents.
-In plants, pollens from one parent is transferred to the stigma of the other parent.
4. Select desirable recombinants
5. Evaluate the selections.

Limitations of conventional breeding

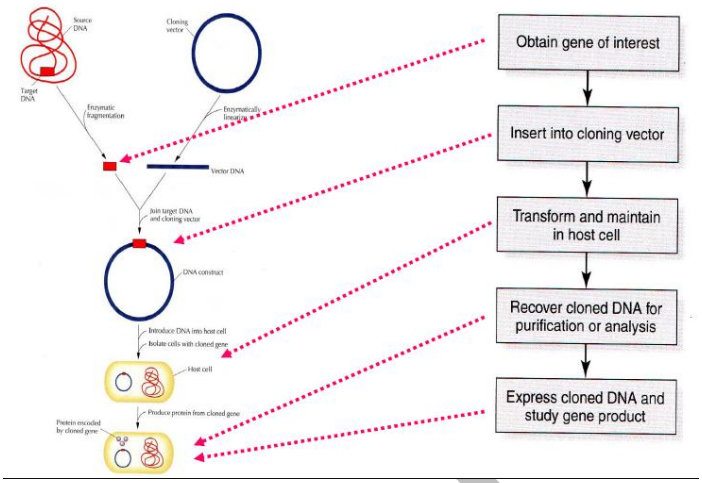
1- Long duration (e.g. plant breeding at least 7-10 years).
2- Limited to crossing within species
-Parents must belong to the same species.
3- Lower selection efficiency
-Sort among enormous variations is not precise.
4-Expensive due to large segregation population

Recombinant DNA Technology

Recombinant DNA technology (gene cloning) is a term used to refer to experimental protocols leading to the transfer of genetic information (DNA) from one organism to another.

General steps in rDNA

1.Obtain the gene of interest (tool: restriction enzymes).
2.Insert the gene into cloning vector (tool: restriction enzymes/ plasmid, bacteriophage, or cosmid).
3.Insert and maintain the vector in bacterial host cell (transformation)
4.Recover cloned DNA for purification and assay (markers).
5.Express cloned DNA and study the product



A. Enzymes as "tools" of molecular biology

Almost all DNA manipulative techniques make use of purified enzymes. Within the cell these enzymes participate in essential processes such as DNA replication and transcription, breakdown of unwanted or foreign DNA (e.g., invading virus DNA), repair of mutated DNA, and recombination between different DNA molecules.

Purified enzymes are therefore crucial to genetic engineering and an important industry has sprung up around their preparation, characterization, and marketing. Commercial suppliers of high purity enzymes provide an essential service to the molecular biologist.

DNA manipulative enzymes can be grouped into four broad classes, depending on the type of reaction that they catalyze:

* Nucleases are enzymes that cut, shorten, or degrade nucleic acid molecules.
* Ligases join nucleic acid molecules together.
* Polymerases make copies of molecules.
* Modifying enzymes remove or add chemical groups.

1- Polymerases

All polymerases synthesize nucleic acids from 5' to 3' using nucleotide triphosphates. Energy is provided by the incoming nucleotide triphosphates.

a) DNA polymerases

DNA polymerases are enzymes that synthesize a new strand of DNA complementary to an existing DNA or RNA template. Most polymerases can function only if the template possesses a double-stranded region that acts as a primer for initiation of polymerization. We can distinguish:

DNA polymerases DNA dependent (or DNA polymerases),

DNA polymerases ARN dependant (or reverse transcriptases)

DNA polymerases that do not require a template (terminal transferases)

α) DNA polymerases DNA dependent (or DNA polymerases)

They make DNA by using DNA as a template. DNA polymerases need a base that is already hybridized or primer. Most often the primer is an oligonucleotide, a single strand that can be chemically synthesized.

All DNA polymerases have:

- A 5' to 3' polymerization activity,

- A terminal transferase activity on double strands with a preference for the incorporation of an adenine. If we incubate a double stranded DNA fragment, presenting a blunt end with a DNA polymerase, we obtain an end with an A coming out at 3'.

Some DNA polymerases have:
-a 3'5' exonuclease activity, this activity is often called correction activity. Indeed, if the polymerase makes a mistake, the last nucleotide is no longer hybridized, the polymerization is blocked.
The 3'5' exonuclease activity removes the unhybridized nucleotide, the polymerization can continue. This 3'5' exonuclease activity also removes the A added in 3' by the terminal transferase activity
- a 5'3' exonuclease activity

Example of DNA polymerases used in molecular biology:

E. coli DNA polymerase I:

It is a 100 kDa enzyme of about 100 KDa that has 5'-3' polymerase activity, 3'-5' exonuclease activity (proofreading activity) and 5'-3' exonuclease activity.
DNA polymerase I is therefore an example of an enzyme with a dual activity-DNA polymerization and DNA degradation. In 1970, Klenow and Henningsen removed the 5'-3' exonuclease activity of DNA polymerase by proteolysis (subtilisin). The obtained protein "Klenow fragment" (76 kDa) is devoid of 5'-3' exonuclease activity but retains the polymerase activity and the 3'-5' exonuclease activity. Since then, this large fragment has been obtained by expression of a truncated gene.

T4 and T7 DNA polymerases :

They have the same activities as Klenow (5'-3' polymerase and 3'-5' exonuclease). The exonuclease activity of T4 DNA polymerase is more important than the exonuclease activity of Klenow, so it will be preferred to Klenow when this activity is required.
The 3'5' exonuclease activity can be chemically removed to improve their performance when the correction is not very important, especially when the DNA is not subsequently cloned. In addition, site-directed mutagenesis replacing a phenylalanine with a tyrosine allows T7 DNA polymerase to better recognize dideoxynucleotides. These modified polymerases marketed under the name sequenase® are used in sequencing reactions.

Thermostable DNA polymerases:

Taq DNA polymerase of Thermus aquaticus

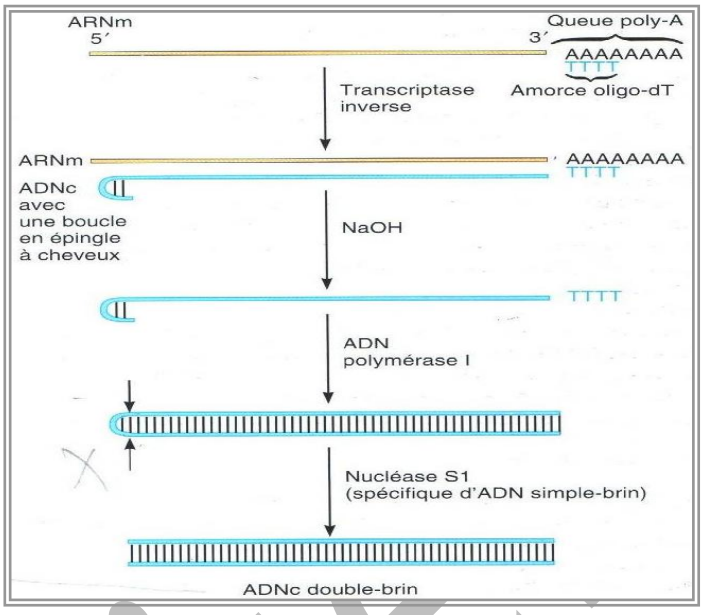
It has 5'-3' polymerase and 5'-3' exonuclease activities. Its advantage is that it is thermostable. This is the special feature of Taq DNA polymerase that makes it suitable for PCR. Since it lacks 3'-5' exonuclease activity, the error rate is about 10^{-5} per duplicated base and the terminal transferase activity is efficient. The 5'-3' exonuclease activity was removed by deleting the N-terminus of Taq DNA polymerase in a manner analogous to what was done on E. coli DNA polymerase I to obtain Klenow. This polymerase has been named KlenTaq or Titanium®Taq. This polymerase is more efficient than Taq DNA polymerase and the amplification products are easier to insert into a cloning vector.

Pfu and Pwo DNA polymerase

They come from Pyrococcus furiosus, a hyperthermophilic archaeon discovered in geothermal springs in Italy, and from Pyrococcus woesei. They have the same sequences and therefore have identical activities. They have 5'-3' polymerase and 3'-5' exonuclease activity but no 5'-3' exonuclease activity. The so-called correcting 3'-5' exonuclease activity reduces the error rate to 10^{-6} per duplicated base.

β) DNA polymerase ARN dependent : reverse transcriptase

Reverse transcriptase is unique in that it uses as a template not DNA but RNA. This enzyme has the same activity as DNA-dependent DNA polymerase, but it makes DNA from RNA. The ability of this enzyme to synthesize a DNA strand complementary to an RNA template is central to the technique called complementary DNA (cDNA) cloning.



Three types of primers can be used:

- an oligo dT in this case we obtain a population of cDNA,
- a random primer, in this case we obtain a population of cDNA whose 5' end is variable
- or a primer specific to a sequence present on an RNA, in this case we obtain cDNAs of a unique sequence corresponding to a single gene from RNA of eukaryotic cells.

These sequences can be amplified by PCR, the successive association of the two techniques has taken the name of RT-PCR.
Reverse transcriptase lacks 3'5' exonuclease activity so that there is no error correction.
Reverse transcriptases are fragile enzymes. To improve synthesis and obtain long cDNA, protein stabilizers can be added.
Different reverse transcriptases are available:
- MMLV and AMV are viral reverse transcriptases.
- SuperScript (Invitrogen): reverse transcriptases such as MMLV and AMV have an associated RNase H activity.
- Fluoroscript (Invitrogen): this reverse transcriptase derived from "superscript" more easily incorporates fluorescent nucleotides. This activity is interesting for making probes to hybridize on DNA chips.

γ) Terminal transferase:

This polymerase does not need a template like other polymerases, it adds nucleotides in 3' to the end of the DNA strand. It adds nucleotides in 3' in the presence of dNTP. If we want to add only one, we add a ddNTP.
It can be used for :
- Making a homopolymeric tail
- Making a sticky end in 3'

b) DNA-dependent RNA polymerases

RNA polymerase recognizes a promoter and synthesizes an RNA complementary to the strand downstream of this promoter. The synthesis is carried out in the 5'-3' direction in the presence of ribonucleotide triphosphates. The gene that interests us must therefore be cloned behind a specific promoter for the RNA polymerase that we want to use. The three main RNA polymerases: T7 RNA polymerase, T3 RNA polymerase and SP6 RNA polymerase. These three polymerases come from the T7, T3 or SP6 phages and each recognizes a specific promoter, so T7 RNA polymerase recognizes the T7 promoter but not the T3 or SP6 promoters.

2- 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:

- Exonucleases remove nucleotides one at a time from the end of a DNA molecule.
- Endonucleases are able to break internal phosphodiester bonds within a DNA molecule.

The main distinction between different exonucleases lies in the number of strands that are degraded when a double-stranded molecule is attacked.

The enzyme called Bal31 (purified from the bacterium *Alteromonas espejiana*) is an example of an exonuclease that removes nucleotides from both strands of a double-stranded molecule. In contrast, enzymes such as *E. coli* exonuclease III degrade just one strand of a double-stranded molecule, leaving single-stranded DNA as the product.

The same criterion can be used to classify endonucleases.

S1 endonuclease (from the fungus *Aspergillus oryzae*) only cleaves single strands, whereas deoxyribonuclease I (DNase I), which is prepared from cow pancreas, cuts both single and double-stranded molecules.

Uses of S1 nuclease

Study of DNA-RNA hybrids to detect introns.
Conversion of sticky ends to blunt ends for cloning.
Removal of loops in cDNA synthesis.

DNase: endonuclease that cleaves DNA into nucleotides or polynucleotides. It hydrolyzes phosphodiester bonds. DNase I is non-specific in that it attacks DNA at any internal phosphodiester bond, so the end result of prolonged DNase I action is a mixture of mononucleotides and very short oligonucleotides.

Uses: in RNA or protein preparations (to eliminate DNA contamination)
in probe labeling with radioisotopes

On the other hand, the special group of enzymes called restriction endonucleases cleave double stranded DNA only at a limited number of specific recognition sites

3) Ligases

T4 ADN ligase

It catalyzes the formation of a phosphodiester bridge between a 3' OH and a 5' phosphate, it needs ATP and divalent ions. It ligates double-stranded DNA.

Use: Ligation of sticky or blunt ends of restriction fragments.

If both ends are dephosphorylated, ligation cannot take place, on the other hand if only one is dephosphorylated, ligation takes place on one of the two strands, the other remains with a nick.

Typically, sticky ends are generated by cutting with restriction enzymes. Most often, the ends to be ligated will be generated by the same enzyme but this is not mandatory. For example, the enzymes Bam HI (G/GATCC), Bgl II (A/GATCT) and Mbo I (N/GATCN) give cohesive ends that can be ligated because they are compatible. However, after certain ligations such as those of a Bam HI end and a Bgl II end, the product cannot be redigested by either of the two enzymes.

Taq DNA ligase has the same activity as T4 DNA ligase, but it requires NAD instead of ATP. It is more stable and works at 45°C.

T4 RNA ligase

Catalyzes the junction between a 5' phosphate of a single-stranded RNA or DNA with a 3' OH (divalent ions and ATP)

DNA ligase of *E. coli*

Double-stranded DNA sticky end ligation. *E. coli* DNA ligase is inefficient at ligating blunt ends or ligating RNA. It uses NAD as a cofactor.

4) Phosphatases and kinases

Phosphatases

The most used is calf intestinal alkaline phosphatase (CIP). It catalyzes the removal of 5' phosphate. It requires zinc and a pH between 9 and 10 to function and is stimulated by magnesium. It is heat-labile, it can be inactivated by incubation at 65°C for one hour. We can also use shrimp alkaline phosphatase (SAP), this phosphatase does not require zinc to be active and can therefore be used in most buffers compatible with restriction enzymes. It is even less stable than CIP and is inactivated by incubation for 15 minutes at 65°C.

Alkaline phosphatases are used for example to dephosphorylate vectors before ligating an insert. Removal of 5'-phosphates is very useful in preventing self-ligation of cleaved DNA vectors

Quick CIP (new england Biolabs) is a heat-labile version of calf intestinal alkaline phosphatase (CIP) purified from a recombinant source.

T4 polynucleotide kinase

Catalyzes the transfer of the gamma-phosphatephosphate from ATP to the 5'OH of DNA (double or single strand) or to RNA.

Uses: - labeling of the 5' end of DNA,

- labeling of radioactive oligonucleotides of synthetic oligonucleotides.
- phosphorylation of PCR fragments for cloning

5) Methylases

Restriction enzymes are produced by bacteria to protect themselves from phages. To prevent bacterial DNA from being digested, the bacteria also produce specific methylases that inhibit digestion. Thus, Eco RI does not cut GAm6ATTC. These methylases can be used to inhibit the digestion of a fragment. Restriction enzymes that recognize the same sequence are not sensitive to the same methylations. We can therefore detect areas on the DNA that are methylated by digesting with two enzymes, one sensitive and the other not. The products will be deposited on an electrophoresis gel and the digestion will be analyzed after a Southern blot. If the band is detected only with the enzyme insensitive to methylation, we can deduce that the area probed is methylated.

6) Restriction endonucleases

The well-known function role of R-M systems is to protect the host cell from invading foreign DNAs

"Immune" system in microorganisms (restriction-modification system): specific methylase/endonuclease couple

Methylated DNA => resistant

Unmethylated DNA => cleavage -> protection against foreign DNA (restriction)

Cut the double-stranded DNA molecule at specific points (palindromes) creating blunt or sticky ends, in a reproducible manner required for gene cloning. The discovery of these 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.

Three different classes of restriction endonuclease are recognized, each distinguished by a slightly different

mode of action. Types I and III are rather complex and have only a limited role in genetic engineering. Type II restriction endonucleases, on the other hand, are the cutting enzymes that are so important in gene cloning.

Nomenclature of restriction enzymes:

This nomenclature does not obey the rules of IUPAC (International Union of Pure and Applied Chemistry), but depends on specific rules which take into account the bacteria from which the restriction enzyme was isolated:

• The first letter, capitalized, represents the initial of the bacterial genus

• The two lowercase letters following the first are representative of the species.

• The Roman numeral following these three letters is the order number of discovery of the enzyme for the same source bacteria.

• The last capital letter is not required for all restriction endonucleases. It is representative of the strain of bacteria from which the enzyme was isolated.

-The names for restriction endonucleases reflect their origin:

EcoRI

- E = genus *Escherichia*
- co= species coli
- R = strain RY13
- I = first endonuclease identified in *E. coli*

HpaI and HpaII

-They are first and second type II restriction endonucleases that were isolated from *Haemophilus parainfluenzae*.

There are also examples of restriction endonucleases with degenerate recognition sequences, meaning that they cut DNA at any one of a family of related sites. HinfI (*Haemophilus influenzae* strain Rf), for instance, recognizes GANTC, so cuts at GAATC, GATTC, GAGTC, and GACTC.

One important feature of sticky end enzymes is that restriction endonucleases with different recognition sequences may produce the same sticky ends. BamHI (recognition sequence GGATCC) and BglII (AGATCT) are examples—both produce GATC sticky ends. The same sticky end is also produced by Sau3A, which recognizes only the tetranucleotide GATC. Fragments of DNA produced by cleavage with either of these enzymes can be joined to each other, as each fragment carries a complementary sticky end.

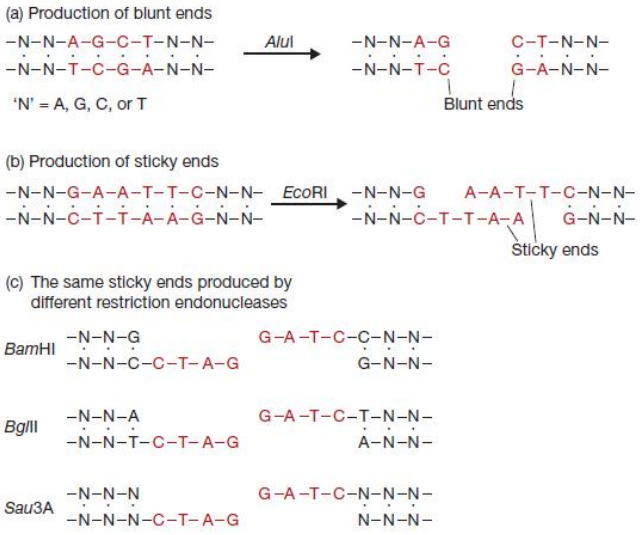


Fig: The ends produced by cleavage of DNA with different restriction endonucleases. (a) A blunt end produced by AluI. (b) A sticky end produced by EcoRI. (c) The same sticky ends produced by BamHI, BglII and Sau3A. BROWN, 2010,