

UNIT III

GENETIC

ENGINEERING

Teacher → Pramod sir

Reference → i) Genetics by Russell.
ii)

Saturday

Date 23/11/2019

* Introduction:

↳ Every living organism has the ability to inherit traits from its parents.

✓ The living cell and organisms are characterized by their remarkable ability to reproduce and transmit characteristics generation after generation & maintain continuity of inherited trait.

✓ Genetic material of any organism is the substance that carries genetic information determining the properties of that organism.

✓ The genetic material is also responsible for transferring genetic material from parent to their off-springs.

* DNA as the Genetic Material:

✓ During 1940 & 1950's several experiments were carried out which clearly indicated and established that the genetic information is present in nucleic acid.

o Griffith's Experiment on Bacterial Transformation (1928)

i) The phenomenon of transformation was discovered by Friedrich Griffith in 1928.

ii) He performed several experiments in order to find out the identity of the genetic material.

He found that the pneumonia causing bacterium Diplococcus pneumoniae exists in two forms:

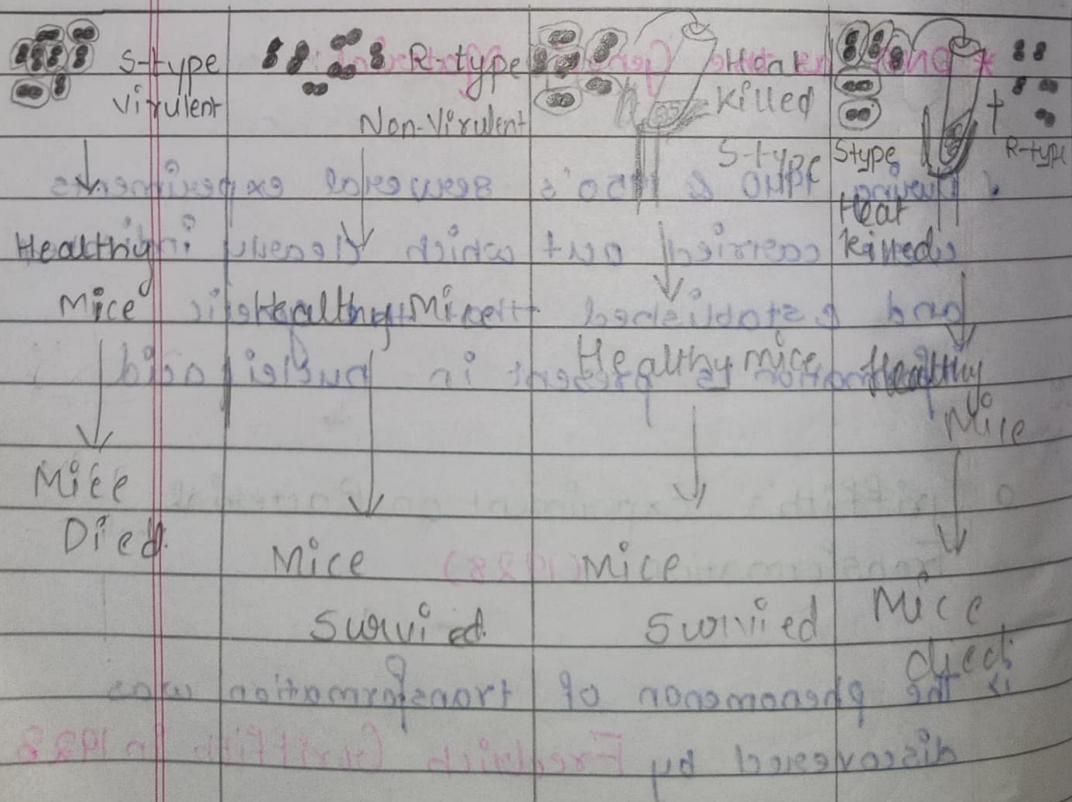
Smooth & Non-Smooth or capsule & non-capsule

Smooth Type (S-type) → It is the virulent strain which is covered by capsule and cause the pneumonia.

Non-Smooth type (R-type) → It is the non-virulent strain which is not covered by capsule.

& are harmless.

iii) The experiment performed by Griffith for the genetic material may be summarized as follows:

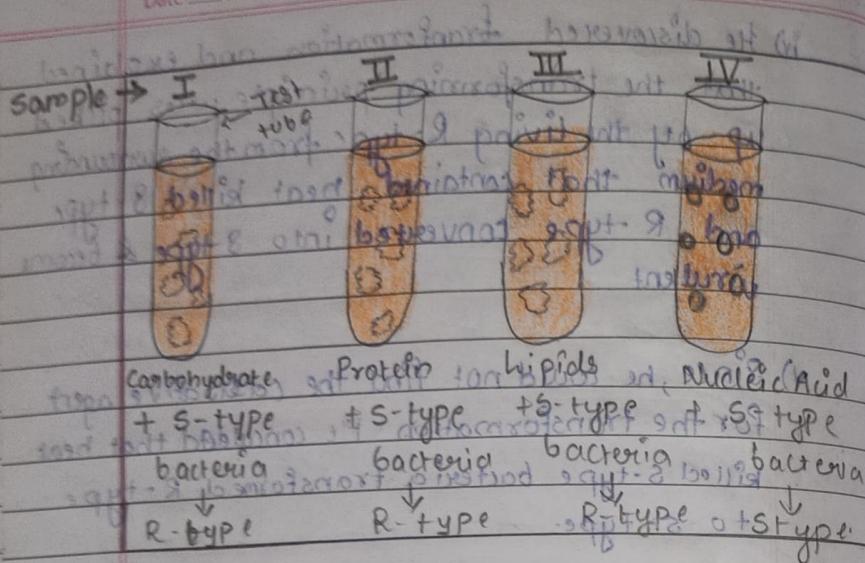


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- v) He discovered transformation and explained that the transforming principle was picked up by the living R-type from the surrounding medium that contained heat killed S-type and R-type converted into S-type & becomes virulent
- v) Since, he could not find the causative agent for the transformation. He concluded that heat killed S-type bacteria transformed R-type to S-type.

Q) Experiment done by Avery, MacLeod & McCarty (1944) to show that DNA is the transforming principle.

- i) The first direct evidence showing that genetic materials is DNA was demonstrated and by Oswald Avery, Colin Macleod and Maclyn McCarty in 1944.
- ii) They were the first to discover and demonstrate that the component of the cell responsible for the phenomenon of transformation of non-virulent to virulent type bacteria is DNA molecule.
- iii) For their experiment they used test tube assay instead of mice.



o Hershey's & Chase Experiments (1952)

- i) Experiments by O. Avery, MacLeod and MacCarty proved that the transforming principle is DNA but all biologist were not convinced.
- ii) Hershey & Chase published a paper that provided more evidence that DNA was the genetic material. They were studying a bacteriophage called P_2 phage.
- iii) Hershey & Chase worked with viruses that infect bacteria i.e. bacteriophage, which have nucleic acid composed of DNA & capsid is made up of proteins.
- iv) They worked to discover whether it carries DNA or protein.
- v) They used radioactive phosphorus (^{32}P) in DNA and radioactive phosphorus (^{35}S) in Protein coat.
- vi) Bacteria → both types.
- vii) Both type of radioactive viruses were allowed to infect E. coli bacteria.

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E-coli infected with ^{32}P -labeled T₂

 ^{32}P DNA

DNA + protein, protein + capsomere x 3
DNA + protein → coat (outer protein) + inner
protein + capsomere AND + capsomere



Blend briefly

After adding a blending agent → Phage
coat and tail machine from solution

Radioactivity recovered → Phage
infect & pass capsomere → ghost
of phage progeny

activity ratio between ^{32}P and ^{35}S under $\times 1000$
aggregation + direction tail coat

^{35}S protein
coat

E-coli infected with
 ^{35}S - labeled T₂

time of infection
ghosts

ghosts
just T₂

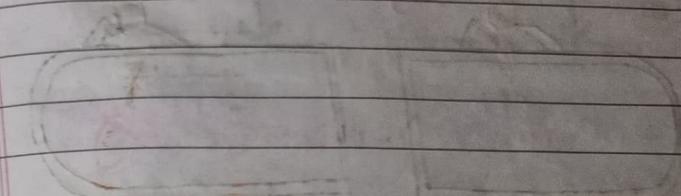
(^{32}P was radioactive with protein)
(^{35}S was radioactive with protein)

Radioactivity recovered
in phage ghosts and
not passed on to

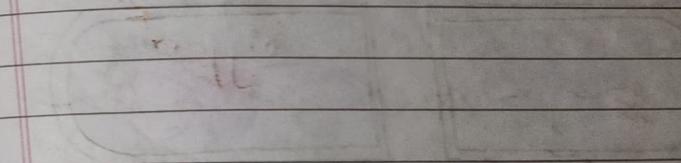
other progeny (ghost for capsomere
+ protein)

vii) They confirmed that DNA is the only genetic material. This fact has universally accepted since then. This indicates that DNA enters the bacterial cells & not proteins. Therefore DNA is the genetic material.

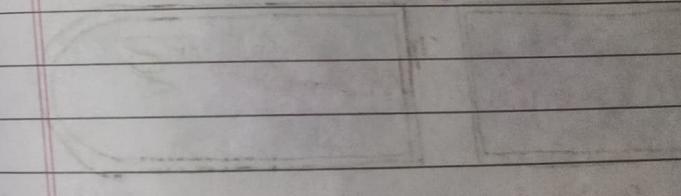
Experiment



In infected bacteria
not infected



In infected bacteria
not infected



EXPERIMENT

EXPERIMENT

ENZYMES

o Nuclease

ii) Nucleases are those enzymes which depolymerizes the nucleic acid.

iii) According to the substrate nuclease are of two namely DNase & RNase.

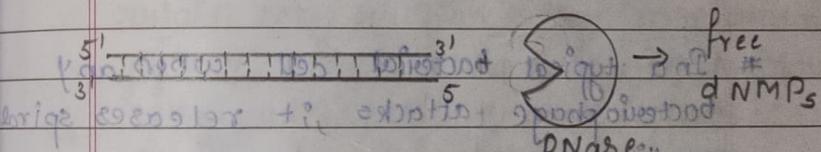
DNase → degrade DNA

RNase → degrade RNA.

iv) According to the mode of action nucleases can also be classified as Exonucleases & Endonucleases.

Consider DNase which contain exonuclease

activity.

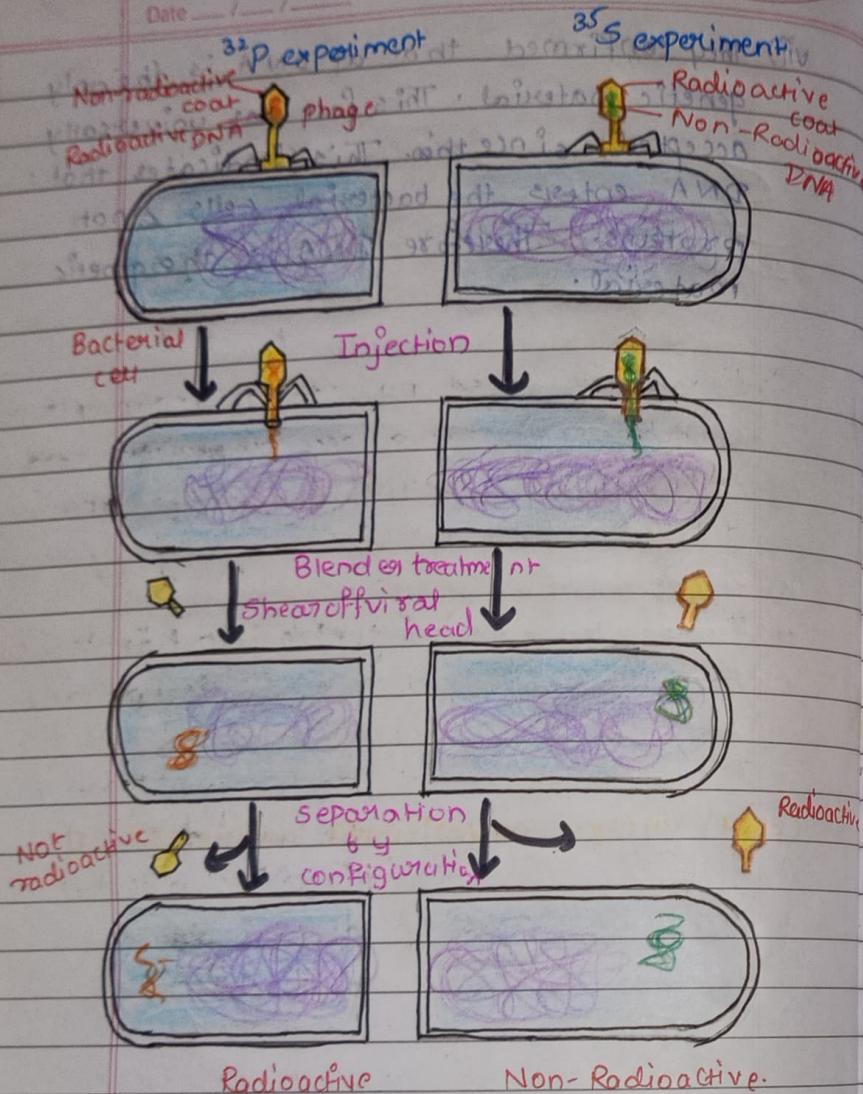


v) These enzymes cleave phosphodiester bond which is closest to their free ends. So by this always exonuclease can remove one nucleotide at a time from free end to the nucleic acid.

This may happens in 5' to 3' or 3' to 5'

direction. In this case if we move 5' to 3' direction, then it is called

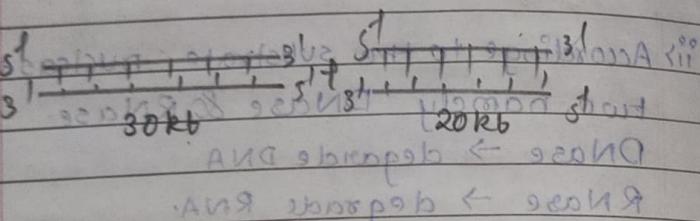
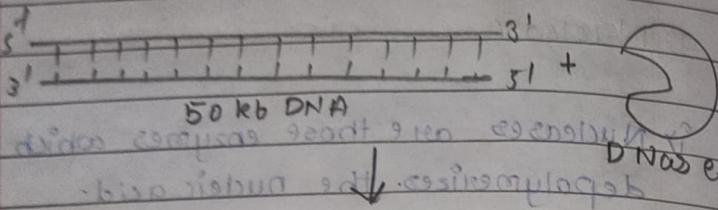
5' to 3' direction. And if we move 3' to 5' direction, then it is called 3' to 5' direction.



HERSHEY-CHASE

EXPERIMENT

Consider DNase with endonuclease activity



These enzymes cleave the phosphodiester bond which is internal of phosphodiester bond and cleaves the bigger DNA into fragments.

□ Restriction Modification system (RMS)

In a typical bacterial cell when any bacteriophage attacks, it releases single nucleic acid.

It was found that viral nucleic acid may be in the form of double stranded or single stranded linear DNA depends upon the type of invading virus.

In order to protect itself from the action of such invading foreign DNA, bacterial

RMS include DNase with endonuclease & exonuclease activity. DNase with endonuclease activity recognizes such foreign invading DNA & cleaves it by breaking internal phosphodiester bond at a particular site or anywhere in the DNA into pieces.

This clearly indicates that such enzymes restricts the survival of foreign DNA with in the bacterial cell hence known as restriction endonucleases / enzymes (RE).

However, to protect own DNA from the action of own RE's a typical bacterial cell do possess modification system (MS).

Using MS bacterial cell make a use of certain enzyme like methyl transferase or glycosyl transferase such enzymes can donate methyl or glucosyl group to specific A or C of a genomic DNA or Plasmid DNA at a particular position.

This kind of modification may be complete or partial. Nevertheless in both the conditions bacteria able to protect its own DNA from getting digested.

By this way RMs plays significant role for the survival of bacterial cell and protection against any foreign DNA.

o Type II Restriction Enzymes

i) It is one of the most essential enzymes which is used exclusively in RDT.

ii) These are the following reasons why

(i) It is extremely specific to its restriction site.

• It cleaves the DNA between H restriction sites.

• It is made up of single polypeptide chain.

• Its molecular mass ranges between 20-100 kDa.

• Type II Restriction Enzyme is very easy to be isolated & purified.

• It is economical as it requires Mg^{2+} as the co-factor.

o Characteristics features:

i) Nomenclature:

→ There is a standard procedure for the nomenclature of type II Restriction Enzyme with the reference to the bacterium from which it is isolated.

For e.g., Eco RI, the nomenclature is as follows:
Where First letter → Genus of the bacteria
Second / third letter → species
Fourth letter → strain
Roman numeral → order of discovery

Eco RI can be described as first restriction enzyme isolated from Escherichia coli belonging to the strain RY13.

↳ Restriction Endonuclease Squeezes using 2nd & 3rd & 4th, 5th pair.

→ It is made up of 4, 6, 8, 10 Base pairs.

→ It shows two-fold axis of rotational symmetry.

→ The sequence is a palindrome in mature.

Foreg :- Restriction site of Eco RI.

5' G A A T T C 3'

3' C T T A A G T 5'

3) Frequency of occurrence:

→ To calculate frequency of occurrence of any restriction site one can use the formula i.e.

$$f = \gamma(4)^n$$

4 → No. of the nitrogenous bases found in DNA
n → No. of base pairs involved in given restriction site.

For e.g.: If restriction site is made up of 4 bp such.
then frequency of occurrence of restriction site in given genomic DNA is:

words ← RATTI ATTCG

prob = $\frac{1}{4}$ i.e. after 16 base pairs

(4)

restriction site is formed at 16th position

Hexan = 4 base pairs apart between enzymes

$$f = \frac{1}{(4)^4} = \frac{1}{256} \text{ i.e. after 256 base pairs}$$

restriction site which made up of 4 base pairs appears theoretically.

Similarly, for 6 and 8 base pairs

$$f = \frac{1}{(4)^6} = \frac{1}{4096} \text{ i.e. after 4096 base pairs}$$

restriction site which made up of 6 base pairs appears theoretically

$$f = \frac{1}{(4)^8} = \frac{1}{65,536} \text{ i.e. after 65,536 base pairs}$$

restriction site which made up of 8 base pairs appears theoretically

→ Based on frequency of occurrence, restriction enzyme can be classified as:

- frequent cutters → It is restriction enzyme whose restriction site is made up of 4 or 6 base pairs.

- rare cutters → It is restriction enzyme whose restriction site is made up of 8 or 10 base pairs.

Eg of frequent cutters:- Sau3A & EcoRI

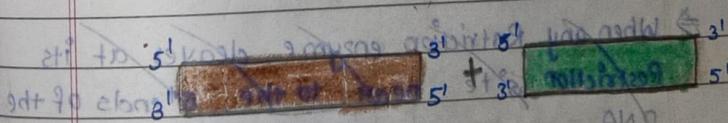
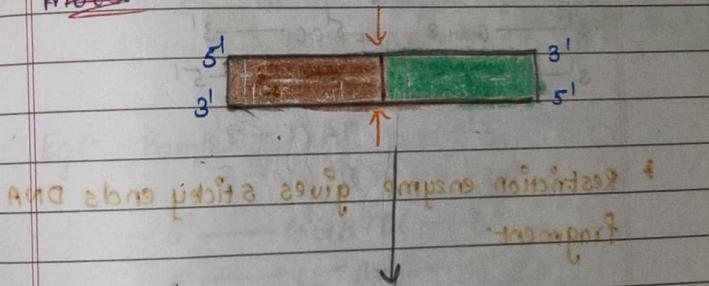
Eg of rare cutters:- Not I

4) Mode of cut / cleavage / digestion

→ When restriction enzyme cleave the DNA at its restriction site it will give rise to either blunt ended or sticky ended fragment; this will depends upon the mode of cleavage.

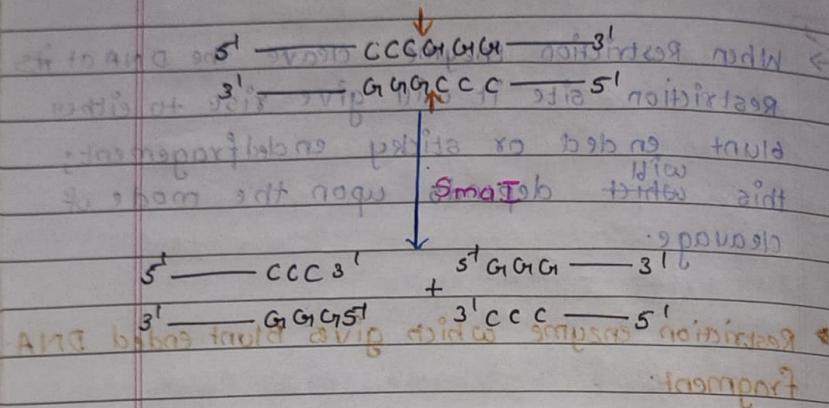
► Restriction enzyme which gives Blunt ended DNA fragment:

→ When any restriction enzyme cleaves at its restriction site at the same position on the both the DNA strand it gives rise to production blunt ended DNA fragment.

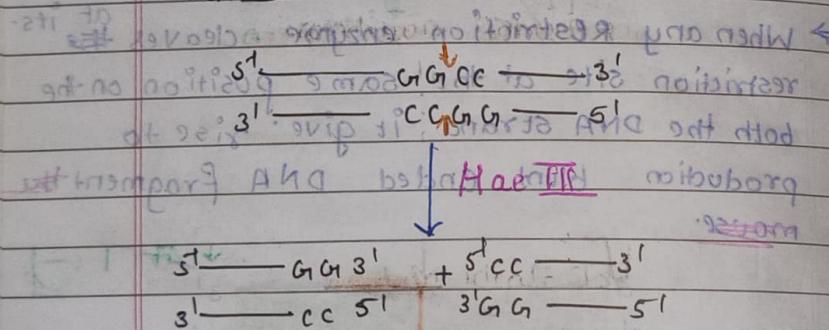


It is evident that after restriction enzyme cleavage the DNA fragments are produced with the same position on both strands.

For e.g.: -> SmaI restriction site to show re



& HaeIII



► Restriction enzyme gives sticky ends DNA fragment.

⇒ When any Restriction enzyme cleaves at its Restriction site near to the 5' ends of the DNA

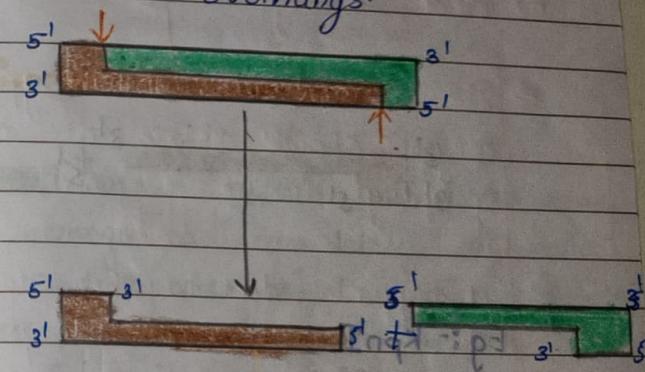
⇒ When any Restriction enzyme cleaves at its Restriction site but at different position on the both the DNA strands it give

rise to production of sticky ended DNA fragment.

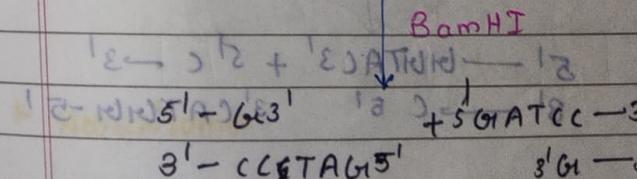
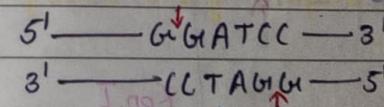
Such RE can be further classified

(A) RE which gives 5' overhang

- When RE cleaves the DNA at its RS near to the 5' ends of the DNA it gives DNA fragments with 5' overhangs.



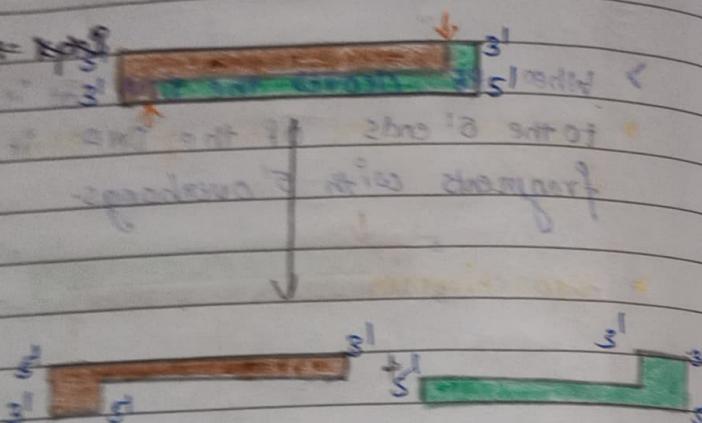
Eg:- BamHI → ATNNNN →



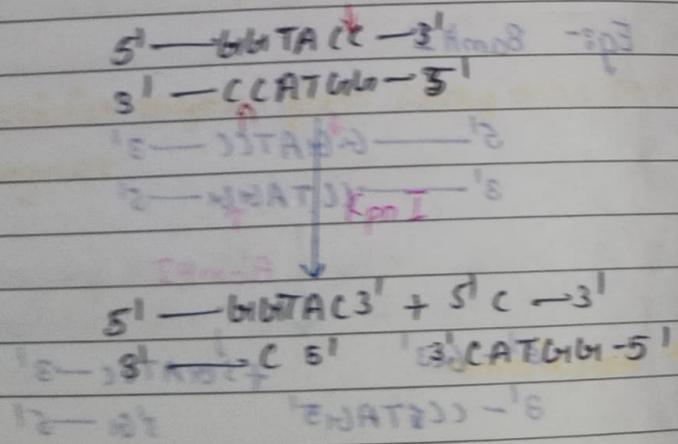
b) RE which gives 3' overhangs

When RE cleaves the DNA at its RS near to the strands of the DNA it gives DNA fragments with 3' overhangs.

Eg:- BpuII



Eg:- KpnI



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Degeneracy

It was found that there are certain Restriction enzyme whose restriction site is degenerative in the nature.

Eg:- HinfI can recognize 5' 5'-GANTC-3' where N stands for any of four NTP Nitrogenous base.

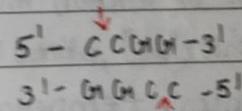
Note:- Here, HinfI recognize Restriction site which is made up of 5 basepair.

Psoschizomorphae

It includes those type II Restriction Enzyme which belongs to completely different genera and species of the micro-organism but recognize same restriction site and cleaves it with the same manner.

Eg:- MspI

~~HAPR~~ HPAI.



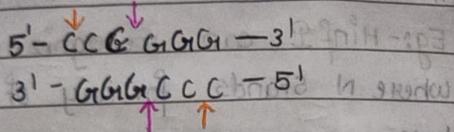
Neoschizomorphae

It includes those type I Restriction Enzyme which belongs to completely different genera and species of the micro-organism but recognizes

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Some restriction site and cleaves it with different manner.

Eg:- Sma I ($\uparrow\downarrow$) Xba I ($\uparrow\downarrow$)



6) Star activity to whom in DNA

⇒ It's a condition where type II restriction enzyme shows relaxation in the specificity in the sequence.

⇒ It happens due to the following reasons:-

- Use of the non-ideal strength buffer
- Excess of Restriction Enzyme
- Excess of DNA
- High Glycerol concentration
- Contamination of reaction mixture by ethanol, DMSO [Dimethylsulphoxide], phenol.
- Non-optimum temperature / pH
- Prolonged incubation time.

DNA ligase

i) It is one of the most essential enzyme used by the cell as well as DNA viruses throughout the process of DNA replication & recombination.

ii) DNA Ligase is also called as molecular suters & molecular glue.

iii) DNA ligase plays very significant role in RDP

iv) DNA ligase which are available to perform ligation reaction under *in vitro* condition are of two different types.

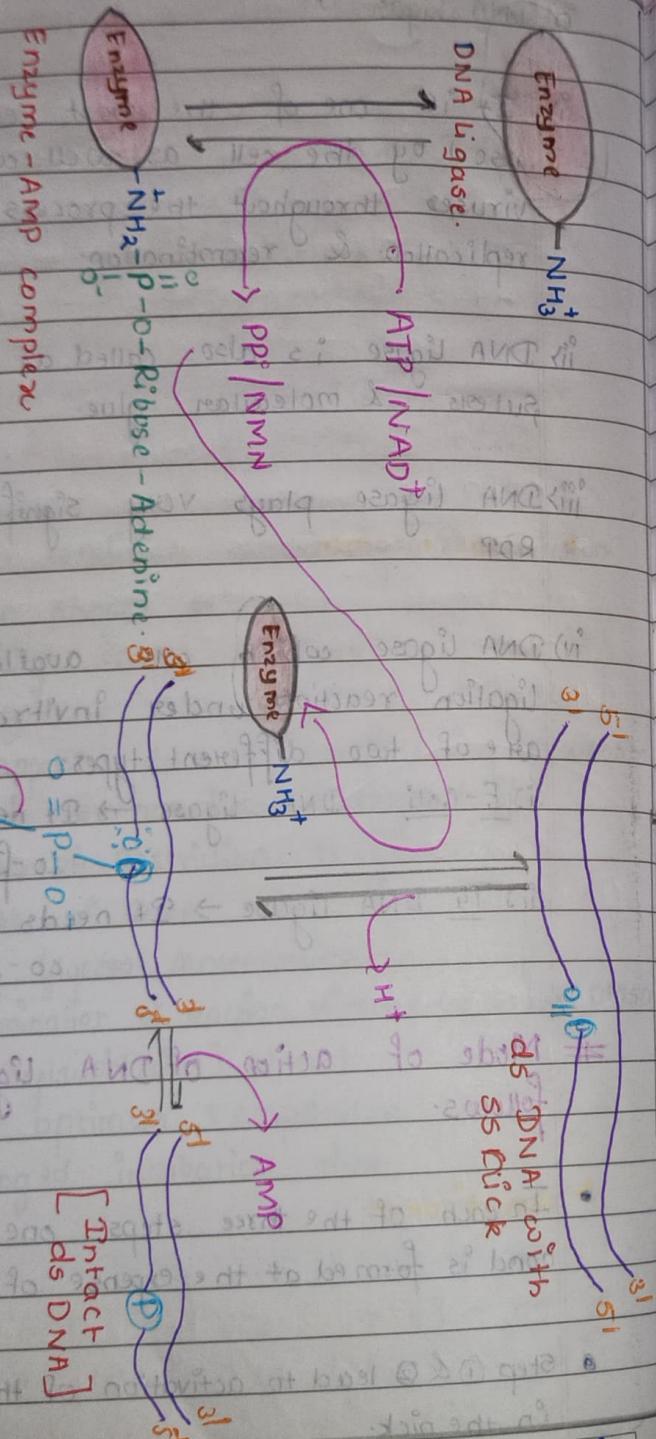
(i) E. coli DNA Ligase → It needs NAD⁺ as co-factor.

(ii) T₄ DNA Ligase → It needs ATP as co-factor.

Mode of action of DNA Ligase is as follows.

- In each of the three steps, one phosphodiester bond is formed at the expense of another.
- Step ① & ② lead to activation of the 5' phosphate in the nick.

Mode of Action of DNA ligase.



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- An AMP group is transferred first to a Lys residue on the enzyme & then to the 5' phosphate in the nick.

- In step ③, the 3'-hydroxyl group attacks this phosphate & displaces AMP, producing a phosphodiester bond to seal the nick.

- In the E. coli DNA Ligase reaction AMP is derived from NAD⁺.

- The DNA ligases isolated from a number of viral & eukaryotic sources use ATP rather than NAD⁺ & they release phosphate rather than Nicotinamide mononucleotide (NMN) in step ①

o DNA Polymerase

- i) The most important enzyme of DNA replication and recombination.

- ii) In prokaryotes, the five different types of DNA polymerase are found
For eg:- I, II, III, IV, V

- iii) Among them Pol I, II, III, are commonly used by the cell.

	I	II	III
$5' \rightarrow 3'$ poly ⁿ	✓	✓	✓
$3' \rightarrow 5'$ exo	✓	✓	✓
$5' \rightarrow 3'$ exo	✓	-	-
Structure	single Polypeptide	single Pepitid	Multimeric complex.

DNA Pol I.

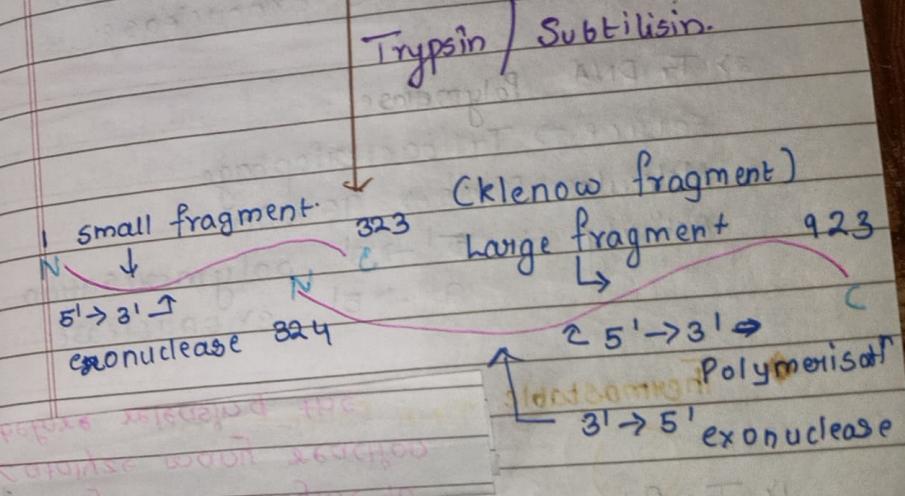
⇒ The search for an enzyme (DNA Pol II) that could synthesize DNA began in 1956 by Arthur Kornberg.

⇒ The molecular mass of DNA Pol I is 109 kDa.

⇒ DNA polymerase I, instead it performs a host of clean-up functions during replication, recombination & repair.

⇒ The polymerase's special function are enhanced by its $5' \rightarrow 3'$ exonuclease activity and distinct from the $3' \rightarrow 5'$ proofreading endonuclease.

⇒ When the $5' \rightarrow 3'$ exonuclease domain is removed, the remaining fragment, the large fragment or Klenow fragment, retains the polymerization and proofreading activities.



⇒ Klenow fragment possesses good processivity as compared to DNA Pol I.

Application of DNA Pol I

- Helps in λ DNA preparation
- Exclusive used in random primer method.
- Nick translation
- Used in Sanger Method
- Helpful to generate blunt end (end filling)
- Effective in End labelling.

Example: $5' \rightarrow 3'$ direction \leftarrow

Q1) T₄ DNA Polymerase

Source → T₄ bacteriophage

Structure → Single Polypeptide chain

Molecular mass → 114 kDa

Activity → $5' \rightarrow 3'$ polymerisation

$3' \rightarrow 5'$ exonuclease.

Properties (highlighted):
① Shows 3' → 5' exonuclease.

Q2) T₁ DNA Polymerase.

Source → T₁ bacteriophage

Molecular Mass → 96 kDa

Activity → $5' \rightarrow 3'$ polymerisation

$3' \rightarrow 5'$ exonuclease.

♦ Thermostable DNA Polymerase.

1) Tag Polymerase.

Source → Thermus aquaticus

Molecular mass → 95 kDa

Activity → $5' \rightarrow 3'$ Polymerisation.

* Remains stable at very high temperature and shows optimum activity at 75°C

2) Pfu DNA Polymerase.

Source → Pyrococcus furiosus

Activity → $5' \rightarrow 3'$ Polymerisation

$3' \rightarrow 5'$ exonuclease.

* It needs Mg²⁺ ions for its activity

Pfu (exō) DNA Polymerase

Shows $5' \rightarrow 3'$ polymerisation but no exonuclease activity. However it shows very high processivity.

4) Vent DNA Polymerase.

Source → Thermococcus litoralis

Possess → $5' \rightarrow 3'$ Polymerisation

$3' \rightarrow 5'$ exonuclease.

* It needs Mg²⁺. Temperature stability very

high upto 80°C. It is composed of two subunits of 20 kDa

5) Vent (exō) DNA Polymerase.

Shows $5' \rightarrow 3'$ polymerisation but no exonuclease activity. However it shows very high processivity.

* Alkaline Phosphatase:

* The source of alkaline phosphatase is bacteria, calf's intestine & shrimps.

* Its function is catalyses the removal of the phosphoryl group from 5' end of the nucleic acid.

* The chemical nature is a dimeric glycoprotein, the molecular mass is 14 kilodalton.

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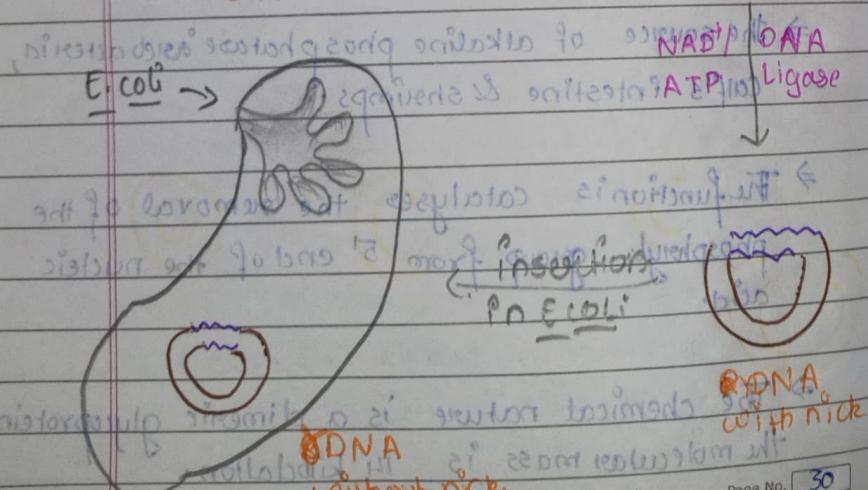
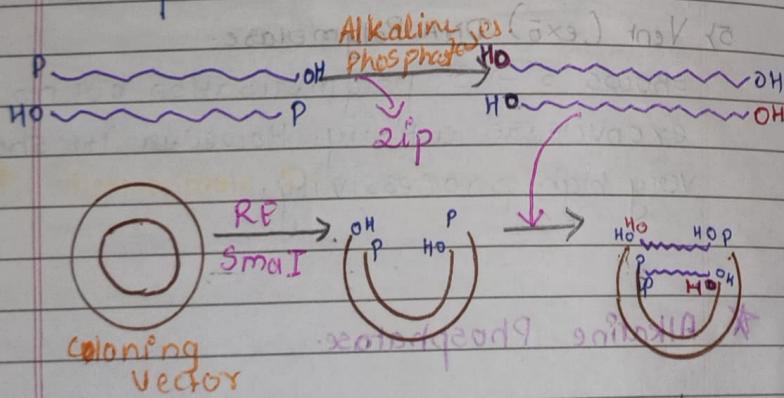
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⇒ This enzyme requires 4Zn^{2+} ions for activity.

Applications of Alk. Phosphatases

- Helps in inhibition of self ligation of cloning vector.
- Used for Gene mapping.
- Plays major role in DNA fingerprinting.

Role of Alkaline phosphatases + I
To prevent self ligation of cloning vector is as follows:



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* T₄ Polynucleotidyl kinase

⇒ The source of T₄ polynucleotidyl kinase is T₄ bacteriophage.

⇒ The structure of T₄ polynucleotidyl kinase is tetramer & the molecular mass is 33 kDa.

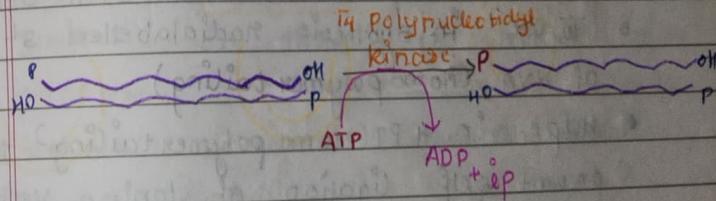
⇒ The function

Functions

- Catalyze transfer of terminal phosphate group of ATP to 5' end of the nucleic acid as follows:

Application

- Exclusively used in synthesis radiolabelled DNA.



* Terminal Deoxynucleotidyl transferase.

⇒ It is also known as template independent DNA polymerase.

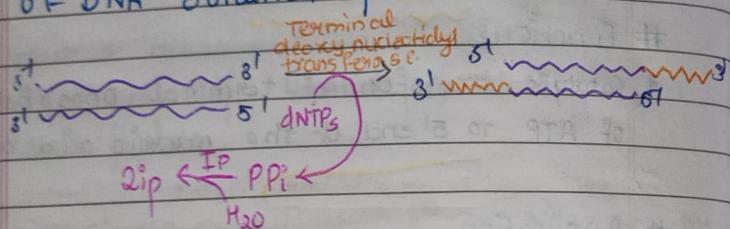
⇒ The source of Terminal deoxynucleotidyl transferase are Bacteria & human WBCs.

⇒ The structure of terminal deoxynucleotidyl transferase is dimer made up of two subunits whose molecular mass is 26.5 kDa & 8 kDa.

⇒ The enzyme need Mg^{2+} / Mn^{2+} / Co^{2+} as a co-factor.

Function: extension of 3' end of template

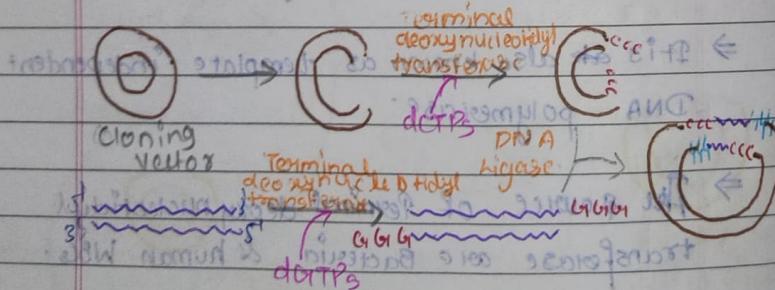
- Catalyzes addition dNMPs to the 3'-OH end of DNA obtained from dNTPs.



Application:

- To create stickyended DNA from Blunt ended DNA
- To use in synthesis radiolabelled 3'-end of DNA
- Helps in HPT (Homo polymer tailing) to prevent self ligation of cloning vector

* Homopolymer tailing (HPT) process (minimum) *



→ In homopolymer tailing (HPT), blunt ended DNA ligation can be turned into sticky ended DNA ligation to enhance success rate of RDP.

→ In this process, blunt ended cleaved cloning vector and foreign DNA can be treated with terminal deoxynucleotidyl transferase in presence of suitable dNTPs.

Note:- dNTPs used for tailing of cloning vector should be complementary to dNTPs used for tailing of foreign DNA.

→ Both DNA's can be subjected for ligation using DNA ligase.

→ This gives rise to formation of heavy chances of recombinant cloning vector having foreign DNA within through the formation of 4 phosphodiester bonds & multiple H-bonds between foreign DNA & clonal vector.

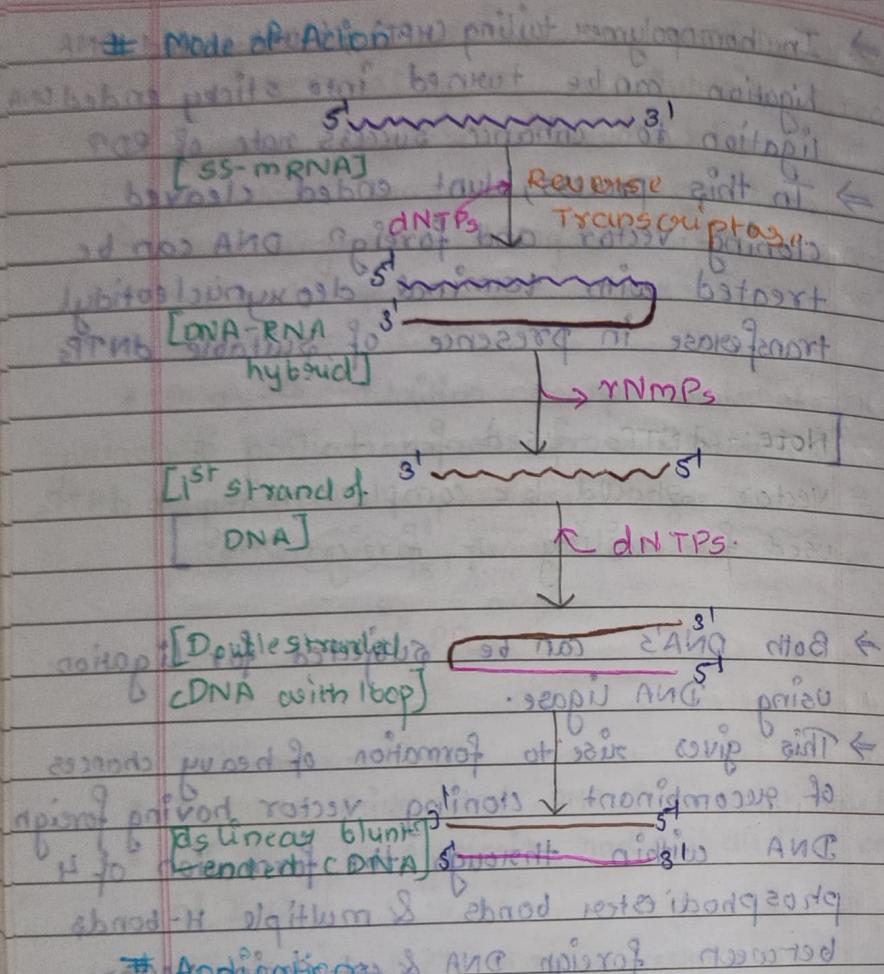
★ Reverse Transcriptase.

⇒ The source is retrovirus for e.g.:-

• Mouse Leukemia Virus (MLV)

• Avian Mylooma Virus (AMV)

⇒ The properties are • RNA dependent DNA Polymerase, RNaseH, & DNA dependent DNA Polymerase activity.



Applications of RT

→ Exclusively used in cDNA library to prepare cDNA

→ Very much helpful in Reverse transcriptase Polymerase chain reaction (RT-PCR)

NOTE :- It is also found that mRNA and activity of RT of MLV is poor as compared to AMV. Hence RT is not applicable to AMV.

- * **Plasmid** :- A genetic structure in a cell that can replicate independently of the chromosomes; typically a small circular DNA strand in the cytoplasm of a bacterium or protozoan.
- * Plasmids are much used in the laboratory for manipulation of genes (transformation).
- * Naturally occurring plasmids are of six different types, they are as follows:-

1) F plasmids

2) R plasmids

3) Col plasmids

2) R PLASMIDS

It carry certain ABC genes, whose gene products provide antibiotic resistance to the host bacterium against that specific antibiotic. For e.g.: Chi^r genes of a plasmid DNA provide chloramphenicol resistance.

3) F PLASMIDS

Its molecular size is approx 99.2 kb. It carries a gene called Pilin which encodes a hydrophobic protein called pilin which comes out of the cell and the monomers of pilin form a single helical

appendages called sex pilus or F-pilus. Cells containing F plasmid are called as F+ or male or donor cells whereas cells lacking F plasmid are called as F- or female or recipient cells. F+ cells can conjugate with F- cells through F pilus and transfers one copy of F-plasmid into the cytoplasm of F- cells. F plasmid may integrate into the host chromosome & then replicate as a part of bacterial DNA.

4) COLICIN PLASMIDS

It has certain bacterial cells carrying col plasmid which encodes one protein called as colicin. Colicin comes out of the col plasmid attacking bacterial cells by causing cell lysis.

For Ex: Due to the presence of col plasmid E. coli acts as a normal intestinal flora of

Human beings.

5) VIR PLASMIDS

It provides pathogenicity to the bacterium.

For Eg: A. tumefaciens & A. rhizogenes carry Ti & Ri plasmid respectively. Due to

the presence of such plasmids both the

bacteria act as a phytopathogen in

normal part of its life cycle.

6) DEGRADATIVE PLASMID

It possesses those genes whose gene products are involved in the degradation of few xenobiotic compounds.

For Ex:- Pseudomonas putida is a superbug this bacteria involved in the hydrocarbon degradation Super bug was developed by Anand Chakrabarty in 1979. This bacteria is used to control oil pollution.

7) Plasmid as a cloning Vectors

- In order to use plasmid as a cloning vector, its molecular size should be more than 10kb.
- With the help of plasmid, foreign DNA of molecular size upto 15kb can be cloned inside the prokaryotic or eukaryotic host cells.
- Any cloning vector should possess following three properties:

• Cloning vector should carry its own origin of replication (Ori) site.

• Cloning vector should carry suitable marker genes.

• Cloning vector should carry unique restriction sites for the corresponding restriction enzymes.

+ **Marker genes / Reporter genes:** Marks or reports the presence of rCNs

They are of 2 types, namely:-

1) Selectable marker

2) Screenable marker.

1) Selectable Marker :-

It include those genes whose gene products helps in the survival of those host cells who possess them, under the selection pressure.

Eg:- Antibiotic resistance genes.

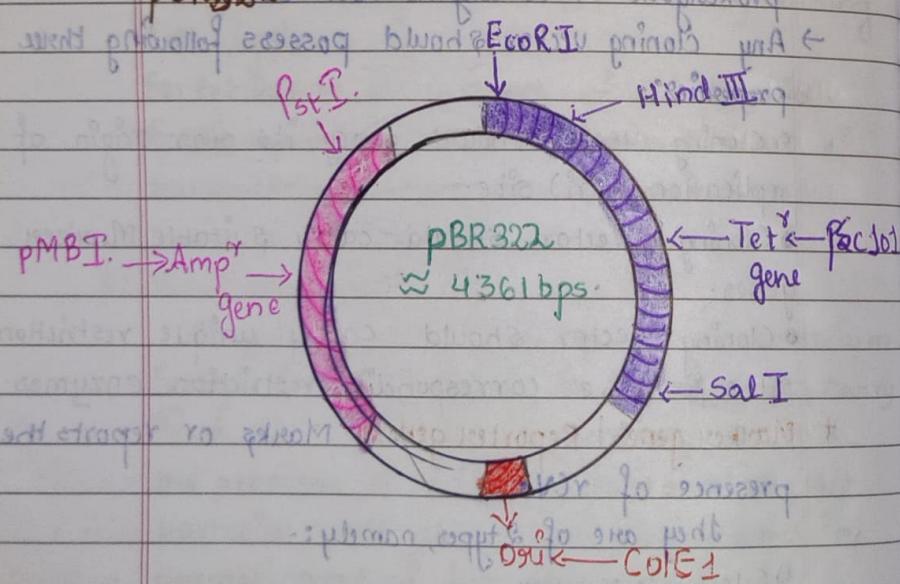
2) Scorable Marker :-

It include those genes whose gene product helps in the differentiation of those cells who possess them from those who didn't possess them.

Eg:- Gus gene (β -glucuronidase) and

lacZ gene (Luciferase).

PBR322 has following restriction sites:



Cloning of PBR322 & Selection of Recombinants

transformed cells via linearization

illigates have importance

a) Treatment of intact circular plasmid of PBR322 with **Pst I** gives rise to cleaved linear PBR322 with interrupted Amp^r gene (as restriction site for **Pst I** found in Amp^r gene)

b) Ligate the obtained cleaved PBR322 with foreign DNA in presence of DNA ligase.

c) This gives rise to the production of self-ligated as well as recombinant pBR322 (rpBR322). [rpBR322 possesses foreign DNA within the coding sequence of Amp^r gene].

d) Transform suitable pro host cells with rpBR322 by means of calcium method.

e) Resulting cells are then subjected to grow on nutrient medium containing Tetracycline. This allows only 'T' bacteria cells to grow on it as they possess Tetracycline resistance (Tet^r gene) received from pBR322. (Tet^r gene is located on Col E 1).

f) Obtained growth pattern of the tetracycline bacteria colonies is then transferred on two in selective medium.

Where first medium contain tetracycline and second selective medium contain tetracycline and ampicillin.

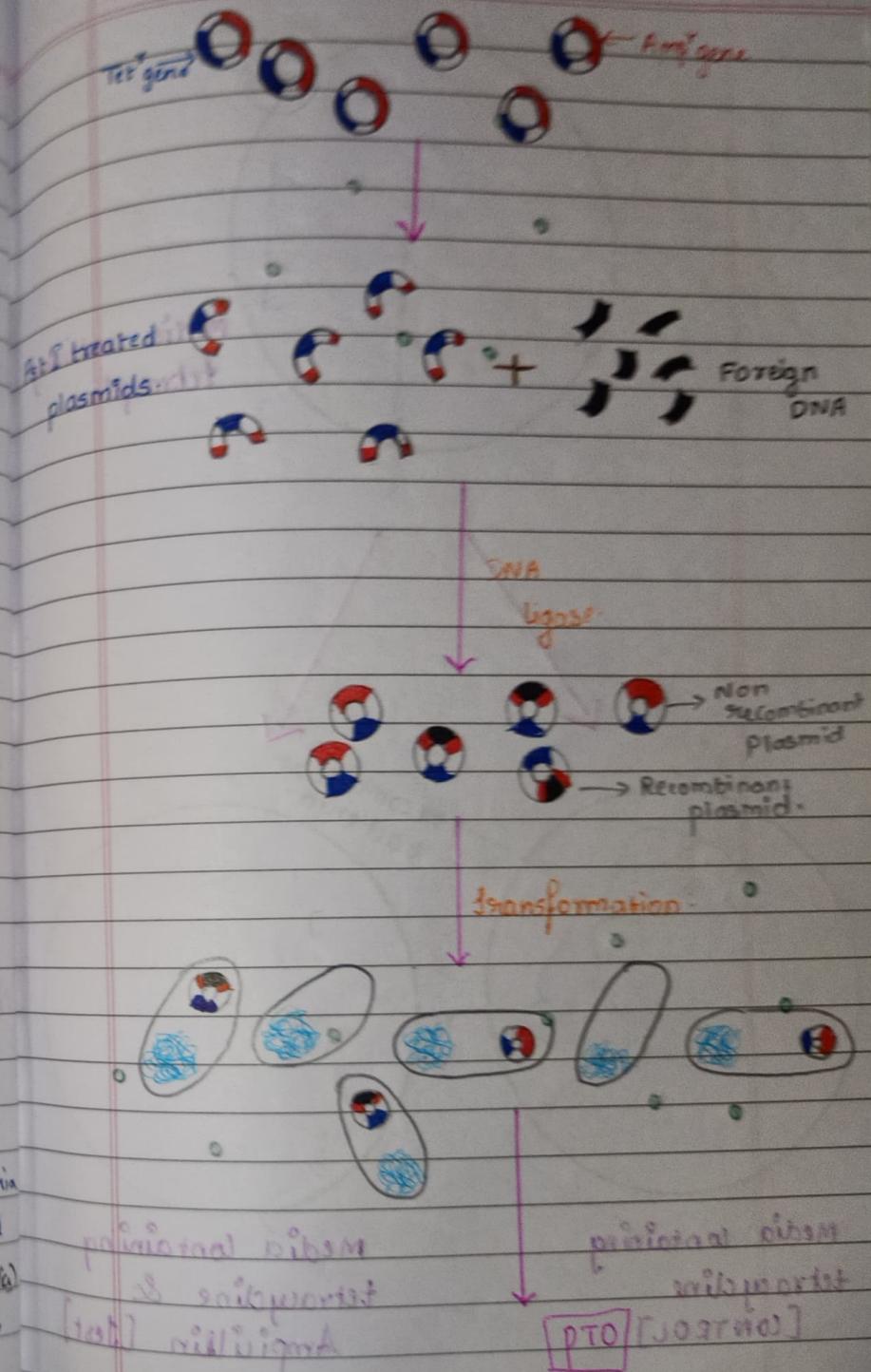
- It was found that, recombinant host cells uptakes pBR822 which possess intact Tet^r gene and interrupted Amp^r gene (due to the insertion of foreign DNA, coding sequence of Amp^r gene is disturbed) hence RT host cells are Amp^r but Tet^r.

- Non-recombinant bacterial host cells uptakes self ligated pBR822 which possess intact Tet^r gene and intact Amp^r gene hence NRT host cells are Amp^r and Tet^r.

- As a result of this RT bacterial host cells grow only on first selective media but not on second (as it contains Amp too).

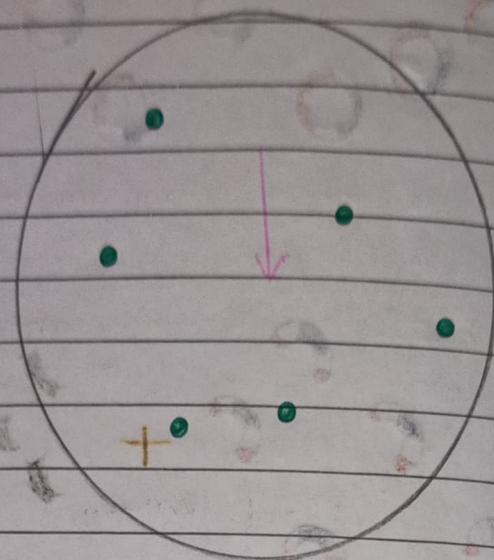
while NRT bacteria host cells grow on both the selective media equally well.

- By comparing both the growth patterns of both the selective media - Recombinant bacteria colonies can be readily identified (as they are unable to grow on second selective media). Such recombinant bacteria colonies are then subjected for sub-culturing.

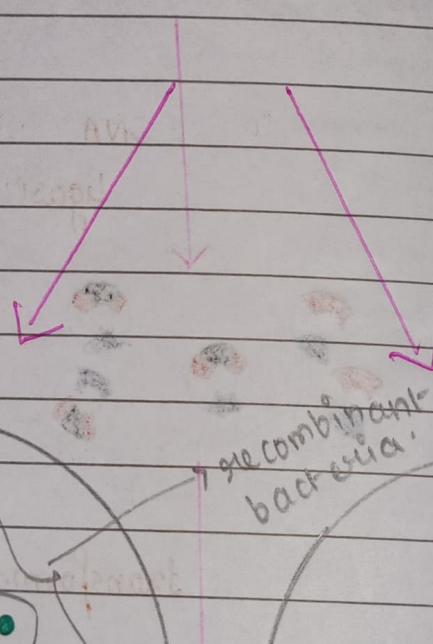


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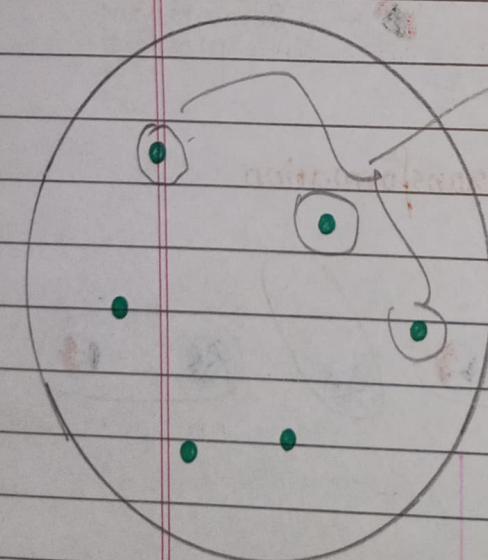
Saathhi



Media containing tetracycline



single recombinant bacteria



Media containing tetracycline
[CONTROL]

Media containing tetracycline &
Ampicillin [test]