Transformation and Selection

Programme: B.Sc (H) Botany
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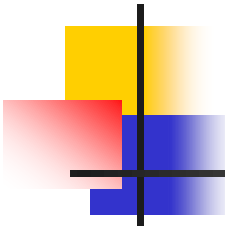
Transformation

- Transformation is the bacterial mechanism for the transfer of genetic material in which free DNA of one genotype is taken in through the cell surface of bacteria of another genotype and is incorporated into the recipient cell chromosome.



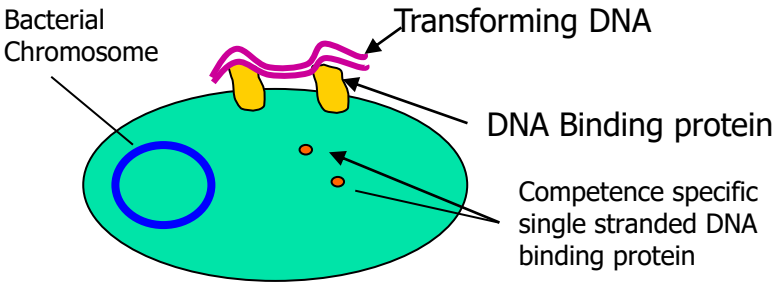
- Not all bacteria take up free-floating DNA in the environment. The genera that generally exhibit transformation include: Bacillus, Streptococcus, Azotobacter, Haemophilus, Neisseria, and Thermus.
- The recipient cells must be competent (able to transform). Competence is a phenotype conferred by one or more proteins. It has been shown that competence occurs late in the exponential phase of bacterial growth. The duration of competence varies from a few minutes in Streptococcus to hours in Bacillus (Krawiec, 2002).

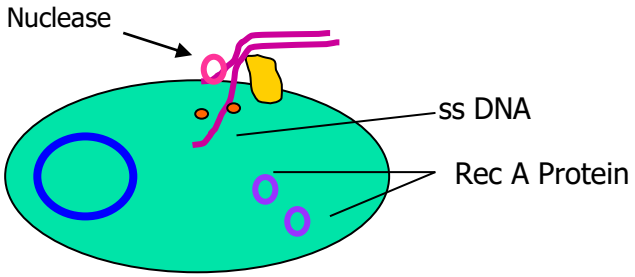
Competence

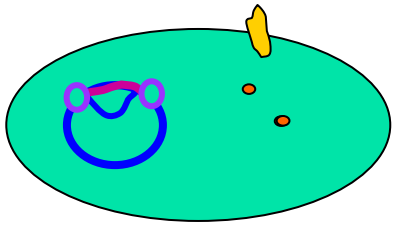
- Most species of bacteria, including *E. coli*, take up only limited amounts of DNA under normal circumstances. In order to transform these species efficiently, the bacteria have to undergo some form of physical and/or chemical treatment that enhances their ability to take up DNA.
- Cells that have undergone this treatment are said to be **competent**.

In nature

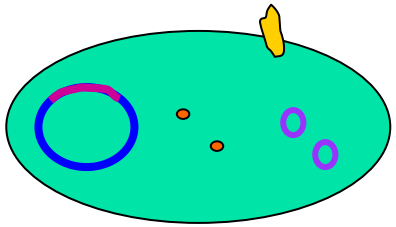
- Double stranded DNA binds to the surface of a competent cell and is cleaved into fragments of about 15 kb. The double stranded DNA, which is still external to the cell, is then separated into single stranded DNA. One fragment of the single stranded DNA is degraded, while the other is transported across the membrane and into the cell (Dubnau et al., 2000). Upon entry into the cell, a single strand of the foreign DNA is incorporated into the recipient cell via recombination (Krawiec, 2002).







Homologous
recombination



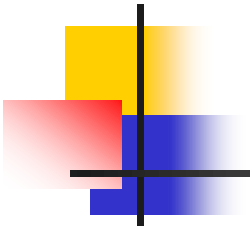
Transformed cell

IN THE LAB

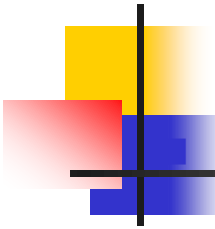
- Transformation is used in the lab in order to assess linkage. Extraction of the donor DNA causes inevitable breakage.
- The exterior surface of the plasma membrane is negatively charged due to the phospholipid head groups. This results in repulsion of the negatively charged DNA

- Its observed that *E. coli* cells that had been incubated in a solution containing divalent cations (often calcium chloride or Magnesium chloride) under cold conditions were more efficient at DNA uptake than unsoaked cells.

- It has been found that growth of Gram negative bacteria in 20 mM Mg^{+} reduces the number of protein to lipopolysaccharide bonds by increasing the ratio of ionic to covalent bonds, which increases membrane fluidity, facilitating transformation. The role of lipopolysaccharides here are verified from the observation that shorter O-side chains are more effectively transformed — perhaps because of improved DNA accessibility.



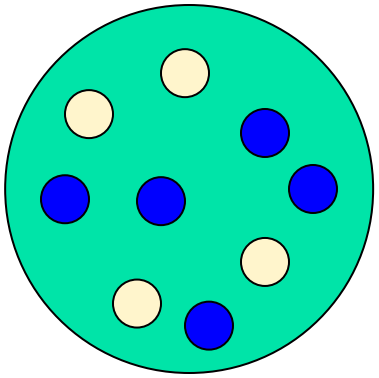
- Check the process in mailed note



- Soaking in CaCl2 affects only DNA binding, and not the actual uptake into the cell. When DNA is added to treated cells, it remains attached to the cell exterior, and is not at this stage transported into the cytoplasm.
- One function of the divalent cation therefore would be to shield the charges by coordinating the phosphate groups and other negative charges, thereby allowing a DNA molecule to adhere to the cell surface.

- It is suggested that exposing the cells to divalent cations in cold condition may also change or weaken the cell surface structure of the cells making it more permeable to DNA.
- The actual movement of DNA into competent cells is stimulated by briefly raising the temperature to 42°C. Once again, the exact reason why this heat shock is effective is not understood.
- The heat-pulse is thought to create a thermal imbalance on either side of the cell membrane, which forces the DNA to enter the cells through either cell pores or the damaged cell wall.

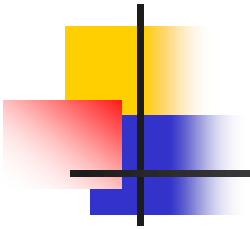
STEP 5. GROWTH ON SELECTION PLATES



Recombinant vector

Non Recombinant
(Self ligated) vector

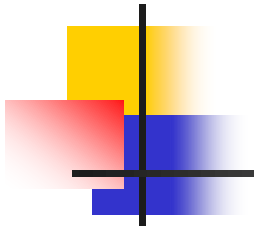
Selection for transformed cells



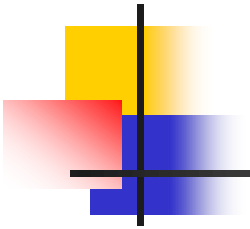
- 1 ng of the plasmid vector can yield 1000–10,000 transformants, this represents the uptake of only 0.01% of all the available molecules.
- 10,000 transformants is only a very small proportion of the total number of cells that are present in a competent culture.

Selection

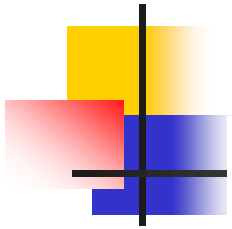
- That means that we must find a way to distinguish a cell that has taken up a plasmid from the many thousands that have not been transformed.
- Uptake and stable retention of a plasmid is usually detected by looking for expression of the genes carried by the plasmid.



- For example, the *E. coli* cells harboring plasmid pBR322 are resistant to the antibiotics ampicillin and tetracycline are normally sensitive to the growth inhibitory effects of the antibiotics.



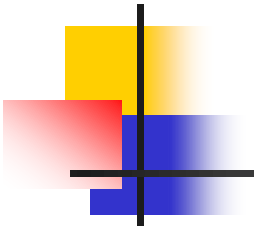
- This is because pBR322 carries two sets of genes, one gene that codes for a b-lactamase enzyme that modifies ampicillin into a form that is non-toxic to the bacterium, and a second set of genes that code for enzymes that detoxify tetracycline.



- After a transformation experiment with pBR322, only those *E. coli* cells that have taken up a plasmid are amp^R tet^R and able to form colonies on an agar medium that contains ampicillin or tetracycline.
- non-transformants, which are still amp_S tet_S, do not produce colonies on the selective medium.

Identification of recombinants

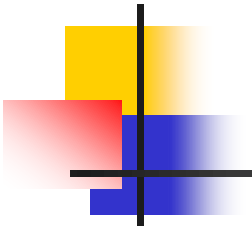
- The next problem is to determine which of the transformed colonies comprise cells that contain recombinant DNA molecules, and which contain self ligated vector molecules
- With most cloning vectors, insertion of a DNA fragment into the plasmid destroys the integrity of one of the genes present on the molecule.



- **Recombinants** can therefore be identified because of **insertional inactivation**
- There are two methods for insertional inactivation

Insertional inactivation of an antibiotic resistance gene

- pBR322 has several unique restriction sites that can be used to open up the vector before insertion of a new DNA fragment.
- *Bam*HI, for example, cuts pBR322 at just one position, within the cluster of genes that code for resistance to tetracycline.



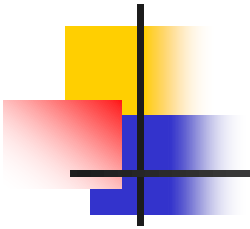
- A recombinant pBR322 molecule, one that carries an extra piece of DNA in the *Bam*HI site is no longer able to confer tetracycline resistance on its host, as one of the necessary genes is now disrupted by the inserted DNA. But it still has the ampicillin resistance.

How to detect the cells which contain the recombinant DNA

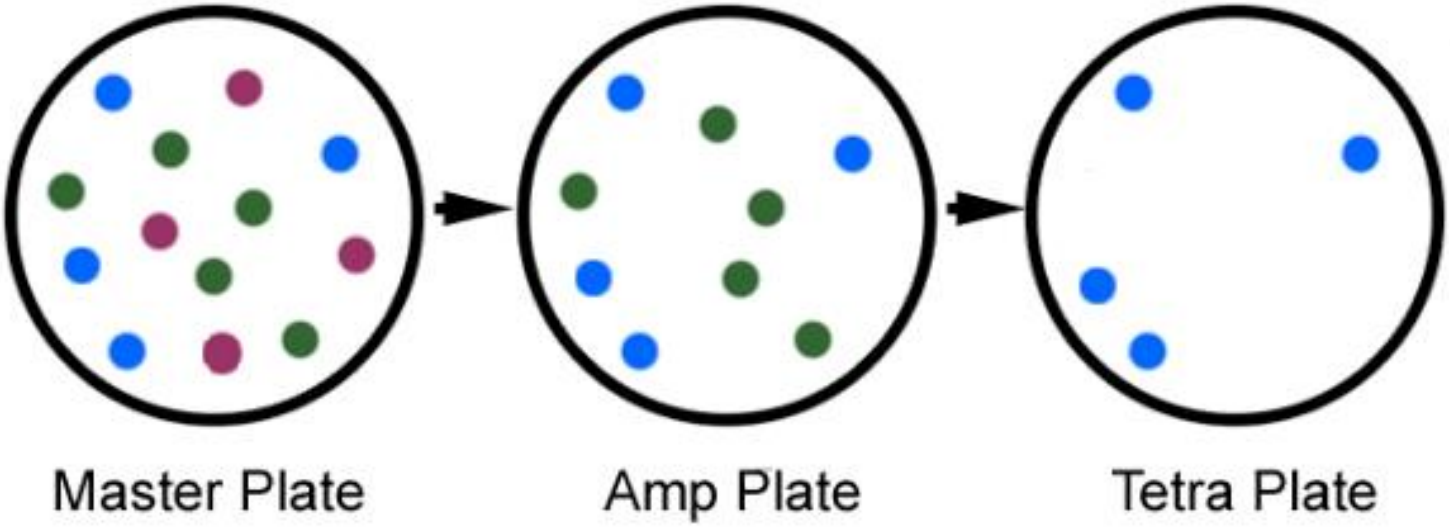
- Screening for pBR322 recombinants is performed in the following way. After transformation the cells are plated onto ampicillin medium and incubated until colonies appear.
- All of these colonies are transformants (untransformed cells are *ampS* and so do not produce colonies on the selective medium), but only a few contain recombinant pBR322 molecules: most contain the normal, self-ligated plasmid.

Replica Plating Method

- To identify the recombinants the colonies are **replica plated** onto agar medium that contains tetracycline. After incubation, some of the original colonies regrow, but others do not.
- Those that do grow consist of cells that carry the normal pBR322 with no inserted DNA and therefore a functional tetracycline resistance gene cluster (*amp*^{R}*tet*^{R}).



- The colonies that do not grow on tetracycline agar are recombinants (*ampRtetS*); once their positions are known on the master plate, samples for further study can be recovered from the original ampicillin agar plate.



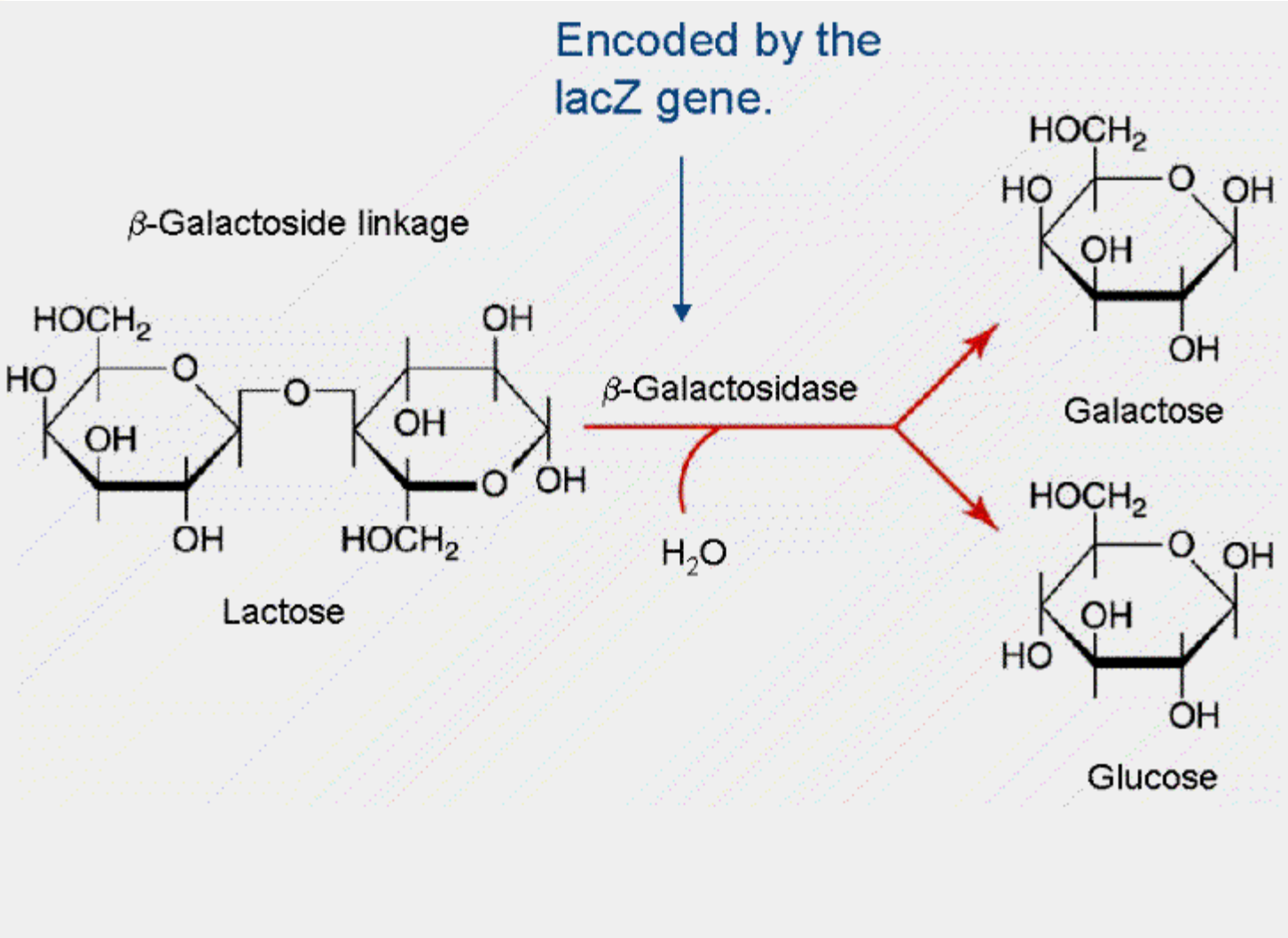
Cells with self ligated vector

Cells with recombinanat

Non transformed Cells

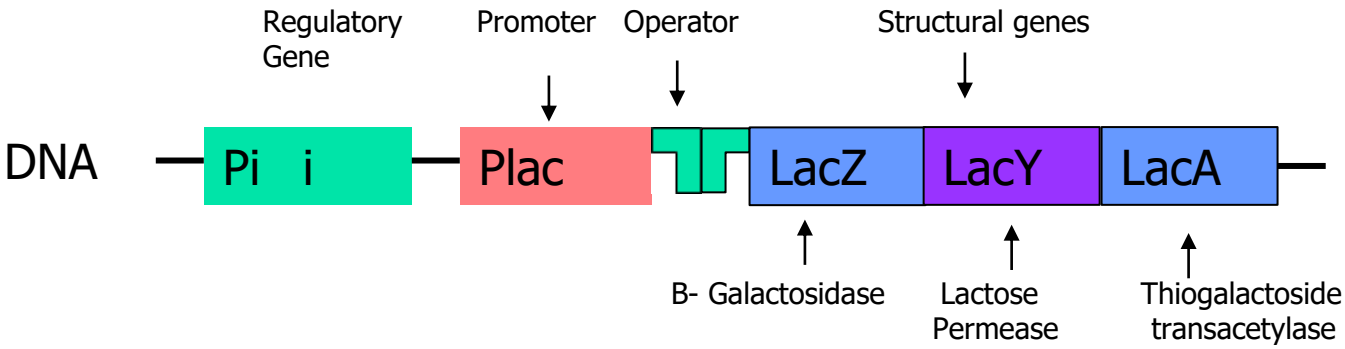
Insertional inactivation of the lacZ′ gene

- Most modern plasmid vectors make use of a different system. An example is pUC8, which carries the ampicillin resistance gene and a gene called *lacZ′*, which codes for part of the enzyme b-galactosidase.
- b-Galactosidase is one of a series of enzymes involved in the breakdown of lactose to glucose plus galactose. It is normally coded by the gene *lacZ*, which resides on the *E. coli* chromosome.

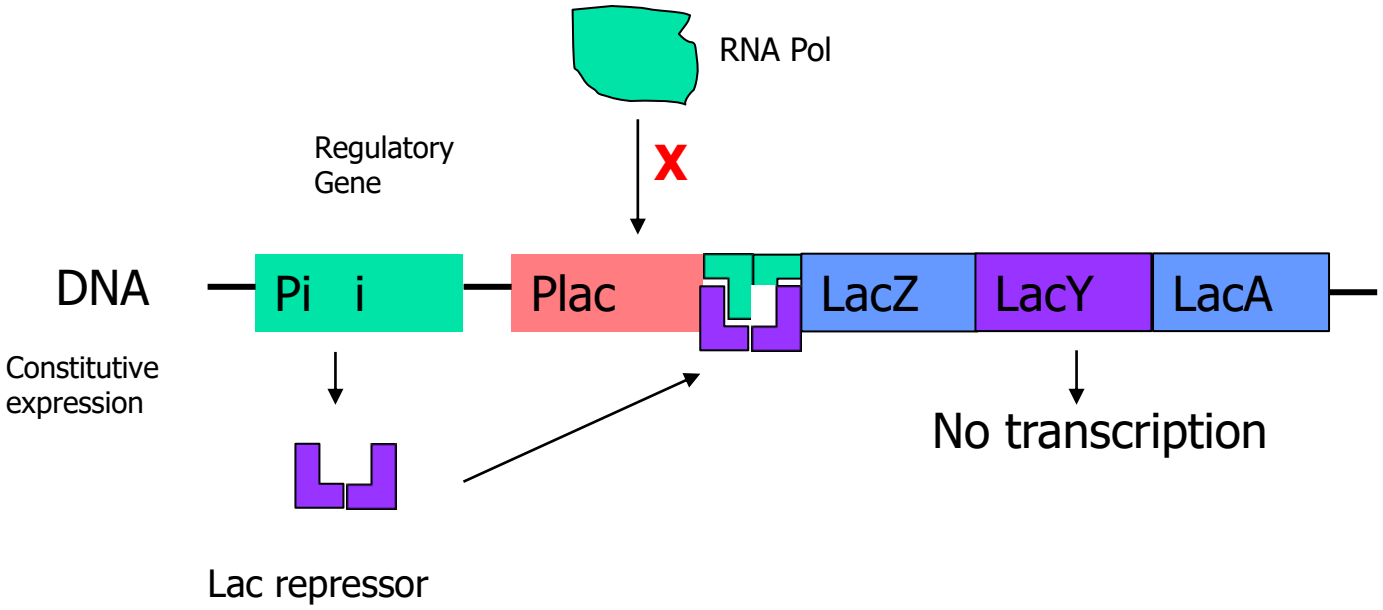


https://msu.edu/course/mmg/205/chap7exercise/galactosidasepage.htm

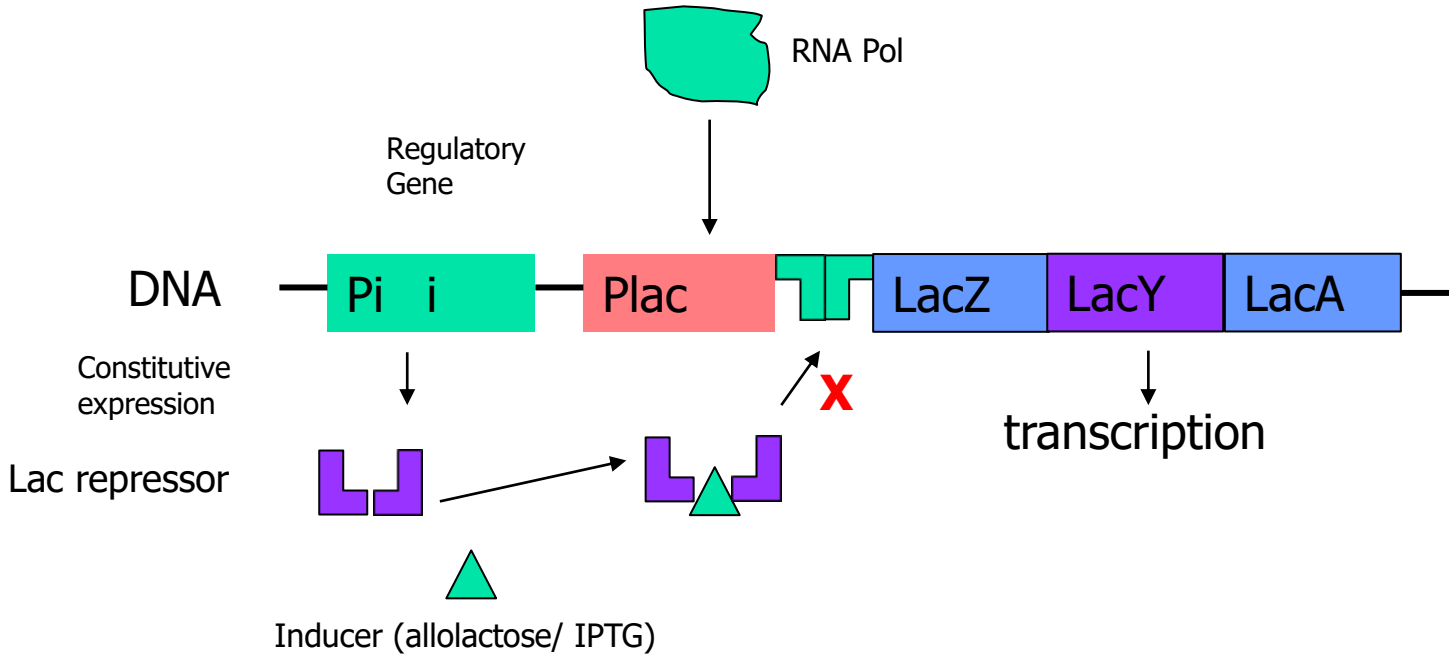
The lac operon and its control

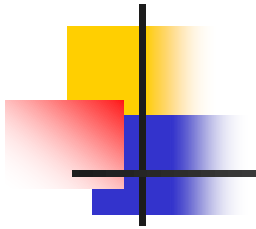


- In an E. Coli cell growing in the **absence of lactose**, a repressor protein binds to the operator, preventing RNA polymerase from transcribing the lac operon's genes. **The operon is OFF.**

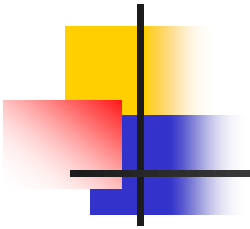

RNA Pol
Regulatory
Gene
X
DNA
Pi
i
Plac
LacZ
LacY
LacA
Constitutive
expression
No transcription
Lac repressor

- When the inducer, **lactose**, is added, its binds to the repressor and changes the repressor's shape so as to eliminate binding to the operator. As long as the operator remains free of the repressor, RNA polymerase that recognizes the promoter can transcribe the operon's structural genes into mRNA. **The operon is ON**


RNA Pol
Regulatory Gene
DNA
Pi
i
Plac
LacZ
LacY
LacA
Constitutive expression
X
transcription
Lac repressor
Inducer (allolactose/ IPTG)

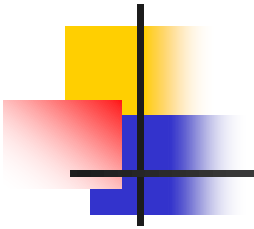


- Some strains of *E. coli* have a modified *lacZ* gene, one that lacks the segment referred to as *lacZ'* and coding for the a-peptide portion of b-galactosidase.



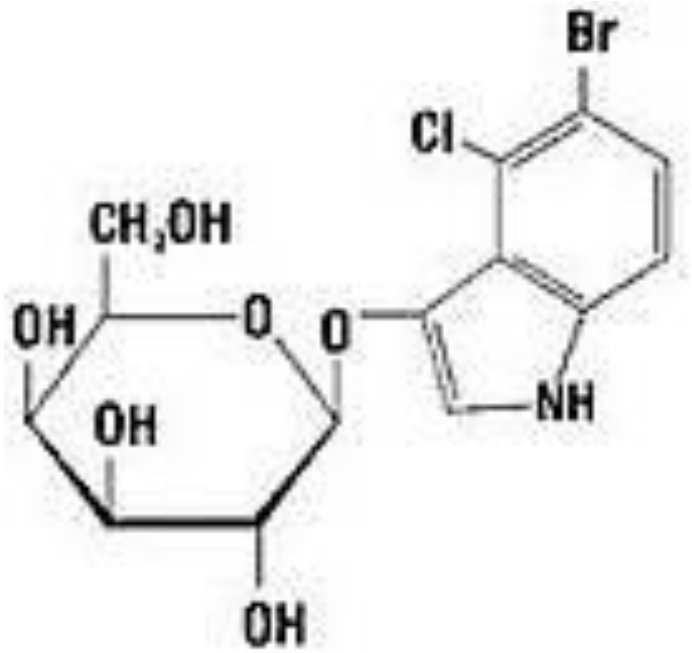
- The host used for cloning is mutant for inability to synthesize b-galactosidase

- These mutants can synthesize the enzyme only when they harbor a plasmid, such as pUC8, that carries the missing *lacZ*′ segment of the gene.

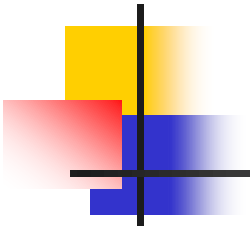


- Cloning with pUC8 involves insertional inactivation of the *lacZ'* gene, with recombinants identified because of their inability to synthesize b-galactosidase
- Cells that harbor a normal pUC8 plasmid are *ampR* and able to synthesize b-galactosidase where as recombinants are also *ampR* but unable to make b-galactosidase. Replica plating method can be used to select the recombinant using the final plate containing lactose.

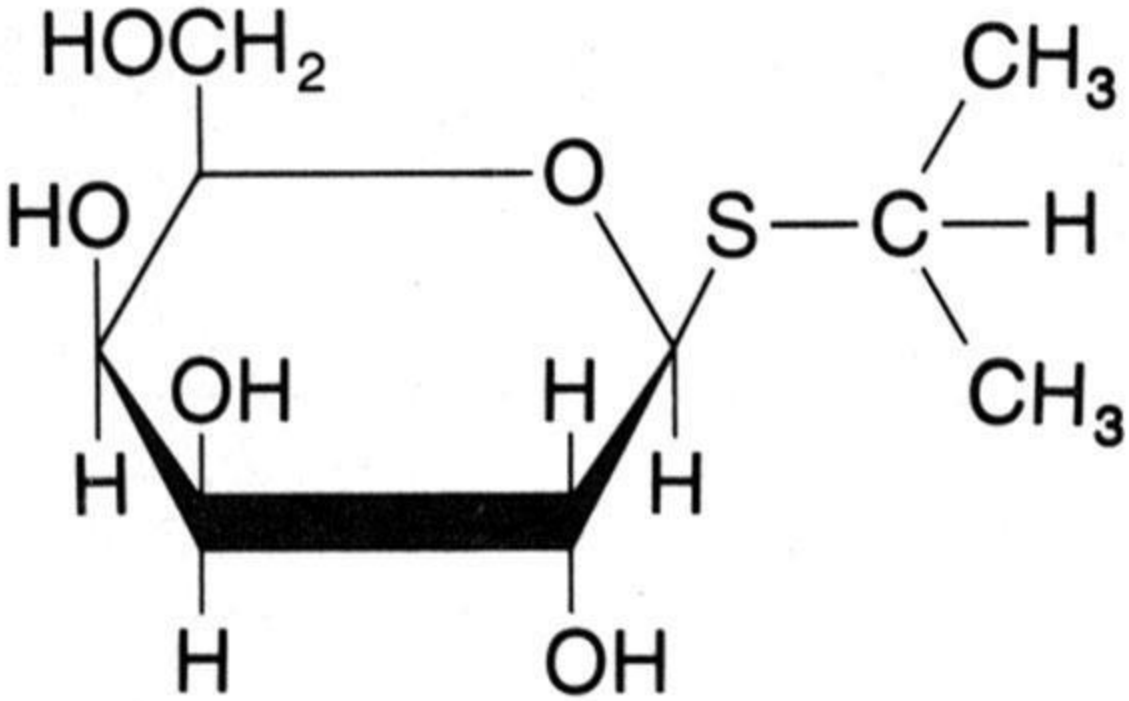
- In lab we test for a slightly different reaction that is also catalyzed by b-galactosidase. This involves a lactose analog called X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) which is broken down by b-galactosidase to a product that is colored deep blue.



X gal a lactose analog

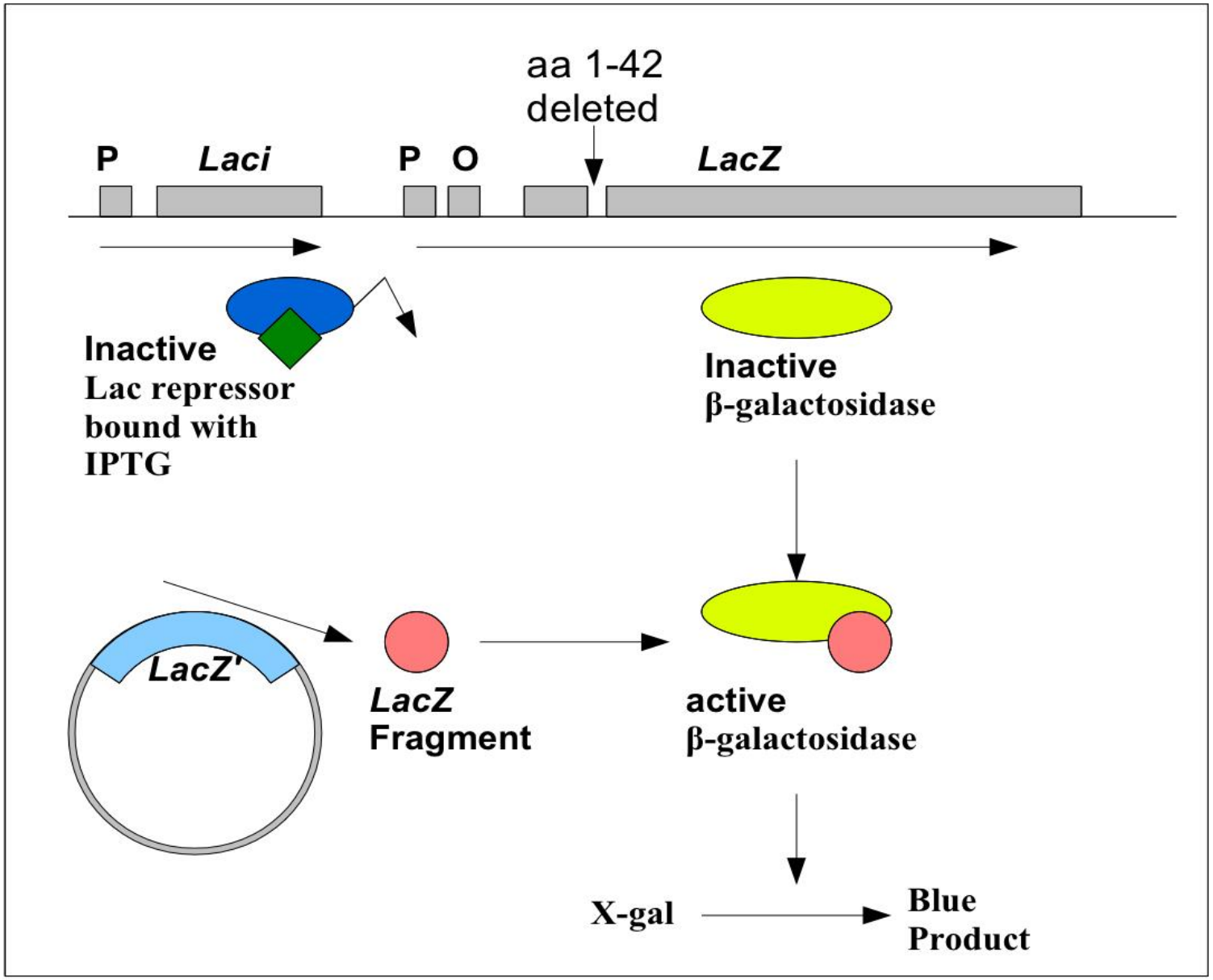


- X-gal is cleaved by ß-galactosidase yielding galactose and 5-bromo-4-chloro-3-hydroxyindole, which is oxidized into **5,5'-dibromo-4,4'-dichloro-indigo**, an insoluble blue product. Thus, if X-gal and an inducer of ß-galactosidase (usually IPTG) is contained within an agar medium on a culture plate, colonies which have a functional lacZ gene can easily be distinguished by their blue color.

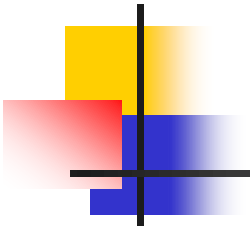


Isopropyl-β-D-thiogalactoside
(IPTG)

https://www.uaz.edu.mx/histo/Biologia/biochem/ch26/iptg.htm

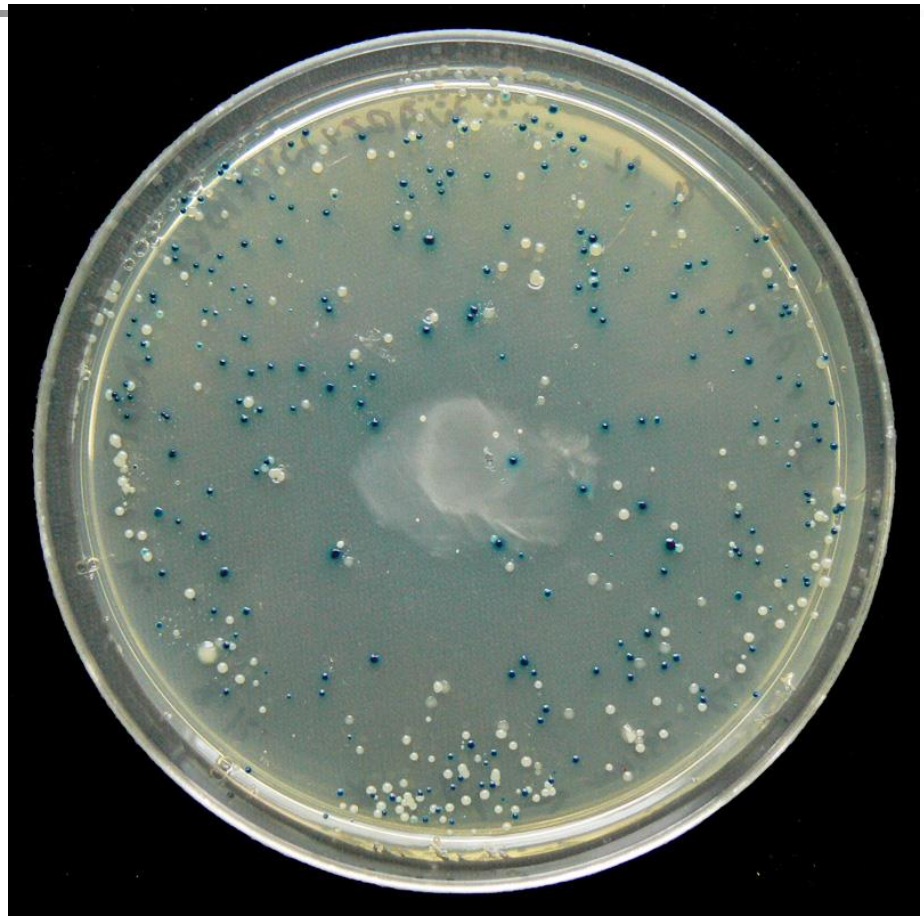


https://en.wikipedia.org/wiki/



- If X-gal (plus an inducer of the enzyme such as isopropylthiogalactoside, IPTG) is added to the agar, along with ampicillin, then non-recombinant colonies, the cells of which synthesize b-galactosidase, will be colored blue,recombinants with a disrupted *lacZ* gene and unable to make b-galactosidase, will be white

- This system, is called **Lac selection or blue and white selection**



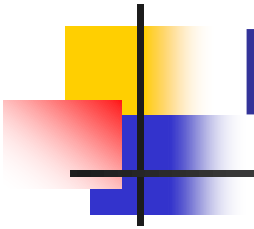
https://en.wikipedia.org/wiki/Blue%E2%80%93white_screen#/media/File:Blue-white_test.jpg

Restriction Enzyme digestion

- Vectors has been created with a number of restriction sites on either side of the insertion point and so may be used to "drop out" the insert to check its size and confirm the presence of insert.
- Electrophoresis separates fragments of DNA into its component sizes. Fragments of the same size travel through the gel in one region, resulting in a "band" of DNA.

- When we compare the band to bands of known size, we can estimate the size of the fragments in our band. Consider the following example.
- In pGEMT vector the enzyme EcoRI has restriction sites on both sides of the insertion point. This means that if we expose our sample to EcoRI, we should end up with two sorts of fragment – one representing the vector (around 3000 bases long, or 3kb) and one representing our insert, which in this case is 700 bases long (0.7kb).

Fig.: Plasmid digested with Bam HI and Eco RI and loaded on 2% gel along with 100bp ladder.



Resources

- NCBI book shelf
- Free web resources