

# **Study of IPTG induced protein expression of H10-T-MPT64-BAP protein in *E.coli* BL- 21 (DE3) RIL strain**

**SAYANTIKA GHOSH**

B.Tech in Biotechnology, Heritage Institute of Technology, Chowbagha Road, Anandapur, Kolkata- 700107.

**Guided by:**

**DR. AMITA GUPTA**

Associate Professor, Department of Biochemistry, University of Delhi (South Campus), Benito Juarez Road, New Delhi- 110021.

## **Abstract**

Expression of cloned genes in prokaryotic system is done to study the function of insert gene. This ability allows the production of large amount of desired protein (H10-T-MPT64-BAP) in host cells (*E.coli* BL21 (DE3) RIL ) which can then be purified and used for various diagnostic applications. For this, one of the basic step which is to be performed is plasmid DNA isolation. Plasmid DNA purification by PEG precipitation method as well by using Genetix kit provides a rapid, simple and inexpensive method of getting purified DNA without RNA contamination. Once isolated, plasmid DNA was incorporated into host bacterium by  $\text{CaCl}_2$  transformation. Lac operon is one of the most efficient prokaryotic system which can be used to regulate the expression of our desired protein. Full expression of Lac operon is found when there is presence of lactose and absence of glucose in the media. Lactose is converted into allolactose by  $\beta$ -galactosidase enzyme which can then act as inducer and bind with Lac repressor to change its conformation. Now Lac repressor cannot bind to the operator region allowing RNA polymerase to express the structural genes. Also when glucose concentration is low, cAMP-

CAP complex formed will allow the efficient binding of RNA polymerase to the promoter region. But allolactose is metabolizable so its concentration will reduce with time. Thus a gratuitous inducer IPTG is being used to induce protein expression. Here our protein is expressed under very strong T7 promoter which can only be recognized by T7 RNA polymerase. T7 RNA polymerase is expressed from T7 gene1 by special *E.coli* host strain (*E.coli* BL21 (DE3) RIL) polymerase under Lac UV5 promoter only in presence of inducer like IPTG. Inspite of this stringent regulation, there is always some basal level expression because of less number of active Lac Repressor being expressed from LacI gene.

**Keywords or phrases:** PEG precipitation method, Genetix kit, CaCl<sub>2</sub> transformation, Lac operon, IPTG, Basal level expression

## Abbreviations

### Abbreviations

PEG	Polyethylene glycol
RNA	Ribonucleic acid
CaCl <sub>2</sub>	Calcium chloride
cAMP	Cyclic adenosine monophosphate
DNA	Deoxyribonucleic acid
CAP	Catabolite activator protein
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
TMG	Methyl-β-D-thiogalactoside

---

## 1 INTRODUCTION

### 1.1 Gene Regulation in Prokaryotes

Genes encode proteins and proteins dictate cell function. Moreover, each step in the flow of information from DNA to RNA to protein provides the cell with a potential control point for self-regulating its functions by adjusting the amount and type of proteins it manufactures.

Two types of sequence-specific DNA-protein interactions are required for regulated prokaryotic transcription. One required DNA-protein interaction determines where transcription begins. It involves the DNA segment called the promoter and the protein

RNA polymerase. When RNA polymerase binds to the promoter DNA, transcription can initiate few bases away from the promoter site. Every gene must have a promoter or it cannot be transcribed. The other type of required DNA–protein interaction regulates whether or not promoter-driven transcription occurs. DNA segments near the promoter serve as protein-binding sites—most of these sites are termed operators—for regulatory proteins called activators and repressors. For some genes, the binding of an activator protein to its target DNA site is a necessary prerequisite for transcription to begin. Such instances are sometimes referred to as positive regulation because it is the presence of the bound protein that is required for transcription (Figure 1). For other genes, preventing the binding of a repressor protein to its target site is a necessary prerequisite for transcription to begin. Such cases are sometimes termed negative regulation because it is the absence of the bound protein that allows transcription to occur.

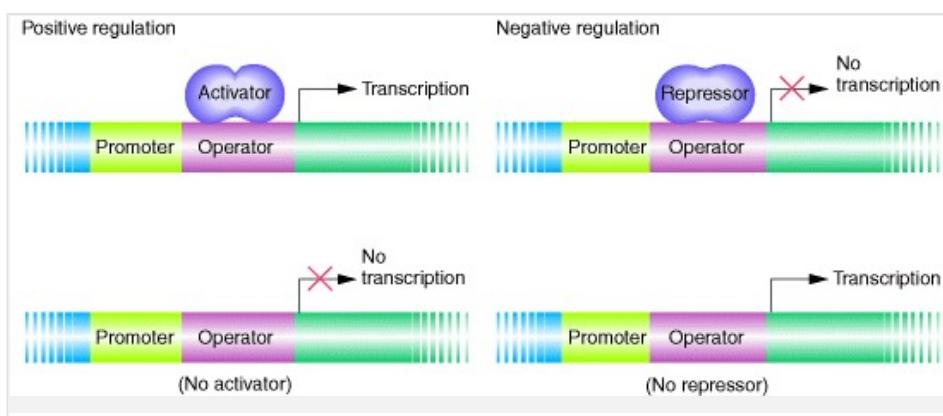


Fig 1 Positive and negative regulation of gene expression. Source: Modern Genetic Analysis

The different consequences of activator and repressor binding to operator DNA. Activator binding is required for transcription. Repressor binding blocks transcription.

For many activator or repressor proteins, the way that DNA binding is regulated is through the interaction of two different sites in the three-dimensional structure of the protein. One site is the DNA-binding domain. The other site, the allosteric site, acts as the toggle switch that sets the DNA-binding domain in one of two modes: functional or nonfunctional. The allosteric site interacts with small molecules called allosteric effectors. An allosteric effector binds to the allosteric site of the regulatory protein in such a way that it changes the structure of the DNA-binding domain. Some activator or repressor proteins must bind to their allosteric effectors to bind DNA. Others can bind DNA only in the absence of their allosteric effectors (Figure 2) [4]. Some genes controlling protein synthesis and much of an organism's central metabolism

are always expressed and are constitutive. In contrast, regulated genes are needed only occasionally. It turns out that the regulation of such genes differs between prokaryotes and eukaryotes [5].

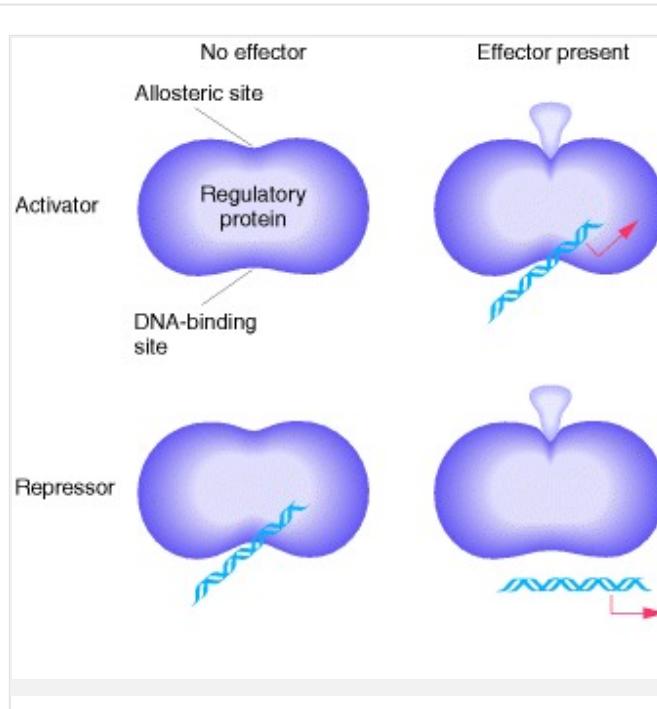


Fig 2 *The effects of allosteric effectors on the DNA-binding activities of activators and repressors*  
Source: Modern Genetic Analysis

To live, cells must be able to respond to changes in their environment. Regulation of the two main steps of protein production — transcription and translation — is critical to this adaptability [1].

## 1.2 Bacterial Operon

Bacteria are typically exposed to an ever-changing environment in which nutrient availability may increase or decrease radically and they respond by altering their gene expression pattern, thus they express different enzymes depending on the carbon sources and other nutrients available to them. It would be wasteful to synthesize, for example, lactose-metabolizing enzymes in the absence of lactose. However, when lactose is the only available carbon source, bacteria must quickly induce lactose-metabolizing enzymes, or else they will die. In bacteria, this sort of genetic regulation is mediated at the level of transcription.

Bacterial genes are organized into operons, or clusters of coregulated genes. In addition to being physically close in the genome, these genes are regulated such that they are all

turned on or off together. Grouping related genes under a common control mechanism allows bacteria to rapidly adapt to changes in the environment [2].

The best-studied examples of operons are from the bacterium *Escherichia coli*, and they involve the enzymes of lactose metabolism and tryptophan biosynthesis. Because the lactose (*lac*) operon shares many features with other operons, its organization and regulation are described in detail below.

### 1.3 The lac Operon

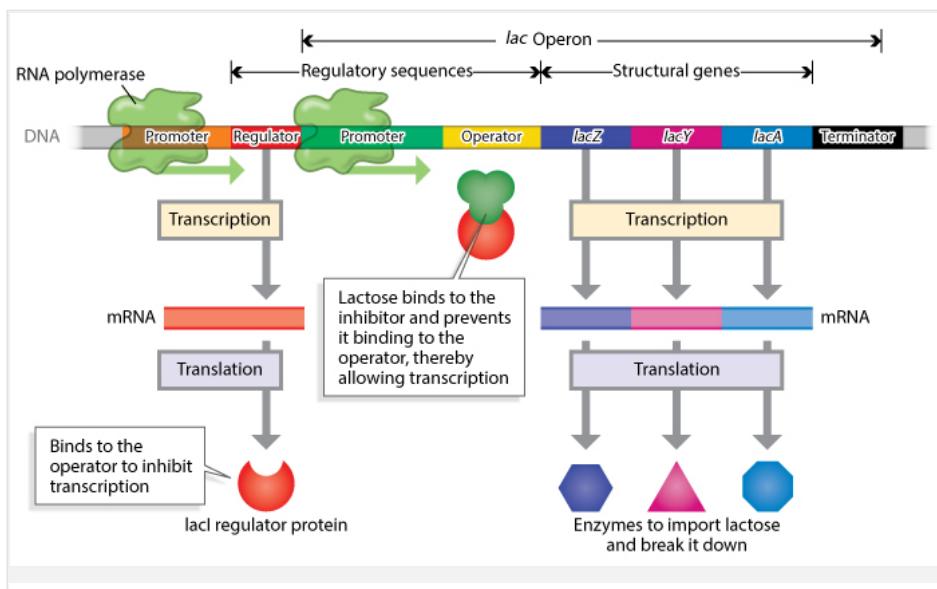


Fig 3 The *lac* operon in *E.coli*. Three lactose metabolism genes (*lacZ*, *lacY*, and *lacA*) are organized together in a cluster called the *lac* operon. The coordinated transcription and translation of the *lac* operon structural genes is supported by a shared promoter, operator, and terminator. A *lac* regulator gene with its promoter is found just outside the *lac* operon. © 2013 Nature Education Adapted from Pierce, Benjamin. *Genetics: A Conceptual Approach*, 2nd ed.

Nobel laureates Francois Jacob and Jacques Monod conducted much of the seminal work describing the lac operon's structure and control mechanisms. The duo noted that the lac operon contains three genes that encode proteins involved in lactose metabolism. These are referred to as lac z, lac y, and lac a. The lac z gene encodes beta-galactosidase, the lac y gene encodes a permease, and the lac a gene encodes the transacetylase enzyme. Together, these gene products act to import lactose into cells and break it down for use as a food source. As in other operons, the genes of the lac operon lie along a contiguous stretch of DNA such

that their expression can be easily coregulated. In addition to these so-called structural genes, the lac operon also contains other sequences that direct the bacterial gene expression machinery.

The organization of genes into an operon allows for simultaneous expression of all the genes that are located in *cis* (i.e., on the same contiguous piece of DNA) in the operon. Several features contribute to this characteristic of operons (Figure 3). First, all of the operon's genes are downstream of a single promoter. This promoter serves as a recognition site for the transcriptional machinery of the RNA polymerase complex. Second, all genes in an operon actually become part of a single messenger RNA molecule, which is subsequently translated into individual protein gene products.

Several other regulatory sequences also ensure coordinated regulation of the lac operon. These include the operator and the terminator. The operator is a special DNA sequence located between the promoter sequence and the structural genes that enables repression of the entire lac operon, following binding by the inhibitor (lac I) protein. Expression of the lac operon is, in fact, regulated by the presence of lactose itself. The ability to turn lactose-metabolizing genes on or off as a group therefore provides an efficient way to quickly adapt to environmental changes. The terminator, on the other hand, instructs the transcriptional machinery to terminate transcription. As such, the promoter serves as a transcriptional start site, the terminator serves as a stop site, and the operator helps determine whether transcription will occur [3].

## 1.4 Negative Regulation in Lac operon

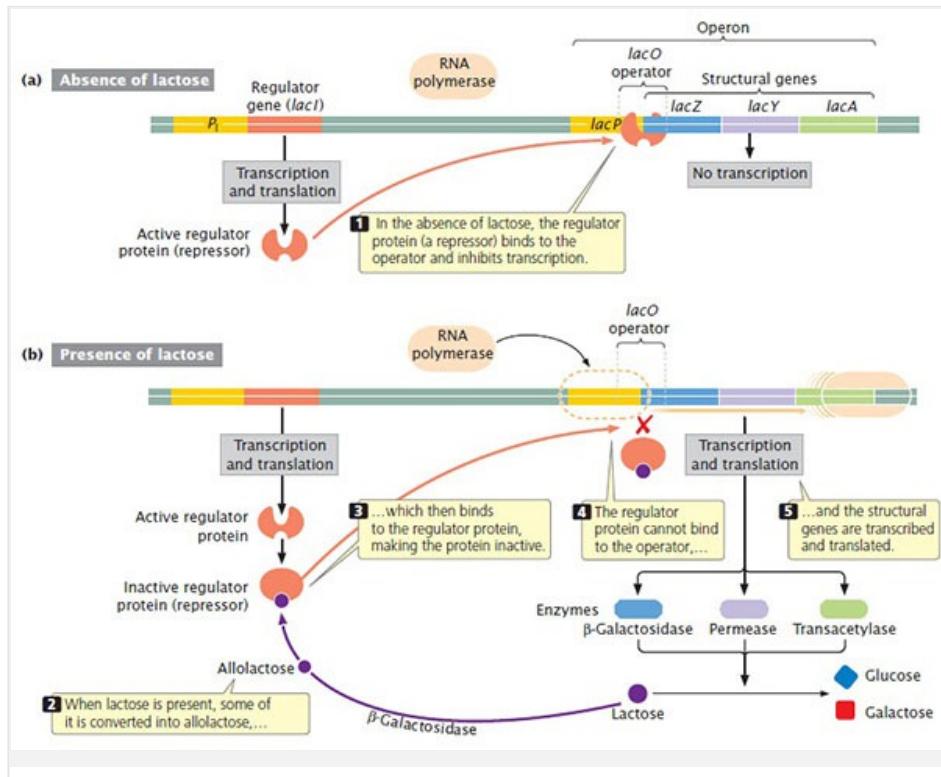


Fig 4 Negative regulation of Lac operon Source: Reference [6]

In the absence of lactose, the lac repressor is bound to the operator region of the lac operon, physically preventing RNA polymerase from transcribing the structural genes. However, when lactose is present, the lactose inside the cell is converted to allolactose. Allolactose serves as an inducer molecule, binding to the repressor and changing its shape so that it is no longer able to bind to the operator DNA. Removal of the repressor in the presence of lactose allows RNA polymerase to move through the operator region and begin transcription of the lac structural genes [6,7,8].

## 1.5 Positive control of Lac Operon

Bacteria typically have the ability to use a variety of substrates as carbon sources. However, because glucose is usually preferable to other substrates, bacteria have mechanisms to ensure that alternative substrates are only used when glucose has been depleted. Additionally, bacteria have mechanisms to ensure that the genes encoding enzymes for using alternative substrates are expressed only when the alternative substrate is available. In the 1940s,

Jacques Monod was the first to demonstrate the preference for certain substrates over others through his studies of *E. coli*'s growth when cultured in the presence of two different substrates simultaneously. Such studies generated diauxic growth curves, like the one shown in Figure 5. Although the preferred substrate glucose is used first, *E. coli* grows quickly and the enzymes for lactose metabolism are absent. However, once glucose levels are depleted, growth rates slow, inducing the expression of the enzymes needed for the metabolism of the second substrate, lactose. The growth rate in lactose is slower, as indicated by the lower steepness of the growth curve.

The ability to switch from glucose use to another substrate like lactose is a consequence of the activity of an enzyme called Enzyme IIA (EIIA). When glucose levels drop, cells produce less ATP from catabolism, and EIIA becomes phosphorylated. Phosphorylated EIIA activates adenylyl cyclase, an enzyme that converts some of the remaining ATP to cyclic AMP (cAMP), a cyclic derivative of AMP and important signalling molecule involved in glucose and energy metabolism in *E. coli*. As a result, cAMP levels begin to rise in the cell (Figure 6) [9].

The lac operon also plays a role in this switch from using glucose to using lactose. When glucose is scarce, the accumulating cAMP caused by increased adenylyl cyclase activity binds to catabolite activator protein (CAP), also known as cAMP receptor protein (CRP). The complex binds to the promoter region of the lac operon (Figure 7). In the regulatory regions of these operons, a CAP binding site is located upstream of the RNA polymerase binding site in the promoter. Binding of the CAP-cAMP complex to this site increases the binding ability of RNA polymerase to the promoter region to initiate the transcription of the structural genes. Thus, in the case of the lac operon, for transcription to occur, lactose must be present (removing the lac repressor protein) and glucose levels must be depleted (allowing binding of an activating protein). When glucose levels are high, there is catabolite repression of operons encoding enzymes for the metabolism of alternative substrates. Because of low cAMP levels under these conditions, there is an insufficient amount of the CAP-cAMP complex to activate transcription of these operons. See Table 1 for a summary of the regulation of the lac operon [10,11].

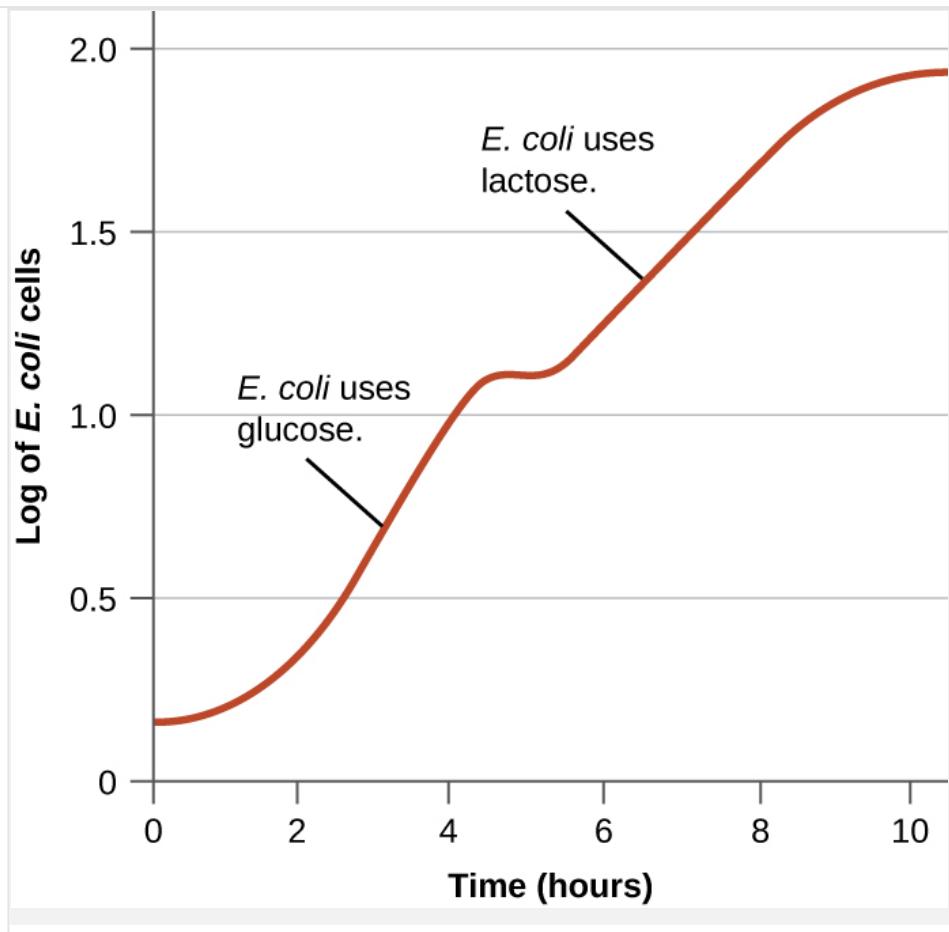


Fig 5 When grown in the presence of two substrates, *E. coli* uses the preferred substrate (in this case glucose) until it is depleted. Then, enzymes needed for the metabolism of the second substrate are expressed and growth resumes, although at a slower rate. Source: Reference [10]

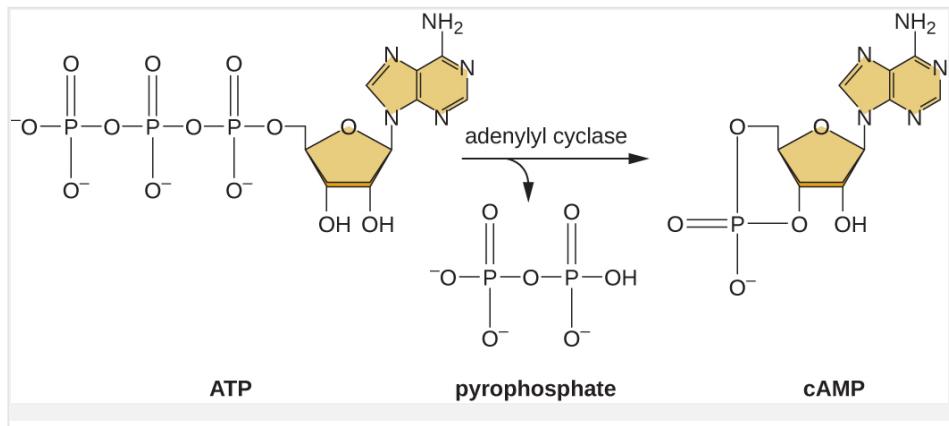


Fig 6 When ATP levels decrease due to depletion of glucose, some remaining ATP is converted to

cAMP by adenylyl cyclase. Thus, increased cAMP levels signal glucose depletion. Source: Reference [10]

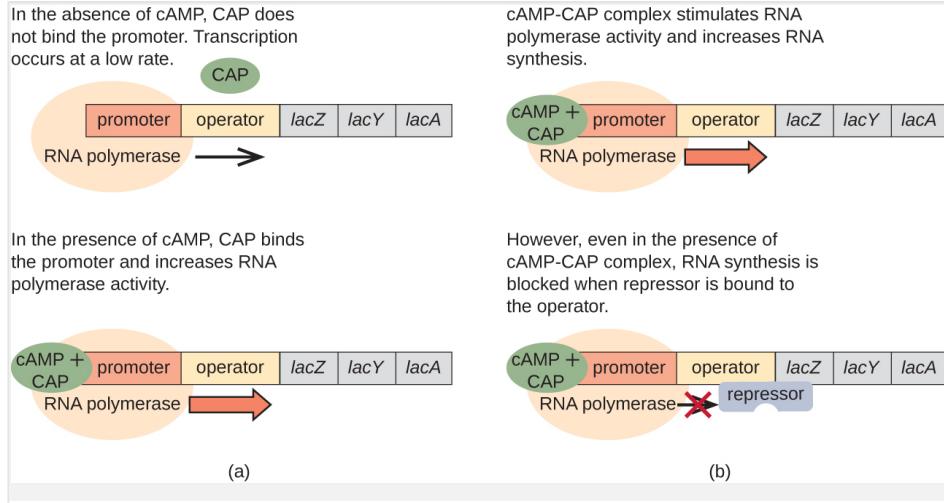


Fig 7 (a) In the presence of cAMP, CAP binds to the promoters of operons, like the lac operon, that encode genes for enzymes for the use of alternate substrates. (b) For the lac operon to be expressed, there must be activation by cAMP-CAP as well as removal of the lac repressor from the operator. Source: Reference [10]

Table 1 Conditions Affecting Transcription of the lac Operon. Source: Reference [10]

Glucose	CAP binds	Lactose	Repressor binds	Transcription
+	-	-	+	No
+	-	+	-	Some
-	+	-	+	No
-	+	+	-	Yes

## 1.6 Induction of Lac Operon

Isopropyl- $\beta$ -D-1-thiogalactopyranoside abbreviated IPTG, is a molecular biology reagent. This compound is used as a molecular mimic of allolactose, a lactose metabolite that triggers transcription of the lac operon. Unlike allolactose, the sulfur (S) atom creates a chemical bond which is non-hydrolyzable by the cell, preventing the cell from “eating up” or degrading the inductant; therefore the IPTG concentration remains constant. These structural analog

of lactose which induces lac operon but are not the substrates for the enzyme  $\beta$ -galactosidase are called gratuitous inducers.

IPTG binds to the lac repressor and releases the tetrameric repressor from the lac operator in an allosteric manner, thereby allowing the transcription of genes in the lac operon, such as the gene coding for beta-galactosidase, a hydrolase enzyme that catalyzes the hydrolysis of  $\beta$ -galactosides into monosaccharides. IPTG intake is independent on the action of lactose permease, since other transport pathways are also involved.

Many regulatory elements of the lac operon are used in inducible recombinant protein systems. IPTG is an effective inducer in the concentration range of 100  $\mu$ M to 1.5 mM. Concentration used depends on the strength of induction required, as well as the genotype of cells or plasmid used – if lac  $I_q$ , a mutant that over-produces the lac repressor, is present, then a higher concentration of IPTG may be necessary.

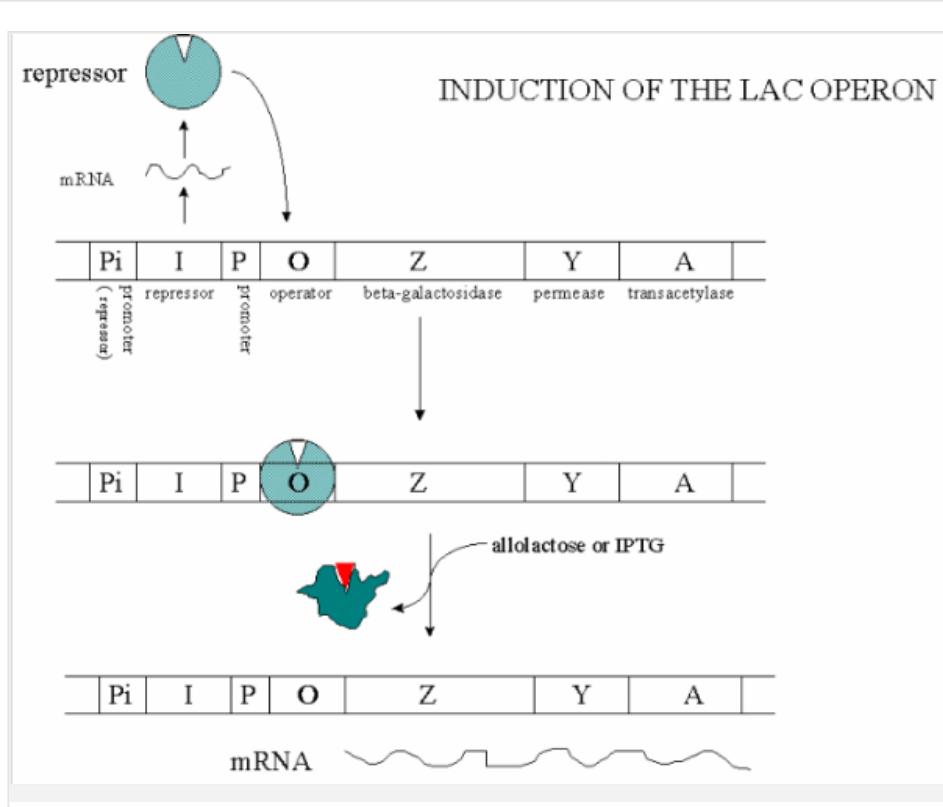


Fig 8 Induction of the Lac operon

## 1.7 OBJECTIVES

### 1.7.1 Overall objective

- To isolate plasmid (pGEM) DNA by PEG precipitation method and by Genetix kit
- To incorporate plasmid DNA into *E.coli* DH5 $\alpha$  strain by CaCl<sub>2</sub> transformation and to calculate its efficiency
- To study IPTG induced protein expression of H10-T-MPT64-BAP protein in *E.coli* BL-21(DE3) RIL strain

## 2 LITERATURE REVIEW

### 2.1 Lac operon

The operon concept (Beckwith, 1987b), introduced by Jacob et al. (1960), has had a profound and lasting effect on the biological sciences.

### 2.2 Feedback Regulation in the Lactose Operon: A Mathematical Modelling Study and Comparison with Experimental Data

A mathematical model for the regulation of induction in the lac operon in *Escherichia coli* is presented. This model takes into account the dynamics of the permease facilitating the internalization of external lactose; internal lactose;  $\beta$ -galactosidase, which is involved in the conversion of lactose to allolactose, glucose and galactose; the allolactose interactions with the lac repressor; and mRNA [13].

### 2.3 Transcription of the lac Operon of *Escherichia coli*

The effects of induction and of transient and catabolite repression on lac-specific RNA in cells of *Escherichia coli* were investigated. The rate of lac-specific RNA synthesis increases linearly for 3 min after induction and then becomes constant [14].

## 2.4 Combinatorial transcriptional control of the lactose operon of *Escherichia coli*

The goal of systems biology is to understand the behaviour of the whole in terms of knowledge of the parts. The lac promoter of *Escherichia coli* offers the possibility of confronting “system-level” properties of transcriptional regulation with the known biochemistry of the molecular *constituents and their mutual interactions*. Here in this paper they have studied the combinatorial control of the lac promoter by the regulators Lac repressor (LacR) and cAMP-receptor protein (CRP). Specifically the analysis indicates that the sensitivity of the inducer response results from LacR-mediated DNA looping, which is significantly enhanced by CRP.

The lac promoter ( $P_{lac}$ ) of *Escherichia coli* is one of the most extensively studied systems of molecular biology. The knowledge and insight gained from these studies have shaped much of how we now think about gene regulation.

It is well known that *E. coli* cells repress the expression of the lac operon when glucose is abundant in the growth medium. Only when the glucose level is low and the lactose level is high is the operon fully expressed. Thus, the regulation of this operon represents an example of “combinatorial control” widely seen in prokaryotes and eukaryotes.

This study presents a proof of concept that the complicated web of interactions that couple repressors, activators, promoters, and DNA loops *in vivo* can be quantitatively dissected, provided that the right modelling together with a precise sequence of experiments on a systematically picked set of mutants are carried out [15].

## 2.5 Lac operon induction in *Escherichia coli*: Systematic comparison of IPTG and TMG induction and influence of the transacetylase LacA.

In majority of the cases induction of lac operon is done using gratuitous inducer IPTG or TMG. So a systematic comparison of lac promoter induction by TMG and IPTG focuses on the aspect of inducer uptake and potential influence of the transacetylase, LacA [16].

## 2.6 Mechanism of promoter repression by Lac repressor–DNA loops

The *Escherichia coli* lactose (lac) operon encodes the first genetic switch to be discovered, and lac remains a paradigm for studying negative and positive control of gene expression. Long-

standing questions persist concerning the actual role of DNA looping in the mechanism of promoter repression. Here they have shown that the distance dependence of repression enhancement is comparable for upstream and downstream auxiliary operators, confirming the hypothesis that repressor concentration increase is the principal mechanism of repression loops. They found as four turns of DNA can be constrained in a stable loop by Lac repressor and RNA polymerase is not trapped at repressed promoters. Finally, they showed that constraining a promoter in a tight DNA loop is sufficient for repression even when promoter and operator do not overlap [17].

### **3 MATERIALS AND METHODS**

#### **3.1 Plasmid DNA isolation**

##### **3.1.1 Plasmid used- pGEM-3Z**

The pGEM-3Z Vector is intended for use as a standard cloning vector, as well as for the synthesis of RNA in vitro. The vector carries the lacZ α-peptide and the multiple cloning region arrangement from pUC18 allowing selection of recombinants by blue/white screening. In addition, the vector contains both the SP6 and T7 RNA polymerase promoters flanking the multiple cloning region

##### **3.1.2 Method used**

###### **3.1.2.1 PEG Precipitation Method**

###### **3.1.2.1.1 Reagents required:**

Tris EDTA Glucose, Alkaline Sodium Dodecyl Sulfate, Potassium acetate, RNase A Mix, Chloroform, Isopropanol, 70% Ethanol, NaCl-PEG Mix, 0.1X Tris EDTA, Dye Mix

###### **3.1.2.1.2 Procedure**

1. Pour plating of LB Agar media containing antibiotic ampicillin ( $\text{Amp}_{100}$ );
2. Streaking for getting isolated colonies;
3. Inoculating isolated colony in ZYM 505 media;
4. Alkaline lysis of the bacterial cell (E.coli DH5α strain) using alkaline SDS;
5. Neutralization of cell lysate and separation of cell debris by centrifugation;
6. Precipitation by polyethylene glycol (PEG);

## 7. Analysis by agarose gel electrophoresis.

Table 2 Composition of ZYM 505 media

Components	Volume (for 10 ml)
ZYT (Tryptophan 1%, YE 0.5%)	9.6 ml
1M MgSO <sub>4</sub>	20 µl
1000X Metals (contains 10 metals)	2 µl
100X 505 (Glycerol + Glucose)	100 µl
50 XM	200 µl
Amp <sub>50</sub>	10 µl

### 3.1.2.2 By using GENETIX kit

#### 3.1.2.2.1 Reagents required

PA1 (resuspension buffer), PA2 ( lysis buffer), PA3 (neutralization buffer), PW4 (wash buffer), elution buffer, spin column.

#### 3.1.2.2.2 Procedure

1. Preparation and clearing of a bacterial lysate using PA1, PA2, PA3 buffer;
2. Adsorption of DNA onto the silica column membrane;
3. Washing and elution of plasmid DNA using PW4 and elution buffer.

## 3.2 CaCl<sub>2</sub> Transformation

3.2.1 Bacterial strain used: *E.coli* DH5 $\alpha$  strain- These are engineered strain used for maximum transformation efficiency. DH stands for Douglas Hanahan. The cells are defined by three mutations recA1, endA1 which help plasmid insertion and lacZM15 which enable blue white screening.

### 3.2.2 Reagents required:

Autoclaved double distilled water, pGEM DNA, LB media, LB agar plate

### 3.2.3 Method used

1. Prepared batch of competent cells was taken and it was mixed with pGEM DNA;
2. Incubate on ice for 30 minutes;
3. Transformation (Heat shock at 42°C for 1min in water bath) and incubate on ice for 2 minutes;
4. Cell recovery method;
5. 200  $\mu$ l of LB media was added to the cells and it was mixed by tapping;
6. Incubate on ice for 45 minutes;
7. 100  $\mu$ l of competent cells was plated in two LB agar plate and it was incubated at 37°C overnight;
8. Transformation efficiency was calculated.

## 3.3 Protein Expression

### 3.3.1 Protein expressed: H10-T-MPT64-BAP

### 3.3.2 Vector used:

Vector pVMExp14367 is a medium copy number T7 promoter-lac operator-based IPTG/lactose inducible expression vector containing sequence encoding N-terminal deca-histidine tag (H10), Tobacco Etch Virus (TEV) protease cleavage site, secretory protein of *Mycobacterium tuberculosis* H37Rv MPT64 and 15 amino acid C-terminal Biotin Acceptor Peptide tag (BAP) (Genbank accession number MG599491). In between the functional tags/protease site, there are glycine-serine rich spacer sequences of appropriate lengths. It was subjected to transformation in *E.coli* BL21 (DE3) RIL strain. The H10-BirA protein was purified

from cytosolic fraction using a two-step protocol involving affinity chromatography on Ni Sepharose Fast Flow resin (NiFF). Furthermore, a robust three-step chromatography pipeline integrated with well-optimized and efficient protocols for TEV protease-based H10 tag removal, and recombinant *E. coli* BirA enzyme-based site-specific in vitro biotinylation has been described to obtain purified proteins (devoid of the N-terminal H10 tag) carrying biotin residue at the C-terminus. Most importantly, the utility of these biotin-tagged recombinant proteins has been exemplified by comparison of passive versus specific protein immobilization in the context of indirect ELISA, and phage display-based affinity selection [12].

### 3.3.3 Reagents required:

Glu(1%), Amp<sub>50</sub>, IPTG (100mM) stock

### 3.3.4 Method used:

1. Inoculate isolated colony from streaked plate of transformed colonies carrying gene of interest H10-T-MPT64-BAP in LB Amp<sub>50</sub> Glu<sub>1%</sub> media;
2. It was kept in incubator shaker at 37°C (250 rpm) for primary culture growth;
3. Primary culture was diluted in the ratio 1:50 in LB Amp50 medium and was kept in incubator shaker at 37°C for secondary culture growth;
4. O.D. was checked from time to time till it was 0.8 to 1.0;
5. Secondary culture was divided into two 3 ml culture and it was labelled as -IPTG and +IPTG;
6. In +IPTG tube 30µl of 100mM IPTG stock was added;
7. It was kept in incubator shaker at 37°C for 90 minutes and again O.D. was checked;
8. 500µl of both -IPTG and +IPTG culture were taken in 1.5ml centrifuge tubes and it was centrifuged at 5000 rpm, RT for 5 minutes;
9. After discarding supernatant, 200 µl of 1X reducing dye was added to it;
10. Both the sample and BRM was given heat shock at 110°C for 5 minutes and then it was vortexed and was given short spin;
11. 5µl of BRM, -IPTG and +IPTG was loaded in duplicates in 12.5% SDS-PAGE.

## 4 RESULTS

### 4.1 Plasmid DNA isolation

#### 4.1.1 PEG Precipitation Method

##### 4.1.1.1 Streaking

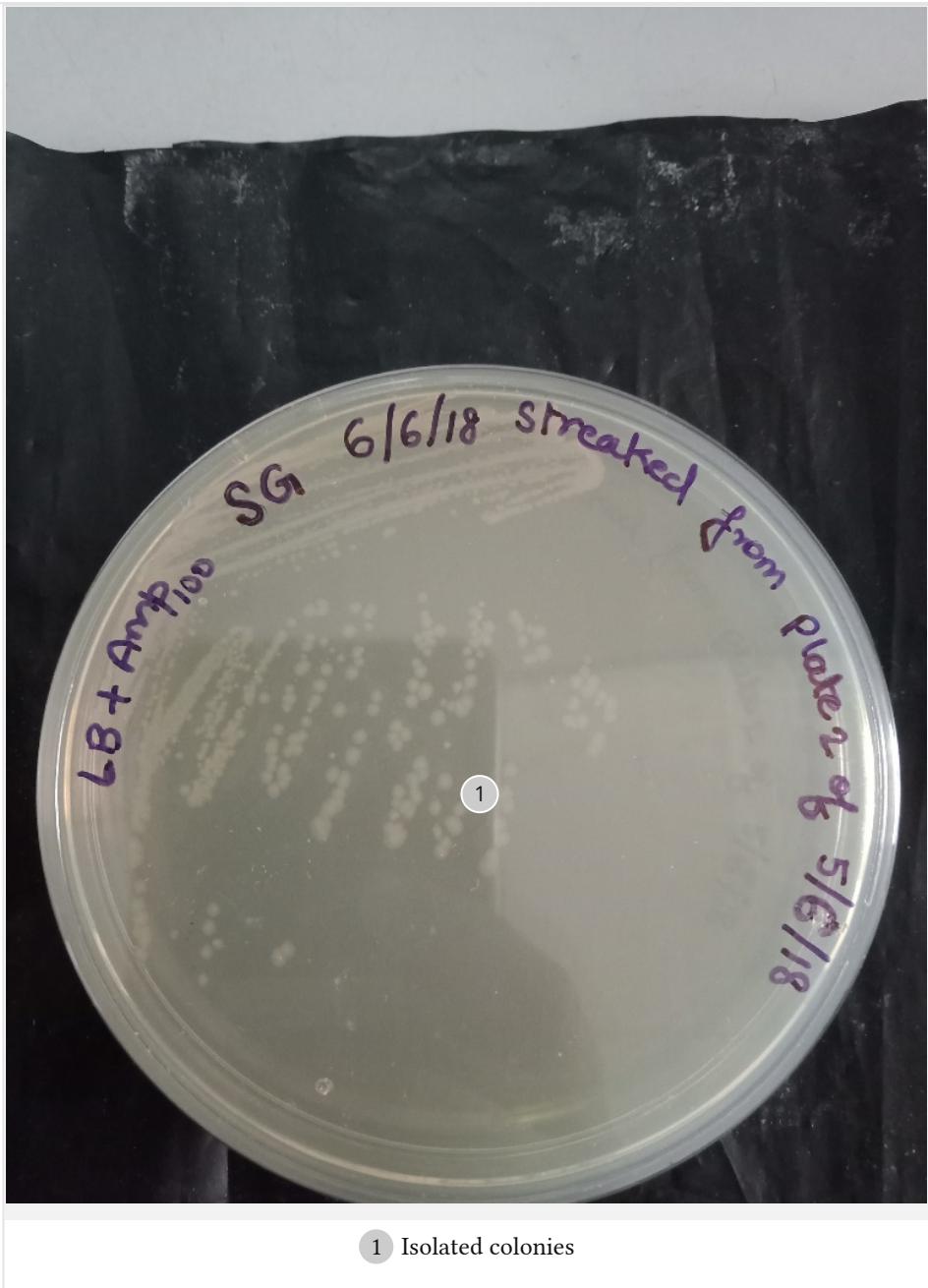


Fig 9 Streaked plate of pGEM DNA transformed into *E.coli* DH5 $\alpha$  cells in LB Amp<sub>100</sub> medium. There were many isolated colonies observed in the plate.

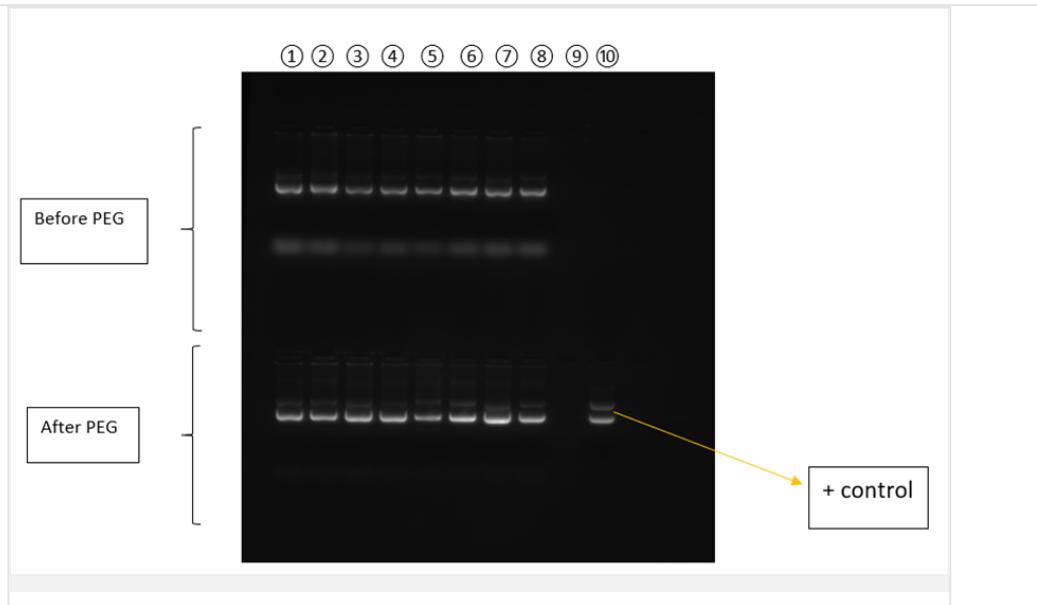


Fig 10 pGEM DNA Isolation Precipitation Method. Intensity of DNA bands increased for after PEG than before PEG. Also there was no RNA contamination observed in after PEG sample.

#### 4.1.2 By using GENETIX kit

##### 4.1.2.1 Agarose gel electrophoresis picture



Fig 11 *pGEM DNA Isolation by using Genetix Kit Method.*

## 4.2 CaCl<sub>2</sub> Transformation

### 4.2.1 Transformed colony plate

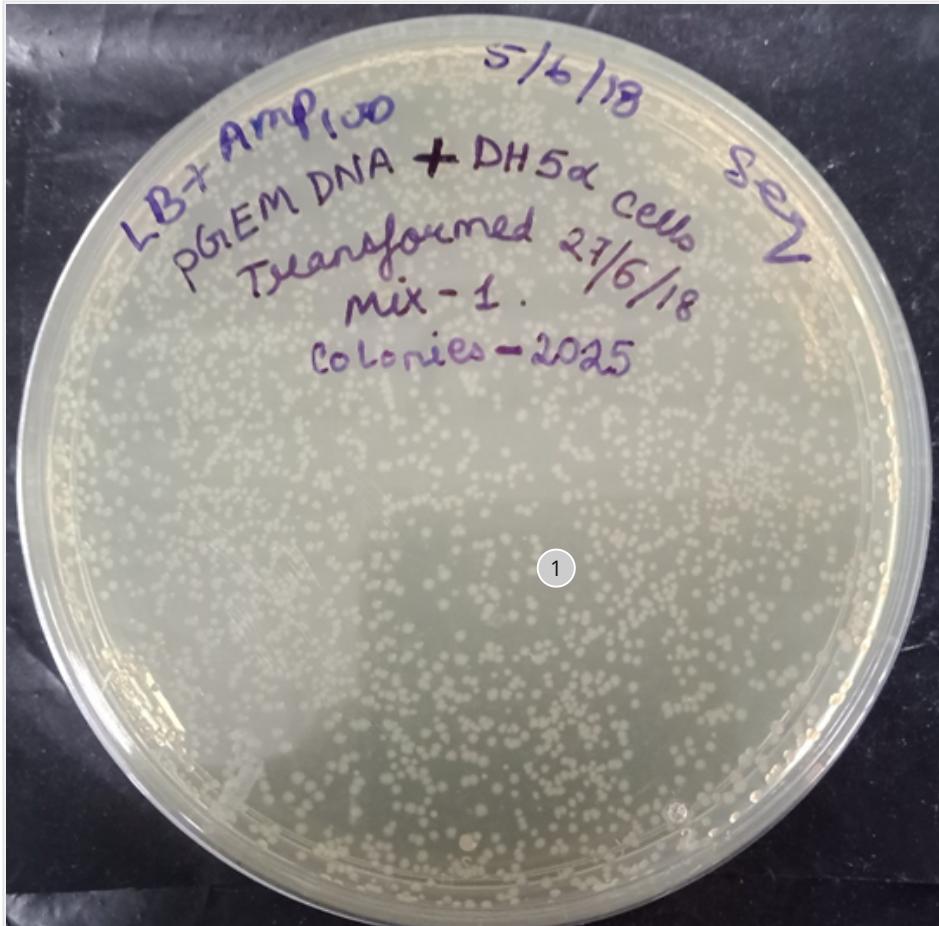


Fig 12 : Large Number Of Colonies of pGEM DNA Transformed into *E.coli* DH5 $\alpha$  cells

### 4.2.2 Calculation of transformation efficiency

pGEM stock concentration = 200 ng/ $\mu$ l

Dilution Factor = 1:1

Final pGEM concentration = 100 ng/ $\mu$ l ( 3  $\mu$ l of pGEM DNA + 3  $\mu$ l of A/c dd H<sub>2</sub>O)

Total number of isolated colonies = 2025 colonies

Transformation Efficiency = Total number of isolated colonies /  $\mu$ g of DNA

Transformation Efficiency = 2025 / ( 100 \* 10<sup>-3</sup>)

= 20.25 \* 10<sup>3</sup>

## 4.3 Protein Expression

### 4.3.1 Streaked Plate



Fig 13 Streaked plate of H10-T-MPT64-BAP transformed into *E.coli* BL21 (DE3) RIL

#### 4.3.2 Primary Culture



Fig 14 Primary culture obtained after overnight incubation

#### 4.3.3 Cultures obtained in absence and presence of IPTG



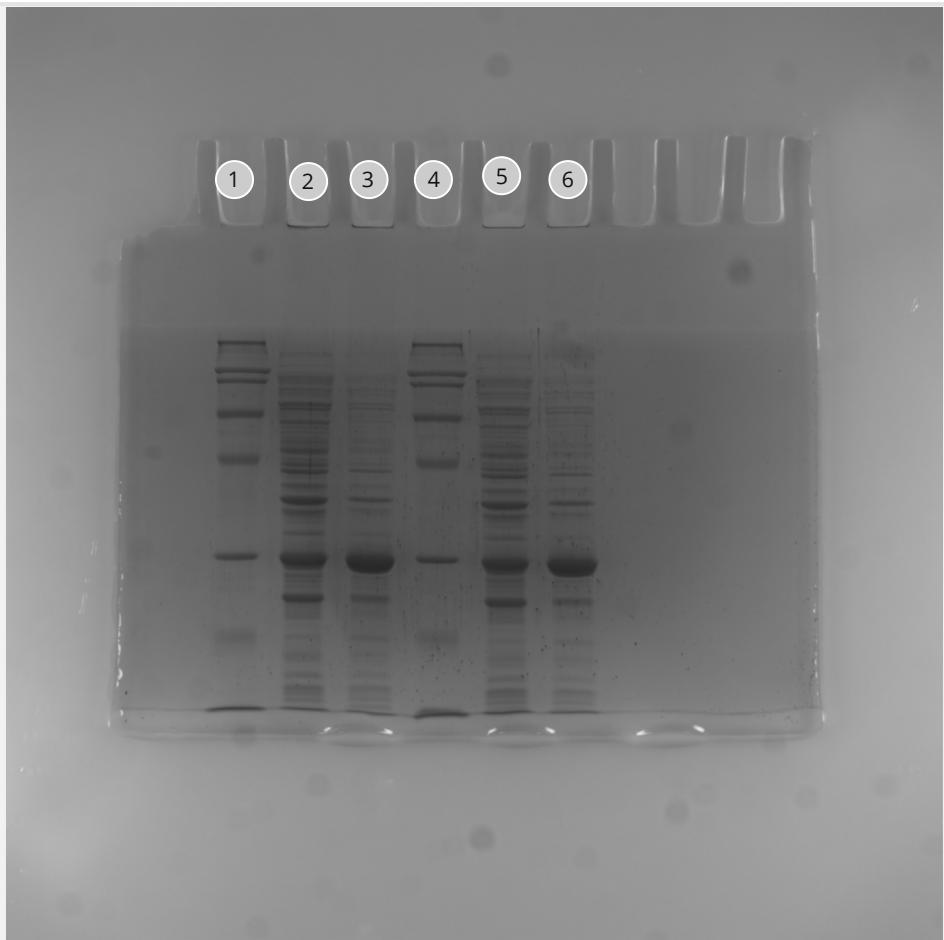
Fig 15 Secondary culture in absence and presence of IPTG inducer

#### 4.3.4 O.D. Readings

Table 3 O.D. Readings for different cultures

Culture	O.D.
<b>Primary Culture</b>	<b>1.598</b>
<b>Secondary Culture</b>	<b>0.6</b>
-IPTG	1.602
+IPTG	1.484

#### 4.3.5 SDS – PAGE Picture



- 1 Lane 1: Broad Range Marker (BRM)
- 2 Lane 2: Samples in absence of IPTG inducer (-IPTG)
- 3 Lane 3: Samples in presence of IPTG inducer (+IPTG)
- 4 Lane 4: Broad Range Marker (BRM)
- 5 Lane 5: Samples in absence of IPTG inducer (-IPTG)
- 6 Lane 6: Samples in presence of IPTG inducer (+IPTG)

Fig 16 SDS-PAGE of Protein expression with and without IPTG induction. H10-T-MPT64-BAP protein (28.04 KDa) were expressed near 31 KDa band of Broad Range Marker (BRM) loaded. There were more amount protein expressed in +IPTG (induced) sample than in uninduced sample. In uninduced sample there were some basal level expression of our desired protein.

## 5 DISCUSSION

Francois Jacob and Jacques Monad proposed a model for an operon, which consisted of a regulator gene, an operator site containing a regulatory DNA sequence, and one or more structural genes. The lac operon was studied in *E. coli*. It contains 3 genes that are needed to produce proteins that are required to break down lactose when it is present in the cell. These 3 genes are Lac Z, Lac Y and Lac A. Each code for B- galactosidase (an enzyme which hydrolyses lactose into glucose and galactose), Permease (an enzyme which promotes lactose

transport across the cell membrane of bacterium) and Transacetylase (an enzyme which transfers acetyl groups from acetyl-coenzyme A to  $\beta$ -galactoside), respectively. Upstream to the structural genes lies promoter and operator. Promoter is the sequence where RNA polymerase will bind to transcribe the three genes. Upstream of the promoter sequence there is another gene. This is the Lac I gene. The Lac I gene is transcribed to make the repressor protein which binds to 3 different operator sequences present at different parts of the Lac Operon. The repressor protein is a tetramer and the binding of this repressor to the operators cause the DNA to be looped around. Once the repressor protein is bound, it stops the RNA polymerase enzyme from transcribing the genes thus effectively it acts as block. When the glucose levels are depleted and the lactose levels are high, the repressor has to be removed in order to transcribe the required genes. This is done by an inducer molecule. This molecule comes from lactose and is Allolactose. Allolactose is synthesised from lactose by the enzyme  $\beta$ -galactosidase, which is also a translational product of the lac gene. The allolactose binds to the repressor protein and causes a conformational change in the repressor which causes it to dissociate from the DNA. Upon binding the allolactose reduces the affinity of the repressor protein for the lac operator by a factor of 1000. RNA polymerase can work as it is not blocked and the Lac Z, Lac Y and Lac A genes are transcribed.

Sometimes promoters are not strong enough to initiate transcription on their own and so require another molecule or complex to help. In the Lac operon, this is done by the CRP – cAMP complex. This is because glucose acts as an inhibitor of the enzyme adenylyl cyclase which is responsible for converting ATP into cAMP. When glucose levels in the cell are low, the levels of cAMP increase as glucose no longer blocks cAMP synthase. This then combines with a CRP protein (sometimes also referred to as the CAP protein) and forms a complex called the CRP-cAMP complex; CRP can activate transcription at more than 100 catabolite-sensitive operons. Thus, this complex then joins to a sequence of nucleotides downstream from the Lac I promoter known as the CRP binding site. The binding increases the affinity of RNA polymerase for the lac promoter sequence and hence it binds and as a result, transcription can take place. When the levels of glucose increase again, the amount of cAMP synthesised is reduced and so the complex levels decrease. This, therefore, inhibits the Lac operon from working because the affinity of the RNA pol II for the lac gene promoter without the activator bound is quite low. So the Lac operon is only active when there is plenty of lactose and there is absence of glucose. But allolactose when present as an inducer its concentration reduces because of being metabolized. There is one more kind of inducer called gratuitous inducer like IPTG which binds with the repressor molecule and changes its conformation and finally preventing the repressor binding with the operator. One advantage with IPTG is that it does act as a substrate to  $\beta$ -galactosidase enzyme and thus its concentration remains constant all through. So when induced with IPTG, H10-T-MPT64-BAP protein (28.04 KDa) was expressed near 31 KDa protein band of BRM in SDS-PAGE. But there was also protein expression in uninduced sample because of the basal level expression or leaky expression. There can be many reasons for leaky expression. They are as follows:

- Lac repressor does not bind so tightly with the operator sequence
- In LB media there is Tryptone which can be a possible source of Lactose and this can act as an inducer for basal level expression

- Also *E.coli* strain BL-21 is deficient in protease so there can be expression of some undesired protein along with our desired protein of the same size (protein aggregation)
- In secondary culture when O.D. comes close to one glucose level becomes very low so cAMP level becomes high. cAMP forms a complex with CAP which helps in efficient recruitment of RNA polymerase and finally in expression

## 6 CONCLUSION AND RECOMMENDATION

H10-T-MPT64-BAP protein was expressed in presence of gratuitous inducer IPTG and was tested in SDS-PAGE.

### Future Perspectives:

Recent advancement studied by Studier shows that protein of interest can also be expressed by Autoinduction other than IPTG induction. In autoinduction there is no need to check for bacterial growth and also to add inducer in the optimised concentration. With this technique more amount of protein can be produced in less time without making it toxic for cells.

## 7 ACKNOWLEDGEMENT

I want to express my sincere gratitude to IAS-INSA-NASI for providing me this opportunity to work and learn as a Summer Research Fellow. Also my deepest gratitude to Dr. Amita Gupta for guiding me in the best possible manner during this tenure. I also thank Dr. Shilpi Saha and Prachi Singh and all my lab mates for helping me throughout my experiments along with clearing my doubts. Lastly I convey my deepest gratitude to my professor Dr. Nandan Kumar Jana for providing my Letter of Recommendation required in the desired format.

## 8 REFERENCES

1. Griffiths AJF, Gelbart WM, Miller JH, et al. Modern Genetic Analysis. New York: W. H. Freeman; 1999.
2. Shuman, H. A., et. al., Microbial genetics: The art and design of genetic screens: *Escherichia coli*, *Nature Reviews Genetics* 4, 419-431 (2003) 10.1038/nrg1087
3. Ralston, A. (2008) Operons and prokaryotic gene regulation. *Nature Education* 1(1):216
4. Jacob, F., & Monod, J. On the regulation of gene activity. *Cold Spring Harbor Symposia on Quantitative Biology* 26, 193–211 (1962)
5. Lawrence, J. G. Shared strategies in gene organization among prokaryotes and eukaryotes. *Cell* 110, 407–413 (2002)
6. Shaw, K. (2008) Negative transcription regulation in prokaryotes. *Nature Education* 1(1):122
7. Jacob, F., & Monod, J. The operon: A group of genes with expression coordinated by an operator.

- Comptes Rendus Biologies 328, 514–520 (1960)
8. Jacob,F., & Monod, J. Genetic regulatory mechanisms in the synthesis of proteins. Journal of Molecular Biology 3, 318–356 (1961)
9. Oxender, D. L., et al. Attenuation in the Escherichia coli tryptophan operon: Role of RNA secondary structure involving the tryptophan codon region. Proceedings of the National Academy of Sciences 76, 5524–5528 (1979)
10. Brown, W., Ralston, A. & Shaw, K. (2008) Positive transcription control: The glucose effect. Nature Education 1(1):202
11. Hirsch, J., & Schleif, R. On the mechanism of action of L-arabinose C gene activator and lactose repressor. Journal of Molecular Biology 80, 433–444 (1973)
12. Verma V, Kaur C, Grover P, Gupta A, Chaudhary VK (2018). Biotin-tagged proteins: Reagents for efficient ELISA-based serodiagnosis and phage display-based affinity selection. PLoS ONE 13(1): e0191315.
13. Yildirim N, Mackey MC. Feedback regulation in the lactose operon: a mathematical modelling study and comparison with experimental data. Biophys J. 2003 May;84(5):2841-51
14. Cooper TG, Magasanik B. Transcription of the lac operon of Escherichia coli. J Biol Chem. 1974 Oct 25;249(20):6556-61
15. Thomas Kuhlman, Zhongge Zhang, Milton H. Saier, Terence Hw. Proceedings of the National Academy of Sciences Apr 2007, 104 (14) 6043-6048; DOI: 10.1073/pnas.0606717104
16. Marbach A, Bettenbrock K. lac operon induction in Escherichia coli: Systemic comparison of IPTG and TMG induction and influence of the transacetylase LacA. J Biotechnol. 2012 Jan; 157(1):82-8.
17. Nicole A. Becker Justin P. Peters Troy A. Lionberger L. James Maher, III. Mechanism of promoter repression by Lac repressor-DNA loops. Nucleic Acids Research, Volume 41, Issue 1, 1 January 2013, Pages 156–166.

Approved Read  
Only