

The Genetics of Beta-galactosidase— Encoded by the *lacZ* gene in *E. coli*— Laboratory Exercises to Illustrate Gene Regulation

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Abstract: Mutations of *E. coli* studied by Jacob and Monod led them to develop a model of transcriptional regulation of the *lac* operon by the *lac* repressor for which they received a Nobel Prize in 1965. This exercise uses *E. coli* strains with mutations in the *lac* operon to demonstrate the regulation of beta-galactosidase production. This exercise is used in a sophomore biology majors genetics and molecular biology laboratory at Purdue University. Students identify the nature of the mutations in each strain based on their determination of the beta-galactosidase activity of each strain. This laboratory enhances the students' understanding of gene regulation.

Introduction

Beta-galactosidase is an enzyme that splits lactose into glucose and galactose; it is encoded by the *lacZ* gene in the *lac* operon of the bacterium *Escherichia coli*. An operon is a set of structural genes transcribed as a single messenger RNA and adjacent regulatory regions that control the expression of these genes. Because beta-galactosidase is a relatively stable enzyme that is easily assayable using the substrate ONPG (o-nitrophenyl-beta-galactopyranoside), it is used in laboratory exercises.

The beta-galactosidase system of *E. coli* was studied by scientists François Jacob and Jacques Monod. From their analysis of mutations within the *lac* operon, they developed a model of transcriptional regulation of the *lac* operon by the *lac* repressor. They formulated a model of genetic regulatory mechanisms, showing how, on a molecular level, certain genes are activated and repressed. They received a Nobel Prize in 1965 for this work.

This laboratory exercise uses *E. coli* strains with different mutations in the *lac* operon to demonstrate the regulation of beta-galactosidase production in *E. coli*. This exercise is used in a three-hour long sophomore level genetics and molecular biology laboratory at Purdue University for biology majors. Students identify the nature of the mutations in each strain based on their determination of the beta-galactosidase activity of each strain. Students examine the genetics and molecular biology of the *lac* operon. This laboratory enhances the students' understanding of gene regulation.

In addition, we discuss the historical background and practical applications of the *lac* operon. We examine the use of the *lacZ* gene as a reporter gene to study gene expression and as a part of cloning vectors such as pUC13.

Note to the student: beta-galactosidase—Not Just Another Enzyme Assay

The operon model put forth by Jacob and Monod with regard to the lactose system of *E. coli* is considered a landmark event in science. Not only did this remarkable work pave the way for further description of genetic regulatory mechanisms (Beckwith 23 March 2006, posted date; Cohen 1995), it also led to the development of numerous molecular biology tools. Every day, modern scientists rely on biochemical assays, cloning vectors and methods to detect gene expression without much thought as to how these tools were developed. There is a great deal to be gained by examining the major historical events in our respective scientific fields. It is difficult to imagine a time when terms such as messenger RNA, operon and inducer had not yet been defined. How fascinating it is to read the original manuscripts that first introduced these terms! The concepts written in textbooks had to come from somewhere. Seeing how the pioneers of science conceived these models can help reinforce the fact that in any course you take, you are not expected to be an expert from day one. There are many small details to be learned in your coursework, but one should not lose sight of the big picture. In reviewing the history of the *lac* operon, the following take-home messages should be evident:

1. **“You can see a lot by just looking.”—Yogi Berra.** Who would have thought that a baseball player would touch on one of the most basic concepts in science? Jacob and

Monod examined data from several experiments performed on the lactose system while developing the operon model. Deductive reasoning and the power of observation are thus, our best friends in the laboratory.

2. **Science is a dynamic field.** In their groundbreaking *Journal of Molecular Biology* publication, Jacob and Monod (1961) believed the repressor mechanism of genetic control applied to enzyme systems in general. It is now known that there are other levels of genetic regulation including positive regulation, translational control and control of transcription termination (Waleh and Johnson 1985; Neidhardt, Ingraham et al. 1990). Even small anti-sense RNAs are believed to play a role in gene regulation (Waleh and Johnson 1985; Hu, Brodie et al. 2005). What one should realize from this is that there is always room for new discoveries and more refined explanations of biological systems.
3. **Better tools through better science and vice versa.** In the 1950s-1960s, the topic of the day was gene regulation and the system of choice was the lactose system. Jacob, Monod and their colleagues took advantage of classical genetic and biochemical techniques to study this system at length. At their disposal were various *lac* mutants, conjugation techniques to create “merozygotes” and several lactose analogs that served as gratuitous inducers and chromogenic substrates. These same analogs, along with the properties of the *lac* operon have expanded the molecular toolkit to include methods to study the inducibility of genes, low-level expression of proteins, cellular localization of proteins, in addition to general cloning vectors. And this is just the short list!

So, when the inevitable question arises, “Why are we doing this lab?” remember these points. If that doesn’t help, the answer to your question will undoubtedly be, “Because I told you to!”

The *lac* Operon from a Historical Perspective

The operon model defined by Jacob and Monod (1961) was a significant development. The suggestion that there can be coordinated control of the expression of multiple genes led researchers to search for similar genetic units controlling other enzyme systems. This made sense considering it had been observed that genes operating a particular metabolic pathway are often found grouped together within the genome. The model also put forth the idea that elements (i.e. genetic control elements and small cytoplasmic molecules) in addition to the structural gene itself affect protein synthesis. Moreover, the article introduces messenger RNA as an intermediate in the transfer of genetic information to the finished protein and the proposal that the regulation of protein synthesis occurs at the genetic level. The latter concept was a subject of debate at the time; it was not known if the rate of protein synthesis was controlled by a cytoplasmic agent or at the genetic level.

What was known was that the synthesis of proteins could be influenced by the presence or absence of external agents. Enzymes were called “adaptive” in that they can be induced or repressed by substrates or metabolites. Before divulging the body of evidence that was used to derive the operon model, Jacob and Monod stated their conclusions up front. They concluded that: in general, enzyme systems were subject to negative control; two new types of genetic determinants, the regulator and operator, are involved in controlling the expression of structural genes; the control of enzyme systems operates at the genetic level. These conclusions were drawn from an analysis of data collected from biochemical assays and mutagenesis experiments performed by Jacob and Monod as well as their colleagues (Jacob and Monod 1961).

Using methods similar to those of this laboratory exercise, Jacob and Monod arrived at the operon model for the regulation of lactose utilization through experiments testing the inducibility of various lactose mutants. See Figure 1 for the operon model. For instance, z^- mutants and y^- mutants lost the ability to synthesize galactosidase and galactoside permease, respectively, regardless of the presence of an inducer. Constitutive mutants, i^- , which were deficient in the *lac* repressor, acquired the ability to synthesize galactosidase and acetylase without induction (Pardee, Jacob et al. 1959; Jacob and Monod 1961). It was shown that i^- mutants could regain their inducibility via complementation with the Fi^+ element. The product of the *i* gene could act in *trans*, leading to an inducible system through the action of a cytoplasmic substance. Although it was first thought that the repressor molecule was RNA (Jacob and Monod 1961), the work of Gilbert and Müller-Hill later showed that the *lac* repressor is a protein (Gilbert and Muller-Hill 1966).

The term “regulator gene” was used to define genes that could control the synthesis of several different proteins. In the *lac* operon, the repressor gene *i* falls into this category, since it can influence the transcription of the remaining operon. It accomplishes this through the interaction with an inducing molecule, which alters its affinity for the site of action, the *lac* operator. Again, through the selection of constitutive mutants (o^c) and complementation with the wild-type allele on an *F'* element, it was determined that the operator gene acts in *cis*; it only affects the transcription of the *zya* genetic element to which it is attached. For instance, a strain with the genotype o^+z^+/Fo^cz^- , remained inducible for β -galactosidase production; whereas a strain with the genotype o^+z^-/Fo^cz^+ was constitutive. These factors: the specificity of the *lac* repressor for genes controlling lactose utilization, the influence of the *lac* repressor on the expression of multiple proteins, the identification of the operator region and the consecutive arrangement of these genes on the chromosome define this system as a “genetic unit of coordinated expression” or operon (Jacob and Monod 1961).

Other Forms of Genetic Regulation

It was originally proposed that negative regulation such as that described for the *lac* operon, under the control of a repressing element, was the universal mode of regulation for enzyme systems. As the area of gene regulation exploded, however, other forms of regulation were discovered. Ironically, it was shown that the *lac* operon is also subject to positive regulation. Oddly enough, this positive control is accomplished through the catabolite *repression* network. (Who said *E. coli* was simple?) Since bacteria can utilize different carbon sources for energy—and one carbon source, such as glucose, might be preferred over the other—there needs to be a way to tell the cell which catabolic pathway to turn on and which to keep off. Briefly, when glucose is present, the conversion of ATP to cAMP is inhibited. When glucose is depleted, the conversion can take place and cAMP levels increase, signaling to the cell that alternative carbon sources can be utilized. A complex between cAMP and a second protein, CAP (catabolite-activating protein), binds to the *lac* promoter, activating transcription (Emmer, deCrombrughe et al. 1970; Zubay, Schwartz et al. 1970; Neidhardt, Ingraham et al. 1990).

The catabolite repression network is just one of many global regulation systems that allow bacteria to sense their environment and respond appropriately. Although interesting, a discussion of all the mechanisms would be too exhaustive. The point here is to alert the student to the fact that gene regulation is not always straightforward. As in the case of the *lac* operon, there can be more than one means of regulating transcription. This system demonstrates both positive and negative regulation. Furthermore, transcription can be controlled not just at the promoter, but also at the end of the transcript through mechanisms affecting transcriptional termination. Enzymes are also often regulated through feedback inhibition in which the end product of a pathway influences the activity

of enzymes earlier in the pathway. If one thinks about the process of synthesizing a protein, there are several steps, from transcription to translation to the final folding and processing of the protein. Regulation can occur (and has been shown to occur) at any of these steps.

Beta-Galactosidase and the *lac* Operon in the Molecular Biology Lab

Why is the *lac* operon so important to molecular biologists? What has it done for our field other than improve the understanding of gene regulation? Set foot in even the most basic biology lab and you will most likely find one of the answers. Probably the most widely used application of the *lac* operon is cloning vectors. We teach our students that a useful cloning vector must have multiple cloning sites, a selectable marker such as an antibiotic resistance cassette, and a suitable means of screening for the correct clone, i.e. the one that contains the insert. Two features of the *lac* operon make it ideal for this purpose: it has a promoter whose expression can be controlled with inducers; it has an easily assayed structural gene, β -galactosidase. When a host strain carrying one such vector is grown on media containing the inducer IPTG and the chromogenic β -galactosidase substrate, X-gal, the colonies are blue. When a fragment of DNA is cloned into the 5' region of the β -gal (*Z*) gene, the production of this enzyme is disrupted and colonies remain white. There are further details to this method that need to be considered. For instance, the *lacZ* gene used in these cloning vectors has a mutation in the 5' end that can be complemented if the host strain carries another mutation in a different part of the *lacZ* gene. Blue-white screening can only be accomplished if the appropriate vector is used in a suitable host. These details are more appropriately discussed in laboratory exercises to demonstrate cloning techniques (Gray, Colot et al. 1982; Messing and Vieira 1982; Vieira and Messing 1982; Yanisch-Perron, Vieira et al. 1985).

The functionality of genes can also be assessed using the *lac* system. For instance, if one wishes to test if the expression of a certain gene is controlled by environmental factors, a gene fusion with a promoter-less β -galactosidase can be used. This is especially useful if the gene product is uncharacterized or is not easily assayed. Along the same lines, the rate of gene expression can also be assessed by performing a β -galactosidase assay. Since this assay is very sensitive, *lac* fusions have been used to measure the expression of low-abundance proteins and to locate a protein within the cell or external space. There are many more specific uses of *lac* fusions. Silhavy and Beckwith provide an excellent review of this topic (Silhavy and Beckwith 1985). *LacZ* used in this way is called a "reporter gene". As the reader can see by the publication date, the list of applications of *lac* fusions has undoubtedly grown, but this at least provides a starting point.

One last use of the *lac* operon that should be mentioned since this has become a topic of importance in recent years is in the field of metabolic engineering. Whether the goal is to model the flux through a particular metabolic pathway or to produce a certain metabolic end product, the metabolic engineer is concerned with **controlling** the rate of enzymatic reactions. Exhaustive measures have been taken to construct tools for doing just that. The *lac* promoter has been used due to its inducibility. To further refine the control, variations of the *lac* promoter have been obtained through point mutations. The mutant promoters may carry additional characteristics such as being insensitive to catabolite repression, as is the case for the *lacUV5* promoter. Higher levels of activity may be attained through the use of *tacI* promoter, another variant of the *lac* promoter (Jensen, Westerhoff et al. 1993; Jensen and Hammer 1998; Keasling 1999). The use of these promoters allows for fine-tuning gene expression, an essential aim in metabolic engineering.

Beta-Galactosidase and the *lac* Operon as a Teaching Tool

The *lac* operon provides a unique opportunity for translating classroom lessons into laboratory exercises. There are a number of different *E. coli lac* mutants available and β -γαλακτοσιδάσε is easily assayed using the chromogenic substrate ONPG. As indicated in the experimental design, the student will be responsible for using **phenotypic** observations to make inferences regarding the **genotype** of the *E. coli* strains with which they are working. This will require an understanding of the regulation of the *lac* operon in order to draw the correct conclusions. As alluded to above, the student is obliged to use the powers of observation and deductive reasoning, rather than merely memorizing facts. More importantly, the notion that multiple genotypes can lead to the same phenotype will be realized.

As a laboratory exercise though, the objectives reach beyond academics and into demonstrating skills that any Biology student should be able to grasp. Upon completion of this exercise, the student will be able to describe the regulation of the *lac* operon and the use of different β -galactosides in assessing its function. In addition, the steps involved in the enzyme assay itself, need to be understood, especially if one is expected to trouble-shoot should problems arise. The β -galactoside assay involves inducing the enzyme with IPTG; releasing the enzyme through cell lysis; detecting the activity with a chromogenic substrate; and finally, measuring the product. In carrying out these steps, the student needs to demonstrate proper use of pipetting devices, organizational and timing skills and operation of a spectrophotometer. One can imagine how this exercise can be expanded to include the demonstration of graphing skills, such as plotting enzyme activity versus time of induction in order to assess reaction rates. It is imperative that today's Biology students are able to translate the skills and lessons learned from chemistry and biochemistry courses to the biology laboratory. Enzyme assays, such as the β -galactosidase assay, are a great way to bridge these subjects.

Student Outline

β -galactosidase Induction in *E. coli*

Objectives:

- To determine which "unknown" *E. coli* strain is inducible and which is constitutive for β -galactosidase production.
- To understand the structure of the *lac* operon and how it operates.
- To understand the difference between induction and catabolite repression.
- To understand the difference between positive and negative control.

After completing this lab, one should be able to:

- Describe how the ONPG assay for β -galactosidase works.
- Given a particular *lac* phenotype, describe an appropriate *lac* genotype.
- Given a *lac* genotype, determine what the *lac* phenotype will be.

Safety concern:

Dispense chloroform in a hood for organic solvents.

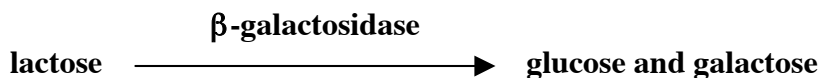
Materials Needed per Pair

20 mL log phase culture of unknown A. *E. coli* (two tubes with 10 mL each)
 20 mL log phase culture of unknown B. *E. coli* (two tubes with 10 mL each)
 30 test tubes
 20-1 mL and 5-5 mL pipettes, 2-10 mL pipettes
 2 mL IPTG 10^{-2} M in H₂O
 8 mL ONPG (4 mg/mL)
 36 mL Na₂CO₃
 Sterile distilled water
 Z buffer (18 mL)
 Chloroform-CHCl₃ and 0.1% SDS in dropper bottles
 Vortex
 Water baths at 37°C and at room temperature
 Spectronic 20 and 3 cuvettes
 Test tube racks

Regulatory systems in *E. coli*

Escherichia coli has approximately 3,000 genes. At any given time, it is making about 1400 different proteins. In the *E. coli* cell, there are only a few molecules of some proteins but there are thousands of molecules of other proteins. *E. coli* is able to alter transcription patterns rapidly in response to environmental stimuli. In many cases enzymes are made quickly when they are needed, and their production ceases when they are not needed. In order to grow efficiently and to respond rapidly to environmental stimuli, cells must be able to regulate gene activity. Bacterial cells often regulate genes coordinately in an operon. An **operon** is a set of contiguous, related genes that are transcribed into a single mRNA and the regulatory regions that control the expression of these genes.

Jacob and Monod first described the operon concept in *E. coli* in 1961. The operon they studied is the *lac* operon, which controls the uptake and utilization of lactose by the cell. Lactose utilization in *E. coli* involves the products of three structural genes: *LacZ* codes for β -galactosidase, an enzyme that hydrolyzes the disaccharide lactose to glucose and galactose, *lacY* encodes a permease, a cell membrane protein that is needed for lactose uptake, and *lacA* encodes an acetylase, whose function is not well understood at the present time.

 β -galactosidase catalyzes the following reaction:

E. coli can rapidly alter the amount of *LacZ* (and *LacY* and *LacA*) proteins that it produces. When growing on carbon sources other than lactose (or if glucose is present), there will only be about two molecules of β -galactosidase per cell. This is because a **regulatory gene** (*lacI*) produces a repressor protein that controls the initiation of transcription of the lac operon. The *LacI* repressor protein physically binds to the **operator** site on the *lac* operon DNA to prevent binding of RNA polymerase to the **promoter**. However, when an **inducer** (lactose) is added to the medium, the inducer will complex with the repressor protein and cause a conformational change that prevents the repressor protein from binding the operator. Since the repressor protein plus inducer complex no longer interacts with the operator, RNA polymerase will be able to bind to the promoter and transcribe the operon. The level of β -galactosidase will rise to over 3,000 molecules per cell.

Glossary of Terms

The following terms will help with the understanding of the operon concept of regulation:

Inducible operon—an operon that is transcribed only in the PRESENCE of a specific small molecule inducer (e. g. lactose).

Repressible operon—an operon that is transcribed only in the ABSENCE of a small molecule co-repressor (e. g. tryptophan).

Constitutive Production—enzyme is produced all the time by the cell, regardless of the presence or absence of inducers or repressors.

Operon—a set of contiguous, related genes that are transcribed into a single mRNA and the regulatory regions that control the expression of these genes.

Promoter—sequence of deoxy-nucleotides (adjacent to "operator") which is the binding site for RNA polymerase.

Operator—a deoxy-nucleotide sequence adjacent to the structural genes that is the site for **REPRESSOR BINDING**.

Regulon—a group of genes and/or operons responding to a common regulatory process, but dispersed over the whole chromosome, and thus transcribed as several mRNA molecules.

Regulatory gene—a gene (sequence which may or may not be adjacent to promoter) that codes for a **REPRESSOR PROTEIN** that has special affinity for the "operator" region.

Negative Control—The presence of a repressor protein prevents transcription, i.e. the repressor turns a gene off.

Positive Control—The presence of an activator protein initiates transcription, i.e. the activator turns a gene on.

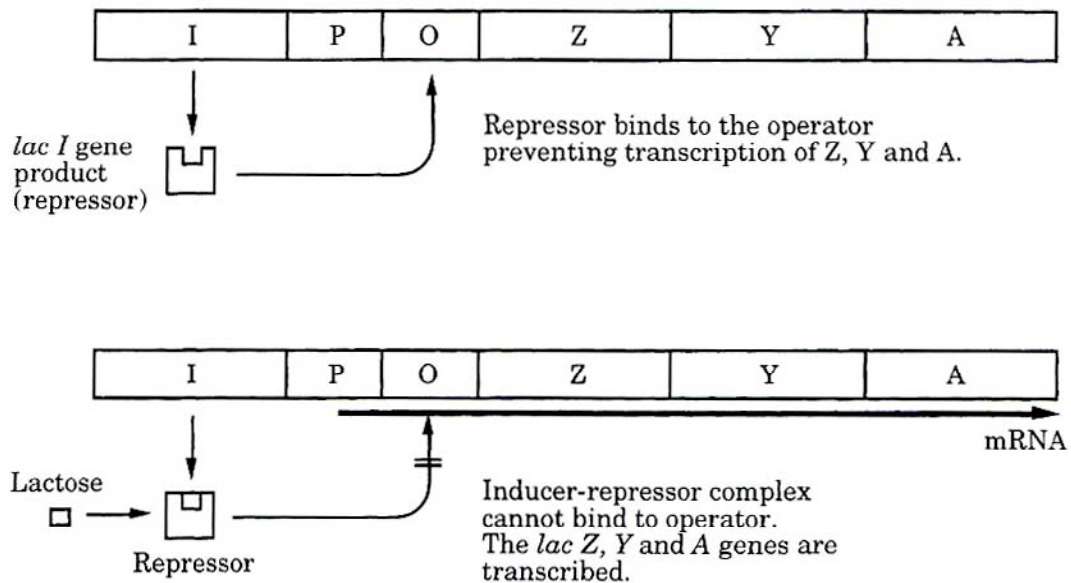
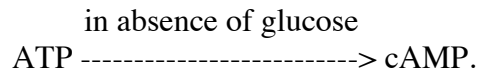
β -GALACTOSIDASE INDUCTION IN *E. COLI*

Figure 1. A schematic of the *lac* operon showing all the key elements. The letters refer to the following; O = the operator; P = the promoter; I = the gene coding for the repressor protein; and Z, Y, A = the structural genes.

Control of the Lac Operon

1. At the **operon level**, it is a **negatively controlled, inducible operon**. Synthesis is shut **off** by the repressor (negative part of control) until the inducer (lactose) is present (inducible part of control). Note that lactose, the inducer, is also the substrate for β -galactosidase.
2. At the **cellular level**, control is **positive**. That is to say an activator is required. Synthesis is **on** in the absence of glucose, because



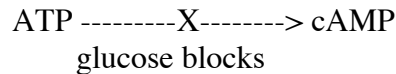
cAMP combines with a catabolite activator protein (CAP, which is encoded by the *crp* gene) to form a CAP-cAMP complex.

The CAP-cAMP complex is **required** for RNA polymerase attachment to the operon. Without RNA polymerase attachment, transcription and thus translation cannot occur. Therefore, the enzymes necessary for lactose hydrolysis are not produced.

Positive Control

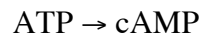
The glucose affect is called **CATABOLITE REPRESSSION** when glucose is present, the cell has a supply of ATP to use and the cell does not break lactose down into glucose and galactose.

1. **When glucose is present**, the *lac* operon is repressed, even in the presence of lactose. This is accomplished by blocking the formation of cAMP.



Without cAMP, the CAP-cAMP complex doesn't form and RNA polymerase doesn't bind to the operon.

2. **When glucose is absent**, the cell first uses all available ATP. When lactose is present, the *lac* operon is turned on.



CAP-cAMP binds to the promotor to allow RNA polymerase attachment and the transcription of the *lac* operon genes.

Sequence of Events

1. **No Glucose**--ATP → cAMP

$$\text{cAMP} + \text{CAP} \rightarrow \text{CAP-cAMP complex}$$
2. Lactose (allolactose) induces the *lac* operon by "removing" the repressor from the operator.
3. The CAP-cAMP complex plus polymerase bind to promotor. The *Lac* operon genes are transcribed from the polycistronic mRNA.
4. The mRNA is translated.
 - Y gene → permease - allows lactose uptake
 - Z gene → β-galactosidase
5. Lactose $\xrightarrow{\beta\text{-galactosidase}}$ Glucose + Galactose.
6. The buildup of the end product, glucose, inhibits this process, blocking Step 1.

The Genetics of the *Lac* Operon

Structural Genes:

- Z^+ = ability to produce β -galactosidase.
 Z^- = lacks ability to produce β -galactosidase.
 Y^+ = ability to produce permease (uptake of inducer).
 Y^- = lacks ability to produce permease (uptake of inducer).
 A = ability to produce acetylase.
 A^- = lacks ability to produce acetylase.

Regulator gene:

- I^+ = **inducible**--produces normal repressor protein that has affinity for the operator and the inducer.
 I^- (also I^c) = **constitutive**--repressor has altered affinity for the operator--cannot bind to the operator.
 I^s = **superrepressed**--altered affinity for **inducer**—The repressor cannot dissociate from operator region.
 I^{ϱ} = **Q refers to quantity**. It is a mutation in the *lacI* gene promoter that results in an increased number of repressor molecules in the cell.

Operator:

- O^+ = wild type, binds repressor.
 O^c = **constitutive**--altered nucleotide sequence--repressor **CANNOT** bind to the operator site.
 O^- = **constitutive**--deletion in repressor BINDING SITE, repressor cannot bind.
 O^{\varnothing} = **deletion** in the proximal area of the operator that includes part of the promoter. Polymerase cannot bind at all.
 CRP^- or p^- = Altered or no CAP binding site. The rate of polymerase binding is lowered and transcription is reduced or goes to zero.
 CAP^+ = wild type catabolic activating protein
 CAP^- = mutant catabolic activating protein will not complex with cAMP, thus rate of polymerase binding is lowered.

When deciding the phenotype of a particular genotype, consider whether the component in question is a **gene product** or an actual **DNA sequence**. The table below

demonstrates several *E. coli* genotypes and which enzymes are produced. If a gene is omitted, assume wild type (+).

Table 1. Enzymes produced by different *E. coli* mutants in the presence and absence of lactose.

Genotype of <i>E. coli</i>	ENZYMES PRODUCED	
	Induced (lactose present)	Not induced (lactose absent)
WILD TYPE $I^+(CAP)P^+O^+Z^+Y^+A^+$	Z, Y, A	none
CONSTITUTIVE $I^+O^cZ^+Y^+A^+$	Z, Y, A	Z, Y, A
SUPER REPRESSED $I^sO^+Z^+Y^+A^+$	none	none
CONSTITUTIVE $I^-O^+Z^+Y^+A^+$	Z, Y, A	Z, Y, A
INDUCIBLE $I^+O^+Z^+Y^-A^-$	Z	none

Method to Study the Relationship Between Different Genes

A partially diploid bacterial cell or **merozygote** (or merodiploid) is constructed through conjugation. The F^+ cells transfer the fertility factor, a plasmid, into an F^- cell. F' are F plasmids which can also carry parts of the chromosome. An F' can carry parts of the *lac* operon. Through this process, an *E. coli* cell can carry two separate and distinct mutations of the *lac* operon. Now, one has to determine which mutant is dominant to decide whether the operon is inducible or repressed. To start the decision-making process, one must decide whether the mutation deals with a diffusible gene product or not. That is, is the mutation in a gene that produces a gene product or is the mutation in a regulatory DNA sequence such as the operator region.

Look at the following examples to determine the phenotype.

$$\begin{array}{rcl}
 \text{Example 1:} & F' I^+ O^+ Z^+ Y^+ & F^- I^s O^+ Z^+ Y^+ \\
 & \text{(inducible)} & \text{(superrepressed)} \\
 & \times & \\
 \text{MEROZYGOTE} & F^- I^+ O^+ Z^+ Y^+ & \\
 & \hline
 & F' I^s O^+ Z^+ Y^+ &
 \end{array}$$

What is the phenotype of this merozygote? What protein products are produced?

I^S is a diffusible gene product that can bind to the operator of both operons that are present. This is called **trans dominant**. Therefore, no enzyme is produced by this cell.

$$\begin{array}{rcc}
 \text{Example 2:} & F^- I^- O^+ Z^+ Y^+ & F^+ I^+ O^+ Z^+ Y^+ \\
 & \text{(constitutive)} & \text{(inducible)} \\
 & \times & \\
 & F^- I^- O^+ Z^+ Y^+ & \\
 \hline
 & F^+ I^+ O^+ Z^+ Y^+ &
 \end{array}$$

What is the phenotype in this case? What protein products are produced?
Recall: *lacI* produces a diffusible protein.

$$\begin{array}{rcc}
 \text{Example 3:} & F^- I^+ O^+ Z^+ Y^+ & F^+ I^+ O^C Z^+ Y^+ \\
 & \text{(inducible)} & \text{(constitutive)} \\
 & \times & \\
 & F^- I^+ O^+ Z^+ Y^+ & \\
 \hline
 & F^+ I^+ O^C Z^+ Y^+ & = \text{CONSTITUTIVE}
 \end{array}$$

O^C is **cis** dominant: NO DIFFUSIBLE PRODUCT IS INVOLVED. The operator is physically a part of the DNA. Even though one operon is inducible, the constitutive one will be producing enzymes all the time, even when the inducible one is not.

$$\begin{array}{rcc}
 \text{Example 4:} & F^- I^S O^+ Z^+ Y^+ & F^+ I^+ O^C Z^+ Y^+ \\
 & \text{(superrepressed)} & \text{(constitutive)} \\
 & \times & \\
 & F^- I^S O^+ Z^+ Y^+ & \text{MEROZYGOTE IS} \\
 \hline
 & F^+ I^+ O^C Z^+ Y^+ & \text{CONSTITUTIVE}
 \end{array}$$

O^C mutation is not sensitive to the repressor, even though I^S is a diffusible.

$$\begin{array}{r}
 \text{Example 5:} \quad F^- I^+ O^C Z^- Y^- \\
 \hline
 F^+ I^S O^+ Z^+ Y^+
 \end{array}$$

What structural genes are produced by the merozygote above?

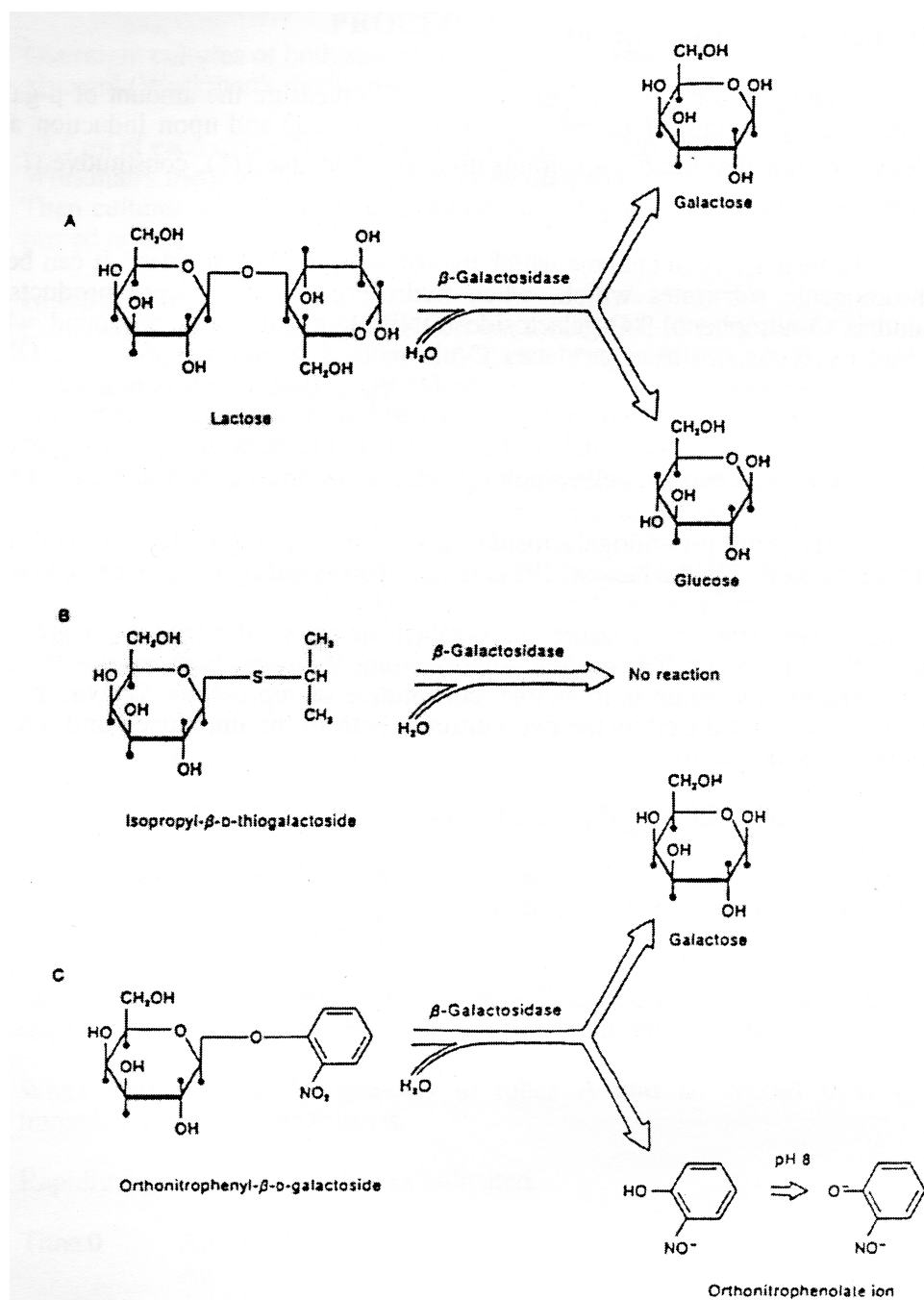


Figure 2 The reaction of β -galactosidase with different β -galactosides.

Design of the Experiment

The purpose of this experiment is to (1) measure the amount of β -galactosidase produced by a strain of *E. coli* in a noninduced state and upon induction and (2) to determine whether the *E. coli* strains used are inducible (I^+), constitutive (I^- or O^c), or repressed (I^s).

β -galactosidase is an enzyme which hydrolyzes β -D-galactosides. It can be measured with chromogenic substrates which, when hydrolyzed, yield colored products. Such a compound is O-nitrophenol- β -D-galactoside (ONPG), a colorless compound which when hydrolyzed by β -galactosidase produces O-nitrophenol, a yellow product. ONP can be measured by its absorption at 420 nm. If the ONPG concentration is in excess, the amount of ONP produced is proportional to the β -galactosidase present. The reaction is stopped by adding concentrated Na_2CO_3 , which shifts the pH to 11, at which point β -galactosidase is inactivated. Also note that the yellow color produced becomes darker in the Na_2CO_3 .

IPTG (isopropyl- β -D-thiogalactoside), a structural analog of lactose, is a more potent inducer than lactose. Unlike lactose, IPTG is not hydrolyzed by the induced enzyme.

This experiment will measure the β -galactosidase synthesized by a growing culture after the addition of IPTG. The student will determine the phenotype of two *E. coli* strains. Determine whether the strain is inducible, constitutive or repressed. Do this by comparing the amount of ONP produced by the two cultures, both in the uninduced and induced states, as a function of time.

Procedure

1. Overnight cultures of both strains were grown in minimal medium + 0.5% glycerol (Werkman's medium).
2. 12.5 mL of each overnight culture was inoculated into 250 mL warm Werkman's medium and allowed to grow on a shaker at 37°C for 2 hours. Then cultures were aliquotted (2 tubes of 10 mL of each strain per pair) and the cultures were placed on ice.
3. BEGIN HERE IN LAB.
Each pair of students should take two test tubes with 10 mL ice cold cell suspension of each *E. coli* type. **BE SURE TO TAKE TWO DIFFERENT *E. coli* TYPES.** There will be four tubes in all. Record strain numbers here and in the laboratory notebook.

Culture No. 1 _____ (referred to below as A)
Culture No. 2 _____ (referred to below as B)

4. Label the tubes with A and B and name. **Immediately** place the tubes in the shaking water bath at 37°C. Allow the cultures to grow 45 minutes.

5. While the cultures grow, prepare 18 tubes, each with 1 mL of Z buffer. Number the tubes as follows:

Tubes 1A, 1A Control, 2A, 4A, 8A, 16A, 32A, 40A, 40A Control

Tubes 1B, 1B Control, 2B, 4B, 8B, 16B, 32B, 40B, 40B Control

6. Have vortex, chloroform, SDS, timers, pipettes ready. Use a fresh pipette for each operation. Adhere to the time schedule indicated. Let the first partner do sample A tubes; the second partner B tubes.

7. At the end of the 45 minute growth period, label one of the A tubes Control A, and one of the B tubes Control B. These cultures will not be induced.

8. When ready to add the inducers to tubes A and B, record time 0. **NOW** immediately proceed as follows.

Rapidly add solutions to tubes as indicated.

Time 0 Add 1 mL of 0.01 M IPTG to tube A
 Add 1 mL of 0.01 M IPTG to tube B
 Add 1 mL sterile distilled water to Control A
 Add 1 mL sterile distilled water to Control B

All tubes remain in 37°C water bath.

Immediately after adding the inducer:

Partner 1
Vortex culture tube then
transfer 1 mL culture from
tube A to tube 1A.
Transfer 1 mL from Control A
to tube 1AC (control).

Partner 2
Vortex culture tube then
transfer 1 mL culture from
tube B to tube 1B.
Transfer 1 mL from Control
B to tube 1BC (control).

9. Immediately add four drops of chloroform + two drops of 0.1% SDS to each of these tubes. **Vortex thoroughly. Careful pipetting and thorough vortexing is very important at each step to get accurate results. Cells must be killed** and membranes disrupted to recover all of the enzyme for the assay! Keep the processed samples at room temperature.

10. At times of 2 minutes, 4 minutes, 8 minutes, 16 minutes, 32 minutes, and 40 minutes remove 1 mL of culture A and place in the appropriately labeled A tube (2A, 4A, 8A, 16A, etc.). **Also**, at times of 2 minutes, 4 minutes, 8 minutes, 16 minutes, 32 minutes, and 40 minutes remove 1 mL of culture B and place in the appropriately labeled B tube (2B, 4B, 8B, 16B, etc.)

As soon as the sample is placed in the appropriate tube, add 4 drops of chloroform and 2 drops of 0.1% SDS. Vortex the sample and store the sample at room temperature until samples from all time points are collected.

Caution: Chloroform is a volatile organic liquid. Dispense chloroform in a hood for organic solvents.

11. Allow the last tubes to remain at room temperature for 5 minutes; then vortex each tube (samples taken from 0 to 40 minutes) vigorously once more. Now add the substrate: 0.4 mL of ONPG (4 mg/mL) to each tube and shake a few times.
12. Place all tubes with samples in a 37°C water bath for 15 minutes.
13. After the 15 minute incubation, add 2 mL of 1 M Na₂CO₃ to each sample tube to stop the reaction and intensify the color.
14. All sample tubes should be clear, not cloudy. If there is cell debris in the tubes, allow it to settle before proceeding. Check with the TA to determine whether centrifugation of samples is necessary.
15. Measure the optical density of the supernatant for each sample. Set the Spectronic 20 to 420 nm, the absorption maximum of the phenolate ion. Use 0.4 mL ONPG plus 2 mL Z buffer plus 2 mL Na₂CO₃ for the blank.

Start reading tubes with the 0 time controls first. If there is a pellet on the bottom, put 4 mL of supernatant into a cuvette without disturbing the pellet and chloroform. Do this for each sample. Record the readings.

If the O.D. of the culture(s) are off scale (too high a reading), as some may well be, dilute the sample in Z buffer before continuing to read. Be sure to record the amount the sample was diluted.

Study Problems

1. What is an inducible operon?
2. What is a repressible operon?
3. What is negative control?
4. What is positive control?
5. The map of the *lac* operon is *I POZY*. The promoter (*P*) region is the site of initiation of transcription through the binding of the RNA polymerase molecule before actual mRNA

production. Mutants of the promoter (P^-) apparently cannot bind RNA polymerase molecule. Certain predictions can be made about the effect of P^- mutations. Use your predictions and your knowledge of the lactose system to complete the table below. Insert a plus where enzyme is produced and a minus where no enzyme is produced.

<u>Genotype</u>	β -galactosidase		Permease	
	No lactose	Lactose	No lactose	Lactose
$i^+p^+o^+z^-y^+/i^c p^+o^+z^+y^-$				
$i^-p^-o^c z^+y^+/i^+p^+o^+z^-y^-$				
$i^+p^+o^0 z^-y^+/i^+p^-o^+z^+y^-$				
$i^+p^-o^+z^+y^+/i^-p^+o^+z^+y^-$				
$i^s p^+o^+z^+y^+/i^-p^+o^+z^+y^+$				

6. What type of mutation could be responsible for a strain that is neither constitutive nor inducible for β -galactosidase? (i.e. the enzyme is never produced). Give two possibilities and the genotype of each strain.

7. For the following genotypes of the *lac* operon, predict whether the structural gene *Z* is constitutively expressed, permanently repressed or not made, or inducible in the presence of lactose.
 - a. $i^+o^-z^+$
 - b. $i^-o^+z^+$
 - c. $i^+o^c z^+$
 - d. $i^-o^+z^+/F' i^+$
 - e. $i^+o^c z^+/F' o^+$
 - f. $i^s o^+z^+$
 - g. $i^s o^+z^+/F' i^+$

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Materials

Recipes

Werkman's minimal medium (per liter)

Solution A= 0.5 M sodium diphosphate

Solution B= 1 M potassium monophosphate

Solution C= 0.1 M calcium chloride

Solution D=1 M magnesium sulfate

Add 100 mL Solution A to 100 mL Solution B. Add 800 mL distilled water. While stirring, add 1 mL Solution C and 1 mL Solution D and 2 g ammonium sulfate.

Adjust to pH 7.0 and autoclave.

The *E.coli* strains used are grown in Werkman's minimal media supplemented with 0.5% glycerol and 0.5 mL of 1% B1 (Thiamine chloride) per liter.

Nutrient Broth (per liter):

1. Into a 2 liter beaker place 3 g beef extract, 5 g peptone, 500 mL distilled water
2. Gently heat (stir) until dissolved (will be colored).
3. Bring the volume to 1000 mL with distilled water.
4. Adjust the pH to 7.0 using 0.1N NaOH or 0.1M HCl as needed.

Nutrient Agar:

Prepare exactly like nutrient broth BUT in step 1 also add 15 g of bactoagar.

Notes for the Instructor: β -galactosidase Induction in *E. coli*

I. Preparation of *E. coli* strains for induction.

We will use three different strains of *E. coli* for this experiment. The strains to be used are:

E. coli 1 constitutive strain ($lacI^-$ mutant)

E. coli 2 $lacZ^-$ repressed strain

E. coli 3 wild type inducible strain

1. Streak freezer stock strains onto nutrient agar plates. Grow overnight at 37°C. Check for growth of strains on X-gal plate. See Appendix C for details.
2. Pick single isolated colonies of each strain and inoculate into 150 mL Nutrient Broth in a 500 mL flask. Grow overnight at 37°C on shakers. These cultures will be used as the stocks for the prerun and for inoculating fresh overnights for classes.
3. The **night before** the experiment it will be necessary to prepare fresh overnight cultures of each strain to be used as inoculum for log phase cultures the next day. Inoculate 1.5 mL of the stock culture into 150 mL Werkman's with glycerol in a 500 mL flask. (Use more if slow growing.) Grow overnight at 37°C on shakers. Prepare one fresh overnight of each strain for each day needed.
4. **1 1/2-2 hours before lab:** To prepare log phase culture inoculate 12.5 mL of saturated overnight culture into 250 mL Werkman's with glycerol in 500 mL flasks **1 1/2-2 hours** before sections begin. Grow at 37°C on shakers until culture is moderately turbid.

For a class of 20 students working in pairs, inoculate two flasks of wild type, one flask of I^- and one flask of Z^- . Be sure to inoculate into flasks containing 250 mL Werkman's media to have enough culture for 20 students.

5. At the time of inoculation, also remove two flasks ONPG and two flasks IPTG from freezer and allow these to thaw on ice.

II. Solutions for β -galactosidase Induction in *E. coli*

isopropyl- β -D-thiogalactoside

IPTG

Make up a 0.01 M solution using sterile distilled water.

- Each pair needs 2 mL. Make 35 mL for 20 students, so there will be a bit extra.
- Store in freezer.
- Wrap in Aluminum foil! Must be stored in dark.

O-nitrophenol- β -D-galactoside

ONPG

Make up a 4.0 mg/mL solution in 0.1 M Na-Phosphate buffer, pH = 7.0.

- Each pair needs 8.0 mL-- make 100 mL per section
- Store in freezer.
- Wrap in Aluminum foil! Must be stored in dark.

- 0.1 M NaPO₄ Buffer preparation (prepare 2 liters).
 - Mix equimolar solutions of:
- | | |
|---|---------|
| 0.1 M Na ₂ HPO ₄ (FW141.96 anhydrous) | 610 mL |
| 0.1 M NaH ₂ PO ₄ (FW137.99 monohydrous) | 390 mL |
| | 1 liter |

Adjust pH to 7, then add 4.0 g ONPG--need not be sterile.

Z buffer (per liter)

16.1 g Na₂HPO₄·7H₂O (0.06 M)
 5.5 g NaH₂PO₄·H₂O (0.04 M)
 0.75 g KCl (0.01 M)
 0.246 g MgSO₄·7H₂O (0.001 M)
 2.7 mL Beta-mercaptoethanol (0.05 M)
 Do not autoclave. Adjust pH to 7.0

Each pair needs 18 mL of Z buffer plus extra for possible dilutions, so prepare 200 mL per 20 students. It is helpful to use dispensers set at 1 mL for this. Keep Z buffer in the hood to minimize the beta-mercaptoethanol exposure.

Chloroform Dispense chloroform into dropper bottles. Have 1 dropper bottle per pair. Have extra chloroform available for class to refill dropper bottles as necessary. Do this in a hood for organics.

0.1% SDS Make up a 0.1% solution SDS. Have one dropper bottle full per pair. Have extra SDS solution available to refill as necessary.

1.0M Na₂CO₃ sodium carbonate, autoclave to sterilize. Each pair needs 36 mL.

sterile water 3 mL in test tubes, 1 tube per pair.

III. Lab Setup for β-galactosidase induction in *E. coli*

Have the following setup and available:

Several beakers and a canister of 1 mL sterile pipettes per group.

Pipette aid for 1 mL pipettes

Spectronic 20s setup with test tube rack, 3 cuvettes, tissues such as Shur-wipes, discard beaker, and wash bottle beside each

Waterbath set at 37°C

Vortex mixers—one per group.

Labeled dropper bottles for chloroform—one per group.

Labeled dropper bottles for SDS—one per group.

Acknowledgments: Many thanks to the thousands of students in Purdue University's Biology 242 who have performed this experiment over the years. May their intellectual curiosity continue to drive modifications of the teaching laboratory exercises.

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Susan J. Karcher received her B.A. in Biochemistry from the University of Wisconsin, Madison in 1972, and her M.S. in 1975, and her Ph.D. in 1979 in Chemistry from the University of California at San Diego. She did post-doctoral work at the University of Washington, Genetics Department. Susan is a tenured assistant professor in the Department of Biological Sciences at Purdue University, where she teaches introductory and upper level genetics and molecular biology laboratory courses and an upper level human genetics lecture course. She hosted the 1995 and 2006 ABLE workshops/conferences and was the editor of the ABLE Proceedings, Volumes 19 to 22. She has presented other major workshops for ABLE on topics including non-radioactive Southern blots, Blue Plants, and PCR.

Kristene L. Henne received her A.A.S. in Radiography from Kankakee Community College in 1995, her B.S. in Biology from Governors State University in 2000, and her Ph.D. from the Department of Biological Sciences at Purdue University in January 2008. At each stage in her career, she taught in some capacity, either as a teaching assistant in a traditional biology laboratory setting or working directly with Radiography students on the hospital floor. She is currently a post-doctoral research associate at St. Jude Children's Research Hospital in Memphis, TN, where she works on the characterization of protein interactions within the toll-like receptor pathway. Kristene believes passionately in the importance of obtaining a broad undergraduate education with a strong understanding of the basics of science and hopes to continue playing a role in education in the future.

Deborah A. Anderson received her B.A. in Biology from Wesleyan University in 1977, and her M.S. in Biology from Purdue University in 1980. She worked as an electron microscopist, in the School of Veterinary Medicine at Purdue University from 1983 to 2001. In 2001 she became an Instructional Coordinator in the Department of Biological Sciences at Purdue University where she runs the freshman and sophomore biology majors laboratory courses.

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Appendix A Answers to Study Problems

1. An inducible operon is under the control of a regulatory molecule or an inducer which acts to block a repressor. By blocking the repressor, transcription can proceed.
2. A repressible operon is shut off and doesn't allow transcription to occur when certain proteins or metabolites are present.
3. Negative control results when the presence of a repressor molecule prevents transcription. The repressor binds to the operator to turn the operon off. When the repressor is absent, the operon is on.
4. Positive control results when an effector or activator molecule initiates transcription. The inability of the effector to bind to the promoter or its absence will turn the operon off.

5.

	<u>β-galactosidase</u>		<u>Permease</u>	
	No lactose	Lactose	No lactose	Lactose
a.	-	+	-	+
b.	-	-	+	+
c.	+	+	-	-
d.	-	-	-	-
e.	-	-	-	-
f.	-	+	-	-
g.	-	-	-	-

6.

	<u>Presence</u>	<u>Absence</u>
	+	-
	-	-
	-	-
	+	+
	+	-
	+	+

7. A mutation in the promoter region of the operator, which prevents polymerase from binding, will result in no enzyme production. The genotype for this is $i^+o^z^+y^+a^+$. An alteration in the CAP-cAMP binding site will also prevent polymerase binding and enzyme production. The genotype for this is i^+p^-

$o^+z^+y^+a^+$. A mutation in the repressor molecule that alters the affinity for the inducer will also prevent enzyme production. In this case, the repressor molecule can not dissociate from the operator region.

The genotype is $i^s o^+z^+y^+a^+$.

8. a. constitutive
- b. constitutive
- c. constitutive
- d. inducible
- e. constitutive
- f. repressed
- g. repressed

Appendix B Sources of strains

Sources of *E. coli* strains for this exercise:

The three strains used in this experiment may be obtained from the Susan Karcher or purchased from sources listed.

1. Any wild type *E. coli*
2. a *lacZ*- *E. coli* strain
3. a constitutive *E. coli lacZ* strain (that is, I^- , repressor minus)

Alternatively, strains may be purchased from the *E. coli* Genetic Stock Center at Yale University (<http://cgsc2.biology.yale.edu/>); The American Type Culture Collection (<http://www.atcc.org/>); Ward's (http://www.wardsci.com/category.asp_Q_c_E_620); or Carolina Biological (<http://www.carolina.com/>).

Appendix C Storing Bacterial Strains

To store bacterial strains indefinitely -70°C is recommended.

The following procedures describe how to prepare bacteria for long term storage (called permanents of bacterial strains). This protocol makes frozen glycerols, to be stored at -20°C or -70°C :

1. Grow an inoculum of the bacterial strain overnight in 1 mL L-broth with antibiotics if applicable.
2. Add 1 mL of a saturated culture of bacteria to 2 mL of sterile glycerol in a small screw-capped vial. Mix thoroughly and freeze in a dry ice-ethanol bath. Store at -20°C or -70°C .

To check that the phenotype of the *E. coli* strain is correct, streak the cultures on an L agar plate containing X-gal. Incubate the plates at 37°C . Streak for single colonies and check that the *lacI*- (constitutive) strain produces deep blue colonies on L x-gal plates. The wild type *E. coli* strain will give colonies that show more blue with longer incubation times. The *lacZ*- strain will have white colonies.

L-broth (also called Luria or L-B -- Luria-Bertani) per liter:

10 g tryptone

5 g yeast extract

5 g sodium chloride

Add water to almost 1 liter. Adjust to pH 7 with sodium hydroxide. Bring volume to 1 liter with water.

For L agar plates: add 15g bacto agar per liter

Autoclave 20 minutes on liquid cycle. Allow the autoclaved L agar to cool to about 45°C before adding X-gal and then pouring plates (about 20 mL/100-mm Petri plate). X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) is added to a final concentration of 80 μg/mL, dissolved in dimethylformamide.