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

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Abstract: 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 workshop describes a laboratory exercise using *E.coli* strains with different mutations in the *lac* operon to demonstrate to students the regulation of beta-galactosidase production in *E. coli*. 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. In addition, we will focus on the historical background and practical applications of the *lac* operon.

Introduction

Note to the student: β-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 can 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 sight 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 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, deCrombrugghe 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 straight forward. 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.

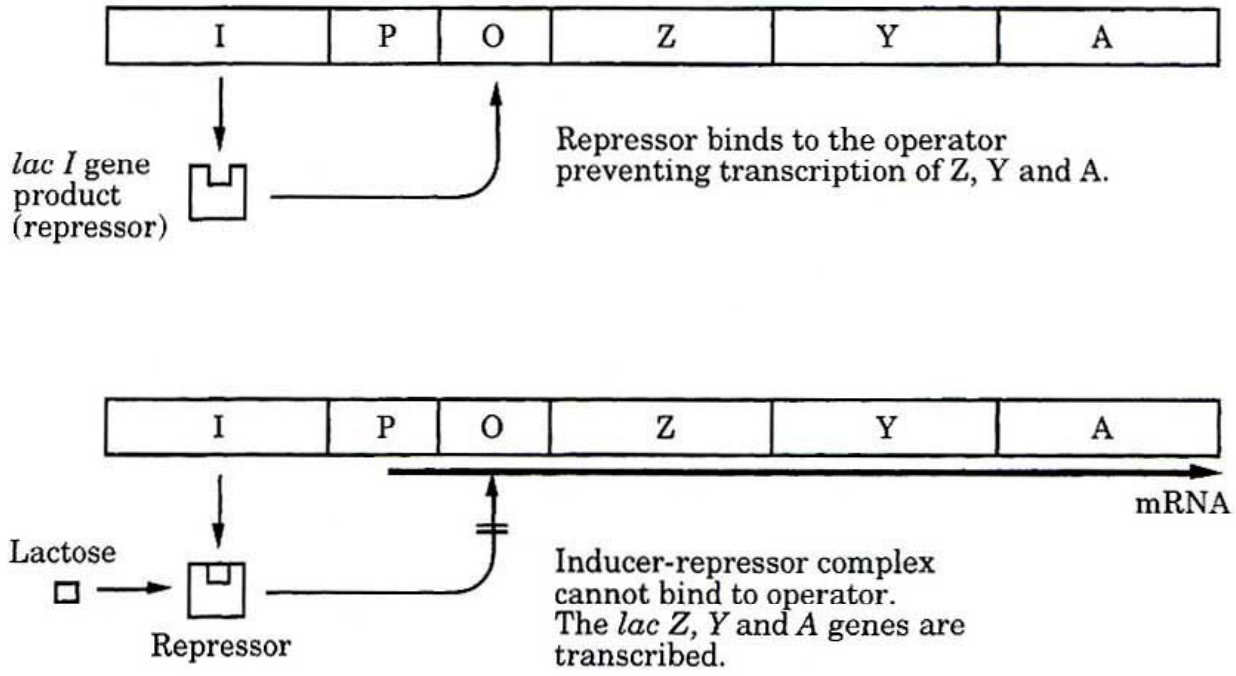

Figure 1. A schematic of the *lac* operon showing all the key elements. The letters refer to the following; O = the operator; P = the promoter; I = the gene coding for the repressor protein; and Z, Y, A = the structural genes.

β-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.

β-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-galactosidase 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-galactosidase 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 be 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.

β-Galactosidase ONPG Assay

1. Grow overnight cultures of *lac*+ and *lac*- strains in minimal medium + 0.5% glycerol (Werkman's minimal medium).
2. Inoculate 12.5 ml of each overnight culture into 250 ml warm Werkman's medium. Grow on a shaker at 37°C for 2 hours. After 2 hours, place 10 ml of each culture into 2 tubes each. Place the 4 tubes on ice.
3. Take two test tubes with 10 ml ice-cold cell suspension of each *E. coli* type.
4. While your cultures grow, prepare tubes each with 1 ml of Z buffer.
5. Have vortex, chloroform, SDS, timers, pipettes ready. Use a fresh pipette for each operation.
6. At the end of the 45-min. growth period, label one of the culture tubes "Control," or "IPTG-".
7. When you are ready to add the inducer to the culture tube (labeled "IPTG+") record time 0, and immediately proceed as follows.
    - Add 1 mL of 0.01 M IPTG to the "IPTG+" culture tube.
    - Add 1 mL sterile distilled water to "IPTG-" culture tube.
    - All tubes remain in 37°C water bath.
    - **Immediately after** adding the inducer:
        - Vortex culture tube, then transfer 1 mL of culture from the IPTG plus culture tube into a time zero culture tube.
        - Do the same for the IPTG minus culture tube.
        - Immediately add four drops of chloroform + two drops of 0.1% SDS to each of these tubes. **Vortex thoroughly**. Place the tubes at room temperature.
8. Take samples from induced and uninduced cultures at noted times up to about 45 minutes.
9. Allow the last tubes to remain at room temperature for 5 minutes; then vortex each tube (0' through 40') vigorously once more.
    - Now add the substrate: 0.4 mL of ONPG (4.0 mg/mL solution in 0.1 M Na-Phosphate buffer, pH = 7.0.) to each tube and shake a few times.
    - Place all tubes with samples in a 37°C water bath for 15 minutes.

10. After the 15' incubation, add 2 mL of 1 M Na_2CO_3 to each sample tube to stop the reaction and intensify the color.
11. All sample tubes should be clear, not cloudy. If there is cell debris in the tubes, allow it to settle before proceeding.
12. Measure the optical density of the supernatant for each sample. Set a Spectronic 20 to 420 nm. This is the absorption maximum of the phenolate ion. Use 0.4 mL ONPG plus 2 mL Z buffer plus 2 mL Na_2CO_3 for the blank. Start reading tubes with 0 controls. Record your readings.

Recipes

Werkman's minimal medium	**Z buffer** (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.	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

For detailed protocols, please contact the authors.

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