

Chapter 8

A Discovery Laboratory Investigating Bacterial Gene Regulation

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Introduction

This laboratory exercise introduces students to the difficult concept of gene regulation, as well as to experimental design and the design of “controls”. During the first week, students use various sugars and an enzyme assay for beta-galactosidase to identify three unknown strains of *E. coli* bacteria: a wild type strain, one lacking the repressor (*I*) and one lacking the beta-galactosidase gene (*Z*). The students start with the enzyme assays first, then discuss the assays and the theory behind the experiment during the first 85 minute incubation step. The entire first week of the exercise can be done in approximately 2.5 hours.

After completing the first experiment, students design their own experiments with controls to study various aspects of the *lac* operon. Their experimental procedures must be discussed with the laboratory instructor before the procedures are performed.

During the second laboratory period, students perform their own experiments, and collect and analyze their data. During the final period, each group of students gives a 5-10 minute presentation on their experimental design and results.

Materials

Shaking incubator or water bath

3 sterile 250 ml flasks

Culture of wild type *E. coli*, strain K12, 6 ml per group: Carolina Biological, #D8-15-5067, or ATCC #23716. Phone 800-638-6597

Culture of *E. coli lac*⁻; 6 ml per group: Carolina Biological, #D8-15-5080, or ATCC #23735. Phone 800-638-6597

Culture of *E. coli lac I*⁻; 6 ml per group: ATCC, #15224. Phone 800-638-6597

Note To Instructor: Label each of the three strains with a letter only, such as A, B, or C. You may grow overnight cultures up to 3 days before use. *Add about 20 ml of fresh medium to each overnight culture and pre-incubate 30-60' prior to use.*

Three 25 ml bottles sterile 5% glucose

One 25 ml bottle sterile 5% sucrose

Three 25 ml bottles sterile 5% lactose

8 ml 1% ONPG per pair students (ortho-nitrophenyl-beta-D-galactoside, Sigma #N1127), a substrate for beta galactosidase; the products are colored.) Prepare in 50 mM sodium

phosphate buffer. pH 7.

1 ml per pair of students, 1% TMG (thiomethylgalactoside, Sigma #M8146), a "super-inducer", which binds to the repressor, and inactivates it.

Shaking water bath at 35-37°C

Vortexes

3 dropper bottles of 1% Sarkosyl detergent (Sigma #L9150). This solubilizes the cell membrane, to allow the ONPG into the cell.

Parafilm

M9 + glycerol medium; 30 ml per group.

Per liter: Mix in a 2 liter flask:

950 ml water

4g glycerol

10 g M9 minimal salts (Sigma #M6030)

0.2 g yeast extract (Sigma #Y4250)

Stir to dissolve; check that the pH is 7.0 - 8.0. Adjust with NaOH or HCl if necessary.

Autoclave.

18 test tubes (per group) that fit the Spectronic-20 [13 X 100 mm].

Spectrophotometer, 1 per 4 students.

Aliquots of M9 + glycerol medium adjusted to each of the following pHs: 4, 5, 6, 7, 8, 9, 9.5

Student Outline:

Introduction:

Bacteria have evolved for efficiency, and for adaptation. Environmental factors may alter a cell's physiology, by temporarily turning gene systems on or off. The *lac* operon in *E. coli* is one of the simplest and best examples of such gene regulation. In this operon, lactose induces the synthesis of the enzymes involved in its breakdown, particularly beta-galactosidase. This enzyme catalyzes the following reaction (Figure 8.1):

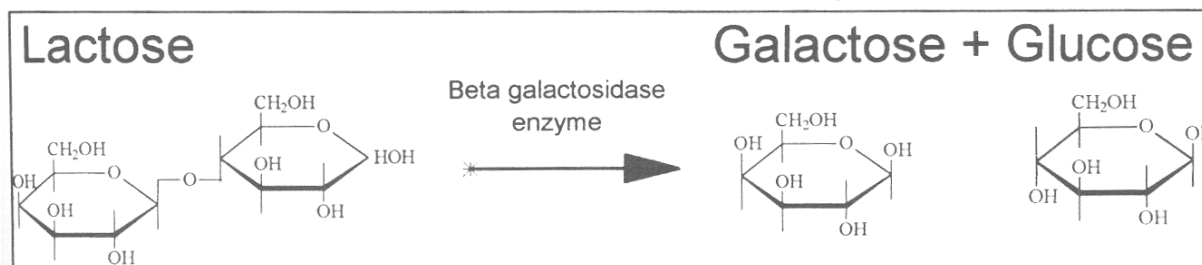


Figure 1: Action of beta-galactosidase

Figure 8.1. Action of beta-galactosidase.

In the absence of lactose, these enzymes are not required, and thus are not synthesized, as it would be inefficient to do so. The primary regulator of the *lac* operon is a "negative control element" called the *lac* repressor. This protein is a negative regulator because when it binds to a gene, it turns the expression of that gene off. (See Figure 8.2.)

When lactose is present in the cell, the repressor binds the sugar and there is a conformational change that inactivates the repressor (the repressor can no longer bind the operator region of the promoter). Under these conditions, the genes of the lactose operon are expressed, and the enzymes required to utilize the lactose are synthesized. This process, whereby an environmental factor turns on the expression of a gene, is called gene or transcriptional *induction*.

The repressor of the lactose operon is coded for by the *lac I gene*. The repressor binds to the operator area, *O*, and blocks the attachment of the RNA polymerase to the promoter site, *P*. RNA polymerase must attach to the promoter site and move through the operator site if the lactose operon is to be transcribed. Thus, for our purposes we will consider the repressor an "on/off switch", whereby when the repressor is bound to the DNA the gene is switched OFF, and when repressor is not bound, it is ON and is transcribed.

The regulation of the lactose operon is not only under the negative control of the repressor, but also under positive control of another protein called the *catabolite activator protein [CAP]*. In the *absence of glucose*, CAP binds to the promoter site, and increases the level of transcription by helping RNA polymerase to bind. In the presence of glucose, CAP fails to bind, and transcription remains low.

I like to use the analogy of a stereo amplifier. The repressor is the "on/off" switch. Repressor binding turns the amp off, and removal of the repressor turns it on. The CAP protein is the volume control. When CAP binds, the volume [rate of transcription] is turned up, assuming that is, that the main switch is ON. If the amp is off, changing the volume has no effect.

This level of regulation is for efficiency. If there is already sufficient glucose in the cell, why invest the energy to manufacture enzymes to create more glucose? Glucose is the preferred energy source, so if it's available, the cell does not need to break down lactose.

The amount of enzyme produced by the *LacZ* gene under various conditions will be measured. In order to do so, we need to be able to measure one of the products of the enzyme's reaction as it is made. Unfortunately, we have no easy assay for glucose or galactose. However, beta-galactosidase will cleave other similar molecules, such as ONPG. One of the cleavage products created when the enzyme operates on ONPG is bright yellow, and thus can be seen easily.

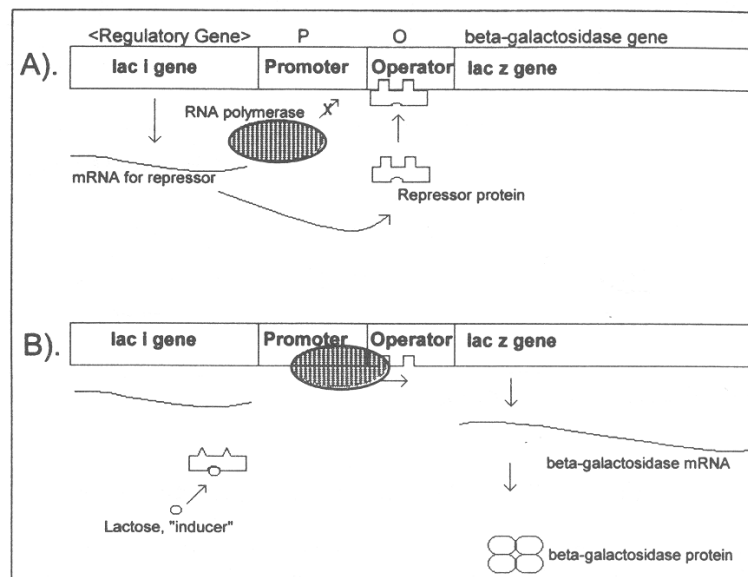


Figure 2: A). Lac operon in the absence of lactose. The repressor is bound to the operator, preventing the RNA polymerase from initiating transcription. B). In the presence of lactose, the sugar binds to the repressor, causing a change in the repressor, which now has a very low affinity for the operator. Without the repressor bound to the operator, polymerase is free to transcribe the *lac z* gene.

Figure 8.2. A). *Lac* operon in the absence of lactose. The repressor is bound to the operator, preventing the RNA polymerase from initiating transcription. B). In the presence of lactose, the sugar binds to the repressor, causing a change in the repressor, which now has a very low affinity for the operator. Without the repressor bound to the operator, polymerase is free to transcribe the *lacZ* gene.

Procedure: First Week

[Things that are not needed for the first week of the exercise, but that you may need for your own experiment are in brackets]

1. Start the experiment at the beginning of the lab period, and discuss the theory behind it during the first 85 minute incubation period.

You will be given cultures of each of three bacterial strains, marked A, B, and C. From the results of this exercise you determine which corresponds to “wild type”, Z^- , and I^- .

2. Add 1.5 ml of glycerol medium to each of 12 small tubes. Use the same 5 ml pipette or P5000 tip to add the medium to all tubes. You need not worry about sterile technique, since the bacteria will not be grown for very long.
3. *Make sure all markings are near the top of the tube, directly on the glass with felt markers provided.*

-Mark all the tubes with your initials.

-Mark 4 tubes with each bacterial strain (four labeled ‘A’, four ‘B’, and four ‘C’)

-Within each lettered strain, mark the tubes as follows:

-Control

-Lac

-TMG

-Glucose.

4. Transfer 0.6 ml of the appropriate bacterial overnight culture to each of the tubes. *Measure as accurately as possible.* Use a separate pipette for each bacterial culture!! Make sure you swirl or mix the culture each time before removing cells, since the bacteria will quickly begin to settle to the bottom.
5. Add 150 microliters (0.15 ml) of the appropriate sugar or TMG to each tube; add distilled water to the CONTROL tubes.
6. Incubate the tubes at 37°C in a shaking water bath for 85 minutes. *The length of this incubation time is important. Do not cut back on this 85 minutes!*
7. [If you’ll be taking quantitative measurements of enzyme for your own experiment, you’ll have to measure the relative amount of bacteria in each tube by taking an O.D. Cover each tube with parafilm, quickly invert to mix, and then immediately place in a Spec-20 and record the absorbance at 550 nm wavelength. The higher the concentration of bacteria in the tube, the higher the absorbance reading will be.]
8. To lyse the cells, add one drop of Sarkosyl detergent to each tube. Briefly vortex each tube at low speed to mix. *Be sure the tube is not pointed toward anyone. Be sure the speed is low enough that there is no spillage from the tube.*

9. Add 0.8 ml 1% ONPG to each tube, and mix each tube again.
10. Incubate for at least 15 minutes at 37 °C, until a rich yellow color develops in some of the tubes. [If you're doing a "time course" (following the reaction over time) you need to terminate the reaction at specific times. Add 1 ml of 1M Na₂CO₃ to stop the reaction.]
11. Place the tubes in front of a white background, and determine the relative density of color in each tube. This is most easily done by grouping the tubes by color, and then recording the colors, such as "slightly yellow", "yellow", "dark yellow", etc. [IF you wish, you can be more quantitative, by testing the density of the color in each tube using the spectrophotometer set at 420 nm. Remember to correct your readings for the different densities of bacteria in each tube.]

Second Week: Design Your Own Experiment:

After having completed the enzyme assays once, design an experiment to answer a question about the regulation of the lac operon. In addition to the reagents included in the first week, the following will be made available:

- Spectrophotometers
- Growth media at pH 4, 5, 6, 7, 8, 9, 9.5
- Glucose, fructose, maltose, and sucrose sugars [Note: If you wish to try combinations of glucose and lactose in the same sample, the glucose must be added 20 minutes prior to the lactose.]
- Growth medium with only half the amount of glycerol
- Incubators at various temperatures, including 4, 37, and 100 degrees C, plus room temperature
- You may design experiments requiring other reagents or equipment; every effort will be made to accommodate your needs.

Note: Be sure you have both positive and negative controls in your experiment. Ask your instructor if you're unsure how to design controls.

Some possible questions you might want to address [you aren't limited to these]:

- How specific is the lac repressor for lactose?
- How does glucose effect the gene induction?
 - What concentration of lactose is required to induce the expression of beta galactosidase? [Add 0.15 ml lactose at concentrations of 0 to 2%; plus 5% as your "positive control". Choose at least 6 concentrations in all]
- How much better is TMG at inducing the lac operon than is lactose [Choose at least 5 TMG concentrations from 0 to 0.5%, plus 1% as your positive control; also include lactose tubes as described above]?
- How long does it take for the cell to respond to new lactose in its environment? [Make sure you take time points out to 85 min.; incubate ONPG for 30 min.]
- How does temperature affect the enzyme reaction rate? [Make sure you include at least 6 temperatures, including 0, 37, and 100 degree points; also make sure you create a

"control" tube for each temperature, such as one with the lac z- bacteria, or without the inducer]

- How does temperature affect the rate of production of the enzyme [make sure you include at least 6 temperatures, including 0, 34, 37, and 41 degrees C.]?
- How is the enzyme reaction rate affected by the concentration of substrate [ONPG]?

You must present your idea, along with procedures and a list of materials needed, to your instructor as soon as possible, either in writing, over email, phone, or in person before performing your experiment.

In your final lab report, do not include your procedures for the first week's lab, but be sure to include your own procedures in detail for the second lab. **Your procedures should be detailed enough so that another person could reproduce your experiment exactly as you did it. If appropriate, graph your results.**

Also, be prepared to present your results to the class the week after you do the experiment. You'll have 5-10 minutes; include any relevant tables or graphs.

QUESTIONS [Include the answers in your write-up]:

1. What is an operon?
2. Explain the difference between positive and negative gene regulators. Which are present in the lac operon?
3. What is the role of each of the following molecules in this lab: TMG, lactose, glucose, ONPG, Sarkosyl, repressor, CAP?
4. Explain what happens, *on the molecular level [what's happening at the operon]*, when bacteria are growing in a medium with lactose? with glucose? with both?
5. Of what advantage to a cell is the negative control of the lac operon?
6. Of what advantage to a cell is CAP?

Appendix A: Instructor Notes On Student Designed Experiments

Do *not* give these notes to the students. Refer to these notes when going over the students' experimental designs with them.

1. How specific is the lac repressor for lactose? Student should test various disaccharides, including lactose, maltose, sucrose. They should find very little induction by other disaccharides. Negative control = no sugar; positive = lactose.
2. How does glucose effect the induction of the lac operon? If glucose is added, cAMP levels should drop, as should transcription of the operon. However, in order to see a significant effect, glucose must be added at least 20 minutes prior to the lactose. Negative control = no glucose [thus no effect], positive control = glucose only; no lactose [maximum effect].
3. What concentration of lactose is required to induce? Choose at least 5 concentrations between 0 [negative control] and 2%, plus 5% as a positive control. Add 0.15 ml lactose at the appropriate concentration. We typically see an increase in enzyme production until a

peak at about 1%, followed by a fall-off. I have not found an explanation for the fall off in the literature, but have hypothesized that as more of the lactose is cleaved, the resulting monosaccharides reduce the cAMP levels, and thus transcription. In fact, when a TMG inducer is substituted for lactose, no such drop off is seen.

4. How much better is TMG at inducing than lactose? See note 3 for lactose section; also choose TMG concentrations from 0 to 0.5%, plus 1% as a positive control.
5. How long does it take for the cell to respond to new lactose in its environment? Make sure you take time points out to 85 minutes. Negative control = 0 minutes.
6. How does temperature alter the enzyme reaction rate? Make two tubes for each temperature: experimental, and negative control [either lac Z- bacteria, or without inducer]. Since the first part of the experiment is to make as much enzyme as possible, use the repressor minus strain. All tubes are incubated at 37 degrees for the first 85 minute incubation. After the addition of sarkosyl, each pair of tubes is pre-incubated at the appropriate temperature for 2 minutes, followed by the addition of ONPG to start the enzymatic reaction. Timing for the second reaction begins with ONPG addition. Make sure you include at least 6 temperatures, including 0, 37, and 100 degree points. This is an interesting experiment! The curve peaks at 37 degrees, falls off, and then starts rising again at about 65 degrees! Were it not for the control tubes, students would conclude that the enzyme is quite active at 100 degrees! With the control, many of the students can reach the conclusion that the ONPG hydrolyzes on its own at high temperatures; the color is not an indication of enzyme activity under those conditions.
7. How does temperature affect the rate of production of the enzyme? Make two tubes for each temperature: experimental, and negative control [either lac z- bacteria, or without inducer]. The experimental tubes must be wild type [K12], so that enzyme production can be induced at the appropriate temperatures. In this experiment, temperature is varied during the first incubation, when the enzyme is being produced. Pairs of tubes should be “pre-incubated” at the appropriate temperatures for 2 minutes prior to adding the inducer [it’s best to use TMG as the inducer]. Timing begins when the inducer is added. After lysing the cells and adding ONPG, all tubes are incubated at 37 degrees for the final incubation, to measure the amount of enzyme present. Students should include at least 6 temperatures, including 0, 34, 37, and 41 degrees C.

Appendix B: Answers to Questions

1. What is an operon? A set of genes that are regulated together, to perform a certain function.
2. Explain the difference between positive and negative regulators. Which are present in the lac operon? A *negative* regulator is one that turns transcription *off* when it binds. A *positive* regulator turns transcription *on*, or *up*, when it binds. The lac operon has both; the repressor is a negative regulator; CAP is a positive regulatory system.
3. What is the role of each of the following molecules in this lab: TMG, lactose, glucose, ONPG, Sarkosyl?

TMG is an inducer [or "superinducer"], which removes the repressor from the operator.

Lactose is the standard "inducer".

Glucose, when it is present, stops CAP from binding, and thus decreases transcription.

ONPG is the indicator, a molecule which turns yellow when cleaved by the enzyme. Thus, the amount of ONPG cleaved is a way to measure the amount of enzyme produced.

Sarkosyl breaks open the cell, so the enzyme and the ONPG may mix.

4. Explain what happens, on the molecular level, when bacteria are grown in a medium with lactose, with glucose, and with both.

When cells are grown in lactose, lactose removes the repressor, and the enzyme is made.

When the cells are grown in glucose, and there is no lactose, the repressor remains bound; in addition, the glucose causes the CAP not to bind; thus there is no transcription.

When cells are grown in both, the lactose removes the repressor, but the CAP does not bind. Thus, there is transcription, but less than in lactose alone.

5. Of what advantage to a cell is negative control of the *lac* operon? With negative control, cells only make enzyme in presence of lactose. Thus, the cell with negative control is more efficient.
6. Of what advantage to a cell is *CAP*? Enzyme production is decreased if glucose is already present. Since glucose is the preferred energy source, the cell does not spend energy breaking down lactose if glucose is present.

K12 is wild type, and will manufacture enzyme when induced by lactose or TMG.

lacI⁻ cannot make the repressor, and thus manufactures enzyme in large quantities under all conditions.

lacZ⁻ cannot make the enzyme itself, so makes no enzyme under any condition.