

Induction of Enzyme Activity in Bacteria: The Lac Operon

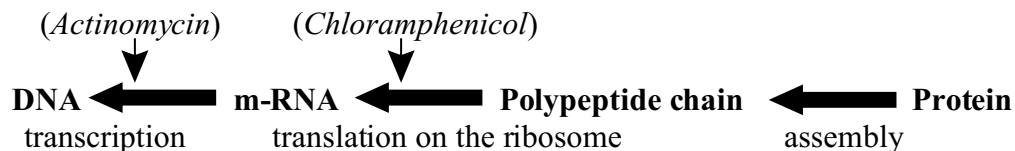
Preparation for Laboratory: Web Tutorial 5 - Lac Operon

Additional background: Freeman "Central Dogma" 227-228, CD 13.1, 13.2

I. Background:

For the last two weeks you have explored the functioning of the enzyme β -galactosidase derived from the bacterium *Escherichia coli*. In *E. coli*, this enzyme is an important catalyst in the breakdown of the 12 carbon sugar lactose into two 6 carbon sugars, glucose and galactose. The breakdown of lactose provides an important source of energy for the bacteria. When *E. coli* are grown in a medium lacking lactose, β -galactosidase is either absent or present only in minute quantities. If lactose is added to the growth medium there is a gradual accumulation of β -galactosidase within the bacterium. This week we will investigate the mechanism by which *E. coli* regulates the intracellular appearance of β -galactosidase.

The production of β -galactosidase is genetically controlled through the pathway shown below:



Many genetically controlled systems are energetically conservative. They remain in an "off" state unless the enzyme is required for a cellular process. Systems of this type are used when a relative slow response is adequate. Other genetically controlled systems are capable of a more rapid response and are in an "on" state unless switched off. **What is the advantage of keeping a system turned off most of the time?**

The production of β -galactosidase involves a number of steps. Each one of these can be probed to try to determine how the cell regulates production. In this laboratory we will try to distinguish between two hypotheses.

Hypothesis 1: Lactose stimulates the synthesis of β -galactosidase by activating the DNA-RNA-protein pathway. This hypothesis suggests that the enzyme is not present, or is present in only very low concentration, in the bacterial cells until lactose is added to the medium. Therefore, the amount of β -galactosidase present in the cell will depend on how long the DNA-protein pathway is active (i.e., it is time dependant).

Hypothesis 2: *E. coli* contains a form of the enzyme β -galactosidase which is present all the time but is inactive in its current configuration. The presence of lactose serves to activate the enzyme molecule. According to this hypothesis, lactose has no influence on the **rate** of β -galactosidase synthesis, but rather serves as an activator for the already present enzyme molecule.

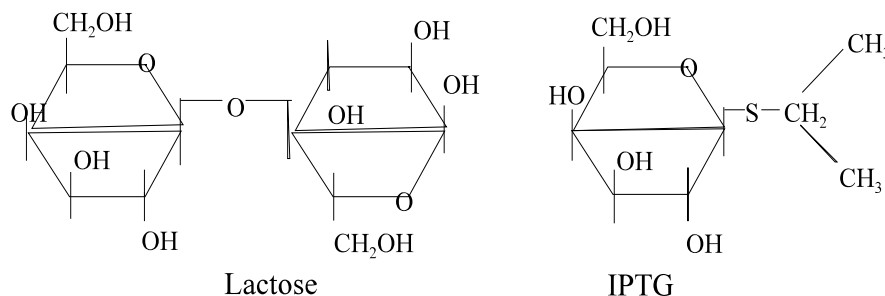
II. Experimental Design:

To differentiate between these two hypotheses we will stimulate the production/activation of β -galactosidase, use an antibiotic at successive time intervals to block protein synthesis, and assay for the amount of β -galactosidase present.

A. Stimulation of the Bacteria:

When bacteria are growing on lactose, the lactose in the medium is eventually used up and the level of intracellular β -galactosidase drops. Our experimental design depends on having a constant level of lactose (the activator). Fortunately, there are several lactose **analogs** or "look-alikes" that also cause the production of β -galactosidase, but unlike lactose they are not broken down by the enzyme. One such molecule is isopropyl- β -D-thiogalactoside (**IPTG**). Its structure and that of lactose are shown below. Note the regions of similarity.

B. Regulation of the DNA pathway:



In this experiment we will use the antibiotic **chloramphenicol** to prevent translation of the message encoded in the m-RNA. Chloramphenicol prevents m-RNA from attaching to the ribosomes. Once chloramphenicol is added to the bacterial culture no additional β -galactosidase will be produced, but any already present will continue to function normally.

Note $\rightarrow \rightarrow$ **1.** If β -galactosidase is produced via the DNA - m-RNA - protein pathway **then** the amount of β -galactosidase in the *E. coli* culture will depend on how long the pathway is active before chloramphenicol is added (i.e., show a time dependency).

$\rightarrow \rightarrow$ **2.** If an inactive form of β -galactosidase is always present **then** the chloramphenicol should have no effect on the amount of β -galactosidase present.

C. The Assay:

As in last week's lab, the assay for the presence of functional β -galactosidase will involve the conversion of colorless **ONPG** (ortho-nitro-pheny-galactosidase) into the yellow compound **ONP** (ortho-nitrophenol).

D. Experimental Cultures:

All of the cultures you set up in your experiment will have the same basic ingredients, including a nutrient medium for growth of the bacteria (tryptone broth, TB), a stimulator (IPTG), a protein synthesis blocker (chloramphenicol) and an inoculum of cells (*E. coli*). Use care in setting up the cultures. The timing of chloramphenicol addition is critical to the success of the experiment.

Setting up cultures: Do not let the caps from culture bottles or tubes touch your hands, clothing or desk top. Do not leave any culture bottle or tube open for any longer than necessary while making a transfer. **Wear gloves.** The required proportions are given in Table 1 below.

1. Place the proper amount of **TB** in a large test tube then add the **IPTG**.
2. Add **chloramphenicol only** to the tubes that get it at **T = 0**.
CAUTION: chloramphenicol **is not added** to tubes 6, 7 and 8 until they have been incubated for 15, 30, or 45 minutes respectively.
3. Start the cultures by adding 1.0 ml of *E. coli* to **all** the tubes **except 2 and 5**.
4. After mixing, incubate the cultures at 37°C for 60 minutes.

Table 1. Ingredients for setting up the culture tubes to study the production of β -galactosidase.

Tube #	TB (ml)	.01M IPTG (ml)	.013M Chloramphenicol (ml) added at Time (T = min)		<i>E. coli</i> culture (ml)	Briefly note the reason for including each treatment
1	9.0	----	----		1.0	
2	9.9	0.1	----		----	
3	8.9	0.1	----		1.0	
4	8.7	0.1	0.2	T = 0	1.0	
5	9.7	0.1	0.2	T = 0	----	
6	8.7	0.1	0.2	T = 15*	1.0	
7	8.7	0.1	0.2	T = 30*	1.0	
8	8.7	0.1	0.2	T = 45*	1.0	

***added during incubation**

During the incubation period you should consider the two hypotheses presented earlier to explain the production of β -galactosidase. For each hypothesis, **predict in the table below which tubes would have what level (none, low, mid, high) of β -galactosidase activity.** Remember that chloramphenicol stops protein synthesis by preventing m-RNA from attaching to the ribosome.

	TEST TUBE #							
	1	2	3	4	5	6	7	8
Predicted ONP level if lactose activates the DNA-protein pathway. (H ₁)								
Predicted ONP level if lactose activates already present enzyme (H ₂)								

Now, justify your predictions below.

Why is it so important to our experiment that the inducing agent, IPTG is *not metabolized*?

Two other commonly used antibiotics are **actinomycin** and **rifampicin** which block the pathway at the level of transcription. **Assume an experiment is run as above, but actinomycin is added to block transcription at successive time intervals. How might the results of this experiment compare to the results of the experiment using chloramphenicol?**

Termination of Incubation

After the cultures have incubated for 60 minutes, for each culture, you will (1) perform an assay to test for β -galactosidase activity (see E, next page), and (2) determine the density of the culture at the beginning of the assay period in order to calculate the number of active β -galactosidase molecules per cell (see F, next page). Both of these procedures must be performed immediately after the 60 minute incubation and **simultaneously**.

Immediately at the end of the 60 minute incubation period

Partner 1 - using transfer pipettes (1-8), place 3 mls of each experimental mixture into a centrifuge tube for the assay (E).

Proceed to part E.

Partner 2 - pour to fill spectrophotometer cuvettes 2/3 full.

Proceed to part F to determine the culture density.

E. Assay for β -galactosidase

1. Quickly add 6 drops of chloroform and 3 drops of SDS (0.1% sodium dodecyl sulfate) to each 3 ml sample and mix each sample on the vortex mixer. The chloroform and SDS kill the cells and disrupt the membranes but do not affect the activity of the enzyme. **Work quickly - you want to kill the cells in all of the cultures at as close to the same time as possible.**
2. Add 0.1 ml of ONPG (.006M) to each culture and mix again.
3. Place the sample tubes in a 37°C water bath for 10 min. to allow the β -galactosidase to catalyze the breakdown of ONPG.
4. Add 3 ml Na_2CO_3 (0.1M) to each sample tube to stop the enzyme catalyzed reaction and intensify the color.
5. Balance centrifuge tubes then centrifuge the samples for 5 minutes to remove the cell debris.
6. For each sample, place approximately 3 mls of the supernatant in a spectrophotometer cuvette. Avoid the pellet and chloroform.
7. Use the Beckman DU Spectrophotometer to measure the optical density of each sample of supernatant at 420nm, against a blank. The blank for Sample 1 is 0.1ml of ONPG in 3ml of TB and 3ml of Na_2CO_3 . The blank for Sample 3 is Sample 2 (2 & 3 are alike except that 2 had no *E. coli*). The blank for Samples 4, 6, 7 & 8 is Sample 5 (5 has same ingredients as 4, 6, 7 & 8 but had no *E. coli*). OD is a measure of the amount of light absorbed by a solution. O-nitrophenol, the yellow product of the breakdown of ONPG, absorbs light maximally at 420nm. Thus OD 420 in this experiment is a measure of the amount of O-nitrophenol present in the sample. How does this measurement relate to activity of β -galactosidase?

F. Determining the number of active β -galactosidase molecules per cell.

1. Use the Beckman DU Spectrophotometer to read the OD of each sample at 600nm. The blank for Sample 1 is tryptone broth, the blank for Sample 3 is Sample 2, and the blank for Samples 4, 6, 7 & 8 is Sample 5 (see 7 above). OD600 is a measure of relative cell density of the culture just before the assay - the denser the culture (ie. the more cells per unit volume), the more light it will absorb.
2. Enter the results from your experiment in the table below. **Then add them to the class data sheet located on the front table.**

	Test Tube #					
	1	3	4	6	7	8
Absorbance (OD) at 420nm						
Absorbance (OD) at 600nm						

III. Data Analysis:

The following formula can be used to calculate the molecules of β -galactosidase per cell.

$$600 \times \frac{\text{OD}_{420}}{t \times \text{vol} \times \text{OD}_{600}} = \text{molecules of } \beta\text{-galactosidase/cell}$$

OD420 is a measure of the amount of o-nitrophenol--the product of β -galactosidase catalyzed breakdown of ONPG (see Fig. 2). Thus OD420 is a measure of β -galactosidase activity in the culture.

OD600 reflects the cell density before the assay

t = time of the assay reaction in min. = 15 min.

v = volume. This is a dilution factor. Since we do not dilute the original culture in the assay mixture,
vol = 1.

600 (mol/cell x min x ml) is a constant that converts the activity per time, per volume, per cell density to molecules of enzyme per cell.

Use **MINITAB** and the **pooled class data** to calculate the rate of enzyme production.

1. Enter the OD600 (C1) and the OD420 (C2) data into minitab and give the columns appropriate headings.

2. Enter tube codes into a column (**C3**) using the *Calc menu* → *make patterned data*
3. Calculate the molecules of β -galactosidase/cell using the *calc menu*. The expression should have the following form:

$$(600 * C2) / (15 * C1)$$

Note C2 = OD420, C1 = OD600

Make sure you understand how this equation is derived from the formula given above.

4. **Did the treatment have a significant effect on the number of molecules produced per cell?** Refer to “What statistic should I use” to determine which test is appropriate. Then use the **Store descriptive stats** function to calculate the mean and SE to use in making an Excel graph.

Assignment: now that you have completed the lab do Freeman CD 14.1 to help you understand how the lac operon is regulated. The sections on negative/positive control will be helpful when you write your discussion.

1. Make a carefully labeled **Excel** graph to illustrate how the **time** at which the reaction was stopped affected the amount of β -galactosidase/cell. Since you will be graphing means be sure to include error bars (use either \pm SE or \pm 95% C.I.). The graph should have a caption that includes the results of the statistical tests, type of error bar, etc. Tube 1 which had no IPTG added should not be included in the figure - it serves as a control. **In the text** compare your experimental treatments to the control and to the time zero treatment.
3. Write a one paragraph results section in which you point out the major features of the figure you have drawn. The focus should be on the effect of time (rather than tube number) on β -galactosidase production. Parenthetically include the results of the statistical test.
4. Write a brief discussion in which you *interpret* the results in light of the two hypotheses. The table you filled in making predictions about the amount of enzyme produced should help you clarify your ideas. In writing the discussion interpret the data and point out contradictions. The suggestions below may help you focus your ideas.
 - a. In stating which of the two hypotheses was supported, restate the hypothesis.
 - b. Explain how the data support one of the hypotheses, but not the other.
 - c. Point out any contradictions. For example, the presence of β -galactosidase in the tube that lacked IPTG (tube 1).
 - d. Try to explain the contradictions.
 - e. Use information from the *CD tutorials* to help explain your results.