

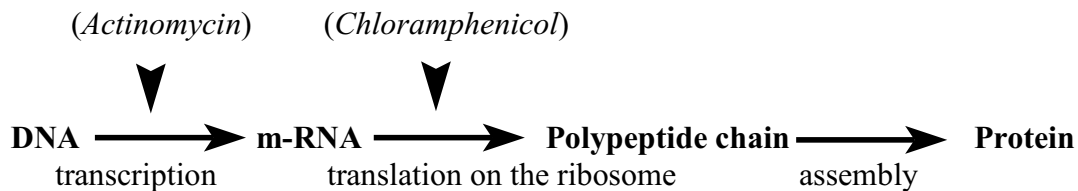
Induction of Enzyme Activity in Bacteria: The Lac Operon

Preparation for Laboratory: Web Tutorial - Lac Operon - submit questions

I. Background:

For the last week you 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 activates β -galactosidase gene expression through 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: Lactose activates existing β -galactosidase proteins. The presence of lactose serves to alter the three dimensional shape of the enzyme converting it to an active form. According to this hypothesis, lactose has no influence on the **rate** of β -galactosidase synthesis, but rather serves to assemble the already present polypeptide chains. This process does **not** depend on how long the DNA-protein pathway is open.

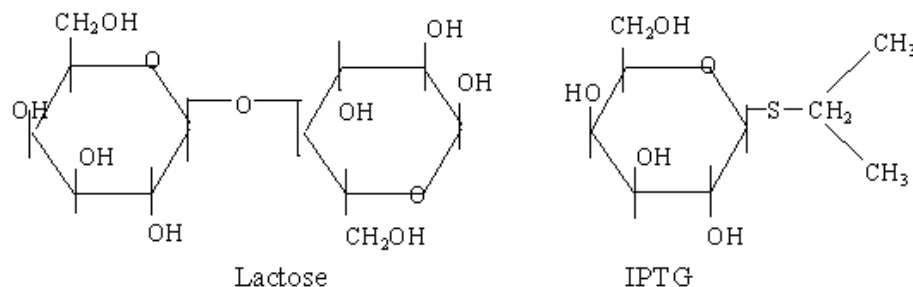
II. Experimental Design:

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,
- assay for the amount of β -galactosidase present.

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.



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. Complete the predictions for the two hypotheses.

Prediction Hypothesis 1 $\rightarrow \rightarrow$ **If** β -galactosidase is produced via the DNA - m-RNA - protein pathway **then**

Prediction Hypothesis 2 $\rightarrow \rightarrow$ **If** an inactive form of β -galactosidase is always present **then**

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).

II. Conducting the Experiment

Setting Up Cultures

All of the cultures you set up in your experiment will have the same basic ingredients:

- **tryptone broth (TB)** a nutrient medium for bacterial growth,
- **IPTG** a stimulator (use 100 μl),
- **chloramphenicol** a protein synthesis blocker (use 200 μl),
- ***E. coli*** the cells in which protein synthesis will occur (use 1000 μl).

The independent variable that you will control is **time** in 15 minute intervals. Use care in setting up the cultures. **The timing of chloramphenicol addition is critical to success.**

Complete table 1 indicating the amount of each ingredient to add so the final volume of each culture is 10ml. A 1000 μl is equivalent to 1 ml. Have the instructor check the final table.

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

Tube #	TB (ml)	.01M IPTG (μl)	.013M Chloramphenicol		<i>E. coli</i> (μl)	Reason for each treatment & relative amount of b-gal (H1/H2) 0, L,M,H,H ⁺ ,H ⁺⁺
			(μl)	Min		
1		----	----	----		
2				0	----	
3				0		
4				*		
5				*		
6				*		
7				*		

*added during incubation

Wear gloves. Do not let the caps from culture bottles or tubes touch your body or desk top. Do not leave any culture bottle or tubes open for any longer than necessary while making a transfer.

- Place the proper amount of tryptone broth (TB) in a large test tube then add the IPTG.
- Add **chloramphenicol only at the indicated times. CAUTION:** chloramphenicol is **not added** to tubes 4 - 7 until they have been incubated for the appropriate times.
- Start the cultures by adding the appropriate amount of *E. coli* to **all** the tubes **except 2**.
- After mixing, incubate the cultures at 37°C for 60 minutes.

Consider the design.

If you want to know that IPTG is required to stimulate the *E coli* to synthesize or assemble β -galactosidase. What two test tubes are necessary?

You will use the hydrolysis of ONPG to ONP as an assay for the amount of β -galactosidase present at the end of 60 minutes. Indicate the relative amount of ONP you would expect given the two hypotheses.

During the incubation period you should consider the two hypotheses presented earlier to explain the production of β -galactosidase. For each hypothesis, sketch the graph that would result from each hypothesis. **Label the axes.** Remember that chloramphenicol stops protein synthesis by preventing m-RNA from attaching to the ribosome.



Figure 1. Predicted ONP level if lactose activates the DNA-protein pathway.



Figure 2. Predicted ONP level if lactose activates already present enzyme.

Briefly justify your figures.

Why is it critical to the experimental design that the stimulator, IPTG is not metabolized by β -galactosidase?

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. Graph how might the results of this experiment might compare to the results of the experiment using chloramphenicol if IPTG activates the DNA-RNA-protein pathway. Use separate lines for each curve.**

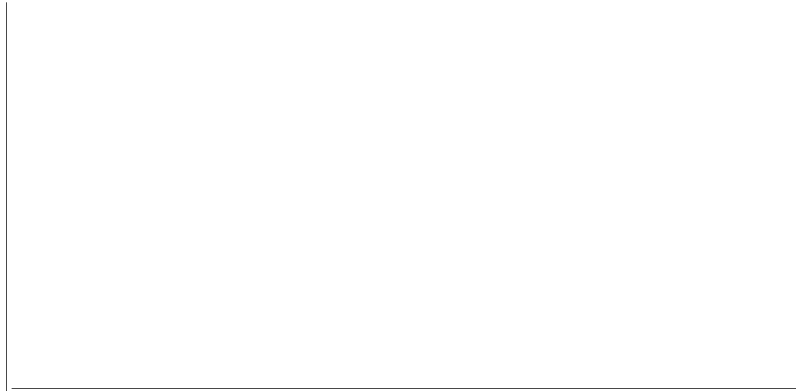


Figure 3. The amount of ONP produced by *E. coli* stimulated with IPTG and treated with either chloramphenicol or actinomycin at 15 minute intervals.

To measure the optical density you need to blank the spectrophotometer with a solution that contains everything except the variable you want to measure. **What is the blank for tube 1 if you want to determine the number of *E. coli*? What is the blank for tubes 3-7?**

Termination of Incubation

After the cultures have incubated for 60 minutes, for each culture, **partner 1** will perform an assay to test for β -galactosidase activity, and **partner 2** will determine the density of the *E. coli* culture at the beginning of the assay period. This will allow you to calculate the number of active β -galactosidase molecules per cell. 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. **Proceed to part A.**

Partner 2 - pour to fill spectrophotometer cuvettes 2/3 full. **Proceed to part B.**

Part A: 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 disrupts their membranes without affecting 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 100 μ l 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 tube 1 is 100 μ l of ONPG in 3ml of TB and 3ml of Na_2CO_3 . The blank for tubes 3-7 is tube 2 (2 has same ingredients as tubes 3-7 but 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 and can be used to calculate β -galactosidase activity.

Part B: 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 tube 1 is tryptone broth, the blank for tubes 3 -7 is tube 2. 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.**

Table 1. Raw data for determining β -galactosidase activity.

	Test Tube #					
	1	3	4	5	6	7
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. = 10 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 treatment codes (you may want to use duration of reaction) into a column (C3) using *Calc menu* ► *make patterned data* ► (text or arbitrary numbers)

3. Calculate the molecules of β -galactosidase/cell using the *calc menu*. The expression should have the following form:

$$(600 * C2) / (10 * C1)$$

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. After determining if the treatment had a significant effect, use *Stats* ► *Store descriptive stats* to calculate and store the mean and SE in columns that can be pasted into Excel for making a graph.

Assignment:

The analysis you are doing this week will become a component of the final paper you write on the properties of the enzyme β -galactosidase. It will be evaluated and returned for incorporation into your final paper. **Use the short report format but exclude the introduction and methods sections.** A few suggestions follow:

Results:

- Make a carefully labeled **Excel** graph to illustrate how the **time** at which the reaction was stopped affected the amount of β -galactosidase/cell.
- **In the text** compare your experimental treatments to the control and to the time zero treatment.
- *Optional* - You may also want to consider how the addition of chloramphenicol affected the relative cell density by graphing the OD600. And analyzing as above. This may help explain some of the changes you got in the amount of β -galactosidase/cell.

Discussion:

Write a brief discussion in which you *interpret* the results in light of the two hypotheses. The graphs you drew in making predictions about the amount of enzyme produced should help you clarify your ideas. The suggestions below may help you focus your ideas.

- In stating which of the two hypotheses was supported, restate the hypotheses.
- Explain how the data support one of the hypotheses, but not the other.
- Point out and try to explain any contradictions. For example, the presence of β -galactosidase in the tube that lacked IPTG or the similarities of several treatments.

Read Freeman “**gene regulation and information flow**” to help you understand how the lac operon is regulated or review the same material using the Freeman CD. The sections on negative/positive control will be helpful when you write your discussion.

Preparation for next week: Web Tutorial - SDS PAGE Gels/Western Blotting