BACTERIAL GENETICS: Labs I & II

The Bacterial Genetics Labs will extend over two laboratory periods. During the first lab, you will set up two different experiments using the bacterium *Escherichia coli*. During the second lab, you will collect and interpret your data. The class as a whole will examine and discuss the data before you write up your conclusions from this lab.

Prelab Assignments:
Before Lab I, read carefully through the entire protocol for Labs I and II and work the **Dilution Problems** included in this exercise. At the beginning of Lab II, you will have a short quiz that will test your understanding of dilutions.
Before Lab II, reread the protocol for Lab II. Also review pp. 261-264 and Figure 12.1, p. 263 in your text (POHS, 5th ed.). Graph the data given in Table 1 (on p. 26) as described on pp. 26-27 (#3 under **Data analysis and write-up**).

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Guidelines for Handling Microorganisms using Sterile (Aseptic) Technique:
The aim of sterile technique is to ensure that airborne fungal spores or human-borne bacteria do not contaminate your experimental cultures.

1. Wipe your hands and the bench surface with 70% ethanol, which is an effective disinfectant, before starting your experiment.
2. Only open and/or unwrap sterilized equipment (petri dishes, pipets, toothpick packages) the moment you intend to use it. Do not place Petri dish or culture flask lids on the bench surface. Instead, hold the lid in one hand while performing the required manipulation with the other hand and replace the lid immediately.
3. When you remove pipets from the metal container, handle only at the wide end. If the tip of the pipet touches anything not sterile (other than your *E. coli* culture), don't use it – get a new sterile pipet. After using a pipet, place it (tip down) in the pipet soaking bucket near the sink. Do NOT place pipets in the sink.

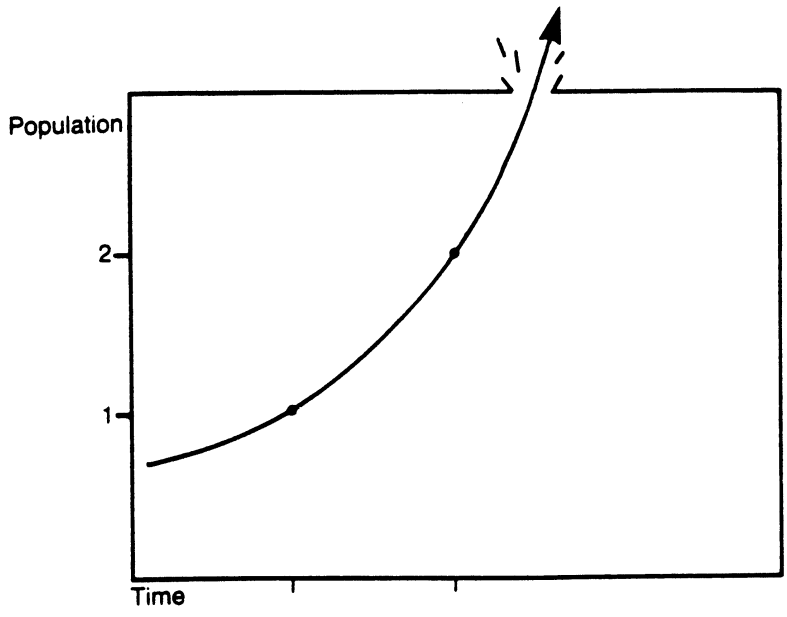
The strain of *E. coli* used in these experiments is not pathogenic. Nevertheless, if you spill any cultures, immediately clean and disinfect the bench surface. Petri dishes containing *E. coli* cultures should be discarded in the red autoclavable bags provided.

Experiment A: Exponential Growth of Bacteria

Introduction: Prokaryotes are placed into one of two domains, either the domain *Bacteria* or the domain *Archaea*. They are both called **prokaryotes** (Greek for "prenucleus") because their genetic material is not bound by a nuclear membrane. Bacterial cells reproduce by a process called binary fission, where the cell duplicates its components and divides into two cells. Under favorable conditions, individual bacteria rapidly proliferate, forming colonies consisting of millions of cells.

Bacterial populations, as well as human populations, exhibit **exponential growth** which is the term used to describe the pattern of population growth in which the number of cells (or the number of individuals in the case of human populations) **doubles** during each unit time period. One of the characteristics of exponential growth is that the rate of increase in cell numbers (or organisms) is slow during the initial stages but increases at an ever-faster rate. This results, in the later stages, in an explosive increase in cell (or organismal) population numbers. (See graph below).

For bacteria, the **generation time** is the time required for a complete growth cycle or, in other words, the generation of two cells from one. Thus the generation or **doubling time** in bacteria is the time required for the cell number to double. In bacteria, this time is highly variable and is dependent upon both nutritional and genetic factors. Under the best nutritional conditions, the generation time of *E. coli* is about 20 minutes. Few bacteria can grow faster than this, and most grow much slower.



Protocol for Exercise A. Work in pairs.

Week 1: The laboratory work for this exercise will include an introduction to pipetting, dilution series and viable cell counts as well as to the mathematics relating to exponential growth and calculation of generation (doubling) time in bacteria. Because of space and equipment limitations, you will not actually generate the data for a growth curve, but you will carry out an exercise involving the standard manipulations that would be used to obtain such data.

The measurement of bacterial cell number can be accomplished in a number of different ways. You will determine the cell concentration in a culture of *E. coli* by doing a **viable cell count**. A viable cell is a cell that is able to divide and form a population of cells. Each pair of students will be given a culture of *E. coli* cells that have been growing overnight at 37oC in a rich medium. The culture will be diluted and aliquots will be plated on rich agar plates.

Before starting, label your tubes by putting tape on each tube and marking on the tape. Remember to **remove all tape** before depositing the tubes in the wash tub at the end of class. This method is used for glassware that will be washed and reused. You can mark directly on the plastic petri dishes because they will not be reused.

Step 1: Do a **serial dilution** of the untreated *E. coli* culture: Fill 7 sterile tubes with 4.5 ml of sterile saline. Transfer 0.5 ml of the undiluted culture to one of the tubes. Mix well, being careful not to spill any of the contents. This is a 10^{-1} (1/10) dilution.

Step 2: Change your pipet and take 0.5 ml from the 10^{-1} dilution and pipet it into the tube marked 10^{-2} dilution. Mix well. This is a 1/100 or 10^{-2} dilution.

Step 3: Next make serial dilutions of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}. Always change pipets between dilutions.

Step 4: Plate 0.1 ml of the 10^{-5} dilution onto each of two agar plates (labeled with your initials and the dilution factor). Dip a glass spreader into alcohol and briefly flame sterilize (allow the alcohol to burn off) using the burners provided. Allow to cool and spread the inoculum over the first plate, turning the plate as needed to obtain complete coverage. Then, spread the inoculum on the duplicate plate.

Step 5: Repeat **Step 4** for the 10^{-6} and 10^{-7} dilutions.

Step 6: Give your plates to an instructor. The plates will be incubated at 37 °C overnight and then stored in a refrigerator (4 °C) until the next class period.

Protocol for Exercise A
Week 2: Data collection
For each dilution, count the number of cells on the duplicate plates. If there are more than a few hundred colonies on the plate, carefully mark off a section (for example 1/4) of the plate and count the number of colonies in that section only, then calculate the number on the entire plate.

Data analysis and lab write up:
1. Set up a table that includes your raw data for each dilution. Average the counts for the duplicates and calculate the number of **viable cells per ml of your original *E. coli* culture** for each of your dilutions. Do the determinations agree with each other? Which determination do you think is the most accurate? Why? Explain any discrepancies in the data.
2. Assume that each colony on the plate represents the descendants of a single, viable *E. coli* cell. Assuming that the generation time of *E. coli* is 90 minutes under these conditions, how many bacterial cells are present in each of these colonies? The plates were incubated at 37°C for about 19 hours. [For this calculation assume that the cells were still in log phase when removed from the incubator and that the lag phase (see #5 below) lasted about an hour.]
3. Table 1 below presents growth curve measurements obtained for the bacterium *Bacillus subtilis.* Cell number was determined using the viable cell count method that you performed on your *E. coli* cultures. Graph these data in three different ways:

Table 1. Results of growth curve measurements of *Bacillus subtilis*.

Time	Cell Number
9:30	1.0×10^7
9:40	1.0×10^7
9:50	1.0×10^7
10:00	1.7×10^7
10:10	2.5×10^7
10:20	4.0×10^7
10:30	6.2×10^7
10:40	1.0×10^8
10:50	1.7×10^8
11:00	2.6×10^8
11:10	4.1×10^8
11:20	5.5×10^8
11:30	6.3×10^8

#1 Using a linear (arithmetic scale) on both X and Y axes. (Time should be on the X axis for each of these graphs.)
#2 Using semi-log paper with a $\log_{10}$ scale on the Y axis and a linear scale on the X axis.
#3 Using a linear scale on both axes and graphing the $\log_{10}$ of the cell number on the Y axis.

If you have access to computer software that can be used for this analysis, computer-generated graphs can be submitted for graphs #1 and #3.

4. Observe your graphs. How do the arithmetic and log plots differ with respect to the shape of the curve and slope of the line?

5. A typical bacterial growth curve can be divided into three phases: (1) lag phase, during which the cells are adjusting to a new supply of nutrients and there is no increase in cell number; (2) logarithmic or exponential growth phase, and (3) stationary phase, where growth rate declines due to exhaustion of nutrients or the accumulation of toxic materials. Mark the lag, logarithmic (aka exponential) and stationary phases on each graph.

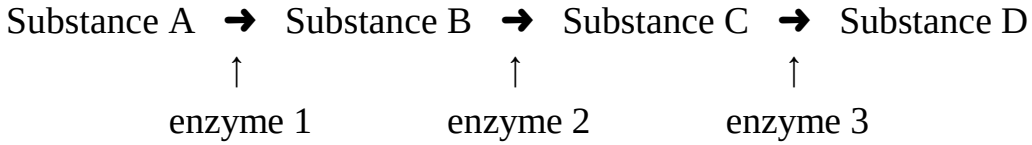
6. From graph #2 or #3, determine the generation (doubling) time of *Bacillus subtilis.*

Experiment B: The Action of Genes in Biochemical Pathways: Beadle and Tatum's classic experiments

Introduction: In this experiment you will be recreating the classic experiment of Beadle and Tatum that led to the **one-gene-one-enzyme** hypothesis (p. 263 of your text; POHS, 5th ed.). This hypothesis provided the first clear insight into the function of genes: It stated that *genes somehow were responsible for the function of enzymes and that each gene apparently controlled the production and activity of one specific enzyme*. Or, in other words, *genes control biochemical reactions by controlling the production of enzymes*. Subsequent to this seminal experiment (which was done in the 1940's), other researchers obtained similar results for other biosynthetic pathways. The one-gene-one-enzyme hypothesis became one of the great unifying concepts in biology because it brought together, in a coherent way, concepts in biochemistry and genetics.

Instead of using the eucaryote *Neurospora* and examining arginine biosynthesis, you will be using the procaryote *E. coli* and sorting out the biosynthetic pathway of tryptophan. The logic of both experiments is the same. We are using bacteria in this experiment because growth of this organism is a convenient indicator of gene function in biochemical pathways.

The metabolic processes underlying bacterial cell growth involve as many as 2000 chemical reactions of a wide variety of types. A specific enzyme catalyzes virtually every chemical reaction. Some of these reactions involve energy transformations; others involve biosynthesis of small molecules, the building blocks of macromolecular polymers (such as DNA, RNA, and proteins). These thousands of enzyme-catalyzed chemical reactions are functionally organized into many different sequences of consecutive reactions called **pathways**, in which the product of one reaction is the substrate of the next reaction. Thus most biosynthetic pathways involve a series of chemical conversions:



In general, culture media can be classified nutritionally as either minimal or rich. **Rich media** are composed of organic materials containing a wide variety of nutrients, far above the minimal requirements of the organism. A **minimal medium** supplies only the minimal nutritional requirements of an organism with no extras added. A wild-type (that is, non-mutant) strain of *E. coli* can

manufacture all of the components it needs for biosynthesis of proteins and nucleic acids. Therefore a wild-type strain can grow on a minimal medium that contains only inorganic salts and a carbon source such as glucose. Mutant strains of *E. coli* that are no longer capable of synthesizing an essential nutrient (such as an amino acid) have been identified. Such strains have an enzyme deficiency that interferes with the synthesis of the essential substance and therefore can grow on minimal medium only when that specific nutrient is provided as a supplement.

Protocol for Experiment B. Work in pairs.

Week 1

You will be provided with the following reagents:

(a) Four strains of E. coli: one that is wild-type (called WT) and three (called *trpB, trpC and trpE*) that have lost the ability to synthesize tryptophan. Each of the mutants is blocked at a different point in the biosynthetic pathway. In other words, each *trp* strain is defective for a different enzyme involved in tryptophan production. Note that anthranilic acid and indole are intermediates in the tryptophan biosynthetic pathway.

(b) 5 tester plates:

Plate 1: minimal medium (MM)
Plate 2: MM plus anthranilic acid
Plate 3: MM plus indole
Plate 4: MM plus tryptophan
Plate 5: rich medium

(c) A package of sterile toothpicks

Step 1: Using a lab marking pen, divide the bottom of each plate into four sectors and label each sector with the identification number of the strain that you intend to streak there. Be sure to put your initials on each plate.

Step 2: Remove a single sterile toothpick and open the dish containing the bacterial strain that you want to streak first. **Lightly touch the surface of a single colony with the fat end of the toothpick.** This procedure will provide more than enough cells for all five tester plates. Replace the lid on the plate.

Step 3: Remove the lid of each tester plate in turn and **very lightly streak** the thick end of the toothpick over the surface of the agar in the ***center*** of the appropriate sector of each tester plate **in numerical order**. Try not to break the surface of the agar. Discard the toothpick into the appropriate waste container.

Step 4: Repeat **Steps 2 & 3** for each *E. coli* strain.

Step 5: Give your plates to an instructor. The plates will be incubated at 37°C for two days and then stored in a refrigerator (4°C) until the next class period.

Protocol for Exercise B
Week 2: Data collection
Score the sectors on each of the six tester plates for **growth** or **no growth**. Identify the control plates and strains and use them for comparison if any of the sectors look ambiguous.

Data analysis and lab write up:
1. What is the purpose of the wild-type strain in this experiment? Why did you streak out each strain on rich medium? On minimal medium with no supplements?
2. Summarize your data in tabular form using a (+) to indicate that growth occurred and a (-) to indicate that no growth occurred.
3. From your data, you should be able to piece together the biosynthetic pathway for tryptophan. Draw out the pathway indicating the position of each substrate and where each mutant strain is blocked in the pathway.
4. The one-gene-one-enzyme hypothesis was eventually changed to the **one-gene-one-polypeptide** hypothesis. Explain why.
5. Do you know of any exceptions to the one-gene-one-polypeptide rule?

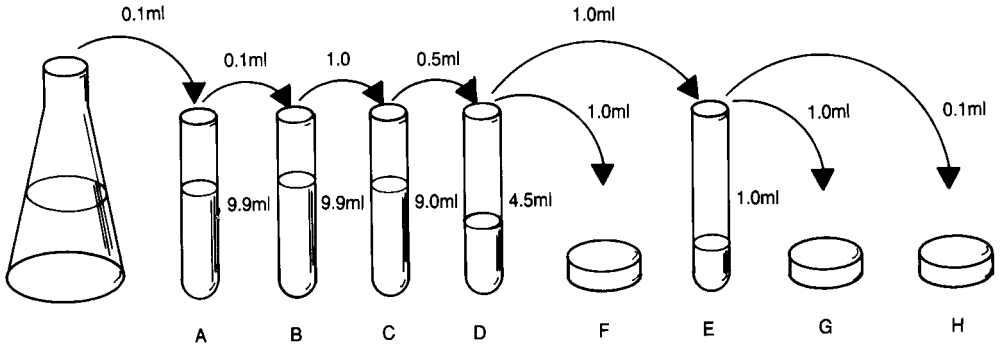
Biology 201- Bacterial Dilution Problems

For these problems, assume each bacterial cell will give rise to one colony.

1- One ml of a sample was mixed with 99 ml of sterile diluent. One ml of this was transferred to nutrient agar. After incubation for 48 h, 241 colonies were present on the plate. How many bacteria were present per ml of the original sample?

2- One ml of a sample was mixed with 99 ml of sterile diluent. One-tenth of a ml (0.1 ml) of this was plated on nutrient agar. After incubation for 48 h, 142 colonies were present on the plate. How many colony-forming units were present per ml of the original sample?

3- Given the dilution series outlined below:



If plate H has 250 colonies,

(a) How many bacteria/ml are in each tube (A-E)?
(b) How many bacteria/ml are in the original culture?
(c) How many colonies will be on plate G and on plate F?

4- Rework question #3, if now there are 30 colonies on plate H.

Definition:

diluent - this is a general term that refers to any liquid medium that is used to dilute a sample.

Answers:

1. 241 colonies/ plate = 241 colonies/ml of diluted culture (=2.41×10^2)

Since you diluted by a factor of 100, you will now have to multiply by 100 to obtain the concentration of colony forming units (CFUs) per ml of original culture:

(2.41×10^2) x 100 = **2.41×10^4 bacteria/ml** of original sample.

2. 142 colonies/0.1 ml =1.42×10^3 colonies/ml of the diluted culture, so this means the original culture had **1.42×10^5** bacteria.

3. (a) tube A: 5×10^7 cells/ml; tube B: 5×10^5 cells/ml; tube C: 5×10^4 cells/ml; tube D: 5×10^3 cells/ml; tube E: 2.5×10^3 cells/ml.

b) there are 5×10^9 cells/ml

c) plate G: 2.5×10^3 colonies; plate F: 5×10^3 colonies.

4. (a) tube A: 6×10^6 cells/ml; tube B: 6×10^4 cells/ml; tube C: 6×10^3 cells/ml; tube D: 6×10^2 cells/ml; tube E: 3×10^2 cells/ml.

(b) there are 6×10^8 cells/ml

(c) plate G: 3×10^2 colonies; plate F: 6×10^2 colonies.