The Power of Bacterial Genetics lies in the ability to study rare events: An investigation of mutagenesis using the model *Escherichia coli*.
OR
Mutations Happen, all the time

Mutagenesis Lab Part 1

This experiment will span several lab periods. As described in this handout, we will first measure spontaneous and induced mutation frequencies for rifS → rif R and lac+ → lac- in E. coli. We will generate rifR and lac- strains and follow up on these mutant lines in different ways. See also ROADMAP to this experiment:
http://fire.biol.wwu.edu/trent/trent/mutagenesisroadmap.pdf

⇛ Before Tuesday 10/23 read carefully through entire handout including Appendices A & B.

- *Your Google doc submission should generally summarize what we are doing but not include specifics (ie of dilution series or plating)*
- *In your lab notebook, draw a diagram or flow chart (see pg 9&10 for examples) outlining and plating steps*

⇛Before Thursday 10/25 work though dilution problems in Appendix D

- *Work these problems suing scientific notation AND without a calculator and put the answers in your lab notebook*
- *Submit a google.doc that summarizes which plates will give you the numerator and which plates will give you the denominator of the mutation frequency (mutant cells/total) for each phenotype.*

Need practice with Scientific Notation? Check out these web sites
http://www.chem.tamu.edu/class/fyp/mathrev/mr-scnot.html
http://www.ieer.org/clssroom/scidrill.html

⇛ Before Thursday 11/1, work up a detailed protocol to measure the rate of reversion (reverse mutation from lac^- → lac^+

- *Your Google doc submission should generally summarize your strategy for detecting reverse mutations*
- *In your lab notebook, draw a diagram or flow chart outlining the experiment details including any dilutions series and plating steps.*

Review in your genetics textbook:

Required Reading:

Trench Warfare in Battle with TB
http://fire.biol.wwu.edu/trent/trent/TBTrenchWarfare.pdf
Weapons of Microbial Drug Resistance Abound in Soil Flora
http://fire.biol.wwu.edu/trent/trent/microbialresistance.pdf

Optional Reading:

Humans as the World's Greatest Evolutionary Force
http://fire.biol.wwu.edu/trent/trent/humans.pdf

In your genetics textbook review:

- spontaneous mutations
- induced mutations and chemical mutagens
- forward and reverse mutations and the AMES test
- gain and loss-of-function mutations
- prokaryotic RNA polymerase
- the *E. coli lac* operon
- minimal medium

Introduction: A mutation is defined as a heritable alteration of DNA. Mutations range from simple nucleotide substitutions to complex chromosomal rearrangements. All mutations can be classified as either spontaneous or induced. Spontaneous mutations are "naturally occurring" mutations that may result from errors in DNA replication or from spontaneous DNA damage resulting from variety of causes. Induced mutations result from the action a mutagen, which is defined as any agent that increases the rate of mutation above the spontaneous frequency. This exercise will examine both spontaneous and induced mutations.

ISOLATION OF SPONTANEOUS MUTATIONS AND EMS-INDUCED MUTATIONS: To a certain extent, the type of experiments a scientist can do is limited by his/her choice of model organisms. One very powerful aspect of bacterial genetics is the ability to set up direct selections to detect very rare genetical events. Using a simple antibiotic selection we will detect rare spontaneous (and less rate induced) mutation events: we will do a **selection** for rifamycin-resistant mutants in untreated and EMS (ethylmethane sulfonate) mutagenized cultures of wild-type *E. coli*. We will also do a **screen** for *lac*$^-$ mutants, that is for mutants that are unable to utilize lactose as a carbon source. (Why aren't we doing a selection for *lac*$^-$ mutants?) We will also measure the frequency of reverse mutations in a culture of *lac*$^-$ cells.

STRAINS USED IN THIS EXPERIMENT:

E. coli B CGSC 5365: wild-type strain from the ***C***oli ***G***enetic ***S***tock ***C***enter at Yale University

E. coli B CGSC 5365 mutagenized with EMS *(ethylmethane sulfonate)*

Mutations happen …….. all the time

After reading this handout, transfer table to lab notebook and fill in blank cells

Genotype wildtype strain	Mutant genotype	Screen or Selection?	Media used to detect mutant	Phenotype(s) of colonies on plates	Gene (s) mutated
rifS	rifR				rpoB
lac+	lac-				lac Z lac Y

TUESDAY OCT 23

PART IA: VIABLE CELL COUNTS (SEE APPENDIX A)

Untreated (not mutagenized) wild-type culture: Do a serial dilution of the untreated *wild-type E. coli* culture: Fill 7 tubes with 4.5 ml of sterile saline. Transfer 0.5 ml of the undiluted culture to one of the tubes. This is a 10^{-1} dilution. Next make serial dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}. Always change pipets and mix well between dilutions.

- Plate 0.1 ml of the 10^{-6} onto an NA (nutrient agar) plate.
- Repeat for the 10^{-7} dilution.
- *Also plate 0.1 ml of undiluted culture so you can see what a continuous lawn of E.coli looks like*
- Place the plates at 37^{o}C overnight

EMS-treated wild-type culture: You will be given an EMS treated culture of wildtype *E. coli*. Do a viable cell count on this culture using the same dilutions as described above. *Your do not need to plate an undiluted culture*.

PART IB: SELECTION FOR *RIF^{R}* MUTANTS

***Rif^{R}* mutants**: Rifamycin (aka rifampicin or rifampin) is a potent inhibitor of *E. coli* RNA polymerase. Mutants of *E. coli* that are resistant to this antibiotic have been isolated and shown to have an altered RNA polymerase.

Untreated (not mutagenized) wild-type culture: To select for spontaneous rifamycin-resistant mutations:

- Spread 0.2 ml of undiluted culture on an NA plate that contains rifamycin (100 μg/ml).
- Set up a total of 2 such plates.
- Place the plates at 37^{o}C overnight

EMS-treated wildtype culture: To select for rifamycin-resistant cells:

- Spread 0.1 ml of each of the following dilutions on an L plate that contains rifamycin (100 μg/ml): undiluted, 10^{-1}, 10^{-2}, 10^{-3}.
- Place the plates at 37^{o}C overnight

PART IC: SCREEN FOR *LAC^{+}* → *LAC^{-}* MUTANTS

lac^{-} mutants: Wild-type *lac^{+}* colonies appear dark red on MacConkey indicator plates. Mutant colonies that are not capable of utilizing lactose as an energy source will appear as white colonies on MacConkey plates.

Untreated (not mutagenized) wild-type culture:

- Spread 0.1 ml of the 10^{-5} dilution on a MacConkey plate.
- Also, spread 0.1 ml of the 10^{-6} dilution on a MacConkey plate.
- *Set up a total of 3 plates of each dilution. See also next page*

- Plates will be placed at 4degC. On Wed PM they will be transferred to a 37deg incubator.

EMS-treated wild-type culture: Follow the instructions for the untreated culture.

THURSDAY OCT 25

PART IIA WORK UP OF DATA

For the viable cell counts, pick a plate with 30-300 colonies and do an accurate colony count. For the rifamycin plates, count all colonies; for the MacConkey plates, just count the number of white colonies on each plate. The total number of cells plated can be determined from your viable cell count, so you don't need to count all of the red colonies. Your lab notebook should contain all of your raw data (i.e. cell counts) as well as your workup of the data.

PART IIB FOLLOW-UP on rif^R candidates

- Get a lesson on streaking (bacterial version only) if you have never done this before. See also instructions on streaking in **Appendix C**.
- Practice streaking for single colonies and let CT see your practice plate before you proceed to the next step.
- Each student should streak out ***two*** candidate *rfrR* mutant colonies on rifamycin plates to confirm the phenotype and to isolate colonies arising from single *E. coli cells*. Pick one induced and one spontaneous mutation and be sure to keep track of the identity of these strains. **Your rifR ID is your drawer number followed by either E (EMS) or S (spontaneous).**
- *A separate handout will describe our follow-up experiments on these mutations.*

MONDAY OCT 29

PART IIC *FOLLOW-UP on lac- candidates:*

- Streak out candidate *lac*$^-$ mutants on a MacConkey plate to confirm the *lac*$^-$ phenotype and to isolate colonies arising from single *E. coli* cells. ***Each student should streak at least two mutant candidates.***
- Start thinking about how to measure the frequency of spontaneous reversion mutations in a culture of *lac*$^-$ cells. Be sure to specify the type of media that you will be using to grow up cultures of lac- cells, to select for revertants and how you will carry out the plating portion of the assay.

Stuff to think about:

1. How do the spontaneous and induced mutation frequencies compare for each gene? Express data in terms of actual mutation frequency: 1 X 10^{-6} mutations per cell per gene
Compare mutation frequencies by indicating ratios:

- spontaneous: lac^-/rif^R
- induced: lac^-/rif^R
- induced/spontaneous lac^-
- induced/spontaneous rif^R

2. Do you expect the mutation frequencies (either spontaneous or induced) to be the same for every gene? How will the frequency of forward mutations compare to reverse mutations.

3. A thorough discussion of the various factors that determine mutation frequency and appearance of a specific mutant phenotype

4. A mutant phenotype can result from a loss-of-function or a gain-of-function mutation. How would you classify the mutant phenotypes that we examined in this lab? For each gene, consider how a loss or gain-of-function would affect the organism. Include a discussion of the mode of action of rifamycin and also discuss what genes could be mutated to produce a *lac*$^-$ phenotype.

5. What is the difference between a selection and a screen? Why was a series of dilutions done for the *lac*$^-$ screen but not for the *rif*^{R} selection? Which is more powerful -- a selection or a screen? In other words, which is more useful for detecting rare events-- such as a very rare mutation?

APPENDIX A 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 the bench suface 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 mouth end. If the other end 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 in the appropriate container. Do NOT place pipets in the sink.
4. 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 autoclavable bags provided. Liquid cultures should be disposed of in the waste flasks provided.

APPENDIX B: Viable Cell Counts

The number of microorganisms in an undiluted broth culture after a 24-hour incubation period is usually in the millions. In order to determine the actual number of organisms in the tube or culture flask, it is necessary to dilute the culture to a point where there are a few hundred organisms per milliliter, or to a point where the number or organisms plated onto (or into) an agar medium will be statistically valid.

Both spread plates and pour plates are utilized to obtain bacterial counts. A spread plate is made by careful, aseptic pipetting of a known volume of sample (usually 0.1 or 1.0 ml) onto an appropriate culture medium and spreading the liquid with a sterile glass spreader. A pour plate is made by adding a known amount of either the original or diluted sample to a melted (50° C) agar tube called an agar tall, mixing the tube and pouring the mixture into a sterile petri plate. When bacteria are plated on either spread plates or pour plates containing a medium that supports growth, only **viable** cells will grow, multiply and form colonies. Each of the colonies is presumed to have arisen from only one cell, although this may not be true if pairs, chains or groups of cells are not completely broken apart before plating. For this reason, the results of cell enumeration in the viable plate count are usually given as **colony-forming units (CFU)/ml**, not as cells/ml. If the number of viable cells on a plate is too great, the colonies will either merge and be impossible to count, or some cells will have insufficient room to form colonies, and the count will be erroneous. At the same time, it is important that the number of cells or colonies not be too few. For statistical validity, it is recommended that **only plates with between 30 and 300 colonies be counted**. Normally, in order to obtain this number, the original culture must be diluted several fold.

Dilutions are usually made ten-fold, hundred-fold or multiples thereof; that is, the most common dilutions are 1/10, 1/100, and 1/1000. As an example, if a ten-fold dilution is to be made, it is feasible to use 0.5 ml of sample in 4.5 ml of diluent or 1.0 ml of sample in 9.0 diluent. The latter gives the fraction:

$$\frac{\text{1 ml of sample}}{\text{1 ml of sample + 9 ml of diluent}} = \frac{\text{1 ml}}{\text{1 ml + 9 ml}} = \frac{1}{10} = 10^{-1}$$

If 100-fold dilutions are to be made, one can use 0.05 ml of a sample in 4.95 ml of diluent, or 0.1 ml in 9.9 ml of diluent, or 1.0 ml in 99 ml of diluent.

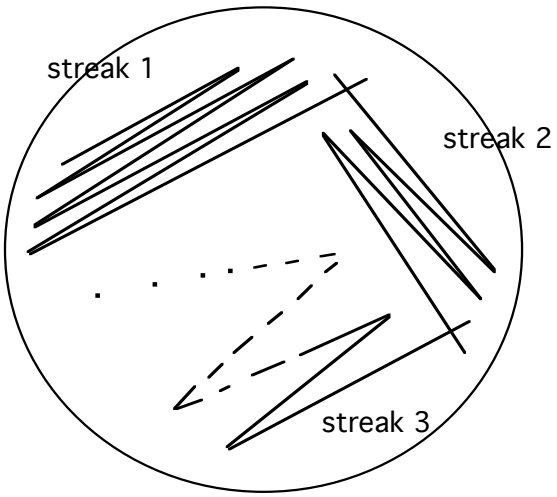
Generally, a dilution of 1/1,000,000 (10^{-6}) is sufficient to decrease the number of viable cells to the point that an appropriate number of colonies will develop on solid medium. A 10^{-6} dilution can be achieved by making three 1:100 dilutions, or six 1:10 dilutions, or a combination of 100-fold and 10-fold dilutions.

APPENDIX C: ISOLATION OF PURE CULTURES (streaking for single colonies)

Structural and biochemical characterizations important in the identification of microorganisms require **pure cultures**. A pure culture theoretically contains a single species of microorganism. There are a number of procedures available for the isolation of pure cultures from mixed populations. A pure culture may be isolated by the use of enrichment or selective media employing specific chemical and/or physical agents in the media that will allow the enrichment or selection of a particular organism over another. More simplified methods for isolation of a pure culture include: (i) spread plating on solid agar medium with a glass spreader and (ii) streak plating with a loop. The purpose of spread plating and streak plating is to isolate individual bacterial cells on a nutrient medium so that during subsequent incubation, a cell grows and divides sufficiently to form a single colony. This allows the assumption that a colony arises from a single bacterium.

Both procedures (spread plating and streak plating) require understanding of the aseptic technique. **Asepsis** can be defined as the absence of infectious microorganisms. However, the term is usually applied to any technique designed to keep unwanted microorganisms from contaminating sterile materials.

The streak plating technique isolates individual bacterial cells on the surface of an agar using a wire loop. The streaking pattern shown in the figure results in continuous dilution of the inoculum to give well separated surface colonies which are produced by single cells. Once again, the idea is to obtain isolated colonies after incubation of the plate.



APPENDIX D: DILUTION PROBLEMS

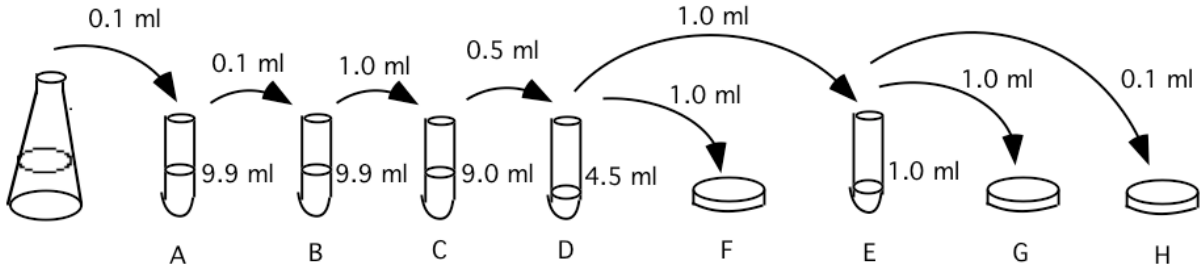
SEE NEXT PAGE BEFORE YOU WORK THESE PROBLEMS.

BE SURE TO GET USED TO WORKING THESE QUESTIONS WITHOUT A CALCULATOR AND USING SCIENTIFIC NOTATION

1. One ml of a sample was mixed with 99 ml of sterile diluent. One ml of this was transferred (using the pour plate method) to nutrient agar. After incubation for 48 h, 241 colonies were present on the plate. How many colony-forming units 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 (using the spread plate method) 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:



A. Give the dilution used for each step (A-E).

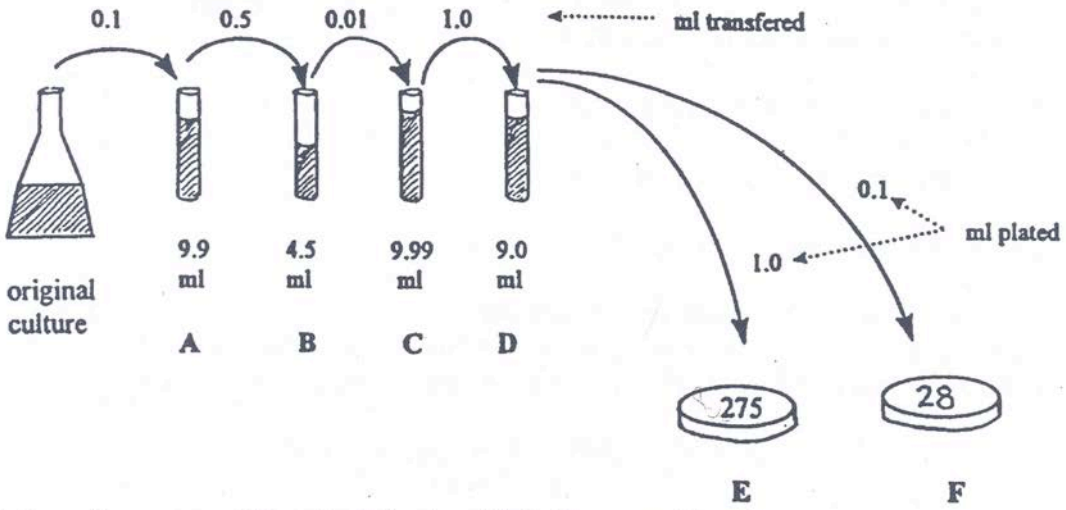
B. What is the total dilution at each step (A-E)?

C. If the original broth culture contains 5 X 10^9 cells ml^{-1},
(i) How many bacteria ml^{-1} in each tube (A-E)?
(ii) How many colonies on each plate (F,G, H)?

D. If tube C contains 60,000 bacteria ml^{-1}, how many bacteria ml^{-1} are in the original culture **and** in the other tubes? How many colonies on each plate?

BACTERIAL DILUTIONS and A FOOL-PROOF WAY TO FIGURE THEM OUT

Look at the dilution scheme below:



Most questions you will be asked about serial dilutions are of two types:

1. The **first type** gives the number of bacterial colonies found on a plate and asks for the number of bacteria per ml in the original culture.

2. The **second type** gives the number of bacteria per ml in the original culture and asks you to devise a serial dilution scheme, so that you will have plates with "countable" numbers of colonies (between 30 and 300).

To solve the **first type** of problem, first determine the **individual dilution factor (IDF)** for each tube using the formula:

$$\text{IDF} = \frac{\text{amount transferred}}{\text{amount transferred + amount already in tube}}$$

For tube A, the IDF $= \frac{0.1}{0.1 + 9.9} = \frac{0.1}{10.0} = 0.01 = 10^{-2}$

For tube B, the IDF $= \frac{0.5}{0.5 + 4.5} = \frac{0.5}{5.0} = 0.1 = 10^{-1}$

For tube C, the IDF $= \frac{0.01}{0.01 + 9.99} = \frac{0.01}{10.0} = 0.001 = 10^{-3}$

For tube D, the IDF $= \frac{1.0}{1.0 + 9.0} = \frac{1.0}{10.0} = 0.1 = 10^{-1}$

Next determine the **total dilution factor** (**TDF**) for the entire dilution series using the formula:

$$\mathbf{TDF = (IDF_A)(IDF_B)(IDF_C)(IDF_D)}$$

For the dilution series above, the TDF for tube A = 10^{-2}

The TDF for tube B = $(10^{-2})(10^{-1}) = 10^{-3}$

The TDF for tube C = $(10^{-2})(10^{-1})(10^{-3}) = 10^{-6}$

The TDF for tube D = $(10^{-2})(10^{-1})(10^{-3})(10^{-1}) = 10^{-7}$

We can assume that each colony of bacteria arose from one living (or viable) cell immobilized on an agar plate. Thus each colony is a clone of cells. We can now determine the number of live bacteria (or Colony Forming Units [CFU]) per ml of original culture by using the formula:

$$\mathbf{CFU/ml = \frac{number\ of\ colonies\ per\ ml\ plated}{Total\ Dilution\ Factor}}$$

As plate E has 275 colonies, the CFU/ml in the original culture:

$$\frac{275\ \text{colonies/ml plated}}{10^{-7}} = 275 \times 10^{7} = 2.8 \times 10^{9}\ \text{CFU/ml}$$

Plate F has 28 colonies, but only 0.1 ml was plated:

$$\frac{28\ \text{colonies/0.1 ml plated}}{10^{-7}} = 280 \times 10^{7} = 2.8 \times 10^{9}\ \text{CFU/ml}$$

***If you use these formulas, you can solve any **type one** serial dilution problem.

To solve the second type of problem, simply rearrange the formula above to solve for **TDF**:

$$\mathbf{Total\ Dilution\ Factor = \frac{number\ of\ colonies/ml\ plated}{CFU/ml}}$$

For example, if you want to have a plate with approximately 30 colonies on it, and the original culture contains 2.8×10^{9} CFU/ml, plug these values into the rearranged equation:

$$TDF = \frac{30}{2.8 \times 10^{9}} = 1.07 \times 10^{-8}$$

An easy way to set up a dilution series like this would be to use 4 tubes, each having an IDF of 10^{-2}, i.e. transfer 0.1 ml into a tube containing 9.9 ml four times. Spread 1.0 ml on the plate, and incubate.