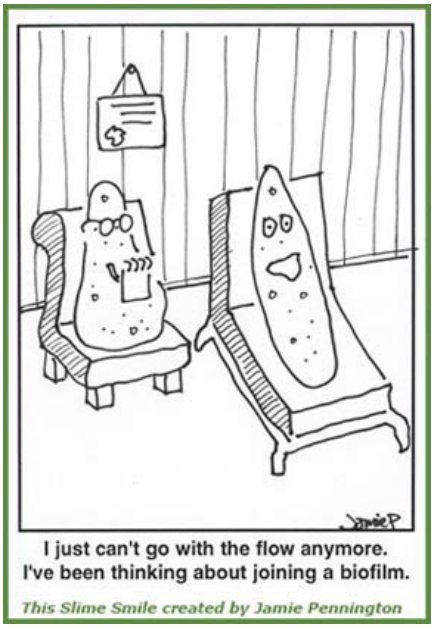
MICROBIOLOGY

BIO 358

LABORATORY EXERCISES

SPRING 2020



Week 1: Media Preparation, Pure Culture Techniques, and Control of Microbial Growth

Media Preparation

INTRODUCTION

A microbiological medium (media, plural) contains the nutrients necessary to support the growth of bacteria, molds, and other microorganisms. It can exist in three consistencies: liquid, solid, and semisolid. In today's exercise, we will be preparing solid media. Solid media are made by adding a solidifying agent, such as agar, gelatin, or silica gel, to a liquid medium. A good solidifying agent is one that is not metabolized by microorganisms, does not inhibit bacterial growth, and does not liquefy at room temperature. Agar and silica gel do not liquefy at room temperature and are metabolized by very few organisms. We will be using agar as our solidifying agent, and this will enable us to grow and isolate colonies of bacteria on the surface of the medium.

MATERIALS AND METHODS – work in **pairs**

One 1-liter beaker
One 1-liter graduated cylinder
One stir bar
Magnetic stir plate
pH meter
NaOH
Two 500-ml bottles
Autoclave tape
Scissors
Stock solution of 100 mg/ml streptomycin
Marker for labeling plates
Petri plates
55˚ C water bath
Striker to light Bunsen burner
Bunsen burner

PROCEDURE

Begin the laboratory period by preparing LB agar containing 100 μg/ml streptomycin for use in **Week 5** when we will be studying phage biology.

1. Place the following into a 1 liter beaker:

    10 g Bacto Tryptone
    5 g yeast extract
    5 g NaCl

2. Add approximately 900 ml distilled water, drop in a stir bar and place the beaker on a magnetic stir plate.

3. After the components fully dissolve, adjust to pH 7.0 with NaOH. Bring the volume to 1 liter by measuring in a graduated cylinder. Mix by dumping the medium back into the beaker.

4. Obtain two 500-ml bottles. Place 500 ml of the medium into each bottle. Weigh out 9 g Bacto Agar for each of the two bottles, and place it inside each. The agar will not dissolve. Swirl it up, place the cap on **without** tightening it completely, and put a small piece of autoclave tape on the cap. **Do not add streptomycin until after autoclaving!**

5. Place your bottles in the autoclave.

6. After the autoclaving is complete, the bottles will be placed in a 55˚ C water bath to cool prior to pouring the media into petri plates. Do not remove the bottles until you are ready to pour as they will soon solidify at room temperature.

7. Add 500 µl of stock solution of 100 mg/ml streptomycin to each 500 ml bottle **after autoclaving (just prior to pouring in plates).** The final concentration of streptomycin will be **100 µg/ml**.

8. Remove the cap of the bottle when you are ready to pour, and briefly flame the neck. Pour approximately 25 ml into each petri plate. There is generally a line on the plates indicating the proper volume to pour.

9. Allow the plates to cool overnight. On the following day, or the next lab period put them in a plastic bag. Label with your group names and lab day; place the bag in the refrigerator.

Pure Culture Technique

When studying the bacterial flora of the body, soil, water, food, or any other part of our environment, we soon discover that bacteria exist in mixed populations. It is only in very rare situations that they occur as a single species. To be able to study the cultural, morphological, and physiological characteristics of an individual species, it is essential that the organism be separated from the other species that are normally found in its habitat. In other words, we must have a **pure culture** of the organism.

The most frequently used technique for isolating bacteria in pure culture is the streak plate. To refresh your pure culture technique, you will isolate a pure culture of *Escherichia coli* strain MC4100, *Staphylococcus epidermidis*, *Bacillus subtilis* **or** *Psuedomonas fluorescens* from liquid culture using the streak plate method. Choose **one species!**

MATERIALS AND METHODS – work **individually**

Liquid culture of *Escherichia coli* strain MC4100 grown at 37˚ C
or
Liquid culture of *Staphylococcus epidermidis* grown at 37˚ C
or
Liquid culture of *Bacillus subtilis* grown at 26˚ C
or
Liquid culture of *Pseudomonas fluorescens* grown at 26˚ C
Inoculating loop
Bunsen burners
Sterile cotton swabs
Small tubes of sterile water
4 Nutrient agar plates (**2 plates** will be used on **Day 1** for the streak plate and the environmental isolate and **2 plates** will be used on **Day 2** for the re-streaking of both cultures)

PROCEDURE

Day 1: The day of your scheduled laboratory period.

Streak plate. **Each person** will perform the streak plate. Firstly, **do not** use the medium you just prepared for this portion of the laboratory exercise. **Do use the media provided by the stockroom**. A properly executed streak plate will facilitate good dilution of the sample, and good separation of individual colonies for further culturing. For the organisms we will be culturing, the quadrant streak will be most effective for producing isolated colonies.

1. Remove an inoculating loop full of bacterial culture of one of the four available species, *Escherichia coli* strain MC4100, *Staphylococcus epidermidis*, *Bacillus subtilis* or *Psuedomonas fluorescens*.

2. Inoculate a Nutrient agar plate using the quadrant streak method as I have illustrated. Incubate the plate in the inverted position at either 37°C or 26˚C for 24 hours. **The plates are inverted to prevent condensation from dropping onto the agar, a possible source of contamination**.

Environmental isolate. **Each person** will perform this portion of the exercise.

1. Isolate microbes from your surrounding environment by taking a sterile swab, dipping it into sterile water, and swabbing a surface in the lab. Swab your isolates onto Nutrient agar.

2. Incubate at **room temperature** until sufficient growth allows you to see individual colonies.

Days 2 and 3

Streak plate. After sufficient growth of the culture, inspect your streak plate for isolated colonies. Carefully subculture an isolated *Escherichia coli* strain MC4100, *Staphylococcus epidermidis*, *Bacillus subtilis* or *Psuedomonas fluorescens* colony onto a second Nutrient agar plate using the quadrant streak. Incubate at 37°C or 26˚C for 24 hours and then inspect the plate on **Day 2 and/or Day 3**. You can toss the initial Nutrient agar plates in the **Biohazard Waste** after you are convinced you have a pure culture.

Environmental isolate. Pick an isolated colony from the Nutrient agar plate, and resteak, or subculture onto a second Nutrient agar plate. Again, incubate at **room temperature**, until well-formed, isolated colonies are visible. After you are convinced that you have a pure culture, toss the Nutrient agar plates in the **Biohazard Waste**.

Control of Microbial Growth: Evaluating Antiseptic and Disinfectant Susceptibilities of Microorganisms

Chemical agents are very useful in controlling microbial growth and survival, and you probably have used a number of them in everyday life. One group of chemicals, classified as **disinfectants**, is used to control microbes on inanimate objects. A second group, **antiseptics**, is used for topical application to body surfaces. The third group contains disinfectants that also do some degree of cleaning, and so are referred to as **sanitizing agents**.

Microbial sensitivity to these agents can be tested using the **agar disc diffusion** technique. In this protocol a culture of the test microbe is spread evenly over an agar plate using a swab. Paper discs containing the agents to be tested for antimicrobial activity are placed directly on the agar plate. The test compounds will diffuse out from the disc creating a gradient. When the plate is incubated, the organism will only be able to grow in parts of the plate without the compound or where the concentration of the compound is low enough so that it does not affect the organism's ability to grow. The clear area of no growth around the disc is called the **zone of inhibition**. Microbes that are **resistant** to the inhibitory affects of a compound will be able to grow right up to the edge of the disc containing the compound.

You will use this procedure to answer a question relating to the efficacy of a disinfectant or antiseptic- 10% bleach, Listerine, 10% Ceepryn, a quaternary amine, or Providone Iodine (Betadine). You will develop a hypothesis, design an experiment to test that hypothesis, and collect and analyze data. Efficacy of the disinfectant or antiseptic is determined by the inhibition of growth on nutrient agar plates. Microorganisms that you may choose from include: *Escherichia coli* strain MC4100, *Staphylococcus epidermidis*, *Bacillus subtilis* and *Pseudomonas fluorescens*. Recall the Gram staining status of these bacteria as this may influence the effectiveness of the antimicrobial agent.

Formulate a **hypothesis** based on your knowledge of the antimicrobial agents and the organisms you are studying. You should have a familiarity with terms such as bactericidal and bacteriostatic and should be aware of the mechanisms by which antiseptics and disinfectants act. Test your hypotheses by **designing an experiment** and collecting the data for analysis.

Laboratory exercise adapted from Investigation into Chemical and Physical Control of Microorganisms by Suzanne Anglehart, Annie Voyles and Bonnie Jo Bratine, Universtity of Wisconsin, La Crosse, submitted to MicrobeLibrary.org.

MATERIALS AND METHODS – work in **pairs**

Overnight nutrient broth cultures of *Escherichia coli* strain MC4100, *Staphylococcus epidermidis*, *Bacillus subtilis* and *Pseudomonas fluorescens*
Cotton swabs
6 Nutrient agar plates **from the stockroom**
Selection of disinfectants and antiseptics as needed (10% bleach, Listerine, 10% Ceepryn, and Betadine)
Sterile filter paper disks (hole punches of Watman paper, similar thickness as the antibiotic disks)
Forceps
Glass Petri dish with 100% ethanol for sterilization
Striker
Bunsen burner
Metric ruler
37˚ C incubator
26˚ C incubator

PROCEDURE

Day 1: The day of your scheduled laboratory period.

First, you need to formulate a **hypothesis**. You will have **six nutrient agar plates** per group, and you can easily put **four chemical-soaked disks** onto each plate. One requirement will be that you will need three trials of each condition, so that you can perform a statistical analysis on your results. Choose a combination of bacterial species and disinfectants/antiseptics to test your **hypothesis**.

You might use Gram-negative and Gram-positive bacteria to assess the differences of a particular disinfectant or antiseptic on cell wall types, or to compare a spore former with a non-spore former. Of the bacteria on the list, which form spores and which do not form spores? You may also assess the activity of various antimicrobial agents on a given species. For example, Lysol claims it is effective against *Staphylococcus*. Does Betadine also kill this species just as well?

1. Using a marking pen to write on the BOTTOM of each plate, divide your plates into four quadrants. This will allow you to test four different compounds by placing a disk in each quadrant. Each disk should be well separated. Label each quadrant so that you know which disk will be placed into it, and be sure to label the plate with your name, the date and the species of bacterium that it will contain.

2. Aseptically insert a sterile swab into the tube containing the selected liquid nutrient broth culture of bacteria, either *Escherichia coli*, *Staphylococcus epidermidis*, *Bacillus subtilis* or *Pseudomonas fluorescens.* Make sure to cover the entire plate with the bacterial culture, and to run the swab around the rim of the plate.

3. Sterilize forceps by dipping in alcohol, then passing through the flame of a burner. Allow the flame to extinguish. With the sterile forceps place the filter paper disks soaked in the chemical agents of your choice in each of the previously labeled quadrants. Be sure that the disks are thoroughly soaked but **well drained** otherwise fluid will flow on the surface of the plate and obscure your results. You can remove excess fluid by carefully dabbing on a Kimwipe. Be sure to sterilize the forceps each time you get a new disk. Carefully record the species of bacteria and the chemical agents used for each quadrant on each plate.

4. Incubate the plates, right side up, at the appropriate temperature for ~24 hours.

Day 2

5. Using a metric ruler, measure the zones of inhibition for the four disks on each plate that you inoculated. Remember that the zone of inhibition is the diameter of the region around the disk that the bacteria did not grow, and that the disk itself is ~ 6 mm. Thus if a particular bacterial species is resistant to the chemical agent, then the zone of inhibition will be 6 mm, **not 0 mm**. Record these results in a well labeled table.

6. For the analysis, use JMP to perform an Analysis of Variance (Anova) on the data to test your null hypothesis that there is no difference between the treatments. If you are not familiar with JMP you can use Excel. In JMP, you can analyze your data by using Fit Y by X. Then from the red arrow select Means/Anova. If there is statistical significance you can then compare all pairs by using Tukey-Kramer HSD (honest significant difference) under the red arrow. Using this post-hoc analysis determine whether you can reject the null hypothesis at the 95% confidence interval.

Lab Report

Only report on the **Evaluating Antiseptic and Disinfectant Susceptibilities of Microorganisms** portion of the exercise. Clearly state your hypothesis and experimental design. Present results in a well-labeled graph or table. Present your statistical analysis. What can you conclude from your experimentation?
Lab reports are due at the beginning of your next laboratory period.

Week 2: Microbiological Assay: Aerobic Versus Anaerobic Utilization of a Limiting Nutrient

INTRODUCTION

When one nutrient in a microbiological medium is present in a limiting concentration and other nutrients are present in excess, the yield of the culture, or maximum growth, is a function of the concentration of the limiting nutrient. This is the principle of a microbiological assay.

In addition to the concentration of a limiting nutrient, the availability of atmospheric oxygen affects the growth yield, or the maximum absorbance of a bacterial culture. In this experiment we will investigate the relationship between the yield obtained per unit of substrate used in the presence of oxygen (respiration) and the growth per unit of substrate used in the absence of oxygen (anaerobic respiration or fermentation).

Adapted from microbiology laboratory exercises developed by Wiltraud Pfeiffer, PhD, UC Davis.

MATERIALS AND METHODS – work in **pairs**

Overnight culture of *Escherichia coli* strain MC4100 grown in LB broth
Sterile M9 minimal medium. This medium is provided for you and contains, per liter, 6g Na_2HPO_4, 3g KH_2PO_4, 0. 5g NaCl , 1g NH_4Cl. After autoclaving, 10 ml of 0.01 M solution of $CaCl_2$, 1 ml of a 1 M $MgSO_4.7H_20$ solution and 1 ml of a 1 mg/ml solution of thiamine are added.
8 Sterile 250 ml flasks containing 100 ml of M9 minimal medium (aerobic cultures)
100-ml bottle of sterile M9 minimal medium (for anaerobic cultures and blank)
8 Sterile culture tubes **for you to dispense** 10 ml of M9 minimal medium (anaerobic cultures)
Sterile glucose: 100 mM, 200 mM, 300 mM, 400 mM, 500 mM, 1 M, and 2 M solutions
Glucose solutions of **unknown** concentration
Disposable Cuvettes
Micropipettors
Spectrophotometers
Computers

PROCEDURE

Day 0: The day prior to your scheduled laboratory period.

1. For the **aerobic** cultures, aseptically add **1 ml** of the sterile glucose solutions to **7** 250-ml flasks containing 100 ml of M9 minimal medium (see **Table 1**). For example, to achieve a final glucose concentration of 1 mM glucose, add 1 ml of the 100 mM glucose solution, for the 2 mM glucose culture add 1 ml of the 200 mM glucose solution, etc. Make sure to label the flasks with your name, glucose concentration and the date using tape and a sharpie.

2. Using a sterile 10-ml pipette and good aseptic technique, dispense 10 ml of M9 medium into each of **8** plastic 12-ml culture tubes. You will use **7** of the tubes for the known glucose concentrations, and **1** 12-ml culture tube for the **unknown** glucose concentration. These will be your **anaerobic** cultures.

3. For the **aneaerobic** cultures, now add **100 µl** of each of the sterile glucose solutions to **7** 12-ml culture tubes containing 10 ml of M9 minimal medium (see **Table 1**). Label tubes using a sharper- no tape is necessary.

Table 1. Glucose solutions for addition to aerobic and anaerobic M9 cultures.

Final Concentration of Glucose	**1 mM**	**2 mM**	**3 mM**	**4 mM**	**5 mM**	**10 mM**	**20 mM**
Add **1 ml** glucose to the **100 ml aerobic** culture	100 mM	200 mM	300 mM	400 mM	500 mM	1 M	2 M
Add **100 µl** glucose to the **10 ml anerobic** culture	100 mM	200 mM	300 mM	400 mM	500 mM	1 M	2 M

4. Obtain your glucose solution of **unknown concentration**. Add **1 ml** to a 250-ml flask containing 100 ml of M9 minimal medium and add **100 µl of the glucose of unknown concentration** to a culture tube containing 10 ml of M9 minimal medium in the 12-ml culture tube. Be sure to properly label each of your culture flasks and tubes! **Note that your bacteria will not grow if they don't have any glucose!**

5. You are now ready to inoculate your cultures with **bacteria**! Add **10 µl** of an overnight culture of ***Escherichia coli* strain MC4100** in LB broth to each of your **16 cultures** using good aseptic technique. Place the 250-ml flasks in the environmental shaker at 37˚ C. Shake at 120 rpm to grow **aerobically**.

6. Cap the culture tubes containing 10 ml of M9 minimal medium tightly, and place them into an incubator set at 37˚ C to be grown **anaerobically**. These standing cultures will not be shaken.

7. Incubate both sets of cultures for ~24 hours.

Day 1: The day of your scheduled laboratory period.

After incubation, determine the amount of growth in each culture by turbidity measurement, an indirect measurement of growth using the spectrophotometer.

8. Transfer a **1 ml** sample from a flask or a tube to a disposable cuvette and measure the turbidity using a spectrophotometer. Set the wavelength to 600 nm. What solution should you use as a blank to set zero absorbance? (Note that you have a 100 ml bottle containing M9 minimal medium without glucose. Use this for your spectrophotometer blank because we are measing cell density, and glucose will not alter the absorbance at 600 nm.) For both the aerobic and anaerobic cultures, make sure that the bacteria are evenly distributed in the vessel. For the flasks you may swirl, and invert the sealed tubes a few times prior to taking the sample. MIX GENTLY so that you do not get air bubbles into the culture, which might adversely affect your results.

9. Construct a growth response curve by plotting the absorbance units as the y-axis and the FINAL molar glucose concentration as the x-axis using JMP or Excel. You should have growth response curves for both the aerobic and anaerobic cultures. (Make sure that your group measures the absorbance values for the **unknown** glucose concentration cultures.)

10. Determine the equation of the line for the linear portion of the dose response curves for both the aerobic and anaerobic cultures.

11. Using the equations describing the linear portions of the aerobic and anaerobic cultures, determine the **unknown** glucose concentration.

12. Compare the efficiency of utilization of glucose aerobically (shaken 100 ml culture) and anaerobically (static 10 ml culture) by determining the following calculations for each condition. Remember that your calculations will only be valid while the glucose is a limited nutrient, i.e., **during the linear portion of the curve**.

    Specific Yield = grams dry weight of bacteria/grams of limiting nutrient (glucose)

    Molar Yield = grams dry weight of bacteria/mole of limiting nutrient (glucose)

    $$\frac{\text{Moles ATP}}{\text{Moles glucose}} = \frac{\text{Molar Yield}}{Y_{ATP}}$$

Some information you might find useful:

A_{600} conversion factor: 1 A_{600} = 0.845 mg dry weight cells/ml
MW glucose = 180 g/mole
Y_{ATP} (grams of dry weight bacteria/mole of ATP) = 10.5 g/mole

Lab Report

The data tables and answers to questions are to be turned in by the end of the laboratory period.

List your absorbance values for the 16 cultures in the table below:

Aerobic Culture	**1 mM**	**2 mM**	**3 mM**	**4 mM**	**5 mM**	**10 mM**	**20 mM**	**Unkn**
A_{600}								
Anaerobic Culture	**1 mM**	**2 mM**	**3 mM**	**4 mM**	**5 mM**	**10 mM**	**20 mM**	**Unkn**
A_{600}								

Summarize your calculations in the following table. Include in your analysis a well-labeled graph, with an informative figure legend, of the final molar glucose concentration versus the A_{600} for both the aerobic and anaerobic cultures. Use **one point** on the linear portion of each curve to fill in the table below. Please show calculations on a separate sheet. Include the calculations and the graphs with your report.

	Aerobic Culture	Anaerobic Culture
Specific Yield		
Molar Yield		
Moles ATP/ Mole Glucose		

QUESTIONS

1. Which unknown glucose solution did you use, and what was its original concentration? Present the equation of the line for the standard curve used to make this determination.

2. Which is more efficient – aerobic growth or anaerobic growth? Explain your answer. How are the bacteria growing if they are not growing aerobically?

3. What is meant by a "limiting nutrient"? In this experiment, at what concentration is glucose no longer limiting?

4. Were the anaerobic cultures truly anaerobic or were they oxygen-limited? Explain your answer.

Week 3: Transposon mutagenesis in *Vibrio fischeri*

INTRODUCTION

Transposon mutagenesis is a powerful genetic tool for studying biological problems in bacteria. In this procedure, random mutations are generated in the chromosome or plasmid of the bacterium and researchers screen for the loss of a particular function. Transposons commonly carry selectable markers, most often antibiotic resistance genes. In this procedure, a collection of mutants is generated, each containing a single transposon insertion. One distinct advantage of transposon mutagenesis is that the selectable marker can be used to locate the site of insertion and can be used to clone the gene of interest.

Transposon mutagenesis is often facilitated by the transfer of a conjugative plasmid containing a transposon to a recipient strain. The donor for this experiment, *E. coli* strain S17-1 contains the genes necessary to assemble the sex pilus and transfer the plasmid pLOFKm containing the mini Tn*10*Km transposon. The origin of replication of pLOFKm is recognized by the π protein, encoded by the *pir* gene, in strain S17-1 that is not found in the *Vibrio fischeri* recipient. Thus the plasmid pLOFKm will not replicate inside the recipient strain and acquisition of the selectable marker will only occur if the transposon inserts into the chromosome of *Vibrio fischeri*. pLOFKm contains an IPTG-inducible promoter upstream of a transposase. The transposon itself contains a kanamycin resistance gene. On the same plasmid, but not in the transposon, there is an ampicillin resistance gene.

The biological problem we will address using transposon mutagenesis is: **What genes are necessary for the production of light by the marine bacterium *Vibrio fischeri?***

This particular wild type *Vibrio* species was isolated out of the gut of a squid from the Oregon Coast. *Vibrio fischeri* is a Gram-negative bent rod, or vibrio, which is a symbiotic bacterium found in the intestines of many marine organisms. It colonizes the light organs of many fish as well as the bobtailed squid *Euprymna scolopes*. We will conjugate the plasmid pLOFKm into the *Vibrio*, selecting for kanamycin resistance conferred from the insertion of the transposon. We will then screen for loss of bioluminescence, or light production.

A couple of things to keep in mind while working on this lab—*V. fischeri* is heat and cold sensitive. **Do not refrigerate any of the plates or samples**. Also, use only the 30˚ C incubator. Since *V. fischeri* is a relatively slow growing bacterium, contamination is a big problem— always use good aseptic technique.

References

1. Herrero *et al*., 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in Gram-negative bacteria. *Journal of Bacteriology* **172**: 6557-6567.

2. Bassler BL, 1999. How bacteria talk to each other: regulation of gene expression by quorum sensing. *Current Opinion in Microbiology* **2**: 582-587.

MATERIALS AND METHODS – work in **pairs**

E. coli strain S17-1(pLOFKm) grown in HI broth with 100 μg/ml ampicillin and 50 μg/ml kanamycin
Vibrio fischeri grown in HI broth containing 50 μg/ml rifampicin
Sterile microcentrifuge tubes for pelleting bacteria and serial dilutions
HI broth in 100 ml bottles
One HI agar plate containing 0.2 mM IPTG
Inoculating loop
Bunsen burners
Strikers
Turntables, ethanol and Pasteur pipettes for spreading
HI agar plates with 100 μg/ml rifampicin and 100 μg/ml kanamycin for mutant selection
HI agar plates with 100 μg/ml rifampicin as a control - one plate per group
HI agar plates with 50 μg/ml kanamycin as a control - one plate per group
HI agar plates as a control – one plate per group
P-1000 Micropipettors
P-100 Micropipettors
Sterile blue tips
Sterile yellow tips
Environmental shaker set to 30˚ C
30˚ C incubator

PROCEDURE

Day 0: The day prior to your scheduled laboratory period.

Mating

1. Pellet the ***E. coli* strain S17-1 (pLOFKm) Km^R, Amp^R (donor)** and ***V. fischeri* Rif^R (recipient)** bacteria by transferring 1 ml each of overnight cultures provided by the stockroom to two separate, sterile 1.5-ml centrifuge tubes. Spin at maximum rpm for 10 seconds.

2. Discard the supernatants.

3. Resuspend the *E. coli* strain S17-1 (pLOFKm) in 100 μl HI broth.

4. Add this to the pelleted *V. fischeri*, and resuspend it. Resuspend gently as the *V. fischeri* are somewhat delicate.

5. Gently pipette the mixture of the two cultures onto an HI plate containing 0.2 mM IPTG (to induce expression of the transposase encoded on the plasmid pLOFKm). Spread the bacterial suspension as little as possible.

6. Place the plate in a location so that you will not have to move it for 15 minutes.

7. **Carefully place the plate right side up in the 30° C incubator, and incubate for 18-24 hours**.

Day 1: The day of your regularly scheduled laboratory period.

Selection

8. After overnight growth, harvest the bacteria using a sterile cotton swab to scrape them off the HI agar. Resuspend the mating mix in a 1.5-ml microcentrifuge tube containing 500 µl HI broth. Bring the total volume of the harvested bacteria to ~1 ml.

9. Perform serial dilutions by pipetting 100 µl of the suspension into 900 µl of HI broth (to make a 10^{-1} dilution). Mix by gentle vortexing. Place 100 µl of the 10^{-1} dilution into a second microcentrifuge tube containing 900 µl of HI broth (to make a 10^{-2} dilution). Continue the serial dilutions to a final dilution of 10^{-6}. Incubate plates right side up.

10. Select for *V. fischeri* transconjugants containing transposon insertions by spreading 100 µl of the 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions on **4** separate HI agar plates containing 100 µg/ml rifampicn and 50 µg/ml kanamycin**. Incubate in the 30° C incubator ~48 hrs until well-formed colonies, approximately 1 to 2 mm in diameter, are visible**.

11. As a **control** to check your selective medium, plate the donor and recipient strains on HI agar and HI agar containing either 100 µg/ml rifampicin or 50 µg/ml kanamycin. Draw a line down the middle of each selective plate, and streak both the donor and recipient on each plate. Each group should have **3** control plates.

Day 3

Phenotypes of the transconjugants

12. Take the HI agar plates containing rifampicin and kanamycin into a darkened room and look for *V. fischeri* transconjugants.

The transconjugants will be colonies of rifampicin resistant *V. fischeri* containing mini Tn*10*Km transposon insertions, and will thus be resistant to both rifampicin and kanamycin. Transposition by Tn*10*Km from the plasmid pLOF/Km after conjugal transfer from the kanamycin resistant donor *E. coli* S17-1(pLOFKm) will confer kanamycin resistance upon the recipient strain. The recipeint *V. fischeri* was initially resistant to rifampicin whereas the donor *E. coli* strain was sensitive to this antibiotic. Thus the donor *E. coli* strain should not grow on a medium containing rifampicin.

Most of the transconjugants on the plates should be bioluminescent. Those that are not bioluminescent are candidates for having transposon insertions in genes necessary for the production of light in *V. fischeri.* However, some of the non-bioluminescent bacteria may be colonies of the donor *E. coli* strain.

Lab Report

Clearly state the objective of the experiment. Present a well-labeled table containing the number of transconjugants on each of the dilutions on selective agar plates, i.e., HI plus 100 μg/ml rifampicin and 50 μg/ml kanamycin. Describe the results of the controls, i.e., whether your donor or recipient strains grew on HI, HI plus rifampicin or HI plus kanamycin.

QUESTIONS

1. How many *V. fischeri* transposon mutants would you need to screen to ensure that you mutagenized every gene on the chromosome? Assume that the *V. fischeri* genome is ~5 Mb or 5 X 10^6 base pairs (bp), and that an average gene is 1 kb, or 1 X 10^3 bp in length.

2. Once you have acquired transposon mutants of *V. fischeri* that do not produce light, how would you identify the genes putatively involved in bioluminescence? Remember that the **genome sequence** of this strain of *V. fischeri* **has not been determined**!

3. Pose an explanation as to why you might see *E. coli* donor strains on the selective plates containing the *V. fischeri* transconjugants on **Day 3**.

Week 4: Microbial Diversity

INTRODUCTION

The normal microbiota, or microbiome plays an important role in human health. The microbiome influences nutrition, resistance to colonization by microbial pathogens, carcinogenesis, obesity and is even thought to affect human behavior. An accurate understanding of these roles, and the nature of the interactions among and between individual members and the host, requires knowledge of the composition of the microbial community. Many studies of environmental microbial communities have demonstrated the limitations of cultivation-dependent methods in determining community composition. Environmental surveys based on acquisition of phylogenetically useful microbial sequences such as that of the 16S rRNA gene (16S rDNA) have revealed a great deal of previously unsuspected bacterial and archaeal diversity. In most instances, the cultivated members represent less than 1% of the total extant population. Broad range small subunit rDNA PCR methods have also revealed cultivation-resistant pathogens in disease settings. Despite the limitations of this approach, most surveys of the human endogenous bacterial microbiota, historically, have relied on cultivation.

Using cultivation techniques, nearly 500 bacterial strains have been recovered from the human subgingival crevice, a particularly well-studied microbial niche. Many of these bacteria are thought to be commensals, and a smaller number, opportunistic pathogens. Local disease, including dental caries, gingivitis, and periodontitis, has prompted most examinations of the oral bacterial flora. These diseases are associated with changes in both local bacterial density and species composition.

In order to investigate the bacterial microbiota of the subgingival crevice, we will use PCR amplification of 16S rDNA sequences in order to identify molecularly these bacterial genera, as well as classical culture techniques. By these methodologies we will initiate characterization of the bacterial diversity within a specimen from the human subgingival crevice. We predict that our results will reveal a significantly broader diversity of bacterial 16S rDNA sequence types (phylotypes) using the cultivation-independent approach, although both methods can identify previously uncharacterized phylotypes and should be viewed as complementary.

This laboratory exercise was adapted from Kroes, I., Lepp, P.W. and D.A. Relman (1999) Bacterial diversity within the human subgingival crevice. *Proceedings of the National Academy of Sciences USA*. **96:** 14547–14552.

MATERIALS AND METHODS – work in **pairs**

Sterile molecular biology grade water
Sterile nutrient broth
Microcentrifuge tubes
Sterile toothpicks
Microcentrifuge racks
Inoculating loops
Microscopes
Microscope kits
Stage micrometers
Materials for performing Gram stain
2 Thermalcyclers with heated lids
Enzyme cold boxes
0.8% agarose in 1 X TAE Buffer, pH 8.0
1 X TAE Buffer, pH 8.0
Electrophoresis apparatuses
6X DNA Gel-Loading Buffer (Sambrook, Frisch and Maniatis)
SYBR Safe DNA gel dye
DNA ladder (NEB 1Kb Plus Ladder)
Gel documentation system
Cultures of *E. coli* strain MC4100 and *Staphylococcus epidermidis* for quality control of LB agar plates containing 100 μg/ml streptomycin prepared in **Week 1**

PROCEDURE

Day 0: The day prior to your schedule laboratory period.

1. Using good aseptic technique, each person will dispense 1-ml of nutrient broth into a microcentrifuge tube.

2. Collect a subgingival specimen from the surface of a tooth using a sterile toothpick as demonstrated in class. Rub the end of the toothpick on the inside of the centrifuge tube containing the nutrient broth.

3. Label the tube with your name and date, and place it in the 37˚ C incubator in a rack. Only one sample will be used for PCR, but both samples can be subjected to Gram staining.

Day 1: The day of your scheduled laboratory period.

4. Set up three PCR mixtures using broadly conserved bacterial 16S rDNA primers.

Table 1. Oligonucleotide primers*

Primers	Sequence (5' - 3')
SDBact0338aA18 †	ACTCCTACGGGAGGCAGC
SDBact0515aA19 ‡	GTGCCSGCMGCCGCGGTAA
SDBact1371aS20	AGGCCCGGGAACGTATTCAC
SDBact1525aS17 ‡	AAGGAGGTGATCCAGCC

*Primers and probes are named according to Oligonucleotide Probe Database convention and are described in Kroes *et al*., 1999. The names incorporate the 59 terminal position (*E. coli* 16S rRNA numbering convention) and primer length in nucleotides.
† Orientation indicates PCR primer sequence
‡ Modified from indicated reference.

PCR Mixture:	Primer Pair:	Annealing Temperature:
1	SDBact0338aA18 (Fwd) SDBact1525aS17 (Rev)	55°C
2	SDBact0515aA19 SDBact1371aS20	55°C
3	SDBact0515aA19 SDBact1525aS17	55°C

5. Set up the three PCR mixtures in a total of 100 µl each in 0.5 ml thin-walled PCR tubes at room temperature. Please note that all components for the PCR are on the front bench, and will not be aliquoted into small volumes.

Template	5 µl of sample (overnight culture in nutrient broth – vortex gently before addition)
Fwd Primer	10 µl of 2 µM stock solution
Rev Primer	10 µl of 2 µM stock solution
10 X Buffer with $MgCl_2$	10 µl
dNTPs	2 µl of 10 mM stock solution
Milli Q sterile H_20	62.5 µl
Total volume:	99.5 µl

6. Start with dispensing the sterile water into each of the thin-walled PCR tubes, then carefully add the other components listed above.

7. Gently mix the above reaction mixture by flicking the tube with your finger or gently vortexing.

8. *Taq* Polymerase, the enzyme, is always added last. Using a micropipettor, pipette 1.0 µl of *Taq* Polymerase enzyme into each tube.

9. Gently mix by flicking or by gently aspirating up and down with a P-100 pipettor and a yellow tip. **Do not vortex!**

10. Program the Thermalcyclers as follows:

<u>For Reactions 1-3</u>

Step 1	Hot Start	94°C	3 minutes
Step 2	Denaturation	94°C	30 seconds
Step 3	Annealing	55°C	30 seconds
Step 4	Extension	72°C	30 seconds
Step 5	Return to Step 2 twenty-nine times for a total of 30 cycles		
Step 6	End		

11. Start the thermalcycler by selecting your program and press <Proceed>. Allow the cycler to reach 94°C **before** you place the tubes in the holes.

12. When it reaches 94°C, put in the tubes and let 'em rip.

13. While the PCR is running pour a 0.8% agarose gel. Start by melting the agarose in the microwave- the <popcorn> setting works well for this. Stop the microwave after a few seconds, and swirl the bottles gently to make sure the agarose is completely melted. Wear a hot mitt and be careful!

14. Allow the agarose to cool slightly on the bench top so that you can pick the bottle up without the hot mitt.

15. Before the agarose begins to solidify, pour ~25 ml into a gel apparatus containing the appropriate comb.

16. Immediately place 3 µl of SYBR Safe DNA gel dye directly into the agarose. Spread thoroughly with the fat end of a yellow pipette tip.

17. Allow agarose to solidify before loading samples.

Microscopy

While you are waiting for the PCRs perform a **Gram stain** on the microbes that you sampled from your subgingival crevice, and grew in an overnight nutrient broth culture. Observe the specimens under brightfield microscopy.

Recall that in 1884 the Danish bacteriologist Christian Gram developed a staining technique that separates bacteria into two groups: those that are Gram-positive and those that are Gram-negative. The procedure is based on the ability of microorganisms to retain the purple color of crystal violet during decolorization with alcohol. Gram-positive bacteria are not decolorized and remain purple. After decolorization, safranin, a red counterstain, is used to impart a pink color to the decolorized Gram-negative bacteria.

Follow the Gram staining procedure illustrated on the handout provided. Of all the staining techniques you will use in the identification of bacterial specimens, Gram staining is the most important tool. Although this technique seems quite simple, performing it with a high degree of reliability requires some practice and experience. Here are two suggestions that can be helpful: first, don't make you smears too thick. Second, pay particular attention to the comments in the decolorization step.

Using the Gram stain you can determine a microbe's cell shape, arrangement of the cells and Gram stain status. Obtain a **stage micrometer** from the front bench and use this along with the ocular micrometer in your microscope to determine the size of the microbes observed in the Gram stain.

Agarose Gel Electrophoresis

1. Remove 20 µl of your completed PCR mixtures and combine with 4 µl 6X DNA Gel-Loading Buffer (1:5 or 1/6 of the total sample volume).

2. Flick the tubes to mix, and load samples into a wells in a 0.8% agarose gel in 1X TAE buffer, pH 8.0. If the well will not hold the full 24 µl, simply fill it to a maximum volume. Don't overload the well so that the sample spills into other wells; this will confuse your results.

3. Prepare the molecular weight marker by combining 1 µl of a DNA ladder (NEB 1Kb Plus Ladder) with 4 µl of sterile water. Add 1 µl of DNA Gel-Loading Buffer and mix. Load the 6 µl of the molecular weight marker into one of the wells in the gel.

4. Turn on the power supply, approximately 15 volts per cm (distance is measured between the two electrodes). You can run most gels at around 120 volts.

5. After the lower dye front is about two-thirds down the gel, turn off the current.

6. Photograph the gel using the Gel documentation system. Consult handout if you have not previously used the Gel documentation system. Communities of 16s rRNA genes should appear as ~1 kb bands. Dispose of the gel in the regular trash.

Quality Control

Your final task of the day will be to make sure that the LB agar plates supplemented with 100 μg/ml streptomycin, that you poured in **Week 1**, are indeed selective.

1. Obtain two of your LB plus streptomycin plates from **Week 1**.

2. Using a sharpie, label them with your name, and the date. On one plate write *E. coli* strain MC4100 and on the second plate write *S. epidermidis*. The *E. coli* strain should be resistant to streptomycin while *S. epidermidis* should be sensitive.

3. Inoculate each species onto its own plate using the quadrant streak.

4. Incubate the cultures at 37˚ C overnight.

5. On **Day 2**, record the results. Is your LB plus streptomycin selective?

Lab Report

Clearly state the objective of the laboratory exercise. Present an image of the gel, including the molecular weight of bands of the DNA ladder (available at the instructor's bench) and present a well-written figure legend to accompany the image. Also draw and describe what you observed by Gram stain using brightfield microscopy. Describe the microbes' **size**, shape, arrangement and Gram stain status. Stage micrometers are on the instructor's bench as well.

QUESTIONS

1. Did you observe mostly prokaryotic or eukaryotic organisms by the Gram staining procedure? How do you know this?

2. Starting from the DNA bands on the gel, how would you identify the community of bacterial species residing on your tooth surface? Remember that each band represents a population of DNA fragments.

3. Assume that you sequenced all of the 16S rDNAs from organisms found in the tooth scraping. If you searched long enough, could you eventually find all the corresponding organisms by *culturing* them? In other words, would you expect the number of unique organisms identified molecularly and those identified by culturing to be the same?

Lab reports are due at the beginning of your next laboratory period.

Weeks 5 and 6: Bacteriophage

Viruses are obligate intracellular parasites. They infect living organisms from all three domains of life on Earth: Bacteria, Archaea and Eukaryotes. Viruses that infect bacteria are known as bacteriophage, or simply phage. Like all viruses, a phage is composed of a protein coat and a core of nucleic acid, which can be either DNA or RNA, single-stranded or double-stranded. All viruses will, however, have only one type of nucleic acid in their genome.

Because they are obligate intracellular parasites, bacteriophage are found where their bacterial hosts dwell. Sewage contains a high bacterial population, and thus also contains many phages, especially those that infect enteric organisms. For the **first** portion of the exercise, **Part A**, we will **isolate phage** that infect *E. coli* from raw sewage.

Bacteriophages are also important tools for the genetic manipulation of bacteria. Horizontal gene transfer, or the transfer of genetic material from one vegetative cell to another, mediated by phage is known as **transduction**. There are **generalized** and **specialized** transducing phage. We will discuss these two types of transduction in lecture.

For the **second** portion of this laboratory exercise, **Part B**, we will use the **specialized transducing phage** λRS45 (1) to transfer a *lacZ* reporter gene fusion, initially in ~30 copies on a multi-copy plasmid, to a chromosomal location in *E. coli* so that only a single copy of the promoter fused to *lacZ* remains. The parent plasmid used for this exercise is pRS551, a derivative of pBR322 with an MB1 origin of replication governing a copy number of ~30 per bacterium. The reporter gene construct is the *LEE1-lacZ* transcriptional fusion containing the promoter of the *LEE1* operon of enteropathogenic *E. coli* (EPEC) in the pRS551-derived pJLM164 plasmid (2). The *LEE1-lacZ* fusion will be transduced into the *Δlac* strain MC4100 using λRS45.

Parts A and B are both **two-week exercises** and will be performed simultaneously, beginning in **Week 5**. An overview of the required steps is presented on the following page.

References

1. Simons, R.W., Houman, F., and Kleckner, N. (1987) Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene* **53**: 85-96.

2. Mellies, JL, Elliott, SJ, Sperandio, V; Donnenberg, MS, and Kaper, JB. (1999) The Per regulon of enteropathogenic *Escherichia coli*: identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). *Molecular Microbiology* **33**: 296-308.

Procedure for isolating bacteriophage from sewage adapted from exercises developed by Wiltraud Pfeiffer, Ph.D., UC Davis.

OVERVIEW OF WEEKS 5 AND 6 LABORATORY EXERCISES

		A. Isolation of Phage	**B. Transduction**
	Day 0	Inoculate MC4100	-
Week 5	**Day 1**	Overlay sewage	Transducing lysate
	Day 2	Purify phage	Transducing phage harvest
	Day 3	Plates in refrigerator	
Week 6	**Day 0**	High titer lysates	Inoculate MC4100
	Day 1	Titration/Host specificity	Transduction
	Day 2	Count plaques/Results on board	Screen transductants
	Day 3	-	Results

Week 5

A. Isolation of Phage from Sewage

INTRODUCTION

Raw sewage contains between ~500 and 15,000 phage particles per ml. We obtain this material from the Portland Wastewater Treatment Plant on N Columbia Blvd. The sewage was filtered with a 0.2 μm filter in order to remove bacteria, but to allow the phage to pass through the filtrate because they are, on average ~50 to 100 nm in size while the bacteria are ~ 1 μm in width.

The phage are combined with a host bacterium, in this case *E. coli* strain MC4100, and placed in top agar on a nutrient agar plate. Lytic phage lyse, or break open the bacterium releasing ~100 to 200 newly synthesized phage particles and form a clearing on the agar plate called a plaque. The plaques result from single phage particle infecting the *E. coli*, and are termed a plaque forming unit, or PFU. Counting the number of plaques on serial dilution plates will indicate the number of PFU/ml in the original sewage sample.

For imaging our phage by electron microscopy (EM), we will isolate a single plaque, hopefully containing a single type of phage particle and amplify its numbers by creating a high titer lysate. The number of PFU/ml for the high titer lysate will be determined. We will precipitate the phage to concentrate and then place them on small copper grids for imaging at the OHSU EM Facility.

Handle the filtered sewage with care, and be sure to wash your hands, or use the hand sanitizer at the end of the exercise.

MATERIALS AND METHODS – work in **pairs**

Filtered sewage
Overnight culture of *E. coli* strain MC4100 F^- *araD139 Δ(argF-lac)U169 rpsL150* (**Str^R**) *relA1 flbB5301 deoC1 ptsF25 rbsR*
LB top agar (contains 0.5 X bacto agar) with 100 μg/ml streptomycin
LB agar plates with 100 μg/ml streptomycin (prepared in **Week 1**)
LB medium in 100 ml bottles
12-ml snap top culture tubes
100 mg/ml streptomycin stock solution
λ diluent (10 mM Tris, pH 7.5, and 10 mM $MgSO_4$)
0.01 M $MgSO_4$
Disposable 5 ml polypropylene tubes with caps
Microcentrifuge tubes
Pasteur pipettes (long) to make spreaders
Sterile Pasteur pipettes (short) for making high titer lysates
Inoculating needles for plaque purification
0.20 μm filters and 5-ml syringes
Sterile cotton swabs
Bunsen burners and turntables
Strikers

100% ethanol
48˚ C bead bath
Nutrient agar plates to check host specificity – **two per group**
Nutrient agar cultures of the following strains to check host specificity: *Bacillus subtilis*, *Staphylococcus epidermidis, Pseudomonas fluorescens, E. coli* strain MC4100
Materials and supplies for electron microscopy

PROCEDURE

Day 0: The day prior to your scheduled laboratory period.

Inoculation of a culture of *E. coli* strain MC4100 in LB broth with 100 μg/ml streptomycin

1. Using good aseptic technique, place 3 ml of LB broth into a 12-ml snap top culture tube.

2. Add 3 μl of a 100 mg/ml stock streptomycin solution for a final concentration of 100 μg/ml streptomycin.

3. With an inoculating loop, place one colony of the MC4100 strain from an LB plate into the LB broth with 100 μg/ml streptomycin.

4. **Incubate overnight at 37˚ C with shaking at 200 rpm**.

Day 1: The day of your scheduled laboratory period.

Overlay sewage

5. Loosen the top of a bottle containing top agar with 100 μg/ml streptomycin, and carefully melt the top agar in the microwave. (Consult the instructor prior to beginning this step.) After it is completely melted, gently tighten the cap, and place in the 48˚ C bead bath.

6. Label 3 LB plus 100 μg/ml streptomycin agar plates 0.1 ml, 0.5 ml, and 1.0 ml. Place them in the 37˚ C incubator to warm.

7. Obtain some filtered sewage (about 2 ml) in a 12-ml snap top tube and place it in the 48˚ C bead bath.

8. Label three 5-ml culture tubes 0.1 ml, 0.5 ml, and 1.0 ml and add 0.3 ml of an overnight culture of *E. coli* strain MC4100 to each tube.

9. To the MC4100 host cells add the 0.1 ml, 0.5 ml, and 1.0 ml volumes of the warmed sewage to the pre-labeled tubes.

10. Pipette 2.5 ml of melted, 48˚ C LB top agar with 100 μg/ml streptomycin into the labeled culture tubes. **Do one tube at a time** so that the agar does not solidify before you are able to pour onto the agar plate!

11. With the cap on, invert the tube of top agar plus host bacteria and phage to mix. **Immediately** pour the entire contents of the tube over the surface of the corresponding, pre-labeled, pre-warmed to 37˚ C bottom agar plate with 100 μg/ml streptomycin.

12. Quickly, but gently rock the plate to spread the agar over the surface then leave the plate on a level surface until the top agar has solidified. Repeat for the other plates.

13. After the plates have hardened, incubate them at 37˚ C for 24 hours.

14. Inoculate a 3-ml culture of *E. coli* strain MC4100 in LB with 100 μg/ml streptomycin for use in the phage purification on **Day 2**. Follow the identical procedure you used on **Day 0** using a 12-ml snap top tube, 3 ml of LB broth and 3 μl of 100 mg/ml streptomycin. Incubate with shaking at 37 ˚ C overnight.

Day 2

Purify phage

15. After incubation, examine the plates for phage plaques. Look to see how many different types of plaques are visible**.**

16. **Record** the number of plaques on the plates and determine the number of PFU per ml of sewage that infected the *E. coli* host.

17. Choose one type of plaque and purify the phage by picking the plaque with an **inoculating needle**. (A large plaque is easier to work with, but a small plaque may turn out to be more interesting because small plaques are usually formed by large phage that can't diffuse as readily.) This is done by sticking the sterilized needle down through the center of the plaque, being careful not to touch surrounding bacteria, and transferring this to 1 ml of λ diluent in a 5-ml tube. **Agitate the needle in the diluent in order to wash off the phage particles.** Allow to stand for 10 minutes at room temperature to allow the phage to diffuse out of the agar.

18. Check the purity of the phage suspension by plating 10^{-2}, 10^{-3}, $10^{-4}$and 10^{-5} dilutions. For example, for a 10:1, or 10^{-1} dilution, add 100 μl of the undiluted phage to 900 μl of λ diluent. Perform serial dilutions to 10^{-5}.

19. Add 0.1 ml of each phage dilution to 0.3 ml of *E. coli* strain MC4100 in a 5-ml tube.

20. **One at a time**, add 2.5 ml of pre-warmed, 48˚C LB top agar with 100 μg/ml streptomycin to the 5-ml tubes containing the *E. coli* and phage dilutions, and pour onto LB agar with 100 μg/ml streptomycin. Follow the same procedure you did in plating the original filtered sewage (**Day 1**, steps 10 to 13).

21. Incubate the plates at 37˚ C overnight after the top agar solidifies.

Day 3

22. Inspect for purity after overnight growth. Do all of the plaques appear identical?

23. **Place the plate(s) with nicely separated plaques, or those of particular interest, in the refrigerator at 4˚ C for use in Week 6**. **Do not allow plates to incubate longer than ~18 hours as they will get contaminated!**

Week 5 - Continued

B. Specialized Transduction

INTRODUCTION

Reporter gene fusions are useful for understanding genetic regulation in both prokaryotic and eukaryotic systems. Single-copy reporter gene fusions are advantageous over multi-copy, plasmid constructs for the study of prokaryotic regulatory events because, in most instances, they better mimic the chromosomal location of the gene being studied. Titration of regulatory proteins, whether activators or repressors, can occur using multiple copies of a reporter gene because there are also multiple copies of a gene's regulatory region, or operators on these DNA segments. The ~30 copies of a regulatory regions associated with the pRS551-derived plasmids (Fig. 1) can bind regulatory proteins, which are generally found in low copy in the bacterium. Regulatory observations from multi-copy systems thus can be misleading because they do not represent the natural, single-copy state at which most genes exist.

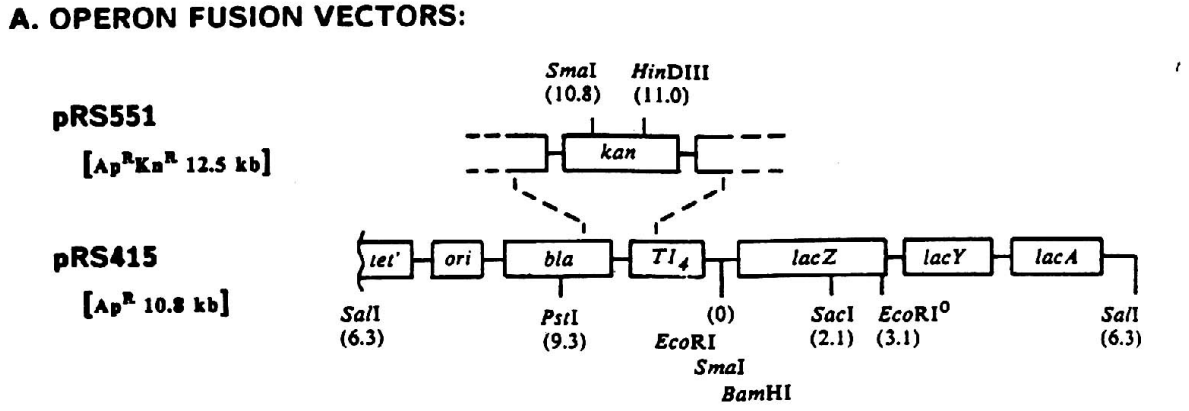


Figure 1. **The pRS415 and pRS551 *lacZYA* reporter gene fusion vectors.** Plasmid pRS551 was constructed by placing the kanamycin resistance cassette (*kan)* between the *bla* gene encoding ampicillin resistance and the four T1 transcriptional terminators.

The genetic system described herein allows reporter gene fusions to be constructed in multi-copy, in this case ~30 copies in pRS551, and then transduced to single-copy into the *E. coli* strain **MC4100**, with the *lac* operon deleted. Thus, after transduction, all of the β-galactosidase activity is derived from the single-copy, reporter gene fusion you have constructed. Briefly, your promoter of interest, in this case the *LEE1* promoter from EPEC, is cloned into the **pRS551** vector using the *Eco*RI and *Bam*HI restriction sites to create plasmid **pJLM164**. We will produce transducing lysates using the **donor strain MC4100 (pJLM164)** with the specialized transducing **phage λRS45**. In this step *LEE1-lacZ* fusion recombines into the phage genome (Fig. 2). A relatively high percentage, approximately 1 in 10,000, of the harvested phage particles will be recombinants containing the *LEE1-lacZ* fusion. The terminal sequences for recombination, those found both in the pJLM164 plasmid and the recombinant lRS45 genome are the *kan* gene and the *lacZYA* locus. The *bla'* indicates that this gene is truncated, and non-functional in the phage, while the *lacZ* gene is also non-functional in the phage genome. Upon

transduction, the resulting bacteria are resistant to kanamycin, ampicillin sensitive and have a functional *lacZYA* operon.

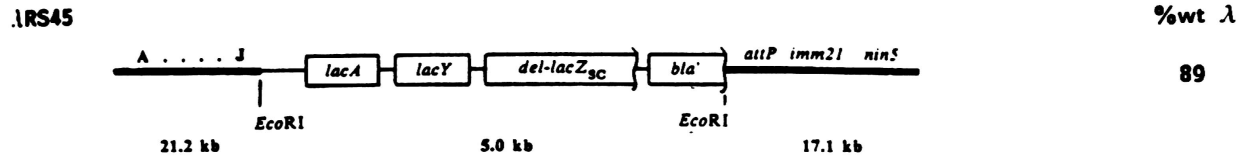


Figure 2. **The λRS45 specialized transducing phage.** Crossover events occur using the *bla'* gene and *lacA* to recombine in *E. coli* strains wt for *recA*, resulting in transductants that are resistant to kanamycin (from the plasmid pRS551), sensitive to ampicillin (*bla'*) and are able to utilize lactose as a carbon source. Site-specific recombination occurs with the *attP* and *attB* sites on the phage and bacterial genomes, respectively.

MATERIALS AND METHODS – work in **pairs**

λRS45 **(Store at 4˚ C)**
E. coli MC4100 (*araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 fblB3501 deoC1 ptsF25 rbsR*)
Overnight culture of MC4100 (pJLM164) amp^R , kan^R - pJLM164 contains a *LEE1-lacZ* fusion
LB plates with 50 μg/ml kanamycin and 30 μg/ml X-gal – 10 plates per group
LB plates with 100 μg/ml ampicillin - one plate per group
20 mg/ml maltose- sterile filtered
0.20 μm filters and 1-ml syringes
Microcentrifuge
Microcentrifuge tubes
48˚ C bead bath
Lots of sterile toothpicks
Numbering forms for plates – 50 spaces per plate
E. coli strains MC4100 amp^S kan^S, MC4100 (pRS551) amp^R kan^R, and MC4100 (pJLM164) amp^R kan^R as controls

PROCEDURE

Day 1: The day of your regularly scheduled laboratory period.

Preparation of phage λRS45 lysates on MC4100 (pJLM164)

1. Centrifuge 1 ml of an overnight culture (grown at 37˚ C with shaking) of the donor strain MC4100 (pJLM164) at 8000 rpm in a microcentrifuge for 5 minutes.

2. Discard the supernatant and resuspend in 0.5 ml 0.01 M $MgSO_4$.

3. In 3 separate, 5-ml snap top culture tubes, combine 50 µl of the donor bacteria with 3 different volumes of λRS45 phage lysate: 0.1 µl, 0.5 µl, and 1.0 µl. (Note the needed dilution to dispense the 0.1 µl volume.) Approximately 1 X 10^7 PFU of phage should be combined with 50 µl of the host bacteria. Therefore, the 3 different volumes of phage lysate will ensure that you have a plate containing the proper ratio of phage to bacteria for adequate phage harvest.

4. Allow the phage particles to adsorb for 10 minutes in the 37˚ C incubator (not the environmental shaker).

5. Place 2.5 ml of melted LB top agar with 100 µg/ml streptomycin (maintained at 48˚ C) into each tube and overlay on LB agar plates. (You will get more even distribution of the top agar across the top of the LB agar plate if you pre-warm the LB plates at 37˚ C a few minutes prior to the overlay.)

6. Allow the top agar to solidify and incubate plates at 37˚ C overnight.

Day 2

Transducing phage harvest

7. After overnight incubation, phage particles are harvested by scraping the top agar from **one** of the plates labeled 0.1, 0.5 or 1.0 using a sterile spatula (carefully dip the spatula in 100% ethanol and flame to sterilize) and placing it in a 1.5 ml microcentrifuge tube. **Avoid harvesting the bottom agar.** The highest phage titer will appear in the region of a plate where a small portion of the bacterial lawn is still visible.

8. Fill the 1.5 ml microcentrifuge tube about half full with the phage-containing top agar.

9. Add λ diluent to 1.2 ml, vortex gently and place at 4˚ C. **This is the refrigerator, not the freezer!**

Week 6

A. Isolation of Phage from Sewage

PROCEDURE

Day 0: The day prior to your scheduled laboratory period.

Preparation of high titer phage lysates

Take plates out of the **refrigerator** from **Day 3 of Week 5** and examine the plaques. Count the number of plaques and determine the number of phage particles in the original 1 ml of broth. This represents the number of phage particles you picked up with your **inoculating needle**. **Record the titer**, or the number of plaque forming units (PFU) per ml.

24. Punch out a plaque from your phage plates by sticking a sterile pasteur pipette into it and sucking the top agar layer into the pipette. Suspend the plaque by gently agitating in 0.5 ml λ diluent in a sterile 12-ml culture tube. Incubate at room temperature for **10 minutes** to allow phage to diffuse into the liquid medium.

25. Add 0.1 ml of the overnight culture of host bacteria, *E. coli* strain MC4100, and incubate together for **10 minutes** at room temperature to allow the bacteriophage to adsorb to the host cells.

26. Add 4 ml LB plus 100 μg/ml streptomycin medium and incubate in the environmental shaker at 37˚ C overnight, until bacterial lysis occurs, indicated by a clearing or loss of turbidity. **You don't want the cultures to incubate too long, so please check in the morning!**

Day 1: The day of your regularly scheduled laboratory period.

Titration of the high titer lysates

27. Split your culture into four microcentrifuge tubes and spin at low speed (8000 rpm) for 10 minutes to remove the bacteria. Place the supernatant back into a sterile 12-ml culture tube.

28. Filter sterilize using a 0.20 μm filter with luer lock on a 5-ml syringe, placing the supernatant into a sterile **15-ml screw top tube**. Filtering is necessary to eliminate bacterial cells that may be contaminating the preparation, but will allow the phage to pass through.

29. Determine the titer by preparing a series of 100-fold dilutions (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-10} , and 10^{-12}) of the phage and plating 100 µl of the last three dilutions with host bacteria as described above (**Day 2 of Week 5**, step 18). You will therefore plate 100 µl of the 10^{-8}, 10^{-10}, 10^{-12} dilutions by adding 2.5 ml of melted top agar and 0.3 ml of an overnight culture of *E. coli* MC4100 to an LB agar plate containing 100 µg/ml streptomycin.

30. Allow the top agar to solidify and incubate at 37˚ C overnight. Come in the following day, **Day 2** to count the plaques. Note the titer of the high titer lysate in **PFU/ml**.

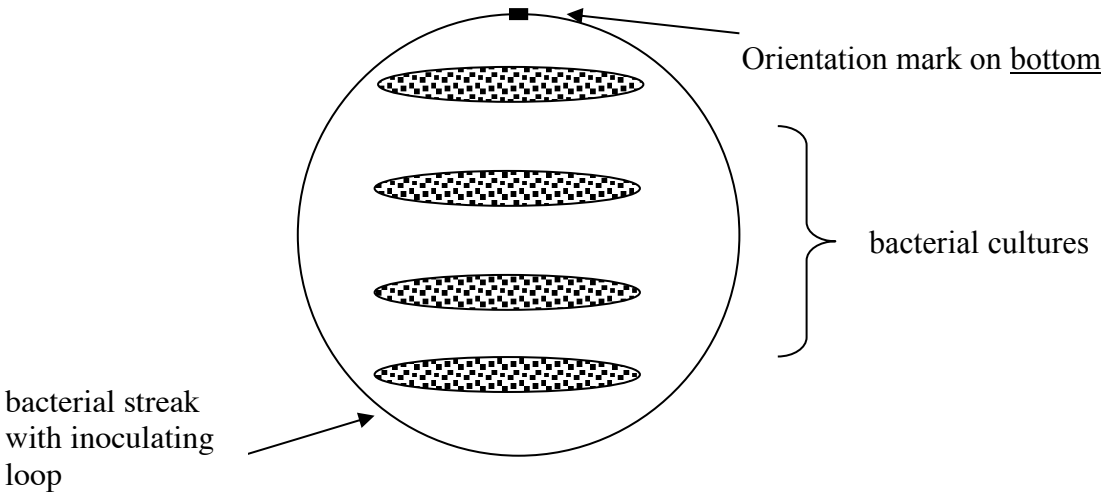
31. **Do not throw away your high titer lysate!** After completing the brief inoculation below to determine host specificity, make sure your tube is labeled with your name, lab day and date, and **store at 4˚ C- in the refrigerator. Placing the phage in the freezer will kill them!**

Host specificity

The host specificity of bacteriophage depends on the ability of the phage to recognize specific host cell surface receptor sites. Phages use these receptors for attachment when initiating an infection. The receptors can be almost any surface structure--pili, flagella, O-side chains of the LPS, transport and binding proteins, teichoic acids of the Gram-positive cell wall, *etc*. The receptor-phage interaction is very specific, involving the chemical properties and the three-dimensional physical structure of the cellular receptor and the phage structure that participates in the attachment.

Once the phage nucleic acid enters the bacterium, replication depends partly on the presence of compatible restriction/modification systems, as well as on the presence of other host functions needed by certain phages. You will test your phage isolate on several different host strains of bacteria to determine how specific your phage is in regard to its host.

32. Using an inoculating loop and good aseptic technique, streak the four different bacterial species (*Bacillus subtilis, Staphylococcus epidermidis, Pseudomonas fluorescens and Escherichia coli* MC4100) across a **Nutrient agar plate** as shown in the diagram below.



33. Also, to be completed on **Day 1**. With a P-100 micropipettor and yellow tip place a drop of your **high titer lysate** containing the phage on each of the bacterial streaks. Be sure to identify which streak represents which organism. Include an orientation mark on the plate, or write the host strains on the bottom of the plate, but not on the lid. **Make a second, identical plate** because you will incubate at two different temperatures.

34. Incubate one plate at **26**˚ **C** and the second at **37**˚ **C** overnight, both plates right side up.

Day 2

35. Record the PFU/ml of the high titer lysate and the results of the host specificity test. P**lace these results on the board** at the front of the class.

Week 6 - Continued

B. Specialized Transduction

PROCEDURE

Day 0: The day prior to your regularly scheduled lab period.

10. Inoculate a 3-ml overnight culture of *E. coli* strain MC4100 in 3 ml of LB with 100 μg/ml streptomycin, grown at 37˚ C with shaking.

Day 1: The day of your regularly scheduled lab period.

Transduction

11. Take the harvested λRS45 out of the 4˚ C refrigerator. Vortex harvested recombinant phage gently, and place the tubes in a microcentrifuge and spin at 8000 rpm for 10 minutes.

12. Pipette the supernatant into a new microcentrifuge tube and then remove the bacteria by forcing the solution through 0.20 μm filter with luer lock on a 1-ml syringe. Filter into a clean, sterile microcentrifuge tube.

13. As with the donor bacteria, prepare the recipient bacteria by centrifuging 1 ml of your overnight culture of MC4100 at 8000 rpm for 5 minutes, removing the supernatant, and resuspending in 0.5 ml of 0.01 M $MgSO_4$. **Note**: Once you have resuspended either the donor or recipient strain in 0.01 M $MgSO_4$, they can be used for several days if stored in the refrigerator.

14. To transfer the recombinant *LEE1-lacZ* fusion from the phage lysate onto the chromosome of the recipient *E. coli* strain MC4100, combine 100 μl of the phage lysate in a 12-ml culture tube with 100 μl of the recipient strain in 0.01 M $MgSO_4$.

15. Set up a 100 μl **"phage only" control** as well as a 100 μl **"bacteria only" control** in the 12-ml snap top culture tubes. Add 100 μl sterile LB to bring all volumes to 200 μl.

16. Allow the recombinant phage to adsorb to the recipient strain for 15 minutes at room temperature.

17. Prepare 6 ml of LB containing 2 mg/ml maltose. **How much of a 20 mg/ml stock solution will you need to add to reach a final concentration of 2 mg/ml in a total volume of 6 ml?** Do your calculation, and check it with me prior to addition! After phage adsorption, add 2.0 ml of LB medium containing 2 mg/ml maltose to each of the three tubes using good aseptic technique. Shake at 37˚ C for 2 hours.

18. With a sterilized glass spreader, spread the transductants on LB agar with 50 μg/ml kanamycin and 30 μg/ml X-gal using the plating scheme presented on the next page.

19. When dry, invert the plates and incubate at 37˚ C overnight.

Plating scheme:

Bacteria & Phage	Bacteria Only	Phage Only
50 µl	50 µl	50 µl
100 µl	100 µl	100 µl
Remainder*	Remainder*	Remainder*

*Plate the "remainder" of the culture by placing it in a 1.5 ml microcentrifuge tube and centrifuging at 13,000 rpm for 10 seconds. Discard all the supernatant except 100 µl. Resuspend the pellet and spread the bacteria on the LB kan X-gal plates.

Day 2

Screening for ampicillin sensitive transductants

In order for the recipient *lac* strain MC4100 to become kanamycin resistant, the recombinant λRS45 DNA must lysogenize into the recipient chromosome. This occurs via integration at the *att* attachment site located on the chromosome of strain MC4100. The recombinant phage particles will lose the ampicillin resistance gene during homologous recombination in the *recA+* host (see the accompanying figures), and thus the transductants will be ampicillin sensitive.

20. Use the 50-colony grids provided to test the transductants' susceptibility to kanamycin and ampicillin. Thus, you will use the LB kan x-gal and LB ampicillin plates for this step. Your transductants should be resistant to kanamycin and sensitive to ampicillin. **Note the phenotype, *i.e.*, the color, of each transductant on the LB kan x-gal plates.** Be sure to include control strains on your two plates. These will include strains MC4100 amp^s kan^s, MC4100 (pRS551) amp^r kan^r, and MC4100 (pJLM164). The relevant genotype of the strain you produced by transduction is MC4100 Φ*LEE1-lacZ*. The hyphen is the symbol indicating a gene fusion and the symbol Φ indicates that the genetic construction is chromosomally located.

Day 3

21. Record your **results** for the transduction, control plates, and ampicillin sensitivity screening in a well-labeled table. **Make sure to save the plates from step 20 for use in Week 7. Place parafilm around them and store in the refrigerator at 4˚ C.**

Lab Report

A. Phage Isolation from Sewage

Include the titers of the original sewage sample, your initial phage isolation and that of the high titer lysate, with the proper units in a well labeled table.

1. Did you see a variety of plaque sizes, or were the plaques fairly homogeneous in size in the initial overlay? What size plaque did you use for your high titer lysate?

2. Describe the host specificity of the phage that you isolated. How did the host specificity of your phage compare to that of others in the class? Was this result expected or unexpected? Why? You might find the following reference useful in explaining your results: http://aem.asm.org/content/78/17/6380.long.

3. Are there potential dangers in using bacteriophage as therapeutic agents? If so, what are they?

B. Specialized Transduction

Present a well-labeled table of the results from the 9 LB kan X-gal plates containing the transduction mixes. Include in your table the results from screening the transductants for kanamycin and ampicillin resistance/sensitivity.

1. Were the results presented in your table expected? Why or why not?

2. In step 15, what do the "phage only" and "bacteria only" tubes control for?

3. Could you use the laboratory *E. coli* strain DH5α containing the plasmid pJLM164 for making lysates of recombinant phage λRS45 containing the *LEE1* regulatory region fused to the *lacZ* reporter gene?

   Hint: the genotype for DH5α is *supE44* Δ*lacU169* (Φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1,* while the genotype of MC4100 is (*araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 fblB3501 deoC1 ptsF25 rbsR*). Note that in genetic nomenclature all genes are wt unless otherwise indicated. For example, *gyrA96* indicates a mutation in the gene encoding the enzyme DNA gyrase. The answer should become clear as you recall the requirements for creating *recombinant* phage.

Lab reports are due at the beginning of your next laboratory period.

Week 7: β-Galactosidase Assays

INTRODUCTION

The *lac* operon of *E. coli* is one of the most widely studied paradigms of gene regulation in biological systems. First described by Jacob and Monod in 1961 (1), it is heralded as the starting point for understanding gene regulation on the molecular level. The product of the *lacZ* gene is the enzyme β-galacosidase, a tetramer of ~464 kDa, and is expressed from the *lacZYA* operon, under the control of the *lac* repressor, encoded by *lacI*. When the *lacZYA* operon is removed from its regulatory region, and fused to a different promoter, transcriptional regulation of a second promoter of interest can be studied.

The enzyme β-galacosidase is a hydrolase that cleaves the disaccharide lactose to glucose and galactose. Expression of the *lacZYA* operon can be induced by the addition of lactose or isopropyl β-D-1-thiogalactopyranoside (IPTG). The compound o-nitrophenyl-β-D-galactoside (ONPG) is also recognized as a substrate and is cleaved to yield galactose and o-nitrophenol, producing a yellow color. When the substrate ONPG is in excess over the enzyme, the production of o-nitrophenol per unit time is directly proportional to the concentration of β-galactosidase. Therefore, the production of the yellow color, observed spectrophotometrically at 420 nm, can be correlated to enzyme concentration.

In his publication (2), Miller defined a repressed *lac* operon as producing 1 unit of activity, while fully induced produced 1000 Miller units. Notice that the β-galactosidase activity is expressed in "Miller units" as opposed to Units with a capital U, because it is not a true specific activity, which correlates cleavage of substrate to mg protein. In the Miller assay, instead of directly quantifying mg protein, cell density is reported spectrophotometrically by taking optical density (OD) readings at 600 nm.

References

1. Jacob, F. and J. Monod. (1961) Gentic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.* **3**: 318-356.
2. Miller, JH (1972) Experiments in Molecular Genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

MATERIALS AND METHODS – work in **pairs**

E. coli strains MC4100, MC4100 (pRS551), MC4100 (pJLM164), and your single-copy *LEE1-lacZ* fusion strain constructed in **Weeks 5** and **6**.
LB broth in 100 ml bottles
50 mg/ml kanamycin
12-ml disposable culture tubes
Environmental shaker set at 37˚ C
Chemicals to prepare Z-Buffer and Stop Solution
500-ml bottles for Z-Buffer and Stop Solution
100-ml bottles
4 mg/ml ONPG in Z-Buffer without β-mercaptoethanol
Chloroform
0.1% SDS
2.0-ml microcentrifuge tubes
Vortexers
Timers
Microcentrifuge
P-1000 Micropipettors
P-100 Micropipettors
Sterile blue tips
Sterile yellow tips
Disposable cuvettes
Spectrophotometers

PROCEDURE

Day 0: The day prior to your scheduled laboratory period.

Inoculation of *E. coli* strains MC4100, MC4100 (pRS551), MC4100 (pJLM164), and your single-copy *LEE1-lacZ* fusion strain (constructed in Weeks 5 and 6) in LB broth containing antibiotics where appropriate.

1. Using good aseptic technique, place 3 ml of LB broth into **4** 12-ml snap top culture tubes. Label the four tubes with the strain names above, and then place your name and the date on all tubes.

2. For all tubes except that for the MC4100 strain, place 3 μl of a 50 mg/ml solution of kanamycin into the medium, for a final concentration of 50 μg/ml. All strains, except MC4100, are resistant to kanamycin. For strain MC4100, place 3 μl of a 100 mg/ml solution of streptomycin into the medium, for a final concentration of 100 μg/ml.

3. Using an inoculating loop and good aseptic technique, place a single colony of the four strains into the four, labeled tubes of media.

4. Make sure the caps are on loosely, and incubate in the environmental shaker at 37˚ C overnight.

5. **Prepare samples of phage isolated from sewage for EM imaging**. Mix 1 ml of your high titer lysate with 250 µl 25% PEG6000-8000/2.5M NaCl in a sterile microcentrifuge tube and place it in the refrigerator overnight.

Day 1: The day of your regularly scheduled laboratory period.

β-Galactosidase assay

6. Prepare **500 ml** of the **Z-Buffer** and **500 ml** of the **Stop Solution** listed below. Note that you will need to calculate the weight of each chemical for the given volumes.

7. Record the OD_{600} values for all four cultures prepared on **Day 0**.

8. Transfer three 100-µl aliquots for each of the four bacterial cultures into 2.0 ml microcentrifuge tubes, for a total of twelve tubes. Each culture will be assayed in triplicate. The results will be averaged, and statistical analysis performed to compare activity from the different genetic constructs.

9. **In the fume hood, add 270 µl β-mercaptoethanol to 100 ml of Z-Buffer.**

10. In succession, add to each of your twelve 100-µl aliquot:

    900 µl Z-Buffer (Be sure the β-mercaptoethanol has been added!)
    10 µl Chloroform
    10 µl 0.1% SDS

11. Vortex each tube exactly 10 seconds (use a timer).

12. Start the reaction by the addition of 200 µl of 4 mg/ml ONPG. Record the exact time.

13. Allow the reaction to proceed until sufficient yellow has been observed. A good rule of thumb is to allow the reaction to go until the yellow is similar to that of a yellow micropipet tip.

14. Stop the reaction by the addition of 500 µl of 1M Na_2CO_3. Record exact time, and **net time** of each reaction in minutes.

15. To pellet the bacterial cell debris, centrifuge reactions at full speed in the microcentrifuge for 3 minutes. Record the absorbance at 420 nm of the supernatant for each reaction using an appropriate blank.

16. Calculate **Miller units** for each of the twelve reactions by using the following formula:

$$\frac{1000 \times (\text{Abs at } 420 \text{ nm})}{T \times V \times (\text{OD at } 600 \text{ nm})} = \text{Miller units of } \beta\text{-galactosidase activity}$$

T = time in minutes
V = volume of culture added in ml

Solutions

Z-Buffer

60 mM $Na_2HPO_4(7H_2O)$ Sodium Phosphate Dibasic Heptahydrate, MW = 268.07
40 mM $NaH_2PO_4(H_2O)$ Sodium Phosphate Monobasic Monohydrate, MW = 137.99
10 mM KCl, MW = 74.55
1 mM $MgSO_4(7H_2O)$, MW = 246.47

Adjust pH to 7.0
Fill to the appropriate volume with diH_2O

Prior to use, add 270 µl of β-mercaptoethanol to a 100 ml aliquot of Z-Buffer, for a final concentration of 50 mM. **Be sure to add the β-mercaptoethanol in the fume hood!**

Stop Solution

1 M Na_2CO_3, MW = 106.00
Fill to the appropriate volume with diH_2O

Preparing Phage for Imaging by Electron Microscopy (Continuation from Weeks 5 and 6)

Time and resources permitting, we will observe the bacteriophage by electron microscopy. We will prepare samples and bring them up to the OHSU Electron Microscopy Lab for processing.

In order to see any virus in the electron microscope, it is necessary to have a concentrated "high titer" suspension of the virus. The titer must be high, at least 10^{10} pfu/ml, in order to assure the presence of some virus in the field being viewed in the electron microscope. At high magnifications, the field is very small. Therefore, the probability of having virus in a particular field being scanned depends on how many virus particles are on the grid.

Because it is difficult to obtain sufficient titers to view the phage by microscopy and the proteins from the medium often skew the images, we will perform a partial purification of the phage using polyethylene glycol (PEG) prior to staining. The procedure for this is listed below.

MATERIALS AND METHODS – work in **pairs**

High titer phage lysate
25% PEG6000-8000 in 2.5M NaCl
50 mM Tris, pH 7.4/, plus 10 mM $MgSO_4$
Carbon formvar grids
Forceps
1% uranyl acetate
Parafilm
Kimwipes

PROCEDURE

PEG Precipitation of Phage

1. Pellet the phage in the high titer lysate by spinning at maximum speed in a microcentrifuge for 10 minutes at 4˚ C. For this step, we will place a microcentrifuge in the 4 C walk-in cold room in B128.

2. Carefully remove the supernatant and resuspend the pellet in 40 µl of 50 mM Tris, pH 7.4/10 mM $MgSO_4$.

3. Follow the Negative Stain Preparation Protocol below.

Negative Stain Preparation

We will use formvar-coated copper wire grids to hold the virus particles, and a 1% uranyl acetate solution in water to stain them. These copper grids are small and delicate, and must be carefully handled with the special fine watchmaker's forceps. The shiny side of the grid is the formvar treated side.

4. Place 40 µl of your partially purified high titer phage lysate onto a small piece of parafilm using a micropipettor and a yellow tip.

5. Using forceps, gently place a carbon-formvar grid, shiny side down, onto the drop of high titer lysate for **5 minutes**.

6. Place three 40 µl drops of sterile water on the parafilm. Wash your sample by placing the grid, shiny side down, on the first drop for **15 seconds**. Repeat the process on the next two drops.

7. Place 40 µl of the 1% uranyl acetate onto the parafilm, and place the grid on the drop for **1 minute**.

8. Wick off the excess liquid from the grid using a Kimwipe, and place it in the carrier for transport up to OHSU. Be sure to write down the location of your grid and the characteristics of your isolated phage.

Lab Report

In **Weeks 5** and **6** exercises, you used transduction to place the *LEE1* promoter of EPEC fused to the *lacZ* reporter gene onto the chromosome of the *lac* deletion strain MC4100. As it is a single-copy fusion construct, we would hypothesize that this gene fusion would produce less β-galactosidase activity than the muli-copy *LEE1-lacZ* fusion construct found in the strain MC4100 (pJLM164). For comparison, the pRS551 parent vector of pJLM164 has an MB1 origin of replication and both are maintained at ~30 copies per cell.

Present your data in a well-labeled graph. Use JMP to perform an Analysis of Variance (ANOVA) on the data to test your null hypothesis that there is no difference between the β-galactosidase activity derived from and the multi-copy *LEE1-lacZ* fusion the strain MC4100 (pJLM164) and the single-copy *LEE1-lacZ* fusion strain you constructed in **Weeks 5** and **6**. If you are not familiar with JMP you can use Excel. In JMP, use the Fit Y by X analysis. Then from the red arrow select Means/Anova, and select Each Pair Student's t test. Using the Student's. Determine whether you can reject the null hypothesis at the 95% confidence interval.

QUESTIONS

1. Did you observe greater β-galactosidase activity for multi-copy versus single-copy *LEE1-lacZ* fusion strains? If so, were your results statistically significant by the Student's t test?

2. Did you observe measurable β-galactosidase activities in the strain MC4100 and MC4100 (pRS551)? Recall that the genotype for MC4100 *araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 fblB3501 deoC1 ptsF25 rbsR,* and that genes are listed only when a mutation or deletion occurs. Thus Δ(*argF-lac*)U169 indicates a deletion from *argF* to *lac*, and U169 is an allele number.

3. If you observed β-galactosidase activity in the MC4100 (pRS551) strain, why did this occur? Note that strain MC4100 has a *lac* deletion and the pRS551 plasmid contains the *lacZYA* genes but no promoter.

Lab reports are due at the beginning of your next laboratory period.