INTRODUCTION

Welcome to the exciting world of Microbial Genetics. MIC428L/528L is going to be your opportunity to experience the hands-on experimental part of microbial genetics. It is important to remember that the field of Microbial Genetics consists of an extremely large area; in this laboratory we will expose you to some of the major theories and techniques used daily in thousands of microbial genetics research labs around the world. In fact, most of the experiments you will perform are used regularly in the research of Dr. Baltrus.

Microbial Genetics 428L/528L is a constantly evolving course. Most students take the lecture (MIC428R/528R) and lab simultaneously. Although the laboratory runs concurrently with the lecture, it is impossible to match the laboratory exercises with the lecture material due to the different rates with which the information can be imparted to you and the limited laboratory periods available. At several points the laboratory utilizes techniques not yet covered in lecture.

LABORATORY GOALS

The overall goal of this laboratory course is to expose you to realistic microbial genetics research. Some laboratory periods will be short. Occasionally you will be required to come in during a non-scheduled lab time or day to continue the experimental protocol. Experience over the past several years re-enforces the maxim that your success and failure is directly related to your preparation and carefulness in the laboratory.

IT IS CRITICAL THAT YOU CAREFULLY READ THE EXPERIMENTAL PLANS PRIOR TO THE LAB PERIODS IN ORDER TO BE PREPARED.

Prior to each new experiment section, you will have a short open book quiz posted on D2L. You will be given a few days to complete the quiz, after having gone over the first section involving that lab. There will be no quiz for experiment section 1 – Common Laboratory Techniques. There will be a quiz for Experiment 8 given later in the class, but not during the first week. Thus, there will be a total of seven (7) quizzes worth 105 points.

- It is important that you do not memorize the specific steps/procedures of the various protocols, as we often modify protocols to improve the results.

- To maximize what you get out of this laboratory course, it is critical that you gain a strong understanding of the general terminology, concepts and methodology used in this laboratory
- Although each group will perform the same experiments, results often differ. Since each group receives identical cultures, DNA, etc., differences observed can be traced back to the care and attention used by the different groups.

- On several occasions you or your partner must come in on a non-regularly scheduled lab period. Unlike people, bacteria never rest, so they grow (or do not grow) dependent on the environment they are exposed to, thus as a microbial geneticist you are now on the schedule of the bacteria you will be working with in the lab.

- IF YOU HAVE TWO (2) CONSECUTIVE LABORATORY PERIODS OF UNEXCUSED ABSENCES THEN YOU WILL BE AUTOMATICALLY DROPPED FROM THE CLASS.

- THERE WILL BE NO MAKE-UP LABORATORIES FOR UNEXCUSED ABSENCES.

   Overall, I hope that you enjoy your journey into microbial genetics, and that it excites and stimulates ideas and questions. Just maybe it might also inspire some of you to become microbial geneticists, or at least further open your eyes to the world of microbiology.

Dr. David Baltrus
### OVERVIEW OF LABORATORY EXERCISES

<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>Topic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Common Laboratory Techniques</td>
</tr>
<tr>
<td>2</td>
<td>Genotype and Phenotype</td>
</tr>
<tr>
<td>3</td>
<td>Mutation and Transformation</td>
</tr>
<tr>
<td>4</td>
<td>Plasmid Isolation</td>
</tr>
<tr>
<td>5</td>
<td>Gene Expression</td>
</tr>
<tr>
<td>6</td>
<td>Transposon Mutagenesis and Compensatory Mutation</td>
</tr>
<tr>
<td>7</td>
<td>Isolation of Chromosomal DNA</td>
</tr>
<tr>
<td>8</td>
<td>Phenotypic Evolution</td>
</tr>
</tbody>
</table>

**INSTRUCTOR:**

Dr. David Baltrus

Office: Marley 821C  
Laboratory: Marley 817/811  
Email: uamic428@gmail.com

**GRADUATE TEACHING ASSISTANTS (GTAs):**

- Section 1  
  Aaron White  
  Shuzo Oita  
  aaronwhite206@email.arizona.edu  
  shuzoo@email.arizona.edu

- Section 2  
  Danae Barker  
  danaebarker@email.arizona.edu

- Section 3  
  Yafei (Jenny) Xu  
  yafeixu@email.arizona.edu
Grading and Lab Reports

GRADING

There are a total of 1,215 points available for 428L/528L.

There are 7 required lab write-ups for this course (Experiment No. 1 requires you to turn in your plates and is worth 50 points).

Lab reports are due two laboratory days after completion of that laboratory unless otherwise noted.

For lab 8, you will put together a 5-minute presentation at the end of the course, but this will be graded as a lab report.

The purpose of the lab reports is to re-enforce experimental concepts. Each lab report is worth 100 points. Thus, there are 750 points available for your lab reports.

There is a written lab midterm exam worth 125 points total.

There is a written lab final exam worth 125 points total.

There are 7 open book quizzes, one to be taken at approximately the time you begin each new experiment section (Excluding experiment no. 1). I will announce the precise times when these quizzes open and close. Each quiz is worth 15 points. Thus, there are 105 points available for your quizzes.

Additionally, 110 points will be awarded by your GTA based on attendance, preparedness for each lab, AND attitude.

<table>
<thead>
<tr>
<th>Lab Reports</th>
<th>750 pts.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab Midterm Exam</td>
<td>125 pts.</td>
</tr>
<tr>
<td>Lab Final Exam</td>
<td>125 pts.</td>
</tr>
<tr>
<td>Experiment Section Quizzes (7 total)</td>
<td>105 pts.</td>
</tr>
<tr>
<td>Lab Effort</td>
<td>110 pts.</td>
</tr>
<tr>
<td><strong>Total available points</strong></td>
<td><strong>1,215 pts.</strong></td>
</tr>
</tbody>
</table>

LAB REPORT FORMAT
Lab reports serve several functions, they:
1. Re-enforce the goals of the experiment you just performed; and
2. Allow us to gauge how well you understand the experiments, how well you are able to interpret the data/results and how you relate your results to goals of the experiment. **The ability to write concisely and intelligently is required.** Writing every detail, hoping you cover what the GTA is looking for is not an acceptable lab report; you should concisely and clearly show your complete understanding of the entire experiment (including but not limited to: methods, results, goals and overall reason for experiment as it relates to microbial genetics).

Although you are working in pairs/partners, **each person** is required to turn in their **own** lab report for each of the required lab reports. Use the format below as a model for **ALL** lab reports for this course. As previously stated, the lab reports need to short, concise and clear and must include:

1. **Title**
2. **Author**
3. **Purpose of the experiment** (1 paragraph) Explain why the experiment was performed and what was the goal(s).
4. **Methodology** (1-2 paragraphs) I do **NOT** want you to re-type the protocol. What I want is a brief description of the logic for the procedure.
5. **Data** Include all data for the experiment. This includes but is not limited to: results, raw numerical data, calculations, etc. For example, what frequency of various mutations did you see?
6. **Conclusions** (1 paragraph) What does the data tell you.
7. **Selected questions** These should help you review the labs and point out key concepts.

**Typed** lab reports must be turned in by the date provided by your GTAs. Lab reports are to be turned in at the **beginning of lab** on the day they are due. Late lab reports will **NOT** be accepted unless you have made prior arrangements with your GTAs. **YOUR GTA HAS COMPLETE DISCRETION OVER WHETHER TO ACCEPT LATE REPORTS, RE-GRADE EXAMS OR REPORTS AND YOUR LAB EFFORT GRADE.**

**GUIDELINES FOR SCHOLASTIC ETHICS**
This course operates under the U of A Code of Academic Integrity as described on the Dean of Student Offices website at the following address:

http://deanofstudents.arizona.edu/codeofacademicintegrity

- Students must write their own reports.
- Students must follow all written and verbal instructions.
- Students must adhere to course requirements as specified in the syllabus.
- Students caught cheating will be given a zero for the quiz, exam or report.
- Students who plagiarize will be given a zero for the course.

SPECIAL NEEDS AND ACCOMMODATIONS

Students needing special accommodations or special services should contact the Learning Disabilities programs/SALT (1010 N. Highland Ave., Tucson, AZ 85721, (520) 621-1242, http://www.salt.arizona.edu/) and/or the Disability Resource Center (1224 E. Lowell St., Tucson, AZ 85721, (520) 621-3268 V/TTY, Fax: (520) 621-9423, uadrc@email.arizona.edu). The needs for specialized services must be documented, verified by these units, and presented to the instructor before the end of the second week of class. We will do everything we can to enhance your learning experience.

WITHDRAWALS

Students withdrawing from this course must notify the instructor prior to nonattendance in classes and execute drop or withdrawal procedures in accordance with the U of A General Catalog. Any student failing to attend class in two or more successive classes is subject to automatic withdrawal if arrangements have not been made previously.

INCOMPLETES

Any incomplete given must be verified with a written agreement with the student that specifies the work to be done and a timetable of completion.

QUESTIONS, COMMENTS AND CRITICISMS

I am happy to discuss any aspect of the lecture/laboratory at any time. Please come see or email me anytime you have questions or conceptual difficulties (or email me and we will schedule a meeting time). I am often present in the lab sessions, so feel free to ask me questions during the time.

My goal for this course is introduce you to the wonderful world of microbial genetic research. Quality research is not easy and often takes time and patience, and sometimes experiments fail for reasons we do not initially understand. However, part of being a good scientist is the ability to dissect experimental protocols to figure out how to improve them.
<table>
<thead>
<tr>
<th>Date</th>
<th>Day</th>
<th>Laboratory Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/9</td>
<td>W</td>
<td>NO LAB</td>
</tr>
<tr>
<td>1/10</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>1/14</td>
<td>M</td>
<td>Experiment No. 1: Common Techniques</td>
</tr>
<tr>
<td>1/15</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>1/16</td>
<td>W</td>
<td>Experiment No. 2: Phenotype and Genotype</td>
</tr>
<tr>
<td>1/17</td>
<td>R</td>
<td>Experiment No. 8: Phenotypic Evolution</td>
</tr>
<tr>
<td>1/21</td>
<td>M</td>
<td>NO LAB</td>
</tr>
<tr>
<td>1/22</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>1/23</td>
<td>W</td>
<td>Experiment No. 2: Phenotype and Genotype</td>
</tr>
<tr>
<td>1/24</td>
<td>R</td>
<td>Experiment No. 3: Mutation and Transformation</td>
</tr>
<tr>
<td>1/28</td>
<td>M</td>
<td>Experiment No. 3: Mutation and Transformation</td>
</tr>
<tr>
<td>1/29</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>1/30</td>
<td>W</td>
<td>Experiment No. 3: Mutation and Transformation</td>
</tr>
<tr>
<td>1/31</td>
<td>R</td>
<td>Experiment No. 4: Plasmid Isolation</td>
</tr>
<tr>
<td>2/4</td>
<td>M</td>
<td>Experiment No. 3: Mutation and Transformation</td>
</tr>
<tr>
<td>2/5</td>
<td>T</td>
<td>Experiment No. 4: Plasmid Isolation</td>
</tr>
<tr>
<td>2/6</td>
<td>W</td>
<td>Laboratory Midterm Exam</td>
</tr>
<tr>
<td>2/7</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>2/11</td>
<td>M</td>
<td>Experiment No. 5: Gene Expression</td>
</tr>
<tr>
<td>2/12</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>2/13</td>
<td>W</td>
<td>Experiment No. 5: Gene Expression</td>
</tr>
<tr>
<td>2/14</td>
<td>R</td>
<td>Experiment No. 6: Transposon Mutagenesis</td>
</tr>
<tr>
<td>2/18</td>
<td>M</td>
<td>Experiment No. 6: Transposon Mutagenesis</td>
</tr>
<tr>
<td>2/19</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>2/20</td>
<td>W</td>
<td>Experiment No. 6: Transposon Mutagenesis</td>
</tr>
<tr>
<td>2/21</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>2/25</td>
<td>M</td>
<td>Experiment No. 6: Transposon Mutagenesis</td>
</tr>
<tr>
<td>2/26</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>2/27</td>
<td>W</td>
<td>Experiment No. 6: Transposon Mutagenesis</td>
</tr>
<tr>
<td>2/28</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>3/2–3/10</td>
<td></td>
<td>Spring Break</td>
</tr>
<tr>
<td>3/11</td>
<td>M</td>
<td>Experiment No. 6: Transposon Mutagenesis</td>
</tr>
<tr>
<td>3/12</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>3/13</td>
<td>W</td>
<td>Experiment No. 6: Transposon Mutagenesis</td>
</tr>
<tr>
<td>3/14</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>3/18</td>
<td>M</td>
<td>Experiment No. 6: Transposon Mutagenesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date</td>
<td>Day</td>
<td>Task</td>
</tr>
<tr>
<td>--------</td>
<td>-----</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>3/19</td>
<td>T</td>
<td>Transposon Mutagenesis</td>
</tr>
<tr>
<td>3/20</td>
<td>W</td>
<td>Experiment No. 6: Transposon Mutagenesis</td>
</tr>
<tr>
<td>3/21</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>3/25</td>
<td>M</td>
<td>Experiment No. 7: Culture Inoculations</td>
</tr>
<tr>
<td>3/26</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>3/27</td>
<td>W</td>
<td>Experiment No. 7: Chromosome Isolation</td>
</tr>
<tr>
<td>3/28</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>4/1</td>
<td>M</td>
<td>Experiment No. 7: Chromosome Ligations</td>
</tr>
<tr>
<td>4/2</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>4/3</td>
<td>W</td>
<td>Experiment No. 7: Transformations</td>
</tr>
<tr>
<td>4/4</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>4/8</td>
<td>M</td>
<td>Experiment No. 7: Plasmid Inoculation</td>
</tr>
<tr>
<td>4/9</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>4/10</td>
<td>W</td>
<td>Experiment No. 7: Plasmid Isolation for</td>
</tr>
<tr>
<td>4/11</td>
<td>R</td>
<td>Sequencing</td>
</tr>
<tr>
<td>4/8</td>
<td>M</td>
<td>Experiment No. 8: Phenotypic Evolution</td>
</tr>
<tr>
<td>4/9</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>4/10</td>
<td>M</td>
<td>Experiment No. 8: Phenotypic Evolution</td>
</tr>
<tr>
<td>4/11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4/15</td>
<td>M</td>
<td>Experiment No. 8: Phenotypic Evolution</td>
</tr>
<tr>
<td>4/16</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>4/17</td>
<td>W</td>
<td></td>
</tr>
<tr>
<td>4/18</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>4/22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4/23</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In this course you will be working with several different bacterial strains. A brief description of some of the strains we will be using is given below.

**Pseudomonas chlororaphis** strain 30-84

This free-living soil-borne bacterium was originally isolated from wheat roots taken from a field where a severe fungal disease of wheat (called take-all) had been naturally suppressed. This bacterium produces three phenazine antibiotics (Fig. 1) that are responsible for the inhibition of the fungal pathogen by this strain and therefore the decline of this fungal disease. The production of these antibiotics inhibits many pathogenic fungi and some bacteria. During the course of studies in Dr. Leland Pierson’s laboratory (formerly at UA, now Texas A&M) on the genetic basis for the regulation of these antibiotics, it was discovered that this bacterium shares several regulatory mechanisms commonly found in both symbiotic and pathogenic microorganisms, including those that interact with plants and those that interact with animals and humans. Thus, this bacterium is a good model for studying the genetic regulation of mechanisms used in both beneficial microbe-host interactions and pathogenic microbe-host interactions.

Fig. 1. Phenazine antibiotics produced by *Pseudomonas chlororaphis* strain 30-84. Phenazine-1carboxylic acid (PCA) is yellow and the two derivatives 2-hydroxy-phenazine-1-carboxylic acid (2OHPCA) and 2-hydroxy-phenazine (2OHPZ) are orange in appearance.

In addition to the production of phenazine antibiotics, strain 30-84 is interesting for several other reasons. Strain 30-84 produces hydrogen cyanide (HCN), a known uncoupler of oxidative respiration in many organisms. Strain 30-84 also produces a fluorescent siderophore. Siderophores are produced by the bacterium under conditions of low iron availability and are secreted into the environment to scavenge iron (Fe). Once they bind Fe, they are transported back into the cell through a specific receptor protein in the cell wall. This strain also produces an extracellular protease (exoprotease). The 30-84Z strain is a derivative of 30-84 where the gene phzB has been disrupted by lacZ. 30-84Z is therefore Phenazine- (Phz-), but will turn blue when grown on Xgal with the timing and amount of LacZ produced controlled by the promoter for phzB.

A couple of good references for these strains and mutants:
**Pseudomonas stutzeri** strain 28a24 (DBL273 and DBL332)

*Pseudomonas stutzeri* is a highly diverse species of great physiological and ecological versatility and widespread geographic distribution. Members of this species have been shown to be involved in nitrification and denitrification processes as well as in the degradation of environmental pollutants. This *P. stutzeri* strain was originally isolated from soil and found to be highly competent for natural transformation. Strains DBL273 and DBL332 will be used during transformation experiments.

**Pseudomonas sp. Leaf58**

*Pseudomonas* strain Leaf58 is closely related to *Pseudomonas putida*, but it distinct enough that it probably warrants consideration as a separate species. This strain is interesting because of its bright yellow/green fluorescent color, a variety of interesting motility/biofilm phenotypes that you may see over the course of this lab, and because it houses a 900kb megaplasmid (for which we have very little understanding of). This strain will be used as the basis for the transposon mutagenesis experiment.

**Pseudomonas syringae pv. tomato** DC3000

This is a model bacterial phytopathogen that is used in many labs to dissect virulence and host responses. This strain is the causative agent of bacterial speck in tomato but has become very useful for lab studies because it’s one of the rare bacterial pathogens that can infect and cause symptoms in Arabidopsis (the lab model system for plant genetics). This parent of this strain was isolated in Norwich UK in 1961, with DC3000 being a rifampicin resistant isolate of this original strain. Virulence *in planta* for DC3000 is due to the presence of a type III secretion system and associated effector proteins as well as secreted toxins such as coronatine. Virulence factors in DC3000 are regulated by a cascade of different transcriptional units, with the HrpR and HrpS proteins activating in conjunction with σ54 to upregulate transcription of HrpL. HrpL is an alternative σ54 factor that binds to a promoter (termed the hrp-box) upstream of virulence genes to trigger expression.

**Escherichia coli** strain DH5

Strain DH5 is an engineered strain of *E. coli* that is widely used for cloning experiments, in fact it is used almost weekly in Dr. Baltrus’s research. Its genotype is given below.

*F.*, *recA1*, *endA1*, *hsdR17*, *supE44*, *thi-1*, *gyrA96*, *relA1*, *(argF-lacZYA)*, *I169*, *80lacZM15*

This strain has 3 very useful characteristics:
1. **RecA.** It is defective in homologous recombination. So, if it contains a plasmid with genes homologous to those on its chromosome NO recombination can occur.

2. **HsdR.** There is NO host restriction system functioning in this strain. Therefore, introduced DNA will not be degraded by restriction enzymes in the cell.

3. **lacZM15.** In *E. coli* the *lacZ* gene encodes for the enzyme β-galactosidase that is only active as a homotetramer (composed of four identical monomers). Each monomer by itself is inactive and is composed of two parts LacZ-alpha and LacZ-omega. The *lacZM15* mutation lacks a small 92 amino acid portion (LacZ-alpha) of the *lacZ* gene that is responsible for causing the β-galactosidase monomers to bind together. Thus, monomers of -β-galactosidase are produced but they cannot bind together to form the active form. Thus, the strain is phenotypically Lac-. However, if a specific type of engineered plasmid is introduced into the strain (such as pUC18) that encodes the missing 92 amino acid portion of *lacZ* (LacZ-alpha), then the β-galactosidase monomers can form the active β-galactosidase tetramer and the strain becomes Lac+. This phenomenon, called -complementation (Fig. 2, pg. 14) is extremely useful in cloning experiments, as you will see.
**Monomers of β-galactosidase (inactive)**

*lacZΔM* mutation lacks a 92 amino acid peptide required for monomer binding.

**Tetramer of β-galactosidase**

Intact *lacZα* gene produces the missing 92 amino acid peptide resulting in monomer binding.

**No insert in MCS**

*lacZα*

92 amino acid LacZα fragment.

**Insert in MCS**

Cloning a DNA fragment into the MCS disrupts the production of the LacZα peptide. Thus the β-galactosidase monomers cannot bind to make the active tetramer.
INTRODUCTION

During this course, it is imperative that you are able to perform accurately laboratory techniques such as streaking plating and dilution plating. In addition, the accurate use of micropipettors is an often overlooked but critical skill. Most of you have performed these techniques in prior laboratory courses and are proficient. Therefore, in this exercise you have the opportunity to demonstrate your proficiency to us. In lieu of a lab report, your plates will be scored for technique.

MATERIALS FOR EXERCISE 1 (per team)

- LB broth culture of a mixture of *Pseudomonas chloroaphis* strain 30-84 & 30-84Z (prepared immediately prior to class)
- 6 LB + X-gal agar plates
- 28°C Incubator

I. STREAK PLATING

When doing genetics, it is important to be able to isolate pure colonies composed of identical clones that do not contain contaminating cells from other populations.

EXPERIMENTAL PROTOCOL

1. Label your plate with your name & section no. Each member of a group will streak a plate independently. Always label the plate on the bottom and not on the lid!

2. Using a sterilized loop, place a loopful of the strain mixture onto one side of the LB agar + X-gal plate.

3. Using the technique shown below (Fig. 1.1), streak the loopful out for single colonies.

4. Place your labeled plates in the box at the front of the room. (Your GTA will look for isolated colonies of both strains)
II. SERIAL DILUTIONS

MATERIALS (per team)

- One of each micropipettor (P-10 or P-20, P-50 or P-100, and P-1000)
- Box of sterile yellow and sterile blue tips
- 4 LB agar + X-gal plates
- *P. chlororaphis*: 4 LB agar + X-gal plates
- Mixed tube of *P. chlororaphis* strain 30-84 and strain 30-84Z prepared immediately prior to class.

Microbial genetics uses very small volumes, on the order of 1µL.

\[
1 \mu\text{L} \text{ (microliter)} = 0.001 \text{ ml (milliliter)} = 1 \times 10^{-6} \text{ L (liters)}
\]

OR

\[
1 \text{ liter} = 1000 \text{ ml} = 1,000,000 \mu\text{L}
\]

Micropipettors are essential for microbial genetics. They are used for physiology, biochemistry, genetics, etc. It is important that you are competent with their usage.
USAGE & CARE OF MICROPIPETTORS

There are several different sizes of micropipettors. Please treat these instruments with care. Their reliable operation is critical for the success of several of your experiments.

PRINCIPLE OF OPERATION

This instrument is designed to be held in the hand and operated by the thumb. The plunger stroke is divided into two parts. The longer calibrated stroke ends at the FIRST STOP, the shorter stroke ends at the SECOND STOP (Fig. 1.2).

VOLUME ADJUSTMENT

1. To select the desired volume, loosen the top locknut by turning it counterclockwise.

2. To reduce the volume, turn the thumb knob clockwise. Turning the thumb knob counterclockwise will increase the volume (Fig. 1.3).

3. Set the desired volume on the digital display to correspond to the arrow mark located on the base of the window frame.

4. The selected volume is fixed by tightening the locknut clockwise (check to see that tightening the locknut has not altered the setting).

---

![Fig. 1.2](image-url)
OPERATING INSTRUCTIONS

1. Attach a clean sterile tip firmly to the barrel of the instrument.

2. Depress the thumb knob to the first stop.

3. Immerse the tip approximately 3 mm into the sample.

4. Smoothly & slowly return the thumb knob to the release position (DO NOT let it snap back).

5. Withdraw the tip from the solution, do not wipe the tip.

6. Place tip against the side of the receiving tube.
MIC et al. 428/528L
Experiment No. 1

7. Smoothly depress the thumb knob to the first stop, pause & then depress the knob to the second stop.

8. With the knob still held at the second stop, withdraw the tip from the receiving tube.

9. Return the knob to the release position (DO NOT let it snap back).

10. Remove the tip by hand or by pushing the tip ejector button.

AIDS TO REPRODUCIBILITY & ACCURACY

1. Try to affect the same speed for both uptake & delivery of all samples.

2. Always depress the thumb knob to the proper stop prior to taking up sample.

3. Insert the tip to the same depth each time & keep the micropipettor as vertical as possible.

EXPERIMENTAL PROTOCOL

You have a tube containing a mixture of two derivatives of *P. chlororaphis*. One derivative is the wild-type strain 30-84 while the second is the -galactosidase reporter strain 30-84Z. Your goal is to isolate each of the strains and determine their population densities (cfu/ml).

Each person does this independently. Label your plates with your name & section number. Using the procedure given below (Fig. 1.4), serially dilute the broth culture and spread onto TWO (2) LB agar + X-gal plates at both $10^7$ and $10^8$. Place your labeled plates in the box at the front of the room. (Your GTA will store the plates until we count them during the next lab period).
Overnight culture of 30-84 & 30-84Z

Sterile 1.5 ml microfuge tubes

10 µl 10 µl 100 µl 100 µl 100 µl

990 µl sterile PBS 990 µl sterile PBS 900 µl sterile PBS 900 µl sterile PBS 900 µl sterile PBS

Dilution: $10^{-2}$ $10^{-4}$ $10^{-5}$ $10^{-6}$ $10^{-7}$

Spread plate 100 µl

Final Dilution: $10^{-7}$ $10^{-8}$

Fig. 1.4
EXPERIMENT NO. 1 – SELECTED QUESTIONS

1. You have performed the indicated series of dilutions. For each, give the titer of the original culture (show your calculations).

A. Tube → 10 µL → 990 µL PBS
   300 µL → 700 µL PBS
   200 µL → 800 µL LB broth
   100 µL → LB agar plate
   Colonies on plate after 24 hr = 2

   Titer of original culture = ________________ cfu/ml.

B. Tube → 100 µL → 900 µL H2O
   1 µL → 9999 µL LB broth
   1 µL → 49 µL LB broth
   50 µL → 950 µL LB broth
   100 µL → LB agar plate
   Colonies on plate after 24 hr = 130

   Titer of original culture = ________________ cfu/ml.

C. Tube → 10 µL → 490 µL PBS
   1 µL → 99 µL PBS
   100 µL → 1900 µL PBS
   500 µL → 500 µL PBS
   100 µL → LB agar plate
   Colonies on plate after 24 hr = 353

   Titer of original culture = ________________ cfu/ml.

2. Why do you plate on duplicate plates for each dilution (think statistics)?
INTRODUCTION

Given their large population sizes and rapid growth rates, microbial populations can adapt quite readily to new environments. Furthermore, their small size means that cells can sense and respond to fine scaled environmental gradients.

During this semester, you will passage one of 12 different Pseudomonas strains in static (not shaking) liquid media. You will observe what these cultures look like each week and will plate out samples of these cultures to identify colony variants that have arisen over this time course.

This experiment was based on a paper (by Mike Travisano and Paul Rainey http://www.nature.com/nature/journal/v394/n6688/abs/394069a0.html) which first investigated this adaptive evolution using Pseudomonas fluorescens strain SBW25. These first experiments showed a rapid diversification of colony types in the same exact media that you have been passaging your cells in. The adaptive radiation occurs because the lack of shaking of the media enables an oxygen gradient to form. Because of this gradient, you can observe mutants of P. fluorescens which form a mat (or pellicle) on the top of the media (high oxygen) and some that are found at the bottom of the media (low oxygen). If you let the cultures sit for long enough, the mat will collapse under its own weight and you’ll see an intermediate mass in the culture. The wrinkly spreader phenotype arises due to cellulose overexpression and modification by P. fluorescens which enables mat formation. At a more direct level, many of the mutations that cause the wrinkly spreader phenotype affect cyclic di-GMP levels, which is a key signaling component across bacteria.

This experiment has been repeated across bacterial, and typically you can find phenotypes that look like wrinkly spreaders after a certain number of passages. The strains you have been passaging are different species of Pseudomonas, which starts off naturally as a variety of colony morphs.

This year we will compare how the evolution of microbial colony shape differs between static and shaking cultures for a variety of different Pseudomonas strains.
Experiment No. 8

Section 1, 1/16/2019 W
Section 2, 1/17/2019 R
Section 3, 1/16/2019 W

MATERIALS

- 2 Test Tubes with 5mL KB media with Strep50
- Plates of bacteria for picking colonies

For this first week, you will pick one colony from the plates provided to 500uL of KB media (provided). You will vortex this tube vigorously, and then add 250uL to each of the 5mL test tubes of KB media supplemented with Streptomycin 50. Take note of what this colony originally looks like (pictures are best). You will place one test tube into a rack in the 28-degree incubator and leave it for 1 week, you will place the other test tube to be put on a shaker for 1 week. After this week, you will “mix” each culture and pipette 50uL into a new test tube containing 5mL of culture liquid. You will then place these test tubes back into the environment that they are evolving in (static or shaker). At this time, you will also perform a dilution series to measure the total population size of each of the bacterial populations on KB plates supplemented with Streptomycin 50 as well as onto LB plates supplemented with Strep50. Please make up the dilution series for each using the provided tubes of 990uL PBS and 900uL PBS, and then spot 10uL of each dilution tube onto a single plate of either KB or LB media. Expect to see colonies around $10^7$ or $10^8$ and adjust dilution series each week based on the results of the prior week. For the remainder of the semester, on W/R you will passage, and on M/T you will observe the plates for colony morphology and color. Over the course of the semester, keep track (pictures are best) of the differences in morphology that arise both in the liquid cultures and on plates. At the end of the course, your group will present your results from this passage experiment to the rest of the class.
PHENOTYPIC VARIATION

Phenotype – the observable properties of an organism, produced by the genotype in conjunction with the environment.

All of the phenotypic characteristics of a bacterium (or any living organism) can be related to the expression of specific genes within the chromosome or extrachromosomal elements such as plasmids. The ability of a bacterium to utilize specific nutrients, to grow in specific niches, to be pathogenic, symbiotic, etc. is dependent upon the appropriate expression of specific genes or groups of genes. The expression of many of these genes is responsive to the bacterium’s environment. Many environmental factors, including nutrition, temperature, and osmolarity influence bacterial gene expression and therefore the phenotype of the bacterium. These are called phenotypic changes.

Remember, *P. chlororaphis* strain 30-84 produces several secondary metabolites such as phenazine antibiotics, HCN, a siderophore and an exoprotease. Keep this in mind as you examine your plates after incubation. We will only look at some of these phenotypes. Strain 30-84Z contains a phzB::lacZ genomic fusion and thus expresses galactosidase in place of phenazines.

MATERIALS (per team)

- LB agar plates of *Pseudomonas chlororaphis* strains 30-84, 30-84Z & 30-84gacA
- 1 LB agar plate and 1 M9 agar plate
- 1 KMB agar plate
- 1 KMB + FeCl3 agar plate (3 ml 0.5 M FeCl3 per liter)
- 1 Skim milk agar plate
- 1 semi-solid motility agar plates
- 1 LB + X-gal agar plate and 1 M9 + X-gal agar plate
- 28°C incubator

EXPERIMENTAL PROTOCOL

I. Examination of the effect of medium type, iron and carbon source

A. Effect of medium type (Fig 2.1)

1. Streak the cultures of *P. chlororaphis* 30-84 onto one (1) LB agar plate and one (1) M9 agar plate. Do the same for the culture of 30-84Z but use plates with X-gal.
2. Incubate the plates at 28°C.

3. Examine the phenotypes after 24 h.

Fig 2.1 Effect of medium type on the soil-borne bacterium *P. chlororaphis* 30-84 and its derivative 3084Z.

**B. Effect of iron**

1. Streak strain 30-84Z onto one (1) KMB+ agar plate and one (1) KMB + FeCl₃ agar plate. Incubate at 28°C. (*KMB = King’s Medium B which is a medium low in available iron)*.

**C. Exoprotease production**

1. Streak strain 30-84, 30-84Z & 30-84gacA onto 1/3 of a skim milk plate. Incubate at 28°C.

**D. Motility**
Using a pipette tip, scrape *P. chloroaphis* 30-84 so that there is a little dab of bacteria on the tip. Stab center of motility plate with this pipette tip. Repeat with 30-84Z & 30-84gacA. BE VERY CAREFUL HANDLING MOTILITY PLATE AND DO NOT TURN MOTILITY PLATE UPSIDE DOWN!!!!!

Section 1, 1/17/2018 R  
Section 2, 1/18/2018 F  
Section 3, 1/17/2018 R

****NOTE THAT YOU NEED TO COME IN BRIEFLY ON AN ODD DAY TO EXAMINE THE PLATES.

1. Examine and RECORD the phenotypes of each bacterial culture on ALL plates, noting any differences. Think about what the results indicate to you about the plasticity of phenotypic expression.
Discussion: How X-gal works.

X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside) is a chromogenic indicator. It was developed based upon the mechanism of action of β-galactosidase which is the enzyme used by bacteria to breakdown the sugar lactose into its constituents glucose and galactose. It consists of a chromophore connected by a β-1,4 linkage to galactose much like how glucose is connected to galactose in lactose. While conjugated to galactose, the chromophore is colorless. However, one the β-1,4 linkage is cleaved by the action of β-galactosidase (the enzyme encoded by the lacZ gene), the released chromophore is blue. Lac− cells do not produce β-galactosidase and cannot cleave X-gal therefore they appear colorless. Lac+ cells, on the other hand, produce β-galactosidase and can cleave X-gal. Therefore they appear blue.

GENETIC VARIATION

Genotype – the genetic makeup of an organism at the nucleic acid level.

The chromosome of a typical *E. coli* bacterial cell is composed of 4,639 kbp (kilo base pairs) or 4,639,221 base pairs of DNA (4.6 x 10⁶ bp) (actual base number depends on the strain). The primary function of the bacterial chromosome (the primary function of the chromosomes of all living organisms) is to serve as a repository for the information that is encoded within the bases of the DNA it contains. In order for this information to be used, specific regions (genes) within the DNA are transcribed by RNA polymerase into messenger RNA (mRNA). This mRNA is subsequently translated by ribosomes along with transfer RNAs (tRNAs) into proteins that carry out the structural and enzymatic processed necessary for the survival and reproduction of the bacterial cell.
In addition, many bacteria contain additional genetic information that resides on self-replicating extrachromosomal elements called **plasmids**. It is often easiest to think of plasmids as accessory pieces of DNA that contain genetic information non-essential to basic cellular metabolism, but which provide the host bacterial cell with additional metabolic or physiological properties. Examples of these properties include the ability to utilize unusual carbon sources or to produce specific toxins important in pathogenesis, etc. Plasmids can be of many different sizes. Some plasmids (**R factors**) carry genes providing resistance to various antibiotics.

![Bacterial Cell](Bacterial_CellDiagram.png)

Surprisingly, although the chromosome serves as a repository for genetic information, it is not an unchanging structure. **Spontaneous mutations** (i.e. changes in the basic DNA sequence) arise at a frequency of ~$1 \times 10^{-8}$ per generation.

Any change in the sequence of bases in the DNA of a cell is called a **mutation**. Many factors in the environment can cause mutations in DNA sequences, including physical factors such as ultraviolet light and radiation; chemical factors such as nitrous acid and benzene; and genetic factors such as transposable elements and some bacteriophages.

These spontaneous mutations may occur within the DNA sequence of the bacterial chromosome or within the DNA sequence of the plasmids contained within the bacterial cell. The majority of mutations are detrimental to the bacterial cell and a cell containing these mutations is selected against. Mutations can also be neutral, that is, they do not alter the fitness of the cell.
Occasionally, a mutation can confer a selective advantage to the cell depending on the particular environmental conditions. In this case, the mutated cell will not only survive, but in many cases, it will replace the original — wild-type or parent strain. Changes in the basic DNA sequences in a bacterial cell are called **genotypic changes**.

**Discussion: mechanism of action of Rifampicin.**

Rifampicin is an antibiotic. It is produced by some species of *Streptomyces*. It binds to the -subunit of RNA polymerase and blocks **initiation** of transcription. Rifampicin does not block elongation of the RNA polymerase complex if transcription has already initiated. Therefore, it has been widely used as a mechanism to block *de novo* transcription during physiological studies.

The structure of the *E. coli* RNA polymerase

RNA polymerase from *E. coli* consists of five protein subunits. It is one of the largest enzymes known and can easily be seen by electron microscopy. Its subunits are encoded by *rpoA* (α), *rpoB* (β), *rpoC* (β′), and *rpoD* (σ). Mutations within *rpoB* are responsible for Rifampicin resistance (RifR).
Lab report due

Section 1, 1/30/2019 W
Section 2, 1/31/2019 R
Section 3, 1/30/2019 W
Phenotypic and Genotypic Variation

1. What was the effect of medium type on pigmentation in strain 30-84 and 30-84Z? What is the difference between these strains?

2. The availability of iron (Fe3+) in the environment is very low (~1 x 10^{-17} M). Therefore, siderophore production by bacteria can be critical for successful growth in nature. Many bacteria in nature produce siderophores, and often one bacterium will out-compete another bacterium for iron. What could explain this observation?

3. How do skim milk plates allow detection of proteases? How does semi-solid motility agar allow for the detection of motility?

Experimental Background

1. Define the following two terms (see link on D2L):

A. Selection.

B. Screening.
Mutation

Mutations normally occur during DNA replication because polymerases make errors while copying the chromosome. Although a large majority of mutations are actually corrected by the polymerases themselves as well as dedicated DNA repair machinery, some occasionally slip through. Mutations can be single base pair changes as well as additions/deletions of nucleotides. Mutations can also occur outside of chromosomal replication as a product of environmental stress (UV light, some antibiotics, etc.). As discussed within the last lab, these mutations are genotypically different from the defined wild type sequence, but likely only have phenotypic effects IF A) the region is transcribed B) the transcribed region is capable of changing a phenotype within the measured environment C) the mutation is non-synonymous.

During growth in liquid culture, bacteria divide in a binary way (one cell gives rise to two cells). If all cells within a culture start out genotypically identical, even if only one mutation occurs during growth, the number of mutant cells at the end of a growth cycle will be determined by when in the growth cycle that mutation occurs. If only ONE mutation occurs over the course of a growth curve, but occurs during an early division, there will be many mutants at the end of growth because each mutant cell will divide several times. Alternatively, if a single mutation occurs during the last possible cell division, there will be only one mutant cell within the whole population. VARIABILITY IN MUTANT NUMBERS AT THE TIME OF MEASURING IS DUE TO DIFFERENT APPEARANCE TIMES OF THE UNDERLYING MUTATIONS DURING GROWTH. Variability across bacterial cultures in the number of mutants roughly follows a Poisson distribution and mutation rates can be estimated by fitting the observed numbers to this mathematical distribution. If a single population is plated out, there is only a set number of mutants within that culture. In this case, any variability is due to randomness and should be normally distributed.

Fig. 1. The Spontaneous Mutation Model. Variability from culture to culture in number of colonies is due to the underlying mutations occurring at different times during cell growth. In each case, there is only one mutant within each population.
Natural Transformation

Many bacteria have the capability to import DNA from the extra-cellular environment and incorporate it into their own genomes. One of the definitive traits of bacteria that are competent for natural transformation is the presence of dedicated proteins and enzymes to transport this DNA into the cell and protect this DNA from degradation. Oftentimes, the trick to making bacterial competent for natural transformation is finding the right environmental conditions to turn on regulatory pathways controlling genes involved in DNA importation. Once the DNA is present within the cell, almost all bacteria have enzymes necessary (i.e. RecA) to recombine this DNA into homologous (matching sequence) regions on the chromosome. As little as 25bp of homologous DNA is sufficient for some level of transformation to occur but even a single nucleotide difference between imported DNA and the chromosome can disrupt this process. Natural transformation can occur without much bacterial growth, and so any variation between plates should be normally distributed.

Streptomycin and Rifampicin Resistance

Streptomycin inhibits bacterial growth by binding to the 16s rRNA subunit, and interfering with the binding of formyl-methionyl-tRNA to the 30s subunit to inhibit protein synthesis. Spontaneous mutations to streptomycin resistance are possible within \textit{P. stutzeri}, and usually occur within the \textit{rpsL} gene. Rifampicin inhibits bacterial growth by binding to the active site of RNA polymerase and preventing transcription. Spontaneous mutations to rifampicin resistance occur quite readily within \textit{P. stutzeri} (>100-fold higher rates than to streptomycin resistance), and usually occur at the \textit{rpoB} gene. The \textit{rpsL} and \textit{rpoB} genes are found in close proximity to each other within the \textit{P. stutzeri} genome.

MATERIALS (per team)

- Overnight culture of 1mL \textit{P. stutzeri} DAB273 in Saltwater LB
- 1mL of Saltwater LB
- 100ng of \textit{P. stutzeri} DBL509 strepR/rifS DNA
- 100ng of \textit{P. stutzeri} DBL455 strepR/rifR DNA
- 100ng of \textit{P. syringae} DAB4 strepR/rifR DNA (a strepR version of strain DC3000)

EXPERIMENTAL PROTOCOL
Mutation

- Dilute *P. stutzeri* DAB273 1:100000 in Saltwater LB (10uL in 1mL followed by 1uL in 1mL). Place 500uL into test tube and place on shaker at room temperature.

- Plate remaining 500uL onto Streptomycin 50 Saltwater LB plates, let dry, and place in 27°C incubator

Transformation

- Take 4 100uL samples of *P. stutzeri* DAB273 overnight culture and mix with either A) 1uL of *P. stutzeri* DBL509 DNA B) 1uL of *P. stutzeri* DBL455 DNA C) 1uL of *P. syringae* DBL5 DNA D) 1uL water

- spot 10uL of each culture on 1 Saltwater LB plate in quadrants, let dry, and place in incubator at 27°C.

MATERIALS (per team)

- Eppendorf tubes and water for performing dilutions
- 1 plain Saltwater LB plate
- 3 Streptomycin 50 Saltwater LB plates
- 1 Streptomycin 50 Rifampicin 50 Saltwater LB plate

Mutation

- Dilute mutation liquid culture 1:10000, 1:100000, 1:1000000, 1:10000000, and plate out 10uL of each on quadrants of Saltwater LB plate. Let dry and place in incubator at 27°C.

- Plate out remaining culture (~490uL) onto Streptomycin 50 Saltwater LB plate, let dry and place in incubator at 27°C.

Transformation

- Resuspend each of the transformation cultures in 1mL of Saltwater LB, mix thoroughly, and plate 10uL of each on quadrants of A) Streptomycin 50 Saltwater LB plate B) Streptomycin 50 Rifampicin 50 Saltwater LB plate
Streak a sample of the DBL455 transformation to single colonies on a Streptomycin 50 SWLB plate

**Section 1, 1/30/2019 W**
**Section 2, 1/31/2019 R**
**Section 3, 1/30/2019 W**

**MATERIALS (per team)**

- 1 Streptomycin 50 Rifampicin 50 Saltwater LB plate
- 1 Streptomycin 50 Saltwater LB plate

- For Mutation and Transformation cultures, count the number of colonies that arises on each type of plate
- If possible, streak (not to singles, just for growth) 10 colonies from the Streptomycin 50 plate (the one where you streaked DBL455 transformation to single colonies) to a Streptomycin 50 Saltwater LB plant and THEN to a Rifampicin 50 Streptomycin 50 Saltwater LB plate.

**Section 1, 2/4/2019 M**
**Section 2, 2/5/2019 T**
**Section 3, 2/4/2019 M**

- Count how many (out of the 10 you streaked) colonies grew on both the strep and strep/rif plates

**Lab report due**

**Section 1, 2/13/2019 W**
**Section 2, 2/14/2019 R**
**Section 3, 2/13/2019 W**
1. Why is it important to dilute your start fluctuation assays with a very small number of cells?

2. When DBL273 is transformed by DNA from DBL455, why can you find both strepR/rifS colonies and strepR/rifR colonies?

3. Is the transformation rate with *P. syringae* DAB4 DNA lower than with *P. stutzeri* DNA? If so (or not) why do you think this is?
INTRODUCTION

Plasmids are small extrachromosomal elements that are separate from the bacterial chromosome but depend on cellular enzymes for replication. Plasmids often confer unique properties on the cells that contain them. Plasmids come in all different sizes, copy numbers, and may contain many different types of genes. Many plasmids have been engineered to be useful in microbial genetics.

Plasmid Nomenclature

1. Plasmids are named beginning with a lower-case p to indicate the name refers to a plasmid and not to a bacterial strain (e.g. pUC18, pKKC259, pKT2, etc.).

2. The letters in front of the plasmid number usually refers to the individuals who engineered the plasmid (e.g. pDBL632 indicates it was constructed by Dr. David Baltrus (the L is for Lab, and this is the 632nd strain in my lab collection).

3. Bacteria containing a known plasmid are named by the strain name followed by the plasmid name in parentheses (e.g. E. coli DH5 (pUC18), or DH5 (pKKCRec1452).

Plasmid DNA Isolation

In comparison to most bacterial chromosomes, plasmids are small in size. They exist in cells as supercoiled (tightly twisted) molecules and are relatively unaffected by many procedures that denature chromosomes and proteins. In fact, we use the fact that plasmids are resistant to shearing (breaking) as part of our isolation methodology.

The basic principle of all plasmid DNA isolations is the same: break open the cells, remove everything you do not want, and what is left is predominantly plasmid DNA. The procedure we will use is a modified alkaline lysis procedure. We will use a kit from Fermentas Corp. for this procedure. The procedure involves re-suspending bacterial cells in a buffer that stabilizes nucleic acids and then a buffer containing a cell wall degrading enzyme (Lysozyme) that lyses the cells by breaking down the peptidoglycan backbone. Proteins are next degraded using an alkaline protease solution under alkaline conditions to denature the chromosomal DNA and proteins. The pH is returned to neutrality which causes the chromosome and proteins to collapse and cell debris is pelleted by centrifugation. The plasmid DNA, being supercoiled and small, remains intact and relatively unaffected. This solution has high salt and causes the ds (double-stranded) plasmid DNA to stick to the spin column. The plasmid DNA is washed of RNA and other contaminants and
dried. Then the column is placed in a sterile tube and the plasmid DNA eluted from the resin in pure water that lacks any contaminating salt. Thus, when you are done, column wash will contain nearly pure plasmid DNA. In this exercise you will compare yields for one high copy number plasmid (pBAM1) with an oriR6K origin of replication.

MATERIALS

- 3 ml LB + Amp₁₀₀ broth overnight of Top10 (pUC18) – high copy number plasmid
- 3 ml LB + Amp₁₀₀ broth overnight of Top10 (pUC18-KSB) – high copy number plasmid that’s larger than “just” pUC18
- Fermentas GeneJetTM Plasmid Miniprep Kit
- Sterile 1.5 ml microfuge tubes
- Micropipettors
- Sterile tips

EXPERIMENTAL PROTOCOL

Isolation of plasmid DNA from bacteria using a modified Fermentas GeneJetTM Plasmid Miniprep Kit Protocol is given on the next page.
Modified Protocol for Fermentas GeneJet\textsuperscript{TM} Plasmid Miniprep Kit

- **Resuspend Cells**
  - Lyse and Neutralize
  - Spin 3 ml of overnight cultures in two (2) 1.5 ml microfuge tubes for 1 min.
  - Drain supernatant and re-suspend each pellet with 125 µl Resuspension Solution and vortex, then combine in single microfuge tube
  - Add 250 µl of Lysis Solution and invert the tube 10 times
  - Add 350 µl of Neutralization Solution and invert the tube 10 times
  - Centrifuge for 7 mins.

- **Bind DNA**
  - Load the supernatant into GeneJet\textsuperscript{TM} spin column
  - Centrifuge for 1 min. and discard flow-through

- **Wash column**
  - Add 500 µl of Wash Solution and centrifuge for 1 min.
  - Discard the flow-through
  - Again, add 500 µl of Wash Solution and centrifuge for 1 min.
  - Discard the flow-through
  - Centrifuge empty column for 2 mins.

- **Elute pure DNA**
  - Transfer column to new sterile 1.5 ml microfuge tube
  - Add 30 µl of 50°C ddH\textsubscript{2}O to the column and incubate for 2 mins.
  - Centrifuge for 5 mins.
  - Collect the flow-through, **KEEP FLOW THROUGH.** Discard column
  - Clearly label your plasmid DNA tubes so you can identify them
Restriction Digestion of Purified Plasmid DNA

1. Digest 5 µL of DNA from each tube of plasmid DNA with BamHI.
   5 µL DNA
   2 µL 10x Reaction 3 Buffer
   2 µL 10x BSA
   10 µL ddH2O
   1 µL BamHI

2. Incubate digestion reaction 37°C water bath for 60 mins.

3. Store digestion at 4°C

4. STORE YOUR PLASMID DNA AT 4°C AS WELL!!

A. Agarose gel electrophoresis of digested DNA

At one point or another, virtually every experiment involving DNA requires electrophoresis. The ability to separate fragments of DNA according to their relative sizes is extremely useful for many applications, from determining approximate sizes, Southern hybridizations, cloning, etc. The basic principles of agarose gel electrophoresis are quite simple.

1. DNA is negatively charged due to its phosphate backbone. Therefore, if placed in an electrolyte solution such as a Tris-Borate-EDTA buffer (TBE buffer) and an electric charge is placed across the buffer, the DNA will move towards the positive electrode.

2. If the DNA is placed in TBE buffer in an agarose gel and a current is supplied, the DNA will move towards the positive electrode, but its rate of movement will be inversely proportional to the log of the length of the fragments (i.e. smaller fragments will move faster through the agarose gel). Agarose is highly purified polysaccharides isolated from seaweed. Theoretically, these principles can be used to determine the approximate molecular weight (i.e. length) of any piece of DNA.
B. **Preparing and running an agarose gel**

For comparative purposes, in this exercise we will load all samples onto a few larger format gels. In future lab exercises, each pair will pour and run their own gels.

Below are the procedures for preparing, loading, running, staining and photographing gels.

**Preparing gels.** Assemble the small horizontal gel tray using the rubber spacers as demonstrated by your GTA. There is pre-melted agarose in the 55°C water bath.

1. Place comb in gel tray. Pour approximately 35-40 ml of melted 1% agarose into the tray (Fig. 3.1). (The goal is to **pour a thin gel**, not a thick one). Let harden (it turns opaque, almost chalky).
2. Carefully remove the comb by rocking it back and forth. Remove the rubber end caps being **CAREFUL** not to tear the wells.
3. Place the gel in the gel box and fill it with 1x TBE buffer until the gel is just submerged.

![Fig. 3.1 Gel Electrophoresis Apparatus](image-url)
Loading gels.
1. Add 2 µL Tracking dye to each BamHI digest (Total volume = 22 µL). DO NOT ADD TRACKING DYE TO YOUR STOCK PLASMID PREPS!!!
2. Take 5µL of your stock, undigested plasmid culture and place in separate tube. Add 15µL water and 2µL Tracking dye (Total volume = 22 µL).
3. Load gel with one lane plasmid digest, one lane undigested plasmid. Include 5 µL Kb ladder and 10 µL λ HindIII as size standards. NOTE: These standards do not require the addition of tracking dye.

Running gels.
1. Carefully place gel top on and connect electrodes to power supply. Run gel at 90V until blue tracking dye is ¼ inch from the bottom of gel. Do not electrocute yourself – major loss of points!

C. Visualization of DNA products on gel using EtBr.

DNA is so small that we cannot visualize it with our own eyes. In order to visualize the DNA on the gel we use a stain called ethidium bromide (EtBr). EtBr is an intercalating agent that fits between the stacked bases of DNA. EtBr fluoresces under UV light. Due to the large number of gels we will add EtBr to the gels in a small volume for staining. We will use the UV gel box and digital camera to capture images of yours gels. The GTAs will help you use the equipment.

ALWAYS WEAR GLOVES WHEN WORKING WITH EtBr BECAUSE IT IS A CARCINOGEN!!!!!!

Notch your gel (check with your GTA) so you can tell which photograph is yours!

EtBr Staining.
1. Using disposable gloves, place gel into a tray containing EtBr in TBE.
2. Let stain for 10 to 20 mins.
3. Visualize bands using a short-wavelength (254 nm) UV light box.
4. Photograph gel using digital camera (make 2 copies of the pictures).
See Appendix 1 for more information on restriction enzymes.
See Appendix 2 at end of exercise regarding molecular weight standards.
APPENDIX 1: RESTRICTION ENZYMES (RESTRICTION ENDONUCLEASES)

Many molecular techniques are based on the now simple notion of cutting and joining DNA molecules. In this exercise, you will be exposed to some of the basic procedures used in molecular cloning. In addition, some of the techniques we have used in previous sections will be used as part of the recombinant experiment you will do. Before we begin, a short discussion of the terms and procedures used in recombinant DNA work is necessary.

There are four classes of restriction enzymes currently known. **Type or Class II** enzymes are primarily used in recombinant DNA work. This class of enzyme recognizes a specific sequence of base pairs in double-stranded DNA and cleaves once on each strand. There are 100’s of known restriction enzymes. We use these enzymes to easily manipulate DNA in cloning experiments.

The other classes not used in recombinant DNA work are:
- Type or Class I enzymes cut DNA randomly, far from their recognition sequences.
- Type or Class III enzymes cut outside of their recognition sequences and require two such sequences in opposite orientations within the same DNA molecule to accomplish cleavage.
- Type or Class IV enzymes recognize modified, typically methylated DNA.

**Enzyme Nomenclature**

Restriction enzymes are named for the bacterium they were isolated from. For example, *Eco*RI was the first restriction enzyme isolated from *Escherichia coli = Eco + RI = EcoRI*. The first three letters are italicized (or underlined) because they are derived from the genus and species name of the bacterium. *Hind*III = third restriction enzyme isolated from strain d of *Haemophilus influenzae*. Commonly used enzymes:

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Bacterium Isolated From</th>
<th>Recognition Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BglII</td>
<td>Bacillus globigii</td>
<td>5’→ A ‘GATCT – 3’&lt;br&gt;3’→ TCTAG ‘A – 5’</td>
</tr>
<tr>
<td>EcoRI</td>
<td>Escherichia coli RY-13</td>
<td>5’→ G ‘AATTC – 3’&lt;br&gt;3’→ CTAA ‘G – 5’</td>
</tr>
<tr>
<td>EcoRV</td>
<td>Escherichia coli J62PLG74</td>
<td>5’→ GAT ‘ATC – 3’&lt;br&gt;3’→ CTA ‘TAG – 5’</td>
</tr>
<tr>
<td>HindIII</td>
<td>Haemophilus influenzae Rd</td>
<td>5’→ A ‘AGCTT – 3’&lt;br&gt;3’→ TTCGA ‘A – 5’</td>
</tr>
<tr>
<td>PstI</td>
<td>Providencia stuartii</td>
<td>5’→ CTGCA ‘G – 3’&lt;br&gt;3’→ G ‘ACGTC – 5’</td>
</tr>
<tr>
<td>SalI</td>
<td>Streptomyces albus G</td>
<td>5’→ G ‘TCGAC – 3’&lt;br&gt;3’→ CAGCT ‘G – 5’</td>
</tr>
<tr>
<td>SmaI</td>
<td>Serratia marcescens</td>
<td>5’→ CCC ‘GGG – 3’&lt;br&gt;3’→ GGG ‘CCC – 5’</td>
</tr>
</tbody>
</table>

As you can see, each one of these restriction endonucleases or enzymes recognizes a different sequence of base pairs. Enzymes such as *Eco*RI and *Hind*III leave 5’overlaps, *Sma*I and *Eco*RV leave blunt ends, and *Pst*I leaves a 3’overhang when it digests DNA.
IMPORTANT: Only DNA fragments with **complementary** ends can be joined together. The exception is blunt-ended fragments, which can be joined to any other blunt-ended fragment. However, the re-joining (ligation) of blunt ended fragments is much less efficient.

**Discussion: Restriction enzyme function**

The restriction enzyme *PstI* will be used as an example. *PstI* is a protein that binds to DNA. When it binds to DNA containing its **recognition sequence** it creates asymmetric nicks in each strand of the DNA. *PstI* happens to create 3’ overhanging ends. Once the DNA is cleaved the affinity between *PstI* and the DNA is weakened, and it dissociates, and the cycle begins again.

---

**APPENDIX 2: DISCUSSION: DNA SIZE STANDARDS**

Measuring fragment sizes using the KB Ladder Standard.
By including size standards on your gel, it is possible to determine the size of any unknown fragment of DNA. The basic idea is that the logarithm of a molecule’s length is proportional to its migration velocity over a broad range. For a typical gel this relationship can be plotted on semilog paper to give the curve shown below (Fig. 3.3).

Thus, the length of any unknown fragment can be determined by co-electrophoresis with other fragments of known length. The fragments of known length (the size standard) can be plotted to yield a standard curve (Fig. 3.3) on which the unknown fragment can be interpolated, resulting in an estimate of its length. In the example shown in the figure, using the size standard has resulted in the curve illustrated. Two fragments of unknown length were also run on the same gel. One migrated 8 mm and the second 15 mm. By interpolating their distances onto the curve, we can tell that the larger fragment is 4,500 bp in length and the shorter one 2,300 bp long. Note that the curve is not linear at the ends. Also, gels are only accurate to + 10%.

We often use one of two size standards, a Kb Ladder or bacteriophage λ DNA which has been digested with HindIII.
Lab report due

Section 1, 2/18/2019 W
Section 2, 2/19/2019 R
Section 3, 2/18/2019 W
1. What physical property allows us to isolate plasmid DNA as opposed to chromosomal DNA?

2. Why should you always include antibiotics in the medium when cultures are grown for plasmid isolation?

3. What is in the Tracking Dye>Loading Buffer?
Bacterial operons are tightly regulated through the action of transcription factors called $\sigma$ factors. The action of these $\sigma$ factors are in turn regulated by inducer and repressor molecules as well as activator proteins. Gene expression within bacterial cells is the product of a multi-layered cascade of regulatory proteins and molecules acting together, with these cascades consisting of multiple interacting/interfering $\sigma$ factors and regulatory molecules.

Virulence gene expression within the bacterial phytopathogen *P. syringae* is the product of a complicated regulatory module consisting of numerous sigma factors and inducers/repressors. At the top of the cascade is the GacAS two component system which triggers expression of the NtrC-like regulators HrpR and HrpS. HrpRS work together to recruit $\sigma^{54}$ to a promoter upstream of the alternative $\sigma^{54}$ transcription factor HrpL. HrpL then binds to a promoter upstream of virulence genes (called the hrp-box) in order to transcribe these genes. Little is known about the environmental signals that ultimately trigger expression of HrpL, other than these signals reflect the cellular environment inside a leaf (low pH, low iron, presence of sugars like fructose/sucrose).

In this lab will use chromosomal *uidA* fusions to HrpRS, HrpL, and Avrpto (a virulence gene) in *P. syringae* strain to investigate the effects of gene knockouts and strain backgrounds on expression of this regulatory cascade. We will also use a plasmid borne *hrpL*:lacZ to investigate the differences between regulation on the chromosome and plasmids as well as differences in reporter genes.

Reporter constructs are utilized to probe regulatory cascades, and interactions between genes therein, to determine how these cascades are affected by both the genetic background (i.e. which genes are active) and the environment. Such reporters are very useful, but one must also consider their limitations. For instance, when constructing phenotypically labeled strains, it’s necessary to take into account where the phenotypic label will be placed. If the reporter gene is placed on a plasmid rather than the chromosome, this may lead to differences in expression even if the same promoter is used. Some regions of the chromosome may be more sensitive to environmental changes than others and if you are just looking for a phenotypic reporter, it’s good practice to try and minimize uncontrolled variance in gene expression. However, this isn’t always possible. One of the major sites for insertion of phenotypic markers in *Pseudomonas* (and many other Gram-negative bacteria) is called the “Tn7 insertion site”. Next experiment we will work with a Tn5 transposon, which inserts fairly randomly throughout the chromosome, but the Tn7 transposon is much more specific. In *Pseudomonas* this transposon usually inserts downstream of the glmS gene (but out of the coding region). Although many studies use this site and transposon to complement mutants from other regions of the chromosome, this site is possibly subject to some changes in regulation regardless of what gene is placed there. Likewise, colonies are spatially structured and may be phenotypically variable. Reporter constructs may be
differentiated in different parts of the colony, even in strains where there are no genotypic differences.

We will explore all of these uses and challenges in today’s lab.

**MATERIALS (per team)**

- 1mL LB media containing *Pseudomonas syringae* strain DC3000
  1) gacA:uidA
  2) hrpRS:uidA
  3) hrpL:uidA
  4) avrpto:uidA
- 1mL LB media containing *Pseudomonas syringae* strain DC3000:ΔhrpRS
  1) gacA:uidA
  2) hrpRS:uidA
  3) hrpL:uidA
  4) avrpto:uidA
- 1mL LB media containing *Pseudomonas syringae* strain DC3000:ΔhrpL
  1) gacA:uidA
  2) hrpRS:uidA
  3) hrpL:uidA
  4) avrpto:uidA
- 1mL LB media containing *Pseudomonas syringae* strain DC3000 +
  1) Tn7::lacZ
  2) plasmid::lacZ (same promoter, just chromosome or plasmid)
- 1mL LB media containing *Pseudomonas stutzeri*
  1) Tn7::lacZ
- 2 KB agar plates + Xgluc
- 2 KB agar plates + Xgal
- 28°C incubator

**EXPERIMENTAL PROTOCOL**

Examination of the effect of medium type and strain background on gene expression on solid agar plates

1. Streak isolates of each *uidA* parent strain onto KB plates containing Xgluc. There are 12 strains total, so split each plate up into 1/6ths to fit every strain.

2. Streak isolates of DC3000 containing *lacZ* in either the Tn7 site or on a plasmid, as well as DC3000 containing *gacA:uidA* onto plates containing Xgal.

3. Make a dilution series of *Pseudomonas stutzeri* (four 1:100 dilutions of starter culture). Plate out 5uL of each dilution onto a KB agar plate containing Xgal.

4. Incubate the plates at 28°C.

5. Examine the phenotypes after 48 h.

Examine and **RECORD** the phenotypes of each bacterial culture on **ALL** plates, noting any differences. Think about what the results indicate to you about the genetics of the regulatory cascade, gene expression, and the ability of each reporter gene to demonstrate gene expression.
Lab Report Due

Section 1, 2/25/2019 M
Section 2, 2/26/2019 T
Section 3, 2/25/2019 M
1. Draw out the regulatory cascade of strain DC3000

2. Do you think gene expression of reporters from a plasmid will be higher or lower than from the chromosome? Why?

3. Do you notice any environmental variability in the *P. stutzeri* dilutions?
INTRODUCTION

In many cases, the loss of a particular phenotype leads to our understanding of how something works. This was the basis of the —Mutant Methodology — pioneered by George Beadle many years ago. The approach of finding a phenotype of interest and then determining what is responsible for the phenotype is often referred to as ‘Forward Genetics.’ This mutational approach is dependent on the occurrence of mutations in the genome of the organism under study. In bacteria, the average mutation rate in the average gene is 0.00000001, or 1 x 10^-8. This is a rather low frequency event and we would have to observe a lot of bacteria in order to have a chance to see a visible mutation. To increase the mutation rate, scientists usually use a mutagen to increase the mutation rate to 1 x 10^-5 to 1 x 10^-3. Therefore, mutagenesis plays a critical role in our understanding of all living things. Even today, with the power of genomics and proteomics, the study of mutants is essential.

In this experiment, you will isolate mutants of *Pseudomonas* that have altered phenotypes and levels of gene expression. What is exciting about this experiment is the opportunity to identify new mutant phenotypes. Most genetic laboratories (prokaryotic and eukaryotic) use transposable elements (TEs) carried by plasmids for mutagenesis experiments.

Plasmids often serve as extremely useful vectors for microbial genetics. Plasmids can be grouped into different classes based on many properties. One useful one is host range. Plasmid host ranges: narrow versus wide

**High copy narrow host range plasmids.** These plasmids can only replicate in specific hosts (e.g. *E. coli*) or in hosts that encode a specific gene product required for replication. This class of plasmids usually exists in large numbers of copies in each cell (30-300).

**Low copy broad host range plasmids.** These plasmids can replicate in a wide range of hosts (e.g. *E. coli, Pseudomonas*, etc.) but maintain themselves at only a few copies per cell (1-4).

Plasmids can often be transferred from cell-to-cell via conjugation. The ability to conjugate into a new host is separate from the ability to replicate in the new host cell. Thus, some plasmids can be conjugated into a different genus of bacteria but will be unable to replicate once they enter the new cell. This seeming suicide by the plasmid resulted in their being termed suicide plasmids. We take advantage of suicide plasmids for transposon mutagenesis. For example, the plasmid we will use, *pRL27*, is a narrow host-range plasmid requires the *pir* gene product π in order to replicate. Thus, it can only replicated in strains of *E. coli* or closely-related bacteria that contain the *pir* gene. We will be using *E. coli* strain S17-1 λpir for matings. The *pir* region has been to this strain added using λ phage. The
Transposable Elements

Transposable elements are distinct pieces of DNA that are capable, as their name implies, of transposing, or —hopping! from one piece of DNA into another. One commonly used Tn for bacterial mutagenesis is the natural transposable element Tn5.

Background on Tn5

Transposon Tn5 is a composite transposon, consisting of 2 identical (except for one base change) inverted repeat sequences (IS50L & IS50R) flanking a core region containing a kanamycin resistance (KmR) gene. The right inverted repeat, IS50R, also contains a gene named tnpA that encodes a transposase enzyme. The transposase in conjunction with the inverted repeat sequences are responsible for allowing Tn5 to —hop or transpose— from one site in a piece of DNA to another site in the same or a different piece of DNA. This transposition is not dependent on RecA-mediated homologous recombination. However, transposons cannot replicate themselves and are dependent on the DNA they are inserted into for replication. We will discuss Tn5 in depth in the lecture course.

Tn5 advantages

1. Many chemical or physical mutagens, such as UV light, cause point mutations that occasionally still allow some gene function to remain. These gene mutations are referred to as ‘leaky’. Mutations created by Tn5 are not ‘leaky’ since Tn5 inserts 5.7 kb of DNA into a gene. Tn5 also can have polar effects on downstream genes, preventing their expression as well.
2. Chemical and physical mutagens often cause multiple mutations. Thus, it is not always simple to know which mutation is responsible for the altered phenotype. Useful transposons such as Tn5 usually transpose once.
3. When Tn5 inserts into the chromosome it creates a KmR marker at that site. Therefore, the mutated region of the chromosome can be cloned directly by selecting for KmR. This has also been called marking a mutation site.

We will use an engineered transposon based on Tn5 in conjunction with the suicide plasmid pRL27 (Fig. 5.1)
Features of pRL27:

1. Contains inverted repeats (IRs). These are the sequences recognized by the transposase encoded by *tnpA*. During transposition, transposase cleaves the dsDNA at each end of the IRs and moves the region between into another piece of DNA (i.e. the chromosome).

2. The Tn lacks the Tn5 *tnpA* transposase gene. This gene is carried by the plasmid outside of the region that is transposed. This means that once transposition has occurred, there is no *tnpA* present to cause any subsequent transpositions to occur, resulting in a very stable mutation.

3. The *tnpA* gene contains several mutations that render the transposase it encodes hyperactive. That means it will transpose the Tn at a much higher frequency than the wild type transposase.

4. Contains neophosphotransferase activity (*npt*) within the transposon. Thus, the location of the insertion is ‘marked’ by kanamycin antibiotic resistance (Kmr).

5. The Tn contains an *oriR6K* origin of replication. This sequence is inactive unless the Tn is present in a cell containing the *pir* gene encoding the π replication protein.

6. The plasmid contains an *oriT* sequence (origin of transfer) that enables it to be ‘mobilized’ into new bacterial cells.
Overview of experiment:

*Pseudomonas* sp. Leaf58 DBL1541 is a rifampicin resistant mutant of a strain originally isolated from the leaf of a wild Arabidopsis plant in Switzerland. We chose this strain initially because it is a fairly useful all purpose strain for exploring a variety of phenotypes as you will see. This strain also naturally contains a 900kb megaplasmid, and we are interested in identifying any phenotypes that the megaplasmid affects.

You will perform the following conjugation ([Fig. 6.2](#)). The net result will be to introduce the modified Tn5 into *Pseudomonas* Leaf58 strain DBL1541. Examine the steps closely as several events must take place for this to work. As convoluted as this may seem initially, it works very well in the lab.

![Figure 6.2 Conjugating pRL27 into Pseudomonas Leaf58 DBL1541](#)

(Biparental Mating) The E. coli donor strain DBL330 contains a pir gene so pRL27 can replicate. It also has tra genes to enable pRL27 to be mobilized into the recipient *Pseudomonas* cell, which lacks the pir gene.

Once inside the recipient, the plasmid cannot replicate and is lost. However, the transposase can transpose a section of pRL27 (between inverted repeats) from the plasmid into the host chromosome before the plasmid is lost. This results in the creation of a Kanamycin (KmR) marked mutation in DBL1541.
MIC et al. 428/528L
Experiment No. 6

MATERIALS

LB + Km25 O/N culture of DBL330(pRL27)
KB O/N culture of P. stutzeri DBL1541
Sterile microfuge tubes
Sterile glass test tubes
LB agar plates (no antibiotics)
10 LB agar + Rif50, Kan25 plates
Sterile nitrocellulose squares
Tabletop centrifuge

Next steps

Sterile toothpicks in glass petri plates
LB + Kan25 plates
KB + Kan25 plates
LB Motility plates
Minimal media plates for auxotrophy
Experimental Protocol

1. Available are O/N cultures of *E. coli* DBL330(pRL27) donor in LB + Kan25, and *Pseudomonas* recipients in KB

These were inoculated from fresh O/Ns early this morning and are shaking at 37°C and 28°C, respectively.

2. For each conjugation mixture, aliquot 500 µL of each into 2 sterile 1.5 ml microfuge tubes (*Fig. 6.3*).

3. Microfuge 1 minute. Remove supernatants by a quick wrist flick into a waste container. Add 200 µL LB, resuspend via gentle pipetting, microfuge 1 additional minute.

4. Re-suspend the cell pellets at the bottom of the tubes in 200 µl KB via gentle pipetting. Spot 50 µL of the suspensions onto nitrocellulose filters on an LB plate. Include a filter with donor alone and recipient alone (*Fig. 6.4*). Once the liquid has been absorbed, incubate plate O/N at 28°C.
1) Using ETOH-flamed forceps, carefully lift and place each filter in labeled large sterile glass tubes. Add 2 ml sterile ddH$_2$O and vortex until the cells are removed from the filters.

2) Spread 100 µl from the suspensions of DBL330 (pRL27) alone onto 1 LB agar + Rif$50$, Kan$25$ plate. Repeat for the Pseudomonas Leaf58 DBL1541 alone tube. Incubate plates @ 28°C.

3) Spread 100 µl from each mating filter suspensions onto 3 LB + Rif$50$, Kan25 plates each (6 plates total). Make a 1:10 dilution of resuspension in 500 µl ddH$_2$O and plate an additional 100 µl onto 2 LB + Rif$50$, Kan25 plates and place plate at 28°C

Store remaining filter suspensions @ 4°C so you can plate more if needed later.
Check plates for the next several lab periods:

**Section 1, 2/20/2019 M to 3/20/2019 W**
**Section 2, 2/21/2019 T to 3/21/2019 R**
**Section 3, 2/20/2019 M to 3/20/2019 W**

---
Using sterile toothpicks, begin to patch at least 500 colonies from your mating plates onto various media (Fig. 6.5 on Next Page) using the grid pattern provided. If you carefully patch your cells onto the grids, then you can save time by replica-pronging the colonies onto the subsequent screening media.

**Replica-pronging Protocol**

We have a limited number of replica-pronging devices. They basically are metal rectangles with a handle on the top and 48 stainless steel prongs on the bottom. Used correctly, you can transfer 48 colonies at once to multiple medium plates. You will use these to maintain your cultures on LB kan plates, and to plate onto skim milk agar to screen for protease activity.

1. Pour 95% ETOH into a glass Petri dish. Dip the pronger into the ETOH and pass it through a flame (do not hold it in the flame).

2. Once the ETOH had burned off, set the pronger lightly onto a plain LB agar plate to cool the prongs (~10 sec.).

3. Carefully line up the pronger on top of the colonies on the first master plate (Pl 1). Jiggle it gently to adsorb cells to the prongs.

4. Lift it up and lightly place on the first screening plate, Skim Milk agar, then passage to another LB + Kan25 plate and place at 37°C. Continue until all plates of that series are done. The last plate to be pronged is another LB + Kan25 plate.

5. Once a set of plates is completed, wash the prongs with water on a paper towel.

6. Replace pronger in ETOH and repeat for the next series of plates (Pl 2, etc.).

7. When done, wash the pronger thoroughly and place it back in the cabinet.

--- Incubate plates @ 28°C.

--- Examine your plates, record what you see. Do you see any unusual colony phenotypes on the LB or KB plates?

Possible phenotypes include colonies that appear less/more “goopy”, have different colors, or those that grow slow. The diameter of the patch can be altered, colony differences on KB media might change etc. Record your findings carefully.

**DO NOT DISCARD YOUR PLATES TOO QUICKLY!**
Remember, many phenotypes are only visible under specific conditions or on specific media. Therefore, the next step is to look for specific phenotypic differences as compared to wild type strain DBL1541. We will use different media as we will be looking for different things.

During this time, you will need to come to the labs to patch your colonies.

NOTE: The plate numbers and patch number determine your mutant’s identification. For example, if patch 37 on plate set 3 looks interesting in some way, then the mutant is 3-37.

Section 1, 3/11/2019 M
Section 2, 3/12/2019 T
Section 3, 3/11/2019 W

Replica stamp colonies onto Motility Plates.
Replica stamp colonies from onto LB + Kan25 plates for propagation
Replica stamp colonies from onto M9 minimal media

Section 1, 3/13/2019 M
Section 2, 3/14/2019 T
Section 3, 3/13/2019 W

Confirm potential motility differences by inoculating another motility plate with potential hyper or hypo motility mutants to. Inoculate multiple mutants onto a single motility plate by picking a sample of that strain from the propagation plate using a toothpick or pipette tip and gently stabbing into the new motility plate. Be sure to also pick a colony that displays a “wild type” phenotype as a control.

Confirm potential auxotrophic mutants by streaking a sample of those mutant strains from the propagation plate to M9 minimal media. It’s possible that you may also see “slow growers” that aren’t necessarily auxotrophs but are phenotypically different nonetheless.

Confirm any other “interesting” phenotypic changes (color change, colony shape change, etc…) by streaking those mutants from the propagation plate onto an LB plate and observing growth of the mutant strain.

Section 1, 3/18/2019 M
Section 2, 3/19/2019 T
Section 3, 3/18/2019 W

Check for auxotrophic mutants on M9 minimal media.
Check motility plates for amotile or hypermotile mutants.
Fig. 6.5 Screening Transposon Mutations

Sterile toothpick

Replica Stamp to Minimal Media, KB, and Motility

P1 1

P1 2

P1 10
MIC et al. 428/528L
Experiment No. 6
---It is imperative as a good scientist that you keep accurate records. A Nobel Prize-worthy mutant means nothing if it cannot be recovered.

---Make copies of the score sheet (see previous page).

---Score your plates. Note the numbers of colonies on each plate and any interesting or unusual phenotypes. Make accurate notes, circle the interesting mutants on the stock LB + Kan25 plates (last ones patched) and refrigerate them.

---Replica-prong all of your mutants from your 10 master plates onto new LB + Kan25 plates as necessary to keep them fresh.

---Re-test your interesting mutants to verify the phenotype. Wait until you have several interesting ones and re-test all at once.

IMPORTANT: THE POSSIBILITY OF SEEING SOMETHING INTERESTING IS DIRECTLY PROPORTIONAL TO THE NUMBER OF EXCONJUGANTS YOU PATCH.

WE WILL BE USING SELECTED MUTANTS FOR OTHER EXERCISES THIS SEMESTER.

BE SURE TO CAREFULLY ANNOTATE YOUR PHENOTYPES, AND KEEP FRESH LB+Kan25 PLATES OF EACH INTERESTING MUTANT.

Please have your collection of interesting mutants (2-5) ready by 3/25 (3/26 T)

Lab Reports due:

Section 1, 3/27/2018 M
Section 2, 3/28/2018 T
Section 3, 3/27/2018 M
<table>
<thead>
<tr>
<th>Patch No.</th>
<th>M9</th>
<th>Motility</th>
<th>KB</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>38</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>44</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>47</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
EXPERIMENT NO. 6 – SELECTED QUESTIONS

1. Once the transposon has transposed from the suicide vector into the chromosome, what keeps it from hopping over and over and over and over and over?

2. Would you expect to see increases and decreases in motility in mutants?

3. You notice that one colony of DBL1541::pRL27 grows slowly on KB plates. What might this tell you about the effect of the transposon insertion?
EXPERIMENT NO.7
ISOLATION OF CHROMOSOMAL DNA

Section 1, 3/25/2019 M
Section 2, 3/26/2019 T
Section 3, 3/25/2019 M

INTRODUCTION

Many types of research that address a myriad of biological questions require the isolation of the genomic DNA of an organism. This is a prerequisite for many experiments, from DNA sequencing analysis of a genome to cloning specific regions or genes to construction of a genomic library of the organism to the performance of metagenomic studies.

In Experiment No. 6 we used a novel modified Tn5 transposon to generate mutants of Pseudomonas with interesting phenotypes.

Review of pRL27

Review the introduction of Experiment No. 6.

The transposon carried by pRL27 has many useful properties.

1. Inverted repeats (IRs).
2. No tnpA transposase gene
3. A hyperactive transposase
4. Neophophotransferase activity (npt = Km).
5. An oriR6K origin of replication.

Experimental overview

We will isolate total genomic DNA from a couple of your mutants and digest it with a restriction enzyme. We will self-ligate the fragments and transform the ligated mix into a special strain of E. coli that contains the pir gene encoding the replication protein. This will allow only the single chromosomal fragment that contains the Tn to replicate as a plasmid. Next, we will isolate the plasmid and use DNA primers specific to the ends of the Tn to sequence into the adjacent chromosomal DNA. The sequence generated will be compared to the GenBank database to try to identify the gene disrupted by the Tn.

The isolation of chromosomal DNA is the critical first step for many experimental procedures. One common goal of all chromosomal isolation protocols is to avoid shearing the chromosomal DNA. The large chromosome is fragile and can be sheared easily into pieces. In fact, in spite of our best efforts, some chromosomal DNA will be sheared—there is
simply no way to avoid it. Most methods utilize a gentle lysis step using a detergent (e.g. SDS) and an enzyme (e.g. Proteinase K) that degrades proteins. Unwanted proteins bound to the DNA are usually removed by the addition of chloroform (CHCL3), a nonpolar chemical that denatures them. Finally, the genomic DNA is precipitated using the nonpolar solvent isopropanol or ethanol (EtOH) and excess salt is removed with a 70% EtOH wash.

**DNA precipitation** is based on the fact that DNA has a strong negatively charged phosphate backbone—it is very polar. If a polar molecule is placed in a less polar solvent such as isopropanol or ethanol, it will try to minimize the amount of surface area exposed to the solvent. Thus it will collapse into a ball, and hence precipitate.

To facilitate chromosomal isolation, we will be using the Gentra Puregene Yeast/Bact. Kit from Qiagen, Inc. ([www.qiagen.com](http://www.qiagen.com)). This kit is speedy and produces good quality chromosomal DNA for restriction digestions.

**You and/or your partner must come in and inoculate 3 ml LB + Km50 overnight cultures (O/Ns) of an interesting mutants. Incubate the tubes shaking at 28°C.**

**MATERIALS**

Two mutants that you wish to try and identify the transposon insertion point (preferably motility and auxotrophs, but any one your mutants works).

1mL KB Kan Liquid

-Inoculate your mutant into the liquid and place on the shaker.

**Section 1, 3/27/2019 W**

**Section 2, 3/28/2019 R**

**Section 3, 3/27/2019 W**

**EXPERIMENTAL PROTOCOL**

We will be following the instructions that come with the Gentra Puregene Yeast/Bact. Kit (next page).
Protocol: DNA Purification from Gram-Negative Bacteria Using the Gentra Puregene Yeast/Bact. Kit

This protocol is for purification of genomic DNA from fresh or frozen samples of 0.5 ml Gram-negative bacterial cultures using the Gentra Puregene Yeast/Bact. Kit.

Things to do before starting

- Preheat water baths to 37°C for use in step 6, 65°C for use in step 18, and 80°C for use in step 5 of the procedure.
- Gram-negative bacterial cultures can be used either fresh or frozen. Typically, an overnight culture contains 1–3 x 10^8 cells/ml. Due to the small genome size of Gram-negative bacteria, up to 3 x 10^8 cells may be used for the protocol. Thus, culture can either be used directly, or, if necessary, concentrated by centrifuging. To concentrate, pellet 1 ml of overnight culture at 13,000–16,000 x g for 1 min. Remove the supernatant, leaving 200 µl residual fluid. Thoroughly suspend the pellet in the residual fluid by pipetting up and down 10 times. Place the sample on ice for immediate use or store frozen at −80°C.
- Frozen bacterial samples should be thawed and equilibrated to room temperature (15–25°C) before beginning the procedure.

Procedure

1. Prepare an overnight culture.
2. Transfer 500 µl of the culture (containing approximately 0.5–1.5 x 10^8 cells) to a 1.5 ml microcentrifuge tube on ice.
3. Centrifuge for 5 s at 13,000–16,000 x g to pellet cells.
4. Carefully discard the supernatant by pipetting or pouring.
5. Add 300 µl Cell Lysis Solution, and mix by pipetting up and down. Incubate sample at 80°C for 5 min to lyse the cells.
   Samples are stable in Cell Lysis Solution for at least 2 years at room temperature (15–25°C).
6. Add 1.5 µl RNase A Solution, and mix by inverting 25 times. Incubate for 15–60 min at 37°C.
7. Incubate for 1 min on ice to quickly cool the sample.
8. Add 100 µl Protein Precipitation Solution, and vortex vigorously for 20 s at high speed.
9. Centrifuge for 3 min at 13,000–16,000 x g.

   The precipitated proteins should form a tight pellet. If the protein pellet is not tight, incubate on ice for 5 min and repeat the centrifugation.
10. Pipet 300 µl isopropanol into a clean 1.5 ml microcentrifuge tube and add the supernatant from the previous step by pouring carefully. Be sure the protein pellet is not dislodged during pouring.

11. Mix by inverting gently 50 times.

12. Centrifuge for 1 min at 13,000–16,000 x g. The DNA will be visible as a small white pellet.

13. Carefully discard the supernatant, and drain the tube by inverting on a clean piece of absorbent paper, taking care that the pellet remains in the tube.

14. Add 300 µl of 70% ethanol and invert several times to wash the DNA pellet.

15. Centrifuge for 1 min at 13,000–16,000 x g.

16. Carefully discard the supernatant. Drain the tube on a clean piece of absorbent paper, taking care that the pellet remains in the tube. Allow to air dry for 5 min. The pellet might be loose and easily dislodged. Avoid over-drying the DNA pellet, as the DNA will be difficult to dissolve.

17. Add 100 µl DNA Hydration Solution and vortex for 5 s at medium speed to mix.

18. Incubate at 65°C for 1 h to dissolve the DNA.

19. Incubate at room temperature overnight with gentle shaking. Ensure tube cap is tightly closed to avoid leakage. Samples can then be centrifuged briefly and transferred to a storage tube.
Place the rest of your DNA in Freezer

**Section 1, 4/1/2019 M**
**Section 2, 4/2/2019 T**
**Section 3, 4/1/2019 M**

**Materials**

Reaction Buffer 3  
BamHI  
Sterile H2O  
Your genomic DNA prep from last week

***It is important to be careful in each step or subsequent ones will not work.***

**Chromosomal Digestions**

Now that we have isolated genomic DNA from several mutants, we need to digest it and re-ligate it to create a plasmid containing the modified Tn and adjacent chromosomal DNA.

**Fig. 1** illustrates the principles of the next step of the experiment. The intact chromosomal DNA will be digested with a restriction enzyme (BamHI) that cleaves within the chromosomal DNA on each side of the Tn at the sequence (5’-GGATCC-3’). There are no BamHI sites within the Tn. The distance from the insertion where BamHI cuts is dependent on the location of the recognition sequence. Therefore, the enzyme may cut close to the Tn or far away. The calculated size a BamHI fragments of DNA is given below.

\[ 4^6 = 4,096 \text{ bps} \]

Where:
4 = number of different bases (A, C, G, or T).
6 = length of the recognition sequence (6 nts)
bps = basepairs

So, on average BamHI will digest the chromosome approximately every 5 kb. This means for a genome of 6 Mb (megabases), a BamHI digestion will create 1,200 fragments (6 Mb = 6,000,000 bps divided by 5,000 bps/cut = 1,200 cuts = number of fragments for a circular piece of DNA).

We will next self-ligate the fragments into circles. The key point is that only one of these
1,200 circles of chromosomal DNA will contain the Tn which has the -dependent R6K origin of replication and KanR. If we transform all of this DNA into an *E. coli* strain that contains the *pir* gene that encodes the replication protein, only the one circular DNA piece will replicate. This plasmid contains the modified Tn plus the adjacent region of the chromosomal DNA!

**Figure 1.**

**PROTOCOL**

1. Set up the following digestion for each of your chromosomal DNAs:

   - 7 µL chromosomal DNA
   - 3 µL 10x Reaction 3 Buffer
   - 18 µL H2O
   - 2 µL *BamHI* enzyme
   - 30 µL total volume

2. Incubate the digestion in the 37°C water bath for 1 hour.

   **NOTE:** Restriction enzymes stocks are extremely sensitive to temperature fluctuations.
They must be kept as close to -20°C as possible at all times. Please remove them from the ice buckets as little as possible, and immediately return them to the freezer when you are done with them.

While the restriction enzyme reaction is working, pour an agarose gel.

After the restriction enzyme reaction has been running for 1 hour, in one lane of the gel, run 6 µL of the completed digestions on the agarose gel you’ve poured. Mix together 2µL of UNCUIT DNA with 4uL of water. In a separate lane of the gel, next to the cut sample, run the uncut sample.

**CLEAN UP OF DIGESTED DNA**

The next step is to perform a ligation reaction, but it lowers the reaction if you try to ligate the DNA while there is still BamHI in the reaction mix (basically, the piece can recut itself). To get rid of BamHI we will clean up the reaction using a basic column kit:

1. To remaining 24µL of restriction digest, add 150 µL GeneJet Wash Solution.
2. Load onto GeneJet column. Spin 1 minute, discard flow-through.
3. Add 750 µL GeneJet Wash Solution. Spin 1 minute, discard flow-through.
4. Spin dry column 2 minutes to dry thoroughly.
5. Add 20 µL 55°C sterile water, let sit 2 mins.
6. Spin column 3 mins. **Keep flow through** as this is your ligated DNA. MAKE SURE YOU PERFORM THIS LAST SPIN INTO NEW 1.5mL EPPENDORF TUBE ***NOT INTO PREVIOUS COLLECTION TUBE***

**LIGATE CLEANED DNA**

Set up ligations with 5 µL of the completed digestions as follows:

14 µL *Bam*HI-digested DNA  
4 µL 5x Ligase Buffer  
2 µL DNA Ligase  
20 µL total volume

3. Incubate ligations at 15°C overnight.

4. Store ligation mixes at 4°C.

**TRANSFORMATION OF *E. coli* WITH LIGATED DNA**
Many bacteria in nature naturally take up exogenous DNA from their environment. This is thought to be a mechanism for acquisition of nucleotides as the DNA is often degraded, and may help bacteria acquire new traits if the DNA provides a beneficial trait. However, the genetic workhorse, *E. coli*, does not naturally take up exogenous DNA. It can be made competent, that is, it can be treated to make it able to take up DNA by the following procedure.

**Procedure for making *E. coli* competent.**

1. Obtain 4mL culture of *E. coli* S17 *pir*, aliquot to a 2 different 2mL Eppendorf tube (if not already in one) and place these tubes on ice for 10 minutes.

2. After 10 minutes, pellet the *E. coli* cells in each tube by spinning in a centrifuge at LOW speed (1,500-2,000g) for 10 minutes.

3. Remove the supernatant from each tube and resuspend the cells gently in 200uL ice-cold 1x wash buffer. Re-pellet the cells as in step 2.

4. Completely remove the supernatant from each tube and gently resuspend the cells in 200uL ice-cold 1x competent buffer.

5. Add either A) 5uL positive control DNA or B) 5uL of your ligation reaction to one tube each. Mix gently for a few seconds.

6. Add 200uL LB media to tube and place on shaker at 37°C. Let shake for 1 day.

**Section 1, 4/4/2019 R YOU MUST COME IN ON AN ODD DAY**

**Section 2, 4/5/2019 F YOU MUST COME IN ON AN ODD DAY**

**Section 3, 4/4/2019 R YOU MUST COME IN ON AN ODD DAY**

1. Spread 400 μL of solution onto LB + Kan25 plates. Incubate at 37°C for 1-3 days.
1. Check transformation plates. Did the positive control work?

2. If colonies on plate from your ligation reaction, pick 2 colonies from each transformation and:
   - Touch a new LB + Kan50 plate, and
   - Inoculate a 3 ml LB + Kan50 broth overnight of each.

3. Incubate the tubes shaking at 37°C and the plates at 37°C.

ISOLATION OF PLASMID DNA FOR SIZING AND SEQUENCING

Now the goal is to isolate the plasmid DNA from the transformed *E. coli* DH5pir cells. This is to allow us to determine:
   1. How large is the plasmid?
   2. The DNA sequence of the chromosomal DNA adjacent to the Tn.

Prepare plasmid DNA from each overnight culture according to the protocol used in Exercise 4.

Please use your best skills during this procedure. Good DNA is essential for successful DNA sequencing. The presence of contaminating salt will inhibit the sequencing reaction resulting in poor or no readable sequence.

Turn in your plasmid preps (well-labeled with name, clone #, etc.). We will determine the concentration of your DNA. The DNA sequencing center requires at least 50 ng/l DNA (400 ng total DNA) for sequence analysis. If necessary, you will have to re-make the plasmid DNA. This is why we keep a plate of the clones.

Lab Report Due:
Experiment No. 7 Selected Questions

1. Would your ligated “plasmids” be able to replicate in ANY strain of *E. coli*?

2. Will most of the *BamHI* cut pieces of the *Pseudomonas* chromosome circularize during ligation? Why won’t most of these be successfully maintained as plasmids in *E. coli*?
Experiment 8 Final Labs

Rationale: Over the course of this semester you and your lab group have been passaging strains in liquid cultures of KB media under both static and shaking conditions. It is very likely that these strains have adapted to these culture conditions and are now morphologically (and potentially physiologically) distinguishable from their ancestors from two months ago. Hopefully you have collected observations and records throughout the course of these passages about these visible changes.

For the final lab, we will compare morphological differences between the ancestral strains and your evolved lines. We will measure two phenotypes that are usually inversely related, motility and biofilm formation, because it is possible that your strains have changed one or both of these traits throughout the course of passage. Biofilms are likely the dominant bacterial form throughout the world, and occur when cells attach to a surface and build complex three dimensional structures above this attachment point. If you have been observing your cultures over the course of passage, you have very likely witnessed a biofilm forming at the liquid/air interface of your cultures after each week of passage, which is visible as a ring of cells stuck to the test tube.

For this last lab, you will measure both biofilm formation and motility for one colony from each of your passaged lines in order to determine whether either of these traits from their common ancestor in either of the populations. Together with your observations over the course of the semester, these data points will form the basis of a short presentation which will serve as your Experiment 8 lab report.

Section 1 4/8/2019 Monday
Section 2 4/9/2019 Tuesday
Section 3 4/8/2019 Monday

Materials:

300uL KB media in an Eppendorf tube.
3 Test Tubes, each with 5mL KB media with strep50
2KB media agar plate with strep50

Procedure:

Prepare 3 total 1.5mL Eppendorf tubes with KB media by pipetting 100uL from the first tube (provided) to two additional tubes.

Pick a single colony from your “static” culture to one of these Eppendorf tubes using your spreader or a pipette tip and resuspend in the 100uL by shaking the bacteria off of the spreader or pipette tip. Do the same for a single colony from your “shaking” culture and from the plate with your strain’s ancestor.

Pipette 50uL from each Eppendorf tube to 1 test tube containing 5mL KB media and place in incubator as you have for the weekly passages.

Streak a sample of the remaining colony resuspensions to single colonies on a KB agar plates and LB agar plates. Streak all three samples to the same plate, such that each takes up ⅓ of the plate. Place plate in incubator.

Section 1 4/10/2018 Wednesday
Section 2 4/11/2018 Thursday
Section 3 4/10/2018 Wednesday

Materials:

1 KB media motility plate

Procedure:

Observe the static and shaking cultures you inoculated last lab session and note any differences in phenotype between your evolved strains and the ancestor (i.e. density, distribution, etc…). Place static cultures back in incubator.

Observe the agar plates that you streaked the strains to singles on last lab session and note any differences in phenotypes between your evolved strains and the ancestor.
Take a small sample from each of your 3 strains and inoculate all three into the motility plate, so that each strain is inoculated into a ⅓ of the plate. Place in 27 degree incubator.

Section 1 4/15/2019 Monday
Section 2 4/16/2019 Tuesday
Section 3 4/15/2019 Monday

Materials:

15mL 1% Crystal Violet solution
15mL PBS solution

Procedure:

Observe the static cultures you inoculated two lab sessions prior and note any differences in phenotype between your evolved strains and the ancestor (i.e. density, distribution, etc...). Save these cultures for biofilm assay below.

Observe the agar plates that you streaked the strains to singles two lab sessions prior and note any differences in phenotypes between your evolved strains and the ancestor.

Observe the motility plate that you inoculated that you inoculated last lab session. If possible, measure halo size (relative is ok but numbers are better if possible) across the three cultures.

Take your static culture tubes and CAREFULLY decant (pour) the liquid portions of the culture into a disposal vial. Carefully add the crystal violet solution to the culture tubes and rotate the tube so that the air/liquid interface from the culture is covered in crystal violet. CAREFULLY decant the liquid crystal violet into a disposal vial. Carefully rinse the tube with PBS solution by gently pouring and/or pipetting into the tube, CAREFULLY decant this liquid into a disposal vial. Observe the tube for where crystal violet has stained all three culture tubes and record any similarities and differences.