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Supplemental Materials

for

Using a Molecular-Genetic Approach to Investigate Bacterial Physiology in a Continuous, Research-Based, Semester-Long Laboratory for Undergraduates

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Appendix 1: Materials, Instruments and Recipes Required

Appendix 1A. Materials and Instruments Required

Materials needed for this experiment are divided into 3 separate categories:

Group Materials: Recommended to be given (per group) in the beginning of the semester and can be accessed by each group during lab session throughout the project in a group drawer or cabinet, if possible.

-Bunsen burner
-Matches
-Jar of 70% Ethanol (Flame Sterilization)
-Wire inoculation wand/sterile toothpicks
-Glass Spreaders
-Beaker of ~100 sterile inoculating sticks
-Microcentrifuge tube rack
-Vacuum
-Propane for Bunsen burner
-Test tube rack
-Marker
-Kimwipes
-Tupperware w/ lids for incubating plates
-Disinfectant bucket
-Tip discard bucket
-650 ml bottles of sterile nanopure water
- Beakers of sterile microcentrifuge tubes, 1 per 2 students

Room Materials: Recommended that materials required throughout the project be kept in classroom throughout the duration of semester to be used by the class, if possible.

- Multiple microcentrifuges
- P20, P200 and P1000 micropipettes (Enough for one of each per group)
- Discard container for inoculating sticks
- Bin for incubating class plates
- Several vortexers
- Boxes of sterile micropipette tips
- Autoclave tape
- Colored labeling tape
- Sharps container
- Biohazard Container
- Scale
-Spatulas
- Plastic weight boats

Daily Materials: Required materials specific for each day in lab (Organized per lab or per student). Daily materials subdivided into materials that should be provided for students and materials that students should already have in an incubator or group container (per group). Materials listed in group/room materials not mentioned in materials required each day.

*If Recommended Lab B not included in project, media listed in "Students Should Have" must be prepared outside of class and provided for the corresponding labs.

Day 1 of class

Recommended Lab A: Viable cell count of *Serratia marcescens*. To be done Individually.

- 5ml overnight cultures of one of *S. marcescens* in nutrient broth, 1 per student pair

- Nutrient agar plates or TSA (can be poured thin to skimp on media), 3 per student

Day 2 of class

Recommended Lab B: Examining the results of Viable cell counts and preparing media and reagents for future use in the lab. To be done individually and in groups.

Per Lab:
- 17 200-250 ml media bottles with autoclavable caps
- 3- 1 liter Erlenmeyer flasks
- 2- 3 liter Erlenmeyer flasks
- Aluminum foil (for making caps on flasks for autoclaving)
- 2- 100 ml grad cylinders
- 1 liter grad cylinders
- Multiple containers of 5 and 10 ml pipettes
- ~2 racks sterile clean culture tubes (16 or 18 mm)
- 100 ml beaker
- Several water baths set at 55°C

Per group:
- 3 nutrient agar plates-for pure streaking *S. marcescens*
- 25 grams agar
- 4 grams sodium chloride
- 15 grams tryptone
- 4 grams yeast extract
- 40 % glucose stock, ~7 mls
- 40 % sodium succinate stock ~4 mls
- 0.276 M K_2SO_4, ~ 2 mls
- 0.528 M $MgCl_2$, ~ 2 mls
- 1M K_2HPO_4 stock, ~ 2 mls
- 1.5 sleeves of Petri plates
- 50 mg/ml kanamycin stock, ~ 1 ml
- 40 X M, 16 mls
- 100 % glycerol, 7 mls
- 15 ml plastic screw cap conical tubes

* This should make all the media (not reagents) needed for the whole project, best stored in a cold room.

Outside of Class:

-LB+ Kanamycin plate (for reviving *E. coli* donor BW20767/pRL27 and *S. marcescens*)

- *E. coli* donor BW20767/pRL27 ((RP4–2-Tc::Mu-1 kan::Tn*7* integrant *leu-63*::IS10 *recA1 zbf-5 creB510 hsdR17 endA1 thi uidA* (Δ*Mlu*I)::*pir*+/pRL27) (Metcalf et al. 1996, Larsen et al., 2002)

- *Serratia marcescens ATCC29632 or any Wild type strain*

Day 3 of class: To begin on day 3 of class if the above recommended labs were assigned. Otherwise, this is day 1 of the Tn mutagenesis experiment as labeled.

Day 1: Conjugation of *S. marcescens* and the *E. coli* BW20767/pRL27 transposon donor

Per Group:
- 1 Pair flat tipped filter forceps per group
- Nalgene analytical 0.45 μm disposable filters (Nalgene Cat No: 147)
- 1 500ml side arm flask and with adapter per group
- 15 ml disposable falcon tube, for storing mating mix
- 50 ml disposable falcon tubes-for 1X M storage
-1.5 ml 40 X M aliquot per group - to make 1 X M
- Sterile water
- LB plates
- Mid log phase *S. marcescens*, 2ml cultures in LB incubated at 28°C, 1 per group.
- Mid log phase -, *E. coli* donor culture (BW20767/pRL27) in 3 ml LB-Kanamycin.

Day 2: Discussion of the genotypes and phenotypes of the donor and recipient strains used, of controls, determination of transposon insertion frequency and selection of transposon (Tn) induced mutants.

Per Group:
-1 50 ml sterile falcon tubes per group - used resuspend the bacteria off the filter following mating
-4 Glucose Mops + kanamycin plates
-5 Glucose Mops plates

Day 3: Analysis of Tn mutant selection dilution plates, calculation of Tn insertion frequency and large scale selection of Tn mutants.

Per Lab:
- 1-2 colony counters or multiple sharpies

Students Should Already Have:
- 2-3 Glucose Mops + kanamycin plates
-*S. marcescens* Tn induced mutant plates
-Freshly streaked parent strains
-Stock of 1X ”M” and 40X ”M”
-Mating mix stored in the refrigerator

Day 4: Continuation of the screen for, and isolation of, pigment mutants.

Students Should Already Have:
-2-3 Glucose Mops + kanamycin
-Streak plates of parent strains
- *S. marcescens* Tn induced mutant plates

Day 5: Phenotypic characterization of pigment mutants on Glucose Mops, Succinate Mops and LB plates.

Students Should Have:
-4-6 glucose Mops plates
-4-6 succinate Mops plates
-4-6 LB plates

Day 6: Class Results of pigment mutant phenotypic characterization and chromosomal preparation discussion.

Per Lab:
-Basket of sterile culture tubes
-5 and 10 ml sterile pipettes and pipettors
-5 mls of a 50 mg/ml kanamycin stock

Students Should Have:
-4-6 glucose Mops plates
-4-6 succinate Mops plates
-4-6 LB plates
-LB broth

Day 7: Chromosomal preparations from Tn induced pigment mutants

Per Lab:
-Bucket of ice
-65°C water bath with microcentrifuge holders
-2.5 g agarose
-500 ml flask and stir bar
-20 mls 50 X TAE (to make 1 L 1 X TAE)
-1 liter grad cylinder and 1 L screw cap storage bottle
-Stirring hot plate

Per Group:
-650 µl MicroBead solution
-110 µl MD1 solution
-220 µl Solution MD2
-1850 µl of Solution MD3
-620 µl of Solution MD4
-110 µl of Solution MD5
-100 µl 6 X DNA gel loading buffer

*Microbead solution and MD1-5 components of MO BIO Laoratories, Inc. Ultraclean Microbial DNA Isolation Kit (Cat No: 1224-50). Other Chromosomal prep kits may be substituted.

*Quantities aliquoted are enough for 2 chromosomal preps per group.

Day 8: Gel electrophoresis and *Bam*HI digests of chromosomal preps from *S. marcescens* Tn mutants.

Per Lab:
-37°C water bath with microcentrifuge tube holders
~2-4 agarose gel electrophoresis apparatuses
-10mg/ml Ethidium bromide stock (mutagen)
-Ethidium bromide waste container
-Nitrile gloves, all sizes
-Spatulas

Per Group:
~5 microcentrifuge tubes
-15µl of *Bam*HI reaction buffer (2 reactions)
-10µl of *Bam*HI enzyme-on ice (2 reactions) * or could be added to each tube by instructor to save on reagents, class size permitting
-50µl sterile nanopure water
-Ice buckets with ice

Students Should Already Have:
-Gels made last period and 1 X TAE
-Chromosomal DNA isolated from *S. marcescens* mutants

Day 9: Ethanol precipitation of *Bam*HI digested chromosomal DNA and ligation reaction set up

Per Lab:
-2 microcentrifuges in cold room
-Ice bucket
-Nitrile gloves, all sizes
-Discard container for supernatant waste
-Water bath in cold room set at 14°C with tube holder enough for ~ 5 tubes per group

Per Group:
-1ml aliquots of 3 M Sodium acetate pH 5.2
-10ml aliquots of 70 % ethanol-on ice
-1ml aliquots of 100 % ethanol
-50 µl of 1 X T4 DNA ligase buffer
-3 µl of T4 DNA ligase

Students Should Already Have:
-*Bam*HI digests of chromosomal DNA

Day 10: Dialysis and transformation of ligation mixes into *E. coli* DH5αλpir electrocompetent cells.

Per Lab:
-Electroporator, per lab
-Forceps

Per Group:
-50mls sterile ice cold 10% glycerol
-Ice bucket
-Sterile plastic petri plates
-2 small (13X100mm) sterile culture tubes each with 1ml SOC media in 37°C water bath
~4 0.2mm gap electroporation cuvettes (appropriate for electroporator used)
-1 60ul aliquot of electrocompetent *E. coli DH5*αλpir per transformation
-sterile 0.025 micrometer nitrocellulose membranes
-Filter forcerps
-Sterile water

Students Should Already Have:
-*Bam*HI digested DNA of *S. marcescens* Tn mutants
-2 LB-kanamycin (50 ug/ml) plates

Day 11: Examination of transformation plates and preparation for plasmid DNA isolations.

Per Lab:
-Sfterile 16 mm culture tubes
- LB-kanamycin broth to be dispensed into sterile tubes

Students Should Already Have:
-2 transformation plates

Day 12: Plasmid minipreps from *E. coli* DH5αλpir transformants and *Bam*HI digests.

Per Lab:
-50 mls 70% Ethanol (to restock student's stocks, if necessary)
-Extra beakers of sterile microcentrifuge tubes
-37°C water bath with microcentrifuge tube rack or "floaties"

Per Group:
-2 ml aliquots of buffer P1 (on ice),
-2 ml aliquots of buffer P2
-3 ml aliquots of buffer P3
-ice buckets with ice
-10 µl of *Bam*HI react 3 buffer (for 5 reactions)
-3 µl of *Bam*HI-on ice (will use 1 µl /reaction)
-50 µl sterile nanopure water

*Buffers P1-P3 are components of Qiagen QIAprep Spin Miniprep Kit (Cat No. 27106). Other Plasmid prep kits may be substituted. (Aliquots are sufficient for 2 plasmid preps per group- adjust as neccessary)

Students Should Have:
-2 transformants overnight cultures in LB Kan broth (50ug/ml)

Day 13: Analysis of *Bam*HI digested plasmid DNA by agarose gel electrophoresis

Per Lab:
-Ethidium bromide waste container
-Exact gene1 KB plus DNA molecular weight ladder
-Aliquots of 6 X DNA gel loading buffer
-0.7 % agarose gels
-1 X TAE with 0.5ug/ml ethidium bromide
-spatulas

Per Group:
- Agarose gel and electrophoresis equipment, 1 to be shared between two groups

Students Should Have:
-*Bam*HI digested plasmids

Day 14: **Whole class analysis of *Bam*HI digested plasmid DNA by gel electrophoresis and discussion of the sequencing reaction, set up sequencing reaction.**

Per Lab:
-37°C water bath with microcentrifuge tube holders

Per Group:
-5µl of BamHI react 3 bufer (for 2 reactions)
-3µl of BamHI on ice (use 1µL per reaction)
-agarose for making gels

Students Should Have:
- 50 x TAE

Day 15: Discussion of the sequencing reaction and how to analyze sequences

Per Lab:
- tpnRL17–1 forward primer (25 uM) aliquot : 5'-AACAAGCCAGGGATGTAACG-3'
-tpnRL13–2 reverse primer (25 uM) aliquot: 5'-CAGCAACACCTTCTTCACGA-3'
-Ethidium bromide waste container
-Exact gene 1kb plus DNA ladder
-1xTAE with 0.5 ug/ml ethidium bromide
-Spatulas

Per Group:
-2 0.2ml PCR tubes

Students Should Already Have:
-0.7% agarose gels

Day 16: Continued sequence analysis on computer and repeating steps to clone Tn insertion if needed.

Per Lab;
-Computers with internet connection

Day 17: Class summary of sequence results, primer design for primer walking of the previously obtained sequence, set up for growth curve analysis of pigment mutants and start growth curve.

Request supplies as needed

Day 18: Growth curve analysis, discussion of calculation of generation times, and primer orders

Request supplies as needed

Day 19: Set up new sequencing reactions for primer walking with new primers and discussion of how to best present this experiment in paper/poster format.

Request supplies as needed

Day 20: Work on figures and assign parts of the poster to different groups

Appendix 1B. Recipes

Recipe Name:

Ingredients:	Quantity:
100% Ethanol	700 ml
Water	300 ml

Recipe Name 50% Glycerol

Ingredients:	Quantity:
Glycerol	50 ml
Water	50 ml

Pipette up and down to remove residual glycerol from pipette. **Filter sterilize**. Recipe based on 100ml.

Recipe Name 1M K2HPO4

Ingredients	Quantity:
Potassium phosphate, dibasic	8.7 g
Water	50 ml

Autoclave to sterilize. Recipe based on 50 ml.

Recipe Name .276 M K2SO4

Ingredients:	Quantity:
Potassium Sulfate	4.8 g
Water	100 ml

Autoclave to sterilize. Recipe based on 100 ml.

Recipe Name: Kanamycin, 50mg/ml

Ingredients:	Quantity:
Kanamycin monosulfate	2.5 g
Water	50 ml

Dissolve Kanamycin in water. **Filter sterilize**. Store in freezer. Recipe based on 50 ml.

Recipe Name: 40X "M"

Ingredients:	Quantity
MOPS	334.8 g
Tricine	28.8 g
Sodium Chloride	116.8 g
Ammonium Chloride	20.4 g
Potassium hydroxide	64 g

Dissolve **no more than 600 mls** nanopure water. Adjust pH to ~ 7.4 with 10 N KOH or HCl. Adjust to final volume if necessary. **Sterilize by filtration**. Recipe makes 1 L. Store at -20C

Recipes continued…..

Recipe Name: 0.528 MgCl2

Ingredients:	Quantity:
Magnesium chloride	10.7 g
Water	100 ml

Autoclave to sterilize. Recipe based on 100 ml.

Recipe Name: 3M Sodium Acetate pH 7.0

Ingredients:	Quantity:
Sodium Acetate	24.6 g
water	100 ml

Dissolve Sodium Acetate in 3/4 volume of water. pH to 7.0. Bring to final volume with water. Autoclave to sterilize. Recipe based on 100 ml.

Recipe Name: 50X TAE

Ingredients:	Quantity:
Tris Base	242 g
Glacial Acetic Acid	57 ml
0.5M EDTA pH 8.0	100 ml
Water	843 ml

Add Tris to ~600ml water. Add EDTA and Acetic Acid. Bring to 1L final volume. Autoclave to sterilize. Recipe based on 1 L.

Recipe Name: LB Broth

Ingredients:	Quantity:
Sodium Chloride	5 g
Tryptone	10 g
Yeast Extract	5 g
Water	1 L

Autoclave to sterilize. Recipe based on 1 L.

Recipe Name: LB-KAN(50)

Ingredients:	Quantity:
Sodium Chloride	5 g
Tryptone	10 g
Yeast Extract	5 g
Kanamycin Sulfate (50mg/ml)	1 ml
Water	1 L

Dissolve dry ingredients in water. Autoclave for 15 minutes at 121C. Allow to cool 45min. **Aseptically add kanamycin.** Recipe based on 1 L. Makes ~ 40 plates.

Recipes continued…..

Recipe Name LB Agar plates

Ingredients:	Quantity:
Agar, Dehydrated	15 g
Sodium chloride	5 g
Tryptone	10 g
Yeast Extract	5 g
Water	1 L

Add ingredients to water. Autoclave on for 15 minutes at 121C. Allow tocool 45 minutes. Swirl to disperse ingredients and pour plates. Makes ~ 40 plates.

Recipe Name: LB Plates Kan(50)

Ingredients:	Quantity:
Agar, Dehydrated	15 g
Sodium chloride	5 g
Tryptone	10 g
Yeast Extract	5 g
Kanamycin Sulfate (50mg/ml)	1 ml
Water	1 L

Mix water and all ingredients except Kanamycin. Autoclave for 15 minutes at 121C. Cool 45 min, and **aseptically add kanamycin**. Swirl to disperse ingredients and pour plates. Makes ~ 40 plates.

Recipe Name: 0.2% Glucose Mops Pi

Ingredients:	Quantity:
Agar	16 g
40X "M"	25 ml
40% Glucose	5 ml
0.276 K2SO4	1 ml
0.528 MgCl2	1 ml
1M K2HPO4 (Pi)	1 ml
Nanopure Water	967 ml

Mix water and Agar and Autoclave for 15 minutes at 121C. Allow to cool 45 min, then add other ingredients aseptically. Swirl to disperse ingredients and pour plates. Makes ~ 40 plates.

Recipe Name: 0.2% Glucose Mops Pi+Kan(50)

Ingredients:	Quantity:
Agar	16 g
40X "M"	25 ml
40% Glucose	5 ml
0.276 K2SO4	1 ml
0.528 MgCl2	1 ml
1M K2HPO4 (Pi)	1 ml
Kanamycin Sulfate (50mg/ml)	1 ml
Nanopure Water	966 ml

Mix water and agar and autoclave for 15 minutes at 121C. Allow to cool 45 min, then **add other ingredients aseptically**. Swirl to disperse ingredients and pour plates. Makes ~ 40 plates.

Recipes continued…..

Recipe Name: 0.2% Succinate Mops Pi

Ingredients:	Quantity:
Agar	16 g
40X "M"	25 ml
40% Succinate	5 ml
0.276 K2SO4	1 ml
0.528 MgCl2	1 ml
1M K2HPO4 (Pi)	1 ml
Nanopure Water	967 ml

Mix water and Agar and Autoclave for 15 minutes at 121C. Allow to cool 45 min, then **add other ingredients aseptically**. Swirl to disperse ingredients and pour plates. Makes ~ 40 plates.

Recipe Name: 0.2% Succinate Mops Pi+Kan(50)

Ingredients:	Quantity:
Agar	16 g
40X "M"	25 ml
40% Succinate	5 ml
0.276 K2SO4	1 ml
0.528 MgCl2	1 ml
1M K2HPO4 (Pi)	1 ml
Kanamycin Sulfate (50mg/ml)	1 ml
Nanopure Water	966 ml

Mix water and Agar and Autoclave for 15 minutes at 121C. Allow to cool 45 min, then add other ingredients aseptically. Swirl to disperse ingredients and pour plates.

Recipe Name: Nutrient Agar Plates

Ingredients:	Quantity
Nutrient Agar	23 g
Water	1 L

Add dehydrated media to water. Autoclave for 15 minutes at 121C. Allow to cool 45 minutes Swirl to disperse agar and plates. Makes ~ 40 plates

Recipe Name: SOC

Ingredients:	Quantity:
Glucose, 20% FS	40 ml
Magnesium sulfate.7H2O, 1M sterile	40 ml
Sodium chloride	0.5 g
Tryptone	20 g
Yeast extract	5 g
Water	920 ml

Mix sodium chloride, tryptone and yeast extract and autoclave to sterilize. Cool for ~ 45 minutes and **asceptically add glucose and magnesium sulfate.**

Protocol for Preparing Electro Competent Cells:

You will need:
1 L sterile LB in a baffled flask
2 L Sterile water
4 Sterile 250 ml rotor bottles with lids
2 sterile 50 ml rotor tubes with lids
A good part of a whole day

Once cells are grown, keep on ice as much as possible!

1. Inoculate 1 colony into 5 mls LB. Grow overnight.
2. Inoculate 1 L of LB + 0.2% glucose with 5mls of overnight culture
3. Grow cells to an OD (600) of ~ 0.6 to 0.8
4. Chill cells 15 to 30 minutes on ice.
5. spin cells down 15 minutes (4K rpm @ 4C), remove as much SN as possible
6. Suspend cells in 1 L ice cold water
7. spin down as in step 5
8. suspend cells in 500 mls ice cold water
9. spin down as in step 5
10. suspend cells in 20 mls ice cold sterile 10% glycerol
11. spin down as in step 5
12. suspend cells in residual 10% glycerol
13. make 120 ul aliquots (enough for 2 transformations)
14. Store at -80C

Appendix 2: Lab Manuals

Appendix 2A. Instructor's Lab Manual*

***This instructor's version of the Lab manual contains all the information the Student's version does as well as additional information needed for lab preparation and suggestions for content delivery. This additional information can be found under the section headings "instructor notes" in italic font at the end of each lab general procedure description.**

***This student's version of the laboratory manual in Appendix 2B is required for each student. It contains general information about the labs to be performed and more specific information on several protocols. Some details are omitted and will be covered in class discussions (see suggestions for these discussions in the instructor's notes).**

Identification of genes involved in prodigiosin production in *Serratia marcescens* by transposon mutagenesis

Introduction to the laboratory (for the instructor)*****. This introduction is much more detailed than the introduction provided in the student's version.

The purpose of this course is to identify genes that are involved in the regulation of the biosynthesis of the pigment prodigiosin in *Serratia marcescens* using transposon mutagenesis. This laboratory course is appropriate as an upper division course for juniors or seniors who have taken an introductory microbiology course and an introductory genetics or molecular biology course. The full length course is designed to take place over 15 week period and requires two 3 hours labs per week. However, the lab manual also includes optional activities that can be added or deleted to alter the length of the course.

This course is designed to be a real time research project that builds from week to week. Students are expected to assimilate concepts introduced throughout the semester and be actively involved in discussions of experimental design, proper controls, trouble shooting of failed experiments, keeping an accurate notebook, etc, so that they gain experience thinking and acting like a researcher. Throughout the semester the students will become familiar with molecular and cloning techniques such as chromosomal and plasmid preparations, restriction enzyme digests, ligations, transformations, and analysis of sequence data. Because this project is designed to foster independent thought by the students, sample calculations and examples are included in the instructor manual, rather than the student manual.

During phase 1 of this project, transposon mutagenesis will be used to generate a pool of *S. marcescens* mutants, some of which show altered pigment production as compared to wild type. Following mutagenesis, students will isolate colonies showing either increased or decreased pigment production, and do phenotypic analysis on various minimal and rich media. During phase 2, students will then clone the transposon insertion site and identify the gene disrupted by the transposon. Although it is likely that some of the genes identified will lie within the previously identified prodigiosin biosynthetic pathway, the students will likely discover novel genes that are involved in the many layers of regulation of prodigiosin production.

The transposon delivery vector, pRL27, used during this experiment is pictured below.

The pRL27 vector encodes a hyperactive transposase gene (tnp) under control of the tetA promoter (tetAp) which is not encoded on the transposon, and thus is lost after transposition, resulting in stable transposon insertions. The transposase cuts at the inverted repeats flanking the transposon, which are indicated by gray bars. OriR6k and the kanamycin resistance cassette (aph) lie within the transposon region. pRL27 is a suicide vector that contains a Pi protein (pir) dependent origin of replication,OriR6k, and can only replicate in bacteria encoding the Pi protein. In non-pir strains such as S. marcescens, kanamycin resistance will only be conferred when the transposon is inserted into the recipient genome because the plasmid will not be maintained. An origin of transfer (oriT) allows the vector to be transferred from the E. coli donor to the recipient strain with high efficiency via conjugation. The E. coli donor strain is a leucine auxotroph, so selection of exconjugants is done on 0.2% glucose MOPS minimal media containing kanamycin. Following the generation of prodigiosin mutants in S. marcescens, the Tn insertion site can be cloned from the chromosome and replicated as a plasmid via the OriR6K origin of replication in the pir+ host, E. coli DH5αλpir (Larsen et al. 2002).

Laboratory student learning objectives

By the completion of this course students should be able to:

1. Describe the methodology and rationale for 2 general approaches to identify genes with a particular function, random mutagenesis and construction of DNA libraries.
2. Describe how to carryout a heterologous expression experiment and how this is used to determine the function of a gene.
3. Describe how to carry out a transposon mutagenesis experiment and why this approach might be used.
4. Describe how to determine how many mutants should be examined in a Tn mutagenesis experiment (or in analyzing a chromosomal library) to ensure complete coverage of the genome using the Poisson distribution.
5. Define the difference between a selection and a screen and describe how and for what purpose each one is carried out.
6. Indicate under what circumstances polar effects could be observed in mutants and how to interpret phenotypes in these cases.
7. Describe the steps in a cloning experiment including the properties and roles of restriction enzymes and DNA ligase.
8. Describe and contrast the methods for introducing exogenous DNA into a bacterial cell, transformation, conjugation and transposition.
9. Carry out and describe the steps in proper DNA manipulation techniques including DNA isolations, precipitations and visualization via gel electrophoresis
10. Describe the sequencing reaction and be able to assemble sequences and choose good sequencing primers.
11. Use bioinformatics programs such as Blast and navigate databases such as NCBI and TIGR.
12. Use sequence information to make predictions about the function of genes

Instructions for the 1st week of class, if assigned, otherwise skip to "Day 1" instructions below, under "Begin the *S. marcescens* transposon mutagenesis experiment".

Day 1 of class

Recommended Lab A: Viable cell count of *Serratia marcescens*. To be done Individually.

Purpose: To review micropipetting and making serial dilutions, aseptic technique and spread plating, and to become familiar with the colony phenotype and growth properties of *S. marcescens.*

General Procedure:

Perform a viable cell count of the *S. marcescens* culture provided. Assume an original concentration of 1 x 10^{9} cells/ml, make the appropriate dilutions in a 1.0 ml final volume using sterile water, and plate only 3 dilutions to get 30-300 colonies/ plate. Half the class should incubate plates at 37°C and the other half should incubate at 28°C for 24-48 hours.

Instructor notes:

- *This lab is recommended for the first day of class because it allows the students to review some basic skills that will be required throughout the lab portion of the course and in future employment situations possibly. It also allows the delay of the Transposon mutagenesis experiment so that students have time to read the assigned journal articles and a class discussion can be carried out in preparation for it. These assignments are in Appendix 4 of the supplementary materials and should be assigned on this first day of class.*

- *An S. marcescens 16-24 hour culture grown in TSB at 28℃ is used. Cultures of S. marcescens should be prepared by inoculating a 5 ml culture of TSB with a single colony and incubating at 28℃ overnight. This should give you a concentration of ~1X10^{9} cells/ml. It is easiest if each pair of students has approximately a 1 ml aliquat of this culture to share.*

- *Students will make serial dilutions in a 1 ml final volume of sterile water and will plate 0.1 ml of 3 of the dilutions to get 30-300 colonies per plate. Assuming an original concentration of 1 X 10^{9} cells/ml, the students should plate 0.1 ml of the 10^{-5}, 10^{-6} and 10^{-7} dilutions for final dilution values of 10^{-6}, 10^{-7}, and 10^{-8}. Assuming an approximate initial concentration of 1 x 10^{9} cells/ml, if student correctly these dilutions, at least one of these plates should yield 30-300 colonies to perform a viable cell count.*

- *Have half of the groups incubate their plates at 28℃ and half at 37℃, so they can discover that pigment production in S. marcescens is temperature dependent.*

- *We recommend letting students struggle a bit on their own to figure out how to carryout the dilution scheme given the provided instructions only. We would also recommend encouraging them to interact with classmates to get to know one another on this 1st day of class, and to collaborate on a dilution scheme. However, it is important that each student create their own dilutions and spread plates to make sure each student gets hands on experience pipetting and manipulating cultures aseptically.*

- *It may be necessary to review basic techniques like micropipetting, aseptic technique and spread plating. This lab provides a good opportunity for this and to determine the skill level of the students in the class.*

- *This is also an excellent time for the instructor to get to know the students by walking around and asking names while students work on their dilution schemes, if the class size permits.*

- *Instructors may want to check off the dilutions schemes before allowing the students perform them.*

Day 2 of class

Recommended Lab B: Examining the results of Viable cell counts and preparing media and reagents for future use in the lab. To be done individually and in groups.

Purpose: To determine student's skill level and experience with aseptic technique, micropipetting, spread plating and making serial dilutions (all very commonly used skills required in this laboratory portion of the course), and to gain experience making media and becoming familiar with its components.

A. Making media

General procedure:

Class will divide into 8 separate groups of no more than 3 students, and each group will prepare one type of the following media. See appendix for media recipes. 8 groups are chosen based on a class size of 20-24. This can be altered according to the class size.

Media to make:

1) 8 x 100 mls LB
2) 8x 100 mls LB-Kanamycin (50 ug/ml)
3) 0.5 L LB agar (~ 20 plates)
4) 1.0 L LB-kan (50 ug/ml) agar (~ 40 plates)
5) 2.0 L 0.2% glucose Mops + Pi + kanamycin agar (~80 plates)
6) 1.5 L 0.2% glucose Mops + Pi agar (~60 plates)
7) 0.5 L 1 % succinate Mops + Pi agar (~ 20 plates)
8) 8 x 10 mls 50 % glycerol

* This should be all the media we will need for the whole semester for a class of ~24 students.

Instructor notes:

- *This lab is also intended to be a review lab on how to make solutions and media, on calculations involved in media making and to get students to think about the components in the media they will be using. It has the extra advantage of delaying the mutagenesis experiment an extra day to allow time for students to complete the journal article assignments prior to beginning the mutagenesis experiment in week 2.*

- *This lab has a lot of preparation that needs to be done ahead of time such as gathering all the glassware and reagents for the media (see Appendix 1B of the supplementary materials). This entire lab will require 3 hours.*

- *It is recommended to discuss making media, the concept of stock solutions, and careful labeling. We recommend having **all** the students be responsible for carrying out any calculations/conversions that might be necessary to make the media.*

- *Ideally the media would be stored (plates at 4 °C) where the students have access to it so that when it is needed, they can be responsible for retrieving and storing their own media for a particular experiment.*

B. Examining viable plate counts-to be done while the media made in part A is in the autoclave

General procedure:

1. Analyze your and your partner's plates. Do you have isolated colonies evenly distributed over the surface of the plate? Do you see a progressive 10 fold decrease in the number of colonies from the lowest dilution plate to the highest dilution plate? Are there any contaminants? Is there a difference in colony phenotype when grown at 28 °C compared to 37 °C? What skills do you need to improve upon or review?

2. Count the colonies on the appropriate plate and calculate CFU/ml of the original culture.

3. Pick a well isolated colony of *S. marcescens* from this plate and perform a streak plate for colony isolation onto a fresh plate of TSA-incubate at 28 °C. Save the plate the colony was streaked from in a sealed Tupperware at 4 °C as a backup source of *S. marcescens* for future experiments.

Instructor notes:

- *We recommend taking time to ask students what they should expect to see on their plates and why at the beginning of the lab period to go over the principles of a viable cell count. Instructors should also take this time to reiterate the importance of the techniques used and to ask the student to evaluate their work based on accuracy of the dilution scheme, of their pipetting, and aseptic technique. For example, instructors might ask their students if they would hire themselves as a quality assurance employee based on what their plates looked like. Instructors might also ask students if they were able to pipette as accurately as they thought they could. *Our experience is that most students are overconfident in their abilities to carry out accurate and aseptic liquid transfers with pipettes and micropipettes and that they usually benefit from a review, although they are reticent to admit that they need it.*

- *Instructors should also use this time to go around the class (if class size permits) and examine each student's plates and go over their techniques with them. Ask students what might have gone wrong (if anything) and offer constructive criticism of their technique so that they leave the lab with instructions on how to improve. This is also an excellent opportunity to get to know the students and their skill levels even more.*

- *Have students determine the actual colony forming units (CFU)/ml of the original culture.*

Sample calculation: # of colonies X dilution factor = CFU/ml or 147 colonies X 10^8 = 1.47 X 10^{10} CFU/ml

- *It may will probably necessary to review what a streak plate is and what its purpose is. This can be done with a demonstration at the front of the class or as a drawing on the chalkboard.*

- *This is also an opportunity to remind students the importance of pure cultures and of knowing what the organism they are working with looks like so that they can spot a contaminant. In fact, an additional benefit of doing these exercises is so that the student can become familiar with the growth and colony morphology of their study organism, S. marcescens.*

- *Encourage students to examine the colonies grown at 28 ℃ compared to 37 ℃. Students should observe that colonies grown at 28 ℃ are deep red to orange colored while those grown at 37 ℃ are cream colored and relatively not pigmented.*

- *This is a good opportunity to introduce the topic of prodigiosin red pigment and how the synthesis is regulated by environmental factors, such as growth temperature for example. Instructors may want to ask the students to come up with explanations as to why the colonies might be pigmented under one condition but not another, and how different genes might be involved in this process, to get them thinking about gene function and regulation.*

Day 3 of class: To begin on day 3 of class **if** the above recommended labs were assigned. Otherwise, this is day 1 of the Tn mutagenesis experiment as labeled.

Begin the *S. marcescens* transposon mutagenesis experiment

Day 1: Conjugation of *S. marcescens* and the *E. coli* *WM20767/pRL27* transposon donor

General Procedure:

1. The class will discuss the Journal article assignments that introduce the topics of *S. marcescens* prodigiosin production and the Transposon delivery system, pRL27.
2. The class will develop a precise protocol for the conjugation and mutagenesis of *S. marcescens* based on the journal article by Larsen et al. and will go over the details of the method.
3. In groups of no more than 3, students will prepare the conjugation experiment by mixing together the *E. coli* WM2672 transposon Donor and *S. marcescens* recipient mid log phase cultures and set up filter mating on LB plates to incubate overnight at 37 °C.

* Save residual donor and recipient cultures at 4 °C for the controls when selecting mutants.

Instructor's notes:

- *This is the first lab that will introduce the method for transposon mutagenesis. This will require a large amount of information to be delivered to the students. Students should already have completed the journal article assignments to prepare them for this, but they likely will not have understood much of it at this point The goal of this lab is to fill in the blanks for the students as to how the transposon mutagenesis works using pRL27 and what it is we are hoping to find out about prodigiosin biosynthesis by doing this. We recommend using the journal articles as a scaffold for leading this discussion. The discussion may take most of the lab period, but allow for about 1 hour to demonstrate and perform the mating experiment.*

- *Prior to beginning the mating experiment, go over the journal article assignments by having a discussion in class where students provide the answers. We recommend using this first journal article class discussion to establish the student led nature of the lab and we encourage instructors to wait for students to respond or to pick on students to respond so that they get used to this style of instruction, which should be maintained throughout the course. The focus of these discussions should be on establishing what is known about the prodigiosin biosynthetic pathway in S. marcescens, how this was determined experimentally and how transposon mutagenesis might yield similar or different results. This is an ideal time to re-introduce the overall questions of the lab and to state the overarching goals the students will be trying to accomplish throughout. This is also an ideal time to reiterate the real research component of this lab, and that in doing research, one often looks to the literature to find established protocols to use on one's own research and before investigating any research questions, one must know what is already understood and what is not. This journal article discussion is designed to introduce students to this process and to make them feel ownership of the research question at hand because they have had to investigate it themselves.*

- *Discuss the basis of the transposon delivery system, pRL27, and how it works. This is a great deal of information for the students so we recommend first discussing the features of the plasmid, what genes are encoded on it and what each of them does. Then, discuss what a transposon is, what are its features, what elements are required for transposition and what parts of the plasmid are involved in this. We recommend drawing a diagram of the plasmid pRL27 on the chalkboard and drawing in each of its pertinent features one at a time while discussing what they do and how they will be involved in the transposon mutagenesis process.*

- *Students will likely be overwhelmed by this information so keep it simple and let them know that this information will be repeated many times with added layers of detail throughout the lab. In the descriptions, it would be more useful to focus on the jobs of each of the parts of the delivery vector pRL27 and what they do, rather than on the details of the names. For example, while discussing the oriR6K, it would be enough to say that this is an origin of replication that is required for the plasmid to be replicated and thus passed on from mother to daughter cell. However, this origin is not active in S. marcescens, so the plasmid cannot replicate in S. marcescens. Discussion of the pir gene and Pi protein can wait until much later in the lab when students will be transforming the cloned Tn insertions into E. coli DH5αλpir and they will need to understand it at a deeper level.*

- *Instructors will likely have to guide the students to coming up with a protocol for the conjugation experiment based on the Larsen et al. journal article. Donor and recipient cultures are mixed at approximately a 1:1 ratio based on cell density using a volume of anywhere from 100 ul of each culture to 1 ml of each culture depending on the cell densities. If the cultures are at an A_{600} between 0.6 and 0.8, using 200 ul of each donor and recipient should work well.*

- *We recommend drawing the conjugation set up on the board while going over the steps as follows: 1) mix donor and recipient volumes as determined based on cell densities in a 15 ml sterile tube. 2) Bring the volume up to approximately 10 mls using sterile 1 x "M" as a buffering solution (see recipes above). 3) Aseptically pour this conjugation mixture into the filtration apparatus and filter through a 0.20um pore size filter to collect the cells on top of the filter. Disposable 0.20 um pore size analytical Nalgene filter funnels (Catalog # 145-0020) atop a side arm flask hooked up to a vacuum source can be used, or a suitable alternative is fine, as long as it is sterile. 4) Using aseptic technique, dismantle the filtration apparatus and remove the membrane filter carefully using sterile forceps. 5) Place the filter CELL SIDE UP, onto the surface of an LB agar plate being careful to not introduce air bubbles between the filter and the plate. *These last steps will have to be demonstrated to the students. 6) Place the LB plate with the filter on it in the 37 ℃ incubator overnight and remove to 4 ℃ in the morning or after approximately 12-16 hours.*

- *After incubation, a light film of creamy colored cell growth should be observed on the entire surface of the filter.*

- *The plates can be stored at 4 ℃ until the next manipulation is required. If this will be longer than 2 days, we recommend storing the plates in an airtight Tupperware with moist paper towels to keep the plates from drying out.*

- *Culture preparation:*

    1. *Several days before this lab, the E. coli WM20767/pRL27) donor strain should be revived from a deep freeze glycerol stock on an LB-Kanamycin plate and incubated at 37℃.*

    2. *The day before this lab, both the E. coli WM20767/pRL27 donor and S. marcescens wild-type recipient should be freshly streaked on appropriate media (LB-Kan for E. coli WM20767/pRL27, and LB or TSA for S. marcescens) and incubated.*

    3. *6 hours prior to the lab, per group of students,*

        - *Donor strain WM20767/pRL27 preparation: inoculate 5 ml broth culture of LB-Kan with an isolated colony of the freshly streaked E. coli WM20767/pRL27 donor and incubate at 37℃ with vigorous shaking. Incubate for 5 to 6 hours or until culture is in mid log phase (A_{600} of 0.4-0.8).*

- *Recipient S. marcescens strain preparation: Inoculate 5 ml broth culture of LB with an isolated colony of freshly streaked S. marcescens and incubate at 37℃ with shaking. At the time of the mating, the cultures should be mixed in a 1:1 ratio based on <u>cell density</u>, not volume. Serratia will grow more quickly than the E. coli, so reduce the incubation time of the Serratia to ~ 3 to 4 hours.*

Day 2: Discussion of the genotypes and phenotypes of the donor and recipient strains used, of controls, determination of transposon insertion frequency and selection of transposon (Tn) induced mutants.

<u>General procedure:</u>

1. Resuspend the conjugation mix of bacteria off of the surface of the filter in approximately 2 mls of 1 x "M" buffer and make 10 fold serial dilutions in 1 X "M" in 1 ml final volumes.

2. Plate 10^{0}- 10^{-3} 10 fold dilutions of the conjugation mix on Glucose Mops + kan plates for selection of Tn induced *S. marcescens* mutants and plate 10^{-3}-10^{-7} dilutions onto Glucose Mops (no kanamycin) plates to determine the concentration of recipient *S. marcescens* cells present in the mating mix. **<u>Save remaining conjugation mix in fridge (4 °C)!!</u>**

3. Plate 100 ul of the residual donor and recipient cultures (stored at 4 °C from previous lab) on each type of media to serve as controls.

4. Incubate all plates at 28 °C in sealed container with moist paper towels.

<u>Students should have:</u>

-6 plates per group of Glucose Mops + kanamycin

-7 plates per group of Glucose Mops no kanamycin

-residual cultures of donor and recipient stored in fridge

Instructor's notes:

- *Prior to beginning of the lab, discuss the principles of a selection. We recommend discussing this by first asking the students to identify what types of cells are present on the filter used for the conjugation surface (and thus will be in the conjugation mix after resuspending in buffer) after the conjugation has occurred. Instructors may need to remind them that most of the cells present are donor and recipient cells, and that a very, very, very small fraction of the cells present are "exconjugants" (those that have actually received the Tn delivery vector, and thus the Tn insertion). Students should be able to come up with some of the phenotypic traits each of the cell types has. The instructor may have to introduce the concept of auxotrophy and introduce the fact that the E. coli donor strain (WM20767/pRL27) is a leucine auxotroph and therefore cannot grow on a minimal medium lacking leucine. This can be followed by asking the students how one might recover the very small fraction of desired cells that <u>are</u> actually Tn induced S. marcescens mutants. This should lead to the idea of selection, which could be contrasted with a "screen" at this point.*

- *Discuss the frequency of Tn insertion and probe students to come up with ways that this could be determined after describing to them that it refers to the percentage of Tn induced mutants relative to the total population of recipient cells. The instructor may want to remind them that they have just carried out*

an experiment (the viable plate count) that allows one to determine the concentration of cells in a population. The goal is to get students to incorporate what they have already learned about using viable cell counts with their new knowledge about selections to come up with the design for determining Tn insertion frequency.

- *Discuss the concept of controls. Once the type of media used for selection has been determined, the instructors may want to pose questions such as, " how do we know for sure that the colonies growing on the glucose Mops + kan plates are actually what we think they are, only Tn induced mutants of S. marcescens?" "What type of experiment could we do to feel more certain about this?" "How do we know that our media is good?" etc…*

- *Instructors will likely have to demonstrate how to aseptically remove the filter from the LB plate and place it into a 50 ml disposable falcon tube or similar tube with a wide mouth. This can be most easily done by pinching one side of the filter with sterile forceps and folding the membrane filter over to grab the other side of the filter simultaneously, forming a "taco" shape. This will allow the filter to be more easily placed into a tube without disturbing the cells on its surface. Once the filter is in the tube, the cells should be thoroughly dislodged from it which can be most easily done by a combination of pipetting the 2 mls of 1 x "M" over the surface of the filter vigorously to wash off the cells and periodically vortexing the solution. The solution should become turbid with cells and the filter can remain in the tube. This mixture of cells is referred to as the conjugation mix and should be stored at 4 ℃.*

- *If desired, instructors may want to assign the chromosomal preparation protocol development assignment (Appendix 5A of the supplementary materials) at this point and discuss sources of info for this so that students will have it ready by the time they will carry out this protocol in lab.*

Day 3: Analysis of Tn mutant selection dilution plates, calculation of Tn insertion frequency and large scale selection of Tn mutants.

General procedure:

1. Examine dilution plates and score controls for growth. What should we be looking for?

2. Determine CFU/ml of Tn induced mutants and determine total *S. marcescens* CFU/ml to determine frequency of Tn insertion. How does this compare to the published values?

3. We will possibly plate more of the conjugation mix to reach desired number of mutants to screen based on the Poisson distribution.

4. Pure streak any pigment mutants on the selection media next to the wild type *S. marcescens* parent strain. Also streak plate one wild type looking *S. marcescens* Tn mutant for comparison. Place all old and new plates in incubator.

Students should have:

-more selection media

-parent strains restreaked fresh from previous labs

-1 X M stocks and 40 X M stocks

-sterile microcentrifuge tubes and racks

-conjugation mix from fridge

Instructor's notes:

- *Direct students to examine their viable count plates in a manner similar to that done (if done) in recommended lab B, looking for a 10 fold decrease in colony number per plate with each increase in dilution, good spread plate technique, contamination…. *Although bacterial contamination is very rare on these selection plates, mold contamination can be a problem if careful aseptic technique isn't used.*

- *Tn insertion frequencies vary depending on the growth phase and density of the donor and recipient cultures used but within the class they should be within the same order of magnitude since all students should have followed the same procedure for conjugation. We recommend having each group write their calculated Tn insertion frequencies on the board for all students to see. This allows spotting any aberrant values that might have come from a miscalculation or incorrectly performing the plate counts. The published value from Larsen et al 2002 is ~1 x 10^{-3}, for comparison.*

- *Students should be instructed to examine plates for any pigment mutants. There will likely be several present if the students look carefully enough. It is easy to overlook the pigmentless mutants since they are white and do not show up well against the media compared to the bright red wild type S. marcescens. Hyper-pigmented mutants may also be present and appear smaller and much darker red in color. Pigment mutants of many shades may appear including white, salmon, peach, orange, hot pink, deep red and any variation in between. Any of these should be streaked for isolation on the selection media for future study. This is a good time to introduce the concept of a screen in contrast to a selection. The selection is for all Tn induced mutants which are kanamycin resistant. The screen is a visual inspection of these kanamycin resistant mutants that are altered in pigment production. The instructor will probably need to remind the students at this point that all the colonies growing on the kanamycin plates represent Tn insertion mutants, but only a small fraction of these mutants have the Tn insertion in a gene that results in a disruption in pigment production. These are the ones we are looking for. The largemajority of the colonies they observe are wild type for pigment production. Students should streak at least one of these wild type pigmented mutants to grow side by side with their pigment mutants for phenotypic comparison.*

- *To get more pigment mutants to study, students should examine their Tn mutant selection plates to determine what dilution of the conjugation mix should yield ~ 200 colonies/plate (this is an easy number of colonies to screen for pigment mutants, students could plate fewer colonies per plate but would then have to plate more plates and use more media). Instructors could introduce the Poisson distribution at this time to illustrate to the students that the Tn insertions are random and that to get complete coverage of the genome, a very large number of mutants have to be screened. For the S. marcescens genome, this number is approximately 20,000 if the desired probability of hitting every gene on the chromosome is 99%, the genome is 5000 kilobases and the approximate size of the genome affected by each insertion is 1.0 kb. This last value assumes an average genes size of 1 kilobase and that the gene density is ~100%. Instructors might wish to plate ~20,000 mutants as a class to achieve this which would mean plating ~ 100 plates at 200 colonies a plate. In our experience, this is not needed to collect a large number of unique pigment mutants, so this may be scaled down according to need. We recommend that each group have at least one unique mutant to work with in the end, which means attempting to isolate 5 or so at this point.*

Day 4: Continuation of the screen for, and isolation of, pigment mutants.

General Procedure:

1. Examine the pigment mutants streaked from the previous lab. Are they pure? What is their phenotype relative to the "wild type pigmented mutant you chose?"

2. Restreak the mutants for isolation if they are not yet pure.

3. Examine the new Tn mutant selection plates and screen these for any new pigment mutants.

4. Pure streak any new pigment mutants alongside the wildtype pigmented Tn mutant for comparison as done before.

5. Incubate all plates in closed Tupperware with moist paper towel as done previously.

Students should have:

-more selection media

-"wild type pigmented mutant restreaked fresh from previous lab

-sterile sticks for streaking

Instructor's notes:

- *At this point, the goal of each group and the class as a whole is to get as many different pigment mutants as possible purified. Groups should carefully document the phenotypes of their mutants with regards to pigment production and should be carefully labeling them and cataloging them in their notebooks (if they have one which is highly recommended). How many mutants each group has will vary and they may be at different stages at this point, with some groups just getting their first pigment mutants streaked and others already having some purified. At the end of this lab period, each group should have at least 1 pigment mutant streaked for purification that they will be able to use in the phenotypic analysis that follows in t he next lab period.*

- *At any point, time permitting, students can go back to the conjugation mix stored at 4 ℃ and plate more mutants to screen for altered pigment phenotypes.*

Day 5: Phenotypic characterization of pigment mutants on Glucose Mops, Succinate Mops and LB plates.

General Procedure:

1. Streak each purified mutant onto a set of Glucose Mops, Succinate Mops, and LB plates using the exact same colony as described. Repeat this onto another set of the same media. Include the wild type pigmented mutant for comparison. Incubate one set at 37 °C and the other set at 28 °C in a closed Tupperware. After 24-48 hours, differences in phenotype between the mutants should be clear. If isolated colonies are all sufficiently large, store them in the fridge.

   Instructor's notes:

   - *The purpose of the phenotypic characterization is to further identify unique mutants as a class and to more fully characterize the effects of the mutation on pigment production. Each isolated pigment mutant should*

be streaked onto minimal media with glucose as a carbon source and onto minimal media with succinate as a carbon source, as well as onto LB. Several mutants that looked similar on glucose Mops media might demonstrate different phenotypes on succinate as a carbon source or on LB. This allows the differentiation of these mutants and indicates something different about the roles of the gene(s) disrupted. Differences among the class pool of mutants might also be evident after incubating them at different temperatures.

- *The instructor might wish to discuss the role of nutrient conditions on pigment production and of temperature on pigment production to preface this part of the experiment, emphasizing that one of the overall goals of the lab is to identify genes involved in pigment production that might have a regulatory role. Examining the pigment phenotypes of the mutants under different growth conditions might help identify some with disruptions in putative regulatory genes.*

- *Students can streak many mutants onto a single plate to save on media. It is recommended to streak the mutants as follows: 1) pick an isolated colony of a mutant with a sterile stick (used for streaking) or a inoculating loop, and touch it to its designated streak spot on the succinate Mops plate. Then using the same stick, and* ***without going back*** *to the original colony picked, touch the glucose Mops plate, and also with the same stick, touch the LB plate. Then, go back to each touched spot and complete the streaks for isolated colonies. This ensures that the exact same colony is being compared on each type of media. Continue on with each additional mutant. Each plate should also have the "wild type" pigmented mutant streaked on it for comparison.*

- *Note: on the above streaks, it is very important to get isolated colonies to compare pigment phenotypes.*

Day 6: Class Results of pigment mutant phenotypic characterization and chromosomal preparation discussion.

General Procedure:

1. Examine the phenotypes of the streaked mutants and compare them each to each other and to the "wild type" pigmented mutant.

2. Carefully document the phenotype of each mutant observed in your notebook and arrange the plates as a class so that the entire class may see the differences in phenotypes of the different pigment mutants on the different media.

3. As a class, determine how many unique mutants the class has isolated based on phenotype alone and compile a table on the chalkboard for all to see. Does each group have a unique mutant? Identify these and be sure to carry on with the following experiments with these unique mutants. If there are not enough, some groups can share the same mutant, but each group should have at least one mutant to work with at this point, but should not have more than 1/person in their group in the event that supplies for downstream steps are limited.

4. Prepare a culture tube with 5 mls of LB-Kanamycin broth for each mutant the group will work with. Carefully label this tube with the corresponding mutant name.

5. Inoculate this tube with an isolated colony of the designated mutant **approximately 16-24 hours prior to the beginning of the next lab period** when you will need this culture.

Students should have:

1. 5 ml LB-kanamycin broth in tubes

2. Sterile sticks for inoculating
3. Glucose and succinate Mops and LB plates

Instructor's notes:

- *At this point, all students should be at the same place in the lab with respect to isolation and characterization of the mutants. At the end of this lab, students will pick which mutants they will attempt to clone the Tn insertion site from in the next phase of the lab.*

- *Instructors should aid students in examining the streaked mutants and advise students to look both at the phenotypes of the cells growing on the surface of the agar as well as the pigment from examining the cultures through the agar side of the plate. It is also recommended to hold the plates up to the light, and to carefully examine pigment in the heavy part of the streak and in isolated colonies to identify differences.*

- *Students should come up with a standard designation for labeling the pigmentation of the mutants that can be adopted by all in the class. This will make comparing phenotypes of different mutants easier.*

- *It is recommended to help the students summarize the whole class results of the mutagenesis experiment at this point, perhaps making a table of the total number of mutants screened as a class, the total number of pigment mutants isolated and the different phenotypes observed. This will help students review where they have been since the beginning of the lab and help bring it all together before they begin the next phase which is more molecular in nature.*

- *We recommend summarizing the progress of the lab so far and introducing the next phase of lab, which is to determine which gene(s) are disrupted in the pigment mutants the students are choosing to work with. Time permitting, it would be helpful at this point to give a brief overview of the molecular steps involved in the next phase, which will begin at the very next lab period: prepare chromosomal DNA, digest DNA with restriction enzyme BamHI, ligate the BamHI fragmented DNA together to circularize it, transform E. coli host to amplify the desired fragment which has the Tn and the flanking disrupted chromosomal DNA, sequence the DNA (See flow chart diagram of these steps in Appendix 8 of the supplementary materials) . This is a skeletal overview and could be drawn on the board just to give the students an idea of how they will be spending the next weeks and to give them a preview of some of the techniques they will learn.*

- *Remind students that they must inoculate the mutants they want to study* **approximately 16-24 hours prior to the beginning of the next lab period,** or make other arrangements for inoculating these to have cultures ready for the chromosomal DNA preparations.

- *Instructors may choose to go over the Chromosomal preparation protocol development assignment at this time to prepare students for the following lab, as there may not be time at the beginning of the following lab to do it before they actually have to start to protocol. This assignment is designed to get students involved in developing the protocols and understanding the steps in them so that they are not just simply following directions. Emphasis in this discussion should be put on the theory behind the steps and discussion how each step works to get progressively more and more pure DNA. Alternatives to steps could also be given to demonstrate to students that there are many ways to achieve a particular outcome, as long as they understand what it is that needs to be done in the process. For example, there are, many published methods for lysing bacterial cells and students may have come up with several of these. This should be discussed with the emphasis on the fact that to get out DNA, one must lyse cells with out degrading DNA and that there are numerous ways to do this.*

Day 7: Chromosomal preparations from Tn induced pigment mutants

General Procedure:

1. Each group will prepare chromosomal DNA from 1-3 of their mutants (depending on resource availability and group size) following the Mo Bio chromosomal prep kit procedure (see protocol below). Pick mutants to prep following a class analysis of all the mutant phenotypes (done on day 6). We want to get as many different types of mutants as possible as a class.

2. Remove 3 µl of each chromosomal DNA preparation to a sterile microcentrifuge tube containing 4 µl of 6 X DNA gel loading buffer and 17 µl of sterile nanopure water. Store this sample (for gel electrophoresis during the next session) and your Chromosomal preps at -20 °C in the designated freezer box. BE SURE TO HAVE LABELED EACH OF YOUR TUBES IN DETAIL WITH DATE, GROUP NAME AND A DESCRIPTION OF THE SAMPLE IT CONTAINS!!!!!!

3. Prepare 0.7% agarose gels and 1 X TAE buffer to use to visualize your chromosomal DNA for the next lab. Store these at 4 °C.

Detailed Protocol for Chromosomal Preps using the MoBIO kit

Please wear gloves at all times!!!!

1. Add 1.8 ml of microbial (bacteria, yeast) culture to a 2 ml Collection Tube (provided) and centrifuge at 10,000 x *g* for 3 min at room temperature. Decant the supernatant completely to remove the media supernatant.

This step concentrates and pellets the microbial cells. In some cases it may take longer to completely pellet the cells. It is important to pellet the cells completely and remove all the culture media in this step.

2. Resuspend the cell pellet in 300 µl of MicroBead Solution and gently vortex to mix. Transfer resuspended cells to MicroBead Tube.

The MicroBead Solution contains salts and a buffer which stabilizes and homogeneously disperses the microbial cells prior to lysis.

3. Add 50 µl of Solution MD1 to the MicroBead Tube.

Solution MD1 contains SDS and other disruption agents required for cell lysis. In addition to aiding in cell lysis, SDS is an anionic detergent that breaks down fatty acids and lipids associated with the cell membrane of several organisms. If it gets cold, it will precipitate. Heating at 60°C will dissolve the SDS and will not harm the SDS or the other disruption agents. In addition, Solution MD1 can be used while it is still warm.

4. To minimize DNA shearing for difficult cells, it is recommended to heat the preps at 65°C for 10 minutes, occasionally bump vortexing for a few seconds every 2-3 minutes.

This optional step can lead to better performance in some cases.

This step creates the combined chemical/ mechanical lysis conditions required to release desired nucleic acids from microbial cells. Many cell types will not lyse without this chemically enhanced bead beating process.

5. Make sure the 2 ml MicroBead Tubes rotate freely in the centrifuge without rubbing. Centrifuge the tubes at

10,000 x *g* for 30 seconds at room temperature. CAUTION: Be sure not to exceed 10,000 x *g* or tubes may break.

The cell debris is sent to the bottom of the tube while DNA is remains in the supernatant.

6. Transfer the supernatant to a clean 2 ml Collection Tube (provided).

7. NOTE: Expect 300 to 350 µl of supernatant.

The volume to expect will vary depending on the size of the original cell pellet from step 1.

8. Add 100 µl of Solution MD2, to the supernatant. Vortex 5 seconds. Then incubate at 4°C (on ice) for 5 minutes.

9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.

Solution MD2 contains a reagent to precipitate non-DNA organic and inorganic material including cell debris and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

10. Avoiding the pellet, transfer the entire volume of supernatant to a clean 2 ml Collection Tube (provided). Expect approximately 450 µl in volume.

The pellet at this point contains non-DNA organic and inorganic materials, including cell debris and proteins. For the best DNA quality and yield, avoid transferring any of the pellet.

11. Add 900 µl of Solution MD3 to the supernatant and vortex 5 seconds.

Solution MD3 is a highly concentrated salt solution. It sets up the high salt condition necessary to bind DNA to the Spin Filter membrane in the following step.

12. Load about 700 µl into the Spin Filter and centrifuge at 10,000 x *g* for 30 seconds at room temperature. Discard the flow through, add the remaining supernatant to the Spin Filter, and centrifuge at 10,000 x *g* for 30 seconds at room temperature. NOTE: A total of 2 to 3 loads for each sample processed are required. Discard all flow through liquid.

DNA is selectively bound to the silica membrane in the Spin Filter device. Contaminants pass through the filter membrane, leaving only the DNA bound to the membrane.

13. Add 300 µl of Solution MD4 and centrifuge at room temperature for 30 seconds at 10,000x *g*.

Solution MD4 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the Spin Filter. This wash solution removes residues of salt, and other contaminants while allowing the DNA to stay bound to the silica membrane.

14. Discard the flow through.

This flow through is waste containing ethanol wash solution and contaminants that did not bind to the silica Spin Filter membrane.

15. Centrifuge at room temperature for 1 minute at 10,000 x *g*.

This step removes residual Solution MD4 (ethanol wash solution). It is critical to remove all traces of wash solution

because it can interfere with down stream DNA applications.

16. Being careful not to splash liquid on the spin filter basket, place Spin Filter in a new 2 ml Collection Tube (provided).

It is important to avoid any traces of the ethanol based wash solution.

17. Add 50 µl of Solution MD5 to the center of the white filter membrane (careful not to poke the membrane with the pipet tip!)

Placing the Solution MD5 (elution buffer) in the center of the small white membrane will make sure the entire membrane is wetted. This will result in more efficient release of bound DNA

18. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.

As the Solution MD5 (elution buffer) passes through the silica membrane, DNA is released, and it flows through the membrane, and into the Collection Tube. The DNA is released because it can only bind to the silica Spin Filter membrane in the presence of salt. Solution MD5 is 10mM Tris pH 8 and does not contain salt.

19. Discard Spin Filter. DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20°C). Solution MD5 contains no EDTA.

Instructor's notes:

- *Each group of students should have a microcentrifuge tube rack with all the appropriate aliquats of the reagents needed and with the required number of bead tubes and spin filters, etc so that the students are not having to share the reagent bottles that come with the kit which risk contamination and would slow the lab down considerably.*
- *Prior to letting students begin the lab, the instructor should show the students the components of the kits so that they can identify them in their racks. The instructor may also wish to point out the instruments required and where they are such as micropipettors (and how to use them for review), microcentrifuges, pipette tip discard etc…*
- *In some cases, the less rigorous lysing method used in this protocol results in a cell supernatant that does not readily spin through the spin filter after the prescribed amount of time in step 12 above, and must be spun for additional time. This is mutant dependent so will likely occur with at least one of the mutants.*
- *It is important to remind students to where gloves (to protect themselves and the DNA) and although they do not have to be working near the flame of the Bunsen burner, they should be careful to avoid contamination. It is worthwhile mentioning that DNA degradation is the major problem in all down stream applications they will carryout so they need to be very cautious with their DNA.*
- *The students should be able to relate the steps they are performing in this protocol to the ones they researched for the chromosomal prep protocol.*
- *It is recommended that students store their chromosomal DNA in a separate box from the sample they prepared with gel loading dye to run on a gel, and to only bring this box out when they need it (after the gel has been run and there could be no confusing the 2 tubes).*

Day 8: Gel electrophoresis and *Bam*HI digests of chromosomal preps from *S. marcescens* Tn mutants.

General Procedure:

1. Set up the required number of gel electrophoresis apparatuses as described to accommodate all the class samples. Each gel should contain at least one lane for the Exact gene 1 KB plus DNA (or other appropriate) molecular weight ladder. The instructor will load the ladder and add the mutagenic DNA dye, Ethidium Bromide.* **To prevent your skin from coming into contact with the gel or gel buffer containing ethidium bromide, you must be wearing nitrile gloves, safety glasses and a lab coat!!!**

2. Run the DNA on the gel until the Brom phenol blue dye front is as the bottom 1/3 of the gel. Visualize DNA using a UV trans-illuminator and include a picture in your notebook.

3. Provided you were able to visualize good quality, high molecular weight chromosomal DNA as described by your instructor, set up a restriction digest of your chromosomal DNA with *Bam*HI as described below. *Remove these digest samples to your freezer box after overnight incubation!!!

4. Store the remainder of the chromosomal DNA in the freezer.

Students should have:

1. 0.7 % agarose gels (made during the previous lab) and 1 x TAE in fridge
2. chromosomal DNA samples prepared to load with gel loading dye in freezer
3. Chromosomal preps stored in freezer

*Bam*HI digest Rxn set up/sample:

To MCF tube, add:

12 µl sterile nuclease free H2O

5 µl BamHI react 3 buffer (NEB)

30 µl Chromosomal DNA

3 µl BamHI enzyme

50 µl total volume

Instructor's notes:

- *The purpose of carrying out gel electrophoresis of the Chromosomal DNA is to visualize it to make sure the DNA is present before carrying on with the remaining cloning steps. This exercise also familiarizes students with an essential technique in molecular biology and allows them to make predictions about what they should see and allows them to actually visualize the DNA they prepped. Prior to beginning the lab, we recommend discussing these objectives and indicating that visualization of DNA on a gel allows one to determine if DNA is there at all, to determine the quality of DNA, the size of the fragments, and to estimate the concentration. This emphasizes the utility of agarose gel electrophoresis in molecular biology and gives them a real application for its use.*

- *Instructors may also wish to discuss the principles of DNA agarose gel electrophoresis at this time and to describe the electrophoresis apparatus set up and how to load the DNA sample.*

- *It may be required to remind the students that they are merely running a very small representative sample of the DNA on the gel which will not be recovered for use-that this is a diagnostic gel rather than a preparative gel, and that their precious DNA that they will be using in downstream steps is what is saved in the freezer. (Students often tend to misunderstand this step and sometimes get their tubes mixed up, loading the pure DNA on the gel and digesting the sample prepped for electrophoresis), which is why we recommend storing the sample they prepared for the gel and the remainder of the chromosomal sample they will need for cloning, in separate boxes.*

- *Bring out the samples for the gel first and get the gels loaded while making sure to remind them to briefly pulse the tubes in the centrifuge for a few seconds to bring the liquid down to the bottom and then to load the **ENTIRE** volume. Instructors may have to demonstrate how to load a gel.*

- *Once the gels are run and the results visualized, bring out the remainder of their chromosomal preps for them to prepare their BamHI digests.*

- *To save BamHI enzyme, the instructor may wish to add this reagent to the student's tubes once all the other components of the BamHI digest have been added, rather than having aliquats of this reagent distributed for each group of students.*

- *At this point, it is highly recommended to describe to the students what the points of digesting the DNA is and how this is getting them closer to identifying what genes have been disrupted by the Tn. We recommend drawing a picture of the chromosomal DNA with the Tn located somewhere in it, and then what this DNA will look like after BamHI digests. It will be important to tell them that we are only interested in knowing what the sequence of the DNA flanking the Tn is and that by digesting the total chromosomal DNA into smaller pieces, we will be able to isolate that smaller fragment and sequence it.*

- *It may also be useful at this point to discuss restriction enzymes and how they work. BamHI is used because we know that it does not cut within the transposon, but, other than that, we do not know where it cuts with in the genome of S. marcescens, so we do not know what size fragment we will create which has the Tn on it.*

- *The BamHI digestion step does not have to be carried out overnight and probably only needs several hours. However, this is a convenient stopping point.*

- *Instructors may also need to review how the Tn got there in the first place and to remind the students that there should only be a single Tn insertion in the chromosome and why this is true.*

- *Finally, it will be important to tell the students that while in the diagram, only one copy of the chromosome is being drawn, that in their tubes, there are millions of copies, and therefore millions of copies of the transposon, but each should be in the exact same spot on the chromosome because the DNA was isolated from a clonal population of cells.*

Day 9: Ethanol precipitation of *Bam*HI digested chromosomal DNA and ligation reaction set up

General procedure:

1. Precipitate the *Bam*HI digested DNA and set up the DNA ligation reaction as described below.

Ethanol precipitation of DNA protocol

1. "Spin down" the liquid of the *Bam*HI digest in the microcentrifuge tube by centrifuging at ~13,000 rpm for several seconds.
2. Estimate the volume of from the digest (should be ~50 µl)
3. Based on this, add 0.1 volumes of 3 M Na Acetate pH 5.2-mix
4. Add 2 volumes 100% ethanol-mix and place at -20 C for ~10 min or on dry ice for ~10 min.
5. Place the samples in the microcentrifuge, spine side out, and centrifuge for 20 minutes at 14,000 rpm in the cold room

6. Immediately but carefully decant the supernatant-the pellet of DNA should be on bottom and side of the spine side of the tube.
7. Immediately but gently add 500 µl of ice cold 70 % ethanol-centrifuge 5 min
8. Immediately but carefully decant the supernatant and leave tube inverted on a kimwipe for several minutes to draw out excess moisture
9. Dry pellet by placing in heat block with lid open for 5-20 minutes
10. Once the pellet appears dry (no more liquid visible upon close inspection), close the tube and prepare the ligation reaction.

Ligation reaction

1. To each tube of precipitated DNA, add 20 µl of 1 X T4 DNA ligase buffer
2. resuspend DNA well by vortexing and/or pipetting up and down- this will take a while, but take your time and focus your efforts on the spine side of the tube where the DNA should be. You will not be able to see it!
3. "spin down the liquid to collect it at the bottom of the tube
4. Add 1 µl of T4 DNA ligase to the tube-mix by flicking gently
5. Spin down
6. Place tube in water bath set at 14°C in the cold room. Make sure all labels are clearly visible and that both the tops and sides of the tubes have been labeled!!!
7. Remove tubes to -20°C freezer box after overnight incubation

Instructor's notes:

- *At this point it is very useful to the students to again summarize what step in the cloning process they are on. We recommend starting each of the proceeding labs with these questions "Where did we leave off last time?" "What is in our tubes at this moment?" and "What needs to happen next to get us where we are going?". We recommend drawing these steps on the board so they can visualize what is happening to the DNA in the tube with each manipulation and where the Tn insertion is at each step. The more clear the instructor can make this, the easier it will be for the students to follow along with all of these small steps in the cloning process. With each small step carried out in each lab, it is important to remind the students where this step is in the big picture, and how it is helping to achieve the ultimate goal of identifying genes involved in prodigiosin biosysnthesis.* ***It is during these cloning steps that students seem to completely lose site of the big picture.***

- *It is important to remind them to work carefully with their DNA to avoid contamination and thus degradation, and to take their time resuspending the precipitated DNA.* ***This is a crucial step! If the DNA does not get adequately resuspended, no ligation can occur and the cloning efficiency will be very low.*** *They should spend at least 20 minutes resuspending the DNA.*

- *If time is limited, they can store the DNA pellets in the freezer or can store the resuspended DNA at 4 ℃*

- *The ligation step does not have to be carried out overnight and probably only needs several hours. However, this is a convenient stopping point.*

- *The instructor may wish to assign the plasmid DNA preparation protocol development assignment (Appendix 5C in the supplementary materials) at this time to give students a chance to complete it prior to doing the plasmid preps in class.*

Day 10: Dialysis and transformation of ligation mixes into *E. coli* DH5alpir electrocompetent cells.

General Procedure:

1. First hour of lab, students will dialyze ligation mix as described below, while preparing the transformation into electrocompetent *E. coli* DH5αλpir.

2. Next 2 hours, students will transform *E. coli* and plate transformants onto LB-kan (50 ug/ml) plates.

Dialyzing the ligation mix:

1. Float a 0.025 um pore nitrocellulose membrane (shiny side up) onto a layer of sterile nanopure water in a sterile petri dish.
2. "Spin down" and then gently pipette your ligation mix onto the surface of the membrane being careful not to submerge the membrane. You may want to practice without your ligation mix first to see how much pressure you can put onto the membrane with the pipette tip with out submerging it.
3. Let the ligation reaction stand undisturbed for ~20 minutes and then carefully pipette the mixture off the membrane into a clean sterile microcentrifuge tube. Store on ice.
4. During the 20 minute wait time, prepare tubes for the transformation as described below.

Transformation: **All steps are to be carried out strictly on ice until the actual electroporation!**

1. Add 60 µl of previously prepared (see Appendix 1B of the supplementary materials) electrocompetent *E. coli* DH5αλpir cells to a sterile microcentrifuge tube **on ice**.
2. Add ½ your ligation mix (~10 µl) to this tube and pipette up and down to mix.
3. Transfer the entire volume of this mixture to the bottom of a prechilled electroporation cuvette as demonstrated. Tap the liquid down so that there are no bubbles or spaces at the bottom of the cuvette.
4. Electroporate the cells as shown and add the electroporated cells to 1ml of prewarmed SOC media in a 16 mm culture tube (see Appendix 1B of the supplementary materials).
5. Recover the cells by incubating at 37 °C with shaking for 1 hour.
6. Plate 100 µl of this cell solution onto LB-Kan plates using aseptic spread plate technique.
7. Centrifuge the remainder of the culture at 6,000 rpm and resuspend the cell pellet in the residual ~ 200 ul supernatant. Plate this entire volume onto LB-kan. Incubate both plates at 37 °C for 24-48 hours.
8. Plate a "cells only" control, by spread plating 60 ul of electrocompetent cells that have not been transformed with DNA onto an LB-Kan plate and incubate along with the transformation plates.
9. Examine the plates after 24 hours. If colonies are visible, remove the plates to 4 °C for further manipulation. If colonies are not yet clearly visible, continue to incubate for an additional 24 hours.

Students should have:

1) LB-kan (50 ug/ml) plates

Instructor's notes:

- *Again, recap where this step is in the big picture and what it is accomplishing. This will require a discussion of plasmids and what the ligated fragments in the tube look like using a diagram. Students should be asked to indicate if all ligated fragments are plasmids? Or which ones are actually plasmids and what makes them a plasmid? To answer these questions, it will be required to review what genes are encoded on the Tn and to discuss their properties. For example, it will be important at this point to remind the students that the oriR6k is encoded on the Tn and that it is this sequence that will allow the*

circularized fragment containing the Tn to replicate in this particular E. coli strain, while the other circularized fragments that do not have the Tn cannot. This should lead naturally into a discussion of the significance of transforming this particular E. coli DH5αλpir strain (Φ80*dlacZ*ΔM15 Δ(*lacZYAargF*) U169 *recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*/λ*pir*) so that the students can understand why the Tn containing fragments can replicate in this *E. coli* strain but not in *S. marcescens (the pir gene in this E. coli strain encodes the Pi protein required for replication at this particular origin, oriR6k). This gene is absent from S. marcescens.*

- *It is also recommended to remind the students that the kanamycin resistance gene is also on the Tn and that this might be useful for the cloning process. The instructor might want to ask the students if they could explain why this is important? The hope is that they would be able to explain its importance in selecting for transformed E. coli.*

- *Dialyzing the ligation mix is not always required but does reduce the chances of arcing during electroporation, which destroys the electroporated sample. If not dialyzing the ligation mix, only use 1 ul at most to transform E. coli by electroporation.*

- *The amount of dialyzed ligation mix used to transform E. coli can be varied from using 5 ul to all of it. The most success has been observed with using all of it. The risk here is that if they use all of it, and the sample arcs during electorporation, they will not have any left to set up another transformation.*

- *The recovery time of 1 hour is somewhat flexible ± 10 minutes depending on need.*

- *Colonies of transformants usually begin to be prominent after approximately 24 hours of incubation. It is important to examine the transformation plates after this period of time to differentiate real transformants from spontaneous mutants that might appear after prolonged incubation. Colonies should look like typical E. coli colonies.*

Day 11: Examination of transformation plates and preparation for plasmid DNA isolations.

General procedure:

1. Examine the transformation plates and look for isolated colonies of *E. coli* DH5αλpir transformants. Compare these plates to the control "cells only" plate to see what no growth should look like. Record your results.

2. Prepare 16 mm culture tubes with 4 mls of LB-kan broth for each transformant chosen to prepare plasmid DNA from. It is recommended to inoculate ~ 2 transformants from each transformation. If different size colonies are observed, inoculate one of each size and keep track of which ones grow in the LB-Kan broth (some may not actually be Kan resistant).

3. Inoculate these tubes with the designated isolated transformant approximately 16 hours prior to the next lab period, and incubate them at 37 °C with vigorous shaking. You will need these cultures to perform a plasmid DNA isolation during the next lab period.

Students should have:

1. LB-kan broth

Instructor's notes:

- *It is important to help students examine their plates to determine if the controls are "clean" (there should be no growth), and to identify transformants. These should be easy to identify as cream colored, round, slightly translucent colonies.*
- *The transformation efficiency is usually quite low and varied. Expect anywhere from 0 to 1000 transformants per transformation. It would be useful to students to explain why the low efficiency exists and that just one transformant is all that is needed.*
- *At this point, students should be able to explain what is in the transformant and why this step was carried out-to isolate and amplify the desired S. marcescens chromosomal fragment which contains the DNA flanking the Tn insertion that resulted in altered pigment production. Again, it will likely be necessary to relate this step to the big picture and to discuss why transformations are carried out in the first place.*
- *If no transformants are recovered, and if time permits, students may want to start over with the cloning steps. Alternately, perhaps some groups will have successfully recovered transformants from more than one ligation and are willing to share, so that each group will have a transformant to work with.*

Day 12: Plasmid minipreps from *E. coli* DH5alpir transformants and *Bam*HI digests.

General Procedure:

1. Prep the plasmid DNA from each transformant using a Qiagen plasmid miniprep kit as described in the qiagen plasmid miniprep handbook. Perform the optional wash with 0.5 ml buffer PB and elute the DNA in 30 ul buffer EB for more concentrated plasmid DNA.
2. Digest 5 ul of the plasmid DNA with *Bam*HI as described below. Incubate the digests in the 37 °C water bath and remove digests to freezer after overnight incubation.
3. Store the remainder of the plasmid prep in -20°C freezer box-we will used this in sequencing reactions after examining the Plasmid DNA using gel electrophoresis.
4. Store 0.75 ml of the remaining transformant culture as a glycerol stock. See directions for making a glycerol stock below.

BamHI digest Rxn set up/sample:

To MCF tube, add:

12 μl sterile dd H2O

2 μl BamHI react 3 buffer

5 μl plasmid DNA

1 μl BamHI enzyme

20 μl total

Making a glycerol stock:

To a 1.5 ml cryovial, add:

0.75 ml of culture

0.75 ml of 50% glycerol

vortex and place in deep freeze freezer at -80 °C

Instructor's notes:

- *It may be useful to ask the students to compare and contrast the steps in a plasmid DNA isolation protocol compared to a protocol for isolating chromosomal DNA and to see if they understand the functions of each of the steps in the Qiagen miniprep kit by analogy. A Plasmid DNA isolation protocol development assignment could also be used here to ensure that the students understand what is happening rather than just following the steps (see Appendix 5C of the supplementary materials).*

- *To save BamHI enzyme, the instructor may wish to add this reagent to the student's tubes once all the other components of the BamHI digest have been added, rather than having aliquats of this reagent distributed for each group of students.*

Day 13: Analysis of *Bam*HI digested plasmid DNA by agarose gel electrophoresis

General procedure:

1. Remove *Bam*HI digests from the freezer and prepare them for loading on a gel (refer to previous gel loading protocol as used for the chromosomal DNA gel electrophoresis.

2. Set up 1 % agarose gels and electrophoresis apparatuses to accommodate all class samples (maybe 4 samples/group? Be sure to allow one extra lane for a molecular weight ladder.

3. Run samples on gels until the Brom phenol Blue dye is approximately 3/4 the distance of the gel and visualize the DNA bands using a UV transilluminator. This should take about 45 minutes if gels are run at 3-5 volts/cm.

4. Look for quality of the plasmid DNA and determine the size and number of the fragments generated from the *Bam*HI digests.

5. Estimate the concentration of DNA in each of the plasmid preparations based on the brightness of the band seen compared to the bands of a quantitative DNA ladder.

6. Revive any transformants that yielded good plasmid DNA

Students should already have or should be prepared in class:

1. aliquots of 6 X DNA gel loading buffer
2. 0.7 % agarose gels already made-stored in fridge
3. 1 X TAE with Ethidium bromide already made-stored in fridge

Instructor's notes:

- *The plasmid isolations work quite well if ~ 1.5- 3.0 ml of a 16-24 hour culture is used.*

- *Usually only one BamHI fragment is generated from the linearization of the circularized DNA containing the Tn and flanking S. marcescens chromosomal DNA. It is important to explain to the students why digestion with BamHI is necessary prior to visualizing the plasmid DNA on the gel. This allows the visualization of the number of BamHI fragments that ligated together prior to circularization to form a plasmid, and allows the size of the band to be more accurately estimated. In*

the event that multiple bands are seen, this is due to the ligation of non-contiguous fragments, only one of which need have the Tn insertion. This plasmid can still be sequenced, however, when analyzing the sequence, one has to identify the position of the BamHI sites that join the non-contigous pieces.

- *We recommend using the Fisher ExACT gene 1 Kb plus DNA ladder for easy estimation of DNA concentration from the gel, but any quantitative ladder with bands ranging from 0.5 to 12 Kb would work.*
- *Images of Agarose gel electrophoresis of plasposon DNA isolated from E. coli transformants and digested with BamHI from students results are shown in Appendix 10 of the supplementary materials.*

Day 14: Whole class analysis of *Bam*HI digested plasmid DNA by gel electrophoresis and discussion of the sequencing reaction, set up sequencing reaction.

General Procedure:

2. As a class, go over the DNA concentration estimations for each plasmid prep and determine if there is enough plasmid DNA to set up a sequencing reaction.
3. Set up sequencing reactions as per sequencing facility instructions. * If using the CSUPERB Microchemical Core facility, add 1 ug of plasmid DNA, 10 pmoles primer and ddH_2O up to 12 ul total in a 0.2 ml PCR reaction tube.
4. Sequence each plasmid template using both the tpnRL 17-1 primer and the tpnRL 13-2 primer.

Instructor's notes:

- *Any sequencing facility will suffice thus the details of the reaction set up described here may be altered according to the need. Due to the number of sequencing reactions being submitted in our hands (usually ~20) sequencing facilities that provide the most economical services for this number of sequences are preferred. It may also be preferable to perform the sequences in house if possible, which might allow students to see the sequencing instruments first hand.*
- *The primers indicated were designed to extend off the ends of the Tn. Their sequences are provided in the* Appendix 1A of the supplementary materials.
- *The DNA concentration estimations are done as a class to make sure everyone is capable of this and that we are generally in agreement on the concentration determined. It is also advantageous to examine the banding patterns of the plasmid digests as a class to see which S. marcescens mutants yielded plasmids with the same banding pattern. If the phenotypes of the mutants are the same, and the plasmid banding pattern is the same, the Tn is likely inserted into the same BamHI fragment on the chromosome. The Tn doesn't necessarily have to be in the exact same location, however, so sequencing these different plasmid clones might still be beneficial.*
- *During this discussion it is recommended to check student's understanding of what these plasmid actually represent and what different plasmid sizes and banding patterns indicates. A different size or banding pattern indicates a different mutant. It may be beneficial to create a large table on the chalk board for students to fill in the phenotypes of the mutants correlating the plasmid banding patterns to see how many unique mutants they might expect.*

Day 15: Discussion of the sequencing reaction and how to analyze sequences

General Procedure:

1. In computer lab, (or on laptops in class) obtain the sequences of each of your plasmids and follow the tutorial to determine the site of the transposon insertion

2. Optional: Determine if will need more plasmid DNA for more sequencing reactions and inoculate cultures if needed.

3. Optional: Assign sequence analysis results figure- must have transposon sandwich sequence in 1 week for primer design. Bring hard copy and also have electronic copy.

Instructor's notes:

- *At this point in the experiment, if sequence from the Tn insertions was obtained, students should be able to determine the identity of the genes disrupted by the Tn insertion, which led to an alteration in pigment production. From this sequence identity obtained form databases, instructors might wish to have the students use the sequence information to construct a map of the insertion site and of the gene context in which it is found. From this, students should be encouraged to make predictions as to how a disruption in that particular gene or genes could result in the pigment phenotypes observed. Many of the mutants will have insertions within the previously characterized S. marcescens pig operon, which is predicted to encode the biosynthetic enzymes for prodigiosin production. However, students may find that the Tn has inserted into other genes such as putative regulators. This provides an opportunity for the students to begin thinking about the physiology of pigment production and to incorporate what is known about it into their predictions. At the time field tests were done, the S. marcescens genome sequence had not yet been published or accessible. Blast searches from Tn induced pigment mutants during field tests of this lab in NCBI and JCVI have resulted in the most frequent hits in genes found in Hahella chejuensis and Serratia proteomaculans. (see an example of these results in student example of laboratory report 2 in Appendix 6B of the supplementary materials).*

- *The remainder of the labs indicated are optional labs that extend the laboratory experience and can easily be incorporated to fill an entire 15 week semester, with the last several weeks being reserved to summarize the class project as a professional scientific poster. We recommend this format as it allows the students to revisit and assemble all that they have done all semester and put it back into the big picture to answer the original question about prodigiosin biosynthesis in S. marcescens. It is also during this time that they can relate the results from the molecular aspects of the project to the physiology of the bacterium. Journal article assignment 2 (described in Appendix 4C of the supplementary materials) is designed to push the students to learn more about the physiology of prodigiosin and how the results they obtained might apply to what is already known. This assignment is also a useful tool to assess student comprehension of the physiology and molecular and genetic techniques at this later time in the semester, after having carried out many of them first hand.*

- *The details of these labs are not provided as a great deal of flexibility can be introduced to suit the needs of the class and to allow repeating steps that might not have worked.*

Day 16: Continued sequence analysis on computer and repeating steps to clone Tn insertion if needed.

General Procedure:

3. Continue analyzing sequence data and construction of Tn insertion map

4. Create a figure with legend to illustrate the gene(s) disrupted by the Tn and indicate at what nucleotide position the Tn inserted.

Day 17: Class summary of sequence results, primer design for primer walking of the previously obtained sequence, set up for growth curve analysis of pigment mutants and start growth curve.

General Procedure:

1. In computer lab or on laptops in class, analyze transposon sandwich and design primers for primer walking. Will pick and order primers during this lab session.

2. Prep more plasmid DNA if needed and analyze on gel.

3. Discuss growth conditions to use to examine the growth phenotypes of the pigment mutants and prepare media and cultures for this.

Day 18: Growth curve analysis, discussion of calculation of generation times, and primer orders

Day 19: Set up new sequencing reactions for primer walking with new primers and discussion of how to best present this experiment in paper/poster format.

Day 20: Work on figures and assign parts of the poster to different groups

Day 21: Examine new *S. marcescens* sequences and assemble sequence contigs

Day 22 through Day 27: Open lab, finishing up lab work and working on posters

Day 27: Poster (s) /presentations due, lab cleanup.

LABORATORY MANUAL APPENDIX A: STUDENT SAFETY CONTRACT

Course_____Instructor _____Semester _____
To ensure an understanding of concepts and the safe execution of laboratory experiments, it is essential that you come to class prepared and follow the rules of laboratory conduct and procedures for discard of materials as outlined below.

LABORATORY CONDUCT Initial after you read each item

- The consumption of food or drinks is not allowed in the laboratory at any time. _____
- Chewing gum or the application of cosmetics is not allowed in the laboratory at any time. _____
- Coats, backpacks, and other personal items are to be stored in the wash room. Keep only those items on the lab bench that is needed to perform experiments. _____
- Clean the lab bench with disinfectant before you begin work and again before leaving the laboratory. _____
- A lab coat must be worn at all times. Do not remove your lab coat from the lab. _____
- Long hair must be tied back to avoid contact with flames of Bunsen burners and potentially infectious materials. ____
- Shoes must be worn at all times. _____
- Hands and arms must be thoroughly washed with soap prior to leaving the laboratory. _____
- Cultures, slides, or other materials may not be taken from the laboratory. _____
- Please obtain prior permission from your instructor for any visitors you will be bringing to class. _____

Laboratory Safety Initial after you read each item

- Report all spills at once to your instructor. Treat all spilled cultures as though they are pathogenic. Your instructor will provide you with information and materials for cleaning up the spill. _____
- Report any injury to your instructor. _____
- Any lab work performed outside of normal lab hours must be done with instructor present. _____
- Eyewash stations are located in Holt 325 and Holt 307. Become familiar with their locations and operation in the event that any chemical splashes in your eyes. _____
- The fire extinguisher is located just inside the main door to the laboratory. _____
- Should the jar of ethanol accidentally ignite, I understand that the most effective method of control is to cap the jar immediately. _____
- MSDS (Material Safety and Data Sheets) which provide safety information for handling chemicals used in the laboratory are located in a binder on the counter. I understand that it is my responsibility to read the MSDS before I handle chemicals. _____

DISCARD PROCEDURES: The following bolded items are used for discard of contaminated materials, including swabs of the skin, throat, or of the environment. Under each bolded item is a list of materials to be discarded in that particular container. Refer to this list frequently to determine the proper method for disposal of contaminated materials. You are responsible for the safe disposal of all materials.

Initial after you read each section.

PIPETTE DISCARD BUCKET (at student desks):

Glass serological pipettes

Glass Pasteur pipettes _____

BIOHAZARD BAG:

Plastic petri plates (labels don't have to be removed)

Gloves

Microcentrifuge tubes

Contaminated paper towels or Kimwipes

NEVER DISCARD ANY GLASS OR SHARPS IN A BIOHAZARD BAG_____

TEST TUBE BASKETS:

Remove all markings and labels from test tubes.

TWIST IN COTTON PLUG FIRMLY and place in slanted baskets.

Make sure the tubes are upright so that cultures will not spill. _____

DISCARD TRAY FOR GLASSWARE: Remove all markings and labels.

Bottles

Flasks

Beakers

Glass Petri Plates-discard agar side down _____

SHARPS CONTAINER: DO NOT ATTEMPT TO RECAP OR BEND NEEDLES

Any broken glassware

Cotton swabs

Cover slips

Razor blades

Capillary tubes

Syringes and needles

Micropipette tips _____

HAZARDOUS CHEMICALS:

Assume that nothing can be disposed of in the sink!

Empty all test tubes, cuvettes, and rinse water into the hazardous waste container provided_____

The rules of laboratory conduct are for your safety and that of other students and laboratory personnel. I have read the above rules, and agree to observe and abide by them. I understand that failure to do so can result in dismissal from the course or a reduction in my course grade.

Appendix 2B. Student's Lab manual

Identification of genes involved in prodigiosin production in *Serratia marcescens* by transposon mutagenesis

Summary of the lab exercises

During the entire lab portion of this course, students will attempt to identify genes involved in prodigiosin (red pigment) biosynthesis in the bacterium, *Serratia marcescens*. The genes encoding the biosynthetic pathway for prodigiosin have been previously identified. However, layers of regulation seem to exist and roles for other, as yet unidentified, genes in this process are likely.

Students will use transposon mutagenesis to make random transposon insertions throughout the genome of *S. marcescens*. The mutants will be screened for alterations in pigment production compared to wild type, and the site of the transposon insertion in these mutants will be determined by cloning and sequencing the insertion site. Students will also carry out experiments to determine the role of the disrupted gene on pigment production and overall physiology of the *S. marcescens* mutants.

Laboratory student learning objectives

By the completion of this course students should be able to:

1. Describe the methodology and rationale for 2 general approaches to identify genes with a particular function, random mutagenesis and construction of DNA libraries.
2. Describe how to carryout a heterologous expression experiment and how this is used to determine the function of a gene.
3. Describe how to carry out a transposon mutagenesis experiment and why this approach might be used.
4. Describe how to determine how many mutants should be examined in a Tn mutagenesis experiment (or in analyzing a chromosomal library) to ensure complete coverage of the genome using the Poisson distribution.
5. Define the difference between a selection and a screen and describe how and for what purpose each one is carried out.
6. Indicate under what circumstances polar effects could be observed in mutants and how to interpret phenotypes in these cases.
7. Describe the steps in a cloning experiment including the properties and roles of restriction enzymes and DNA ligase.
8. Describe and contrast the methods for introducing exogenous DNA into a bacterial cell, transformation, conjugation and transposition.
9. Carry out and describe the steps in proper DNA manipulation techniques including DNA isolations, precipitations and visualization via gel electrophoresis
10. Describe the sequencing reaction and be able to assemble sequences and choose good sequencing primers.
11. Use bioinformatics programs such as Blast and navigate databases such as NCBI and TIGR.
12. Use sequence information to make predictions about the function of genes

Instructions for the 1st week of class, if assigned, otherwise skip to "Day 1" instructions below, under "Begin the *S. marcescens* transposon mutagenesis experiment".

Day 1 of class

Recommended Lab A: Viable cell count of *Serratia marcescens*. To be done Individually.

Purpose: To review micropipetting and making serial dilutions, aseptic technique and spread plating, and to become familiar with the colony phenotype and growth properties of *S. marcescens.*

General Procedure:

Perform a viable cell count of the *S. marcescens* culture provided. Assume an original concentration of 1 x 10^9 cells/ml, make the appropriate dilutions in a 1.0 ml final volume using sterile water, and plate only 3 dilutions to get 30-300 colonies/ plate. Half the class should incubate plates at 37°C and the other half should incubate at 28°C for 24-48 hours. .

Day 2 of class

Recommended Lab B: Examining the results of Viable cell counts and preparing media and reagents for future use in the lab. To be done individually and in groups.

Purpose: To determine student's skill level and experience with aseptic technique, micropipetting, spread plating and making serial dilutions (all very commonly used skills required in this laboratory portion of the course), and to gain experience making media and becoming familiar with its components.

B. Making media

General procedure:

Class will divide into 8 separate groups of no more than 3 students, and each group will prepare one type of the following media. See appendix for media recipes. 8 groups are chosen based on a class size of 20-24. This can be altered according to the class size.

Media to make:

9) 8 x 100 mls LB
10) 8x 100 mls LB-Kanamycin (50 ug/ml)
11) 0.5 L LB agar (~ 20 plates)
12) 1.0 L LB-kan (50 ug/ml) agar (~ 40 plates)
13) 2.0 L 0.2% glucose Mops + Pi + kanamycin agar (~80 plates)
14) 1.5 L 0.2% glucose Mops + Pi agar (~60 plates)
15) 0.5 L 1 % succinate Mops + Pi agar (~ 20 plates)
16) 8 x 10 mls 50 % glycerol

* This should be all the media we will need for the whole semester for a class of ~24 students.

B. Examining viable plate counts-to be done while the media made in part A is in the autoclave

General procedure:

1. Analyze your and your partner's plates. Do you have isolated colonies evenly distributed over the surface of the plate? Do you see a progressive 10 fold decrease in the number of colonies from the lowest dilution plate to the highest dilution plate? Are there any contaminants? Is there a difference in colony phenotype when grown at 28 °C compared to 37 °C? What skills do you need to improve upon or review?

2. Count the colonies on the appropriate plate and calculate CFU/ml of the original culture.

3. Pick a well isolated colony of *S. marcescens* from this plate and perform a streak plate for colony isolation onto a fresh plate of TSA-incubate at 28 °C. Save the plate the colony was streaked from in a sealed Tupperware at 4 °C as a backup source of *S. marcescens* for future experiments.

Day 3 of class: To begin on day 3 of class **if** the above recommended labs were assigned. Otherwise, this is day 1 of the Tn mutagenesis experiment as labeled.

Begin the *S. marcescens* transposon mutagenesis experiment

Day 1: Conjugation of *S. marcescens* and the *E. coli* *WM20767/pRL27* transposon donor

General Procedure:

1. The class will discuss the Journal article assignments that introduce the topics of *S. marcescens* prodigiosin production and the Transposon delivery system, pRL27.
2. The class will develop a precise protocol for the conjugation and mutagenesis of *S. marcescens* based on the journal article by Larsen et al. and will go over the details of the method.
3. In groups of no more than 3, students will prepare the conjugation experiment by mixing together the *E. coli* WM2672 transposon Donor and *S. marcescens* recipient mid log phase cultures and set up filter mating on LB plates to incubate overnight at 37 °C.

* Save residual donor and recipient cultures at 4 °C for the controls when selecting mutants.

Day 2: Discussion of the genotypes and phenotypes of the donor and recipient strains used, of controls, determination of transposon insertion frequency and selection of transposon (Tn) induced mutants.

General procedure:

1. Resuspend the conjugation mix of bacteria off of the surface of the filter in approximately 2 mls of 1 x "M" buffer and make 10 fold serial dilutions in 1 X "M" in 1 ml final volumes.

2. Plate 10^{0}- 10^{-3} 10 fold dilutions of the conjugation mix on Glucose Mops + kan plates for selection of Tn induced *S. marcescens* mutants and plate 10^{-3}-10^{-7} dilutions onto Glucose Mops (no kanamycin) plates to determine the concentration of recipient *S. marcescens* cells present in the mating mix. **Save remaining conjugation mix in fridge (4 °C)!!**

3. Plate 100 ul of the residual donor and recipient cultures (stored at 4 °C from previous lab) on each type of media to serve as controls.

4. Incubate all plates at 28 °C in sealed container with moist paper towels.

Students should have:

-6 plates per group of Glucose Mops + kanamycin

-7 plates per group of Glucose Mops no kanamycin

-residual cultures of donor and recipient stored in fridge

Day 3: Analysis of Tn mutant selection dilution plates, calculation of Tn insertion frequency and large scale selection of Tn mutants.

General procedure:

1. Examine dilution plates and score controls for growth. What should we be looking for?

2. Determine CFU/ml of Tn induced mutants and determine total *S. marcescens* CFU/ml to determine frequency of Tn insertion. How does this compare to the published values?

3. We will possibly plate more of the conjugation mix to reach desired number of mutants to screen based on the Poisson distribution.

4. Pure streak any pigment mutants on the selection media next to the wild type *S. marcescens* parent strain. Also streak plate one wild type looking *S. marcescens* Tn mutant for comparison. Place all old and new plates in incubator.

Students should have:

-more selection media

-parent strains restreaked fresh from previous labs

-1 X M stocks and 40 X M stocks

-sterile microcentrifuge tubes and racks

-conjugation mix from fridge

Day 4: Continuation of the screen for, and isolation of, pigment mutants.

General Procedure:

1. Examine the pigment mutants streaked from the previous lab. Are they pure? What is their phenotype relative to the "wild type pigmented mutant you chose?"

2. Restreak the mutants for isolation if they are not yet pure.

3. Examine the new Tn mutant selection plates and screen these for any new pigment mutants.

4. Pure streak any new pigment mutants alongside the wildtype pigmented Tn mutant for comparison as done before.

5. Incubate all plates in closed Tupperware with moist paper towel as done previously.

Students should have:

-more selection media

-"wild type pigmented mutant restreaked fresh from previous lab

-sterile sticks for streaking

Day 5: Phenotypic characterization of pigment mutants on Glucose Mops, Succinate Mops and LB plates.

General Procedure:

2. Streak each purified mutant onto a set of Glucose Mops, Succinate Mops, and LB plates using the exact same colony as described. Repeat this onto another set of the same media. Include the wild type pigmented mutant

for comparison. Incubate one set at 37 °C and the other set at 28 °C in a closed Tupperware. After 24-48 hours, differences in phenotype between the mutants should be clear. If isolated colonies are all sufficiently large, store them in the fridge.

Day 6: Class Results of pigment mutant phenotypic characterization and chromosomal preparation discussion.

General Procedure:

1. Examine the phenotypes of the streaked mutants and compare them each to each other and to the "wild type" pigmented mutant.

2. Carefully document the phenotype of each mutant observed in your notebook and arrange the plates as a class so that the entire class may see the differences in phenotypes of the different pigment mutants on the different media.

3. As a class, determine how many unique mutants the class has isolated based on phenotype alone and compile a table on the chalkboard for all to see. Does each group have a unique mutant? Identify these and be sure to carry on with the following experiments with these unique mutants. If there are not enough, some groups can share the same mutant, but each group should have at least one mutant to work with at this point, but should not have more than 1/person in their group in the event that supplies for downstream steps are limited.

4. Prepare a culture tube with 5 mls of LB-Kanamycin broth for each mutant the group will work with. Carefully label this tube with the corresponding mutant name.

5. Inoculate this tube with an isolated colony of the designated mutant **approximately 16-24 hours prior to the beginning of the next lab period** when you will need this culture.

Students should have:

4. 5 ml LB-kanamycin broth in tubes
5. Sterile sticks for inoculating
6. Glucose and succinate Mops and LB plates

Day 7: Chromosomal preparations from Tn induced pigment mutants

General Procedure:

5. Each group will prepare chromosomal DNA from 1-3 of their mutants (depending on resource availability and group size) following the Mo Bio chromosomal prep kit procedure (see protocol below). Pick mutants to prep following a class analysis of all the mutant phenotypes (done on day 6). We want to get as many different types of mutants as possible as a class.

6. Remove 3 µl of each chromosomal DNA preparation to a sterile microcentrifuge tube containing 4 µl of 6 X DNA gel loading buffer and 17 µl of sterile nanopure water. Store this sample (for gel electrophoresis during the next session) and your Chromosomal preps at -20 °C in the designated freezer box. BE SURE TO HAVE LABELED EACH OF YOUR TUBES IN DETAIL WITH DATE, GROUP NAME AND A DESCRIPTION OF THE SAMPLE IT CONTAINS!!!!!!

7. Prepare 0.7% agarose gels and 1 X TAE buffer to use to visualize your chromosomal DNA for the next lab. Store these at 4 °C.

Detailed Protocol for Chromosomal Preps using the MoBIO kit

Please wear gloves at all times!!!!

1. Add 1.8 ml of microbial (bacteria, yeast) culture to a 2 ml Collection Tube (provided) and centrifuge at 10,000 x *g* for 3 min at room temperature. Decant the supernatant completely to remove the media supernatant.

This step concentrates and pellets the microbial cells. In some cases it may take longer to completely pellet the cells. It is important to pellet the cells completely and remove all the culture media in this step.

2. Resuspend the cell pellet in 300 µl of MicroBead Solution and gently vortex to mix. Transfer resuspended cells to MicroBead Tube.

The MicroBead Solution contains salts and a buffer which stabilizes and homogeneously disperses the microbial cells prior to lysis.

3. Add 50 µl of Solution MD1 to the MicroBead Tube.

Solution MD1 contains SDS and other disruption agents required for cell lysis. In addition to aiding in cell lysis, SDS is an anionic detergent that breaks down fatty acids and lipids associated with the cell membrane of several organisms. If it gets cold, it will precipitate. Heating at 60°C will dissolve the SDS and will not harm the SDS or the other disruption agents. In addition, Solution MD1 can be used while it is still warm.

8. To minimize DNA shearing for difficult cells, it is recommended to heat the preps at 65°C for 10 minutes, occasionally bump vortexing for a few seconds every 2-3 minutes.

This optional step can lead to better performance in some cases.

This step creates the combined chemical/ mechanical lysis conditions required to release desired nucleic acids from microbial cells. Many cell types will not lyse without this chemically enhanced bead beating process.

5. Make sure the 2 ml MicroBead Tubes rotate freely in the centrifuge without rubbing. Centrifuge the tubes at 10,000 x *g* for 30 seconds at room temperature. CAUTION: Be sure not to exceed 10,000 x *g* or tubes may break.

The cell debris is sent to the bottom of the tube while DNA is remains in the supernatant.

6. Transfer the supernatant to a clean 2 ml Collection Tube (provided).

7. NOTE: Expect 300 to 350 µl of supernatant.

The volume to expect will vary depending on the size of the original cell pellet from step 1.

8. Add 100 µl of Solution MD2, to the supernatant. Vortex 5 seconds. Then incubate at 4°C (on ice) for 5 minutes.

9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g*.

Solution MD2 contains a reagent to precipitate non-DNA organic and inorganic material including cell debris and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications.

10. Avoiding the pellet, transfer the entire volume of supernatant to a clean 2 ml Collection Tube (provided). Expect approximately 450 µl in volume.

The pellet at this point contains non-DNA organic and inorganic materials, including cell debris and proteins. For the best DNA quality and yield, avoid transferring any of the pellet.

11. Add 900 µl of Solution MD3 to the supernatant and vortex 5 seconds.

Solution MD3 is a highly concentrated salt solution. It sets up the high salt condition necessary to bind DNA to the Spin Filter membrane in the following step.

12. Load about 700 µl into the Spin Filter and centrifuge at 10,000 x *g* for 30 seconds at room temperature. Discard the flow through, add the remaining supernatant to the Spin Filter, and centrifuge at 10,000 x *g* for 30 seconds at room temperature. NOTE: A total of 2 to 3 loads for each sample processed are required. Discard all flow through liquid.

DNA is selectively bound to the silica membrane in the Spin Filter device. Contaminants pass through the filter membrane, leaving only the DNA bound to the membrane.

13. Add 300 µl of Solution MD4 and centrifuge at room temperature for 30 seconds at 10,000x *g*.

Solution MD4 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the Spin Filter. This wash solution removes residues of salt, and other contaminants while allowing the DNA to stay bound to the silica membrane.

14. Discard the flow through.

This flow through is waste containing ethanol wash solution and contaminants that did not bind to the silica Spin Filter membrane.

15. Centrifuge at room temperature for 1 minute at 10,000 x *g*.

This step removes residual Solution MD4 (ethanol wash solution). It is critical to remove all traces of wash solution because it can interfere with down stream DNA applications.

16. Being careful not to splash liquid on the spin filter basket, place Spin Filter in a new 2 ml Collection Tube (provided).

It is important to avoid any traces of the ethanol based wash solution.

17. Add 50 µl of Solution MD5 to the center of the white filter membrane (careful not to poke the membrane with the pipet tip!)

Placing the Solution MD5 (elution buffer) in the center of the small white membrane will make sure the entire membrane is wetted. This will result in more efficient release of bound DNA

18. Centrifuge at room temperature for 30 seconds at 10,000 x *g*.

As the Solution MD5 (elution buffer) passes through the silica membrane, DNA is released, and it flows through the membrane, and into the Collection Tube. The DNA is released because it can only bind to the silica Spin Filter membrane in the presence of salt. Solution MD5 is 10mM Tris pH 8 and does not contain salt.

19. Discard Spin Filter. DNA in the tube is now ready for any downstream application. No further steps are required.

We recommend storing DNA frozen (-20°C). Solution MD5 contains no EDTA.

Day 8: Gel electrophoresis and *Bam*HI digests of chromosomal preps from *S. marcescens* Tn mutants.

General Procedure:

1. Set up the required number of gel electrophoresis apparatuses as described to accommodate all the class samples. Each gel should contain at least one lane for the Exact gene 1 KB plus DNA (or other appropriate) molecular weight ladder. The instructor will load the ladder and add the mutagenic DNA dye, Ethidium Bromide.* **To prevent your skin from coming into contact with the gel or gel buffer containing ethidium bromide, you must be wearing nitrile gloves, safety glasses and a lab coat!!!**

2. Run the DNA on the gel until the Brom phenol blue dye front is as the bottom 1/3 of the gel. Visualize DNA using a UV trans-illuminator and include a picture in your notebook.

3. Provided you were able to visualize good quality, high molecular weight chromosomal DNA as described by your instructor, set up a restriction digest of your chromosomal DNA with *Bam*HI as described below. *Remove these digest samples to your freezer box after overnight incubation!!!

4. Store the remainder of the chromosomal DNA in the freezer.

Students should have:

4. 0.7 % agarose gels (made during the previous lab) and 1 x TAE in fridge
5. chromosomal DNA samples prepared to load with gel loading dye in freezer
6. Chromosomal preps stored in freezer

*Bam*HI digest Rxn set up/sample:

To MCF tube, add:

12 µl sterile nuclease free H2O

5 µl BamHI react 3 buffer (NEB)

30 µl Chromosomal DNA

3 µl BamHI enzyme

50 µl total volume

Day 9: Ethanol precipitation of *Bam*HI digested chromosomal DNA and ligation reaction set up

General procedure:

1. Precipitate the *Bam*HI digested DNA and set up the DNA ligation reaction as described below.

Ethanol precipitation of DNA protocol

11. "Spin down" the liquid of the *Bam*HI digest in the microcentrifuge tube by centrifuging at ~13,000 rpm for several seconds.
12. Estimate the volume of from the digest (should be ~50 µl)
13. Based on this, add 0.1 volumes of 3 M Na Acetate pH 5.2-mix
14. Add 2 volumes 100% ethanol-mix and place at -20 C for ~10 min or on dry ice for ~10 min.
15. Place the samples in the microcentrifuge, spine side out, and centrifuge for 20 minutes at 14,000 rpm in the cold room
16. Immediately but carefully decant the supernatant-the pellet of DNA should be on bottom and side of the spine side of the tube.
17. Immediately but gently add 500 µl of ice cold 70 % ethanol-centrifuge 5 min

18. Immediately but carefully decant the supernatant and leave tube inverted on a kimwipe for several minutes to draw out excess moisture
19. Dry pellet by placing in heat block with lid open for 5-20 minutes
20. Once the pellet appears dry (no more liquid visible upon close inspection), close the tube and prepare the ligation reaction.

Ligation reaction

8. To each tube of precipitated DNA, add 20 µl of 1 X T4 DNA ligase buffer
9. resuspend DNA well by vortexing and/or pipetting up and down- this will take a while, but take your time and focus your efforts on the spine side of the tube where the DNA should be. You will not be able to see it!
10. "spin down the liquid to collect it at the bottom of the tube
11. Add 1 µl of T4 DNA ligase to the tube-mix by flicking gently
12. Spin down
13. Place tube in water bath set at 14°C in the cold room. Make sure all labels are clearly visible and that both the tops and sides of the tubes have been labeled!!!
14. Remove tubes to -20°C freezer box after overnight incubation

Day 10: Dialysis and transformation of ligation mixes into *E. coli* DH5alpir electrocompetent cells.

General Procedure:

3. First hour of lab, students will dialyze ligation mix as described below, while preparing the transformation into electrocompetent *E. coli* DH5αλpir.

4. Next 2 hours, students will transform *E. coli* and plate transformants onto LB-kan (50 ug/ml) plates.

Dialyzing the ligation mix:

5. Float a 0.025 um pore nitrocellulose membrane (shiny side up) onto a layer of sterile nanopure water in a sterile petri dish.
6. "Spin down" and then gently pipette your ligation mix onto the surface of the membrane being careful not to submerge the membrane. You may want to practice without your ligation mix first to see how much pressure you can put onto the membrane with the pipette tip with out submerging it.
7. Let the ligation reaction stand undisturbed for ~20 minutes and then carefully pipette the mixture off the membrane into a clean sterile microcentrifuge tube. Store on ice.
8. During the 20 minute wait time, prepare tubes for the transformation as described below.

Transformation: **All steps are to be carried out strictly on ice until the actual electroporation!**

10. Add 60 µl of previously prepared (see Appendix 1B of the supplementary materials) electrocompetent *E. coli* DH5αλpir cells to a sterile microcentrifuge tube **on ice**.
11. Add ½ your ligation mix (~10 µl) to this tube and pipette up and down to mix.
12. Transfer the entire volume of this mixture to the bottom of a prechilled electroporation cuvette as demonstrated. Tap the liquid down so that there are no bubbles or spaces at the bottom of the cuvette.
13. Electroporate the cells as shown and add the electroporated cells to 1ml of prewarmed SOC media in a 16 mm culture tube (see Appendix 1B of the supplementary materials).
14. Recover the cells by incubating at 37 °C with shaking for 1 hour.
15. Plate 100 µl of this cell solution onto LB-Kan plates using aseptic spread plate technique.
16. Centrifuge the remainder of the culture at 6,000 rpm and resuspend the cell pellet in the residual ~ 200 ul supernatant. Plate this entire volume onto LB-kan. Incubate both plates at 37 °C for 24-48 hours.

17. Plate a "cells only" control, by spread plating 60 ul of electrocompetent cells that have not been transformed with DNA onto an LB-Kan plate and incubate along with the transformation plates.
18. Examine the plates after 24 hours. If colonies are visible, remove the plates to 4 °C for further manipulation. If colonies are not yet clearly visible, continue to incubate for an additional 24 hours.

Students should have:

2) LB-kan (50 ug/ml) plates

Day 11: Examination of transformation plates and preparation for plasmid DNA isolations.

General procedure:

4. Examine the transformation plates and look for isolated colonies of *E. coli* DH5αλpir transformants. Compare these plates to the control "cells only" plate to see what no growth should look like. Record your results.

5. Prepare 16 mm culture tubes with 4 mls of LB-kan broth for each transformant chosen to prepare plasmid DNA from. It is recommended to inoculate ~ 2 transformants from each transformation. If different size colonies are observed, inoculate one of each size and keep track of which ones grow in the LB-Kan broth (some may not actually be Kan resistant).

6. Inoculate these tubes with the designated isolated transformant approximately 16 hours prior to the next lab period, and incubate them at 37 °C with vigorous shaking. You will need these cultures to perform a plasmid DNA isolation during the next lab period.

Students should have:

1. LB-kan broth

Day 12: Plasmid minipreps from *E. coli* DH5alpir transformants and *Bam*HI digests.

General Procedure:

5. Prep the plasmid DNA from each transformant using a Qiagen plasmid miniprep kit as described in the qiagen plasmid miniprep handbook. Perform the optional wash with 0.5 ml buffer PB and elute the DNA in 30 ul buffer EB for more concentrated plasmid DNA.

6. Digest 5 ul of the plasmid DNA with *Bam*HI as described below. Incubate the digests in the 37 °C water bath and remove digests to freezer after overnight incubation.

7. Store the remainder of the plasmid prep in -20°C freezer box-we will used this in sequencing reactions after examining the Plasmid DNA using gel electrophoresis.

8. Store 0.75 ml of the remaining transformant culture as a glycerol stock. See directions for making a glycerol stock below.

BamHI digest Rxn set up/sample:	Making a glycerol stock:
To MCF tube, add:	To a 1.5 ml cryovial, add:
12 μl sterile dd H2O	0.75 ml of culture
2 μl BamHI react 3 buffer	0.75 ml of 50% glycerol
5 μl plasmid DNA	vortex and place in deep freeze freezer at -80 °C
1 μl BamHI enzyme	
20 μl total	

Day 13: Analysis of *Bam*HI digested plasmid DNA by agarose gel electrophoresis

General procedure:

7. Remove *Bam*HI digests from the freezer and prepare them for loading on a gel (refer to previous gel loading protocol as used for the chromosomal DNA gel electrophoresis.

8. Set up 1 % agarose gels and electrophoresis apparatuses to accommodate all class samples (maybe 4 samples/group? Be sure to allow one extra lane for a molecular weight ladder.

9. Run samples on gels until the Brom phenol Blue dye is approximately 3/4 the distance of the gel and visualize the DNA bands using a UV transilluminator. This should take about 45 minutes if gels are run at 3-5 volts/cm.

10. Look for quality of the plasmid DNA and determine the size and number of the fragments generated from the *Bam*HI digests.

11. Estimate the concentration of DNA in each of the plasmid preparations based on the brightness of the band seen compared to the bands of a quantitative DNA ladder.

12. Revive any transformants that yielded good plasmid DNA

Students should already have or should be prepared in class:

4. aliquots of 6 X DNA gel loading buffer
5. 0.7 % agarose gels already made-stored in fridge
6. 1 X TAE with Ethidium bromide already made-stored in fridge

Day 14: Whole class analysis of *Bam*HI digested plasmid DNA by gel electrophoresis and discussion of the sequencing reaction, set up sequencing reaction.

General Procedure:

2. As a class, go over the DNA concentration estimations for each plasmid prep and determine if there is enough plasmid DNA to set up a sequencing reaction.

3. Set up sequencing reactions as per sequencing facility instructions. * If using the CSUPERB Microchemical Core facility, add 1 ug of plasmid DNA, 10 pmoles primer and ddH_2O up to 12 ul total in a 0.2 ml PCR reaction tube.

4. Sequence each plasmid template using both the tpnRL 17-1 primer and the tpnRL 13-2 primer.

Day 15: Discussion of the sequencing reaction and how to analyze sequences

General Procedure:

1. In computer lab, (or on laptops in class) obtain the sequences of each of your plasmids and follow the tutorial to determine the site of the transposon insertion

2. Optional: Determine if will need more plasmid DNA for more sequencing reactions and inoculate cultures if needed.

3. Optional: Assign sequence analysis results figure- must have transposon sandwich sequence in 1 week for primer design. Bring hard copy and also have electronic copy.

Day 16: Continued sequence analysis on computer and repeating steps to clone Tn insertion if needed.

General Procedure:

3. Continue analyzing sequence data and construction of Tn insertion map

4. Create a figure with legend to illustrate the gene(s) disrupted by the Tn and indicate at what nucleotide position the Tn inserted.

Day 17: Class summary of sequence results, primer design for primer walking of the previously obtained sequence, set up for growth curve analysis of pigment mutants and start growth curve.

General Procedure:

1. In computer lab or on laptops in class, analyze transposon sandwich and design primers for primer walking. Will pick and order primers during this lab session.

2. Prep more plasmid DNA if needed and analyze on gel.

3. Discuss growth conditions to use to examine the growth phenotypes of the pigment mutants and prepare media and cultures for this.

Day 18: Growth curve analysis, discussion of calculation of generation times, and primer orders

Day 19: Set up new sequencing reactions for primer walking with new primers and discussion of how to best present this experiment in paper/poster format.

Day 20: Work on figures and assign parts of the poster to different groups

Day 21: Examine new *S. marcescens* sequences and assemble sequence contigs

Day 22 through Day 27: Open lab, finishing up lab work and working on posters

Day 27: Poster (s) /presentations due, lab cleanup.

LABORATORY MANUAL APPENDIX A: STUDENT SAFETY CONTRACT

Course_____Instructor _____Semester _____
To ensure an understanding of concepts and the safe execution of laboratory experiments, it is essential that you come to class prepared and follow the rules of laboratory conduct and procedures for discard of materials as outlined below.

LABORATORY CONDUCT Initial after you read each item

- The consumption of food or drinks is not allowed in the laboratory at any time. _____
- Chewing gum or the application of cosmetics is not allowed in the laboratory at any time. _____
- Coats, backpacks, and other personal items are to be stored in the wash room. Keep only those items on the lab bench that is needed to perform experiments. _____
- Clean the lab bench with disinfectant before you begin work and again before leaving the laboratory. _____
- A lab coat must be worn at all times. Do not remove your lab coat from the lab. _____
- Long hair must be tied back to avoid contact with flames of Bunsen burners and potentially infectious materials. ____
- Shoes must be worn at all times. _____
- Hands and arms must be thoroughly washed with soap prior to leaving the laboratory. _____
- Cultures, slides, or other materials may not be taken from the laboratory. _____
- Please obtain prior permission from your instructor for any visitors you will be bringing to class. _____

Laboratory Safety Initial after you read each item

- Report all spills at once to your instructor. Treat all spilled cultures as though they are pathogenic. Your instructor will provide you with information and materials for cleaning up the spill. _____
- Report any injury to your instructor. _____
- Any lab work performed outside of normal lab hours must be done with instructor present. _____
- Eyewash stations are located in Holt 325 and Holt 307. Become familiar with their locations and operation in the event that any chemical splashes in your eyes. _____
- The fire extinguisher is located just inside the main door to the laboratory. _____
- Should the jar of ethanol accidentally ignite, I understand that the most effective method of control is to cap the jar immediately. _____
- MSDS (Material Safety and Data Sheets) which provide safety information for handling chemicals used in the laboratory are located in a binder on the counter. I understand that it is my responsibility to read the MSDS before I handle chemicals. _____

DISCARD PROCEDURES: The following bolded items are used for discard of contaminated materials, including swabs of the skin, throat, or of the environment. Under each bolded item is a list of materials to be discarded in that particular container. Refer to this list frequently to determine the proper method for disposal of contaminated materials. You are responsible for the safe disposal of all materials.

Initial after you read each section.

PIPETTE DISCARD BUCKET (at student desks):

Glass serological pipettes

Glass Pasteur pipettes _____

BIOHAZARD BAG:

Plastic petri plates (labels don't have to be removed)

Gloves

Microcentrifuge tubes

Contaminated paper towels or Kimwipes

NEVER DISCARD ANY GLASS OR SHARPS IN A BIOHAZARD BAG_____

TEST TUBE BASKETS:

Remove all markings and labels from test tubes.

TWIST IN COTTON PLUG FIRMLY and place in slanted baskets.

Make sure the tubes are upright so that cultures will not spill. _____

DISCARD TRAY FOR GLASSWARE: Remove all markings and labels.

Bottles

Flasks

Beakers

Glass Petri Plates-discard agar side down _____

SHARPS CONTAINER: DO NOT ATTEMPT TO RECAP OR BEND NEEDLES

Any broken glassware

Cotton swabs

Cover slips

Razor blades

Capillary tubes

Syringes and needles

Micropipette tips _____

HAZARDOUS CHEMICALS:

Assume that nothing can be disposed of in the sink!

Empty all test tubes, cuvettes, and rinse water into the hazardous waste container provided_____

The rules of laboratory conduct are for your safety and that of other students and laboratory personnel. I have read the above rules, and agree to observe and abide by them. I understand that failure to do so can result in dismissal from the course or a reduction in my course grade.

Appendix 3: Assessment of student learning objectives

Appendix 3A. Pre/Post Project Assessment

Survey to be given on the first day of class and on the day of the final exam to determine student learning throughout course. Please fill out as carefully and thoroughly as you can for 10 extra credit points. Please answer each question. If you do not know the answer, write "I don't know". Thank you.

Selection/screening

1. You have just created a population of bacterial cells that each carry a mutation in a different location on the chromosome. You are trying to find the few cells in that population in which the mutation disrupts the ability to produce a bright yellow pigment. How would you find these particular mutants, using a selection, or a screen? Describe how you would find these mutants in the population.

Determining the function/identification of a gene

Proline is an amino acid that all organisms require. Some bacteria, such as *E. coli*, possess the genes required to synthesize proline such that in the absence of proline in their growth media, they can synthesize their own, and still be able to grow. Bacteria that do not possess these genes must be provided proline in the media for growth.

2. Describe how you would find the genes for proline biosynthesis in a bacterium using a chromosomal library.

3. Describe how you would identify genes for proline biosynthesis using transposon mutagenesis.

4. You have cloned a gene X into a plasmid and suspect that gene X encodes the ability of the bacterium to use valine as a carbon source for growth. How could you determine if this is the real function of gene X using a heterologous expression experiment?

5. You have constructed a chromosomal library of *E. coli* with plasmids containing 10 kilobase inserts. The chromosome of *E. coli* is 4.6×10^6 base pairs. Using this library, you are looking for genes for purine biosynthesis. How many library clones will you have to examine to be 99% certain that every gene on the chromosome has been examined? Describe the calculation that you would use to determine this and carry out the calculation if you can.

6. If you have a conjugative plasmid that you want to introduce into a population of bacteria, what would be the best method to introduce it, mating (conjugation), transformation, or transposition?
7. You have isolated a mutant with an insertion in gene *htxA* of the *htx* operon, which has the following organization: *htxABCDEFG*. This insertion results in the loss of ability to metabolize hypophosphite. Does this mean that gene *htxA* is solely responsible for hypophosphite metabolism? Explain other possible interpretations.

8. Draw the diagram of a bacterial gene on a chromosome and indicate all the signals required for its accurate expression into a protein. Label these signals and indicate where they would generally be found on a gene.

Appendix 3B. Pre/Post Project Assessment Answer Key

The answer key of learning assessment is composed of 2 answers for each question. The first answer is a student example of an incorrect answer written before the project, on the firs day of class. The second answer is a correct answer written by the same student at the end of the semester, after completing the full length laboratory project.

Selection/screening

1. You have just created a population of bacterial cells that each carries a mutation in a different location on the chromosome. You are trying to find the few cells in that population in which the mutation disrupts the ability to produce a bright yellow pigment. How would you find these particular mutants, using a selection, or a screen? Describe how you would find these mutants in the population.

   ***Initial Incorrect Answer*: Use gene knockout to knock the gene out that codes for the pigment. Once the set of genes are located, then test the genes using insertion into different DNA. If the pigment is produced, then you know where the genes are located. Use the isolation streaking technique to find mutated organisms in the population.**

   ***Final Correct Answer:* If a transposon containing antibiotic resistance was used to create this new population of bacterial cells, the pigment mutants must have the transposon and the antibiotic resistance gene to that antibiotic in their DNA. To select for the pigment mutants only, grow the culture on a media that contains the antibiotic that the resistance for is present in the transposon. This would be selection. To screen for the mutants you could look over the original plate if mutants to find any colonies with mutations in pigment compared to the wild type** bacteria.

Determining the function/identification of a gene

Proline is an amino acid that all organisms require. Some bacteria, such as *E. coli*, possess the genes required to synthesize proline such that in the absence of proline in their growth media, they can synthesize their own, and still be able to grow. Bacteria that do not possess these genes must be provided proline in the media for growth.

2. Describe how you would find the genes for proline biosynthesis in a bacterium using a chromosomal library.

   ***Initial Incorrect Answer*: If there are two bacterial strains present and they are not provided with proline in their media and some survive yet other die, the ones that survived show that they have the gene to synthesize proline, but if they die it means they don't. Those that don't cant be screened to see where the mutation have occurred which will provide us with gene that is responsible for the production of proline.**

   ***Final Correct Answer:* Digest and ligate the bacteria at multiple sites in your bacteria and make your chromosomal library. Some of the genes in the chromosomal library will be the ones to synthesize proline. Take the gene fragments and put them in an organism that doesn't synthesize proline but requires it to live, then allow the organism to grow on a media that doesn't have proline and see if it grows or not. If the organism grows then the gene fragment it got was the one that is responsible for proline synthesis. Sequence the DNA of the organism and find the insertion site.**

3. Describe how you would identify genes for proline biosynthesis using transposon mutagenesis.

   ***Initial Incorrect Answer*: I'm not sure.**

Final Correct Answer: **Use a transposon with an antibiotic resistance to randomly delete genes in the genome. Then select for mutants by plating on a plate with the antibiotic to make sure the organism you select actually is a mutant. Then screen for mutants that could not synthesize proline. Isolate the mutants, isolate their chromosomal DNA, digest chromosomal DNA and create plasmids you could insert into *E. coli* to multiply the plasmids. Isolate the plasmids and sequence them, looking for the insertion sequence. this will show you where the plasmid was inserted.**

4. You have cloned a gene X into a plasmid and suspect that gene X encodes the ability of the bacterium to use valine as a carbon source for growth. How could you determine if this is the real function of gene X using a heterologous expression experiment?

Initial Incorrect Answer**: Don't know.**

Final Correct Answer: **Find a bacterial species that can't use valine as a carbon source. Use transposon mutagenesis to insert gene X into the bacterial chromosomal DNA. Grow bacteria on a media with valine as its only carbon source and see if it grows. To make sure the transposon is having the effect, the inserted plasmid could also have an antibiotic resistance in itand the bacteria could be plated on a plate with valine as the only carbon source and have an antibiotic on it.**

5. You have constructed a chromosomal library of *E. coli* with plasmids containing 10 kilobase inserts. The chromosome of *E. coli* is 4.6 x 10^6 base pairs. Using this library, you are looking for genes for purine biosynthesis. How many library clones will you have to examine to be 99% certain that every gene on the chromosome has been examined? Describe the calculation that you would use to determine this and carry out the calculation if you can.

Initial Incorrect Answer**: 4.6 X $10^6/10^4$**

Final Correct Answer: Use the Poisson distribution **N=ln(1-0.99)/ln((1-1KB)/4.6X10^7) = 2.12 X 10^4 clones**

6. If you have a conjugative plasmid that you want to introduce into a population of bacteria, what would be the best method to introduce it, mating (conjugation), transformation, or transposition?

Initial Incorrect Answer **: Transformation**

Final Correct Answer: **Conjugation.**

7. You have isolated a mutant with an insertion in gene *htxA* of the *htx* operon, which has the following organization: *htxABCDEFG*. This insertion results in the loss of ability to metabolize hypophosphite. Does this mean that gene *htxA* is solely responsible for hypophosphite metabolism? Explain other possible interpretations.

Initial Incorrect Answer**: No because there are other factors such as the growth factors, environment, media it was grown in or other mutations that might have occurred which caused the ability of metabolism to be lost.**

Final Correct Answer: **No. Not necessarily. *htxA* could affect the transcription of the rest of the genes in the operon. This is called polarity.**

8. Draw the diagram of a bacterial gene on a chromosome and indicate all the signals required for its accurate expression into a protein. Label these signals and indicate where they would generally be found on a gene.

***Initial Incorrect Answer*:: ??**

Final Correct Answer: **A correct answer shows a stretch of DNA with the -10 and -35 regions of the promoter labeled. The student will have indicated the ribosomal binding site, the +1 transcriptional start site and the translational cues (start and stop codons) for each individual gene within the operon.**

Appendix 4: Journal Article Assignments

Appendix 4A. Journal Article Assignment 1

Read the following articles that are posted on the course website then answer the following questions. Be prepared to discuss these questions in class.

Article 1: Biosynthesis of carbapenem antibiotic and prodigiosin pigment in Serratia is under quorum sensing control.

N. R. Thomson, M. A. Crow, S. J. McGowan, A. Cox and G. P. C. Salmond.
Molecular Microbiology (2000) 36(3), 539±556
* For this article, focus on how the genes for prodigiosin biosynthesis were identified

Article 2: The Serratia gene cluster encoding biosynthesis of the red antibiotic, prodigiosin, shows speciesand strain-dependent genome context variation. Abigail K. P. Harris,1 Neil R. Williamson,1 Holly Slater,1 Anthony Cox,1 Sophia Abbasi,1 Ian Foulds,1 Henrik T. Simonsen,2 Finian J. Leeper2 and George P. C. Salmond1
Microbiology (2004), 150, 3547–3560

*For this article, focus on the organization and identity of the genes that encode prodigiosin production.
Answer these questions using the above 2 articles.

1. What is prodigiosin?

2. What genes are known to be involved in prodigiosin biosynthesis based on these articles? How are the genes organized? What functions do they encode?

3. How were these genes identified? Try to summarize the general experimental approach and indicate any terms or concepts you did not understand.

Article 3: Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria.

Rachel A. Larsen, Marlena M. Wilson, Adam M. Guss and William W. Metcalf. Arch Microbiology (2002) 178 :193–201
Answer these questions using Article 3.

4. Describe the important features of the transposon delivery vector described in this paper. What does it do and how does it work to generate mutations?

5. How were transposon induced pigment mutants acquired in the work described in this paper? Describe the actual experimental approach by outlining the steps involved (look at how the authors put the transposon into Xanthobacter and then how they found the cells deficient in pigment production. Try to reconstruct a general protocol for this mutagenesis experiment.

6. Indicate all the terms and methodology that you did not understand.

Appendix 4B. Journal Article Assignment 1 Answer Key

Student sample provided as an answer key.

1. What is prodigiosin?

Prodigiosin is a red pigment produced by *Serratia marescens* and other bacteria. Prodigiosin is a linear tripyrrole secondary metabolite of *Serratia*. The physiological role of prodigiosin is unknown, but the pigment has been shown to have antibacterial, antifungal and immunosuppressive activity, making it interesting clinically.

2. What genes are known to be involved in prodigiosin biosynthesis based on these articles? How are the genes organized? What functions do they encode?

The prodigiosin biosynthetic pathway is organized as a fourteen gene operon ordered *pigA* through *pigN*. The genes encode the proteins that catalyze prodigiosin synthesis. Prodigiosin is formed by combining the bipyrrole unit, MBC, with a different monopyrole (2-undecylpyrrole) called MAP. PigI, PigG, PigA, PigE and PigF are involved in MBC synthesis. Pig J, Pig H, PigC and PigN are involved in MAP synthesis. The role of PigL, PigB, PigD, PigK and PigM are unknown. It is thought that one or more may have a role in the condensation of Map and MBC, to produce prodigiosin.

3. How were these genes identified? Try to summarize the general experimental approach and indicate any terms or concepts you did not understand.

A chromosomal library representing all *Serratia marcescens* genes was created from *Serratia* chromosomal DNA. A chromosomal library is created by partially digesting chromosomal DNA to create varying sized fragments and then cloning the fragments into a vector, in this case a cosmid. The cosmids were introduced into *Erwinia carotovera* via transduction. *Erwinia* mutants were then screened for heterologous expression of the *S. marcescens* prodigiosin genes. In this case screening was simply looking for red colonies and verifying that the red pigment was prodigiosin.

The cosmid was recovered and sequenced by primer walking, allowing them to identify potential prodigiosin biosynthetic genes. The cosmid was then transformed into *E. coli.* Tranformants that were Amp resistant and produced the red pigment prodigiosin (pPig4 clones) were isolated. Transposon mutagenesis was used to create colorless pPig4 colonies. Colonies became colorless due to transposon insertion in a gene required for prodigiosin synthesis, thus disrupting its function. DNA sequences adjacent to or flanking the transposon insertion sites were analyzed using bioinformatics.

So, first potential genes are identified by artificially expressing a trait in a host strain. Then individual genes required for expression of the trait are identified by introducing mutations that disrupt gene function.

4. Describe the important features of the transposon delivery vector described in this paper. What does it do and how does it work to generate mutations?

There are several important characteristics of the pRL27 transposon delivery system. First it contains a hyperactive transposase under control of the tetA promoter that is 1,000X more active than wild type. The *tet:tnp* gene fusion is located outside of the transposon so is lost after transposition, resulting in stable transposon insertions. It encodes an origin of transfer gene that allows the plasmid to be transferred into recipient strains by conjugation with *E. coli* donor strains. It encodes kanamycin resistance as a selectable marker and an origin of replication (*ori*R6K) that allows cloning of the transposon insertion site. Finally, *ori*R6K requires the π protein encoded by the *pir* gene to replicate. In non-*pir* strains, drug resistance will only be conferred if the transposon inserts into the recipient genome.

The transposon is introduced into the recipient strain by mating with an *E. coli* donor strain. The transposase inserts the transposon into recipient chromosome, causing a single mutation.

5. How were transposon induced pigment mutants acquired in the work described in this paper? Describe the actual experimental approach by outlining the steps involved (look at how the authors put the transposon into Xanthobacter and then how they found the cells deficient in pigment production. Try to reconstruct a general protocol for this mutagenesis experiment.

Donor and recipient cells were grown to mid-exponential phase, were mixed and collected on a 0.45µm filter. The filter containing the cells was incubated overnight at 37C on a rich media. After incubation, cells were re-suspended and dilutions were plated on selection media containing an antibiotic. Mutants were screened for pigment production that varied from wild type.

Protocol:

Mix *E. coli* donor strain and *S. marcesces* grown to OD600~0.8 1:1 in a 2 ml final volume and filter mix onto a 0.45 µm nalgene filter. Place filter on LB plate and incubate overnight at 37C.

The next day, collect filters from LB plates. Re-susped cells off of filter into sterile media. Plate 10 fold dilutions of cells onto LB and LB Kan50 plates. Incubate overnight at 37C. Look for non-pigmented or hyperpigmented mutants and determine mutation frequency.

6. Indicate all the terms and methodology that you did not understand.

I think I understood the articles.

Appendix 4C. Journal Article Assignment 2

Conduct a literature search for a journal article more recent than 2005 that describes the identification of genes involved in prodigiosin biosynthesis. Look up the article and then write a summary that includes:

1) The question being explored/hypothesis

2) The experimental methods used to answer these questions

3) The results of the experiments

4) Final conclusions of what was learned

5) How this result might relate to the lab project in our class.

Appendix 5: Protocol Development Assignments

Appendix 5A. Chromosomal DNA Prep Protocol Development Assignment

We will be purifying Chromosomal DNA from *Serratia marcescens* mutants during this lab project. We will discuss the methods, theory and protocol for the DNA isolation procedures you found in class. To prepare for this, complete the protocol development assignment, which should include the following:

1. A detailed protocol written out in either step-wise fashion or in a flow chart. This should include required reagents and their properties.

2. A brief but detailed statement as to what each step achieves in the process of separating plasmid DNA from the cell. You should be able to describe what is happening at each step and how each particular treatment works.

3. At least 2 references for the indicated protocol. The references should be stated and can come from journal articles, online resources or the "Current protocols in Molecular Biology". The protocol you write down can be a synthesis of several or can be from just one of these sources.

* Note- a "kit based" protocol is provided to you in the lab manual. We will be using this protocol in class. I want you to find additional protocols that are not kit based and determine what each of the steps is achieving in the kit and how alternative methods achieve the same results without the kit components. This is so you will understand what is happening during the isolation procedure and so that you will not be simply following directions.

I advise making 2 copies, one to turn in and one to keep during the discussion in class.

Appendix 5B. Chromosomal Prep Protocol Development Assignment Answer Key
Student sample provided as answer key.

Procedure:

1) **Centrifuge at ~3600 rpm bacteria broth culture to form a pellet of cells for harvest and discard supernatant.**
   This allows you to concentrate the bacteria into a pellet in order to separate the cells from the rest of the broth. Reducing the volume you are working with is easier to do and requires fewer reagents.
2) **Resuspend pellet in Tris buffer with lysozyme and EDTA**
   The Tris is a buffering agent that will maintain the pH at a range of 7-9. The buffer is used to keep the solution in this range to prevent DNA degradation due to unfavorable pH's as well as to keep added enzymes in a range that makes the enzymes the most effective.
   Lysozyme lyses the cell by breaking the NAG-NAM bonds in the peptidoglycan layer.
   EDTA protects the DNA from degradation by nucleases by depleting local metal ions in solution need by nucleases to degrade DNA.
3) **Add SDS and mix by gently inverting tube.**
   SDS solublizes the membrane and denatures proteins. SDS has a polar sulfate head and a non polar carbon chain, giving it a similar structure to lipids in membranes. Its similarity to lipids allows it to enter the membrane and disrupt non-covalent bonds.

Anions of SDS also bind to amino acids of proteins. This imparts a greater negative charge and causes proteins to unfold do to the electrostatic repulsion.

4) **Incubate at 65C for an hour, inverting every 15 min.**
   This allows for an increase in the amount of membrane disruption and protein denaturing, by giving it plenty of time to do so, and repeatedly mixing the solution so that all SDS, and lysozyme diffuse throughout the cells. Shaking to harshly could break DNA.
5) **Add equal volumes of phenol and chloroform and centrifuge**
   This seperates the DNA from the rest of the solution by solublizing the DNA in the top layer (aqueous phase) while the rest of the solution of lysed cells remain in the lower layer. Pipetting this top layer off isolates the DNA. Multiple washes with phenol/chloroform advised.
6) **Add aliquots of chloroform and isoamyl alcohol, centrifuge, and extract top layer.**
   This removes excess phenol from the solution by simple diluting phenol concentrations and washing. Recommended to be done twice.
7) **Layer with volume of ethanol twice the amount of solution. Swirl pipet tip in solution.**
8) This will isolate the strands of DNA from the solution by precipitating the DNA out of the solution. Swirling the pipet tips results in the adhering of the DNA to the pipet tip. Giving a prepped and isolated DNA sample.
9) **Dry Sample**
   Ethanol will evaporate, so if the DNA sample is allowed to dry out you will have a dry DNA sample. Although for this procedure, it might be ideal to keep it in a small volume of solution.

References were turned in with assignment, but not provided in answer key.

Appendix 5C. Plasmid Prep Protocol Development Assignment

We will be purifying Plasmid DNA from *E. coli* transformants during this lab project. We will discuss the methods, theory and protocol for the DNA isolation procedures you found in class. To prepare for this, complete the protocol development assignment, which should include the following:

1. A detailed protocol written out in either step-wise fashion or in a flow chart. This should include required reagents and their properties.

2. A brief but detailed statement as to what each step achieves in the process of separating plasmid DNA from the cell. You should be able to describe what is happening at each step and how each particular treatment works.

3. At least 2 references for the indicated protocol. The references should be stated and can come from journal articles, online resources or the "Current protocols in Molecular Biology". The protocol you write down can be a synthesis of several or can be from just one of these sources.

* Note- a "kit based" protocol is provided to you in the lab manual. We will be using this protocol in class. I want you to find additional protocols that are not kit based and determine what each of the steps is achieving in the kit and how alternative methods achieve the same results without the kit components. This is so you will understand what is happening during the isolation procedure and so that you will not be simply following directions. With this plasmid prep protocol, pay close attention to the differences and similarities between the steps for isolating chromosomal DNA compared to isolating plasmid DNA.

I advise making 2 copies, one to turn in and one to keep during the discussion in class.

Appendix 5D. Plasmid Prep Protocol Development Assignment Answer Key

Student sample provided as answer key.

1) Culture specific bacteria in LB broth with a type of antibiotic that the bacterial mutants are resistant to because of the plasmid.
    a. This allows you to select for the specific type of mutant that has been altered with your plasmid. In our case it would be kanamyecin.
2) Fill microcentrifuge tube with bacterial culture from broth and centrifuge for 1 minute, dumping the supernatant and drain. Refill tube with bacterial broth and repeat process.
    a. This increases the total number of concentrated cells to maximize the amount of plasmid DNA to be isolated.
3) Add cold solution containing gluscose, EDTA and Tris to resuspend cell pellet by repipetting.
    a. Glucose: Increases the osmotic pressure outside of the cell. This prevents the cell from absorbing to much water because of low osmotic pressure and bursting before we want it to.
    b. Tris: A buffering agent that maintains the pH of the solution to about 8. To prevent proteins that are pH sensitive from denaturing.
    c. EDTA: Protects DNA from degradation by DNases by binding, and depleting environment of divalent cations that are needed for degradative enzymes to break apart DNA.
4) Add solution containing NaOH and the detergent SDS. Cap and invert the microcentrifuge tubes gently about 5 times. Then let them sit for about 5 minutes.
    a. NaOH: The alkaline molecule ruptures the cell. It weakens the H-Bonds involved the in cell membrane to increase the fragility of the cell. This weakening of H-bonds also helps in breaking DNA into single strands.
    b. SDS: Has a very similar structure to lipids in the membrane. It is capable of inserting itself in the membrane of a cell because of its polar head and non-polar tale regions, which further weakens the cell membrane and helps break apart the lipid bilayer. It also is capable of solublizing proteins by inserting itself into the proteins as well. This disrupts the proteins and denatures their structurally specific enzymatic functions.
    c. Inverting gently allows the solution to be spread throughout the suspension without sheering the DNA and destroying the plasmids.
5) Add cold solution of acetic acid and potassium acetate. Invert 5 times, gently. Let incubate 10 min. on ice.
    a. Acetic acid: Allows the DNA strands to renature by neutralizes the pH of the solution, therefore undo the weakening of the H-bonds done by the NaOH.
    b. Potassium acetate removes the SDS from solution by precipitating it out along with the cellular debris. The partially renatured chromosomal DNA is also removed by precipitation. Therefore leaving the plasmid DNA in solution.
    c. Inverting gently allows the solution to be spread throughout the suspension without sheering the DNA and destroying the plasmids.
6) Centrifuge for 5 minutes. Transfer supernatant to fresh microcentrifuge tube.
    a. This forms a pellet of the precipitant. Therefore easily allowing the supernatant containing the plasmid DNA to be isolated and removed from the cellular debris and chromosomal DNA.
7) Add isopropanol. Let sit for 2 minutes.
    a. This separates the remaining plasmid DNA from any soluble proteins by precipitating the nucleic acids and not the proteins in solution. Therefore purifying the DNA.
8) Centrifuge for 5 minutes. Pour of supernatant.
    a. This further isolates plasmid DNA from anything else, further purifying the DNA.
9) Add cold 70% ethanol. Cap and invert several times. Centrifuge for 1 minute. Pour out supernatant.
    a. Ethanol helps further purify the sample by solublizing and removing any remaining salts and SDS.
10) Allow tube to dry for 5 minutes. Add TE (tris+EDTA) to the tube.
    a. Tris: This stabilizes the pH of the plasmid DNA, by acting as a buffer.
    b. EDTa: Further helps protect the DNA from degradation by DNases by binding, and depleting environment of divalent cations that are needed for degradative enzymes to break apart DNA.
11) **Plasmid DNA is purified and ready for use.**

Appendix 6: Laboratory Report Assignments

Appendix 6A. Laboratory Report Assignments 1 & 2

Laboratory Reports should be written in the format of a peer reviewed scientific journal article with the following sections:

1.Title-a detailed descriptive title

2. Abstract- a concise description of the questions addressed, the methodology used and the results observed.

3.Introduction- a concise review of the pertinent literature summarized to lead the reader to the questions that remain on the particular research topic. For example, student's introductions should discuss prodigiosin, what it is, why it is important, what is known about its synthesis and *S. marcescens*. The last paragraph should describe the objectives of this study and should establish why they are a worthwhile endeavor. This section should contain in text citations of the pertinent journal articles.

4. Materials and Methods- This should be a detailed description of the methods used. See journal articles for examples on how to write this section.

5. Results- This should be comprised of a brief summary of the questions addressed, how they were investigated and the results observed. All data and results should be formatted in a table or figure as appropriate with appropriately formatted figure legends and titles. See journal articles for examples of these. The data should also be accompanied by text describing the results.

6. Discusion-This section is where you EXPLAIN ad interpret your results. What do they mean, and how do they address your initial objectives? What broader conclusions can be drawn and what questions remain?

7. References- see ASM journal articles for appropriate formatting.

These should be well done pieces of work that have been carefully proofread and edited. The objective is for someone of similar educational background but not necessarily familiar with this project to be able to read your report and understand what you did, why you did it, what you found and what it means.

Appendix 6B. Laboratory Report 1 Answer Key
Student sample provided as answer key.

Mutagenesis of *Serratia marcescens* by conjugation to knock-out gene expression for production of prodigiosin

With a calculated total number of 3.04E+09 colonies of *Serratia marcescens* plated from the mating mix, 1.35E+05 colonies of *Serratia marcescens* were mutated and transposed with the Kanamycin resistance gene, resulting in a transposition frequency of 4.44E-05 by the species E. coli/pRL27. A variety of different phenotypes were characterized in the mutants ranging from dark red to pink to clear. Some consistently lacked pigment and some had a variety of pigment reduction depending on plate media. Some mutants had a hyper pigmentation; those hyper pigmented also developed a gold sheen on some media, but not on other media. No hyper pigmentation was observed on hyper pigmented mutants on LB agar media. A total of six pigment mutants were isolated and characterized on different media and two were selected for future genetic analysis, one hyper pigmented mutant with gold sheen and one mutant lacking pigment.

INTRODUCTION

Serratia marcescens is a gram-negative bacterium that has been isolated from soil, water, plants and air. It is pathogenetic to humans, plants and insects. It is resistant to several drug treatments, making it particularly concerning from a clinical perspective (1). *Serratia marcescens* is the major producer of prodigiosin (Pig) a chemical which gives it a noticeable red pigment. This red pigment is present in *S. marcescens* that are isolated from the environment, but not usually in clinical isolates (2). Hence it is believed that prodigiosin has no essential role in the survival of the cell, since cells can live without it, but it may rather work as a overflow for waste product from primary metabolism, therefore is a secondary metabolite (2). Pig has shown to be produced between trophophase and idiophase of the cell cycle (2) and is commonly located on the cell envelope (3). It is made up of the precursors MAP and MBC (1). Prodigiosin has a wide clinical application with biological properties such as antibacterial, antimalarial, antifungal, immunosupressive, and antiprotozoal properties. It also induces apoptosis in cancer cells (3). Production has been found to be affected by media composition, pH and temperature. The Pig operon has been fully mapped, but the genes that affect the production of prodigiosin outside of the pig operon have not yet been identified (3).

In this study, we randomly, mutated *Serratia marcescens* chromosomal DNA via conjugation to affect the production of the red pigment prodigiosin. Two of these mutants DNA will later be analyzed to determine which regions of their DNA have been changed to induce or promote the production of prodigiosin in order to identify more genes responsible for the regulation of prodigiosin in 6 mutants that were isolated.

METHODS AND MATERIALS

To identify genes that have a role in the expression of prodigiosin in *Serratia marcescens*, we randomly mutated its chromosomal DNA via conjugation to knock out genes that have some function in prodigiosin expression to be later identified in chromosomal analysis. Using an *E. coli* culture containing the plasmid pRL27 *(E.coli*/pRL27), in mid log phase, we transferred pRL27 into the WT *Serratia marcescens*, also in mid log phase, in a 1x"M" solution.

The *E. coli* used is a particular species that makes a pili that can transfer its own plasmid DNA into other cells, known as formation or conjugation. The pRL27 plasmid contains the gene for resistance to the antibiotic Kanamycin (Kan). pRL27 randomly inserts the kanamycin resistance gene into recipient cell's DNA, thereby disrupting a random gene and giving the recipient cell Kan resistance. This plasmid is a suicide vector because it cannot replicate outside of the *E.coli*/pRL27 cell since *S. marcescens* doesn't have the π proteins required for

plasmid replication in the recipient cells. These cells were filtered onto 0.45 µm filter paper and incubated on a LB agar plate at 35˚C for 1 day.

The filter was transferred filter to 50mL falcon tube with 1mL 1x "M" to make a "Mating Mix" that was used to make serial dilutions to a factor of $1x10^{-7}$. Dilutions 10^{-7} to 10^{-4} were plated on MOPS agar and dilutions 10^{-3} to 10^{0} were plated on MOPS+Kan agar as well as WT *S. marcescens* and *E. coli* controls. All were incubated at 27˚C. We conducted plate counts to determine the transposition frequency. Mutants were then selected based on phenotypic characterization and restreaked onto MOPS+Kan agar with controls and incubated at 27˚C.

Unfortunately, our MOPS+Kan that we restreaked on didn't contain phosphate, therefore there was no growth of our mutants and the previous series of plates were questionable as to whether they were still alive. So we went back to our mating mix and made a dilution of 10^{-1}, that was plated onto fresh MOPS+Kan again. 6 mutants were selected from the plates based on reduced or increased pigmentation and restreaked to form isolated colonies. Isolated colonies of these mutants were streaked onto LB, MOPS +succinate and MOPS +glucose agar plates. From these, two final mutants (9b and 8A) were chosen for genetic analysis at a later time.

RESULTS

Table 1: Transposition frequency of kanamycin resistant into wild-type *Serratia marcescens* by *E. coli*/pRL27

Total Number colonies	Number Kan Resistant Colonies	Tn Frequency	Published Frequency
3.04E+09	1.35E+05	4.44E-05	1.00E-03

Calculation: 1.35E+05/3.04E+09=4.44E-05

With a calculated total number of 3.04E+09 colonies of *Serratia marcescens* plated from the mating mix, 1.35E+05 colonies of *Serratia marcescens* were mutated and transposed with the Kanamycin resistance gene. Resulting in a transposition frequency of 4.44E-05, which is significantly lower than the calculated frequency of 1.00E-03. Meaning that either our calculations of the Tn frequency has some errors in it, or that our protocol had some errors in it and requires improvement in the future to achieve the proper efficiency.

Table2: Phenotypes of *S. marcescens* conjugated mutants on LB , 0.2% glucose MOPS and 1% succinate MOPS. (*) indicates mutants that were selected for genetic analysis, yet to be conducted.

Mutant	Morphology		
	LB Agar	MOPS + Succinate Agar	MOPS + Glucose Agar
7A	Thick, light pink, large, semi translucent colonies	Peach, small, not translucent colonies	Salmon colored small/medium not translucent colonies
8A*	Semi translucent white, slight yellow, large and thick colonies	Translucent, white, yellow Medium, thin colonies	Translucent white, slight yellow medium/small colonies
8B	Thick, very dark red, large, not translucent colonies	Faint pink, flat and thin Translucent colonies	Translucent, pink pinpoint colonies
9A	Dark orange with dark red large not translucent colonies	Faint pink, flat and thin translucent colonies	Dark red, w/gold sheen, not translucent, medium colonies
9B*	Dark red, large and thick colonies not translucent colonies	Orange with gold sheen, thick , not translucent	Dark red with gold sheen, small not translucent colonies
9C	Thick red/ orange, not translucent	Faint light pink, mainly white, thin, translucent colonies	Clear, translucent, pinpoint colonies

From our second series of plating for mutants from our mating mix, we discovered 6 different phenotypic distinct mutants, as shown in Figure 1 and described in Table 2. Some lost all pigmentation and were either translucent and either clear or white. Mutants 7A and 8A lacked pigment in all 3 media LB agar, MOPS+succinate, and MOPS glucose (LB/succ/glu). While one mutant (8B) lacked pigment in the media, except in the presence of glucose where it had a light pink pigmentation. Some mutants (9A and 9B) developed a gold sheen on Glucose media, along with the control bacteria. This could be a natural affect of glucose on the bacteria, or more likely that we plated a colony which appeared to have a normal pigment on MOPS media, but had a noticeably different effect on glucose media. Only one of these mutants (9B) maintained its gold sheen on succinate media, while one (9A) turned a faint pink color and the control turned the wild type phenotype dark orange. Mutant (9C) appeared an orange/red color on LB only, while on succ/glu it lacked pigment.

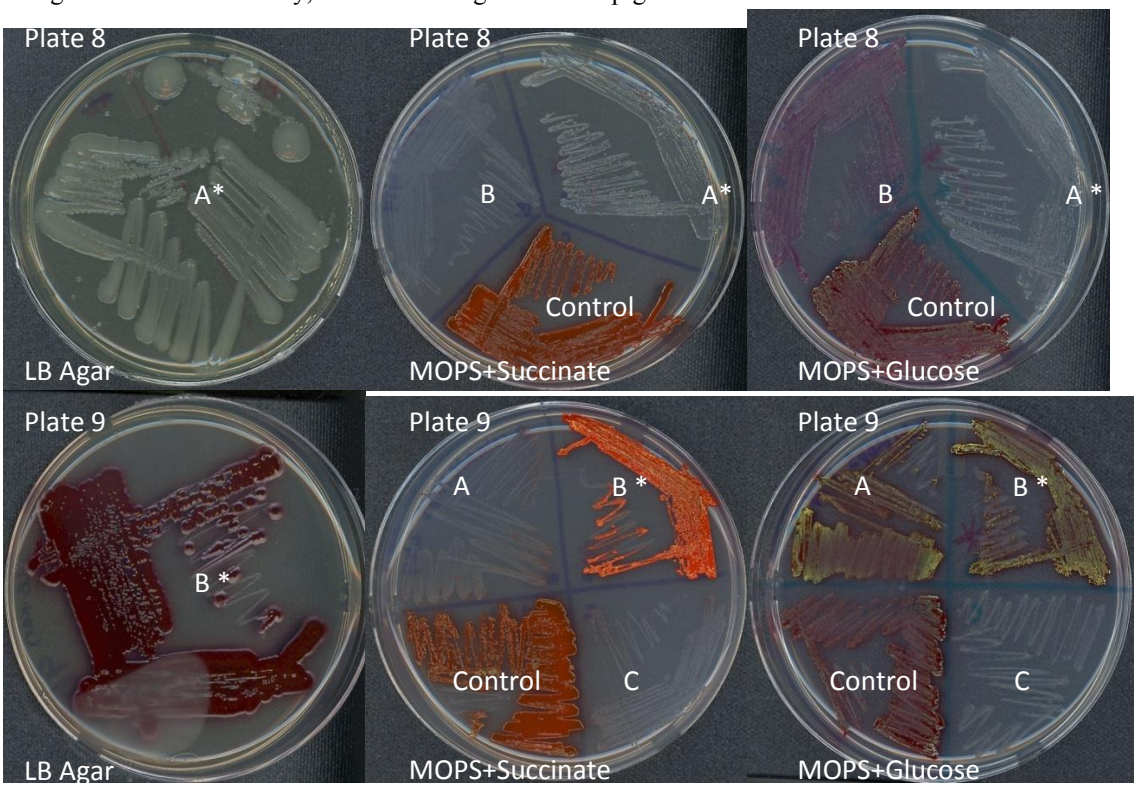

Figure 1: Phenotypes of *Serratia marcescens* conjugated mutant by *E. coli*/pRL27 on LB , 0.2% glucose MOPS and 1% succinate MOPS agar media. (*) indicates mutants that were selected for genetic analysis, yet to be conducted.

DISCUSSION

In our mutation of *Serratia marcescens* with *E. coli*/pRL27 we effectively changed the pigmentation from wild-type species of *Serratia marcescens.* Some mutants pigment was removed while some mutants showed hyper pigmentation. Since in every media it was exposed to, mutant 8A always showed no pigment, therefore there is no indication of prodigiosin production. The loss of pigment in mutant 8A could indicate that a gene vital in the production of prodigiosin had been altered and no longer functional.

The loss of pigment in mutants 8A, 8B and 9C may be result of a gene knock-out of prodigiosin promoter/activator region activated by the presence of absence of certain nutrients in the *S. marcescens* mutant. Considering that prodigiosin production is a secondary metabolite and a byproduct of primary metabolism waste (2), it is possible that this promoter/activator is responsible for pigment production when exposed to or deficient in certain different nutrients. For example, mutant 9C had a faint pigmentation or no pigmentation in minimal media +

succinate or glucose. But in the LB media, it had a thick red/orange pigment. This could mean that in the gene for pigment production in the presence of a nutrient that isn't glucose or succinate, is still active, but some gene for the mechanism of pigment production in the absence of this nutrient has been altered and no longer functional, and therefore preventing production of prodigiosin.

Mutant 8B retained its dark pigment in rich media, but was reduced in glucose media and absent in succinate. It's possible that the gene for the activator for pig production under nutrient stress had been knocked, but overall pigment production was still functional under ideal conditions, similar to 8A, 8B, and 9C. The difference in this mutant is that pigment production wasn't entirely knocked out in glucose. So the gene for activation in the presence of glucose isn't knocked out entirely, but the activation of the gene has been reduced, resulting in partial prodigiosin production.

Mutants 9A and 9B displayed hyper pigmentation. Mutant 9B displayed an increase of pigmentation in LB and glucose media, and a bright orange pigment in succinate media, which suggests that a gene encoding a repressor binding site region, or maybe the gene for production of the repressor molecule for prodigiosin production with ideal nutrients resources, was knocked out. An elimination of a repressor molecule or its binding site would result in an overproduction of prodigiosin and give the colonies darker pigmentations as long as it had a supply of the required nutrients. The fact that it was hyper pigmented in rich media as well as in glucose may suggest that glucose is required for at least one production pathway of prodigiosin. The orange pigment in succinate is not the result of any mutation considering that our control streaking is also an orange color. This means that S. marcescens could naturally turn orange in the presence of succinate minimal media. Yet mutant 9B has a stronger orange pigment than the control, therefore could still producing more prodigiosin that the control.

Mutant 9A has the same orange pigment, but it is much weaker and mostly lacks pigment. This could be a similar knock-out as 9B, except that this mutant probably knocked out a gene that still overproduced prodigiosin, but can't synthesize as many molecules itself and requires them to be provided in its nutrient source.

A strong gold sheen was observed on mutants 9A and 9B on glucose minimal media. This gold sheen is suspected to be a byproduct of prodigiosin production of glucose media because it was observed on both control streaks on plate 9 and plate 8. If this is true, since the production of prodigiosin was de-repressed, the appearance of the gold sheen increased along with the pigment. The fact that no gold sheen was found on hyper pigmented colonies in rich LB media indicates that the gold sheen production may be a byproduct nutrient stress on the bacteria. Further testing will be required, along with genetic analysis to determine the actual genes modified and their molecular consequences.

Mutants 8A and 9B were selected from the six total mutants isolated for genetic analysis later.

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Appendix 6B. Laboratory Report 2 Answer Key

Student sample provided as answer key.

Genetic sequencing and identification of genes mutated by conjugation resulting in the change of pigment expression by the altered production of prodigiosin in *Serratia marcescens*

Serratia marcescens bacteria were mutated via conjugation by *E. coli*/pRL27 to randomly knock out genes to effect the expression of prodigiosin red pigment. In this study 2 pigment mutant strains were selected to identify the genes deleted which resulted in this change in prodigiosin expression. Mutants were selected based on their loss of pigmentation (GG8a) and development of hyper-pigmentation (GG9b). Chromosomal DNA of mutants were broken down into fragments of DNA by a BAM HI digest and converted into plasmids using a DNA ligase. The plasmids were transformed into *E. coli* DH5 α λ pir via electroporation and the plasmids containing the genetic sequence mutanted that resulted in the change in pigment expression were selected for by plating onto agar containing kanamycin, an antibiotic in which resistance was encoded for in the plasmids pGG8a and pGG9b that contain the pigment mutation. Plasmid DNA was isolated and sequenced to identify what genes were mutated which resulted in the change of prodigiosin production. It was determined that *S. marcescens* mutant GG8a's loss of all red pigment was a result of a mutation in the gene HCH 06024, a conserved hypothetical protein contained in an operon with genes encoding for enzymes involved in the synthesis of phosphoenolpyruvate which is a intermediate of glycolysis. It was also determined that *Serratia marcescens* mutant GG9b's hyper-pigmentation was a result in a mutation in gene Spro 4689, which encodes for a phosphate transporter which did not appear contained in an operon.

INTRODUCTION

S. marcescens is a gram-negative bacterium that has been isolated from soil, water, plants and air. It is pathogenic to humans, plants and insects. Its resistant to several drug treatments makes it particularly concerning from a clinical perspective (1). *S. marcescens* is a major producer of prodigiosin (Pig) a chemical which gives it a noticeable red pigment. This red pigment is present in *S. marcescens* that are isolated from the environment but not usually in clinical isolates (2). Hence it is believed that prodigiosin has no essential role in the survival of the cell since cells can live without it, but it may rather work as an overflow for waste product from primary metabolism, therefore is a secondary metabolite (2). Pig has shown to be produced between trophophase and idiophase of the cell cycle (2) and is commonly located on the cell envelope (3). Pig is made up of the precursors MAP and MBC (1). Prodigiosin has a wide clinical application with biological properties such as antibacterial, antimalarial, antifungal, immunosuppressive, antiprotozoal properties and also induces apoptosis in cancer cells (3). Production has been found to be affected by media composition, pH and temperature. Still, little is known about the enzymes or especially the genes involved in the production of this pigment (3).

In a previous study *S. marcescens* bacterium were mutated via conjugation by *E. coli*/pRL27 to randomly delete genes to effect the expression of prodigiosin red pigment. 6 strains of prodigiosin pigment mutants were isolated into purified colonies. The chromosomal DNA of these mutants was altered to knock-out genes that encoded for proteins that had a function in the expression of prodigiosin. In this study, of these 6 purified mutant strains 2 were selected to identify the genes deleted which resulted in this change in pig expression by forming plasmids out of the mutated DNA regions, these plasmids were then cultured in *E. coli* and then purified to be sequenced for genetic analysis. Sequencing of plasmid DNA was compared to known DNA sequences to identify the protein genes that were deleted.

METHODS AND MATERIALS

Pig mutants GG8a and GG9b were selected for DNA analysis to determine the location and identity of the gene mutation responsible for the change in expression. On LB agar media mutant GG8a displayed total loss of red pigmentation and possessed only a white pigment. On LB agar media mutant GG9b displayed an increase of red pigmentation, possessing a deep dark red color and developed a glossy golden sheen on the surface of the colonies.

Cultures of mutants were collected from LB broth and each mutant's chromosomal DNA was isolated using a commercial MoBIO chromosomal DNA prep kit. A sample of the DNA isolation was tested via agarose gel electrophoresis to confirm that chromosomal DNA was indeed isolated, detect any signs of DNA shearing from the

process, as well as determine a rough estimate of the size and mass of the DNA sample. Electrophoresis was conducted at 100V for 15 minutes and then changed to 60V for 45 minutes. Gel electrophoresis indicated failure in isolation of chromosomal DNA of mutants GG8a and GG9b.

Protocol for chromosomal DNA isolation was modified using alternative lysis methods provided by MoBIO Laboratories troubleshooting guide to minimize the amount of DNA shearing during the isolation of DNA. A second agarose gel electrophoresis was conducted using a sample of the modified chromosomal DNA isolation at 100V for 30 minutes. After the gel electrophoresis confirmed the successful DNA isolation, the isolated chromosomal DNA of prodigiosin mutants of *S. marcescens* were digested with the BAM endonuclease restriction enzyme to asymmetrically cleave at the chromosome at specific palandromic 5`-GGATCC-3` sequences called BAM sequences, a process known as a BAMHI digest. The DNA samples were loaded into a solution of sterile nanopure water, BAM buffer and the BAM enzyme. Samples were incubated at 37ºC overnight and then frozen until next step in procedure.

After degradation of mutants chromosomal DNA at BAM sites, the BAM enzyme and buffer was precipitated out of solution. Based on volume of digest, 0.1 volumes of 3M sodium acetate at pH 5.2 mix added to DNA solution along with 2 volumes of 100% ethanol and placed into a -70ºC ice bath for 10 minutes. Solution was then centrifuged for 20 minutes at 14,000 rpm in a cold room then immediately decanted supernatant leaving DNA at bottom of tube as pellet. 70% ice cold ethanol was immediately added to pellet and the centrifuged for 5 minutes at 14,000 rpm in cold room. Supernatant then carefully decanted and inverted over a kimwipe for several minutes then placed in heat block with lid off to dry out the pellet.

Once precipitated the chromosomal DNA of mutants GG8a and GG9b were ligated with T4 DNA ligase in order to bind the BAM ends of each DNA segment covalently to other BAM ends of the same segment in order to form circular segments of DNA. The piece of circular DNA which contained the gene with the mutation also contained an origin of replication site (Ori R6K) that was included in the plasmid which was conjugated into the *S. marcescens* wild type to cause the mutation. This Ori R6K allows for the initiation of replication of the circular DNA segment, therefore making it a plasmid that is capable or replicating itself. To ligate the DNA into plasmids 1x T4 DNA ligase buffer was mixed into each tube of precipitated DNA by continuously pipetting solution. DNA ligase was added and mixed by flicking the tube, pulsed for 5 seconds and placed in a 14ºC bath overnight. 14ºC baths is not ideal temperature for DNA ligase enzymatic activity, but any higher temperature would have risked Hydrogen bonds to break before DNA ligase could anneal BAM regions together by forming covalent bonds.

Ligation mix was gently pipetted onto surface of nitrocellulose membrane floating in a petri dish of nanopure water. This dialysis of ligation mix was allowed to stand for 20 minutes and then carefully transferred into microcentrifuge tubes on ice, around 20 μ L total.

Ligation mix was transformed to *E. coli* DH5 α λ pir electrocompetent cells by electroporation. First *E. coli* DH5 α λ pir cells were required to be prepped. *E. coli* DH5 α λ pir cells were resuspended in ice cold 10% glycerol and kept on ice at all times. The *E. coli* DH5 α λ pir resuspension was centrifuged at 6,000 rpm for 5 minutes and supernatant immediately poured off then placed on ice. This process was done a total of 3 times, keeping on ice at all times. During last decanting of supernatant 120 μ L of supernatant was left in suspension.

Entire 20 μ L of ligation mix was added to prepped electrocompetent *E. coli* DH5 α λ pir cells on ice, pipetted multiple times to mix. Entire volume of *E. coli* DH5 α λ pir cells and ligation mix solution was added to an electroporation cuvette. Any bubbles in electroporation cuvette were removed by tapping vigorously. Cells electroporated at a time constant of 5.38, then added to prewarmed SOC media. Electroporated cells in SOC were then incubated at 35ºC while shaking for 1 hour. 100 μ L of the solution was then platted onto LB-Kan agar plate. Remainder of solution was centrifuged at 6,000rpm and resuspended in residual supernatant and entire volume of resuspension was plated onto LB-Kan agar plates. Both plate were incubates at 35ºC.

2 isolated colonies of transformed cells on LB-Kan agar were inoculated into 5mL of LB broth and incubated at 35ºC in an incubation rocker. Plasmid DNA was isolated from *E. coli* DH5 α λ pir+pGG8a and *E. coli* DH5 α λ pir+GG9b transformed cells using QIAspin Miniprep Kit following protocol provided in QIAprep Miniprep Handbook. To confirm effectiveness of plasmid DNA isolation samples of plasmid DNA were tested by agarose gel electrophoresis.

After confirmation of successful plasmid DNA isolation 86ng of each samples plasmid DNA was loaded into 2 PCR tubes. Volume of plasmid DNA that was transferred to PCR tubes in order to load 86ng of plasmid DNA was determined based on comparison of sample plasmid DNA to DNA molecular weight ladder brightness intensity to determine an approximate concentration of the sample. One PCR tube was loaded with tn 27 forward primer and the other was loaded with tn 27 reverse primer. Tubes were then filled to a final volume of 12 μ L with nanopure

sterile water. PCR tubes containing plasmid DNA pGG8a and pGG9b were sent to SDSU Micromedical Core Facility for genetic sequencing. DNA sequences were then assembled by forming transposon sandwichs to identify chromosomal DNA sequence of *S. marcescens* that was mutated by the insertion of plasmid pRL27 into chromosomal DNA. DNA sequence of S. marcescens that was mutated by the insertion of pRL27 was compared to the JVCI Comprehensive Microbial Resource online database to identify the genes that were knocked out by the insertion of pRL27.

RESULTS

The first gel electrophoresis of chromosomal DNA indicated that DNA was in fact isolated, but smeared bands of DNA down the gel indicated that the isolation procedure resulted in massive shearing that destroyed the chromosomal DNA of mutants therefore not acceptable (Figure 1A).

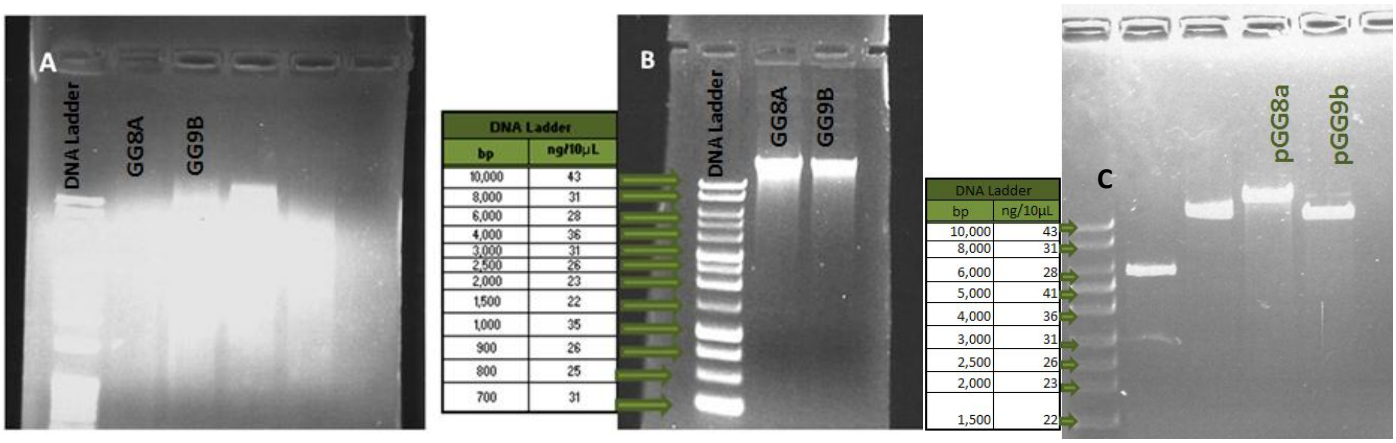

Figure 1: A) Agarose gel electrophoresis of 1st chromosomal DNA isolation resulting in shearing of DNA of mutants GG8a and GG9b using Kodak Gel Logic Camera. **B)** Agarose gel electrophoresis of 2nd chromosomal DNA isolation with modified protocol to minimize shearing of mutant GG8a and GG9b DNA using Kodak Gel Logic Camera. **C)** Agarose gel electrophoresis of plasmid DNA isolation of pGG8a and pGG9b from *E. coli* DH5 α λ pir using Kodak Gel Logic Camera.

Table 1: Summary data of gel electrophoresis of 1st chromosomal DNA isolation resulting in shearing of DNA of mutants GG8a and GG9b, 2nd gel electrophoresis using modified protocol to minimize shearing of mutants GG8a and GG9b and final gel electrophoresis of plasmid DNA isolation of pGG8a and pGG9b from *E. coli* DH5 α λ pir. Size and concentrations of DNA are rough estimations based upon comparison to DNA molecular weight ladder.

DNA Isolation	Pigment Mutant	Quality of DNA	Concentration of DNA (ng/µL)	Size of DNA (kb)
1st Chromosomal DNA Prep	GG8a	sheared	n/a	n/a
	GG9b	sheared	n/a	n/a
2nd Chromosomal DNA Prep	GG8a	Good/minimal shearing	24	14
	GG9b	Good/minimal shearing	19	14
Plasmid DNA Isolation	pGG8a	Good no shearing	86	11
	pGG9b	Good no shearing	68	10

The second electrophoresis resulted in bright bands with some signs of smearing, which suggests that some DNA may have been degraded, but the minimal amount of shearing present was within tolerable limits(Figure 1B) and was therefore excepted for further experimentation for both GG8a and GG9b. The position of the bands for both GG8a and GG9b were above the highest marker on the DNA molecular weight ladder and were estimated to be

around 14,000 base pairs in size. The brightness of GG8a was around 4 times as bright as the 31ng/10µL molecular ladder, it was estimated that GG8a was around 24ng/µL. The brightness of GG9b was around 3 times brighter than the 31ng/10µL molecular ladder, it was estimated that GG9b was around 19ng/µL.

The gel electrophoresis of the isolated plasmid DNA resulted in defined bands with little signs of DNA shearing due to the isolation process in both pGG8a and pGG9b (Figure 1-C). Once again the bands appeared above the highest DNA molecular weight ladder. pGG8a was estimated to be around 11 kilobases in size while pGG9b was estimated to be around 10 kilobases in size. By comparison of the brightness of the samples plasmid DNA bands and the molecular weight DNA ladder it was estimated that pGG8a was around 86ng/µL and pGG9b was around 68ng/µL.

Table 2: Results of transformation of plasmids pGG8a and pGG9b into electrocompetent *E.coli* DH5αλpir cells plated onto LB+kanamycin agar media.

Transformation	Ligation Used (µL)	Expected growth on LB+Kan agar	Growth on LB+Kan agar
E.coli DH5αλpir+ pGG8a	20	2-4	TNTC
E. coli DH5αλpir+ pGG9b	20	2-4	500 colonies

Transformation of plasmids of mutant DNA pGG8a and pGG9b was much more successful than was expected. The entire volume of ligation was transformed into *E. coli* DH5αλpir by electroporation instead of doing 2 separate ligation which increased or risk of losing all of the plasmid samples if transformation failed. It was expected to see only a couple of colonies of transformed *E.coli* DH5αλpir+ pGG8a and *E. coli* DH5αλpir+ pGG9b. The amount of growth observed was much more than initially expected. *E.coli* DH5αλpir+ pGG8a growth on LB+Kanamycin agar was too numerous to count and *E. coli* DH5αλpir+ pGG9b was around 500 colonies.

The sequencing of plasmid DNA of pGG8a and pGG9b resulted in the successful identification of the forward and reverse sequencing of pGG9b, but only the forward sequencing of pGG8a. Therefore the DNA sequence before and after the insertion of pRL27 was identified in mutant GG9b, but only the sequence after the insertion of pRL27 in mutant GG8a was obtained. Both sets of data were able to be blasted to determine which genes were deleted to cause the pigment mutation in both GG8a and GG9b.

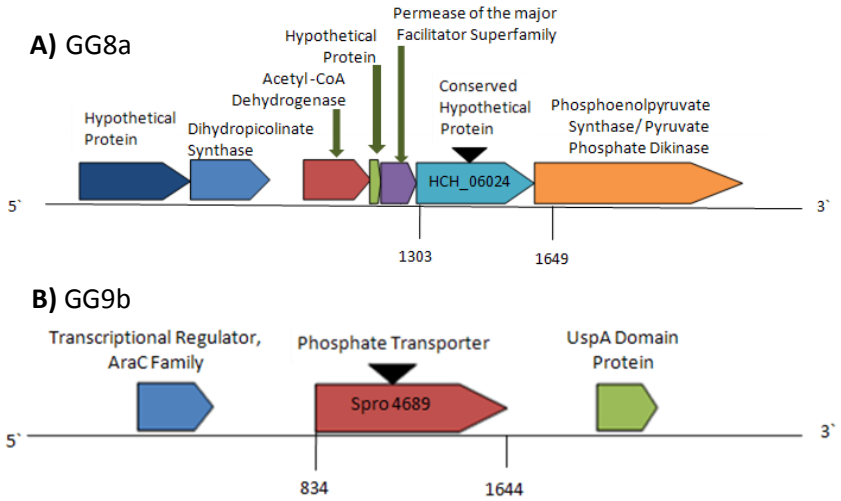

Figure 2: Gene maps for the plasmids obtained from transformation of *Serratia marcescens* into *E.coli* DH5α/λpir. **A)** Depicts the gene map for the mutation site in mutant GG8a. The insertion point of the transposon is indicated by the black arrow and occurs at base 1303. **B)** Depicts the gene map for the mutation site in mutant pGG8a. The insertion point of the transposon is indicated by the black arrow and occurs at base 867 of the gene. Also this gene is in an operon that is related to metabolism.

Table 3: Summary data of Figure 2 for gene maps of mutants GG8a and GG9b of Serratia marcescens pigment mutants. Query length indicates length of sequence searched in JVCI CMR online database to identify genes mutated. Insertion site refers to where plasmids pRL27 was inserted to cause mutation in the gene to alter pigment expression.

Mutant:	Gene mutated:	Query Length (bp)	Insertion Site	Encoded Protein
GG8a	HCH 06024	744	1303	Conserved Hypotheitcal Protein
GG9b	Spro 4689	1646	867	Phosphate transporter

The blast of the transposon sandwiches in JCVI Comprehensive Microbial Resource online database resulted in matches for each pigment mutant. The sequence of DNA that was blasted was 744 nucleotides long for GG8a. The gene knocked out in GG8a was identified in *Hahella chejuensis* KCTC 2396 chromosomal DNA, designated as HCH 06024 and is a conserved hypothetical protein that appears as a component of an operon. The plasmid pRL27 was inserted at nucleotide 1303 to knock out the gene HCH 06024. In GG9b the length of sequence blasted was 1646 bases in length. The gene knocked out in GG9b was identified in *Serratia proteamaculans* chromosomal DNA as the gene for Spro 4689 and is described as a phosphate transporter. Spro 4689 does not appear to be in any operon. Plasmid pRL27 was inserted at nucleotide 876 to knock out the Spro 4689 gene.

DISCUSSION

The reason none of our Serratia marcescens mutants DNA sequence blasts resulted with any matches with *S. marcescens* is because even though the genome for *S. marcescens* has been mapped, it had not yet been published in the JCVI Comprehensive Microbial Resource online database. Since the same sequences in *S. proteamaculans* and *H. chejuensis* were isolated from the *S. marcescens* mutant samples, it can be concluded that these proteins are also present in *S. marcescens.* Although there arrangement in operons may be different, it was presumed that their roles are similar so therefore their organization in operons would be relatively similar.

It can be concluded that mutant GG8a's mutation of a conserved hypothetical protein was the cause of GG8a's total loss of pigment. Since this protein is identified as a hypothetical protein, the function of this protein has not yet been identified. But the fact that it is conserved means that has been found in many different species including species that don't produce prodigiosin suggests that this proteins role isn't directly involved in the production of prodigiosin. Instead it is a protein in which its removal has downstream consequences on the production of prodigiosin. The fact that this HCH 06024 protein is incorporated within an operon that also encodes for phosphoenolpyruvate synthase suggests that it is involved in the glycolysis metabolitic pathway. Since prodigiosin is considered a metabolic waste product, it is probable that the deletion of this HCH gene disrupted some aspect of glycolysis which therefore stopped the production of a substrate which was converted into metabolic waste as prodigiosin. Considering that the mutants were able to continue replication when plated on new media, the knock-out of HCH 06024 did not disrupt all metabolic pathways, so either *S. marcescens* possessed an adaptation to its glycolitic pathway to function without the HCH 06024 protein or *S.marcescens* utilized another metabolic pathway other than glycolysis when it's glycolytic is knocked out.

It can be concluded that mutant GG9b's mutation of a phosphate transport protein was the cause of GG9b's hyper-pigmentation due to an over-expression of prodigiosin. It is possible that the inability to uptake adequate levels of phosphate results in the over expression of prodigiosin. This is a likely explanation since separate experimentation on *Serratia* 39006 has confirmed that phosphate limitation is a cause of prodigiosin operon up regulation. Mutations in the Pst transport system, the major route for inorganic phosphate uptake under phosphate-limiting conditions, physiologically mimics the phenotype of phosphate limitation by an increase in prodigiosin expression (4). So it can be concluded that *Serratia marcescens* hyper-pigment mutant GG9b was a result of lack of inorganic phosphate in the cell due to the deletion of a phosphate transferase channel protein.

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Appendix 7: Example Exam questions and student's answers addressing lab theory

1. After performing a mating experiment in which a plasmid carrying an ampicillin antibiotic resistance gene is transferred from a leucine auxotroph *E. coli* donor to a *Serratia marcescens* (leucine +) recipient, what type of media would you use to select for transconjugates, and what controls would you include and what are their anticipated phenotypes. Explain your media choice. (8)

"Use a minimal media without leucine but which has ampicillin in it." Plate the *E. coli* and the *S. marcescens* donor and recipient on it. Neither should grow."

2. Consider our Tn induced mutants from lab. Which of the following explains why we can be sure that each of our mutants has only a single transposon insertions? (Circle all that apply).

A. The transposase gene is not expressed in *S. marcescens*

B. The transposon is a mutant variety that can hop in but cannot hop out

C. Our mutants probably do have multiple Tn insertions but this is not a problem

D. The Tn delivery vector cannot replicate in *S. marcescens*

E. The Tn does not encode its own transposase

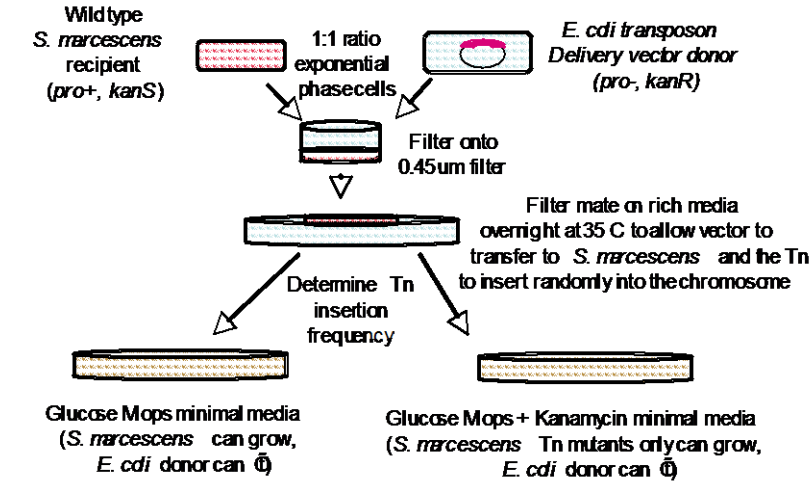
Correct answers are D and E

3. In lab, how did we select for *S. marcescens* transposon induced mutants and how does this selection work?

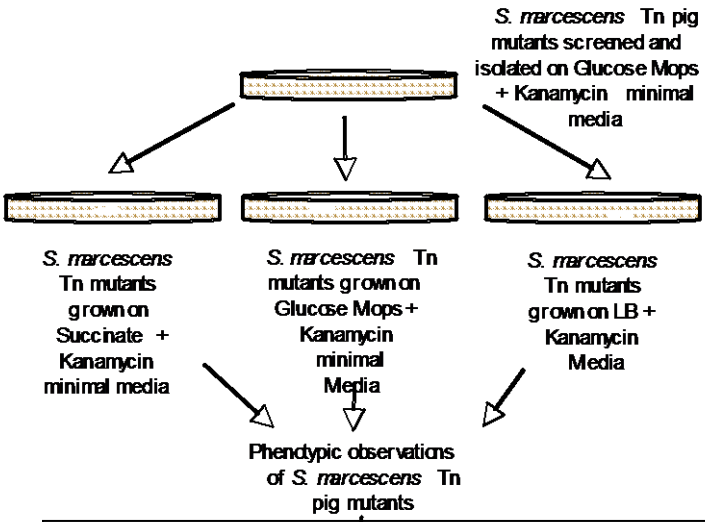
"we used a minimal media with kanamycin in it so that only the *S. marcescens* with a transposon in it could grow (the Tn encodes the kanamycin resistance). *E. coli* cannot grow because it cannot grow on this minimal media even though it is kanamycin resistant."

Appendix 8: Flow chart diagram of the steps in the Tn mutagenesis and cloning procedure in *S. marcescens*

Phase 1

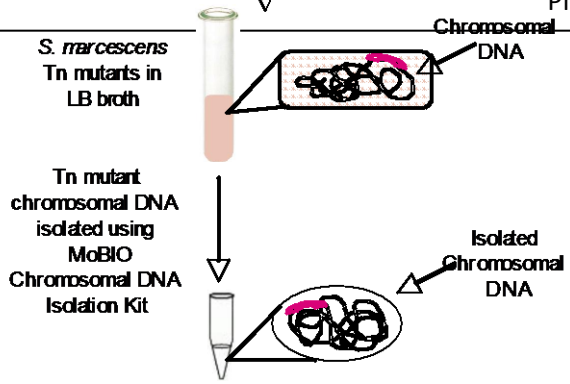


Determining the frequency of transposition

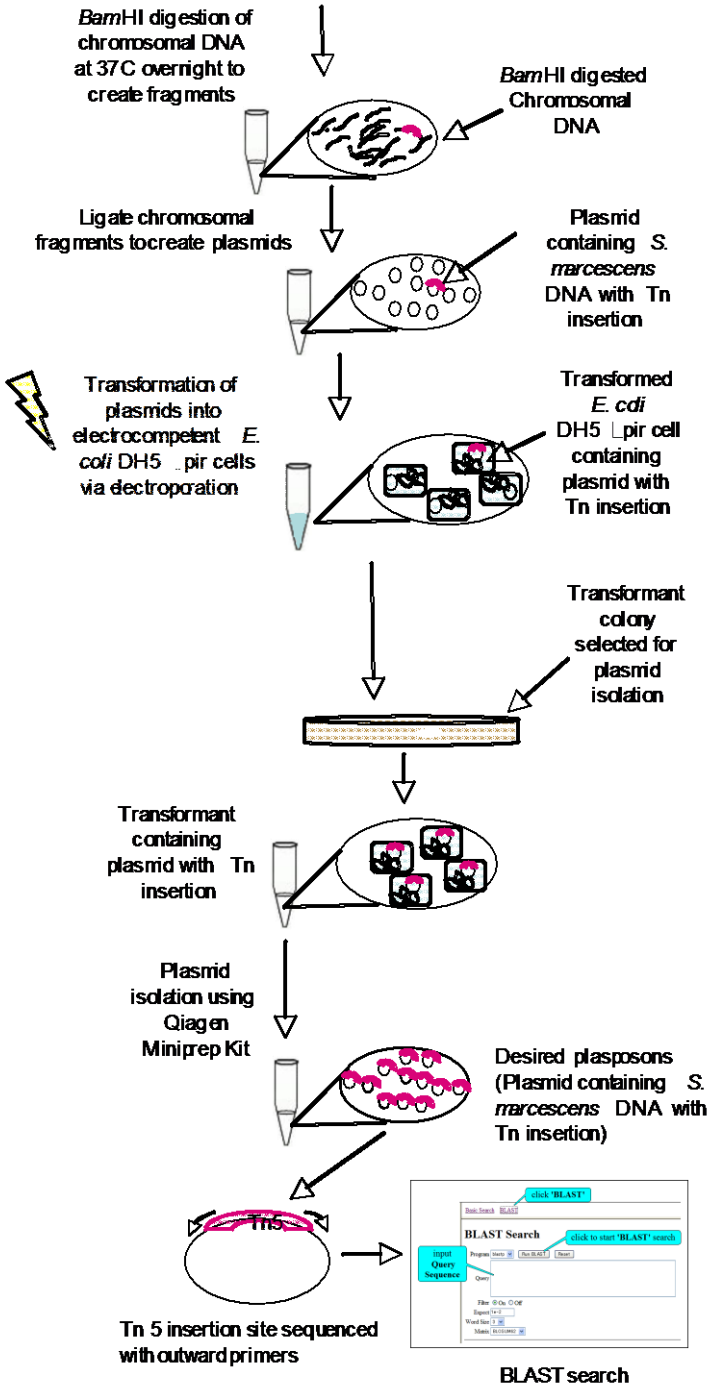


Purification and phenotypic characterization of Tn induced pigment mutants

Phase 2



Cloning the Tn insertion site



Cloning the Tn insertion site continued

Sequence analysis of the insertion site

Appendix 9: Agarose gel electrophoresis results of *Bam*HI digested Plasposon DNA isolated from *E. coli* transformants

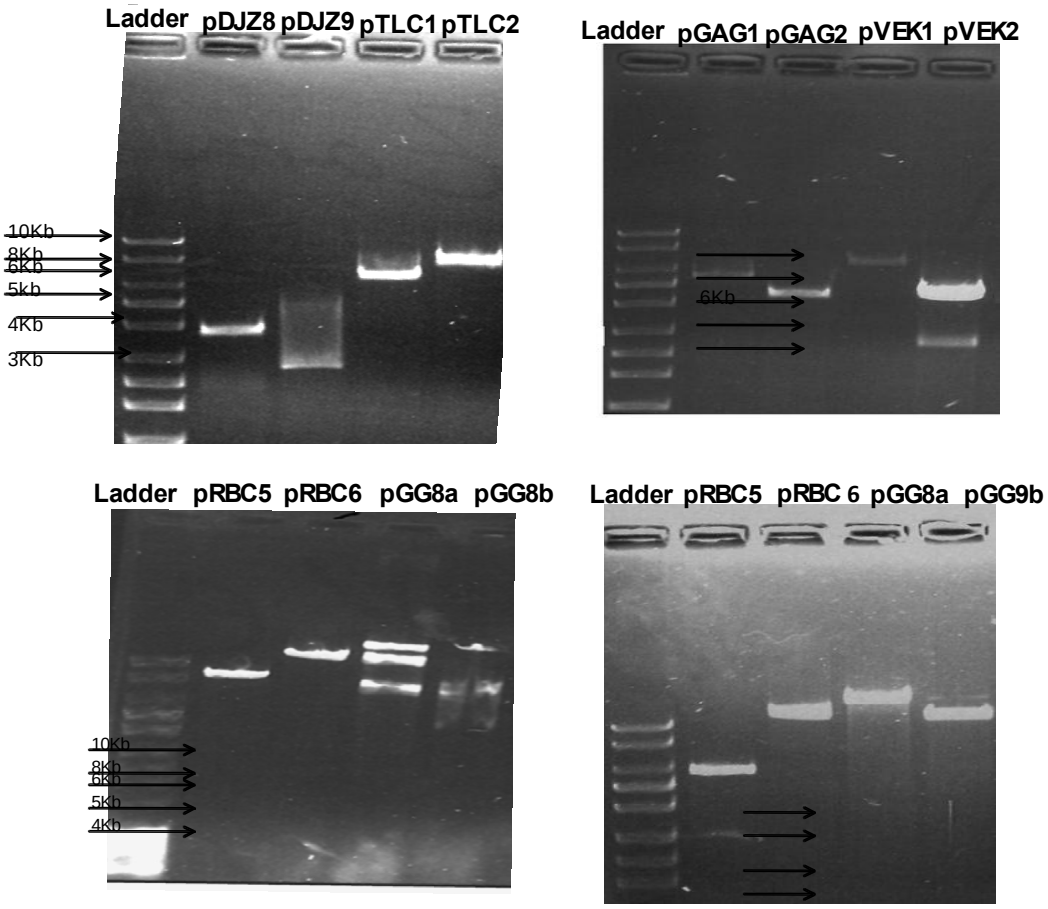


Figure 1: Gel electrophoresis of *Bam*HI digested plasposons containing *Serratia marcescens* chromosomal DNA with Tn insertions. A plasmid digest was run on 1% agarose gels for 60 minutes at 100 mV. A single band indicates a single *Bam*HI chromosomal fragment was isolated.