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### BIOM 411.01: Experimental Microbial Genetics Laboratory

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BIOM 411  
Experimental Microbial Genetics Laboratory  
Course Syllabus  
Spring 2014  
Class Hours: 2:10 – 4:00 MW  
Class Location: HS 405

Instructor: Professor William Holben  
Teaching Assistant: Franny Gilman  
TA Office: HS 503  
TA Office Hours: 4:00 – 5:00 MW; or by appointment  
TA E-mail Address: [frances.gilman@umconnect.umt.edu](mailto:frances.gilman@umconnect.umt.edu)

### A. DESCRIPTION

This course provides an overview and hands-on exploration of the methods employed in classical microbial genetics and recombinant DNA technology. The focus will be on natural conjugation plasmids encoding of 2,4-D biodegradation genes from indigenous soil bacterial strains into specialized recipient strains. These recipients lack either all of the genes in the upper pathway for 2,4-D biodegradation to 3-oxoadipate (*tfdA, B, C, D, E, F*), or just the rate-limiting initial *tfdA* gene, but encode the basal metabolic genes needed for complete mineralization of 3-oxoadipate to CO<sub>2</sub> in their chromosome. Laboratory technique emphases are on microbiology and molecular ecology techniques including pipetting, plating, mating, metagenomic and plasmid DNA isolation, restriction digests, restriction mapping, and quantitative PCR.

### B. ORGANIZATION

Students will work in groups of two and independently through out this course. Each class will begin with a 10-15 minute overview from the Instructor and/or TA of the day's experiments, the experimental design, and underlying mechanistic basis of techniques and main goals will be discussed. The remaining class time will be spent on execution of the planned laboratory experiments. The experiments can be complicated and the time is short, so each student will need be **FULLY PREPARED AT THE BEGINNING** of each class and to maintain a **DETAILED LABORATORY NOTEBOOK**.

### C. COURSE TOPICS

1. Bacterial Conjugation
2. Plasmid Purification and Preparation
3. Restriction Digests

4. Gel Electrophoresis and Restriction Mapping
5. Metagenomic DNA Purification from soil
6. Quantitative Polymerase Chain Reaction (qPCR)

#### **D. GRADING AND COURSE REQUIREMENTS**

Each student will be required to maintain their own laboratory notebook which will be graded twice during the lab course. Each day in lab you should write your name, the date, and the title of that day's experiment at the top of the page. You should then state the general purpose of the work you will be doing that day (2-5 sentences). The remainder of your notes from that day will include all details, calculations, observations, variations in protocol and all other details for each lab. Two short lab reports will be submitted during this class, one from the Conjugation experiments and one from the Restriction Mapping and Quantitative PCR experiments. Thus, maintaining a well-organized laboratory notebook is not only required for grading and general laboratory practice, but will be highly beneficial for writing your lab reports. Attendance is a large portion of the class and lab should not be missed unless the student is verifiably sick, has pre-scheduled absences for acceptable reasons, or for emergencies. Each student's grade will be broken down as follows:

Lab Notebook	20%
Conjugation Report	30%
Mapping and qPCR Report	30%
Attendance/Participation	20%

#### **E. LABORATORY REPORT FORMAT**

The purpose of the laboratory report is to communicate experimental work in writing. This is a necessary skill, regardless of the field of science you are in. The goal is to help students practice expressing their ideas and communicating them in a professional manner. Thus, the format of the laboratory reports will be similar to that in peer-reviewed scientific literature. Please seek out help during office hours if you are new to writing lab reports, I'm happy to get you going.

1. The lab report must be printed out, stapled, and submitted to the TA during the class period it is due.
2. The lab reports will have a title page that includes the course number and name, the student's name, date, and a descriptive title.
3. Acceptable lab reports will be double-spaced in an easily readable 12-point font with each page numbered at the bottom. The lab reports should be in the page range of 6-10 pages (double spaced, not including title page or references).
4. Reports will be in grammatically correct English without spelling errors (use your spell-check!).

5. Figures will be labeled as “Figure 1”, etc. and Tables as “Table 1”, etc. and can be imbedded in the text or attached as an appendix to the back of the report. Each figure will have a legend that describes the figure in sufficient detail. All tables will have a title at the top of the table. Font size for legends and descriptions can be smaller but a minimum of 10-point font.
6. References cited will be indicated by sequential numbers e.g. (1), (2,3) in the body of the report, with a bibliography of references cited at the end of the report using ASM format ([http://aem.asm.org/site/misc/journal-ita\\_org.xhtml#03](http://aem.asm.org/site/misc/journal-ita_org.xhtml#03)) (only cite primary and peer reviewed sources – journal articles, book chapters, avoid citing websites).
7. The sections of the reports will be as follows:
  - A. **Abstract** – Around 5 sentences that recaps the intro, methods, results, and discussion (write this portion last).
  - B. **Introduction** – Background information on the theory behind the experiment, system of study, questions/hypotheses that will be investigated, and general approach to answering them.
  - C. **Materials and Methods** – Step by step (in prose) description of the experimental methods. You can cite this lab manual, but be sure to describe any modifications made to any protocol.
  - D. **Results** – State the results of the experiments with descriptive figures and tables and accompanying explanation. Do not analyze the results in terms of how they relate to the questions being asked in the study, just state the results.
  - E. **Discussion** – Analyze the results in regard to how they answer the questions that you were asking. Draw conclusions, state limitations and how they relate to those conclusions, and how you would overcome such limitations in future experiments.

## F. ACADEMIC HONESTY

Appropriate ethical behavior in the classroom is required of every University of Montana student. Academic misconduct is subject to an academic penalty by the course instructor and/or a disciplinary sanction by the University. All written assignments in this class must be completely original.

**Definition: Academic Dishonesty** “cheating” and “plagiarism”, the theft of ideas and other forms of intellectual property – published or unpublished.

**Definition: Plagiarism** is the use of another writer’s words or ideas without acknowledging the source. Plagiarism also means “passing off a source’s information, ideas, or words as your own by omitting to cite them, which makes it an act of lying, cheating, and stealing.”

**Definition: *Cheating*** is defined as obtaining or attempting to obtain, or aiding another to obtain credit for work, or any improvement in evaluation of performance, by any dishonest/deceptive means. All students need to be familiar with the *Student Conduct Code*, which is available for review at [life.umt.edu/vpsa/student\\_conduct.php](http://life.umt.edu/vpsa/student_conduct.php)

### **Accommodations**

I am happy to work with students and Disability Services for Students (DSS) to make accommodations that facilitate students' class participation and learning. Please see me on the first day of this class to plan for any such accommodations.

### **G. LAB INFORMATION AND PROTOCOLS**

#### **2, 4-Dichlorophenoxyacetic acid:**

2,4-Dichlorophenoxyacetic acid is a commonly used herbicide both in the United States and world-wide. It is a synthetic chemical compound that prevents the growth of broadleaf plants, but not grasses. This quality makes it especially useful on golf courses, turf, lawns, other manicured grasslands, pastures, hayfields, cereal grain crops, and corn crops. When this chemical is manufactured cheaply it is often left contaminated with dangerous dioxins that leave this herbicide carcinogenic. However, 2,4-D, itself, is not generally considered a carcinogen. This compound is rarely found to contaminate ground water and is readily decomposed by many different kinds of bacteria in the soil. 2,4-D has been detected in very low concentrations in some soils and surface waters in the United States and is currently banned in many Nordic countries. The metabolic pathway involved in breaking down 2,4-D is widely studied. For example, the gene we will focus on during the qPCR experiments is the *tfdA* gene, which is responsible for the initial steps of the break down of this chemical. The figure below (from Top, et al., 1995) shows the pathway of break down in strain JMP 134.

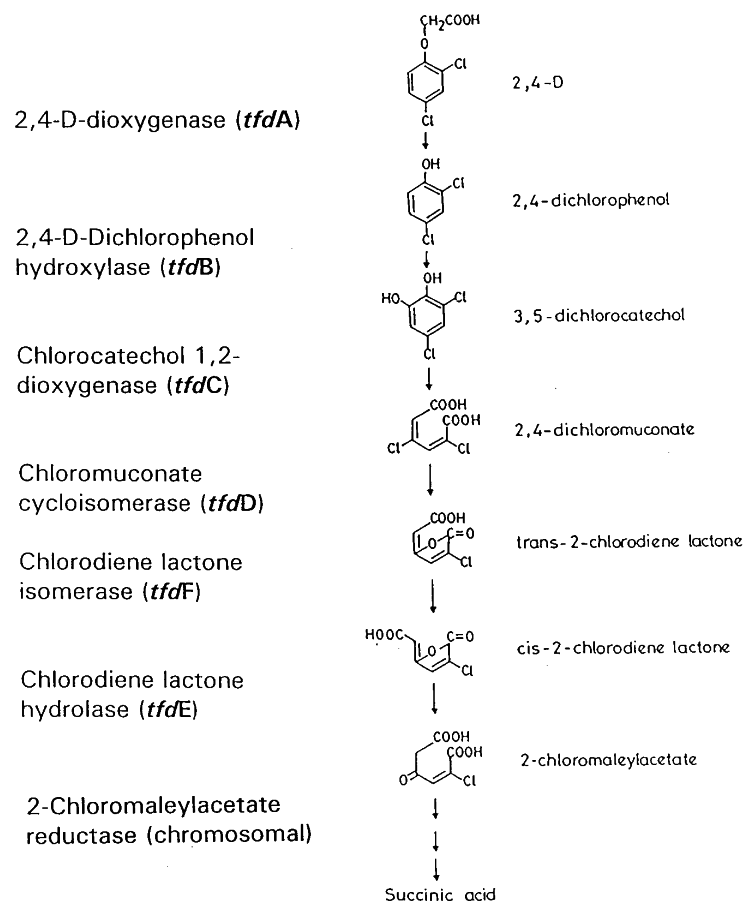


FIG. 1. Pathway for degradation of 2,4-D in strain JMP134. The figure is adapted from publications by Bhat et al. (5) and Fukumori and Hausinger (15) with permission from the publisher.

## A Few Papers from Holben and Collaborators to Get You Going:

1. **Baelum J, Jacobsen CS, Holben WE.** 2010. Comparison of 16S rRNA gene phylogeny and functional *tfdA* gene distribution in thirty-one different 2,4-dichlorophenoxyacetic acid and 4-chloro-2-methylphenoxyacetic acid degraders. *Systematic and Applied Microbiology*. **33**: 67-70.
2. **Baelum J, Nicolaisen MH, Holben WE, Strobel BW, Sorensen J, Jacobsen CS.** 2008. Direct analysis of *tfdA* gene expression by indigenous bacteria in phenoxy acid amended agricultural soil. *The ISME Journal*. **2**: 677-687.
3. **Baelum J, Prestat E, David MM, Strobel BW, Jacobsen CS.** 2012. Modeling of Phenoxy Acid Herbicide Mineralization and Growth of Microbial Degraders in 15 Soils Monitored by Quantitative Real-Time PCR of the Functional *tfdA* Gene. *Applied and Environmental Microbiology*. **78**: 5305-5312.
4. **Holben WE, Schroeter BM, Calabrese VG, Olsen RH, Kukor JK, Biederbeck VO, Smith AE, Tiedje JM.** 1992. Gene probe analysis of soil microbial populations selected by amendment with 2,4-dichlorophenoxyacetic acid. *Applied and Environmental Microbiology*. **58**: 3941-3948.

5. **Ka JO, Holben WE, Tiedje JM.** 1994. Genetic and phenotypic diversity of 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacteria isolated from 2,4-D-treated field soils. *Applied and Environmental Microbiology*. **60**: 1106-1115.
6. **Top EM, Holben WE, Forney LJ.** 1995. Characterization of Diverse 2,4-Dichlorophenoxyacetic Acid-Degradative Plasmids Isolated from Soil by Complementation. *Applied and Environmental Microbiology*. **61**: 1691-1698.

### About the Samples:

Top soil no more than 2 inches below the surface was collected from the Larchmont Golf Course in Missoula, MT on 2/13/14. This soil has a history of being treated with 2,4-D as a weed suppressant. Prior to the start of the lab course, the soil sample was split in to a control, low 2,4-D treatment (50 µg/ml), and high 2,4-D treatment aliquot (500 µg/ml). The first 2,4-D treatment was on 2/20/14. The second treatment was on 3/7/14. All aliquots were incubated at RT until the start of the lab class.

### Three Experiments in Brief:

- 1) Conjugation experiment. The recipient strains required for this experiment include: JMP228 (2,4-D-) (an *Alcaligenes eutrophus* strain that does not contain the plasmid carrying genes to degrade 2,4-D) and JMP 228/pBH501aE (an *Alcaligenes eutrophus* strain that contains a plasmid with the initial *tfdA* gene in the 2,4-D degradation pathway largely deleted and replaced with an *nptII* gene cassette (encoding kanamycin resistance (kan<sup>R</sup>)). By combining the indigenous soil bacterial fraction from soils enriched (or not) with 2,4-D with these recipient strains in a plate-mating format we will be able to “trap” and characterize 2,4-D biodegradative plasmids from indigenous soil taxa. The purpose of this lab is to facilitate and detect conjugation and genetic transfer of metabolic genes from indigenous bacteria to the recipient strains. This lab exercise will also reaffirm the natural occurrence of conjugation and the constant genetic transfer occurring within microbial communities.
- 2) Restriction digest and plasmid mapping experiment. Plasmids obtained from conjugation experiment will be digested with multiple restriction enzymes to generate a restriction map. Theoretically, all of these plasmids are not the same and thus will create different mapping profiles when digested these enzymes. Many standard molecular techniques will be taught during this exercise: including restriction digests, gel electrophoresis, fragment size determination and restriction mapping.
- 3) Quantitative PCR of the *tfdA* gene. Metagenomic DNA extracted from soils at the end of 2,4-D treatment will be used as template. Quantitative PCR will allow us to track the abundance of this gene, which is required for 2,4-D degradation as a function of degree of 2,4-D selection for degraders within the soil. This lab exercise will give students hands on experience in

metagenomic DNA isolation from environmental samples (soil), preparing PCR mastermixes reactions, and interpreting qPCR results.

### **Protocols to be used:**

**Experiment 1: *In situ* conjugation experiment – Students work in pairs (one student works with JMP228, the other with JMP228/pBH501aE). Each student uses each soil type (control, low treatment, high treatment).**

#### Conjugation 1

Materials Needed From Media Lab: Two LB flasks to start cultures (25ml) of LB rif (100µg/ml) and two flasks of LB +rif +kan (both 100µg/ml); 20 sterile tubes/bottles filled with 10 ml LB 1/10 (half to be used at step 7); at least 2 scales; P1000s with 1 box tips per student; p100s with 1 box tips per student; P10s (one box of tips per student); 60 sterile 1.5 ml eppi tubes; 30 sterile tubes filled with 9 ml saline solution (0.85% NaCl); rotary shaker; sterile centrifuge tubes; 20 empty sterile test tubes; 85 LB plates +cycloheximide (300 µg/ml); hockey sticks and ethanol; 10 aliquots of sterile water (10 test tubes with 5ml)

Materials To Be Used From Holben Lab: Sorvall SS34; laminar flow hood; 30°C incubator

1. TA starts overnight culture 2 days before lab (3/15/14) – two flasks of JMP228 in LB 100µg/ml rif; two flasks of JMP228pBH501aE LB rif kan (both 100 µg/ml)
2. TA one day before lab - Dilute overnight culture of recipient strain [JMP228 or JMP228(pBH501aE)] 1:1,000 in LB 1/10 by placing 10µl culture into sterile tube containing 10 ml LB 1/10. (Make 5 of each recipient strain – 10 tubes total).
3. Dilute soil samples 10-fold in sterile saline solution (0.85%NaCl) by adding 1g soil to 9 mL saline solution. Shake on a rotary shaker for 1 h.
4. Allow large soil particles to settle for 30 minutes.
5. Carefully decant supernatant into sterile centrifuge tubes.
6. Collect soil bacterial cells via centrifugation in a Sorvall SS34 rotor at 10,000 rpm for 10 min.
7. Discard supernatant and resuspend soil bacterial pellet in 0.1 volume of 1/10 strength LB broth (LB 1/10).
8. Mix one ml of this suspension with 1 ml of the overnight culture of the recipient strain [JMP228 or JMP228/pBH501aE] (from step 2).
9. Move 1 ml of this mixture to a sterile 1.5 ml eppi tube and centrifuge at top speed for 3 min.
10. Remove supernatant and pipette into another sterile eppi tube.
11. Take 200µl of that supernatant add to the tube with the soil bacterial pellet



12. Resuspend the pellet in the 200µl of recipient supernatant by pipetting up and down.
13. Remove 10 µl and mix with 90µl sterile saline solution to make a 1:10 dilution of the concentrated mating mixture.
14. Pipette 100 µl of the concentrated mating mixture and 100µl of the 1:10 dilution of mating mixture onto two different LB plates, and spread with an ethanol flamed hockey stick, leave open to dry on bench top. (12 experimental LB plates per pair)
15. Incubate all plates inverted overnight or until next lab period at 30°C to allow conjugation to occur on the plate surface.

### Conjugation 2

Materials Needed From Media Lab: spatulas (at least 10) and beakers of ethanol; 60 sterile tubes of 5ml saline soln (0.85% NaCl); 240 sterile tubes of 9ml saline soln (0.85% NaCl); hockey sticks and ethanol; 150 MMO +2,4-D (500µg/ml) +rif (100µg/ml) plates cycloheximide (300 µg/ml); 150 MMO +2,4-D (500µg/ml) +rif (100 µg/ml) +kan (100µg/ml) + cycloheximide (300 µg/ml); P100s and P1000s one box tips per student

Materials To Be Used From Holben Lab: stock solutions to make MMO media (MMO stocks, 2,4-D stock); 30°C incubator

1. Aseptically scrape colonies from each experimental plate (12 per pair/6 per student) using an ethanol flame sterilized spatula. Touch spatula to surface of plate and gently drag across to gather cell material.
2. Resuspend cells in 5ml of sterile saline (dip spatula into test tube and twirl in between fingers to release cell material).
3. Serially dilute (in sterile saline) out to  $10^{-4}$  by moving 1 ml from previous dilution into next dilution tube containing 9 ml saline.
4. Spread plate 100µl of the 1X, -1, -2, -3, and -4 dilution samples onto MMO plates. If you're the student using the JMP228 strain use MMO/rif plates; if you are the student using the JMP228/pBH501aE strain use MMO/rif/kan plates.
5. Incubate 4 days at 30°C.

### Conjugation 3

Materials Needed from Media Lab: 30 plates MMO +2,4-D +rif; 30 plates MMO +2,4-D +rif + kan.

Materials To Be Used From Holben Lab:  
stock solutions to make MMO media (MMO stocks, 2,4-D stock); 30°C incubator

1. Examine cultures from Conjugation 2

2. Re-streak (up to 3 from each soil type) on appropriate media (MMO rif = JMP228; MMO rif/kan = JMP228pBH).
3. Incubate 30°C until next lab period; refrigerate over spring break.

#### Conjugation 4

Materials Needed From Media Lab: disposable plastic loops preferred or sterile toothpicks (at least 70); 25 sterile test tubes of 5ml MMO +2,4-D +rif liquid media; 25 sterile test tubes of 5ml MMO +2,4-D +rif kan liquid media.

Materials To Be Used From Holben Lab: stock solutions to make MMO media (MMO stocks, 2,4-D stock); 30°C incubator

1. Pick transconjugant colonies using sterile toothpicks. Pick a total of 5 colonies from each recipient cell type, total of 10 picked colonies between pairs. (touch end of toothpick to colony) (picking from plates with 30-300 colonies is ideal)
2. Inoculate in to 5 ml MMO liquid media and incubate at 30°C until next lab period. (drop toothpick into test tube or twirl dispo loops) (MMO rif = JMP228; MMO rif kan = JMP228pBH).

**Experiment 2: Restriction digest and plasmid mapping experiment – students still working in pairs, each student works with one recipient cell type**

Plasmid Prep – Qiagen Spin Mini Prep

Materials Needed From Media Lab: plasmid clean up kit – Qiagen Spin Miniprep Kit (50 preps needed). P1000s and one box tips per student; As many table top microcentrifuges you have available; 60 sterile 1.5ml microcentrifuge tubes; space in -20 freezer.

Materials To Be Used From Holben Lab: centrifuge

1. Pick three cultures of transconjugants from either soil type (each student does 3 plasmid preps; 6 preps in a pair).
2. Follow protocol given by Qiagen...
3. Pellet 5 ml bacterial overnight culture by centrifugation at >8000 rpm (6800 x g) for 3 min at room temperature (15–25°C).
4. Completely resuspend pelleted bacterial cells in 250 µl Buffer P1 and transfer to a microcentrifuge tube. Ensure that no clumps of cells remain.
5. Add 250 µl Buffer P2 and mix by inverting the tube 10–12 times until the solution turns completely blue. Incubate for 5 min. Do not allow lysis to proceed for more than 5 min.
6. Add 350 µl Buffer N3 and mix immediately and thoroughly by inverting the tube 10–12 times until the solution turns colorless.
7. Centrifuge for 10 min.
8. Apply the supernatant from step 6 to the QIAprep spin column by decanting or pipetting. Centrifuge for 30–60 s and discard the flow-through, or apply vacuum to the manifold to draw the solution through the QIAprep spin column and switch off the vacuum at source.
9. Wash the QIAprep spin column by adding 500 µl Buffer PB. Centrifuge as in step 7, or apply vacuum to draw the solution through the QIAprep spin column as in step 7.
10. Wash the QIAprep spin column by adding 750 µl Buffer PE. Centrifuge as in step 7, or apply vacuum to draw the solution through the QIAprep spin column as in step 7. Transfer the QIAprep spin column to the collection tube.
11. Centrifuge for 1 min to remove residual wash buffer.
12. Place the QIAprep spin column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 60 µl Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of the QIAprep spin column, let it stand for 1 min, and centrifuge for 1 min.
13. Place plasmid DNA in to -20 freezer until next lab period.
14. TA will quantify plasmid DNA for you.

## Restriction Digests

Materials Needed From Media Lab: 500µl sterile eppi tubes (30); p2s (one box filter tips per student if available); p200s (one box filter tips per student if available); heat block or water bath set at 37°C will need to incubate 28 tubes; another heat block or water bath set at 65°C; -20 freezer space

Materials To Be Used From Holben Lab: water bath/heating block; EcoRI, BamHI, and Hind III.

1. You will be setting up restriction digests on two of your plasmid DNA samples (3 per student)
2. You will be doing 7 different digests on each sample (EcoRI; HindIII; BamH I; EcoRI with HindIII; HindIII with BamHI; EcoRI with BamHI; and one with all three)
3. Make 30µl reaction mixtures for the above described reactions

DNA	1µg (use 1µl)	or	DNA	1 µl
Enzyme	1 µl		Enzyme A	1 µl
10X Buffer	3 µl		Enzyme B	1 µl
sterile H <sub>2</sub> O	25 µl		10X Buffer	3 µl
			sterile H <sub>2</sub> O	24 µl

or

DNA	1µg (use 1µl)
Enzyme A	1 µl
Enzyme B	1 µl
Enzyme C	1 µl
10X Buffer	5 µl
sterile H <sub>2</sub> O	23 µl

4. Add enzyme last when making mixture, keep enzymes on ice
5. Do not vortex mixture, mix by pipetting up and down. Set pipettor to 30µl and pipette up and down 10 times. Spin down in centrifuge to collect at bottom.
6. Incubate reactions at 37°C until the end of the day (TA will remove samples if necessary).
7. Freeze reactions at -20 until next lab period

## Gel Electrophoresis and Mapping

Materials Needed From Media Lab: Buffer for gel; agarose for gel (make 1.5% gels); prepare 3 large gels ahead of time with 2, 16 well combs; sterile 500 $\mu$ l eppi tubes (45); 1 kb ladder (20 lanes worth); gel loading dye (170 $\mu$ l); power source for gels; p20s or p10s and one box tips per student

Materials To Be Used From Holben Lab: Large gel rigs; EtBr for gels; transilluminator and camera

1. Set up 3 gel rigs with 3 gels with two 16-well combs in each.
2. Add 6 $\mu$ l of 5X loading dye to each digest. Mix gently, spin briefly to collect in bottom of tube.
3. For practice gel add 30 $\mu$ l sterile water with 6 $\mu$ l loading dye) spin briefly to collect in bottom of tube.
4. Load 25 $\mu$ l of sample from step 3 into practice gel.
5. When ready, load 25  $\mu$ l of each sample (in the order given by TA), keep track of which gel and which wells you have loaded.
6. TA loads 10 $\mu$ l 100bp ladder into each gel
7. Set gels to 100V and let run for appropriate length of time.
8. Capture gel image upstairs in the Holben lab and print gel image.
9. Proceed to mapping discussion and exercise same day or next lab period

### **Experiment 3: Quantitative PCR of the *tfdA* gene**

#### DNA Extraction

Materials Needed From Media Lab: scales (2); weigh boats (15); spatulas; vortexes (as many as you have); table top centrifuges (a few would be great); use of refrigerator for 4°C incubations; p100 and one box tips per student – do not need to be full; p100 and one box tips per student – do not need to be full

Materials To Be Used From Holben Lab: MOBIO Powersoil DNA Isolation Kit (use endpoint soil sample; 2 extractions per student; 20 extractions) divide all kit solutions into appropriate number of aliquots; Vortex adaptor

1. To the PowerBead Tubes provided, add 0.25 grams of soil sample.
2. Gently vortex to mix.
3. Check Solution C1. If Solution C1 is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60 µl of Solution C1 and invert several times or vortex briefly.
5. Secure PowerBead Tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1-24) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.  
Note: If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.
6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge 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.
7. Transfer the supernatant to a clean 2 ml Collection Tube (provided).  
Note: Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.
8. Add 250 µl of Solution C2 and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean 2 ml Collection Tube (provided).
11. Add 200 µl of Solution C3 and vortex briefly. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean 2 ml Collection Tube (provided).
14. Shake to mix Solution C4 before use. Add 1200 µl of Solution C4 to the supernatant and vortex for 5 seconds.
15. Load approximately 675 µl onto a Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow through and add an

- additional 675 µl of supernatant to the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Note: A total of three loads for each sample processed are required.
16. Add 500 µl of Solution C5 and centrifuge at room temperature for 30 seconds at 10,000 x g.
  17. Discard the flow through.
  18. Centrifuge again at room temperature for 1 minute at 10,000 x g.
  19. Carefully place spin filter in a clean 2 ml Collection Tube (provided). Avoid splashing any Solution C5 onto the Spin Filter.
  20. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).
  21. Centrifuge at room temperature for 30 seconds at 10,000 x g.
  22. Discard the Spin Filter. The DNA in the tube is now ready for any downstream application. No further steps are required.
- We recommend storing DNA frozen (-20° to -80°C). Solution C6 contains no EDTA. To concentrate the DNA see the Hints & Troubleshooting Guide.

### Quantitative PCR

Materials Needed From Media Lab: iQ sybrgreen super mix; synthesize primers; pcr water; BSA; PCR plates and plate seal?; 14 test tubes of sterile DI or MQ water (5mls ea.); sterile 500µl eppi tubes (45 tubes each qPCR day); p2s and one box sterile filter tips per student if available; P200s and one box sterile filter tips per student if available; ice and ice buckets; table top centrifuges

\*Primers order 0.05mMol: tfdAF 5'-GAG CAC TAC GCR CTG AAY TCC CG-3'  
 tfdAR 5'-GTC GCG TGC TCG AGA AG-3'

Materials To Be Used From Holben Lab: icylcer, PCR plates and plate seal; 16S primers

Use *C. necator* JMP134 (harbors class I tfdA gene) for standards

1. Dilute DNA 1:10 by adding 10µl DNA to 90µl sterile water in 500µl eppi tube
2. Mix by vortexing. Centrifuge briefly to collect sample. Keep all DNA samples and all PCR components on ice
3. Note: You will run all samples in triplicate
4. Make a Master Mix. Each student should have 2 DNA samples (low 2,4-D treated soil; high 2,4-D treated soil)  
 General Master Mix for 1 rxn: (this is subject to change depending on the concentration of primers we use)  
 F Primer      1 µl F primer (10 pmol/ml)  
 R Primer      1 µl R primer (10 pmol/ml)

- |           |                     |
|-----------|---------------------|
| BSA       | 1 µl BSA (10 µg/ml) |
| SybrGreen | 12.5 µl Sybr Green  |
| PCR Water | 8.5 µl              |
| (Template | 1 µl)               |
5. Vortex master mix and centrifuge to collect mixture
  6. Add 24 µl of master mix to your designated wells on the PCR plate, you do not need to change the tip
  7. Add 1 µl of your sample to your designated wells on the PCR plate, change tip every time
  8. Take turns carefully loading samples in to plates, TA will load standards. Standards were previously diluted to  $10^7$ ,  $10^6$ ,  $10^5$ ,  $10^4$ , and  $10^3$  copies/µl)

PCR Conditions:

15 min at 95

50 cycles of

60 s at 95

30 s at 64

60 s at 72

7 min at 72

melt curve

**RECIPES**

Gels:

In to gel bed: 1600 ml 1X TAE plus 1.6 ml EtBr 125 µg/ml

Full gel: 155 ml 1XTAE plus 2.32 g gene pure agar plus 155 µl 125 µg/ml EtBr

MMO 2,4-D plates (1 L):

40 ml MMO A

900 ml MQ water

15 g Bacto Agar

autoclave

add 25 mls MMO B

10 mls MMO C

1.25 ml 2,4-D soln (at 400 mg/ml)

add appropriate antibiotics

and bring to appropriate volume with sterile MQ water if necessary



# Experimental Microbial Genetics Syllabus and Lab Manual

Spring 2015, Instructor: Bill Holben, TA: Franny Gilman

Lab#	Date	Topic
X	Mon 3/16	No Lab – Science Fair
1	Wed 3/18	Overview of lab
2	Mon 3/23	DNA extraction
3	Wed 3/25	16S QPCR
X	Mon 3/30	SPRING BREAK NO LAB
X	Wed 4/1	SPRING BREAK NO LAB
4	Mon 4/6	Conjugation 1
6	Wed 4/8	Conjugation 2
7	Mon 4/13	Conjugation 3
8	Wed 4/15	<i>tfdA</i> QPCR; Note Book Check #1
9	Mon 4/20	Conjugation 4
10	Wed 4/22	Plasmid Prep
11	Mon 4/27	Restriction Digest; Lab Report 1 on Conjugation
12	Wed 4/29	Gel Electrophoresis
13	Mon 5/4	(Last week of classes) Restriction Mapping
14	Wed 5/6	Discuss Results
X	Mon 5/8	(Finals Week Starts) Final Lab report on Mapping and QPCR; Note Book Check #2