MICROBIAL GENETICS LAB (BIO-377/577)
FALL 2013

Place and Time: Wednesdays 1:50 – 5:50.
Lab discussions: HS 266, Wednesdays, 1:50 to ~2:40.
Lab rooms: HS 240, 163 (Bioseparations-Proteomics lab), and 165. Most of the work will be done in the HS 240 Microbial Genetics - Biotechnology Lab, which will be available for ongoing experiments.

Instructor: Dr. Toivo Kallas

Office: Halsey 245 (phone 424-7084; e-mail: kallas@uwosh.edu)
webpage: http://www.uwosh.edu/faculty_staff/kallas

Office hours: Mon 1:50 – 3:50, Tu 3:00 – 5:00. Other times by appointment. Anytime by phone or e-mail. If I am not in, please leave a message or check the lab rooms (HS 238, 240, or 163/145 Bioseparations-Proteomics Labs)

Lab manuals

Kallas, T. 2003 (under revision). *Experiments in Microbial Genetics*, UW-Oshkosh. Pending revision of the lab manual, we will use the 2003 Lab Manual and handouts as needed. The 2003 manual and an appendix of commonly used protocols are posted on the D2L site.

(recommended): Miller, J.R. 1992. *A Short Course in Bacterial Genetics: Lab Manual*, Cold Spring Harbor Laboratory Press. Provides background information and protocols for *in vivo* experiments with *Escherichia coli.*

Other materials

***Important* -- Lab notebook** for recording ongoing **objectives**, **results**, and **conclusions** of experiments. USB memory stick for saving computer data files.

Locks & pipetors

A set of 3-4 micro-pipetors will be issued to each lab group together with drawers and locks. Each group is responsible for returning their pipetors in good condition at the end of the semester. Students will be responsible for repair or replacement of damaged or lost pipetors. Replacement costs are $200 - $250.

Desire2Learn (D2L) Site

Materials including the lab manual and supplementary protocols will be posted on the class D2L site (**Microbial Genetics Lab Bio-377/577**). The lecture course D2L site (Microbial Genetics, Bio 375/575) will also be accessible to lab students.

Other useful microbial genetics laboratory references

Miller, J. H. 1992 *A Short Course in Bacterial Genetics: Handbook*, Cold Spring Harbor Laboratory.

Sambrook, J and Russell, D. 2000 *Molecular Cloning: A Laboratory Manual* 3rd ed., Cold Spring Harbor Laboratory.

Primrose, S. B. and Twyman, R. M. 2006, 2012. *Principles of Gene Manipulation and Genomics*. Blackwell, Oxford.

(Note that many useful resources are available via web sites such as those listed below.)

Some genetics resources, Websites

1. **Class D2L site**, described above.
2. **American Society for Microbiology (ASM)** home page: http://www.asmusa.org.
3. **DOE Joint Genomics Institute (JGI)**: http://www.jgi.doe.gov/JGI_microbial/html/index.html (Microbial genome databases and a great resource for genome analysis including BLAST searches.)
4. **ExPASy Molecular Biology Server:** http://www.expasy.ch/. (A very useful site for molecular biology, genomics, and proteomics included predicted peptide mass fingerprints.)
5. **NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION (NCBI):** http://www.ncbi.nlm.nih.gov/. (This site includes the GenBank and other DNA, protein, and genomic databases and extremely useful search programs such as "BLAST." Includes the PubMed, MEDLINE literature database.)
6. Within **NCBI**, note for example **PubMed** (http://www.ncbi.nlm.nih.gov/pubmed/) for literature database searches and **PubChem** (http://pubchem.ncbi.nlm.nih.gov/) for structures and information about small molecules including metabolites, antibiotics, and inhibitors.
7. **TIGR** (The Institute for Genomic Research): http://www.tigr.org.
8. **Kazusa** Genome Research Institute: http://www.kazusa.or.jp.
9. ***E. coli* Genetics Stock Center**: http://cgsc.biology.yale.edu/. (a nice site for gene names, maps, etc.)
10. ***E. coli* Genome Center**: http://www.genome.wisc.edu
11. **The RCSB Protein DATA Bank**: http://www.rcsb.org/pdb/. (Site from which to download ".pdb" files of coordinates for viewing and manipulating protein and DNA sequence 3D structures).
12. **PyMOL:** http://pymol.sourceforge.net/ (Site for downloading the PyMOL program for very nice viewing and manipulation of protein and molecular 3D structures on Mac and Windows platforms.)
13. **Webcutter** (a site for on-line restriction site analysis): http://www.firstmarket.com/cutter/cut2.html
14. **Net Primer** (a site that allows downloaded or on-line design of PCR primers. They also carry "Plasmid Premier" a program for plasmid design): http://www.premierbiosoft.com/netprimer.html
15. **BioBIKE** (Biological Integrated Knowledge Environment): http://ramsites.net/~biobike/ (Provides integrated databases and access to a 'non-expert' programming language for bioinformatics investigation of biological databases).
16. **CyanoBIKE** (Cyanobacterial Biological Integrated Knowledge Environment): http://cyanobike-community.csbc.vcu.edu/ (graphical interface programming environment for access to integrated cyanobacterial genome databases, manipulation and data mining).
17. **KEGG** (Kyoto Encylopedia of Genes and Genomes): http://www.genome.jp/kegg/ (A very useful bioinformatics resource for linking genomes to biological systems and environments.)
18. **New England Biolabs**, Restriction Enzyme Database (NEB-REB): http://rebase.neb.com.
19. **Promega** Corporation (Madison, WI): http://www.promega.com/
20. **UWO (Polk) Library:** http://www.uwosh.edu/library/ (Polk Library provides access to a variety of useful literature databases such as Medline and Web of Science and carries on-line, full-text subscriptions of several relevant journals including *Science, the Nature Journals,* Elsevier Journals via Science Direct, and the *American Chemical Society (ACS) Journals*. Follow on-screen instructions or see me.)

Course Overview and Learning Objectives

Our objectives are:
1) To provide 'hands-on' experience in the investigation and manipulation of microorganisms and their genes.
2) To develop the ability to think critically and devise genetic strategies that might be used to address interesting biological problems.

A variety of experimental approaches will be introduced. Most experiments will use *Escherichia coli*, which is used in some stage of virtually every molecular genetic investigation or genetic engineering application of prokaryotic or eukaryotic organisms. Some experiments will use the cyanobacteria *Synechococcus* PCC 7002 or *Synechocystis* PCC 6803. Cyanobacteria perform ~25% of global photosynthesis, they produce oxygen for life on earth, remove CO_2 from the atmosphere, and hold great potential for biofuels applications.

Experiments will include chemical and transposon mutagenesis, analysis of mutants, gene transfer, and strain construction. Molecular genetic procedures such as chromosomal and plasmid DNA isolation, DNA amplification by standard or quantitative polymerase chain reaction (PCR or qPCR), hybridization, gene cloning, and restriction site analysis will also be introduced.

Students will have the latitude to pursue selected topics in greater depth as 'mini-projects.' All students will work on one of the mini-project options or on a mini-project of their own design. Mini-project might include construction of 'knockout' or site-directed mutations to investigate specific genes, reverse-transcriptase, real-time quantitative PCR (RT-qPCR) for expression analysis of selected genes, or possible microarray hybridizations for global gene expression analysis.

Graduate students will be expected to devise a detailed experimental protocol that could be used to construct a useful bacterial strain, a recombinant DNA molecule, or answer a specific biological question. All students are expected to submit an outline of major steps to be performed and materials needed for their miniprojects.

Experiments & projects. We will begin the semester with a couple of classical, *in vivo* bacterial genetics experiments (chemical mutagenesis with ethylmethane sulfonate, EMS, and transposon mutagenesis with a Tn10 derivative) designed to generate large collections of mutant bacteria. Because ***"Mutations Define Genes,"*** these classical approaches are still extremely useful, particularly when combined with molecular strategies. Students wishing to gain further experience with *in vivo* gene manipulations will have the option of pursuing further experiments in this area. Otherwise, students will subsequently work on molecular genetic experiments (such as characterization of Tn10 insertions in plasmids from the above transposon mutagenesis experiments) and then for the remainder of the semester on one of the mini-projects listed below, or possibly on a mini-project of their own design.

Some mini-project options

1. Cyanobacterial 'knockout' mutants. We will discuss the creation of knockout mutations to investigate gene/protein function and as a way to construct useful microbial strains. Several very interesting PCR-based (e.g. Frigaard et al., 2004 *Methods in Molec. Biol.*) and 'Gibson Assembly' (Gibson and Russello, 2012 *NEB Expressions*) methods are now available for generating mutantions and gene modifications without the need for 'cut and paste' gene cloning. This project might involve selection of one or more target genes, design of PCR primers, and construction of PCR products that contain an antibiotic-resistance cassette (e.g. for Km-resistance) inserted into the coding regions of the selected genes. These knockout constructs would be introduced into the cyanobacterium *Synechococcus* PCC 7002 by transformation. If time allows, the potential knockout cyanobacterial strains would be tested for integration of the inactivated genes and their possible consequences.

Examples of interesting mutants that have been constructed are SdhB (by Sheriff Otun and Brandon Brummeyer), YrdC (by Justin Zangl), GlgA1 (Brandon Thomas and Andrea Felton), GlgA2 (Ola Aremu), and ApcF (Sarah Black). SdhB is defective in succinate dehydrogenase, which may have important roles in electron transfer. YrdC is defective in a 'YrdC-like' regulator of CO_2 uptake and grows extremely poorly at low CO_2. GlgA1 and GlgA2 are defective in synthesizing the major glycogen carbohydrate storage polymer of cyanobacteria. ApcF is defective in a light-harvesting protein. For reasons to be explained, both the GlgA1-GlgA2 and ApcF mutants were created to increase the yield of isoprenoid bioproducts.

Many other knockout mutations would be interesting. For example, we now believe that a CpcB mutant, of the major phycocyanin, light-harvesting complex of cyanobacteria, would be more useful than the ApcF light-harvesting mutant. By knocking out CpcB and most or the light-harvesting complex, cells would not shade each other, and should grow to a higher density and produce more isoprene per culture volume. Andrea Felton has initiated work on a CpcB knockout. GlgC for ADP-Glucose pyrophosphorylase, and SpsA for sucrose phosphate synthase, would be other interesting targets, the inactivation of which should increase carbon flux to isoprenoid bioproducts.

2. Site-directed mutagenesis of selected genes or regulatory sites. Directed mutagenesis is a cornerstone of modern genetic analysis designed to probe regulatory networks and protein structure-function in biological processes. We have successfully used the Invitrogen "Quick Change" PCR-based mutagenesis strategy that allows construction of specific mutations in genes carried on any plasmid. In this experiment, students would design primers and create specific mutations in selected cyanobacterial genes – for example ones implicated in electron transport regulation or that might be useful for improved, isoprenoid, hydrocarbon production. The modified genes would be characterized by DNA sequencing and, if time allows, introduced into cyanobacteria for replacement of the native gene with the mutant allele.

3. Yellow Fluorescent Protein (YFP) reporter plasmid to investigate gene regulation. We have nice plasmid, pAQ1Ex-PcpcB-YFP, that contains a gene (YFP) for a yellow fluorescent protein under the control of a strong, light-harvesting protein (pcpB) promoter. This project would involve PCR amplification of a selected gene promoter region and its integration into the YFP plasmid to replace the pcpB promoter. The goal is to construct a 'reporter' plasmid where a selected promoter controls the expression of the YFP gene. When introduced into *Synechococcus* 7002 cyanobacteria, such 'reporter' plasmids would be very useful for testing the regulation and strength of selected promoters. Such promoters might be used, for example, to control the expression of isoprenoid, hydrocarbon sysnthesis genes.

4. Reverse-transcriptase, quantitative PCR (RT-qPCR) analysis of gene expression. We have a nice instrument (ABI StepOne) for Real-Time quantitative PCR, which allows the quantitative detection of DNA sequences (e.g. from medical or environmental samples) or RNA transcripts. RT-qPCR is often used to confirm selected global gene expression data or independently for the detailed analysis of selected genes. We would select a few *Synechococcus* genes, perhaps in conjunction with data from a microarray experiment, for detailed analysis. This experiment might also involve the design of 'TaqMan' primers and probes (to be discussed) for highly specific qPCR or RT-qPCR. Target genes of interest could be ones that we have introduced or modified for isoprenoid, hydrocarbon biosynthesis in *Synechococcus* cyanobacteria.

5. RT-qPCR analysis of gene and/or plasmid copy number. We are involved in a project to produce isoprene, a valuable chemical feedstock and biofuel precursor, in the cyanobacterium *Synechococcus* PCC 7002. A question of interest is the copy number of introduced or over-expressed genes designed to promote isoprene synthesis. This can be addressed by qPCR. A related question has to do with the copy number of a large plasmid, pAQ4, that carries interesting stress response genes but that appears to be lost from some strains of *Synechococcus*. The presence of this plasmid and its copy number can be addressed by qPCR.

6. Oligonucleotide microarray analysis of global gene expression. Oligonucleotide microarrays are made by massive, parallel, "on-chip" synthesis of tens of thousands of "probes" consisting of short (e.g. 60 base) DNA sequences. Through the UW Madison Biotechnology Center and NimbleGen, we have two "generations" of oligonucleotide arrays for the cyanobacterium *Synechococcus* 7002. Each array carries 72,000 probes. Each of the ~3000 predicted genes in the genome are represented for most genes by either

3 or 7 probes per gene repeated 3 times per array. Upstream, untranslated regions of selected genes are covered by high-density probes. These allow the identification of transcription start sites and operons and thus regions important in gene regulation. In this experiment we would isolate RNAs from a control cyanobacterium and a mutant cyanobacterium, e.g. one with a defect in photosynthetic electron transport, or from an isoprenoid producing cyanobacterium vs. a control strain. These RNAs would be converted to cDNAs, labeled, and hybridized against the arrays. As time allows, these data would be analyzed to pick out genes that are differentially expressed in the experimental strain relative to the control. Such work can provide insight into how cyanobacteria regulate metabolic pathways and how they respond to environmental signals. The first steps in these exeperiments – the rapid isolation of high-quality RNA – are the same as those for RT-qPCR. Students have successfully done microarray experiments in the past, but their feasibility depends on time commitment and also the availability of funds.

7. Synthetic gene construct for β-pinene synthesis in cyanobacteria. A set of codon-optimized genes (GPPS and mono-TPS) has been designed and synthesized to enable synthesis in *Synechoccoccus* 7002 cyanobacteria of β-pinene, a precursor for jet fuel (work of MS student Rhiannon Carr). To achieve pinene synthesis in the cyanobacterium, a constitutive or regulatable strong promoter needs to be integrated upstream of these genes and the gene construct needs to be targeted to either a plasmid or chromosomal site in *Synechococcus*. Versions on this theme would be the objectives of this project.

8. A gene to alleviate the vitamin B_{12} requirement of *Synechoccocus* 7002 cyanobacteria. These cyanobacteria cannot synthesize vitamin B_{12}, and thus B_{12} needs to be included in the growth medium to enable methionine biosynthesis (the only step for which B_{12} is required). The requirement for vitamin B_{12} can be alleviated by introducing a *metE* gene that encodes an alternative (non-B_{12} requiring) enzyme for methionine biosynthesis (Don Bryant, Penn State, personal communication). A *metE* gene from *E. coli* should work. This project would involve PCR amplification of a *metE* gene, inclusion of a cyanobacterial promoter, and targeting to a plasmid or chromosomal site (currently being pursued by Rhiannon Carr). The *metE* gene also opens interesting possibilities for use as a selectable marker.

Additional mini-project options are described in the lab manual and in a handout to be discussed later.

Grading and Requirements

Students will work collaboratively in groups of two or three. ***Master's students or advanced undergraduates may be asked to serve as team leaders/mentors for less experienced students.*** Requirements are listed below. Most of the grade will be based on lab reports handed in individually by each student (including 10% for successful results or reasonable efforts made toward obtaining positive results). The main criteria for grading will be clarity of presentation and understanding of procedures and concepts. Two small and one mini-project lab reports are required. The first report may be re-submitted for a higher score. Additional reports may be used to replace low scores.

Lab notebooks: Students are expected to maintain a detailed laboratory notebook where objectives, results, and conclusions (successes and failures) of experiments are recorded. Lab notebooks may be reviewed midway during the semester. Up to ~10% of the grade may be based on lab notebooks.

Grading

Lac and GFP genotype tests & predictions	due September 11	20
Lab report 1 -- Mutagenesis with EMS	due September 27	100
Undergraduate students: Mini-project outline	Preliminary, Sept 27 Final, October 18	50
Graduate students: Project protocol	Preliminary, Sept 27 Final, October 18	(100)
Lab report 2 -- Transposon mutagenesis	October 25	100
Mini-project lab report	December 14	200
Project presentation	December 11	50
Lab notebook and lab maintenance		50
Total		570/(630)

Lab reports: Guidelines for lab reports are included in the lab manual as are questions specific to each experiment. Target dates for lab reports are shown in the schedule. *All lab reports are due by the last day of the semester. Late assignments will be marked down progressively.*

Grades: 90-100% =A, 80-90% =B, 70-80% = C, 60-70% = D, less than 60%=F. Grades of A^+, A^-, B^+, B^-, C^+, C^-, D^+, and D^- will be used, at the discretion of the instructor, for borderline scores. 10% of the grade will be based on maintenance of clean and safe conditions in the lab.

Graduate students: are expected to submit a detailed experimental protocol as described above, take the lead on an independent 'mini-project,' show a more comprehensive understanding of the material, and answer additional questions in lab reports.

Attendance: Students are required to attend the weekly discussion meetings. Advance notice of unavoidable absences is expected. Lab rooms will be accessible at all times for ongoing work.

Academic integrity: We operate under the principle of "academic integrity" expected at this university. UW System guidelines state: "*Students are responsible for the honest completion and representation of their work, for the appropriate citation of sources and for respect of others' academic endeavors*." (s. UWS 14.01, Wis. Adm. Code). Cheating or obstruction of the efforts of others will not be tolerated in any form. Students caught cheating will receive an F grade on the exam or assignment and may be subject to further disciplinary action. ***Note in particular that this honor system applies during take-home exams and assignments. Please do not be tempted to represent the work of others as your own. This constitutes cheating (plagiarism) and will be treated as described above.***

Tentative Schedule

The starting dates of experiments or 'mini-projects' are shown. Mini-projects as well as experiments will typically take several weeks to complete and consist of several sequential components. These experiments do not require constant attention and it may be possible to perform parts of more than one concurrently with the aid of your lab partners. The actual schedule will depend on class and individual interest. I encourage students to seek me out to discuss the progress of experiments and interpretation of results.

Week	Experiment	Description
0 Sept. 4>	*E. coli* strain tests & introduction	**Lac and GFP genotype tests & predictions.** Tests and confirmation of *E. coli* strains and reagents for the EMS mutagenesis experiment
0-1 Sept 9>	**1. (required)** **(**handout & Miller-3, p 135)	**Mutagenesis with EMS.** Mutagenesis of an *Escherichia coli* strain expressing a jellyfish green fluorescent protein (GFP) to obtain auxotrophic, fluorescent, and lactose repressor mutants.
	2. (optional) (Miller-6, p157)	Screening for nonsense mutations. Use of nonsense suppressor strains of *E. coli* to screen for nonsense mutations in the lactose repressor gene.
2 Sept 23>	**3. (required)** **(**handout & Miller-27, p343)	**Transposon mutagenesis.** Use of transposon Tn10 (carried on a suicide bacteriophage) to obtain "knockout" mutations in *E. coli* plasmids carrying genes for the lactose operon and the green fluorescent protein (GFP).
3 Sept 27		**Preliminary mini-project outlines or protocols due** **Lab report #1 due**
3-4 Oct 7>		**Complete transposon mutagenesis experiment**
5 Oct 18>		**Mini-project outlines or protocols due** **Begin work on mini-projects:** *for example*
	3b. (mini-project option-1, see lab manual) TK-1	**Molecular analysis of transposons in plasmid DNA.** Isolation of plasmids containing transposon insertions. Restriction and possible sequence analysis to identify location of transposon insertion.
	4. (mini-project option-2) **(**Miller-28)	**Or, Transposon insertions near a gene of interest.** Use of the pool of transposon insertion from experiment 3 and bacteriophage P1 transduction to obtain a transposon tag near a gene of interest.
		Or, one of the mini-projects described above.
		Or, a student designed mini-project of your own. Design a protocol for an experiment that addresses a specific question in microbial genetics, or that would allow the construction of a useful strain or plasmid – then work on this project!
12		**November 27 – December 1, Thanksgiving break!**
8-14		Mini-projects and completion of work in progress
14 Dec 11		**December 11 – Mini-project presentations**
14 Dec 13		**Mini-project reports due**
Dec 13		**End of semester celebration, Fratello's!**