Biotechnology Lab (Bio-390/590, 2 credits)
Spring 2017

Place and time: Wednesdays 1:50 – 5:50.
Lab discussions: Wednesdays, 1:50 for ~ 30-50 minutes, HS 367
Lab rooms: Most of the lab work will be done in the HS 240 Biotechnology Lab & HS 163-145 Bioseparations-Proteomics Labs. Students will have access at all times via keypads.

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: M 1:50 – 2:50, Tu 3:30 – 5:30, Thu 11:30 – 12:30. 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 Labs). (S.A.F.E. trained – all students are welcome.)

Lab manual. Kallas, T. 2017. *Experiments in Biotechnology* – under revision. Pending revision, we will use protocols and flow charts for individual experiments, as well as the 2003 manual for reference as needed. These and a Lab Manual appendix of commonly used protocols are posted on D2L.

Other materials. Lab notebook -- *Very Important!* Hardcopy or electronic version for recording **objectives**, **results**, and **conclusions** of experiments. USB 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 ~$250.

Some Biotechnology/Molecular Genetics Lab References

1. Primrose, S. B. and Twyman, R. M. 2006, 2012. *Principles of Gene Manipulation and Genomics*. Blackwell, Oxford.
2. Sambrook, J and Russell, D. 2000. *Molecular Cloning: A Laboratory Manual* 3rd ed., Cold Spring Harbor Laboratory.
3. Many other resources are available via web sites such as those listed below.

Desire2Learn (D2L) Site. Materials including protocols, flow charts, and lab manual will be posted on the class D2L site (**Biotechnology Lab, Bio-390/590**). The lecture course D2L site (Bio 389/589) will also be available to lab students.

Biotechnology 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. **Human Genome Research Institute**: http://www.genome.gov/
10. **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).
11. **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.)
12. **SWISS-PROT**, University of Geneva, Switzerland: http://expasy.hcuge.ch/sprot/sp-docu.html (Site from which to download the Swiss-PDB viewer program for protein 3D structures).
13. **Frontdoor to PROTEIN EXPLORER**: http://molvis.sdsc.edu/protexpl/frntdoor.htm (Site for online use of the PROTEIN EXPLORER program for protein structure viewing & manipulation).
14. **SINCRIS** information server for crystallography: http://www.lcmp.jussieu.fr/sincris-top/ (A nice site for information and access to programs and databases for viewing and manipulating biomolecules).
15. **Webcutter** (a site for on-line restriction site analysis): http://www.firstmarket.com/cutter/cut2.html
16. **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
17. **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).
18. **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).
19. **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.)
20. ***Nature Biotechnology*:** http://www.nature.com/nbt/, (available on-line via Polk Library)
21. ***Trends in Biotechnology***: http://www.trends.com/tibtech/default.htm (via Science Direct, Polk Libr.)
22. **New England Biolabs**, Restriction Enzyme Database (NEB-REB): http://rebase.neb.com.
23. **UW-O (Polk) Library**: http://www.uwosh.edu/library/ (Polk Library provides access to a variety of literature search databases and carries on-line, full-text subscriptions to several relevant journals including *Science, the Nature Journals* (including *Nature* and *Nature Biotechnology, Trends Journals* via Science Direct, and the *American Chemical Society (ACS) Journals*. Follow on-screen instructions or see me.

Course Overview and Learning Objectives

To gain practical, 'hands-on' experience in techniques of microbial cell culture, product recovery, gene manipulation, and data analysis that are fundamental to many areas of biotechnology. 2) To gain experience in critical thinking and experimental design that may be used to address interesting questions and achieve practical objectives in biology or biotechnology. Topics will include work on aspects of molecular databases and DNA sequence analysis; DNA amplification by polymerase chain reaction (PCR); gene cloning and assembly of genetic constructs; gene expression analysis, fermenter culture; protein production, purification, and analysis; and possible DNA sequencing. During the latter part of the semester, students work on 'mini-projects' of choice that will be pursued in greater depth. In the 'mini-projects,' students address a question in biotechnology or devise a procedure that may lead to development of a useful microbial strain, bio-product, or process. Students may choose mini-projects from a list of topics or design projects related to specific areas of interest.

Graduate students will be expected to devise a more detailed experimental protocol that could be used to develop a useful microbial strain, produce a useful bio-product, or answer a specific biological question by means of a 'biotechnology strategy.' All students are expected to submit an outline of major steps to be performed and materials needed for their mini-projects.

Experiments and projects. We will begin the semester with an experiment to characterize a plasmid, pOSH37/GFP (which encodes an engineered 'fusion' protein, TRX-GFP-ISF, that contains segments of the proteins thioredoxin, the jellyfish Green Fluorescent Protein, and an iron-sulfur protein). The plasmid will be introduced by electroporation into a bacterial expression strain (*E. coli* AD494-DE3) to 'overproduce' the fusion protein. *E. coli* ScarabXpress-T7lac (www.scarabgenomics.com) may be used as an aternative expression host, which is advertised as a leaner and 'sportier' strain for protein expression. After plasmid and strain characterization, we will work on experiments to express (produce), purify, and analyze the protein by gel electrophoresis (SDS-PAGE) -- and possibly by mass spectrometry if our mass spectrometers are back on-line. The GFP tag provides a nice visual (fluorescent!) marker for tracking the protein.

Undergraduate Grading and Requirements

Students will work collaboratively in groups of two or three. ***Master's students or advanced undergraduates may serve as team leaders/mentors for less experienced undergraduates.*** Requirements are listed below. Much of the grading will be based on lab reports handed in individually by each student as described below. The main criteria for lab report grading will be progress toward successfully meeting objectives, clarity of presentation, and understanding of procedures and concepts. Two small and one major (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. Notebooks may be reviewed midway during the semester. 10% of the grade may be based on lab notebooks.

Undergraduate Assignments and Grading

Assignments	**Due date**	**Points**
Plasmid analysis warm-up exercise	February 10	20
Lab report 1 -- Transformation of *E. coli* with plasmid pOSH37/GFP and restriction analysis	February 24	100
Genome analysis, gene expression, gene fusion exercise (the same assignment may be used in both the lecture and lab sections)	March 1	50
Mini-project topic	March 8	10
Mini-project outline and provisional protocol	March 17	50
Lab report 2 -- Expression and affinity purification of a GFP fusion protein from plasmid pOSH37/GFP	March 29	100
Mini-project lab report	May 10	200
Project presentation	May 10	50
Lab notebook and lab maintenance		50
Total points		630

Lab reports. Guidelines for lab reports are included in the lab manual as are questions specific to the pre-planned (non-project) experiments. 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.

Attendance. All students are required to attend the weekly discussion meetings. Lab rooms will be accessible at all times for ongoing work. Students intending to work evenings or weekends need to obtain an after-hours pass from the Biology Office.

Academic integrity. Interaction and collaboration among students is encouraged but each student is responsible for submitting his/her own work. Students should be aware of the principle of "academic integrity" expected at this and other universities. The 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."* Cheating or obstruction of the efforts of others will not be tolerated in any form. Students caught cheating will receive an F grade and may be subject to further disciplinary action. ***Note in particular that this honor system applies during take-home assignments. Please do not be tempted to represent the work of others as your own.***

Additional Information and Requirements for Graduate Students

Graduate students are expected to complete all assignments required of undergraduates as well as additional assignments as described below.

Graduate Student Assignments and Grading

Assignments	**Due date**	**Points**
Plasmid analysis warm-up exercise	February 10	20
Lab report 1 -- Transformation of *E. coli* with plasmid pOSH37/GFP and restriction analysis	February 24	120
Genome analysis, gene expression, gene fusion exercise (the same assignment may be used in both the lecture and lab sections)	March 1	60
Mini-project topic	March 8	10
Mini-project outline and provisional protocol	March 17	50
Lab report 2 -- Expression and affinity purification of a GFP fusion protein from plasmid pOSH37/GFP	March 29	120
Graduate students report: 1) protein 3D structure analysis exercise or 2) mass spectrometry to identify the TRX-GFP-ISF fusion protein or another protein of interest (if mass spectrometers are available).	May 3	50
Mini-project lab report	May 10	200
Project presentation	May 10	50
Lab notebook and lab maintenance		50
Total points		730

Graduate Student Grading Policy. 92-100% =A, 90-92% =A-, 88-90% =B+, 82-90% =B, 80-82% =B-, 75-80% =C+. Grades of C or lower are considered failing for graduate students. Grades may be curved if necessary. Graduate students are expected to submit a detailed experimental protocol, submit an additional analysis assignment, take the lead on an independent 'mini-project,' show a more comprehensive understanding of the material, and answer additional questions on lab reports.

Graduate Student Objectives

1) Data Analysis and Critical Thinking. Special emphasis will be placed on the ability of graduate students to understand and interpret data and think analytically and critically about information necessary to understand and perform lab experiments. Graduate students are further expected to develop the ability to design experiments or develop procedures to address interesting problems in biotechnology or produce useful bio-products. To assess accomplishment of this objective, graduate students **will address additional questions in lab reports** and will **submit a detailed protocol describing the objectives, materials, and procedure they will use to address a problem in biotechnology or produce a useful bio-product.**

2) Clear and Logical Expression in Writing. Graduate students will be expected to write more clearly, logically and correctly than undergraduates. To assess accomplishment of this objective, **graduate students will be held to a higher standard on lab reports.** Their lab reports will be in the form of short, scientific papers that are clearly written and describe objectives or hypotheses tested, results found including necessary data tables and figures, and conclusions drawn. They will also **conduct an additional assignment and submit an additional report on either protein 3D or global gene expression analysis**. These assignments should show a more detailed understanding of the subject than those by undergraduates.

3) Clear and Logical Oral Presentation. Graduate students will be expected to clearly and logically present the objectives, results, and conclusions of their mini-project in an oral presentation. This objective will be **assessed by the quality of the graduate student oral presentation** of their mini-project research at the end of semester. This presentation should be well organized and logically presented. Graduate students are expected to show a deeper understanding of the topic presented, field questions, provide thoughtful answers, and in general will be held to a higher standard in their presentations than will undergraduates.

4) Ability to Synthesize Information and Develop Creative Work. Graduate students will be expected to use information discussed and experiments performed in the lab class, and/or found in the scientific literature, to synthesize and develop new and creative work (e.g. genetic strategies) to tackle important and interesting questions in biology. To assess accomplishment of this objective, **graduate students will develop a detailed experimental protocol** describing the objectives, materials, and procedure to be used to address a problem in biotechnology as described above. The ability to synthesize information and develop creative work **will be further evaluated in their oral presentation of the mini-project.**

5) Leadership. Graduate students will be expected to take a leadership role in the laboratory class. They will be **expected to lead a lab group and mentor undergraduate partners**, participate actively in class discussions, ask questions and show a deeper understanding of the concepts and procedures involved. They will be expected to **help undergraduates understand often difficult concepts and laboratory procedures.** To assess accomplishment of this objective, I will keep track of graduate student leadership and work with undergraduates. Assessment of such leadership activities may elevate or lower the final grade of graduate students, particularly those at the borderline between grades.

Some Mini-Project Options – Undergraduate and Graduate Students

1. Genome analysis, gene-construction, and protein expression

Objective: To express a gene of interest from a genome sequence database (e.g. cyanobacterial or *E. coli* databases). Students may choose a gene for a protein of their choice, design PCR primers for amplification of these genes, order the primers, use these primers to amplify the gene, clone the PCR product into a plasmid vector (e.g. pET32a, Novagen, Madison, WI), and express the cloned gene to produce the desired protein. If the cloned gene is fused to the thioredoxin (TRX) gene on plasmid pET32a, metal-chelating affinity chromatography may be used to purify the fusion protein (if it is soluble), and the protease thrombin may be used to separate the protein of interest from the thioredoxin affinity tag. Students may further characterize the recombinant plasmids by sequencing and analysis of the protein product. ***All students are expected to complete the database/bioinformatics/gene construct design segment of this project (Genome exercise 1).***

2. Overproduction and purification of recombinant *Thermus aquaticus* or *Pyrococcus furiosus* thermal-stable DNA polymerases

Objective: To grow a fermenter culture of an *E. coli* strain that has been engineered to overproduce one of these DNA polymerases, induce expression of the plasmid-borne polymerase gene, purify by FPLC (Fast Protein Liquid Chromatography), and test the polymerase in a PCR reaction. The purified protein may be characterized in a variety of ways including MALDI mass spectrometry (if available).

3. Possible 'RNA-Seq' analysis of cyanobacterial global gene expression

Objective: To discover genes and regulatory elements involved, for example, in adaptation to specific growth, environmental, or bioproduction conditions. This is a possible option depending on availability of funds. Further information will be provided if this option becomes available.

'RNA-Seq' Example (Isoprene bioproducts): Matt Nelson has introduced codon-optimized isoprene synthase (*IspS*) and IDI isomerase (*IDI*) genes into *Synechococcus* 7002 and engineered this strain to produce isoprene, a valuable feedstock chemical for synthetic rubber and liquid biofuels. This strain produces isoprene at a high rate but grows slowly. It would be interesting to investigate the impact of *IspS-IDI* transgenes (foreign genes) on global gene expression in the *Synechococcus (IspS-IDI)* strain. These data could identify genes that are upregulated in response to isoprene. The promoter regions of these genes could then be used to obtain inducible, high-level expression of isoprene synthase and other genes to maximize isoprene production. The first steps in these experiments – the rapid isolation of high-quality RNA – are the same as those for RT-qPCR. Students have successfully done microarray global gene-expression experiments in the past. The feasibility of 'RNA-Seq' experiment depends on time commitment and availability of funds.

4. Reverse-transcriptase, quantitative PCR (RT-qPCR) analysis of gene expression. Objective: To investigate the expression levels of selected *Synechococcus* genes as a function of growth or environmental conditions, or in bioproduction strains. We have a nice instrument (ABI StepOne) for Real-Time quantitative PCR (qPCR), quantitative detection of DNA sequences or RNA transcripts. RT-qPCR is often used to confirm selected global gene expression data or independently for detailed analysis of selected genes. We would select a few *Synechococcus* genes for detailed analysis. 'TaqMan' probes (to be discussed) would be used for these qPCR experiments. *(Several students in previous Biotech labs have done nice RT-qPCR projects to investigate selected gene expression patterns in cyanobacteria.)*

Example: Bioproducts - connections, RT-qPCR analysis of MEP pathway genes:

Objective: As mentioned, we have introduced *IspS* and *IDI* genes into *Synechococcus* PCC 7002 cyanobacteria to produce isoprene. The precursor for isoprene, dimethylallyl diphosphate (DMAPP) is synthesized via the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. Thus conditions that increase MEP pathway activity should increase isoprene production. This project would involve RNA extractions from *Synechococcus* and 'TaqMan' primers and probes in RT-qPCR experiments to track the expression levels of selected MEP pathway genes under different growth or bioproduction conditions. For example *Synechococcus* 7002 grows rapidly at high light intensity (full sunlight) and 100% CO_2. We will have two, 0.5 Liter, Phenometrix ePBR photobioreactors that can be programmed for day-night and temperature cycles from which samples could be extracted for RT-qPCR analysis.

MS student Rhiannon Carr has recently developed a simplified protocol for RNA extractions.

5. Metabolic engineering of cyanobacteria for increased isoprene-terpene production.

Objective: To increase carbon capture and carbon flow into the MEP pathway for increased isoprene-terpene production. One strategy is to increase the copy number of native genes, or genes from other organisms, or synthetic genes for carbon capture and the MEP pathway leading to isoprene and terpenes. For example:

- Introduce added genes RubisCo *rbcLXS* genes for carbon fixation into a chromosomal site in *Synechococcus* PCC 7002 or in the ultra-fast growing *Synechococcus* UTEX 2973 strain.
- Introduce added MEP pathway genes, such as the *DXS* gene in *Synechococcus* PCC 7002 or *Synechococcus* UTEX 2973.

6. Cyanobacterial knockout mutants to increase bioproduct production.

Objective: To investigate the impacts of mutations in metabolic pathway or regulatory genes on electron transfer reactions, gene expression, and/or bioproduct production. Students in recent Microbial Genetics or Biotech labs have constructed 'knockout' mutations of cyanobacterial electron transport, metabolic, or regulatory genes. Such mutations provide insights into solar energy conversion and metabolic pathways of cyanobacteria, which will be important for engineering bioproduct production in these organisms. These projects might involve genetic construction of mutant cyanobacteria or characterization of existing bio-engineered cyanobacteria with respect to impacts on electron transfer reactions (with the BioLogic JTS-10 spectrophotometer), gene expression (by RT-qPCR), or the production of bioproducts such as isoprene (tested by Gas Chromatography – Mass Spectrometry or a real-time, Fast Isoprene Sensor).

Examples: Several interesting and efficient PCR-based (e.g. Frigaard et al., 2004 *Methods in Molec. Biol.*) and 'Gibson Assembly' (Gibson and Russello, 2012 *NEB Expressions*) methods are available for generating mutations and gene modifications. Examples of interesting mutants that have been generated by previous students (or are in progress) are: YrdC, GlgA1, GlgA2, GlgC, SpsA, ApcF, and CpcB. YrdC is defective in a 'YrdC-like' regulator of CO_2 uptake and grows very poorly at low CO_2. GlgA1 and GlgA2 are defective in synthesizing the major glycogen carbohydrate storage polymer of cyanobacteria. GlgC is defective in glycogen and soluble sugar synthesis. SpsA cannot synthesize sucrose. ApcF and CpcB are defective in a light-harvesting proteins. For various reasons, all of these mutants may increase the yield of bioproducts such as isoprene.

There are many other knockout mutations that would be interesting to make and test in *Synechococcus* PCC 7002 or in the ultra-fast growing *Synechococcus* UTEX 2973 cyanobacteria.

7. Gene regulation to control the expression of potentially toxic bioproducts genes.

Objective: To investigate the use of regulated promoters to control gene expression for increased bioproduct production. Projects might involve constructing plasmids where a promoter region of potential interest is linked to a 'reporter' gene such as the gene *YFP* gene for a yellow fluorescent protein (YFP). Promoter activity can then be assessed by the intensity of YFP fluorescence.

- For example, a temperature-regulated bacteriophage regulator-promoter (cI857-pR) is a gene regulator that MS student Meghan Raebel has pursued. Meghan has linked the cI857-pR regulator both to a *YFP* reporter gene and to a *DXS* gene for a MEP pathway enzyme. Cyanobacteria that carry these genetic constructs show approximately a 4-fold increase in YFP expression at 40°C vs 32°C. Those that carry the regulated *DXS* gene together with isoprene production genes show

approximately a 3-fold increase in isoprene production at the high temperature. Further work with the cI857-pR regulator could involve random mutagenesis to increase the level and dynamic range of gene expression – as measured by YFP fluorescence.

- Another interesting promoter and regulatory gene is a nickel (Ni) regulated promoter. We have a plasmid (pNF1609, Cheah…Peebles, 2013 *Biotechnol. Progress*) that carries a Ni-regulated *nrsRS-PnrsB* regulator-promoter sequence. This could be amplified by PCR and joined to a *YFP* reporter or a potentially toxic gene such as the *DXS* gene.

8. Synthetic genes for β-pinene synthesis in cyanobacteria.

Objective: To achieve regulated expression of β-pinene, a precursor for jet fuel. A problem is that β-pinene, at some levels, is toxic to the cyanobacteria (work of former honors student Valerie Wagner). A set of codon-optimized genes (GPPS and mono-TPS) has been designed and integrated into *Synechoccoccus* 7002 cyanobacteria to enable β-pinene synthesis (work of MS student Rhiannon Carr). These genes are strongly expressed at the mRNA level but pinene production has not been detected. Further investigation is needed and a possible solution might be to control the expression of these genes from a regulated promoter. Ideas along these lines could be the objectives of this project.

9. Characterization of ultra-fast growing *Synechococcus* UTEX 2973 cyanobacteria and metabolic engineering of isoprene production.

Objective: To understand growth, metabolic activity, and the potential for bioproducts production in UTEX 2973. The ultra-fast growing *Synechococcus* UTEX 2973 cyanobacteria have a doubling time of ~2 hours in contrast to a doubling time of ~4 hours in *Synechococcus* PCC 7002 (previously one of the fastest growing cyanobacteria). Thus UTEX 2973 should produce 8-times as much biomass (and potentially 8-times as much bioproduct) in a 12-hour period relative to *Synechococcus* 7002. Further, UTEX 2973 can form colonies in 2 to 3 days relative to 7 to 14 days for *Synechococcus* 7002. This is a potentially huge advantage for metabolic engineering, CO_2 capture, and bio-products production.

- Growth characteristics can be investigated with two, 0.5 liter Phenometrix ePBR photobioreactors that can be programmed for day-night, temperature, and light intensity cycles with automated cell density measurements.
- Isoprene production can be engineered by using PCR and Gibson Assembly methods to target e.g. *IspS* and *IDI* genes (ones that were initially designed for *Synechococcus* 7002, or genes specifically optimized for UTEX 2973) into chromosomal neutral sites in *Synechococcus* UTEX 2973. One challenge is that UTEX 2973 cannot be readily transformed by electroporation, and thus genes need to be introduced by tri-parental mating (conjugation), which is very efficient but requires extra steps.
- Isoprene production would be measured by GC-MS or with a real-time, Fast Isoprene Sensor.

(Undergraduate students Andy Dorner and Kyle Kettner worked on aspects of this project and Kyle is continuing some of this in his honors thesis.)

10. CRISPR/Cas9 modifications of cyanobacterial genes.

Objective: To develop or test CRISPR/Cas9 tools for efficient, 'markerless' modifications of genes in the cyanobacteria *Synechococcus* UTEX 2973 or PCC 7002. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and the CRISPR-associated (Cas) gene 9 for a sequence-specific

endonuclease have been developed as remarkably efficient tools for 'genome editing' (i.e. genome modifications) in bacteria, archaea, and eukaryotes including cyanobacteria (Wendt et al. 2016). This and other journal articles will be posted in D2L for reference. The primary goal of a biotech lab project would be to implement a CRISPR/Cas9 strategy to create a 'markerless' genetic modification (e.g. a knockout mutation) in a cyanobacterium or other organism of interest.

Additional mini-project options are encouraged! Further options will be described in a 'Mini-Project Ideas' handout to be discussed later.

Tentative Schedule – Undergraduate and Graduate Students

Tentative schedules of are shown for initial experiments that will be done by the entire class. The latter part of the semester will be devoted to individual or group mini-projects. Experiments will usually take several weeks to complete and consist of several sequential steps. Most experiments do not require constant attention and it may be possible to perform some parts concurrently with the aid of 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 data interpretations.

Week	Experiment or Project	Components
1 - 2	**Experiment 1: Transformation of *E. coli* with plasmid pOSH37/GFP and restriction analysis** Introduction of plasmid pOSH37/GFP into *E. coli* expression hosts. Selection of transformants by antibiotic resistance and green fluorescence. Confirmation of plasmid identity by plasmid isolation and restriction analysis and PCR. The *E. coli* (pOSH37/GFP) strain produced in this experiment will be used to produce the GFP fusion protein to be purified in Exp #2.	- Plasmid electrotrans-formation of *E. coli* - Plasmid isolation and analysis by restriction digestion, PCR, and gel electrophoresis
2 - 4	**Genome analysis, gene-expression exercise.** DNA sequence analysis and *in silico* plasmid construction via sequence databases and Gene Construction Kit software. Select a gene for a protein of interest. Design PCR primers to construct a plasmid that carries the gene and may be used to express its protein product. **Project options:** Design and order PCR primers, amplify and clone the gene and express the encoded protein. Construct the recombinant plasmid and analyze by restriction digestion, PCR, and possible DNA sequencing. Possible further analysis of expressed proteins by gel electrophoresis (SDS-PAGE) and MALDI or ESI mass spectrometry.	- Internet databases - primer design - Gene Construction Kit (GCK) software - PCR amplification - Gene cloning - Restriction analysis - Gene expression - SDS-PAGE analysis of protein production - DNA sequencing
4 - 7	**Experiment 2: Expression and affinity purification of a GFP fusion protein from plasmid pOSH37/GFP.** Culture of *E. coli* (pOSH37/GFP, from exp #1 above), gene expression,	- Cell culture - Gene & protein expression - Cell harvest & breakage

	production of the fluorescent GFP fusion protein, affinity purification, thrombin cleavage to separate the iron-sulfur protein segment, analysis by gel electrophoresis (SDS-PAGE), possible immuno-blotting, and possible MALDI or ESI mass spectrometry.	- Affinity chromatography - Thrombin cleavage - SDS-PAGE (possible immunoblot)

7 - 14	**Mini-projects**	
14	**Project presentations – May 10**	
10 -14	Complete work in progress **End of semester celebration at Fratello's! (May 12)**	