Biotechnology lab (Bio-390/590, 2 credits)
Spring 2012

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 & the 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 & Th 3:30 – 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 manual. Kallas, T. 2012 (under revision). *Experiments in Biotechnology*. UW Oshkosh. Pending revision of the lab manual, we will use the 2003 Lab Manual and supplementary protocols as needed. These and the 2003 Lab Manual appendix of commonly used protocols are posted on the D2L site.

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.

Some useful Biotechnology lab references

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

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. Twyman, R. M. 2004. *Principles of Proteomics.* Bios Scientific.
4. Veenstra, T. D. and Yates, J. R. 2006. *Proteomics for Biological Discovery.* Wiley.

Desire2Learn (D2L) Site. Materials including the lab manual and supplementary protocols will be posted on the class D2L site (**Biotechnology Lab, Bio-390/590**). The lecture course D2L site (Bio 390/590) will also be accessible to lab students. To access, go to the UW Oshkosh home page, > then click, "D2L, Desire2Learn." On the **D2L** login page, enter the username and password that you use for UW Oshkosh e-mail.

Some 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. (Includes the database for the genome sequence of the cyanobacterium *Synechocystis* PCC 6803.)
9. ***E. coli*** **Genome Center**: http://www.genetics.wisc.edu:80/index.html
10. **Human Genome Research Institute**: http://www.genome.gov/
11. **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. **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).
14. **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).
25. **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).
16. **Webcutter** (a site for on-line restriction site analysis): http://www.firstmarket.com/cutter/cut2.html
17. **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
18. **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).
19. **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).
20. **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.)
21. ***Nature Biotechnology*****:** http://www.nature.com/nbt/, (available on-line via Polk Library)
22. ***Trends in Biotechnology***: http://www.trends.com/tibtech/default.htm (via Science Direct, Polk Libr.)
23. **New England Biolabs**, Restriction Enzyme Database (NEB-REB): http://rebase.neb.com.
24. **Promega** Corporation (Madison, WI): http://www.promega.com/.
25. **UW-O (Polk) Library**: http://www.uwosh.edu/library/ (Polk Library provides access to a variety of literature databases such as Medline and Web of Science 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 objectives

To gain practical, "hands-on" experience in some of the techniques of cell culture, product recovery, *in vitro* manipulation of genes, and data analysis that are fundamental to many areas of biotechnology. 2) To gain experience in critical thinking and experimental design to address interesting problems in biology or biotechnology. Topics include: analysis of DNA sequence databases, DNA amplification by polymerase chain reaction (PCR), gene cloning and expression techniques, cell culture, protein production and purification, DNA and protein gel electrophoresis, protein identification by MALDI-TOF (matrix assisted laser desorption ionization, time of flight) and/or ESI (electrospray ionization) LC-MS/MS (liquid chromatography, tandem) mass spectrometry, and possible DNA sequencing. 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. Students are expected to devise an experimental protocol related to their mini-project.

Experiments & projects. We will begin the semester with an experiment to introduce a plasmid (pOSH37/GFP, which encodes an engineered 'fusion' protein containing parts of the proteins thioredoxin, the jellyfish Green Fluorescent Protein, and an iron-sulfur protein), into a bacterial expression strain (*E. coli* AD494(DE3)) so that we can 'overproduce' the fusion protein. We will also introduce the same plasmid into another *E. coli* host strain (ScarabXpress-T7lac, www.scarabgenomics.com) so that we can compare the expression of the encoded TRX-GFP-ISF fusion protein in these two hosts. ScarabXpress-T7lac is supposed to be a leaner and 'sportier' strain for protein expression. We will then purify and analyze the protein, by protein gel electrophoresis (SDS-PAGE) and MALDI or ESI mass spectrometry. The GFP tag provides a nice visual (fluorescent!) marker for tracking the protein. Subsequently, students will work on one of the following projects or a project of their own design. Additional miniproject options will be provided separately.

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 (pET32a, Novagen, Madison, WI), and express the cloned gene to produce the desired protein. If the cloned gene has been translationally 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 attempt to characterize the recombinant plasmids by sequencing and by analysis of the protein product. ***All students are expected to complete the computer/database/bioinformatics segment of this project.***

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

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

3. Oligonucleotide array analysis of cyanobacterial global gene expression

Objective: To discover genes and regulatory mechanisms involved in redox signaling, responses to oxygen radicals, adaptation to changing environments, or other parameters. Through an arrangement with the UW Madison Biotechnology Center and NimbleGen, we have designed and synthesized 4-plex oligonucleotide arrays for the marine, coastal cyanobacterium *Synechococcus* PCC 7002. Each slide contains four 0.5 x 1.0 cm microarrays each containing ~72,000 probes. In **array design 1**,

most of the ~3000 genes in the *Synechococcus* genome are covered with 7 probes repeated three times on each array. In addition there are ~6000 high-density "tiling" probes covering upstream untranslated (UTR) regions of ~200 genes of interest for the purpose of mapping transcription start sites. In **array design 2**, most genes in *Synechococcus* are covered with 3 probes repeated three times on each array, but high-density UTR probes are included for all of the predicted genes.

Example 1: RNAs might be isolated from wild type *Synechococcus* and one or two electron transfer mutants grown under selected conditions. Mutants PetB-R214H and PetC1-Δ2G both slow electron transfer but only PetB-R214H overproduces oxygen radicals. Another mutant, NdhF, is defective in NAD(P)H dehydrogenase. These RNAs would be converted to cDNAs, labeled, and hybridized against the 4-plex microarrays. Subsequent work would then involve exploring computer programs such as ArrayStar and/or Bioconductor "R" to analyze these data to pick out sets of genes that are differentially regulated in the mutants versus the control. *(RNA extractions for some of these experiment have been performed by students in recent Microbial Genetics and Biotech labs and students -- and beautiful data have been obtained.)*

Example 2: Matt Nelson has introduced isoprene synthase (*IspS*) and IDI isomerase (*IDI*) genes into a *Synechococcus* strain and has gotten 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 very interesting to investigate global gene expression in the *Synechococcus (IspS-IDI)* strain.

4. Reverse-transcriptase, quantitative PCR (RT-qPCR) analysis of gene expression.

Objective: To investigate the expression levels of a few selected *Synechococcus* genes as a function of environmental conditions or in mutants. We now have a very nice instrument (ABI StepOne) for Real-Time quantitative PCR, which allows the quantitative detection of DNA sequences (e.g. from medically or environmentally important samples) or RNA transcripts. RT-qPCR is often used to confirm selected microarray gene expression data or independently for the detailed analysis of selected genes. We would select a few *Synechococcus* genes, perhaps in conjunction with the preliminary microarray data, for detailed analysis. This experiment might include a comparison of SyberGreen dye and "TaqMan" probes (to be discussed) for real-time PCR. *(Several students in the Spring 2009 Biotech lab did nice RT-qPCR projects to investigate selected gene expression patterns in cyanobacteria shifted from optimal photosynthetic conditions to dark anaerobic conditions.)*

5. Biofuels connections, RT-qPCR analysis of MEP pathway gene expression.

Objective: To investigate the expression levels of selected 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway genes in the cyanobacterium *Synechococcus* sp. PCC 7002. As mentioned, we have introduces *IspS* and *IDI* genes into this cyanobacterium to produce isoprene. The precursor for isoprene, dimethylallyl diphosphate (DMAPP) is synthesized via the MEP pathways. Thus conditions that lead to increased MEP pathway activity might lead to increased isoprene production. This project would involve RNA extractions from *Synechococcus* and "TaqMan" primers and probes in RT-qPCR reactions to track the expression levels of selected MEP pathway genes under different growth conditions. For example *Synechococcus* 7002 grows rapidly at high light intensity (full sunlight) and 100% CO_2 as well as under more moderate conditions.

6. Isotope-assisted mass spectrometry and protein expression analysis.

Objective: To discover proteins and regulatory mechanisms involved in redox signaling, responses to oxygen radicals, or adaptation to environmental stresses such as exposures of cyanobacteria to high-light intensity. Understanding of these will be important for biofuels applications. This experiment might complement microarray experiment such as those described above. It would provide information about proteins that are differentially expressed in experimental versus control cyanobacteria. This is interesting because proteins do most of the actual work of cells and gene expression levels do not always reflect protein levels in cells. The experiment involves growing control and mutant cyanobacteria separately in media containing light (N^{14}) and heavy (N^{15}) isotopes of

nitrogen, harvesting and mixing these cultures, breaking and fractionating the cells, digesting the mixed proteins with trypsin, and subjecting the protein fragments (peptides) to liquid chromatography and tandem mass spectrometry (LC-MS/MS). For reasons that will be explained, this allows both the identification of individual proteins from complex mixtures of proteins, and quantification of relative protein expression levels. *(This work was successfully initiated by students in the 2008 Biotech lab.)*

7. Inhibitors and photosynthetic electron transport pathways.

Objective: To investigate the impacts of recently synthesized inhibitors of photosynthesis on electron transfer reactions and gene expression responses in cyanobacteria. Several students have been involved in the synthesis and preliminary characterization of inhibitors of photosynthesis (collaboration with Drs. Brant Kedrowski and Linfeng Xie, Chemistry). These inhibitors (abbreviated NQNO, 4-1HQ, and iso-4-1HQ) act on sites within the cytochrome *bf* electron transfer complex of photosynthesis, which is involved in solar energy conversion and redox signaling reactions. Lab projects might include investigating the impacts of these inhibitors on electron transport (with our BioLogic JTS-10 kinetics spectrophotometer) and on gene expression via RT-qPCR or microarrays.

8. Cyanobacterial mutants, electron transport activities, and gene expression.

Objective: To investigate the impacts of mutations in cyanobacterial electron transport or regulatory genes on electron transfer reactions and gene expression by RT-qPCR or possible microarrays. Several students in the 2010 - 2011 Microbial Genetics Labs have constructed or are working on "knockout" mutations of cyanobacterial electron transport or regulatory proteins. These mutations may provide insights into energy conversion pathways and adaptation of cyanobacteria to e.g. high light intensity, which will be important for solar-energy driven production of biofuels or bioproducts in these organisms. These projects might involve completing the genetic construction of the mutant cyanobacteria, characterizing the impacts on electron transfer reactions with the BioLogic JTS-10 spectrophotometer, and investigating impacts on gene expression by RT-qPCR or microarrays.

Two such interesting mutants are SdhB (not yet completed) and YrdC (completed by Justin Zangl). SdhB will be defective in succinate dehydrogenase, which may have an important roles in electron transfer pathways. YrdC is defective in a 'YrdC-like' regulator of CO_2 uptake and grows extremely poorly at low CO_2.

9. More biofuels connections, mutations that may increase isoprene production.

Objective: To investigate the impact of alternative, regulatable promoters, decreased light harvesting, and inactivation of competing carbon pathways on isoprene production. Several students in the 2011 Microbial Genetics Lab initiated these studies. One project (You Yang and Ehren Snyder) involves modifcation of a plasmid to allow easy 'swapping out' of promoter sequences to test for increased and regulatable expression of *Isps* and *IDI* genes for isoprene production. Another project involves inactivation of glycogen synthase *glgA1* (Brandon Thomas) and *glgA2* (Ola Aremu) genes to limit carbon flux into competing glycogen synthase pathways. A final project involves inactivation of an *apcF* gene (Devin Hundt) to knock out the phycobilisome light harvesting complex and thus allow cells to grow to a higher density and increase per volume production of isoprene. If any of these genetic modification projects are successful, it will then be interesting to test their impact on gene expression, isoprene synthase protein expression, and isoprene production.

Additional mini-project options are possible and encouraged! Further options are described in the lab manual and in the "mini-project ideas" 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 serve as team leaders/mentors for less experienced undergraduates.*** Requirements are listed below. Most of the grade will be based on lab reports handed in individually by each student as described below. The criteria for 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.

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.

Graduate students are expected to take the lead on an independent "mini-project" (such as those listed here or in the lab manual), show a more comprehensive understanding of the material, complete the additional requirements listed above, and answer additional questions in lab reports.

Assignments	**Due date**	**Points**
Report: Exp #1, Transformation of *E. coli* with plasmid pOSH37/GFP and restriction analysis	February 22	100
Genome analysis-gene expression exercise (the same report may be used in both the lecture & lab classes)	February 29	50
Report: Exp #2, Expression and affinity purification of a GFP fusion protein from plasmid pOSH37/GFP.	March 15	100
Graduate students: Are expected to 1) work on identifying the TRX-GFP-ISF fusion protein or another protein by mass spectrometry or 2) complete a protein 3D structure analysis exercise	April 26	(50)
Mini-project outline and provisional protocol	March 1	50
Mini-project lab report	May 10	200
Project presentation	week of May 10th	50
Lab notebook and lab maintenance (to be determined)		
Total (undergraduate/graduate)		550/(610)

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.***

Tentative schedule of experiments (Spring 2012)

The starting weeks of experiments or "mini-projects" are shown. "Mini-projects" as well as standard 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 or Project	Components
1	**Exp #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. 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 and gel electrophoresis
2	**PROJECT #1 (option): Genome analysis/gene-construction/expression.** Analysis of sequence databases via the internet. Selection of an open-reading-frame (gene) for a protein of interest. Design of PCR primers, amplification, cloning, and expression of the gene. Analysis of recombinant plasmids by restriction digestion and DNA sequencing. Possible analysis of expressed proteins by gel electrophoresis (SDS-PAGE) and MALDI or ESI mass spectrometry. ***(All students will work on the database analysis portion. The actual lab portion is a mini-project option.)***	- 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	**Exp #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, 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 immunnoblotting, and possible MALDI or ESI mass spectrometry.	- Culture - Induction of gene expression for protein overproduction - Cell harvest & breakage - Affinity chromatography (possibly by FPLC) - Thrombin cleavage - SDS-PAGE (possible

		immunoblot) - Possible MALDI or ESI mass spectrometry

Week	Experiment or Project	Components
6	**PROJECT #2 (option): Overproduction and purification of recombinant, thermal-stable DNA polymerases.** Fermenter culture of *E. coli* expressing *Thermus aquaticus* or *Pyrococcus furiousus* DNA polymerases, enzyme purification by FPLC chromatography, and testing of polymerase activity in a polymerase chain reaction (PCR). Possible protein analysis by gel electrophoresis (SDS-PAGE) and MALDI or ESI mass spectrometry.	- Fermenter culture - Enzyme purification by FPLC - Functional (PCR) assay for polymerase activity - *Production of a ton of valuable Taq DNA polymerase!*
6	**PROJECT #3 (option): Oligonucleotide array analysis of cyanobacterial global gene expression:** Described above.	- Cyanobacterial cultures - RNA isolation & DNA removal - cDNA synthesis & labeling - Array hybridization & scanning - Data analysis, bioinformatics, & statistics
6	**PROJECT #4 (option): Real-Time quantitative PCR analysis of gene expression (RT-qPCR)**	- Cyanobacterial cultures - RNA isolation & DNA removal - Design of primers and 'TaqMan' probes for selected genes - RT-qPCR experiments - Data analysis
6	**PROJECT #6 (option): Isotope-assisted mass spectrometry and protein expression analysis:** Described above.	- Cyanobacterial cultures grown in N^{14}- and N^{15}-NO_3 - Cell breakage & separation of membrane & soluble proteins - Trypsin digestion - LC-MS/MS - Data analysis, bioinformatics, & statistics
6	**PROJECT: Other mini-project options described above, in the mini-project handout, or student-designed mini-projects**	
9-14	Completion of work in progress ***End of semester celebration at Fratello's! (May 11)***	