

Systems Biology Strategies and Technologies for Understanding Microbes, Plants, and Communities

Analytical Strategies for the Study of Plants, Microbes, and Microbial Communities

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Towards an Understanding of Proteins that Govern the Structure and Function of *Synechocystis* sp. PCC6803 and Multiple *Cyanotheca* Strains

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Project Goals: The primary goal of this project is to apply a systems biology approach to understand the network of genes and proteins that govern the structure and function of *Cyanobacteria*. These microorganisms make significant contributions to harvesting solar energy, planetary carbon sequestration, metal acquisition, and hydrogen production in marine and freshwater ecosystems. *Cyanobacteria* are also model microorganisms for studying the fixation of carbon dioxide through photosynthesis at the biomolecular level. Importantly, this project addresses critical U.S. Department of Energy science needs, provides model microorganisms to apply high-throughput biology and computational modeling, and takes advantage of EMSL's experimental and computational capabilities.

In the initial phase of this project, proteomics characterizations of *Synechocystis* sp. PCC6803 and *Cyanotheca* sp. ATCC 51142 were performed to improve our understanding of the proteome makeup of these model organisms under normal and perturbed growth conditions. We have developed the most complete quantitative proteome analysis of *Synechocystis* sp. PCC6803 under various critical environmental perturbations applying a high sensitivity mass spectrometry approach spanning 33 physiological conditions. The resulting proteome dataset consists of 22,318 unique peptides,

corresponding to 2,369 unique proteins, covering 65% of the predicted proteins. Quantitative analysis of changes in protein abundance under environmental perturbations has led to the identification of the key proteins required for the maintenance of cellular fitness necessary for cell survival.

We also examined the impact of diurnal rhythms on the protein level of *Cyanotheca* 51142. We identified a total of 3,616 proteins with high confidence, which accounts for ~68% of the predicted proteins based on the completely sequenced *Cyanotheca* 51142 genome. About 77% of identified proteins could be assigned to functional categories. Quantitative proteome analysis uncovered that ~3% of the proteins exhibit oscillations in their abundance under alternating light-dark conditions. The majority of these cyclic proteins are associated to central intermediary metabolism, photosynthesis as well as biosynthesis of cofactors. Our data also suggest that diurnal changes in activities of several enzymes are mainly controlled by turnover of related cofactors and key players but not entire protein complexes.

While *Cyanotheca* sp. ATCC 51142 continues to represent a model organism for proteomics investigations, six additional *Cyanotheca* either have finished, or draft genome sequences. This number of strains, having genome sequences, allows for comparison of *Cyanotheca* at the level of the core genome and core proteome. While the core genome predicts the common phenotype of *Cyanotheca*, the core proteome represents the actual protein phenotype characteristic of *Cyanotheca*. As such, all strains were cultured in the laboratory under growth conditions best representing their natural environments and proteins extracted from cells have been analyzed by LC-MS/MS. Results from over 460 LC-MS/MS analyses have been used to develop proteomics databases for strains PCC8801, PCC8802, PCC7424, PCC7425, PCC7822, and ATCC51142. An additional proteomics database for ATCC51472 is pending on the completion of a draft genome sequence for this strain. The Proteomics database for the more distantly related *Synechosystis* sp. PCC6803 was also included in this analysis to better constrain the core proteome to represent *Cyanotheca*. While the core proteome of *Cyanotheca* is composed of a large percentage of proteins involved in energy production, translation, and amino acid production, a significant portion of the core proteome is also made up of proteins having no predicted function, or only a general assigned function. Of interest was the observation of hypothetical and conserved hypothetical proteins suggesting the importance of these proteins in defining the general lifestyle of *Cyanotheca*, yet also suggesting the need for additional functional characterization of these proteins to better understand *Cyanotheca* from a systems biology perspective.

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Deciphering Microbial Community Dynamics via Observational and Experimental 'Metatranscriptomics': Developments and Applications

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http://openwetware.org/wiki/DeLong_Lab

Project Goals: One of our central project goals is to develop and refine methods for studying microbial community gene expression in the environment, referred to here as 'metatranscriptomics'. There are a variety of diverse applications of these new metatranscriptomic methods. The approach can be used to verify that hypothetical ORFs identified in metagenomic projects are indeed transcribed and expressed. In surveys, 'observational metatranscriptomics' can be used to survey both the nature and abundance of different RNA species (including ribosomal RNA, messenger RNA, and small non-coding RNAs) existing within any given microbial community. Finally, 'experimental metatranscriptomics' can be leveraged to reveal transcriptional responses of microbial communities to environmental perturbation, as well as to discover the specific metabolic pathways involved in matter and energy cycling. In the course of developing methods and protocols for metatranscriptomics, we have explored many of these specific applications. We report here on a few examples, including the refinement of the metatranscriptomic protocols and analyses of biological and technical replicates, the discovery of new microbial small RNAs, and the identification of metabolic pathways involved in the turnover of high molecular weight dissolved organic matter (DOM) in the marine environment.

Development and quantitative evaluation of an rRNA subtraction protocol

Metatranscriptomes generated by pyrosequencing have great potential use for describing functional processes and attributes of complex microbial communities. Meeting this potential requires protocols that maximize mRNA recovery by reducing the relative abundance of ribosomal RNA, as well as systematic comparisons to identify methodological artifacts and test for reproducibility across datasets. We

developed a protocol for subtractive hybridization of small and large subunit RNAs using sample-specific probes, that is applicable across diverse environmental samples. To test the method, rRNA-subtracted and unsubtracted transcriptomes were pyrosequenced from several different bacterioplankton communities ocean, representing ~350 Mbp of metatranscriptomic data. The new subtractive hybridization method reduced bacterial rRNA transcript abundance by 40 to 58%, increasing recovery of non-rRNA sequences up to fourfold. To evaluate this method, we established criteria for detecting sequences replicated artificially via pyrosequencing errors and identified such replicates as a significant component (6 to 39%) of total pyrosequencing reads. Following replicate removal, statistical comparisons of reference genes (identified via BLASTX to NCBI-nr) between technical replicates and between rRNA-subtracted and unsubtracted samples showed low levels of differential transcript abundance (< 0.2% of reference genes). However, gene overlap between datasets was remarkably low, with no two datasets (including duplicate runs from the same pyrosequencing library template) sharing greater than 17% of unique reference genes. These results suggest that current levels of pyrosequencing capture a small subset of total mRNA diversity, underscoring the importance of rRNA subtraction to enhance sequencing coverage across the functional transcript pool.

Novel small RNAs revealed by metatranscriptomics

Previous metatranscriptomic studies have suggested that many cDNA sequences share no significant homology with known peptide sequences, and therefore might represent transcripts from uncharacterized proteins. We found that a large fraction of cDNA sequences detected in a metatranscriptomic datasets are comprised of well-known small RNAs (sRNAs), as well new groups of previously unrecognized putative sRNAs (psRNAs). These psRNAs mapped specifically to intergenic regions of microbial genomes recovered from similar habitats, displayed characteristic conserved secondary structures, and were frequently flanked by genes that suggested potential regulatory functions. Depth-dependent variation of psRNAs generally reflected known depth distributions of broad taxonomic groups, but fine-scale differences in the psRNAs within closely related populations suggested potential roles in niche adaptation. Genome-specific mapping of a subset of psRNAs derived from predominant planktonic species like *Pelagibacter* revealed recently discovered as well as potentially new regulatory elements. Our analyses show that metatranscriptomic datasets can reveal new information about the diversity, taxonomic distribution and abundance of sRNAs in naturally occurring microbial communities, and suggest their involvement in environmentally relevant processes including carbon metabolism and nutrient acquisition.

Metabolic pathways of DOM turnover revealed by metatranscriptomics

To better measure and model the microbial processes associated with the turnover of DOM in the sea, we performed metatranscriptomic analyses in experimental microcosms that were amended with DOM. High molecular weight DOM from surface waters of the North Pacific Subtropical Gyre near station ALOHA was concentrated by ultrafiltration

tion using a 1 nm pore membrane filter, and added to unfiltered seawater microcosms. The twenty liter microcosms were maintained at *in situ* temperatures and light intensities, and sampled periodically over the course of a 27 hour incubation period. In conjunction with metagenomic datasets obtained at the beginning and end of the experiment, samples for metatranscriptomic analyses were collected over the time course of the experiment in both the unamended control, DOM enriched sample. Subsequent analyses revealed the timing, potential biochemical pathways, microbial species, and potential organic carbon compound intermediates associated with HMW DOM degradation. The results suggested a successional cascade of microbial species related to stepwise metabolic transformations involved in microbially mediated oxidation of DOM in the sea.

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Identification Characterization of Methanosarcinaceae Cell Surface Proteins

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Project Goals: The goal of this research project is to gain a better understanding of the microorganisms that capture, store and mobilize energy, processes that occur naturally in the Earth's biosphere. We are investigating the molecular biology and biochemistry of several model methane producing archaea and associated hydrogen producing syntrophic bacteria. As participants of anaerobic food chains, they aid in the conversion of complex plant and animal polymers to a variety of small molecular weight carbon intermediates (e.g., alcohols, short chain fatty acids, and various aromatic compounds) to hydrogen, methane and carbon dioxide. However, still lacking is a clear definition of key metabolic pathways, energy conserving complexes, and cell architectures needed to use the above compounds for methane/hydrogen end product formation. We are developing tools to study these anaerobic microorganisms to assess and model hydrogen and methane production. This includes development of community resources for mRNA enrichment/sequencing methods for transcript analysis of model microbes, application of proteomic methods to further define the unique biology, and exploratory metabolomic studies to document the metabolic intermediates in these model microbes. Development and application of such tools will allow better assessment, and modeling of these poorly understood and underutilized hydrogen and methane producing microorganisms for future exploitation.

The cell envelopes of many archaeal species contain a proteinaceous lattice termed the surface layer or S-layer. It is typically composed of only one or two abundant, often post-translationally modified proteins that self-assemble to form a highly organized cell surface-exposed array. Little is known about these proteins in any methanogenic archaean. Surprisingly, over a hundred proteins were annotated to be S-layer or surface associated components in the *Methanosarcina acetivorans* C2A and *Methanosarcina mazei* Gö1 genomes, reflecting limitations of current bioinformatics predictions. To experimentally address what proteins are present, we devised an *in vivo* biotinylation technique to affinity tag all surface-exposed proteins. This overcame several challenges in working with these fragile microorganisms. The two *Methanosarcina* species were adapted to growth under N₂ fixing conditions to minimize the level of free amines that would interfere with the NHS-label acylation chemistry used. A 3-phase separation procedure was then employed to isolate the intact labeled cells from any lysed-cell derived proteins. The Streptavidin affinity enrichment was followed by stringent wash to remove non-specifically bound proteins, and LC-MS-MS methods were employed to identify the labeled surface proteins. In *M. acetivorans* C2A and *M. mazei* Gö1 the major surface layer proteins were identified to be the MA0829 and MM1976 gene products, respectively. Each of the proteins were shown to exist in multiple forms by using SDS-PAGE coupled with glycoprotein-specific staining, and by interaction with the lectin, Concanavalin A. Of the less abundant surface-exposed proteins identified, the presence of all three subunits of the thermosome suggests that the archaeal chaperonin complex is both surface- and cytoplasmically-localized. The above-described techniques provide an alternative strategy to isolate and characterize cell envelope proteins in these archaea.

In related studies we are characterizing the molecular and structural properties of the above surface layer proteins. The *M. acetivorans* MA0829 protein possesses two domains of unknown function that are 78% identical and 86% similar. X-ray crystallography is being used to gain insight into this structure whereby crystallization screening has yielded crystals that diffract to 2.4 Å. Structure solution using selenomethioine-labeled protein is in progress. Finally, bioinformatics searches have revealed the distribution of related surface layer proteins in the Methanosarcinaceae and in other archaeal species.

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Coupling Function to Phylogeny via Single-Cell Phenotyping

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Project Goals: 1) Develop new technology for presorting functional populations and analyze them at the single cell level for both phenotypic and genomic parameters. 2) Apply this approach to populations from Lake Washington sediments to couple functional and genomic datasets at the single cell level.

Rapid advances in modern molecular methods such as the whole genome community sequencing (WGCS) approach open new ways to study microbial ecology. While application of the high-throughput sequencing could result in a blueprint of genomic content of the ecosystem of interest, generally it provides little information about ecological significance of the newly detected functions. To truly understand the role of microbes in the environment, the genomic sequences should be reconsidered in the context of physiological data. Integration of single-cell physiological measurements with genomic data in order to elucidate the functional role of yet uncultivable microbes is the major focus of our current research. Overview of our approaches is presented in Figure 1. We use respiration as a core metabolic function to describe methylotrophic capabilities of microbial cells from two natural environments: freshwater lake sediment (Lake Washington) and salt water (Saanich Inlet). Subsequently, cells tested positive for specific functions are targeted for further genomic explorations via whole genome amplification, PCR-surveys for functional genes and whole genome sequencing.

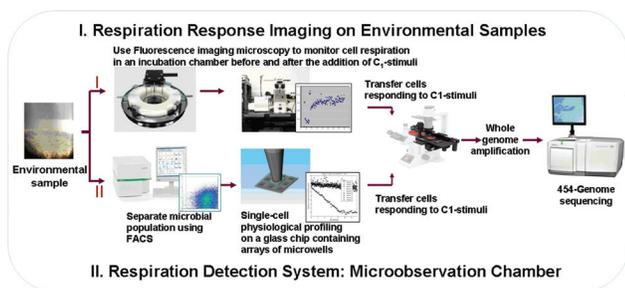


Figure 1. Proposed approaches to couple physiological function with genomics

This single cell platform is a new way to uncover or refine the function for yet uncultured members of natural microbial communities.

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Proteomics Driven Analysis of Microbes, Plants and Microbial Communities

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Project Goals: This project employs comprehensive global and directed proteomic analyses of microbes, plants and microbial communities to enhance the scientific understanding through improved annotation of genomic sequences, elucidation of phenotypic relationships between environmentally important microorganisms, characterization of higher organisms, characterization of the metabolic activities within microbial communities, and identification of post-translationally modified proteins.

Inherent to exploiting microbial function or utilizing plants as biofuels is the detailed understanding of the physiology of the cell. These cellular functions are dictated by the proteins expressed in the cell, their localization and their modification state. This project exploits the technological and informatics advances in the proteomics pipeline at PNNL (as described in the poster by Anderson et al) to address organism-specific scientific objectives developed in conjunction with biological experts for a number of different microbes and plants. In our poster, we highlight the ability to use proteomics data for genome annotation of microbes and fungi, characterization of microbial communities, advances in the characterization of protein phosphorylation state, and the identification of new proteins important to photosynthesis, and the determination of protein localization in stem, root and leaf tissues of poplar.

Genome sequences are annotated by computational prediction of coding sequences, followed by similarity searches such as BLAST, which provide a layer of (possible) functional information. While the existence of processes such as alternative splicing complicates matters for eukaryote genomes, the view of bacterial genomes as a linear series of closely spaced genes leads to the assumption that computational annotations which predict such arrangements completely describe the coding capacity of bacterial genomes. However, proteomic experiments have shown the expression in *Pseudomonas fluorescens* Pf0-1 of sixteen non-annotated

protein-coding regions, of which **nine were antisense to predicted genes**, six were intergenic, and one read in the same direction as an annotated gene but in a different frame. The expression of all but one of the newly discovered genes was verified by RT-PCR. Few clues as to the function of the new genes were gleaned from informatic analyses, but potential orthologs in other *Pseudomonas* genomes were identified for eight of the new genes. The 16 newly identified genes improve the quality of the Pf0-1 genome annotation, and the detection of antisense protein-coding genes indicates the under-appreciated complexity of bacterial genome organization.

Unique to proteomic studies, the elucidation of post-translational modifications and protein localization lead to a richer understanding of the biological system. We have developed advanced technologies to fractionate proteins from microbial subcellular fractions and have applied this technology to mixtures of dissimilar microbes. The applications of this technology to microbial communities will result in a reduction of the sample complexity and increase characterization of the community. Heme moieties play an important role in microbial respiration, yet to date have remain recalcitrant to proteomic characterization. Application of refined separation strategies have resulted in samples enriched in heme containing proteins and thus aid in the identification of these proteins.

Proteomics characterization is also used to understand more complex systems such as plants and microbial communities. *Populus* is the fastest growing tree species in North America and has been identified as a potentially important crop species for converting plant biomass to liquid fuels. *Populus* species are broadly adapted to nearly all regions of the U.S., and hybrid clones have demonstrated 10 dry tons per acre productivity on a commercial scale. Still, improvements in growth rate, cell wall composition, drought tolerance, and pest resistance are required before this species reaches its potential as an energy crop. We have used proteomics technologies to map the protein expression patterns between root, leaf and stem tissues.

Termites degrade and thrive on lignocellulose with help from the bacterial microbiome harbored within their guts. Recent metagenomic analyses have begun to shed light on the genetic potential of the termite hindgut community, but little is known about which genes are expressed to support the symbiotic relationship. Here, we analyzed the metaproteome of the bacterial community resident in the hindgut paunch of the wood-feeding 'higher' *Nasutitermes* species and identified 886 proteins, 197 of which have known enzymatic function. Using these enzymes, we reconstructed known metabolic pathways to gain a better understanding of carbohydrate transport and metabolism, nitrogen fixation and assimilation, energy production, and amino acid synthesis in this endosymbiotic microbiome.

Additional information and supplementary material can be found at the PNNL proteomics website at <http://oberproteomics.pnl.gov/>

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181 Advances for High Throughput, Comprehensive and Quantitative Proteomics and Metabolomics Measurements; Enabling Systems Biology

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Project Goals: This project is developing and applying new and greatly expanded quantitative, high throughput proteomics and metabolomics capabilities for studying diverse microbial systems and communities, plants, and ecosystems of increasing levels of complexity. Capability advances this past year centered on extending modified protein coverage through the integration of bottom-up and top-down measurements, the development of multiplexed activity-based proteomics, developing broad metabolome measurement coverage, and the integration of proteomics and metabolomics measurements with genomic information. In concert with other measurements and information, these developments are addressing deficiencies in the coverage of biochemical components provided by current measurement capabilities, and thus providing the detailed data and the quality needed to enable truly effective systems biology approaches. FWP number: 40601

Understanding microbial and bioenergy-related systems requires knowledge of the array of proteins and their complement of post-translational modifications, as well as knowledge of the large (and often unknown) range of metabolites and other cellular components. Among the basic challenges associated with gaining this understanding are identifying and quantifying large sets of proteins, modified proteins, and metabolites whose relative abundances

typically span many orders of magnitude, and doing so in a sufficiently high throughput manner. Compounding these analytical challenges is the largely unknown extent and nature of many protein modifications, and the high chemical and structural diversity of metabolites.

PNNL is developing and applying high throughput mass spectrometry-based measurement technologies and associated informatics tools applicable to a broad range of biological studies, many of which are presently conducted in collaboration with a number of BER Genomic Sciences researchers (see poster by M.S. Lipton et al.). Our high throughput proteomics/metabolomics analysis pipeline is based on high resolution nano-liquid chromatography separations combined with high mass measurement accuracy mass spectrometry measurements. This poster highlights several developments that build upon this foundation:

1. Providing much broader protein coverage, including modification states to which current measurements are effectively 'blind'. We developed new approaches that combine top-down and bottom-up measurements to extend quantitative proteome coverage to a large range of protein modification states, and that integrate measurements from targeted post-translationally modified sub-proteomes. As part of these efforts, we combined a "RePlay" chromatography method for on-line reanalysis of the separated proteome components with an ultra-fast post-column pressure digestion system to attain nearly continuous mass spectrometer utilization and simultaneous acquisition of both top-down and bottom-up proteomics data from a single analysis, which avoids many of the present ambiguities associated with data interpretation. In conjunction with this approach, we are exploiting the increased throughput of bottom-up measurements (see below, and poster by R.D. Smith et al.) to provide detailed measurements for targeted sub-proteomes. We also are commencing development of new informatics approaches to integrate these complementary data sets.

2. Multiplexed activity-based proteomics. To augment the more detailed proteomics measurements noted above, we are implementing measurements that directly measure enzyme *activities* rather than abundances, and thus measurements that account for changes in protein modification, structure, localization etc. The approach involves the synthesis of *in vitro* or *in vivo* multiplexed (for different activities) and isotopically coded chemical probes that can be applied simultaneously to discover and quantitatively follow enzymatic activities. The approach allows isolation, enrichment, and analysis of large sets of labeled 'signature' peptides that in turn enable protein identification, as well as provide direct and quantitative data on a large range of biological activities in any targeted biological system.

3. Increasing '-omics' coverage by broad nanoLC measurements of the metabolome. We have adapted the new high throughput nanoLC-ion mobility-MS platforms noted below to obtain broad and quantitative measurements of the broad range of metabolites and small molecule components of biological systems. These measurements provide much more comprehensive data sets, which are needed to support

the development of computational models for biological systems and effective systems biology approaches.

A new fast separation liquid chromatography-ion mobility-mass spectrometry platform has been developed (and several versions now implemented) that benefits all of the above efforts by providing high levels of data quality in conjunction with an order of magnitude increase in measurement throughput (see poster by R.D. Smith et al.). The information garnered from improved global coverage of protein modifications and metabolites, and obtainable with increased throughput, is expected to have a profound impact on our ability to develop computational models of biological systems.

Gaining the full benefits of these extended measurement capabilities requires a significantly different and extended computational infrastructure. Thus, we have expanded the PNNL informatics pipeline to incorporate a suite of data analysis tools, data consolidation applications, and statistical packages, as well as visualization software for data interpretation. Through the development of these new tools and the enhancement of existing tools, we have implemented a framework that will support integration of the enhanced proteomics and metabolomics data sets. This framework further supports integration of genomics data from public repositories and provide the needed infrastructure to interoperate with the GTL Knowledgebase.

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A New Platform for Much Higher Throughput, Comprehensive, and Quantitative Proteomics and Metabolomics Measurements and Data Analysis

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Project Goals: This project aims to develop and apply greatly improved capabilities for proteomics and metabolomics. In this context, we have developed and demonstrated a new fast separation liquid chromatography/ion

mobility/mass spectrometry platform for quantitative high throughput proteomics and metabolomics measurements that achieves high levels of data quality in conjunction with an order of magnitude increase in measurement throughput. In addition to significantly improved sensitivity and extremely large data generation rates, the new platform provides the basis for effectively generating and combining data from multiple measurements to attain broader coverage of both protein modification states and chemically diverse metabolomes. The platform considerably speeds large-scale applications and thus enables previously impractical studies, e.g., of diverse microbial systems, communities, and ecosystems. FWP number: 40601

A major challenge underlying the successful development and application of systems biology approaches is the large numbers of measurements needed to accommodate experimental constraints, e.g., derived from available sample sizes, variability in measurements, and/or practical measurement throughput limitations. Although much greater than feasible with classical approaches (using 2D-PAGE), the proteomics measurement throughput now provided by LC-MS- and LC-MS/MS-based approaches is grossly inadequate for characterizing very large numbers of samples, e.g., involving many perturbations, or spatially and/or temporally distinct samples. Metabolomics is similarly constrained, and faces additional challenges because of the broad chemical diversity of metabolites and the greater difficulties associated with identification. Additionally, sample recovery and enrichment methodologies can limit proteome and metabolome coverages.

To address these shortcomings, we developed a new platform at PNNL that demonstrates greatly improved measurement throughput, sensitivity, robustness, and quantitative capability for proteomics and metabolomics measurements in a range of biological research applications.

The new measurement platform incorporates fast multiplexed nanocapillary LC separations coupled via a greatly improved electrospray ionization interface to an ion mobility spectrometer (IMS) stage interfaced to a high speed, accurate mass, and broad dynamic range time-of-flight mass spectrometer (TOF/MS). The automated fast nanocapillary LC system incorporates high pressure LC pumps, an autosampler, and a multiplexed 4-column fluidics system. Each 10-cm-long capillary LC column is operated at 10,000 psi to provide both fast and high resolution separations. Electrospray ionization (ESI) generated ions are accumulated at the end of the second stage of a dual electrodynamic ion funnel trap before being injected into an IMS separation drift tube stage where peptide or metabolite ion separations occur on a time scale of <50 msec. To increase IMS-TOF/MS sensitivity, we developed a novel multiplexing approach that increases the number of ion injection pulses into the IMS separation stage by >30-fold, and thus the S/N levels achievable in a given analysis time, without any loss of separation or MS data quality. Downstream of the IMS separation drift tube, spatially dispersed ion packets are efficiently collected by another electrodynamic ion funnel and

transferred for analysis to an orthogonal acceleration TOF/MS analyzer stage. A high-performance data acquisition system based on a high speed analog-to-digital converter developed to ensure high mass accuracy, high dynamic range measurements is being used in conjunction with a real-time multi-dimensional spectral averaging capability developed under a new CRADA with Agilent Technologies.

Detailed evaluation of the LC-IMS-TOF/MS platform has confirmed significantly improved performance compared to the best currently available proteomics platforms. The new platform provides more than an order of magnitude increase in data generation rates, and initial studies confirm more than an order of magnitude improvement in sensitivity, as well as lower limits of detection. Further improvements in performance are expected from the use a more intense ion source based upon an advanced ESI multi-emitter design used in conjunction with a dual stage ion funnel interface. These advances are being complemented by the development of a new informatics pipeline for rapidly processing and analyzing the greatly expanded data volumes. In combination with improved informatics tools, application of the new platform is expected to enable much more comprehensive coverage of proteins (and e.g., modified proteins) and chemically diverse metabolites

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183 Stable Isotope Probing of RNA Combining Phylogenetic Microarrays and High Resolution Secondary Ion Mass Spectrometry to Link Composition and Function in Microbial Systems

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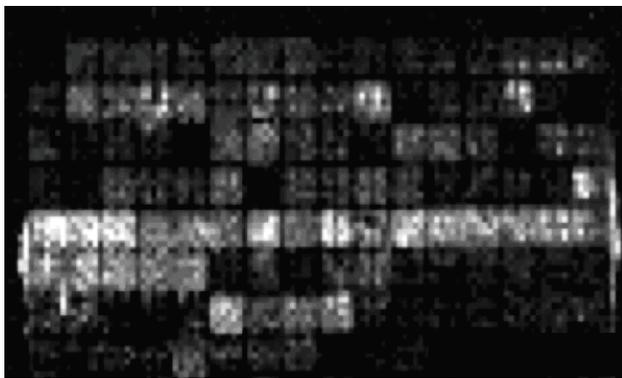
Project Goals: To develop nano-scale stable isotope probing, complex community meta-transcriptomic analysis, and the translation of genome scale data into biogeochemical and metabolic flux network models.

A fundamental goal in microbial ecology is to understand the biogeochemical role of individual microbial taxa in their natural habitat. This rather simple concept is in actuality a complex problem because 1) most microbes remain uncul-

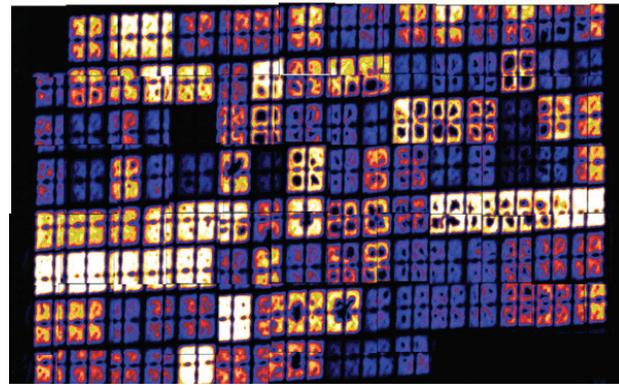
tivated and 2) the majority of microbial communities are very diverse. The former makes the direct testing of isolated strains for biogeochemical activity a limited approach. The latter impacts culture-independent methods like metagenomics as many biogeochemical processes cannot be directly inferred from sequence data alone, even when assembly of complete genomes is possible.

Our approach to this problem (Chip-SIP) involves the combination of high-density phylogenetic microarrays (“chips”) and stable isotope probing (SIP) to directly link identity and function. Microbial communities are incubated in the presence of stable isotope-enriched substrate (s), RNA is extracted and hybridized onto a microarray synthesized on a conductive surface, and the array is imaged using high resolution secondary ion mass spectrometry (SIMS) with a Cameca NanoSIMS 50 to detect isotopic enrichment. We have successfully validated this approach utilizing RNA from a single pure culture with varying degrees of isotopic enrichment using two different substrates (^{15}N -labeled ammonium and ^{13}C -labeled glucose). We show that isotopic enrichment of individual probe spots as detected by nanoSIMS is positively correlated with fluorescence as detected by a traditional microarray scanner (figure 1). This allows the relationship between hybridization efficiency and relative isotopic enrichment to be determined. Further, we have successfully detected ^{15}N enrichment in an estuarine bacterial community following incubation with ^{15}N - NH_4 , demonstrating the utility of the method in mixed natural communities.

Current efforts are aimed at elucidating the major players in nitrogen fixation and carbon transformation in the gut of the wood-eating passalid beetle *Odontotaenius disjunctus*. The gut of this organism is spatially segregated into at least 4 distinct compartments (foregut, midgut, anterior hindgut, and posterior hindgut; see figure 2) each differing physically, chemically and microbiologically. We hypothesize that the sequential biogeochemical activities required to derive energy from lignocellulosic materials are partitioned across the gut sections. We are employing the Chip-SIP approach to determine which organisms at each gut location are involved in these processes.



Fluorescence by microarray scanner



^{13}C enrichment by nanoSIMS

Figure 1: Visual comparison of ^{13}C -enriched RNA from *Pseudomonas stutzeri* hybridized to an array comprised of *Pseudomonas*-specific probes, showing corresponding signal between fluorescence (top) and ^{13}C isotopic enrichment by nanoSIMS (bottom)

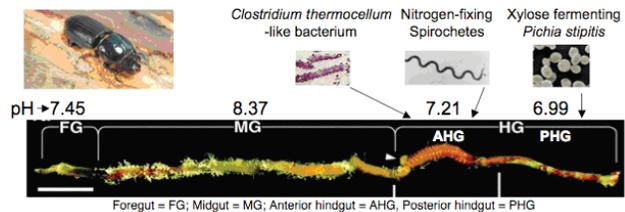


Figure 2: Dissected *Odontotaenius disjunctus* beetle gut showing the 4 sections (foregut, midgut, anterior hindgut and posterior hindgut); adapted from Nardi et al., Arthropod Struct. Dev. 35 (2006) 57-68. Scale-bar = 10 mm

184 Synthetic Genomics: Progress on Construction of a Synthetic Bacterial Cell

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Project Goals: Synthesize a minimal mycoplasma genome that has all of the machinery for independent life. Our goal in this aspect of the program is to create a minimal bacterial cell based on *Mycoplasma genitalium*, which has the smallest genome of any bacterial cell that can be grown in pure culture. A minimal cell contains only essential genes and can be grown in pure culture under defined conditions. It lacks synthetic capacity for small molecules or metabolites that can be supplied in the medium. Thus it is stripped down to core functions for macromolecular synthesis and cell division. The rationale for this is that through creation and analysis of a cell with perhaps fewer than 400 protein coding genes we will be better able to learn the first principals of cellular life. Such a cell would have less than one tenth as many genes as *Escherichia coli* and the lack of complexity would enable an uncluttered perspective on how cells work.

Bacteria and yeast have been widely used as hosts for cloning segments of DNA from a variety of organisms. Cloning of large DNA segments is limited by size and toxicity to the host. Reports of *Escherichia coli* DNA clones larger than three hundred kilobases have been infrequent, whereas yeast has been commonly used to clone megabase-sized DNA. We cloned whole bacterial genomes from *Mycoplasma genitalium*, *Mycoplasma pneumoniae*, and *Mycoplasma mycoides* as circular centromeric plasmids in yeast. Once cloned, the bacterial genomes can be modified using efficient, well-established methods for DNA manipulation in yeast. Next, for one of those cloned genomes, *M. mycoides*, we introduced such modified genomes back into a different bacterial cell, *Mycoplasma capricolum*, by genome transplantation. To do this, restriction barriers had to be overcome. These methods should enable future transplantation of a synthetic genome, and also facilitate the engineering of bacteria with poorly developed genetic systems.

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Characterization of Microbial Strains Important in Biofuels and Biomass Conversion

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Project Goals: To explore the genomic and physiological properties of stable bacterial co-cultures important for biomass conversion and biofuels production in consolidated bioprocessing schemes.

Comparative Genomics of *Clostridia*

Genomic sequencing of 20+ *Clostridia* strains related to

biofuels production and biomass conversion were sequenced, including multiple strains from Cluster III thermophilic and mesophilic cellulolytic *Clostridium* species and multiple strains of saccharolytic *Thermoanaerobacter* species. This dataset represents a significant improvement in the genomic knowledge base of bacteria important to biofuels production. The genomes of two strains of *Thermoanaerobacter*, *T. pseudethanolicus* 39E and *Thermoanaerobacter* sp. X514, have been finished and comparative genomics analysis has been conducted. Experimental studies have shown that when either of these *Thermoanaerobacter* strains are grown in coculture with *Clostridium thermocellum* LQRI, cellulose degradation rates and ethanol production yields are increased compared to the *C. thermocellum* monoculture. Furthermore, this effect is greater for the X514 co-culture compared to the 39E co-culture. Comparative genomics and experimental analysis revealed several potential mechanisms by which such physiological effects could manifest. First, it was noted that X514 encodes a complete *de novo* Vitamin B₁₂ biosynthesis operon whereas 39E encodes only a partial operon. Experimental analysis showed that X514 monocultures are largely insensitive to the addition of exogenous B₁₂, while ethanol yields are severely impacted in 39E monocultures when no exogenous B₁₂ is added. This effect can be alleviated in 39E when 2-3X B₁₂ is added to the culture. This effect is magnified in coculture with *C. thermocellum*, suggesting that X514 synthesizes its own B₁₂ while 39E does not and that B₁₂ is a critical nutrient in determining ethanol yields. Metabolic flux analysis also revealed that absolute flux through the central carbon metabolism pathway is greater in X514 than in 39E. Finally, 39E and X514 encode distinctly different xylose uptake systems and the X514 genes in general are more highly expressed under xylose growth conditions compared to 39E. Thus, substrate uptake, metabolic flux rates and vitamin synthesis likely contribute greater to variable ethanol production and cellulose degradation rates in *Thermoanaerobacter*-*C. thermocellum* co-cultures.

Hydrogen Production from *Desulfovibrio vulgaris*-*Clostridium cellulolyticum* Cocultures

Experimental analysis shows that the stable coculture of *D. vulgaris*-*C. cellulolyticum* produces significantly higher concentrations of molecular hydrogen when grown on cellulose compared to *C. cellulolyticum* monocultures. Functional genomic and experimental analyses were conducted to identify the mechanisms behind this observation. SEM images suggest that cellular binding to cellulose fibers is greater in the coculture than in the *C. cellulolyticum* monoculture, suggesting that the addition of *D. vulgaris* increases binding to the cellulose fibers and may in turn increase cellulose degradation rates. Preliminary microarray analysis also shows that *C. cellulolyticum* cellulosome genes are more highly expressed in co-culture than in monoculture as well as NiFe-hydrogenase genes and other genes related to hydrogen production.

Transcriptional Profiles of X514 at Different Carbon Substrates

The transcriptional profiles of *Thermoanaerobacter* sp. X514 at different carbon substrates have been conducted. Experimental studies show that X514 is able to metabolize

hexose (glucose, fructose, ribose, galactose and so on), pentose monosaccharides (including xylose) and some complex carbohydrates (sucrose, cellobiose, starch). When X514 are grown in glucose, xylose, fructose and cellobiose, the corresponding genes in carbon uptake system are more highly expressed. Moreover, X514 metabolized these four sugars by Embden-Meyerhof-Parnas (EMP) pathway and pentose phosphate (PPP) pathway. X514 encodes carbohydrate active enzymes for catabolism of fructose, xylose and cellobiose. In contrast to glucose metabolism, growth on fructose, xylose and cellobiose resulted in upregulation of carbohydrate metabolism genes which shift carbon fluxes head towards ribose. These observations suggest that when X514 is grown on fructose, xylose and cellobiose, more ribose should be synthesized as the substrate of nucleotide and amino acid metabolism. Experimental analysis shows that energy metabolism of X514 on fructose is more active than that on other sugars, with higher concentrations of ethanol, acetate and lactate generated. Furthermore, the V-type ATPase genes and a large number of genes involved in inorganic ion transport and metabolism (such as sodium-translocating decarboxylase enzyme genes, Na⁺/H⁺ antiporter and sodium/hydrogen exchanger genes and so on) were significantly up-regulated. The data indicate that under fructose growth conditions, electrochemical ion gradient at the cytoplasmic membrane is much more actively established than when grown on other sugars. Thus, more ATP should be generated under these conditions. For the alcohol generation, the results show the three characterized *adh* genes are all expressed at similar levels when grown on these four sugars. But for the additional six lineage-specific *adh* genes, the expression levels greatly varied under different growth conditions, indicating differential expression of the *adh* genes in X514 under different growth conditions.

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Nitrate Reduction and Functional Characterization of *c*-type Cytochromes in *Shewanella*

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Project Goals: As part of the GTL program, our research is focused on nitrate reduction and functional

characterization of *c*-type cytochromes in *Shewanella* *Shewanella* species are characterized by their respiratory versatility and psychrophility. Their ability to utilize a wide range of electron acceptors for respiration is due to a large number of *c*-type cytochromes in their genome. The dissimilatory metal reduction capacity of *Shewanella* and *Geobacter* provides a potential opportunity for the efficient bioremediation and electricity generation.

The NapC- and CymA-Dependent Nitrate Reduction in *Shewanella*

Nitrate respiration systems are highly diverse among *Shewanella* species. Bioinformatics analysis revealed three types of nitrate reduction systems in *Shewanella* genomes. *S. oneidensis* MR-1 harbors only the CymA-dependent nitrate reductase and the NapGH ubiquinol oxidase. Most *Shewanella* species, including *S. putrefaciens* W3-18-1, have both CymA- and NapC-dependent nitrate reductases, as well as the NapGH ubiquinol oxidase. The *S. baltica* strains have both the CymA- and NapC-dependent nitrate reductases but lack the NapGH ubiquinol oxidase. MR-1 appears to be atypical because it lacks both *napC* and *nrfBCD*, whose gene products act to transfer electrons from the quinol pool to terminal reductases NapA and NrfA. In *E. coli*, NapC and NrfBCD are essential for catalyzing reductions of nitrate to nitrite and the subsequent reduction of nitrite to ammonium, respectively. Our previous results revealed that CymA is likely to be a functional replacement of both NapC and NrfBCD in the nitrate and nitrite reduction in MR-1.

Our results suggest that the two-step manner of nitrate reduction found in MR-1 may be common among *Shewanella* species. Deletion of the *nap1* (*napDAGHB*) or *nap2* (*napDABC*) operon did not significantly affect cell growth, but the double mutant could not grow on nitrate, suggesting that the two *nap* operons are functionally redundant. In addition, the in-frame of *cymA* and *napC* of W3-18-1 deletion mutants did not show severe growth inhibition on nitrate, though deletion of *cymA* resulted in the loss of nitrate and nitrite reduction and growth in MR-1. Furthermore, the *cymA* deletion mutant showed little growth on nitrite in contrast to the *napC* deletion mutant, indicating that CymA was involved in nitrite reduction in both W3-18-1 and MR-1. The *cymA* gene from W3-18-1 complements the MR-1 *cymA* in-frame deletion mutant and allows reduction of ferric ions, nitrate, and nitrite when expressed *in trans*. The *napC* gene from W3-18-1 can also complement the MR-1 *cymA* deletion mutant and allows ferric iron reduction but it failed to allow nitrite reduction. These results support the hypothesis that the NapC-dependent and CymA-dependent periplasmic nitrate reduction systems allow an efficient dissimilatory reduction of nitrate and nitrite. Deletion of *narP* and *narQ* resulted in the growth inhibition on nitrate, suggesting that nitrate reduction is also regulated by the NarQP two-component system in W3-18-1. Our competition assays showed that W3-18-1 had a competitive advantage over MR-1 when grown together on nitrate.

Characterization of C-type Cytochromes and Their Role in Anaerobic Respiration in *Shewanella*

The arsenal of *c*-type cytochromes is also highly diversified across the 21 sequenced *Shewanella* genomes and only twelve of the 41 *c*-type cytochrome of *S. oneidensis* MR-1 are present in all other sequenced strains. Only a few *c*-type cytochromes have been characterized. To discern the functions of unidentified *c*-type cytochrome genes in bacterial energy metabolisms, we generated 37 single mutants with an in-frame deletion of each individual cytochrome gene in MR-1. Reduction of a variety of electron acceptors was measured and the relative fitness was calculated for these mutants based on competition assays. This revealed that SO0610, SO1777, SO2361, SO2363, and SO4360 were important under aerobic growth conditions, and that most *c*-type cytochromes play a more important role in anaerobiosis. The *petC* gene appeared to be important to both aerobiosis and anaerobiosis. Our results regarding functions of CymA and MtrC are consistent with previous findings. We also assayed the biofilm formation of these mutants and results indicate that SO4666 might be important for pellicle formation.

S. putrefaciens W3-18-1 lacks orthologues for the secondary metal reductase and accessory proteins (MtrFED) of *S. oneidensis* MR-1. Sputw3181_2446 encodes a decaheme *c*-cytochrome, orthologous to the outer membrane primary metal reductase OmcB of MR-1 (60% similarity) while another reductase similar to OmcA in MR-1 was also found in W3-18-1. Sputw3181_2445 encodes an 11-heme *c*-type cytochrome OmcE, which only shares 40% similarity with the decaheme cytochrome OmcA. Single and double in-frame deletion mutants of *omcB* and *omcE* were generated for functional characterization of *omcE* and metal reduction in W3-18-1. Reduction of solid-phase Fe (III) and soluble Fe (III) in *S. putrefaciens* W3-18-1 was mainly dependent on OmcB under anaerobic conditions (with 50 mM lactate as electron donors and Fe₂O₃, α-FeO (OH), β-FeO (OH) and ferric citrate as electron acceptors. W3-18-1 catalyzed a more rapid reduction of α-FeO (OH) as compared to MR-1, suggesting that other genes may be involved in Fe (III) reduction in W3-18-1. As previously observed in MR-1, the deletion of both OmcE and OmcB led to a severe deficiency in reduction of solid-phase Fe (III) in W3-18-1 and an even greater deficiency in the reduction of soluble iron. The *omcB* and *omcE* genes of W3-18-1 have been expressed with the pBAD vector in *E. coli*. Heme staining assays also demonstrated that the disappearance of specific protein bands in the SDS-PAGE gels were consistent with *omcB* and *omcE* deletion in three mutant samples. These results suggest that *omcE* and *omcB* are actually expressed as cytochrome proteins and could play a central role in metal reduction in *S. putrefaciens* W3-18-1.

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Expression, Purification, and SAXS Structural Studies of the *Caulobacter* Chromosomal Segregation Machinery Complexes and Components

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Project Goals: The purpose of this project is to: 1) map the protein-protein interaction network of *Caulobacter crescentus* including the implementation of TAP tagging to identify interaction partners for proteins that form biomolecular complexes; 2) isolate these complexes by co-expression in *E. coli*, overexpression in *Caulobacter* and/or cell-free systems and to automate these steps to achieve high-levels of throughput for complexes that will be applied on a pathway- to genome-scale; 3) characterize these complexes biochemically, functionally, and structurally at the molecular to cellular level.

Caulobacter crescentus is stalked α-proteobacteria with significant demonstrated potential as a bioremediative agent, as well as being a well-characterized model organism for microbial systems biology. Approaches for the expression, purification, stabilization, and complex formation have been developed that allow for a high-success rate for the production of high-value target proteins and protein complexes. Chromosomal segregation during cell division is highly coordinated spatially and temporally through a set of proteins whose interactions drive the process of properly positioning the chromosomes to the two cells poles. In *Caulobacter* this utilizes the ParA and ParB proteins that interact with *parS* (proximal to the origin of replication). Additionally in *Caulobacter* these proteins interact with the novel cell polarity protein, PopZ, to attach the daughter chromosomes to the two cell poles through interactions of PopZ with ParB and *parS* (Bowman *et al.*, 2008). We express PopZ, ParA, and ParB individually in *E. coli* in sufficient quantities for structural and functional studies. SAXS studies on these proteins individually and in complex with each other reveals sets of interactions for these proteins. SAXS and native gel studies on PopZ alone indicates that in solution this ~20 kD proline-rich polypeptide forms a large assembly of ~ 300 kD which can be disassembled with urea and reassembled by urea removal *in vitro*. ParB forms a homo-dimer that becomes more compact with the addition of *parS*. ParA is an ATPase that oligomerizes upon ATP binding and interaction with ParB (Figure 1), and is thought to provide the force required to move the chromosomes to their proper position. The ParA/ParB/*parS* complex demonstrates a significant increase in the radius of gyration upon addition of ATP. The SAXS data have generated models for ParB, ParA and their complexes that can be compared and fitted with crystal structures of the components. These approaches, combined with the genetic, biochemical, and microscopic

data, are utilized to address structural and functional studies on a number of protein complexes involved in cell polarity, cell cycle control, transcriptional regulation, and bioremediation.

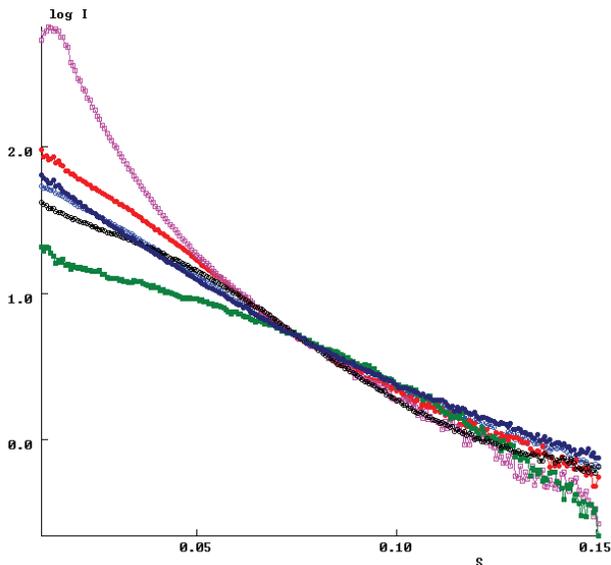


Figure 1. SAXS experiments on ParA, ParB, and *parS* demonstrate oligomerization upon addition of ATP (purple curve) compared with the sample without ATP (red curve). We thank Greg Hura of the ALS SIBYLS beamline 12.3.1 for assistance with the SAXS experiments.

Biological Systems Interactions

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PNNL Foundational Scientific Focus Area— Biological Systems Interactions

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Project Goals: The primary FSFA objectives include—develop a mechanistic understanding of metabolic interactions among key members of microbial mats using

the tools of genomics and systems biology; understand the collective energy, carbon, and nutrient processing in laboratory-based microbial systems that contributes to their stability and efficient utilization of resources using a systematic application of -omics (transcriptomic, proteomic, and metabolomic) approaches; determine interspecies co-adaptations and functional innovations that contribute to robustness and functional efficiency; explore microbe-microbe and microbe-environment interactions that control genome evolution; determine the functional content of the mobile pool of genes in microbial mats and corresponding mechanisms by which they are disseminated; and understand cellular strategies that permit a system of interacting organisms to control the excess generation of reactive oxygen species to promote adaptive responses that enhance their survival; and systems biology investigations of the culturable lichen *Cladonia grayi* to understand mechanisms of resilience against environmental stress.

The PNNL Genomic Science Foundational Scientific Focus Area (FSFA), initiated at the beginning of FY10, is addressing critical scientific issues on microbial interactions, investigating how microorganisms interact to carry out, in a coordinated manner, complex biogeochemical processes such as the capture and transfer of light and chemical energy. The primary research emphasis will be on associations between autotrophic and heterotrophic microorganisms with the additional objective of obtaining a predictive understanding of how interactions impart stability and resistance to stress, environmental fitness, and functional efficiency. The main scientific objectives of the FSFA include: development of a mechanistic understanding of interactions among key members of microbial autotroph-heterotroph associations (AHA) using the tools of genomics and systems biology; understanding the collective energy, carbon, and nutrient processing in AHAs that contributes to their stability and efficient utilization of resources; probing interspecies co-adaptations and functional innovations that contribute to robustness and functional efficiency and exploring the types of microbe-microbe and microbe-environment interactions that control genome evolution; understanding cellular strategies that permit a system of interacting organisms to control the excess generation of ROS to promote adaptive responses that enhance their survival; and systems biology investigations of the culturable lichen *Cladonia grayi* to understand mechanisms of resilience against environmental stress.

Autotroph-heterotroph microbial associations formed the foundation of the biosphere nearly 3 billion years ago with oxygenic photosynthetic prokaryotes (cyanobacteria) and their associated heterotrophic partners colonizing shallow ocean zones. The photolithotrophs use sunlight for energy to fix CO₂ and N₂ and produce O₂, H₂, and organic molecules that supported the growth and metabolism of their heterotrophic partners that facilitate recycling of carbon and nutrients. Autotroph-heterotroph associations are common planet-wide, representing metabolically interactive, self-sustaining communities that are often pioneering and can represent the only biota in extreme environments. These associations are well-adapted to a range of harsh conditions

that include extremes of temperature, salinity, desiccation, irradiance, high O₂, and nutrient deprivation. Microbial associations, inclusive of photolithotrophs (e.g., light energy) and chemolithotrophs (e.g., inorganic chemical energy), are highly relevant to DOE mission areas including bioenergy, carbon cycling/sequestration, and contaminant fate and transport. Further, interacting microorganisms provide key services such as carbon, nutrient, and metal cycling to the biosphere, have considerable potential for a wide range of biotechnological applications, and present challenging and exciting new basic research opportunities.

The PNNL FSFA is utilizing genome-enabled systems biology approaches on three levels—molecular, cellular, and community—to elucidate the underlying design principles of microbial associations, emphasizing interactions between microorganisms for which there are established or hypothesized interdependencies. As part of this approach, the FSFA is developing the experimental tools and data necessary to quantitatively understand and predict causal relationships between environmental change, microbial associations, and cellular functions. The FSFA is using a combined top-down/bottom-up approach where bioinformatics-based genome functional predictions are made using a range of tools and resources; evaluations of evolutionary and ecological adaptation processes are made at the genome scale, high-throughput expression analyses and functional genomics are used to uncover key genes and proteins as well as metabolic and regulatory networks. The bottom-up component uses genetic, physiological, and biochemical approaches to test or verify predictions made by the top-down approaches. The top-down experimental component includes the generation of large amounts of data from biological perturbation experiments that support computational analyses to develop models of various cellular networks. The FSFA is utilizing a series of lab-based model systems consisting of constructed consortia with engineering potential, natural communities, and consortia derived from natural communities for hypothesis testing. Natural communities include microbial mats, biofilms, and lichens. These systems include associations that have evolved to permit successful colonization of extreme environments through effective utilization of solar and chemical energy and scarce nutrients. Research involving these systems will guide our ability to understand and predict how biological associations function with a high degree of efficiency and resiliency. A significant advantage is afforded by using a combination of mechanistic and systems-level investigations of representative associations cultivated in the laboratory under controlled conditions and analyses of natural assemblages using analytical and computational tools of systems biology.

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Transcriptional Regulation of *Shewanella* Central Carbon Metabolism by HexR

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Project Goals: This project was started as a component of the *Shewanella* Federation studies aimed at better understanding of the ecophysiology and speciation of this important genus. It is now continued in the framework of the PNNL Foundational Scientific Focus Area (FSFA) “Biological Systems Interactions” (PI, J. Fredrickson). This FSFA has a scientific focus on understanding interactions between microbes using systems biology approaches including state-of-science technologies, and it is focused on interactions between microbes and their extracellular environments, with an emphasis on acquiring an understanding of microbial autotroph-heterotroph associations. The predictive understanding of these biological systems will be acquired by integrating experimental and computational approaches that exploit the expertise from multiple disciplines working in a synergistic manner. Genomics and proteomics approaches will be applied to predict gene occurrence and function and to identify, quantify, and characterize individual proteins and complexes. Microbial physiology, ecology, biochemistry, metabolic profiling and genetic approaches will be used to verify predictions and test specific hypotheses.

The absence of genes encoding phosphofructokinase in all sequenced *Shewanella* leads to the Entner-Doudoroff (ED) and pentose phosphate (PP) pathways being the major routes of sugar utilization rather than a glycolytic route for central carbohydrate metabolism (CCM). Not surprisingly, such a redistribution of catabolic flux is associated with a completely different regulatory strategy compared to those used for classical *glycolytic* metabolisms found in other proteobacteria such as *Escherichia coli*. Using a comparative genomics approach, we have identified a novel *Shewanella* regulon that is controlled by HexR and that encompasses ~30 genes from the CCM pathways, as well as the deoxy-nucleoside and glycine utilization. The HexR-binding motif was predicted to be a 17-bp palindromic sequence with the consensus tTGTAATwwwATTACa. Assay of purified HexR protein by electrophoretic mobility shift analysis confirmed recognition of the predicted binding motifs by this regulator. The ED pathway intermediate, 2-keto-3-deoxy-6-phosphogluconate, functions as a HexR antagonist releasing it from its target operator. Analysis of the relative

position of the HexR binding sites and candidate promoters in multiple *Shewanella* genomes suggested a dual mode of HexR action; negative regulation (repression) of some of the target genes and positive regulation (activation) of others. This observation is in agreement with the expression patterns of 27 predicted HexR regulon genes observed in the ~200 *S. oneidensis* MR-1 microarray experiments available in the M3D database (<http://m3d.bu.edu/>).

Overall, three distinct groups of highly correlated HexR-regulated genes were revealed: (i) *zwf-pgl-edd-eda, pykA, tal-pgi, gapA2*; (ii) *phk, deoAB, cdd, nqrABCDE*; and (iii) *ppsA, gapA3, gcvTHP*. Remarkably, the third group of genes showed a strong anti-correlation with the first two groups supporting the proposed dual mode of HexR regulation. This observation was directly supported by qPCR-based comparison of the expression of HexR regulon genes in the wild-type and a targeted *hexR* deletion mutant of *S. oneidensis*. The most significant differences in WT vs. mutant gene expression patterns were observed between genes involved in catabolic pathways and in gluconeogenesis (repressed or activated, respectively). For example, of the two genes, *pykA* and *ppsA*, that encode enzymes catalyzing phosphoenolpyruvate to pyruvate interconversion in opposite directions, the former is repressed whereas the latter is activated by HexR. Comparison of growth phenotypes of mutant and wild type strains on various carbon sources (N-acetylglucosamine, glycerate, inosine, and lactate) showed that *hexR* deletion leads to an inability of *S. oneidensis* to grow on lactate as a single carbon source. This finding confirmed the observed positive mode of action of the HexR regulator on the gluconeogenic gene, *ppsA*, whose activity is known to be essential for the growth of *E. coli* on lactate. The detailed results of our HexR regulon reconstruction, including the predicted transcription factor binding sites, are presented in a recently developed RegPrecise database (<http://regprecise.lbl.gov>).

Additional physiological studies and metabolomic profiling analyses are in progress to further investigate the role of HexR in the regulation of CCM in *Shewanella*. The HexR regulon in *Shewanella* may be considered as a partial functional replacement of a classical 6-fructose-phosphate regulon FruR, which is known to control fructose utilization and CCM in *E. coli*. The sequenced *Shewanellae* lack FruR and are not able to grow on fructose. Reconstruction and comparative analysis of HexR regulons was expanded to a broader set of genomes from γ - and β -proteobacteria and some Firmicutes contributing to better understanding of evolutionary history of HexR and its role in the regulation of CCM.

These studies demonstrate the value of applying comparative genomics and complementary experimental analyses to predict and validate regulatory networks in previously uncharacterized biological systems. As part of the new Foundational Science Focus Area project team led by the Pacific Northwest National Laboratory we intend to continue applying such strategies to explore regulatory networks in individual species and communities.

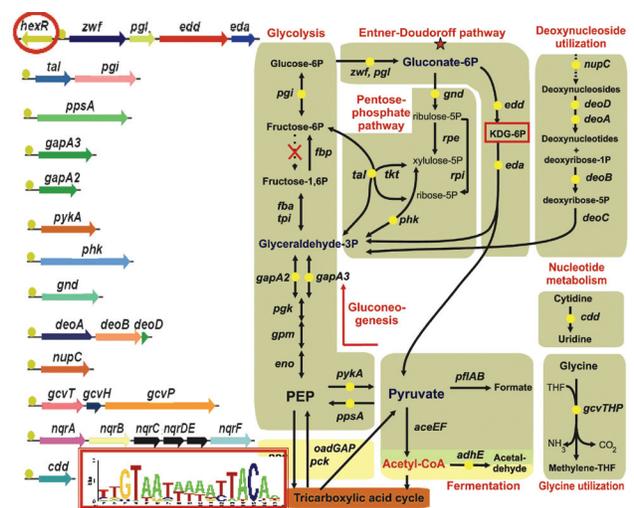


Fig. 1. The reconstructed HexR regulon and target pathways in *Shewanella oneidensis* MR-1.

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Finding Function for Fungal Glycoside Hydrolases

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Project Goals: The goal of this project is to develop a pipeline for functional annotation of fungal glycoside hydrolases.

Plant biomass is efficiently broken down and used a source of carbon by many microbes, including fungi. Many enzymes that are involved in the deconstruction of cellulose and hemicelluloses are members of a large group of enzymes called glycoside hydrolases (GHs). On average, the genomes of filamentous fungi contain well over 100 genes encoding different GH family enzymes. Currently, GHs are classified into families based on sequence and predicted structure. GH families may contain multiple enzymatic activities. The goal of our project is to develop a pipeline for functional characterization of GHs. Functional information GHs is generated by genome annotation, proteomic analysis of the secretome and enzymatic assays of expressed GHs of interest. We have cultured on different substrates and subsequently processed a variety of fungal secretomes for proteomic analysis. We compare protein expression profiles of GHs across different culture substrates. Finally, we have compared the activity and stability of expressed *Aspergillus niger* enzymes produced by different hosts: *E. coli*, *Pichia* and *Aspergillus niger*. Our data allow us to add functional data to annotations of fungal

GHs there were previously characterized based solely on predicted amino acid sequence analysis.

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High-Temperature Chemotrophic Microbial Communities of Yellowstone National Park: Metagenomics Provides a Foundation for Dissecting Microbial Community Structure and Function

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Project Goals: The goals of this project are to study microbial interactions in model geothermal microbial communities. Extreme geochemical conditions and high temperature result in low-diversity microbial communities where metagenomic sequence data can be used to dissect microbial community structure and function.

Microbial communities are a collection of interacting populations. However, a significant fraction of our knowledge base in microbiology originates from organisms grown and studied in pure culture, in the absence of other members of the community who may compete for resources or provide necessary co-factors and or substrates. Moreover, many of the organisms studied in pure culture have not necessarily represented the numerically dominant members of microbial communities found in situ. The advent of molecular tools (e.g., genome sequencing) has provided opportunities for assessing the predominant and relevant indigenous organisms, as well as their likely function within a connected network of different populations (i.e., community). High-temperature microbial communities are often considerably less diverse than mesophilic environments and constrained by dominant geochemical attributes such as pH, dissolved oxygen, Fe, sulfide, and or trace elements including arsenic and mercury. Consequently, the broader goal of our work is to utilize extreme high-temperature geothermal environments including acidic Fe-oxidizing communities as model systems for understanding microbial interactions among community members. Recent metagenomic sequencing of high-temperature, acidic Fe-mats of Norris Geyser Basin, Yellowstone National Park (YNP), conducted as part of a DOE-Joint Genome Institute (Community Sequencing Project) reveal communities dominated by novel members of the *Archaea*, bacterial members of the deeply-rooted Order Aquificales as well as other less-dominant Bacillales and Clostridiales. Phylogenetic and functional analysis of metagenome sequence is providing an excellent foundation for establishing the role of individual populations in a net-

work of interacting community members, and for directing hypotheses regarding the importance of specific biochemical pathways responsible for material and or energy cycling. For example, we are using metagenome sequence in combination with information available from reference strains to identify protein-coding sequence of importance in the oxidation and or reduction of Fe, S, O, and As, as well as central C metabolism (including fixation of CO₂). Genes coding for proteins with hypothetical or putative roles in electron transfer, C-capture and C-transformation have been prioritized for design of quantitative-reverse transcriptase-PCR (Q-RT-PCR) primers to evaluate functional capacity quantitatively in both pure-culture and subsequent mixed communities. Future proteomic and transcriptomic analyses, as an element within the PNNL Foundational Scientific Focus Area, will focus on both pure-culture experiments under different electron donor and acceptor conditions, as well as natural thermophilic mats. Proteomic results will be used to assess and confirm the importance of specific proteins and to improve microbial community models. Application of genomic, proteomic, and metabolic information to dissect microbial community structure and function is tractable within high-temperature geothermal systems in part due to the relative simplicity of the community and the dominance of several key geochemical variables (i.e. pH, Fe, O₂).

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Comparative Genome Analyses of Members of the Ecologically Versatile Genus *Shewanella*: Searching for Sequence Signatures That Reflect Environmental Adaptation

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Project Goals: This project is a component of the *Shewanella* Federation and, as such, contributes to the overall goal of applying the genomic tools to better understand the ecophysiology and speciation of respiratory-versatile members of this important genus.

Shewanellae are an environmentally important group of bacteria whose members have been isolated from many different ecosystems (fresh and marine waters and sediments, a deep sea iron mat, subsurface sedimentary rock, and squid gland) that vary in atmospheric pressure, temperature, and salinity. These organisms thrive in red-ox interfaces in the environment and are well known for their versatile respiratory capability, using over 20 different compounds as electron acceptors. Complete genome sequences have been obtained for 19 *Shewanellae*. The strains were selected for sequencing based on their phylogenetic distance, some representing closely related sub-species clusters and others being more distantly related. Overall they represent a genetic gradient in which speciation and environmental adaptation

can be studied. A recent comparative analysis of the genome sequences, phenotypic characteristics, and proteomic expression profiles of the first ten strains sequenced showed that phenotypic and genotypic similarities largely correlated with phylogenetic distances despite the evidence of laterally transferred genes (1). Some of the phenotypic and genotypic traits were more conserved with increasing evolutionary distance (i.e. predicted metabolic pathways) than others (i.e. protein expression patterns).

Our comparative analysis has extended to 19 completed *Shewanella* genome sequences, including more distantly related strains that are obligately marine or that thrive in lower or higher temperatures than those previously studied. The protein sequences from all 19 strains have been analyzed for their domain content (Pfam, TIGRFam) in order to detect differences (functional, protein family compositions) that correlates with the environment in which the strains were isolated. Protein families involved in responses to environmental factors (chemotaxis proteins, two-component regulators, signaling proteins) appear to be large and more diverse among the *Shewanellas*. A curated table of orthologous proteins has been generated for the sequenced shewanellae allowing for categorization of *Shewanella* proteins as core (present in all strains), dispensable (absent in one or more strains), or strain-specific. A significant amount of curation relating to gene calling and function assignments has been done for this dataset, which is available in the *Shewanella* Knowledgebase (2). Of the 16612 orthologous groups of *Shewanella* genes (redundant genes removed), 11% are core genes, 54% dispensable genes and 35% unique genes, the two latter categories encoding genes involved in adaptation to and survival in select environments.

In addition to studying the presence and absence of genes, we are now searching the *Shewanella* genomes for evidence of selective changes in the sequences that can be linked to our knowledge of specific functions or specific environmental conditions of the strains. Sequence changes at non-synonymous vs. synonymous sites have been identified to find genes that are under a purifying or a diversifying selection pressure. Amino acid replacement ratios, radical vs. conservative changes, have also been determined (3). Such codon usage analyses have been used to identify genes that are under biochemical or ecological constraints. We have also calculated the Codon Adaptation Indexes for the *Shewanella* sequences, an estimate of the synonymous codon usage bias and of gene expression levels. The *Shewanella* ortholog table is used as a framework for our studies allowing us to separate the genes into sets that are common to all of the *Shewanellae* or that vary among the strains giving them their unique characteristics. The sequence changes are also evaluated relative to the gene product functions, locations, and protein family memberships.

Our comparative analysis of members of the *Shewanella* genus is forming the foundation for studying other groups of related organisms as well as consortia of microbes in selected environments.

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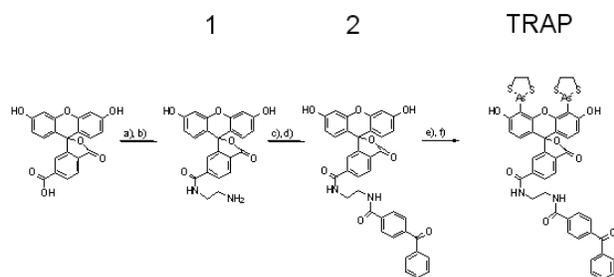
In-Vivo Trapping and Structural Interrogation of Transient Protein Complexes

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Project Goals: Identification of new imaging methods that permit high-throughput measurements of protein complexes that will allow the development of predictive models for bioenergy applications and with regard to how microbes respond to environmental change.

Protein cross-linking, especially coupled to mass-spectrometric identification, is increasingly used to determine protein binding partners and protein-protein interfaces for isolated protein complexes. The modification of cross-linkers to permit their targeted use in living cells is of considerable importance for studying protein-interaction networks, which are commonly modulated through weak interactions that are formed transiently to permit rapid cellular response to environmental changes. We have therefore synthesized a targeted and releasable affinity probe (TRAP) consisting of a biarsenical fluorescein linked to benzophenone that binds to a tetracysteine sequence in a protein engineered for specific labeling (Scheme 1). Here, the utility of TRAP for capturing protein binding partners upon photoactivation of the benzophenone moiety has been demonstrated in living bacteria and eukaryotic cells. In addition, ligand exchange of the arsenic-sulfur bonds between TRAP and the tetracysteine sequence to added dithiols results in fluorophore transfer to the crosslinked binding partner. Following isolation of protein complexes, the facile release of TRAP from the original binding site permits the identification of the proximal binding interface through mass spectrometric fragmentation and computational sequence identification.



Scheme 1. *Synthesis of TRAP.* A) EDC, Et₃N, NHS, dry DMF, 30 min; B) *N*-Boc-ethylenediamine, 16 h; C) 20 % TFA/CH₂Cl₂, 2 h; D) 4-Benzoylbenzoic acid, EDC, NHS, iPr₂EtN, DMF, 16 h; E) HgO, TFA, 70 °C; F) 1) AsCl₃, PdOAc, iPr₂EtN, NMP, 4 h; 2) EDT, 20 % acetone/H₂O (overall yield: 2 %).

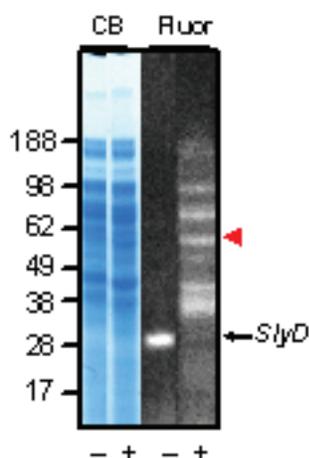


Figure 1: *Selective in-vivo labeling of chaperon SlyD and Photo-dependent Trapping of Binding Partners that Include Elements of [Ni-Fe]-Hydrogenase Maturation Pathway.* Coomassie Blue (CB) protein stain (left panel) and fluorescence image (right panel) before (-) and following (+) photodependent cross-linking. Positions of SlyD and HypB (red arrow), previously shown to bind SlyD, are indicated.

One example of the utility of using TRAP to identify molecular pathways associated with the maturation of [Ni-Fe]-hydrogenases involves the introduction of a tetracycysteine tag into the chaperone SlyD expressed in *Shewanella*, permitting its selective labeling (Figure 1). Following photoactivation and reduction, the fluorescence TRAP moiety is readily transferred to binding partners – resulting in a considerable simplification in the identification of the unique molecular interfaces following proteolytic digestion and mass spectrometric analysis (Figure 2).

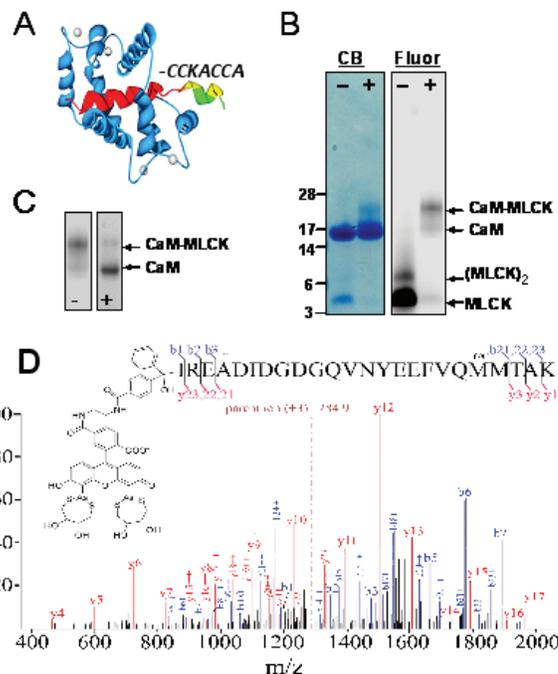


Figure 2: *Mass Spectrometric Identification of Interfacial Binding Site Following Crosslinking and Fluorophore Transfer.* (A) Depiction of the structural interface between tagged MLCK protein (red) containing engineered TRAP binding site CCKACCA (yellow and green) in complex with binding partner CaM (blue). (B) Coomassie Blue (CB) protein stain and fluorescence from TRAP reagent (Fluor) prior to (-) and following (+) photo-induced crosslinking. (C) Cross-linked complex before (-) and following (+) transfer of TRAP to binding partner upon addition of DTT (5 mM). (D) Mass spectrum of TRAP-bound peptide, where all b (blue) and y (red) fragment ions are identified. Site of TRAP binding in CaM bait protein is shown above mass spectrum.

Conclusions: We have synthesized a new class of photocrosslinker (i.e., TRAP) that can be targeted to a tetracycysteine tag to capture (that is, trap) protein binding partners upon light activation following the straight-forward introduction of a unique tetracycysteine binding sequence onto a protein of interest. The simplicity of this method will facilitate the high-throughput identification of protein complexes in a range of different organisms. The small size of TRAP compared to other targeted and multifunctional crosslinkers enables facile identification of the site of cross-linking by searching for the added molecular weight of the transferred crosslinker using commonly available mass spectrometers and publically available software. Optimal conditions associated with cross-linking complexes of interest are facilitated by the high-fluorescence yield of the TRAP reagent, enabling facile visualization of TRAP fluorescence on SDS-PAGE gels.

Future Measurements: TRAP will be used in combination with targeted in vivo photocrosslinking and mass spectrometry to identify interfacial binding sites, permitting an understanding of how environmental conditions affect protein-protein interaction networks. Coupled with the use of complementary multiuse affinity reagents (MAPs)

that permit the visualization of protein localization within microbial communities, we propose to use these reagents to identify regulatory elements that modulate energy flux through key metabolic pathways associated with biomass and the generation of biofuels.

Reference

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Plant-Microbe Interfaces

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Plant-Microbe Interfaces

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<http://PML.ornl.gov>

Project Goals (Abstracts 194-205): Understand the genome-dependent molecular and cellular events involved in establishing and maintaining beneficial interactions between plants and microbes. *Populus* and its associated microbial community serve as an initial test system for understanding how these molecular events manifest themselves within the spatially, structurally, and temporally complex scales of natural systems. To achieve this goal, we will focus on 1) characterizing the natural variation in *Populus* microbial communities within complex environments, 2) elucidating *Populus*-microbial interactions at the molecular level and dissecting the signals and pathways responsible for initiating and maintaining microbial relationships, and 3) performing metabolic and genomic modeling of these interactions to aid in interpreting the molecular mechanisms shaping the *Populus*-microbial interface.

Rapid progress in biological and environmental sciences has been enabled by the availability of genome sequences and the tools and technologies involved in interpreting genome function. As our understanding of biological systems grows,

it becomes increasingly clear that the functional expression of individual genomes is affected by an organism's environment and the community of organisms with which it associates. The beneficial association between plants and microbes exemplifies a complex, multi-organism system that is shaped by the participating organisms and the environmental forces acting upon it. These plant-microbe interactions can benefit plant health and biomass production by affecting nutrient uptake, influencing hormone signaling, effecting water and element cycling in the rhizosphere, or conferring resistance to pathogens. Studying the integral plant-microbe system in native, perennial plant environments, such as *Populus* and its associated microbial community, provides the greatest opportunity for discovering plant-microbial system functions relevant to DOE missions related to bioenergy and carbon-cycle research and understanding of ecosystem processes.

The functional attributes of *Populus* depend on the microbial communities with which it associates. Bacteria and fungi can be found within *Populus* tissues and closely associated with the roots in the rhizosphere. Understanding these communities, and the interfaces between organisms, is critical to realizing fundamental scientific knowledge that may enable increased plant productivity, ecosystem sustainability, disease resistance, drought tolerance, and ecosystem carbon budgets. This interface can also influence the processes, or mechanisms, by which adaptive traits arise from genetic variation and community function. Microbial rhizosphere structure, plant root bacterial and fungal colonization patterns, and the microbe-plant signaling pathways inherent in each type of association are all found within *Populus* and can be functionally translated hierarchically across scales into ecosystem patterns and processes.

Understanding the mechanisms by which plants and microbes interact represents a grand challenge facing biological and environmental science. How microbial selection and colonization occurs, what reciprocal benefits are bestowed upon the plant and microbe, and how these interactions ultimately affect, and are affected by, the environment are just some of the intrinsic scientific questions. The multiple spatial and temporal scales involved in these interfaces, the complexity of the component systems, and the need for better tools that use and build upon growing genomics resources to probe and interpret these combined systems represent some of the essential technical challenges. The variety and magnitude of these challenges are only offset by the impact and benefit of overcoming these challenges and in applying this understanding to issues as diverse as efficient energy transformation and carbon cycling.

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Student Presentation

Plant-Microbe Interfaces: Characterization of Cell Surface Properties in *Azospirillum brasilense* Wildtype Cells and Che1 Pathway Mutants Using Atomic Force Microscopy

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<http://PMI.ornl.gov>

Project Goals: See goals for abstract 194.

In order to compete in complex microbial communities, bacteria must quickly sense environmental changes and adjust cellular functions for optimal growth. Such responses are detected and initiated by various two-component signal transduction pathways. Chemotaxis-like signal transduction pathways, which are typically responsible for modulating the swimming motility patterns of chemotactic bacteria, have also been implicated in the modulation of other cellular responses, including cell-to-cell aggregation. Microbial cell-to-cell aggregation is an important behavior which serves to enhance cell survival in adverse environmental conditions. It is particularly advantageous for microbes to organize into aggregative communities not only for protection against predation or antimicrobials, but also for associative metabolic interactions. Plant associative bacteria harbor mechanisms which lead to the remodeling of cell surface and extracellular structures to promote cell-to-cell aggregation and plant microbial interactions. Recently, the Che1 chemotaxis signal transduction pathway from the alpha-proteobacterium *Azospirillum brasilense* was shown to modulate the propensity of cells for cell-to-cell aggregation that leads to flocculation under certain growth conditions. *A. brasilense* are soil diazotrophic bacteria that colonize the roots of many economically important grass and cereal species. Under conditions of high aeration and limiting availability of combined nitrogen, *A. brasilense* cells differentiate into aggregating cells and form dense flocs that are visible to the naked eye. Flocs are formed by cells embedded in a dense polysaccharide matrix and by cell-to-cell aggregation. Optical and electron microscopy (EM) approaches have been previously applied to compare and identify specific cell surface changes that accompany aggregation and flocculation in *A. brasilense* wild-type strain Sp7 and its Che1 pathway mutant strain derivatives that are affected in their propensity to flocculate under nutritional and aeration stresses. However, no specific extracellular structure could be identified using these techniques, despite the preliminary observation

of changes in extracellular polysaccharide (EPS) production detected by growing colonies in the presence of Congo Red. Although optical and EM techniques have revealed many insights into bacterial aggregative behavior, resolution limitations and fixative procedures can inhibit visualization of extracellular structures. Therefore, atomic force microscopy (AFM) was selected as a unique alternative to imaging *A. brasilense* Che1- dependent flocculation behavior at nanometer resolution in an effort to directly visualize changes in cell surface properties that correlate with flocculation. In this study, we investigated *A. brasilense* Sp7 and its Che1 mutant strain derivatives, $\Delta cheA1$ and $\Delta cheY1$, utilizing AFM imaging techniques to gain insight into molecular and regulatory role of Che1 in cell-to-cell aggregation and flocculation. We demonstrate that AFM identifies a distinctive remodeling of the cell surface and extracellular matrix, likely via changes in EPS production, in the $\Delta cheA1$ and $\Delta cheY1$ strains concomitant with flocculation under nitrogen-limiting conditions and high aeration.

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Plant-Microbe Interfaces: Initial Proteome Characterization of the *Populus* Rhizosphere Community

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<http://PMI.ornl.gov>

Project Goals: See goals for abstract 194.

A complex consortium of microorganisms lives in the plant rhizosphere – a narrow region of soil surrounding the roots. The rhizospheric microbial community feeds on proteins and sugars released by the roots and plant material sloughed off from the roots. In return, the community helps plants to acquire water, nitrogen, and other minerals, and to suppress disease. Here, we describe the development of proteomics methods for understanding this symbiotic relationship. Samples were acquired from both greenhouse-grown and wild *Populus*, the latter including specimens growing in clay and in sandy soil in the Caney Fork State Park in Middle Tennessee (see poster by Schadt et al. for more details). Proteins were extracted from fine roots and associated microorganisms, followed by shotgun proteomics analysis using 2-dimensional liquid chromatography and tandem mass spectrometry. Peptides and proteins were identified by searching mass spectral data against a composite protein sequence database comprised of *Populus* proteins and

genomes of relevant sequenced microorganisms. Because the membership and physiology of the rhizosphere community are influenced by growth conditions and genotypes of *Populus* and environment variables associated with soil, results from these different sites and specimens provided a broad survey of the proteomes of *Populus* rhizosphere community. We identified a core rhizosphere proteome present across all samples and sets of proteins unique to soil types or *Populus* growth conditions. Experiments to improve the depth of the community proteome coverage through optimization of protein extraction methods and employment of high-performance mass spectrometers are ongoing. The observations from these field samples will be followed up with measurements on controlled, reconstituted small soil-less or soil-based model systems. Informatics tools to search proteomic data using the metagenomic sequence data collected from the same samples are also being developed.

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Plant-Microbe Interfaces: Development of a Knowledgebase for Exploring Plant-Microbe Interactions Using Metabolic Reconstructions

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<http://PML.ornl.gov>

Project Goals: See goals for abstract 194.

The Plant-Microbe Interfaces knowledgebase provides an integration framework for microbial, fungal and plant genome data along with information about the characteristics of plants, soils and weather conditions from field studies, their geographical location, diversity and the phenotypic characteristics of microorganisms from soil and plant samples at each location. This macro level information on the samples will be later supplemented by proteomics, metabolomics and transcriptomics data. In this study we have utilized the database to explore metabolic signatures for different classes of organisms interacting with *Populus*. In natural environments a tripartite interaction among plants, fungi, and bacteria is essential to plant growth. On one hand, the intimate relationships within the plant/bacteria/mycorrhizal fungus network supplies plants with nutrients, promotes their growth, and increases their resistance to stress. On the other hand, pathogenic fungi and bacteria can be harmful to plants and can lead to diseases and to altered production of desired traits. Additionally, some microorganisms, also known as biocontrol agents, can protect plants by reducing the number of pathogenic microorganisms. Molecular mechanisms underlying phenotypic differences among plant-associated microorganisms are not clear. The

microbial species responsible for beneficial and pathogenic effects on plant growth that have been sequenced provide an opportunity to discover the genomic determinants of the various phenotypes through comparative analysis of the genomes. Metabolic capabilities inferred from genome annotations can be especially helpful in fingerprinting phenotypic differences at the level of enzymes and metabolic pathways. In this study we perform a comparative analysis of phenotypes by developing Pathway Genome Databases (PGDBs) for two fungal species, *Eremothecium gossypii* ATCC 10895 (a plant pathogen) and *Laccaria bicolor* S238N-H82 (a plant symbiont) and a set of sequenced bacterial species including four endophytes (*Pseudomonas putida* W619, *Stenotrophomonas maltophilia* R551-3, *Enterobacter* sp. 638, *Methylobacterium populi* BJ001), six plant pathogens from the genera *Agrobacterium*, *Pseudomonas*, and *Burkholderia*, and five species from the same genera that are used for biocontrol of phytopathogens.

PGDBs were generated by the Pathologic program from the Pathway Tools software. Because the quality of the metabolic reconstruction by this software depends on the genome annotation, we have developed an automated pipeline to improve the enzyme annotation and to make it consistent across studied organisms. The primary input file to the pipeline contains all of an organism's RefSeq files downloaded from the NCBI website. This file is parsed to build input files for Pathologic and to augment them with the enzyme information from the KEGG orthology annotation of the organism's genome. The PGDB is built with Pathologic running in batch mode, and the PGDB is then refined, to predict transcription units and transporters. Additionally, MySQL tables are created to characterize each protein coding sequence in the genome by a product name, EC numbers, pathways, and protein domains. The domain annotations are generated by searching each sequence against a set of databases (CDD, Pfam, SMART, TIGRFAM, and COG) using RPSBLAST. Thus, the pipeline allows us to quickly incorporate the latest annotation information into the KnowledgeBase, supports metabolic reconstructions, provides a means for improving their quality, and facilitates a comparative analysis of the organisms.

A preliminary analysis of the information generated by the pipeline has revealed some interesting metabolic differences between pathogenic and beneficial microbes at the level of specific enzymes and metabolic pathways. Across analyzed microbial species, biological control agents have a significantly larger number (~10-20%) of metabolic enzymes and pathways when compared with either plant pathogens or endophytes ($p < 0.05$). No statistically significant differences, however, were observed when plant pathogens were compared with plant endophytes, indicating a closer metabolic relationship between these phenotypes. In addition to increased metabolic versatility, all of the analyzed biocontrol agents encode in their genomes two specific enzymes that clearly distinguish their metabolic capabilities from the analyzed pathogenic bacteria and endophytes. One enzyme, 1,6-dihydroxycyclohexa-2,4-diene-1-carboxylate dehydrogenase (EC 1.3.1.25), is involved in benzoate degradation, namely in the conversion of benzoate to catechol. It is pres-

ent in biocontrol agents but is not found in any pathogen. The other enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase (EC 3.5.99.7), is absent from endophytes, but is common for biocontrol bacteria. This enzyme catalyzes the conversion of ACC, a precursor of ethylene synthesis in plants, to α -ketobutyrate and ammonia. A variety of beneficial effects on plant growth has been linked to this enzyme, including enhanced nodulation and increased resistance to stress.

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Plant-Microbe Interfaces: Novel Navigation Techniques to Study Plant-Microbe Associations Utilizing Google Maps API

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<http://PMI.ornl.gov>

Project Goals: See goals for abstract 194.

Efficient integrative analysis, navigation and visualization of information are important prerequisites to understanding dynamic functional interactions between plants and microbes in the environment. The information that must be collected and analyzed includes a diverse set of data characterizing plants, microbe and their environments at different levels of resolution. The collected data will include characteristics of plants, soils and weather conditions from field studies, their geographical location, diversity and phenotypic characteristics of microorganisms from soil and plant samples at each location. This macro-level sample information will be supplemented by proteomics, metabolomics and transcriptomics data, and by genome annotations of the sequenced microorganisms. These micro-level measurements will be also supplemented by results of statistical analyses and by computational predictions produced by developed mathematical models. The unique diversity of the collected data and their composite nature necessitates a more flexible way of managing the information in the project knowledgebase than commonly used relational databases and related visualization and navigation tools.

To address the complex nature of the collected information in studies of plant-microbe associations, a novel navigation and visualization technique based on Google Maps Application Programming Interface (API) is being developed. This approach will allow us to combine a comprehensive mashup of the collected data, high speed visualization and a facilitated supplementation of the data by available knowledge from public Internet resources. The mashup

tool provides novel navigation techniques to quickly and efficiently locate and zero-in on interesting plant-microbe associations. The web application will integrate data from different sources and explore experimental datasets that are based upon a common geographical and biological sample space. The technology helps users to generate ideas by identifying relationships and associations both within plants and microbes, and between plants and microbes. The tool provides a sophisticated and intuitive graphical user interface to interactively browse the data using different navigation parameters. It provides for a rich interactive user-experience for users to; 1) filter data by biological, geographical or categorical traits of the collected samples; 2) correlate and compare data based on the user selected parameters; and 3) identify interesting trends and patterns in the collected datasets. Particularly, the tool is used to explore a relationship between soil characteristics, plant genotypic and phenotypic characteristics, and microbial phenotypic, metagenomic and metaproteomic characteristics in field-based studies of *Populus* associated microbial communities. The web application is accessible from <http://pmi.ornl.gov>.

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Plant-Microbe Interfaces: Functional Analysis of Phytochrome Signaling in *Populus*

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<http://PMI.ornl.gov>

Project Goals: See goals for abstract 194.

In addition to providing energy for photosynthesis, light also modulates the spatial and temporal responses of plants to their surrounding environment. Plants have evolved multiple mechanisms to modulate their growth and development in response to the spectral quality of light. One such mechanism involves red (R) and far-red (FR) light perception by phytochromes (PHY), where a reduction in R:FR due to vegetative shading results in 'shade avoidance response' (SAR). The SAR is characterized by rapid elongation of stem internodes and upward reorientation of leaves. The role of PHYs in shade avoidance is widely studied in the model plant *Arabidopsis thaliana*. In *Arabidopsis*, phytochromes are encoded by PHYA, PHYB, PHYC, PHYD and PHYE; of these, PHYB plays an important role in mediating responses to plant-plant competition. Here we report initial characterization of the PHY gene family from *Populus trichocarpa*. Unlike *Arabidopsis*, the *Populus* genome encodes three PHY genes namely, PtPHYA, PtPHYB1 and PtPHYB2. In order to gain insight into the role of PtPHYs in light signaling, the transcripts of the three PtPHYs in different plant tissues were measured by quantitative RT-PCR. Consistent

with the light labile nature, PtPHYA transcript was the least abundant of all three PHYs studied. The expression of PtPHYB1 was highest in female flowers and that of PtPHYB2 was highest in the phloem. In *Arabidopsis*, SAR is characterized by upregulation of key response genes such as *ATHB4*, *HFR1* and *PIF3*. *Populus* homologues of *ATHB4* and *HFR1* as well as PtPHYB1 and PtPHYB2 showed significant upregulation in response to low R:FR ratio. These results suggest that the mechanism of PHY signaling is partly conserved between *Arabidopsis* and *Populus*. The gene regulatory networks involved in SAR are being studied by microarray analysis on *Populus* exposed to lower R:FR light. Finally, phytochromes are protein kinases and are believed to affect the downstream responses by interacting with other proteins. In order to identify the PHY interacting proteins, *Populus*-specific PHYs were cloned as C-terminal and N-terminal green fluorescent protein (GFP) and hemagglutinin (HA) tagged constructs. The protein localization was then studied by expressing the GFP-fusion protein in sweet pea leaf mesophyll protoplasts. The candidate PtPHY interacting proteins will be identified by protein-protein interaction assays in leaf mesophyll protoplasts. This work will lead to a conceptual model of phytochrome-mediated responses to shade avoidance and to a more detailed understanding of light-induced signaling cascades in *Populus*.

200 Plant-Microbe Interfaces: Transcript and Protein Evidence for Novel Small Protein Genes in *Populus*

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Project Goals: See goals for abstract 194.

Small proteins less than 200 amino acids in length encoded in short open reading frames (sORF) have major functions. Most of the small proteins characterized so far play important roles in cell-to-cell signal transduction. Hundreds or even thousands of novel sORF genes may await discovery in each plant species. The goal of this research is to systematically study the functional genomics of small proteins in relation to signal transduction involved in plant-microbe interactions. We hypothesize that small mobile proteins mediate long-distance signal transduction via the phloem/xylem channels. A systems biology approach is being used to realize three objectives: 1) discovery of sORF candidate genes using bioinformatics, transcriptomics and proteomics;

2) molecular characterization of the candidate genes using full-length gene cloning, sub-cellular localization and movement, and protein-protein interactions; and 3) functional characterization of the candidate genes using signal transduction assays and regulation of gene expression.

Our transcriptomics and proteomics research revealed thousands of sORF genes expressed in *Populus* leaf tissue under normal and drought conditions. Our recent comparative genomics analysis identified some interesting small protein candidates that are potentially involved in signal transduction via the phloem/xylem channels. Proteomics data were also analyzed for the presence of these small proteins. Protein extracts of leaves were prepared, and shotgun proteomics measurements were performed using an LC-MS-MS approach. By comparing experimental tandem mass spectra against a combined database containing current *Populus* gene annotations plus novel small protein candidates, we obtained proof-of-principle confirmation that small proteins are amenable to detection using these protocols. To identify more sORF candidate genes, sequencing of transcriptome and proteome in *Populus* phloem/xylem sap, as well as root system, including rhizosphere microorganisms (e.g., inoculated by fungus *Laccaria*), will be carried out.

For molecular characterization, small protein genes are being cloned as C- or N-terminal GFP (green fluorescent protein) and HA (hemagglutinin) tag constructs. The recombinant protein will be used for in vivo localization assays. The sub-cellular localization of the proteins will be studied by expressing the GFP-tagged constructs in the leaf mesophyll protoplasts. The inter-cellular long-distance movement of small proteins in plants will be monitored by imaging of fluorescent and radiolabeled protein probes. To characterize the role of the small proteins in mediating the plant microbe interactions, candidate genes will be analyzed by making transgenic *Populus* plants and/or hairy roots with altered gene expression (i.e., over-/down-regulation). In addition, the role of the small protein candidates will be studied using protoplast-based signaling assays.

201 Plant-Microbe Interfaces: Deciphering Plant-Microbe Signaling with Integrated Networks

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<http://PMI.ornl.gov>

Project Goals: See goals for abstract 194.

The mechanisms by which symbiotic fungi engage host plant systems and induce local and systemic resistance

to pathogens, and promote growth and other beneficial effects remains poorly understood. A goal of our project is to develop experimental and computational methods to discover the signaling cascades and subsequent biochemical and molecular mechanisms driving these mutually beneficial interactions between host plants and fungal symbionts. A systems-level approach will be used to address this goal by: i) constructing a coexpression network map to characterize host plant transcriptional profiles in response to environmental perturbations with and without fungal symbionts; ii) developing computational methods to map regulatory and metabolic pathways implicated in plant-microbe mutualism; and iii) integrating proteome, metabolome, and transcriptome profiles to refine understanding of crucial genes and molecular mechanisms involved in these interactions.

To address these tasks, we have developed a model system where *Arabidopsis thaliana* was grown alone or co-cultivated with the root colonizing mycorrhiza-like fungi *Piriformospora indica*. An abiotic stress coexpression network map was created for *A. thaliana* that consisted of subnetworks (modules) enriched with gene products contributing to signaling, drought, heat, salt, and UV-B perturbations. We hypothesized that these modules would demonstrate robustness to environmental perturbation when *A. thaliana* was co-cultivated with *P. indica*, since this fungus has been shown previously to enhance host plant yield under abiotic stress conditions. After confirming that *P. indica* hyphae were associated with host plant roots using microscopy, plants with and without *P. indica* were subjected to ambient (25 °C) and heat (38 °C) treatments. Regardless of treatment, co-cultivation with *P. indica* induced gene expression in the heat shock and ROS (reactive oxygen species) signaling subnetworks. Co-cultivated plants exposed to the heat treatment maintained other subnetworks similar to ambient temperature expression levels, whereas heat treated plants cultured in the absence of the fungal symbiont had significant expression level changes for all subnetworks relative to ambient temperature controls. Biochemical quantification of ROS confirmed the network-derived signaling result and suggests that *P. indica* manipulates this signaling pathway to buffer host plants against abiotic perturbations.

To further refine our ability to discover biochemical and molecular mechanisms of plant - fungal mutualisms, expression changes in over 200 currently known co-expression modules reflecting tightly coordinated pathways in *A. thaliana* between inoculated and control samples are being systematically investigated. Rearrangements in the co-expression modules in response to mutualistic interactions, specifically genes that are co-expressed in the subnetworks, are being mapped. These genes will be targeted for further analytical studies as potential candidates involved in inter-specific signaling pathways. Finally, a Bayesian statistical framework, calibrated on several well-studied *A. thaliana* molecular mechanisms to integrate proteome, metabolome and transcriptome data, is being used to decipher novel molecular mechanisms corresponding to the co-expression subnetworks that are found to be important in response to interactions with *P. indica*. The overall experimental and

computational approach will be extended to future plant/microbiome communities investigated in this project.

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Plant-Microbe Interfaces: Characterization of Native Microbial Communities in the Roots and Rhizosphere of *Populus deltoides*

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<http://PMI.ornl.gov>

Project Goals: See goals for abstract 194.

The beneficial associations between plants and microbes exemplify complex, multi-organism systems that are shaped by the participating organisms and the environmental forces acting upon them. These plant-microbe interactions can benefit plant health and biomass production by affecting nutrient uptake, influencing hormone signaling, effecting water and element cycling in the rhizosphere, or conferring resistance to pathogens. The rhizosphere of *Populus* species represents an ideal system for understanding the natural diversity of these associations, as well as the molecular details that result in function. *Populus*, and more generally the Salicaceae (willow family) to which they belong, are host to bacterial endophytes and to two prominent types of mycorrhizal fungi, arbuscular endomycorrhizae (AM) formed by Glomalean fungi, and ectomycorrhizae (EM) formed by Ascomycetes and Basidiomycetes.

Broad-based efforts to understand the natural diversity of microbial associates of *Populus deltoides*, *P. trichocarpa*, and their natural and artificial hybrids in both native and controlled habitats have been initiated. As a pilot study for this work, a population of *P. deltoides* as it occurs along the Caney Fork River was sampled in the early fall of 2009. Two *P. deltoides* stands were sampled, representing both upland and bottomland ecotypes and soil conditions that commonly occur in this region. This study is also serving as a “testbed” for methods development that will be applied more broadly for similar studies in the coming growing season. These efforts include four related foci: 1) Community assessment based on rRNA genes using pyrosequencing and other methods to describe the variation within and between individual trees in these two environments (described here); 2) Efficient cultivation, typing and physiological characterization of representative associates (described in poster by Pelletier et al.) that can be used as models for further molecular interaction studies; 3) Methods development for localization and quantification of microbial associates within

diverse *Populus* tissue-types; and 4) Methods development for single-cell manipulations that will compliment targeted cultivation, as well as metagenomic and metaproteomic efforts (described in poster by Retterer et al).

As part of this study, we are conducting 454 based pyrosequencing to describe both bacterial and fungal root and rhizosphere associations of the Caney Fork populations. Rhizosphere populations were examined from field samples that were washed in buffered saline. Mycorrhizal and bacterial tissue-associated populations are being examined on the same surface sterilized root samples. Extensive efforts to optimize both surface sterilization methods and subsequent DNA extraction showed that efficient sterilization could be achieved by combining hydrogen peroxide and sodium hypochlorite based washing followed by commercially available DNA extraction methods. Using these methods, approximately 50-100ng DNA per mg root tissue was extracted and was readily amplifiable with PCR based approaches. Existing methods targeting the V1-V2 region of the 16S rRNA genes have shown that root and rhizosphere associated communities are extremely diverse, comprising thousands of OTUs per sample. Similar to past studies we show that these communities are dominated by alpha-proteobacteria. Comparisons of variation within individual tree samples, between trees from similar environments, and between ecotypes are in process pending the completion of final samples from these collections. Our initial efforts in developing efficient methods for fungal community assessment have focused on descriptions of the D1/D2 region. This region can be targeted with conserved primer sets, allowing alignments over the entire diversity of fungi, but is also variable enough to allow for robust assessment of populations to the family level or below. Initial tests with multiple barcoded primer variants show that efficient and unbiased assessment may be achievable with the developed methods.

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Plant-Microbe Interfaces: The Role of Plant Genotype and Phenotype in Regulating the Symbiotic Microenvironment

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<http://PML.ornl.gov>

Project Goals: See goals for abstract 194.

A number of signals broadly classified as phytohormones (auxin, cytokinin and ethylene) and secondary metabolites

(flavonols, alkaloids and polyamines) have been known to affect plant-microbe associations, however, the molecular underpinnings of how the signals are transduced into plant level phenotypic changes (e.g. lateral root proliferation, induction of systemically-acquired resistance) is poorly understood. Genotypic variability in plant carbon and nitrogen metabolism greatly alters both the intracellular and extracellular metabolite profiles and thus determines the biochemical microenvironment in which microbial (fungal and bacterial) symbionts exist. Our previous biochemical analyses of pure species of black cottonwood (*Populus trichocarpa*), eastern cottonwood (*P. deltoides*), their interspecific hybrids, other *Populus* sp. and their transgenic and cisgenic mutants provide a diverse variety of clones with tailored differences in primary and secondary metabolism that can be exploited to probe plant-microbe interactions. We hypothesize that enhanced production of metabolites of primary C and N metabolism in *Populus* roots, including simple sugars, organic acids, and amino acids, promotes colonization by ectomycorrhizal fungi and endophytic bacteria. We also hypothesize that unique secondary metabolites produced by *Populus* roots function as selective agents that both promote and inhibit specific microbial species. Here we present the current status of *Populus* genetic resources that have been characterized or created through transgenesis to provide the basis for future studies on *Populus*-microbe associations.

Populus trichocarpa (T) and *P. deltoides* (D) differ in their profiles of secondary metabolites and these metabolic differences segregate in successive generations such that quantitative trait loci associated with metabolite production (mQTL) can be mapped. We have created dense genetic marker maps and mapped several hundred mQTL for both leaf and root metabolites in two pseudo-backcross (TDxD') pedigrees, including family 13 and family 52124. The identification of 38 definitive and 117 suggestive mQTL for root metabolites in family 13 included the location of an mQTL hotspot on linkage group X that regulates the production of several key secondary metabolites, including salicortin, salireposide, and several phenylpropane glycosides (vimalin-like). Five candidate genes were selected within the mQTL region and constructs were designed for their up- and down-regulated expression. These cisgenic transformants with putatively increased and decreased secondary metabolism are scheduled to be available for characterization early in 2010 and will be available to test the effects of altered metabolism on plant-microbe associations. Additionally, the F2 backcross progeny with extremely high (and low) production of secondary metabolites that led to the identification of 239 mQTL associated with 105 metabolites in family 52124 are available and can also be selected to determine the effects of altered metabolism on plant-microbe associations.

We have shown that a key difference in secondary metabolites among *Populus* species is the nature and concentration of hydroxycinnamate-quininate/shikimate esters that are present and are likely to affect microbial colonization. The role of such metabolites in colonization can be assessed by the selection of *Populus* species with diverse profiles of such metabolites. Additionally, we are generating *P. deltoides*

clones that have been down-regulated for all genes in the lignin biosynthetic pathway, including hydroxycinnamate-quininate transferase (HCT) and coumarate-3-hydroxylase (C3H), that all have direct and indirect effects on the concentrations of these metabolites, given that they serve as storage compounds for the up-stream lignin precursors that have inhibitory effects on microbes. In addition to these clones, we have determined the metabolic phenotype of a number of activation-tagged *P. tremula x alba* clones that have elevated or greatly depleted concentrations of these metabolites. Microbial species that are successful colonizers must be able to tolerate the free mono- and diphenolic acids and their quinate/shikimate esters. These *Populus* clones can serve as the background plant material to determine what classes of compounds promote or inhibit key plant-microbe associations.

We have selected the early colonization events of the *Populus-Laccaria* association, which is the model perennial tree-fungal association with both organisms having had their genomes sequenced and thus have available a broad array of genomic resources. *Laccaria bicolor* is an ectomycorrhizal fungus that routinely colonizes *Populus*. The metabolic signaling responses involved in the establishment of the association is being characterized by analyzing the time-course of metabolomic and transcriptomic (array-based) responses of both organisms reared under *in vitro* culture, contrasted with greenhouse pot culture with samples in both studies collected every 2 weeks up to 8 weeks in the *in vitro* study, and up to 12 weeks in the pot culture study. Additionally, the role that the poplar genotype has in the metabolic responses involved in the establishment of the plant-microbe association is being investigated by altering the *Populus* host, including *P. deltoides*, *P. trichocarpa*, and three *P. trichocarpa x deltoides* hybrids. Once putative signals are identified, their roles will be assessed by initiating assays of indirect contact between *Populus* roots and *Laccaria in vitro* (i.e., a cellophane membrane allows molecular cross-talk through diffusible metabolites, such as auxins, without physical contact) at early time points. Similarly, the effects of the direct contact of a limited number of putative signals at very early time points will also be studied. The metabolite signals and microarray responses will be correlated in co-expression networks to identify novel signaling pathways that regulate the *Populus-Laccaria* association.

Recent reports suggest that root growth induced in *Populus* interacting with *Laccaria* required polar auxin transport as well as auxin signaling through *Populus* auxin response regulator proteins. Many sequenced microbial genomes carry genes of auxin biosynthetic pathway, but it is unclear to what extent these signaling pathways are universal or specific in establishing a symbiotic relationship between a specific microbe and host genotype. We hypothesize that certain auxin response factor proteins belonging to the *Aux/IAA* and *ARF* families play a direct role in establishment and/or signal transduction post-establishment. We are testing this hypothesis by co-culturing specific microbial strains with PCR-confirmed *Populus* RNAi lines specific to genes from the *Aux/IAA* and *ARF* gene families. A micropropagation protocol established to generate whole plantlets from

Populus shoot tips is being used to also test candidate endophytic and rhizosphere microbes identified from *Populus* field surveys and will be tracked by imaging and molecular profiling methods. The efforts to identify the molecular factors are being complemented with measurements of the levels and impact on altered levels of auxin in co-cultivation experiments. These plant materials will be harvested at various timepoints and characterized at the transcriptomic and proteomic levels in order to understand the mechanisms of hormone cross-talk relaying the plant level outcomes due to the microbial association. This presents a pipeline for micro-scale screening of where, when, and how microbes associate with the host plant.

204 Plant-Microbe Interfaces: Isolation and Characterization of Cultivable Members of the *Populus* Rhizosphere-Endosphere Community

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Project Goals: See goals for abstract 194.

As part of the newly initiated ORNL Plant-Microbe Interfaces Science Focus area, we are using broad-based methods to characterize the natural diversity of microbial associates of *Populus* and elucidate the molecular mechanisms by which these diverse organisms interact. The goal of the isolation and screening efforts for this project is to develop methods for efficient cultivation, typing, and physiological characterization of representative associates that can be used as model organisms for further molecular interaction studies. In a pilot study for this work, we sampled a population of *P. deltoides* as it occurs along the Caney Fork River in Tennessee in the early fall of 2009. Two *P. deltoides* stands were sampled, representing both upland and bottomland ecotypes and soil conditions that commonly occur in this region. From these samples, a number of diverse fungal and bacterial associates have been isolated from *Populus* rhizosphere and surface sterilized root tissues using broadly compatible media and direct plating methods. These isolates are being screened for phylogenetic identity with bacterial and fungal rDNA primers, and for traits of interest involved in plant-microbe interactions using molecular and biochemical assays, including nitrogen fixation (*nifH*), indole-3-acetic acid synthesis pathways (*iaa*), salicylate degradation genes (*nahJ* and *nahW*), and fungal oxalate production (*oahA*).

The exchange of chemical signaling molecules through either direct contact or diffusion has been demonstrated in a few well studied plant-microbe model systems to effect establishment and maintenance of symbiotic interactions. These signals (e.g., phytohormones, antimicrobials, quorum sensing compounds) affect a wide range of phenotypic responses that can influence plant-microbe and microbe-microbe interactions, including production of antimicrobials, exopolysaccharides, exoenzymes, motility, and conjugation. Microbes isolated from the *Populus* rhizosphere and endosphere are being screened for the production of small signaling molecules by GC-MS metabolomics of culture supernatants from isolates grown in the presence and absence of plant-derived exudates. Isolates are also being screened for known and novel homoserine lactone-derived quorum sensing compounds.

These cultivation and screening efforts are being complemented with improved methods for resynthesizing microbial relationships with *Populus* in greenhouse and tissue culture based assays that are critical components of proving the function of these microbial associates. In addition to conventional plate-based isolation methods, efforts are focused on single-cell microbial isolation and characterization techniques and the development of relevant technologies that will ultimately facilitate the genetic characterization of a greater portion of the endophytic and rhizospheric communities. We anticipate that multiple new microbial species will be characterized and advanced toward further studies.

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Plant-Microbe Interfaces: Application of Microfluidic Technologies for Microbial Isolation, Cultivation, Characterization, and Emulation of the Plant-Rhizosphere Microenvironment

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<http://PMI.ornl.gov>

Project Goals: See goals for abstract 194.

Isolation, cultivation and characterization of microbes from complex samples, derived from soil, rhizosphere and plant material, are essential for defining the plant microbiome.

Technologies that allow the physical and chemical manipulation of fluid volumes that approach the single cell level are critical to such processes and offer a means to understand the full range of genetic and phenotypic variation within plant associated microbial and mycorrhizal communities. Microfluidic platforms, created using soft lithographic and advanced nanofabrication techniques, have been developed and their utility for physically isolating single cells in microbead emulsions and creating complex chemical gradients for the study of microbial taxis has been demonstrated.

Micro-scale alginate beads were created using a microfluidic multiphase droplet generation system. By combining a solution of model cells within the aqueous/alginate phase, the system was successfully used to encapsulate individual and small numbers of GFP-expressing *E. coli*. Both internal and external gelation processes were examined for their effects on cell viability and bead stability. The successful isolation of individual cells within the alginate microbeads and subsequent cultivation of these isolates demonstrates the potential of this system for the small scale clonal expansion of microbial cells captured from more complex mixtures of cells taken from natural samples. As this technology is developed, its implementation in the field and application to the characterization of field samples will play a critical role in assessing the genetic and phenotypic diversity present within the *Populus* microbiome.

Once microbial species are isolated via conventional or emerging cultivation techniques, characterization of their phenotypic response to specific molecular signals found within the plant rhizosphere may be correlated with fluctuations in their population. Such fluctuations may correspond to both developmental and phenotypic stages of plant growth. Understanding microbial taxis in response to components of plant exudate/xylem sap, characterized via mass spectrometry during such stages, may provide insight into what role cell motility and taxis plays on microbial recruitment and colonization. A microfluidic platform that allows exquisite spatial and temporal control of chemical gradients while allowing sustained imaging and tracking of individual bacteria has been developed. This system will be used to examine taxis of model microbes and natural isolates as they become available.

The Predictive Microbial Biology Consortium

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Genemap-MS: Stable Isotope Assisted Metabolite Profiling of *Synechococcus sp.* 7002

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Project Goals: Development of a metabolomics-centric platform for comprehensive validation and expansion of genome annotations.

Computational homology-based annotations of sequenced genomes of microbes provide an overview of their metabolic capabilities. Inherent uncertainties in homology-based functional annotations, presence of a significant fraction of genes of unknown function in annotated genomes along with a large number of enzymatic activities without a known corresponding gene limit the extent of genome annotations. Comprehensive profiling of cellular metabolites offers an attractive opportunity for the validation and expansion of genome annotations since the presence of specific metabolites indicates the presence of related enzymatic activities.

The first essential prerequisite for the exploitation of metabolites as indicators of enzymatic activities is the ability to identify these metabolites in complex metabolite profiles. Liquid chromatography coupled to electrospray time-of-flight mass spectrometry (LC-ESI-TOFMS) provides chromatographic separation among metabolites in complex mixtures and mass spectra with high mass accuracy. Metabolites can be identified by comparing their accurate mass, retention time, and fragmentation (MS/MS) spectra against chemical standards. Complex metabolite profiles often contain numerous features which do not correspond to any available and analyzed chemical standard. These features can be analyzed by assigning putative empirical formulas based on accurate mass and isotopic profiles (applicable to small compounds - $m/z < \sim 200$) and inferring partial structural information from MS/MS spectra.

To expand the unambiguous assignment of empirical formulas to larger metabolites (up to $m/z \sim 500$), uniform labeling of *Synechococcus sp.* 7002 cultures was performed with stable isotopes ^{13}C and ^{15}N . Characteristic shifts in masses of features in ^{13}C - and ^{15}N -labeled metabolite profiles discriminated background signals from metabolites originating from the metabolism of *Synechococcus sp.* 7002. Additionally, these shifts determined the number of carbons and nitrogens in specific metabolites thus discriminating between alternative candidate empirical formulas, which were determined from accurate mass of features in an unlabeled control dataset only. This approach facilitated the

inference of a large number of putative compounds with validated empirical formulas. A fraction of these was assigned to specific metabolites based on the correspondence of their accurate mass and retention time to chemical standard.

Draft metabolic network of *Synechococcus sp.* 7002 was reconstructed using Pathway Tools software (BioCyc). This reconstruction is based on genome annotation but also accounts for additional enzymatic activities or spontaneous reactions inferred from the topology of the metabolic network (gap filling according to reference metabolic pathways). Comparison of the draft network against the set of confirmed empirical formulas showed that many of the unique empirical formulas have no correspondence to any of the 805 metabolites in the draft network. Moreover, a number of the confirmed empirical formulas do not correspond to any metabolite in MetaCyc or KEGG databases.

Following the analysis of MS/MS spectra, the identity of a subset of empirical formulas without any correspondence in MetaCyc or KEGG could be assigned to dipeptides of glutamate (at the N-terminus) with one of multiple hydrophobic amino acids. An intermediate of an alternative biosynthetic pathway, with only one of the pathway's reactions assigned to a gene in the genome, was also identified using MS/MS spectra. Another interesting finding is what appears to be a dead-end metabolite according to KEGG database. This metabolite is present predominantly in the metabolite profile of the culture media, which is significantly less complex than the profile of the cell extract.

Metabolite profiling is an attractive approach for comprehensive interrogation of cellular metabolism. Presence of specific metabolites may serve as an indicator of the presence of specific enzymatic activities or metabolic pathways. Comprehensive metabolite profiling may thus serve as the first step in the validation of genome annotations and identification of candidate enzymatic activities or pathways missing from the genome annotation. Combination of metabolite profiling with genetic/environmental perturbations and transcriptomics/proteomics may further zoom in on specific genes related to specific enzymatic activities or metabolic pathways.

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Applications of GeoChip for Analysis of Different Microbial Communities

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Project Goals: As part of the GTL program, our research is focused on expanding and improving the GeoChip, a comprehensive functional gene array, and to use this array to detect and monitor microbial communities.

Microarray technology provides the opportunity to identify thousands of microbial genes or populations simultaneously. The objective of this study was to further develop and apply a comprehensive functional gene array (GeoChip) to detect and monitor microbial communities. GeoChip 2.0 is a functional gene microarray which allows for the simultaneous detection of >10,000 genes involved in the geochemical cycling of C, N, and S, metal reduction and resistance, and organic contaminant degradation. Based on GeoChip 2.0, a new generation, GeoChip 3.0 has been developed, which has several new features. First, GeoChip 3.0 covers more gene groups including antibiotic resistance, energy processing, and additional functional genes involved in C, N, P, and S cycling. Second, the homology of automatically retrieved sequences by key words is verified by HUMMER using seed sequences so that unrelated sequences are removed. Third, a universal standard has been implemented so that data normalization and comparison of different microbial communities can be conducted. Fourth, a genomic standard is used to quantitatively analyze gene abundance. In addition, GeoChip 3.0 includes phylogenetic markers, such as *gyrB*. Finally, a software package has been developed to facilitate management of probe design, data analysis, and future updates. This version of GeoChip was a recipient of a 2009 R&D100 Award from *R&D Magazine*, which recognizes the 100 most innovative scientific and technical breakthroughs of the year. Additional expansion is currently underway and will include genes related to stress response, virulence factors, human-microbiome, and phage genes.

The GeoChip has been used to examine dynamic functional and structural changes in microbial communities from many different environments. Here, examples of studies utilizing the GeoChip to examine microbial communities at contaminated sites are presented. These studies illustrate the ability of the GeoChip to provide direct linkages between microbial genes/populations and ecosystem processes and functions. These three studies examined areas within the U.S. DOE's Field Research Center (FRC) in Oak Ridge,

TN. (1) Microbial communities within a pilot-scale test system established for the biostimulation of U (VI) reduction in the subsurface by injection of ethanol were examined using GeoChip 3.0. Functional community dynamics were examined during a period of nitrate exposure. After exposure to nitrate the diversity and richness increased several fold but quickly returned to pre-nitrate levels. Detrended correspondence analysis (DCA) indicated a shift in the overall community structure after nitrate exposure but the community began to return to pre-exposure structure once nitrate was removed. The relative abundance of several nitrogen cycling genes showed an increase immediately after nitrate exposure, including ammonification, denitrification, and nitrogen fixation genes indicating a stimulation of these communities.

(2) In the second study from the FRC, analysis of groundwater monitoring wells along a contamination gradient using GeoChip 2.0 revealed less overlap between wells with different levels of U and NO₃ contamination. While diversity of nitrate-fixation genes decreased in NO₃-contaminated wells, the diversity of metal reduction and resistance genes did not correlate with metal concentrations. Signal intensity did, however, increase in heavily contaminated wells, indicating a larger percentage of organisms with metal-related genes. Sulfate-reduction genes had greater diversity and greater signal intensity in more contaminated wells. Individual principle component analyses (PCA) of the gene diversity and geochemistry of the wells separated them in similar ways. CCA indicated that pH was an important variable that correlated with gene diversity in the lowest-contamination well, while NO₃ and U correlated with the most highly contaminated well. Overall, contaminant level appears to have significant effects on the functional gene diversity along the contaminant plume at the FRC.

(3) A third study is currently underway using GeoChip 3.0 to examine functional gene changes in a U (VI) contaminated area after introduction of a slow-release-substrate (SRS), designed to provide a long-term electron donor for U (VI) reduction. Preliminary results indicate a stimulation of microbial communities. These studies demonstrate the analytical power of the GeoChip in examining microbial communities. This is the first comprehensive microarray available for studying the functional and biogeochemical cycling potential of microbial communities.

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Pipeline for Large-Scale Purification and Identification of *Desulfovibrio vulgaris* Membrane Protein Complexes

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http://pcap.lbl.gov

Project Goals: To develop and apply a pipeline for the high-throughput isolation and identification of *Desulfovibrio vulgaris* Hildenborough membrane protein complexes in cultures grown under standard conditions, and to characterize changes in these complexes brought about by environmentally relevant stressors.

As a component of the LBNL ENIGMA Program, an important task of the Protein Complex Analysis Project (PCAP) is to develop and apply methodologies for the identification of membrane protein complexes isolated from the sulfate reducing bacterium *Desulfovibrio vulgaris* Hildenborough (*D. vulgaris*). Given its demonstrated ability to reduce heavy metals, *D. vulgaris* is projected to play an important role in the management of contaminated sites. To optimize the use of *D. vulgaris* at these sites it will be critical to know how environmental changes affect its performance. To gain insight into these questions, we have developed a pipeline to isolate and identify stable membrane protein complexes present in cultures grown under standard conditions, stationary phase conditions, and in the presence of environmental stressors. Through these efforts we hope to assemble the data needed to characterize stress-induced changes in the relative abundance, composition, and stoichiometry of *D. vulgaris* membrane protein complexes. This data, in turn, will be used to model its stress response pathways and optimize the bioremediation capabilities of this microbe.

Membrane protein complexes pose unique purification and analysis challenges. Largely due to the requirement for detergent solubilization, stable isolation of homogeneous intact membrane protein complexes typically requires separation conditions that are different from those used for water soluble proteins. For this task we have been employing a “tagless” strategy optimized for purifying membrane proteins and then identifying them by mass spectrometry (MS). As opposed to strategies employing affinity tags for the purification of target molecules, use of a tagless strategy will enable us to obtain global views of stress-induced changes involving membrane proteins in *D. vulgaris* cultures grown under a variety of conditions.

In the pipeline, *D. vulgaris* cell membranes isolated from large-scale (100 liter) cultures are first treated with

a relatively mild detergent suited for the extraction of inner-membrane proteins. The residual membranes of this gram-negative bacterium are subsequently treated with a second more active detergent to solubilize proteins of the outer-membrane. Each membrane extract is then independently processed. To purify candidate complexes of the inner- and outer-membrane fractions, ion exchange (IEX) and molecular sieve chromatography are used. Fractions obtained from these procedures are further analyzed using SDS and blue native gel electrophoresis to isolate candidate complexes and obtain molecular weight estimates. To prepare samples suitable for MS analysis, whole lanes are cut from blue-native PAGE gels, placed horizontally along the stacking sections of denaturing gels and subjected to a second dimension of SDS PAGE. Potential complex subunits manifest themselves as bands or spots providing insight into the composition of the native complex. Spots removed from these gels are subjected to in-gel digestion and analysis by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) or liquid chromatography and matrix assisted laser desorption time of flight mass spectrometry (LC-MALDI-MS/MS). Protein identification is achieved by searching a custom *D. vulgaris* database using the Mascot or Protein Pilot search engine.

We are currently completing a baseline membrane protein complex dataset derived from *D. vulgaris* large-scale cultures grown under standard conditions (mid-log phase). In addition to providing a catalog of *D. vulgaris* membrane protein complexes, this data will serve as an essential reference for the detection and characterization of changes in the complexes of cultures subjected to the aforementioned stressors.

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High Throughput Identification, Purification and Structural Characterization of Soluble Protein Complexes in *Desulfovibrio vulgaris*

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Project Goals: One of the main goals of the PCAP component of the LBNL ENIGMA SFA is to develop high throughput pipelines to purify and identify protein complexes and structurally characterize them by EM. *Desulfovibrio vulgaris* was selected as a model bacterium because of its bioremediation potential in immobilizing

toxic heavy metals in soils. Our overall workflow consists of (i) a multidimensional separation of the soluble proteome using tagless strategy; (ii) identification of putative complexes by quantitative mass spectrometry (MS); (iii) further purification of selected complexes; and (iv) structural analysis by EM. In some cases, the stoichiometry of protein complexes is studied using novel mass spectrometry techniques that enable analysis of non-covalent assemblages (native MS). Our major focus is to increase the throughput of MS and EM single-particle analyses and to develop methods for an automated assignment of protein complex components. Currently we are implementing automated collection of EM data, particle boxing, and analysis of structural variation, and the engineering of new support-film technologies for better EM sample preparation to preserve quaternary structure in a conformationally homogeneous state. In the future, we will also take advantage of the SAXS expertise within ENIGMA and incorporate this technology into our structural characterization of complexes.

One of the main goals of the PCAP component of the LBNL ENIGMA SFA is to develop high throughput pipelines to purify and identify protein complexes and structurally characterize them by EM. *Desulfovibrio vulgaris* (*DvH*) was selected as a model bacterium because of its bioremediation potential in immobilizing toxic heavy metals in soils at the DOE sites by rendering them practically insoluble upon conversion to the low red-ox state. Our overall workflow consists of (i) a multidimensional separation of the soluble proteome; (ii) identification of putative complexes by quantitative mass spectrometry (MS) followed by bioinformatics analysis; (iii) further purification of selected complexes; and (iv) structural analysis by EM. In some cases, the stoichiometry of protein complexes is studied using novel mass spectrometry techniques that enable analysis of non-covalent assemblages (native MS). In future, we will also take advantage of the SAXS expertise within ENIGMA and incorporate this technology into our structural characterization of complexes.

To identify protein complexes, we have introduced and established a tagless strategy, which is based on the premise that the great majority of stable protein complexes will survive intact separation through a series of orthogonal chromatographic methods. Under this scenario, co-migrating polypeptide components of protein complexes generate overlapping elution profiles at each stage of protein separation. Changes in the relative concentration of each polypeptide (i.e., their elution profiles across the final chromatographic step) are measured with the aid of isotopic dilution mass spectrometry (MS) and iTRAQ reagents (Dong et al., 2008). Various data analysis approaches are being developed to automate assignment of the identified polypeptides to putative complexes. To date, ~64% of the target protein complex separation space has been analyzed, resulting in the identification of over 900 polypeptides. The great majority of the polypeptides are engaged in intermolecular interactions, as evidenced by more than 70% demonstrating significantly higher elution volume (at least 2x) in size exclusion chromatography (SEC) than expected from the

molecular weight of the polypeptide predicted from genome sequence. In addition to heteromeric complexes, the tagless strategy allows detection of homomers, which are not easy to recognize by other methods (e.g., TAP). Overall, at least 45 heteromeric and over 550 homomeric complexes have been identified so far. To address the challenges posed by a co-elution of non-related polypeptides with the legitimate components of complexes, we are evaluating monitoring elution profiles at the two final stages of protein complex separation (hydrophobic interaction chromatography and SEC), as opposed to the single step (SEC) used so far. The resulting 2D polypeptide elution map is expected to provide higher resolution data and consequently to increase confidence in protein complex assignments.

To determine molecular structures, we selected 16 complexes identified by the tagless strategy with molecular weights 400 - 1,000 kDa and subjected them to single-particle EM analysis. Half of the complexes studied proved stable enough to produce high-quality 3-D reconstructions with a resolution of ~2 nm (Han et al., 2009). This success rate for obtaining structures is about 10 times greater than that of previous "proteomic" screens. We have found that there are a surprisingly large number of differences in the quaternary structures of complexes isolated from *DvH* compared to those of homologous proteins from other microbes. These differences occur so frequently that structures determined for complexes in other micro-organisms are likely to be inadequate as templates for modeling the biochemical networks within a given microbe of interest. By extension, we suspect that it may also be the case that complexes change structure frequently under different physiological conditions and future work will address this possibility. Our major focus now is to increase the throughput of EM single particle structural analysis. This effort currently includes the implementation of automated data collection, particle boxing, and analysis of structural variation (Shatsky et al., 2009), and the engineering of new support-film technologies for better EM sample preparation to preserve quaternary structure in a conformationally homogeneous state.

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Protein Complex Analysis Project (PCAP): Large-Scale Identification of Protein-Protein Interactions in *Desulfovibrio vulgaris* Using Tandem-Affinity Purification

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Project Goals: Develop a high-throughput platform for chromosomal modifications of microbes of interest to DOE. Identify protein-protein interactions in the model sulfate reducer *Desulfovibrio vulgaris* using tandem affinity purification. Develop computational tools and models to characterize sulfate-reducing bacteria.

Most cellular processes are mediated by multiple proteins interacting with each other in the form of multi-protein complexes and not by individual proteins acting in isolation. One of the major goals of LBNL ENIGMA SFA is to develop a comprehensive knowledgebase of protein complexes and protein-protein interactions (PPI) in microbes of interest to DOE. As part of the Protein Complex Analysis Project component of ENIGMA, *Desulfovibrio vulgaris*, a sulfate reducing bacterium (SRB) found to exist in several DOE waste sites, has been used as a model to screen for PPI using a high throughput tandem affinity purification (TAP) approach. The challenges in working with *D. vulgaris* are typical of organisms of interest to DOE. While likely the best characterized SRB, *D. vulgaris* is an obligate anaerobe and possessed very limited genetic tools. Our approach therefore required the development of a high throughput pipeline to enable the creation of a library of genetically engineered strains, which builds upon generic principles of recombination of non-replicating gene replacement constructs ("suicide" constructs). We have applied this pipeline to the creation of *D. vulgaris* strains bearing TAP-tagged alleles designed for tandem affinity purification of individually tagged bait proteins; however, the versatility of this approach enables its potential application for chromosomal modifications of the majority of microbes of interest to DOE.

Previously we reported the development of large-scale single- and double- crossover chromosomal integration platforms for generating TAP-tagged strains of *D. vulgaris*. Transformation of plasmids into *D. vulgaris* is inefficient and multiple strategies, including constructing a restriction endonuclease host strain mutant that enhanced transformation of stable plasmids into *D. vulgaris* have been explored. However the single greatest improvement in transformation

and integration of suicide constructs has resulted from the transition from the TOPO-Gateway[®] scheme to the Sequence and Ligation Independent Cloning (SLIC) technique for suicide construct generation. Success rates for suicide construct generation improved from 50% to more than 80%, transformation and integration of constructs into *D. vulgaris* from 34% to 65% and ~60 % of these isolates were found to express a TAP-tagged fusion protein detected by IP-western. Currently, we have a library of over 700 suicide plasmid constructs which have been employed for the generation of over 300 tagged SRB strains of which ~200 baits have been analyzed by TAP to date.

Here we present details of our high throughput pipeline for strain engineering along with results of the subsequent TAP analysis of these engineered strains. The interactions presented cover a range of biological processes, including energy conservation (ATP Synthase, Ech hydrogenase), sulfate reduction (dissimilatory sulfite reductase, adenylylsulfate reductase) and protein secretion (YajC-HflCK complex), and include both novel and previously predicted interactions.

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Systems Approach in a Multi-Organism Strategy to Understand Biomolecular Interactions in DOE-Relevant Organisms

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<http://gaggle.systemsbio.net/projects/doe-archaea/2007-04/>
<http://maggie.systemsbio.net>
<http://baliga.systemsbio.net>

Project Goals: Bolster through high-end state-of-art systems approaches, developed specifically for the study of archaeal organisms, the comprehensive analysis of multi-protein complexes in DOE-relevant organisms.

Rational re-engineering of biology for the purpose of bioremediation, bioenergy or C-sequestration requires deep understanding of all functional interactions of relevant components within native cell (s). Many of these functional interactions are conserved across diverse species to different

degrees depending on their evolutionary distance. We are conducting integrative analysis of genomic architecture and composition, transcriptome and proteome structure/function, protein-protein and protein-DNA interactions and metabolic networks to find keystone complexes and specialized circuit architectures for important application-relevant genes within four archaeal organisms. These organisms have enormous potentials from the standpoint of H₂ production, N₂ fixation, and C-sequestration; they include an anaerobic thermophile (*Pyrococcus*), an acidophilic and aerobic thermophile (*Sulfolobus*); a hydrogenotrophic methanogen (*Methanococcus*), and a photoheterotrophic halophile *Halobacterium* NRC-1. A key aspect of our approach is to use the power of systems biology to delineate the process of nucleation, assembly, and turnover of key complexes. Here, we report comparative analysis of dynamically changing transcriptome structures of the four archaea with special emphasis on the conditional activation of unconventional transcriptional promoters within conserved genes and operons.

Note: Computational and experimental results from this study will be freely available upon publication at <http://maggie.systemsbiology.net/>. All of the software tools developed in this project have been made freely available at <http://gaggle.systemsbiology.net/projects/doe-archaea/2007-04/>.

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Metabolic Transformations and Chemical Differences

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Project Goals: Use autocorrelation for the analysis of metabolites using liquid chromatography coupled to mass spectrometry.

Defining the complete metabolic reconstruction for even a simple organism requires detailed knowledge of enzyme function, activity, and biochemistry. Uncharacterized enzymes and unknown metabolic pathways limit the utility of inferring metabolic capability based on genome sequencing and annotation. Homology-based protein function predictions are limited by evolutionary processes that result in conserved domains, and the complexity of biochemistry is so diverse that only a small portion has been defined. Therefore, improving genome annotations and furthering our knowledge of biochemistry is of tremendous importance to take advantage of the potential of genome sequencing.

The detection and analysis of metabolites using liquid chromatography coupled to mass spectrometry (LC/MS)

has the potential to define metabolic pathways, understand the regulation of substrate utilization, measure biomass composition, and much more. The primary challenge associated with this analytical platform is the interpretation of the thousands of molecular features detected in a typical dataset. There are three hurdles which define this crux: 1) a large fraction of the detected features are associated with uncharacterized metabolites, 2) standard methods for the detection of known metabolites are not widespread, and 3) optimum methods have not been defined for feature identification from raw data.

Although the scale biochemistry is vast, the emergence of primary and secondary metabolites is due to a limited number of elementary chemical-differences relating compounds. In this work, we explore a finite number of chemical transformations, and examine chemical-difference-space to understand alterations in metabolism. This is accomplished using autocorrelation, an established method in data analysis with a very high sensitivity for detecting correlated relationships. Here, we have applied autocorrelation to interpret raw mass spectrometry data and then linked the global correlation spectra to chemical differences. This has enabled a global analysis of LC/MS data without relying on feature identification. This analysis was applied to *Sulfolobus sulfataricum*, an archaea that is of interest in creating biofuels, and early results indicate that discrete chemical differences can be measured and interpreted.

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Towards Localization of Functionality in *Desulfovibrio vulgaris* by Electron Microscopy

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Project Goals: The twin goals of this project are 1. to develop an integrated set of high throughput pipelines to identify and characterize multi-protein complexes in a microbe more swiftly and comprehensively than currently possible and 2. to use these pipelines to elucidate and model the protein interaction networks regulating stress responses in *Desulfovibrio vulgaris* with the aim of understanding how this and similar microbes can be used in bioremediation of metal and radionuclides found in U.S. Department of Energy (DOE) contaminated sites.

Bacteria display a spatially and temporally defined three-dimensional organization of their macromolecular complex inventory that allows them to grow, divide, and respond to

environmental cues. As part of the PCAP component of the LBNL ENIGMA Program, our overall goal is to develop tools that can be used to define the localization of such complexes within the cell.

Desulfovibrio vulgaris Hildenborough (DvH) is an anaerobic sulfate-reducing bacterium (SRB). It is used as a model organism for the study of environmental bioremediation of heavy metal and radionuclide contamination. Its ability to efficiently reduce toxic heavy metals such as uranium and chromium is of particular interest to the DOE for use in high-risk metal contaminated sites, and it can also provide novel insights on the range of metabolic pathways available in microbes. Protein expression levels and subcellular localization of proteins and macromolecular complexes may change in response to various environmental factors such as exposure to the target toxins, and may also differ among bacterial cells of identical genetic origin within a given culture. We aim to use our localization technology to understand how protein abundance and spatial distribution relate to the cell's normal metabolism and how changes in these factors are involved in the cell's response to changes in the environment.

For some of the very large complexes that display a distinctive size and shape, structural approaches for identification will allow complex localization, for example by identifying single-particle-EM derived 3D structures in whole mount cryo-tomograms. A high-resolution single particle analysis of the highest molecular weight protein structures purified from DvH cells has been conducted by the PCAP EM project, characterizing structures of 16 individual molecular machines at a resolution compatible with electron tomography (Han et al., PNAS 2009). It was hoped that these 'templates' could be used to localize and monitor the differing numbers of single particle structures within the tomographic datasets of intact DvH cells at distinctive stages of its growth and under different stress conditions.

While our cryo-electron tomography studies of DvH have been quite productive in characterizing aspects of overall morphology, they have so far yielded less information about cytoplasmic composition than we had initially hoped. The resolution of the tomograms within the DvH cytoplasm is noticeably lower than the resolution obtained in tomographic datasets from cells of other microbes of similar size. It was not clear if this lack of information is due to a lower number of internal molecular machines, i.e. ribosomes, GroEL, RNA polymerase etc, within the DvH cells, or if the cytoplasm of DvH is for some unknown reason idiosyncratically more electron dense than in other cells, which would decrease the contrast and thus limit our ability to identify cytoplasmic complexes.

The fledgling technique of vitreous cryo-sectioning has been adopted to elucidate this problem. A number of various cell types, including DvH, yeast, *E. coli* and *Caulobacter* have all been successfully high-pressure frozen in specialized copper tubes, sectioned and imaged in the frozen-hydrated state. Variations in the density of the extracellular medium, such as with dextran that is often used as a cryo preservative, provide a mechanism of contrast matching to complement

other measures of cell mass density. A number of tentative tomographic datasets have been recorded from the sections, and data comparison is ongoing.

For smaller proteins, where shape or size is no longer a unique identifier, we can apply tag-based labeling approaches to identify the precise location of the tagged protein. To this end we have explored the SNAP-tag labeling system for DvH. This approach overcomes problems of using GFP derived-fusion proteins in an obligate anaerobe. Labeled cells can be examined at the light microscope level, and can also be photoconverted to provide contrast for EM visualization at much higher resolution. Over the last year we have overcome several obstacles that had plagued us in earlier work, and we recently were able to fluorescently label 13 out of 25 strains. Successful labeling was judged by fluorescence microscopy, SDS gel electrophoresis and plate reader analysis. Using photoconversion of fluorescent signals, followed by resin-embedding, we have begun to map out a variety of proteins at higher resolution. Several of the fluorescent cell lines have been successfully photoconverted and imaged at the TEM level to identify high-precision subcellular location of the tagged proteins. Interestingly we found cell-to-cell differences in labeling signal strength, which we attribute to real differences in protein expression. We also found cell-to-cell differences in extracellular metal reduction activity.

Energy Dispersive X-ray Analysis (XEDS) has also been used to track the evolution of internal and external electron dense material, which becomes visible through the life cycle of an anaerobic DvH culture. Large internal elemental sulfur balls as well as both internal and external Iron sulfide bodies have been identified and characterized.

Ultimately, in addition to describing the distribution of protein complexes within the cell, by comparing cell-to-cell differences in abundance and subcellular localization with uneven extracellular metal reduction activity we hope to be able to gain insight into the distribution and hence function of candidate proteins, assumed to play a role in extracellular metal reduction and other aspects of the cell's redox chemistry.

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Environmental Microbiology Core Research on Stress Response Pathways in Metal-Reducers ENIGMA:VIMSS:ESPP

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Project Goals: The environmental microbiology core of the ESPP project is the source of environmental data and samples that determine the stressors that will be studied, provides the environments for growing the organisms to be tested, simulates stressed environments, and verifies the conceptual models to determine how these stress regulatory pathways control the biogeochemistry of contaminated sites.

Field Studies

Previous research specifically points toward SRB as environmentally relevant experimental systems for the study of heavy metal and radionuclide reduction, and our recent data has detected *Desulfovibrio* sequences at the FRC and Hanford 100H. To effectively immobilize heavy metals and radionuclides, it is important to understand the cellular responses to adverse factors observed at contaminated subsurface environments, such as mixed contaminants and the changing ratios of electron donors and acceptors. In a recent study, we focused on responses to Cr (VI). At Hanford 100H as part of our ERSP project we injected 40 lbs of HRC (polylactate) as a slow release electron donor in August 2004. Until March 2008 reducing conditions were maintained, along with undetectable levels of Cr (VI) (Hubbard et al, 2008; Faybishenko et al., 2008). During this time the environment was dominated by sulfate reducers and we were able to detect *Desulfovibrio vulgaris*-like organisms with direct fluorescent antibody. Last year we injected 10 lbs of HRC at the same site to determine if there is a 'memory' response and observed H₂S production after only 23 h. Once the community stabilizes we will begin push-pull stress tests in the field with NO₃ and monitor functional gene, community structure, and stress responses as compared with previously published models by our group with pure cultures. We are also isolating consortia and determining the dominant community structure to compare with our lab studies.

In order to characterize microbial community dynamics associated with Cr (VI) biostimulation at the Hanford 100-H area, both groundwater and sediment communities are being tracked via SSU rRNA gene sequences. Stainless steel-mesh columns packed with Hanford sediments are placed down-well and microbial communities are allowed to establish. Four wells are being monitored, and baseline communities have been analyzed for the sediment-associated populations. Predominant populations include: *Acetivibrio*; *Geobacter*; *Ruminococcus*, *Alkaliflexus*, *Thiohalomonas*, *Acidovorax*, *Aquaspirillum*, and *Herbaspirillum*. Groundwater sample analysis is in progress, and biomass loads appear to be lower for groundwater compared to sediments. Rarfaction curves indicate that coverage has not been saturated; therefore, pyrosequencing efforts are also underway. Once baseline groundwater and sediment populations are determined during stimulation with HRC, community dynamics will be tracked during an in situ nitrate perturbation. Methanogen enrichments have yielded slow-growing cultures. A hydrogenotrophic enrichment is dominated by *Methanocella paludicola* (90% similar), and an acetoclastic enrichment is predominated by *Methanosarcina barkeri* (99% similar).

We are also using the GeoChip 3.0, a comprehensive functional gene array contains ~25,000 probes covering ~47,000 sequences for 292 gene families involved in the geochemical cycling of C, N, and S, metal reduction and resistance, and organic contaminant degradation was used to examine microbial communities both at Hanford and at Oak Ridge within a pilot-scale test system established for the biostimulation of U (VI) reduction in the subsurface by injection of ethanol. Sediment from eleven different sampling wells, representing two different treatment zones within this system, was evaluated. The results showed that different microbial communities were established in different wells and high gene overlap was observed from wells within the same treatment zone. Higher microbial functional gene number, diversity and abundance were observed within the active bioremediation zone. The microbial community structure was highly correlated with the hydraulic flow rate and geochemical conditions of the treatment zone, especially pH, manganese concentration and electron donor level. In a different study of the same system, functional community dynamics were examined during a period of oxidation by nitrate. After exposure to nitrate the diversity and richness increased several fold but quickly returned to pre-nitrate levels. The relative abundance of several nitrogen cycling genes showed an increase immediately after nitrate exposure, including ammonification, denitrification, and nitrogen fixation genes indicating a stimulation of these communities after nitrate exposure.

Desulfovibrio spp. and consortia genomes. *Desulfovibrio* FW1012B was isolated from contaminated groundwater during biostimulation for U (VI) reduction in Oak Ridge, TN. Genome sequencing of isolate *Desulfovibrio* FW1012B, tentatively *Desulfovibrio oakridgensis*, was completed in late spring of 2009 at the DOE's Joint Genome Institute at Lawrence Berkeley National Lab in conjunction with Los Alamos National Lab, Oak Ridge National Lab, and Montana State University. The G+C content of the chro-

mosome is 66.5%. Based on the draft, incomplete sequence, automated gene scanning software predicted a total of 3,737 protein-encoding genes, 3,190 of which match best to KEGG-annotated genes in Proteobacteria genomes. The closest organisms with fully sequenced genomes are *Desulfovibrio vulgaris* Miyazaki (dvm) at 740 hits, *Desulfovibrio desulfuricans* G20 (dde) at 479 hits, *Desulfovibrio vulgaris* DP4 (dvl) at 375 hits and *Syntrophobacter fumaroxidans* (sfu) at 196 hits. A further 333 predicted protein-encoding genes had no match which could signify novel and/or unique genes. Analysis of the data raises some interesting questions regarding the metabolism of the organism. Unlike most *Desulfovibrio* species, *D. oakridgensis* appears to have a fully intact glycolysis pathway, a pyruvate dehydrogenase, and a nearly intact citric acid cycle. α -ketoglutarate is made by an NADP⁺ dependent isocitrate dehydrogenase rather than NAD⁺ dependent which creates and lacks a putative malate dehydrogenase. The presence of nitrogenase supports our observations that the organism can assimilate atmospheric nitrogen, and the lack of an aldehyde dehydrogenase coincides with the inability to use ethanol as electron donor and carbon source. Still, other questions are left unanswered. We are also sequencing a number of Hanford isolates that show association with *Desulfovibrio* at Hanford: *Geobacter metallireducens*, *Pseudomonas stutzeri*, and *Desulfovibrio vulgaris*.

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Laboratory Models for the Study of Community Interaction, Functional Stability, and Survival

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Project Goals: Model microbial communities can bridge the experimental gap between the simplicity of monocul-

ture and the complexity of open environmental systems. We are developing laboratory communities of increasing phylogenetic, functional, and spatial complexity in order to: 1) identify extended genomic and regulatory networks and assess their importance to processes in the field, 2) determine how community organization influences activity and resilience, 3) determine how evolution affects community structure and activity over extended periods of adaptation, as is relevant to extended nutrient stimulation of field sites and the consequences of previous field site manipulation on achieving remediation goals.

Initial studies examined a relatively simple two-species community in which a sulfate reducer (*Desulfovibrio vulgaris* Hildenborough) was paired with a hydrogen consuming methanogen (*Methanococcus maripaludis*) in suspended steady-state cultures. This two-member community represents a critical step in microbial food webs that control organic matter mineralization. Only when the two species are growing together (syntrophic growth) can organic substrates be oxidized in the absence of electron acceptors such as sulfate. Significant change in community activity was associated with transition from suspended to attached (biofilm) growth states, forming different exopolymers and producing more methane. Methane production was also influenced by the spatial distribution of the two populations, pointing to the importance of initial conditions of biofilm formation for community structure and activity. Although flux balance models were in generally good agreement with experimental data, growth rate and yield of the two-member community increased significantly with long-term maintenance in the lab, indicating that adaptive evolution must be considered in extended manipulation of field sites. In addition, *D. vulgaris* from independently evolving communities had varying competitive abilities when paired with coevolved or foreign *M. maripaludis*, suggesting that there are different adaptive mechanisms and that species interactions affect the course of evolution. Complementary transcriptional and proteome analyses of the two-member community showed that *Desulfovibrio* uses largely independent energy generation pathways during syntrophic growth and sulfate-respiration, and further indicated the importance of using model communities to identify key genetic and physiological processes not expressed in monoculture.

In separate studies, we examined *D. vulgaris* adaptation to salt stress, a common environmental stressor. The most significant improvements in salt tolerance occurred between 500 and 1000 generations in a long-term evolution experiment. Transcriptional and ongoing genome sequencing analyses indicate that the contributing mutations were fixed by 1000 generations, with little subsequent increase in salt tolerance. Ongoing studies are now characterizing the contribution specific mutants identified in the salt-evolved *Desulfovibrio*, and *Desulfovibrio* recovered from evolved two-member communities, to altered phenotypes.

We are also developing higher communities and supporting technologies to understand interactions of increasing complexity, using the two-species community as the foundation for a tri-culture by adding the acetate oxidizing *Geobacter*

sulfurreducens as well as tri-cultures of *Clostridium cellulolyticum*, *D. vulgaris*, and *G. sulfurreducens* established in steady state chemostats using cellobiose and different electron acceptors. These higher-complexity communities more closely capture the multiple physiological states of metal-reducing bacteria in the environment, where they are dependent upon associated fermenters for nutrients and reducing equivalents. Parallel studies using steady-state chemostats established from Cr (VII) contaminated groundwater from Hanford, WA simulate the ongoing lactate injection experiments at Hanford, and allow us to better define key populations and interactions that lead to emergent system level properties of nutrient amended subsurface environments. Together these laboratory efforts are designed to improve process control and predictive understanding of environmental microbial activities relevant to DOE priorities.

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VIMSS Systems Biology Knowledge Base

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Project Goals: The VIMSS Systems Biology Core group seeks to generate and integrate multiple functional genomic data sets in order to create a comprehensive framework for understanding the biology of the sulfate reducing environmental microbe *Desulfovibrio vulgaris* Hildenborough.

Background: The VIMSS Systems Biology Core group seeks to generate and integrate multiple functional genomic data sets in order to create a comprehensive framework for understanding the biology of the sulfate reducing environmental microbe *Desulfovibrio vulgaris* Hildenborough. The group is responsible for high throughput experiments, data management, data integration, data analysis, and comparative and evolutionary genomic analysis of the data for the VIMSS project. We have expanded and extended our existing tools sets for comparative and functional genomics to deal with new data produced by the VIMSS ESPP2 members. The Systems Biology Core is developing methods to store and analyze diverse data sets including: microarrays, ChIP-chip arrays, tiling arrays, proteomics, metabolomics, metabolic flux, phylochips, metagenomics sequencing, genome sequencing, growth curves, phenotype arrays, bar-coded knock out strain collections and links to existing literature and web based resources. Our analysis has been incorporated into our comparative and functional genomics website MicrobesOnline (<http://www.microbesonline.org>) and made available to the wider research community. By

taking advantage of data integration across diverse functional and comparative datasets, we have been able to pursue large research projects in evolutionary and systems biology studies.

Systems Biology Experiments: The Systems Biology Core is currently prioritizing the functional annotation of SRB genomes and the creation of a complete systems-level investigation into the physiology of DvH. To accomplish these tasks in the most robust and reproducible manner possible, we have created a robotic setup to automate most of the sample prep, growth curve fitness and phenotype assays and data collection. To functionally annotate SRB genomes, we are systematically generating large sequence-defined transposon libraries in *D. desulfuricans* G20 and DvH. These libraries are “bar-coded” with unique DNA tags which will enable the parallel monitoring of strain fitness in thousands of SRB mutants. By screening across hundreds of growth conditions and monitoring per gene fitness effects, we will be able to assign phenotypic outcomes to each gene.

A complete systems-level investigation into DvH physiology requires a complete parts list of all transcribed elements in the genome. Towards this aim, we are using high-density tiling microarrays and next-generation sequencing technologies to precisely define the transcriptome of DvH. By combining high density tiling arrays with the sequencing data, we will be able to define transcription starts, operon structure, terminator sequences, improve promoter motif predictions, and identify potential antisense transcripts and small RNA genes.

The methods and techniques we have developed are applicable over a broad range of microbes and we will have to capacity to reproduce several of these experiments in related species, allowing the results to be analyzed in the broader context of *Desulfovibrio* evolution.

Data Integration: Data management, integration and distribution are critical functions for all large projects. A primary goal of the Systems Biology Core is to capture all experimental data from the ESPP2 investigators, including relevant metadata, raw data and processed data, and to make these data sets available through intuitive queries. Our group has developed Experimental Information and Data Repository (<http://vimss.lbl.gov/EIDR/>) and the MicrobesOnline database to provide this functionality. Researches have access to datasets from biomass production, growth curves, image data, mass spec data, phenotype microarray data and transcriptomic, proteomic and metabolomic data. New functionality has been added for storage of information relating to mutant strains, transposon knockout libraries and protein complex data, in addition to new visualization for assessing existing data sets such as the phenotype microarrays.

RegTransBase and RegPrecise: We have built tools and resources for studying regulation in bacteria and archaea using comparative genomics approach. In addition to working on a high quality semi-manual regulon inference in a wide range of species we are building several on-line resources covering different aspects of regulation. RegTransBase, a database of regulatory interactions from literature

collected by a group of experts, currently includes 5100 annotated articles describing 12 thousand experiments. RegPrecise describes manually curated computational predictions of regulons in bacterial genomes done by comparative genomics. RegPredict is a set of highly integrated web tools for fast and accurate inference of regulons. All regulation-related resources are based on the MicrobesOnline data.

The MicrobesOnline Database: The MicrobesOnline database (<http://www.microbesonline.org>) currently holds over 1000 microbial genomes and will be updated semi-annually, providing an important comparative and functional genomics resource to the community. New functionality added this year includes the addition of fungal genomes and the framework for adding additional eukaryotic genomes, an updated user interface for the phylogenetic tree based genome browser that allows users to view their genes and genomes of interest within an evolutionary framework, improved tools to compare multiple microarray expression data across genes and genomes, phylogenetic profile searches using our high quality species tree, and addition of external microarray data from the Many Microbial Microarrays Database for bacteria and Yeast. Additionally we have begun adding metagenomic data to MicrobesOnline.

MicrobesOnline continues to provide an interface for genome annotation, which like all the tools reported here, is freely available to the scientific community. To keep up with the rapidly expanding set of sequenced genomes, we have begun to investigate methods for accelerating our annotation pipeline. In particular we have completed work FastHMM and FastBLAST, methods to speed up the most time consuming process of our analysis pipeline, homology searching through HMM alignments and all against all BLAST. These methods now enable us to deal with the many millions of gene sequences generated from metagenomics. And our FastTree program allows us to create phylogenetic trees for all gene families, even those with over 100,000 members, so that all genes can be studied within an evolutionary framework.

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Environmental Stress Pathway Project: Study of *Desulfovibrio vulgaris* Hildenborough

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Project Goals: The anaerobic, sulfate reducing bacterium *D. vulgaris* Hildenborough, provides an avenue to examine important microbial metal reducing functions in the environment. A systematic understanding of the physiology of such organisms provides invaluable insights into key metabolic and regulatory networks mechanisms and their evolution. The Environmental Stress Pathway Project (ESPP) builds upon the genetic tools, systems biology and analytical methodologies developed for *D. vulgaris* to obtain an in-depth knowledge of the metabolic capabilities, stress response, adaptation and regulatory networks of physiological states and factors that impact its physiology with respect to its environment. Projects outlined in this poster include cells wide studies, transposon library analyses, targeted studies of regulatory factors and signal transduction systems.

The anaerobic, sulfate reducing bacterium *D. vulgaris* Hildenborough, provides an avenue to examine important microbial metal reducing functions in the environment. A systematic understanding of the physiology of such organisms provides invaluable insights into key metabolic and regulatory networks mechanisms and their evolution. The Environmental Stress Pathway Project (ESPP) builds upon the genetic tools, systems biology and analytical methodologies developed for *D. vulgaris* to obtain an in-depth knowledge of the metabolic capabilities, stress response, adaptation and regulatory networks of physiological states and factors that impact its physiology with respect to its environment. Projects outlined in this poster include cells wide studies, transposon library analyses, targeted studies of regulatory factors and signal transduction systems.

Functional genomics continues to provide a valuable strategy to gain a broad cell wide understanding of *D. vulgaris* physiology. Having applied these measurements to specific stress response assessment, studies are now being focused on understanding *D. vulgaris* biofilms. Both transcript and protein expression profiles demonstrated that biofilm cells have an altered flux of carbon and energy compared to planktonic cells, which may influence metal-interacting capacity and survivability. In addition planktonic and biofilm cells were also exposed to different concentrations of

dissolved oxygen to establish if exposure had any effects on cells and/or biofilm formation. Results from these studies provide insight to better control the growth of sulfate-reducing biofilms.

To improve the functional annotation of *D. vulgaris* and related genomes, large sequence-defined transposon libraries have been generated in both *D. vulgaris* and *D. desulfuricans* G20. These libraries are “barcoded” with unique DNA tags to enable the parallel monitoring of strain fitness in thousands of mutants. Several broad and targeted studies have been conducted using these libraries. Furthermore, a complete systems-level investigation into *D. vulgaris* physiology requires a complete parts list of all transcribed elements in the genome. To address this a high-density tiling microarray and next-generation sequencing technologies are being used to precisely define the transcriptome of *D. vulgaris*.

A key focus of ESPP is to elucidate the structure and evolution of molecular networks in *D. vulgaris*. Towards this aim, a systematic examination of the two component signal transduction pathways is being undertaken. Two component systems trigger responses to a variety of stress and environmental signals. Approximately 70 such systems are annotated in *D. vulgaris*. Using a library of tagged two component system proteins (Histidine Kinases and Response regulators) high throughput protein-DNA interaction strategies are being adopted to map the two component regulatory network. These studies are being complimented using gene deletion mutants in Histidine kinases. The *D. vulgaris* genome also encodes Crp-Fnr like genes that are known to serve as positive transcription factors and play an important role in response to environmental stresses. To examine the function, regulation, and possible networks of these regulators, individual knockout mutants in all four Crp-Fnr genes are being studied. Findings from these studies will be summarized.

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The Metalloproteomes of Microorganisms are Largely Uncharacterized: A Component of the MAGGIE Project

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Project Goals: The overall goal of the MAGGIE project is to provide robust GTL technologies and comprehensive characterization to efficiently couple gene sequence and

genomic analyses with protein interactions and thereby elucidate functional relationships and pathways. The operational principle guiding MAGGIE objectives is that protein functional relationships involve interaction mosaics that self-assemble from independent protein pieces that are tuned by modifications and metabolites, including metals. The objective is therefore to comprehensively characterize protein complexes on a genome-wide basis, including metal-protein complexes, underlying microbial cell biology.

The overall goal of the MAGGIE project is to provide robust GTL technologies to efficiently couple gene sequence and genomic analyses with protein interactions and thereby elucidate functional relationships and pathways. This involves a comprehensive characterization on a genome-wide scale of protein complexes and protein-cofactor complexes. Metals have been found to play essential roles as cofactors in many enzymes and proteins involved in nearly all cellular processes. However, the true extent of the metalloproteome of any organism remains unknown. It is not possible to predict the types of metal that an organism uses or the number and/or types of metalloprotein encoded in its genome sequence because metal coordination sites are diverse and not easily recognized. In this study, we used *Pyrococcus furiosus*, a hyperthermophilic archaeon that grows optimally at 100°C, as the model organism to gain insight into metalloprotein diversity. The key questions were, what elements does the organism assimilate from its normal laboratory growth medium, can techniques be devised to identify novel metalloproteins, and how specific is metal incorporation into proteins? Conventional non-denaturing liquid chromatography and high-throughput tandem mass spectrometry were used to separate and identify proteins, respectively, and metals were identified by inductively coupled plasma mass spectrometry (ICP-MS). Statistical and algorithmic methods were used to identify potentially novel metalloproteins. A total of 345 metal peaks were identified after fractionation of the cytoplasmic extract of *P. furiosus* through two levels of column chromatography, 160 of which could not be ascribed to any known or predicted metalloprotein. The peaks observed included metals known to be utilized by *P. furiosus* (Fe, Ni, Co, and Zn) and metals that *P. furiosus* was not previously known to take up (U, Pb, Ge, Mo, Mn, and V). Similar chromatographic and metal analyses were performed using the cytoplasmic extracts of two other microorganisms, *Sulfolobus solfataricus* and *Escherichia coli* (grown on their conventional laboratory media). These revealed peaks of yet other types of unanticipated metals. Several of the unassigned metal peaks in the *P. furiosus* analyses were purified to give a single protein using conventional liquid chromatography fractionation. This led to the identification of multiple new types of Ni- and Mo-containing proteins, the properties of which will be presented. The presence of novel V, Mn, Pb, U, and Ge proteins will also be discussed in terms of whether their incorporation into proteins by the organism appears to be intentional or unintentional. These results indicate that metalloproteomes are much more extensive than previously recognized, and likely involve both biologically conventional

and unanticipated metals with implications for a complete understanding of cell biology.

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Access to Shape and Assembly of Macromolecular Complexes in Pathways Using Small Angle X-Ray Scattering

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Project Goals: The operational principle guiding MAGGIE (Molecular Assemblies, Genes, and Genomics Integrated Efficiently) objectives can be succinctly stated: protein functional relationships involve interaction mosaics that self-assemble from independent macromolecular pieces that are tuned by modifications and metabolites. Several metrics and tools have been developed in MAGGIE which identify, capture, characterize and predict the effects of macromolecular assemblies. One of our developed tools is small angle X-ray scattering (SAXS). MAGGIE was the first to recognize and apply SAXS as a proteomic scale tool by coupling robotic fluid handling with the extreme brightness of synchrotron light.

The operational principle guiding MAGGIE (Molecular Assemblies, Genes, and Genomics Integrated Efficiently) objectives can be succinctly stated: protein functional relationships involve interaction mosaics that self-assemble from independent macromolecular pieces that are tuned by modifications and metabolites. Several metrics and tools have been developed in MAGGIE which identify, capture, characterize and predict the effects of macromolecular assemblies. One of our developed tools is small angle X-ray scattering (SAXS). MAGGIE was the first to recognize and apply SAXS as a proteomic scale tool by coupling robotic fluid handling with the extreme brightness of synchrotron light.

SAXS characterizes the shape and assembly of macromolecules in solution. Information both on routine cellular processes and cell state are often carried in the shape and assembly of macromolecules on the length scales measured by SAXS. As we have enabled data collection at a rate of 96 samples in 4 hours on practical concentrations and volumes for most macromolecules a new scale of structural characterization has been enabled. Facile access to shape and conformation of macromolecules in a large number of

contexts which are encountered during cellular processes has had significant impact on our understanding of pathways and mechanisms for information transduction. Often macromolecules interact with several metabolites and specific combinations cause large length scale re-arrangements.

Pathways which maintain genomic stability, protein re-naturation and most recently components of energy generation have been targets of our approach. Our purification and data collection success rate has been significantly enhanced using proteins from our 3 target extremophile organisms. In addition to providing higher stability proteins and complexes we hope to learn the mechanisms these organisms utilize to accomplish these ubiquitous tasks under the challenging conditions in which they thrive. These may provide design principles for engineering pathways of interest which are of ultimate interest to GTL.

In addition to utilizing SAXS as a tool to characterize shape and assembly of pathway components, members may be identified which are tractable for other biophysical techniques. Multimerization state influences concentrations required for many techniques. Large flexible regions may prohibit crystallization. Specific alterations to sequence or post translational modifications may stabilize a conformation which enhances the likelihood of success by other techniques.

The developed infrastructure for SAXS is a unique resource for GTL and has already been utilized by hundreds of research labs around the country. Future target pathways and relevant components will be directed by the bioinformatics core of MAGGIE. In addition, the extension of SAXS to membrane proteins is currently being explored.