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Biological Hydrogen Production
Systems Biology and Metabolic Engineering Approaches

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Characterization of H_2 Producing Microbial Communities of the Wood-Feeding Beetle *Odontotaenius disjunctus* by Multi-Scale Measurement of Metabolic Function

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Project Goals: Our research aims to develop an integrated analysis of energy flow in complex microbial communities. We are combining biogeochemical, stable isotope probing, metatranscriptomic/metagenomic, and computational approaches, to understand nutrient cycling and biofuel (H_2) production in complex microbial communities. A comprehensive understanding of such communities is needed to develop efficient, industrial-scale processes for microbial H_2 production and lignocellulose degradation. Our ultimate goal is the development of multi-scale models that can predict ecological and biochemical relationships within multi-trophic microbial systems.

One of our model systems is represented by the hindgut of the passalid beetle *Odontotaenius disjunctus* (Fig. 1A). This wood-feeding beetle is able to survive on a low nutrient diet through symbiotic interaction with its gut microbiome, which provides nutrients from plant polymer decomposition and nitrogen through fixation. Both of these processes result in the production of hydrogen gas.

The digestive system of the passalid beetle is compartmentalized in 4 morphologically differentiated regions (Fig. 1D); each has been characterized with our integrated approach, which consists of:

1. Culture independent techniques used to assess the diversity and composition of the microbial communities inhabiting each gut region by using the PhyloChip, together with analysis of community gene expression by qPCR, and sequencing of enriched cDNA libraries.
2. Use of Chip-SIP, a high-sensitivity, high-throughput stable isotope probing (SIP) method performed on a phylogenetic array to directly measure functional roles of uncultivated microorganisms.
3. Construction of Fosmid libraries for enzymatic assays and sequencing to search for the genes involved in the transformation of specific substrates. We have optimized HMW-DNA extraction and library construction protocols for their application to the beetle gut microbiome.
4. Micron-scale measurement of physicochemical gradients (O_2, H_2, pH) on the distinct gut regions of the passalid beetle using microelectrodes (Fig 1C).
5. Characterization of changes in the chemical composition of digested/undigested substrate (oak wood) using Fourier Transform Infrared Spectroscopy (FTIR).

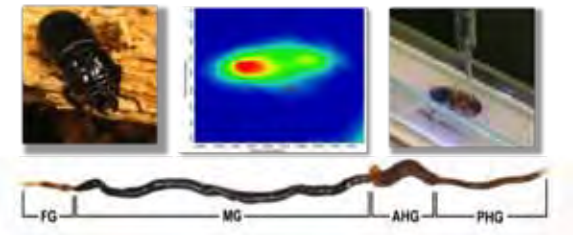

Figure 1. A) The passalid beetle, B) SIR image of undigested oak wood, C) arrangement of the dissected beetle and H_2 microsensor, D) the compartmentalized gut of the passalid beetle, F=Foregut, MG = Midgut, AHG= Anterior hindgut, and PHG =Posterior Hindgut.

By combining these complementary approaches we are attempting to determine the primary processes and organisms contributing to energy flow in this natural system. We have characterized the microbial communities of the passalid beetle, obtaining the highest levels of diversity in the Foregut (FG), followed by Posterior Hindgut (PHG), Midgut (MG), and Anterior Hindgut (AHG). *nifH* expression analysis showed that N_2 fixation was highest in the AHG and sequencing of *nifH* genes identified the dominant N_2 fixing group as members of the Porphyromonadacea (Clostridiales). Concentrations of O_2 and H_2 varied across the different gut compartments; the highest nifH gene expression and lowest bacterial diversity spatially correspond to regions with high concentrations of H_2 production and O_2 is at its minimum.

Using FTIR, we have also characterized changes in the composition of the beetle's substrate (Oak wood); results suggest cellulose and hemicellulose content declines as the substrate passes through the sequential gut regions. We are currently screening fosmid libraries, and performing analysis of enriched cDNA libraries and Chip-SIP microarrays.

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Anoxic Carbon and Nitrogen Metabolism in Photosynthetic Microbial Mats as Revealed by Metatranscriptomic and NanoSIMS Studies

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Photosynthetic microbial mats found in coastal environments are complex, stratified microbial communities that fix CO_2 during the day and ferment the fixed carbon under anoxic conditions at night, generating significant amounts of H_2 and organic acids. Fermentation of accumulated photosynthate may be required to provide energy for anoxic N_2 fixation. To understand C and N flux through these mats at a single cell and community level and to relate this flux to the observed rates of H_2 evolution and N_2 fixation, we have analyzed the upper 2 mm of cyanobacterial mats collected from Elkhorn Slough, CA, which have been shown to possess high rates of fermentation and N_2 fixation by metatranscriptomic and NanoSIMS studies.

We characterized the active microbial community from two mats samples collected under dark, anoxic conditions using metatranscriptomic sequencing to define the metabolic pathways for fermentation and N_2 fixation. Clone library and 454 pyrosequencing of the rRNA expressed by this community demonstrated that the active community was dominated by filamentous cyanobacteria including populations closely related to *Microcoelus chthonoplastes* and a novel diazotrophic cyanobacterial isolate, ESFC-1. Approximately 388,000 transcripts (average length ~390 bp) were obtained by sequencing amplified cDNA. Of these transcripts, ~199,000 transcripts (51%) were classified as protein coding sequences by comparison to the SEED database (bit score>40). Transcripts related to *Cyanobacteria* and *Chloroflexales* represent >70% of the total classified sequences in the transcriptome, suggesting that representatives of these phyla are active in the mats under anoxic conditions.

Metabolic reconstruction of the *Microcoleus chthonoplastes* genome identified a complete pathway for fermentation of glycogen to lactate, acetate, formate, ethanol and H_2. Fragment recruitment of metatranscriptomic reads indicated that all the genes in this fermentation pathway were expressed, consistent with organic acid and H_2 evolution measurements. A large number of metatranscriptomic reads affiliated with the *Chloroflexales* were annotated as acetate/lactate permeases and polyhydroalkonoate synthases, suggesting that the *Chloroflexales* may metabolize the organic acids produced by the cyanobacteria further and also synthesize carbon storage compounds to be utilized in photoheterotrophic growth. Assembly of the metatranscriptomics reads revealed that partial *nif* operons were reconstructed with the structural nitrogenase genes (*nifHDK*) for *Microcoleus,* ESCF-1 and an uncultivated member of the *Chloroflexales*, suggesting that these populations were responsible for the observed N_2 fixation activity.

These metatranscriptomic studies were complemented by NanoSIMS measurement of mat samples collected from Elkhorn Slough and incubated with isotopically labeled substrates. Incubation of mat samples with $^{13}HCO_3^-$ provided evidence for the fixation and storage of carbon by *Microcoleus* populations during the day and release of the stored carbon under anoxic conditions. Addition of ^{13}C-labeled acetate resulted in isotopic enrichment of the *Chloroflexi*, providing further evidence for the anoxic food web hypothesis generated by analysis of the mat metatranscriptome. Incubation of the mats with $^{15}N_2$ demonstrated that cyanobacterial populations related to ESFC-1 were highly active in N_2 fixation under dark anoxic conditions; however no evidence was found for incorporation of $^{15}N_2$ into *Microcoleus* and *Chloroflexi.*

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Development and Validation of Novel Techniques to Understand and Address Limitations to Algal Hydrogen and Biofuels Photoproduction

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Project Goals: To develop techniques that will provide a deeper understanding of algal H_2 metabolism and accelerate the development of future photobiological H_2-producing catalysts and organisms.

Photobiological H_2 production from water is a clean, nonpolluting and renewable technology. The efficiency of light conversion into H_2 by biological organisms is theoretically high (about 10%). However, the system is currently limited by biochemical and engineering constraints, including the extreme O_2 sensitivity of the biological hydrogenases and the low availability of reductants to the hydrogenase due to the predominance of competing metabolic pathways. In Task 1, we developed a Rhodobacter-based H_2-sensing assay that is useful for conducting high-throughput screening for algal H_2-production mutants under fermentative conditions. The assay incorporates an agar overlay of H_2-sensing *R. cap-*

sulatus cells that fluoresce in response to H_2 produced by an agar underlay of *C. reinhardtii* colonies. The assay is sensitive enough to distinguish one H_2-producing colony from out of thousands of non H_2-producing colonies as present on a single Petri dish. We will focus next on adapting the assay for operation under photobiological H_2-production conditions and use it to screen for microbial libraries with increased H_2 photoproduction. In parallel work, we have transferred an exogenous hydrogenase from *Clostridium acetobutylicum,* along with its attendant assembly proteins, into the H_2-reporter bacterial strain. This bacterium produces hydrogen from the exogenous hydrogenases and emits a fluorescent signal in response to its H_2 production. We will focus our work on using this system to screen for O_2-tolerant [FeFe]-hydrogenases generated through directed-evolution techniques.

In green algal chloroplasts, ferredoxins (FDXs) play a central role in the allocation of low-potential electrons from photosynthesis to the hydrogenase enzyme, as well as to numerous competing assimilatory pathways. These pathways include CO_2 fixation, nitrite reduction, glutamate synthesis, sulfite reduction, cyclic electron transport around Photosystem I, and reduction of thioredoxin for regulation of biosynthetic pathways. Under certain physiological states, these processes have the potential to compete with the hydrogenases for electrons. Moreover, there are 6 potential FDXs that could interact with the hydrogenase enzymes (HYDA1 and HYDA2) and it is not clear yet which one is the favored natural electron donor to hydrogenases under different physiological conditions. To address the issue of competitive metabolic pathways with H_2 production, Task 2 has used a two-hybrid assay to screen *C. reinhardtii* expression libraries, with HYDA2 and HYDA1 as well as the 6 FDXs as baits, followed by pull-down assays to confirm *in vitro* the results obtained *in vivo*. We have also examined pair-wise interactions between each hydrogenase and FDX isoform to identify specific protein-protein interactions. These approaches have resulted in the identification of novel protein-protein interactions that are leading to the development of a map of the network centered on ferredoxins and involving multiple metabolic pathways in *Chlamydomonas*.

Publications

1. Wecker MSA, Meuser JE, Posewitz MC, Ghirardi ML. Design of a new biosensor for algal H_2 production based on the H_2-sensing system of *Rhodobacter capsulatus*. *International Journal of Hydrogen Energy* 2011, 36(17):11229-11237.
2. Peden E, Boehm M, Davis RA, Old W, Ghirardi ML Dubini A. Identification of the chloroplastic ferredoxins metabolic network in *Chlamydomonas reinhardtii. In preparation.*

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Development of *Cyanothece* as a Model Organism for Photobiological Hydrogen Production

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http://sysbio.wustl.edu/Pakrasi/projects/hydrogen.php

Project Goals: The aim of this project is to develop the cyanobacterium *Cyanothece* as a model organism for photobiological hydrogen production. Members of the genus *Cyanothece* are unicellular oxygenic prokaryotes with the ability to fix atmospheric nitrogen. Our long-term goal is to develop a deep understanding of the metabolism of these microbes as it pertains to H_2 evolution. Specifically, we are using genome sequencing, transcriptomics, proteomics, metabolomics, mutagenesis, biochemical analysis and physiological approaches, all of which are encased in a systems biology framework.

Comparative genomics and hydrogen production in Cyanothece: Analysis of the complete genome sequences of six *Cyanothece* strains (ATCC 51142, PCC 7424, PCC 7425, PCC 7822, PCC 8801, and PCC 8802) indicates that this genus maintains a plastic genome, incorporating new metabolic capabilities while simultaneously retaining archaic metabolic traits, a unique combination which provides the flexibility to adapt to various ecological and environmental conditions (Bandyopadhyay et al., 2011). Our study uncovered the presence of a large and contiguous nitrogenase gene cluster in four of the five newly sequenced strains. These strains were also analyzed for their ability to fix nitrogen and produce hydrogen. High rates of aerobic nitrogen fixation and hydrogen production were observed in five of the six strains, distinguishing *Cyanothece* as a genus of unicellular, aerobic nitrogen-fixing cyanobacteria. The contiguity of the nitrogenase gene cluster in *Cyanothece* sp. 7425 is interrupted by a 2.5Mbp insertion and this cluster exhibits a rearrangement of the *nif* genes as seen in some of the anaerobic nitrogen fixing *Synechococcus* strains. *Cyanothece* 7425 also appears to have lost its aerobic nitrogen-fixing ability and exhibits nitrogen fixation and hydrogen production only under anaerobic conditions. In addition, our study revealed the presence of 3 linear chromosomal elements in *Cyanothece* 7822. These linear elements, like the linear chromosome in

Cyanothece 51142, harbor genes involved in various carbohydrate metabolism pathways and represent a unique feature of the genus *Cyanothece* compared to other sequenced cyanobacteria. A comparison of the genomes of the six *Cyanothece* strains revealed the presence of several pathways involved in fermentative metabolism, traits usually observed in non-oxygenic microbes. The ability of this genus to maintain a suboxic intracellular environment for a significant part of a diurnal cycle is unique and allows the *Cyanothece* cells to provide a platform for both aerobic and anaerobic metabolic processes. Our analysis suggests that members of the genus *Cyanothece* can be appealing as model organisms for studies pertaining to biohydrogen production.

Metabolomic studies: We have developed a web-based platform (MicrobesFlux) for generating and reconstructing metabolic models for annotated microorganisms. The MicrobesFlux is able to load the metabolic network (including enzymatic reactions and metabolites) of over 1,100 species from KEGG database (Kyoto Encyclopedia of Genes and Genomes) and then automatically converting it to a metabolic model. The platform also provides diverse customized tools, such as gene knockouts and introduction of heterologous pathways, for users to redefine the model network. The reconstructed metabolic network can be formulated to a constraint-based flux model to predict and analyze the carbon fluxes in the metabolisms. The simulation results can be output in SBML format (The Systems Biology Markup Language). The MicrobesFlux is an installation-free and open-source platform that enables biologists with little programming knowledge to develop metabolic models for newly sequenced microorganisms. Our system allows users to construct metabolic networks of organisms directly from the KEGG database. It also provides users with predictions of microbial metabolism via flux balance analysis. This prototype platform can be a springboard to advanced and broad-scope metabolic modeling of complex biological systems by integrating other "omics" data or ^{13}C-assisted metabolic flux analysis results. This platform is being used for generating metabolic models of the *Cyanothece* strains and for predicting gene knockouts and pathway modifications. MicrobesFlux is available at http://tanglab.engineering.wustl.edu/static/MicrobesFlux.html.

Proteomic Studies: A comparative analysis of the proteomes of the six *Cyanothece* strains was carried out to identify similarities and differences in the metabolism of the strains grown under nitrogen sufficient conditions. Among the five strains, the largest number of proteins (3967) could be detected in *Cyanothece* PCC7424 and the lowest (2728) in *Cyanothece* PCC7425. Our results reveal considerable similarities in proteins involved in conserved metabolic and biochemical pathways such as photosynthesis, respiration, N_2-fixation, H_2-production as well as proteins involved in housekeeping functions. Out of a total of 1369 predicted orthologs common to all *Cyanothece* strains, 644 have been detected in the comparative study. However, considerable differences were also evident, particularly in *Cyanothece* PCC7425 where significantly higher numbers of proteins (682 proteins) were uniquely expressed. This may suggest that proteins in *Cyanothece* PCC7425 diverged separately compared to the other strains, possibly as a result of specific selection pressure and subsequent adaptation to environment. Furthermore, in addition to commonly shared phycobilisome (PBS) proteins, we identified 3 phycobiliproteins and 2 PBS-linker polypeptides expressed specifically in PCC7822, PCC7424 and PCC8801 together with phycoerythrin (PE)-operon protein, suggesting their association with PE assembly. While homologs of PSI core proteins PsaAB were identified in all six strains, expression of another PsaB protein (Cyan7822_4989) was only detected in PCC7822.Our results elucidate proteomic footprints of six *Cyanothece* strains, and provide useful targets for future applications in biotechnology.

To determine cellular factors involved in H_2 production, we employed label-free quantitative proteomics in *Cyanothece* ATCC 51142 and PCC 7822 under eight culture conditions. Rates of H_2 production were measured for each condition and compared to changes of protein abundances. Our analysis provides systems level understanding about nitrogenase-mediated H_2 production under aerobic condition providing useful insights into how *Cyanothece* might be optimized for H_2 production.

Publication

1. Bandyopadhyay A, Elvitigala T, Welsh EA, Stöckel J, Liberton M, Min H, Sherman LA, Pakrasi HB (2011). Novel metabolic attributes of *Cyanothece*, a group of unicellular nitrogen fixing cyanobacteria. *mBio* 2 (5): e00214-11. doi:10.1128/mBio.00214-11.

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Contrasting the Metabolic Capabilities of *Cyanothece* 51142 and *Synechocystis* 6803

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Project Goals: The current project uses systems biology to define the metabolic networks involved in the photobiological production of advanced biofuels and/or their chemical precursors by cyanobacteria. By encasing transcriptomics, metabolic profiling, mutagenesis, physiological analysis and genome-scale metabolic modeling in a systems biology framework, our goals are: i) to develop genome-scale models of two widely used cyanobacterial

species, *Cyanothece* 51142 and *Synechocystis* 6803, ii) to refine these models based on systems level information obtained from experimental studies (i.e., transcriptome, metabolome, etc.), and iii) to genetically modify these strains based on model predictions, experimentally evaluate their performance and subsequently enhance the production yield of desired products.

Table 1: *Synechocystis* 6803 iRS706 and *Cyanothece* 51142 iRS764 model statistics

	***Synechocystis 6803 iRS*706 model**	***Cyanothece 51142 iRS*764 model**
Included genes	706	764
Proteins	604	660
Single functional proteins	258	247
Multifunctional proteins	192	210
Protein complexes	3	4
Isozymes	27	30
Multimeric proteins	107	149
Others[a]	17	20
Reactions	1,844	1,910
Metabolic reactions	1,834	1,900
Transport reactions	5	5
GPR associations		
Gene associated (metabolic/transport)	1,798	1,836
Spontaneous[b]	18	19
Nongene associated (metabolic/transport)	18	40
Exchange reactions	5	5
Metabolites	1,894	1,862
Cytoplasmic	1,889	1,857
Extracellular	5	5

[a]Others include proteins involve in complex relationships, e.g. multiple proteins act as protein complex which is one of the isozymes for any specific reaction.

[b]Spontaneous reactions are those without any enzyme as well as gene association.

Cyanobacteria are an important group of photoautotrophic organisms that can synthesize valuable bio-products by harnessing solar energy. Cyanobacteria contribute significantly to biological carbon sequestration, O_2 production and the nitrogen cycle. They exhibit robust growth under diverse environmental conditions and have minimal nutritional requirements. They are also endowed with high photosynthetic efficiencies and diverse metabolic capabilities that confer the ability to convert solar energy into a variety of biofuels and their precursors. However, less well studied are the similarities and differences that exist between the metabolic capabilities of different species of cyanobacteria that may contribute toward their niche biological functions and their suitability as microbial production systems. Towards goal (i) above, here we introduce and compare genome-scale models for two phylogenetically related cyanobacterial species, namely *Cyanothece 51142 i*RS764 and *Synechocystis 6803 i*RS706. Model *i*RS764 is comprised of 764 genes, 1,910 reactions and 1,862 metabolites, whereas model *i*RS706 spans 706 genes, 1,844 reactions and 1,894 metabolites. As many as 1,628 reactions and 1,702 metabolites are common in these two models. All reactions are elementally and charge balanced and localized in three different intracellular compartments (i.e., cytoplasm, carboxysome and thylakoid lumen). GPR (gene-protein-reaction) associations are also established based on the functional annotation information and homology prediction. We contrast flux balance analysis results under different physiological conditions, (i.e., phototrophic, chemotrophic and mixotrophic) and explore their impact on biomass formation, nitrogen fixation and hydrogen yield of these two cyanobacterial species.

Additionally, towards goal (ii) above, we have begun characterizing genetic elements active at different phases of cyanobacterial culture, including during exponential growth and stationary phase. We have identified putative promoters that are highly active at stationary phase and have constructed a library of expression vectors that target genes to areas of the genome that are highly transcribed during stationary phase.

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Photophysiology and Energetics of Light-Driven H_2 Production by *Cyanothece sp.* ATCC 51142

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Project Goals: The PNNL Biofuels Scientific Focus Area (BSFA) conducts fundamental research of microbial photoautotrophs with specific emphasis on photosynthetic energy conversion, reductant channeling, and central carbon metabolism. Subsequent to that are the mechanisms of reductant generation and partitioning in cyanobacteria and how relevant pathways are affected by external environmental factors and macronutrient concentrations. We specifically explore the conditions and mechanisms whereby reducing equivalents are funneled to H_2 as well as the physiological and biochemical bases for light-driven proton reduction. Generally, H_2 evolution in cyanobacteria is catalyzed by hydrogenase and/or nitrogenase enzymes and is inhibited by the presence of O_2 or other competing electron acceptors such as CO_2 and N_2. In *Cyanothece* 51142, however, H_2 production occurs simultaneously with photosynthetic release of O_2 under continuous light conditions as a direct result of nitrogenase activity in the absence of exogenous nitrogen and carbon sources. We hypothesize that, under these conditions, sustained and uninterrupted H_2 production depends upon electrons derived from photocatalytic water splitting.

Cyanothece sp. ATCC 51142 is a unicellular cyanobacterium which can tolerate substantial amounts of O_2 during the operation of its dinitrogenase enzyme, despite the strong inhibitory effect of O_2 upon the catalytic mechanism of this enzyme. As the dinitrogenase enzyme has an auxiliary function to reduce protons to H_2 as well as its primary role in N_2 fixation, the organism is capable of producing H_2 gas

in the presence of O_2. Nevertheless, *Cyanothece* 51142 is well known for its dynamic physiology which can temporally separate its dinitrogenase activity from the O_2-evolving process of oxygenic photosynthesis. As H_2 is an attractive candidate as a clean renewable fuel, it has been a scientific imperative to elucidate strategies whereby the catalysis of H_2 production can be driven efficiently by the energy in sunlight. Here we show, for the first time, the continuous production of H_2 by a unicellular photosynthetic organism with concomitant evolution of O_2.

Under continuous illumination with a feedback-controllable dual-wavelength light-enclosure, steady-state chemostat cultures of *Cyanothece* 51142 were maintained under NH_4^+-limitation in a custom photobioreactor. Despite the high expression of proteins and transcripts for the dinitrogenase system, no H_2 production could be detected during chemostat growth. Instead, the production of H_2 was shown to be easily induced by these cells upon interruption of the steady-state growth. When transferred from the NH_4-limited chemostat to sealed anaerobic tubes incubated under constant illumination, H_2 production was induced after an initial 12 h phase of O_2 accumulation. The dynamic pattern of alternating H_2 and O_2 evolution showed a periodicity of 24 h, reminiscent to previous descriptions of the temporal separation of N_2 fixation and photosynthesis. However, the level of each gas that accumulated did not significantly decrease between each production phase, and thus it became of interest to monitor the fine-scale dynamics of this process.

By halting the supply of NH_4^+ to the photobioreactor, chemostat growth was interrupted, and again H_2production activity was induced. However, facilitated by the high time-resolution instrumentation of the reactor, in-situ measurements enabled the striking observation that H_2 production can occur concomitantly with photosynthetic O_2 evolution in this unicellular organism. Within 1 h following the halt of NH_4^+ supply, H_2 production appeared with a rate which accelerated steadily over the next 24 h, reaching a maximal productivity of 400 μmol H_2/mg Chl/h. Within 6 h of the treatment, the rate of O_2 evolution had declined to a steady rate of 100 μmol O_2/mg Chl/h. However, following the peak of maximal H_2 production rates, the rate of net O_2 production rose, and the rates for both gases began to oscillate every 12 h, displaying an inverse correlation. However, throughout 100 h of continuous illumination, both H_2 and O_2 production activities maintained a positive net evolution rate, eventually dampening toward stable rates of 125 and 90 μmol/mg Chl/h, respectively. Additionally, dynamic changes in CO_2 evolution were observed, positively correlated with the rising and falling of the H_2 production rate.

By additionally halting the CO_2 supply during the chemostat interruption, *Cyanothece* 51142 induced a H_2 production activity which now displayed a *positive correlation* with changes in O_2 evolution rates, no longer following a 24 h periodicity. The light-dependence of the process was demonstrated by brief periods of darkness within the photobioreactor, leading to an immediate cessation of H_2 production, as well as by performance of in-situ light-saturation curves which showed dose dependence for both gases. Interestingly, the PS II-specific inhibitor DCMU was only capable of suppressing H_2 production in the long-term (hours as opposed to minutes). The yield of H_2 was partially diminished by the presence of increasing amounts of O_2, N_2, or CO_2, suggesting that other electron sinks may compete with the proton-reducing function of nitrogenase when they are available.

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Genomic, Genetic, and Cytological Analysis Reveals Biased Inheritance of PatN, a Negative Regulator of Heterocyst Differentiation in *Nostoc punctiforme*

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Project Goals: Heterocyst-forming cyanobacteria, such as *Nostoc punctiforme*, are applicable for cost-effective photo-biohydrogen production, providing the frequency of heterocysts in the filaments can be increased by genetic manipulation and the metabolic end product H_2, is uncoupled from growth. We are applying "omic", genetic and cytological analyses to define the signal transduction pathway(s) leading to pattern heterocyst differentiation.

We hypothesize that if the frequency of heterocysts, sites of nitrogen fixation and hydrogen evolution, can be increased approximately 3-fold above the less than 10% normally found in filaments, then heterocyst-forming cyanobacteria would be applicable for cost effective photo-biohydrogen production. In cyanobacteria of the family Nostocaceae, the heterocysts are present singly (apart from other heterocysts) in the filaments and separated by 10 to15 vegetative cells. In *Nostoc punctiforme*, the heterocyst frequency of 8% in the free-living state is increased to 30-35% when in symbiotic association with terrestrial plants, such as the hornwort *Anthoceros* spp. and the angiosperm *Gunnera* spp. We are applying genetic, transcriptomic and proteomic analyses with wild-type and mutant strains to identify the regulatory circuits of free-living heterocyst differentiation and how those circuits have been co-opted during symbiotic growth.

We previously proposed a two stage—biased initiation, competitive resolution—model for the initiation of heterocyst differentiation (Meeks and Elhai, 2002). The biased initiation stage of the model holds that all cells in a filament sense the signal of combined nitrogen limitation (most likely elevated concentrations of 2-oxoglutarate), but only some

cells can respond to the signal and initiate differentiation. The vegetative cells that can respond occur as a cluster of 2 to 4 cells. What condition specifically allows cells to respond is unknown. The second stage is resolution of the cluster of differentiating cells to a single cell that continues differentiation. Competitive resolution is the consequence of interactions between positive regulatory elements (HetR, HetF) and a negative regulatory element (PatS); overexpression of HetR or HetF, or deletion of PatS results in multiple contiguous heterocysts differentiating at a site in the filament.

We have isolated a mutant strain that displays a heterocyst frequency of about 30% in the free-living growth state. The heterocysts are present singly at relatively regular intervals along the filament, similar to the spacing pattern seen in the symbiotic growth state. This spacing pattern would be near optimal for function, with at least two photosynthetic vegetative cells supplying reductant as photosynthate to each heterocyst for subsequent hydrogen production from nitrogenase or a bidirectional hydrogenase.

The mutated gene has been designated *patN* and, in contrast to other genes involved in positive (e.g. *hetR*, *hetF*) or negative (*patS*) regulation of heterocyst differentiation, *patN* is found only in the genomes of the heterocyst-forming cyanobacteria sequenced to date. Transcription of *patN* is constitutive and PatN localizes to the cytoplasmic membrane in vegetative cells. In filaments supplemented with ammonium the localization is dynamic, with PatN undergoing a round of partitioning and differential inheritance with each cell division, so that the cell with the newest septum receives the majority of PatN. Deletion of the heterocyst patterning gene *patA* is epistatic to deletion of *patN*, and *patA* transcription increases in a *patN* mutant suggesting PatN may function by limiting the level of *patA*. Based on the unequal distribution of PatN among cells in a filament prior to nitrogen deprivation, and the increase in heterocysts observed upon deletion of *patN*, we propose that cells with low levels of PatN at the time of nitrogen starvation may be biased to initiate heterocyst differentiation. Understanding function of PatN is the first step to manipulating *N. punctiforme* to express the symbiotic growth phenotype apart from the plant partner in photo-production of biohydrogen.

Reference

1. Meeks, J. C. and J. Elhai. 2002. Regulation of cellular differentiation in filamentyous cyanobacteria in free-living and plant-associated symbiotic growth states. Microbiol. Mol. Biol. Rev. 66:94-121.

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Biohydrogenesis in the Thermotogales

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Project Goals: (see below)

The bacterial order Thermotogales consists of obligate fermentative anaerobes, most that grow optimally in the range of 65-80°C. These bacteria are characterized by their unique "toga"-like outer membrane, which plays a role in breaking down a wide range of complex polysaccharides. Thermotogales are also capable of producing hydrogen with a high yield approximately twice that of mesophilic bacteria (3-4 mol H_2/mol glucose compared to 1-2). The objectives of this project are:

1. **Examine the regulation of substrate catabolic proteins and pathways as this relates to carbon partitioning, disposition of reducing power, and H_2 generation in *Thermotoga maritima* (*Tma*).**
2. **Dissect catabolic and regulatory pathways using genetic approaches based on past success with other hyperthermophiles.**
3. **Thermotogales biodiversity arises from adaptive specialization that expands on a conserved minimal genome; physiological characterization of selected novel traits will be done to expand understanding of biohydrogenesis.**

***Thermotoga* genetics.** During the past year our lab has focused on advancing targeted integration technology for *Thermotoga maritima* cell line construction combined with studies on the metabolism of this organism as it pertains to redox homeostasis. An additional promoter, *Msed_1271p* (pilin), has been developed to drive expression of *T. maritima pyrE* that provides expanded versatility to the *T. maritima* genetic system. qRTPCR quantitation of promoter strength has been conducted based on the transcript abundances of *groES*, *Msed_1271*, *hisI*, *ldh*, *rpoD* and transgene *kan* providing insight into transgenic cell lines. While other genetic markers remain in play, an emphasis has been placed on the use of natural and synthetic alleles of *pyrE*, therefore multiple cycles of *pyrE* transformation and recombination have been conducted to paramatize the process. Advances in studies on *T. maritima* metabolism include genetic screens for redox homeostasis mutants and new information on metal reduction that provide insights into regulation of hydrogen formation.

Metal reduction by *T. maritima*. We have discovered that *T. maritima* actively forms magnetic iron (magnetite) using

insoluble synthetic ferrihydroxides in a process that is driven by sugar fermentation. Metal reduction is a preferred electron sink that shifts excretion of reductant from synthesis of hydrogen gas to formation of ferrous iron. Negative staining transmission electron microscopy demonstrates synthesis of pili during this process. Genes implicated in this process have been targeted for inactivation to test predictive models.

Phylogenetic analyses revealed likely *ompA* and *ompB* homologs among Thermotogales species. Since OmpA1 and OmpB are the two dominant structural proteins of the *T. maritima* toga and all known species of the Thermotogales have togas, we examined the genome sequences of other members of the Thermotogales to identify possible homologs of these proteins. The closest OmpB pBLAST sequence outside the Thermotogales was from *Vibrio mimicus* with an E value of 8×10^{-6} indicating that currently there are no closely related sequences outside the Thermotogales in the GenBank database. OmpB orthologs are present in *Thermosipho melanesiensis*, *Fervidobacterium nodosum*, and most species of the *Thermotoga* genus. However no OmpB homologs were found in *Thermotoga lettingae*, *Thermosipho africanus*, *Kosmotoga olearia, Petrotoga mobilis*, and Thermotogales bacterium mesG.Ag.4. The *ompB* orthologs lie in syntenic regions containing four genes: *secG-tyrS* (tyrosyl-tRNA synthetase)-*ompA1-ompB*. The organisms that lack homologs to *ompB* also have this syntenic region, but have a gene downstream of their *ompA1* homolog that encodes a protein with no homologs in the GenBank nonredundant database (Fig. 3). The exception is that the *K. olearia* protein and the Thermotogales bacterium mesG.Ag.4 protein are homologous to one another, but to no other sequences. All these non-homologous protein sequences were examined for porin characteristics and all had many porin characteristics, except that they do not appear to be globular proteins. Though these proteins are not recognizable homologs of *T. maritima* OmpB, they may be analogs serving the same function.

Mesophilic member of the Thermotogales. A novel mesophilic member of the Thermotogales designated MesG1.Ag.4.2 was isolated from sediments from Baltimore Harbor, Maryland, USA. Cells of strain MesG1.Ag.4.2 were non-motile ovoids with sheath-like structures (togas). The strain was Gram-negative and grew optimally at 37°C with a doubling time of 16.5 h (specific growth rate = 0.06 h^{-1}) on xylose at 37°C. It grew best on carbohydrates, but proteinaceous compounds also supported growth. This is the first describes species of the mesotoga lineage and provides new possibilities for the development of genetic methods for the Thermotogales.

Thermotoga species differ with respect to sugar utilization. Four hyperthermophilic members of the bacterial genus *Thermotoga* (*T. maritima, T. neapolitana, T. petrophila,* and *T.* sp. RQ2) share a core genome of 1470 open reading frames (ORFs), or about 75% of their genomes. Nonetheless, each species exhibited certain distinguishing features during growth on simple and complex carbohydrates that correlated with genomic inventories of specific ABC sugar transporters and glycoside hydrolases. These differences were consistent with transcriptomic analysis based on a multi-species cDNA microarray. Growth on a mixture of six pentoses and hexoses showed no significant utilization of galactose or mannose for any of the four species. *T. maritima* and *T. neapolitana* exhibited similar monosaccharide utilization profiles, with a strong preference for glucose and xylose over fructose and arabinose. *T.* sp. RQ2 also used glucose and xylose, but was the only species to utilize fructose to any extent, consistent with a phosphotransferase system (PTS) specific for this sugar encoded in its genome. *T. petrophila* used glucose to a significantly lesser extent than the other species. In fact, the XylR regulon was triggered by growth on glucose for *T. petrophila*, which was attributed to the absence of a glucose transporter (XylE2F2K2), otherwise present in the other Thermotoga species. This suggested that *T. petrophila* acquires glucose through the XylE1F1K1 transporter, which primarily serves to transport xylose in the other three Thermotoga species. The results here show that subtle differences exist among the hyperthermophilic Thermotogales that support their designation as separate species.

TM1300 locus in the *T. maritima* genome. The TM1300-1338 locus in the *T. maritima* genome contains a number of hypothetical proteins, including several that correspond to ORFs of less than 100 aa. Analysis of this locus has revealed that several putative bacteriocins and toxin-antitoxins (HicAB) are encoded, suggesting a key role in post-transcriptional regulation and possible anti-microbial actions. The gene encoding TM1312 has been expressed and confirmed to be a toxin (ribonucleases). Purification of TM1316, a putative cyclic peptide and suspected bacteriocin, is underway. Also, gel shift assays have been used to investigate two transcriptional regulators in this locus. For example, TM1330 appears to be involved in regulation of the region upstream of TM1316.

Publications in the past year citing DOE GTL Support

1. Petrus, A. K., K. S. Swithers, C. Ranjit, S. Wu, H. M. Brewer, J. P. Gogarten, L. Pasa-Tolic, and K. M. Noll. Genes for the major structural components of Thermotogales species' togas revealed by proteomic and evolutionary analyses of OmpA and OmpB homologs. PloS One (submitted)
2. Nesbø, C. L., D. M. Bradnan, M. Dlutek, A. K. Petrus, J. Foght, W. F. Doolittle, and K. M. Noll. *Mesotoga prima* gen. nov., sp. nov., the first described mesophilic species of the Thermotogales. Extremophiles (submitted)
3. Swithers, K. S., et al. Genome sequence of *Kosmotoga olearia* strain TBF 19.5.1., a thermophilic bacterium with a wide growth temperature range, isolated from the Troll B oil platform in the North Sea. J Bacteriol. 193: 5566-5667. 2011.
4. Swithers, K. S., et al. Genome sequence of *Thermotoga* sp. strain RQ2, a hyperthermophilic bacterium isolated from a geothermally heated region of the seafloor near Ribeira Quente, the Azores. J Bacteriol. 193:5869-5870. 2011.
5. Boucher, N., K. M. Noll. Ligands of thermophilic ABC transporters encoded in a newly sequenced genomic region of *Thermotoga maritima* MSB8 screened by differential scanning fluorimetry. Appl. Environ. Microbiol. 77:6395-6399. 2011.
6. Frock, A.D., S.A. Gray and R.M. Kelly. Hyperthermophilic Thermotoga species differ with respect to specific carbohy-

drate transporters and glycosidase hydrolases. Appl Environ Microbiol (accepted pending minor revisions)

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Mutational Analysis of the Enzymes Involved in the Metabolism of Hydrogen by *Pyrococcus furiosus*

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Project Goals: The overall goal is to incorporate a systems-level approach to improve our understanding of microbial regulatory and metabolic networks related to hydrogen production through the development of new model organisms for microbial hydrogen production. We are using targeted approaches for the identification and characterization of enzymes and biochemical pathways relevant to biological hydrogen production. Advancing fundamental scientific knowledge in these areas are critical to characterizing enzymatic structure and function, modeling regulatory and metabolic networks, and engineering of enzymes and organisms to improve biological hydrogen production for a future hydrogen economy.

The hyperthermophilic archaeon *Pyrococcus furiosus* grows optimally near 100˚C by fermenting carbohydrates to acetate, carbon dioxide and hydrogen gas (H_2). In the presence of elemental sulfur (S^0) it produces hydrogen sulfide rather than H_2. *P. furiosus* contains three distinct hydrogenases, which are enzymes that metabolize H_2. Two are cytosolic and are termed SHI and SHII. Both use NADP(H) as an electron carrier and each is encoded by a 4-gene operon. The third hydrogenase, MBH, is membrane-bound and uses the redox protein ferredoxin as an electron carrier. It is encoded by a 14-gene operon. In a previous study (1) we constructed deletion strains lacking the operons encoding SHI or SHII or both and showed that they exhibited no obvious phenotype under the usual growth condition (at 98˚C using maltose as the carbon source). This study has now been extended to include biochemical analyses and growth studies under a variety of conditions using the ΔSHI and ΔSHII deletion strains together with strains lacking a functional MBH or more specifically, lacking the catalytic subunit (MbhL) of MBH. While deletion of either one or both cytosolic hydrogenases does not produce any obvious growth phenotype, the levels of hydrogenase activity in the cytoplasmic extracts are not affected in the ΔSHII strain but are strongly reduced in the ΔSHI strain (<10% compared to the parental strains). In the SHI and SHII double deletion strain, the cytosolic hydrogenase activity was below the detection limit. These data indicate that SHI is responsible for most of the hydrogenase activity in the cytoplasm. In contrast, the strain lacking the membrane-bound hydrogenase catalytic subunit showed no growth in the absence of S^0. This confirms the hypothesis that, in the absence of S^0, MBH is the enzyme that produces H_2. Moreover, these results show that in the absence of S^0 *P. furiosus* can only dispose of reductant generated from sugar oxidation in the form of H_2 gas. The deletion strain devoid of all three hydrogenases (ΔSHI ΔSHII Δ*mbhL*) grows only in the presence of S^0 and did not produce any detectable H_2. When the hydrogenase deletion strains and their parental strains were grown in the presence of limiting S^0 (0.5 g/L vs 2 g/L), both S^0 is reduced and H_2 is produced in the parental strains (ca. 50% H_2 produced compared to no added S^0). Interestingly, in Δ*mbhL*, a significant amount of H_2 is produced (ca. 20% compared to the parental strains) and growth was compromised (ca. 50% lower cell yield as compared to the parental strains). Therefore, the H_2 produced in Δ*mbhL* must be catalyzed by SHI, showing that this cytosolic 'uptake' hydrogenase can also produce H_2 from NADPH *in vivo*, even though this reaction is thermodynamically unfavorable. However, SHI cannot compensate for the absence of MBH since it does not enable significant growth of the Δ*mbhL* strain. We propose that the *in vivo* function of SHI is to recycle H_2 and provide a link between external H_2 and the intracellular pool of NADPH needed for biosynthesis. *P. furiosus* only uses a low potential ferredoxin in its glycolytic pathway, which is linked to MBH for the disposal of all reducing equivalents as H_2, generating an ion gradient for ATP generation in the process. The ability to recycle H_2 might have a distinct energetic advantage in the environment, but it is clearly not required for heterotrophic growth of the organism under the usual laboratory conditions.

Reference

1. Lipscomb, G. L., Stirrett, K., Schut, G. J., Yang, F., Jenney, F. E, Jr., Scott, R. A., Adams, M. W. W. and Westpheling, J. (2011) Natural competence in the hyperthermophilic archaeon *Pyrococcus furiosus* facilitates genetic manipulation: use for constructing multiple markerless deletions of genes encoding two cytoplasmic hydrogenases. *Appl. Environ. Microbiol.* 77, 2232-2238

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Pathway of Fermentative H_2 Production by Sulfate-Reducing Bacteria

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Project Goals: The abundant soil anaerobes, the sulfate-reducing bacteria (SRB) of the genus *Desulfovibrio*, produce H_2 from organic acids. These apparently simple pathways have yet to be clearly established and their elucidation forms the goals for our research efforts. Information obtained may facilitate the exploitation of other anaerobes not yet readily amenable for examination by molecular tools and will be useful in consideration of practical applications. Energy metabolism, flux through the pathways, and regulation are likely to be limiting factors in channeling reductant for H_2 production, the process which we are examining. Our research has focused on: (1) the determination of the path of electrons during pyruvate fermentation growth in order to identify possible electron sinks, (2) confirmation of the enzymes that are essential during pyruvate fermentation, and (3) determination of the role of the pyruvate enzymes during lactate respiration.

Strains of *Desulfovibrio* can ferment organic acids in the absence of additional terminal electron acceptors and produce rather large amounts of H_2. We believe a study of the limitations to H_2 production in our model organism, *Desulfovibrio alaskensis* G20 (Hauser et al, 2011) may be informative to decipher the flow of electrons in those organisms chosen for industrial application for H_2 production.

We proposed to tease apart the contribution of fermentation to the respiratory energy budget to determine the dependence of the bacterium on this process. In studying growth, we have realized an important role for the utilization of fumarate via the fumarate reductase complex (Dde_1258 – Dde_1256) during various growth modes. *D. alaskensis* G20 is able to grow robustly on fumarate through disproportionation after adaptation when subcultured from cells grown on lactate/sulfate. However, growth on fumarate is inhibited by formate, lactate, pyruvate, H_2, or CO_2 indicating that the use of fumarate by the cells may be a secondary or tertiary option as an electron acceptor. Interestingly, proteomic analysis reveals that even during growth on lactate/sulfate or by pyruvate fermentation, the proteins of the fumarate reductase complex are expressed at relatively abundant levels. Yet, a very small proportion of reductant appears as succinate in these culture conditions.

Mutant analysis of the genesencoding the fumarate reductase complex, the quinone-reducing complex, and the type-1 tetraheme cytochrome c_3 indicate possible components playing a role in the flow of electrons, as all are unable to grow on fumarate. In addition, several mutants lacking formate dehydrogenase isozymes have an impaired growth phenotype on fumarate. Microarray, proteomic, and metabolite analysis is currently underway to determine changes that may be critical to robust growth of *D. alaskensis* G20 by disproportionation of this substrate and in the channeling of reductant for H_2 production.

References and Publications

1. Hauser, L. J., M. L. Land, S. D. Brown, F. Larimer, K. L. Keller, B. J. Rapp-Giles, M. N. Price, M. Lin, D. C. Bruce, J. C. Detter, R. Tapia, C. S. Han, L. A. Goodwin, J-F. Cheng, S. Pitluck, A. Copeland, S. Lucas, M. Nolan, A. L. Lapidus, A. V. Palumbo, and J. D. Wall. (2011) The Complete Genome Sequence and Updated Annotation of *Desulfovibrio alaskensis* G20. *J Bacteriol.* 191:4268–4269.
2. Keller, K. L., and J. D. Wall. (2011) Genetics and molecular biology of the electron flow for sulfate respiration in *Desulfovibrio*. *Front Microbiol.* 2:1-15.

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Pathways and Regulatory Network of Hydrogen Production from Cellulose by *Clostridium thermocellum*

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Clostridium thermocellum, a thermophilic, ethanogenic, and cellulolytic anaerobe, produces a complex extracellular cellulolytic organelle called the cellulosome. The cellulosome contains various depolymerizing enzymes that are arrayed on a protein scaffold and effectively degrades complex cellulosic substrates. During cellulose fermentation, the bacterium evolves hydrogen at a high rate. Analysis of its genome sequence reveals the existence of at least three putative hydrogenases (CtHydA1, CtHydA2 and CtHydA3) central to hydrogen metabolism. Furthermore, at least 20 genes are potentially related to hydrogen metabolism. The bacterium is thus remarkably versatile in employing various enzymes, some of which are potentially novel, for hydrogen metabolism. The versatility indicates the significance of this biological process. Yet little is known concerning the pathway for hydrogen production and the underlying regulatory mechanism/network that control these hydrogenase and the related genes as well as cellulolytic process and other metabolic pathways in the organism.

Due to the novelty of CtHydA3, it was selected for initial study. Genes encoding CtHydA3 (Ct_3003), a ferredoxin-like protein (Ct_3004), and three FeFe-hydrogenase maturation proteins (CtHydE, CtHydF, and CtHydG) have been cloned into three plasmids and co-transformed into *E. coli* strain Rosetta (DE3) and BL21 (DE3) for heterologous expression. In addition, a 6X His-tag sequence was fused to either the C- or N-terminus of CtHydA3. Protein immunoblots confirmed the expression of both the C- and N-terminus His-tagged CtHydA3 (73 kDa band) in *E. coli* Rosetta, but not in *E. coli* BL21, likely due to differences in codon usage between *C. thermocellum* and *E. coli*. The recombinant protein was affinity-purified with *in vitro* activity linking to reduced methyl viologen. However, the low yield of the purified protein prevented its biochemical characterization. The *E. coli* Rosetta strain also displayed a high background hydrogenase activity. We have since initiated transformation using an *E. coli* FTD strain (DE3) lacking all background hydrogenase activity with the intent to determine cofactors requirements and directionality (H_2 production or H_2 uptake) of CtHydA3 in *E. coli* cell-free extract. The outcome will reveal the physiological functions of CtHydA3 during cellulose metabolism.

To identify transcription factors controlling metabolic pathways, we developed an affinity purification method by immobilizing promoter DNA sequences to a solid support. DNA-binding proteins from the *C. thermocellum* cell lysate, obtained by growing on cellobiose or crystalline cellulose and eluted from the affinity columns, were identified by the MALDI-TOF or LC-MS-MS techniques. We thus identified a REX-like protein that regulates the expression of a hydrogenase gene and many other genes, indicating that it is a global regulator. It is likely the first global regulator experimentally verified in this bacterium. We also carried out the proteomic analysis and identified the cellulosomal proteins and non-cellulosomal glycosyl hydrolases that are up- or down-regulated when the cells were grown on the cellulose substrate vs. cellobiose.

The studies will provide important insights into the pathway and regulatory mechanism/network controlling hydrogen metabolism and cellulolysis as well as other pertinent metabolic pathways in this very intriguing cellulolytic and thermophilic bacterium, which catalyzes the rate-limiting cellulose-degradation reaction in a single-step process of biomass conversion (or CBP, Consolidated Bioprocessing). Detailed understanding of the pathway and regulatory mechanism/network will ultimately provide rationales for engineering, alternating, or deregulating the organism for biomass conversion to liquid and hydrogen fuels.

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The Ins and Outs of Algal Metal Transport

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Project Goals: (see abstract)

Metal transporters are a central component in the interaction of algae with their environment. They represent the first line of defense to cellular perturbations in metal concentration, and by analyzing algal metal transporter repertoires, we can get insight into a fundamental aspect of algal biology. The ability of individual algae to thrive in environments with unique geochemistry, compared to non-algal species commonly used as reference organisms for metal homeostasis, provides an opportunity to broaden our understanding of biological metal requirements, preferences and trafficking. *Chlamydomonas reinhardtii* is the best developed reference organism for the study of algal biology, especially with respect to metal metabolism; however,the diversity of algal niches necessitates a comparative genomic analysis of all sequenced algal genomes. A comparison between known and putative proteins in animals, plants, fungi and algae using protein similarity networks has revealed the presence of novel metal metabolism components in Chlamydomonas including new iron and copper transporters. This analysis also supports the concept that, in terms of metal metabolism, algae from similar niches are more related to one another than to algae from the same phylogenetic clade.