Final Scientific/Technical Report

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Project Title: Development of Cyanothece as a New Model Organism for Biological Hydrogen Production

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Executive Summary

The potential for developing commercially viable microbial H_2-production systems as a renewable source of biofuel has been limited by the need for an anaerobic environment to enable photobiological H_2-production in capable bacterial and algal species. In this project, we have shown that the cyanobacterium *Cyanothece* sp. ATCC 51142 has the capacity for highly efficient H_2-production under natural aerobic conditions. The marine cyanobacterium *Cyanothece* sp. ATCC 51142 has a diurnal metabolic cycle; photosynthesis and carbon fixation occur during daylight hours and then at night, high rates of respiration create a suboxic intracellular environment that enables O_2-sensitive processes to occur, including N_2-fixation and H_2-production. We developed a two-stage approach to monitor H_2-production by *Cyanothece* 51142. In the first stage, we grew the bacteria aerobically in an alternating 12 hour light/dark cycle. A second 'incubation' stage was then carried out in which we took cells from the end of a 12 hour light growth period and incubated them in air-tight vials for a further 12 hours under continuous illumination. Analysis of the headspace in the vial revealed high specific rates of H_2-production (>150 μmol of H_2 per mg chlorophyll per hour) during this incubation period. Furthermore, the rate of H_2-production could be enhanced by growing the cells in the presence of high levels of CO_2 or glycerol.

We also confirmed that H_2-production was mediated by the nitrogenase system found in these *Cyanothece* cells. Interestingly, in the absence of molecular N_2, nitrogenase systems channel all available electrons towards H_2-production. Accordingly, when we incubated glycerol-supplemented *Cyanothece* 51142 cells in the absence of N_2, the rate of H_2-production increased up to 467 μmol of H_2 per mg chlorophyll per hour, which is an order of magnitude greater than

those rates previously observed in other wild type H_2 producing model photosynthetic microorganisms under anaerobic conditions. As glycerol and CO_2 are both abundantly available as industrial waste products, the fact that they substantially enhance aerobic H_2-production suggests that *Cyanothece* 51142 is a potentially viable system for producing biohydrogen as a renewable fuel source.

During this project, we have conducted a detailed systems level analysis of this interesting cyanobacterial system, and have created a rich knowledgebase for the use of *Cyanothece* cells as efficient biohydrogen producers in large scale.

Specific Aims of Awarded Project

The objective of this proposal was 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 used genome sequencing, microarrays, proteomics, mutagenesis, biochemical analysis and physiology, all of which was encased in a systems biology framework. We propose to initiate the project with the following specific aims:

I. To evaluate the hydrogen production potentials of different *Cyanothece* strains.
   - A. To determine the capacity of hydrogen production by *Cyanothece* strains under various physiological conditions.
   - B. To examine the metabolic potentials of different *Cyanothece* strains using a comparative genomics approach.
   - C. To validate predicted metabolic potentials by ^{13}C based metabolic flux analysis.

II. To develop genetic tools for facile metabolic manipulation of *Cyanothece*.

III. To use transcriptomic, proteomic and fluxomic approaches for systems level understanding of hydrogen production by *Cyanothece*.
   - A. To examine the dynamics of *Cyanothece* transcriptome using whole genome microarray analysis.
   - B. To determine the behavior of the cellular proteomes using quantitative approaches.
   - C. To integrate transcriptomic, proteomic and fluxomic studies to gain insights into factors regulating *Cyanothece* growth and hydrogen metabolism.

Project Activities and Results

The project team successfully completed the aims of the project and disseminated results via a website, academic journals, and conference proceedings.

Hydrogen production by Cyanothece: We established a two stage system for photobiological H_2 production in the unicellular cyanobacterium *Cyanothece* ATCC 51142. Using this system, we demonstrated high rates of nitrogenase-mediated photobiological H_2 production under aerobic conditions. H_2 production was dependent on the cellular glycogen reservoir and could be enhanced with an external carbon source like high CO_2 and glycerol. A batch culture of *Cyanothece* 51142 in the presence of glycerol could produce more than 900 ml of H_2 per liter of culture over a period of two days. These rates are several folds higher compared to the rates reported for any other wild type model H_2 producing strain. H_2 production in *Cyanothece* 51142 was largely driven by light even in the presence of glycerol, suggesting that the observed rates were not solely due to glycerol fermentation. However, photosystem II did not seem to be

involved in the process as observed from studies in the presence and absence of the photosystem II inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea). The strain is being analyzed further at the systems level to unravel the mechanism of H_2 production and to obtain insights into possible ways of further improving yields. H_2 production has also been optimized in six other sequenced *Cyanothece* strains. Analysis of nitrogenase activity and H_2 production in these strains revealed the ability of aerobic nitrogen fixation and H_2 production in five of the six *Cyanothece* strains. *Cyanothece* PCC 7425 exhibited H_2 production only under anaerobic incubation conditions.

Cyanothece ATCC 51472 and PCC 7822 were assayed for H_2 production as well as for the storage of carbon (in the form of glycogen, PHA and EPS) in detail. *Cyanothece* 7822, but not 51472, produces copious amounts of EPS and PHA and both strains make substantial levels of glycogen. Cultures were grown under ambient and elevated (0.4%) CO_2 concentrations, as well as in the presence of glycerol. *Cyanothece* 51472 grows well under high light (400 $\mu mol.m^{-2}s^{-1}$). Both the species had enhanced growth under increased CO_2 and under photomixotrophic conditions (with glycerol), and glycogen content was enhanced in 51472 under increased CO_2 concentration. Glycerol enhanced the nitrogenase activity and glycogen content in both the species. The level of H_2 evolved was positively correlated with higher levels of intracellular glycogen in both *Cyanothece* strains. In addition, our results demonstrated that *Cyanothece* sp 7822 was more efficient than 51472 in H_2 production both under photoautotrophic and photomixotrophic conditions. Although both strains produce high levels of H_2 (150-200 µmoles H_2 evolved/mg Chl/h), neither strain is as productive as *Cyanothece* 51142.

Comparative genomics: Complete genome sequences of six *Cyanothece* strains (ATCC 51142, PCC 7424, PCC 7425, PCC 7822, PCC 8801, and PCC 8802) are currently available and one more (ATCC 51472) is in the process of completion at the DOE Joint Genome Institute. The sequencing revealed the presence of one linear chromosome in *Cyanothece* 51142 and 3 linear elements in *Cyanothece* 7822. This feature is unique to *Cyanothece* strains compared to other sequenced cyanobacteria and suggest the presence of distinctive metabolic traits in members of this group. A comparison of the genomes of the six *Cyanothece* strains revealed the presence of several pathways analogous to non-oxygenic microbes in these strains, an observation which complies with their ability to maintain a suboxic intracellular environment for a significant part of a diurnal cycle. These characteristics suggest that the group *Cyanothece* can be appealing as model organisms for studies pertaining to biohydrogen production.

Cyanothece genetics: *Cyanothece* 7822 was successfully transformed using a single-stranded DNA technique and a *nifK* knockout mutant was generated. A stable line of this mutant is being further analyzed. In addition, we constructed a mutant *(ΔhupL,* a deletion of the gene encoding the large subunit of uptake hydrogenase) in *Cyanothece* 7822 by inserting a neomycin/kanamycin antibiotic resistance cassette in the *hupL* gene. The growth rate of the mutant strain was about half the rate of the wild type strain in BG11 medium with nitrate, and the *ΔhupL* strain grew very poorly in nitrate-free BG11. In contrast to uptake hydrogenase mutants in filamentous cyanobacterial strains we observed no H_2 production and no nitrogenase activity in *ΔhupL* mutant. The *in vitro* uptake hydrogenase activity is zero in the mutant. The mutant cells were rounder and fatter in morphology and 1.7 times larger in volume relative to the wild type cells. The knockout of *hupL* caused a defect of nitrogenase in terms of conversion of N_2 to NH_3, with a concomitant defect in H_2 production. In these unicellular cells, HupL appears to play an important role in balancing *in vivo* N_2 fixation and H_2 production processes.

Metabolomic and Fluxomic studies: The previous ^{13}C-assisted metabolism analyses have identified unique metabolic features (*i.e.* citramalate pathway and CO_2 fixation regulations) in *Cyanothece* 51142. Based on these discoveries, a constraint-based genome-scale flux balance model for *Cyanothece* 51142 metabolism is under development. The model can be used not

only to analyze functional pathways under different cultivation conditions, but also for *in silico* genetic manipulations (*e.g.* knock-out and over-expression) and to predict metabolic behavior in mutant strains. To facilitate our study on other cyanobacterial species, our model development mainly focuses on a general platform for metabolic network reconstruction and flux balance analysis. Such user-friendly and website-based software is able to automatically reconstruct genome scale metabolic network for different cyanobacterial species based on the "KEGG" database. Then the customers can manually redefine functional pathways and flux boundaries. Users can also select their own objective functions (such as maximum biomass productions, minimal enzyme usage, etc). In addition, the software can perform dynamic flux analysis by integrating kinetics and fluxomics via static optimization approach. This software can be potentially used for comparing the physiologies of different cyanobacterial species and providing the guidelines for rational design of metabolic network for CO_2 fixation and H_2 production.

Proteomic Studies: The proteomes of six *Cyanothece* strains (ATCC 51142, PCC 7822, PCC 7424, PCC 7425, PCC 8801 and PCC 8802) were analyzed and compared to identify proteins common to all strains as well as unique proteins characteristic of an individual strain. The observed coverage of predicted proteins (based upon genome annotation) ranged from about 47% of the predicted genome in *Cyanothece* PCC 7822 to 67% in *Cyanothece* ATCC 51142. We also performed dynamic proteomic profiling, the first time for any photosynthetic organism, to determine the changes in protein abundance in phases in the diurnal process during which hydrogen production is maximal.

Publications:

1. Bandyopadhyay, A., Stöckel, J., Min, H., Sherman, L. A. and Pakrasi, H. B. (2010) High rates of photobiological H_2 production by a cyanobacterium under aerobic condition. Nature Comm., 1:139. doi: 10.1038/ncomms1139.
2. Wu, B., Zhang, B., Feng, X., Rubens, J. R., Huang, R., Hicks, L. M., Pakrasi, H. B. and Tang, Y. J. (2010) Alternate Isoleucine Synthesis Pathway in Cyanobacterial Species. Microbiology, 156: 596-602. doi: 10.1099/mic.0.031799-0.
3. Feng, X., Bandyopadhyay, A., Berla, B., Page, P., Wu, B., Pakrasi, H. B. and Tang, Y. J. (2010) Mixotrophic and photoheterotrophic metabolisms in *Cyanothece* sp. ATCC 51142 under continuous light. Microbiology, 156: 2566 – 2574. doi: 10.1099/mic.0.038232-0.
4. Sherman, L. A., Min, H., Toepel, J. and Pakrasi, H. B. (2010) Better living through *Cyanothece* - unicellular diazotrophic cyanobacteria with highly versatile metabolic systems. Adv Exp Med Biol., 675: 275-290. doi: 10.1007/978-1-4419-1528-3_16.
5. Min, H. and Sherman, L. A. (2010) Hydrogen production by the unicellular, diazotrophic cyanobacterium *Cyanothece* sp. strain ATCC 51142 under conditions of continuous light. Appl Environ Microbiol. 76:4293-4301. doi: 10.1128/AEM.00146-10.
6. Bandyopadhyay, A., Elvitigala, T., Welsh, E., Stöckel, J., Liberton, M., Min, H., Sherman, L. A. and Pakrasi, H. B. (2011) Novel metabolic attributes of *Cyanothece*, a group of unicellular nitrogen fixing cyanobacteria. mBio, 2: 1-10. doi: 10.1128/mBio.00214-11.
7. Feng, X., Page, L., Rubens, J., Chircus, L., Colletti, P., Pakrasi, H. B. and Tang, Y. J. (2011) Bridging the Gap between Fluxomics and Industrial Biotechnology. J. Biomed. Biotech. 460717. doi: 10.1155/2010/460717.
8. Bandyopadhyay, A., Elvitigala, T., Liberton, M. and Pakrasi, H. B. (2013) Variations in the rhythms of respiration and nitrogen fixation in members of the unicellular diazotrophic cyanobacterial genus *Cyanothece*. Plant Physiol., 161: 1334-1346. doi: 10.1104/pp.112.208231.

9. Zhang, X., Sherman, D.M. and Sherman, L. A. (2014) The uptake hydrogenase in the unicellular diazotrophic cyanobacterium *Cyanothece* sp. strain PCC 7822 protects nitrogenase from oxygen toxicity. J. Bacteriol. 196: 840-849. doi: 10.1128/JB.01248-13.
10. Aryal, U. K., Callister, S. J., McMahon, B., McCue, L. A., Brown, J., Stöckel, J., Liberton, M., Mishra, S., Zhang, X., Nicora, C. D., Angel, T., Koppenaal, D. W., Smith, R. D., Pakrasi, H. B. and Sherman, L. A. (2014) Proteomic profiles of five strains of oxygenic photosynthetic cyanobacteria of the genus *Cyanothece*. J. Proteome Res., 13: 3262-3276. doi: 10.1021/pr5000889.

Intellectual Property:

There were no inventions, patent applications, or licensing agreements created through this project.