

II.H.1 Hydrogen from Water in a Novel Recombinant Oxygen-Tolerant Cyanobacterial System

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Objectives

- Develop an O₂-tolerant cyanobacterial system for sustained and continuous light-driven hydrogen production from water.

Technical Barriers

This project addresses the following technical barriers from the Hydrogen Production section (3.1) of the Hydrogen, Fuel Cells and Infrastructure Technologies Program Multi-Year Research, Development and Demonstration Plan:

- (AH) Rate of Hydrogen Production
- (AI) Continuity of Photoproduction

Technical Targets

This project is performing genetic-engineering studies on cyanobacteria, the outcome of which will lead to a much more O₂-tolerant cyanobacterial system for continuous photolytic H₂ production from water.

Therefore, this project will facilitate the construction of an efficient and continuous H₂ production system that meets the following DOE 2011 milestone: producing one cyanobacterial recombinant to evolve H₂ through an O₂-tolerant NiFe-hydrogenase (4Q, 2011).

Accomplishments

- The Sargasso Sea Project, a pilot study for VI's Sorcerer II Expedition, generated 1.045 billion bps of non-redundant deoxyribonucleic acid (DNA) sequences, from which 1.2 million new genes including 782 new rhodopsin-like photoreceptors were identified and 1800 genomic species including 148 new bacterial species were revealed (VI).
- The Sorcerer II Global Ocean Sampling Expedition generated 6.3 billion bps of non-redundant sequences; identified 6.12 million proteins (covering nearly all known prokaryotic protein families and doubling the number of current proteins); and revealed 3995 global ocean sampling (GOS)-only clusters that have no homology to known protein families (VI).
- We identified putative novel NiFe-hydrogenases through the metagenomic analysis of the Sargasso Sea microbial community. One of the putative novel NiFe-hydrogenases has been heterologously expressed (VI).
- We transferred *Thiocapsa* O₂-tolerant hydrogenase genes into cyanobacteria through homologous DNA recombination, and the transferred hydrogenase has been heterologously expressed (VI).
- We transferred *Rubrivivax* O₂-tolerant hydrogenase genes into *E. coli* as the host initially, and demonstrated the expression of the hydrogenase catalytic subunit (NREL).



Introduction

Photobiological processes are attractive routes to renewable H₂ production. With the input of solar energy, photosynthetic microbes such as cyanobacteria and green algae carry out oxygenic photosynthesis, extracting energy from water oxidation. The resulting energy can couple to a hydrogenase system to yield H₂. However, one major difficulty is that most hydrogen-evolving hydrogenases are inhibited by O₂, which is an inherent byproduct of oxygenic photosynthesis. The rate of H₂ production is thus limited. Certain photosynthetic

bacteria are reported to have an O₂-tolerant evolving hydrogenase, yet these microbes do not split water, requiring other, more expensive feedstocks.

To overcome these difficulties, we propose to construct novel microbial hybrids by genetically transferring the O₂-tolerant hydrogenase from other bacteria into cyanobacteria. These hybrids will use the photosynthetic machinery of the cyanobacterial hosts to perform the water-oxidation reaction with the input of solar energy, and couple the resulting reducing equivalents to the O₂-tolerant bacterial hydrogenase, all within the same microbe. This proposal therefore addresses one of the key technological hurdles to cost-effective photobiological production of H₂, overcoming the sensitivity of the hydrogenase enzyme to O₂, which limits the production of H₂ in the current algal systems.

Approach

Our goal is to construct a novel microbial hybrid taking advantage of the most desirable properties of both cyanobacteria and other bacteria, to serve as the basis for technology to produce renewable H₂ from water. To achieve this goal, we use the following two approaches. The first approach is to transfer known O₂-tolerant hydrogenases from anoxygenic photosynthetic bacteria *Thiocapsa* and *Rubrivivax* to cyanobacteria. Since a very limited number of the O₂-tolerant hydrogenases is available, our second approach is to identify novel O₂-tolerant hydrogenases from environmental microbial communities and transfer them into cyanobacteria.

Results

Venter Institute: This task was not funded by DOE during 2006, and the following progress was made with internal funding from the Venter Institute. To apply the first approach, we transferred both structural and essential accessory genes of *Thiocapsa* O₂-tolerant NiFe-hydrogenase, such as *hynS*, *hynL*, and *hynD*, into the *Synechococcus* genome. This O₂-tolerant hydrogenase has been heterologously expressed in the host upon isopropyl β-D-1-thiogalactopyranoside (IPTG) induction, as demonstrated by Western blotting results. As part of the second approach, the Venter Institute's Sorcerer II Global Ocean Sampling Expedition generated 6.3 billion bps of non-redundant DNA and generated 6.12 million proteins (nearly covering all known prokaryotic protein families and doubling the number of current proteins). Through metagenomic analysis of the ocean microbial DNA sequences by using 14 hydrogenases Hidden Markov Models (HMMs) we constructed, we identified a novel NiFe-hydrogenase with strong homology to *Thiocapsa* O₂-tolerant hydrogenase. Its structural and accessory genes were traced and cloned from the Sargasso Sea environmental samples, and they were successfully hetero-expressed

as demonstrated by Western blotting results. This study represents the first conversion of an environmental DNA into a hydrogenase.

NREL: This task was not funded by DOE in FY 2006. The NREL principal investigator returned to NREL in April 2007 to resume the project. Progress made during this short performance period is described below. Prior to creating the cyanobacterial recombinant, we tested *E. coli* as the host to express the O₂-tolerant hydrogenase from *Rubrivivax*. Genes encoding the hydrogenase catalytic large (CooH) and small (CooL) subunits were cloned into a duet vector pCDFDuet-1 (Figure 1A). The CooH catalytic subunit is labeled with a Strep II tag to facilitate its affinity purification and detection. When expressed in *E. coli*, tagged CooH (42 kDa) is detected in cell-free extract following IPTG induction (Figure 1B). Identity of the 42 kDa protein as CooH was further confirmed via protease digest followed by mass spectrometry analysis. The ultimate goal of the project is to construct a cyanobacterial recombinant. Work is ongoing to clone and transform the *Rubrivivax* hydrogenase structural genes into *Synechocystis* host for cyanobacterial H₂ production.

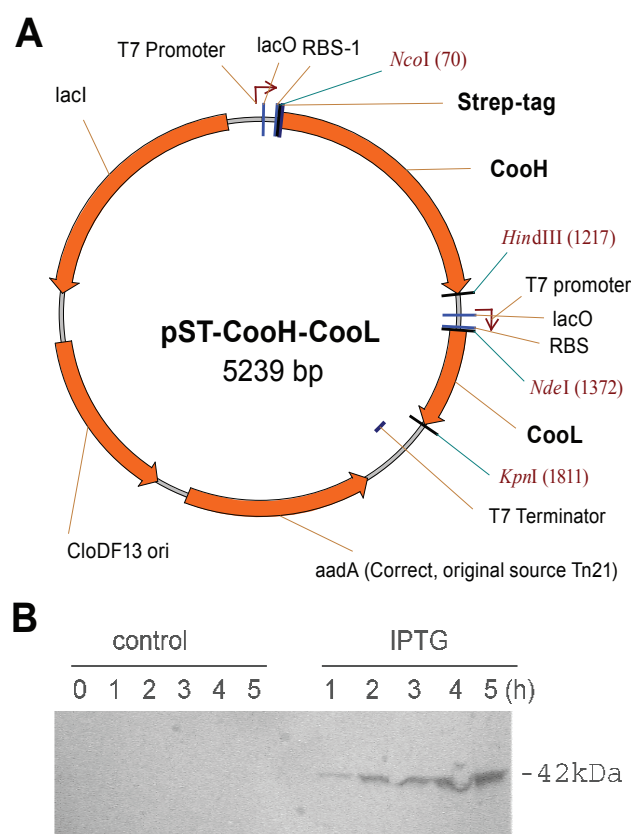


FIGURE 1. (A) pCDFDuet-1 plasmid harboring the labeled CooH and CooL gene construct; (B) *E. coli* expressing the 42 kDa CooH protein for various durations (hours) following IPTG induction. Control samples were transformed with plasmid yet did not receive IPTG.

Conclusions and Future Directions

From the Venter Institute's research, we conclude that environmental microbes have significant potential in carrying new genes or pathways that may improve efficiency of renewable energy production. To date, an environmental DNA has been successfully converted into a novel NiFe-hydrogenase; and the *Thiocapsa* O₂-tolerant hydrogenase has been transferred into cyanobacterium and its hetero-expression in the host has been confirmed by Western blotting. These findings provide critical inputs for our efforts to construct a cyanobacterial recombinant to evolve H₂ through an O₂-tolerant NiFe-hydrogenase.

The NREL research leads to the conclusion that we have succeeded in modifying the *cooH* gene encoding the catalytic subunit of the *Rubrivivax* O₂-tolerant hydrogenase with an affinity tag to facilitate its purification and detection. Both Western blot and mass spectrometry analysis confirmed the heterologous expression of *cooH* in *E. coli*. These encouraging findings position us to begin the transformation experiments using *Synechocystis* as the host.

The areas that we plan to pursue in FY 2007 are:

1. To purify and characterize the novel NiFe-hydrogenase we identified from the Sargasso Sea microbial community (VI).
2. To transfer more accessory genes of *Thiocapsa* O₂-tolerant hydrogenase into cyanobacteria, and perform functional analysis of the hydrogenase that is hetero-expressed in cyanobacteria (VI).
3. To insert the Strep II-tagged as well as non-tagged *Rubrivivax* hydrogenase (*cooLXUH*) into the pPETE_σD plasmid (Dr. R. Burnap of Oklahoma State University) for delivery into *Synechocystis sp.* PCC6803 (NREL).
4. To systematically express *Rubrivivax* hydrogenase structural genes (*cooMKLXUH*) and accessory genes (*hypABCDEF*) for expressing foreign O₂-tolerant hydrogenases in *Synechocystis sp.* PCC6803 (NREL).

FY 2007 Publications/Presentations

1. Q. Xu, G. Maroti, S. Yooseph, Y. Tong, H. O. Smith, and J. C. Venter. Identification and Functional Analysis of NiFe-hydrogenases from the Sargasso Sea Microbes. Presented in Genome, Medicine, and the Environment Conference, 2006, Poster 52.
2. "The construction of a *Synechocystis* recombinant system for solar hydrogen production", oral presentation at the 2nd BioSolarH2 Mini-symposium, Princeton University, NJ (July 2006, P.C. Maness).
3. "Expression of an O₂-tolerant evolving hydrogenase in *E. coli*", poster presentation at ACS annual meeting, San Francisco, CA (September 2006, J. Yu).
4. "Expression of an O₂-tolerant evolving hydrogenase in *E. coli*", oral presentation at the 16th Western Photosynthesis Conference, Pacific Grove, CA (January 2007, J. Yu).
5. Ghirardi, ML, MC Posewitz, PC Maness, A Dubini, J Yu, and M Seibert. Hydrogenases and hydrogen photoproduction in oxygenic photosynthetic organisms. 2007 *Ann. Rev. Plant. Biol.* 58, 71-91.
6. Rusch DB, Halpern AL, Sutton G, Heidelberg KB, Williamson S, Yooseph S, Wu D, Eisen JA, Hoffman JM, Remington K, Beeson K, Tran B, Smith H, Baden-Tillson H, Stewart C, Thorpe J, Freeman J, Andrews-Pfannkoch C, Venter JE, Li K, Kravitz S, Heidelberg JF, Utterback T, Rogers YH, Falcon LI, Souza V, Bonilla-Rosso G, Eguiarte LE, Karl DM, Sathyendranath S, Platt T, Birmingham E, Gallardo V, Tamayo-Castillo G, Ferrari MR, Strausberg RL, Neilson K, Friedman R, Frazier M, Venter JC. The Sorcerer II Global Ocean Sampling Expedition: Northwest Atlantic through Eastern Tropical Pacific. 2007. *PLoS Biol.* 5(3):e77, 398-431.
7. Yooseph S, Sutton G, Rusch DB, Halpern AL, Williamson SJ, Remington K, Eisen JA, Heidelberg KB, Manning G, Li W, Jaroszewski L, Cieplak P, Miller CS, Li H, Mashiyama ST, Joachimiak MP, van Belle C, Chandonia JM, Soergel DA, Zhai Y, Natarajan K, Lee S, Raphael BJ, Bafna V, Friedman R, Brenner SE, Godzik A, Eisenberg D, Dixon JE, Taylor SS, Strausberg RL, Frazier M, Venter JC. The Sorcerer II Global Ocean Sampling Expedition: Expanding the Universe of Protein Families. 2007. *PLoS Biol.* 5(3):e16, 432-466.