

II.F.1 Hydrogen from Water in a Novel Recombinant O₂-Tolerant Cyanobacterial System

Qing Xu (Primary Contact) and
Hamilton O. Smith

J. Craig Venter Institute (JCVI)
9704 Medical Center Drive
Rockville, MD 20850
Phone: (301) 795-7248; Fax: (240) 268-4004
E-mail: qxu@venterininstitute.org

DOE Technology Development Manager:
Roxanne Garland

Phone: (202) 586-7260; Fax: (202) 586-2373
E-mail: Roxanne.Garland@ee.doe.gov

DOE Project Officer: Lea Yancey
Phone: (303) 275-4944; Fax: (303) 275-4788
E-mail: Lea.Yancey@go.doe.gov

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National Laboratory Collaborator:
Pin-Ching Maness and Jianping Yu
National Renewable Energy Laboratory (NREL),
Golden, CO

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Objectives

- Develop an O₂-tolerant cyanobacterial system for sustained and continuous light-driven H₂-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

Characteristics	Current Status	2011 Target	2018 Target
Duration of continuous H ₂ photoproduction in air	Zero to 30 seconds in air	Produce a cyanobacterial recombinant evolving H ₂ through an O ₂ -tolerant hydrogenase	H ₂ production in air for 30 min

Accomplishments

- We searched the metagenomic data of the global ocean sampling for novel O₂-tolerant hydrogenases. One of these novel hydrogenases has been cloned from environmental deoxyribonucleic acid (DNA) samples and its structural and accessory genes have been heterologously expressed in *Thiocapsa roseopersicina*, which generated a functional O₂-tolerant hydrogenase. This study represents the first report of conversion of a piece of environmental DNA into a functional novel NiFe-hydrogenase, and it provides a completely new approach for screening novel hydrogenases from environmental microbes (JCVI).
- We transferred the structural and accessory genes of *Thiocapsa* O₂-tolerant NiFe-hydrogenase into cyanobacterium *S. sp* PCC7942 through homologous DNA recombination. We demonstrated that *Thiocapsa* hydrogenase is expressed in *S. sp* PCC7942 upon isopropyl-beta-D-thiogalactopyranoside (IPTG) induction and it's located in the membrane fraction of the host. We established an efficient transfer system for integrating the genes of foreign hydrogenases into the cyanobacterium and for heterologously expressing these hydrogenases in the host (JCVI).
- We cloned the O₂-tolerant *Rubrivivax* hydrogenase genes in a plasmid suitable for integration into the genome of *Synechocystis*. We also codon optimized and cloned four hydrogenase accessory genes into a replicated plasmid suitable for expression in *Synechocystis* (NREL).
- We generated antibodies against the large and the small subunits of the *Rubrivivax* hydrogenase. We also verified that both subunits were indeed expressed in *E. coli* when transformed with these two genes (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 yielding 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 feedstock.

To overcome these difficulties, we work to construct novel microbial hybrids by genetically transferring an 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 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 hydrogen in 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 only a very limited number of O₂-tolerant hydrogenase is available, our second approach is to identify novel O₂-tolerant hydrogenases from environmental microbial communities and transfer them into cyanobacteria.

Results

JCVI Part I. Transferring O₂-tolerant Hydrogenase Genes from *Thiocapsa* into Cyanobacteria. Our goal for this part of the work is to transfer the O₂-tolerant NiFe-hydrogenase *Hyn* from *Thiocapsa roseopersicina* into cyanobacteria, as we described in the previous report, we cloned two structural and one accessory genes of this hydrogenase (*hynS*, *hynL*, and *hynD*) and integrated these genes into the chromosome of *Synechococcus* through homologous DNA recombination. In this year, we transferred additional accessory genes *hupK*, *hypC1*, and *hypC2* into the recombinant *Synechococcus*. Figure 1 shows the map of the whole gene cassette that was integrated into the chromosome of *Synechococcus*. IPTG-inducible heterologous expression of this *Thiocapsa* hydrogenase was detected in the cyanobacterial host by Western blotting, in which hydrogenase-specific rabbit polyclonal antibodies were used. To optimize conditions for IPTG-induction, we tested different IPTG concentrations such as 5, 20, 100, and 200 μM. Our Western blotting result demonstrates that 20 μM IPTG would be sufficient to induce expression of *Thiocapsa* hydrogenase. We also examined different lengths of induction times. Our result shows that induction of *Thiocapsa* O₂-tolerant hydrogenase reached the highest amounts at 12 hours while substantial amounts of the hydrogenase could be detected at 6 hours.

We further characterized hetero-expressed *Thiocapsa* hydrogenase and examined its cellular location in the recombinant cyanobacterial strain. *Thiocapsa* hydrogenase carries a signal peptide in its small subunit and it is a membrane-bound hydrogenase in its original host *Thiocapsa roseopersicina*. To investigate whether hetero-expressed *Thiocapsa* hydrogenase is a membrane-bound hydrogenase in the recombinant strain, we prepared whole cell extracts, crude membrane fractions, and supernatant fractions from IPTG-induced cyanobacterial cells. Western blotting was performed by using these prepared samples. Our result (Figure 2) shows that the hetero-expressed was in whole cell extract and crude membrane fraction of the recombinant *Synechococcus* cells, but not in its supernatant fraction. No *Thiocapsa* hydrogenase



FIGURE 1. Phylogenic representation of the hydrogenase gene cassette that was integrated into cyanobacterium *S. sp* PCC7942. P_{trc} : an IPTG-inducible promoter; *Hyn*'s structural genes: *hynS* and *hynL* (in green); *Hyn*'s essential accessory genes: *hynD*, *hupK*, *hypC1* and *hypC2* (in red).

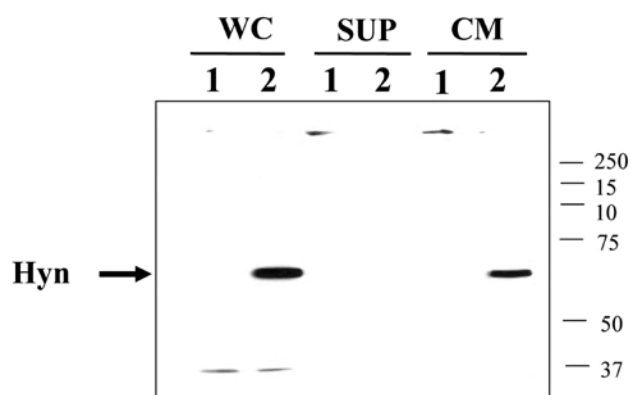


FIGURE 2. Examination of the location of heterologously-expressed *Thiocapsa* O₂-tolerant hydrogenase in the recombinant *S. sp* PCC7942 strain. Strains used are: 1. *S. sp* PCC7942 WT and 2. recombinant cyanobacterium *S. sp* PCC7942 Hyn3 strain. WC: whole cell extract; SUP: supernatant; and CM: crude membrane fraction. The sizes of molecular weight standards are marked on the left side of the protein gel. Rabbit serum specific for *Thiocapsa* hydrogenase HynL was used for the Western.

was detected in wild type strain, as expected. These results indicate the hetero-expressed hydrogenase is a membrane-bound hydrogenase in the recombinant *Synechococcus* strain.

JCVI Part II. Identifying Novel O₂-tolerant Hydrogenases from Marine Microbes in the Global Ocean. The goal for this part of the work is to identify novel O₂-tolerant hydrogenases from the ocean and transfer them into cyanobacteria. JCVI's Sorcerer II Global Ocean Sampling Expedition generated 6.3 billion bps of non-redundant DNA and 6.12 million proteins (nearly covering all known prokaryotic protein families and doubling the number of current proteins). To take advantage of this huge amount of genomic information, we searched novel hydrogenases through analysis of the metagenomic data. As reported previously, a novel NiFe-hydrogenase with strong homology to *Thiocapsa* O₂-tolerant hydrogenase has been cloned from the environmental DNA samples. Its structural (*hynS/L*) and accessory genes (*hupH* and *hynD*), as demonstrated by room temperature - polymerase chain (PCR) and Western blotting results, were heterologously expressed in the foreign host of *Thiocapsa roseopersicina* strain GB112131, a mutant strain with all the three native hydrogenases knocked out. We functionally analyzed this heterologously expressed hydrogenase, which demonstrated both H₂-evolution and H₂-uptake activities. We further quantitatively analyzed its hydrogenase activities under *in vitro* conditions. Reduced methyl viologen was used as the electron donor for the hydrogenase in the H₂ evolution assay, and H₂ produced was measured by gas chromatograph. The result (Figure 3A) shows that the calculated H₂ evolution activity of the novel hydrogenase is 0.26 μl

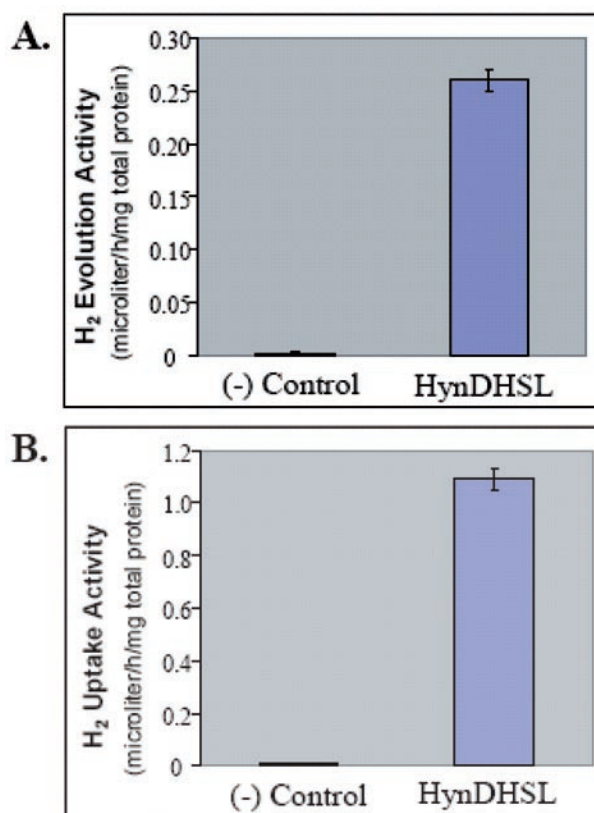


FIGURE 3. Detection of the hydrogenase activities of hetero-expressed novel hydrogenase under *in vitro* conditions. Panel A: Measurement of *in vitro* H₂ evolution activity of the hetero-expressed novel hydrogenase. Panel B: Measurement of *in vitro* H₂ uptake activity of the hetero-expressed hydrogenase. (-) control (negative control): *T. roseopersicina* GB112131 ($\Delta hynSL$, $\Delta hupSL$ and $\Delta hoxH$) strain; and HynDhSL: GB112131 strain carrying the genes of novel hydrogenase, *hynDhSL*.

$\text{H}_2 \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ protein. In the *in vitro* H₂ uptake assay, we used oxidized benzyl viologen as an artificial electron receptor, and we determined that its H₂-uptake activity was 1.09 μl H₂ $\cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ protein (Figure 3B). All these results demonstrate that we have converted a piece of environmental DNA from ocean water into a functional novel hydrogenase with both H₂-evolution and H₂-uptake activities.

NREL: Our ultimate goal is to construct a cyanobacterial recombinant harboring the O₂-tolerant hydrogenase from *Rubrivivax gelatinosus*. To introduce *R. gelatinosus* casa bonita shrubby hydrogenase genes into the model cyanobacterium *Synechocystis* 6803, we are using a plasmid vector provided by Robert Burnap (Oklahoma State University) that features (1) light-regulated transcription, (2) a spectinomycin antibiotic resistance marker, and (3) neutral-site for homologous recombination. Four structural genes encoding the *Rubrivivax* hydrogenase, *coolXUH*, have been successfully amplified from the *Rubrivivax* genomic

DNA via PCR, and cloned into the pPETE_sigD plasmid by replacing its *sigD* gene (data not shown). Work is underway to transform this plasmid into a *Synechocystis* host which lacks all background hydrogenase activity. Meanwhile, we are developing techniques to verify *Rubrivivax* hydrogenase expression at the protein levels. We have generated antibodies against the *Rubrivivax* hydrogenase small subunit CoolL and the catalytic large subunit CooH via genetic immunization (Strategic Diagnostics, Inc). We then tested CoolL and CooH protein expression in a transgenic *E. coli* transformed with a total of 10 genes: four *Rubrivivax* hydrogenase structural genes (*coolXUH*), and six accessory genes (*hypABCDEF*), in collaboration with Florida International University. As shown in Figure 4, a putative CoolL band and a putative CooH band are clearly visible in western blot demonstrating both protein expression in *E. coli* (bands 1 & 2). The molecular weight of the CooH band was higher than predicted (42 kDa) in transgenic *E. coli* (lane 2), suggesting possible post-translational modification. We also performed western blot in the native *R. gelatinosus* host as a control under conditions where *Rubrivivax* hydrogenase are either un-induced or induced (in carbon monoxide, CO). Lanes 3 and 4 clearly indicate both proteins accumulate upon induction in CO, conforming to their respective predicted molecular weight. Data from Figure 4 thereby verify that the hydrogenase

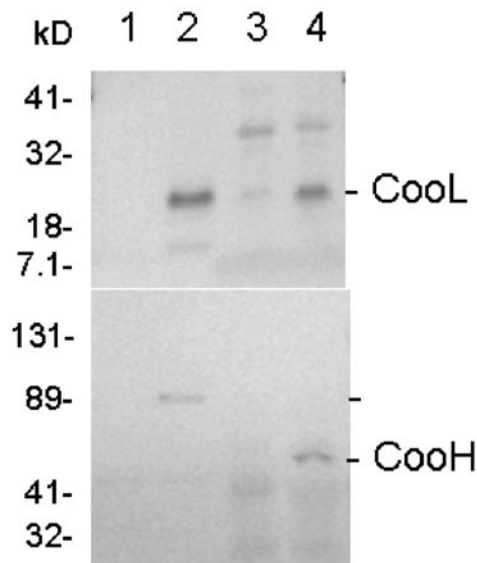


FIGURE 4. Western blot showing the expression of *Rubrivivax* hydrogenase subunits CoolL and CooH in transgenic *E. coli*. Lanes 1. non-transformed *E. coli* host strain; 2. *E. coli* transformed with 10 *R. gelatinosus* hydrogenase genes; 3. non-induced *R. gelatinosus* membrane; and 4. CO-induced *R. gelatinosus* membrane. Note that the CooH band in transgenic *E. coli* (lane 2) migrated more slowly than the native protein (lane 4), suggesting post-translational modification in the foreign host. kD: kilo Daltons.

antibodies are highly specific. The same experiments will be carried out to probe CooL and CooH expression in *Synechocystis* once we obtain a transformant.

Conclusions and Future Directions

JCVI: 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. We succeeded in converting an environmental DNA into a novel functional NiFe-hydrogenase with both H₂-evolution and H₂-uptake activities, which proved the concept that environmental enzymes potentially useful for energy production may be studied in a culture-independent manner. We transferred two structural and four accessory genes of *Thiocapsa* O₂-tolerant hydrogenase into *Synechococcus*. The optimized condition for IPTG-inducible expression of this hydrogenase in the foreign host has been determined. We have demonstrated that the *Thiocapsa* hydrogenase expressed in *Synechococcus* is a membrane-bound hydrogenase.

NREL: The NREL research leads to the conclusion that we have cloned the *Rubrivivax* hydrogenase genes in a plasmid suitable for integration into the genome of *Synechocystis*. We succeeded in generating *Rubrivivax* hydrogenase antibodies for future testing of its expression in the *Synechocystis* host. We have demonstrated that both large and small subunits of the *Rubrivivax* hydrogenase are produced in a transgenic *E. coli* strain.

Our future directions

- Analyze thermal and oxygen stabilities of the novel functional NiFe-hydrogenase that was heterologously expressed in *Thiocapsa* (JCVI).
- Transfer more accessory genes of *Thiocapsa* O₂-tolerant hydrogenase into cyanobacteria to heterologously express a functional hydrogenase in cyanobacteria (JCVI).
- Continue to optimize conditions and test the transformation and expression of *Rubrivivax* hydrogenase in the *Synechocystis* host (NREL).

FY 2008 Publications/Presentations

1. Q. Xu, et al. "Identification of novel NiFe-hydrogenases from the microbial communities in the Sargasso Sea", the 8th International Hydrogenase Conference, Breckenridge, CO, August 5-10, 2007.
2. Q. Xu, et al. "construct a cyanobacterial recombinant for solar H₂ production", Gordon Research Conference on photosynthesis, South Hampton, Massachusetts, June 22-27, 2008.

3. M. L. Ghirardi, A. Dubini, J. P. Yu, P. C. Maness. Natural systems (photosynthesis and hydrogenases), manuscript submitted to Chemical Review (*invited*).
4. P. C. Maness: “The construction of a *Synechocystis* recombinant system for solar H₂ production” 8th International Hydrogenase Conference, Breckenridge, CO, August 5-10, 2007.
5. P. C. Maness: “Biological H₂ production research at NREL”, Postech International Symposium on H₂ energy, Pohang, Korea, November 16, 2007 (*invited*).
6. P. C. Maness: “Biohydrogen from photosynthetic microbes”, 108th American Society for Microbiology Annual Meeting, Boston, MA, June 1-5, 2008 (*Invited*).
7. Q. Xu and P. C. Maness. DOE Annual Hydrogen Program Review Meeting, Washington, D.C., June, 2008. Presentation PD# 32.
8. J. P. Yu: “Heterologous expression of an O₂-tolerant evolving hydrogenase”, Gordon Research Conference on Iron-sulfur enzymes, Colby-Sawyer College, New London, New Hampshire, June 8-13; and Gordon Research Conference on photosynthesis, Mt. Holyoke College, South Hampton, Massachusetts, June 22-27, 2008.