II.F.1 Hydrogen from Water in a Novel Recombinant O_2-Tolerant Cyanobacterial System

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Objectives

- Develop an O_2-tolerant cyanobacterial system for sustained and continuous light-driven H_2-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 O_2-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 O_2-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* O_2-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 O_2-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 H_2 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 H_2. However, one major difficulty is that most hydrogen-evolving hydrogenases are inhibited by O_2, which is an inherent byproduct of oxygenic photosynthesis. The rate of H_2 production is thus limited. Certain photosynthetic bacteria are reported to have an O_2-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 O_2-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 O_2-tolerant bacterial hydrogenase, all within the same microbe. This addresses one of the key technological hurdles to cost-effective photobiological production of H_2, overcoming the sensitivity of the hydrogenase enzyme to O_2, 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 H_2 from water. To achieve this goal, we use the following two approaches. The first approach is to transfer known O_2-tolerant hydrogenases from anoxygenic photosynthetic bacteria *Thiocapsa* and *Rubrivivax* to cyanobacteria. Since only a very limited number of O_2-tolerant hydrogenase is available, our second approach is to identify novel O_2-tolerant hydrogenases from environmental microbial communities and transfer them into cyanobacteria.

Results

JCVI Part I. Transferring O_2-tolerant Hydrogenase Genes from Thiocapsa into Cyanobacteria. Our goal for this part of the work is to transfer the O_2-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 uM. Our Western blotting result demonstrates that 20 uM 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* O_2-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).

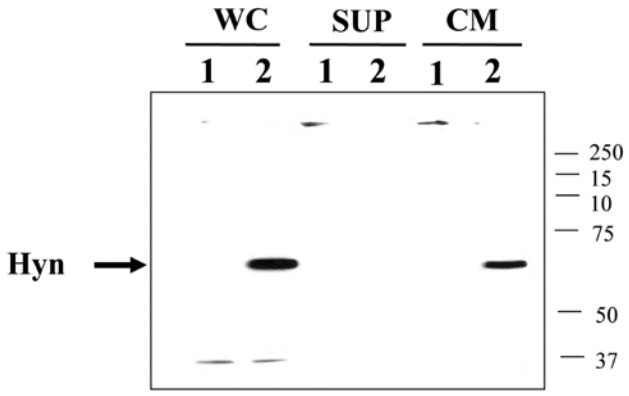

FIGURE 2. Examination of the location of heterologously-expressed *Thiocapsa* O_2-tolerant hydrogenase in the recombinant *S. sp* PCC7942 strain. Strains used are: **1.** S*. sp* PCC7942 WT and 2**.** recombinant cyanobacterium *S. sp* PCC7942 Hyn3 strain. WC: whole cell extract; SUP: supernatant; and 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.

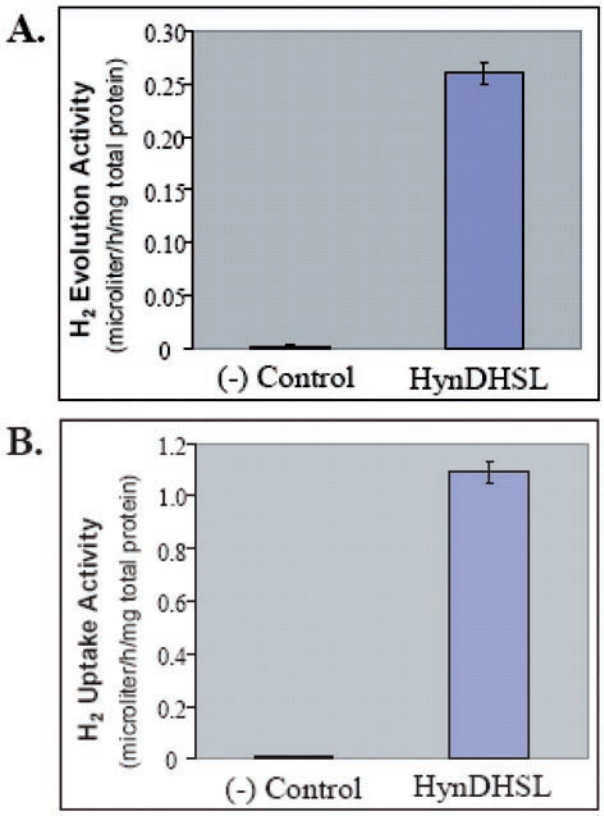

FIGURE 3. Detection of the hydrogenase activities of hetero-expressed novel hydrogenase under *in vitro conditions.* Panel A: Measurement of *in vitro* H_2 evolution activity of the hetero-expressed novel hydrogenase. Panel B: Measurement of *in vitro* H_2 uptake activity of the hetero-expressed hydrogenase. (-) control (negative control): *T. roseopersicina* GB112131 (Δ*hynSL,* Δ*hupSL* and Δ*hoxH*) strain; and HynDHSL: GB112131 strain carrying the genes of novel hydrogenase, *hynDHSL.*

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 O_2-tolerant Hydrogenases from Marine Microbes in the Global Ocean. The goal for this part of the work is to identify novel O_2-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* O_2-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 H_2-evolution and H_2-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 H_2 evolution assay, and H_2 produced was measured by gas chromatograph. The result (Figure 3A) shows that the calculated H_2 evolution activity of the novel hydrogenase is 0.26 μl H_2*h^{-1}*mg^{-1} protein. In the *in vitro* H_2 uptake assay, we used oxidized benzyl viologen as an artificial electron receptor, and we determined that its H_2-uptake activity was 1.09 μl H_2*h^{-1}*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 H_2-evolution and H_2-uptake activities.

NREL: Our ultimate goal is to construct a cyanobacterial recombinant harboring the O_2-tolerant hydrogenase from *Rubrivivax gelatinosus*. To introduce *R. gelatinosus* casa bonita shrubbery 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 CooL and the catalytic large subunit CooH via genetic immunization (Strategic Diagnostics, Inc). We then tested CooL 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 CooL 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 antibodies are highly specific. The same experiments will be carried out to probe CooL and CooH expression in *Synechocystis* once we obtain a transformant.

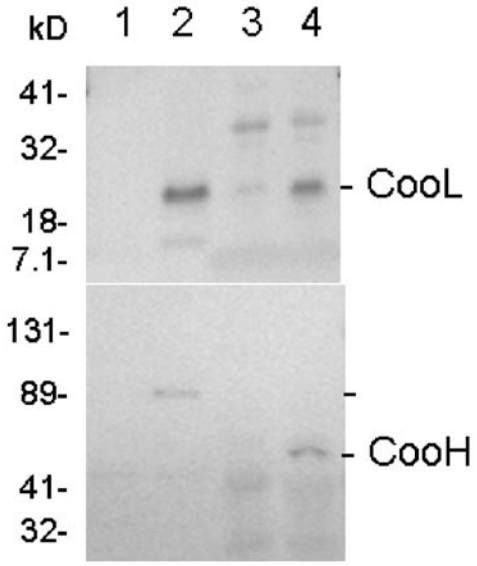

FIGURE 4. Western blot showing the expression of *Rubrivivax* hydrogenase subunits CooL and CooH in transgenic *E. coli.* Lanes 1. non-transformed *E. coli* host strain; 2. *E. coli* transformed with 10 *R. gelatinosus* hydrogeanse genes;3. non-induced *R. gelatinosus* membrane; and 4. CO-induced *R. 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.

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 H_2-evolution and H_2-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* O_2-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* O_2-tolerant hydrogenase into cyanobacteria to heterologouly 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 H_2 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 H_2 production" 8th International Hydrogenase Conference, Breckenridge, CO, August 5-10, 2007.

5. P. C. Maness: "Biological H_2 production research at NREL", Postech International Symposium on H_2 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 O_2-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.