II.H.4 Hydrogen from Water in a Novel Recombinant Oxygen-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 (3.1.4) of the Fuel Cell 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

JCVI

- Transferred the genes of the *Thiocapsa roseopersicina* stable hydrogenase to *Synechococcus elongatus* PCC 7942 and detected activity from the heterologously-expressed hydrogenase.
- Cloned a novel NiFe-hydrogenase from Sargasso Sea environmental deoxyribonucleic acid (DNA), expressed it in *T. roseopersicina*, and detected activity from the purified protein in 1% oxygen.
- Transferred the genes of this novel environmental hydrogenase into *S. elongatus*, and detected activity from the heterologous hydrogenase.

NREL

- Cloned the O_2-tolerant *Rubrivivax gelatinosus* hydrogenase genes in two plasmids suitable for expression in *Synechocystis* sp. PCC 6803. One plasmid will integrate *Rubrivivax* genes into the genome of *Synechocystis* via the homologous recombination mechanism. Genes cloned into the second plasmid will be expressed off the plasmid. Expression from both plasmids is driven by a native promoter in *Synechocystis*.
- Protein western blots revealed the expression of three *Rubrivivax* hydrogenase subunits using the above two expression systems.

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 propose 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 roseopersicina* and *Rubrivivax gelatinosus* to cyanobacteria. Since only a very limited number of O_2-tolerant hydrogenases are available, our second approach is to identify novel O_2-tolerant hydrogenases from environmental microbial communities and transfer them into cyanobacteria.

Results

JCIV: Expression of HynSL hydrogenase from *Thiocapsa roseopersicina* in Cyanobacteria

We have introduced the O_2-tolerant hydrogenase from *T. roseopersicina* into cyanobacteria along with various combinations of accessory genes. These constructs were introduced into a mutant strain of *Synechococcus elongatus* PCC 7942 (Hoxmut) which lacks the HoxYH hydrogenase and has no native hydrogenase activity. The *T. roseopersicina* hydrogenase structural genes, *hynSL*, were introduced into Hoxmut along with the known *T. roseopersicina* accessory genes in plasmids pHyn4, pHyn5, and pHup (Figure 1). Proteins encoded by the accessory genes are necessary to assemble a functional active site for NiFe hydrogenases. While not all accessory genes are known for *T. roseopersicina* because the genome has not been sequenced, the available 16 genes were co-expressed in cyanobacteria to determine whether a hydrogenase with a functional active site could be assembled.

The *T. roseopersicina* hydrogenase was expressed upon isopropyl β-D-1-thiogalactopyranoside (IPTG) induction in the cyanobacterial strains (Figure 2), but no active hydrogenase was produced (Figure 3). However, when the accessory genes from a novel environmental hydrogenase (construct Wav10 described in the following) were co-expressed with the *T. roseopersicina* genes in Hyn4 or Hyn5, a *T. roseopersicina* hydrogenase with a fully assembled active site was produced and possessed activity upon *in vitro* assay (Figure 3).

Identifying Novel O_2-Tolerant Hydrogenases from Marine Microbes and Expression in Cyanobacteria

We have previously identified sequences of large and small NiFe hydrogenase subunits from the Global Ocean Sampling expedition. One of these environmentally-derived NiFe hydrogenase sequences was expressed in *T. roseopersicina* and found to encode a functional hydrogenase. When the genes encoding the small and large subunits of the hydrogenase and the adjacent accessory genes were transferred to the Hoxmut strain of the cyanobacterium *S. elongatus* (strain RC41, Figure 2), an active hydrogenase expressed (Figure 3). The heterologously-expressed hydrogenase possessed roughly

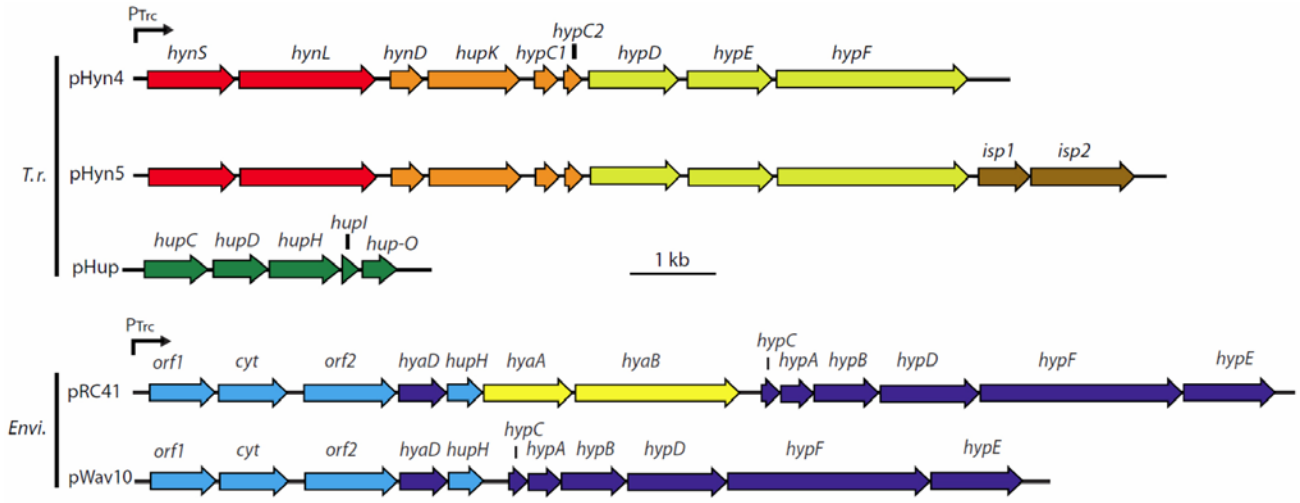

FIGURE 1. Constructs used to express *T. roseopersicina* (T. r.) and environmental (Envi) hydrogenases in cyanobacteria.

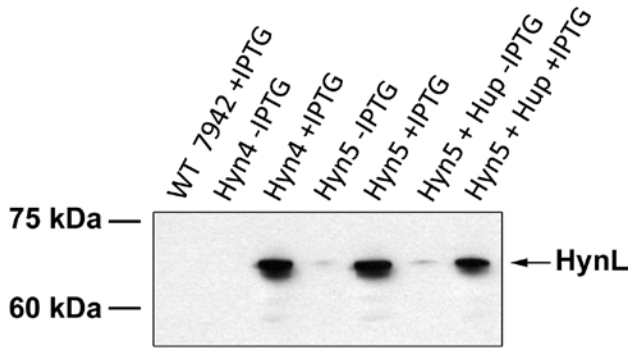

FIGURE 2. Expression of *T. roseopersicina* hydrogenase in cyanobacteria. Proteins from wild-type and HynSL-expressing strains were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blots were performed using antiserum raised against HynL.

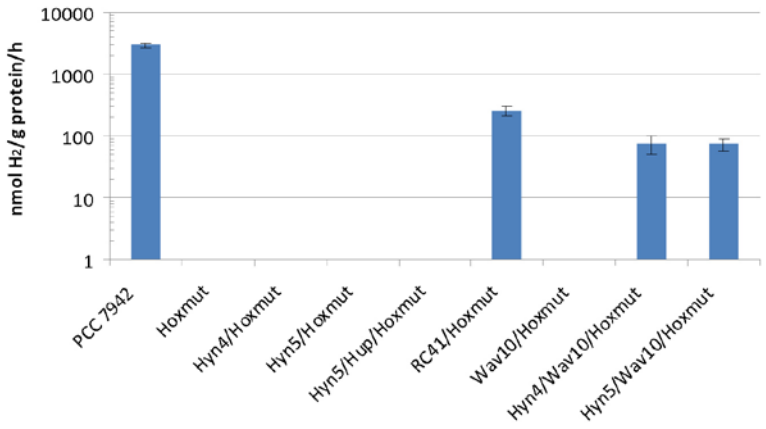

FIGURE 3. In vitro H_2 evolution assays were performed on crude cell extracts from cyanobacterial strains containing *T. roseopersicina* constructs (Hyn4, Hyn5, Hyn5/Hup) or constructs Hyn4 and Hyn5 in combination with the environmental hydrogenase accessory gene construct (Wav10). Cells were induced with isopropyl ß-D-1-thiogalactopyranoside, lysed, and assays were performed under Ar atmosphere using the synthetic electron donor methyl viologen.

one-tenth the activity of the native cyanobacterial hydrogenase (Figure 3). With the *T. roseopersicina* HynSL hydrogenase described above, this is the first report describing heterologous expression of active NiFe hydrogenases in cyanobacteria.

Purifying Hydrogenases Expressed in *T. roseopersicina* and Testing O_2-Tolerance

We have previously described purification of the *T. roseopersicina* HynSL hydrogenase. Using a similar technique, we have also purified the environmental hydrogenase expressed in a *T. roseopersicina* host. We then determined whether the purified enzymes could maintain activity in the presence of O_2 using the hydrogen-deuterium exchange assay. The environmentally-derived hydrogenase maintained 20% activity in 1% O_2, 5% activity in 3% O_2, and did not possess activity at 5% O_2. This indicates that the novel, environmental hydrogenase is active in the presence of O_2.

NREL: The goal for this part of the work is to construct a cyanobacterial recombinant harboring the O_2-tolerant hydrogenase from *Rubrivivax gelatinosus*, using *Synechocystis* sp. PCC 6803 as the model host. We used two plasmids to accomplish this goal. Plasmid pPETE_sigD was provided by Prof. Robert Burnap (Oklahoma State University) which enables light-regulated gene expression and homologous recombination. We cloned four structural genes *cooLXUH* encoding the *Rubrivivax* O_2-tolerant hydrogenase into the pPETE_sigD plasmid followed by its transformation into a *Synechocystis* mutant host lacking background hydrogenase activity (Hox^-; provided by Prof. Teruo Ogawa). Protein western blot confirmed the expression of *Rubrivivax* hydrogenase subunits CooH (catalytic subunit), CooL (small subunit), and CooU (unknown function) in one of the three LXUH transgenic lines tested (Figure 4A). Expression of CooX is likely but not yet confirmed due to poor quality of its antibody. Since the transfer of up to 12 genes is likely needed to express a functional *Rubrivivax* hydrogenase, we employed a broad host range plasmid pRL1342 (provided by Prof. Peter Wolk, Michigan State University) to express additional genes. We constructed and transformed in the *Synechocystis* Hox^- strain the plasmid carrying codon-optimized *Rubrivivax cooL* gene alone (pRL1342_L), or *cooL* as well as the *hypA* (hydrogenase maturation; pRL1342_LA) genes. As shown in Figure 4B, CooL protein is detected in both transgenic lines, demonstrating the utility of this broad host range plasmid in expressing foreign genes in *Synechocystis*. It is likely that the HypA protein was also expressed in the +LA line, which will be confirmed once we have the HypA-specific antibody.

Conclusions

JCVI: We have heterologously expressed two NiFe hydrogenases in cyanobacteria, one from *T. roseopersicina* and the other cloned from environmental DNA, and demonstrated that they are expressed with fully assembled and functional active sites. We have also purified these hydrogenases and verified that the environmentally-derived hydrogenase

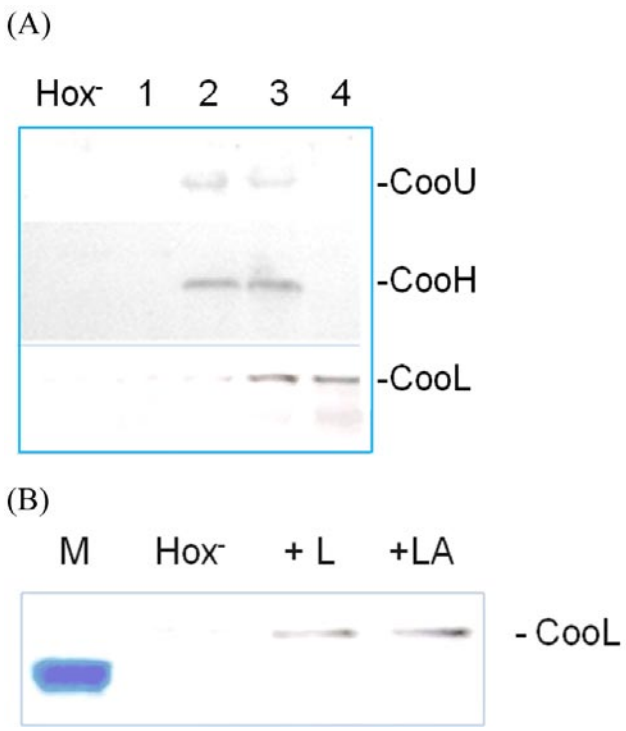

FIGURE 4. (A) Protein western blot showing the co-expression of the *Rubrivivax gelatinosus* hydrogenase subunits CooL, CooH, and CooU in a transgenic line (#3) of *Synechocystis* host (Hox$^-$). (B) Protein western blot showing the expression of *Rubrivivax* hydrogenase subunit CooL in both Hox$^-$transgenic lines transformed with the pRL1342 plasmid harboring either *cooL* (+L) or *cooL* and *hypA* (+LA) genes. M: molecular weight marker. *Synechocystis* Hox$^-$ served as the untransformed control.

maintains activity in the simultaneous presence of up to 3% O_2. This work demonstrates that novel hydrogenases with potentially useful properties can be isolated from environmental DNA.

NREL: The NREL research confirmed the expression in *Synechocystis* of three genes, via homologous recombination, encoding subunits of the O_2-tolerant hydrogenase from *Rubrivivax*. Moreover, we also detected the expression of a *Rubrivivax* hydrogenase subunit via a plasmid-based approach in *Synechocystis* which could be used to express additional genes for building the recombinant.

Future Directions

- Re-engineer plasmid constructs and demonstrate increased expression of hydrogenase in vitro (JCVI).
- Test electron mediator requirement of hydrogenase expressed in cyanobacteria strains with increased hydrogenase expression (JCVI).
- Optimize conditions and transfer additional hydrogenase structural and maturation genes of *Rubrivivax* to express a functional O_2-tolerant hydrogenase in *Synechocystis* (NREL).

FY 2010 Publications/Presentations

1. Ghirardi, M.L., S.N. Kosourov, P. C. Maness, S. Smolinski, and M. Seibert. (2009). "Hydrogen Production, algal." Wiley Encyclopedia of Industrial Biotechnol.

2. Eckert, C., A. Dubini, J. Yu, P. King, M. Ghirardi, M. Seibert, and P.C. Maness. 2010. Hydrogenase genes and enzymes involved in solar hydrogen production. Accepted for publication. *In* the book "State of the art and progress in production of biohydrogen". Bentham Science Publishers.

3. Maróti, G., Y. Tong, S. Yooseph, H. Baden-Tillson, H.O. Smith, K.L. Kovács, M. Frazier, J.C. Venter, and Q. Xu. (2009). Discovery of a [NiFe]-hydrogenase in Metagenomic Sargasso Sea DNA: Cloning and Functional Study in *Thiocapsa roseopersicina*. Appl. Environ. Microbiol. 75: 5821-5830.

4. Vanzin, G., J.P. Yu, S. Smolinski, V. Tek, G. Pennington, and P.C. Maness. (2010). Characterization of genes responsible for the CO-linked hydrogen production pathway in *Rubrivivax gelatinosus*. Appl. Environ. Microbiol. 76: 3715-3722.

5. Vargas, W.A., P.D. Weyman, Y. Tong, H.O. Smith, and Q. Xu. A NiFe-Hydrogenase from *Alteromonas macleodii* with Unusual Stability in the Presence of Oxygen and High Temperature. (Appl. Environ. Microbiol., Submitted).

6. Weyman, P.D., W.A. Vargas, R. Chung, H.O. Smith, and Q. Xu. Heterologous Expression of a Novel NiFe-Hydrogenase in *E. coli.* (J. Bacteriol., Submitted).

7. Discovery of Novel O_2-tolerant NiFe-hydrogenases from environmental microbes to construct a cyanobacterial recombinant for solar H_2 production. 13th International Symposium on Phototrophic Prokaryotes, Montreal, Canada, August 9–14th, 2009. (Q. Xu).

8. Photobiological hydrogen production in the cyanobacterium *Synechocystis* sp. PCC 6803. 13th International Symposium on Phototrophic Prokaryotes. Montreal, Canada, August 9–14th, 2009 (Maness, P. C).

9. Genetic engineering of *Synechocystis* sp. PCC 6803 for sustained hydrogen production. Poster presentation. 19th Western Photosynthesis Conference, Pacific Grove, CA. January 7–10, 2010 (P. C. Maness).

10. Expression of oxygen-tolerant hydrogenases in *Synechococcus elongatus*. 10th Cyanobacterial Molecular Biology Workshop. June 11-15, 2010, Lake Arrowhead, CA. (P. D. Weyman).

11. 2010 DOE Hydrogen Program Review – Washington, D.C., June 2010, Oral Presentation PD039 (P. D. Weyman and P. C. Maness).