II.H.3 Hydrogen from Water in a Novel Recombinant Oxygen-Tolerant Cyanobacterial System

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Fiscal Year (FY) 2011 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

FY 2011 Accomplishments

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- We achieved higher hydrogenase activity from the environmentally derived hydrogenase expressed in cyanobacteria by adding additional transcriptional regulation to the gene cluster.
- The cyanobacterial ferredoxin was found to serve as an electron mediator to the environmentally derived hydrogenase.

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- We developed genetic tools to manipulate the genome of *Rubrivivax gelatinosus* CBS and generated an affinity-tagged hydrogenase active in hydrogen production. This development will simplify hydrogenase purification for characterization in the long run.
- The *Rubrivivax* hydrogenase was expressed and purified from a *Synechocystis* host. The recombinant hydrogenase was shown to contain all four subunits (CooLXUH) forming a stable complex during aerobic growth of *Synechocystis*, albeit with no activity.
- Four *Rubrivivax* NiFe-hydrogenase maturation genes (*hypFCDE*) were transferred to *Synechocystis*, and HypE protein expression was confirmed. Expression of the maturation genes is a prerequisite for assembly of an active *Rubrivivax* NiFe-hydrogenase in *Synechocystis*.

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, using sunlight energy to extract reducing equivalents from water. The resulting reducing equivalents can be fed 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, and require other more expensive feedstocks.

To overcome these difficulties, we propose to construct novel microbial hybrids by genetically transferring O_2-tolerant hydrogenases 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. By overcoming the sensitivity of the hydrogenase enzyme to O_2, we address one of the key technological hurdles to cost-effective photobiological H_2 production which currently limits the production of hydrogen in 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

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Previously, we reported the successful expression in cyanobacteria of active NiFe hydrogenases. These NiFe hydrogenases included the stable hydrogenase from *Thiocapsa roseopersicina* and a novel, environmentally-derived NiFe hydrogenase, HynSL, (previously named HyaAB). Although active hydrogenases were obtained indicating that all the required accessory proteins were expressed, the activity was low. We hypothesized that improved plasmid design may increase activity. The original expression plasmid, pRC41, expressed the environmentally-derived hydrogenase under the regulation of one promoter at the beginning of the 13 gene construct (Figure 1a). With such a long transcript (~13-kb), genes encoded at the end of the operon, such as the *hyp* genes, may not be expressed at sufficiently high levels to allow for maximal activity of the environmental hydrogenase in cyanobacteria. These genes at the end of the operon are required for maturation of the hydrogenase and must be transcribed at the optimal level.

In order to achieve higher expression throughout the gene cluster, we have re-engineered the expression plasmid to create pRC41-4 with an additional three promoters spaced throughout the operon (Figure 1a). The redesigned plasmid is also smaller after removing genes that we found did not contribute to hydrogenase maturation or function. Each new promoter sequence is preceded by a terminator sequence to create shorter transcripts that may be more stable.

When the plasmids were transferred to an *E. coli* strain lacking its native hydrogenases, strains containing the redesigned plasmid, pRC41-4, and the single promoter control, pIY003, produced hydrogenase activity at similar levels in *E. coli* (Figure 1b). These plasmids were then successfully transferred to the cyanobacterium *S. elongatus* PCC 7942, and hydrogenase activity was measured (Figure 1c). Strain RC41-4 had approximately three-fold higher hydrogenase activity than the RC41 strain. We are currently working to increase this expression through further transcriptional modification.

To test whether HynSL could accept electrons from the native cyanobacterial ferredoxin, PetF, we obtained purified PetF from our collaborator at NREL and used it in place of methyl viologen in our *in vitro* hydrogenase assays. We tested extracts from our HynSL-expressing cyanobacterial strains in assays with PetF, but because of low HynSL expression levels, no hydrogen was detected (data not shown). However, when we used extracts from an *E. coli* strain expressing HynSL at a much higher level than the cyanobacterial strains, we were able to detect hydrogen produced via the PetF electron mediator (Table 1). This result provides evidence that the cyanobacterial ferredoxin can serve as an electron mediator for hydrogen production in strains over-expressing HynSL.

TABLE 1. Cyanobacterial Ferredoxin (PetF) can act as an electron mediator for the novel environmental hydrogenase, HynSL, expressed in *E. coli.*

E. coli Strain	Treatment	nmole H_2/mg protein/ h
Empty Vector	Dithionite + PetF	0.00
pRC41	Dithionite	0.04
pRC41	Dithionite + PetF	0.16

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The overarching goal for the NREL work is to construct a cyanobacterial recombinant harboring the O_2-tolerant hydrogenase from *Rubrivivax gelatinosus,* using *Synechocystis* sp. PCC 6803 as a model host. A prerequisite for success is to gain better understanding of the *Rubrivivax* hydrogenase and its underlying maturation machinery to ensure transfer of the correct genes into *Synechocystis*. As such, we successfully developed genetic tools to manipulate the genome of *Rubrivivax*. We generated an affinity-tagged *Rubrivivax* hydrogenase with hydrogen production activity similar to the wild type enzyme (Figure 2a), and protein immunoblot confirmed expression (Figure 2b). This outcome could simplify hydrogenase purification for characterization and for comparison to the heterologously expressed *Rubrivivax* hydrogenase in *Synechocystis*. Using these genetic tools, we also successfully generated a *Rubrivivax* mutant lacking the *hypE* gene, which is putatively involved in the assembly of the *Rubrivivax* hydrogenase. However, the mutant displayed a wild-type level of hydrogenase activity, suggesting the presence of

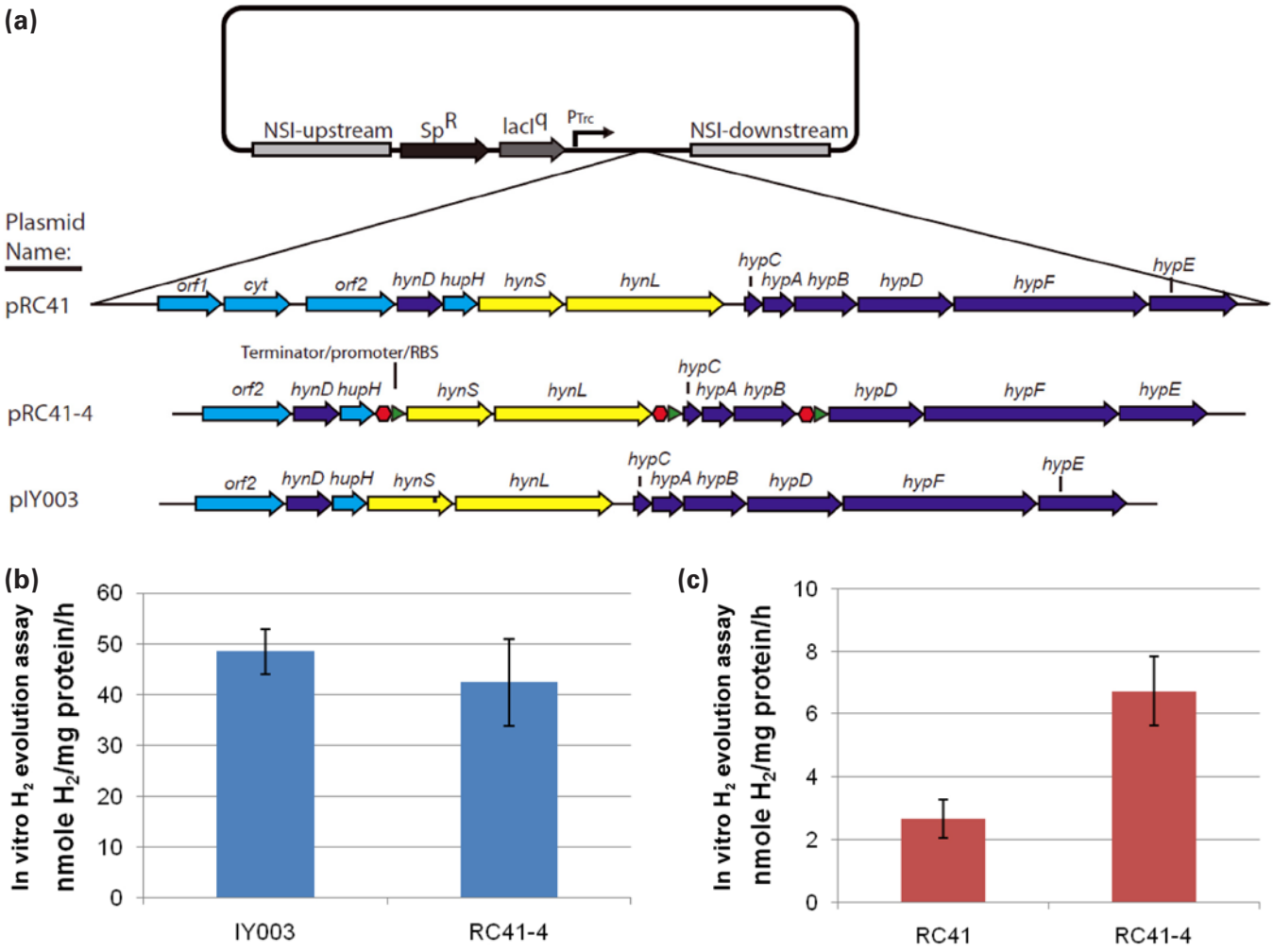

FIGURE 1. Re-engineered plasmids to express the environmentaly derived hydrogenase HynSL. (a) The map of the original expression plasmids, pRC41, and modified plasmids, pIY003 and pRC41-4. Colors depict hydrogenase structural genes, HynSL (yellow), genes encoding essential maturation factors (dark blue), non-essential factors (light blue). Plasmids pIY003 and pRC41-4 are similar to pRC41 but is shortened by two genes. Plasmid pRC41-4 also has three additional terminators (red octagons) and promoters (green arrows). (b) *In vitro* H_2 evolution assay from *E. coli* strains expressing the environmental hydrogenase from strains containing plasmid pIY003 and pRC41-4. (c) *In vitro* H_2 evolution assay from cyanobacterial strains expressing the environmental hydrogenase from strains RC41 and RC41-4.

multiple copies of the *hypE* gene (data not shown). Work is underway to sequence the genome of *Rubrivivax*, which will guide construction of the recombinant *Synechocystis*.

Working toward building the cyanobacterial recombinant, four genes (*cooLXUH*) encoding the multi-subunit *Rubrivivax* O_2-tolerant hydrogenase have been transformed in *Synechocystis*. The recombinant hydrogenase was purified, and protein immunoblot confirmed that all four subunits were expressed and formed a complex during aerobic growth of *Synechocystis*, albeit with no activity (Figure 3). This finding prompted us to additionally express the hydrogenase maturation genes, *hypABCDEF*, which are likely involved in the assembly and maturation of the *Rubrivivax* hydrogenase. We generated a construct containing the *Rubrivivax hypFCDE* genes and demonstrated integration of *hypFCDE* into the genome of *Synechocystis*. Protein immunoblot confirmed the expression of the HypE protein, suggesting the upstream HypFCD proteins are also expressed. However, no hydrogenase activity was detected in the *Synechocystis* recombinant line (already harboring the *cooLXUH*) lacking its native hydrogenase. The expression of *hypAB* is underway.

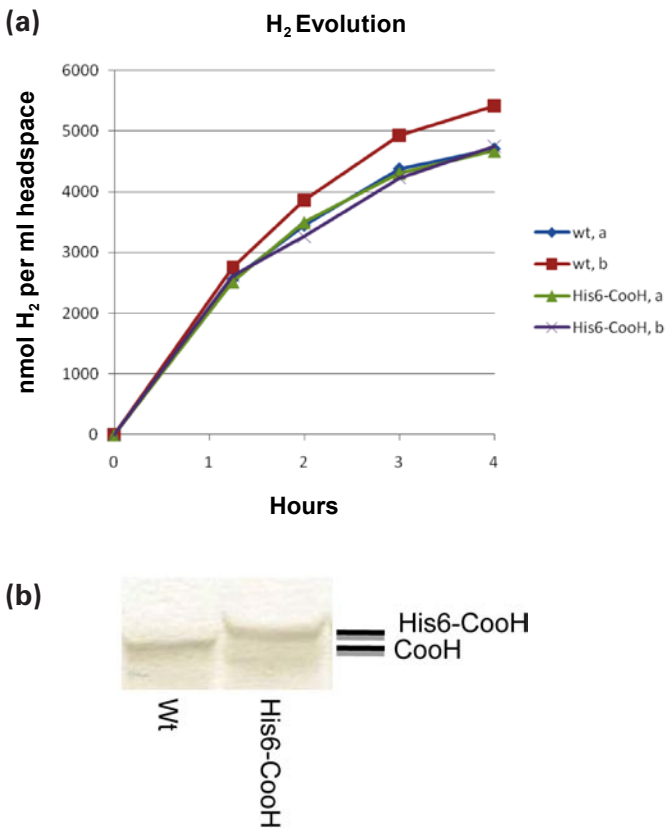

FIGURE 2. Hydrogen production in wild-type and affinity-tagged hydrogenase in *Rubrivivax gelatinosus* CBS. (a) wild-type and mutant *Rubrivivax* expressing His6-CooH were incubated with CO at time zero, and hydrogen evolution was monitored over time. (b) Protein immunoblot revealed that only the affinity-tagged version of the hydrogenase (His6-CooH) was expressed in the mutant cell line.

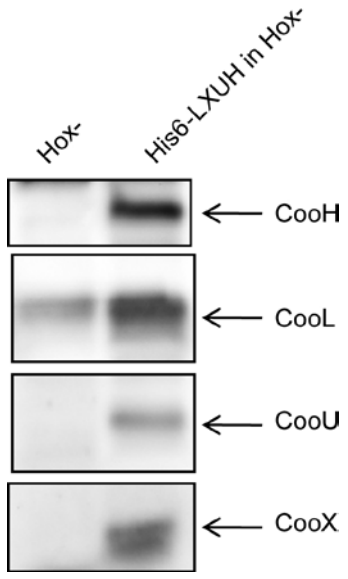

FIGURE 3. Protein immunoblot reveals expression of *Rubrivivax gelatinosus* CBS hydrogenase structural genes in a *Synechocystis* host.

Conclusions and Future Directions

Conclusions

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- Increased activity of the environmentally-derived hydrogenase can be achieved by modifying the transcriptional regulation of the gene operon.
- Cyanobacterial ferredoxin can act as an electron mediator to the environmentally derived hydrogenase.

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- Using the genetic tools developed in *Rubrivivax*, we generated an active affinity-tagged hydrogenase to facilitate purification for characterization. Using these tools, we also successfully generated a *hypE* knock-out mutant in *Rubrivivax* to probe HypE function. However, the mutant displayed a wild-type level of hydrogenase activity, suggesting the presence of multiple copies of this maturation gene.
- Four *Rubrivivax* hydrogenase maturation genes (*hypFCDE*) were incorporated into the genome of a transgenic line of *Synechocystis* already harboring the *Rubrivivax* hydrogenase *cooLXUH* genes. We confirmed expression of HypE protein, albeit with no hydrogenase activity. Nevertheless, this *Synechocystis* strain will serve as the platform to express additional *Rubrivivax* hydrogenase maturation genes.

Future Directions

- We will continue to modify the environmentally-derived hydrogenase cluster to increase hydrogenase activity in cyanobacteria (JCVI).
- We will identify additional hydrogenase maturation genes using the sequenced genome of *Rubrivivax*. Once confirmed, these maturation genes of *Rubrivivax* will be transferred to express a functional O_2-tolerant hydrogenase in *Synechocystis* (NREL).
- We will optimize growth conditions to maximize the expression of the heterologous hydrogenase in *Synechocystis* (NREL).

FY 2011 Publications/Presentations

1. Carrieri, D., K. Wawrousek, C. Eckert, J. Yu, and P.C. Maness. 2011. The role of the bidirectional hydrogenase in cyanobacteria. Biores. Technol. In press.

2. 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.

3. Weyman, P.D., W.A. Vargas, R.Y. Chung, Y. Chang, H.O. Smith, and Q. Xu. 2011. Heterologous Expression of a Novel NiFe-Hydrogenase in E. coli. J. Bacteriol. 157: 1363-74.

4. Vargas, W.A., P.D. Weyman, Y. Tong, H.O. Smith, Q. Xu. 2011. A [NiFe]-hydrogenase from *Alteromonas macleodii* with unusual stability in the presence of oxygen and high temperature. Appl. Environ. Microbiol. 77: 1990-1998.

5. Weyman P.D., W.A. Vargas, Y. Tong, J. Yu, P.C. Maness, H.O. Smith, Q. Xu. 2011. Heterologous Expression of *Alteromonas macleodii* and *Thiocapsa roseopersicina* [NiFe] Hydrogenases in *Synechococcus elongatus*. PloS One. 6:e20126.

6. "The CO oxidation and H_2 production system in the photosynthetic bacterium *Rubrivivax gelatinosus* CBS." Gordon Research Conference, Molecular Basis of One-carbon Metabolism. August 1–6, 2010 (K. Wawrousek).

7. "The CO oxidation and H_2 production system in the photosynthetic bacterium *Rubrivivax gelatinosus* CBS." The 15th International Congress on Photosynthesis. August 22–27, 2010. Beijing, China (J. Yu).

8. "Hydrogen production by *Rubrivivax gelatinosus* CBS Coo hydrogenase." 20th Western Photosynthesis Conference, Pacific Grove, CA. January 9, 2011 (K. Wawrousek).

9. "Expression of oxygen-tolerant hydrogenases in *Synechococcus elongatus*". Molecular Bioenergetics of Cyanobacteria: From Cells to Community. April 10–15, 2011, San Feliu de Guixols, Spain. (P.D. Weyman).

10. 2011 DOE Hydrogen Program Annual Merit Review – Washington, DC, May 2011, Oral Presentation PD039 (P.D. Weyman and P.C. Maness).