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

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

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Current Status</th>
<th>2011 Target</th>
<th>2018 Target</th>
</tr>
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<tbody>
<tr>
<td>Duration of continuous H$_2$ photoproduction in air</td>
<td>Zero to 30 seconds in air</td>
<td>Produce a cyanobacterial recombinant evolving H$_2$ through an O$_2$-tolerant hydrogenase in air for 30 min</td>
<td>H$_2$ production in air for 30 min</td>
</tr>
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</table>

FY 2011 Accomplishments

JCVI

- 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.

NREL

- 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 coupled 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**

**JCVI**

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, pLY003, 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*.

<table>
<thead>
<tr>
<th>E. coli Strain</th>
<th>Treatment</th>
<th>nmole H/mg protein/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty Vector</td>
<td>Dithionite + PetF</td>
<td>0.00</td>
</tr>
<tr>
<td>pRC41</td>
<td>Dithionite</td>
<td>0.04</td>
</tr>
<tr>
<td>pRC41</td>
<td>Dithionite + PetF</td>
<td>0.16</td>
</tr>
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**NREL**

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
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.
Conclusions and Future Directions

Conclusions

JCVI

- 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.

NREL

- 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 knockout 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 O₂-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


