II.D.4 Hydrogen from Water in a Novel Recombinant Oxygen-Tolerant Cyanobacterial System

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Project End Date: January 30, 2014

Overall Objective

Develop an O_2-tolerant cyanobacterial system for sustained and continuous light-driven H_2 production from water.

Fiscal Year (FY) 2013 Objective

Increase activity of environmentally derived hydrogenase by at least five-fold.

Technical Barriers

This project addresses the following technical barriers from the Hydrogen Production section (3.1.5) of the Fuel Cell Technologies Office Multi-Year Research, Development, and Demonstration Plan:

(AP) Oxygen Accumulation

Technical Targets

Barrier AP: O_2 Accumulation	2011 Status	2015 Target	2020 Target
Duration of continuous photoproduction in full sunlight	2 min	30 min	4 h

FY 2013 Accomplishments

- Increased hydrogen evolution activity for our environmentally derived hydrogenase expressed in cyanobacteria by 20-fold compared to our original strain.
- Developed an affinity-tagged purification scheme for use with the environmentally derived hydrogenase.
- Created plasmids with different combinations of cyanobacterial promoters to find optimal levels of transcription of hydrogenase accessory proteins.

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* 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

Previously, we reported the successful expression in cyanobacteria of active, oxygen-tolerant 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 hydrogenase was detected indicating co-expression of all required accessory proteins, the activity of this strain (RC41) was 10-fold lower than the native hydrogenase activity (strain 7942, Figure 1). To increase activity we developed two strategies. The first involved increasing the number of promoters to achieve higher transcription and higher levels of the hydrogenase activity in the cyanobacteria (strain RC41-4, Figure 1). The second strategy involved modification of the amino acid residues predicted to ligate the iron sulfur (FeS) clusters in the small subunit. Each of these strategies resulted in a four-to-five-fold increase in activity. When combined in the same plasmid, hydrogen evolution activity increased by 20-fold (strain CM12, Figure 1).

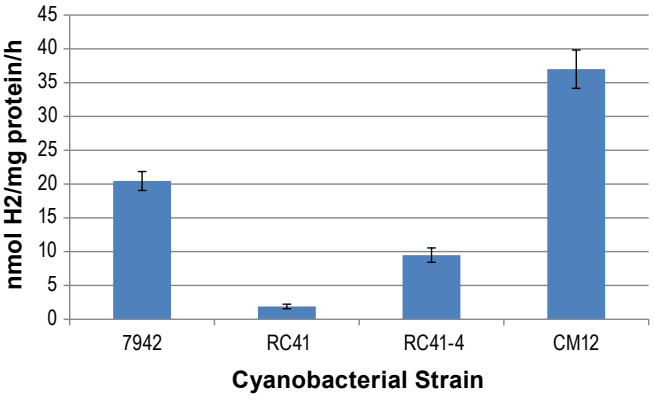

FIGURE 1. Comparison of hydrogenase activity of selected cyanobacterial strains. See text for strain details. Shown is the H_2-evolution activity assayed in an *in vitro* assay using reduced methyl viologen as the electron donor. Error bars describe one standard deviation from the mean of triplicate biological replicates.

Many of our strategies to engineer hydrogenase to function better in cyanobacteria involve substitutions of the amino acid residues composing the hydrogenase. To better study the effect of these substitutions, an easy method of protein purification is desirable so increases in activity can be directly correlated with changes to the protein. To facilitate protein purification, we designed an affinity-tagged version of our hydrogenase using the StrepII tag which binds tightly to the Streptactin chromatography resin. We achieved purification of the hydrogenase with increases in purification of over 100-fold using this technique (Table 1). Because we added the StrepII tag to the end of the hydrogenase small subunit, HynS, it also allowed us to visualize this protein on a western blot (Figure 2). Suprisingly, we did not purify an appreciable amount of the immature HynS, although this protein was observed in the crude extract. The large subunit (HynL) can also be examined using antisera available in our laboratory. As expected, only the mature, proteolytically cleaved version is obtained in the purified fraction (Figure 2).

TABLE 1. Comparison of affinity-tagged versions of the wild type environmentally derived hydrogenase (Construct 1) and the FeS cluster ligation substitution (Construct 2).

	Specific activity (nmole H_2 produced/ mg protein/h)	Fold purification
Construct 1 (crude)	369	
Construct 1 (purified)	41,450	112
Construct 2 (crude)	765	
Construct 2 (purified)	134,214	176

Previous results suggested that there is an optimal level of expression for the accessory proteins that mediate hydrogenase maturation. To find the optimal configuration, we generated 27 versions of our hydrogenase expression plasmids that contain combinations of three different promoters at three different sites. The three promoters have different strengths and include Rbc (low level expression), Trc (intermediate expression), and PsbA (high level expression). We have successfully created all 27 plasmids (Figure 3), and experiments to test activity in cyanobacteria are currently ongoing.

CONCLUSIONS AND FUTURE DIRECTIONS

- Using a combination of two different strategies, we have increased hydrogenase activity by 20-fold compared to our initial published activity levels.
- We have successfully designed a strategy for affinity purification of the hydrogenase and have used this strategy to obtain fractions with greater than 100-fold increased specific activity.

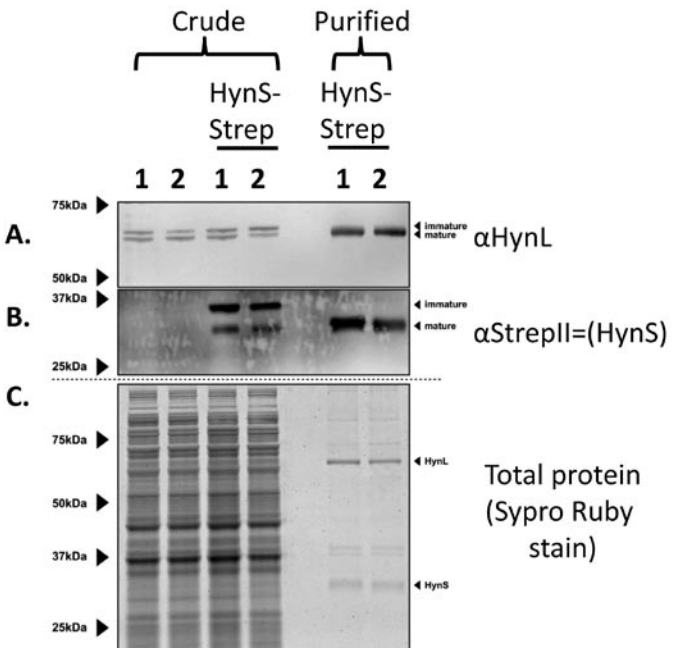

FIGURE 2. Affinity-tagging of HynS and purification of the hydrogenase complex. Samples include untagged and HynS-strep tagged versions of two constructs: the wild type environmentally derived hydrogenase (1) and the FeS cluster ligation mutant (2). A. Western blot with anti-HynL antisera. B. Western blot with anti-StrepII antisera to visualize HynS. C. Total protein visualized with Sypro Ruby.

- We have created a series of plasmids with promoters of different strengths to find the optimal levels of expression of the hydrogenase accessory proteins.

FY 2013 PUBLICATIONS/PRESENTATIONS

1. Weyman, PD. "Using synthetic biology to develop photosynthetic microorganisms into alternative energy factories", Nov. 2012, Swarthmore College. (Invited Speaker)

2. Weyman, PD. "Synthetic Biology Approaches to Develop Algae for Food Production." NASA Synthetic Biology Workshop on Food Production in Space, Ames Research Center, September 2012.

3. Weyman, PD. "An Expanded Role for Synthetic Genomic Approaches in Microbiology." Frontiers in Large Scale Organism and Pathway Engineering. DOE Joint Genome Institute. Walnut Creek, CA. April 29–30, 2013.

4. Weyman, PD. "Hydrogen from water in a novel recombinant oxygen-tolerant cyanobacterial system", EERE Fuel Cell Technologies Office, Annual Merit Review. May 17, 2013, Arlington, VA.

5. Yonemoto IY, Materri CM, Nguyen TA, Smith HO, Weyman PD. Dual organism design cycle reveals small subunit substitutions that improve [NiFe] hydrogenase hydrogen evolution. *Journal of Biological Engineering (*2013) 7:17.

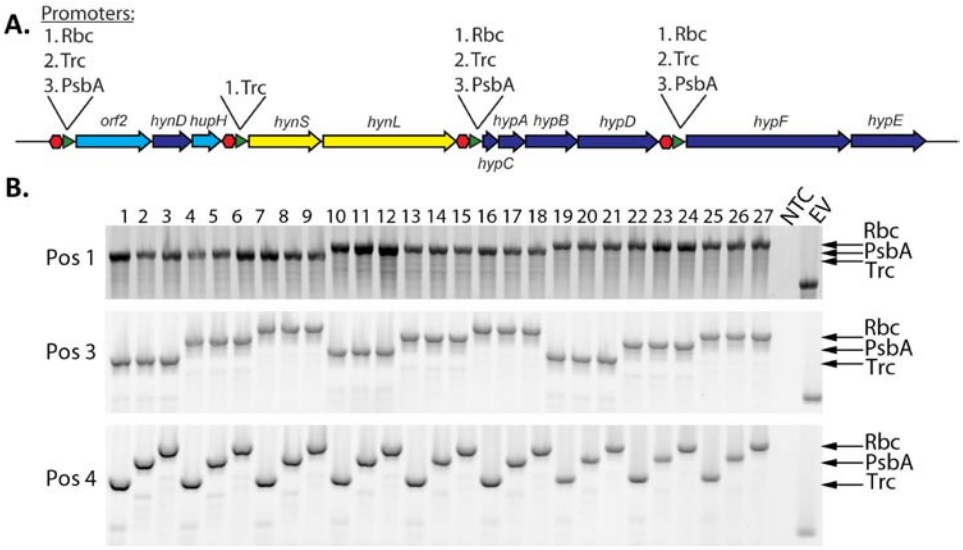

FIGURE 3. Promoter-modification plasmid design and confirmation. A. Three different promoters (Rbc, Trc, and PsbA) were inserted in all possible combinations at three different sites (positions 1, 3, and 4). B. Polymerase chain reaction results confirming correct assembly of the plasmids.