II.E.2 Improving Cyanobacterial O_2-Tolerance Using CBS Hydrogenase for H_2 Production

Pin-Ching Maness (Primary Contact), Carrie Eckert, and Jianping Yu

National Renewable Energy Laboratory (NREL)
15013 Denver West Parkway
Golden, CO 80401
Phone: (303) 381-6114
Email: pinching.maness@nrel.gov

DOE Manager

Katie Randolph
Phone: (720) 356-1759
Email: Katie.Randolph@ee.doe.gov

Project Start Date: May 1, 2005
Project End Date: Continuation and direction determined annually by DOE

Overall Objectives

- Decipher the maturation machinery of the O_2-tolerant hydrogenase in *Rubrivivax gelatinosus* to transfer the correct number of genes to build a cyanobacterial recombinant
- Construct a cyanobacterial recombinant by expressing four hydrogenase genes and six maturation genes from *R. gelatinosus* for sustained H_2 production
- Demonstrate H_2 production in the cyanobacterial recombinant during photosynthesis for photolytic H_2 production

Fiscal Year (FY) 2015 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 barrier from the Hydrogen Production section (3.1.4) of the Fuel Cell Technologies Office Multi-Year Research, Development, and Demonstration Plan

(AP) Oxygen Accumulation

TABLE 1. Technical Targets

Characteristics	Units	2011 Target	2015 Target	2020 Target	Ultimate Target
Duration of continuous H_2 production at full sunlight intensity	Time units	2 min	30 min	4 h	8 h

FY 2015 Accomplishments

- *Rubrivivax gelatinosus* CBS (hereafter CBS) contains two sets of maturation genes, *hyp1* and *hyp2* with unknown roles. We have generated CBS mutants lacking either or both *hyp1/hyp2*. Phenotypical analysis revealed that *hyp1* is essential for assembling the O_2-tolerant, H_2-evolving hydrogenase and *hyp2* is for the H_2-oxidizing uptake hydrogenase. The findings confirm that only *hyp1* genes (*hyp1FCDEAB*) will be genetically transferred into *Synechocystis* along with the hydrogenase genes to assemble the O_2-tolerant CBS hydrogenase.
- To ensure balanced protein expression between CBS and recombinant *Synechocystis*, we refactored the promoter/ribosome binding site (RBS) constructs and detected enhanced expression of most CBS proteins (CooLUH and HypBCE) in *Synechocystis* based on the available antibodies in hand. The outcomes serve as the first step toward active CBS hydrogenase activity in *Synechocystis*.

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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 solar energy to extract reducing equivalents (electrons) from water. The resulting reducing equivalents can be fed to a hydrogenase system yielding H_2. However, one major barrier is that most hydrogen-evolving hydrogenases are inhibited by O_2, which is an inherent byproduct of oxygenic photosynthesis. The rate and duration of H_2 production is thus limited. Certain photosynthetic bacteria are reported to have an O_2-tolerant, H_2-evolving hydrogenase, yet these microbes do not split water and require other more expensive feedstock.

To overcome these technical barriers, 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 (Barrier AP) to cost-effective photobiological H_2 production, which currently limits the production of H_2 in photolytic systems.

APPROACH

Our goal is to construct a novel microbial recombinant, 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 and solar energy. To achieve this goal, we transfer known O_2-tolerant hydrogenase from CBS to the model cyanobacterium *Synechocystis* sp. PCC 6803.

RESULTS

Probing Hydrogenase Maturation Machinery in CBS

The overarching goal is to construct a cyanobacterial recombinant harboring the O_2-tolerant hydrogenase from CBS using *Synechocystis* sp. PCC 6803 as a model host for sustained photolytic H_2 production. A prerequisite for success is to gain better understanding of the CBS hydrogenase and especially its underlying maturation machinery to ensure transfer of the correct genes into *Synechocystis* to confer hydrogenase activity. CBS genome was sequenced and annotated in FY 2013 via collaboration with Michigan State University and Pacific Biosciences. Using the Basic Local Alignment Search Tool – Protein (BLASTP) tool, we uncovered in CBS genome a second set of hydrogenase maturation genes (*hyp2*), which is different from the set of hydrogenase maturation genes found earlier (*hyp1*). This raises the question as to which set of maturation genes is responsible for building an active CBS hydrogenase and hence needs to be co-transformed along with the CBS hydrogenase into *Synechocystis*. As such we conducted site-directed mutagenesis and obtained CBS mutants lacking either *hyp1* or *hyp2* or both. Genetic strategy and PCR verification of mutant generation are shown in Figure 1. Analysis of H_2 production, CO uptake, and H_2 uptake profiles in wild type and mutants revealed that *hyp1* genes are essential for assembly and maturation of the CBS O_2-tolerant hydrogenase. This finding thereby confirms that only *hyp1* (*hyp1FCDEAB*) will be co-transformed into *Synechocystis* along with the CBS hydrogenase structural genes (*cooLXUH*) to confer CBS hydrogenase activity.

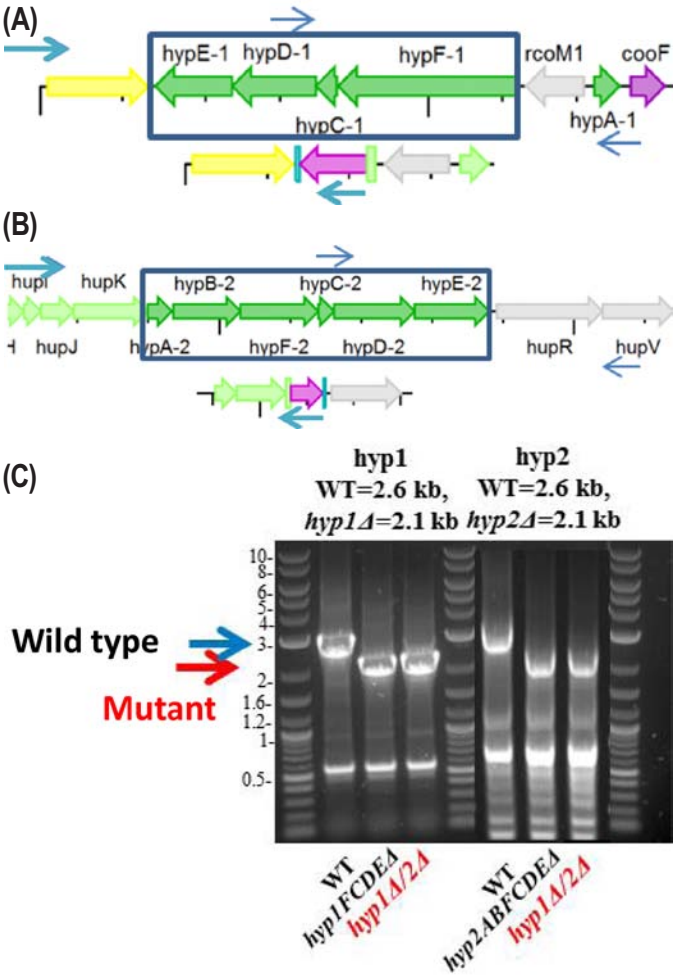

FIGURE 1. Genetic strategy for deletions of *hyp1* (A) and *hyp2* (B) in *Rubrivivax gelatinosus* CBS. Blue arrows denote 5-ft- and 3-ft-end primers for PCR verification; (C) PCR verification of *hyp1*/*hyp2* deletion mutant generation using primer set noted in (A) and (B).

Expression of the CBS Hydrogenase in *Synechocystis*

One strategy to increase H_2 production is to increase the amount of active CBS hydrogenase proteins expressed in *Synechocystis* by using stronger promoters (enhancing gene expression) and its cognate RBS (enhancing protein expression). We have acquired the antibodies to confirm the latter. In this new modular design, both *hyp1* genes and *cooLXUH* are driven by the stronger *Ptrc* promoter (as comparing to the *psbA* promoter) and Trc RBS known to enhance expression in *E. coli* and functional in cyanobacteria [1]. PCR data (not shown) confirmed the correct integration of both constructs in the neutral genome sites of *Synechocystis*. Based on protein western blots using the available antibodies in hand, we indeed detected enhanced expression of all the proteins (denoted by purple arrows) when comparing to the weaker *petE* and *psbA* promoters (Figure 2). Most protein levels are comparable to

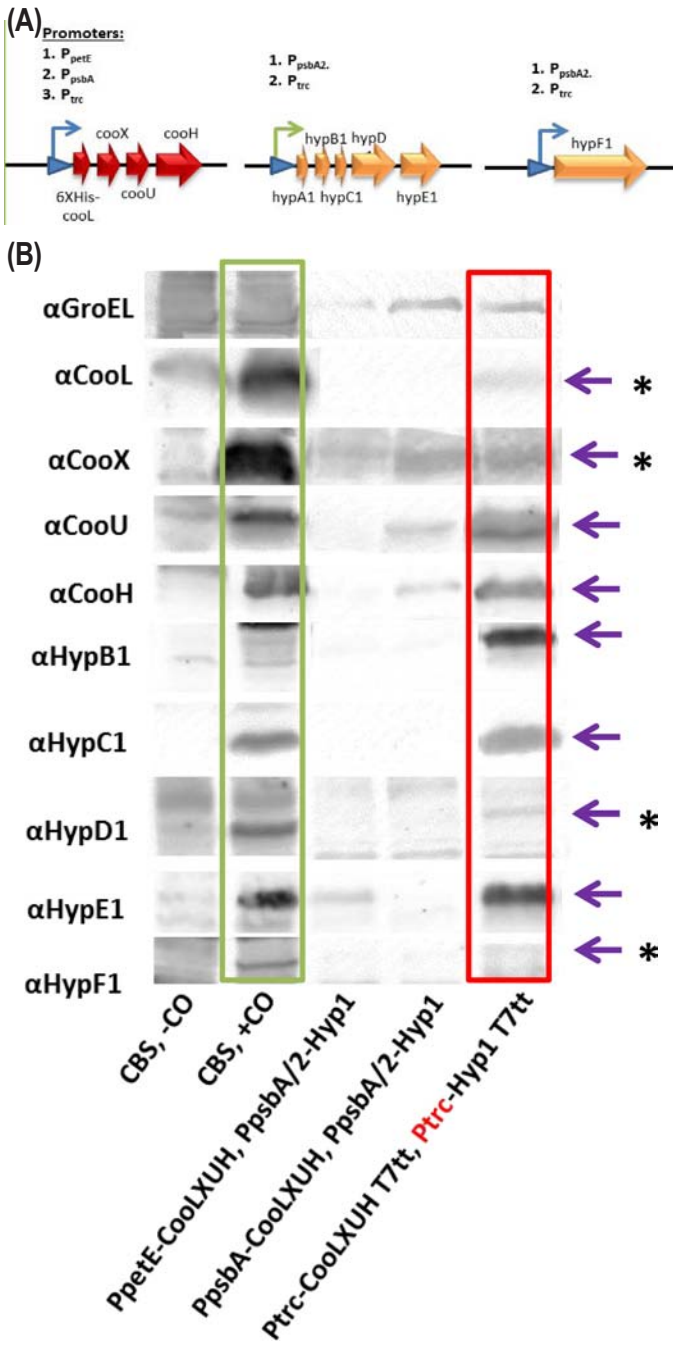

FIGURE 2. (A) Diagram illustrating promoters and tuning strategy for cloning into *Synechocystis*, and (B) balanced protein expression verified via protein western blots of various *Rubrivivax gelatinosus* CBS proteins expression levels in CBS native host and in recombinant *Synechocystis*, the latter driven by three different promoters.

those in native CBS (CO inducible) except those denoted with asterisks. The outcomes serve as the first step toward active CBS hydrogenase activity in *Synechocystis*. The recombinant *Synechocystis* however didn't display any in vitro hydrogenase activity when assayed using sodium dithionite as the reductant and methyl viologen as the mediator. Parallel transformation was also conducted in *E. coli* for troubleshooting. A culprit for the lack of activity could be the missing two subunits of CBS hydrogenase, CooM and CooK, both are membrane proteins and therefore more difficult for expression in foreign host. Work is ongoing for optimization and co-expression of *cooMK* in *Synechocystis* and in *E. coli* to afford hydrogenase activity.

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

- CBS mutants analysis lacking either or both *hyp1/hyp2* revealed that only *hyp1* is essential for assembling the O_2-tolerant, H_2-evolving hydrogenase and that only *hyp1* genes (*hyp1FCDEAB*) will be genetically transferred into *Synechocystis* along with the hydrogenase genes to assemble the O_2-tolerant CBS hydrogenase.
- We refactored the promoter/RBS constructs and obtained a *Synechocystis* strain expressing all 10 CBS genes (*cooLXUH* and *hyp1FCDEAB*). We indeed detected enhanced expression of most CBS proteins in the *Synechocystis* recombinant. Yet the latter still lacks active hydrogenase activity.

Future Directions

- We will generate knockout mutant in CBS lacking *cooM*, *cooK*, or both to probe their functionality in assembling the active CBS hydrogenase activity.
- Optimize codons, build genetic constructs containing *cooM*, *cooK*, or both for expression in *Synechocystis* and in *E. coli* (for troubleshooting), and determine hydrogenase activity.

FY 2015 PUBLICATIONS/PRESENTATIONS

1. Wawrousek, K., S. Nobles, J. Korlach, J. chen, C. Eckert, J. Yu, and P.C. Maness. 2014. "Genome annotation provides insight into carbon monoxide and hydrogen metabolisms in *Rubrivivax gelatinosus*." PLoS One. DOI: 10.1371.

2. Eckert, C.E. "Improving cyanobacterial O_2-tolerance using CBS hydrogenase for hydrogen production." Poster presentation at the DOE Hydrogen and Fuel Cells Annual Merit Review, June 9, 2015, Washington, DC (PD095).

REFERENCE

1. Huang, H.-H., D. Camsund, P. Lindblad, and T. Heidorn. 2010. "Design and characterization of molecular biology tools for a synthetic biology approach towards developing cyanobacterial biotechnology." Nucleic Acids Res. 38: 2577–2593.