

II.E.2 Improving Cyanobacterial O₂-Tolerance Using CBS Hydrogenase for H₂ Production

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Project End Date: Continuation and direction determined annually by DOE

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 O₂-tolerant, H₂-evolving hydrogenase and *hyp2* is for the H₂-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 O₂-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*.



Overall Objectives

- Decipher the maturation machinery of the O₂-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 H₂ production
- Demonstrate H₂ production in the cyanobacterial recombinant during photosynthesis for photolytic H₂ production

Fiscal Year (FY) 2015 Objectives

- Develop an O₂-tolerant cyanobacterial system for sustained and continuous light-driven H₂ 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

INTRODUCTION

Photobiological processes are attractive routes to renewable H₂ 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 H₂. However, one major barrier is that most hydrogen-evolving hydrogenases are inhibited by O₂, which is an inherent byproduct of oxygenic photosynthesis. The rate and duration of H₂ production is thus limited. Certain photosynthetic bacteria are reported to have an O₂-tolerant, H₂-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 O₂-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 O₂-tolerant bacterial hydrogenase, all within the same microbe. By overcoming the sensitivity of the hydrogenase enzyme to O₂, we address one of the key technological hurdles (Barrier AP) to cost-effective photobiological H₂ production, which currently limits the production of H₂ 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 H₂ from water and solar energy. To achieve this goal, we transfer known O₂-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 O₂-tolerant hydrogenase from CBS using *Synechocystis* sp. PCC 6803 as a model host for sustained photolytic H₂ 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 H₂ production, CO uptake, and H₂ uptake profiles in wild type and mutants revealed that *hyp1* genes are essential for assembly and maturation of the CBS O₂-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.

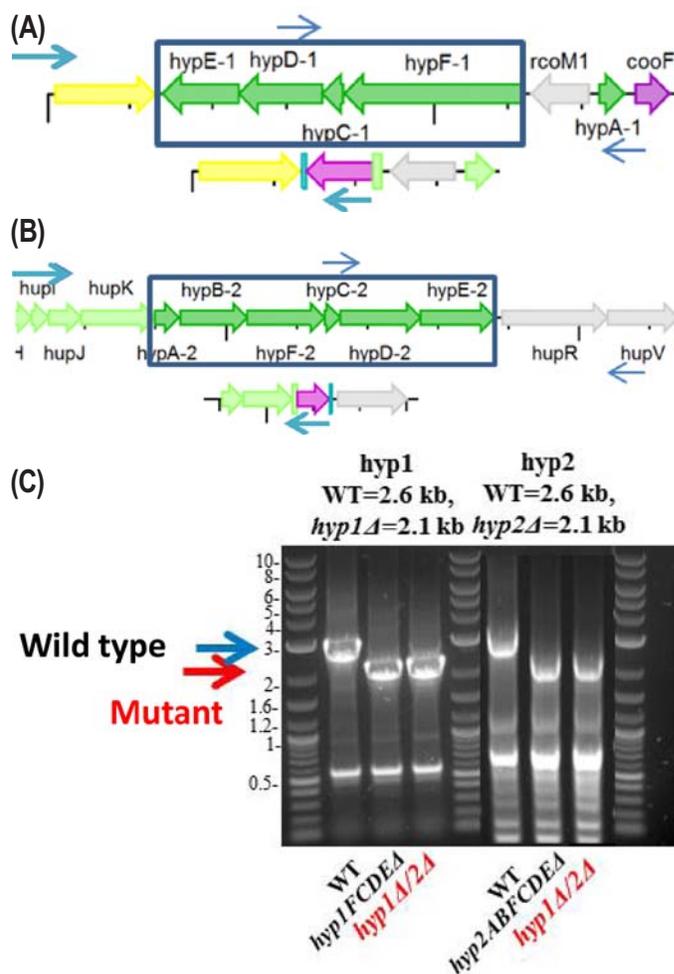


FIGURE 1. Genetic strategy for deletions of *hyp1* (A) and *hyp2* (B) in *Rubrivivax gelatinosus* CBS. Blue arrows denote 5-ft- and 3-ft-end primers for PCR verification; (C) PCR verification of *hyp1/hyp2* deletion mutant generation using primer set noted in (A) and (B).

Expression of the CBS Hydrogenase in *Synechocystis*

One strategy to increase H₂ 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 *P_{trc}* 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

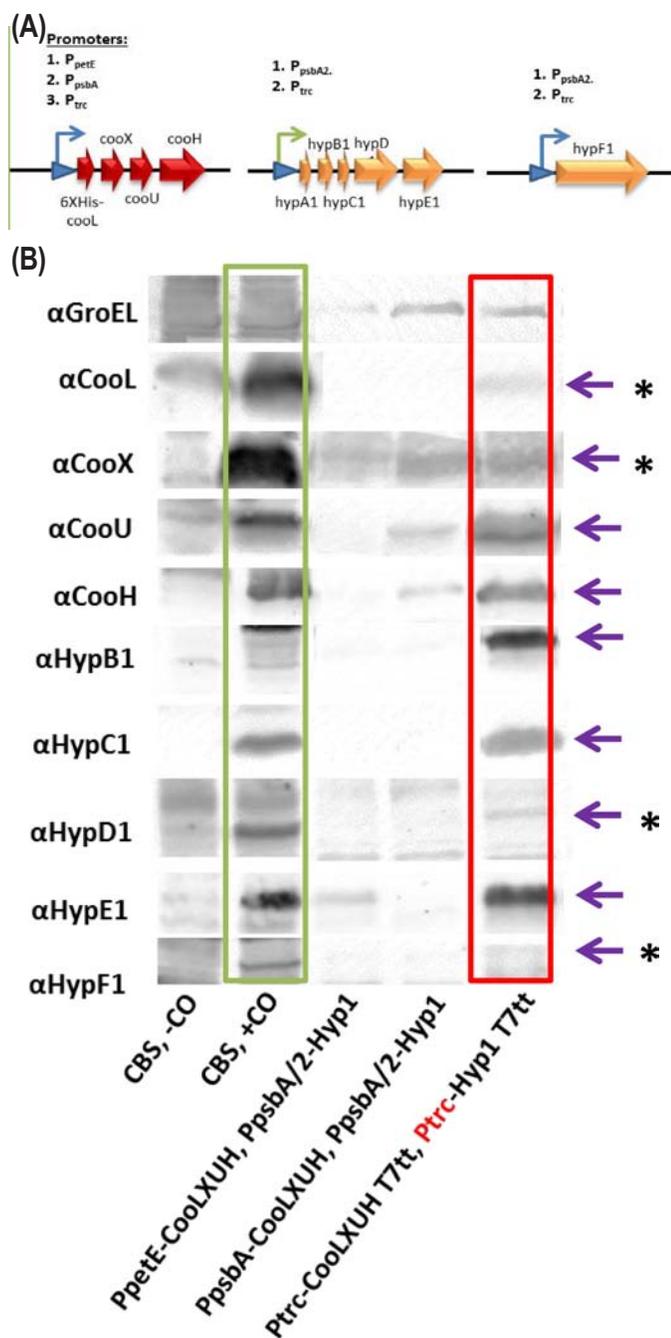


FIGURE 2. (A) Diagram illustrating promoters and tuning strategy for cloning into *Synechocystis*, and (B) balanced protein expression verified via protein western blots of various *Rubrivivax gelatinosus* CBS proteins expression levels in CBS native host and in recombinant *Synechocystis*, the latter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