II.D.5 Probing O_2-Tolerant CBS Hydrogenase for Hydrogen Production

Pin-Ching Maness (Primary Contact), Scott Noble, 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@go.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 an optimal cyanobacterial recombinant.
- Construct a cyanobacterial recombinant by expressing four hydrogenase genes and six maturation genes from *Rubrivivax gelatinosus* for sustained H_2 production.
- Demonstrate H_2 production in the cyanobacterial recombinant during photosynthesis for photolytic H_2 production.

Fiscal Year (FY) 2013 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 Office Multi-Year Research, Development, and Demonstration Plan:

(AP) Oxygen Accumulation

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 2013 Accomplishments

We sequenced the genome of *Rubrivivax gelatinosus* Casa Bonita strain (hereafter "CBS"), which uncovered a second set of hydrogenase maturation operon *hyp2*. We created *hypE* single and double mutant strains in *hyp1* and/or *hyp2* loci. Based on mutant analysis, we concluded that HypE proteins from the two loci can complement each other, and that no other gene in the CBS genome can substitute for the HypE function. Yet gene expression profiles based on quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) data suggest a role of *hyp1* to be consistent with assembling the CBS hydrogenase, which guides its expression in *Synechocystis*.

- We tested a strong *psbA* promoter to drive the expression of CBS hydrogenase genes in a *Synechocystis* recombinant. When compared to the original weaker *petE* promoter, hydrogenase protein levels increased by 16- to 44-fold with the strong *psbA* promoter. We have subsequently constructed a *Synechocystis* recombinant harboring nine CBS hydrogenase and related genes driven by the strong *psbA* and *psbA2* promoters. Work is underway to express *hypF1* in the recombinant driven by the *psbA* promoter.

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 feedstocks.

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 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 and solar energy. To achieve this goal, we will transfer the known O_2-tolerant hydrogenase from *Rubrivivax gelatinosus* 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 *Rubrivivax gelatinosus* 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 its underlying maturation machinery in CBS to ensure transfer of the correct genes into *Synechocystis* to confer hydrogenase activity. Annotation of the CBS genome revealed a complex picture of life using CO as the carbon and energy source, including CO sensor/transcriptional regulator and CO dehydrogenase, H_2-sensing hydrogenase, evolving hydrogenase (the "CBS hydrogenase"), and uptake hydrogenase. In addition to the set of hydrogenase maturation genes found earlier (*hyp1*), a second complete set of hydrogenase maturation genes (*hyp2*) was found within the uptake hydrogenase operon. This raises the question as to which set of maturation genes is responsible for building an active CBS hydrogenase and needs to be co-transformed along with the CBS hydrogenase into *Synechocystis*. Using qRT-PCR to probe gene expression, we found that *hyp1* genes (*A1, B1,* and *D1*) are specifically induced by CO (but not H_2), similar to that of the CBS hydrogenase, while *hyp2* genes (*C2, D2,* and *E2*) are specifically induced by H_2, but not CO (Figure 1). The data are consistent with a role of *hyp1* genes for the maturation of the evolving CBS hydrogenase, and a role of *hyp2* genes for the maturation of the uptake hydrogenase, which is not of interest to this project.

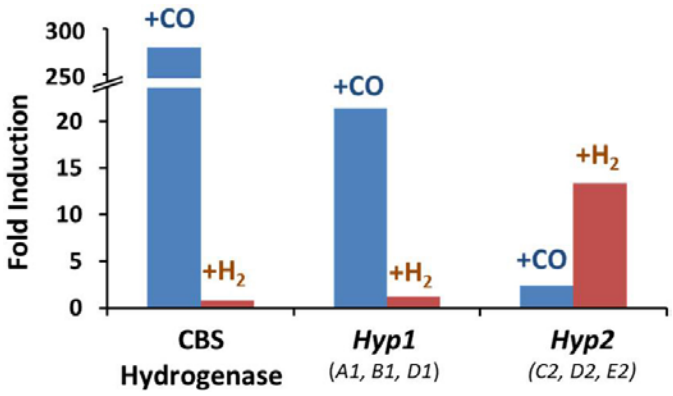

FIGURE 1. Quantitative RT-PCR of CBS maturation genes in various gas substrates. WT: wild type. Fold changes are based on control cultures grown in argon gas.

These gene expression data could not rule out the possibility that *hyp2* genes or any additional genes in the CBS genome are capable of assisting the maturation of the CBS hydrogenase. To address this question we took a genetic approach and created Δ*hypE1*, Δ*hypE2*, and Δ*hypE1*:Δ*hypE2* mutant strains. The double mutant has clearly lost the ability to utilize CO to support growth, indicating that it lacks a functional CBS hydrogenase and that there is no other gene in the CBS genome that can substitute for the HypE function. Interestingly, either Δ*hypE1* or Δ*hypE2* strains can grow in CO. This case study provides evidence that *hyp1* and *hyp2* genes are complementary—that either copy is able to mature the CBS O_2-tolerant hydrogenase. Yet based on the qRT-PCR evidence, initial work will focus on co-expressing only *hyp1* genes with the CBS hydrogenase in *Synechocystis* for photolytic H_2 production.

Expression of the CBS Hydrogenase in *Synechocystis*

Working toward building the cyanobacterial recombinant, we have used a weak *petE* promoter to drive the expression of CBS hydrogenase genes in *Synechocystis.* Lack of consistent H_2 production in the transgenic *Synechocystis* may have been resulted from low hydrogenase protein levels. To address this issue, we tested a strong *psbA* promoter to drive the hydrogenase genes *cooLXUH.* As shown in Figure 2, *psbA* promoter dramatically enhanced CBS hydrogenase protein expression in the cyanobacterium by 16- to 44-fold. Subsequently we have reconstructed a *Synechocystis* recombinant containing nine CBS hydrogenase and related genes, with *psbA* promoter driving the expression of *cooLXUH* (encoding CBS hydrogenase), and *PpsbA2* driving *hyp1ABCDE*. Work is ongoing to clone *hypF1* driven by *psbA* promoter to afford hydrogenase activity in *Synechocystis*.

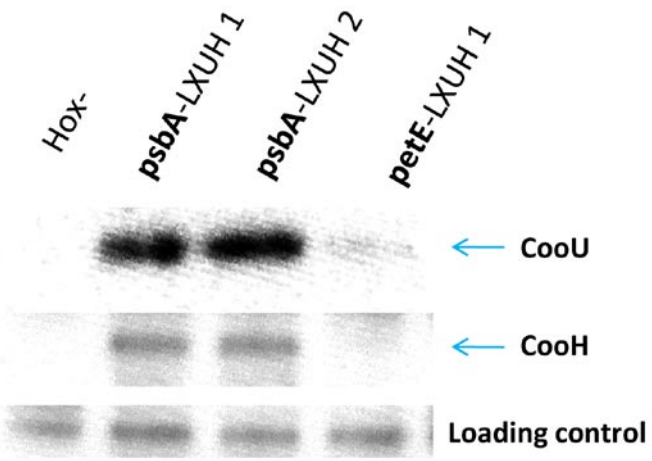

FIGURE 2. The strong *psbA* promoter enhanced the expression of CooLXUH in *Synechocystis*. Shown is a Western blot for CBS CooU and CooH subunits. Hox- is the background parent strain into which either the *psbA-LXUH* or *petE-LXUH* constructs were transformed. CooU and CooH were both expressed at higher levels from *psbA* promoter over *petE* promoter, with 16- and 44-fold increases, respectively.

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

- Either *hyp1* or *hyp2* genes are capable of assembling the CBS O_2-tolerant hydrogenase based on mutant analysis. Yet qRT-PCR data are consistent with a role of *hyp1* in the maturation of CBS hydrogenase. Initial work will focus on expressing *hyp1* genes along with CBS hydrogenase in *Synechocystis*.
- Use of the strong *psbA* promoter dramatically enhanced CBS hydrogenase protein expression levels in *Synechocystis*. A *Synechocystis* recombinant has been constructed expressing nine CBS hydrogenase and related genes, driven by the strong *psbA* and *psbA2* promoters. Transformation of *hypF* is underway driven by *psbA* promoter in order to confer hydrogenase activity.

Future Directions

- We will further investigate the roles of CBS *hypE1* and *hypE2* in hydrogenase maturation by directly examining CO uptake and H_2 production in the single and double mutants.
- We will apply the strong *psbA* promoter to enhance expression of additional CBS hydrogenase genes in *Synechocystis* for consistent hydrogen production. We will optimize growth and induction conditions to boost CBS hydrogenase activity in the recombinant *Synechocystis*.

FY 2013 PUBLICATIONS/PRESENTATIONS

1. Eckert, C.; Boehm, M.; Carrieri, D.; Yu, J.; Dubini, A.; Nixon, P.J.; Maness, P.C. 2012. "Genetic analysis of the Hox hydrogenase in the cyanobacterium *Synechocystis* sp. PCC 6803 reveals subunit roles in association, assembly, maturation, and function." *J. Biol. Chem.* 287: 43502-43515.

2. Ghirardi, M.L.; King, P.W.; Mulder, D.W.; Eckert, C.; Dubini, A.; Maness, P.C.; Yu, J. 2013. "Hydrogen production by water photolysis." *Microbial Bioenergy*, Advances in Photosynthesis and Respiration series (Govindjee and T. Sharkey, eds.), in press.

3. Maness, P.C. 2012. "Genetic engineering in the cyanobacterium in *Synechocystis* sp. PCC 6803 for solar H_2 production." Presentation at the 14th International Symposium on Phototrophic Prokaryotes, August 5–10, 2012, Porto, Portugal.

4. Maness, P.C. 2013. "Probing O_2-tolerant CBS hydrogenase for hydrogen production." Presentation at the DOE Hydrogen and Fuel Cells Program Annual Merit Review, May 16, 2013, Arlington, VA (PD095).