II.E.4 Probing O_2-Tolerant CBS Hydrogenase for Hydrogen Production

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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) 2014 Objective

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

Technical Barrier

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

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 2014 Accomplishments

- Based on amino acid homology comparison of the newly sequenced genome, we uncovered additional hydrogenase maturation genes in *Rubrivivax gelatinosus* Casa Bonita strain (hereafter "CBS"). They are *slyD* and *carAB*; the former is responsible for Ni insertion and the latter for the synthesis of carbon-nitrogen ligand. Both are likely involved in assembling the CBS hydrogenase active site. *Synechocystis* contains *carAB*, but not *slydD*; hence, only *slyD* will be genetically transferred into *Synechocystis* to assemble the O_2-tolerant CBS hydrogenase.
- We used the strong *psbA* promoter to drive the expression of CBS hydrogenase maturation gene *hypF*, in a *Synechocystis* recombinant already expressing CBS hydrogenase and five of the maturation genes (*hypABCDE*). When compared to the original weaker *petE* promoter, HypF protein levels increased by near 9-fold with the strong *psbA* promoter. Yet, the *Synechocystis* recombinant still failed to yield hydrogenase activity, warranting the refactoring of the genetic construct to afford activity.

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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 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 by Michigan State University and Pacific Biosciences. Using the Basic Local Alignment Search Tool – Protein (BLASTP) tool, we uncovered a second set of hydrogenase maturation genes (*hyp2*) in the CBS genome, 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 needs to be co-transformed along with the CBS hydrogenase into *Synechocystis*. As such we conducted a detailed comparison of CBS *hyp1* with *hyp2*, as well as with other known hydrogenase maturation proteins. These data will provide the blueprint to guide genetic engineering effort toward constructing a *Synechocystis* recombinant harboring O_2-tolerant hydrogenase activity. The homology comparison in Table 1 using BLASTP reveals that CBS *hyp1* is quite different from CBS *hyp2* based on low level of identity (34–58%). Data from Table 2 show the homology comparison of CBS *hyp1* and CBS *hyp2* with the *hyp* genes known to be involved in the maturation of a well-studied uptake hydrogenase in *Ralstonia eutropha* (Re). The higher level of identity between Re *hyp* genes with CBS *hyp2* (60% to near 80%), but not with *hyp1,* clearly supports that CBS *hyp2* genes are involved in the maturation of the uptake hydrogenase in CBS, while the CBS *hyp1* genes are involved in the maturation of the O_2-tolerant evolving hydrogenase.

TABLE 1. Homology Comparison (% amino acid identity) of CBS Hyp1 with Hyp2 Proteins

CBS Hyp Proteins	Hyp1 vs. Hyp2 (%)
HypA	34.5
HypB	53.6
HypC	39.2
HypD	50.9
HypE	53.8
HypF	37.2

TABLE 2. Homology Comparison (% amino acid identity) of the Hyp proteins from *Ralstonia eutropha* with the respective Hyp1 and Hyp2 proteins from CBS

Ralstonia eutropha	CBS Hyp1 (%)	CBS Hyp2 (%)
HypA	31.0	65.5
HypB	52.3	67.4
HypC	38.4	59.5
HypD	47.4	77.3
hypE	54.0	74.4
HypF	38.4	60.2

Moreover, after careful search of the CBS genome, we identified a single *slyD* homolog which displays 33% amino acid identity to its counterpart in *E. coli,* which was used as the model system to probe maturation of NiFe-hydrogenases. *Synechocystis* genome does not contain a SlyD homolog based on a BLASTP search. Studies in *E. coli* suggest that carbamoyl phosphate synthase (encoded by *carAB*) is necessary for hydrogenase maturation. This enzyme complex synthesizes carbamoyl phosphate, the precursor for carbon-nitrogen ligand, which is an integral component of the hydrogenase active site. A search of the CBS genome revealed *carA* and *carB* homologs with 64% and 70% identity, respectively, to the homologs in *E. coli*. CarA and CarB homologs with 51% and 60% identity, respectively, are present in *Synechocystis.* Therefore, we may need to minimally evaluate the expression of CBS *slyD* in addition to the *hyp1* genes in order to obtain a more active CBS hydrogenase in *Synechocystis*.

Expression of the CBS Hydrogenase in *Synechocystis*

One strategy to increase H_2 production is to increase the amount of active CBS hydrogenase expressed in *Synechocystis* by using stronger promoters. We have systematically re-engineered the *Synechocystis* recombinant using the strong *psbA* promoter to drive the expression of

cooLXUH (encoding CBS hydrogenase) and *psbA2* promoter to drive the expression of *hyp1ABCDE*. The resultant recombinant is *psbA-LXUH/psbA2-ABCDE*. Moreover, we incorporated the CBS maturation gene *hypF* in the above recombinant driven by the strong *psbA* promoter. We first produced a plasmid where the weak promoter *petE* was replaced with *psbA* promoter, with its replacement confirmed by restriction digest analysis and sequencing. We then transformed this plasmid into *Synechocystis* (*psbA-LXUH/psbA2-ABCDE)* and confirmed the integration of *psbA-hypF* into the *Synechocystis* genome via colony polymerase chain reaction (PCR) in 8 out of 18 colonies tested (Figure 1A). Expression of HypF protein was determined via protein immunoblots. We detected that HypF expression was enhanced from 1.7- to 8.9-fold in these strains (*psbA-LXUH/psbA2-ABCDE/psbA-hypF*) when driven by the strong *psbA* promoter, when compared to expression using the weak *petE* promoter (Figure 1B). Yet, the engineered strain failed to yield hydrogenase activity.

To troubleshoot, expression levels need to be carefully quantified for further improvements. Therefore, we performed quantitative protein immunoblots of CooLXUH and Hyp1EF (based on available antibodies) to compare levels of each CBS protein in CBS to that in *psbA-LXUH/psbA2-hypABCDE/psbA-hypF.* It was clear from the results that only CooU, CooH, and HypE1 were expressed at detectable levels in the *Synechocystis* strain, and levels were much lower than those seen in CBS, the latter loaded with two-third less protein (data not shown). This imbalanced protein expression prompted us to redesign the expression construct (Figure 2) that employs the strong *trc* promoter from *E. coli,* which has shown up to 80 times higher expression levels in *Synechocystis* when compared to native promoters [1]. In addition, we also integrated a consensus ribosomal binding site from *Synechococcus* 7002 (cpcB gene) shown to have high levels of expression in *Synechocystis* [2] in front of each gene to be expressed to maximize translational efficiencies. Because we may need to break up the expression constructs

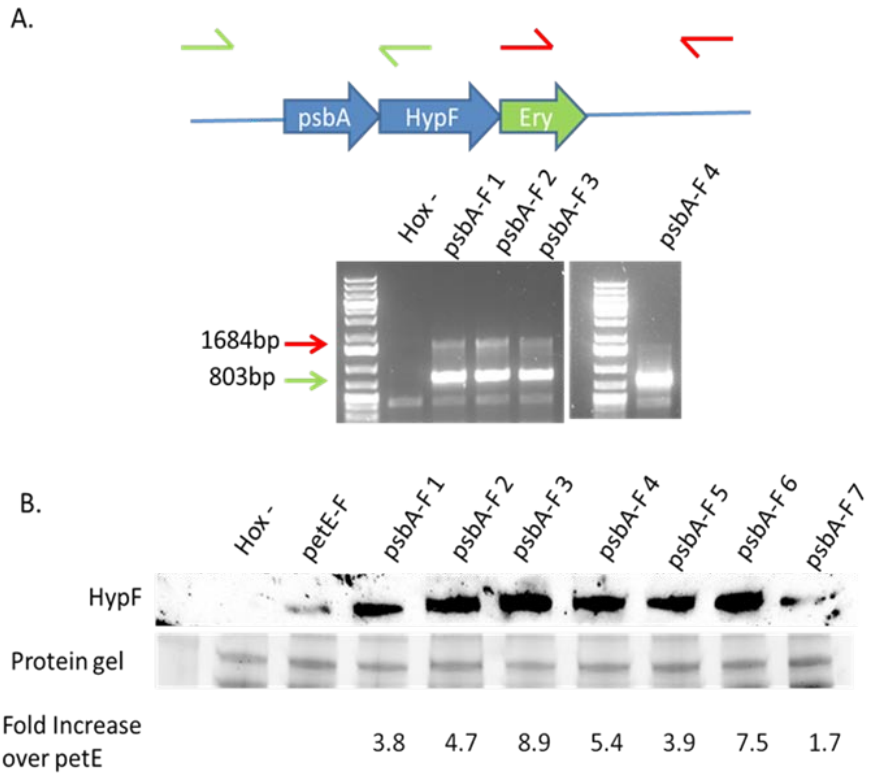

FIGURE 1. Integration and Enhanced Expression of HypF in ***psbA-LXUH/psbA2-ABCDE/psbA-hypF*** Strains—Panel A shows colony PCR confirming the integration of *psbA-hypF* into the *Synechocystis* genome. Primers upstream, downstream, and within the integrated construct (red and green arrows denoting forward and reverse primers) should produce two products of 803 bp and 1648 bp in size. PCR of colonies confirmed the correct integration of the construct into the *Synechocystis* genome. Panel B shows a western immunoblot against the CBS HypF protein. The new recombinants containing the *psbA-hypF* construct displayed a higher level of expression compared to that of the control with *petE-hypF*. Fold increase was calculated by normalizing to a constant band in the protein gel and comparing levels of *psbA-hypF* to the *petE-hypF* recombinant.

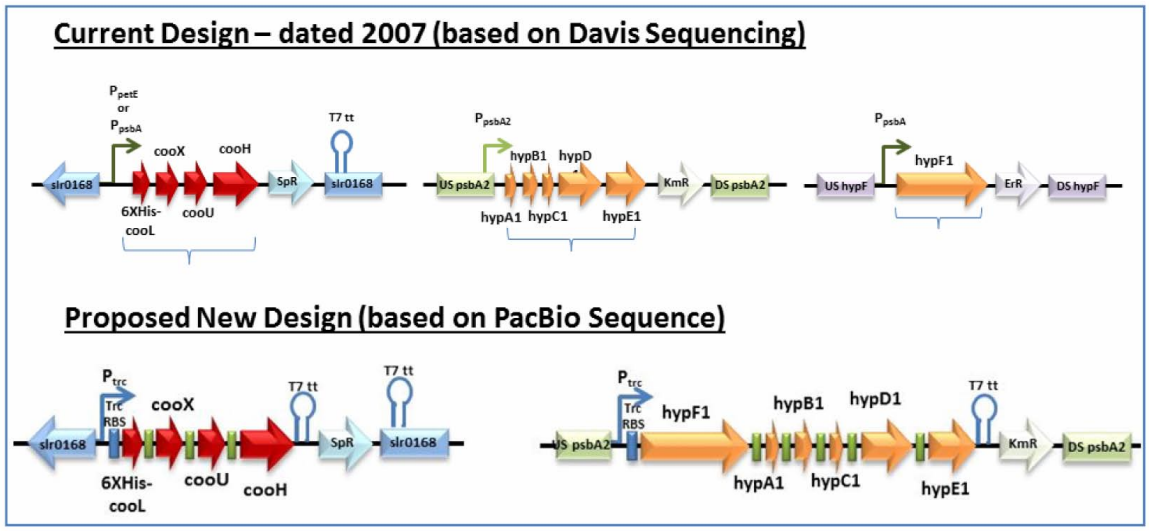

FIGURE 2. Current and Proposed New Design to Express the CBS Hydrogenase and Maturation Genes in *Synechocystis*

into smaller number of genes expressed per promoter, we have also inserted unique restriction sites between each gene to allow for the insertion of additional promoters if necessary. The new design is modular, affording flexibility in tuning and balancing gene expression individually, on a need basis, to ultimately afford O_2-tolerant hydrogenase activity in *Synechocystis*.

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

- Amino acid homology comparison reveals that *hyp1* and *hyp2* are dissimilar, with *hyp2* displaying high level of homology with the *hyp* genes in *R. eutropha*, known to assemble its H_2-uptake hydrogenase. As such, *hyp1* likely assembles the O_2-tolerant CBS hydrogenase. *slyD*, which has a putative role in Ni insertion, was identified in CBS also, but not in *Synechocystis*. Initial work will focus on expressing *hyp1* and *slyD* genes along with CBS hydrogenase in *Synechocystis*.
- Use of the strong *psbA* promoter dramatically enhanced CBS hydrogenase maturation protein HypF expression levels by near 9-fold in *Synechocystis*, yet the recombinant still lacks hydrogenase activity. We propose to re-engineer the CBS gene construct to incorporate strong *trc* promoter and proven ribosome binding sites to afford hydrogenase activity.

Future Directions

- Further investigate the roles of CBS *hyp1* and *hyp2* in hydrogenase maturation by directly examining CO uptake and H_2 production in the *hyp1* and *hyp2* single and double mutants.
- Transform the new designs containing *cooLXUH*, followed by *hyp1* operons to ensure balanced protein expression. If the protein expression is not balanced, we will troubleshoot by inserting more frequent *trc* promoters in between each gene to tune expression. In conjunction with this iterative approach, we will perform in vitro hydrogenase activity assay using reduced methyl viologen as the mediator.

FY 2014 PUBLICATIONS/PRESENTATIONS

1. Ghirardi, M.L.; King, P.W.; Mulder, D.W.; Eckert, C.; Dubini, A.; Maness, P.C.; Yu, J. 2013. "Hydrogen production by water biophotolysis." *Microbial Bioenergy: Hydrogen Production*, Advances in Photosynthesis and Respiration series (D. Zannoni and R. De Philippis, eds.), p. 101-135.

2. Maness, P.C. "Genetic analysis of the Hox hydrogenase in the cyanobacterium *Synechocystis* sp. PCC 6803." Oral presentation at the 10th International Hydrogenase Conference, July 8-12, 2013, Szeged, Hungary.

3. Maness, P.C. "Hydrogen from water in a novel recombinant O_2-tolerant cyanobacterial system." Presentation at the Hydrogen Production Tech Team Review. July 30, 2013, PNNL, Richland, WA.

4. Maness, P.C. "Improving cyanobacterial O_2-tolerance using CBS hydrogenase for hydrogen production." Presentation at the DOE Hydrogen and Fuel Cells Annual Merit Review, June 19, 2014, Washington, DC (PD095).

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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.

2. Abe, K., K. Miyake, M. Nakamura, K. Kojima, S. Ferri, K. Ikebukuro, and K. Sode. 2014. Engineering a green-light inducible expression system in *Synechocystis* sp. PCC 6803. Microbial Biotech., 7: 177-183.