

II.G.3 Hydrogen from Water in a Novel Recombinant Oxygen-Tolerant Cyanobacterial System

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Fiscal Year (FY) 2012 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 barriers from the Hydrogen Production section (3.1.4) of the Fuel Cell Technologies Program Multi-Year Research, Development and Demonstration Plan:

(AH) Rate of Hydrogen Production

(AI) Continuity of Photoproduction

Technical Targets

Characteristics	Current Status	2011 Target	2018 Target
Duration of continuous H ₂ photoproduction in air	Zero to 30 seconds in air	Produce a cyanobacterial recombinant evolving H ₂ through an O ₂ -tolerant hydrogenase	H ₂ production in air for 30 min

FY 2012 Accomplishments

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- Re-engineered transcriptional regulation of hydrogenase to achieve four-fold higher activity (from 0.5 to 2 nmol H₂*mg protein⁻¹*h⁻¹).
- Examined the effect of transcriptional modifications on hydrogenase maturation and found that proper regulation of accessory genes is essential for optimal hydrogenase maturation.
- Created mutants of the environmentally-derived hydrogenase small subunit with four-fold improved activity that is biased toward hydrogen evolution.

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- Determined that the putative maturation genes *hypABCDEFG* likely are involved in the maturation of the Casa Bonita strain (CBS) O₂-tolerant hydrogenase. This is supported by similar expression profiles in response to CO for both maturation genes and the hydrogenase structural genes.
- A *Synechocystis* recombinant harboring 10 CBS genes was constructed (including four hydrogenase genes and six maturation genes), with the expression of both HypE and HypF verified by protein immunoblots. A very low level of *in vitro* hydrogenase activity was detected in the recombinant compared to zero-hydrogenase activity in the untransformed control.



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 sunlight

energy to extract reducing equivalents from water. The resulting reducing equivalents can be fed to a hydrogenase system yielding H₂. However, one major difficulty is that most hydrogen-evolving hydrogenases are inhibited by O₂, which is an inherent byproduct of oxygenic photosynthesis. The rate of H₂ production is thus limited. Certain photosynthetic bacteria are reported to have an O₂-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 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 to cost-effective photobiological H₂ 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 H₂ from water. To achieve this goal, we use the following two approaches. The first approach is to transfer known O₂-tolerant hydrogenases from anoxygenic photosynthetic bacteria *Thiocapsa roseopersicina* and *Rubrivivax gelatinosus* to cyanobacteria. Since only a very limited number of O₂-tolerant hydrogenases are available, our second approach is to identify novel O₂-tolerant hydrogenases from environmental microbial communities and transfer them into cyanobacteria.

Results

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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 hydrogenases were expressed indicating co-expression of all required accessory proteins, the activity was low. We hypothesized that improved plasmid design may increase activity. The original expression plasmid, pRC41, expressed the environmentally-derived hydrogenase under the regulation of one promoter at the beginning of the 13 gene construct (Figure 1A). With such a long transcript (~13-kb), genes

encoded at the end of the operon, such as the *hyp* genes, may not be expressed in sufficient quantity to allow for maximal activity of the environmental hydrogenase in cyanobacteria. These genes at the end of the operon are required for maturation of the hydrogenase and must be transcribed at the optimal level.

In order to achieve higher expression throughout the gene cluster, we have re-engineered the expression plasmid to create an additional three promoters spaced throughout the operon (Figure 1A). We also inserted a sequence encoding the StrepII peptide tag onto the N-terminus of the last gene in the cluster, *hypE*, so that we could monitor expression at the end of the gene cluster.

The presence of one additional promoter preceding the last three genes in the cluster, Nstrep3 (Figure 1A) did not significantly improve hydrogenase activity in cyanobacteria, but it did increase expression of the last gene in the cluster, *hypE* (Figure 1C). The redesigned plasmid with three additional promoters spread throughout the gene cluster, Nstrep5, had approximately four-fold increase in activity over the single promoter version, Nstrep1. This increase in activity was consistent with an increase in total abundance of HynL and HypE protein (Figure 1C). Overall, in both Nstrep1 and Nstrep 5 constructs, most of the HynL protein was found in the pre-processed or immature form, suggesting that optimization of hydrogenase maturation remains a key challenge for heterologous expression. One surprising result was that construct Nstrep3 had increased processing of PreHynL to HynL without a corresponding increase in activity (Figure 1C). This suggests that relative strengths of the promoters we employ to achieve optimum expression must be optimized.

In a parallel approach to increasing activity in the environmentally-derived hydrogenase, we made three mutants that differ in the amino acids that ligate the iron-sulfur clusters to the hydrogenase small subunit. In the first mutant (Figure 2A diagram 2), an amino acid near the proximal cluster was modified from histidine to cysteine which is expected to change the ligation of the cluster to the protein. In the second mutant (Figure 2A diagram 3), an amino acid near the medial cluster was changed from proline to cysteine. This is expected to change the cluster from 3Fe4S to 4Fe4S. Research in other labs suggests that this mutation will remove energy barriers in the flow of electrons from the electron transfer site to the catalytic center. The third mutant combines both of these single mutations into one single strain (Figure 2A diagram 4). We found that neither of the single mutations increased activity individually, but when combined, hydrogen evolution activity increased by approximately four-fold (Figure 2B). To determine if this was a specific change in enzyme bias or a more general increase in the activity of the enzyme, we also measured hydrogen uptake activity. We found that while uptake activity was slightly elevated in constructs 2 and 3, the increase was not

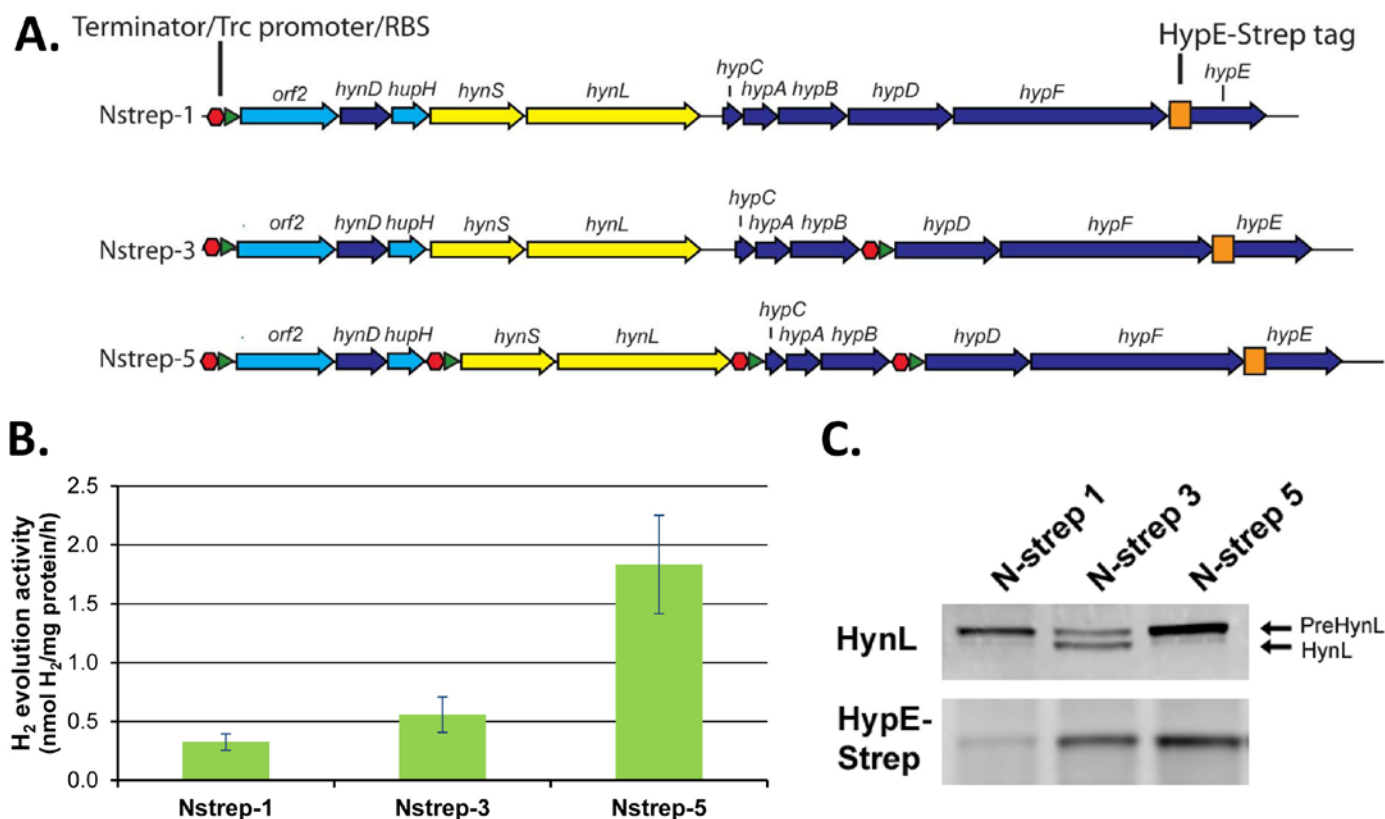


FIGURE 1. Transcriptional re-engineering of the environmentally-derived hydrogenase: (A) Diagram of hydrogenase structural and accessory genes, terminator/promoter cassettes (red hexagon/green triangle), and *hypE* N-terminal StrepII-tag (orange square). (B) *In vitro* hydrogen evolution activity of transcriptionally-modified constructs described in A. (C) Western blots of cyanobacterial extracts from B using anti-HynL or anti-StrepII antisera.

significant and the uptake activity of the mutants was similar to wild type (Figure 2C).

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The overarching goal of the NREL work is to construct a cyanobacterial recombinant harboring the O₂-tolerant hydrogenase from *Rubrivivax gelatinosus* CBS (hereafter “CBS”) using *Synechocystis* sp. PCC 6803 as a model host for sustained photolytic hydrogen 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. One strategy to probe the function of the six putative *hypABCDEF* maturation genes is to test if their expression follows the same induction profile as the CBS hydrogenase genes (*cooMKLXUH*), the latter is specifically induced by CO. RT-qPCR was used to examine transcript levels and fold changes of these genes under various conditions. In this case, we chose to grow CBS in three gaseous conditions: with argon gas (un-induced condition), with CO (induced condition), or with CO₂ (a product of CO oxidation). As seen in Figure 3, the *hypA*, *B*, and *D* genes are specifically induced in the presence of

CO, by as high as ~450 fold (*hypB*) compared to the argon gas control, and the *cooH* gene was used as a positive control. Very little transcript abundance was detected in CO₂ atmosphere. While *hypC*, *E*, and *F* are not shown in Figure 3, transcription of these genes was also specifically induced by CO; however, the fold change in mRNA level could not be calculated because the *hypC*, *E*, and *F* transcripts were not abundant enough to be detected in the un-induced condition (in argon gas). Protein immunoblots also confirmed the expression of both HypE and HypF proteins only upon induction by CO (data not shown). These results clearly show that the *hypABCDEF* genes are specifically induced in the same condition in which the CBS O₂-tolerant hydrogenase is induced (both by CO), and strongly suggests that these six *hyp* genes are involved in the production of an active CBS O₂-tolerant hydrogenase and should therefore be transferred into *Synechocystis* host along with the CBS hydrogenase.

Working toward building the cyanobacterial recombinant, we have generated a *Synechocystis* strain containing the four codon-optimized CBS hydrogenase genes (*cooLXUH*). Using this strain as the recipient, we first transformed five codon-optimized *hypABCDE* genes into its genome. Correct gene insertion was verified by polymerase

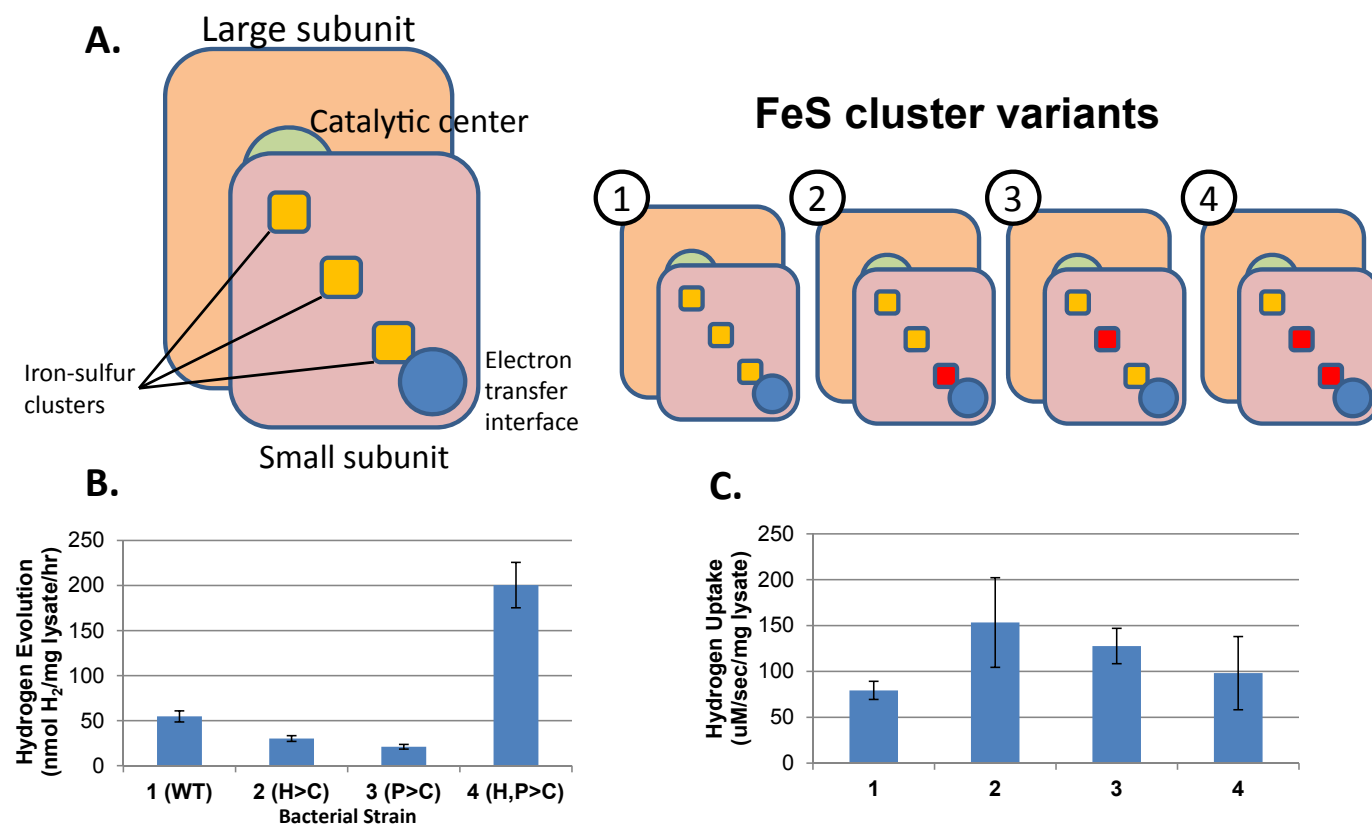


FIGURE 2. Modification of activity bias in mutants of hydrogenase small subunit: (A) Diagram of point mutations. Construct 1 shows the wild type, 2 shows a His to Cys mutation in the proximal cluster, 3 shows a Pro to Cys mutation in the medial cluster, and 4 shows the double mutant with both mutations from 2 and 3. (B) *In vitro* hydrogen evolution activity in extracts from *E. coli* strains expressing the wild type environmental hydrogenase or strains containing the point mutants. (C) *In vitro* hydrogen uptake activity in extracts from *E. coli* strains expressing the wild type environmental hydrogenase or strains containing the point mutants.

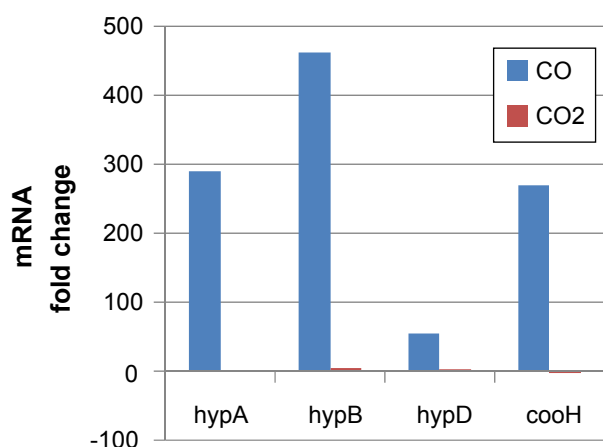


FIGURE 3. Expression profiles of the CBS hydrogenase maturation genes. RT-qPCR was used to detect changes in *hypABCDEF* gene transcript abundance. An un-induced control under argon gas was used to calculate fold change of transcripts using the $\Delta\Delta C_t$ method. The *cooH* gene, encoding the hydrogenase catalytic subunit, was used as a positive control.

chain reaction (data not shown) and the expression of HypE was verified by protein immunoblot (Figure 4A). This was

then followed by transforming the codon-optimized CBS *hypF* gene into the above recombinant, with HypF expression also confirmed via immunoblot (Figure 4B). A schematic of the genotype of the *Synechocystis* strain heterologously expressing 10 CBS genes is illustrated in Figure 4C. The recombinant yielded very low level of hydrogenase activity, *in vitro*, when assayed using methyl viologen reduced by sodium dithionite. Albeit low, the untransformed control exhibited no hydrogenase activity.

Conclusions

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- By increasing the frequency of promoters driving the expression of hydrogenase genes, we have achieved four-fold higher activity from the environmentally-derived hydrogenase expressed in cyanobacteria.
- We mutated the DNA sequence of the hydrogenase small subunit to change the ligation of FeS clusters. The resulting mutant has increased activity in the direction of hydrogen production.

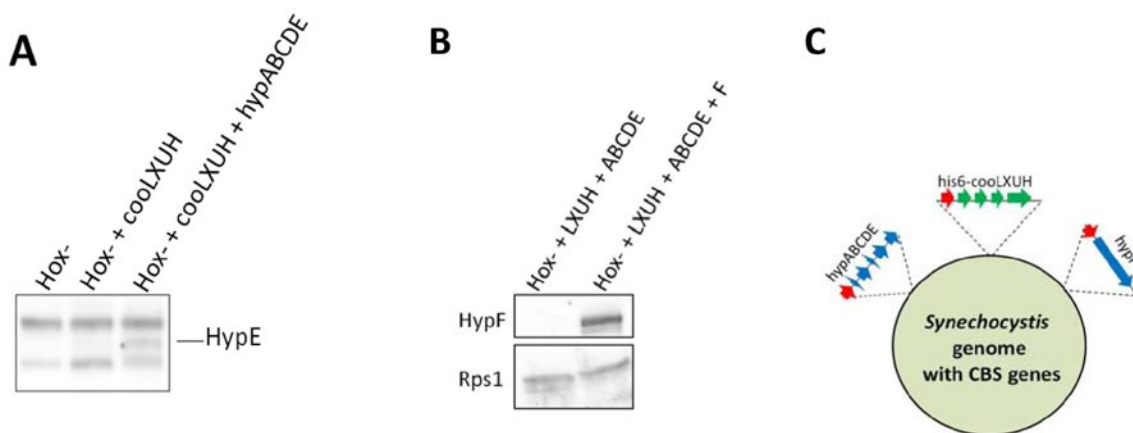


FIGURE 4. (A) Expression of HypE in the recombinant *Synechocystis* strain harboring *coolXUH* and *hypABCDE* as revealed by immunoblot. (B) Expression of HypF in the recombinant *Synechocystis* strain harboring *coolXUH*, *hypABCDE*, and *hypF*, as revealed by immunoblot. (C) Schematic of CBS hydrogenase genes integrated into the genome of *Synechocystis*. Red arrows denote promoters, green arrows are the CBS hydrogenase structural genes *coolXUH*, and blue arrows represent the putative hydrogenase maturation genes, *hypABCDE*.

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- Using both RT-qPCR and protein immunoblots, we successfully demonstrated that the CBS *hypABCDE* genes are likely involved in the assembly and maturation of the CBS O₂-tolerant hydrogenase. The maturation genes and hydrogenase genes display similar induction profiles upon the addition of CO, but not in the argon and CO₂ gas controls.
- We constructed a *Synechocystis* recombinant harboring all ten CBS genes: four hydrogenase genes and six maturation genes. The recombinant displayed low levels of hydrogenase activity when assayed with methyl viologen reduced by sodium dithionite. Albeit low, its untransformed control displayed no hydrogenase activity.

Future Directions

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- We will continue to modify the environmentally-derived hydrogenase by using additional promoters and by varying the relative promoter strengths throughout the gene cluster to optimize maturation of the enzyme.
- We will combine transcriptional modification approaches and point mutant approaches to further increase hydrogenase activity.

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- We will identify additional hydrogenase maturation genes using the sequenced genome of CBS. Following initial characterization of gene expression and function, candidate maturation genes will be transferred into *Synechocystis* host to express a functional O₂-tolerant hydrogenase.
- We will engineer stronger promoter and shorter transcript size, and optimize growth conditions to maximize the expression of the heterologous hydrogenase in *Synechocystis*.

FY 2012 Publications/Presentations

- Carrieri, D., K. Wawrousek, C. Eckert, J. Yu, and P.C. Maness. 2011. The role of the bidirectional hydrogenase in cyanobacteria. *Biores. Technol.* 102: 8368-8377.
- Hu, P., J. Lang, K. Wawrousek, J. Yu, P.C. Maness, and J. Chen. 2012. Draft genome sequence of *Rubrivivax gelatinosus* CBS. *J. Bacteriol.* 194: 3262.
- 2012 DOE Hydrogen Program Annual Merit Review – Washington, DC, May 2010, Oral Presentation PD039 (Weyman and Maness).
- Noble, SL, Wawrousek K, Yu J, Maness PC. (2012 June) “Genetic Engineering of *Synechocystis* sp. PCC 6803 for Sustained Hydrogen Production” Poster presentation at Gordon Research Conference on Iron-Sulfur Enzymes, Mount Holyoke College, MA (Noble).