# II.F.4 Photobiological Hydrogen Research*

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## Objectives

To develop advanced and cost-effective technologies for bio-hydrogen production, we will enhance the existing microbial systems by:

- Cloning the subunits and structural genes (*cooLH* and *cooUX* respectively) of the $O_2$-tolerant NiFe-hydrogenase from a photosynthetic bacterium *Rubrivivax gelatinosus CBS* strain in collaboration with NREL scientists.
- Cloning the active site maturation genes (*hypA-F*) of the NiFe-hydrogenase from *R. gelatinosus CBS* in collaboration with NREL.
- Transforming the structural and subunits genes along with the maturation genes of the NiFe-hydrogenase from *R. gelatinosus CBS* into an *E. coli*.
- Determining the minimum number of genes required to express a fully functional *CBS* hydrogenase in the *E-coli* host.
- Purifying and characterizing the recombinant NiFe-hydrogenase from *E. coli*.
- Performing bioreactor studies to optimize *E-coli* hydrogen production.

## Technical Barriers

This project addresses the following technical barriers from the Biological Hydrogen Production section of the Hydrogen, Fuel Cells and Infrastructure Technologies Program Multi-year Research, Development and Demonstration Plan:

(AF) Lack of Naturally Occurring Microorganism Characterization

(AM) Rate of Hydrogen Production

(AP) Systems Engineering

## Technical Targets

The key target of the proposed research is to advance the status of biological hydrogen production by developing NiFe-hydrogenase enzymes with enhanced tolerance to $O_2$ and expressing them in industrially robust hosts, such as *E-coli*. Achievement of this target will contribute to the "Lower cost methods for producing and delivering hydrogen," a Critical Path technology necessary for developing hydrogen infrastructure, as identified by DOE's Hydrogen Program: "Develop advanced renewable photoelectrochemical and biological hydrogen generation technologies. By 2018, verify the feasibility of these technologies to be competitive in the long term."

## Accomplishments

- Cloning of *cooM* which encodes the membrane-anchoring protein of the hydrogenase along with *cooK* and *hypAB* into a compatible vector with Duet expression vectors (in collaboration with NREL scientists).
- Double plasmid transformation: Co-transformed both the subunit and structural genes (*cooXLUH*) of the *CBS* hydrogenase along with four of its active site maturation genes (*hypCDEF*) in two different compatible duet vectors into a zero hydrogenase background *E-coli* host.
- Expression of an inactive hydrogenase in *E. coli* by transforming the double plasmids which carry the structural (*cooXU*), subunits (*cooL* and *cooH*) and the maturation genes (*hypCDEF*): The expression of *cooH*, encoding the large subunit protein of the CBS hydrogenase was assessed by western blotting.
- Triple Plasmid Transformation: Co-transformed the third plasmid which carries two of the maturation genes (*hypAB*) along with *hypCDEF* and *cooXLUH* into a zero background *E-coli*.

- Detection of the expression of the small subunit protein (*CooL*) of the *CBS* hydrogenase in the triple transformed, zero background *E-coli*.
- Optimization of the cell growth conditions is underway.

## Introduction

Microbial $H_2$ production is an attractive alternative to conventional processes because it limits the emission of carbon dioxide, which leads to the greenhouse effect. The primary challenge of the biological hydrogen program is to lower the cost of hydrogen by increasing the capability and the hydrogen production capacity of microorganisms. This research and development project aims at the development of a cost-effective biochemical system for hydrogen production by *E. coli*, a robust industrial microorganism that is easy to grow and genetically manipulate.

Optimizing photobiological hydrogen production will require an active, efficient, and $O_2$-resistant hydrogenase to be expressed in appropriate hosts. Because $O_2$ is a strong inhibitor of microbial $H_2$ production, expression of an $O_2$-tolerant NiFe hydrogenase in *E. coli* will also facilitate eventual expression of the hydrogenase in a cyanobacterium by NREL scientists. The NiFe-hydrogenase from the purple photosynthetic bacterium *R. gelatinosus CBS* is resistant to $O_2$-inactivation (half-life of 21 hours in vivo) [1] and hence holds promise for eventually developing a commercial biological $H_2$ production process. The CBS hydrogenase is a heterodimeric protein without a C-terminal extension and is homologous to the hydrogenase 3 from *E-coli* [2].

## Approach

Basic research is needed to understand the mechanisms of expression of the $O_2$-tolerant and fully efficient hydrogenases to increase the hydrogen production capability of microbes and water-splitting organisms such as cyanobacteria. Since the latter microorganisms are producing oxygen as a byproduct of water decomposition, the oxygen sensitivity is one of the major challenges for the photobiological hydrogen production by such kind of microbes. We have previously proposed the need to determine the minimum number of genes of $O_2$-tolerant NiFe-hydrogenase from *R. gelatinosus CBS* to achieve expression of a fully functional enzyme in *E-coli* as well to apply the information to cyanobacterial recombinant systems. In the past, the subunit structural genes (*cooLH* and *cooXU* respectively) of the hydrogenase were detected and isolated by NREL scientists. The large subunit *cooH* was cloned with or without strep II tag to allow determination of the expression level of the enzyme and subsequent purification of the active enzyme via affinity chromatography.

According to the latest publications, biosynthesis of the NiFe-hydrogenase active site is a complex process involving the action of the maturation proteins HypA through HypF [3]. These genes of the CBS hydrogenase are homologous to the assembly genes of hydrogenase 3 from *E. coli.* However, the maturation genes from *E. coli* may not be helpful in the assembly of the active site of the CBS hydrogenase. On the other hand, *E. coli* does not express some of the structural genes that the *R. gelatinosus* hydrogenase requires, which may be necessary for the expression of the active enzyme in *E. coli*. To obtain a correctly folded, fully-functional NiFe-hydrogenase in *E. coli,* we may need to transform all of the Hyp genes along with the *CooXLUH* construct because this construct carries the large subunit gene, *cooH*, which is necessary for expression of a functional hydrogenase.

## Results

In the first year of the project we completed the cloning process of the subunit and structural genes (*cooLH, cooXU*, and *cooK*) of the $O_2$-tolerant NiFe-hydrogenase of *R. gelatinosus* in different Duet expression vectors under the T7 promoter. The active site maturation genes (hypA to hypF) of the CBS hydrogenase were also cloned into different Duet vectors. During that year we initiated cloning of the largest structural gene *CooM*, which encodes the membrane-anchoring protein of the hydrogenase. In order to confirm the cloning efficiency and to avoid unwanted mutations, all plasmids prepared were fully sequenced. To understand if the CBS hydrogenase can use *E. coli*'s maturation machinery we co-transformed the large subunit and small subunit genes (*cooH and cooL*) along with the other structural genes (cooXU) into both BL21(DE3*) and a zero-background *E. coli* strain grown under the standard hydrogenase expression conditions. However, after IPTG induction, neither $H_2$ gas nor hydrogenase activity was detected.

In the past year, since the transformation of *cooXLUH* into a *BL21(DE3*)* and zero-background E-coli, *MC4100FTD(DE3*)* did not show hydrogen production or hydrogenase activity, the immediate goal of the research was to transform and express the active site assembly genes (*HypA* to *HypF*) along with the subunit and structural genes (cooXLUH) of the *CBS* hydrogenase constructed by NREL collaboration. After the transformation of the plasmids into the zero-background *E-coli* host we performed the following experiments to assess the minimum gene requirement for the expression of hydrogenase from *R. gelatinosus CBS*:

- Transformation of the double (*cooXLUHand hypCDEF)* and triple plasmids (*cooXLUH*, *hypAB and hypCDEF*) of the *CBS* hydrogenase genes in Duet vectors was achieved and the transformation efficiency was confirmed by agarose gel assays.
- Protein expression of the large subunit (CooH) of the *CBS* hydrogenase in the double transformed *E-coli* MC4100FTD(DE3*) was detected by western blotting (Figure 1). *R. rubrum* hydrogenase antibody (a gift by Dr. Steve Singer) was used for the immuno-detection.
- Protein expression of the small subunit (CooL) of the *CBS* hydrogenase in a triple transformed *E-coli* was detected by western blotting (Figure 2). Anti-CooL antibody has been produced and used against the small subunit of the CBS hydrogenase by our NREL collaborators.
- The largest structural gene cooM along with cooK and hypAB from *R. gelatinosus CBS* were cloned into a compatible vector with the Duet expression system developed by NREL.
- Hydrogenase activity was tested by using methyl viologen. No activity was detected in either double or triple transformed MC4100FDT(DE3*).
- The head-space hydrogen accumulation was tested by a gas chromatograph. No hydrogen gas was detected in either the double or triple transformed MC4100FTD(DE3*).

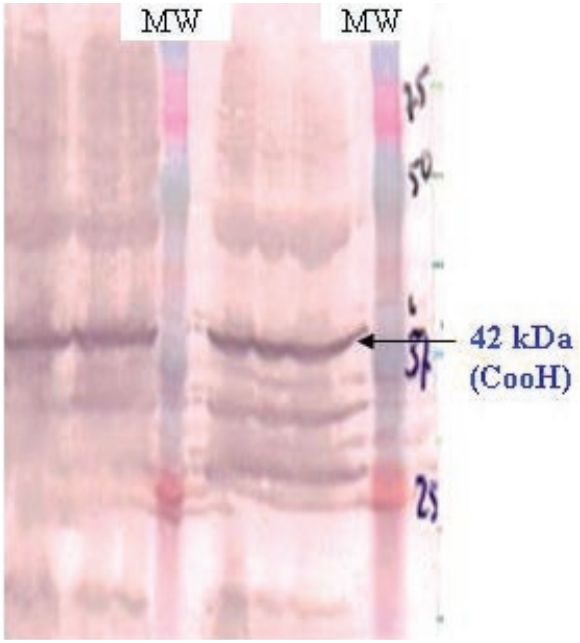


**FIGURE 1.** Detection of the expression of the large subunit of the *CBS* hydrogenase in a double transformed MC4100FTD(DE3*). Except for the standards, all other lanes were loaded with different amounts of the same sample.

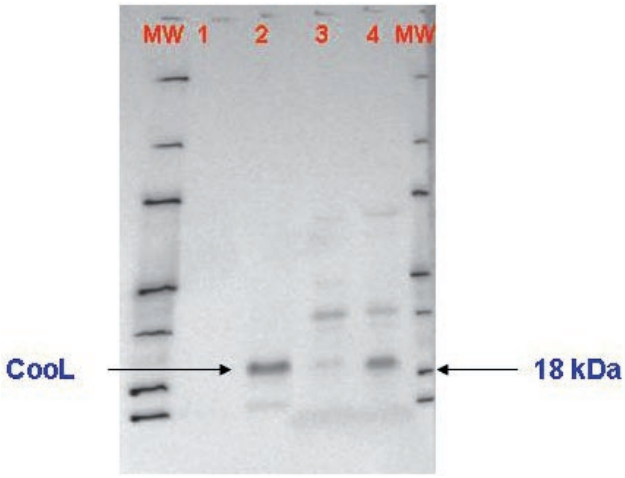


**FIGURE 2.** Detection of the expression of the small subunit of the CBS hydrogenase in a triple transformed MC4100FTD(DE3*). Lane 1: non-transformed *E. coli* host strain; Lane 2: *E. coli* transformed with 10 *R. gelatinosus* hydrogeanse genes; Lane 3: non-induced *R. gelatinosus* membrane; Lane 4: CO-induced *R. gelatinosus* membrane.

## Conclusions and Future Directions

Although much progress was made in terms of gene cloning and transformation into *E-coli* hosts of various combinations of gene constructs and expression of the large and the small subunits of the $O_2$-tolerable CBS hydrogenase, no enzyme activity has been observed yet. Since the co-transformations of all of the structural and maturation genes has not been completed yet, we are continuing our investigation of the minimum number of necessary genes for the expression of the fully functional CBS hydrogenase in *E-coli.*

The following tasks will be pursued in the third year of the research:

- Continue the expression studies of the *CBS* hydrogenase in *MC4100FTD(DE3*)*.
- Asses the expression of the hydrogenase genes of CBS in an *E-coli* strain both at the ribo nucleic acid level and protein level.
- Complete the co-transformations of the remaining genes (*cooMK*), which may be needed for expression of the active hydrogenase in the zero-background *E-coli* as well as into the new *E-coli* strains (e.g. *Rosetta 2*).
- Sub-clone the previously constructed hydrogenase genes from the *CBS* strain into diverse expression systems to express the fully efficient hydrogenase in *E-coli*.
- Purify and characterize the recombinant NiFe-hydrogenase from *E. coli.*
- Study $H_2$ generation under fermentation conditions to assess $H_2$ production capability of the recombinant enzyme in *E-coli.*

## FY 2008 Publications/Presentations

**1.** *Photobiological Hydrogen Research*, Vekalet Tek and George Philippidis, DOE 2008 Annual Merit Review, Arlington, VA, June 9-13, 2008.

**2.** Tek, V. and Philippidis, G., Cloning and Preliminary Expression Studies of NiFe-Hydrogenase from *R.gelatinosus CBS* in *E-coli*. The 8 th International Hydrogenase Conference, Hydrogenase and Hydrogen Production 2007, August 5-10, 2007, Breckenridge, CO.

**3.** Yu, Jianping, Vanzin, G., Tek, V., Smolinski, S. and Maness, P.C. Expression of an O2-Tolerant Evolving Hydrogenase in *E-coli* and in *Synechocystis 6803.* The 8 th International Hydrogenase Conference, Hydrogenase and Hydrogen Production 2007, August 5-10, 2007, Brekenridge, CO.

**4.** Maness, PC., Yu, Jianping, Vanzin, G., Tek, V. and Smolinski, S. The Construction of a *Synechocystis* Recombinant System for Solar $H_2$ Production. Oral Presentation. The 8 th International Hydrogenase Conference, Hydrogenase and Hydrogen Production 2007, August 5-10, 2007, Brekenridge, CO.

## References

**1.** Maness, P.C, Smolinski, S., Dillon, A.N., Heben, M.J., and Weaver, P.F. Applied and Environ. Microbiology. 2002, Vol. 68, No. 6: 2633-2636.

**2.** Maness, P.C., Huang, J., Smolinski, S., Tek, V., and Vanzin, G. Applied and Environmental Microbiology, 2005, Vol. 71, No 6: 2870-2874.

**3.** Leach, M.R. and Zamble, D.B. Current Opinion in Chemical Biology, 2007, 11:159-165.