II.H.5 Photobiological Hydrogen Research*

George Philippidis, Ph.D. (Primary Contact) and Vekalet Tek, Ph.D.
Applied Research Center
Florida International University (FIU)
10555 W. Flagler Street, EC 2100
Miami, FL 33174
Phone: (305) 348-6628; Fax: (305) 348-1852
E-mail: George.Philippidis@arc.fiu.edu

DOE Technology Development Manager:
Roxanne Garland
Phone: (202) 586-7260; Fax: (202) 586-9811
E-mail: Roxanne.Garland@ee.doe.gov

DOE Project Officer: Jill Gruber
Phone: (303) 275-4961; Fax: (303) 275-4753
E-mail: Jill.Gruber@go.doe.gov

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*Congressionally directed project

Objectives

- Isolate the structural genes encoding the subunits of the O_2-tolerant NiFe-hydrogenase from the *Rubrivivax gelatinosus CBS* strain developed by the National Renewable Energy Laboratory (NREL).
- Isolate the active site maturation genes of the CBS hydrogenase in collaboration with NREL.
- Clone both the structural and the maturation genes of the CBS hydrogenase into an *E. coli* host.
- Determine the minimum number of genes required to express a fully functional CBS hydrogenase in the host.
- Purify and characterize the recombinant NiFe hydrogenase from *E. coli*.
- Perform 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:

(AH) Rate of Hydrogen Production

(AI) Continuity of Photoproduction

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 technology necessary for developing a hydrogen infrastructure, as identified by DOE's Hydrogen Program.

Accomplishments

- Completed the cloning of the putative NiFe active site maturation genes (HypA through HypF) of the CBS hydrogenase.
- Completed the cloning of the structural genes cooK, cooL, CooX, CooU and CooH encoding the NiFe hydrogenase of *R. gelatinosus CBS*. Cloning of cooM, which encodes the membrane-anchoring protein of the hydrogenase, is underway.
- Co-transformed *E. coli* with both the large- and small-subunit genes of the CBS hydrogenase along with its structural genes.
- Expressed an inactive hydrogenase in *E. coli* by transforming the subunit genes (cooXLU) along with the large subunit cooH.
- Manipulated the zero-background *E. coli* strain to use as an alternative to the currently used BL21(DE3) to address the lack of hydrogenase activity.

Introduction

Hydrogen gas is a renewable energy carrier that addresses both the issues of energy security and energy independence, while preserving the quality of the environment. Microbial H_2 production is an attractive alternative to conventional processes. This R&D project aims at the development of a cost-effective biochemical system for hydrogenase production via *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

The immediate goal of this research is to clone the active site assembly genes (HypA to HypF) and the structural genes cooK, cooL, cooX, cooU and cooH of the *R. gelatinosus CBS* O_2-tolerant NiFe-hydrogenase previously identified by NREL scientists. These genes will be transformed into *E. coli* hosts to express a fully functional hydrogenase. The large subunit of the novel NiFe-hydrogenase cooH will be 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.

Biosynthesis of the NiFe hydrogenase active site is a complex process involving the action of the auxiliary 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 and the structural 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

The structural genes (cooXLU, cooH, cooK) and the active site assembly genes (hypA through hypF) of the *R. gelatinosus* CBS hydrogenase have been cloned into Duet protein-expression vectors under the control of the T7 promoter. The cooH encodes the large subunit of the hydrogenase, which harbors the NiFe-metal center of the enzyme. Cloning of the largest structural gene CooM, which encodes the membrane-anchoring protein of the hydrogenase, is underway. 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 to assemble a hydrogenase, we co-transformed the large subunit gene cooH along with the other structural genes into a zero background *E. coli* strain grown under the standard hydrogenase expression conditions. However, after isopropyl β-D-1-thiogalactopyranoside (IPTG) induction neither H_2 gas nor hydrogenase activity was detected. Head-space gas concentrations were quantified by gas chromatography and the hydrogenase activity assays were performed by using methyl viologen as an artificial electron acceptor. Agarose gels confirmed the cloning of the maturation genes HypF-HypCDE (Figure 1) and the cloning of one of the structural genes of the CBS hydrogenase, cooK, into the Duet expression vectors (Figure 2). Figure 3 represents confirmation of both (1) the cloning of the structural gene constructs cooLXU and cooH into the Duet expression vector and (2) the transformation efficiency of this plasmid in the *E. coli* host.

Conclusions and Future Directions

Progress was made ahead of schedule in terms of gene cloning and transformation into *E. coli* hosts of various combinations of gene constructs. However, no enzyme activity was observed. Since the co-transfomations of all the structural and maturation genes is not completed yet, we are continuing our investigation of the minimum number of genes necessary for the expression of the fully functional CBS hydrogenase in *E. coli.*

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

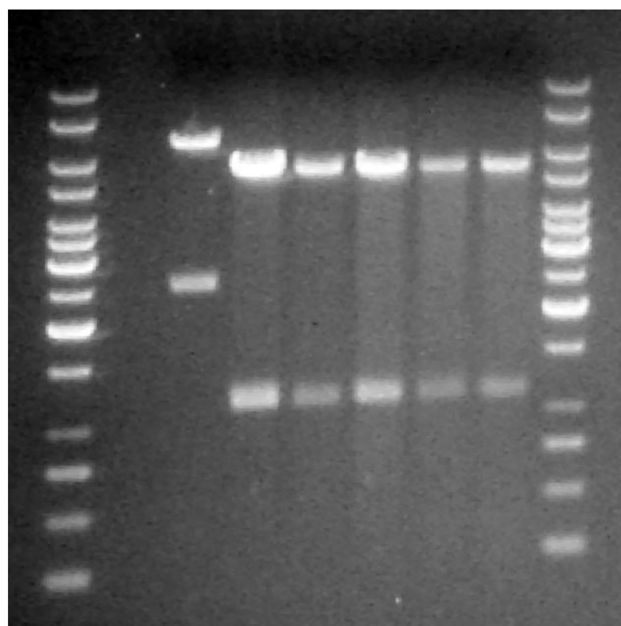

FIGURE 1. Confirmation of Cloning of HypFCDE in a Duet Expression Vector (line #3 from the left)

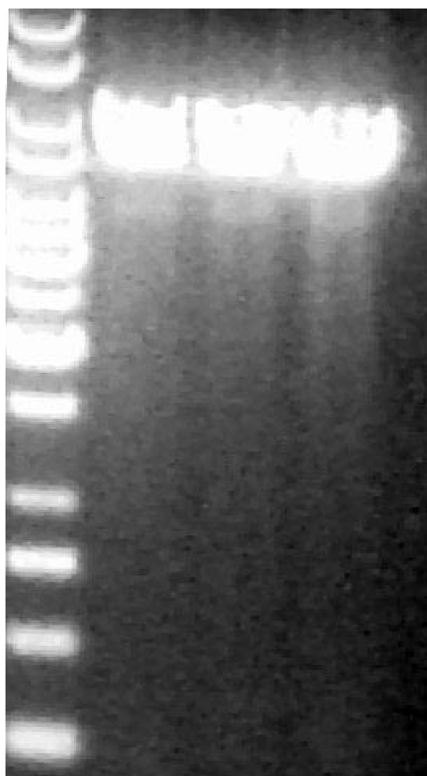

FIGURE 2. Confirmation of Cloning of cooK in a Duet Expression Vector (lines # 2, 3, and 4 from the left)

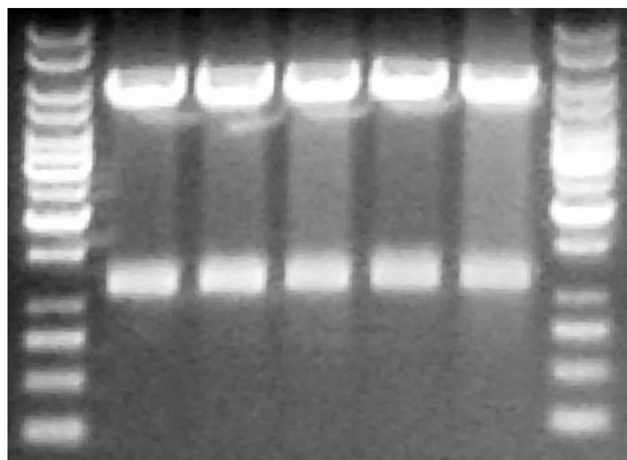

FIGURE 3. Confirmation of the Transformation Efficiency of cooHXLU into *E. coli* (line #3 from the left)

- Complete the cloning of cooM, which may be needed for expression of the active hydrogenase; new expression vectors may be required because of the large size of cooM (4 kb).
- A zero-background *E. coli* strain, modified by our collaborators at NREL, will be used as an alternative to the *E. coli* host currently used.
- Complete the co-transformations of the remaining genes into the new *E. coli* strain.
- Purify and characterize the recombinant NiFe-hydrogenase from *E. coli.*
- Study H_2 generation under fermentation conditions to assess H_2 production capability.

FY 2007 Publications/Presentations:

1. *Photobiological Hydrogen Research*, Vekalet Tek and George Philippidis, DOE 2007 Annual Merit Review, Arlington, VA, May 15-18, 2007.

2. *Expression of an O_2-tolerant evolving hydrogenase in E. coli and in Synechocystis 6801.* Jianping Yu, Gary Vanzin, Vekalet Tek, Sharon Smolinski and PinChing Maness, 16th Western Photosynthesis Conference, Pacific Grove, CA, Jan. 4–7, 2007.

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.