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Photobiological Hydrogen Research

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Abstract

The goal of this R&D project is to identify the **structural** and **active site** maturation genes of an O_2-tolerant [NiFe]-hydrogenase, which are critical to optimal expression of the enzyme in *E. coli.* The hydrogenase was derived from the non-sulfur purple photosynthetic bacterium ***Rubrivivax gelatinosus CBS*** developed by NREL. This work will contribute to the development of a more efficient and robust system for photosynthetic hydrogen production in *E. coli*, a robust industrial microorganism. Expression in *E. coli* will also facilitate eventual expression of the hydrogenase in cyanobacteria. *CBS* hydrogenase is a heterodimeric protein without a C-terminal extension and is assumed to share maturation process characteristics with *E. coli* hydrogenase 3.

To date, we have cloned and transformed hydrogenase assembly genes (*hypA-F*) along with the subunit (*cooLH*) and the structural genes (*cooXUK*) of the NiFe-hydrogenase into a zero background *E.coli* (*MC4100FTD(DE3**). CooH carries the active metal center, whereas CooL encodes its small subunit of the enzyme. Transformation of *cooM*, the largest gene encoding the membrane-anchoring protein of hydrogenase, is underway. We have been optimizing the growth conditions of *E-coli* to express a fully active recombinant hydrogenase from *R.gelatinosus CBS*. To now, we have detected the expression of the subunit proteins of the *CBS* hydrogenase in the zero background, triple transformed *E-coli.*

Objectives

Overall

Identify which **structural** and **active site** maturation genes of the O_2-tolerant [NiFe]-hydrogenase from the bacterium ***Rubrivivax gelatinosus CBS*** are critical to optimal expression of the enzyme in *E. coli.*Expression in *E.coli* will facilitate eventual expression of the hydrogenase in *cyanobacteria* at NREL.

May 2007 – June 2009

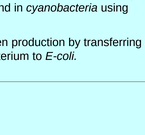
Clone the largest structural gene *cooM* of the hydrogenase into duet expression vectors under the T7 promoter.

Transform the clones in Duet vectors (*cooXLUH*, *cooMK*, *hypABCDEF*), along with the hydrogenase large subunit into a proper *E.coli* host, such as *MC4100FTD(DE3*),* to express an active hydrogenase.

Optimize the growth conditions of *MC4100FTD(DE3)** for the expression of a fully efficient hydrogenase in *E-coli.*

Determine the efficiency of the recombinant hydrogenase from *CBS* in *MC4100FTD(DE3*)* by testing for the presence of hydrogen and for hydrogenase activity.

Detect and purify the fully efficient recombinant hydrogenase in *E-coli.*



Approach Overview

Optimizing biological hydrogen production requires understanding the enzymatic pathways through which H_2 is formed at the molecular level. Work in this project is divided into (1) fundamental aspects designed to understand the protein expression system of the O2 tolerant [NiFe]-hydrogenase from the photosynthetic bacterium ***R. gelatinosus CBS*** in an *E. coli* host and (2) applied aspects focusing on H_2 production by *E.coli.*

Task 1.0 — July 2006-May 2007

• Clone structural and the subunit genes encoding for the large subunit of hydrogenase (*cooH*) into Duet Vectors: *cooM, cooK, cooL, cooU, and cooX*
-One of the genes carries strep-II tag (cooH: large subunit of hydrogenase)
-Confirm cloning by DNA sequencing of the plasmids

Task 2 .0 — Sep. 2006-August 2007

•Clone active site assembly genes into Duet Vectors: *HypA* through *hypF*
(putative transcriptional regulator gene; not needed in IPTG-inducible T7 polymerase system)
-Confirm cloning by DNA sequencing of the plasmids.
For fully active hydrogenase in *E. coli* active site maturation is vital.

Task 3 .0 — March 2007-Nov. 2007

•Transform the cloned hydrogenase genes into proper *E. coli hosts.*
-Confirm transformation efficiency by agarose gel.
-Test H_2 gas production by gas chromatography.
-Test hydrogenase activity by methyl viologen assay.
-Confirm hydrogenase expression by western blotting and MS analysis.
- Co-transform plasmids carrying the essential genes for expression of active hydrogenase and H_2 production.

Con't: Approach Overview

Task 4.0 — May 2007-May 2008

•Optimize cell conditions to express the fully active oxygen-tolerant hydrogenase in E-coli.

•Determine the minimum number of hydrogenase genes required for fully efficient hydrogenase expression

•Purify the recombinant hydrogenase from E-coli

•Characterize the purified enzyme

Task 5.0 — May 2007-May 2008

▪Optimize H_2 Production by Physiological Means (Fermentation Techniques)

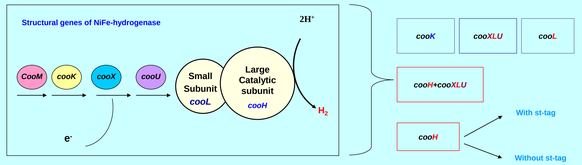
•Uniqueness of the Overall Approach

•Hydrogenases in *E-coli* and water-splitting cyanobacteria are very sensitive in the presence of oxygen. To surmount this problem and produce microbial H_2 via an efficient enzyme, we need to transfer and express an oxygen-tolerant hydrogenase from the photosynthetic bacterium in *E-coli* and in *cyanobacteria* using molecular biology and biochemistry tools.

•Developing advanced bacteria for fermentative and photosynthetic hydrogen production by transferring and expressing a more oxygen-tolerant hydrogenase from a photosynthetic bacterium to *E-coli.*

Technical Accomplishments/Progress/Results

Task 1-3:Overall Clones of the O_2-Tolerant [NiFe]-Hydrogenase from *R. gelatinosus CBS* in Duet Vectors.



Maturation genes of the hydrogenase were cloned into Duet vectors and transformed into a zero background *E-coli*, *MC4100FTD(DE3*).*

Technical Accomplishments/Progress/Results

Task 3.a: Double plasmid transformation of the hydrogenase genes into *MC4100FTD(DE3*)*

▪**We co-transformed the Duet plasmids which carry the hydrogenase genes: The maturation genes (*hypCDEF*) along with the subunit genes (cooH, cooL), and structural genes (*cooUX* of the *CBS* hydrogenase have been co-transformed into *MC4100FTD147 (DE3)*.***

▪**We confirmed the transformation by DNA gel assay. Transformation efficiency: 100 %**

▪**Headspace gas analysis by GC showed no detectable H_2 gas.**

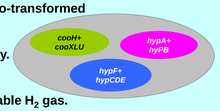
▪**Hydrogenase testing (MV assay) showed no detectable enzyme activity**.

•T7 Polymerase gene was transferred into host strain MC4100FTD147(DE3*) via phage lysogenization by our collaborators at NREL. Zero-background strain was used as an alternative to BL21(DE3*), which was originally selected as an *E. coli* host for the protein expression in this project.

Technical Accomplishments/Progress/Results

Task 3.b: Triple transformation of the hydrogenase genes into *MC4100FTD(DE3*)*

▪**Triple plasmid transformation: The maturation genes (*HypAB*, *hypCDEF*) along with the subunit and structural genes (*cooH, cooL, cooXU*) of the oxygen-tolerant NiFe-hydrogenase were co-transformed into *MC4100FTD147 (DE3)*.***
▪**Three Duet vectors carried the 10 hydrogenase genes**
-We confirmed the transformation by using DNA gel assay. Transformation efficiency: 100 %



-Headspace gas analysis by using GC showed no detectable H_2 gas.

Task 4.0: Hydrogenase Expression in a triple transformed *E-coli* Strain

▪**The activity of the hydrogenase enzyme was tested by methyl viologen - no activity.**

▪**The protein expression of the large subunit has not been detected yet.**

- The protein expression of the small subunit was detected by western blotting (Fig.3)

Technical Accomplishments/Progress/Results

Task 3: Transform the Triple Plasmids which carry the Hydrogenase genes into a Zero background *E-coli*

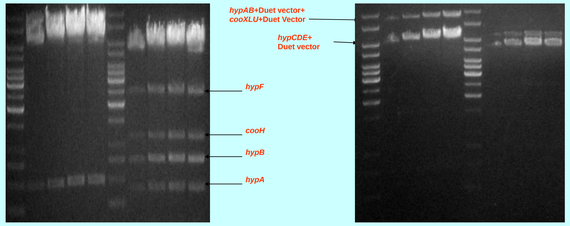


Fig.1-Agarose Gel Assays of the Hydrogenase genes to Test the Triple Transformation into *MC4100FTD(DE3*).*

Technical Accomplishments/Progress/Results

Task 4a: Detection of the Large Subunit (CooH, 42 kDa) of the Non-tagged CBS Hydrogenase in a Double Transformed E-coli, *MC4100FTD(DE3*),* by Western Blotting .

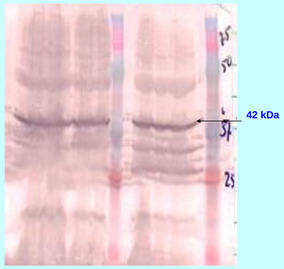


Fig.2.

The large subunit of the hydrogenase was detected in a double transformed *MC4100FTD(DE*)* by western blotting.

▪Polyclonal anti-rabbit hydrogenase antibody was produced against the *R. rubrum* hydrogenase *(*kindly provided us by Dr.Steve Singer)

Technical Accomplishments/Progress/Results

Task 4.b: Detection of the expression of the small subunit CooL of the *CBS* hydrogenase in *MC4100FTD147(DE3*).*

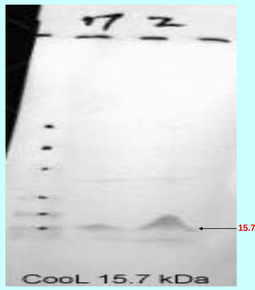


Fig.3.

The small subunit CooL of the hydrogenase was detected in a zero background, triple transformed (10 hydrogenase genes in three duet vectors) *E-coli MC4100FTD(DE3*)* by western blotting with NREL collaboration. The specific anti-hydrogenase antibody against the small subunit was produced by the NREL collaborators.

Remaining Work (FY2008-09)

•Complete cloning
-*CooM* (may be needed for active hydrogenase expression) will be cloned into an appropriate vector other than the Duet vectors.

•Complete co-transformations, including *cooK* and *cooM*, if they are still needed
-To test how many genes of the hydrogenase are needed to obtain a fully functional enzyme and to produce microbial hydrogen, we will co-transform the remaining structural genes of *CBS* hydrogenase into a proper *E. coli*.
-CooK and cooM, in addition to the triple plasmids, will be co-transformed into *MC4100FTD147(DE3*).*
•Co-transform the entire clones of the hydrogenase into alternative *E-coli strains*
-Entire genes of the hydrogenase will be co-transformed into *BL21(DE3*) and/or* Rosetta after evaluation of the *MC4100FTD(DE3*)* results

•Optimize the conditions of protein expression for hydrogenase
-Express active hydrogenase in *BL21(DE3*)* and/or Rosetta
-Express active hydrogenase in *MC4100FTD147(DE3*)*

•Assess the expression of hydrogenase genes in *E-coli* by mass spec analysis
- In order to detect how many hydrogenase genes are expressed in double and triple transformed *E-coli* hosts, we will subject the we will subject the protein samples to mass spec analysis.
•Purify and Characterize Hydrogenase
- Following expression of active hydrogenase we will purify and characterize the enzyme from *E. coli*.
•Optimize H_2 Production by Physiological Means
- We will study H_2 generation under fermentation conditions.

Summary

Task 1.0
• Cloning of cooM, which encodes the membrane anchoring protein of the O2-tolerant NiFe-hydrogenase from *R. gelatinosus CBS,* into a Duet expression vector is pending.

Task 2.0
•This task was completed last year (ahead of time)

Task 3.0
•Double Transformation:
The subunit genes, cooL and cooH without strep-II-tag, and the structural genes, *cooXU*, along with the the maturation genes(hypCDEF) were co-transformed into *MC4100FT147(DE3).*
After the IPTG induction, double transformed (8 genes in two different Duet vectors) *E-coli* did not generate H_2 gas and did not show hydrogenase activity.
•Triple Transformation:
Two of the maturation genes (*hypAB*, which were previously constructed in a Duet vector) along with *hypCDEF* were used to transform *MC4100FTD(DE3*).*

Task 4.0
Expression of the small subunit CooL of the *CBS* hydrogenase was detected in triple-transformed *E-coli* by western blotting (NREL collaboration).
Expression of the large subunit, CooH without st-II-tag, in double transformed *E-coli* by western blotting (NREL collaboration).
Following **tasks 1-3** we will purify and characterize the membrane-bound anaerobic hydrogenase of *E. coli*.
Preparation of the cell membrane of *R. gelatinosus CBS* as a tool.

Relevance
Help to answer the fundamental questions necessary for assessing the feasibility of advanced biological hydrogen production technologies.

Approach
Develop advanced microbes by transferring oxygen-tolerant hydrogenase genes for fermentative and photobiological hydrogen production.

Technical Accomplishments and Progress
Cloned and transformed 10 genes of hydrogenese into *E-coli* and verified expression of the subunit genes.

Benefits from this Project

•This project aims at determining the minimum number of auxiliary and structural genes required for the expression of a fully functional NiFe-hydrogenase from *R.gelatinosus CBS* in *E. coli*.

•New genetic constructs developed in this project will be used in cyanobacterial expression work at NREL.

•The research will improve our understanding of how solar-driven, water-splitting cyanobacteria can become hydrogen-producing vehicles.

•Once the molecular manipulations are complete, H_2 production via fermentation using *E. coli* and cyanobacteria will serve as a technology platform for commercialization.

Presentations

Poster Presentations:
•**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.
•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.

Oral presentation:
Maness, PC., Yu, Jianping, Vanzin, G., **Tek, V**. and Smolinski, S. The Construction of a *Synechocystis* Recombinant System for Solar H2 Production. Oral Presentation. The 8 th International Hydrogenase Conference, Hydrogenase and Hydrogen Production 2007, August 5-10, 2007, Brekenridge, CO.

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