Photobiological Hydrogen Research

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Project ID: PDP 7

Overview

Timeline

- Start: June 2006
- Finish: May 2009
- 65 % Complete

Budget

DOE: $565,000 (3-years)
FIU Cost share: $141,252
Total: $706,252

Barriers

(Y) Rate of Hydrogen Production
(Z) Continuity of Photoproduction
(AA) Systems Engineering

Partners

NREL
- PinChing Maness
- Dr. Maria Ghirardi
- Dr. Jianping Yu

- Project Lead: Dr. George Philippidis

Objectives

Overall	Identify which **structural** and **active site** maturation genes of the O_2- tolerant NiFe-hydrogenase from the photosynthetic 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.
March 2007-April 2008	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 O_2 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
Completed: 85 %

- **Clone structural 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
Completed: 100 %

- **Clone active site assembly genes into Duet Vectors: From *hypA to hypF***

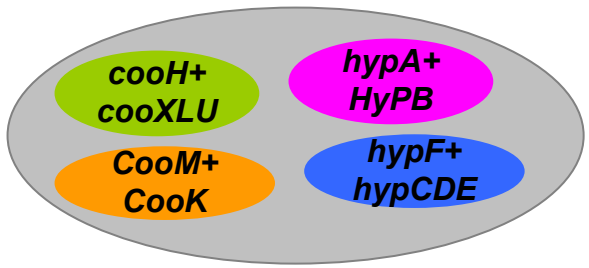
(putative transcriptional regulator gene, *lytR*: not needed in IPTG-inducible T7 polymerase system)
-Confirm cloning by DNA sequencing of the plasmids.
-For the fully active hydrogenase in *E. coli* , active site maturation is vital.

Task 3 .0

March 2007-Nov. 2007
Completed : 40 %

- **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 the expression of the active hydrogenase and the H_2 production.



Approach Overview (cont'd)

Task 4.0

May 2007-May 2008
Completed: 10 %

- Optimize cell conditions to express the fully active *CBS* 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

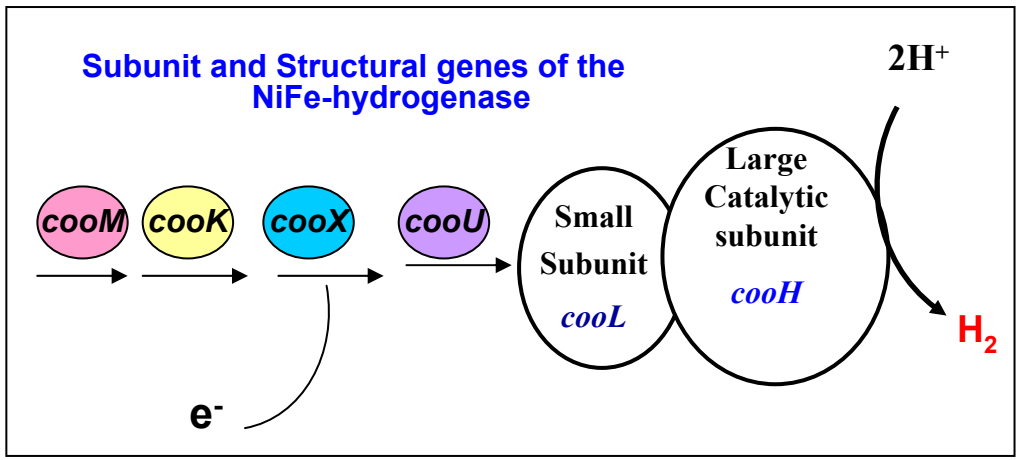
•Uniqueness of the Overall Approach

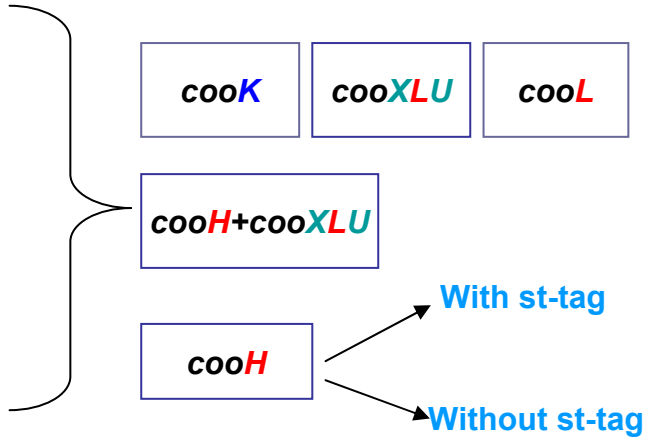
- Hydrogenases in E-coli and water-splitting cyanobacteria are very sensitive in the presence of oxygen. To overcome this problem and produce microbial H_2 via an efficient enzyme, we need to transfer and express an oxygen-tolerant hydrogenase from the photosynthetic bacterium in *E-coli* and in cyanobacteria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

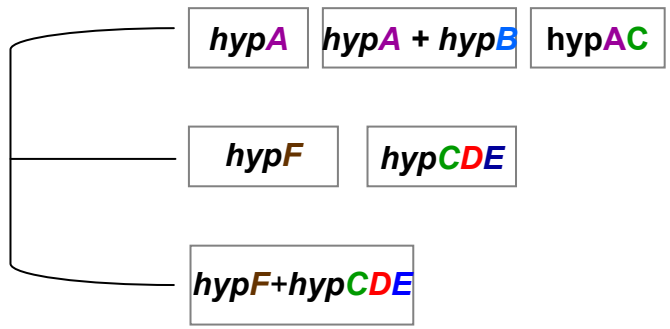
Task 1- 3.

Fig.1. 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*).*

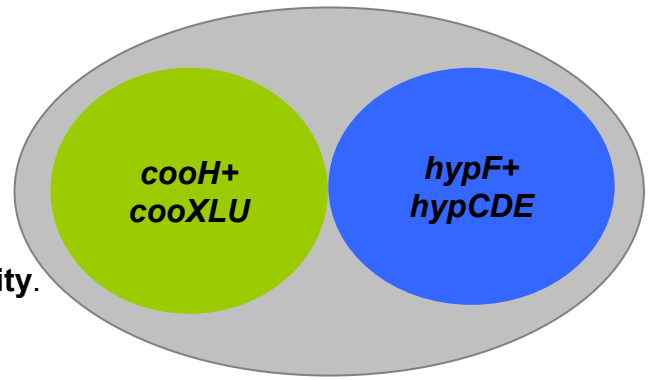


Technical Accomplishments/Progress/Results

Task 3.0 (cont'd)

•Double 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 the structural genes (*cooUX* of the *CBS* hydrogenase have been co-transformed into *MC4100FTD147 (DE3*)*- see Fig. 2.
- 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 *FTD147 (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.

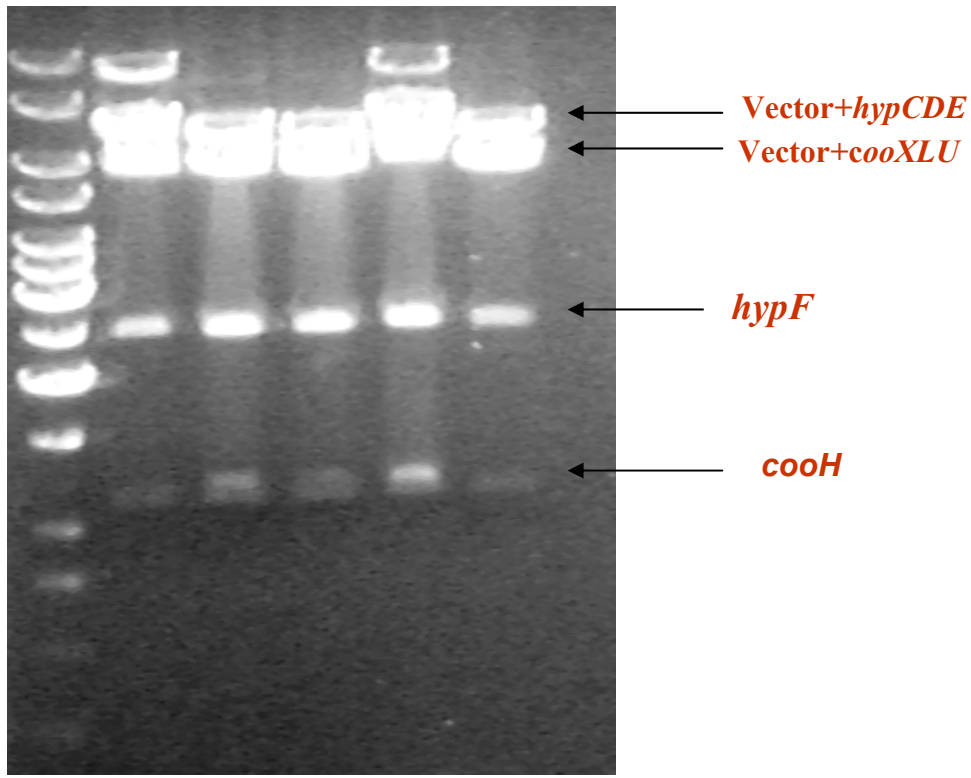
Fig.2.0 Agarose gel shows the transformation efficiency of the double plasmids in a zero background *E-coli*

Technical Accomplishments/Progress/Results

Task.3.0 (cont'd)

Fig.2. Agarose Gel Assay to Test the Transformation Efficiency of the Hydrogenase genes in *MC4100FTD(DE3*)* (Double Transformed).

Two different duet plasmids carrying the maturation genes (*hypCDEF*), the subunit genes (*cooLH*), and the structural genes (*cooUX*) of the *CBS* hydrogenase were transformed into a zero background *E-coli*, *MC4100FTD(DE*)*.

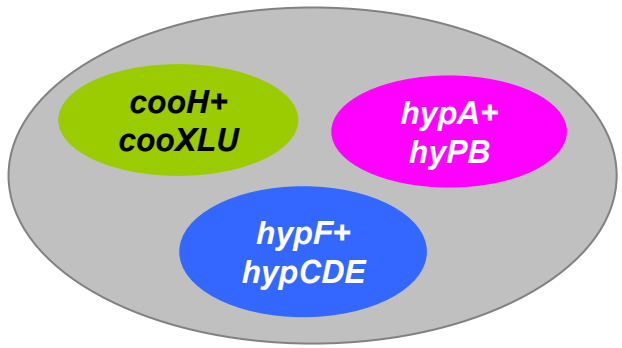


Technical Accomplishments/Progress/Results

Task. 3.0 (cont'd):

•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 (Fig.4)
- 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

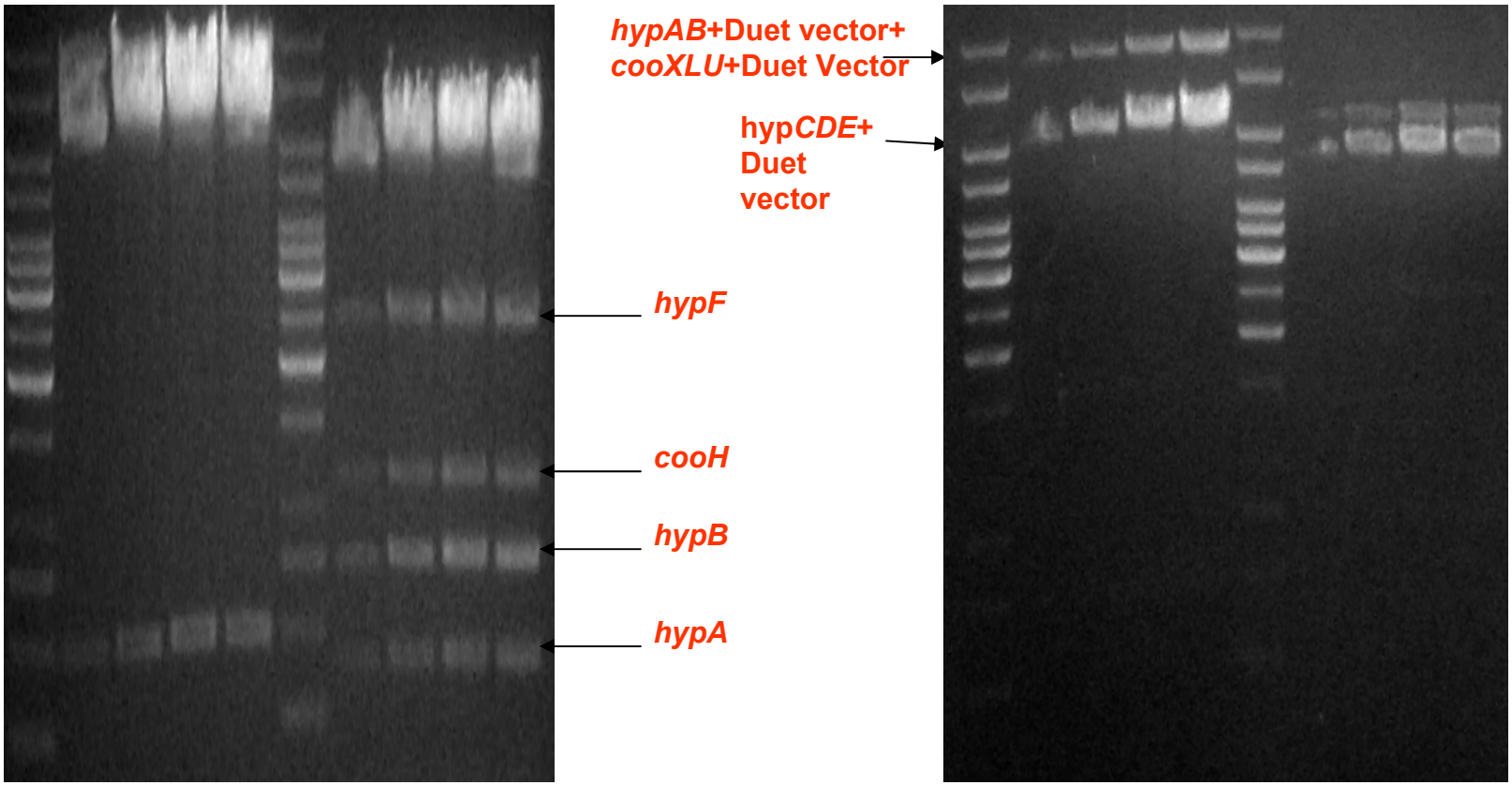
Hydrogenase Expression in a triple transformed *E-coli* Strain

- 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.5).

Technical Accomplishments/Progress/Results

Task.3.0 (cont'd)

Fig.3. Agarose Gel Assays of the Hydrogenase genes to Test the Triple Transformation into *MC4100FTD(DE3*)* .



Technical Accomplishments/Progress/Results

Task 4.0 (cont'd)

- **Membrane preparation of *R. gelatinosus CBS* to use as a tool**

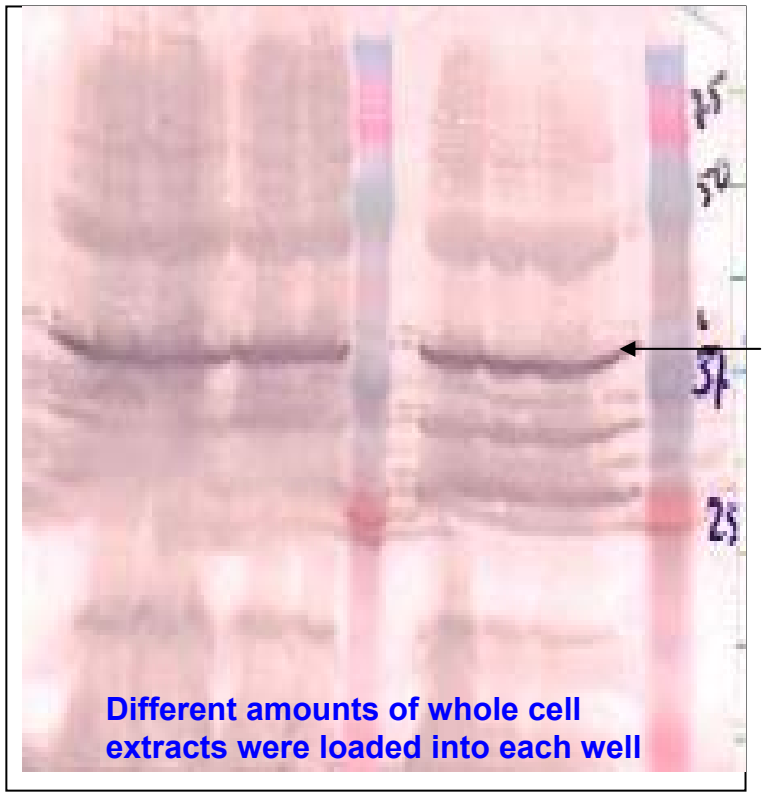
- The cell membrane was prepared and used to characterize the recombinant hydrogenase in *E-coli* by comparing it with the wild type hydrogenase from *CBS*.

- Oxygen-tolerant hydrogenase (wild type enzyme) in the original host is a fully active protein.

Technical Accomplishments/Progress/Results

Task 4.0(continue) :

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

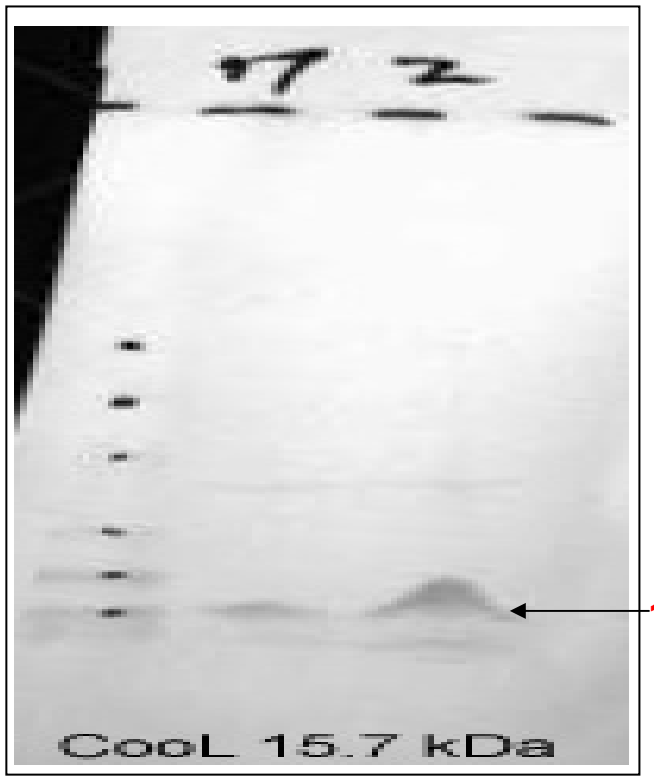


- The large subunit of the hydrogenase was detected in double transformed *MC4100FTD(DE*)* by western blotting.
- Polyclonal anti-rabbit hydrogenase antibody was produced against the *R. rubrum* hydrogenase (kindly provided us by Dr.Steve Singer).

Technical Accomplishments/Progress/Results

Task. 4.0. cont'd)

Fig.5: Detection of the expression of the small subunit *CooL* of the *CBS* hydrogenase in *MC4100FTD147(DE3*).*



15.7 kDa
cooL

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)

•**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 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*)* or Rosetta after evaluation of the *MC4100FTD(DE3*) results.*

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

•**Assess the expression of the 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 protein samples to mass spec analysis.

Remaining Work (FY2008-2009) (cont'd)

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.

Project Summary

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.

Summary (cont'd)

•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

•Cloning of the maturation genes of the *CBS* hydrogenase into Duet vectors was completed last year.

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 *hypCDE*F were transformed into *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.