DOE Hydrogen Program

Hydrogen from Water in a Novel Recombinant Oxygen-Tolerant Cyanobacterial System

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J. Craig Venter INSTITUTE

Overview

Timeline

- Project start: 5-01-05
- Project end: N/A
- Percent complete: 10%

Budget

- Total project funding
  - $720K (Contractor share)
- Funding for FY06
  - $0 for the Venter Institute
  - $26K for NREL
- Funding for FY07
  - $175K for the Venter Institute
  - $100K for NREL

Barriers

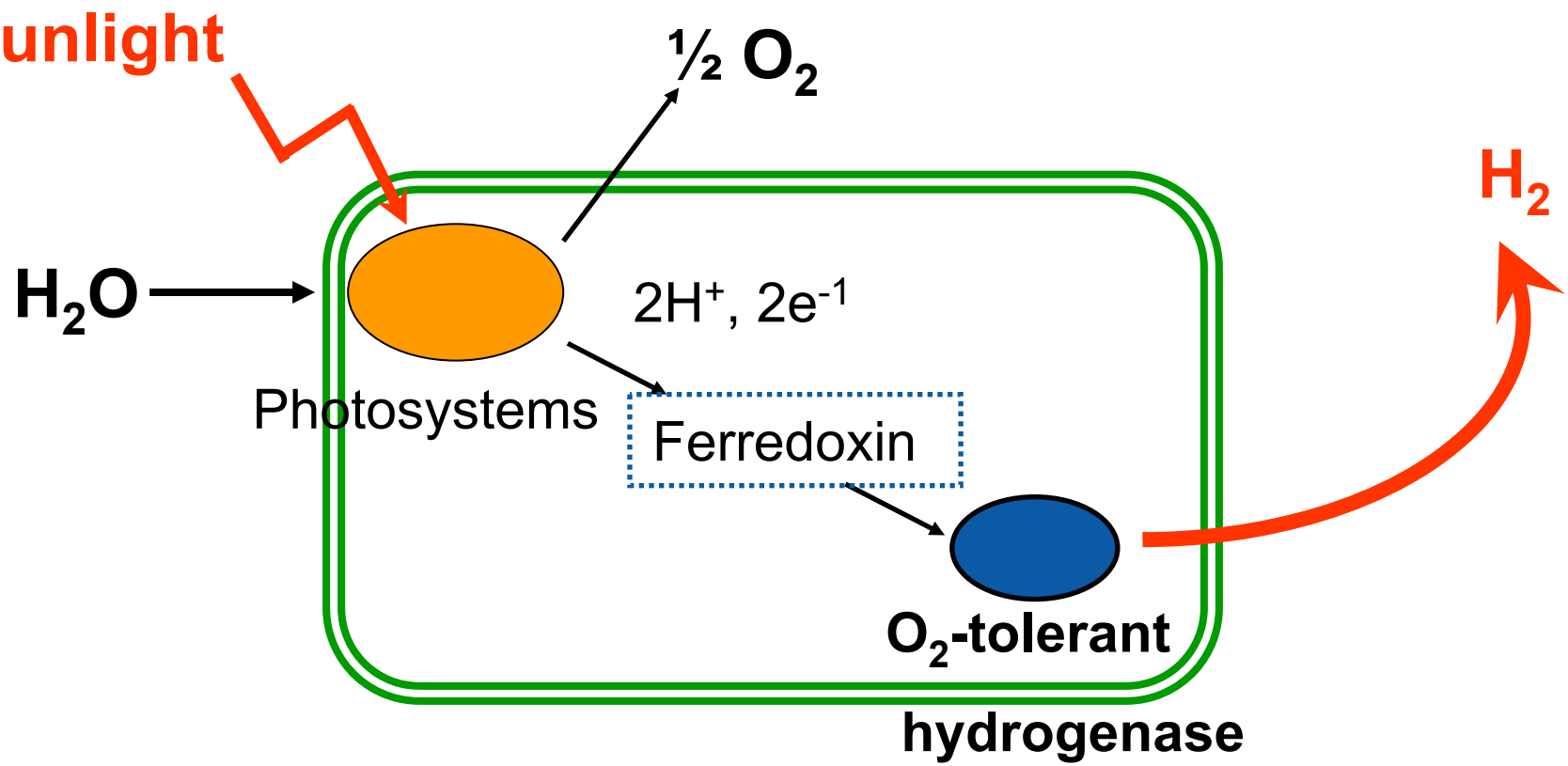
- Barriers addressed
  - Barrier Z, continuity of H_2 photo-production

Partner

- National Renewable Energy Laboratory

Objective

Develop an O_2-tolerant cyanobacterial system for continuous light-driven H_2 production from water



- The overall goal is to produce a cyanobacterial recombinant to produce H_2 continuously

Approaches to address hydrogenase O_2-sensitivity barrier

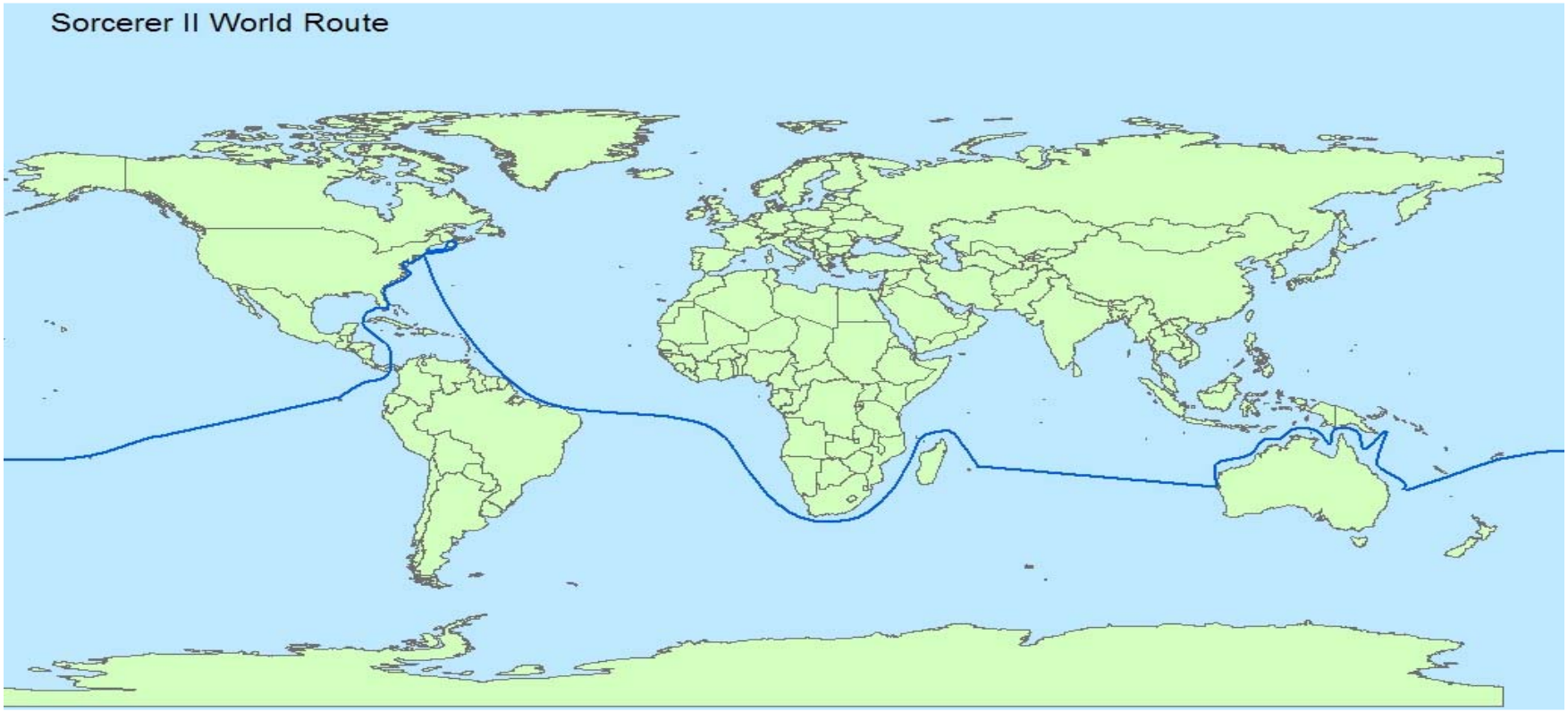
- Problem: Cyanobacteria have the ability to split water photolytically into O_2 and H_2, but their hydrogenases are highly O_2-sensitive. In contrast, certain bacteria have O_2-tolerant H_2-evolving hydrogenases, but they can not use water as the electron donor.

- Approach: transfer O_2-tolerant hydrogenases into cyanobacteria
    1. Identifying novel O_2-tolerant hydrogenases from VI's sampling in international waters and transferring them into cyanobacteria
    2. Transferring known O_2-tolerant hydrogenases into cyanobacteria

Technical Approach 1 (Venter Institute)

- Identification of novel O_2-tolerant hydrogenases from environmental microbes in international water

  (This task was not funded by DOE during 2006, and the following progress was made with internal funding from the Venter Institute.)



Sorcerer II Expedition: an Global Ocean Sampling Project accomplished at the Venter Institute

Technical Accomplishments/ Progress

The Sargasso Sea Project: A Pilot Study for VI's Sorcerer II Expedition

- Generated 1.045 billion base pairs of non-redundant sequences
- Found 1800 genomic species, including 148 new bacterial species
- Identified 1.2 million new genes, including 782 new rhodopsin-like photoreceptors

The Sorcerer II Global Ocean Sampling Expdition

- Generated 7.7 million sequencing reads equivalent to 6.3 billion bps of non-redundant sequences
- Identified 6.12 million proteins (covering nearly all known prokaryotic protein families) that nearly double the number of current proteins
- Revealed 3995 GOS-only clusters that have no homology to known protein families
- The rate of new protein families discovered is linear with the addition of new sequences, implying we are still far from discovering all the protein families in nature.

Environmental microbes have significant potential in carrying new genes or pathways that may improve efficiency of renewable energy production.

Technical Accomplishments/ Progress

Searched 1.2 million peptides in the Sargasso Sea Dataset for putative novel NiFe-hydrogenases

- 20 peptide hits representing the sequences from 10 NiFe-hydrogenases were identified by using 14 Hidden Markov Models (HMMs) of hydrogenases that we constructed.

- Three of 10 NiFe-hydrogenases are known hydrogenases, which are

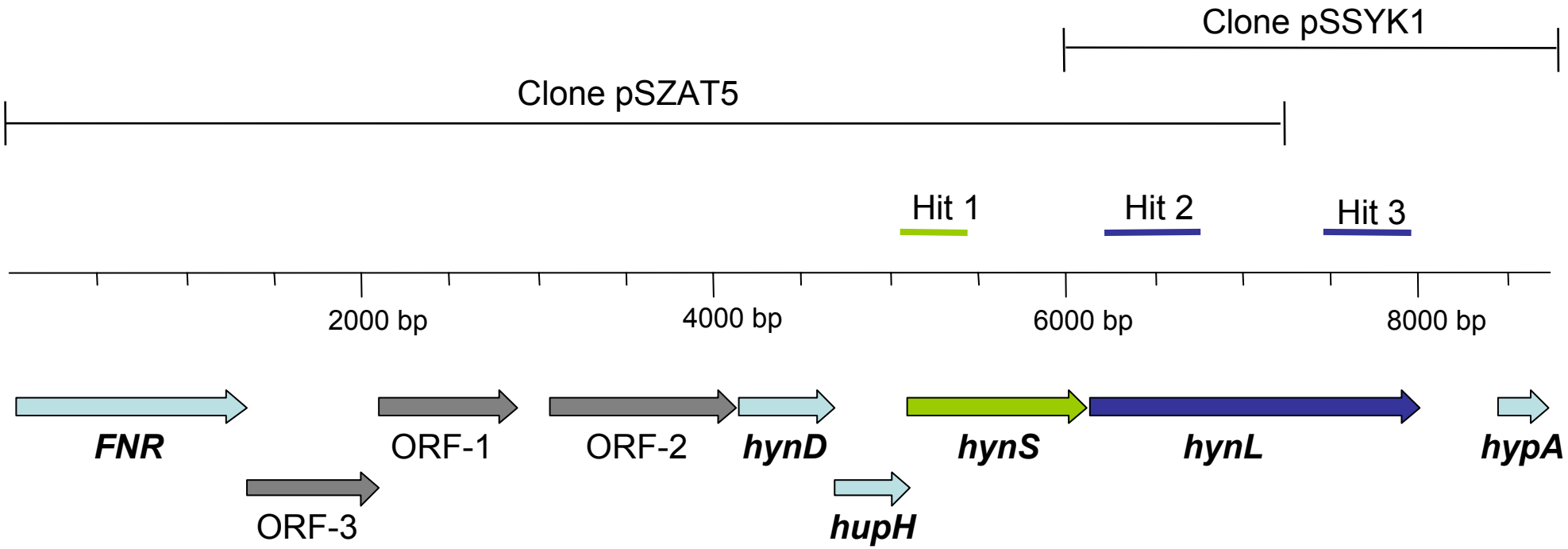
  Shewanella oneidensis quinone-reactive H_2ase (8 hits)
  Citrobacter freundii H_2ase-1 (2 hits)
  E. coli/S. enterica H_2ase-3 (1 hit)

- The remaining 7 H_2ases appear to be novel, with homology to

  E. coli/S. enterica H_2ase-2 (3 hits)
  E. coli/S. enterica H_2ase-3 (1 hit)
  E. coli/S. enterica H_2ase-4 (1 hit)
  Gloeothece sp. uptake H_2ase (1 hit)
  Thiocapsa roseopersicina O_2-stable H_2ase (3 hits)

Technical Accomplishments/ Progress

Cloned genes of a putative novel hydrogenase from the Sargasso Sea with strong homology to a *Thiocapsa* O_2-tolerant hydrogenase



hynL: Large subunit of novel hydrogenase

hynS: Small subunit of novel hydrogenase

hynD: NiFe-hydrogenase maturation factor

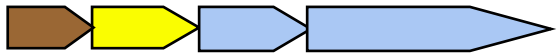
hupH and *hypA*: NiFe-hydrogenase expression protein

Systems Used to Heterologously Express the Novel Hydrogenase

Host organism: *Thiocapsa roseopersicina*

- Well defined wild-type strain contains 3 different hydrogenases.
- Hydrogenase biosynthesis is understood in detail.
- A suitable mutant (*ΔhynSL, ΔhupSL, ΔhoxH*) is constructed.
- Genetic techniques have been developed for this organism.

Expression vector pAmDHSL, for expression of

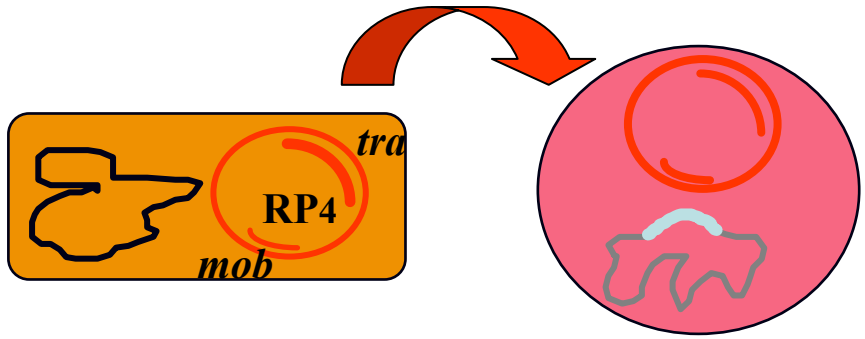


- pAmDHSL can self-replicate in *T. roseopersicina*
- It is transferred from *E. coli* into *T. roseopersicina* through conjugation.

Hydrogenase activity of *T. roseopersicina*

WT strain vs. the mutant (*ΔhynSLΔhupSLΔhoxH*)

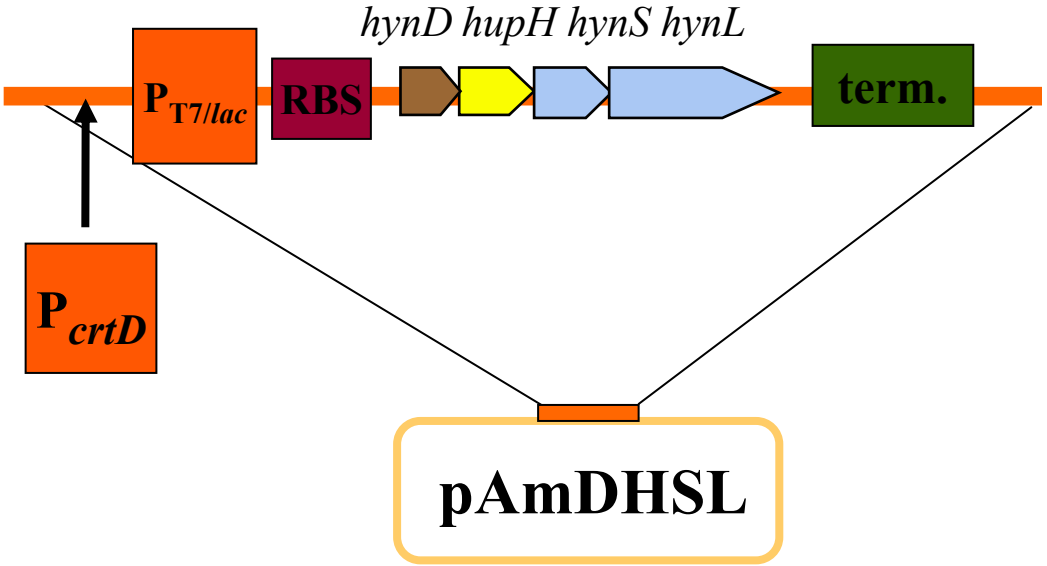
Conjugational system

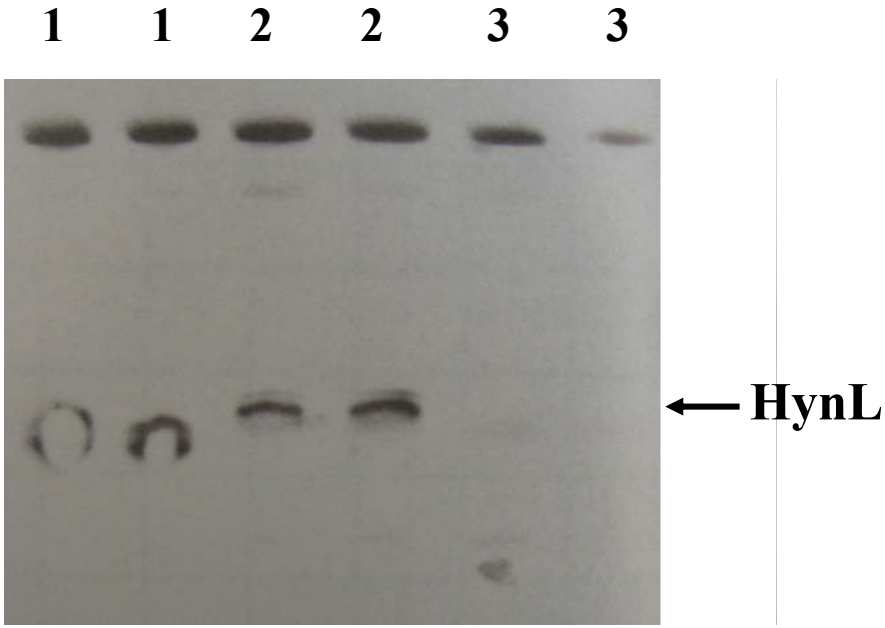


E. coli ***T. roseopersicina***

Technical Accomplishments/ Progress

The novel hydrogenase from environmental samples in the Sargasso Sea is heterologously expressed in *Thiocapsa roseopersicina*





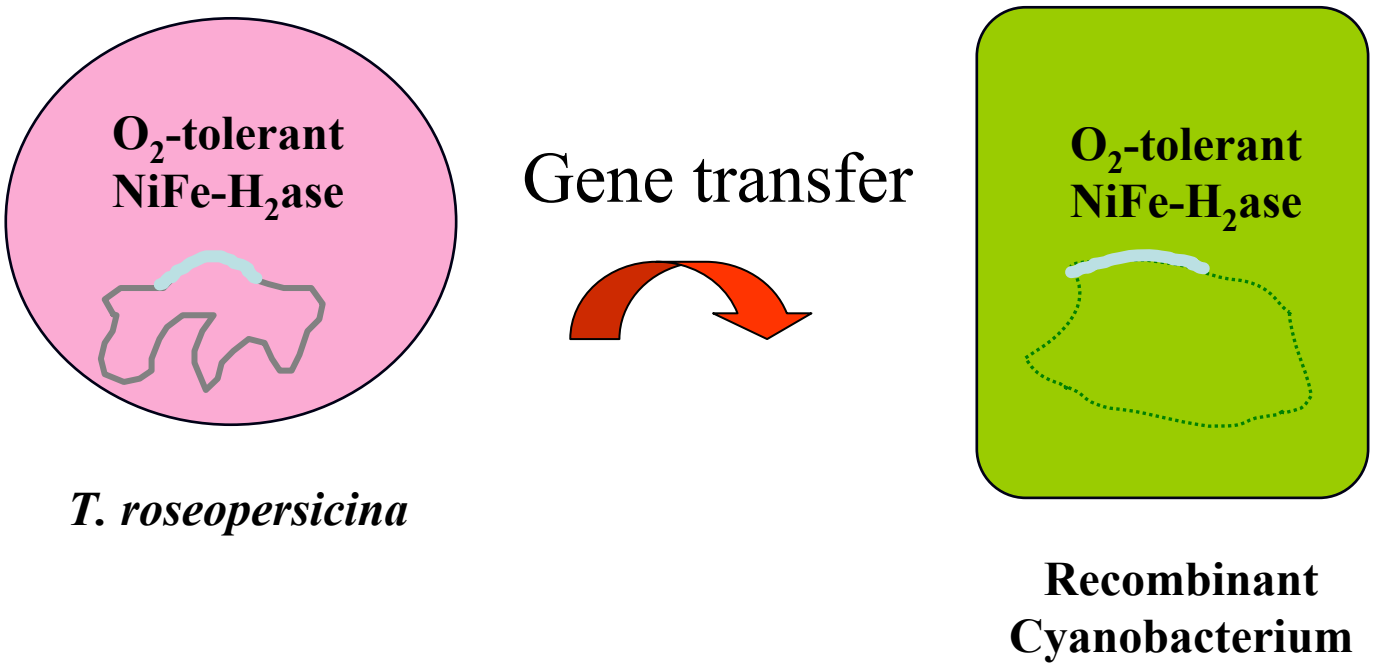
1. *T. roseopersicina* wild-type strain (BBS), positive control
2. *T. roseopersicina* ΔHyn, ΔHup, ΔHox strain containing pAmDHSL (*hynDhupHhynSL*)
3. *T. roseopersicina* ΔHyn, ΔHup, ΔHox strain (GB112131), negative control

Rabbit polyclonal antibody specific for *T. roseopersicina* HynL was used for Western blotting

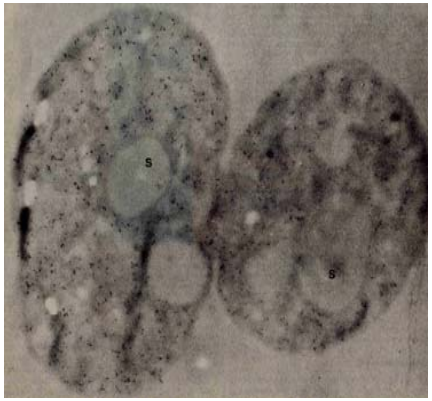
Technical Approach 2.1 (Venter Institute)

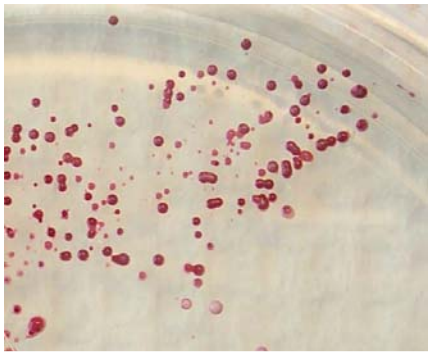
Transferring a known O_2-tolerant NiFe-hydrogenase from bacterium *Thiocapsa roseopersicina* into cyanobacterium *Synechococcus* PCC7942

(This task was not funded by DOE during 2006, and the following progress was made with internal funding from the Venter Institute.)

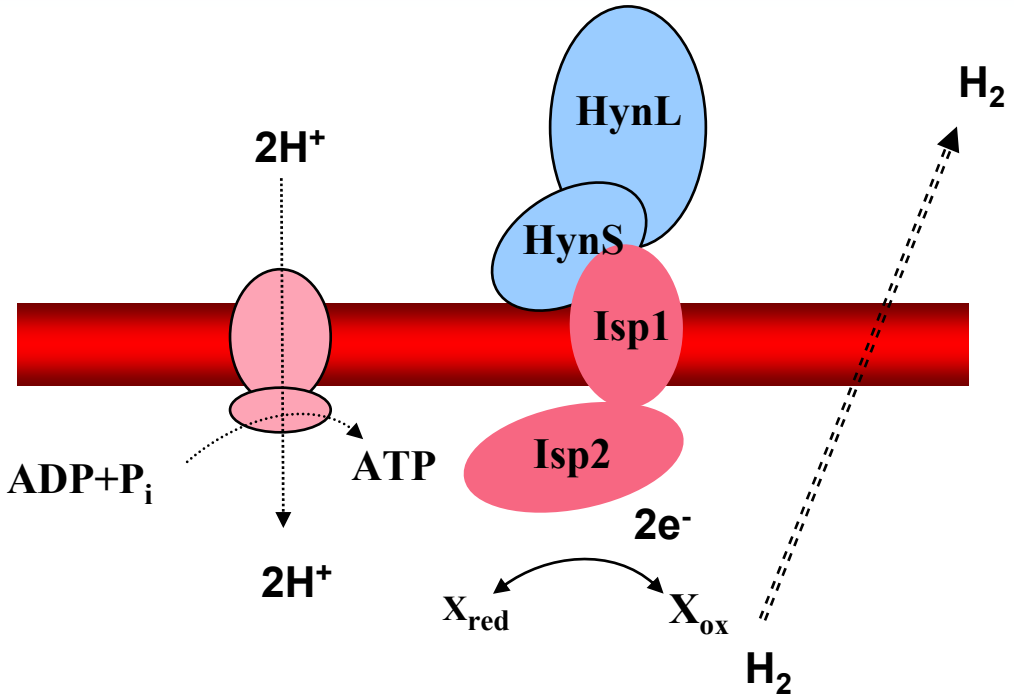


Phototrophic purple sulfur bacteria *Thiocapsa roseopersicina* carries an O_2-tolerant hydrogenase (Hyn)





T. roseopersicina

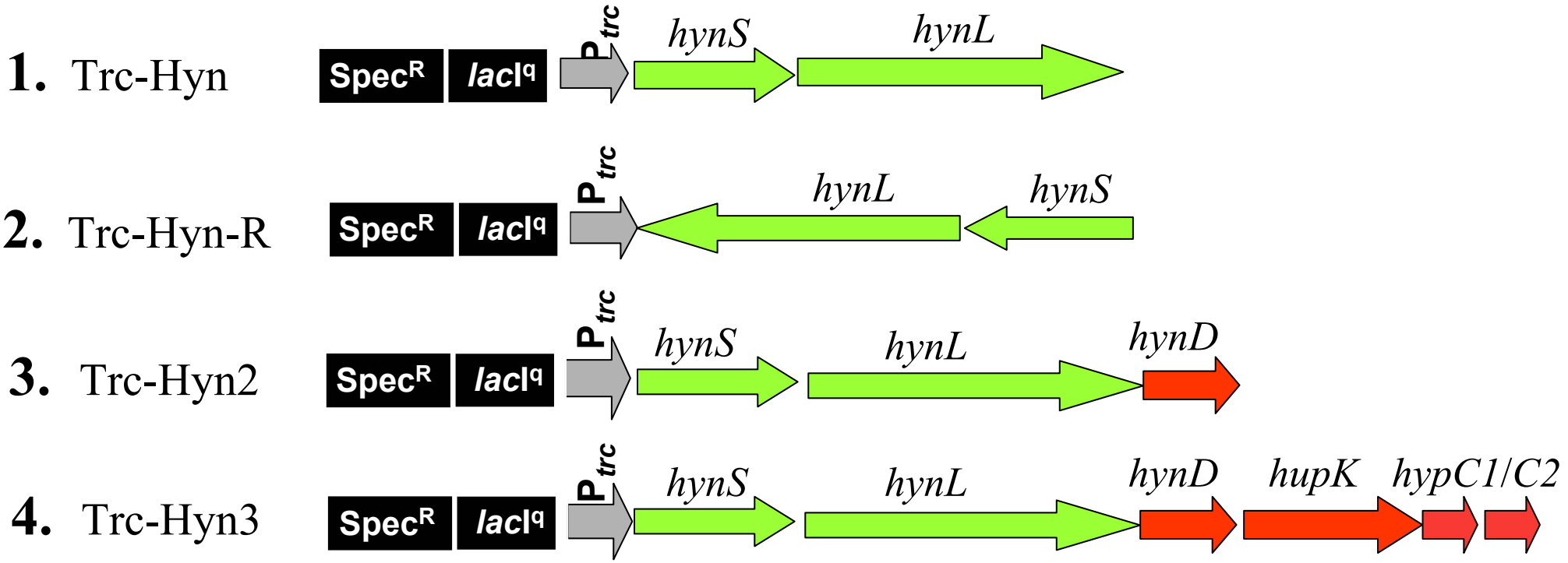


High O_2 / thermal stability, and resistance to proteolysis

- Structural subunit: HynS and HynL
- Electron transfer subunit: Isp1 and Isp2

Technical Accomplishments/ Progress

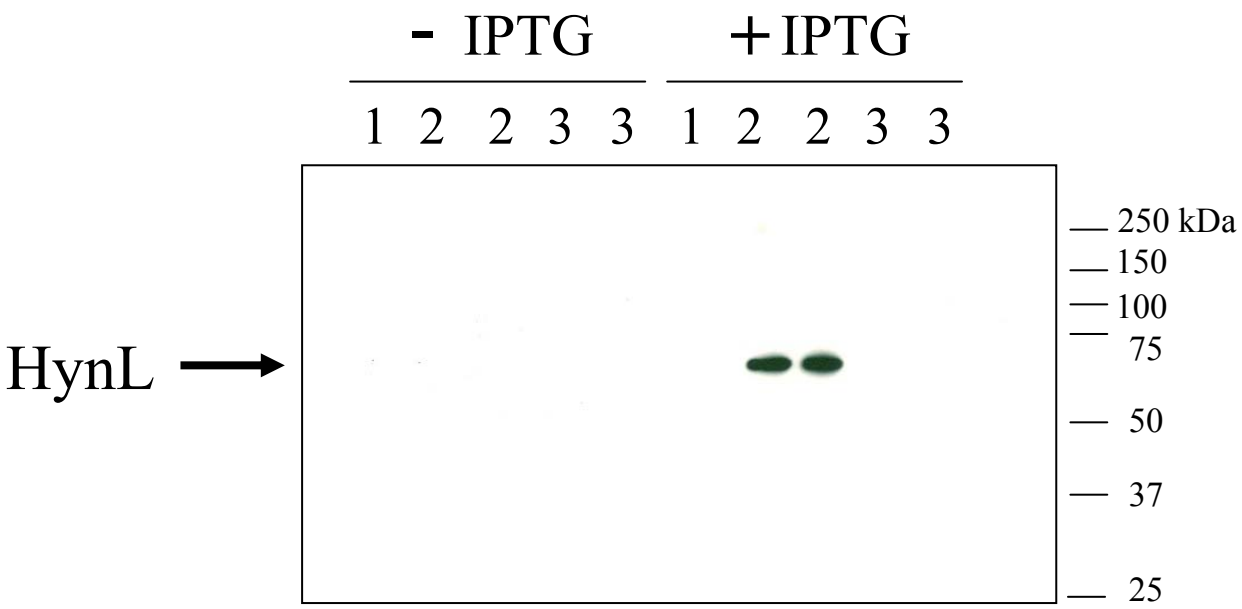
Transferred Hyn's structural and accessory genes into the genome of cyanobacterium *Synechococcus sp* PCC7942 strain



$P_{Trc:}$ an IPTG-inducible promoter; *hynS* and *hynL: Hyn's* structural genes (green); *hynD*, *hypC1*, *hypC2*, and *hupK:* accessory genes essential for Hyn's assembly (red)

Technical Accomplishments/ Progress

Confirmed IPTG-inducible expression of O_2-tolerant hydrogenase Hyn in constructed recombinant *Synechococcus sp* PCC7942 strains



Polyclonal antibody specific for HynL was raised and used for Western blotting

1. *Synechococcus sp* PCC7942 wild type strain
2. *Synechococcus sp* PCC7942 Trc-Hyn
3. *Synechococcus sp* PCC7942 Trc-R-Hyn

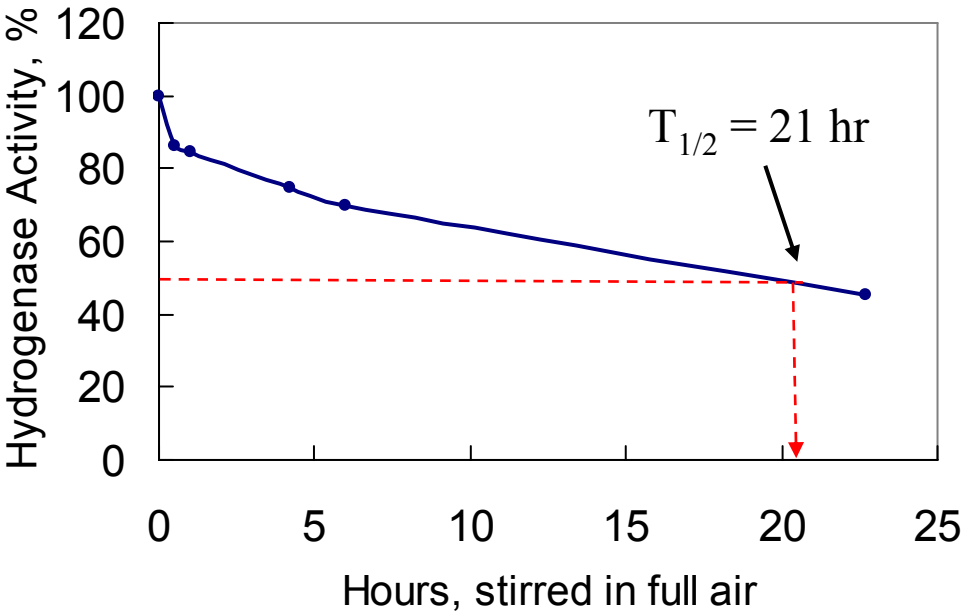
Technical Approach 2.2 (NREL)

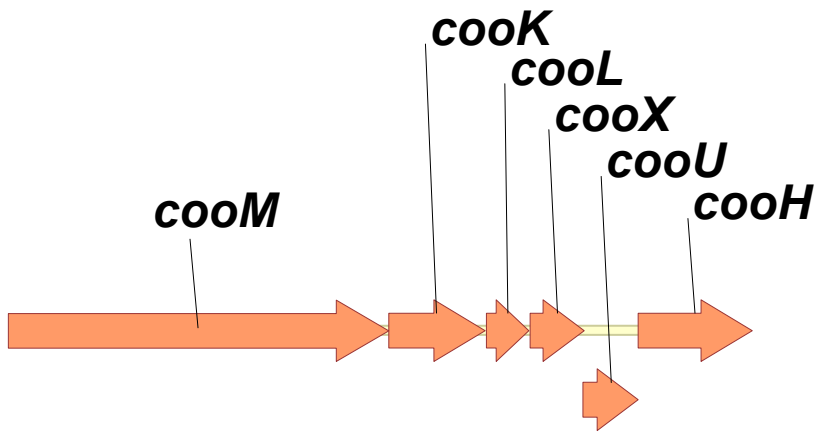
- Goal: Transfer the O_2-tolerant hydrogenase from *Rubrivivax gelatinosus* CBS into the cyanobacterial hosts *Synechocystis sp.* PCC6803

➢ ***Purple non-sulfur photosynthetic bacterium Rx. gelatinosus CBS was isolated from soils in the metro Denver area***

➢ ***Rubrivivax evolving hydrogenase displays a half-life of 21 hours in air***

➢ ***Structural and accessory genes encoding the Rubrivivax hydrogenase have been cloned***



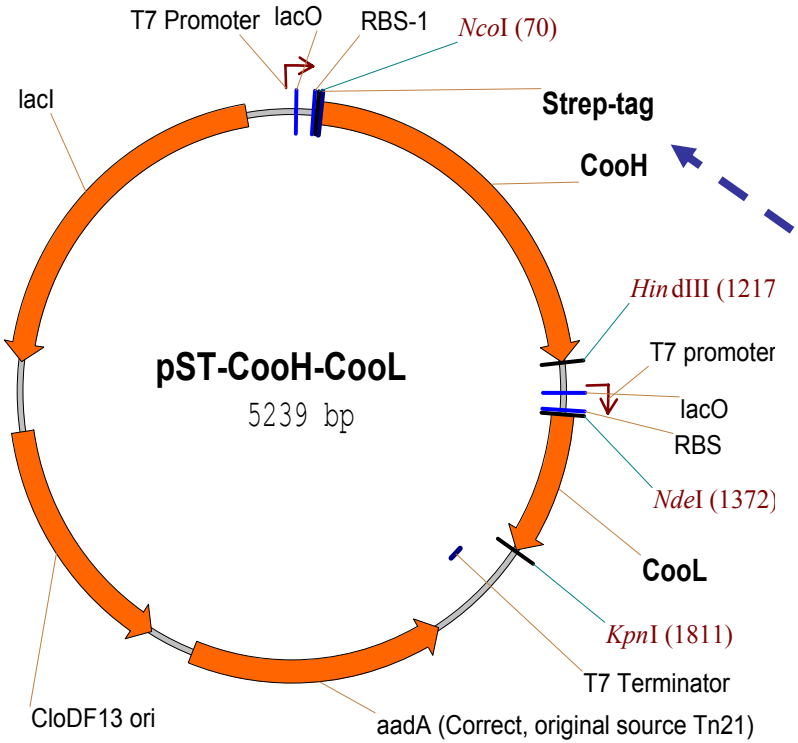


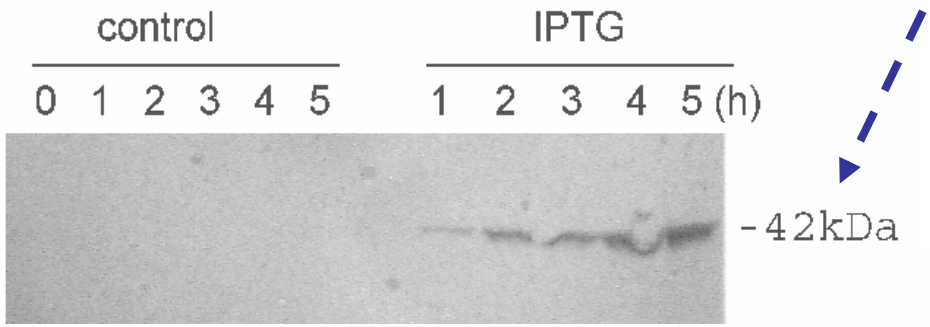
Structural subunits: CooL and CooH

Electron-transfer subunits: CooX and CooU

Technical Accomplishments/ Progress

Expression of *Rubrivivax* Hydrogenase (CooLH) in *E. coli*





- Hydrogenase subunits: CooL (small subunit) and CooH (large catalytic subunit) are cloned into a duet vector pCDFDuet-1
- The CooH catalytic subunit is labeled with a **Strep II tag** to facilitate affinity purification
- When expressed in *E. coli*, **tagged CooH (42kDa) is detected** in cell-free extracts following IPTG induction
- Protease digestion/mass spectrometry confirmed the 42 kDa protein as CooH

Future Work (Venter Institute)

- **Remainder of FY2007:**
  - Purify hetero-expressed novel NiFe-hydrogenase (identified from the Sargasso Sea environmental samples) from *T. roseopersicina*
  - Clone more accessory genes of *Thiocapsa* O_2-tolerant hydrogenase into cyanobacterial expression vector pTrc-NSI

- **FY2008:**
  - Continue to transfer the accessory genes of *Thiocapsa* O_2-tolerant hydrogenase into cyanobacterium *Synechococcus* PCC7942
  - Characterize purified novel hydrogenases that was identified from the Sargasso Sea environmental samples

Future Work (NREL)

- **Remainder of FY2007:**
  - The Strep II-tagged as well as non-tagged *Rubrivivax* hydrogenase (CooLXUH) will be inserted into the pPETE_sigD plasmid (Dr. R. Burnap of Oklahoma State Univ.) for delivery into *Synechocystis sp.* PCC6803

- **FY2008:**
  - Systematically express *Rubrivivax* hydrogenase structural genes (*coo*MKLXUH) and accessory genes (*hyp*ABCDEF) for expressing foreign O_2-tolerant hydrogenases in *Synechocystis sp.* PCC6803

Summary

- **Approach 1 (Venter Institute)**

1. 1.2 millions of peptide sequences in the Sargasso Sea dataset were searched for NiFe-hydrogenases by using 14 hydrogenases HMMs we constructed. 10 NiFe-hydrogenases were identified from this search (VI).
2. One novel NiFe-hydrogenases identified has strong homology (64% identity and 75% similarity) to *Thiocapsa* O_2-tolerant hydrogenase Hyn. Its structural and accessory genes have been cloned and sequenced. This novel hydrogenase was successfully hetero-expressed in *T. roseopersicina*, as demonstrated by Western blotting results. This study represents the first conversion of an environmental DNA into a hydrogenase (VI).

- **Approach 2 (Venter Institute and NREL)**

1. Both structural and essential accessory genes of *Thiocapsa* O_2-tolerant NiFe-hydrogenase, such as *hynS*, *hynL*, *hynD*, *hypC1*, *hypC2*, and *hupK*, have been transferred into the genome of cyanobacterium *Synechococcus sp* PCC7942 (VI).
2. *Thiocapsa* O_2-tolerant hydrogenase Hyn is heterologously expressed in *Synechococcus sp* PCC7942 upon IPTG induction, as demonstrated by Western blotting in which HynL-specific polyclonal antibodies were used (VI).
3. The structural genes encoding the *Rubrivivax* O_2-tolerant hydrogenase have been modified with a Strep II affinity tag to simplify purification. These genes were cloned in a pCDFDuet-1 vector and the CooH catalytic subunit was heterologously expressed in *E. coli* as the host initially, as demonstrated by Western blotting. Work is underway to express *Rubrivivax* hydrogenase genes in the cyanobacterium *Synechocystis sp.* PCC 6803 (NREL).