DOE Hydrogen Program

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

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Project ID #
pdp_18_xu

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J. Craig Venter
INSTITUTE

Overview

Timeline

- Project start date: 5-01-05
- Project end date: 4-30-10
- Percent complete: 60%

Budget

- Total project funding
  - DOE share: $1.09M for JCVI
  - DOE share: $1.26M for NREL
  - JCVI cost-share: $720K
- Funding received for FY08
  - $500K for JCVI
  - $600K for NREL
- Funding for FY09
  - $0 for JCVI
  - $0 for NREL

Barriers

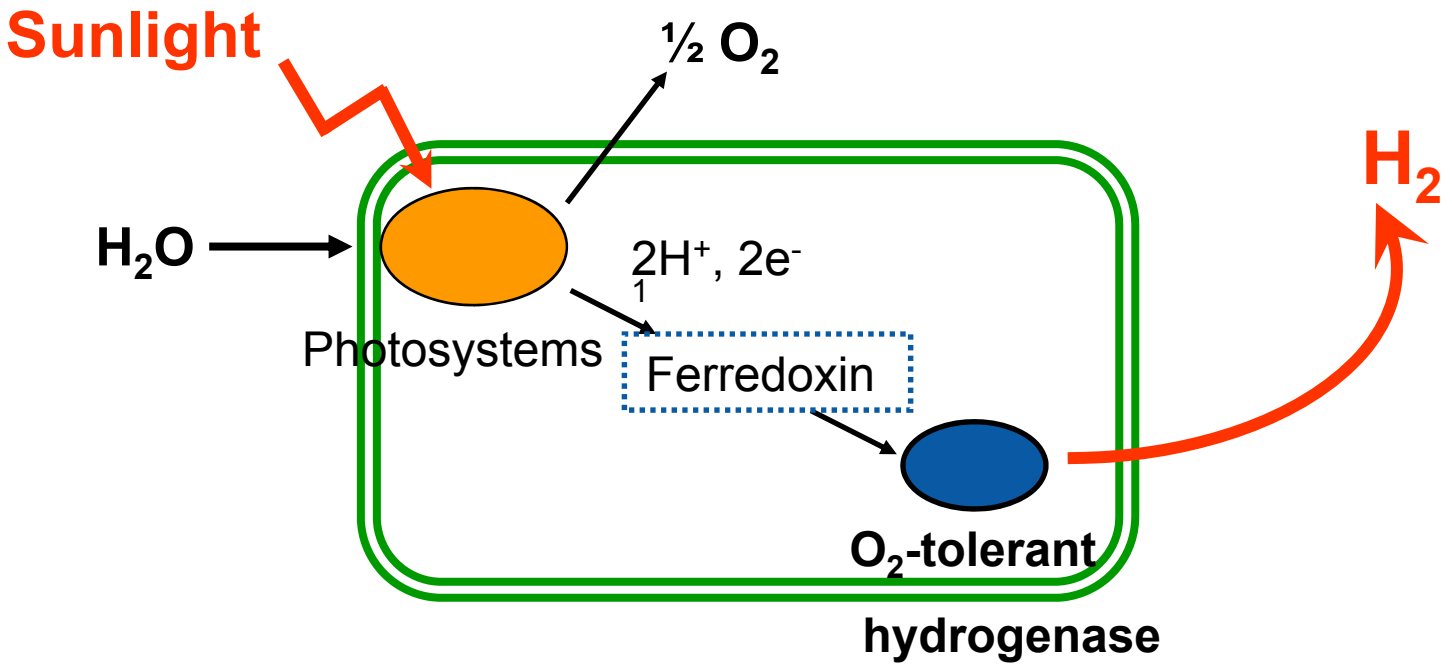
- Barriers addressed
  - Production Barrier Z: Continuity of H_2 production

Partners

- J. Craig Venter Institute
- National Renewable Energy Laboratory

Objective-Relevance

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



Characteristics	2009 Status	2011 Target	2018 Target
Duration of continuous photoproduction	Zero to 30 seconds in air	Produce one cyanobacterial recombinant evolving H_2 through an O_2-tolerant NiFe- hydrogenase	Demonstrate H_2 production in air in a cyanobacterial recombinant

Milestones

Task 1. (JCVI and NREL)

Month/Year	Milestone	% Comp
Dec-08	Verify hydrogenase expression in cyanobacteria	JCVI, 100% NREL, 100%
Apr-09	Purify O_2-tolerant hydrogenases	JCVI, 100% NREL, 30%*
Sept-09	Verify hydrogenase functionality in oxygen	JCVI, 30% NREL, 30%

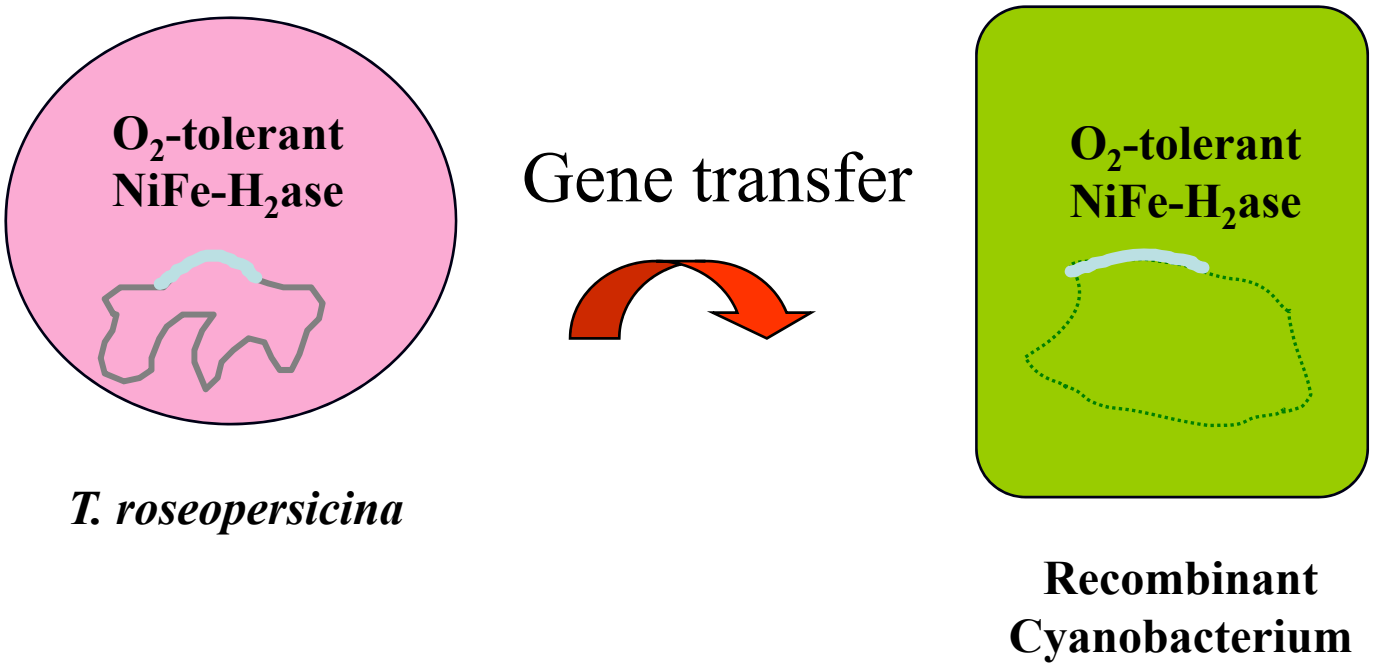
* DOE Program Manager Roxanne Garland agreed for NREL to delay this milestone till 9/09.

Task 2. (JCVI)

Month/Year	Milestone	% Comp
Apr-09	Identify hydrogenase gene sequences through metagenomic analysis of marine microbes from the oceans.	JCVI, 100%
Sept-09	Identify novel functional hydrogenases from the oceans	JCVI, 100%

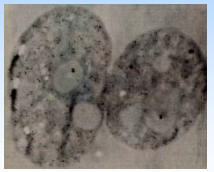
JCVI-Technical Approach

Task 1.1. Transferring a known O_2-tolerant NiFe-hydrogenase from *T. roseopersicina* into cyanobacterium *Synechococcus sp* PCC7942

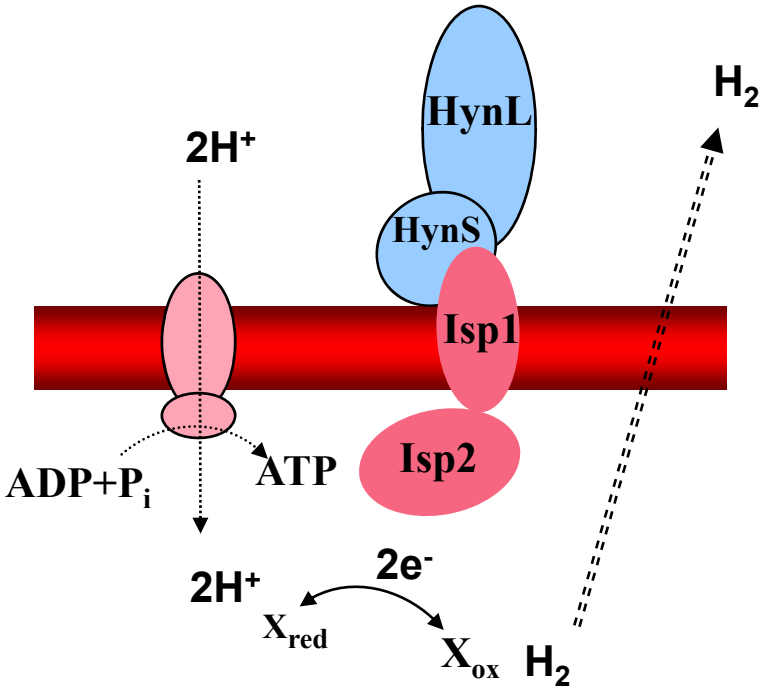


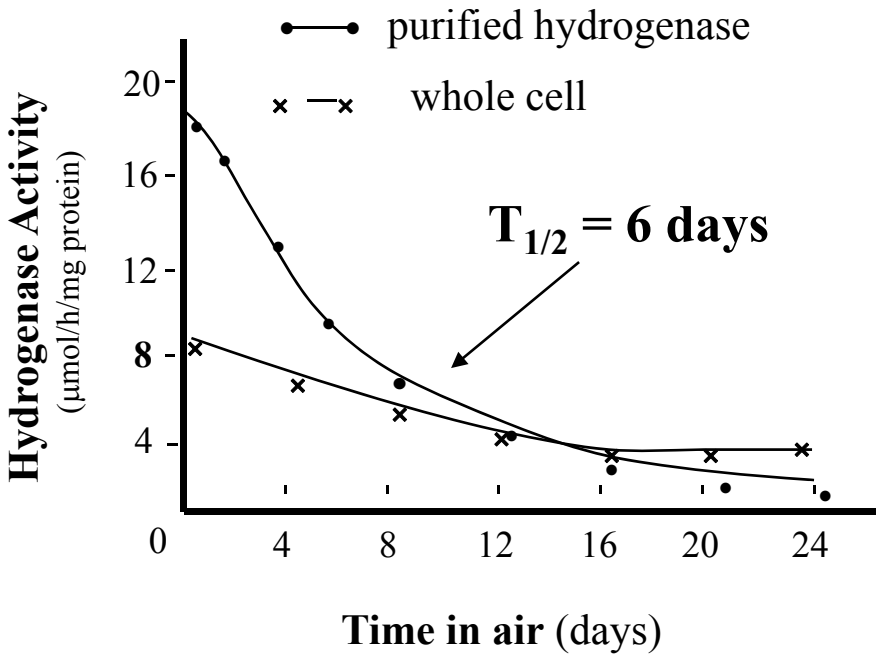
- JCVI approach is complementary to that of NREL in harnessing two of Nature's O_2-tolerant hydrogenases and their transfer into two model cyanobacteria

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



- Phototrophic purple sulfur bacterium *Thiocapsa roseopersicina* carries an O_2-tolerant and thermal-stable hydrogenase (HynSL).
- The *Thiocapsa* hydrogenase HynSL displays a half-life of 6 days in air.
- Structural and accessory genes encoding the *Thiocapsa* hydrogenase are identified.





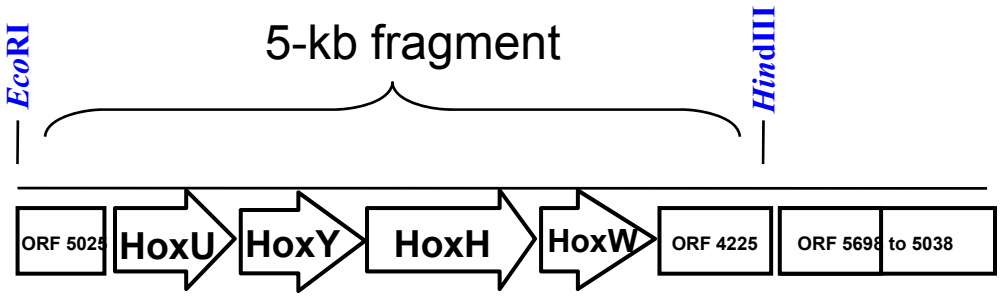
- Structural subunit: HynS and HynL
- Electron transfer subunit: Isp1 and Isp2

Biochimica et Biophysica Acta 523:335-343 (1978)

JCVI -Technical Accomplishments

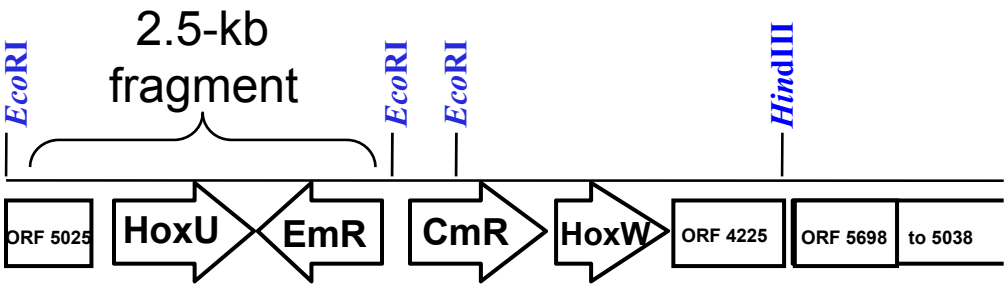
Constructed a cynobacterial *Synechococcus sp* PCC7942 strain with its native hydrogenase Hox knockout

Hox gene Locus in wild type 7942



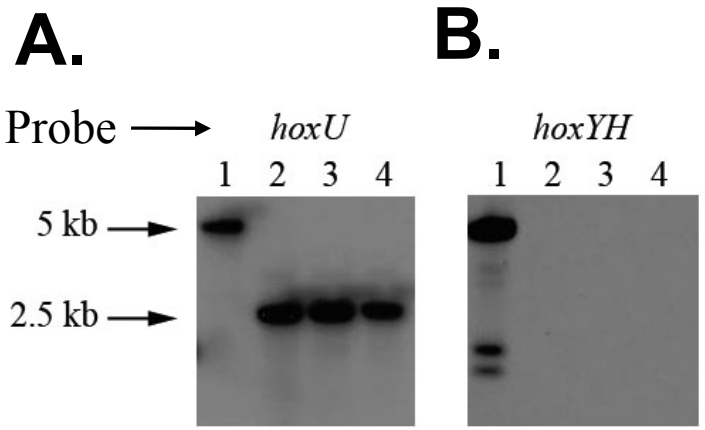
Hox Y and HoxH encode structural subunits of native hydrogenase Hox.

Hox gene Locus in the knockout mutant



Hox Y and HoxH genes were knockout (EmR/CmR: selection markers).

Southern Blotting confirmed absence of Hox genes in the knockout mutant



Lane1. Wild-type 7942 DNA
Lane 2-4. Mutant DNA (Hoxmut-4, 6, 7)

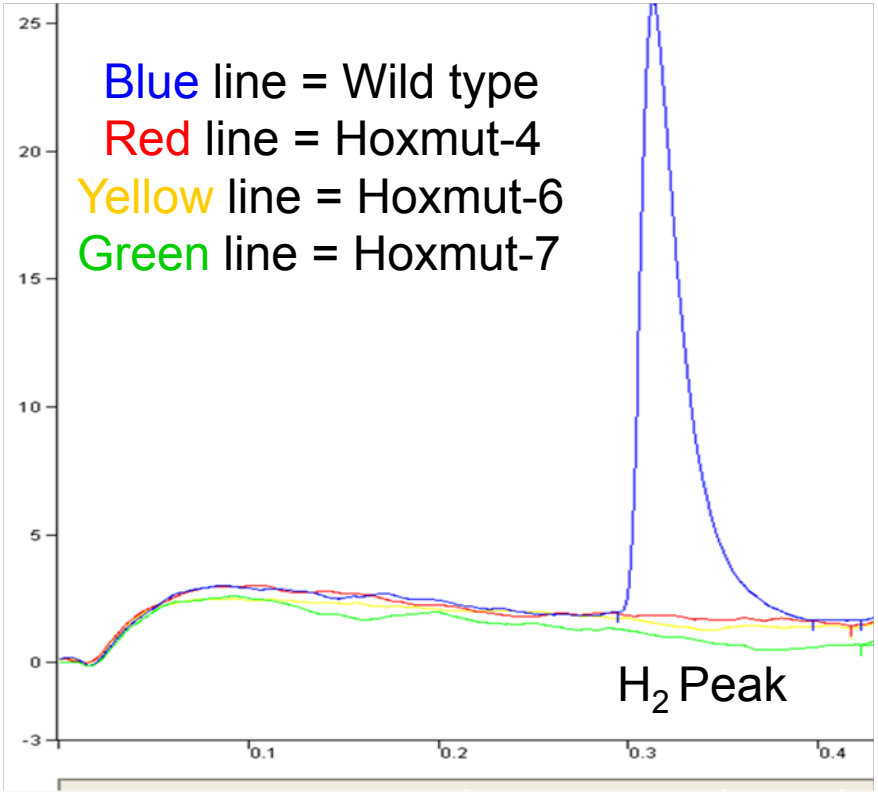
(DNA was digested with *Eco*RI and *Hin*dIII)

A probe for hoxU hybridized to a 5 kb fragment in the wild-type and a 2.5-kb fragment in the mutant(Hoxmut). Whereas, a hoxYH probe did not hybridize to Hoxmut.

Hox knockout mutant of cyanobacterium *Synechococcus* 7942 was constructed for transferring genes of *Thiocapsa* O_2-tolerant hydrogenase.

JCVI -Technical Accomplishments

Confirmed that the Hox knockout mutant (Hoxmut) of *Synechococcus sp* PCC7942 does not evolve hydrogen



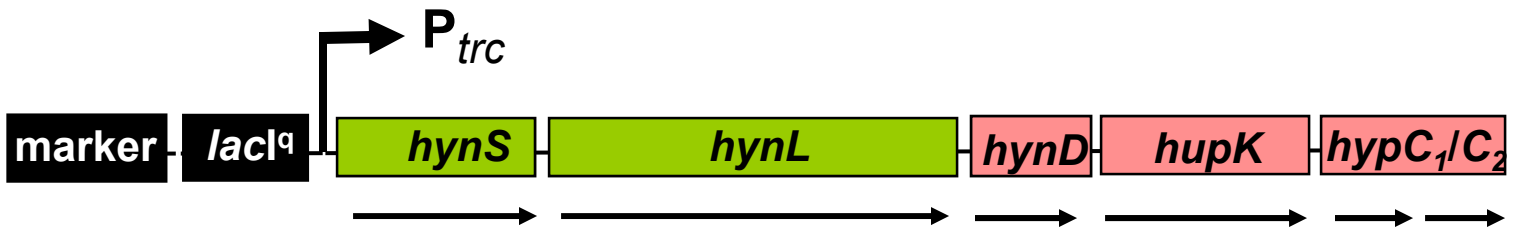
Strain	Hydrogenase Activity (nmole H_2/ mg Chl/hr)
Wild type 7942	156 ±12
Mutant Hoxmut-4	0
Mutant Hoxmut -6	0
Mutant Hoxmut -7	0

- GC analysis shows that only wild type *Synechococcus* 7942 generated a hydrogen peak. No hydrogen was detected in three clones of the Hox knockout mutant, Hoxmut-4, 6, and 7.

The Hox knockout mutant without any hydrogenase activities is ready to be used as a cyanobacterial recipient for transferring genes of *Thiocapsa* O_2-tolerant hydrogenase.

JCVI - Technical Accomplishments

Transferred genes of *Thiocapsa* O_2-tolerant hydrogenase (Hyn) into cyanobacterium *Synechococcus sp* PCC7942



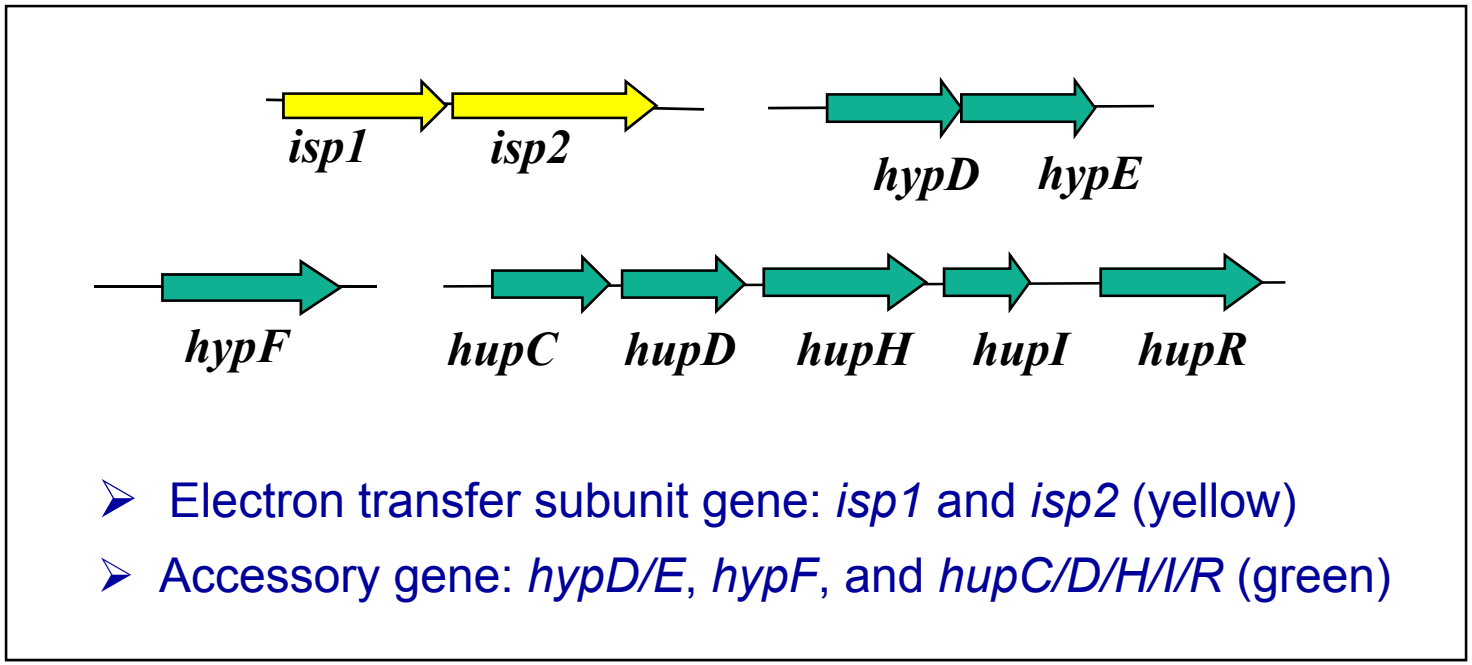
P_{Trc}: an IPTG-inducible promoter
Structural genes: *hynS* and *hynL*, encoding small and large subunits
Essential accessory genes: *hynD*, *hupK*, $hypC_1$ and $hypC_2$

- Through homologous DNA recombination, Hyn's genes were integrated into the cyanobacterial genome, as confirmed by PCR and Southern blotting.
- After being transferred into *S. sp* PCC7942, expression of the *Thiocapsa* hydrogenase is under control of an IPTG-inducible promoter.

Two structural and four essential accessory genes of the *Thiocapsa* O_2-tolerant hydrogenase have been transferred into cyanobacterium *Synechococcus* PCC7942

JCVI - Technical Accomplishments

Cloned more genes of *Thiocapsa* O_2-tolerant hydrogenase for transfer into *Synechococcus* PCC7942 Hoxmut

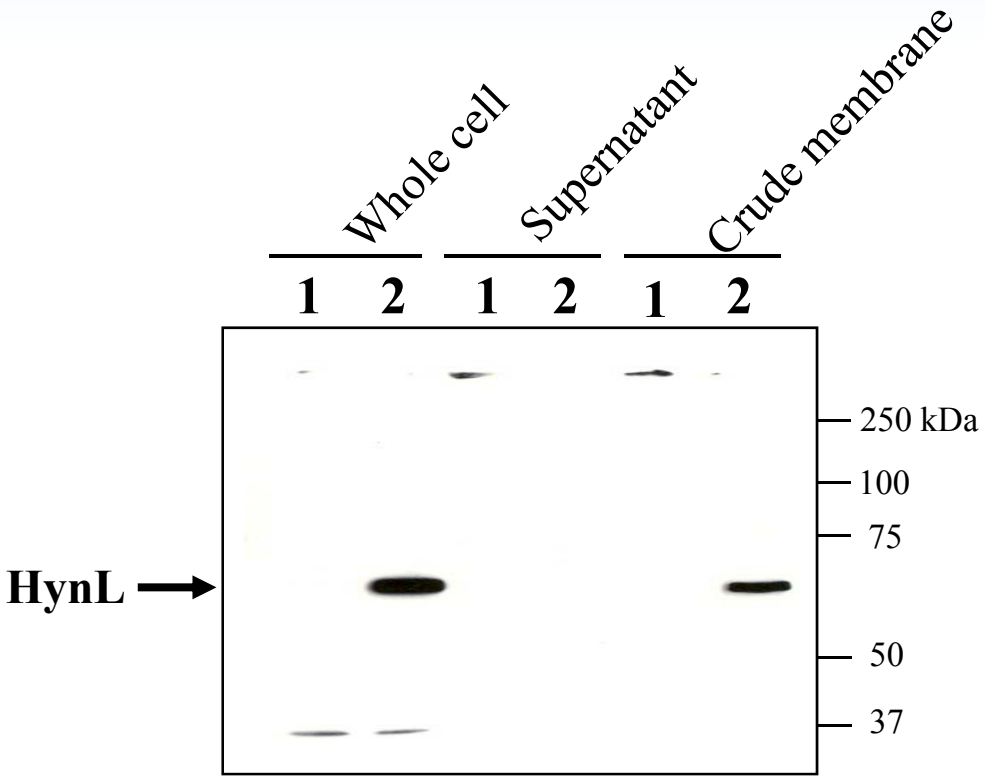


- Electron transfer subunit gene: *isp1* and *isp2* (yellow)
- Accessory gene: *hypD/E*, *hypF*, and *hupC/D/H/I/R* (green)

All genes that encode accessory proteins for assembling the *Thiocapsa* hydrogenase were cloned into cyanobacterial expression vectors. Transferring these genes into cyanobacterium is in progress.

JCVI - Technical Accomplishments

Confirmed expression of O_2-tolerant hydrogenase Hyn and examined its location in recombinant *S. sp* PCC7942



1. Wild-type *S. sp* PCC7942
2. Recombinant *S. sp* PCC7942 strain

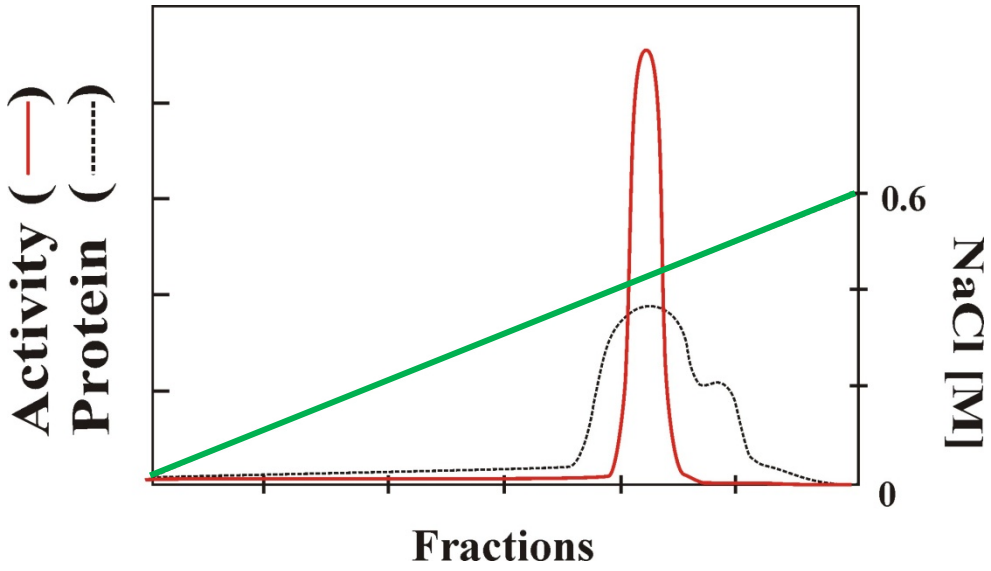
- Polyclonal antibodies specific for hydrogenase subunit HynL were raised as detection tools.
- IPTG-inducible expression of O_2-tolerant hydrogenase Hyn was detected by these antibodies in the recombinant *Synechococcus.*
- Cellular location of heterologously expressed Hyn was examined. Hyn was translocated to the cell membrane through the membrane targeting and translocation pathway.

Reached Milestone "Verify hydrogenase expression in cyanobacteria" **(12/08)**

JCVI - Technical Accomplishments

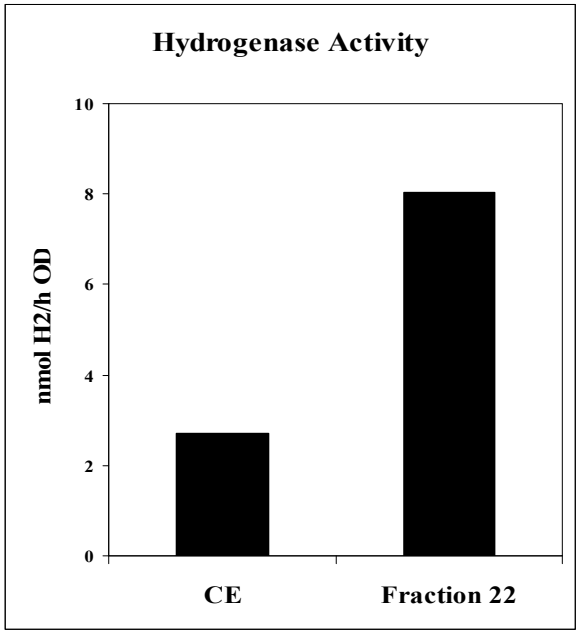
Purified *Thiocapsa roseopersicina* O_2-tolerant hydrogenase

Chromatography of *Thiocapsa* crude extract and H_2 evolution activity assay



- Red = H_2 evolution activity
- Black = Protein concentration
- Green=NaCl gradient 0 - 0.6 M

(The hydrogenase was eluted at NaCl ≈ 0.4 M)



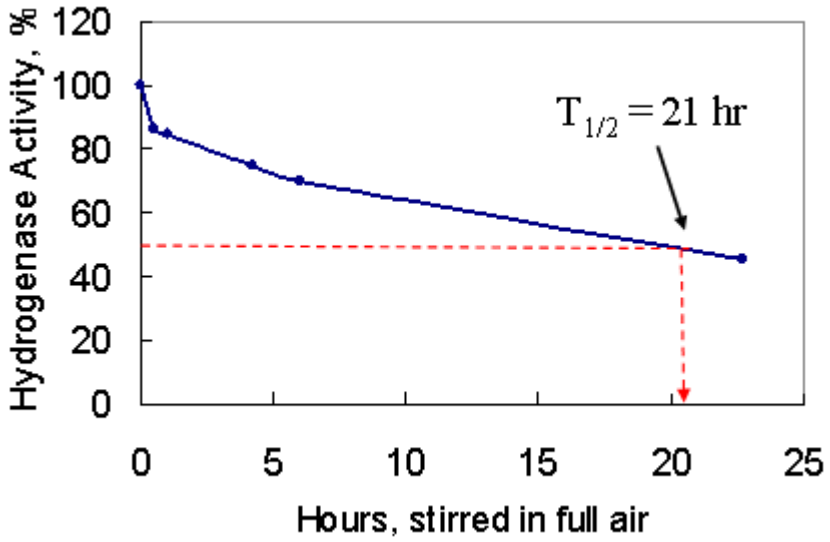
- CE: Crude extract
- Fraction 22 = Purified hydrogenase

Reached Milestone "Purify *Thiocapsa* O_2-tolerant hydrogenases" **(04/09)**

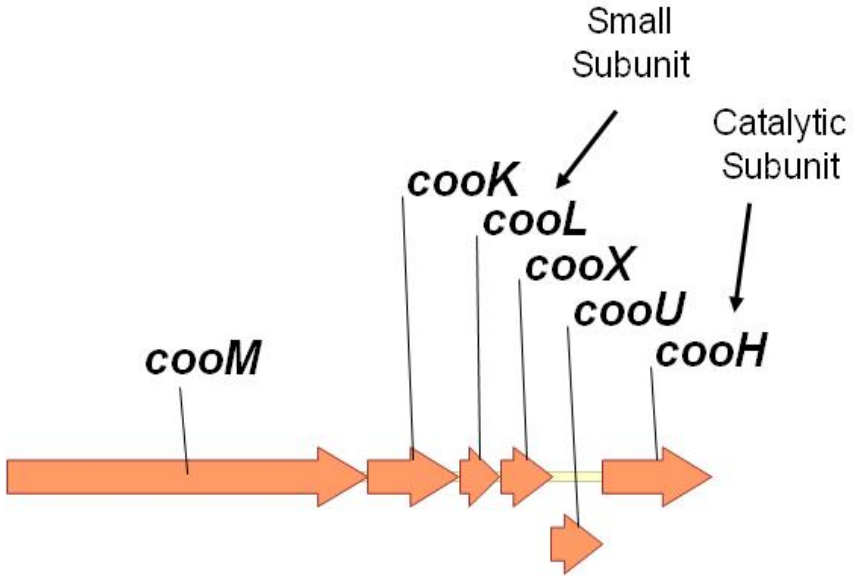
Approach (NREL)

Task 1.2. Transfer an O_2-tolerant NiFe-hydrogenase from the bacterium *Rubrivivax gelatinosus* CBS (hence "CBS") (isolated by NREL) into the cyanobacterium *Synechocystis* sp. PCC6803

CBS Hydrogenase half-life in air: 21 hr



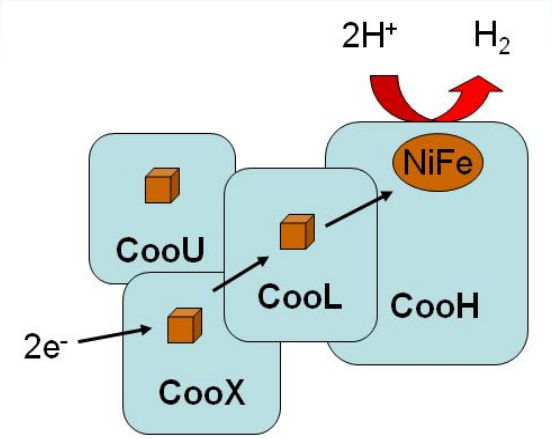
Hydrogenase genes were cloned



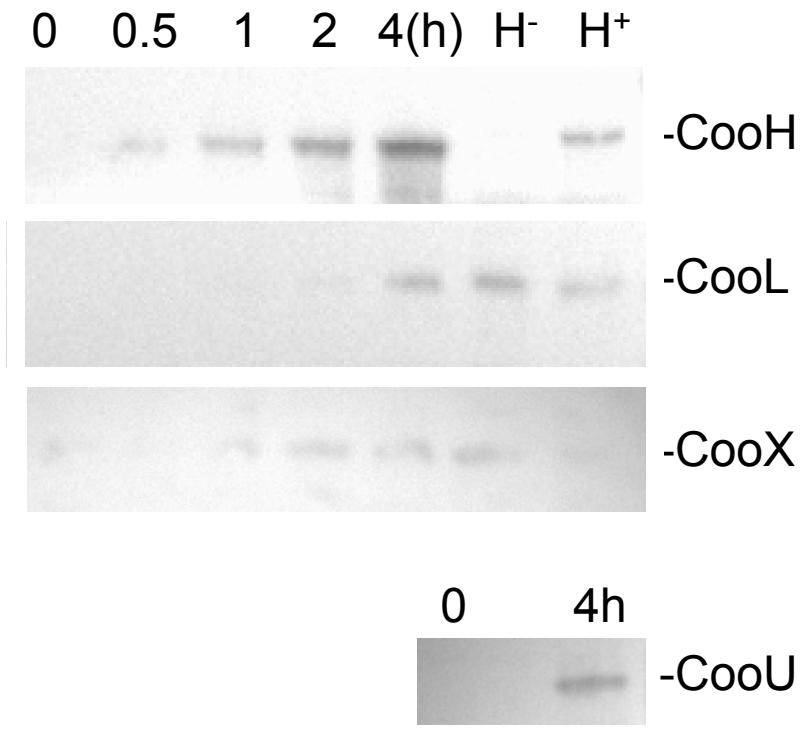
NREL approach is complementary to that of JCVI in harnessing two of Nature's O_2-tolerant hydrogenases and their transfer into two model cyanobacteria

NREL – Technical Accomplishments

Develop Antibody Tools for Detection of CBS Hydrogenase



Subunit	Putative Function
CooL	Small subunit, electron transfer
CooX	Electron transfer
CooU	Unknown
CooH	Catalytic subunit, H_2 production

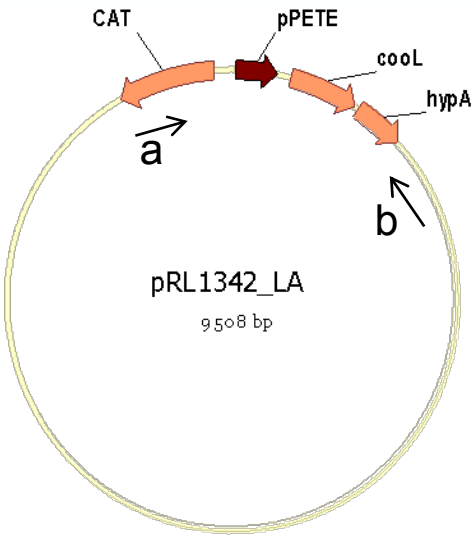


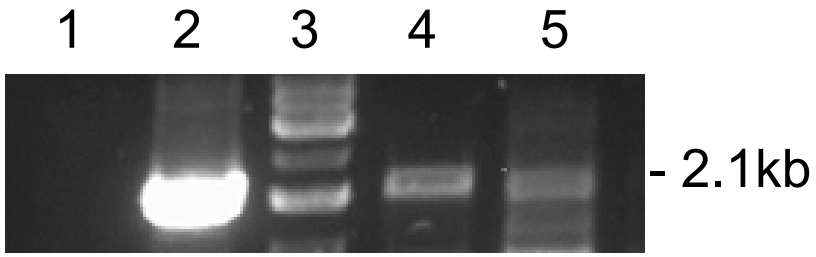
- Antibodies against the four subunits of the CBS hydrogenase were generated.
- CBS wild type culture was treated with CO for up to 4 hours.
- A CooH mutant (H^-) and a complemented strain (H^+) were also treated with CO for 4 hours
- Demonstrated the antibodies were specific for each of the subunits.

With these tools we can detect expression of CBS hydrogenase in *Synechocystis*

NREL – Technical Accomplishments

CBS Hydrogenase Small Subunit Transformed in *Synechocystis*



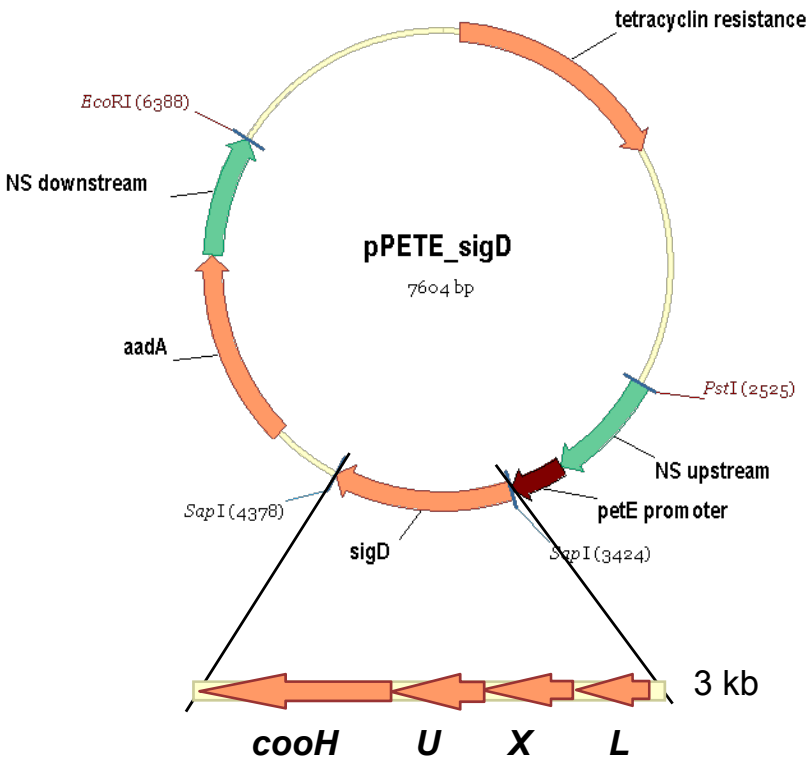


- Construct BHR plasmid pRL1342_LA carrying CBS *hypA* (maturation) and *cooL* (small subunit) genes. The parental plasmid pRL1342 is from Peter Wolk at Michigan State University.
- PCR using "a-b" primer pair confirmed the presence of *cooL, hypA,* and *CAT* genes (2.1kb) in transformed *Synechocystis* strains.
- Lane 1, WT as negative control; lane 2, plasmid pRL1342_LA as positive control; lane 3, MW marker, lane 4 and 5, WT and HoxH$^-$ transformed with the pRL1342_LA plasmid
- **Confirmed the transformation of CBS *hypA* and *cooL* genes in the *Synechocystis* host.**

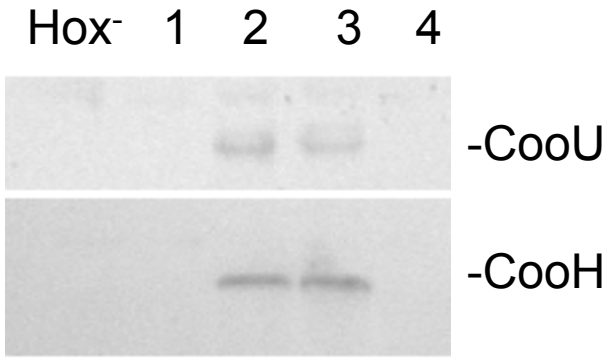
Completed Milestone "*Verify hydrogenase expression in cyanobacteria*" (12/08).

NREL – Technical Accomplishments

CBS Hydrogenase Large Subunit Expressed in *Synechocystis*



- CBS genes *cooLXUH* were transformed into *Synechocystis* host strain Hox$^-$ using the pPETE_sigD plasmid (Rob Burnap, Oklahoma State University)
- Protein expression was controlled by the plastocyanin *petE* promoter
- Four transformed lines were tested and two lines (#2, and #3) showed expression of two CBS hydrogenase proteins including the large catalytic subunit.



Completed Milestone "*Verify hydrogenase expression in cyanobacteria*" (12/08).

JCVI - Technical Approach

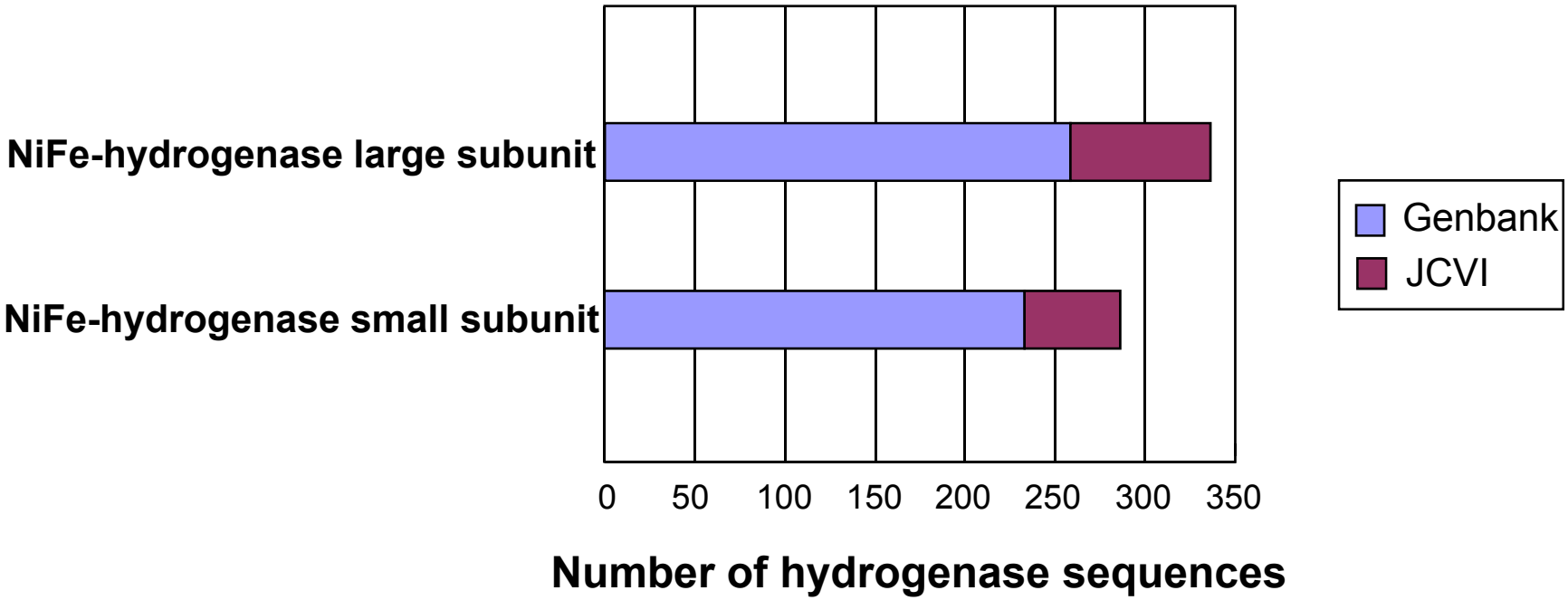
Task 2. Identifying novel O_2-tolerant hydrogenases through metagenomic analysis of marine microbes in the global ocean and transferring the hydrogenases into cyanobacteria

Sorcerer II Expedition: a Global Ocean Sampling Project accomplished by JCVI

- This approach is complementary to two approaches in the Task 1 about harnessing nature's O_2-tolerant hydrogenases and their transfer into cyanobacteria

JCVI - Technical Accomplishments

Searched metagenomic data of the Global Ocean Sampling (GOS) for novel NiFe-hydrogenases

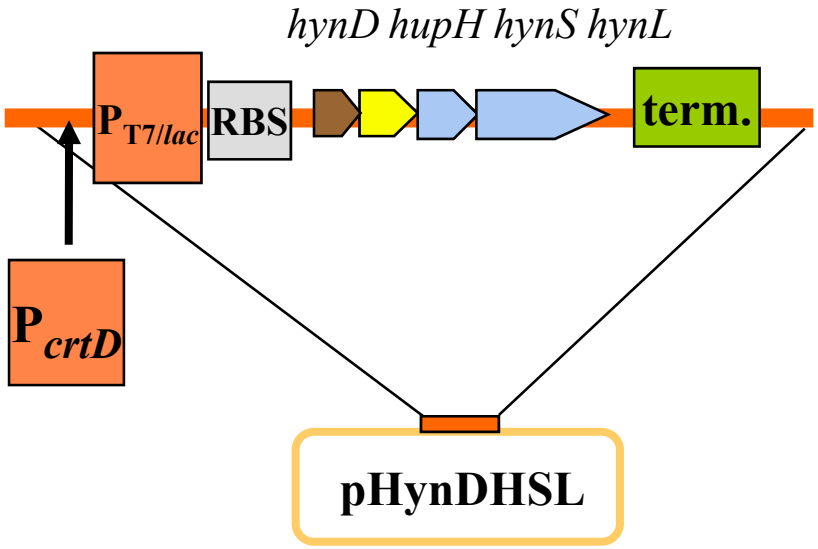


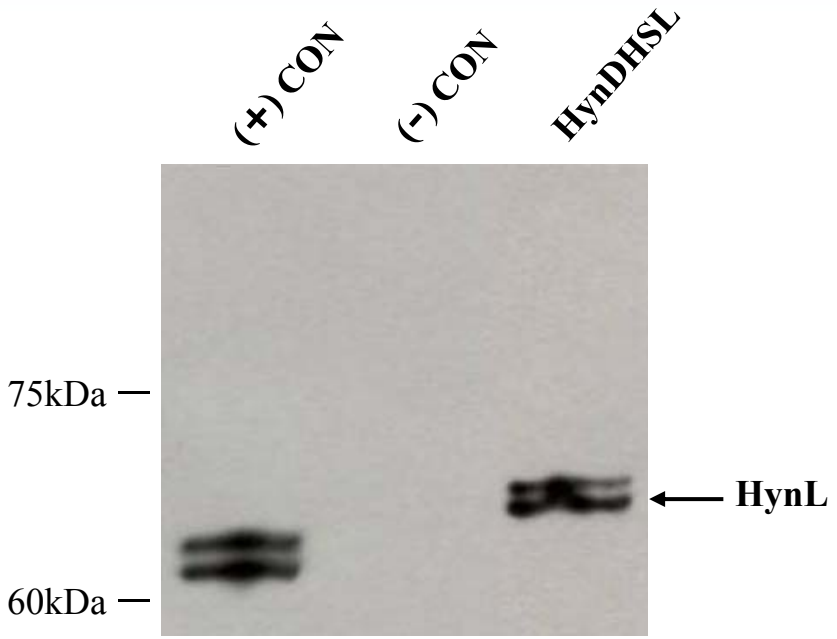
- GOS's metagenomic data were searched for novel NiFe-hydrogenases. We found 76 large subunit and 52 small subunit sequences.
- These numbers indicate significant addition by GOS compared to the public database Genbank (261 large and 234 small NCBI nr sequences),.

Reached Milestone "Identify hydrogenase gene sequences through metagenomic analysis of marine microbes from the oceans" **(04/09)**

JCVI - Technical Accomplishments

Cloned and expressed the genes of a novel environmental NiFe-hydrogenase with 60% similarity to *Thiocapsa* O_2-tolerant hydrogenase





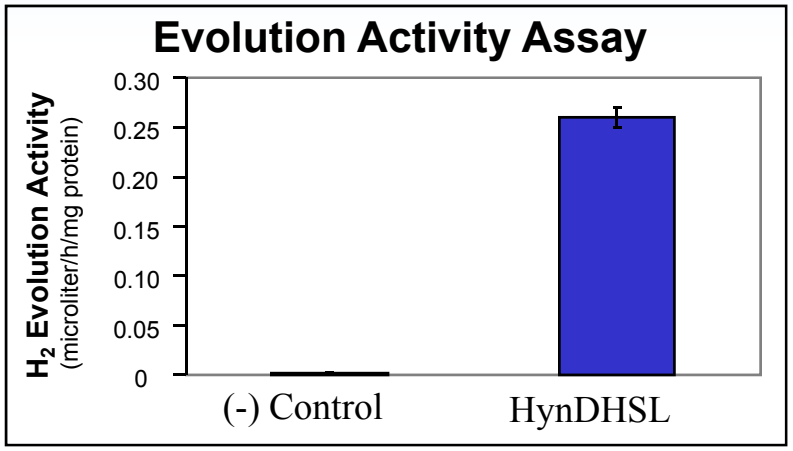
- *hynS*/hyn*L*: hydrogenase structural genes
- *hynD/hupH*: hydrogenase accessory genes
- P_{crtD}: a promoter from *T. roseopersicina*

(+) CON: *T. roseopersicina* wild-type strain
(-) CON: *T. roseopersicina* knockout mutant
HynDHSL: *T. roseopersicina* with pHynDHSL

- Structural and accessory genes of a novel hydrogenase were cloned from environmental DNA.
- The construct pHynDHSL carrying *hynD*/*hupH*/*hynS*/hyn*L* was transferred into *T. roseopersicina*
- Expression of transferred hydrogenase genes is controlled by *T. roseopersicina* promoter P_{crtD}.
- Western blotting detected expression of the novel hydrogenase HynL in the foreign host of *T. roseopersicina.*

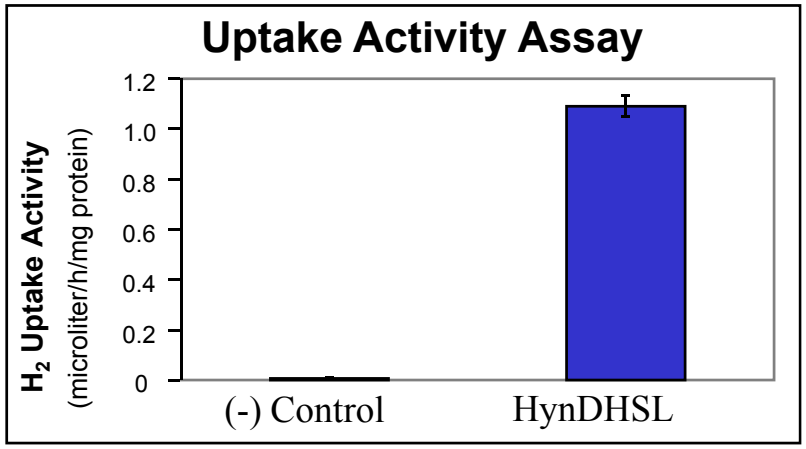
JCVI - Technical Accomplishments

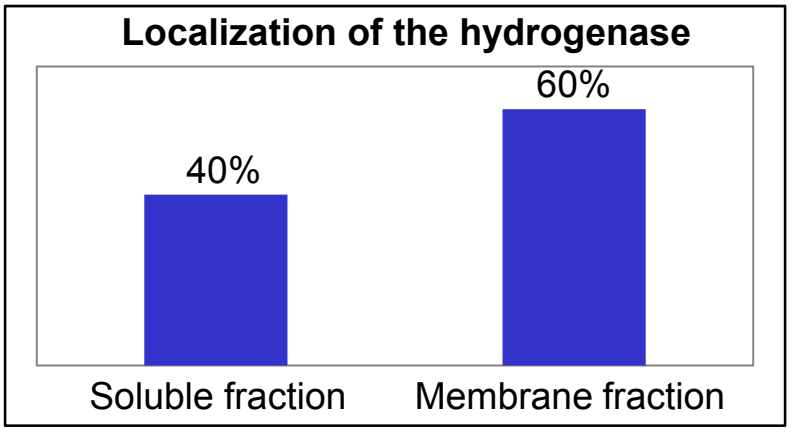
Analyzed the Novel Hydrogenase that was Heterologously Expressed in the *T. roseopersicina* Host



(-) Control: *T. roseopersicina* knockout mutant

HynDHSL: Transferred with pHynDHSL



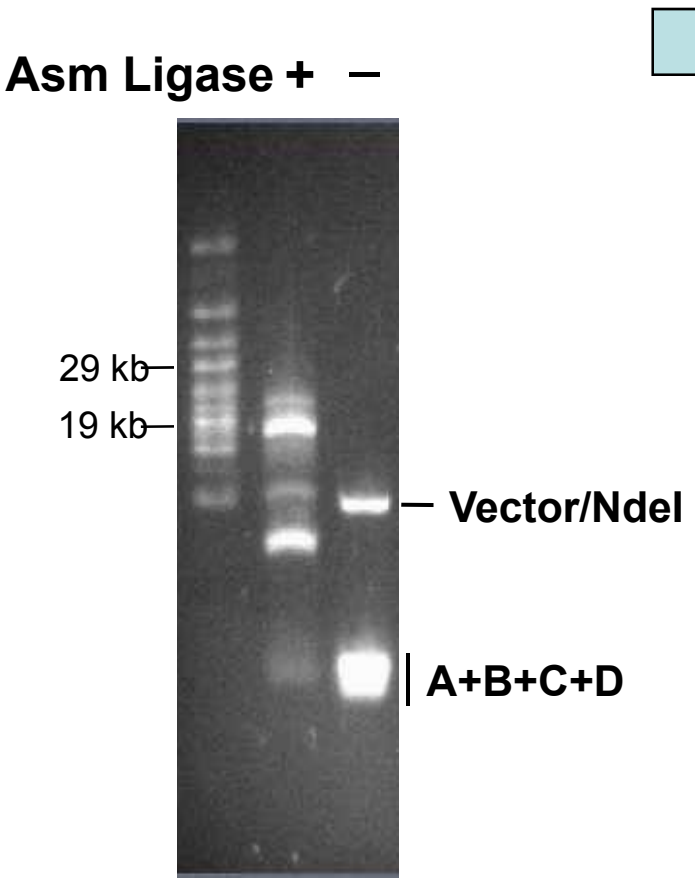


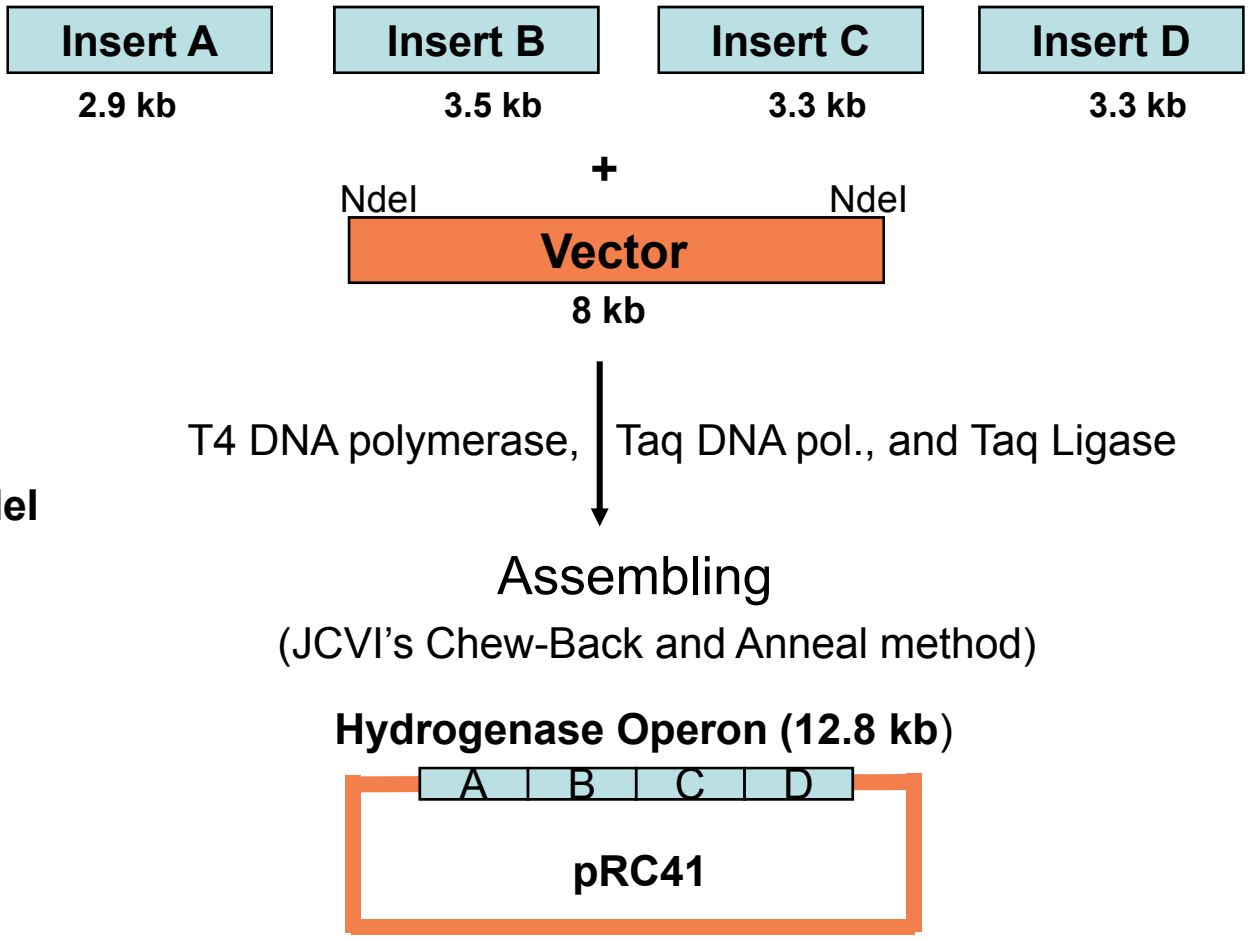
- The novel environmental hydrogenase showed both H_2-evolution and uptake activities.
- The heterologously expressed hydrogenase is loosely membrane-bound.
- The novel hydrogenase showed extraordinary stability.

Reached milestone "Identify novel functional hydrogenase from the ocean"**(09/09)**

JCVI - Technical Accomplishments

Assembled and cloned the entire gene operon of the novel environmental hydrogenase for transferring into cyanobacterium





Accuracy of pRC41 was confirmed by RE digestion, PCR, and DNA sequencing. The genes of novel environmental hydrogenase were transferred into *E. coli* and cyanobacterium PCC7942.

Collaborations

- University of Szeged, Hungary
  - Expressing novel environmental hydrogenase in *Thiocapsa*
- Vanderbilt University
  - Expressing O_2-tolerant hydrogenases in cyanobacteria
- Florida International University
  - Expression of CBS hydrogenase in *E. coli*
- Chinese Academy of Sciences
  - Bioprospecting for novel hydrogenases

Summary

- **JCVI**

1. The O_2-tolerant hydrogenase from *Thiocapsa* was purified through FPLC.
2. Rabbit antibodies were produced, which can be used to specifically detect expression of *Thiocapsa* and environmental hydrogenases.
3. The *Thiocapsa* O_2-tolerant hydrogenase was transferred into *S. sp* PCC7942. A membrane bound hydrogenase was expressed in the host upon IPTG induction.
4. The GOS sequences were searched for novel NiFe-hydrogenases. 76 large subunit and 52 small subunit sequences were found.
5. A novel NiFe-hydrogenase was cloned from the Sargasso Sea environmental DNA, and it was expressed in a foreign host (*T. roseopersicina)*, showing both H_2 evolution and uptake activities.
6. The novel hydrogenase showed extraordinary stability and it is loosely membrane-bound.
7. The genes of this novel hydrogenase were transferred into *E. coli* and *Synechococcus*. Heterologous expression is in progress.

- **NREL**

1. Antibody tools developed which enabled the detection of CBS hydrogenase protein expression in *Synechocystis*.
2. Four CBS hydrogenase genes transferred, and two hydrogenase subunit proteins expressed in *Synechocystis*.

Future Work

- **JCVI**

  - Verify hydrogenase functionality in oxygen
  - Continue to analyze the recombinant cyanobacterium that was transferred with the novel environmental hydrogenase
  - Continue to analyze the recombinant cyanobacterium that was transferred with *Thiocapsa* O_2-tolerant hydrogenase

- **NREL**

  - Verify *cooXUH* genes and CooL protein in the transformed *Synechocystis* host
  - Transform CBS with affinity tagged hydrogenase construct to purify protein and verify functionality in O_2