

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

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Project ID # PD32

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# Overview

## Timeline

- Project start date: 5-01-05
- Project end date: 4-30-10
- Percent complete: 40%

## Budget

- Total project funding
  - DOE share: \$1.26M for NREL
  - DOE share: \$1.62M for JCVI
  - JCVI share: \$720K
- Funding received for FY07
  - \$400K for JCVI
  - \$300K for NREL
- Funding for FY08
  - \$500K for JCVI
  - \$600K for NREL

## Barriers

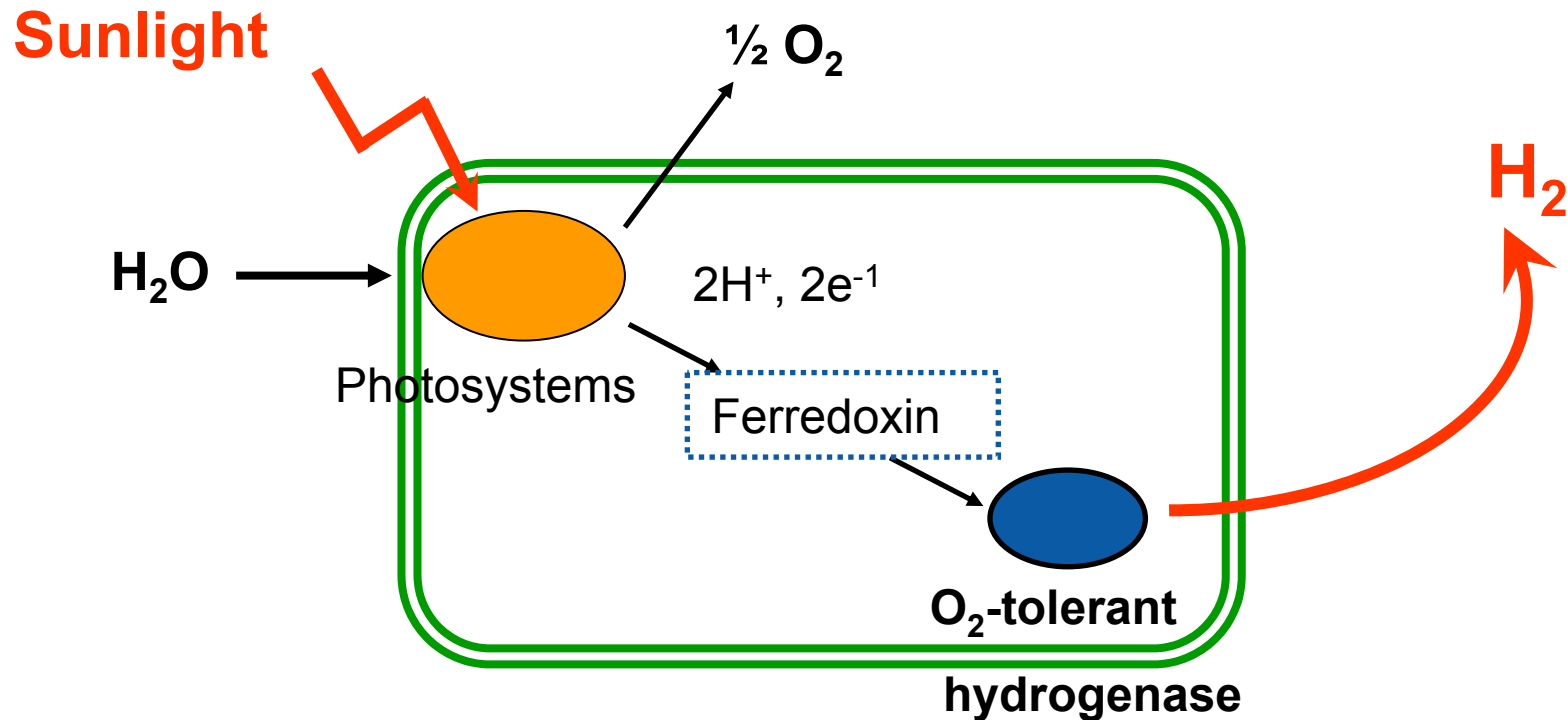
- Barriers addressed
  - Production Barrier Z:  
Continuity of H<sub>2</sub> production

## Partners

- J. Craig Venter Institute
- National Renewable Energy Laboratory
- Univ. Szeged, Hungary
- Vanderbilt University

# Objective

Develop an O<sub>2</sub>-tolerant cyanobacterial system for continuous light-driven H<sub>2</sub> production from water



**Approach:** transfer O<sub>2</sub>-tolerant hydrogenases into cyanobacteria to overcome the hydrogenase O<sub>2</sub>-sensitivity issue

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# Milestones

<b>Month/Year</b>	<b>Milestone</b>
June-09	<i>Task 1.</i> Identify hydrogenase gene sequences through metagenomic analysis of marine microbes from the oceans.
Sept-09	<i>Task 1.</i> Identify novel functional hydrogenases from environmental samples
Sept-07	<i>Task 2.</i> Make plasmid constructs for transferring hydrogenase genes into cyanobacteria
Dec-08	<i>Task 2.</i> Verify hydrogenase expression

# Technical Approach (JCVI)

**Task 1.** Identifying novel O<sub>2</sub>-tolerant hydrogenases through metagenomic analysis of marine microbes in the global ocean and transferring the hydrogenases into cyanobacteria



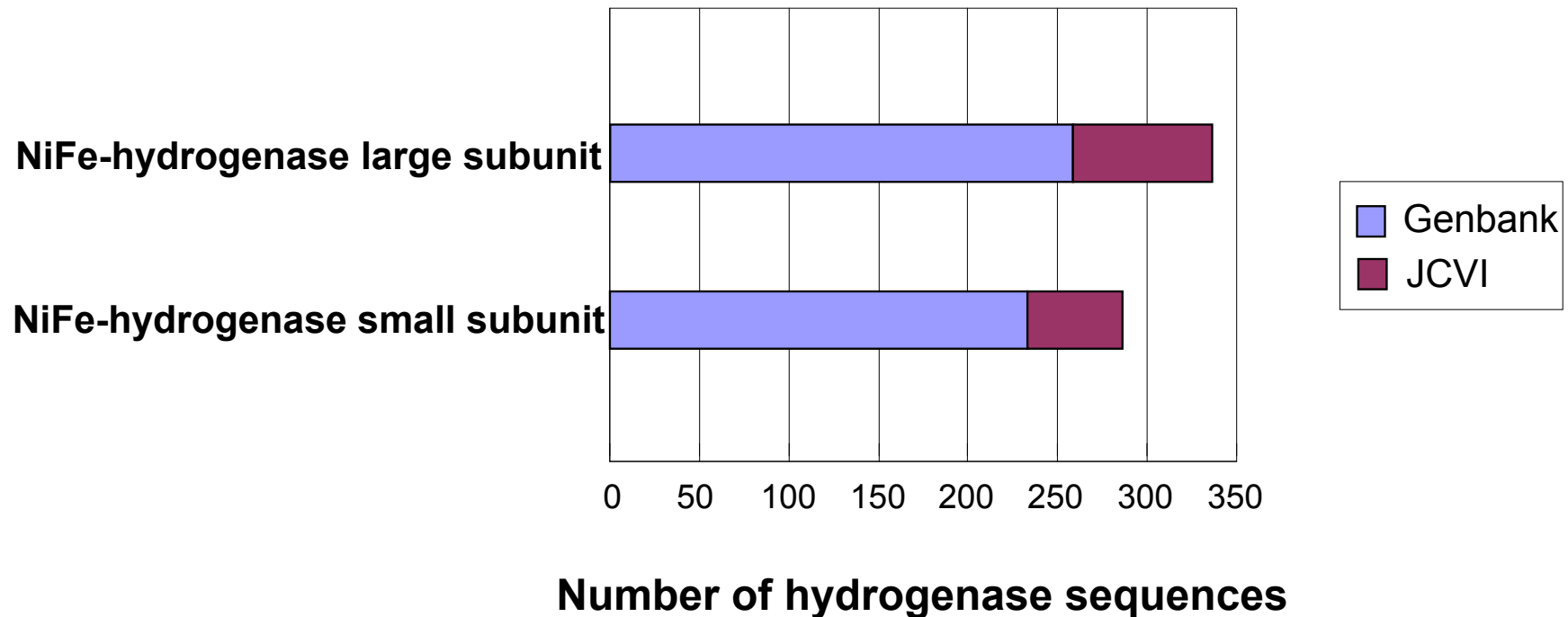
**Sorcerer II Expedition: a Global Ocean Sampling Project accomplished by the Venter Institute**

**J. Craig Venter**

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# Technical Accomplishments - JCVI

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

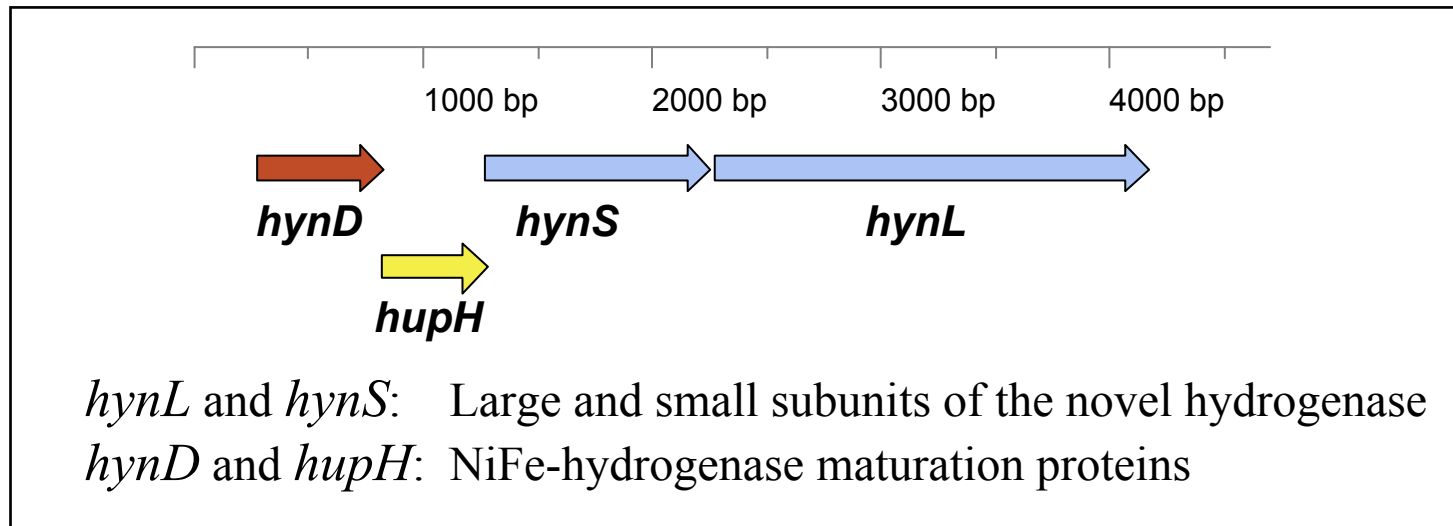


## From the Sargasso Sea sampling Site:

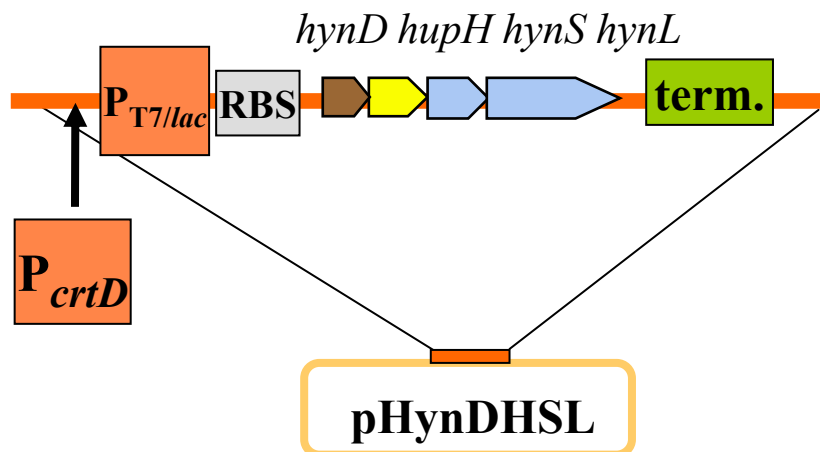
- Seven novel NiFe-hydrogenases were identified by using Hidden Markov Models.
- One novel hydrogenase shows strong homology to *T. roseopersicina* O<sub>2</sub>-stable hydrogenase and has been studied in details.

# Technical Accomplishments - JCVI

Cloned and expressed the genes of a novel environmental NiFe-hydrogenase homologous to a known O<sub>2</sub>-tolerant hydrogenase from *Thiocapsa roseopersicina* (60% identity to the large subunit and 64% to the small subunit)



Expression vector pHynDHS� transferred to *T. roseopersicina*:

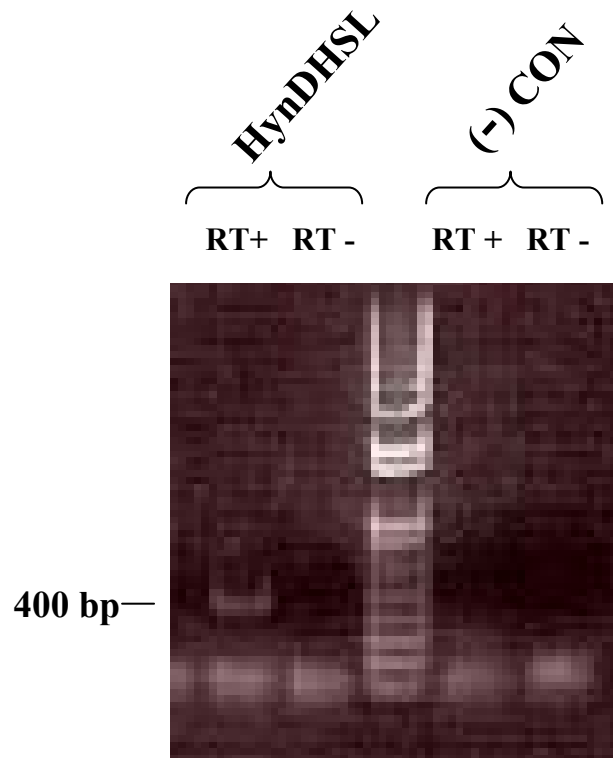


- Transferred into *Thiocapsa* through conjugation
- Self-replicated in *T. roseopersicina*
- Expression of Hyn's genes *hynD/hupH/hynS/hynL* is driven by promoter P<sub>crtD</sub>

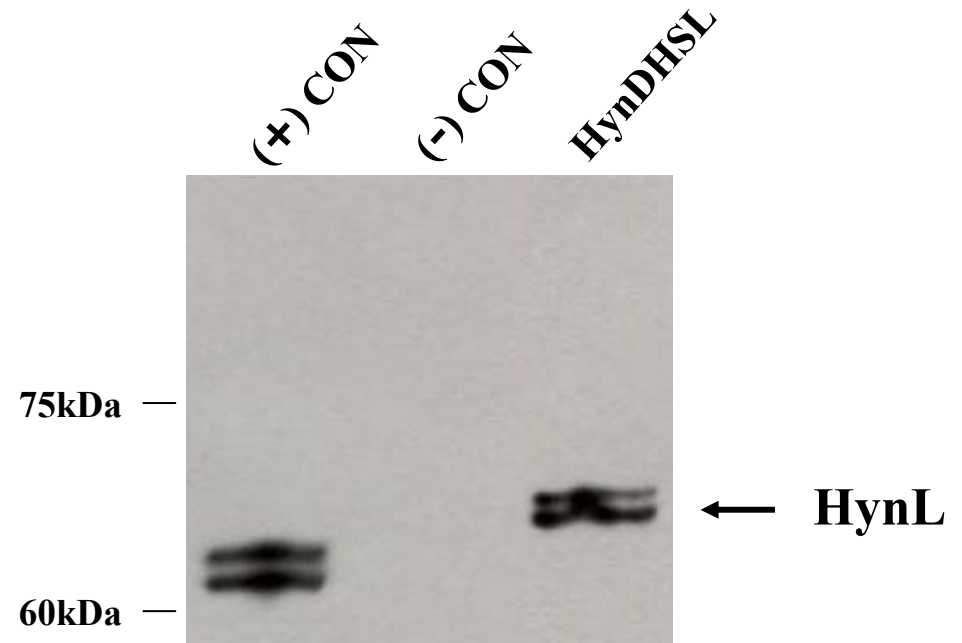
# Technical Accomplishments - JCVI

Detected heterologous expression of the novel NiFe-hydrogenase in *T. roseopersicina* on the levels of RNA and protein

## RT-PCR Confirmation



## Western Blotting Confirmation



(+) CON: *T. roseopersicina* wild-type strain

(-) CON: *T. roseopersicina* GB112131 strain ( $\Delta$ Hox,  $\Delta$ Hyn, and  $\Delta$ Hup)

HyndHSL: *T. roseopersicina* GB112131 strain containing pHynDHSL

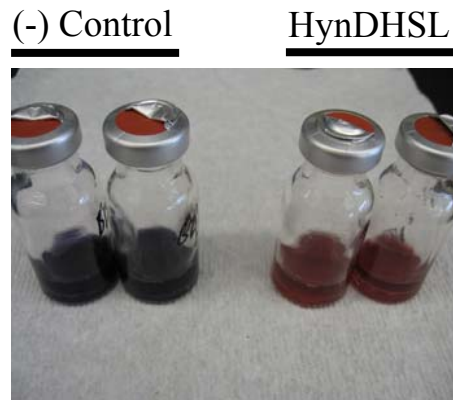
Rabbit polyclonal antibody specific for *T. roseopersicina* HynL was used for Western blotting



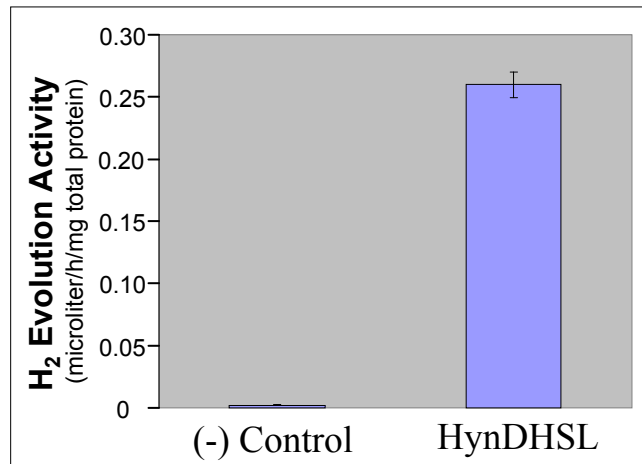
# Technical Accomplishments - JCVI

Detected H<sub>2</sub>-evolution and H<sub>2</sub>-uptake activities of the novel hydrogenase that was heterologously expressed in *T. roseopersicina*

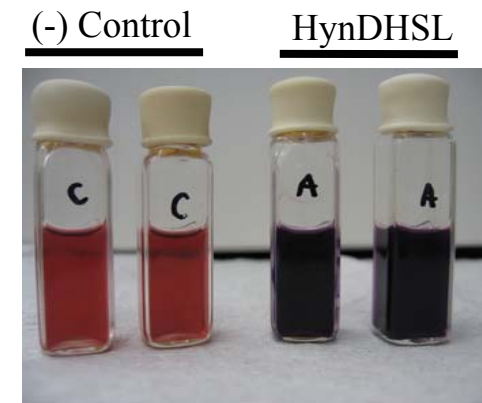
## *in vitro* H<sub>2</sub> evolution activity assay



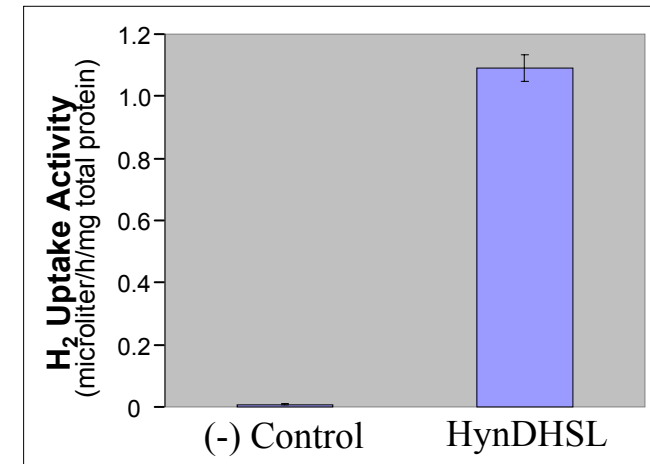
Artificial electron donor: reduced methyl viologen (blue)



## *in vitro* H<sub>2</sub> uptake activity assay



Artificial electron receptor: oxidized benzyl viologen (colorless)



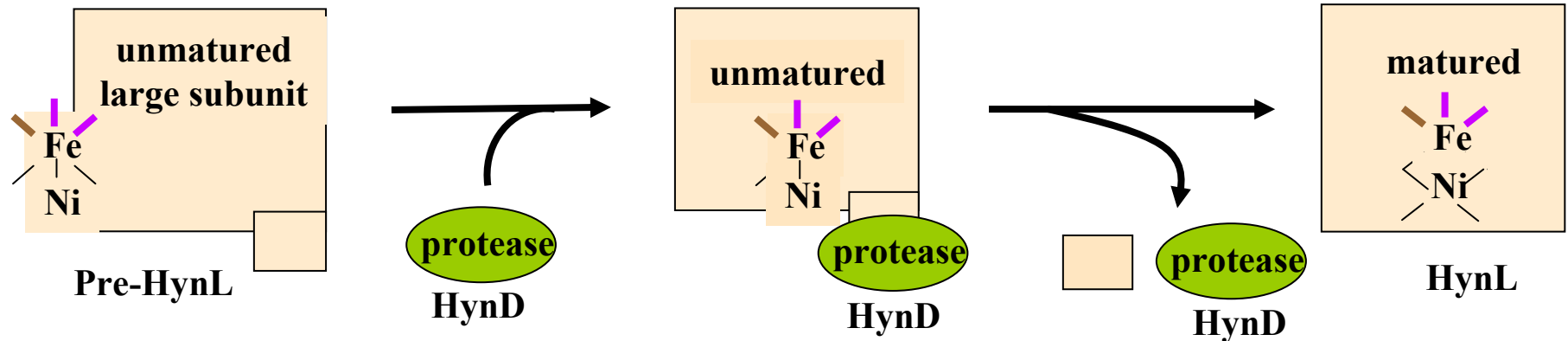
(-) Control: *T. roseopersicina* GB112131 strain (( $\Delta$ Hox,  $\Delta$ Hyn, and  $\Delta$ Hup)

HynDHSL: *T. roseopersicina* GB112131 transformed with pHynDHSL

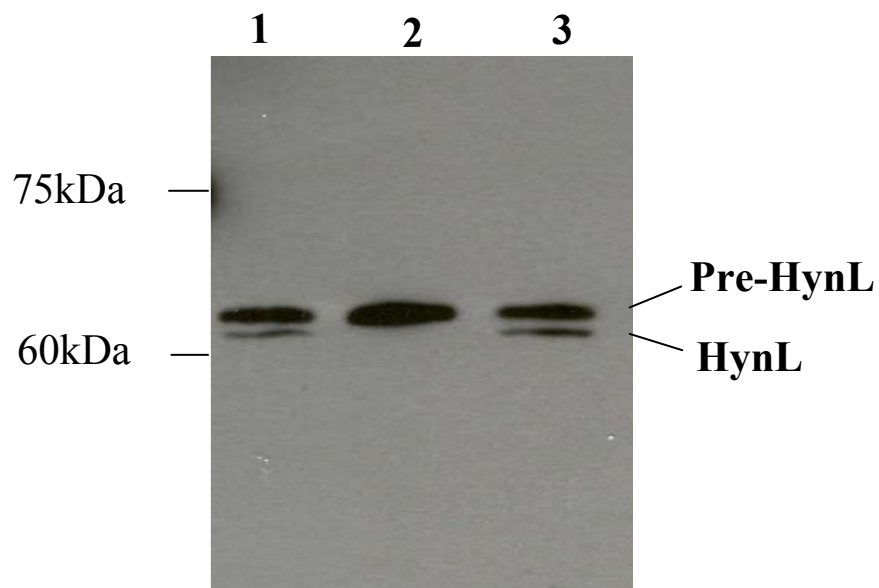
# Technical Accomplishments - JCVI

Heterologous novel *hynD* complemented *Thiocapsa hynD* in *hynD* mutant

HynD is a protease that cleaves the C-terminal end of unmaturred large subunit Pre-HynL



Restored the cleavage of Pre-HynL:

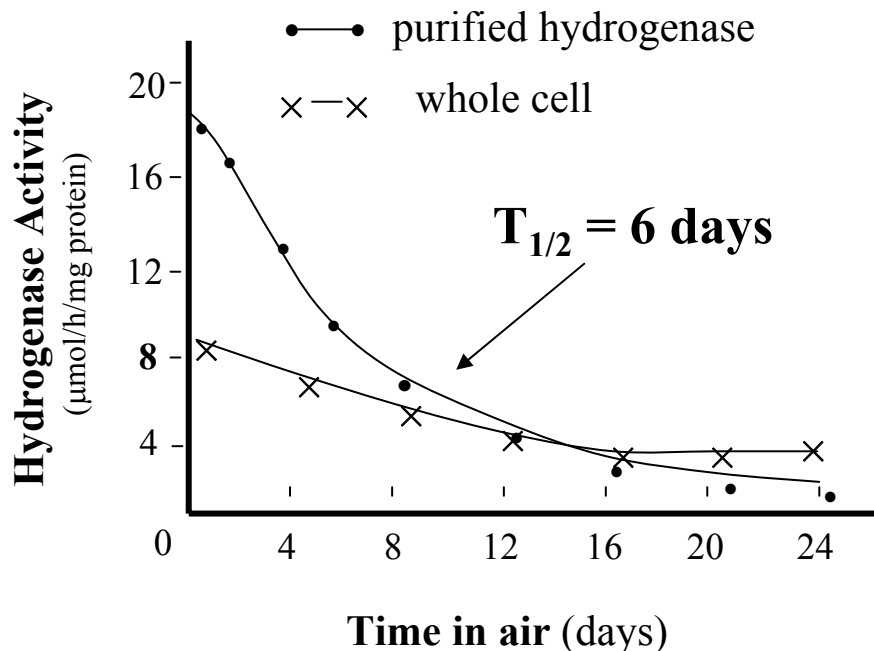


1. *T. roseopersicina* wild type strain.
2. *T. roseopersicina hynD* mutant ( $\Delta hynD$ ).
3. *T. roseopersicina hynD* mutant ( $\Delta hynD$ ) complemented with heterologous novel *hynD*.

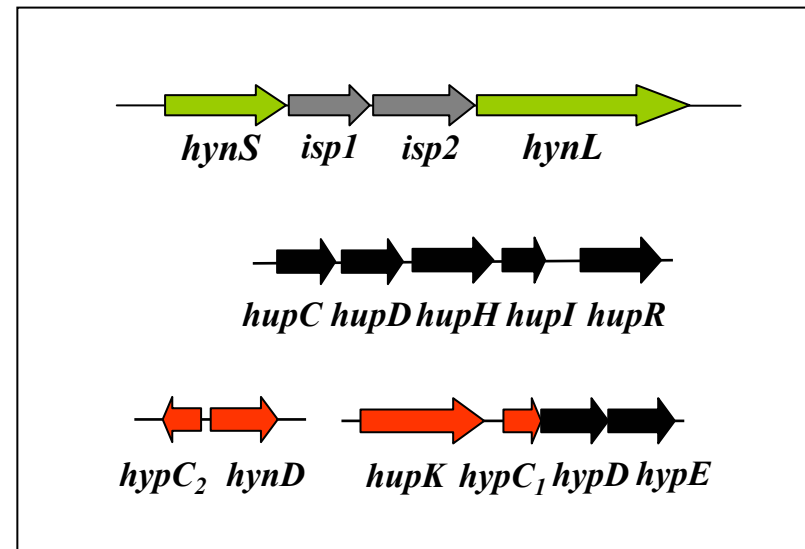
# Technical Approach (JCVI)

## Task 2.1. Transferring a known O<sub>2</sub>-tolerant NiFe-hydrogenase from *T. roseopersicina* into cyanobacterium *Synechococcus sp* PCC7942

- Phototrophic purple sulfur bacterium *Thiocapsa roseopersicina* carries an O<sub>2</sub>-tolerant and thermal-stable hydrogenase (Hyn).
- The *Thiocapsa* hydrogenase Hyn displays a half-life of 6 days in air.
- Structural and accessory genes encoding the *Thiocapsa* hydrogenase are cloned.



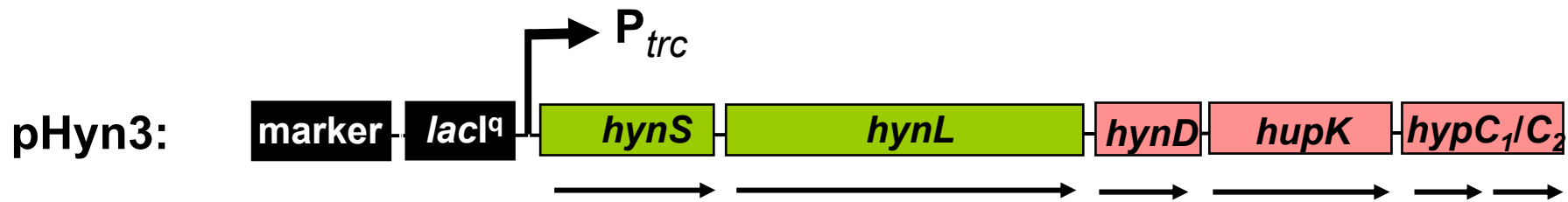
*Biochimica et Biophysica Acta* 523:335-343 (1978)



- Structural subunit gene: *hynS* and *hynL*
- Electron transfer subunit gene: *isp1* and *isp2*
- Accessory gene: *hynD*, *hupK*, *hypC1* and *hypC2*

# Technical Accomplishments - JCVI

Constructed a vector system to transfer the structural and accessory genes of *Thiocapsa* O<sub>2</sub>-tolerant hydrogenase Hyn to *S. sp* PCC7942



P<sub>Trc</sub>: an IPTG-inducible promoter;

Hyn's structural genes: *hynS* and *hynL* (in green);

Hyn's essential accessory genes: *hynD*, *hupK*, *hypC<sub>1</sub>* and *hypC<sub>2</sub>* (in red)

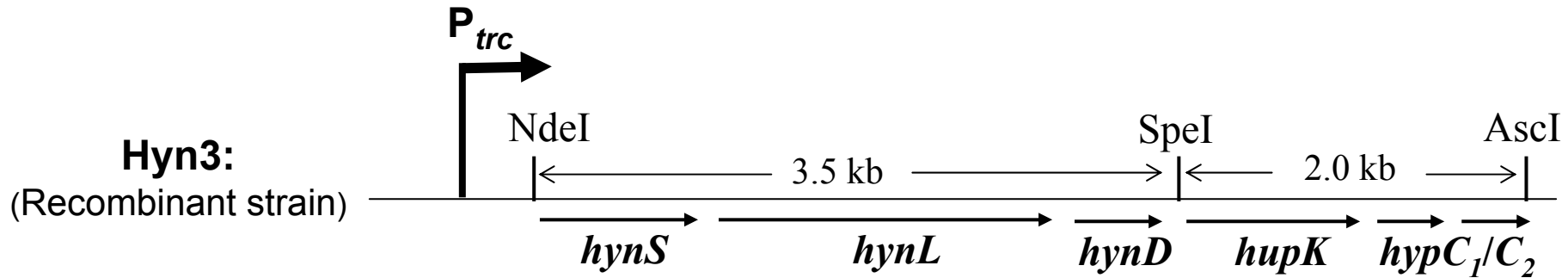
- Through homologous DNA recombination, the hydrogenase Hyn's genes can be integrated into the cyanobacterial chromosome.
- After being transferred into *S. sp* PCC7942, expression of the hydrogenase genes is under control of an IPTG-inducible promoter.

**Reached the Milestone (09/07):** *Thiocapsa* hydrogenase genes have been cloned into an expression vector to create construct pHyn3.

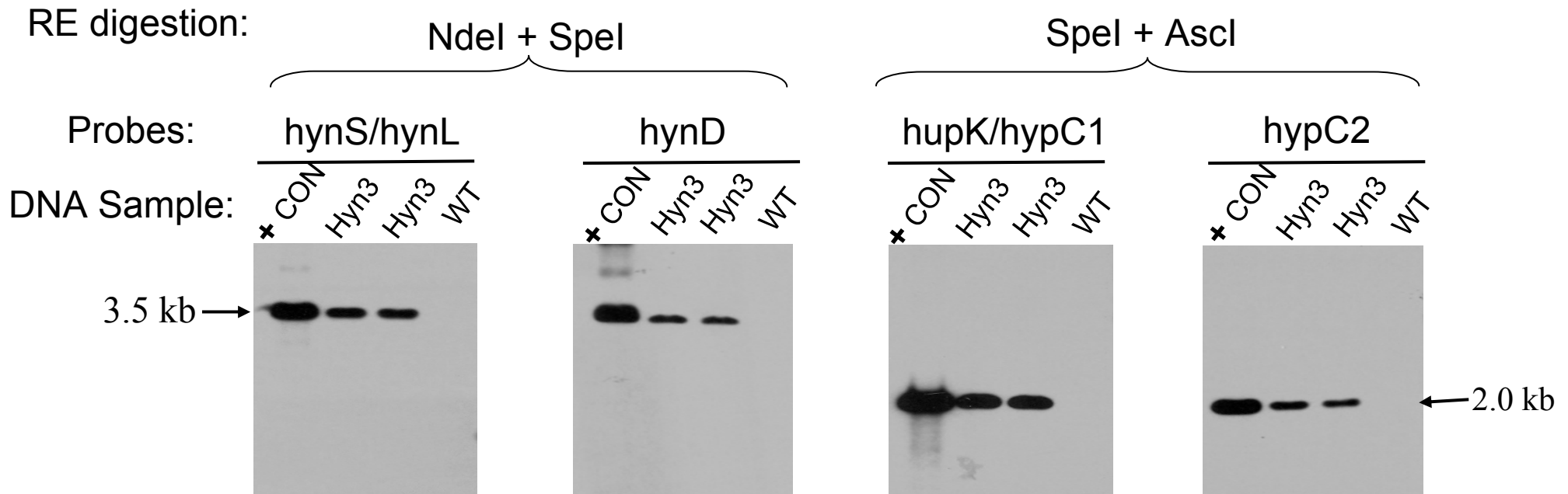
# Technical Accomplishments - JCVI

Integrated *Thiocapsa* hydrogenase Hyn's structural and accessory genes into the chromosome of cyanobacterium *S. sp* PCC7942

Southern Blotting Confirmed the integration of Hyn's genes in recombinant strains



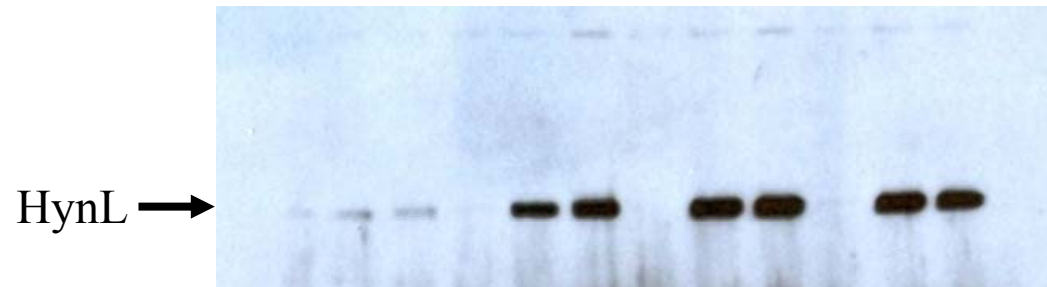
*Thiocapsa* Hyn's gene cassette integrated into the chromosome of *S. sp* PCC7942



# Technical Accomplishments - JCVI

Confirmed IPTG-inducible expression of O<sub>2</sub>-tolerant hydrogenase Hyn in recombinant *Synechococcus sp* PCC7942 strain Hyn3

+ IPTG (uM)	5			20			100			200		
Strains	1	2	2	1	2	2	1	2	2	1	2	2



1. *S. sp* PCC7942 wild type strain
2. Recombinant *S. sp* PCC7942 Hyn3

Western blotting was performed by using rabbit polyclonal antibody specific for HynL.

**Reached the Milestone (12/08):** *Thiocapsa* hydrogenase expression has been verified in recombinant *S. sp* PCC7942 strain Hyn3.

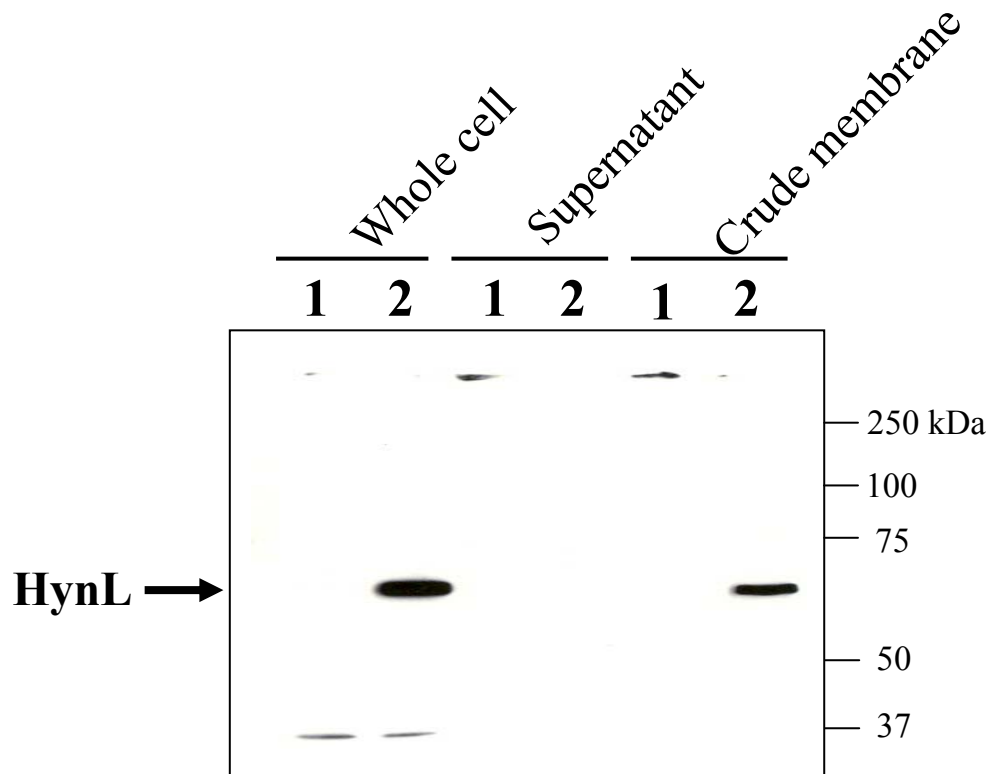
# Technical Accomplishments - JCVI

Examined the location of heterologously expressed O<sub>2</sub>-tolerant hydrogenase in a recombinant *S. sp* PCC7942 Hyn3 strain

Twin-arginine motif in signal peptide of HynS

**RRXFXL**

It can be recognized by membrane targeting and translocation pathway.

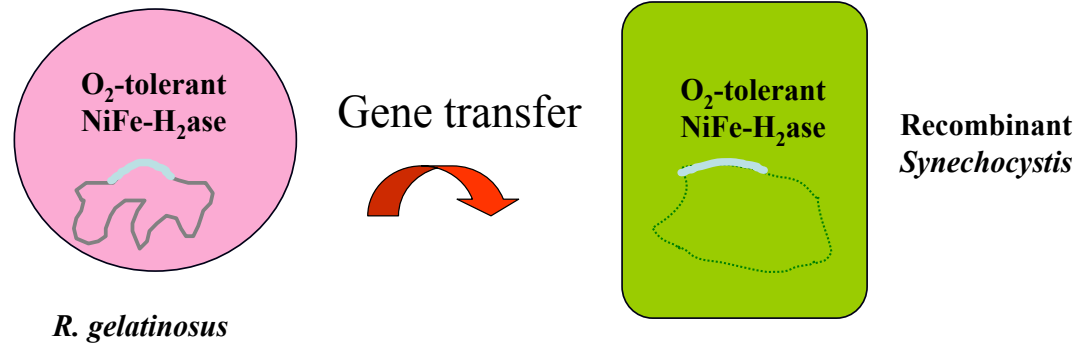


1. *S. sp* PCC7942 wild type strain
2. Recombinant *S. sp* PCC7942 strain Hyn3

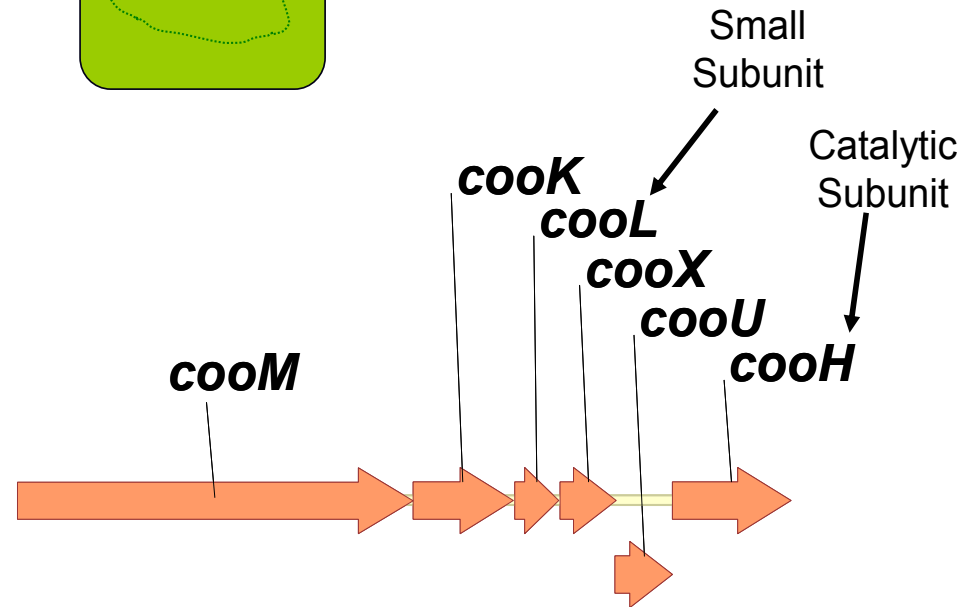
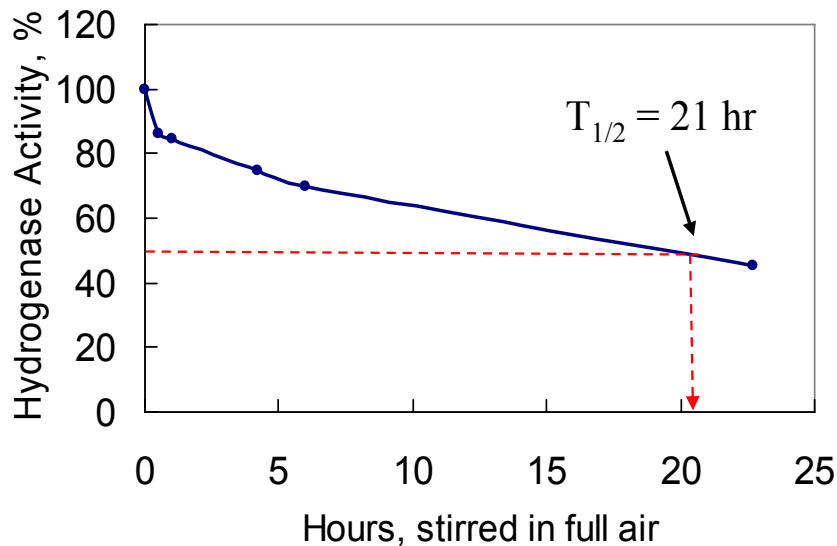
**Hetero-expressed Hyn is a membrane-bound hydrogenase in the Hyn3 strain.**

# Technical Approach (NREL)

Task 2.2. Transfer an O<sub>2</sub>-tolerant NiFe-hydrogenase from the bacterium *Rubrivivax gelatinosus* CBS into the cyanobacterium *Synechocystis* PCC6803



Hydrogenase half-life in air: 21



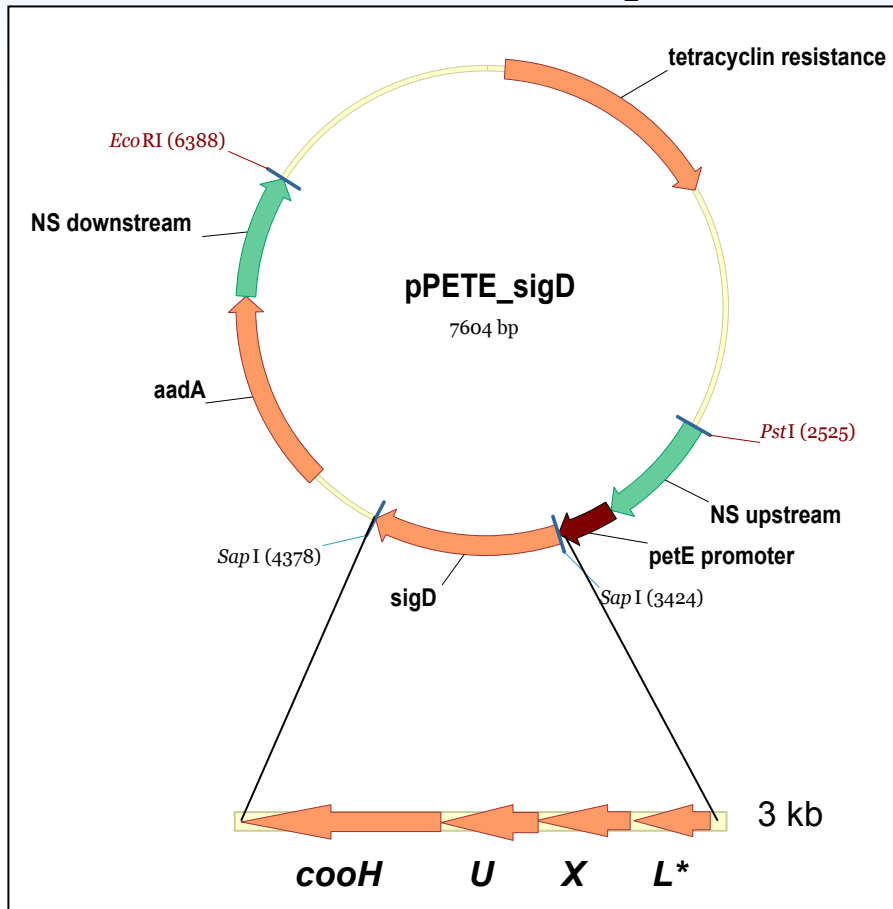
Hydrogenase genes are cloned

NREL will simultaneously develop *E. coli* as the host: (1) serving as a model with more available tools, and (2) DOE funding issue, with funds from Florida Intl. Univ.



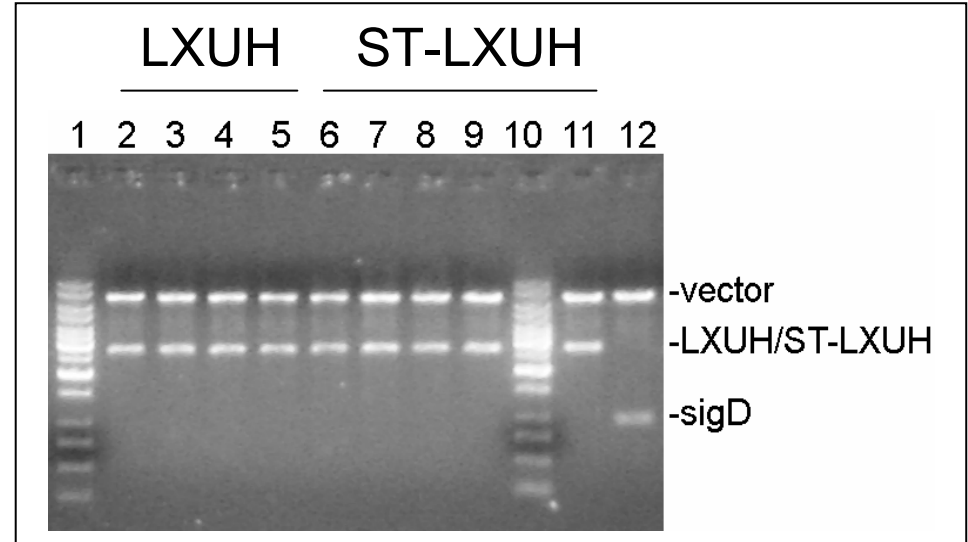
# Technical Accomplishment - NREL

## Cloned hydrogenase genes for transfer into *Synechocystis* 6803



### pPETE features

- copper-regulated (*petE* promoter)
- neutral-site for integrating foreign genes into its chromosomes



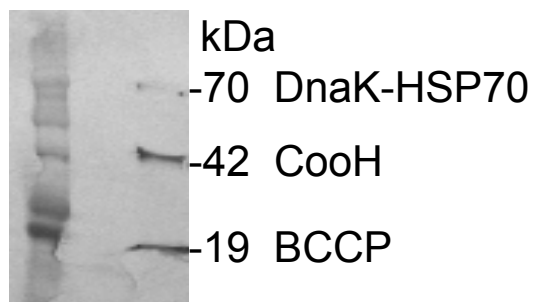
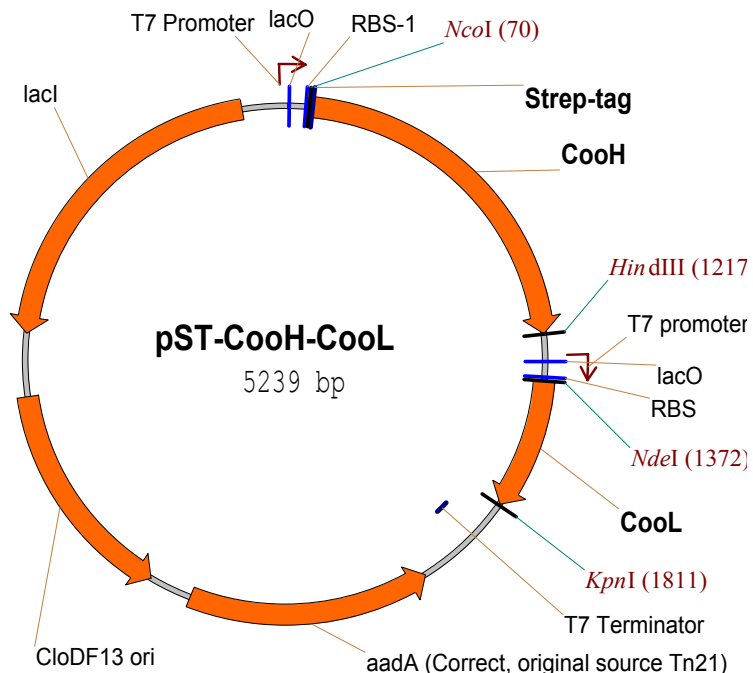
\*With and without affinity tag

**Meeting Milestone (9/07):** Hydrogenase genes *cooLXUH* were cloned in the *Synechocystis* 6803 expression vector (Prof. Burnap, Oklahoma State Univ.)

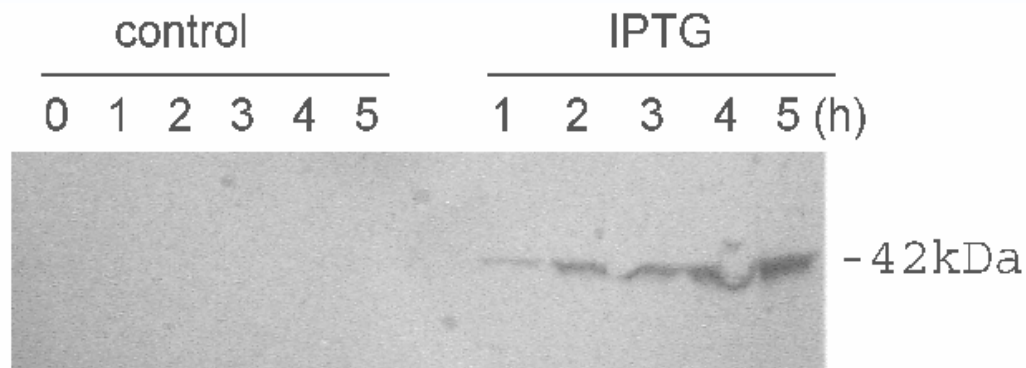
- Also confirmed an alternative plasmid **pRL1342**, suitable for expressing foreign genes in *Synechocystis* (Prof. C. P. Wolk, Michigan State Univ.)

# Technical Accomplishment - NREL

## Hydrogenase Catalytic Subunit Expressed in *E. coli*



Amino Black staining

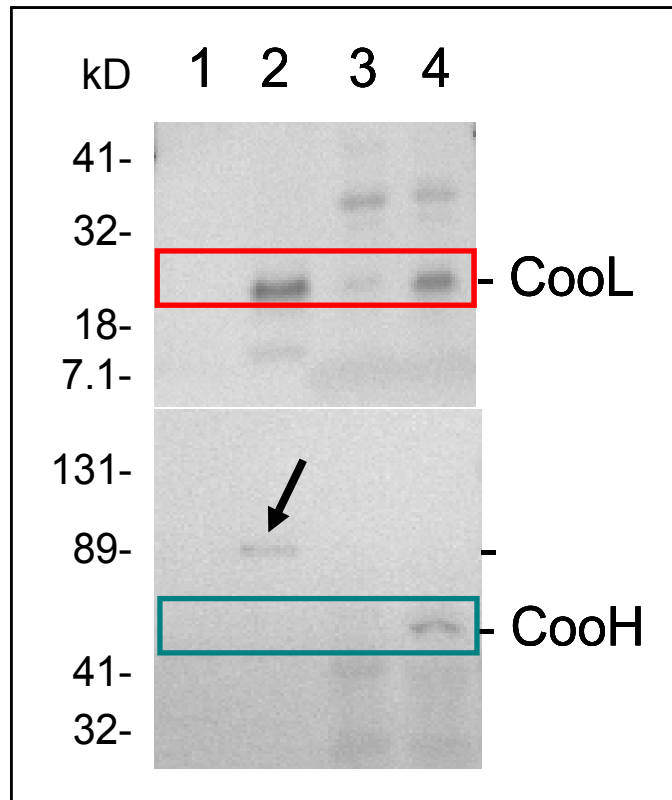


- *cooH* is fused with a Strep II affinity tag for affinity purification
- CooH (42 kDa) is detected in *E. coli* following IPTG induction
- Tagged protein was purified via affinity chromatography and stained with Amino Black
- Protease digestion/mass spectrometry revealed the 42 kDa protein as CooH, the hydrogenase catalytic subunit

# Technical Accomplishment - NREL

## Hydrogenase Small Subunit Expressed in *E. coli*

- A hydrogenase-free *E. coli* strain (F. Sargent) was modified for IPTG induction
- This FTD147(DE3) strain was transformed with **10** *R. gelatinosus* hydrogenase structural and assembly genes (*coolXUH* and *hypABCDEFGF*)\*
- Hydrogenase antibodies generated and hydrogenase protein expression verified in this transgenic *E. coli*.
- These antibodies will be used to confirm hydrogenase expression in *Synechocystis*



Lanes

1. *E. coli* control
2. *E. coli* transformed with *R. gelatinosus* hydrogenase genes
3. *R. gelatinosus*, hydrogenase not induced
4. *R. gelatinosus*, hydrogenase induced

**Toward meeting Milestone (12/08):**  
Tools development to verify  
hydrogenase expression

\* Collaboration with Florida Intl. Univ. (PDP 7)

# Future Work

- **JCVI**

- Further characterize the novel environmental hydrogenase that was heterologously expressed in *Thiocapsa roseopersicina*
- Transfer more accessory genes of *Thiocapsa* O<sub>2</sub>-tolerant hydrogenase into cyanobacterium *S. sp* PCC7942 to express a active hydrogenase in the host

- **NREL**

- The *R. gelatinosus* CBS hydrogenase genes will be transformed into *Synechocystis* (pPETE plasmid) and protein expression verified using their respective antibodies.
- Test hydrogenase expression using alternative plasmid (pRL1342)

# Summary

- **JCVI**

1. The GOS sequences were searched for novel NiFe-hydrogenases. A novel NiFe-hydrogenase homologous to *Thiocapsa* O<sub>2</sub>-tolerant hydrogenase was cloned from the Sargasso Sea samples.
2. Environmental DNA encoding this hydrogenase was converted into a functional hydrogenase with both H<sub>2</sub> evolution and uptake activities.
3. The heterologous novel protease HynD complemented *Thiocapsa* HynD in *Thiocapsa hynD* mutant.
4. The genes of *Thiocapsa* O<sub>2</sub>-tolerant NiFe-hydrogenase were transferred into *S. sp* PCC7942. A membrane-bound hydrogenase was expressed in the recombinant cyanobacterium upon IPTG induction.

- **NREL**

1. Cloned four O<sub>2</sub>-tolerant hydrogenase genes into a pPETE plasmid for expression in *Synechocystis*
2. Verified that O<sub>2</sub>-tolerant hydrogenase were expressed in a transgenic *E. coli* strain