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 H₂ production

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

Develop an O₂-tolerant cyanobacterial system for continuous light-driven H₂ production from water

Approach: transfer O₂-tolerant hydrogenases into cyanobacteria to overcome the hydrogenase O₂-sensitivity issue
## Milestones

<table>
<thead>
<tr>
<th>Month/Year</th>
<th>Milestone</th>
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<tbody>
<tr>
<td>June-09</td>
<td><em>Task 1.</em> Identify hydrogenase gene sequences through metagenomic analysis of marine microbes from the oceans.</td>
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<tr>
<td>Sept-09</td>
<td><em>Task 1.</em> Identify novel functional hydrogenases from environmental samples</td>
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<tr>
<td>Sept-07</td>
<td><em>Task 2.</em> Make plasmid constructs for transferring hydrogenase genes into cyanobacteria</td>
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<td>Dec-08</td>
<td><em>Task 2.</em> Verify hydrogenase expression</td>
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**Technical Approach (JCVI)**

**Task 1.** Identifying novel $O_2$-tolerant hydrogenases through metagenomic analysis of marine microbes in the global ocean and transferring the hydrogenases into cyanobacteria

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*Sorcerer II Expedition: a Global Ocean Sampling Project accomplished by the Venter Institute*
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$_2$-stable hydrogenase and has been studied in details.
Cloned and expressed the genes of a novel environmental NiFe-hydrogenase homologous to a known O$_2$-tolerant hydrogenase from *Thiocapsa roseopersicina* (60% identity to the large subunit and 64% to the small subunit)

Expression vector pHynDHSL 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 $P_{crtD}$
Detected heterologous expression of the novel NiFe-hydrogenase in *T. roseopersicina* on the levels of RNA and protein

**Technical Accomplishments - JCVI**

**RT-PCR Confirmation**

<table>
<thead>
<tr>
<th>HynDHS</th>
<th>(-) CON</th>
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<tr>
<td>RT+</td>
<td>RT-</td>
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</table>

400 bp

**Western Blotting Confirmation**

<table>
<thead>
<tr>
<th>(+) CON</th>
<th>(-) CON</th>
<th>HynDHS</th>
</tr>
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<tbody>
<tr>
<td>RT+</td>
<td>RT-</td>
<td>RT+</td>
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</tbody>
</table>

75kDa

60kDa

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

(-) CON: *T. roseopersicina* GB112131 strain (ΔHox, ΔHyn, and ΔHup)

HynDHS: *T. roseopersicina* GB112131 strain containing pHynDHS

Rabbit polyclonal antibody specific for *T. roseopersicina* HynL was used for Western blotting
Detected H₂-evolution and H₂-uptake activities of the novel hydrogenase that was heterologously expressed in *T. roseopersicina*

*in vitro* H₂ evolution activity assay

(-) Control  |  HynDHSL

Artificial electron donor: reduced methyl viologen (blue)

*in vitro* H₂ uptake activity assay

(-) Control  |  HynDHSL

Artificial electron receptor: oxidized benzyl viologen (colorless)

(-) Control: *T. roseopersicina* GB112131 strain ((ΔHox, ΔHyn, and ΔHup)

HynDHSL: *T. roseopersicina* GB112131 transformed with pHynDHSL
HynD is a protease that cleaves the C-terminal end of unmatured large subunit Pre-HynL.

- **Pre-HynL**
  - [FeNi]
  - **protease**
  - HynD

- **unmatured**
  - [FeNi]
  - **protease**
  - HynD

- **matured**
  - [FeNi]
  - **protease**
  - HynL

**Restored the cleavage of Pre-HynL:**

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

![Image of 3 lanes with molecular weight markers (75kDa and 60kDa) and protein bands labeled Pre-HynL and HynL.](Image)
Task 2.1. Transferring a known O$_2$-tolerant NiFe-hydrogenase from *T. roseopersicina* into cyanobacterium *Synechococcus sp* PCC7942

- Phototrophic purple sulfur bacterium *Thiocapsa roseopersicina* carries an O$_2$-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.

![Graph showing hydrogenase activity over time in air](image)

**Hydrogenase Activity (μmol/h/mg protein)**

- **T$_{1/2} = 6$ days**
- Purified hydrogenase
- Whole cell

**Time in air (days)**

*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*
Construct a vector system to transfer the structural and accessory genes of *Thiocapsa* O$_2$-tolerant hydrogenase Hyn to *S. sp* PCC7942

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.
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

**Hyn3:**
(Recombinant strain)

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

**Probes:**
- hynS/hynL
- hynD
- hupK/hypC1
- hypC2

**DNA Sample:**
- CON
- Hyn3
- WT

**RE digestion:**
- NdeI + SpeI
- SpeI + Ascl
Confirmed IPTG-inducible expression of O$_2$-tolerant hydrogenase Hyn in recombinant *Synechococcus sp* PCC7942 strain Hyn3

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

### Table

<table>
<thead>
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<th>20</th>
<th>100</th>
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<td>2</td>
<td>1</td>
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<td>2</td>
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</tbody>
</table>

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

**Reached the Milestone (12/08):** *Thiocapsa* hydrogenase expression has been verified in recombinant *S. sp* PCC7942 strain Hyn3.
Examined the location of heterologously expressed O₂-tolerant hydrogenase in a recombinant *S. sp* PCC7942 Hyn3 strain

**Technical Accomplishments - JCVI**

Twin-arginine motif in signal peptide of HynS

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$_2$-tolerant NiFe-hydrogenase from the bacterium *Rubrivivax gelatinosus* CBS into the cyanobacterium *Synechocystis* PCC6803

Hydrogenase half-life in air: 21 hours

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

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

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*

- *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
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 *hypABCDEF*)

Hydrogenase antibodies generated and hydrogenase protein expression verified in this transgenic *E. coli*. These antibodies will be used to confirm hydrogenase expression in *Synechocystis*.

**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$_2$-tolerant hydrogenase into cyanobacterium *S. sp* PCC7942 to express an 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* O₂-tolerant hydrogenase was cloned from the Sargasso Sea samples.

2. Environmental DNA encoding this hydrogenase was converted into a functional hydrogenase with both H₂ evolution and uptake activities.

3. The heterologous novel protease HynD complemented *Thiocapsa* HynD in *Thiocapsa hynD* mutant.

4. The genes of *Thiocapsa* O₂-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 O₂-tolerant hydrogenase genes into a pPETE plasmid for expression in *Synechocystis*

2. Verified that O₂-tolerant hydrogenase were expressed in a transgenic *E. coli* strain