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

Qing Xu, Philip D. Weyman, and Hamilton O. Smith, J. Craig Venter Institute
Pin-Ching Maness, Jianping Yu, and Karen Wawrousek, NREL

June 10, 2010

Project ID
PD039

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Overview

Timeline
- Project start date: 5-01-05
- Project end date: 8-31-2011
- Percent complete: 80%

Budget
- Total project funding
  - DOE share: $1.62M for JCVI
  - DOE share: $1.26M for NREL
  - JCVI cost-share: $720K
- Funding received for FY09
  - $100K for JCVI
  - $220K for NREL
- Funding for FY10
  - $300K for JCVI
  - $86K for NREL

Barriers
- Barriers addressed
  - Production Barrier Z: Continuity of H₂ 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

**Chemical Reaction:**

\[ \text{H}_2 \text{O} \rightarrow \frac{1}{2} \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \]

**Diagram:**

- Sunlight
- Water (H$_2$O) enters Photosystems
- Generating 2H$^+$, 2e$^-$
- Ferredoxin
- O$_2$-tolerant hydrogenase
- Produces H$_2$

**Table:**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>2009 Status</th>
<th>2011 Target</th>
<th>2018 Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of continuous photoproduction</td>
<td>Zero to 30 seconds in air</td>
<td>Produce one cyanobacterial recombinant evolving H$_2$ through an O$_2$-tolerant NiFe-hydrogenase</td>
<td>Demonstrate H$_2$ production in air in a cyanobacterial recombinant</td>
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</tbody>
</table>
## Milestones

### Task 1. (JCVI and NREL)

<table>
<thead>
<tr>
<th>Month/Year</th>
<th>Milestone</th>
<th>% Comp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sept-09</td>
<td>Purify hydrogenases</td>
<td>JCVI, 100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NREL, 100%</td>
</tr>
<tr>
<td>Apr-10</td>
<td>Determine electron mediator requirement</td>
<td>JCVI, 50%</td>
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<tr>
<td></td>
<td></td>
<td>NREL, 50%</td>
</tr>
<tr>
<td>Sept-09</td>
<td>Verify hydrogenase functionality in oxygen</td>
<td>JCVI, 100%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NREL, 100%</td>
</tr>
<tr>
<td>Apr-10</td>
<td>Construct cyanobacterial hybrid to express an active <em>Thiocapsa</em> hydrogenase</td>
<td>JCVI, 90%</td>
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<tr>
<td></td>
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<td>NREL, 50%</td>
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</tbody>
</table>

### Task 2. (JCVI)

<table>
<thead>
<tr>
<th>Month/Year</th>
<th>Milestone</th>
<th>% Comp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sept-09</td>
<td>Identify novel functional hydrogenases from the oceans</td>
<td>JCVI, 100%</td>
</tr>
<tr>
<td>Apr-10</td>
<td>Screening for a new O$_2$-tolerant hydrogenase</td>
<td>JCVI, 100%</td>
</tr>
<tr>
<td>Apr-10</td>
<td>Construct a cyanobacterial hybrid to express an active environmental hydrogenase</td>
<td>JCVI, 50%</td>
</tr>
</tbody>
</table>
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 and thermal-stable 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 and Electron Transfer Subunits

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

![Chemical Reaction Diagram](image)

**Diagram Description:**

- $2H^+ + ADP + P_i \rightarrow ATP$
- $2H^+ + 2e^- \rightarrow H_2$
- $X_{red} \rightarrow X_{ox} \rightarrow H_2$

**Graph:**

- **Y-axis:** Hydrogenase Activity (µmol/h/mg protein)
- **X-axis:** Time in air (days)
- **Data Points:**
  - Purified hydrogenase: $T_{1/2} = 6$ days
  - Whole cell

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*Biochimica et Biophysica Acta 523:335-343 (1978)*
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 $\approx 0.4$ M)

**Reached Milestone** “Purify *Thiocapsa* $O_2$-tolerant hydrogenases” *(09/09)*
Transferred all genes of the *Thiocapsa* O$_2$-tolerant hydrogenase (Hyn) into *Synechococcus* PCC7942 Hoxmut

- Structural genes: *hynS/hynL* (red); Electron transfer subunit genes: *isp1* and *isp2* (brown)
- Accessory genes: *hynD/hupK/hypC1/C2* (orange), *hypD/E/F* (green) and *hupC/D/H/I/R* (yellow)

- Through homologous DNA recombination, genes in different combinations were integrated into the cyanobacterial genome, as confirmed by PCR and Southern.
- The *Thiocapsa* hydrogenase is expressed under the control of an IPTG-inducible promoter in the mutant strain Hoxmut, in which the native hydrogenase Hox was knocked out.
Heterologously expressed an active O2-tolerant Thiocapsa hydrogenase in the recombinant cyanobacterium S. e. PCC7942

JCVI - Technical Accomplishments

1. Hoxmut – IPTG; 2. Hoxmut +IPTG; 3. Recombinant strain (1) –IPTG; 4. Recombinant strain (1) +IPTG; 5. Recombinant strain (2) –IPTG; 6. Recombinant strain (2) +IPTG

- IPTG-inducible expression (A) of O$_2$-tolerant hydrogenase Hyn was detected in the recombinant Synechococcus using specific antibodies.
- IPTG-inducible hydrogenase activity (B) was detected by in vitro H2-evolution assay.
Task 1.2. Transfer an O\textsubscript{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\textsubscript{2}-tolerant hydrogenases and their transfer into two model cyanobacteria
Obtained *Synechocystis* transformants in the Hox- zero-H₂ background host.

Three CBS hydrogenase subunits (CooLUH) were expressed with gene integration in *Synechocystis* via homologous recombination.
NREL – Technical Accomplishments

- Hydrogenase Maturation Gene Expressed in *Synechocystis*

- Constructed a plasmid-based expression system with CooL and likely HypA (maturation) proteins expressed.
- Hydrogenase maturation genes *hypABCDEF* were cloned for expression.
Purified O$_2$-tolerant CBS Hydrogenase

Size Exclusion Chromatography (Sephacryl S-200)

27 fold purification, 5.7% yield
Purified hydrogenase retained 60% activity in 13% O$_2$.

Completed Milestone “Purify CBS O$_2$-tolerant hydrogenase (9/09)
**Task 2.** Identifying novel $O_2$-tolerant hydrogenases through metagenomic analysis of marine microbes in the global ocean and transferring the hydrogenases into cyanobacteria
Cloned and expressed the genes of a novel environmental NiFe-hydrogenase with 60% similarity to *Thiocapsa* O₂-tolerant hydrogenase

- *hynS/hynL*: hydrogenase structural genes
- *hynD/hupH*: hydrogenase accessory genes
- *P*crtD: a promoter from *T. roseopersicina*

![Diagram](image)

- **(+)** 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/hynL* 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*. 
Analyzed the Novel Hydrogenase that was Heterologously Expressed in the *T. roseopersicina* Host

- After purification, the activity of the novel environmental hydrogenase was enhanced 14 times.
- The novel hydrogenase showed extraordinary thermo-stability.
- The purified hydrogenase retained ~30% activity in 1% O2.
Assembled and cloned the entire gene operon of the novel environmental hydrogenase for transferring into cyanobacterium.

Accum image of the diagram:

- Asm Ligase + –
- Vector/NdeI
- 29 kb
- 19 kb
- Insert A: 2.9 kb
- Insert B: 3.5 kb
- Insert C: 3.3 kb
- Insert D: 3.3 kb
- T4 DNA polymerase, Taq DNA pol., and Taq Ligase
- Assembling
  (JCVI’s Chew-Back and Anneal method)
- Hydrogenase Operon (12.8 kb)
- Vector
- 8 kb
- pRC41

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.
Expressed an active environmental hydrogenase in the host of the cyanobacterium S.e. PCC7942

A. Western Blotting

B. H2-evolution activity assay


- IPTG-inducible expression (A) of novel environmental NiFe-hydrogenase HyaAB was detected in the recombinant Synechococcus PCC7942 using specific antibodies.
- IPTG-inducible hydrogenase activity (B) was detected by in vitro H2-evolution assay.
Collaborations

- University of Szeged, Hungary
  - Expressing novel environmental hydrogenase in *Thiocapsa*

- Vanderbilt University
  - Expressing $O_2$-tolerant hydrogenases in cyanobacteria

- Qingdao Institute of Bioenergy and Bioprocess Technology
  - Sequencing the CBS genome
Proposed Future Work

**JCVI**
- Re-engineer plasmid constructs and demonstrate increased expression of hydrogenase *in vitro* (FY10 and 11).
- Verify hydrogenase activity in cyanobacteria *in vivo* and assess ability to make hydrogen from water (FY10 and 11).
- Test electron mediator requirement of hydrogenase expressed in cyanobacteria with increased hydrogenase expression (FY10 and 11).

**NREL**
- Express additional CBS hydrogenase maturation genes and measure hydrogenase activity in *Synechocystis* host (FY10 and 11).
- Begin purification of the affinity-tagged CBS hydrogenase to test its functionality in O$_2$ (FY10 and 11).
Summary

**JCVI**

1. The $O_2$-tolerant hydrogenase from *Thiocapsa* was purified through FPLC. Its linkage with cyanobacterial ferredoxin has been confirmed *in vitro*.

2. The genes of the *Thiocapsa* $O_2$-tolerant hydrogenase were transferred into *S. PCC7942* and activity from the heterologously-expressed hydrogenase was detected.

3. A novel NiFe-hydrogenase was cloned from the Sargasso Sea environmental DNA, expressed *in T. roseopersicina*, and showed activity in the presence of low levels of 1% oxygen.

4. The genes of this novel hydrogenase were transferred into *E. coli* and *Synechococcus*, and activity from the heterologously-expressed hydrogenase was detected.

**NREL**

1. Developed two different expression systems and expressed at least three CBS hydrogenase subunits and one maturation subunit in *Synechocystis*.

2. CBS native hydrogenase was purified. Developed an affinity system that enables faster purification of CBS hydrogenase for characterization.