Improving Cyanobacterial O$_2$-Tolerance using CBS Hydrogenase for H$_2$ Production

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(PI; Presenter)

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Overview

Timeline

- Project start date: FY05 (not funded in FY06)
- Project end date: 10/2014*

Barriers

Barriers addressed
- Oxygen Accumulation (AP)

Budget

- FY13 DOE Funding: $350K
- Planned FY14 DOE Funding: $350K
- Total DOE Project Value: $2.21M

Partners

- Dr. Phil Weyman, J. Craig Venter Institute
- Dr. Jin Chen, Michigan State University
- Dr. Jonas Korlach, Pacific Biosciences

*Project continuation and direction determined annually by DOE
**Objective/Relevance**

**Objective:**
Develop a robust O$_2$-tolerant cyanobacterial system for light-driven H$_2$ production from water while increasing system durability. The long-term goal is to be O$_2$ tolerant for 8 hours (during daylight hours).

**Oxygen Accumulation (Barrier AP):**
Along with H$_2$, photolytic microbes such as algae and cyanobacteria co-produce O$_2$, which inhibits the activity of hydrogenase, the enzyme responsible for H$_2$ production.

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**Technical Target**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Unit</th>
<th>2011 status</th>
<th>2015 Target</th>
<th>2020 Target</th>
<th>Ultimate Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of continuous H$_2$ production at full sunlight intensity</td>
<td>Time Unit</td>
<td>2 min</td>
<td>30 min</td>
<td>4 h</td>
<td>8h</td>
</tr>
</tbody>
</table>
Objective/Relevance: Project Overview

Relevance:

- **Task 1**: Probe hydrogenase maturation machinery in CBS.
- **Task 2**: Expression of the CBS hydrogenase in *Synechocystis*

CBR Hydrogenase Half-life in Air: 21 h

NREL’s approach harnesses Nature’s O₂-tolerant hydrogenase and its transfer into a model cyanobacterium with genetic tools.
**Approach/Milestone**

**Task 1: Probe Hydrogenase Maturation Machinery in CBS**

**Approach:** CBS contains two sets of hydrogenase maturation genes, \( hyp1 \) and \( hyp2 \). This task aims to determine which set should be co-transformed with the hydrogenase structural genes to afford \( O_2 \)-tolerant hydrogenase activity in *Synechocystis*. The approaches are:

- Homology comparison of \( hyp1 \) with \( hyp2 \), and with other known \( hyp \) genes
- Probing the function of \( hyp1 \) and \( hyp2 \) maturation genes via expression profiles & gene knockout
- Uncovering more hydrogenase maturation-related genes.

**O\(_2\)**-tolerant hydrogenase (evolving H\(_2\))

<table>
<thead>
<tr>
<th>Hydrogenase</th>
<th>( cooM )</th>
<th>( K )</th>
<th>( L )</th>
<th>( X )</th>
<th>( U )</th>
<th>( H )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( hyp1 )</td>
<td>hypB</td>
<td>A</td>
<td>F</td>
<td>C</td>
<td>D</td>
<td>E</td>
</tr>
<tr>
<td>Maturation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

**H\(_2\)**-uptake hydrogenase (oxidizing H\(_2\))

- \( hyp2: \) Maturation genes
- Uptake hydrogenase
## Milestones

### Task 1: Probe Hydrogenase Maturation Machinery in CBS

<table>
<thead>
<tr>
<th></th>
<th>FY 14 Milestone – all regular</th>
<th>Completion Date</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1</td>
<td>Identify the CBS homologues of three hydrogenase maturation genes, <em>slyD</em> and <em>carAB</em>, through a targeted search of the CBS genome and comparison with those in <em>E. coli</em>, using Blast tool with 35% amino acid sequence identity</td>
<td>12/13</td>
<td>Complete</td>
</tr>
<tr>
<td>Q2</td>
<td>Provide detailed analysis of the CBS genome for genes encoding the O₂-tolerant evolving hydrogenase, uptake hydrogenase, and the two sets of hydrogenase maturation genes, <em>hyp1</em> and <em>hyp2</em>. These data will provide the blueprint to guide genetic engineering effort toward constructing a <em>Synechocystis</em> recombinant harboring O₂-tolerant hydrogenase activity.</td>
<td>3/14</td>
<td>Interim milestone; Complete</td>
</tr>
<tr>
<td>Q3</td>
<td>Through deletion of <em>hyp2ABFCDE</em> either in the wild type or Δ<em>hyp1FCDE</em> background, determine if <em>hyp2</em> increases total hydrogenase activity by 15% based on <em>in vitro</em> assay using reduced methyl viologen and is required for optimal hydrogenase activity; if so, <em>hyp2</em> will be co-transformed with <em>hyp1</em> into <em>Synechocystis</em> to assemble a more active CBS hydrogenase for increased H₂ production.</td>
<td>6/14</td>
<td>Delayed from Q2 to Q3; On Track</td>
</tr>
</tbody>
</table>
Task 1 – Technical Accomplishments

Uncovered Additional Maturation Genes: slyD and carAB in CBS Genome

- *E. coli* is the working model for proteins involved and mechanism for NiFe-hydrogenase maturation
- In *E. coli*, slyD plays a role in Ni binding and Ni insertion into the hydrogenase NiFe-active site.
- A slyD homolog was identified in CBS, but not in *Synechocystis*, hence might warrant the expression of slyD in *Synechocystis*.
- carAB homologs are present in both CBS and *Synechocystis*, which synthesize the CN ligand for the hydrogenase NiFe-active site.

<table>
<thead>
<tr>
<th>CBS</th>
<th>Identity to <em>E. coli</em> (%)</th>
<th>Identity to <em>Synechocystis</em> (%)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>SlyD</td>
<td>33</td>
<td>No identical homolog</td>
<td>Ni insertion</td>
</tr>
<tr>
<td>CarA</td>
<td>64</td>
<td>51</td>
<td>Synthesis of CN ligand for NiFe-active site</td>
</tr>
<tr>
<td>CarB</td>
<td>70</td>
<td>60</td>
<td>Synthesis of CN ligand for NiFe-active site</td>
</tr>
</tbody>
</table>

➢ Summary: Complete FY14 Q1 Milestone. slyD, but not carAB, is a valid candidate for expression in *Synechocystis*. 
Task 1 – Technical Accomplishments

Homology Comparison of CBS hyp1/hyp2 Genes

- CBS Hyp1 and Hyp2 proteins display low identity based on homology comparison.
- CBS Hyp2 proteins are more similar to the respective Hyp proteins in *Ralstonia eutropha* (*Re*), the latter proteins are known to assemble an uptake hydrogenase in *Re*.
- CBS *hyp1* genes cluster near the O₂-tolerant hydrogenase and *hyp2* genes near the H₂-uptake hydrogenase.
- CBS hydrogenase and *hyp1* genes have similar induction profile, both are induced by CO (but not by H₂). The uptake hydrogenase and *hyp2* are both induced by H₂ (but not by CO) – 2013 AMR presentation.
- CBS hydrogenase likely has related function with *hyp1*, but not *hyp2*.

**CBS hydrogenase reaction in CBS:**

\[
\text{CO} + \text{H}_2\text{O} \rightarrow \text{H}_2 + \text{CO}_2
\]

<table>
<thead>
<tr>
<th>CBS Hyp Proteins</th>
<th>% Identity Hyp1 vs. Hyp2</th>
<th>Ralstonia eutropha</th>
</tr>
</thead>
<tbody>
<tr>
<td>HypA</td>
<td>34.5</td>
<td>CBS Hyp1 (%)</td>
</tr>
<tr>
<td>HypB</td>
<td>53.6</td>
<td>HypA 31.0</td>
</tr>
<tr>
<td>HypC</td>
<td>39.2</td>
<td>HypB 52.3</td>
</tr>
<tr>
<td>HypD</td>
<td>50.9</td>
<td>HypC 38.4</td>
</tr>
<tr>
<td>HypE</td>
<td>53.8</td>
<td>HypD 47.4</td>
</tr>
<tr>
<td>HypF</td>
<td>37.2</td>
<td>hypE 54.0</td>
</tr>
</tbody>
</table>

|                | CBS Hyp2 (%)       |
|                | HypA 65.5          |
|                | HypB 67.4          |
|                | HypC 59.5          |
|                | HypD 77.3          |
| hypE           | 74.4               |
| HypF           | 60.2               |

- Summary: Complete FY14 Q2 interim Milestone. The data suggest *hyp1*, but not *hyp2*, should be co-transformed with CBS hydrogenase into *Synechocystis*. 
Task 1 – Technical Accomplishments
Generate hyp1 and hyp2 Deletion Mutants in CBS – On Going

• We aim to delete either hyp1FCDE or hyp2ABCDEF which provides more direct evidence if they work in concert to assemble CBS hydrogenase (Q3 Milestone).
• Two deletion constructs were verified with mutant screening ongoing – integrating a large DNA knockout region could be a challenge.

**hyp1FCDE (4.87 kb) deletion**

![Deletion diagram]

**hyp2ABFCDE (4.81 kb) deletion**

![Deletion diagram]

**Summary:** Verified two deletion constructs via restriction digest.
Approach/Milestone

Task 2 – Expression of the CBS hydrogenase in *Synechocystis*

- **Approach:** Transfer the $O_2$-tolerant CBS hydrogenase and its maturation genes (hyp1) into a *Synechocystis* host with no background $H_2$ production.
  - Optimize hydrogenase activity via tuning the expression levels of hydrogenase and hyp1 maturation genes.
  - Transform additional hyp genes if needed (slyD, hyp2).
  - Resolve the hydrogenase subunit composition (CooLXUH) in *Synechocystis*
    - Native CBS hydrogenase is a hexamer. We plan to transform only the four soluble subunits (CooLXUH) into *Synechocystis* as the subcomplex displayed *in vitro* hydrogenase activity in previous work.
## Approach/Milestone

### Task 2 – Expression of the CBS hydrogenase in *Synechocystis*

<table>
<thead>
<tr>
<th>FY13 Milestones – all regular</th>
<th>Completion Date</th>
<th>Status</th>
</tr>
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<tbody>
<tr>
<td><strong>3.23.2-1</strong> Improve expression of CBS hydrogenase genes by two-fold via manipulating promoter strength (presented 2013 AMR)</td>
<td>7/13</td>
<td>Complete</td>
</tr>
<tr>
<td><strong>3.23.2-2</strong> Construct a <em>Synechocystis</em> recombinant with stronger promoters to drive the expression of 10 CBS genes (four hydrogenase structure genes and six maturation genes) and show at least two-fold improvement in the expression of either the CBS maturation protein HypF or the hydrogenase protein CooX in order to assemble a more active CBS hydrogenase in <em>Synechocystis</em></td>
<td>9/13</td>
<td>Complete</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>FY14 Milestones – all regular</th>
<th></th>
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</thead>
<tbody>
<tr>
<td><strong>Q3</strong> Verify that more than 30% of the CooLXUH proteins are present as a tetramer of each of the four proteins in a <em>Synechocystis</em> recombinant expressing 10 CBS hydrogenase and related maturation genes, using an affinity pull-down assay/protein Western blotting method.</td>
<td>6/14</td>
</tr>
<tr>
<td><strong>Q4</strong> Double the CBS hydrogenase activity over the baseline rate of 10 nmol H$_2$/ml culture/h in whole cells of a <em>Synechocystis</em> recombinant, assayed in vitro with reduced methyl viologen.</td>
<td>9/14</td>
</tr>
</tbody>
</table>
Task 2 – Technical Accomplishments

**psbA promoter Enhanced Maturation Protein Expression in *Synechocystis***

- Promoter tuning will increase hydrogenase activity in *Synechocystis*.
- Improved HypF protein expression by more than two-fold via replacing the *petE* promoter with the stronger *psbA* promoter.
- Yet the recombinant did not yield any *in vitro* hydrogenase activity using the reduced methyl-viologen based assay.

> Summary: Complete FY13 Q4 Milestone. Confirmed stronger promoter indeed yields more CBS HypF protein in the *Synechocystis* recombinant, and the need to tune the other proteins.
Hydrogenase Subunit Composition in Recombinant *Synechocystis*

- FY14 Q3 Milestone aims to determine that 30% of the recombinant hydrogenase is a CooLXUH tetramer in *Synechocystis*, with the rationale that a tetramer will have higher hydrogenase activity.
- The hydrogenase subunit CooL has been modified with an affinity 6XHis tag, needed for pull-down assay for the above composition analysis, but the tag might interfere with hydrogenase activity in *Synechocystis*.
- A knock-in of 6XHis tag-*cooL* was integrated into the CBS genome successfully and the tag has no effect on *in vivo* hydrogenase activity in CBS.

**Summary:** His affinity tag has no effect on hydrogenase activity, and outcome meets toward FY14 Q3 Milestone.
Promoter Tuning to Optimize Hydrogenase Expression in Recombinant *Synechocystis*

- Quantitative protein immunoblots is ongoing to compare hydrogenase and Hyp proteins levels in native CBS and in recombinant *Synechocystis* – FY13 AMR Reviewer’s suggestion
- Promoter reconstruction is underway, by using strong *psbA* and *trc* promoters of varying frequency to improve hydrogenase activity in the *Synechocystis* recombinant (Q4 Milestone).

**Promoter Tuning Strategy**

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Carrie Eckert
NiFe hydrogenase is a logical gene candidate....However, the rationale was not explained clearly, and it is unclear why the growth assay is a good proxy for HypE1 and E2 hydrogenase (Hyd) activities.

- **Response:** qRT-PCR data suggest that HypE1 is consistent with a role in CO metabolism (leading to H₂ production, but growth in CO depends on HypE2), and HypE2 with a role in H₂ uptake (leading to cell growth). We therefore tested the effects of gene deletions on growth in either CO or H₂ alone; which forms the rationale of using the growth assay as the proxy for their respective role.

\[
\text{O}_2\text{-tolerant Hyd: } \text{CO} + \text{H}_2\text{O} \xrightarrow{\text{E}1} \text{H}_2 + \text{CO}_2
\]

\[
\text{H}_2\text{-uptake Hyd: } \text{H}_2 + \text{CO}_2 \xrightarrow{\text{E}2} \text{cell mass}
\]

Gene expression is one factor that can lead to low enzyme activity, but so can inefficiencies in protein folding. It is suggested that a near-term experimental objective should include comparing the heterologously expressed protein in Synechocystis with the native protein isolated from Rubrivivax (if it is possible to scale up)....

- **Response:** We have planned in FY14 and beyond to compare the native and the recombinant version of the O₂-tolerant CBS hydrogenase as to activity, O₂ tolerance, protein aggregates, truncated proteins, and especially expression levels in both CBS and the *Synechocystis* recombinant, as a means of proper protein folding and expression tuning to identify the rate-limiting steps to guide further optimization.

There are parallel paths to improving oxygen tolerance and increasing hydrogen yield, but nothing has been integrated.

- **Response:** The most critical parameter is to construct a cyanobacterial recombinant with O₂-tolerant hydrogenase activity, after which the other parameters (truncated antenna chlorophylls, competing pathways, and dissipation of proton gradient) will be integrated to build a model strain.
Collaborations

• **Task 1. Probe hydrogenase maturation machinery in CBS**
  Drs. Jin Chen (Michigan State Univ.; Office of Science Funding) and Jonas Koralch (Pacific Biosciences; free service)

• **Task 2. Expression of the CBS hydrogenase in *Synechocystis***
  Dr. Phil Weyman, J. Craig Venter Institute
  — JCVI has constructed a *Synechococcus* recombinant expressing an environmental hydrogenase. NREL will perform experiments to determine the linkage of the hydrogenase with *Synechocystis* ferredoxin, the latter reduced photosynthetically.
  — The successful outcomes will validate that foreign hydrogenase could link to the host photosynthetic pathway in a cyanobacterium for light-driven H₂ production. JCVI and NREL will publish together.
Remaining Challenges and Barriers

Probe Hydrogenase Maturation Machinery in CBS

• Identify the key maturation genes to correctly assemble the CBS hydrogenase
  — slydD is a valid candidate for heterologous expression.
  — hyp1 and/or hyp2 deletion will provide more direct evidence if hyp1 is sufficient. Need to optimize hyp1/hyp2 deletion when integrating a large knockout region (4.87 kb hyp1 and 4.81 kb hyp2).

Expression of the CBS hydrogenase in Synechocystis

• Tuning expression levels of the CBS hydrogenase structural and maturation genes in Synechocystis
  — Test strong promoters and more frequent placement to drive transcription
• Demonstrate in vitro and in vivo H₂ production, the latter linking to the host photosynthetic pathway
  — CBS hydrogenase is able to link to photosynthetically reduced ferredoxin from a cyanobacterium, in vitro, as previously demonstrated.
Proposed Work

Task 1. Probe hydrogenase maturation machinery in CBS

- Continue to generate the hyp1 (Δhyp1FCDE) or hyp2 (Δhyp2ABFCDE) deletion mutants and characterize growth profiles (in CO or H₂) and hydrogenase activity to unravel their functions in assembling the CBS hydrogenase (FY14).
- The outcome will provide more direct evidence the role of hyp2 genes in assembling an active CBS hydrogenase in Synechocystis (FY15).
- Express CBS slyD in the Synechocystis recombinant (FY15).

Task 2. Expression of the CBS hydrogenase in Synechocystis

- Continue to tune expression levels of the hydrogenase and Hyp1 proteins in native CBS and in the Synechocystis recombinant guided by promoter engineering, with stronger promoter and more frequent placement to improve hydrogenase activity (FY14)
- Perform affinity pull-down assay to determine activity and hydrogenase subunit composition in the above recombinant (FY14).
- Optimize growth conditions (light intensity, growth medium, stage of growth) and determine in vitro hydrogenase activity and O₂ tolerance (FY15).
- Link CBS hydrogenase to the host Synechocystis photosynthetic pathway for light-driven H₂ production (FY15).
Summary

Task 1. Probe hydrogenase maturation machinery in CBS

• Uncover slyD in CBS, but not in *Synechocystis*. slyD is a nickel insertion maturation gene, which may warrant its expression in *Synechocystis*.
• CBS hyp2 is more similar to the hyp genes in *R. eutropha*, which assemble an uptake hydrogenase. We will therefore focus on expressing only hyp1 in *Synechocystis* in FY14.
• Work is ongoing to generate CBS hyp1 and hyp2 mutants by screening colonies obtained from initial transformation.

Task 2. Expression of the CBS hydrogenase in *Synechocystis*

• Improved the expression of the CBS maturation protein HypF by up to nine-fold, when a strong *psbA* promoter was used in lieu of the weak *petE* promoter.
• Determined that affinity tagging of the CBS CooL hydrogenase subunit has no effect on hydrogenase activity. The tag is needed to resolve CBS hydrogenase subunit composition via a pull-down assay.
• Work is ongoing to tune and optimize CBS gene expression in *Synechocystis* via promoter engineering to obtain active hydrogenase activity and the hydrogenase subunit compositions.