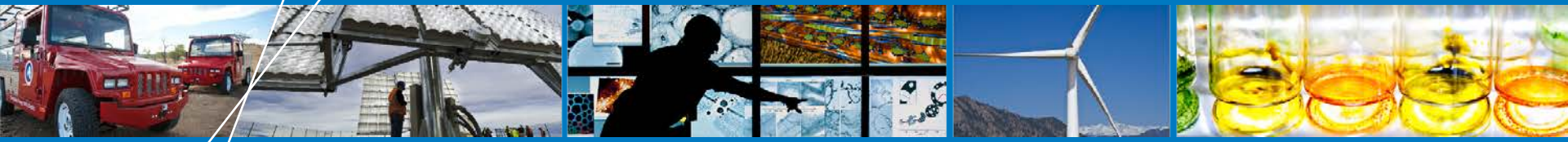
NREL
NATIONAL RENEWABLE ENERGY LABORATORY

Probing O_2-tolerant CBS Hydrogenase for Hydrogen Production



2013 Annual Merit Review and Peer Evaluation Meeting;
May 16, 2013

Pin-Ching Maness, National Renewable Energy Laboratory (NREL PI; Presenter)

Phil Weyman, J. Craig Venter Institute (Key Collaborator)

Project ID #: PD095

This presentation does not contain any proprietary, confidential, or otherwise restricted information

NREL is a national laboratory of the U.S. Department of Energy, Office of Energy Efficiency and Renewable Energy, operated by the Alliance for Sustainable Energy, LLC.

Overview

Timeline

- Project start date: FY05
- Project not funded in FY06
- Project end date: 10/2013*
- Percent complete: N/A

Barriers

Barriers addressed

- Oxygen Accumulation (AP)

Budget

- Total project funding: $1.86M
- Funding received in FY12: $350K
- Planned funding for FY13: $350K

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

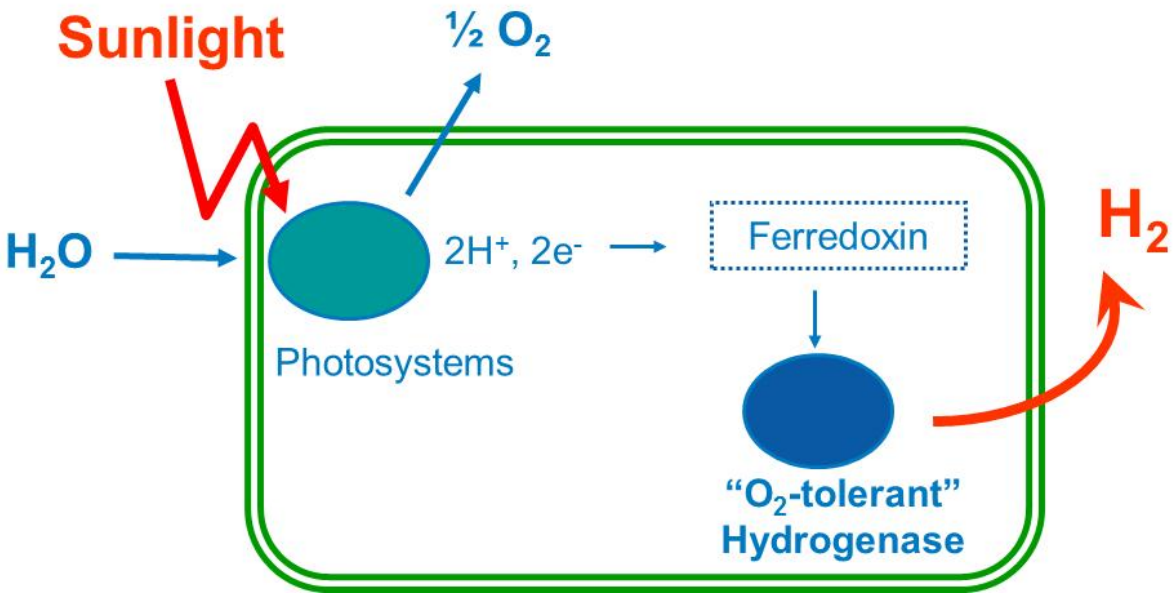
- **Oxygen Accumulation (Barrier AP):** Along with H_2, photolytic microbes such as algae and cyanobacteria co-produce O_2, which inhibits the activity of hydrogenase, the enzyme responsible for H_2 production.

Technical Target

Characteristics	Unit	2011 status	2015 Target	2020 Target	Ultimate Target
Duration of continuous H_2 production at full sunlight intensity	Time Unit	2 min	30 min	4 h	8h

Objective/Relevance

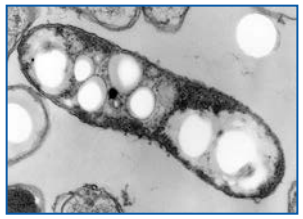
Develop a robust O_2-tolerant cyanobacterial system for light-driven H_2 production from water while increasing system durability. The long-term goal is to be O_2 tolerant for 8 hours (during daylight hours).



Cyanobacterial Recombinant

Objective/Relevance

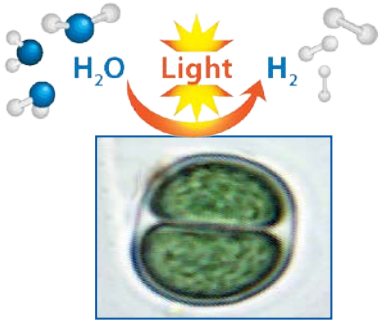
Project Overview



***Rubrivivax gelatinosus* CBS ("CBS")**

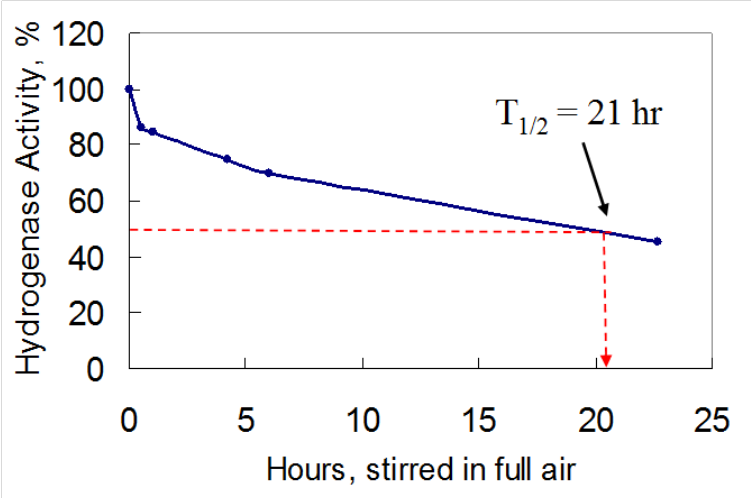
O_2-tolerant Hydrogenase

Maturation proteins



Synechocystis

CBS Hydrogenase Half-life in Air: 21 h



Relevance:

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

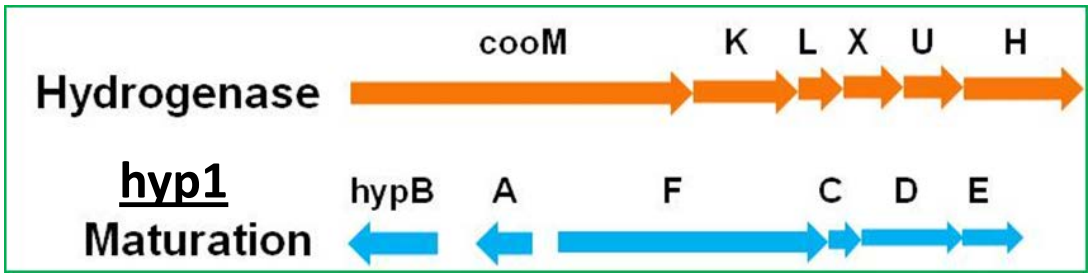
NREL approach is complementary to that of JCVI in harnessing two of Nature's O_2-tolerant hydrogenases and transferring into two model cyanobacteria.

Approach/Milestone

Task 1: Probe Hydrogenase Maturation Machinery in CBS

Approach: To assemble an active CBS hydrogenase in *Synechocystis*, it is necessary to know hydrogenase maturation machinery in CBS in order to transform the proper maturation genes. Work in Task 1 entails:

- Sequencing CBS genome (to uncover additional maturation genes); and
- Probing the function of maturation genes via expression profiles & gene knockout



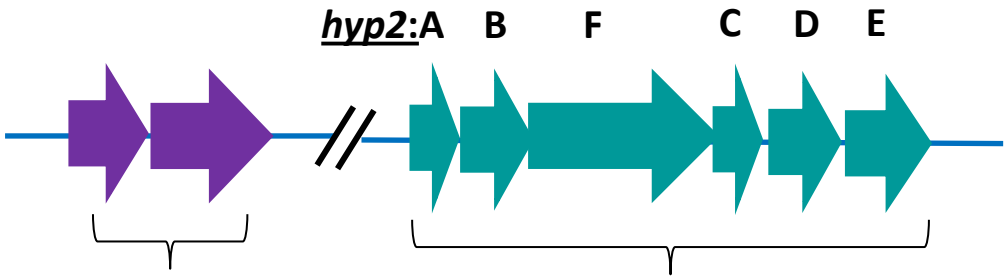
Already cloned hydrogenase structural genes (**evolving H_2**) and *hyp1* maturation genes.

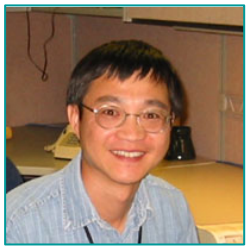
	Milestone	Completion Date	Status
3.23.1-1	Use quantitative RT-PCR to determine CO induction pattern of at least two hyp2 genes to indicate whether the operon is induced under similar conditions as the hydrogenase genes	1/13	Completed
3.23.1-2	Verify function of at least two putative hydrogenase maturation genes that are needed for hydrogenase activity and H_2 production, based on analysis of strains with gene deletions	5/13	On Track

Task 1 – Technical Accomplishments

Uncovered Additional Maturation Gene (*hyp2*) in CBS Genome

- The CBS genome is sequenced and annotated via collaboration with Michigan State Univ. (Office of Science funding) and Pacific Biosciences (free service).
  - 5.1 Mb; 3,582 genes
- Genome analysis uncovered a H_2 "uptake" hydrogenase (oxidizing H_2) and a second set of maturation genes **(*hyp2*)** with homology to *hyp1*.
- Work in Task 1 therefore aims to determine which *hyp* genes (*hyp1* and/or *hyp2*) are more important to assemble an active CBS hydrogenase (**evolving H_2**) in *Synechocystis* to improve continuity of H_2 production in O_2.





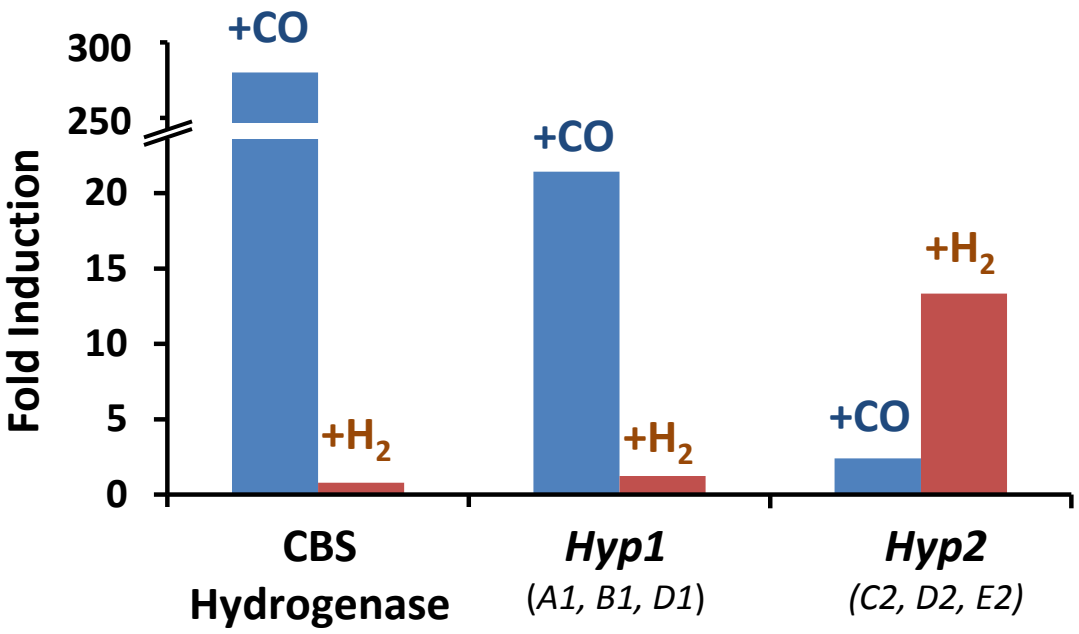
Jianping Yu

Scott Noble

Task 1 – Technical Accomplishments

Quantitative RT-PCR to Probe Function of *hyp1*/*hyp2* Genes in CBS

- Quantified gene expression using qRT-PCR to determine which set of *hyp* genes should be co-transformed with CBS hydrogenase into *Synechocystis*.



CBS hydrogenase reaction in CBS:
$CO + H_2O \rightarrow H_2 + CO_2$

- CBS hydrogenase and *hyp1* were induced similarly, likely have related function.
- The data suggest *hyp1* should be co-transformed with CBS hydrogenase into *Synechocystis*.

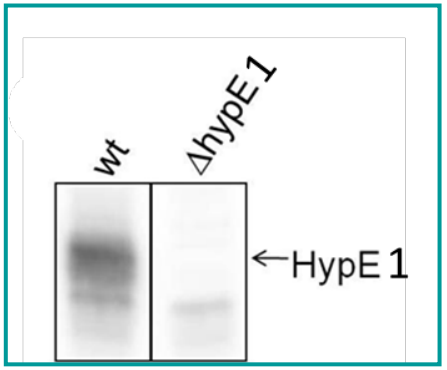
	Milestone	Completion Date	Status
3.23.1-1	Use quantitative RT-PCR to determine CO induction pattern of at least **two hyp2** genes to indicate whether the operon is induced under similar conditions as the hydrogenase genes	1/13	Completed

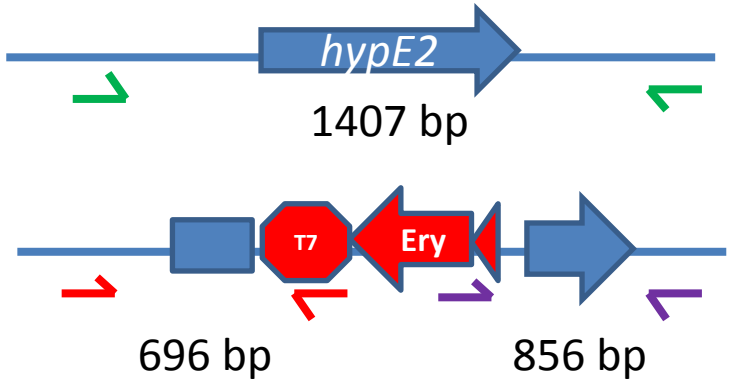
Task 1 – Technical Accomplishments

Generated *hypE1* and *hypE2* Single/Double Mutants in CBS

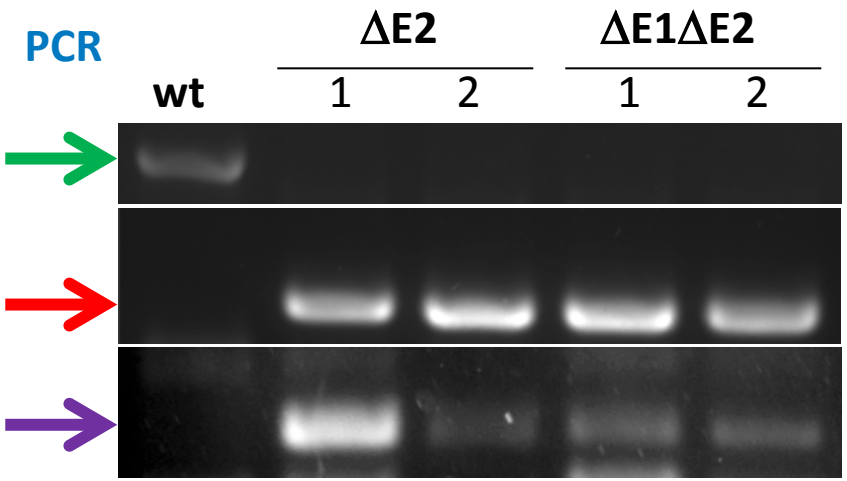
- PCR and protein western results confirmed three CBS mutants: Δ*hypE1*, Δ*hypE2*, and Δ*hypE1*Δ*hypE2*
- Effect of mutations will reveal whether *hyp1* and *hyp2* work in concert to assemble CBS hydrogenase.

Protein western of ΔE1





PCR



	Milestone	Completion Date	Status
3.23.1-2	Verify function of at least two putative hydrogenase maturation genes that are needed for hydrogenase activity and H_2 production, based on analysis of strains with **gene deletions**	5/13	On Track

Task 1 – Technical Accomplishments

hyp1 and *hyp2* Genes are Complementary in CBS

CBS hydrogenase reaction in CBS: $CO + H_2O \rightarrow H_2 + CO_2$

Cell growth in CO or H_2

Strains	CO	H_2
WT	+	+
Δ*hypE1*	+	+
Δ*hypE2*	+	+
Δ*hypE1*Δ*hypE2*	-	To be determined

+: Cell growth
- : No cell growth

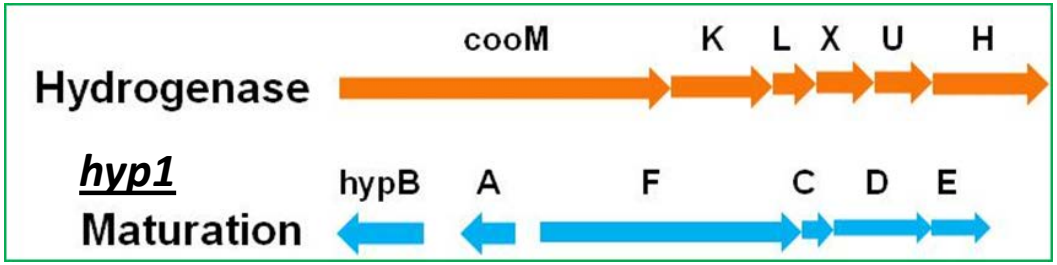
Novel discovery that *hyp1* and *hyp2* genes are complementary; *hyp2* may provide a new genetic resource to assemble an active CBS hydrogenase in *Synechocystis*.

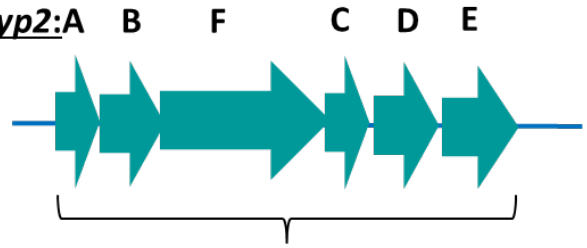
	Milestone	Completion Date	Status
3.23.1-2	Verify **function** of at least **two** putative hydrogenase maturation genes that are needed for hydrogenase activity and H_2 production, based on analysis of strains with gene deletions	5/13	On track

Approach/Milestone

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

- **Approach:** Transfer the O_2-tolerant CBS hydrogenase and its maturation genes (*hyp1* and/or *hyp2*) into a *Synechocystis* host with no background H_2 production.





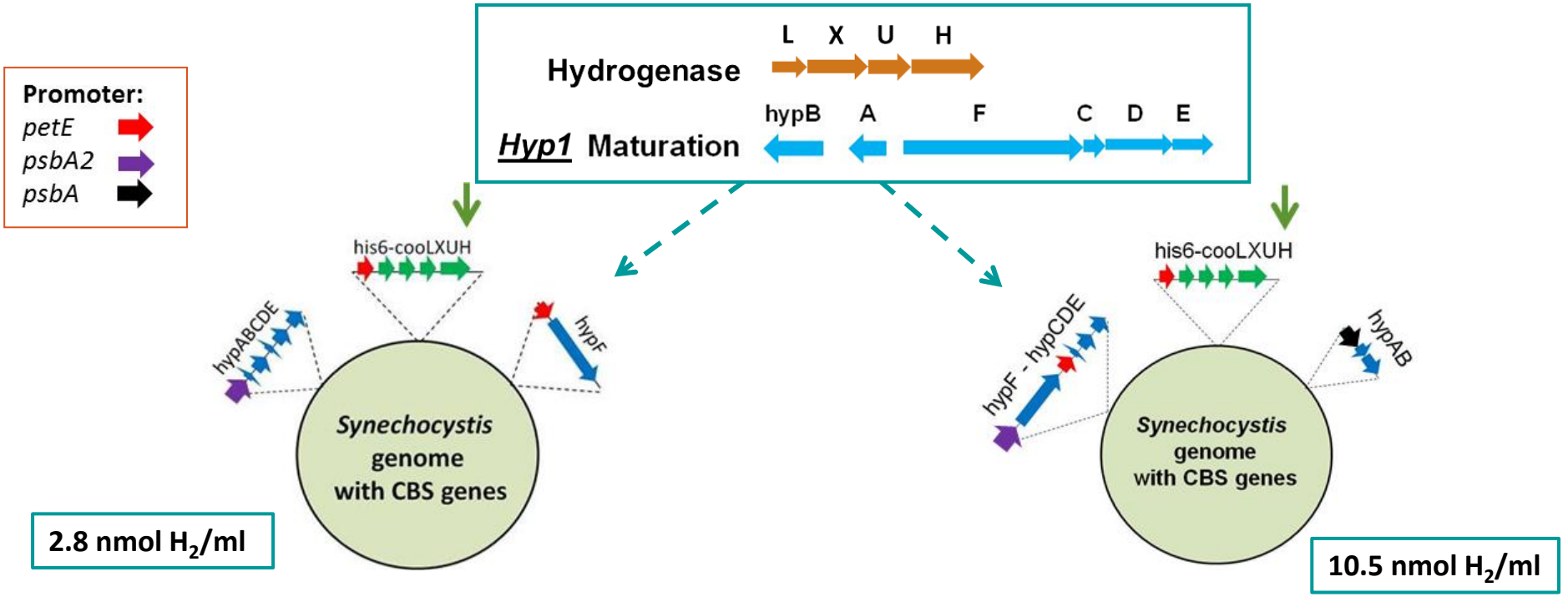
hyp2: Maturation genes

	Milestones	Completion Date	Status
3.23.2 (FY12)	Generate a *Synechocystis* recombinant evolving H_2 via the CBS hydrogenase	9/12	Completed
3.23.2-1 (FY13)	Improve expression of CBS hydrogenase genes by two-fold via manipulating promoter strength	7/13	Completed
3.23.2-2 (FY13)	Improve CBS hydrogenase activity by two-fold over the baseline rate of 5 nmol H_2/ml culture/hr from whole cells of a *Synechocystis* recombinant, assayed with methyl viologen	9/13	On track

Task 2 – Technical Accomplishments

Generated Two *Synechocystis* Recombinants with Hydrogenase Activity

- Generated two *Synechocystis* S6803 recombinants (*cooLXUH* + *hyp1ABCDEF*) yet both displayed low hydrogenase activity.
- **Culprit:** Low hydrogenase activity is likely due to weak *petE* promoter.

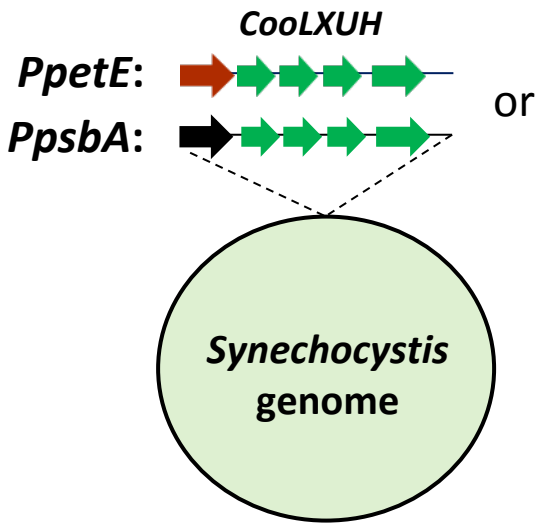


	Milestones	Completion Date	Status
3.23.2 (FY12)	Generate a *Synechocystis* recombinant evolving H_2 via the CBS hydrogenase	9/12	Completed

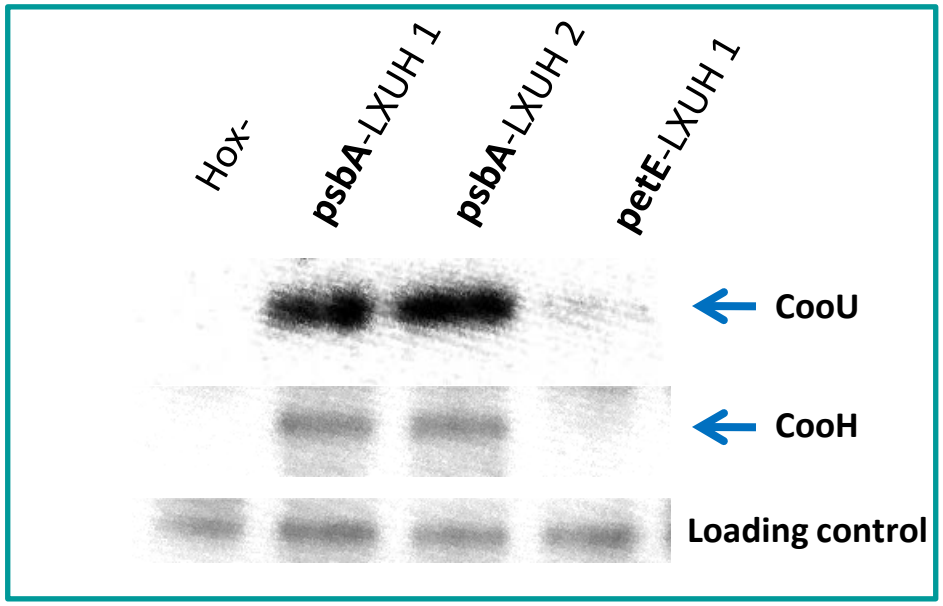
Task 2 – Technical Accomplishments

Improved Hydrogenase Protein Expression with Stronger Promoter

- CBS hydrogenase genes (*cooLXUH*) were transformed into *Synechocystis* using two promoters of different strength.
- Switching to a strong *psbA* promoter (black arrow) increased protein expression: increased hydrogenase CooH catalytic subunit by **44-fold** and CooU subunit by **16-fold**, compared to that of *petE* promoter (red arrow)**.**



Protein Western Blots

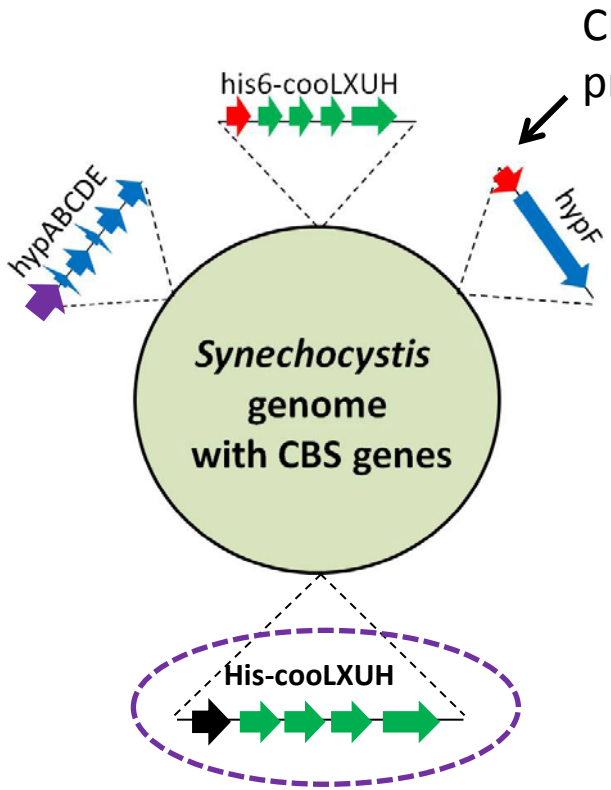


	Milestone	Completion Date	Status
3.23.2-1 (FY13)	Improve expression of CBS hydrogenase genes by two-fold via manipulating promoter strength	7/13	Complete

Task 2 – Technical Accomplishments

Use *psbA* promoter to Enhance Hydrogenase Expression in *Synechocystis*

- Work has begun to express an extra copy of *psbA-his6-cooLXUH* (purple circle) in the *Synechocystis* recombinant already expressing 10 CBS hydrogenase and related genes.
- An ideal insertion site is at the loci of the PHB-encoding genes to redirect electron sink to H_2.
- We also plan to enhance *hypF* expression by replacing the *petE* promoter with *psbA* promoter.
- *In vitro* hydrogenase activity will be determined in the recombinant using reduced methyl-viologen based assay.



Promoter:
petE
psbA2
psbA

On track to complete Milestone 3.23.2-2: improve CBS hydrogenase activity by two-fold in a *Synechocystis* recombinant (9/13)

Collaborations

- **Task 1. Probe hydrogenase maturation machinery in CBS**

  Drs. Jin Chen (Michigan State Univ.; Office of Science Funding) and Jonas Korlach (Pacific Biosciences; free service)

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

  Dr. Phil Weyman, J. Craig Venter Institute

  – JCVI provided NREL its proprietary plasmids (pRC41 and pRC41-4) harboring the environmental hydrogenase and the maturation genes. Work is ongoing at NREL for its expression in *Synechocystis* S6803.

Proposed Work

Task 1. Probe hydrogenase maturation machinery in CBS

- Continue to characterize growth profiles in CO or H_2 of the single and double CBS mutants (Δ*hypE1*, Δ*hypE2*, and Δ*hypE1*Δ*hypE2*) to unravel their functions in assembling the CBS hydrogenase (FY13).
- Measure hydrogenase enzyme activity (assayed with reduced methyl viologen) in the above mutants (FY13, FY14). The outcome will guide which, if any, *hyp2* gene is needed along with *hyp1* to assemble an active CBS hydrogenase in *Synechocystis*.

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

- Using the *Synechocystis* recombinant as the host (*cooLXUH-hyp1ABCDEF*), express a second copy of *cooLXUH* (CBS hydrogenase) driven by the strong *psbA* promoter (FY13)
- Measure *in vitro* hydrogenase activity in the above transformant using reduced methyl viologen (FY13).
- Optimize growth conditions (light intensity, growth medium, stage of growth) and determine *in vitro* hydrogenase activity and O_2 tolerance (FY13, FY14).
- Link CBS hydrogenase to the host *Synechocystis* photosynthetic machinery for light-driven H_2 production (FY14).

Summary

Task 1. Probe hydrogenase maturation machinery in CBS

- CBS genome annotation reveals a second set of hydrogenase maturation genes *hyp2*, with high homology with previously discovered *hyp1*, the latter clustered near the CBS hydrogenase.
- qRT-PCR revealed that *hyp1* likely is involved in assembling the CBS hydrogenase (evolving H_2) based on their similar expression profile.
- CBS *hypE1* and *hypE2* single and double mutants were generated. Physiological growth studies suggested that *hyp1* and *hyp2* are complementary to each other and may provide another genetic resource for assembling CBS hydrogenase in *Synechocystis*.

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

- Two *Synechocystis* recombinants were constructed harboring 10 CBS hydrogenase and maturation genes, yet with low hydrogenase activity.
- We improved the expression of CBS hydrogenase by 16-44 fold when a strong *psbA* promoter was used in lieu of the weak *petE* promoter.
- Work is ongoing to transform the *Synechocystis* recombinant with a second copy of CBS hydrogenase genes (*cooLXUH*) driven by the strong *psbA* promoter, aimed at boosting hydrogenase activity in *Synechocystis*.