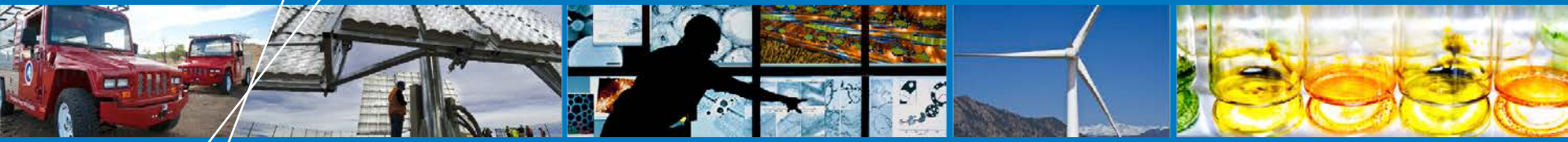
NREL
NATIONAL RENEWABLE ENERGY LABORATORY

Improving Cyanobacterial O_2-Tolerance using CBS Hydrogenase for H_2 Production



2014 Annual Merit Review and Peer Evaluation Meeting

June 19, 2014

Pin-Ching Maness, National Renewable Energy Laboratory

(PI; Presenter)

Project ID #: PD095

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

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

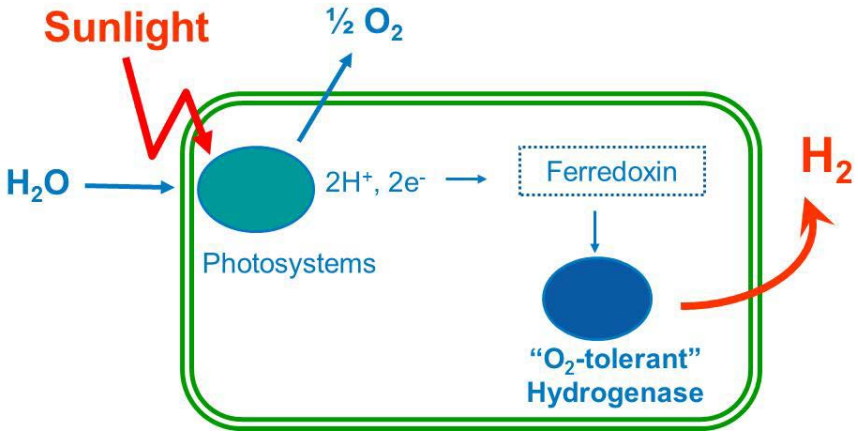
Objective/Relevance

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.

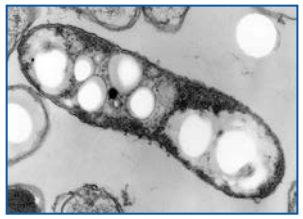


Cyanobacterial Recombinant

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

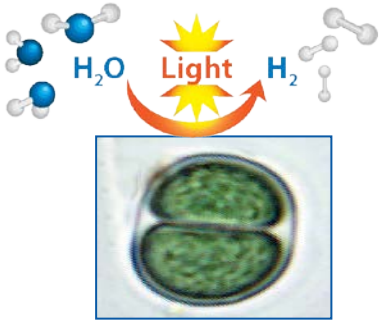
Objective/Relevance: Project Overview



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

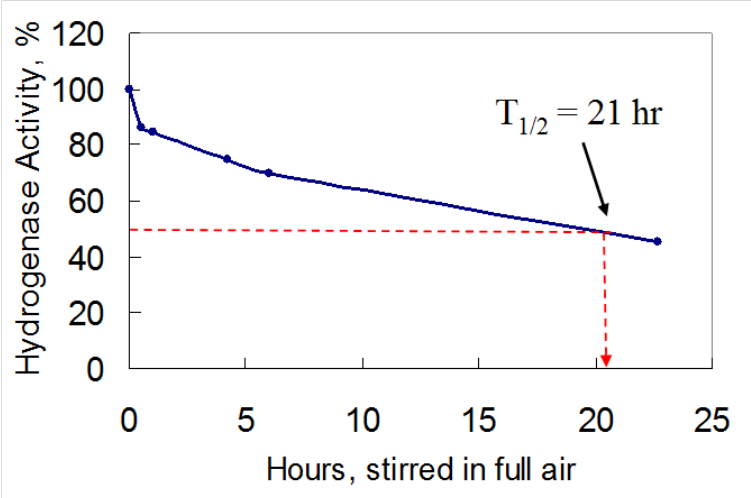
O_2-tolerant Hydrogenase

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's approach harnesses Nature's O_2-tolerant hydrogenase and its transfer into a model cyanobacterium with genetic tools.

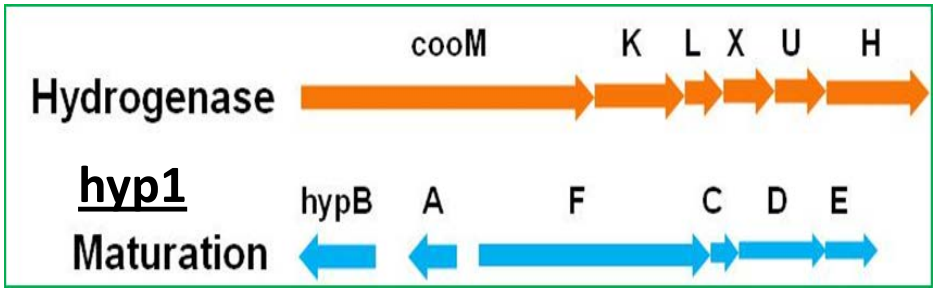
Approach/Milestone

Task 1: Probe Hydrogenase Maturation Machinery in CBS

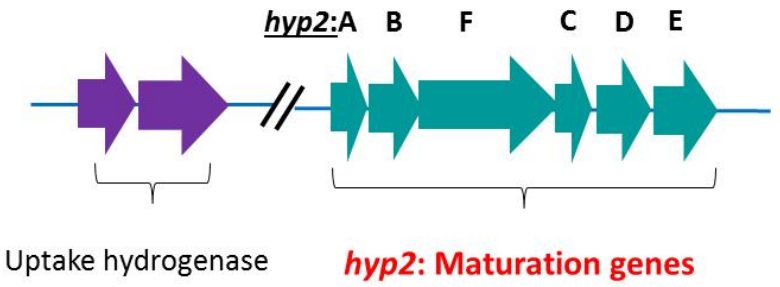
Approach: CBS contains two sets of hydrogenase maturation genes, *hyp1* and *hyp2*. This task aims to determine which set should be co-transformed with the hydrogenase structural genes to afford O_2-tolerant hydrogenase activity in *Synechocystis*. The approaches are:

- 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)



H_2-uptake hydrogenase (oxidizing H_2)



Milestones

Task 1: Probe Hydrogenase Maturation Machinery in CBS

	FY 14 Milestone – all regular	Completion Date	Status
Q1	Identify the CBS homologues of three hydrogenase maturation genes, *slyD* and *carAB*, through a targeted search of the CBS genome and comparison with those in *E. coli*, using Blast tool with 35% amino acid sequence identity	12/13	Complete
Q2	Provide detailed analysis of the CBS genome for genes encoding the O_2-tolerant evolving hydrogenase, uptake hydrogenase, and the two sets of hydrogenase maturation genes, *hyp1* and *hyp2*. These data will provide the blueprint to guide genetic engineering effort toward constructing a *Synechocystis* recombinant harboring O_2-tolerant hydrogenase activity.	3/14	Interim milestone; Complete
Q3	Through deletion of *hyp2ABFCDE* either in the wild type or Δ*hyp1FCDE* background, determine if *hyp2* increases total hydrogenase activity by 15% based on *in vitro* assay using reduced methyl viologen and is required for optimal hydrogenase activity; if so, *hyp2* will be co-transformed with *hyp1* into *Synechocystis* to assemble a more active CBS hydrogenase for increased H_2 production.	6/14	Delayed from Q2 to Q3; On Track

Task 1 – Technical Accomplishments

Uncovered Additional Maturation Genes: *slyD* and *carAB* in CBS Genome

Carrie Eckert

- *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.

CBS	Identity to *E. coli* (%)	Identity to *Synechocystis* (%)	Function
SlyD	33	**No identical homolog**	Ni insertion
CarA	64	51	Synthesis of CN ligand for NiFe-active site
CarB	70	60	Synthesis of CN ligand for NiFe-active site

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

- 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 O_2-tolerant hydrogenase and *hyp2* genes near the H_2-uptake hydrogenase.
- CBS hydrogenase and *hyp1* genes have similar induction profile, both are induced by CO (but not by H_2). The uptake hydrogenase and *hyp2* are both induced by H_2 (but not by CO) – 2013 AMR presentation.
- CBS hydrogenase likely has related function with *hyp1*, but not *hyp2*.

CBS Hyp Proteins	% Identity Hyp1 vs. Hyp2
HypA	34.5
HypB	53.6
HypC	39.2
HypD	50.9
HypE	53.8
HypF	37.2

Ralstonia eutropha	CBS Hyp1 (%)	CBS Hyp2 (%)
HypA	31.0	65.5
HypB	52.3	67.4
HypC	38.4	59.5
HypD	47.4	77.3
hypE	54.0	74.4
HypF	38.4	60.2

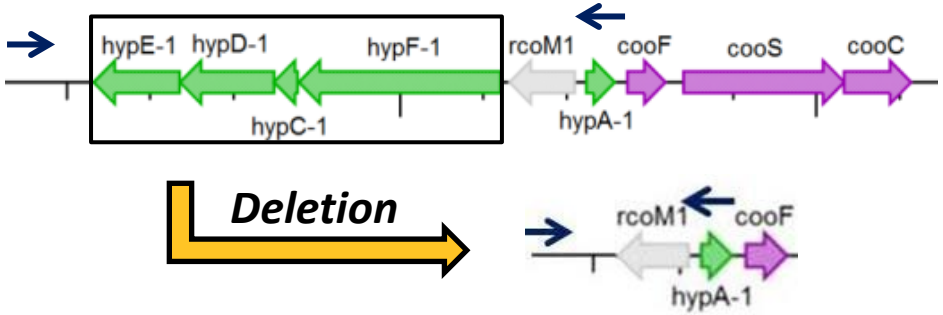
➢ **Summary: Complete FY14 Q2 interim Milestone. The data suggest *hyp1*, but not *hyp2*, should be co-transformed with CBS hydrogenase into *Synechocystis*.**

Task 1 – Technical Accomplishments

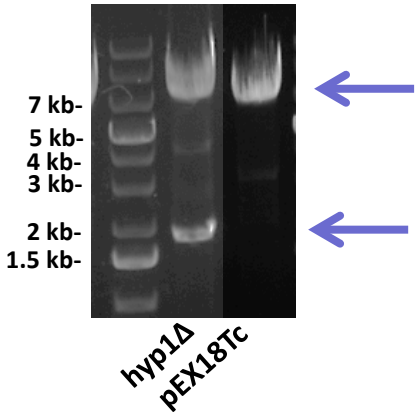
Generate *hyp1* and *hyp2* Deletion Mutants in CBS – On Going

- 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**

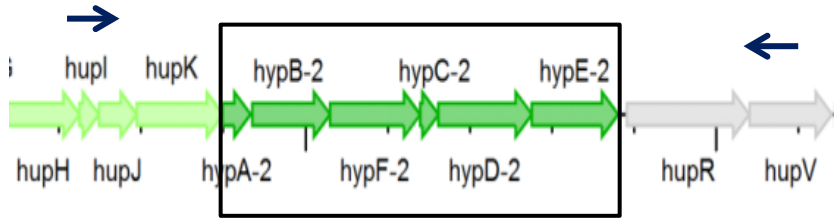


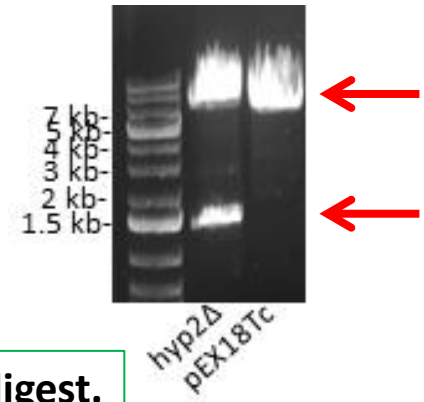
SphI Digest



pEX18Tc: 6.4 kb
***hyp1ECDFΔ*:**
6.7, 1.8 kb

***hyp2ABFCDE* (4.81 kb) deletion**





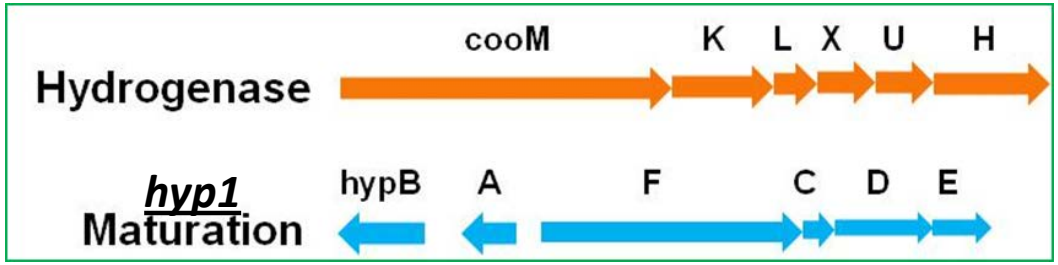
pEX18Tc: 6.4 kb
***hyp2ABFCDEΔ*:**
7.1, 1.4 kb

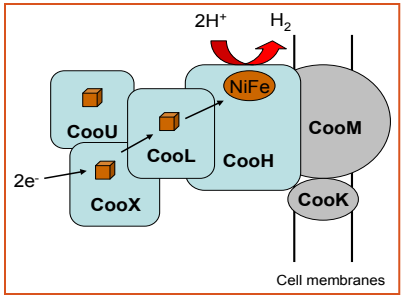
➢ **Summary: Verified two deletion constructs via restriction digest.**

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*

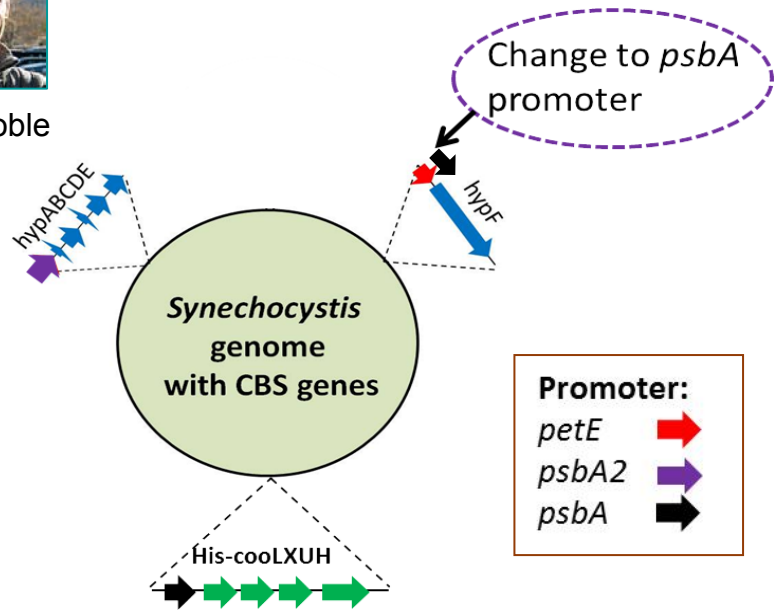
	FY13 Milestones – all regular	Completion Date	Status
3.23.2-1	Improve expression of CBS hydrogenase genes by two-fold via manipulating promoter strength (presented 2013 AMR)	7/13	Complete
3.23.2-2	Construct a *Synechocystis* recombinant with stronger promoters to drive the expression of 10 CBS genes (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 *Synechocystis*	9/13	Complete
	FY14 Milestones – all regular		
Q3	Verify that more than 30% of the CooLXUH proteins are present as a tetramer of each of the four proteins in a *Synechocystis* recombinant expressing 10 CBS hydrogenase and related maturation genes, using an affinity pull-down assay/protein Western blotting method.	6/14	On track
Q4	Double the CBS hydrogenase activity over the baseline rate of 10 nmol H_2/ml culture/h in whole cells of a *Synechocystis* recombinant, assayed in vitro with reduced methyl viologen.	9/14	On Track

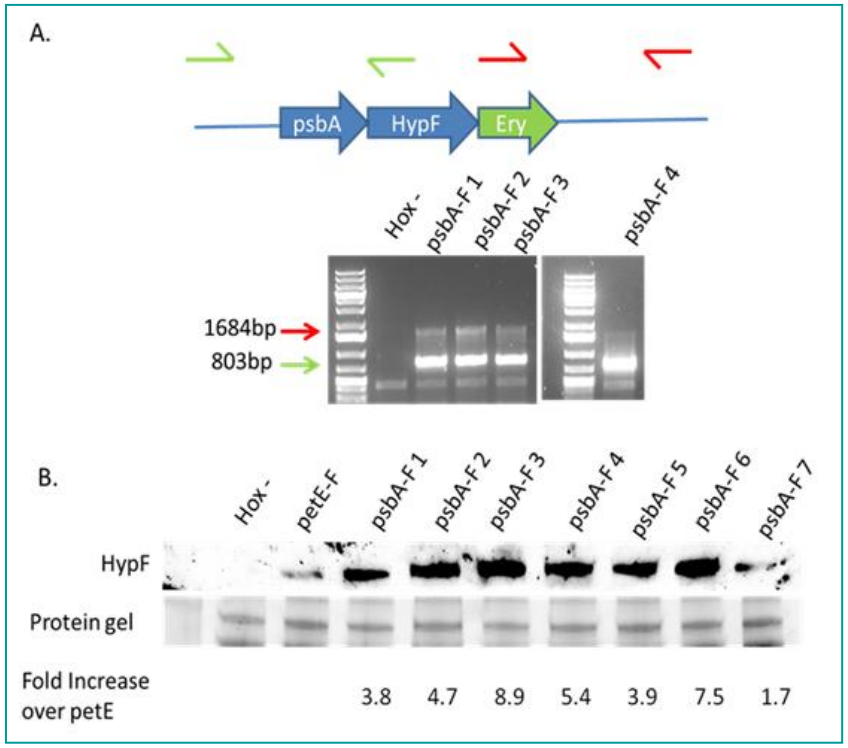
Task 2 – Technical Accomplishments

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.

Scott Noble



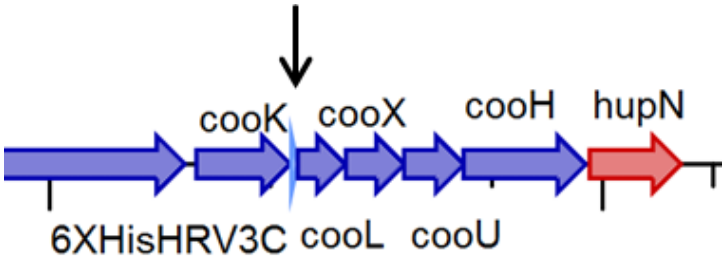


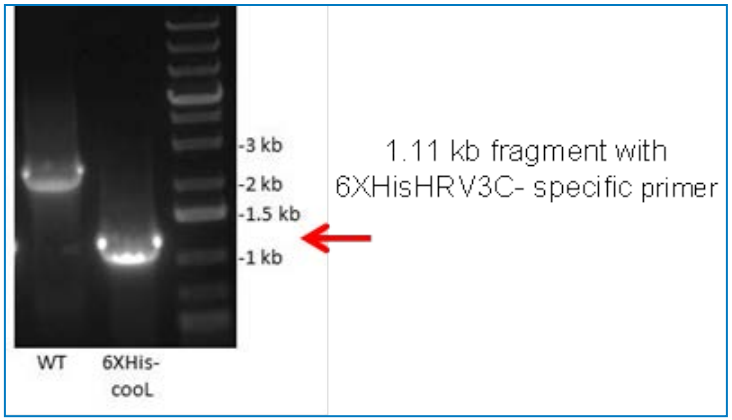
➢ **Summary: Complete FY13 Q4 Milestone. Confirmed stronger promoter indeed yields more CBS HypF protein in the *Synechocystis* recombinant, and the need to tune the other proteins.**

Task 2 – Technical Accomplishments

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.





Carrie Eckert

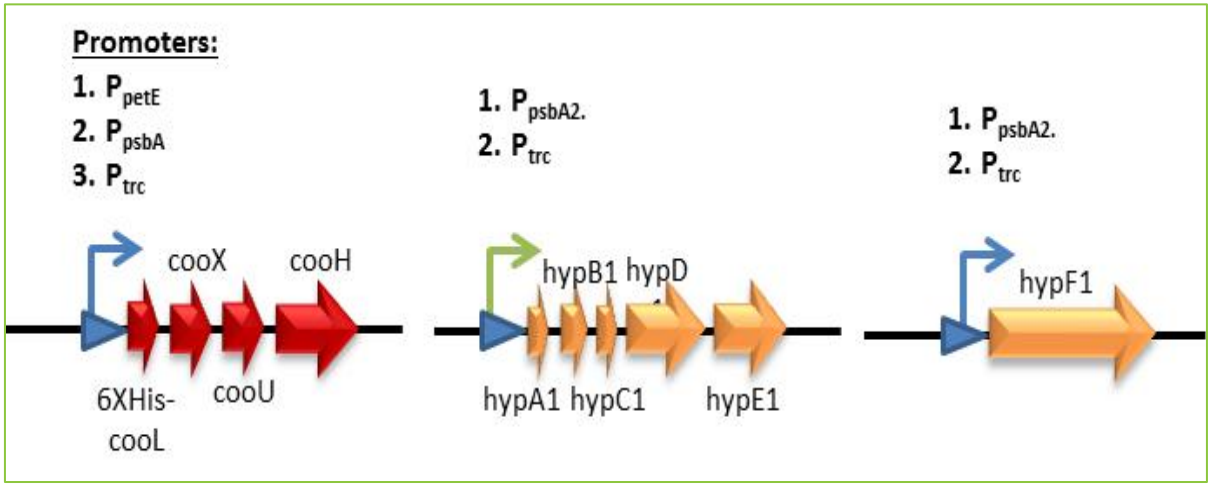
➢ **Summary: His affinity tag has no effect on hydrogenase activity, and outcome meets toward FY14 Q3 Milestone.**

Task 2 – Technical Accomplishments

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



Carrie Eckert

Response to Reviewers' Comments

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 H_2 production, but growth in CO depends on HypE2), and HypE2 with a role in H_2 uptake (leading to cell growth). We therefore tested the effects of gene deletions on growth in either CO or H_2 alone; which forms the rationale of using the growth assay as the proxy for their respective role.

O_2-tolerant Hyd: $CO + H_2O \xrightarrow{E1} H_2 + CO_2$

H_2-uptake Hyd: $H_2 + CO_2 \xrightarrow{E2}$ 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 O_2-tolerant CBS hydrogenase as to activity, O_2 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 O_2-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 Korlach (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 H_2 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* H_2 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* (Δ*hyp1FCDE*) or *hyp2* (Δ*hyp2ABFCDE*) deletion mutants and characterize growth profiles (in CO or H_2) 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 *sly*D 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 O_2 tolerance (FY15).
- Link CBS hydrogenase to the host *Synechocystis* photosynthetic pathway for light-driven H_2 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 S*ynechocystis* via promoter engineering to obtain active hydrogenase activity and the hydrogenase subunit compositions.