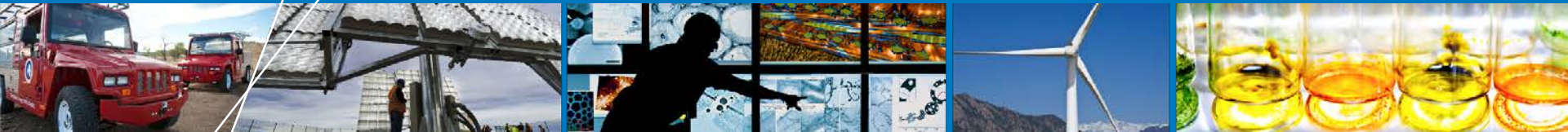


Improving Cyanobacterial O₂- Tolerance using CBS Hydrogenase for H₂ Production



2015 Annual Merit Review and Peer Evaluation Meeting

June 9, 2015

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: 10/2015*

Budget

- FY14 DOE Funding: \$350K
- Planned FY15 DOE Funding: \$350K
- Total DOE funds received to date: \$2.66M

Barriers

Barriers addressed

- Oxygen Accumulation (AP)

Partners

- NREL Team Members: Carrie Eckert and Jianping Yu
- 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

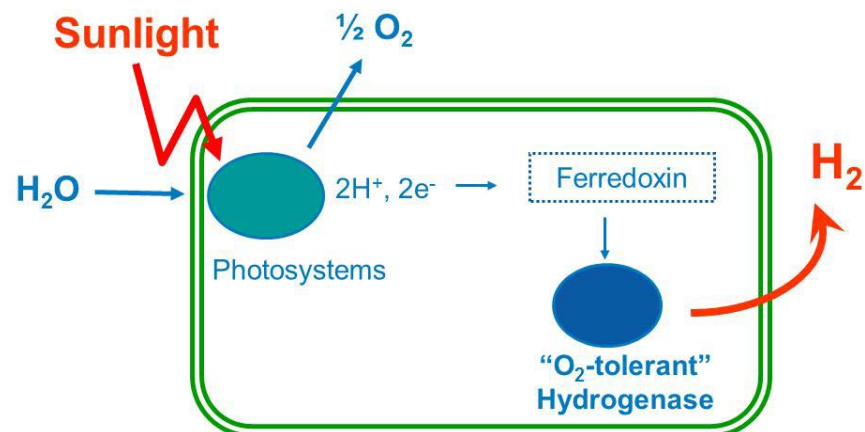
Objective/Relevance

Objective:

Develop a robust O₂-tolerant cyanobacterial system for light-driven H₂ production from water while increasing system durability. The long-term goal is to be O₂ tolerant for 8 hours (during daylight hours).

Oxygen Accumulation (Barrier AP):

Along with H₂, photolytic microbes such as algae and cyanobacteria co-produce O₂, which inhibits the activity of hydrogenase, the enzyme responsible for H₂ production.

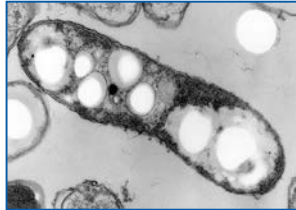


Cyanobacterial Recombinant

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

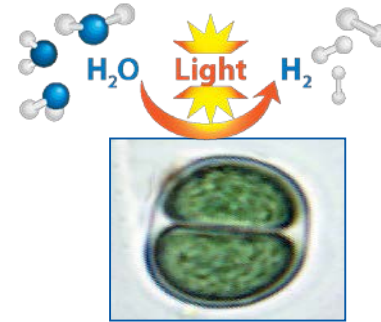


Rubrivivax gelatinosus
CBS ("CBS")

O₂-tolerant
Hydrogenase

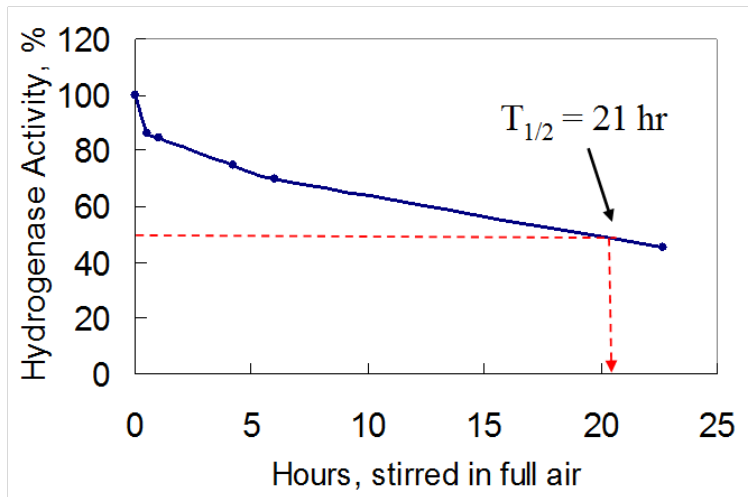


Maturation
Proteins



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 O₂-tolerant hydrogenase and its transfer into a model cyanobacterium with genetic tools.

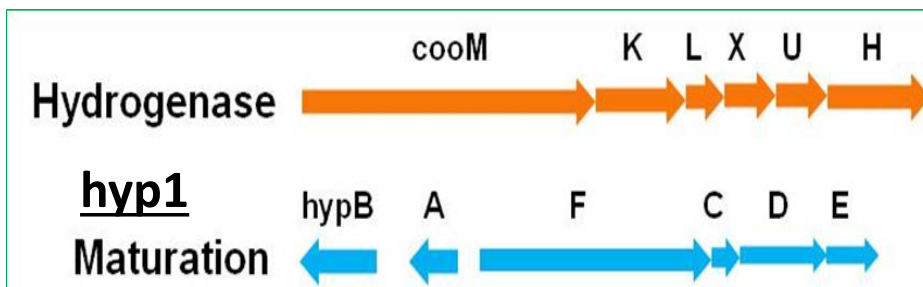
Approach/Milestone

Subtask 1: Probe Hydrogenase Maturation Machinery in CBS

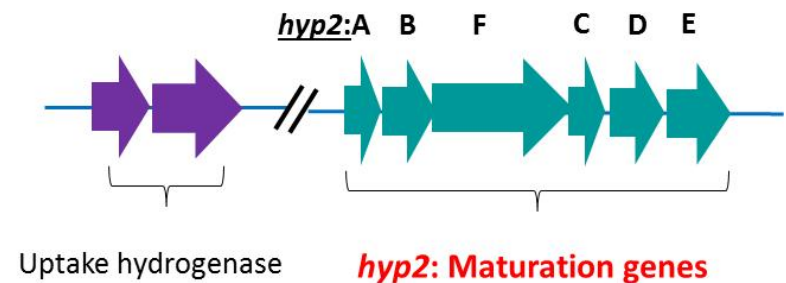
Approach: CBS contains two sets of hydrogenase maturation genes, *hyp1* and *hyp2*. This task aims to determine which set should be co-transformed with the hydrogenase structural genes to afford O₂-tolerant hydrogenase activity in *Synechocystis*. The approaches are:

- 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 – the latter is the focus in FY15.
- Uncovering more hydrogenase maturation-related genes and their deletions for functionality - deletion is the FY15 focus.

O₂-tolerant hydrogenase (evolving H₂)



H₂-uptake hydrogenase (oxidizing H₂)



Milestones

Subtask 1: Probe Hydrogenase Maturation Machinery in CBS

	FY 14/15 Milestone – regular	Completion Date	Status
FY14 Q4 and FY15 Q1	Through deleting <i>hyp1FCDE</i> genes in the wild type background with the presence of <i>hyp2</i> genes, determine if <i>hyp1FCDE</i> deletion decreases the total hydrogenase activity by 50% or more based on <i>in vivo</i> assay of H ₂ production in whole cells, as is required for optimal hydrogenase activity (FY14, Q4 CPS Agreement Milestone), and obtain a construct for a <i>hyp2ABFCDE</i> deletion mutant to measure the impact on hydrogenase activity; the outcome will determine if <i>hyp2</i> will be co-transformed with <i>hyp1</i> into <i>Synechocystis</i> to assemble a more active CBS hydrogenase for increased H ₂ production (FY15, Q1).	9/2014 12/2014	Complete

- $\Delta hyp1$ single mutant generation and characterization in FY14 Q4
- $\Delta hyp2$ single mutant and $\Delta hyp1\Delta hyp2$ double mutant generation and characterization in FY15 Q1.



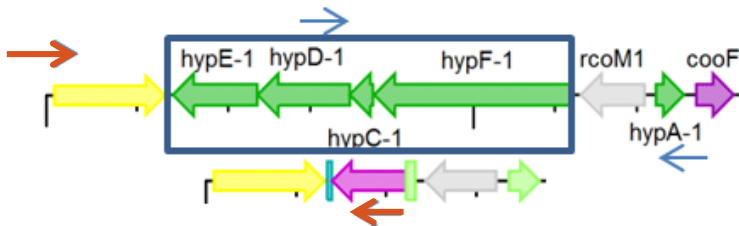
Carrie Eckert

Task 1 – Technical Accomplishments

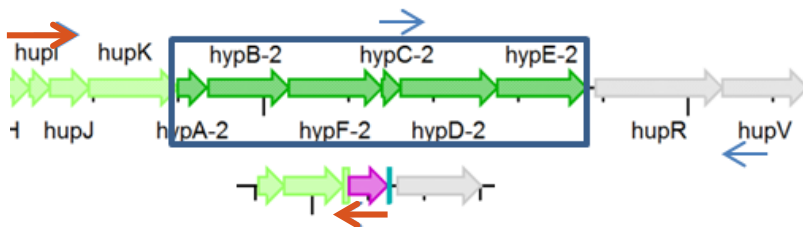
Generate *hyp1*, *hyp2*, and *hyp1/hyp2* Deletion Mutants in CBS

- We aim to delete *hyp1* and/or *hyp2* which provides more direct evidence of their roles in assembling CBS O₂-tolerant hydrogenase (FY14/15 Milestones).

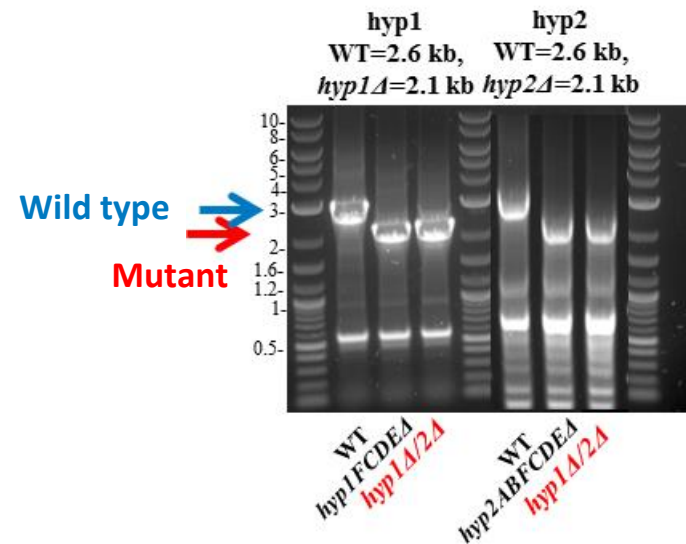
hyp1FCDE (4.87 kb) Deletion Strategy



hyp2ABFCDE (4.81 kb) Deletion Strategy



Obtained $\Delta hyp1$ and $\Delta hyp2$ single mutants, and $\Delta hyp1/\Delta hyp2$ double mutant based on PCR products.



➤ Summary: these mutants will be used to probe their role in hydrogenase maturation.

Task 1 – Technical Accomplishments

Probe hydrogenase activities in mutants

CBS O₂-tolerant Hydrogenase Reaction: $\text{CO} + \text{H}_2\text{O} \rightarrow \text{H}_2 + \text{CO}_2$

CBS Uptake Hydrogenase Reaction: $\text{H}_2 + \text{CO}_2 \rightarrow \text{cell growth}$

Strains	H ₂ Production	CO Uptake	H ₂ Uptake
Wild Type	100%	100%	100%
$\Delta hyp1$	45.7%	67.7%	151%
$\Delta hyp2$	151%	133.5%	0%
$\Delta hyp1/\Delta hyp2$	0%	0%	0%

- ***hyp1* deletion**
 - Delayed H₂ production by >4 h; decreased H₂ production by 54.3%, yet no effect on H₂ uptake
- ***hyp2* deletion**
 - No effect on O₂-tolerant hydrogenase activity, loss of H₂ uptake led to an increase in net H₂.
- ***hyp1/hyp2* double deletion**
 - All hydrogenases activities are lost, no H₂ production (from CO) or H₂ uptake.
- ***hyp1* is more specifically needed to assemble the O₂-tolerant hydrogenase while *hyp2* is for the H₂ uptake hydrogenase.**

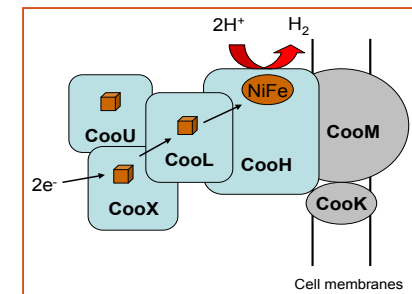
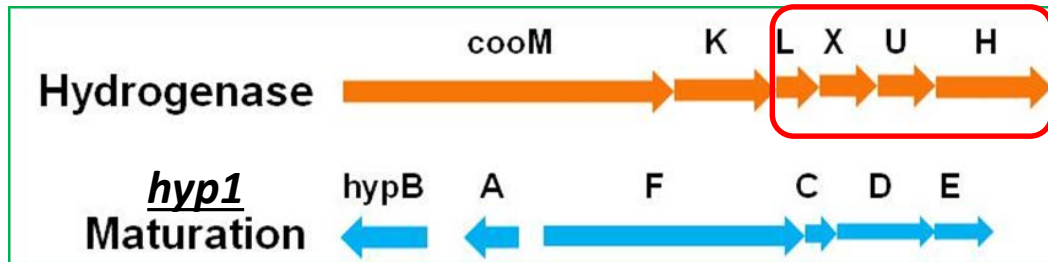
➤ **Summary: Complete FY14/15 Milestones. Only *hyp1* genes should be transformed into *Synechocystis* for H₂ production.**

Approach/Milestone

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

Approach: Transfer the O₂-tolerant CBS hydrogenase (CooLXUH) and its maturation genes (*hyp1*) into a *Synechocystis* host (*hox*⁻) with no background H₂ production.

- Redesign modular gene constructs with stronger promoter and ribosomal binding site (RBS) to boost gene and protein expression.
- Balance protein expression: optimize hydrogenase activity via tuning the expression levels of hydrogenase and *hyp1* maturation genes by comparing protein levels in native CBS and in *Synechocystis* recombinants.
- Transform *slyD* if needed, found in CBS but not in *Synechocystis*, which aids in nickel insertion into NiFe-hydrogenase based on the *E. coli* model.



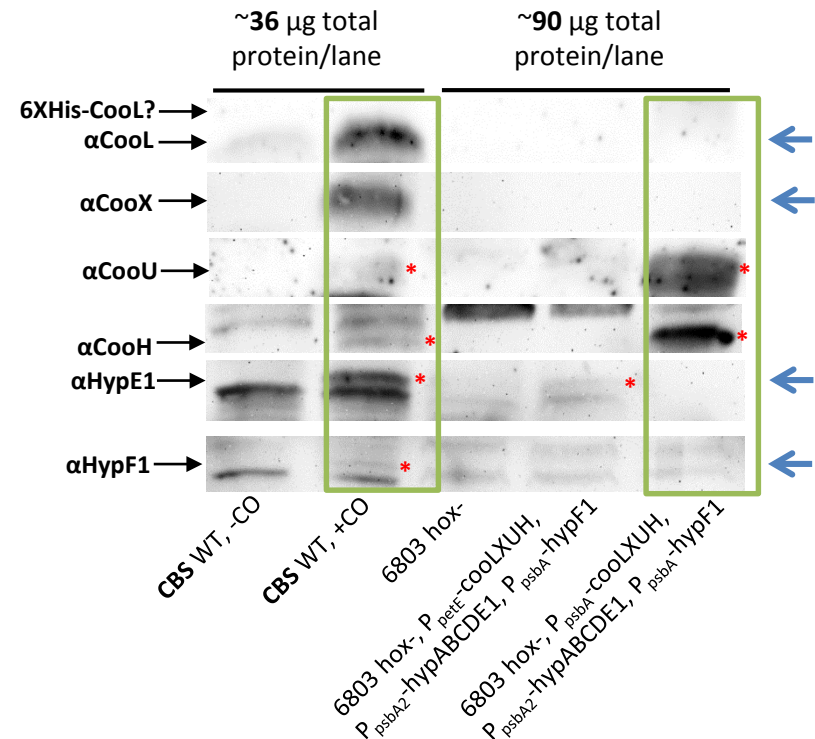
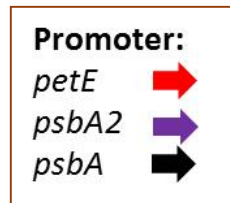
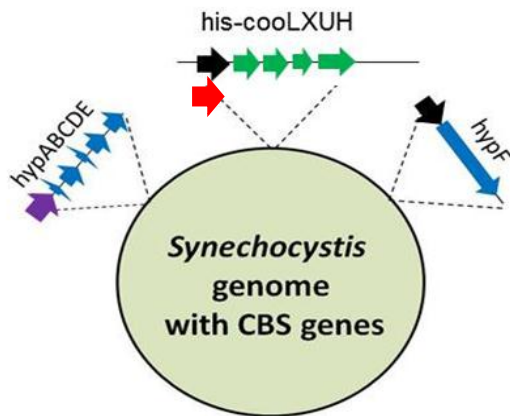
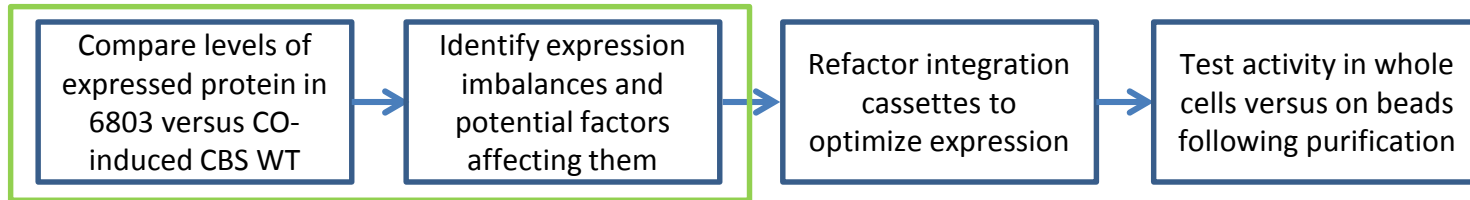
Approach/Milestone

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

	FY14 Milestones – all regular	Completion Date	Status
FY14 Q3	Compare levels of the hydrogenase and maturation proteins expressed in CBS (CO-induced) vs. those in the two <i>Synechocystis</i> recombinants to guide the tuning and balance of protein expression levels in the <i>Synechocystis</i> recombinants.	6/14	Complete
FY14 Q4	Redesign construct to tune and balance expression levels of the <i>coolXUH</i> hydrogenase and <i>hyp1ABCDEFGF</i> maturation genes, transform the newly designed and constructed <i>coolXUH</i> and demonstrate improved and balanced expression of CoolXUH in a <i>Synechocystis</i> recombinant, at 75% level, by comparing protein levels with those of the native CBS host.	9/14	Design Complete; balance expression delayed to FY15Q2
	FY15 Milestones – all regular		
Q2	Determine balanced protein expression using Western blotting by comparison of CoolXUH and Hyp1FABCDE in CBS (CO induced) and new <i>Synechocystis</i> recombinant containing Ptrc-coolXUH-T7tt and Ptrc-hyp1FABCDE-T7tt, using 75% balanced protein expression as the benchmark, which will dictate a need for refactoring promoters and ribosome binding sites to improve expression.	3/15	Complete
Q3	Obtain CBS hydrogenase activity 2-fold over the baseline rate of 10 nmol H ₂ /ml culture/h in whole cells of a <i>Synechocystis</i> recombinant, assayed in vitro with reduced methyl viologen.	6/15 (Go/No-go)	On Track

Task 2 – Technical Accomplishments

Detected Imbalance of Protein Expression



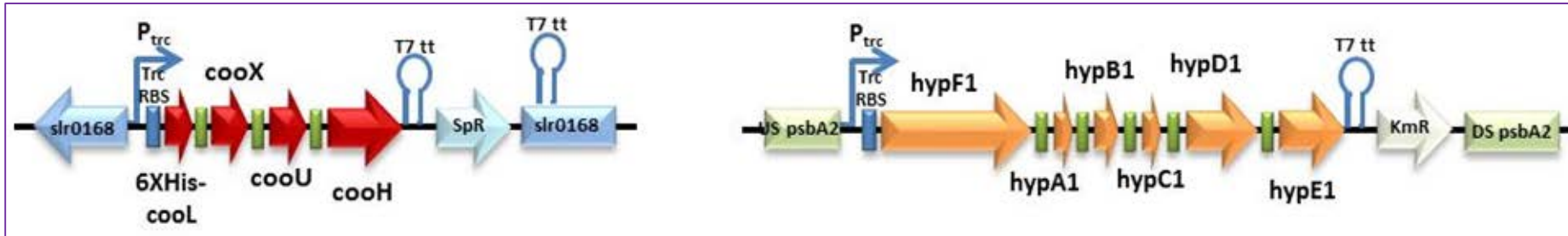
Imbalanced protein expression

- CoolL, CooX, and HypF1 were NOT detected in recombinant *Synechocystis*, albeit 3X more protein loading.

➤ **Summary: Complete FY14 Q3 Milestone. Imbalanced protein expression prompts the redesign of modular gene constructs using stronger promoter and better RBS.**

Task 2 – Technical Accomplishments

New Modular Design for Balanced Protein Expression in *Synechocystis*



- Redesign gene constructs based on new CBS genome sequence, a collaboration of PacBio, Inc. Michigan State University, and NREL (two publications).
- The new design is modular, affording multiple and more frequent insertion sites (■) for the strong *P_{trc}* promoters and ribosome binding sequences (RBS) to enhance both gene and protein expression.
- *hypF* will be expressed at the same genome location as *hypABCDE* to avoid any potential positional effect.

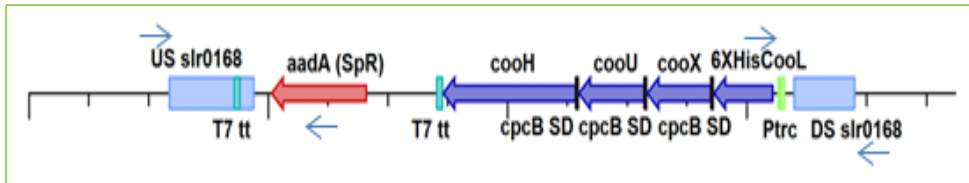
➤ **Summary: Complete FY14 Q4 Milestone. The redesign constructs will be transformed into *Synechocystis* in FY15 for balanced protein expression and affording hydrogenase activity.**

Task 2 – Technical Accomplishments

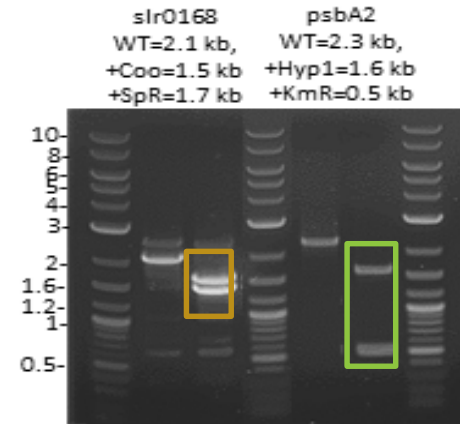
Construct *Synechocystis* Expressing CBS Genes

- Generated *Synechocystis* recombinants by integrating both CBS *cooLXUH* and *hyp1FABCDE* into its genome.

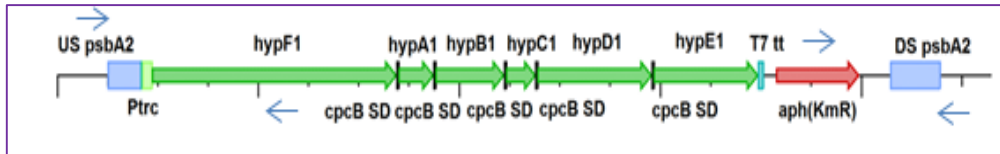
cooLXUH driven by *Ptrc* promoter, integrated into *slr0168* neutral site



Integrating Maturation Genes



hyp1FABCDE driven by *Ptrc* promoter, integrated into *psbA2* site



Integrating Hydrogenase Genes

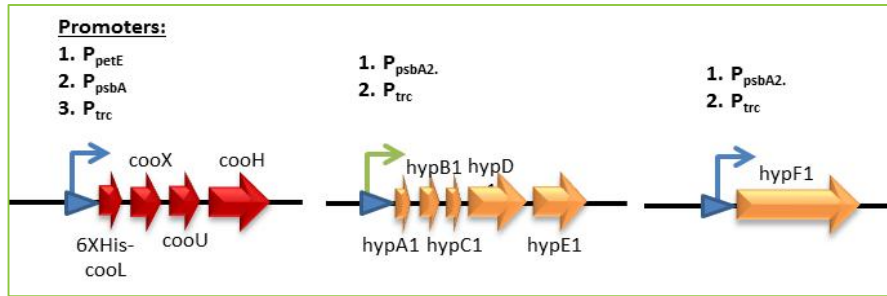


- **Summary:** The *Synechocystis* recombinant will be used to determine balanced protein expression (Q2 Milestone) and hydrogenase activity (Q3 Go/No-Go).

Task 2 – Technical Accomplishments

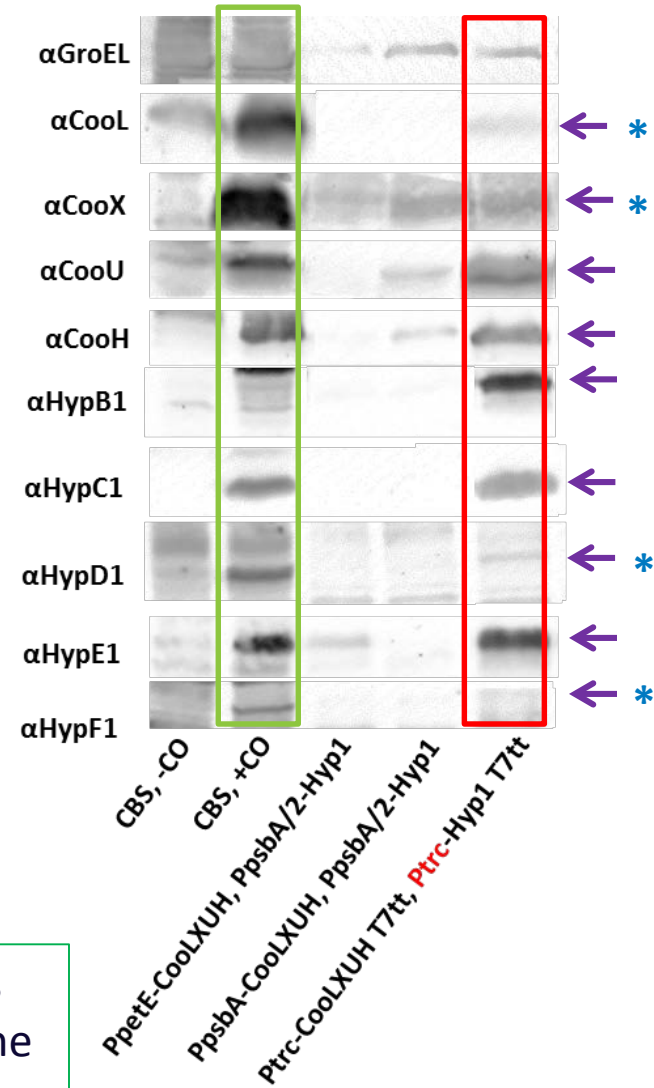
Balanced Protein Expression

Promoter Tuning Strategy:



- New P_{trc} constructs: increased most Coo and Hyp1 proteins compared to previous constructs (weaker promoters)
- CooU, CooH, HypB1, HypC1, and HypE1: equal or higher levels than CBS (+CO).
- CooL, CooX, HypD1, and HypF1(*): lower than levels in CBS +CO.
- **No detectable MV-linked H₂ in *in vitro* assays**
- **Solution:** Integrate new promoter/RBS combinations that should improve both expression and activity.

➤ **Summary:** demonstrate on average 75% balanced proteins expression of hydrogenase and maturation machinery in the new design with strong promoter (FY15 Q2 Milestone).



Response to Reviewers' Comments

It is perfectly fine to work on a piece of the whole in parallel, but context would be useful to assess whether the whole is worthwhile to begin with.

- **Response:** Agreed, this project in the past is a component of a larger portfolio including Univ. of California (Melis) in improving light absorption efficiency, and NREL (Ghirardi) in dissipation of proton gradient to increase electron transfer to H₂ production, sulfur deprivation, and photobioreactor production systems.

There are multiple notable accomplishments...But it is unclear why the (hydrogenase) tetramer was selected over the hexamer.

- **Response:** *In vitro* work had showed that the tetramer is active in H₂ production. The remaining two proteins (CooMK) are membrane bound and will therefore require more effort to optimize their heterologous expression, an out year goal.

More detail is requested on plans to “demonstrate in vitro and in vivo hydrogen production, the latter linking to the host photosynthetic pathway.”

- **Response:** To demonstrate activity *in vitro*, we will use sodium dithionite as the reductant and methyl viologen as the electron mediator to determine hydrogenase activity in the recombinant *Synechocystis* (already lacking its native hydrogenase enzyme). *In vivo* rates will be measured by whole cell H₂ production under either photosynthetic condition or dark fermentative conditions without exogenously added reductant/electron mediator.

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 H₂ production. JCVI and NREL will publish together.

Remaining Challenges and Barriers

Probe Hydrogenase Maturation Machinery in CBS

- *slyD* is a candidate maturation gene for heterologous expression. We will begin its deletion in CBS to probe function.

Expression of the CBS hydrogenase in *Synechocystis*

- One remaining challenge is to demonstrate *in vitro* and *in vivo* H₂ production, the latter links to the host photosynthetic pathway
 - CBS hydrogenase is able to link to a photosynthetically reduced cyanobacterial ferredoxin, *in vitro*, as previously demonstrated by NREL.
- *CooM* and *CooK* are likely required to afford hydrogenase activity as a hexameric complex (*CooMKLXUH*) – FY14 Reviewers' suggestion.
 - We will delete *cooM* and *cooK* in CBS to probe their functions, followed by expression in a *Synechocystis* recombinant already expressing 10 CBS genes (*hyp1* and *cooLXUH*).

Proposed Work

Task 1. Probe hydrogenase maturation machinery in CBS

- Prepare a manuscript for publication, documenting the functions of *hyp1* ($\Delta hyp1FCDE$), *hyp2* ($\Delta hyp2ABFCDE$), and $\Delta hyp1/\Delta hyp2$ double mutants, based on evidence of growth profiles (in CO or H₂), gene induction profiles (RT-PCR), and hydrogenase activity to unravel their functions in assembling the various hydrogenases in CBS (FY15).
- Delete *slyD* in CBS to probe its function and if warranted, express *slyD* in the *Synechocystis* recombinant (FY15, FY16).

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

- Determine *in vitro* hydrogenase activity in the recombinant *Synechocystis* by optimizing growth conditions (growth medium, stage of growth) (FY15).
- Integrate new promoter/RBS combinations that should improve both expression and activity (FY15).
- Delete *cooM* and *cooK* in CBS to probe their functions (FY16).
- If warranted, express *cooM* and *cooK* in the *Synechocystis* recombinant already expressing *cooLXUH* and *hyp1ABFCDE* (+/-*slyD*) (FY16)

Technology Transfer Activities

Technology-to-market or technology transfer plan or strategy

- Photobiological H₂ is still a more long-term technology due to the challenges of O₂ sensitivity and the cost of photobioreactors. However once realized, it has immense application in producing renewable H₂ from sunlight and water, both are abundant resources on earth.

Plans for future funding

- Respond to funding opportunities announcement from DOE FCTO FOA, DOE ARPA-E, or DOD.

Patents, licensing

- Not yet. Any genetic strategies and new plasmids design (genetic tools) developed in CBS should have intellectual property potential.

Summary

Task 1. Probe hydrogenase maturation machinery in CBS

- Generated a series of mutants in CBS: *hyp1* ($\Delta hyp1FCDE$), *hyp2* ($\Delta hyp2ABFCDE$), and $\Delta hyp1/\Delta hyp2$ double mutants.
- Mutants characterization revealed that *hyp1* is responsible for assembling the CBS O₂-tolerant hydrogenase (Coo) and *hyp2* for the H₂-oxidizing hydrogenase (Hup), based on evidences of *in vivo* H₂ production, H₂ and CO uptake profiles, and cell growth in CO or H₂ (+CO₂).
- *hyp2* can partially complement the function of *hyp1*, but with a delay. *hyp1*, however, cannot replace the function of *hyp2*.

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

- Demonstrated imbalanced protein expression by comparing those in native CBS vs. in recombinant *Synechocystis*, the latter with weaker promoters (*PpsbA2* and *PpetE*).
- Refactored new modular gene constructs and obtained *Synechocystis* recombinants expressing 10 CBS genes (*hyp1* and *coolXUH*), driven by a strong promoter (*Ptrc*) and integrated ribosomal binding sites.
- Demonstrated balanced protein expression, in average, by comparing native CBS with recombinant *Synechocystis*.
- However, the recombinant has no hydrogenase activity. A similar recombinant is also constructed in *E. coli* for troubleshooting.
- Work is ongoing to probe functions of CBS *slyD*, *cooM*, and *cooK* in CBS and evaluate their potential expression in *Synechocystis* to afford hydrogenase activity.