FUEL CELL TECHNOLOGIES OFFICE FINAL REPORT

"IMPROVING CYANOBACTERAIL O_2-TOLERANCE USING CBS HYDROGENASE FOR HYDROGEN PRODUCTION"

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Started in FY 2010; ended in FY 2016

Executive Summary

Cyanobacterial H_2 production is a viable path to renewable H_2 with water serving as the electron donor and sunlight the energy source. A grand challenge is the sensitivity of the underlying hydrogenase to O_2, the latter an inherent byproduct of oxygenic photosynthesis. This challenge has been identified as a technical barrier in the Fuel Cell Technologies Office (FCTO) Multi-year Research, Development and Deployment Plan. One solution is to express in cyanobacterium an O_2-tolerant hydrogenase to circumvent this barrier. We have uncovered an O_2-tolerant hydrogenase from a photosynthetic bacterium *Rubrivivax gelatinosus* CBS (Casa Bonita Strain; hereafter "CBS") with a half-life near 21 h when exposed to ambient O_2. We sequenced the CBS genome and identified two sets of maturation machineries *hyp1* and *hyp2*. Transcripts expression analysis and mutagenesis revealed that *hyp1* is responsible for the assembly of the O_2-tolerant CO-oxidation (Coo) hydrogenase and *hyp2* is involved in the maturation of a H_2-uptake hydrogenase. The structural genes encoding the O_2-tolerant hydrogenase (*cooLXUH*) and maturation genes *hyp1FABCDE* were therefore cloned and expressed in the model cyanobacterium *Synechocystis* sp. PCC 6803. We obtained several recombinants displaying hydrogenase activity in a *Synechocystis* host lacking background activity, suggesting that the CBS hydrogenase is active in *Synechocystis*. Yet the activity is extremely low. To ensure balanced protein expression, we systematically optimized heterologous expression of 10 CBS genes by using stronger promoters and better ribosome binding site. Moreover we attempted the expression of *cooM* and *cooK* genes, verified to be important in CBS to afford activity. CooM is a very large protein and both CooM and CooK are membrane-associated. These properties limited our success in expressing both genes in *Synechocystis*, although they were both expressed in *E. coli* yet with no activity. This project was terminated in FY2016 due to the inability to generate a *Synechocystis* strain with consistent and active H_2 production activity. Nevertheless the research has led to a few high-impact publications and numerous conference presentations to increase global visibility of the DOE-funded research.

Background and Previous Research

Hydrogen (H_2) is a clean fuel that when used in a fuel cell only generates water as the byproduct. If produced from renewable resources, H_2 has the potential to address the adverse environmental impact of petroleum-derived fuels while ensuring energy security. Numerous microbes have the innate ability to produce H_2, catalyzed by hydrogenase enzymes, as a means to dissipate excess electrons to attain redox homeostasis. An ideal H_2-prodcution process would be via photosynthesis with water serving as the electron donor and sunlight providing the energy source. Indeed numerous algae and cyanobacteria can carry out photolytic H_2 production during photosynthesis (1, 2). Yet the process is not sustainable in light due to the sensitivity of the hydrogenase to O_2, the latter an inherent byproduct of oxygenic photosynthesis. The topic of algal H_2 production research was supported by FCTO from 2000-2015 to develop an O_2-tolerant algal system for sustained H_2 production, for which numerous approaches were attempted and improvements accomplished to increase O_2-tolerance of its native FeFe-hydrogenase. Cyanobacteria are comparable model photosynthetic hosts; they are prokaryotes hence with more amenable genetic tools and are adaptive to high salt environment. Most cyanobacteria contain NiFe-hydrogenases, albeit also sensitive to O_2, the inhibition by O_2 is reversible once returning to anaerobic status (3, 4). One such model host is *Synechocystis* sp. PCC 6803 (hereafter "*Synechocystis*") which contains a bidirectional hydrogenase Hox with a half-life of mere 2 min in air (5), albeit the inhibition by O_2 is reversible.

To circumvent the O_2-sensitivity of the *Synechocystis* H_2-production system, one potential solution is to transform *Synechocystis* with genes encoding a NiFe-hydrogenase that is inherently more O_2 tolerant.

One such candidate is the NiFe-hydrogenase from the photosynthetic bacterium *Rubrivivax gelatinosus* CBS (hereafter "CBS" for Casa Bonita Strain). CBS was originally isolated from soil in Denver by NREL researchers (6, 7). CBS contains an active CO-to-H_2 pathway according to the equation: $CO + H_2O \rightarrow CO_2 + H_2$. The hydrogenase participating in this reaction is induced by CO, and has been shown to be more tolerant to O_2 than its counterpart NiFe-hydrogenase, the former has a half-life of 21 h when stirred in ambient air (21% O_2) (6). Genes encoding this O_2-tolerant NiFe-hydrogenase along with its putative maturation machinery have been cloned in CBS (8, 9). These tools and knowledge therefore position the NREL team to construct a cyanobacterial recombinant expressing the O_2-tolerant hydrogenase from CBS for sustained photobiological H_2 production, a long-term goal of this FCTO project. Figure 1 depicts the scheme for the overall concept.

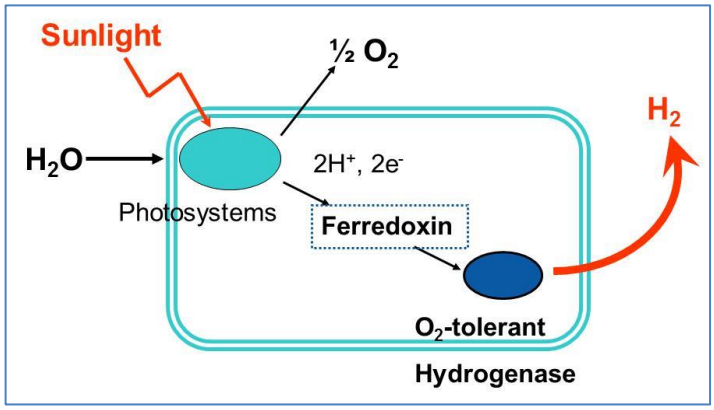


Figure 1. A cyanobacterial-bacterial recombinant system expressing a foreign O_2-tolerant hydrogenase for sustained photolytic H_2 production.

Objectives of the FCTO Project

The NREL project funded by FCTO (FY 2010 – FY 2016) has two overarching objectives for sustained photolytic H_2 production. The first objective is to identify the maturation machinery required to assemble the O_2-tolerant hydrogenase in CBS. This objective is being addressed in Task 1: Probe hydrogenase maturation machinery in CBS. The second objective is to engineer the robust cyanobacterium *Synechocystis* to express the O_2-tolerant CBS hydrogenase for sustained photolytic H_2 production, using genes identified in the first objective as well as other genetic modifications as necessary. This objective is being addressed in Task 2: Expression of the CBS hydrogenase in *Synechocystis*.

Prior to the AOP project, the NREL team was awarded a three-year project via FCTO competitive grant solicitation teaming with J. Craig Venter Institute (JCVI). The title of the award is "Hydrogen from water in a novel recombinant cyanobacterium." Due to issue with Congressional budget appropriation, the award was delayed until Q3 of FY 2007 (end date Q4 of FY2009). In this funding period, major advances were made toward developing genetic tools and cloning strategies for gene transfer from CBS into *Synechocystis*. Some notable progresses include: (1) cloning of the CBS hydrogenase genes *cooLH* and *cooLXUH* for expression in both *E. coli* and in *Synechocystis.* For the latter host, we used a plasmid that was inconsistent in stably expressing the CBS genes in *Synechocystis*. After lengthy troubleshooting we identified the lack of a terminator as the root cause. We redesigned the plasmid which led to successful transformation during the AOP project period; (2) testing a conjugation strategy using a plasmid that can facilitate gene transfer via mating to express the CBS *cooMK* hydrogenase genes and *hypAB* maturation genes. After lengthy troubleshooting, we abandoned this approach since the improved plasmid from (1) can accommodate our effort; and (3) generating antibodies to verify protein expression. We tested

several sources and eventually identified the best antibodies leading to progress made during the AOP project period. The tools and techniques developed during this project period expedite the research progress toward the AOP project, which is described in more details below.

1. Task 1. Probe Hydrogenase Maturation Machinery in CBS

The milestones covered during this project period, and their completion dates are outline in Table 1.

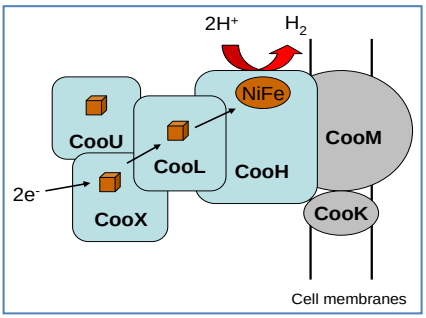
Task 1. Milestone Table for Probing Hydrogenase Maturation Machinery in CBS.

	Milestones	Completion Date
FY 2010	Test antibodies specificity and determine hydrogenase compositions	12/09
FY 2010	Determine subunit composition and induction kinetics of native hydrogenase	3/10
FY 2010	Determine hydrogenase subunit in a recombinant system	9/10
FY 2011	Probe function of two putative hydrogenase maturation genes in assembling the CBS O_2-tolerant hydrogenase	4/11
FY 2012	Identify two additional hydrogenase maturation genes via quantitative PCR and the analysis of CBS genome	5/12
FY 2013	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
FY 2013	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
FY 2014	Identify the CBS homologues of three genes additionally involved in hydrogenase maturation in *E. coli*, *slyD* and *carAB*, through a targeted blastp comparison of the putative CBS genes against those in *E. coli*, using ~35% amino acid sequence identity to verify homology.	12/13
FY 2014	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

	Milestones	Completion Date
FY 2014/FY 2015	Through deleting *hyp1FCDE* genes in the wild type background with the presence of *hyp2* genes, determine if *hyp1FCDE*Δ decreases the total hydrogenase activity by 50% or more based on *in vivo* assay of H_2 production in whole cells, as is required for optimal hydrogenase activity, and obtain a construct for *hyp2ABFCDE* deletion mutant to measure the impact on hydrogenase activity; the outcome will determine if *hyp2* will be co-transformed with *hyp1* into *Synechocystis* to assemble a more active CBS hydrogenase for increased H_2 production	12/14

1.1. Cloning of the genes encoding O_2-tolerant CBS Hydrogenase and its subunit compositions.

A critical criterion to probe the genetic maturation machinery in CBS is to clone the CBS O_2-tolerant hydrogenase-encoding genes as well as its clustered hydrogenase maturation genes. Since CBS is a novel NREL isolate, no genome sequence was available at project start, and obtaining a sequence was another goal of this project. Via transposon-based random mutagenesis and screening, we generated several CBS mutants lacking the ability to produce H_2. Sequencing upstream and downstream of the insertion sites revealed the gene sequences encoding the multi-subunit O_2-tolerant CBS hydrogenase CooMKLXUH (Coo: CO oxidation), with *cooH* encoding the catalytic subunit and *cooL* its redox partner donating electrons to CooH subunit (Figure 2). Homology comparison suggests that CBS hydrogenase is a hexamer (10, 11); hence it is highly likely all six genes encoding CooMKLXUH are required to confer activity in a foreign host such as *Synechocystis*. Sequencing and characterization of the CBS hydrogenase-encoding genes led to a publication in Applied and Environmental Microbiology in 2010 entirely funded by FCTO (8).



Subunit	Putative Function
CooM	Membrane anchoring
CooK	Proton translocation
CooL	Small subunit, electron transfer
CooX	Electron transfer
CooU	Unknown
CooH	Catalytic subunit, H_2 production

Figure 2. Putative compositions and electron-transfer pathway (left) and functions (right) of the hexameric O_2-tolerant hydrogenase in CBS. Coo: CO-oxidation.

Protein immunoblot (or protein western blot) is a powerful technique to probe hydrogenase expression either in native CBS or in *Synechocystis*, which requires the generation of subunit-specific antibodies. The respective amino-acid sequences of CooMKLXUH were submitted to YenZym (San Francisco).

YenZym used a computer algorithm to predict regions conferring the best antigenicity, followed by expressing the short peptides in *E. coli*. The peptides were then purified from *E. coli* and used to immunize two rabbits. The resulting serum from both rabbits was affinity purified to obtain high quality antibody. Data from Figure 3 show western blots of their specificity toward the native hydrogenase in CBS. Bands for CooLXUH proteins appeared at their respective molecular weights (41 kDa for CooH, 16 kDa for CooL, 19 kDa for CooX, and 20KDa for CooU). These antibodies were suitable to test their expression in *Synechocystis* transformed with these genes.

Preliminary results (not shown) indicate that CooM and CooK, two membrane anchor proteins, are more difficult to work with than the more soluble proteins (CooLXUH). This is a common challenge for membrane proteins. The apparent molecular mass of CooK is 25 kD on the western blot, while the deduced value based on predicted amino acid sequence is 32 kD. The apparent molecular mass of CooM is around 95kD on the western blot, while the deduced value is 126 kD, a fairly large membrane protein. It is common that large membrane proteins appear in a smear in gel electrophoresis (SDS PAGE), and that membrane proteins do not migrate at the expected molecular mass either. Nevertheless both proteins only appeared in the presence of CO. ***The above outcomes successfully completed the Milestone: Test antibodies specificity and determine hydrogenase compositions (Q1, FY 2010).***

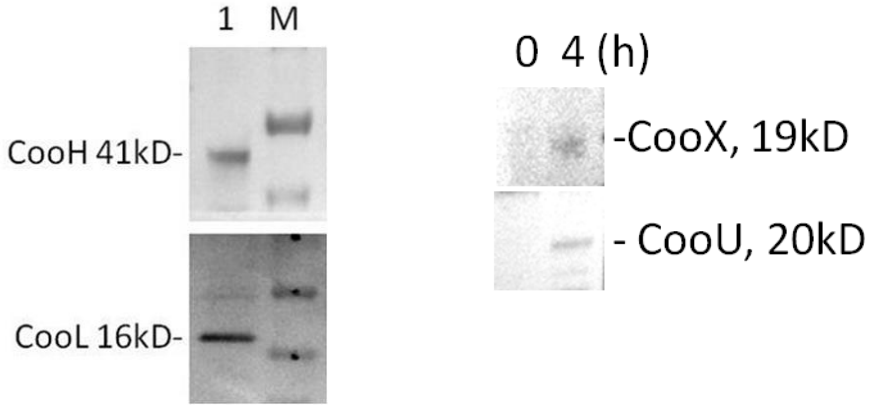


Figure 3. Protein immunoblot showing the detection of CooH, CooL, CooX, and CooU proteins in CBS treated with CO. 0, 1, 4 represent the hours after CO addition to the CBS culture headspace. M: molecular weight markers.

Meanwhile, we subjected the CBS wild-type (WT) strain to CO treatment. Data from Figure 4 illustrate the expression kinetics of CooH, CooL, CooU, and CooX over time upon exposure to 20% CO. With these, proteins appeared at their respective molecular weight, and only in the presence of CO. ***These findings completed the Milestone*: Determine subunit composition and induction kinetics of the native hydrogenase *(Q2, FY 2010)*.**

1.2. Develop a genetic system in CBS.

More in-depth knowledge of the biochemistry of the CBS native O_2-tolerant hydrogenase (structure and its NiFe active site) is essential for its comparison with the recombinant enzyme when expressed in *Synechocystis*. We successfully developed a genetic system in CBS which allows us to modify any gene of choice on the chromosome. We constructed a plasmid that would deliver a His-tagged *cooH* gene into WT CBS and confer kanamycin resistance in the transformed strain. Meanwhile a *sacB* gene was

included in the plasmid that enables a counter selection which kills any cell that maintains this plasmid when sucrose is supplied. The combination of kanamycin resistance and sucrose tolerance therefore successfully selected only transformed strains that have lost the plasmid and yet kept the chromosomally integrated His-*cooH.* Developing a genetic system in CBS is a major achievement, with which we could insert an affinity tag to the hydrogenase gene for its purification via affinity chromatography. We could also knockout gene of interest to reveal its function. Using this system, we fused a His6 affinity tag (with HRV3C cleavage site) to the N-terminus of CooH.

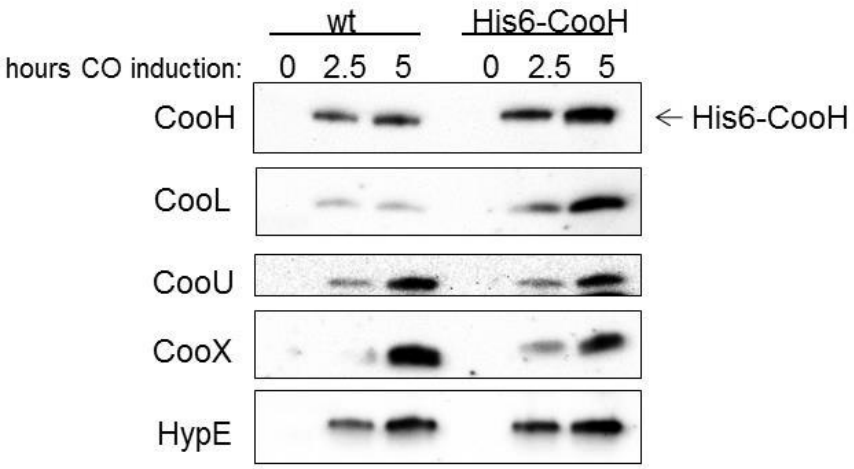


Figure 4. Induction of *Rubrivivax gelatinosus* CBS hydrogenase subunits CooH, L, U, and X and maturation protein HypE upon CO treatment, in both wild-type (wt) and recombinant (His6-CooH) strains. 20% CO was added to each sample at time zero, and samples were taken at 0, 2.5, and 5 hours post-CO induction. Samples were then analyzed by protein immunoblot with antibodies to the CooH, L, U, X, and HypE proteins, respectively.

This recombinant strain has thus far been quite stable genetically, with its hydrogenase activity similar to that of the WT strain, as judged by H_2 evolution in the presence of CO (Figure 5A). Protein immunoblots also show that all of the CooH in the recombinant strain is His6-CooH, as evidenced by the electrophoretic mobility shift due to the presence of the His6 tag, not a mix of WT and mutant CooH (Figure 5B). The immunoblots (Figure 4) demonstrate that hydrogenase subunits CooL, X, U, and H are all present in the recombinant His6-CooH strain, and with similar expression levels compared to those in the WT strain. ***These findings completed Milestone: Determine hydrogenase subunit in a recombinant system (Q4, FY 2010).***

1.3. Probe hydrogenase maturation machinery in CBS

It is well established that maturation of the NiFe-active site recruits a suite of maturation proteins for the insertions of Ni, Fe, CO, and CN ligands (12-15). Transposon mutagenesis coupling with gene walking had revealed six putative hydrogenase maturation genes (*hypFCDEAB*) (green arrows) clustered near the CBS O_2-tolerant *coo* hydrogenase (red arrows) (Figure 6). It is therefore important to determine if these *hyp* genes are indeed involved in the assembly and maturation of the CBS O_2-tolerant hydrogenase. If confirmed, their co-transformation into *Synechocystis* will be needed to confer hydrogenase activity.

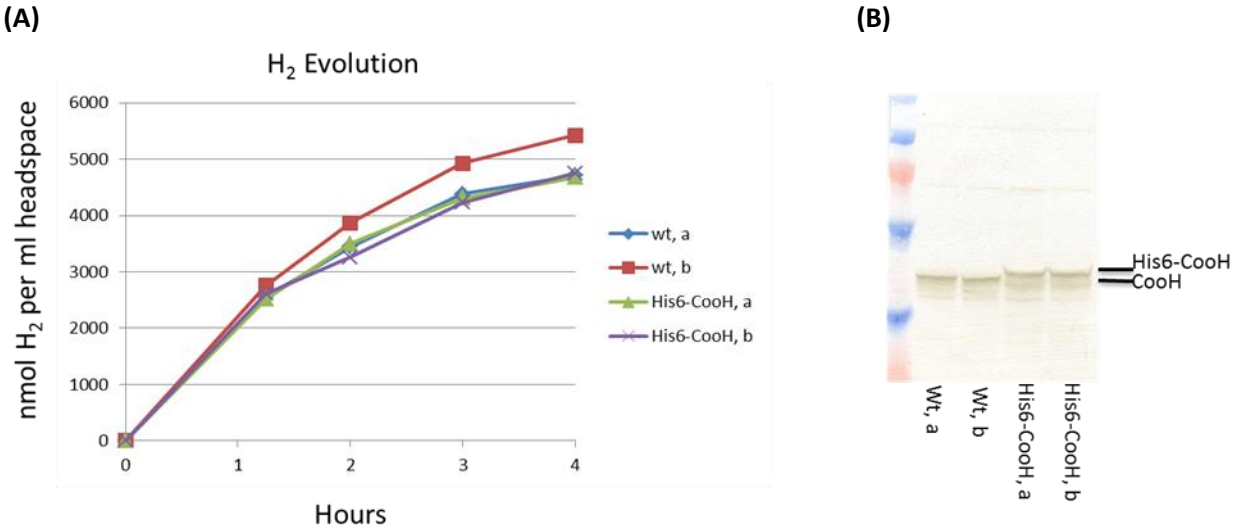


Figure 5. (A). Duplicate samples of wild-type CBS and His6-CooH CBS were induced with CO at time zero, and H_2 evolution was measured in the culture headspace. (B). Samples were taken at the end of this experiment and protein levels were analyzed by immunoblot, probing with the CooH antibody.

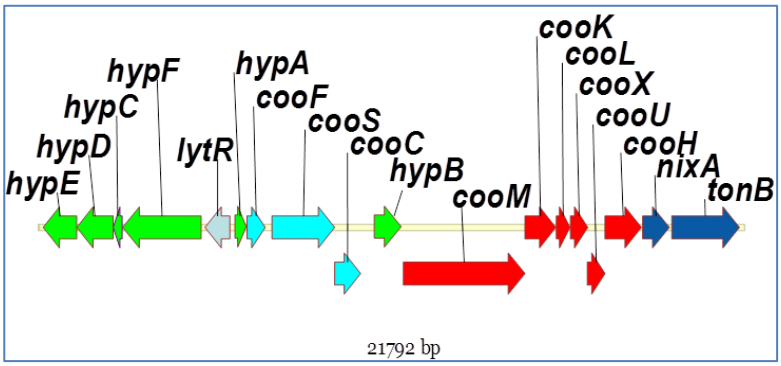


Figure 6. The arrangement of the CBS maturation genes (*hyp*) and hydrogenase structural genes (*coo*) on the CBS genome.

1.3.1 Hyp1 Expression Profiles. We have generated antibodies against both HypE and HypF proteins. Both HypE and HypF are induced by CO (Figures 4 and 7), similar to the CBS hydrogenase, suggesting that *hypE* and *hypF* are likely involved in the assembly and maturation of the CBS hydrogenase. ***The outcome completed the Milestone: Probe function of two putative hydrogenase maturation genes in assembling the CBS O_2-tolerant hydrogenase (Q3, FY 2011).***

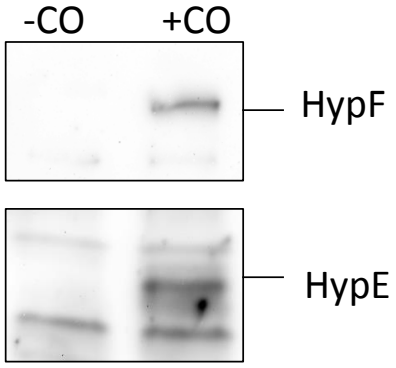


Figure 7. Immunoblot of CBS HypE and HypF proteins. Cells were incubated in the absence or presence of CO for 4 hours before cell lysis and 10 ug of soluble protein was used for analysis by immunoblot.

Instead of generating more costly antibodies, we used qRT-PCR (quantitative reverse transcription-polymerase chain reaction) to examine fold-changes in transcript abundance of *hypA*, *hypB*, and *hypD* genes under CO and CO_2, the latter serving as a control. We grew CBS with argon gas in the headspace (un-induced condition), CO in the headspace (induced condition), or CO_2 in the headspace (CO_2 is the product of CO oxidation) as a second un-induced control. The un-induced control in argon gas was used to calculate the transcript fold-change. As shown in Figure 8, the *hypA*, *B*, and *D* genes are specifically induced in the presence of CO, and the *cooH* gene was used as a positive control. While *hypC*, *E*, and *F* were not shown, transcription of these genes was also specifically induced by CO. However, their fold-change in mRNA level could not be calculated because the *hypC*, *E*, and *F* transcripts were not abundant enough to be detected in the un-induced condition (argon gas control), thus the fold-change compared to the un-induced condition could not be calculated. These results clearly show that the *hypABCDEF* genes are specifically induced in the same condition in which the CBS O_2-tolerant hydrogenase is induced (both by CO), and strongly indicates that these six *hyp* genes are involved in production of the CBS O_2-tolerant hydrogenase. ***The outcomes completed the Milestone: Identify two additional hydrogenase maturation genes via quantitative PCR and the analysis of CBS genome (Q3, FY 2012).***

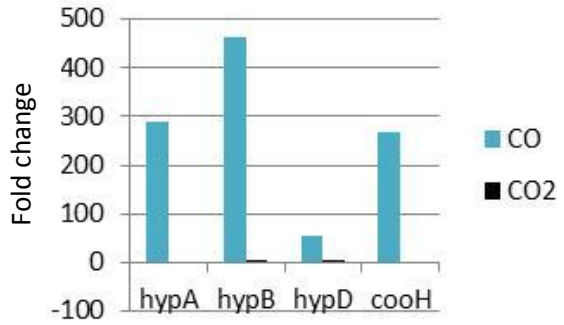


Figure 8: CO-induced hydrogenase maturation gene expression. qRT-PCR was used to detect changes in *hypABCDEF* gene transcript abundance.

1.3.2. CBS Genome sequencing and identification of *hyp2*. Sequencing CBS genome is an invaluable tool that will allow us to uncover additional genes that may be involved in the generation of an active CBS hydrogenase. We sought after a collaboration with Michigan State Univ. (Dr. Jin Chen) in sequencing the CBS genome at no cost to NREL. Genomic CBS DNA was isolated at NREL, and sequenced using Illumina technology at Michigan State University. The short, sequenced DNA fragments (40-60 base pairs) were then assembled into 1,240 scaffolds. The CBS genome is estimated to be 4.1M bp (million base-pairs), which is consistent with those of other purple non-sulfur photosynthetic bacteria. Over half of the genome is on a scaffold of 8 kb (kilo-base) or larger, and half of the 1,240 scaffolds are larger than 2.2 kb. We identified two scaffolds with hydrogenase maturation genes. Scaffold one contains a putative homolog of the maturation genes *hupK, hypA, hypB,* and partial *hypF* (N-terminus) genes. Scaffold two contains putative homologs of *hypC*, *hypD, and hypE and* partial *hypF* (C-terminus) genes. We used the National Center for Biotechnology Information (NCBI) tool ORF (open reading frame) Finder to analyze all possible ORFs within these two scaffolds and identified partial or complete ORFs for a total of seven maturation genes (Figure 9). By aligning the two *hypF* fragments, we predicted the presence of 525 nucleotides, missing between these two scaffolds. We confirmed that these two scaffolds are indeed contiguous and encode a full-length *hypF* ORF by PCR using various primers and obtaining PCR products of the correct sizes (data not shown). A primer is a short strand of DNA that serves as a starting point for DNA synthesis. It "primes" DNA replication because the enzymes that catalyze this process, DNA polymerase, can only add new nucleotides to an existing strand of DNA. qRT-PCR primers were designed and tested against the seven new hydrogenase maturation genes *hypABCDEF-2* (*hyp2*, to be distinct from *hyp1*) and *hupK,* the latter a scaffold protein for NiFe-hydrogenase metal cofactor. mRNA levels of

hupK and *hypD-2* were determined by qRT-PCR in Argon (control), CO/H_2 and CO_2/H_2 conditions (Figure 10). It was found that the induction pattern of these genes matches that of the uptake hydrogenase large subunit *hupL* and is distinct from that of the *hyp1* genes. These findings support the conclusion that *hyp1* genes are induced by CO and *hyp2* genes by H_2. We hence identified a new genomic region in CBS that contains at least 7 additional maturation genes (*hyp2*) via genome analysis, and confirmed regulation of these maturation genes and primary function are distinct from the *hyp1* maturation cluster. ***Collectively these findings completed the milestone***: ***Identify two additional hydrogenase maturation genes via quantitative PCR and the analysis of CBS genome (Q3, FY 2012).*** These findings led to a publication in the Journal of Bacteriology in 2012 (9), entirely supported by FCTO.

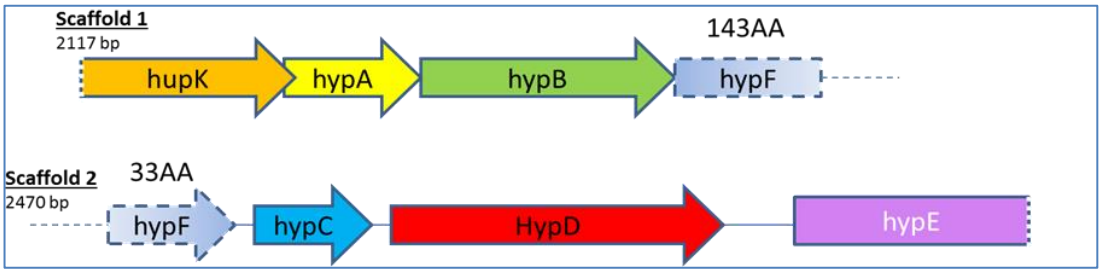


Figure 9. Identification of a new set of hydrogenase maturation genes, *hyp2*. A depiction of the ORFs present on the two scaffolds, 1 & 2. *hupK* and *hypE* are partial ORFs. *hypF* has two partial ORFs with an N-terminal sequence on scaffold 1 and a C-terminal sequence on scaffold 2.

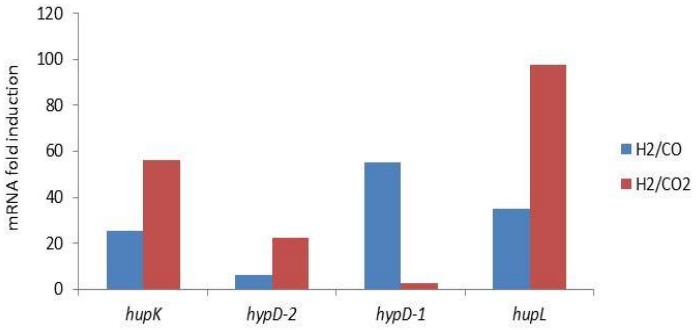


Figure 10. Expression pattern of *hyp2* cluster hydrogenase maturation genes. qRT-PCR showing the relative levels of induction of *hupK, hypD-2, hypD-1,* and *hupL. hupK, hypD-2* and *hupL* are induced under both H_2/CO and H_2/CO_2 whereas *hypD-1* is induced specifically by H_2/CO.

1.3.3. Characterizations of *hyp1* and *hyp2* genes and deletions of *hypE1* and/or *hypE2* in CBS. The discovery of two sets of hydrogenase maturation genes prompted the question as to which set is essential for the maturation of the CBS O_2-tolerant hydrogenase, to be co-transformed with hydrogenase-encoding genes into *Synechocystis*, which forms the rationale of this work. We exposed WT CBS in either CO or H_2 and measured induction of *hyp1* (*hypA1*, *B1, D1*) and *hyp2* (*hypC2, D2, E2*) using CBS *cooH* hydrogenase as a positive control. We found that *hyp1* expression requires the presence of CO, consistent with the CBS *cooH* gene, while H_2 alone is sufficient for *hyp2* expression (Figure 11). We therefore conclude that *hyp1* cluster is involved in the maturation of the O_2-tolerant, H_2-evolving hydrogenase while *hyp2* cluster is responsible for the H_2-uptake hydrogenase (Figure 10, *hupL*), not of interest to this project. ***These results completed the Milestone: 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 (Q2, FY 2013).***

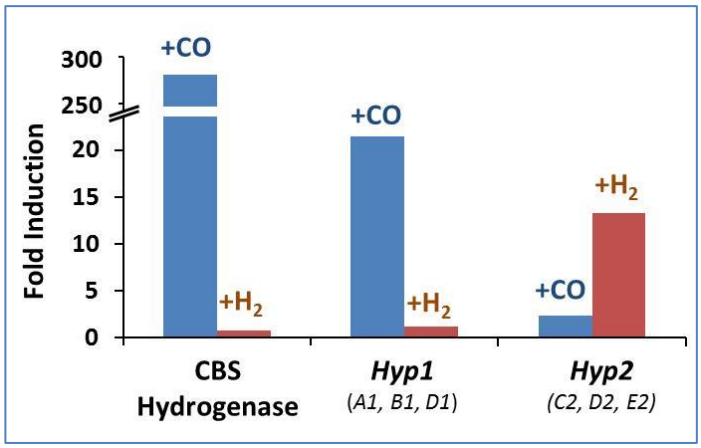


Figure 11. Quantitative RT-PCR of CBS maturation genes in various gases substrates. Fold-changes are based on control cultures grown in argon gas.

Although qRT-PCR data strongly suggest the distinct roles of *hyp1* and *hyp2*, the most definitive answer as to their contribution in assembling the O_2-tolerant hydrogenase requires single and double deletions of *hyp1* and/or *hyp2* genes, the goal of the FY 2013 Q3 Milestone. A genetic system was previously developed in CBS to enable homologous recombination, which we exploited to create targeted gene deletions in CBS. We specifically deleted the putative *hypE1* gene known to be involved in hydrogenase maturation (14), and replaced it with a gene encoding resistance to the antibiotic kanamycin, as depicted in Figure 12A. The strain containing the *hypE1* deletion is referred to as the "*ΔhypE1*" strain. Complete replacement of the *hypE1* gene with the KanR gene was confirmed by PCR (Figure 12B and 12C). Finally, protein western blot analysis confirmed a complete loss of the HypE protein in the CBS Δ*hypE* strain (Figure 12D).

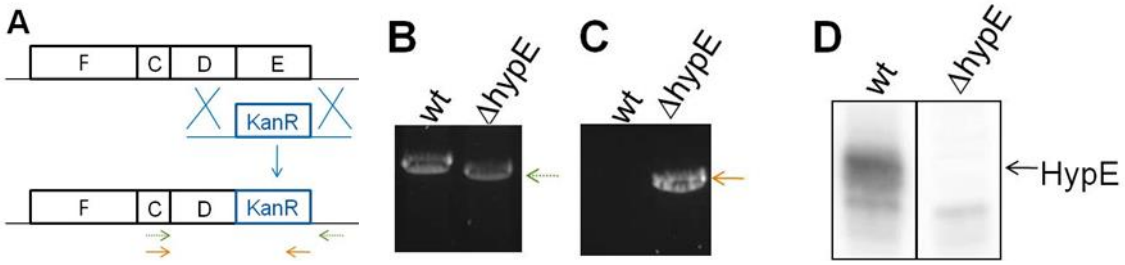


Figure 12: A) Diagram of gene replacement scheme in CBS. Homologous recombination was used to replace the *hypE* gene, "E", with a gene encoding resistance to kanamycin, "KanR". Primer pairs used to verify gene replacement are marked with colored arrows. B), C) PCR was used to verify the replacement of the *hypE* gene in the recombinant strain. In panel B, the DNA amplified using primers that recognize the *hypC* gene and a region downstream of *hypE/KanR* was a slightly smaller size in the ΔhypE strain than in the WT (wild-type) host strain, indicating replacement of the *hypE1* gene with the *KanR* gene. Primers recognizing the *hypC* gene and the *KanR* gene only amplified DNA from the recombinant Δ*hypE* strain, as expected. D) Western blot analysis revealed that the HypE protein is expressed in wild-type CBS but not in the mutant Δ*hypE* strain, confirming the *hypE* gene knockout. The *hypE* gene is later renamed as *hypE1*.

However, H_2 production in the Δ*hydE1* strain was similar to that in WT, suggesting *hypE2* may compensate for the loss of *hypE1*. As such we attempted and successfully deleted *hypE2* gene in either

the WT or Δ*hypE1* background hence yielding single and double mutants of Δ*hypE1,* Δ*hypE2,* and Δ*hypE1*:Δ*hypE2* strains (Figure 13). Either Δ*hypE1* or Δ*hypE2* strains can grow in CO, suggesting the presence of an active CBS O_2-tolerant hydrogenase. However, the double mutant cannot grow in CO, indicating a loss of the O_2-tolerant hydrogenase activity. This finding clearly implies that *hyp1* and *hyp2* genes are complementary, that either copy is able to assemble the CBS O_2-tolerant hydrogenase. ***This finding completed milestone: 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 (Q3, FY 2013).*** Since *hyp1* genes and CBS hydrogenase genes (*cooLXUH*) displayed similar expression profile, that is, both are induced by CO but not H_2, it guided later work to focus on co-expressing *hyp1* with *cooLXUH* in *Synechocystis* to confer hydrogenase activity.

1.3.4. CBS genome resequencing, identification of additional hydrogenase-maturation genes, and deletion of *hyp1* and/or *hyp2* operons.

CBS genome resequencing. Previous effort in sequencing CBS genome (4.1 M bp) using Illumina technology led to numerous short and fragmented DNA pieces and even after reassembly still resulted in 1,240 scaffolds with sequencing gaps that cannot be closed due to missing fragments (see 1.3.2). These short reads of fragmented genes make genome annotation cumbersome. To circumvent this challenge, we collaborated at no cost with Michigan State Univ. (PI Jin Chen) and PacBio, Inc. (PI Jonas Korlach), using their proprietary SMRT Sequencing technology, especially suited for DNA with high G+C content (CBS genome has a G+C content > 75%). The sequencing and assembly led to only three contigs with respective sizes of 4.7 million, 362K, and 241K base pairs (bp). After additional *de novo* genome assembly, we generated a 5.1 M bp bacterial chromosome that contains all the 4,910 genes serving as the CBS genetic blue print. The new sequence also reveals the presence of two satellite DNA elements plus uncovering missing fragments missing from the Illumina reads, which accounted for the increase in genome size. This work was published in the journal of PlosOne in 2014 (16). We therefore were equipped with a searchable database to probe all the putative hydrogenase genes (up to 158), their maturation machineries, H_2-sensing, and CO-sensing genes in CBS (more details in (16)).

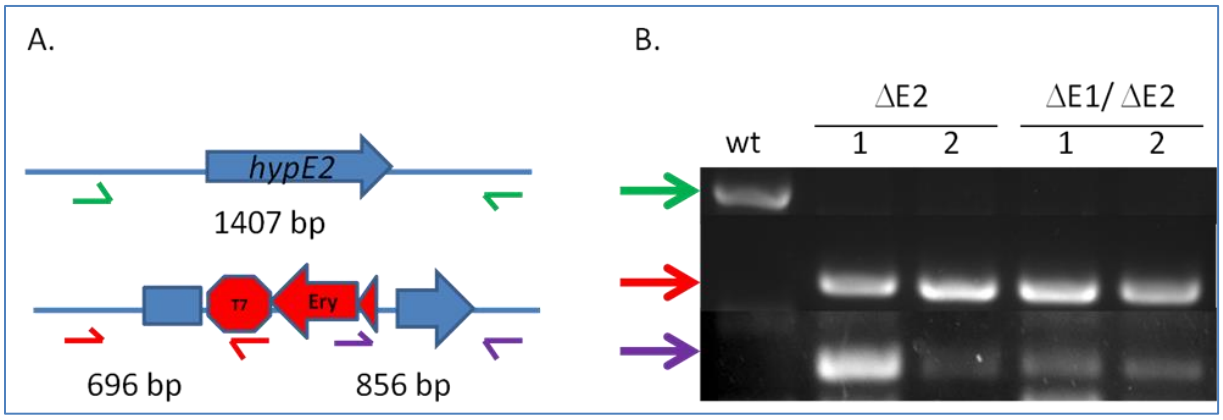


Figure 13. Generation of Δ*hypE2* and Δ*hypE1:*Δ*hypE2* single and double mutants. Panel A shows the PCR scheme used to test for the incorporation of the Erythromycin (Ery) resistance gene into wild type (WT) CBS *hypE2*. Panel B shows the confirmation of two Δ*hypE2* single mutant strains, and two Δ*hypE1*:Δ*hypE2* double mutant strains by the loss of the WT PCR product (green arrow), and the appearance of the 5' PCR product (red arrow) and the 3' PCR product (purple arrow).

Identification of additional hydrogenase-maturation genes. In searching for additional maturation genes in CBS, *E. coli* provides the working model of proteins and mechanisms involved in the assembly and maturation of NiFe-hydrogenases. In addition to HypA-F, SlyD, a protein that functions as a molecular chaperon and a Ni-binding protein, is also necessary for optimal hydrogenase maturation especially in *E. coli* (17, 18). Specifically, SlyD interacts with HypB to accelerate GTP hydrolysis leading to Ni delivery to the hydrogenase active site. After careful search of the CBS genome annotation (PacBio), we identified a single *slyD* homolog which displays 33% amino acid identity to its counterpart in *E. coli* as revealed by the Basic Local Alignment Search Tool – Protein (BLASTP) tool (Table 2). Studies in *E. coli* suggest that carbamoyl phosphate (CP) synthase (encoded by *carAB*) may also be necessary for hydrogenase maturation (19). This enzyme complex synthesizes CP, the precursor for the CN ligand which is an integral component of the hydrogenase active site. The donation of CN ligand from CP to the hydrogenase active site involves concerted actions of the maturation proteins HypFCDE. A search of the CBS genome annotation revealed *carA* and *carB* homologs with 64% and 70% identity, respectively, to the homologs in *E. coli* (Table 2). Both SlyD and CarAB may play important roles in the assembly and maturation of CBS hydrogenase and are likely necessary for activity in *Synechocystis*. *Synechocystis* genome does not contain a SlyD homolog based on BLASTP search. However, a CarA and CarB homolog with 51% and 60% identity, respectively, are present in *Synechocystis* (Table 2). Therefore, we may need to transfer the CBS *slyD* in addition to the *hyp1* genes in order to obtain a more active CBS hydrogenase in *Synechocystis*. ***This analysis is outlined in the below Table and completed the Milestone: Identify the CBS homologues of three genes additionally involved in hydrogenase maturation in E. coli, slyD and carAB, through a targeted blastp comparison of the putative CBS genes against those in E. coli, using ~35% amino acid sequence identity to verify homology (Q1, FY 2014).***

Table 2. Homology comparison of SlyD, CarA and CarB.

CBS	Identity in *E. coli*	Identity in *Synechocystis*	Function
SlyD	33%	None present	Ni insertion
CarA	64%	51%	CN ligand for active site assembly
CarB	70%	60%	CN ligand for active site assembly

Now that we have an improved map of CBS genome (PacBio), we conducted a detailed genome annotation to uncover additional hydrogenases and maturation genes, aimed to serve as the genetic blueprint to construct an active *Synechocystis* recombinant. We identified two regions of the genome containing two different NiFe-hydrogenases, a membrane-bound uptake hydrogenase (Hup; H_2 oxidation) and its associated maturation genes/regulatory factors (*hyp2*), as well as our CO-linked Coo hydrogenase (H_2 evolution) and its associated factors (*hyp1*; as previously described). Homology comparison in Table 3 reveals that CBS *hyp1* is quite different from the CBS *hyp2* based on low level of identity (34 – 58% identity). Data from Table 4 show the homology comparison of the CBS *hyp1* and CBS *hyp2* with the *hyp* genes known to be involved in the maturation of a well-studied uptake hydrogenase in *Ralstonia eutropha* (Re) (20, 21). The higher level of identity between Re *hyp* genes with CBS *hyp2* (60 to near 80%), but not with *hyp1,* clearly supports that CBS *hyp2* genes are involved in the maturation of the uptake hydrogenase in CBS, while the CBS *hyp1* genes are involved in the maturation of the O_2-tolerant evolving hydrogenase. Combining these comparisons with operon gene structure and transcriptional expression data performed previously, we concluded that the *hyp1* genes in the *coo* operon are the relevant *hyp* genes required for heterologous expression and activity of the O_2-tolerant Coo hydrogenase in *Synechocystis.* ***The outcomes completed the Milestone: 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 (Q2, FY 2014).

Deletion of *hyp1* and/or *hyp2* operons and determination of hydrogenase activities. To provide more definitive evidence for roles of *hyp1* and *hyp2* operons, we built constructs for deletion of *hyp1FCDE* and *hyp2ABFCDE* to be integrated alone or in combination into WT CBS for analysis of hydrogenase activity in these mutants. Previous use of a markerless knockout design failed to yield mutants, due to possible issues with integration to remove such a large knockout region (4.81 kb *hyp2* and 4.87 kb *hyp1*). To increase the probability of integration and selection we redesigned the deletion constructs (Figure 14A and B) to include antibiotic resistance cassettes (to be integrated in an orientation with transcriptional terminators to avoid polar effects). The same design was also used to generate *hyp2* deletion mutant in CBS. Transformation into WT CBS resulted in the successful generation of *hyp1FCDEΔ* and *hyp2ABFCDEΔ* single deletion strains, and *hyp1/hyp2* double deletion (*hyp1FCDEΔ/hyp2ABFCDEΔ)* mutants as verified by PCR (Figure 14C).

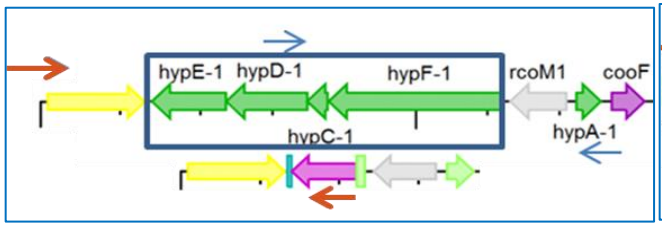
Table 3. Homology comparison (% identity) of CBS Hyp1 with Hyp2 proteins.

	Hyp1 vs. Hyp2 (%)
HypA	34.5
HypB	53.6
HypC	39.2
HypD	50.9
HypE	53.8
HypF	37.2

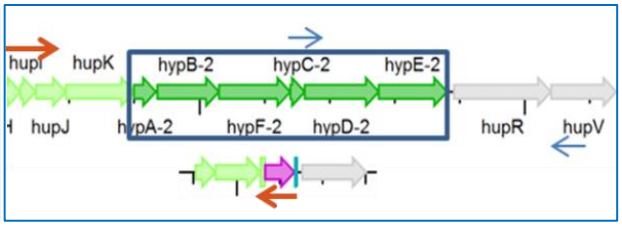
Table 4. Homology comparison (% identity) of the Hyp proteins from *Ralstonia eutropha* with the respective Hyp1 and Hyp2 proteins from CBS.

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

***(A) hyp1FCDE* (4.87 kb) Deletion Scheme**



***(B) Hyp2AB FCDE* (4.81 kb) Deletion Scheme**



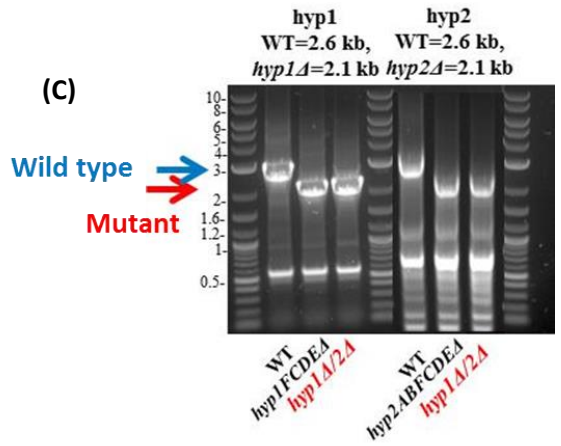


Figure 14. Deletion constructs for CBS *hyp1* using kanamycin marker (A) and *hyp2* using ampicillin marker (B); (C) PCR products using forward and reverse primers denoted in (A) and (B) for verification of *hyp1*, *hyp2* single and double deletion mutants. The primers denoted by blue arrows yield WT PCR products while the red arrows yield mutant PCR products.

We then determined effects of *hyp* deletions in hydrogenase activities in CBS *hyp1FCDEΔ, hyp2ABFCDEΔ,* and *hyp1FCDEΔ/hyp2ABFCDEΔ* double deletion strains. We measured *in vivo* CO-induced net H_2 production (catalyzed by the O_2-tolerant hydrogenase, target enzyme of this project) and net H_2/CO uptake compared to WT. As we showed previously, when cells were grown anaerobically in growth media containing malate as the carbon source, the *hyp1FCDE* deletion had no effect on H_2 uptake, the latter catalyzed by the H_2-uptake hydrogenase. In cultures induced with CO, the *hyp1FCDE* deletion resulted in a marked 4 h delay in H_2 production compared to WT over an 8 hour period, with a loss of near **55%** of the O_2-tolerant *coo* hydrogenase activity (Table 5). Since CO is required to activate expression of the Coo hydrogenase and Hyp1 maturation factors (Figure 11), this delay in H_2 production suggests that Hyp1 maturation factors are necessary for "timely" maturation and resultant activity of the Coo O_2-tolerant hydrogenase under CO. Additionally, it seems that the Hyp2 maturation factors can compensate for the loss of Hyp1FCDE, albeit with a delayed H_2 production pattern since Hyp2 proteins are likely constitutively expressed at a low level, with increased expression induced by H_2, a phenomenon reported in the highly similar *R. eutropha* MBH operon (22). Interestingly, the *hyp2ABFCDEΔ* strain exhibited both increased rate of and peak H_2 production compared to WT, which also correlated with increased CO uptake. In addition, there was no significant H_2 uptake when H_2 was added to the cultures. Therefore, the loss of Hyp2 maturation proteins does not have a negative impact on the O_2-tolerant Coo hydrogenase activity, although there is an effect on the uptake hydrogenase activity that results in the increased net H_2 production in the Hyp2-deficient strain. When both *hyp* operons are disrupted, all the H_2 metabolic activities were abolished. The cells show no CO uptake, H_2 production, or H_2 uptake, verifying that these are the only functional *hyp* operons in CBS. Data from the double deletion strain further validate our research plan that only *hyp1* genes should be transformed into *Synechocystis* to confer hydrogenase activity. ***Generation of the single and double mutants and the loss of near 55% hydrogenase activity upon deletion of hyp1 (but not hyp2) completed the Milestone: Through deleting hyp1FCDE genes in the WT background in the presence of hyp2 genes, determine if hyp1FCDEΔ decreases the total hydrogenase activity by 50% or more based on in vivo assay of H_2 production in whole cells, as is required for optimal hydrogenase activity, and obtain a hyp2ABFCDE deletion mutant to measure the impact on hydrogenase activity; the outcome will determine if hyp2 will be co-transformed with hyp1 into Synechocystis to assemble a more active CBS hydrogenase for increased H_2 production (Q4, FY 2014 and Q1, FY 2015).*** **The outcomes also satisfactorily completed the research for Task 1.**

Table 5. Relative *in vivo* H_2 production/uptake and CO uptake, in wild type versus *hyp* mutants in CBS.

Strains	H_2 Production	CO Uptake	H_2 Uptake
Wild Type	100%	100%	100%
hyp1FCDEΔ	45.7%	67.7%	151%
hypABFCDE2Δ	151%	133.5%	0%
hyp1Δ/hyp2Δ	0%	0%	0%

Task 2. Expression of the CBS Hydrogenase in *Synechocystis*.
The overarching goal of Task 2 is to heterologously express the CBS hydrogenase and maturation genes in *Synechocystis* for sustained photolytic H_2 production. The milestones covered during this project period and their completion date are outline in Table 6.

Table 6. Task 2 Milestone Table.

	Milestones	Completion Date
FY 2010	None	NA
FY 2011	Confirm expression of the CBS hydrogenase maturation proteins in recombinant *Synechocystis* toward producing an active hydrogenase with increased oxygen tolerance.	9/11
FY 2012	Generate a *Synechocystis* recombinant evolving H_2 via the CBS hydrogenase.	9/12
FY 2013	Improve expression of CBS hydrogenase genes by two fold via manipulating promoter strength	2/13
FY 2013	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
FY 2014	Compare levels of the hydrogenase and maturation proteins expressed in the native CBS (CO-induced) vs. those in the two *Synechocystis* recombinants to guide the tuning and balance of protein expression levels in the *Synechocystis* recombinants	6/14
FY 2015	Determine balanced protein expression using Western blotting by comparison of CooLXUH and Hyp1FABCDE in CBS (CO-induced) and new *Synechocystis* recombinant containing P_{trc}-*cooLXUH*-T7tt and P_{trc}-	3/15

	Milestones	Completion Date
	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.	
FY 2015	Obtain CBS hydrogenase activity 2-fold over the baseline rate of 10 nmol H_2/mL culture in whole cells of a *Synechocystis* recombinant, assayed in vitro with reduced methyl viologen	6/15 (Go-No Go) – not completed; project close-out.
FY 2016	Determine hydrogenase activity in recombinant Synechocystis expressing CBS maturation and hydrogenase (*cooLXUH*) genes including *cooM*, a membrane subunit of the hydrogenase. Co-expression of CooM subunit may be needed to assemble an active hydrogenase in *Synechocystis* and will explain the lack of activity in earlier effort when only the *cooLXUH* genes and maturation genes were attempted	12/15 – not completed; project close-out.

2.1. Transformation of the CBS hydrogenase genes.

A pre-requisite for transforming CBS hydrogenase genes into *Synechocystis* is to develop genetic transformation for the latter host. We obtained a pPETE-E plasmid designed for homologous recombination in *Synechocystis* from Rob Burnap in Oklahoma State University. We further improved the plasmid stability by inserting a terminator downstream of the cargo genes (foreign genes of interest, such as CBS genes) for insertion into *Synechocystis*. Moreover, we obtained a hydrogenase-free *Synechocystis* host (*hox*-) from T. Ogawa (Personal Comm.) so that any H_2 production in this host will be entirely attributed from the foreign hydrogenase expression. The plasmid construct and homologous recombination scheme is shown in Figure 15. We later replaced the Strep-affinity tag with a His-affinity tag since the His-tag itself is less costly and works equally well as the Strep-tag. We performed PCR to verify that full-length insert are incorporated into the specific site of the host genome. We verified both upstream (Figure 16, lane 4) and downstream (on a different gel – not shown here) integration, as well as full-length insert (Figure 16, lane 5). The major PCR bands appeared at expected molecular masses, demonstrating His6-*cooLXUH* homologous recombination into the *Synechocystis hox*$^-$ genome. The CooH and CooU proteins were clearly detected in the transformed Hox$^-$ strain using protein immunoblot (Figure 17). CooL and X were likely expressed, since they should be transcribed before CooU and H. One rationale is that CooL and CooX are present in low protein concentrations in the cells. In an effort to increase the amounts of CooL, X, U, and H proteins in the cell, these genes were subsequently codon-optimized for optimal protein expression in *Synechocystis*.

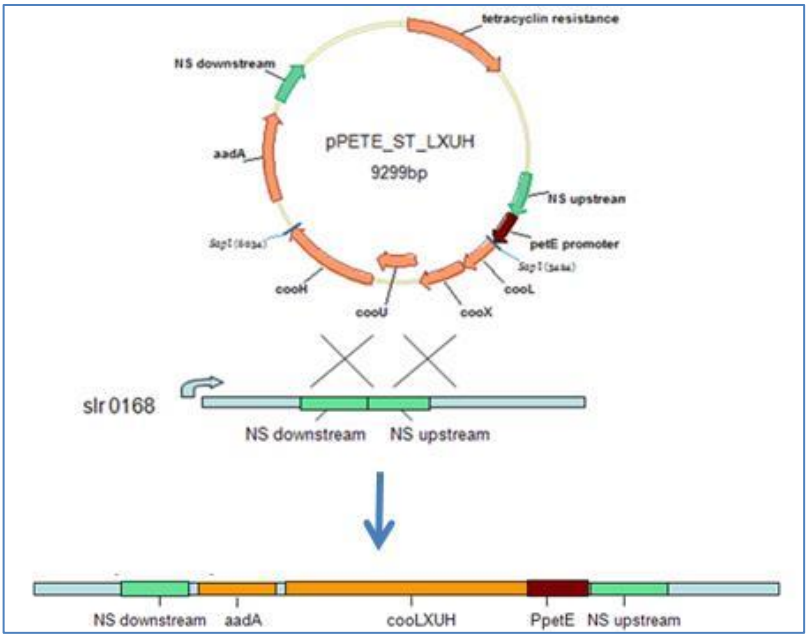


Figure 15. Schematic of integration of Strep-tag *cooLXUH* (ST_LXUH, tagged on CooL N-terminus) into the *Synechocystis* genome at the gene slr0168, a neutral site (NS). Homologous recombination mediated by the NS upstream and NS downstream sequences leads to insertion of foreign DNA. *aadA*: encoding for spectinomycin resistance.

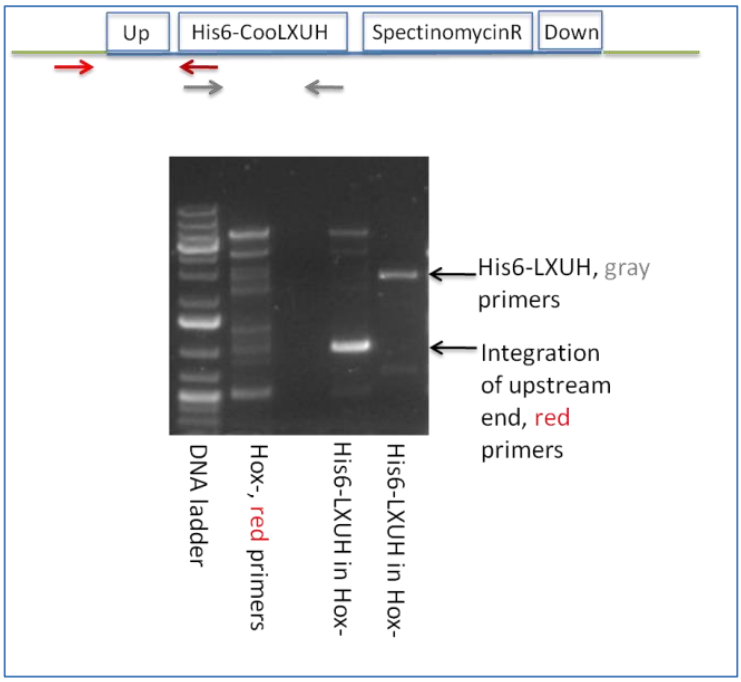


Figure 16. PCR verification of site-specific integration of the His-tagged *cooLXUH* fragment into the genome of *Synechocystis* 6803 Hox$^-$ strain.

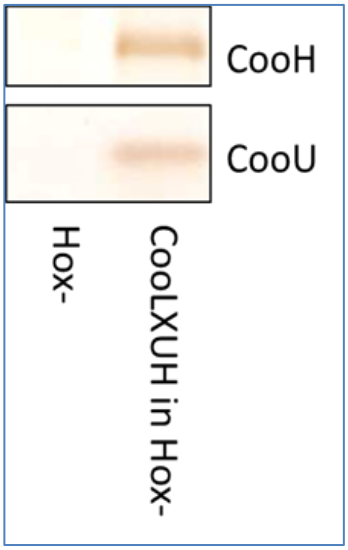


Figure 17. Immunoblot of heterologously expressed CBS hydrogenase proteins with His6-*cooL* in a Hox$^-$ *Synechocystis* sp.PCC 6803 host.

2.2. Transformation of the CBS Maturation genes and hydrogenase activity measurements.

2.2.1. Synechocystis recombinant construction. We believe it is also necessary to transfer the CBS *hyp1ABCDEF* genes to *Synechocystis,* to ensure proper assembly and maturation of the CBS hydrogenase. We codon-optimized the CBS hydrogenase maturation genes *hyp1ABCDE* and *hypF1*. The codon-optimized genes for *hypABCDE* have been cloned into the pPsbA2 plasmid (kindly provided to us by Arizona State University), allowing for their integration into *psbA2* site (a neutral site) of the *Synechocystis* genome. *hypABCDE* genes were then integrated into the genome of a recombinant *Synechocystis* already expressing the CBS *cooLXUH* hydrogenase genes. PCR was used to verify correct insertion of these genes (data not shown), and expression of HypE was verified by immunoblot (Figure 18A). Since the *hypE* gene is the last gene to be expressed in the operon, detection of the HypE protein suggests concomitant expression of HypA, B, C, and D. We then transformed *hypF* gene into the above strain (insert into the *Synechocystis* native *hypF* site) with expression of the HypF protein confirmed in Figure 18B, thus obtaining a *Synechocystis* recombinant harboring all ten CBS genes, *cooLXUH* and *hypABCDEF* (Figure 18C). ***These results completed Milestone: Confirm expression of the CBS hydrogenase maturation proteins in recombinant Synechocystis toward producing an active hydrogenase with increased oxygen-tolerance (Q4, FY 2011).***

In a similar approach, we also generated a second *Synechocystis* recombinant harboring *cooLXUH* with the CBS maturation genes *hypFCDE* integrated into the *Synechocystis psbA2* site and CBS *hypAB* into the host native *hypF* site (Figure 19B). The two recombinant strains of *Synechocystis* expressing 10 CBS hydrogenase genes displayed low levels of *in vitro* hydrogenase activity determined with the redox mediator methyl viologen (MV) reduced by sodium dithionite (Figure 19 C, D). Yet the activity was difficult to reproduce, likely attributed to low expression level of the heterologously expressed hydrogenase comparing to those in the native CBS host. This may lead to poor assembly/maturation and hence no or low activity of the hydrogenase complex. This finding guided later work using stronger promoter to boost CBS gene expression in the *Synechocystis* host. ***Nevertheless the detection of low levels of hydrogenase activity in the two Synechocystis recombinants completed the Milestone: Generate a Synechocystis recombinant evolving H_2 via the CBS hydrogenase (Q4, FY 2012).***

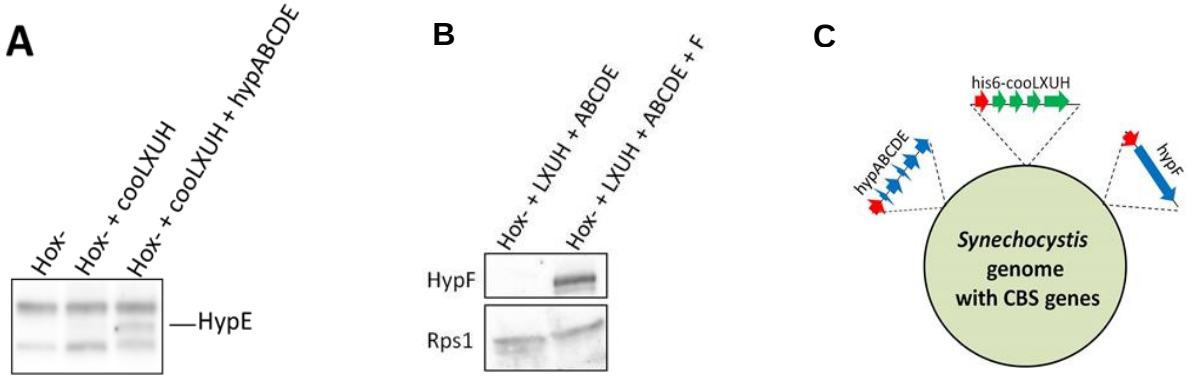


Figure 18: (A) Immunoblot of soluble *Synechocystis* protein extracts from cells without or with the CBS *hypABCDE* genes. 10ug of soluble protein was loaded in each lane of the gel, and antibody that recognizes HypE was used for immunoblot analysis; (B) HypF is expressed in the recombinant *Synechocystis* strain harboring *cooLXUH*, *hypABCDE*, and *hypF*, as revealed by immunoblot; (C) Schematic of CBS hydrogenase gene integrated into the genome of *Synechocystis*. Red arrows denote promoters, green arrows are the CBS hydrogenase structural genes *cooLXUH*, and blue arrows represent the putative hydrogenase maturation genes, *hypABCDEF (*all *hyp1)*.

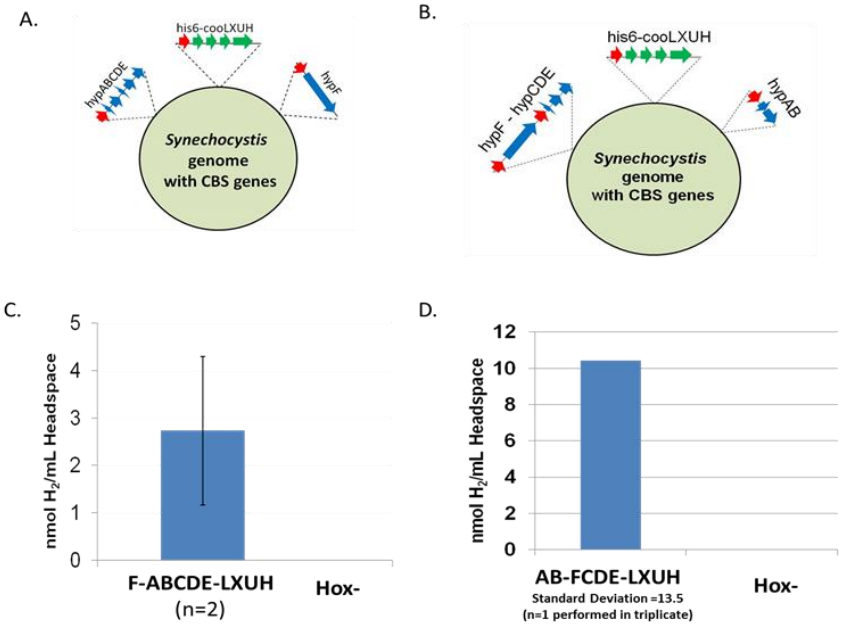


Figure 19. Hydrogen production in two different *Synechocystis* recombinants. Diagrams of the two *Synechocystis* recombinants harboring CBS hydrogenase genes: (A) F-ABCDE-LXUH and (B) AB-FCDE-LXUH. Promoters are depicted with red arrows, maturation genes with blue arrows and structural hydrogenase genes with green arrows. *In vitro* hydrogenase assays were performed on both strains and with H_2 in the gas phase detected by GC. Panel C depicts the averaged results of two experiments and D depicts the averaged result of one experiment performed in triplicate. The recipient *Synechocystis* host (Hox-) lacks background H_2 activity.

2.2.2. Stronger promoters to boost gene expression. To improve gene expression, we generated a new construct designed to express the CBS hydrogenase structural genes *cooLXUH* driven by the strong *psbA* promoter and transformed into *Synechocystis.* CooU and CooH protein levels via protein western blots were compared to a previous strain with *cooLXUH* driven by the weaker *petE* promoter. We found that indeed the *psbA* promoter increased *cooU* expression by 16 fold and *cooH* expression by 44 fold, compared to that of *petE* promoter (Figure 20). ***These findings completed FY 2013 Milestone: Improve expression of CBS hydrogenase genes by two fold via manipulating promoter strength (Q2, FY 2013).*** The findings guide the use of *psbA* promoter to drive the expression of the CBS maturation genes.

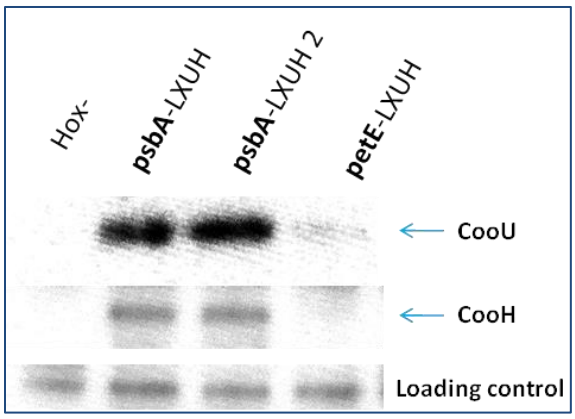


Figure 20. CBS hydrogenase protein expression profiles in *Synechocystis* hox- driven by either *petE* or *psbA* promoter. Shown is a protein western blot probed with antibodies for CBS CooU and CBS CooH.

The above findings prompted us to systematically re-engineer the *Synechocystis* recombinant using the strong *psbA* promoter to drive the expression of *cooLXUH* (Figure 20) and *psbA2* promoter to drive the expression of *hypABCDE*. The resultant recombinant is *psbA-LXUH/psbA2-ABCDE* (data not shown). We

then incorporated the CBS maturation gene *hypF* in the above recombinant driven by the strong *psbA* promoter. We first produced a plasmid where the weak promoter *petE* was replaced with *psbA* promoter, with its replacement confirmed by restriction digest analysis and sequencing (data not shown). We then transformed this plasmid into *Synechocystis* (*psbA-LXUH/psbA2-ABCDE)* and confirmed the integration of *psbA-hypF* into the *Synechocystis* genome via colony PCR in 8 out of 18 colonies tested (Figure 21A). Expression of HypF protein was determined via protein immunoblots. We detected that HypF expression was enhanced from 1.7- to 8.9-fold in these strains (*psbA-LXUH/psbA2-ABCDE/psbA-hypF*) when driven by the strong *psbA* promoter, when compared to expression using the weak *petE* promoter (Figure 21B). ***The outcomes completed FY 2013 Milestone "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 (9/2013).***

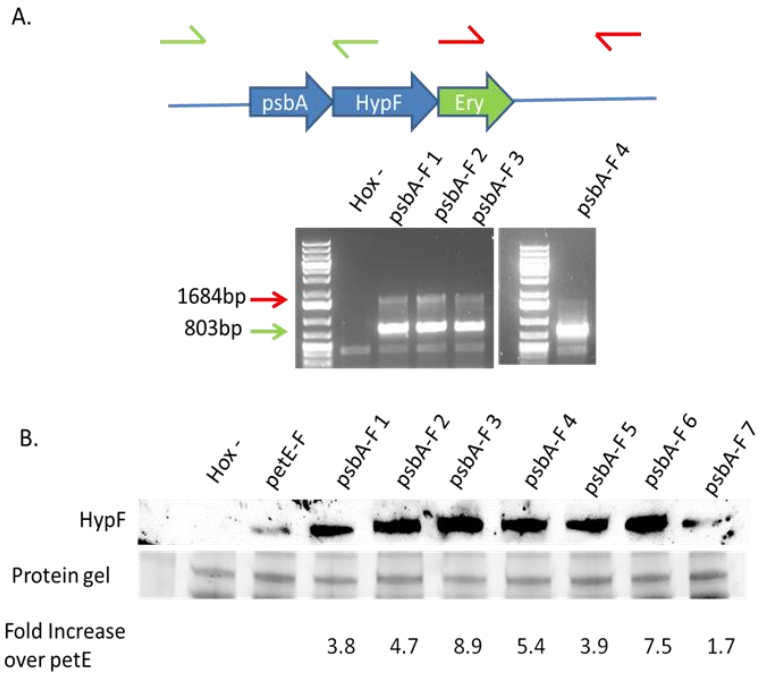


Figure 21. Integration and Enhanced Expression of HypF in *psbA-LXUH/psbA2-ABCDE/psbA-hypF* strains. Panel A shows colony PCR confirming the integration of *psbA-hypF* into the *Synechocystis* genome. Primers upstream, downstream and within the integrated construct (red and green arrows denoting forward and reverse primers) should produce two products of 803bp and 1648bp in size. PCR of colonies confirmed the correct integration of the construct into the *Synechocystis* genome. Panel B shows a western immunoblot against the CBS HypF protein. The new recombinants containing the *psbA-hypF* construct displayed a higher level of expression compared to that of the control with *petE-hypF*. Fold increase was calculated by normalizing to a constant band in the protein gel and comparing levels of *psbA-hypF* to the *petE-hypF* recombinant.

2.2.3. Balanced protein expression. Previous efforts have led to 16- to 44-fold higher levels of CBS hydrogenase (CooH and CooU) expression in *Synechocystis* by switching from the weak *petE* promoter to the stronger *psbA2* and/or pea plant *psbA* promoters, yet no hydrogenase activity was detected. To troubleshoot, expression levels need to be carefully quantified for further improvements. Therefore, we performed a quantitative protein immunoblots of CooLXUH and HypEF1 in CO-induced CBS lysates in the

following *Synechocystis* recombinant strains: (1) P_{petE}-6XHis-CooLXUH/P_{psbA2}-Hyp1ABCDE/ P_{psbA}-HypF1, and (2) P_{psbA}-6XHis-CooLXUH, P_{psbA2}-Hyp1ABCDE/P_{psbA}-HypF1 (Figure 22). Standard GroEL served as the loading control for normalization. We only were able to analyze CooLXUH and HypE1 and HypF1 since we do not have antibodies to the other Hyp1 proteins. It was clear from the Western blotting results that only CooU, CooH and HypE1 were expressed at detectable levels in the *Synechocystis* strains (red asterisks of the two recombinants) and levels were much different from that seen in CBS, the latter loaded with two-third less protein.

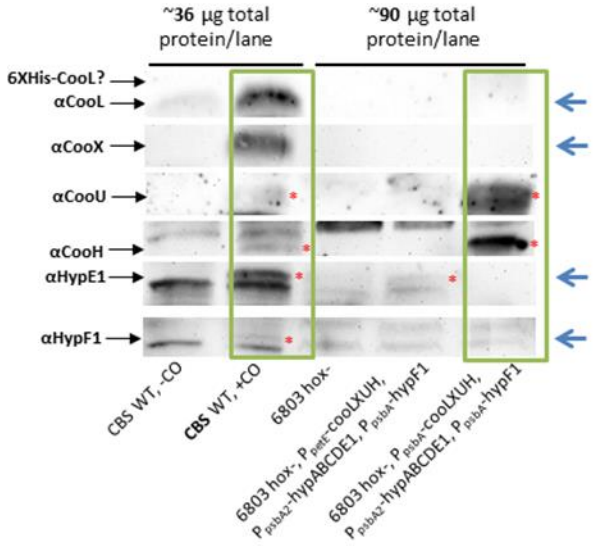


Figure 22. Comparative protein western blots of CooLXUH, HypE1, and HypF1 proteins in CBS (CO-induced) vs. those expressed in the two *Synechocystis* recombinants. Red asterisks and blue arrows mark expressed and un-expressed proteins, respectively, in the recombinants.

Analysis of expression constructs revealed a number of issues that could lead to the imbalanced protein expression. Therefore, we began a redesign of expression constructs to incorporate a demonstrated stronger promoter from *E. coli*, *trc* (23) (NREL-JCVI joint publication) according to Figure 23. The *trc* promoter has shown up to 80 times higher expression levels in *Synechocystis* when compared to native promoters (24). In addition, we also integrated a consensus ribosome binding site (RBS) from *Synechococcus* 7002 (*cpcB* gene) shown to have high levels of expression in *Synechocystis* (25) in front of each gene to be expressed to maximize translational efficiencies. Because we may need to break up the expression constructs into smaller number of genes expressed per promoter, we have also inserted unique restriction sites between each gene to allow for the insertion of addition promoters if necessary. The new design is modular, affording flexibility in tuning and balancing gene expression individually, on a need basis. Initial tests of improved relative expression/activity can be conducted in *E. coli,* and once verified, can be transferred to *Synechocystis* for integration and testing.

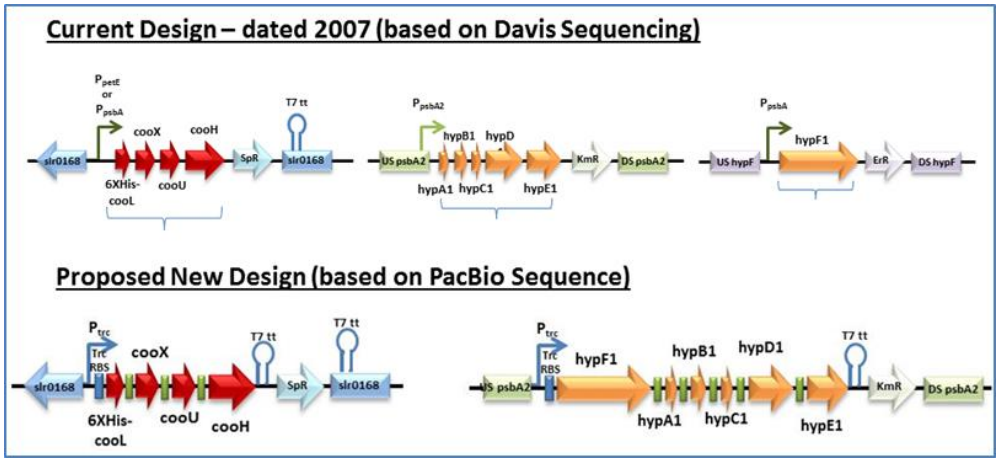


Figure 23. Original and new design to express the CBS hydrogenase and maturation genes in *Synechocystis*. RBS: ribosome binding site.

These expression cassettes for P_{trc}-6XHisCooLXUH and P_{trc}-Hyp1FABCDE were synthesized by DNA2.0 and integrated into *Synechocystis*. An altered P_{trc}-CooLXUH without the 6XHis tag was also constructed and integrated into *Synechocystis*, as well as a combined integrant with P_{trc}-CooLXUH/P_{trc}-Hyp1FABCDE. These strains were grown along with the previous P_{petE} and P_{psbA} integrants and CBS +/-CO and tested for MV-linked hydrogenase activity and Western blotting. When comparing CBS protein levels in recombinant *Synechocystis* to the CBS (+CO) sample, we were able to detect overall more balanced expression of the proteins, with an average that was at least 75% of levels observed in CBS (Figure 24)**.** ***This outcome completed the Milestone: Determine balanced protein expression using Western blotting by comparison of CooLXUH and Hyp1FABCDE in CBS (CO-induced) and new Synechocystis recombinant containing P_{trc}-cooLXUH-T7tt and P_{trc}-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 (Q2, FY 2015).***

Despite much improved expression of all detectable CBS proteins in the strain containing the new P_{trc} constructs over the previous P_{petE} and P_{psbA} constructs (Figure 23), hydrogenase activity was still not detectable in this new strain. One culprit could be that the first gene in each operon, *cooL* and *hypF1*, were expressed at much lower levels than those in native CBS. To overcome this challenge, we replaced the P_{trc} promoter and RBS with an altered P_{psbA} promoter (P_{psbA*}) and a verified strong version 4 RBS (V4RBS), the latter was shown to have improved expression in a NREL BETO Project (Personal Comm.). The two modified constructs were transformed and integrants obtained driving the expression of both *cooLXUH* and *hyp1FCDEAB* genes in *Synechocystis*. Protein immunoblots shown that this new recombinant displayed the highest, most balanced expression of the 10 heterologously expressed Coo and Hyp1 proteins (Figure 25). Yet the recombinant failed to yield hydrogenase activity albeit with dramatic improvements in protein expression.

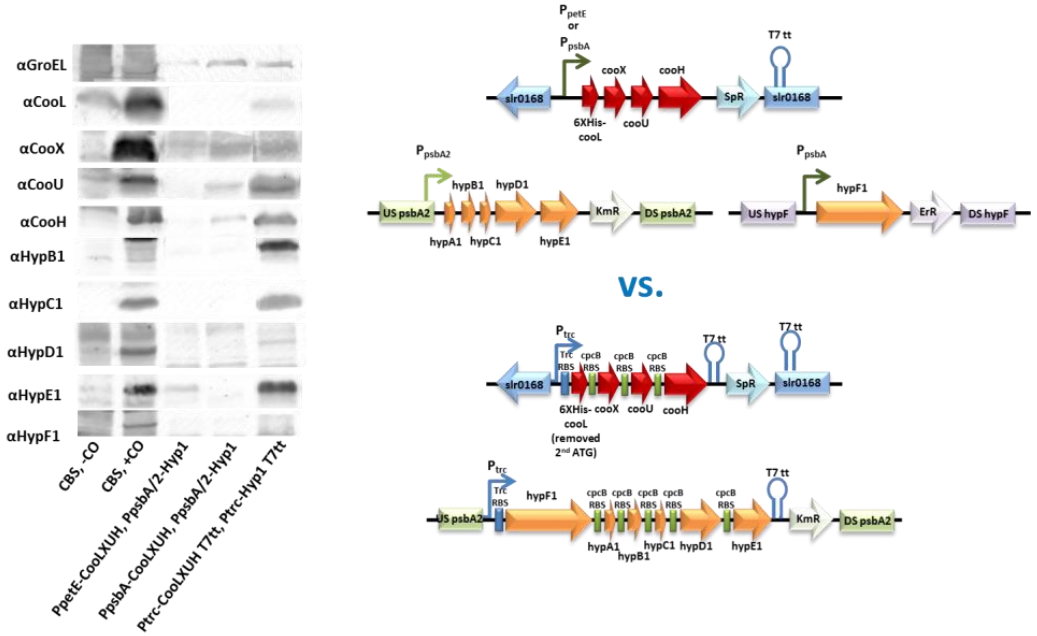


Figure 24. *Synechocystis* sp. PCC6803 CBS coo and hyp1 expression, old vs. new constructs and vs. CBS.

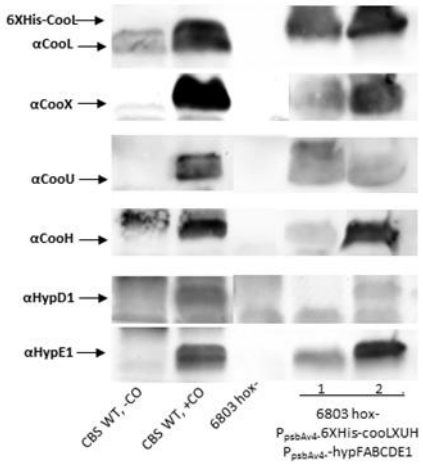


Figure 25 Western blot showing expression levels of new PpsbA* v4 RBS 6XHis-CooLXUH, Hyp1FABCDE *Synechocystis* expression strains versus CBS.

2.2.4. Co-expression of cooM and cooK in E. coli and in Synechocystis. A culprit to the lack of activity could be the absence of CooM and CooK, two membrane-associated proteins that likely serve as a scaffold for correct formation of a hexameric hydrogenase enzyme (10, 11) (Figure 2). As such we generated CBS mutants lacking either *cooM* or *cooK* genes, or both. Deletion of *cooK* had a minimal effect on *in vitro* hydrogenase activity while deletion of *cooM* had a more pronounced defect. Yet deletion of either *cooK,* or *cooM,* or both produced much less H_2*, in vivo*, following CO addition, and with a delay in H_2 production compared to WT (data not shown). These observations confirm that both CooM and CooK play a role in optimal hydrogenase activity in CBS and should be expressed in *Synechocystis* to afford activity. We also expressed them in *E. coli* as a control for troubleshooting, if needed.

We designed cloning strategies to add *cooM* or *cooK,* or both to the *E. coli* P_{trc} expression vectors as well as $P_{psbA^* v4RBS}$-CooM(K) into a vector targeting an additional neutral site utilized at NREL (Wang and Yu, unpublished). We overcame the cloning difficulty in *E. coli* due to large size of CooM (130 kD) and both CooM and CooK are membrane proteins, and obtained a vector of 6XHis-*cooLXUHMK* in *E. coli* (using *trc* promoter). This vector was transformed into *E. coli* cells along with a pACYC177 vector containing *hyp1FABCDE*. Expression of 6XHis-CooLXUHMK +/- Hyp1FABCDE (0.4 mM IPTG, 24h, RT) via Western blot/Pulldown analysis shows that all proteins are expressed and at least a portion of all Coo and Hyp1 proteins are soluble including CooM and CooK (Figure 26, S lanes). Moreover, all Coo and Hyp1 proteins were detectable in affinity bead pull-down fraction with the exception of CooK, suggesting that Hyp proteins are additionally associating with the active site in CooH, likely in the process of assembling its NiFe-active site. Despite soluble expression of all of the CBS proteins identified as necessary for activity in CBS, there was still no detectable MV-linked *in vitro* hydrogenase activity in any fraction. To aid in activity, we increased the volume of the expression culture by 5-fold, employed anaerobic induction (26) and with added $NiCl_2$ (providing Ni for the NiFe-hydrogenase), as well as concentrated the protein with affinity beads. Yet all these efforts failed to yield hydrogenase activity in recombinant *E. coli*.

Since we were unable to obtain vectors (in *E. coli*) for the *Synechocystis* integration constructs, a shuttle vector was designed and assembled in yeast, in which *cooM/K* will not be expressed. Our rationale is that expression of *cooM*/*K* may be toxic to the cells due to overburdening of its genetic machineries from expressing foreign genes of large size. Assembly was verified by colony PCR and the yeast containing verified vectors were used to amplify via PCR a 7.8-kb fragment successfully from the vector containing flanking homology regions for recombination, a chloramphenicol resistance cassette, and *cooMK*. This linear fragment was transformed into *Synechocystis* already containing both 6XH*is-cooLXUH*

and *hyp1FABCDE,* yet no transformant was obtained after multiple attempts. This unsuccessful pursuit led to the close-out of the project in Q2 of FY 2016. Nevertheless we have developed new genetic tools, techniques, and knowledge in hydrogenase cloning, expression, and activity of an O_2-tolerant hydrogenase.

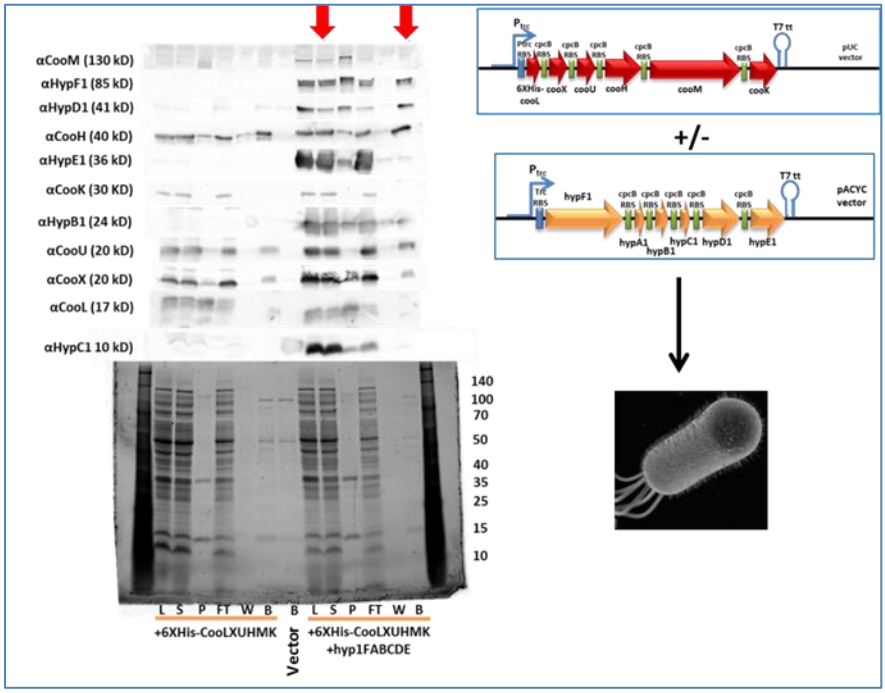


Figure 26. Expression and Pulldown of CBS hydrogenase genes in *E. coli.* Whole cell lysate (L), Soluble fraction (S), Insoluble fraction (P), flow through following incubation of soluble fraction with beads (FT), bead washes (W), beads (B). The center lane (Vector) represents residual *E. coli* proteins bound to beads from a strain carrying vectors only (no CBS genes).

Conclusions

In our effort to assist DOE FCTO in overcoming the technical barrier for photolytic H_2 production, we identified an O_2-tolerant hydrogenase and its maturation machinery in CBS. We sequenced the CBS genome, a novel microbe, and identified additional hydrogenase and its associated maturation machinery. We constructed *Synechocystis* recombinants harboring all ten CBS 10 genes and even after additional optimization to ensure balanced protein expression, we failed to detect CBS hydrogenase activity in *Synechocystis*, which ends the project. Nevertheless we have developed genetic tools for both CBS and *Synechocystis*, gained insight into its H_2-production network and the underlying regulations. These tools and techniques will be applied in future projects to help address the Nation's need in renewable energy based on H_2. One future opportunity could be using CBS as the water-gas shift catalyst to convert the CO component in gasified biomass (i.e. synthesis gas, containing mainly CO and H_2) into additional H_2, potentially of interest to FCTO. We already demonstrated that deletion of *hyp2* leads to increased net H_2 production due to the elimination of the H_2-uptake reaction. Another opportunity could be to gain in-depth understanding as to how CBS redirect its carbon and electron flow upon deletions of *hyp1*, or *hyp2*, or both. This topic could be of interest to DOE Office of Science

programs. The outcomes could serve as the blueprint to guide genetic engineering strategies to further improve H_2 production.

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