

Direct Solar Biohydrogen: Part II Annual Report – June 2007 to May 2008

Investigators

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Abstract

The overall objective of this research project is to develop a photosynthetic organism for the efficient conversion of sunlight and water into hydrogen fuel. The splitting of water to generate the required electrons and protons, however, also generates molecular oxygen which then poisons the intended catalyst for hydrogen production, an Fe-Fe hydrogenase enzyme. Our main focus, at this time, is therefore the evolution of an oxygen tolerant hydrogenase. In addition, we are studying the requirements for both *in vivo* and *in vitro* expression of active hydrogenases. This latter information will be important in moving quickly to engineer the hydrogen producing organism after the required enzyme is developed.

Using cell-free synthesis to produce the mutated hydrogenases allows them to be evaluated in a rapid and efficient manner. However, when extensive mutagenesis of a hydrogenase with a relatively simple structure only produced enzymes with more activity but with no improvement in oxygen tolerance, we turned to a hydrogenase with a more complex structure. This, in turn, necessitated significant improvements in the methods used for expression and evaluation of the mutated enzymes. These improvements are now being implemented. For example, reoptimization of the expression gene and the cell-free reagents provided a significant improvement in the cell-free hydrogenase titers. These, in turn, overshadowed a background activity observed in the colorimetric activity assays. We have also discovered two previously unrecognized factors that are required for hydrogenase activation. In addition, significant progress has been made in the development of an ultra-high throughput hydrogenase screening protocol using fluorescently activated cell sorter (FACS) technology. This technology will enable the screening of millions of candidates in the search for oxygen tolerant hydrogenases. Finally, we are making good progress in improving the *in vivo* expression and maturation of these complicated enzymes in *E.coli*. This system uses maturation helper proteins from *Shewanella oneidensis* to help activate hydrogenases from *Chlamydomonas reinhardtii* and from *Clostridium pasteurianum*. This feat suggests that this complex expression and maturation system can indeed be moved to different organisms, which bodes well for the future feasibility of engineering the photosynthetic hydrogen producer.

Introduction

Our overall objective, shown schematically in Figure 1, is to engineer a new microorganism that captures sunlight and channels the energy directly to the formation of hydrogen. As the solar energy is captured by the photolysis center (photosystems I and II), water is split into protons, electrons, and molecular oxygen. The electrons are transferred directly to the electron carrying protein, ferredoxin, which then transfers them to the hydrogenase enzyme that produces the hydrogen. Also illustrated in Figure 1 is the current major challenge for the project. The side product, oxygen, inactivates all known hydrogenases. Thus, the first and most significant challenge for this project is to evolve a natural hydrogenase enzyme to become oxygen tolerant. As will be described, this search has proven to be more difficult than expected. It has been necessary to switch from the evolution of a smaller, simpler hydrogenase to a larger and more complex enzyme with better potential for protecting the oxygen sensitive active site. We are also developing new screening technology using emulsions in which each water droplet is an individual bioreactor to provide dramatically higher throughput for the evaluation of greater numbers of altered hydrogenases. In parallel, we are learning more about the requirements for hydrogenase activation. These insights allow us to improve the cell-free expression system for more effective hydrogenase evolution. They also are guiding the optimization of *in vivo* hydrogenase expression and maturation to provide information that will help us design the photosynthetic hydrogen producer.

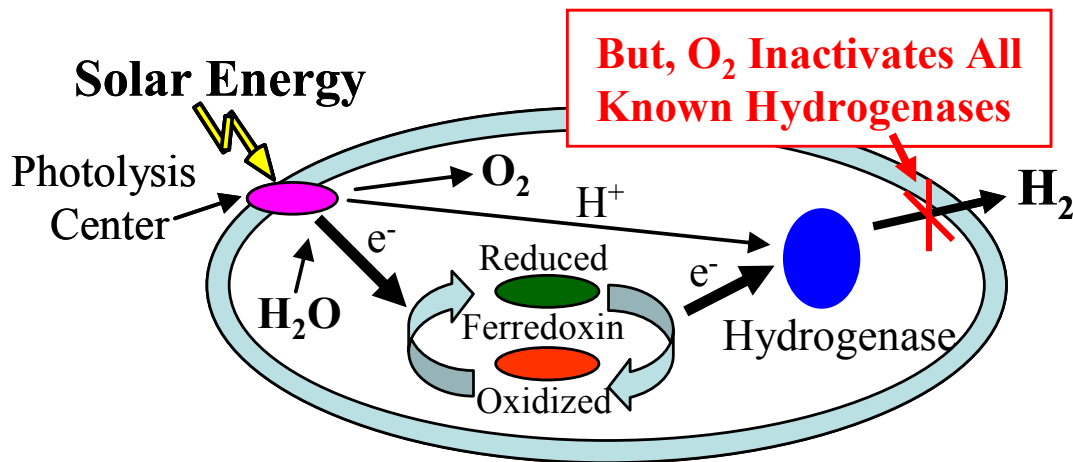


Figure 1: Diagram of proposed *Synechocystis* bacterium engineered to support a short, efficient pathway for the conversion of sunlight and water into molecular hydrogen. As shown, the dominant barrier to this technology is the availability of an oxygen-tolerant hydrogenase enzyme.

Background

Strategy for Evolving Hydrogenases. Hydrogenases are very complicated enzymes typically classified as either [NiFe]- or [FeFe]-hydrogenases. We have focused on the [FeFe]-hydrogenases as these enzymes provide much faster catalytic rates for hydrogen production. The 3-D structure has been determined for the [FeFe] hydrogenase, Cpl, from *Clostridium pasteurianum* (see Figure 2).

The CpI hydrogenase has four [4Fe-4S] iron-sulfur clusters proposed to function as an electron transport chain to the deeply buried, oxygen-sensitive catalytic active site known as the H-cluster. The H-cluster is a complicated [6Fe-6S] complex, structured as a [4Fe-4S]-S_{cys}-[2Fe-2S] cluster, in which the [2Fe-2S] cluster is stabilized by carbon monoxide and cyanide ligands as well as a dithiolate molecule. Recent molecular diffusion computational studies reveal two channels through which oxygen likely diffuses.[1] Our working hypothesis is that the protein structure can be modified to exclude oxygen from the active site while still allowing protons to enter and hydrogen to exit. We are now employing a process called “protein evolution” to evolve an oxygen tolerant hydrogenase (Figure 3).

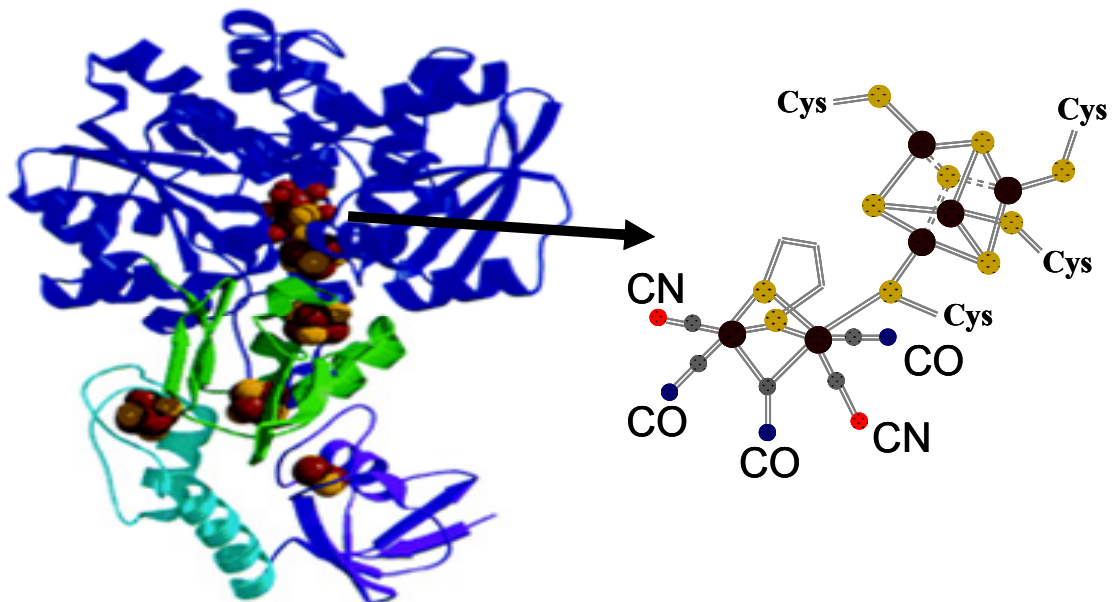


Figure 2: The structure of the CpI hydrogenase with an expanded diagram of the hydrogenase active site. Note that the oxygen sensitive active site (the expanded area) is well surrounded by protein structure suggesting that structural modifications can result in oxygen exclusion.

Although the diagram in Figure 3 is simple, protein evolution can be a long and difficult endeavor. It is particularly important to be able to quickly express and evaluate the hundreds of thousands, or even millions, of candidates that may need to be evaluated. This could be done with living organisms, but the process would be very difficult and tedious. We currently use cell-free protein expression to produce the hydrogenase candidates directly from the products of polymerase chain reactions (PCR). This method does not require the isolation of individual colonies, their growth under anaerobic conditions, or cell breakage and protein purification. The initial *in vitro* synthesis and evolution of an active hydrogenase enzyme in cell-free reactions was a significant challenge. Specific obstacles included expression of the active form as well as the development of rapid high-throughput methods for quantifying active hydrogenase yields. Additional rigorous requirements include use of an anaerobic environment as well as inclusion of maturation enzymes, initially suggested by the National Renewable Energy Laboratory (NREL).[2] Additionally, it was necessary to devise a consistent

method for precise oxygen exposure so that relative degrees of oxygen tolerance could be evaluated. Fortunately, we have addressed these challenges and now have a fully functional system for hydrogenase evolution using cell-free protein synthesis.

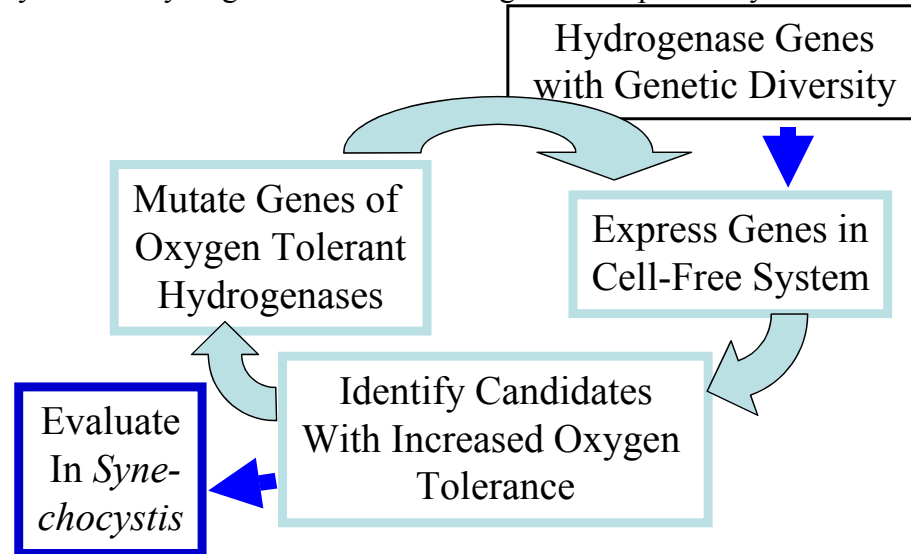


Figure 3: A diagram of the protein evolution concept. The native hydrogenase genes are mutated to provide a library of modified genes encoding for altered proteins. These proteins are then screened to find the rare mutations that increase oxygen tolerance. These improved enzymes are then mutated in subsequent rounds to increase oxygen tolerance. Promising candidates can be tested in the photosynthetic organism during this process to better define the requirements for the final enzyme.

As will be described later in this report, initial work on this project focused on the smaller and simpler hydrogenase from *Chlamydomonas reinhardtii*, HydA. This enzyme was indeed simpler to produce and to study. It therefore served as an excellent model for the initial part of the project since cell-free hydrogenase expression and activation was a significant challenge. However, a screen of 80,000 candidates only produced enzymes with more activity and no enzymes with improved oxygen tolerance. A closer look at the estimated structure of HydA suggested a reason for this. The structure of the enzyme probably does not surround the active site completely enough to ever exclude oxygen. The focus of the project was therefore shifted at the beginning of this year to concentrate on the evolution of the larger and more complex CpI hydrogenase shown in Figure 2.

Competition for Developing Oxygen Tolerant Hydrogenases. The competitive landscape has actually become more favorable in the last year as established companies and research groups have been caught up in the biofuels-from-glucose craze. We have heard that the NREL/Colorado School of Mines project has stopped their activities in searching for an oxygen-tolerant hydrogenase. It also appears that Solazyme is focusing on different projects. We are still concerned, however, about a patent that covers mutations made in the “hydrogen channel” as estimated by a computer model of the protein structure.

With the recent advances in our program described below, we are in an excellent position to discover hydrogenases with significant oxygen tolerance. However, with the recent proliferation of papers and patent applications, we may run into problems if our mutants coincide with some of the predictions in the other applications. Our approach is certainly novel and is unique and is now more powerful than ever. We must hope that we will discover unique changes that couldn't have been predicted by the computer modeling. This is likely, but not a certainty. Nonetheless, we are still in a good position to build a strong proprietary position given the power of our approach.

Results

Summary

- Validated cell-free screen by isolating more active HydA hydrogenases; but no improvement in oxygen tolerance after screening 80,000 candidates. Switched to Clostridial CpI mutants.
- Further improved cell-free CpI expression to enable protein evolution screening.
- Improving microtiter plate oxygen exposure protocols.
- Developing new technology for new ultra high throughput screening.
- Used *in vitro* hydrogenase maturation to identify required factors. Discovered another new requirement that was not recognized or predicted.
- Improved *in vivo* activation of hydrogenases.

Validating the Cell-Free Search Approach. Because of the complexity of the cell-free hydrogenase mutation, expression, and evaluation system, it was important to obtain evidence that the system was performing sufficiently well to recognize improved hydrogenases. It was therefore encouraging that we were able to isolate mutants of the *Chlamydomonas* HydA1 hydrogenase with improved activity. Figure 4 shows hydrogenase activities for two such mutants. This indicates that the cell-free protein evolution platform is indeed capable of isolating interesting new mutants. It also

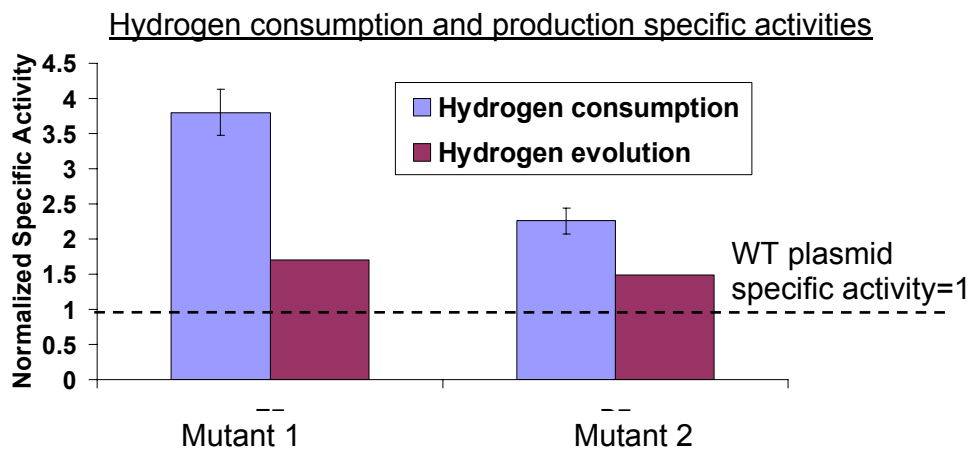


Figure 4: Improved activities of two mutants isolated in the screen searching for oxygen-tolerant HydA hydrogenases.

supports the idea that the HydA1 hydrogenase is not a good candidate for evolving oxygen tolerance since that was the primary focus of the search, and no improvement was found after evaluating 80,000 mutants. A closer look at the assumed protein structure of this hydrogenase suggests that it may not be a good candidate for developing oxygen tolerance. The region of the enzyme near the active site has an open structure so that the H-cluster active site is relatively exposed. This interpretation is supported by its higher oxygen sensitivity relative to that of the Clostridial CpI hydrogenase which has a more enclosed structure. Thus, the HydA1 molecular architecture may make it very difficult to find mutants that have sufficiently altered the protein structure to hinder oxygen diffusion into the active site.

In contrast, the *Clostridium pasteurianum* CpI hydrogenase is a larger protein with a large domain structure with 4 Fe-S clusters that more completely surrounds the active site (Figure 2). These Fe-S clusters are similar to those in ferredoxin and are probably already oxygen tolerant. Most likely, they serve to convey electrons into the active site. It is much more likely that this enzyme can be evolved to oxygen tolerance since the oxygen sensitive active site is completely surrounded by protein structure. CpI was not pursued initially because its more complex structure makes it much harder to properly fold and activate. Initial cell-free activities were very low. However, these issues have been addressed as described below, and the program is now focused on evolving CpI.

Improving Cell-free Production of the CpI Hydrogenase. Initially, very low amounts of active CpI hydrogenase were produced from the linear DNA templates used to evaluate hydrogenase mutants. The assay used to measure hydrogenase activity reduces methyl viologen to form a blue color. However, this assay is potentially subject to background oxido-reductase activity that would confuse our results. It is therefore important to produce sufficient hydrogenase activity to swamp out any background. Furthermore, the initial yields of active CpI hydrogenase from the cell-free reactions were highly variable. In order to reduce this variability and increase the yield of active CpI hydrogenase, the cell-free system was analyzed and several modifications made: (1) the 5' coding sequence of the CpI gene was optimized for better expression, (2) cell-free reagent mixtures were optimized and the preparation procedures were also optimized and carefully documented, and (3) purified GAM protein was added during cell-free protein synthesis to stabilize the linear DNA templates.

Work with previous proteins, including the HydA1 hydrogenase, had shown the importance of optimizing the 5' coding sequence of the expression gene to avoid secondary structure in the messenger RNA thereby improving the rate of translation initiation. When this approach was applied to the CpI hydrogenase, a dramatic improvement in the production of active hydrogenase resulted. (Figure 5)

There are many different reagents that are added to the cell extract during cell-free protein synthesis and many of these are grouped into stock mixtures that are prepared in advance and then stored as frozen solutions. Previously, protocols for their preparation had not been carefully optimized and documented. As a result, different reagent batches produced different amounts of active hydrogenase, and, in some cases did not produce

any. It was important to carefully and immediately adjust the pH of all solutions, to flash freeze reagents for storage and to minimize the number of freeze thaw cycles, to standardize the order of reagent addition and the mixing protocols, and to use different acids and bases for pH adjustment. With these improvements, CpI hydrogenase activities from cell-free expression more than doubled and were also much more consistent (see Figure 5).

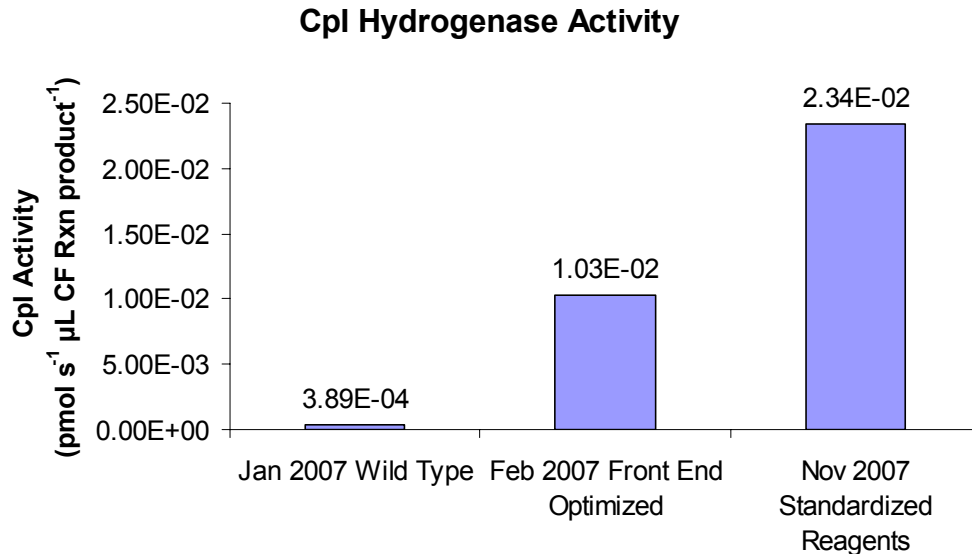


Figure 5: To obtain sufficient cell-free hydrogenase activity for effective mutant screening, we optimized the 5' coding region of the CpI hydrogenase gene and also optimized the preparation and storage procedures for the reagent mixtures.

GAM is a protein that stabilizes linear DNA in cell-free reactions by inhibiting the RecBCD complex activity.[3] We developed a cell-free expression process and a rapid and effective purification process to produce the Gam protein since it was not commercially available. An *in vivo* expression process was also evaluated, but the purification was significantly less effective than for the cell-free produced material. Upon the addition of purified GAM, cell-free yields of hydrogenase expressed from linear DNA templates approximately doubled.

Improving the Activity Assay and Oxygen Inactivation Procedure. The methyl viologen colorimetric assay is done in an anaerobic chamber with an atmosphere of 2% hydrogen and the balance nitrogen. Two assays are run for each cell-free reaction. First, a portion of the cell-free reaction product is diluted in Tris buffer, pH 8, and the activity determined by following the rate of blue color (reduced methyl viologen) development as the hydrogenase moves electrons from hydrogen to oxidized methyl viologen. In the second assay, a portion of the cell-free reaction product is diluted in the same Tris buffer and a carefully titrated volume of air-saturated Tris buffer is added to expose the hydrogenase to a controlled quantity of oxygen. After waiting 10 minutes for deactivation to occur, methyl viologen is added to determine the remaining hydrogenase

activity. Dividing the activity from the second assay by the pre-exposure activity gives a measure of the oxygen tolerance of the hydrogenase.

After achieving higher yields of active CpI hydrogenase, we began to use the methyl viologen assay to screen through the mutants produced with overlapping PCR mutagenesis. These were designed to evaluate the importance of the amino acid side chains in the oxygen diffusion channels proposed by Cohen et al. [1]. However, we encountered problems with consistency in the oxygen deactivation step and also saw that a background activity increased after oxygen exposure. As mentioned above, the ability to make much greater levels of hydrogenase activity allowed the dilution of the cell-free product to a point that the background activity was tolerable. We then needed to focus on the oxygen exposure procedure as that appeared to be a significant source of variability.

Upon investigation, it became evident that the method used to bring the oxygen-containing buffer into the anaerobic chamber was problematic. Essentially, the amount of oxygen in the buffer was not being controlled. We have therefore improved and standardized: (1) how the buffer is air-saturated, (2) how it is brought into the anaerobic chamber, and (3) how the buffer is stored in the anaerobic chamber before use. Through a combination of improving the production of CpI hydrogenase activities and also improving our sample dilution, assay, and oxygen exposure procedures; we can now generate oxygen inactivation curves such as that shown in Figure 6. This type of consistency will now allow us to screen CpI mutants for improved oxygen tolerance with much more confidence that we can detect incremental improvements.

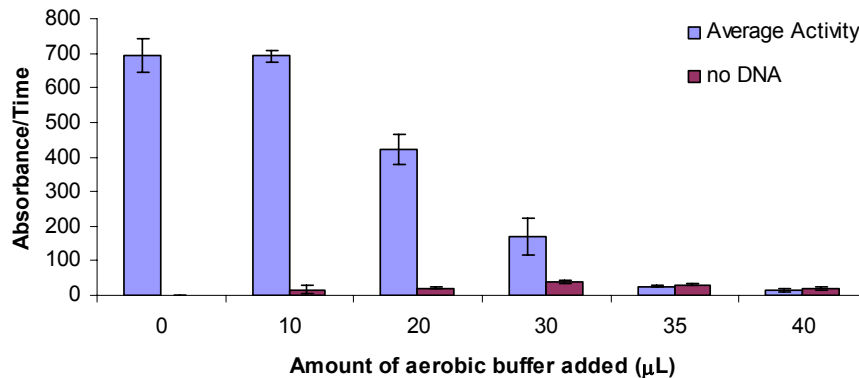


Figure 6: Hydrogenase activity (the rate of increase in reduced methyl viologen) as a function of the volume of air saturated buffer added to the diluted cell-free reaction product. The “noDNA” controls indicate the background activity which, unfortunately, is activated by oxygen exposure.

Developing an Ultra-High Throughput Screen for Oxygen Tolerant Hydrogenases. The experience gained in evolving the HydA1 hydrogenase to higher activity validated the cell-free evolution platform, but it also taught us that we will need to screen many more candidates than we thought, perhaps millions. We have therefore developed an ultra-high throughput screen to identify and recover oxygen tolerant hydrogenase mutants. The screen uses fluorescence-activated cell sorting (FACS) to analyze mutants at a rate of up to 10^7 /hour. This represents an enormous improvement over the microtiter

plate-based screens that our group and others have employed. Development of the complicated assay procedure has been difficult, but a series of incremental breakthroughs now has us on the verge of being able to begin screening millions of mutants for oxygen-tolerance.

In vitro compartmentalization (IVC) [4] is a powerful new cell-free protein synthesis-based screening technology that encapsulates mutant genes, their products, and the products of enzymatic reactions within femtoliter-scale emulsion droplets. While this technique has been used to successfully evolve a number of less complex enzymes, our lab's expertise allows us to apply IVC to complex proteins, such as the CpI hydrogenase, that other groups cannot express with cell-free protein synthesis. Hydrogenase is a very difficult protein to screen for: its substrates (protons, electrons, and H₂) are small and impossible to bind or functionalize, making the design of a selection or screen very challenging. After testing a number of potential embodiments of IVC and repeated iterations of refinements, we have developed a method that we believe is capable of screening millions of mutant hydrogenases per hour in an extremely reagent-efficient manner.

Mutants are screened as follows: Biotinylated mutant genes and biotinylated anti-tag antibodies are incubated with streptavidin-coated beads at a low gene:bead ratio such that most beads bind many antibodies but no more than one mutant gene. The beads are then added to CFPS reaction mixture and immediately emulsified into a surfactant-stabilized oil mixture, isolating each bead in a stable, femtoliter-sized reaction compartment. Billions of isolated protein synthesis reactions then proceed in parallel, and the tagged protein products bind to the antibodies on the bead attached to their encoding DNA, establishing a physical genotype-phenotype linkage. The emulsions are broken and the CFPS mixture washed away from the beads. The beads are then exposed to oxygen sufficient to deactivate wild-type hydrogenase. Following this step, only enzymes with improved oxygen-tolerance retain activity. The beads are mixed in a buffer containing a substrate which becomes fluorescent upon reduction, and the beads are then re-emulsified in oil. The analyte is hydrophobic and binds nonspecifically but tightly to the hydrophobic polystyrene beads. Upon exposure to hydrogen, the hydrogenases that have retained activity will cause their beads to be fluorescent so they can be recognized by the cell sorting instrument. 10⁷ beads per hour can be interrogated for fluorescence by FACS, and fluorescent beads can be sorted and recovered. PCR amplification of the genes still attached to the beads results in a mutant library enriched for genes that encode improved hydrogenases, which can be further mutated, recombined, or diluted to single molecules and reamplified in microtiter plates for further characterization and sequencing.

The data shown in Figure 7 indicates that we have a viable system. The number of events (i.e, beads) is plotted on the Y-axis (normalized to the peak maximum for each type of bead) and the fluorescence intensity is plotted on the X-axis. The peaks to the left indicate the background activity observed from beads with the gene for chloramphenicol acetyl transferase (CAT) attached. This is our negative comparison. The peak on the right is obtained from beads with the wild type CpI hydrogenase gene attached. These

beads are approximately 20 fold brighter showing that we now have an analytical signal that clearly differentiates beads with hydrogenase activity. The 20-fold factor will also allow us to expose the hydrogenase mutants to oxygen and still detect beads that have retained only a portion of their initial activity.

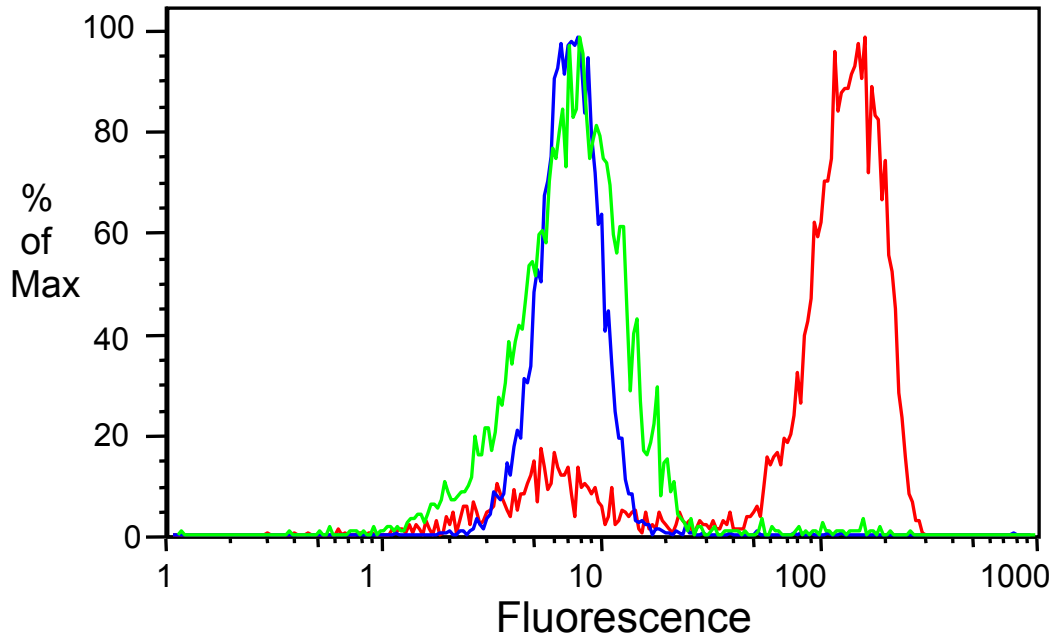


Figure 7: The assessment of bead fluorescence using a FACS device clearly differentiates the beads that have expressed the CpI hydrogenase (the peak to the right) from the beads that have not (the left peak)

We are currently performing model selections to fully validate the procedure before beginning to screen mutants in earnest. The failure of our group and others to isolate oxygen-tolerant hydrogenases with lower-throughput approaches indicates that this is an extremely challenging problem that will only be solved with extremely powerful screening platforms such as this one.

Identifying cofactors for the in vitro maturation of [FeFe] hydrogenase. After an oxygen-tolerant hydrogenase has been identified, we will need to express and activate it in a photosynthetic organism such as a strain of *Synechocystis*. In this endeavor, it will be important to understand the importance both of the maturation proteins and of the small molecules required for hydrogenase activation. As described, we know that at least three helper proteins are required. The literature suggests that S-adenosyl methionine, GTP, Fe^{+2} , and S^{-2} are also required. Last year, we showed, for the first time, that NAD^+ was also beneficial. This year we continued to characterize the small molecule requirements because of observations that *in vitro* hydrogenase maturation was variable and incomplete. Using anaerobically-prepared *E. coli* cell extract that contains the heterologous *S. oneidensis* [FeFe] hydrogenase maturases (HydE, HydF, and HydG), we are able to post-translationally activate the *C. reinhardtii* HydA1 hydrogenase. Although this is not the hydrogenase targeted for evolution to oxygen tolerance, it provides a good experimental reagent for studying active site assembly because of its more open structure.

The accessibility of the active site region allows us to efficiently activate the enzyme after the apoprotein has been fully expressed and purified.

A recent publication demonstrates that combining a crude cell extract with heterologous hydrogenase and an extract with heterologous maturases results in activating the [FeFe] hydrogenase without the addition of any cofactors such as SAM or GTP.[6] The system utilized, however, could not measure absolute yields of active hydrogenase. Furthermore, the cell extracts used were neither dialyzed nor reconstituted with Fe^{+2} and S^{-2} . We therefore decided to modify our *in vitro* system to more clearly define the impact of the cofactors we previously identified.

Our current *in vitro* hydrogenase maturation platform uses cell extract containing anaerobically-expressed hydrogenase maturases. The extract is also dialyzed against a pH buffer to remove any small molecules that could serve as cofactors. The apoprotein (immature hydrogenase) is the HydA1 hydrogenase polypeptide with an N-terminal His-tag to allow convenient purification from the cell-free reaction in which it was synthesized. In this cell-free reaction, the required cofactors are not added and the hydrogenase is not activated. The inactive hydrogenase is then added to the dialyzed cell extract containing the maturases. Minimal hydrogenase activity results unless the required cofactors are present.

Radiolabeling hydrogenase maturases to identify the substrates for [FeFe] hydrogenase H-cluster formation. Although the structure of the [FeFe] hydrogenase H-cluster has been determined, the process by which this active site is assembled remains unknown. Interestingly, the [NiFe] hydrogenase has a similar active site that also contains non-protein carbon monoxide ligands and cyanide ligands. Radiolabeling studies with the [NiFe] hydrogenase maturases have shown that carbamyl phosphate likely serves as the substrate for the cyanide ligands. [7] However, carbamyl phosphate additions have consistently failed to stimulate maturation of the HydA1 Fe-Fe hydrogenase.

Preliminary studies have been done to see if a portion of the cofactors $^{14}\text{CH}_3\text{-SAM}$, $^{14}\text{COOH-SAM}$, $^{35}\text{S-SAM}$ (produced from L- ^{35}S -methionine), and L-[U- ^{14}C]-tyrosine covalently attach to one of the [FeFe] hydrogenase maturases. Other possible cofactors including ^{14}C -carbamyl phosphate and a mixture of L-[U- ^{14}C]-amino acids have also been examined. However, neither carbamyl phosphate nor the other 19 amino acids (excluding tyrosine) have a beneficial effect on HydA1 *in vitro* maturation. For our tests, the maturases present in the anaerobic cell extract are incubated under anoxic conditions. Both native and denaturing gel electrophoresis in conjunction with autoradiography have shown that the methyl portion of SAM may covalently attach to one of the maturases, specifically HydF, which has a mass of 43 kD (Figure 8). Further work is required, however, to confirm that the ^{14}C from $^{14}\text{CH}_3\text{-SAM}$ is attaching specifically to HydF and not to another native *E. coli* protein with a similar molecular weight. Moreover, it has recently been suggested that the H-cluster precursors may already exist on the maturases being tested.[6] Thus, the tested substrates may still function in H-cluster assembly, and a different system will be needed to examine this theory.

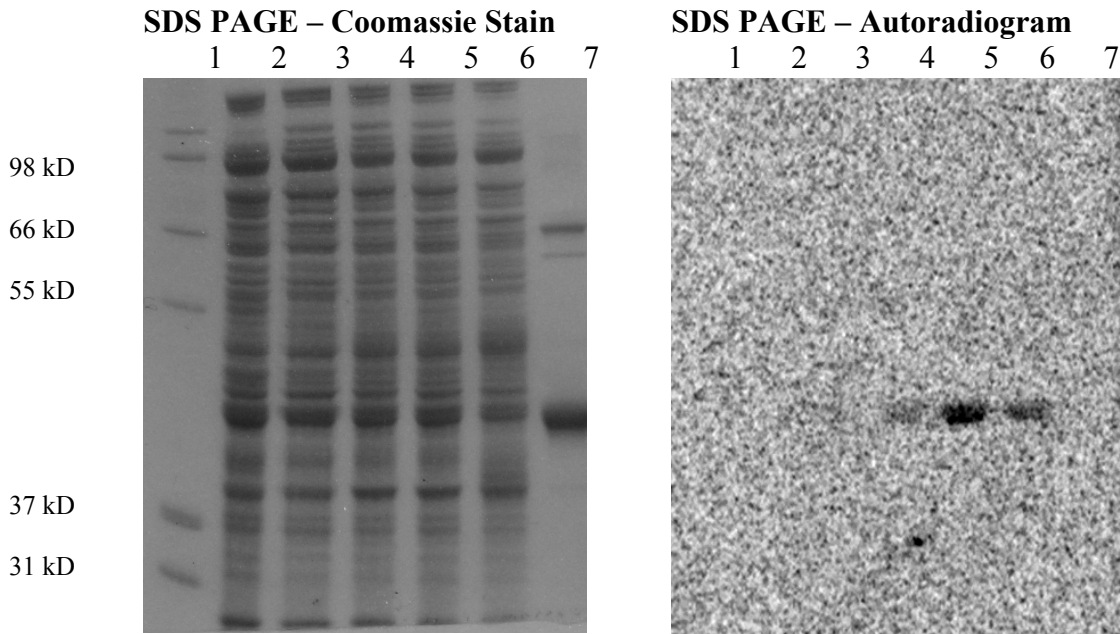


Figure 8. SDS-PAGE and autoradiography of radiolabeled proteins from *E. coli* cell extract following *in vitro* incubation with S-adenosyl-L- ^{14}C -methionine. Lane 1 is the Mark12TM protein ladder. Lane 2 is dialyzed KC6 cell extract. Lane 3 is dialyzed BL21(DE3) pACYCDuet negative control cell extract (no maturases). Lanes 4 and 5 are non-dialyzed and dialyzed, respectively, BL21(DE3) pACYCDuet-1 hydGxEF anaerobic cell extract. Lane 6 is dialyzed BL21(DE3) pACYCDuet hydGx hydEF aerobic cell extract. Lane 7 is purified N-His₆-HydF. Radiolabeled proteins were imaged using autoradiography after 5 days of exposure to a storage phosphor screen and the autoradiogram was aligned with the Coomassie blue stained gel using radioactive markers

In vivo expression and maturation of [FeFe] hydrogenase. Preliminary *in vivo* co-expression studies have been done using the *lac* promoter to control expression of the T7 RNA polymerase which then transcribes the genes encoding the maturases and the hydrogenase. Addition of inorganic iron and L-cysteine to complex growth media improves active HydA1 hydrogenase yields up to 50-fold relative to our initial system (Figure 9) [5]. Addition of glucose to the media, however, results in decreased yields of active hydrogenase. We have also examined longer periods of aerobic expression prior to switching to anoxic conditions, but less active hydrogenase was produced.

Progress

We continue to make significant progress on this project, but also continually are forced to reassess the difficulty of the challenges. It was fortunate that the project began with a more tractable hydrogenase, the HydA1 enzyme, as it allowed us to develop the ability to make cell extracts capable of hydrogenase expression, and it also allowed us to build the technology framework for the cell-free screening platform. However, the necessary progression to the more complex hydrogenase CpI, then required continued

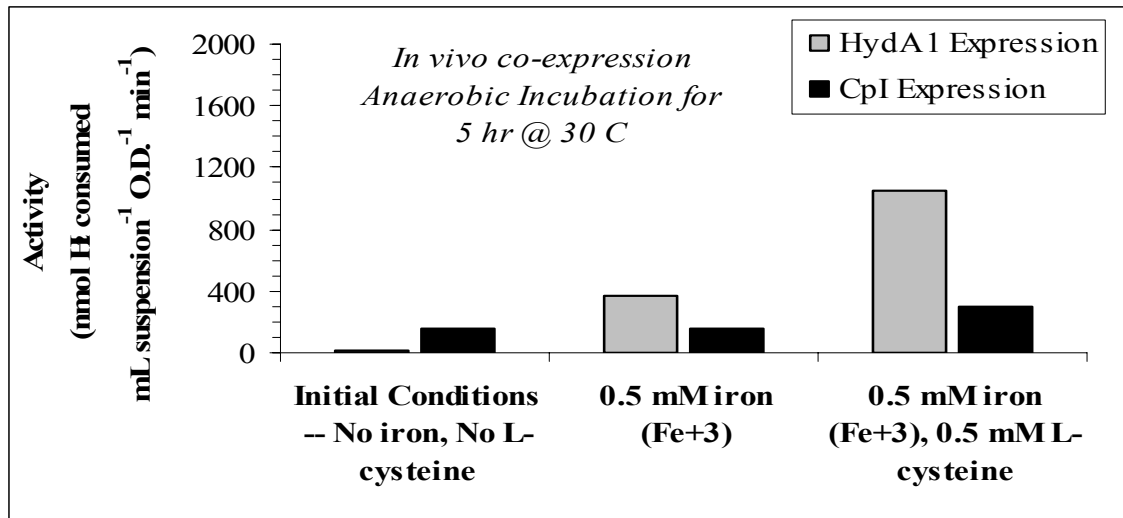


Figure 9. Doubly heterologous in vivo co-expression of [FeFe] hydrogenase and the required maturases, HydE, HydF, and HydG. The HydA1 (*C. reinhardtii*) or CpI (*C. pasteurianum*) hydrogenase was expressed along with the maturases from the HydGxEF operon (*S. oneidensis*) in the *E. coli* strain BL21(DE3). Both iron [Fe(NH₄)₂-citrate] and L-cysteine were added to particular samples as indicated above. Initial conditions were those used by Boyer et al. [5]

development both of the expression platform and of the screening technology. This has progressed nicely, but nonetheless, the project is now behind the projected timeline.

A related realization is that we will need to screen many more mutants than anticipated. This motivated the development of the emulsion based screening technology to allow the rapid evaluation of millions of candidates. This again was more difficult than anticipated, but we appear to have the method in hand. This is a very exciting development and now gives our program an additional level of unique capability.

In parallel, we have determined the maturases and co-factors required for hydrogenase activation and have begun work to gain experience with heterologous hydrogenase expression and maturation. This will set the stage so that we can quickly express our oxygen tolerant mutants in a photosynthetic organism to demonstrate hydrogen production from sunlight.

Future Plans

This is the last year of GCEP funding and a renewal will not be requested. I will be exploring other funding opportunities, both public and private. In the meantime, we will continue to work to evolve an oxygen tolerant hydrogenase and to express it in a photosynthetic organism.

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