# Engineering for the Direct Biological Conversion of Sunlight to Hydrogen

**Investigators**
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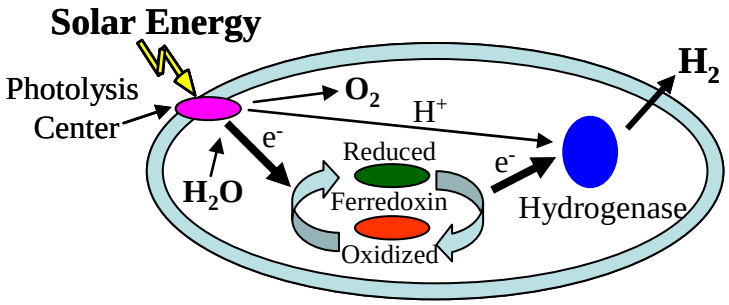
**Abstract**

The overall objective of this project is the biological conversion of sunlight to hydrogen for the economical, large-scale production of this potentially important fuel. Process cost analysis indicates the need for efficient energy conversions (about 20%), for economical and reliable large surface area solar collectors that double as bioreactors, and for the efficient collection and separation of the product gases (hydrogen and oxygen). As indicated by the diagram, our approach seeks to engineer a photosynthetic microorganism to activate a short and efficient pathway that first uses the organism's photolysis centers to split water and then combines the mobilized electrons and protons to produce molecular hydrogen. This latter reaction is catalyzed by a complex enzyme, an [Fe-Fe] hydrogenase, that contains a complicated series of iron-sulfur centers. The key reaction center is oxygen sensitive, but molecular modeling of the protein structure and published data for other hydrogenases suggest the feasibility of evolving the [Fe-Fe] hydrogenase to become oxygen tolerant while maintaining its high catalytic rates.




Since the hydrogenase evolution task will determine the feasibility of the entire project, it was the focus of the first funding period. Protein evolution requires the generation of genetic diversity (gene mutations) and the efficient identification of altered enzymes with desired functional attributes. In this case, the complexity of the hydrogenase enzyme suggests that the synthesis, folding, and evaluation of this enzyme will also be complicated. The extreme oxygen sensitivity of the native enzyme also poses serious challenges. For efficient identification of the very rare improved hydrogenases, we needed to develop methods for the efficient production, isolation, and evaluation of hundreds of thousands of candidates in an environment with low oxygen activity. Cell-free protein synthesis (CFPS) was chosen as the technology platform for this evolution as CFPS offers many advantages for this challenging protein evolution. However, no one had previously produced an enzyme of this complexity using CFPS.

The project was successful in establishing a high throughput evolution platform for discovering oxygen tolerant hydrogenases. We also gained considerable knowledge about the requirements for activating these hydrogenases. These insights will be

important for the efficient production of our evolved hydrogenase enzyme in the hydrogen producing organisms. The advances were accomplished by a series of coordinated subprojects:

*CFPS of the Fe-S Protein, Ferredoxin.* The photosynthetic bacterium, *Synechocystis sp.* PCC 6803, has become a model organism for studying bacterial photosynthesis. It uses a ferredoxin protein to transport electrons. We chose to develop cell-free methods for the production of this ferredoxin. It is an important protein for the project and provided experience in the cell-free production of Fe-S proteins. We produced high yields of active ferredoxin in cell-free reactions using an *E.coli* cell extract (for the first time), and we also developed a more convenient activity assay and an improved purification protocol. In addition to gaining valuable experience with this type of protein, we can now use *E.coli* to efficiently produce large quantities of active ferredoxin for testing improved hydrogenases.

*Cell-Free Production of Active [Fe-Fe]Hydrogenases*. The [Fe-Fe] hydrogenases as a class offer the high specific activities required for economical biohydrogen production. However, they are also extremely oxygen sensitive and have a complicated active site composed of one 4Fe-4S center and an adjacent 2Fe-2S center stabilized by carbon monoxide, cyanide, and an unusual dithiolate molecule. Specialized proteins have co-evolved in nature to assist in assembling and activating these reaction centers. Fortunately, these [Fe-Fe] hydrogenase maturation helper proteins were identified in 2004. The specific proteins that were first reported proved to be ineffective for our needs, but homologous genes were cloned from a distantly related bacterium, *Shewanella oneidensis*. The products of these genes catalyzed active hydrogenase production in *E.coli* and in cell-free reactions (for the first time). For the CFPS work, it was necessary to establish an anaerobic "laboratory" consisting of a cell disruption device, a centrifuge, a microplate spectrophotometer, and other assorted equipment within anaerobic glove boxes. Fermentation and cell extract preparation protocols were developed with an aerobic cell growth period followed by an anaerobic helper protein production and activation period followed by an anaerobic cell extract preparation protocol. This procedure now reliably provides productive cell extracts.

Cell-free synthesis of active hydrogenase has been increased dramatically by: a) activating cell extracts using an anaerobic incubation with iron and sulfur sources, b) adding 2 mM S-adenosyl methionine (SAM), and c) optimizing reaction temperature and duration. Up to 40% of the expressed enzyme can be activated under the best conditions. These activities are more than sufficient for establishing an enzyme evolution procedure for the *Chlamydomonas* HydA1 Fe-Fe hydrogenase.

*Developing Screening Procedures for Cell-Free Evolution.* To maintain the connection between each mutated gene and its gene product, we needed to dilute our mutated gene libraries so that a single DNA molecule is placed in each reaction chamber. This DNA molecule is then amplified to produce sufficient copies to serve as the DNA template for the cell-free reaction. Although single-molecule PCR (sm-PCR) procedures were described in the literature, they were not effective for us. Success only resulted

after the development of improved DNA purification methods and PCR protocols. To simplify the search for improved hydrogenases, methods were developed so that the product of the single molecule PCR reaction could be used directly for hydrogenase expression and so that purification of the hydrogenase candidate was not required. Although some background activity was observed in initial experiments, optimized hydrogenase activation overwhelmed it. As the last step, procedures for partial oxygen inactivation have been developed and are now being tested.

*Summary*. In spite of the complexity of the Fe-Fe hydrogenases, procedures have now been developed for the cell-free expression and evolution of these important enzymes.

**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 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, we have made major progress toward this goal during the grant period.

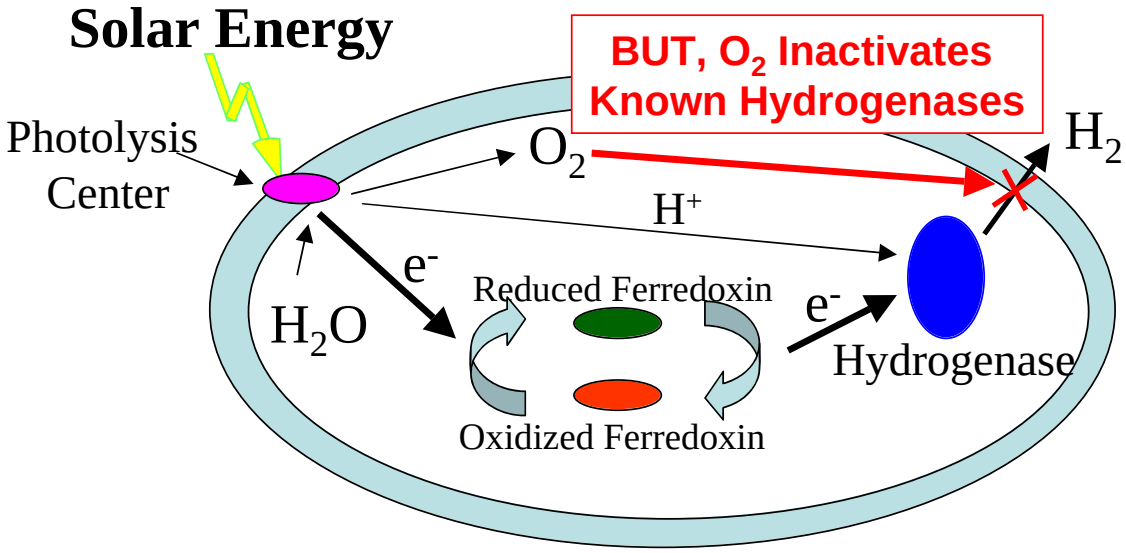



**Figure 1:** Diagram of proposed *Synechocystis* bacterium engineered to support a short, efficient pathway for the conversion of sunlight and water into molecular hydrogen. As shown, the dominant barrier to this technology is the availability of an oxygen-tolerant hydrogenase enzyme.

**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 a few [FeFe] hydrogenases including one (CpI) from *Clostridium pasteurainum*. The CpI hydrogenase has four 4Fe-4S 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 unique 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 currently implementing a process called "protein evolution" to evolve an oxygen tolerant hydrogenase (Figure 2).

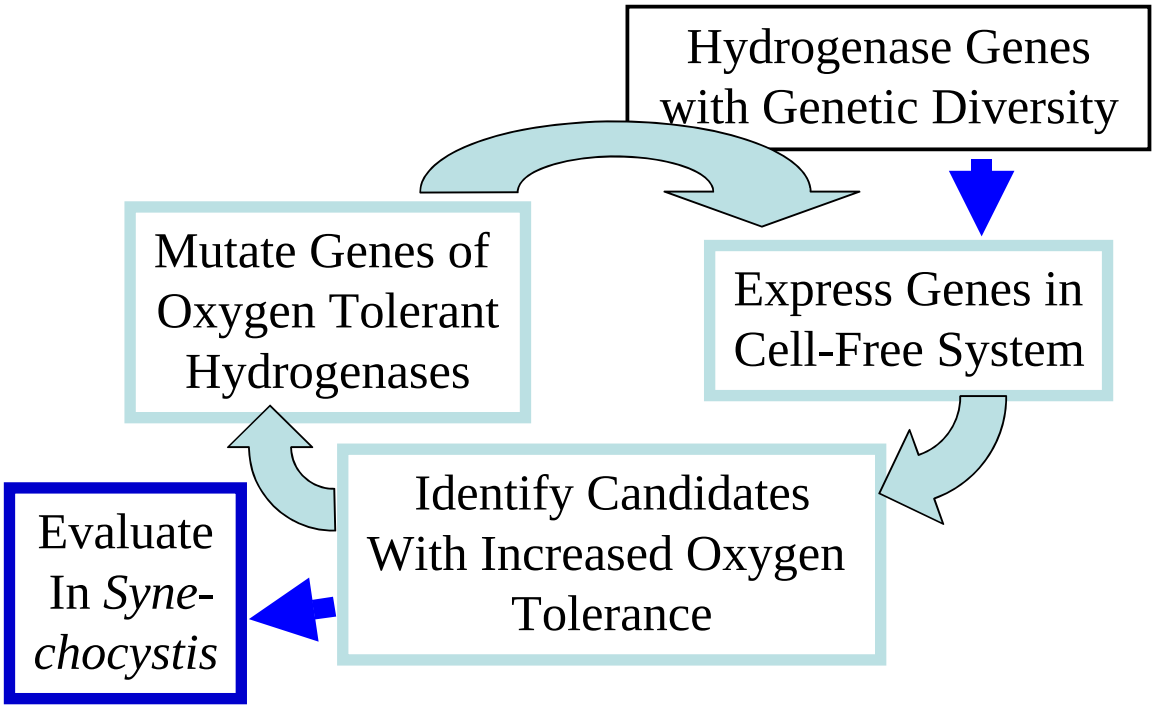



**Figure 2:** A diagram of the protein evolution concept. The native hydrogenase genes are mutated to provide a library of modified genes encoding for altered proteins. These proteins are then screened to find the rare mutations that increase oxygen tolerance. These improved enzymes are then mutated 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.

Although the diagram in Figure 2 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 of candidates that may need to be evaluated. This could be done with living organisms, but the process would be long 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. However, the *in vitro* synthesis of an active hydrogenase enzyme in a cell-free reaction has thus far been a significant challenge. Specific obstacles have included expression of the active form as well as the development of a rapid high-throughput means to quantify active hydrogenase yields. Additional rigorous requirements include use of an anaerobic environment as well as inclusion of maturation enzymes, initially identified by the National Renewable Energy Laboratory (NREL)[2].

**Research Results**

The project has focused on establishing the necessary facilities and on evolving an oxygen tolerant hydrogenase using cell-free expression technology. The first and most important feasibility barrier was the cell-free expression of active hydrogenase. This has been accomplished for two different Fe-hydrogenases, enabling entrance into the enzyme evolution phase of the project.

*Cell-free expression of Synechocystis ferredoxin*. We reasoned that the cell-free expression of the *Synechocystis* ferredoxin would be useful both to gain experience with an iron-sulfur protein and to develop technology for producing the ferredoxin needed for the hydrogenase evolution program. We consulted with Japanese researchers who had recently expressed ferredoxin *in vivo* in *E.coli*. They assured us that it was impossible to express active ferredoxin in cell-free reactions. However, with our cell-free system, the expression of the *Synechocystis* ferredoxin was surprisingly straightforward both for anaerobic and aerobic production. Fully active ferredoxin was produced[3]. Cysteine was the preferred sulfur source and ferrous ammonium sulfate the preferred iron source for the Fe-S center. Although an operon in the *E.coli* genome had been shown to be beneficial for *in vivo* expression, its overexpression in the cell extract provided no significant advantage for cell-free synthesis. An improved assay for ferredoxin function was developed, and a streamlined purification procedure was implemented. All of these advances will be useful when we produce the *Synechocystis* ferredoxin for screening hydrogenase mutants.

In addition, by observing protein production vs. activation kinetics, we learned that lower temperatures improve active ferredoxin production, and that activation can occur significantly after polypeptide production. This was further confirmed by denaturing active ferredoxin and showing that it efficiently refolds when added to a cell-free reaction mixture. These results have now been published[3]. The lessons have been beneficial as it has turned out that lower temperature expression also benefits hydrogenase formation and that hydrogenase also can fold and assemble significantly after the polypeptide is produced.

*Constructing optimized genes for cell-free hydrogenase expression*. In this program, we have focused on two different hydrogenases. The first is from *Clostridium pasteurianum* and has the relatively complicated structure shown in Figure 3. The second is from *Chlamydomonas reinhardtii*. It is a smaller protein with only the active site Fe-S center. Both genes have a number of codons that are rarely used by *E.coli* and initial attempts at expression resulted in low yields. Since the presence of rare codons can have a significantly negative effect on cell-free expression, both genes were totally assembled from synthetic oligonucleotides with overlapping homologies using a series of PCR primer extension reactions. The oligonucleotides were designed to use *E.coli* preferred codons. Because PCR reactions sometimes introduce genetic mutations, the resulting genes were completely sequenced, and several errors were detected and then repaired in subsequent reactions. Both of the resultant genes have been cloned into plasmids behind a T7 promoter for expression both *in vivo* and in cell-free reactions.

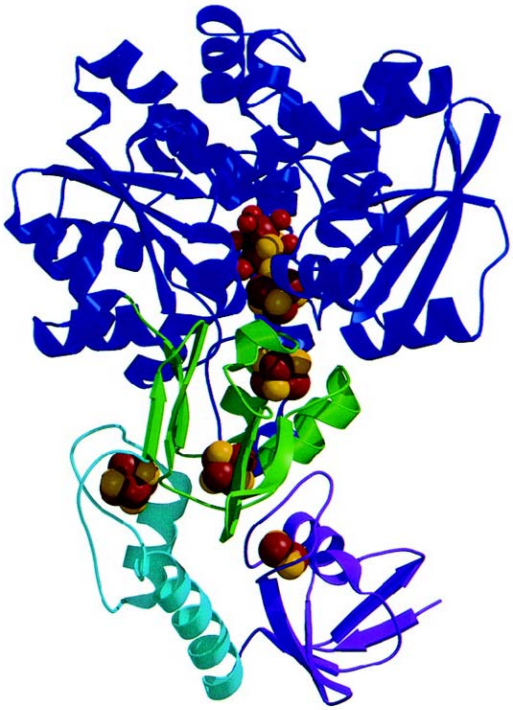


**Figure 3**: 3-D structure of the FeFe hydrogenase from *Clostridium pasteurainum*.

*Establishing anaerobic cell-free methods and infrastructure.* Considerable efforts were expended to establish cell-free expression of active hydrogenases. A variety of cell-free reaction modifications were made to encourage hydrogenase folding and activation. For example, carbamoyl phosphate was added as a source of the cyanide and carbon monoxide required for stabilization of the active site iron-sulfur center. Based on the work with ferredoxin, ferrous ammonium sulfate was added as the iron source and the cysteine concentration was increased to increase the supply of sulfur. None of these changes were effective. Reasoning that *C. pasteurainum* might have specific helper

proteins required for hydrogenase assembly, cell extracts were prepared anaerobically from Clostridial cultures, and these extracts were mixed with the *E.coli* extracts. This provided a hint of activity, but it was very small and highly variable. It is important to note here that producing and maintaining the cell extracts under anaerobic conditions was technically very demanding. Consequently, a new procedure for cell extract preparation was required. To facilitate this process, an anaerobic glove box was equipped with a high-pressure cell homogenizer, a centrifuge, and a chilled water circulation system to keep in-process solutions cold and also to remove the heat generated by the homogenizer and centrifuge. This new installation has been instrumental in project success. We also discovered that new cell extract storage procedures were required. The plastic vials used for other extracts are sufficiently permeable to oxygen, even at –80°C, to slowly degrade the performance of these anaerobically-prepared cell extracts. We now place the plastic vials within glass vials before storage. Development of these techniques and infrastructure has taken significant time and attention, but has proven essential for production of reliable results.

*Establishing reliable in vivo hydrogenase expression.* A 2004 publication from the Seibert/Ghirardi group at the National Renewable Energy Lab (NREL) described the identification of helper proteins that assist with hydrogenase folding[2]. They showed that the products of the hydEF and hydG genes in *Chlamydomonas reinhardtii* could assist with the folding of the Chlamydomonas HydA1 Fe-only hydrogenase. They demonstrated this by co-expressing the hydrogenase gene and the two helper gene segments in *E.coli*. However, the hydrogenase activity that they produced was very low and was also extremely variable. The system they reported was not nearly adequate for hydrogenase evolution. We obtained their plasmids but were unable to reproduce their results. However, the NREL investigators also pointed out that genes similar to hydEF and hydG existed in a variety of other organisms, including the gram-negative bacterium, *Shewanella oneidensis*. This is an organism that has been actively studied here at Stanford by the Spormann group.

The homologous genes in *S.oneidensis* exist in a single operon with the gene order HydGXEF as shown in Figure 4. The X gene is a possible open reading frame (expression element) with unknown function. The entire operon was cloned into a plasmid behind the T7 promoter, and a commercial *E.coli* host, BL21(DE3), was transformed with the resulting plasmid. Success was assessed by the ability of anaerobically harvested cells to produce hydrogen after the initial transformants were further transformed with a second plasmid encoding the Chlamydomonas hydrogenase, HydA1. Initial results were negative, but further analysis showed that most of the original transformants contained helper protein plasmids that were not correctly assembled. Only 3 out of 24 isolates had the correct plasmid size and composition. When these three isolates were transformed for hydrogen production, one displayed significantly more hydrogen production than the untransformed organism suggesting *in vivo* production of active hydrogenase. This was the desired plasmid. Further work showed that the X gene could be deleted without reducing hydrogenase activation. The HydGXEF plasmid also stimulated significant *in vivo* activation of the more complicated CpI hydrogenase from *Clostridium pasteurianum*.


**Figure 4:** Helper gene order in the pACYC HydGxEF plasmid used to complement *in vivo* and cell-free expression of hydrogenases.

*Cell-free expression of active hydrogenases.* Although the hydrogenase activities produced *in vivo* were reproducible and significantly greater than background activity, they were also low. Still, these positive results confirmed that we could produce active helper proteins in our cultures since cells without the helper proteins produced no hydrogenase activity. After demonstrating *in vivo* activation, the same BL21(DE3) host transformed with the HydGXEF plasmid was used to prepare cell extracts. Initial success was modest but significant. This was the first time that hydrogenase activity had been reproducibly generated in a cell-free reaction. Subsequent reactions showed that both the *C. pasteurianum* CpI and the *Chlamydomonas* HydA1 hydrogenases could be produced in active form although the HydA1 enzyme displayed more activity.

Since the first demonstration of cell-free production and activation of hydrogenases, significant effort has been expended to increase the yield of active hydrogenase protein. The cell extract growth and preparation procedures have been significantly improved, as has the cell-free reaction. Figure 5A shows that the combination of these improvements has resulted in a 30-fold improvement in activity for the CpI hydrogenase and a 17-fold activity increase for the HydA1 hydrogenase. Activity is indicated by the initial slope of the $A_{578}$ increase as oxidized methyl viologen is converted to the reduced form by the hydrogenase. Although the synthesis of new polypeptide is essentially over in the first two hours, activity continues to increase for several hours. The improvements have been realized due to improved extract preparation, optimization of cell-free reaction conditions, and reconstitution of cell-free extracts. These improvements are discussed below.

*Extract Preparation.* Considerable effort was expended to improve the extract for more active helper protein function. Variations to the cell extract preparation procedure have included the use of: different vectors for recombinant expression of the HydEFG maturation enzymes, different *E. coli* strains, and various anaerobic induction times, temperatures, and procedures. The most productive extracts were first grown aerobically, then induced for helper protein production, and then shifted to anaerobiosis. At the same time, iron and sulfur sources were added to the culture to assist in helper protein assembly, and the temperature was reduced for an overnight period of slow protein expression and folding. Finally, special cell harvest techniques were developed to maintain the cells under anaerobic conditions. All of these measures were required to obtain cell extracts with high activity for the cell-free expression of active hydrogenase.

*Cell-free Reaction Conditions.* Optimization of cell-free reaction conditions has also led to an increase in active protein yields. Several variables were found to have significant effects. Reducing temperature from 37 °C to 27°C increased the amount of active protein that accumulated even though the total protein accumulation decreased. This is illustrated by comparing the upward trend in active protein yields evident from Figure 5A with the downward trend in total protein yields illustrated in Figure 5B. Additionally, supplementation of the cell-free reactions with 2 mM S-adenosyl methionine has increased the amount of active protein accumulated.

*Reconstitution.* The most beneficial change made to the cell-free production protocol has been the introduction of a pre-incubation step for further activating the cell extract with iron and sulfide. Recent publications have indicated that hydrogenase helper proteins can be produced aerobically *in vivo* and later activated by incubation with sources of iron and sulfur[4,5]. We hypothesized that our extracts may also contain inactive helper proteins that may benefit from a similar incubation period before translation of hydrogenase polypeptide. Cell-free reactions were conducted after anaerobic pre-incubation of the cell extract with 1 mM dithiothreitol (DTT), 1 mM ferrous ammonium sulfate, and 1 mM sodium sulfide at room temperature for 1 hr. This preincubation led to significant improvements in active hydrogenase yields (Figure 5). DTT was added to provide a reducing environment, while ferrous ammonium sulfate and sodium sulfide were added as sources of iron and sulfur, respectively. Additionally, experiments were conducted in which the cell extract was reconstituted following exposure to aerobic atmospheric conditions. This cell extract was capable of producing significant amounts of active hydrogenase. These observations suggest that not only can the HydEFG maturation enzymes in the cell extract be reconstituted to their active forms, but also that oxygen does not appear to irreversibly inactivate these proteins. These insights may prove to be very important when we seek to produce active hydrogenase in the photosynthetic organism.

*Aerobic Extracts.* As we previously discussed, the preparation protocol for hydrogenase-specific cell extract is far more complex than the standard cell extract procedure, mostly because of the necessity for maintaining strict anaerobiosis. Given the *in vitro* reconstitution results, we have attempted to simplify the cell extract preparation protocol by eliminating the need for anaerobic conditions except for the final activation step. Following this *in vitro* reconstitution step, these aerobically-produced hydrogenase-specific cell extracts are capable of producing active hydrogenase (Figure 5). However, active protein yields are currently somewhat lower than those obtained using cell extracts produced anaerobically. The potency of both anaerobic and aerobic cell extracts is likely limited by the concentration or activity of the helper enzymes expressed during growth. Published observations also indicate that production of the HydEFG proteins is difficult in *E. coli*.[2] We are currently exploring alternate strains, vector arrangements, and growth and expression procedures to improve helper protein expression and activation in the cell extracts.

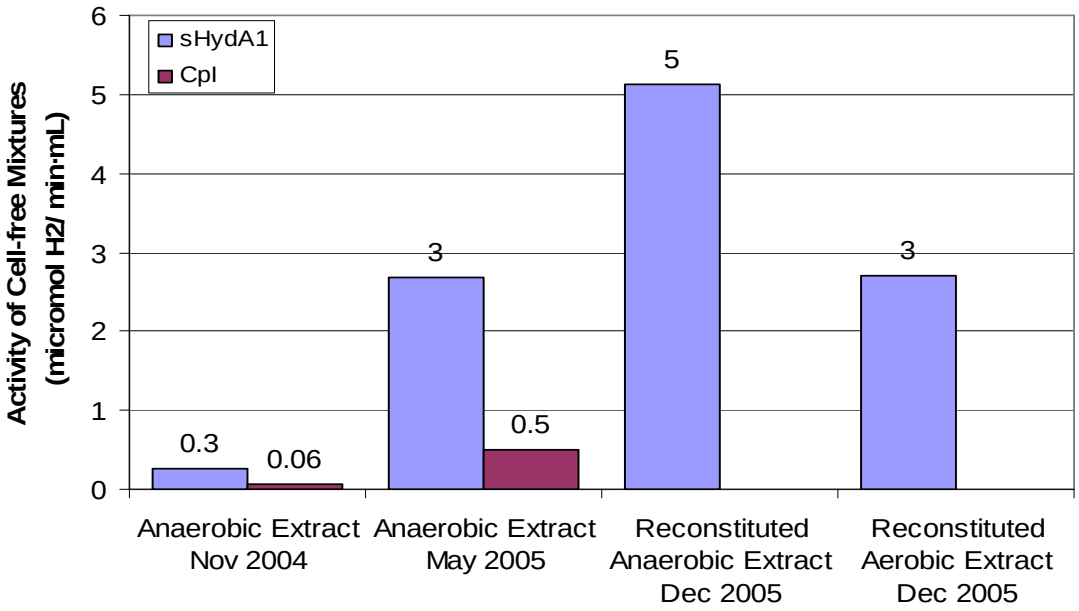



**Figure 5A:** Improvements in production of active hydrogenase proteins

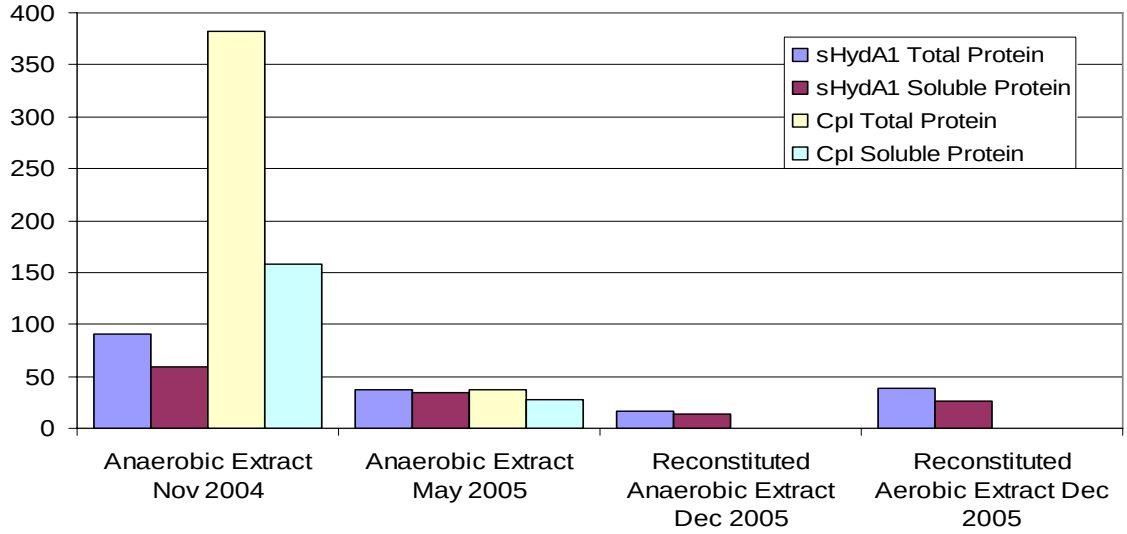



**Figure 5B:** Hydrogenase protein expression from the same experiments shown in Figure 5A. Note that protein accumulation levels decreased as activity levels rose. Thus, the new conditions are significantly more effective in folding and activating the hydrogenases.

Figure 5B shows a very surprising result. The significant improvements in activity have actually been produced from significantly less expressed protein, especially for the CpI hydrogenase, which has five Fe-S centers instead of only one for the HydA1 hydrogenase. These results suggest that there is still significant opportunity for increasing hydrogenase activity production from cell-free reactions. **However, the more**

**important assessment is that the activities presently being obtained are more than sufficient to allow us to screen mutant libraries for oxygen-tolerant hydrogenases**.

*Demonstrating Hydrogen Production with the Cell-Free Produced Hydrogenases.* The activities indicated in Figure 5 are extremely exciting and encouraging. However, all of these measurements were made by using hydrogen consumption to reduce methyl viologen thereby increasing light absorbance (reduced methyl viologen is a blue color). It is far more relevant to measure the forward, hydrogen-producing reaction. Figure 6 shows initial assessments of hydrogen production by the CpI hydrogenase from cell-free reactions. Both reduced methyl viologen and reduced *Synechocystis* ferredoxin were evaluated as electron donors.

The unexpected and very favorable result from this experiment is that the background activity for hydrogen production is very low. This suggests even more strongly that we now have sufficient cell-free hydrogenase expression and activation to enable the hydrogenase evolution program.

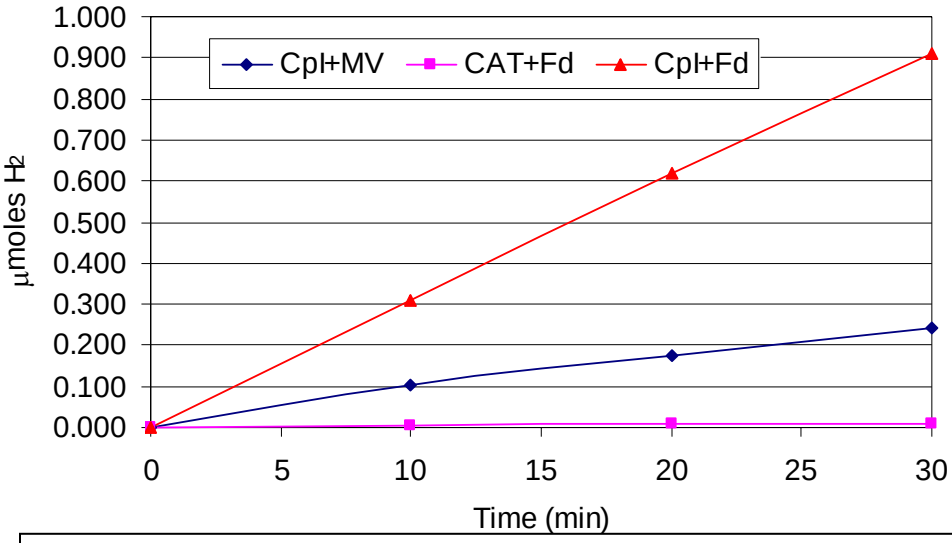



**Figure 6**: Hydrogen production by the CpI hydrogenase produced in cell-free reactions. MV is methyl viologen; 30µl of the crude cell-free reaction product was added to 1ml of 2mM methyl viologen solution. Fd is ferredoxin; 100µl of the crude cell-free reaction was added to 1ml of 50µM reduced ferredoxin. CAT (chloramphenicol acetyl transferase) is the product in the control reaction and 150µl of this crude cell-free reaction product was added to 1ml of 50µM reduced ferredoxin.

*Construction of the Mutant Hydrogenase Library*. We have overcome a number of obstacles standing between us and an efficient, high-throughput screen for improved oxygen-tolerant hydrogenase variants. One of the more significant challenges has been establishing the ability to amplify single molecules of DNA using the polymerase chain reaction (PCR). This is required so taht we can produce a single mutated hydrogenase in each cell-free reaction in order to separately evaluate each candidate. Single-molecule

amplification pushes the sensitivity of PCR to the limit, and is, not surprisingly, a delicate undertaking. If the single copy of the gene is destroyed, stuck to the wall of a tube, or otherwise lost, the amplification will fail.

Although a few labs have reported successful single-molecule PCR (sm-PCR) in the literature, we had a difficult time developing a reliable protocol for amplifying our gene target. A number of variables had to be optimized, including template purification and dilution techniques, PCR cycling protocol, and concentrations of buffers, oligo-nucleotides, primers, and polymerase. Numerous types of polymerases, buffers, and additives were tested for suitability. A few improvements stood out as being particularly important as we progressed from our initial reactions dominated by aberrant products to our current clean amplifications. First, the target gene was constructed to allow the use of a single primer that anneals at both ends of the gene. This reduced the dominant formation of primer-dimer side products. Additionally, a number of single primers had to be evaluated before identification of one that gave reliable results free of primer-dimers. The second major improvement involved a more rigorous purification protocol during template DNA preparation. Single-molecule PCR consistently failed when DNA was purified by the standard technique of agarose gel purification, suggesting that residual agarose was poisoning the PCR reactions. Switching to polyacrylamide gel purification led to a vast improvement. The PCR recipe has also been altered to ensure that the products can be directly used as templates for the cell-free protein synthesis reactions that will convert the amplified DNA mutant library into a protein library for testing.

Amplification from single molecules has now been demonstrated statistically. A certain percentage of reactions are expected to contain no molecules of DNA, and this fraction can be predicted with a Poisson distribution. We have shown agreement between the theoretically predicted fraction of wells that do not amplify due to lack of template and our observed results. To further validate the technique, a collection of genes that are identical except for the location of a restriction site introduced at various sites was also constructed. After diluting and amplifying a mixture of these constructs using sm-PCR, endonuclease digestion of the products yielded only two bands thus showing that the amplification originated from a single molecule of DNA (Fig 7).

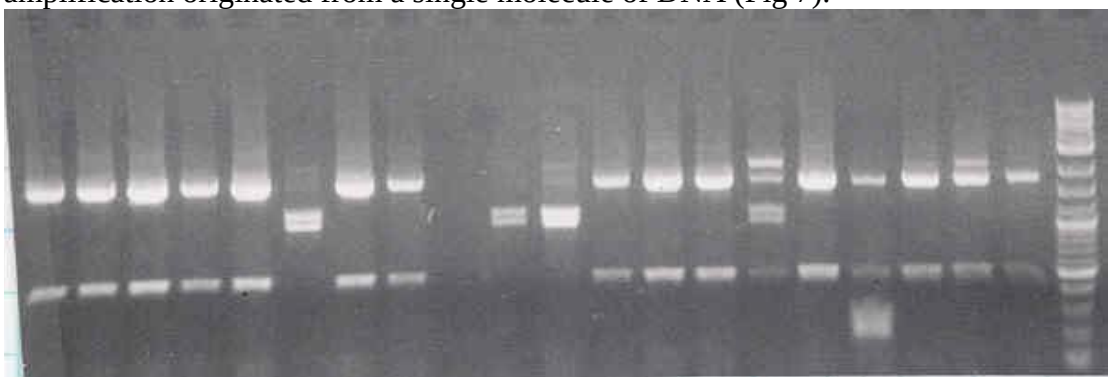


**Figure 7:** Digested sm-PCR amplifications of a mixture of two constructs with the same restriction sequence in different sites on the gene. Most lanes show either one pattern or the other, indicating a single parent for the amplification.

Error-prone PCR has been implemented to introduce mutations into the wild-type hydrogenase gene from *Chlamydomonas reinhardtii* to generate the diversity of genes that we will screen for improved variants. The protocol we chose to create this library of mutant genes involves the incorporation of nucleotide analogs into the newly synthesized DNA chains, which then associate non-selectively with multiple nucleotide bases in later rounds, resulting in base pair changes. Six different libraries covering a range of mutational loads have been created and characterized. Too many mutations per gene would likely result in a high fraction of mutants being misfolded and inactive; too few mutations will limit the diversity of mutants screened. We have chosen a library that represents a good compromise between these considerations for the first phase of the screen. A rationally-designed library or a new library generated from the first partially-improved mutants we identify will be used in subsequent screening phases.

*Preparing for the evolution of oxygen-tolerant hydrogenases.* The advances outlined above now enable the beginning of evolution of the hydrogenase. Further improvements to the system are also anticipated, but the present extracts are sufficient to allow the program to begin. The screening strategy is shown is Figure 8. Genetic diversity will be generated as described above and/or by conducting family shuffling between the CpI and HydA1 genes. We will then dilute this DNA library and place approximately 20 molecules of DNA into each well of 96-well microtiter plates. These will be amplified by PCR to high enough concentrations to be used as DNA templates for cell-free production of the mutated hydrogenases.

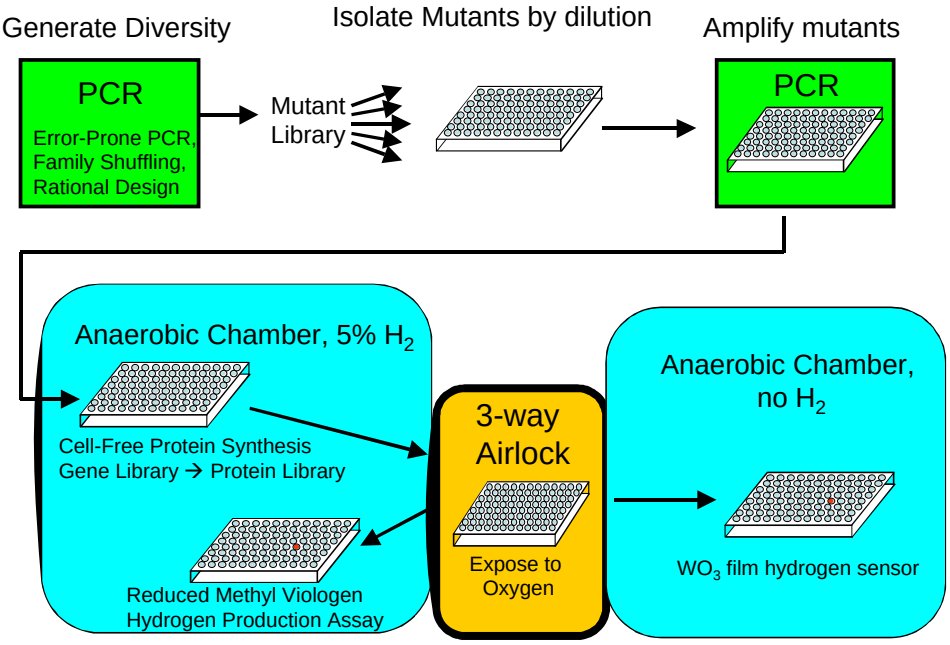



**Figure 8**: Diagram showing the protein evolution protocol and relevant equipment for identifying oxygen-tolerant hydrogenases.

At this point, the cell-free protein synthesis reactions can be conducted. The microtiter plates containing the DNA templates will be moved into the anaerobic glove box. Experience suggests that the PCR amplification product can be used directly without purification. Thus, the cell extract and other reagents will be mixed into wells of a reaction plate and the appropriate amount of linear DNA templates added. A significant portion of each DNA template solution will be retained to facilitate recovery of the DNA that encodes for a new and desirable enzyme.

When the cell-free reaction is completed, the reaction products (still in the microtiter plate) will be exposed to a controlled oxygen partial pressure for a controlled time period by passage of the plate into a 3-way air lock. There the atmosphere will be exchanged into the desired oxygen content and then flushed out again at the end of the exposure time. Early experiments suggest that the unpurified hydrogenase in the cell-free reaction mixture is 90% inactivated following a 3-minute exposure to a 7% oxygen mixture. The plate will then be moved back into the anaerobic glove box. Finally, the hydrogenase activity will be determined using reduced methyl viologen as the electron donor. This initial screen will be straightforward since a simple color change will indicated retained hydrogenase activity. A 96-well colorimeter has been installed inside the glove box to facilitate the assays.

Promising genes will be amplified and expressed again to generate more hydrogenase and will then be tested with ferredoxin as the electron donor to make sure that ferredoxin coupling has not been compromised by the mutation(s). The ferredoxin driven assay will be conducted by transferring the plate into a different anaerobic chamber where hydrogen is not present (the right hand chamber in Figure 8). Reduced ferredoxin will be used as the electron donor, and activity will be measured by hydrogen production. We will use tungsten oxide-coated glass as a hydrogen sensor since the sensor changes to a blue color in the presence of hydrogen. A lab at UC Santa Barbara has experience in fabricating these sensors[6], and they have assisted us in making several sensors the size of 96-well plates.

**Conclusions**

In this first three-year project, we have developed methods for the cell-free production of complex Fe-Fe hydrogenases. We have also developed methods for single molecule PCR to allow rapid evaluation of mutated hydrogenases. These accomplishments now provide a technology platform to enable the rapid evolution of oxygen-tolerant hydrogenases. In addition, we have assembled facilities, developed methods, and accumulated insights that provide a strong foundation for developing an organism, bioreactor, and integrated process for converting sunlight into hydrogen fuel.

When the evolved hydrogenase is available, considerable metabolic engineering of the photosynthetic bacterium will be required. Helper protein expression will be required for hydrogenase activation, and helper protein, hydrogenase, and ferredoxin accumulation levels will require optimization. Another challenge is making sure that the protons are available for hydrogen generation. Although the pH stabilization functions in the

organism will assist with this, it may be necessary to introduce "uncoupler proteins" that allow protons to leak across a lipid bilayer membrane. At this point, bioreactors will be designed to support these organisms while collecting sunlight and facilitating gas harvesting. When these are scaled to large coverage area "solar hydrogen farms", this technology promises economical production of hydrogen fuel without significant environmental impact. It will probably first be used for local customers until hydrogen storage and transport technologies can be improved. The extent of its deployment will depend upon evolving economic trends, but it has the potential to supply a major fraction of the fuel needs for our nation.

## Publications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