BIOLOGICAL H_2 FROM FUEL GASES AND FROM WATER

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Abstract

When photosynthetic bacterium *Rubrivivax gelatinosus* CBS-2 was cultured under an atmosphere containing CO, a unique water-gas shift pathway is quickly induced. This bacterium is able to convert CO (and H_2O) into H_2 (and CO_2). The terminal step involves a CO-linked hydrogenase. This hydrogenase is unique in its tolerance to O_2, with a half- life of 21 hours when stirred in full air. By measuring H_2-D_2O (H-D) exchange reaction with a capillary mass spectrometer, we determined that this hydrogenase is also partially functional in an atmosphere containing up to 3% O_2, carrying out the H-D exchange reaction at a linear rate throughout the 10-min measurement. This level of tolerance renders this hydrogenase suitable for scale-up application in which O_2 is present simultaneously. In order to sustain long-term H_2 production, we need to further understand the components involved and rate-limiting steps of the bacterial water-gas shift pathway. To achieve this goal, we performed mutant isolation and obtained seven mutants with enhanced CO shift rates, which are worthy of further investigations. Using a different approach to elucidate the components involved in CO shift pathway, we determined that once photo-reduced, a soluble ferredoxin of both a bacterial and of a blue-green algal source can link to the bacterial hydrogenase from CBS-2 to support H_2 production. This finding strongly suggests that a ferredoxin could be involved in mediating H_2 production from CO oxidation in *Rx gelatinosus* CBS-2.

Introduction

Photosynthetic bacteria are versatile in their modes of H_2 metabolism. They have four terminal enzymes mediating the H_2 metabolism: nitrogenase; an uptake hydrogenase; a fermentative hydrogenase; and a CO-linked hydrogenase. Nitrogenase is derepressed under N-free condition and catalyzes H_2 production while an abundance of energy is available (Stewart, 1973). This enzyme system is light-dependent and consumes 4 moles of ATP per mole of H_2 produced. A classical uptake hydrogenase has been isolated from numerous photosynthetic microbes and its main function is to oxidize H_2 to support CO_2 fixation (Colbeau *et al*., 1983). A hydrogenase linking to formate oxidation has also been identified and its main function is to dissipate excess reducing equivalents under fermentative dark growth mode (Gorrell and Uffen, 1977; Schultz and Weaver, 1982). This enzyme is extremely O_2 sensitive with a half-life of less than 1 min in full air; it equilibrates at 10% partial pressure of H_2 (Maness and Weaver, unpublished data), and is therefore not suitable for scale-up application.

The fourth enzyme, the CO-linked hydrogenase (refer to as "hydrogenase" for the rest of the document), is the most unique among all hydrogenases reported. It was first reported by Uffen in two strains of photosynthetic bacteria to shift CO (and H_2O) in darkness to H_2 (and CO_2) (Uffen, 1981). We have since isolated 450 strains of photosynthetic bacteria possessing this water-gas shift pathway. Among them, *Rubrivivax gelatinosus* CBS-2 is most unique in that its hydrogenase is highly resistant to O_2, with a half-life of 21 hours when whole cells were stirred in full air. When the membrane-free, partially purified hydrogenase was stirred in full air, a half-life of 4.5 hr was observed, clearly indicating that its O_2 tolerance lies in the intrinsic nature of the enzyme conformation, not owing to higher respiratory rates contributed by whole cells. This enzyme has a rate constant of over 60,000 and is energetically more favorable toward the H_2 evolution direction. Compared to most other hydrogenase systems, the CO-linked pathway is most robust and has great potential for commercial applications.

One requirement for economical H_2 production is to further characterize its O_2 tolerance. Although previous data reveal that the hydrogenase has a half-life of 21 hrs when stirred in full air, this experiment was conducted by pre-exposing whole cells in air, followed by assaying H_2 production anaerobically. Since O_2 was not present during the assay, the results only indicate that the hydrogenase is reversible with regards to O_2 inactivation. We need to determine whether hydrogenase can actually function with the simultaneous presence of O_2, which would provide insight as to the immediate impact upon O_2 addition, along with duration and rates of the enzyme in O_2. We have been using an assay method including a reducing agent and an electron mediator, both of which exclude the addition of O_2 during assays. To meet the requirement of including O_2 during the assay, we developed an H_2-D_2O (H-D) exchange assay. Hydrogenases from various sources have been reported to catalyze an H-D exchange reaction yielding HD without using a reducing agent, nor a mediator (San Pietro, 1957; Fauque *et al*., 1988; Vignais *et al*., 1997). This method is suitable to determine hydrogenase activity in the presence of O_2. Using this method, we found that the simultaneous presence of O_2 inhibited hydrogenase activity by 20% at 3.3% O_2 and 40% by 13% O_2. Yet, more than 90% of the original activity was restored when O_2 was removed subsequently. However, this batch assay does not yield kinetic information as to the immediate impact of O_2 on the enzyme activities and whether the loss was due to enzyme operating at a lower but constant level, or at a high initial rate which then fell off.

In this report, we document the development of a continuous sampling system using a capillary mass spectrometer that resolves these issues.

Another goal of this project is to gain a better understanding of the components involved in the overall water-gas shift reaction to identify the rate-limiting step(s) so the shift rates can be further enhanced. The biological water-gas shift pathway is a multi-step reaction involving at least two enzymes (CO dehydrogenase and hydrogenase) and four or more mediators shuffling electrons through the chain reaction (Ludden *et al*., 1996). There are three approaches to study the components involved in the shift reaction. The first one is to biochemically purify all the components, and then, through reconstitution experiments, determine what role each component plays in re-establishing the overall reaction. This approach is time-consuming and requires extensive effort on purification of the membrane-bound proteins. The second approach is to genetically obtain mutants deficient in one or more of the intermediates and determine their ability to carry out the overall reaction. This report documents an ongoing effort in generating mutants to meet this goal. The third approach is the most easily attainable among the three in the near term. This method is to construct an *in vitro* assay containing photosynthetic membranes from spinach, bacterial hydrogenase from CBS-2, and any physiological electron mediators of interest that might link the reaction, assuming the mediator can be photo-reduced by the spinach membranes. Any evidence of linkage *in vitro* would suggest that the mediator could play a role *in vivo* also. We chose to examine ferredoxin from two sources as the candidate mediator since ferredoxin is known to serve as a natural electron mediator in many low-redox potential reactions including hydrogenase in all life forms. This paper details the construction of such an *in vitro* system and reports the successful linkage of ferredoxin in mediating the CO-linked hydrogenase from *Rx. gelatinosus* CBS-2.

Materials and Methods

Growth Condition

Rubrivivax (formerly *Rhodocyclus*) *gelatinosus* CBS-2 was cultivated in modified RCV medium (Weaver *et al*., 1975) supplemented with Na-malate (28mM) and yeast extract (0.05%, w/v) (BG medium) along with 17% CO to fully induce the water-gas shift pathway. Photosynthetic cultures were illuminated with a band of 40W incandescent lamps with approximately 100W/m^2 of light intensity reaching the culture surface.

H_2-D_2O Exchange Reaction

Whole cells of CBS-2 (5 day old) cultured as above were spun down and suspended in 20 mM potassium phosphate buffer (pH 7.5) to a final concentration of 29.4 mg cell dry wt (cdw)/ml. A 15-ml aliquot of the cell suspension was placed inside a 100-ml Erlenmeyer flask. To collect signal, a capillary tube was inserted, through a 22G needle, into a neoprene stopper and positioned right above the culture surface. The headspace contains 3% H_2. Background levels of HD was recorded for 5 min, followed by the addition of 5 ml D_2O (Sigma-Aldrich) to initiate the H-D exchange reaction. Mass 3 for HD was continuously monitored for changes during the kinetic isotope exchange experiment, collected by a Balzers Quadstars data gathering software. The capillary mass spec (Balzers) was equipped with a Pfeiffer high vacuum system (TCP015).

Mutant Isolation

Log-phase CBS-2 culture was randomly mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (NTG, 20μg/ml) for 35 min to achieve 50% cell killing. The mutant was then inoculated into fresh RCV medium containing penicillin (150μg/ml) in the presence of 17% CO to kill only those cells capable of shifting CO. After 8-10 hrs of incubation in light, the culture was spun down (8,000 × g, 15 min) to remove excess penicillin. The pellet was resuspended in 1-ml BG medium followed by adding penicillinase (one crystal, < 1 mg) to remove residual antibiotics. The suspension was inoculated into fresh BG medium for photosynthetic growth for the next round of penicillin killing. We repeated this penicillin killing/CO selection procedure three times to enrich for the population of CO^- species. This culture is now designated as CBS2NTG3P.

A log-phase CBS2NTG3P culture was serially diluted in PBS and spread onto nutrient agar surface containing malate to yield approximately 150 to 200 colonies per plate. These colonies were then picked onto two sets of agar plates (pH 8.0) for replica plating, with one set containing Na-acetate (40 mM) along with CO (17%) while the other set containing Na-acetate and Na-bicarbonate (0.1%). After one week of photosynthetic growth, the patterns of both sets were compared to look for colonies that grow well on acetate/$NaHCO_3$, but do not grow on acetate/CO, an indication of lacking the CO-shift pathway. Colonies fitting that criterion were then transferred to BG liquid medium for photosynthetic growth.

Photoreductant Linkage

Stored-bought spinach was deveined to yield ~ 200g of leaves. The leaves were blended in 500 ml of B1 buffer (0.4M NaCl, 2 mM $MgCl_2$, 0.2% BSA, and 20 mM Tricine, pH 7.5) for 10 seconds at low speed, then two times at full speed. The blended extract was filtered through 16 layers of cheesecloth to remove unbroken cells. The filtrate was centrifuged at 300 × g for 1 min, followed by 4000 × g for 10 min. The resulted precipitates containing thylakoid membranes were resuspended in ~10 ml of K4 buffer containing 400 mM sucrose, 15 mM NaCl, 5 mM $MgCl_2$ and 20 mM K-phosphate (pH 7.0). Chlorophyll was determined by extracting 10μl of the membranes into 10 ml 80% acetone and measuring absorption using the equation (8.02 x A663nm) + (20.21 x A645nm) yielding chlorophyll in mg per ml. Membranes prepared this way have very good water-splitting activity.

To prepare membranes containing hydrogenase activity, four-day old CBS-2 culture was resuspended in 50 mM HEPES buffer (pH 7.5) to approximately 10 mg cdw/ml. The suspension was subjected to sonication for 5 min at 80% output (model W 375, Heat Systems-Ultrasonics, Inc). Unbroken cells were removed by centrifugation at 35,000 × g for 15 min and the supernatant was used for the linkage experiment.

Photoreductant linkage experiment was conducted according to Fry *et al.* (1977) and Rao *et al*. (1978) in a Clark-type O_2 electrode system (Yellow Spring, OH). The reaction mix contains, in final concentrations: 1.5 ml K4 buffer (pH 7.0), glucose (25 mM), glucose oxidase (5 units/ml), catalase (500 units/ml), ethanol (1%), thylakoid membranes (~ 20 μg chlorophyll), and 0.3 ml hydrogenase extract. A baseline activity was recorded in light (5V output, Unitron, Inc.)

followed by additions of either methyl viologen (10 μM) or ferredoxins from *Clostridium pasteurianum* (8.5 μM) or from *Spirulina sp*. (5 μM), obtained from Sigma Chemical Co.

Results and Discussion

Effect of O_2 on the Kinetics of Hydrogenase Activity

After evaluating configurations of the various mass spectrometers at NREL, we concluded that a capillary mass spectrometer is most suited for kinetic measurements of low quantity of HD continuously. CO-linked hydrogenase has a very high forward/backward ratio (near 50) of reaction rates measured with methyl viologen for both directions, suggesting that the enzyme prefers H_2 production reaction versus H_2 oxidation reaction. However, the H-D exchange reaction is initiated by a heterolytic cleavage of H_2 molecule to a proton and a hydride prior to exchanging with the deuterium ions in the liquid medium. Based on these criteria, a capillary mass spectrometer would meet our needs since it provides continuous sampling of reactor headspace at near ambient pressure without requiring any carrier gas, which normally would dilute the HD signal significantly.

Figure 1 shows the kinetics of HD production when 3% H_2 was in the atmosphere. The exchange reaction occurred almost immediately upon the addition of D_2O to the bacterial suspension and it proceeded with a linear rate for at least 10 min. Prior measurements indicated that the exchange reaction reached equilibrium after 10-min period (data not shown). Therefore, to study O_2 inhibition, O_2 would have to be added at zero time so any decrease in rates can be attributed entirely to O_2 inactivation, not from reaction reaching equilibrium. Consequently, to quantify O_2 tolerance, we divided the same bacterial suspension into several aliquots and each received D_2O along with various amounts of O_2 to initiate the exchange reaction. Data from Fig. 1 indicate that even though the hydrogenase activity was inhibited with increased levels of O_2, the H-D exchange reaction rate was linear with the simultaneous presence of various amounts of O_2. This result clearly suggests that the hydrogenase was operational in O_2. Most hydrogenases are inhibited irreversibly by even a trace amount of O_2. Our results indicate that CO-linked hydrogenase is not only tolerant, it is also partially functional in the presence of O_2, which has not been reported in literature. This unique feature allows for the genetic transfer of this hydrogenase into a cyanobacterium leading to H_2 production with the simultaneous production of O_2 derived from photosynthetic water-splitting reaction.

Isolation of mutants affected in the water-gas shift reaction

As discussed earlier, one approach to understanding the water-gas shift reaction is to generate mutants affected in the shift reaction and then study the impact on rates. Our strategy of mutant isolation is based on the fact that photosynthetic growth on Na-acetate requires exogenous CO_2, which can be provided entirely via CO shift reaction. Any mutant deficient in the CO shift reaction therefore will not generate enough CO_2 to support growth in acetate with CO while the wild type would. Following the mutagenesis, selection and enrichment scheme according to Methods and after screening approximately 1400 colonies, we identified seven colonies that grew well in acetate/CO_2, but not in acetate/CO, indicating their CO shift pathway could be impaired. However, when these mutants were grown in liquid medium containing malate/yeast

extract/CO, GC analysis of the headspace revealed H_2 production. This conflicting finding prompted us to examine rates of H_2 production in two reactions using GC analysis. We first measured H_2 production from CO, a reaction that requires every component of the CO to H_2 pathway to function. We then measured H_2 production from Na-dithionite and methyl viologen, a reaction that requires only the terminal hydrogenase to be functional. Results are shown in Table 1. Unexpectedly, comparing with parent CBS-2, all mutants show enhanced rates of H_2 production from either CO or reduced methyl viologen. Three of them: E63, G42 and T21 had the most significant increase, presenting great values for enhanced H_2 production if incorporated in an applied system.

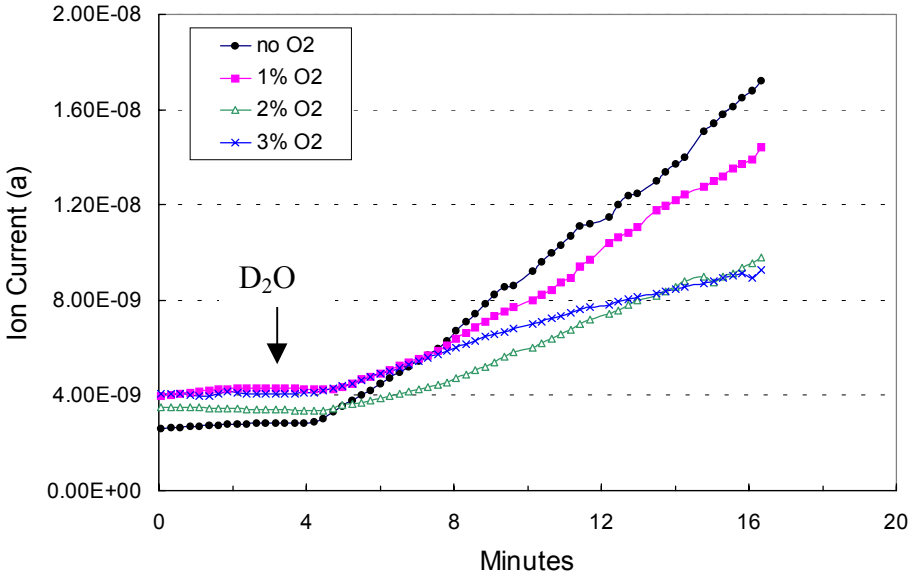

Figure 1. Effect of O_2 on kinetic exchange activity in whole cells of *Rx. gelatinosus* CBS-2

Linking bacterial hydrogenase with a photoreduced ferredoxin

To examine if ferredoxin indeed plays a role in our system, we constructed an *in vitro* assay according to Methods. We used a soluble ferredoxin from either *Clostridium pasteurianum* (bacterial source) or *Spirulina sp.* (blue-green algal source), since ferredoxin of prokaryotic source probably is most compatible with a photosynthetic bacterium.

Figure 2 shows that when an artificial electron mediator such as methyl viologen was used, H_2 production occurred almost immediately when light was turned on, yielding a rate of 1.4 μm H_2/hr/mg chl. When a physiological electron mediator such as *clostridial* ferredoxin was used instead, similar linkage was observed, although at a much lower rate (90 nm H_2/hr/mgchl). When ferredoxin from a blue-green algal source was used, a slightly higher rate was observed (135 nm H_2/hr/mgchl). The linkage is strictly light-dependent. No activity was detected when mediator was not included. These results clearly support our assumption that a ferredoxin-like protein is most likely involved in mediating electron transfer to CBS-2 hydrogenase, whether photoreduced by spinach photosystems or by the CO-oxidation reaction.

Table 1. Isolation of mutants with enhanced CO shift activities

	CO to H_2 (% Activity)	Hydrogenase (% Activity)
D44	134.1	210
D63	111.2	214
E63	160.7	253.2
F52	140	175.2
G42	168.1	273.8
K44	107.1	168.7
T21	156	211
CBS-2 (parent)	100	100

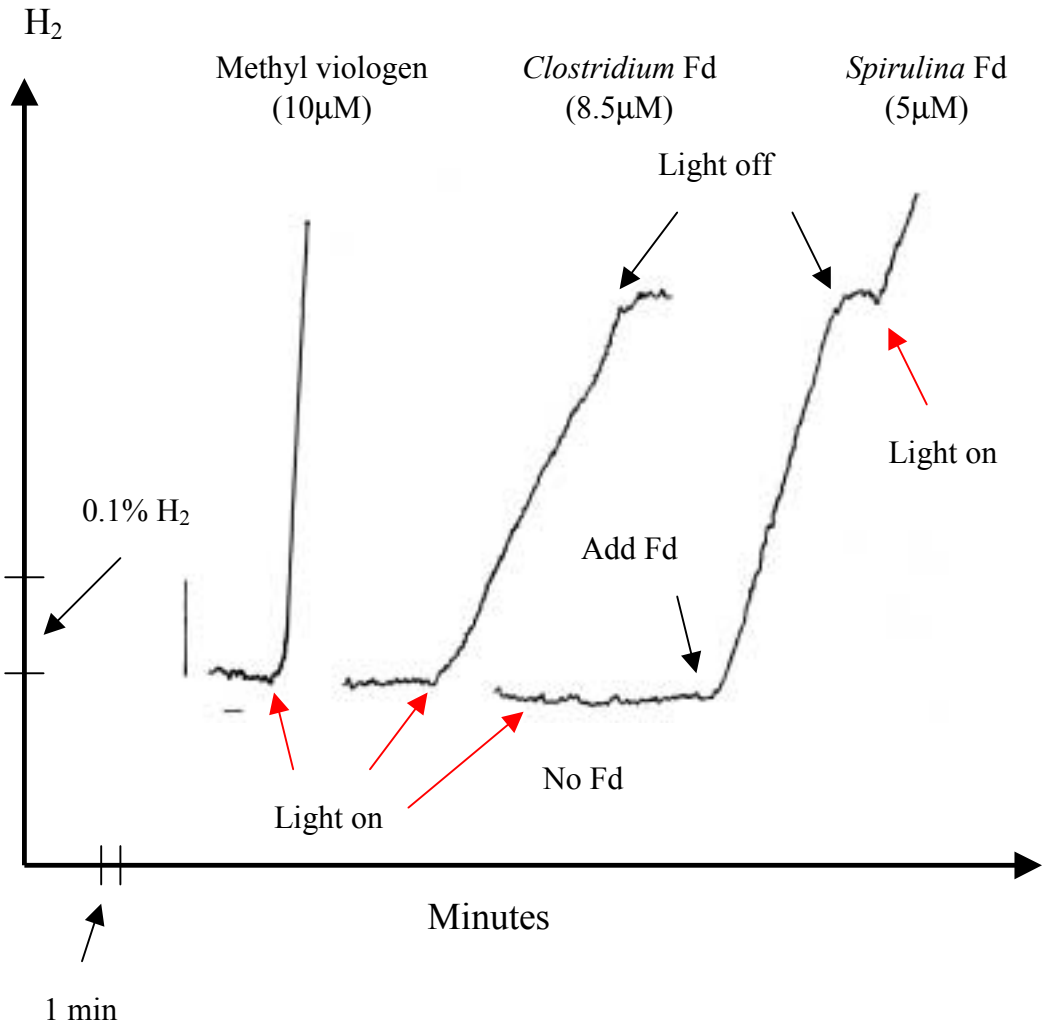

Figure 2. The photo-linkage of spinach thylakoids, CBS-2 hydrogenas, and various electron mediators. Fd: ferredoxin

We used a crude membrane fraction as the source of hydrogenase activity. These membranes presumably also contain other mediators native to the hydrogenase enzyme. Reduced methyl viologen is known to interact directly with the active site of the enzyme bypassing other native components, which explains its high linkage rate. On the other hand, reduced ferredoxin would still need to interact with other mediators before electrons are finally shuffled to the active site of the enzyme. This process will certainly account for the lower reaction rate.

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Our result is supported by findings in the literature. Many evolving hydrogenases have been reported to accept electrons from reduced ferredoxin to mediate H_2 production (Adams *et al.,* 1981). Kemner and Zeikus (1994) reported the involvement of a ferredoxin in the CO-oxidizing: H_2 evolving activity in the acetotroph *Methanosarcina thermophila*. Based on genetics evidence, a ferredoxin-like protein has also been proposed by Ludden and his coworkers (1996) to be involved in CO shift reaction of another photosynthetic bacterium *Rhodospirillum rubrum*.

Conclusions

The thrust of this project is the demonstration of an O_2-tolerant hydrogenase from a novel photosynthetic bacterium *Rx. gelatinosus* CBS-2. Knowing that most hydrogenases are irreversibly inhibited even by a minute quantity of O_2, the CO-linked hydrogenase from CBS-2 is indeed unique with a half-life of 21 hours when stirred in full air. Using a very sensitive kinetic H-D sampling methodology, this report demonstrates that this hydrogenase is also partially functional in O_2. This phenomenon has not been reported in literature with other hydrogenases. H-D exchange reaction solely relies on the proton and deuterium ions interacting with the active site of the enzyme to carry out the H_2-oxidation and H_2-production reactions. Since no reductant nor mediator is involved in the H-D exchange reaction, the tolerance to O_2 is entirely contributed to the intrinsic nature of the active site of the enzyme itself, not owing to an activation of the enzyme by an added reductant or the reduced mediator interacting with O_2. The actual demonstration of a working hydrogenase in O_2 is a significant breakthrough.

The development of the methodology for continuous sampling of HD formation is also a valuable contribution. Operating at near ambient pressure, the capillary leak provides continuous sampling of the HD signal without using any carrier gas. This configuration renders this assay to be extremely sensitive and can be used to evaluate other hydrogenase enzyme for its functionality in the presence of O_2.

Using a scheme designed to isolate mutant with impaired CO shift rates, we acquired seven mutants with enhanced activities instead. We need to re-evaluate this selection/enrichment technique itself as to why the opposite of the concluded result was obtained. One possible

explanation is that the above procedure could have selected for mutants deficient in the CO_2-uptake pathway instead; therefore, cells would have to overly express their CO shift reaction to compensate. Even though we do not fully understand the nature of these mutants, their enhanced rates do present opportunity in an applied system to achieve elevated levels of H_2 production.

Our photoreductant linkage experiment clearly indicates that ferredoxin could play an important role in mediating H_2 production from CO oxidation. This finding is consistent with reports in the literature. The fact that the ferredoxin from a blue-green algal source is more effective than that of a bacterial source is encouraging. This finding makes it possible to transfer bacterial hydrogenase into a cyanobacterium in which the bacterial hydrogenase can then utilize the native ferredoxin of the host to mediate H_2 production reaction *in vivo*.

Future Work

The above kinetic HD determination was conducted with whole cells, which could respire and thus lower the O_2 tension. To complete the O_2-tolerance determinations without complications of cellular respiratory reaction, we need to measure kinetic HD production in O_2 with purified enzyme instead. This requires purified enzyme with sufficient quantity and improved purity. We will continue to develop various detergent extraction steps to improve yield by solubilizing more of the membrane-bound hydrogenase, and subjecting it to chromatography columns more specific to the hydrogenase itself to enhance purity. The purified enzyme can also be used in the photoreductant linkage experiment to further understand the electron mediator involved. Presently the crude membranes used also contain other cofactor besides the hydrogenase. Using a purified enzyme, we will test if it can couple directly with a photo-reduced ferredoxin without contributions from other cofactors. Having seven mutants with enhanced shift rates in our library of culture collection, we plan to determine their enzyme stability over repeated sub-culturing and against selection pressure. Only those stable mutants will have potential applications in an applied system. Once their stability is determined, it may be worthwhile to determine, at genetic levels, the nature of the genotype that accounts for the enhanced rates; namely whether the changes occur at regulation levels or in the hydrogenase gene itself. Eventually, we like to test these mutants in a bioreactor to examine if indeed they would produce elevated levels of H_2 for scale up application.

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