## FINAL PROGRESS REPORT

**Title:** **Fundamental Studies of Recombinant Hydrogenases**

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This research addressed the long term goals of understanding the assembly and organization of hydrogenase enzymes, of reducing them in size and complexity, of determining structure/function relationships, including energy conservation via charge separation across membranes, and in screening for novel $H_2$ catalysts. A key overall goal of the proposed research was to define and characterize minimal hydrogenases that are produced in high yields and are oxygen-resistant. Remarkably, in spite of decades of research carried out on hydrogenases, it is not possible to readily manipulate or design the enzyme using molecular biology approaches since a recombinant form produced in a suitable host is not available. Such resources are essential if we are to understand what constitutes a "minimal" hydrogenase and design such catalysts with certain properties, such as resistance to oxygen, extreme stability and specificity for a given electron donor. The model system for our studies is *Pyrococcus furiosus,* a hyperthermophile that grows optimally at 100°C, which contains three different nickel-iron [NiFe-] containing hydrogenases. Hydrogenases I and II are cytoplasmic while the other, MBH, is an integral membrane protein that functions to both evolve $H_2$ and pump protons. Three important breakthroughs were made during the funding period with *P. furiosus* soluble hydrogenase I (SHI). First, we produced an active recombinant form of SHI in *E. coli* by the co-expression of sixteen genes using anaerobically-induced promoters. Second, we genetically-engineered *P. furiosus* to overexpress SHI by an order of magnitude compared to the wild type strain. Third, we generated the first 'minimal' form of SHI, one that contained two rather than four subunits. This dimeric form was stable and active, and directly interacted with a pyruvate-oxidizing enzyme with any intermediate electron carrier.

### 1. Production of the Native Tetrameric Form in *E. coli* of Hydrogenase I (SHI) of *P. furiosus.*

Hydrogenases are extremely complex, air-sensitive enzymes that achieve catalysis using a binuclear nickel-iron cluster that contain cyanide and carbon monoxide (see **Figure 1**). This cluster is covalently bound to the protein and receives electrons for proton reduction via multiple iron-sulfur (FeS) clusters. A very complicated maturation process is required to synthesize the [NiFe]-catalytic center and insert it into the apoenzyme (see **Figure 1**). We successfully produced in *Escherichia coli* the recombinant form of *P. furiosus* SHI using novel expression vectors for the co-expression of thirteen *P. furiosus* genes (four structural genes encoding the hydrogenase and nine encoding maturation proteins). Remarkably, the native E. coli maturation machinery will also generate a functional hydrogenase when provided with only the genes encoding the hydrogenase subunits and a single protease (frxA) from *P. furiosus*. Another novel feature is that their expression was induced by anaerobic conditions, whereby *E. coli* was grown aerobically and production of recombinant hydrogenase was achieved by simply changing the gas feed from air to an inert gas ($N_2$). The recombinant enzyme was purified and shown to be functionally similar to the native enzyme purified from *P. furiosus*, including its high stability towards oxygen ($t_{1/2} > 20$ hr in air) and the use of NADP(H) as an electron carrier. An enzyme that evolves hydrogen from NADPH holds promise for large-scale harvesting of molecular hydrogen from renewable biomass, and the methodology to produce the key hydrogen-producing enzyme has now been established. The results of this work were published in *PLoS One.*
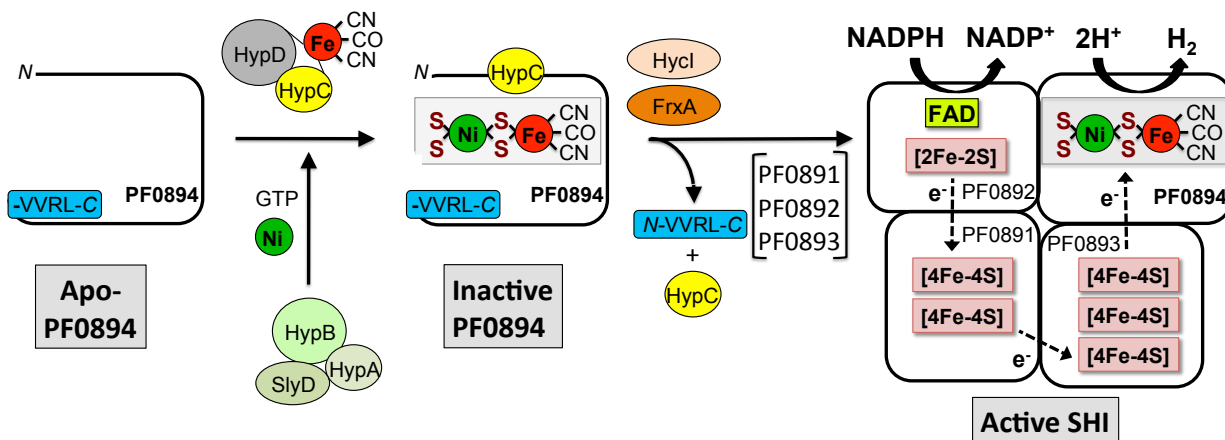
**Figure 1. Biosynthesis of *P. furiosus* hydrogenase I (SHI)**

## 2. Production of a Minimal Forms in *E. coli* of Hydrogenase I (SHI) of *P. furiosus.*

The heterotetrameric holoenzyme form of *P. furiosus* SHI is encoded by four genes, PF0891-PF8094 (**Figure 1**). With a heterologous expression system in hand, we have designed an expression construct for the dimeric (PF0893-PF0894) and the monomeric (PF0894) forms. The recombinant *E. coli* strain containing the monomer did not exhibit hydrogenase activity, but that expressing the dimeric version did produce $H_2$ using an artificial electron donor. This dimeric form of the hydrogenase was less thermostable than the native enzyme ($t_{1/2}$ ~ 24 hr at 90°C) but was still a very stable protein ($t_{1/2}$ ~ 1 hr at 90°C), thereby demonstrating the advantages of using hyperthermophilic enzymes for such studies. In addition, the dimeric form could no longer accept electrons from the physiological donor NADPH, which was not surprising since the other two subunits (PF0891 and PF0892) are required for interaction with NADP(H) (see **Figure 1**). Upon purification of the SHI-dimer by size exclusion chromatography two peaks of hydrogenase activity were observed, one at ~80 kDa corresponding to the dimer (PF0893-PF0894) and one with a mass of ~50kDa. We assume this corresponds to the monomer (PF0894) and is produced by the recombinant strain when the second subunit (PF0893) is either not added or is proteolytically digested by *E. coli*. The 50kDa peak was further purified and yielded a fraction with hydrogenase activity and a Fe:Ni ratio of ≤1, which is expected for the monomeric form. The Fe:Ni value for the dimer is 13 (see **Figure 1**). Further characterization will be needed of the recombinant *E. coli* strain that appears to be producing not only the active dimeric form but also the monomeric form of *P. furiosus* SHI. A monomeric form is unprecedented for a NiFe-hydrogease and obtaining it would represent a major breakthrough.

## 3. Attempts to Obtain Soluble and Membrane Bound Recombinant Forms in *E. coli* of the Membrane Bound Hydrogenise (MBH) of *P. furiosus*.

Having successfully developed an anaerobic expression system in *E. coli* (based on the anaerobically-induced $P_{HYA}$ promoter) to heterologously produce recombinant *P. furious* SHI, our goal was to use the same system to generate in *E. coli* the membrane-bound hydrogenase (MBH) of *P. furiosus.* However, MBH is an exceedingly complex enzyme that both evolves $H_2$ and pumps sodium ions. It is encoded by a 14-gene operon. Bioinformatic analyses indicate that five of the genes (PF1432-PF1436) encode a soluble hydrogenase segment (SMBH), three genes encode a membrane anchor (MA, PF1429-PF1431), and six genes encode a $Na^+/H^+$ antiporter (AP, PF1423-1428). Our goal was to recombinantly produce SMBH (5 genes), SMBH+MA (8 genes) and the complete enzyme system, SMBH+MA+AP (14 genes). All three constructs were expressed under anaerobic conditions together with the nine hydrogenase processing genes of *P. furiosus* (HypCDAB-HycI, HypEF, SlyD-FrxA), and a pRIL plasmid to overcome the rare codon bias in *E. coli.* Unfortunately, none of the three constructs had any detectable hydrogenase activity when grown using *E. coli* expressing SHI as a positive control.

QPCR analyses indicated that the three MBH constructs were being transcribed, and immunoanalysis of the whole cell extract from all three recombinant strains using antibodies to PF1432, the catalytic subunit of MBH, confirmed the presence of PF1432 protein. Hence we concluded that active and assembled *P. furiosus* MBH cannot be produced in *E. coli*.

**4. Over-Production of the Native Tetrameric Form in *P. furiosus* of Hydrogenase I (SHI) of *P. furiosus*.**

A major breakthrough was the development of a genetics system in *P. furiosus* in another on-going project and this was used to develop expression systems for the hydrogenases. This gave us the capability for both heterologous (in *E. coli*) and homologous (in *P. furiosus*) system to engineer *P. furiosus* hydrogenases. Our first objective was to obtain a *P. furiosus* strain in which SHI was significantly over-expressed and where the protein contained an affinity tag for rapid purification. To achieve this we utilized a parental strain that had a deletion in a gene (*pyrF*) required for uracil biosynthesis in *P. furiosus.* This requires uracil for growth and is resistance to the uracil biosynthetic pathway inhibitor, 5-fluoroorotate (5-FOA). A 'knock-in' cassette was constructed containing the upstream (UFR) and downstream (DFR) flanking regions of the promoter region for SHI ($P_{SHI}$), together with *pyrF* (driven by the promoter for glutamate dehydrogenase, $P_{GDH}$), a terminator sequence (T1) and replacing $P_{SHI}$ with $P_{SLP}$, the promoter for the S-layer protein (PF1399), and the strep-II tag (8 amino-acids) at the N-terminus of the PF0891 subunit of SHI. $P_{SLP}$ is one of the most highly expressed genes in *P. furiosus* based on our DNA microarray data. The construct is shown in **Figure 2**. The recombinant selection is based on the acquisition of uracil prototrophy in defined medium lacking uracil (recombinants are also sensitive to 5-FOA). Recombinants gave rise to the expected PCR product compared to the parental D*pyrF* strain and this was confirmed by DNA sequencing (**Figure 2**)*.

The specific hydrogenase activity of cytoplasmic extracts of wild type *P. furiosus* cells and the Δ*pyrF* strain were 0.77 ± 0.5 units/mg. The values for the two strains where SHI was over-expressed (OE-SHI) by $P_{SLP}$ were 8.2 ± 0.2 units/mg, representing an increase of more than an order of magnitude. The dramatic overexpression of SHI protein in the OE-SHI strains was confirmed by immuoanalysis using antibodies to PF0894 (the catalytic subunit of SHI). Given the extremely complex maturation process that is involved in synthesizing the hydrogenase, the results show that the wild-type level of the maturation machinery is able to process >10-fold more SHI than it usually does. This was somewhat surprising since no attempt was made to up-regulate these processing genes. DNA microarray analysis of the OE-SHI strains are planned to determine if the expression of one or more of the unlinked processing genes are affected by over-expression of the SHI structural genes. In contrast to SHI produced in wild-type *P. furiosus,* which requires five chromatography steps to obtain pure proteins, Step-II-Tagged Over-Expressed SHI (STOE-SHI) was purified in two steps, using a DEAE-column and a Strep-Tactin affinity column, to electrophoretic homogeneity. From 25 g (wet weight) of the recombinant *P. furiosus* cells we obtain 11 mg of pure STOE-SHI, compared to 0.6 mg of SHI from the same amount of wild type cells using our traditional purification procedure. The results of this work were published in *J. Biological Chemistry*.

**5. Development of a Second Selection for Genetic Manipulation in *P. furiosus*.**

While obtaining the Δ*pyrF* strain of *P. furiosus* was a major breakthrough that enabled genetics in this organism, we sought an additional and more robust selection method that could also be used with a variety of rich growth media (that inadvertently contain uracil). Based on a report with *Thermococcus kodakorensis,* we chose PF1623, which is involved in polyamine biosynthesis. A knockout creates a strain that requires agmatine for growth, a compound not present in any rich growth media. The PF1623 deletion was made by double crossover recombination between the ΔpyrF/ΔSH1 host and a PCR product containing the *pyrF* gene (driven by $P_{GDH}$) and the UFR and DFR of PF1693. The resulting ΔPF1623/ΔSH1 mutant requires at least 2 mM agmatine to support normal growth even in a rich medium.
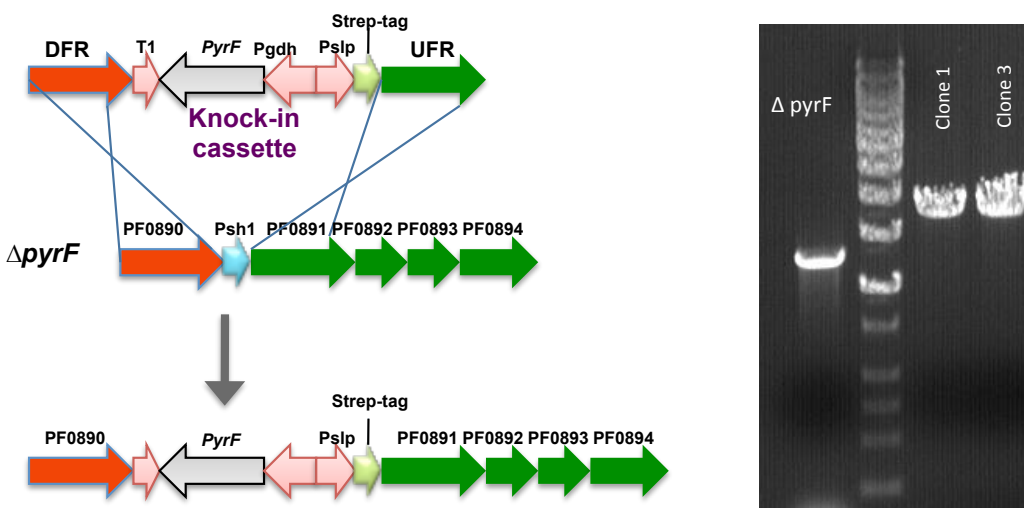
**Figure 2.** Knock-in construct for over-production of SHI (*left*) and PCR products obtained from parental Δ*pyrF* strain and two recombinant OE-SHI strains (*right*).

The double deletion ΔPF1623/ΔSH1 strain was used in a straightforward procedure to overexpress various mutant forms of SHI in *P. furiosus*. A DNA fragment containing the PF1623 gene, the SHI construct (with the appropriate promoter and affinity tags) and the UFR and DFR of PF1623 is obtained by overlapping PCR. This is transformed into the double mutant. Transformants have the ability to grow in media without the addition of agmatine. This allowed the construction of the His$_{10}$-tagged dimer (His-PF0893-PF0894) form of SHI. The heterodimeric form is highly active (150 units mg) in H$_2$ production using the artificial electron donor methyl viologen) and thermostable (t$_{1/2}$, 0.5 hour at 90°C). Moreover, the heterodimer does not use NADPH and instead can directly utilize reductant supplied by pyruvate ferredoxin oxidoreductase from P. furiosus. The SHI heterodimer and POR therefore represent a two-enzyme system that oxidizes pyruvate and produces H$_2$ in vitro without the need for an intermediate electron carrier.

## PEER-REVIEWED PUBLICATIOND RESULTING FROM THIS RESEARCH

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2.  Baker, S. E., Hopkins, R. C., Blanchette, C. D., Walsworth, V. L., Sumbad, R., Fischer, N. O., Kuhn, E. A., Coleman, M., Chromy, B. A., Létant, S. E., Hoeprich, P. D., Adams, M. W. W., and Henderson, P. T. (2009) "Hydrogen production by a hyperthermophilic membrane-bound hydrogenase in soluble nanolipoprotein particles" *J. Am. Chem. Soc.* 131, 7508-9
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4.  Chandrayan, S. K., McTernan, P. M., Hopkins, R. C., Sun, J., Jenney, F. E. and Adams, M. W. W. (2012) "Engineering the hyperthermophilic archaeon *Pyrococcus furiosus* to overproduce its cytoplasmic [NiFe]-Hydrogenase" *J. Biol. Chem.* **287,** 3257-3264
5.  Hopkins, R. C., Sun, J., Jenney, F. E., Chandrayan, S. K., McTernan, P. M. and Adams, M. W. W. (2011) "Homologous expression of a subcomplex of *Pyrococcus furiosus* hydrogenase that interacts with pyruvate ferredoxin oxidoreductase" *PLoS ONE* **6,** e26569