Title: Novel Hydrogen Production Systems Operative at Thermodynamic Extremes

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Final Report

We have employed a suite of molecular, bioinformatics, and biochemical tools to interrogate the thermodynamically limiting steps of H₂ production from fatty acids in syntrophic communities. We also developed a new microbial model system that generates high H₂ concentrations (over 17% of the gas phase) with high H₂ yields of over 3 moles H₂ per mole glucose. Lastly, a systems-based study of biohydrogen production in model anaerobic consortia was performed to begin identifying key regulated steps as a precursor to modeling co-metabolism. The results of these studies significantly expand our ability to predict and model systems for H₂ production in novel anaerobes that are currently very poorly documented or understood.

Aim 1. Determine the mechanism of hydrogen production from acyl-CoA intermediates during fatty and aromatic acid metabolism. *Clostridium kluyveri* has a soluble butyryl-CoA:electron transfer flavoprotein oxidoreductase system that couples the energetically favorable reduction of crotonyl-CoA to butyryl-CoA by NADH with the unfavorable reduction of ferredoxin by NADH. The genome of *Syntrophomonas wolfei* contains nine acyl-CoA dehydrogenase genes, one of which is associated with genes for the alpha and beta subunits of a hypothetical electron transfer flavoprotein. The products of this latter gene could be a butyryl-CoA:ETF oxidoreductase that catalyzes reverse electron transfer using a bifurcation mechanism as shown for the *C. kluyveri* enzyme.

1A. Does *S. wolfei* contain a butyryl-CoA:ETF oxidoreductase similar to that present in *C. kluyveri*? We partially purified the butyryl-CoA dehydrogenase from *S. wolfei* by anion exchange chromatography and a salt step-gradient under anaerobic conditions to test the hypothesis that the enzyme catalyzes the concomitant reduction of crotonyl-CoA and ferredoxin with NADH as the electron donor. The highest activity (7,100 nmol min⁻¹ mg protein⁻¹) was detected in a fraction, which eluted with 200 mM NaCl after anion exchange chromatography. NADH oxidation was not detected when crotonyl-CoA and NADH were added. The addition of FAD and fractions eluted with higher salt concentrations (>.35 M NaCl) did not result in increased NADH oxidation over background. Subunit composition was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. High concentrations of a large subunit having an apparent molecular mass of 68 kDa were present in *S. wolfei* grown axenically on crotonate. These results are consistent with the presence of a butyryl-CoA dehydrogenase/electron transfer flavoprotein complex as found in *C. kluyveri*. In support of this, genomic analysis of *S. wolfei* revealed that the 68-kDa large subunit may be encoded by either SYN_2052 or SYN_0384.

1B. Metabolism of *S. wolfei* revealed by proteomics. Multiple homologues of the enzymes involved in beta-oxidation were detected in *S. wolfei* cells. Eight of the nine butyryl-CoA dehydrogenases encoded on the genome were detected in the proteome. The most abundant butyryl-CoA dehydrogenase is encoded by Swol_2052. Three other butyryl-CoA dehydrogenases, encoded by Swol_0268, Swol_0384, and Swol_2126, are abundant at the same concentration during growth in butyrate. The butyryl-CoA dehydrogenases encoded by Swol_1933 were only detected under syntrophic growth on butyrate. Swol_1933 is in a gene cluster with a CoA transferase, an enoyl-CoA hydratase, a 3-hydroxybutyryl-CoA dehydrogenase, and an acetyl-CoA acetyltransferase, all of which were detected in the proteome and more abundant when the methanogen was present. Acetate kinase, encoded by Swol_0768, and phosphotransacetylase, encoded by Swol_0767, is detected at similar levels during all conditions, showing that they are constitutively expressed. The peptides corresponding to the gene cluster

Swol_1483-Swol_1486, encoding for a butyryl-CoA dehydrogenase, an enoyl-CoA hydratase, a 3-hydroxybutyryl-CoA dehydrogenase, and an acetate kinase, were not consistently detected. These studies revealed the core pathway for metabolism of four carbon acids in this model syntrophic bacterium.

Aim 2. Develop new model microbial hydrogen-producing systems: the novel anaerobe, *Anaerobaculum hydrogeniformans* strain OS1. The ability of *A. hydrogeniformans* strain OS1 to generate H₂ at concentrations up to 17% suggests a previously undescribed system for H₂ production from carbohydrates (e.g., not involving NADH).

2A. A formal taxonomic study of *Anaerobaculum hydrogeniformans* strain OS1 was performed. We conducted detailed fatty acid profile analysis of the three species in the genus *Anaerobaculum*, *A. hydrogeniformans*, *A. thermoterrenum* DSM 13490, and *A. mobile* DSM 13181. Strain OS1 and *A. thermoterrenum* were shown to contain the phospholipids PL4 and PL5 but these were undetected in *A. mobile*. These plus other findings define *A. hydrogeniformans* strain OS1 as a new species. It represents the type species of the genus. A manuscript describing *Anaerobaculum hydrogeniformans* OS1 was recently published and it has been deposited in the DSMZ culture collection (DSM 22491) and the ATCC collection (BAA-1850).

2B. Range of environmental conditions for cell growth. The production of hydrogen from waste materials that could be low cost substrates for commercial biohydrogen production was investigated. H₂ production by *A. hydrogeniformans* from brewery wastewater, sweet sorghum juice, cheese whey wastewater, raw sewage and anaerobic digester sludge was evaluated. Clarification of brewery wastewater, cheese whey wastewater, raw sewage, and anaerobic digester sludge by autoclaving, centrifugation, and pH adjustment resulted in higher H₂ yields than unclarified wastes. The maximum rate of hydrogen production was 13 ml H₂ L⁻¹ h⁻¹ with glucose as the substrate and 9 ml H₂ L⁻¹ h⁻¹ with clarified brewery wastewater. Final gas phase, hydrogen levels were 955 ml H₂ L⁻¹ and 623 ml H₂ L⁻¹ with glucose and clarified brewery wastewater as substrates, respectively. When grown in a mineral medium with glucose as the substrate, *A. hydrogeniformans* produced 116 ml H₂ g⁻¹ COD reduced. When grown on the clarified feedstocks the following H₂ yields were obtained: clarified raw sewage, 91.2 ml H₂ g⁻¹ COD; clarified cheese whey wastewater 17.1 ml H₂ g⁻¹ COD; sweet sorghum juice 10.3 ml H₂ g⁻¹ COD; clarified brewers wastewater 6.68 ml H₂ g⁻¹ COD; and anaerobic digester sludge 2.81 ml H₂ g⁻¹ COD. Significantly, our work shows that *A. hydrogeniformans* can be used to produce H₂ from waste feedstocks while significantly reducing COD in all wastewaters tested (84-99 % reduction in COD). With this information it is now possible to develop a targeted approach for process design and medium optimization for future large-scale H₂ production facilities. The yield of H₂ per mole of glucose remained high, 3 to 4 moles H₂ per mole glucose, under all growth conditions.

2C. Strain physiology and glucose metabolism. We determined the optimal fermentation conditions to elucidate the limiting factors for *A. hydrogeniformans* when grown on glucose. Labeling studies indicated that a modified Embden-Meyerhof-Parnas is present in *A. hydrogeniformans*. Cultures grown with [1-¹⁴C]-D-glucose had only small amounts of radioactivity in the headspace after near depletion of glucose. Over 95% of the label remained in the liquid phase after acidification. Thus, strain OS1 does not convert the 1-C of glucose to CO₂ as do organisms that have the Entner-Doudoroff or pentose phosphate pathways. We detected significant amounts of ¹³CO₂ in cultures grown on [3-¹³C]-D-glucose. NMR labeling studies did not detect significant labeling of the methyl group of acetate when cells were grown on [1-¹³C]-D-glucose. Cultures grown with [1-¹³C]-D-glucose or [3-¹³C]-D-glucose were subjected to GC-MS analysis. Acetate with a one mass unit increase was detected in cultures grown with [1-¹³C]-D-glucose, but not in those grown with [3-¹³C]-D-glucose, compared to culture growth with unlabeled glucose. These labeling studies are consistent with the operation of the Embden-Meyerhof-Parnas pathway and not by the Entner-Doudoroff or pentose phosphate pathways.

Enzyme activities also indicate the operation of a modified Embden-Meyerhof-Parnas pathway. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was not detected in *A. hydrogeniformans* using NAD^+ or NADP^+ as electron acceptors. However, an enzyme activity for a glyceraldehyde-3-phosphate:ferredoxin oxidoreductase (GAPOR) was detected ($41 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$). The oxidation of pyruvate to acetate is accomplished by a pyruvate:ferredoxin oxidoreductase ($2150 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$), phosphotransacetylase ($785 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$) and acetate kinase ($1270 \text{ nmol min}^{-1} \text{ mg protein}^{-1}$). Regarding electron and proton disposal, hydrogenase activity was present in both soluble (cytoplasmic) and insoluble (membrane fractions). Formate dehydrogenase activity was not detected. These studies establish the basis to explore the energetics and thermodynamics of strain OS1 metabolism.

2D. Metabolomic analysis of OS1 physiology. Metabolites produced by *A. hydrogeniformans* during different stages of growth were evaluated using services at Metabolon, Inc., Durham, NC. All of the intermediates of the Embden-Meyerhof-Parnas pathway were detected with the exception of glyceraldehyde-3-phosphate and 1,3-bisphosphoglycerate. Glyceraldehyde-3-phosphate has been shown to be an unstable metabolite, which makes its detection difficult. The production of 1,3-bisphosphoglycerate would not be expected in an organism that contains GAPOR, which directly oxidizes glyceraldehyde-3-phosphate to 3-phosphoglucerate. Key metabolites of the Entner-Doudoroff pathway, such as 6-phosphoglycerate and 2-keto-3-deoxy-6-phosphogluconate (KDPG), were not detected. These studies represent the first report of a eubacterial species that oxidizes glucose via a ferredoxin-dependent pathway, such as that previously thought to occur only in hyperthermophilic archaeal species.

2E. Genome sequencing, annotation, OS1 metabolic reconstruction. A genome sequence analysis of *A. hydrogeniformans* strain OS1 was performed in collaboration with the University of Washington Genome Center in St. Louis, MO. The genome draft sequence consists of one large contig containing 2,127,725 base pairs of DNA, one circular plasmid of about 5 KB, plus several small contigs that may or may not be within a small DNA region spanning a genomic repetitive sequence. There are 2271 orfs of which 2237 are contained in the large contig. These include 2208 protein coding orfs plus an additional 47 orfs of tRNA's, 2 rRNAs and 14 apparent sRNA's. The DNA sequence was annotated, manually inspected, and deposited into genbank with accession number gi:226331783. An updated and refined version of the strain OS1 genome sequence is being prepared for submission in the summer of 2012. A metabolic reconstruction of the *A. hydrogeniformans* strain OS1 genome was prepared and it revealed the central pathway for glucose catabolism to acetate plus many features of cell biosynthesis from sugar pathway intermediates.

Aim 3. Perform systems-based studies of biohydrogen production in model syntrophic consortia and in pure cultures to identify key regulated steps. We examined genes and gene systems involved in syntrophic H_2 production from fatty acids using *S. wolfei* as a model system.

3A. Transcript profiling of biohydrogen production. Analysis of the genome of *S. wolfei* revealed multiple ways to produce H_2 including a membrane-bound hydrogenase, a soluble NADH-dependent hydrogenase, and a highly conserved, membrane-bound FeS oxidoreductase may be involved in the reverse electron transfer reactions leading to H_2 production. To determine if any of these NADH-dependent hydrogenases play an important role in syntrophic metabolism, we used quantitative reverse transcriptase PCR (Q-RT-PCR). RNA was harvested and isolated from mid-exponential phase *S. wolfei* grown in co culture with *Methanospirillum hungatei* on the substrates butyrate or crotonate and in pure culture on crotonate. All of the hydrogenases and the FeS oxidoreductase were expressed under all the conditions tested. The NADH-dependent hydrogenase gene, Swol_1017, was also highly expressed under syntrophic growth conditions. This leads us to predict that the NADH-dependent hydrogenase encoded by Swol_1017, is important for syntrophy.

3B. Protein profiling of biohydrogen production by *S. wolfei*. We analyzed the proteome of *S. wolfei* grown axenically on crotonate and syntrophically with *M. hungatei* with either crotonate or butyrate as the substrate. The proteomic analysis was done in collaboration with Dr. Gregory Hurst at Oak Ridge National Laboratory. Approximately 1090 unique peptides were detected in total across three growth conditions, 912 gene products were detected under axenic, crotonate grown conditions, of which 156 peptides were detected only under axenic conditions. A total of 656 gene products were detected when *S. wolfei* was grown on crotonate with *M. hungatei*, of which 21 peptides were unique to this growth condition. Eight hundred and eighty two peptides were detected in butyrate grown cells with *M. hungatei*, and 125 of those were only found in this growth condition. Five hundred and sixty eight gene products were detected under all three conditions, while 78 of those appear to be constitutively expressed (less than 0.5x fold change over all conditions). Only a relatively small set of proteins, 34 gene products, were detected when *S. wolfei* was grown with the methanogenic partner *M. hungatei*.

Three potential routes for reverse electron transfer were hypothesized for *S. wolfei*, a bifurcating butyryl-CoA dehydrogenase, an electron transfer flavoprotein:quinone oxidoreductase (Fix system) and a membrane-bound FeS oxidoreductase with adjacent electron transfer flavoproteins. The set of electron transfer flavoproteins, encoded by Swol_0696 and Swol_0697, which are adjacent to Swol_0698, which encodes the putative membrane-bound FeS oxidoreductase, were among the most abundant proteins detected in the proteome. The proteomic analysis indicates that the membrane-bound FeS oxidoreductase system rather than Fix is the dominant mechanism used for electron flow during beta-oxidation.

Polypeptides for all three *S. wolfei* hydrogenases were detected in all growth conditions. Thus, this syntroph appears to use multiple systems to make hydrogen, including a Fe-only hydrogenase, a membrane-bound hydrogenase, and a putatively confurcating Fe-only hydrogenase. Hydrogenase activity has been detected in both the membrane and the soluble portion of the cells of *S. wolfei*, during axenic and syntrophic growth on multiple substrates, demonstrating that the hydrogenases detected in the proteome are in fact active.

3C. Computational approaches to study hydrogen production. We used computational tools to further explore and model the above organisms. Metabolic reconstructions of *S. wolfei* genomic information were generated and refined to provide a framework to better understand and predict the genes/systems needed for H₂ production in this model syntrophic bacterium. Potential transcript units were proposed to begin distinguishing how the various acyl-CoA dehydrogenases, electron transfer flavoproteins (ETF), and other oxidoreductases genes are controlled by cell culture conditions. We also identified paralogous enzyme systems potentially utilized by other syntrophic microorganisms based on their genomic DNA sequences. These studies address the genes systems needed for the most critical H₂ production reactions. They also increase our understanding of the regulatory and metabolic networks used in nature for H₂ production. In the longer term it is anticipated that the findings generated in this study can be used to engineer applied applications of hydrogen production.

Papers and other products delivered:

There are eight publications resulting in part from this project plus one manuscript currently under review. Two additional manuscripts are in preparation that will describe the proteome of *S. wolfei* grown syntrophically with special emphasis on the enzymes systems needed for syntrophic hydrogen production, plus a study describing biohydrogen production from waste feedstocks by *A. hydrogeniformans*.

Publications:

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3. Maune, M. W., and R. S. Tanner. 2012. *Anaerobaculum hydrogeniformans* sp. nov., a novel anaerobe that produces hydrogen from glucose. *International Journal of Systematic and Evolutionary Microbiology*, 62:832-838.
4. McInerney, M. J., J. R. Sieber, and R. P. Gunsalus. 2011. Microbial syntrophy: ecosystem-level biochemical cooperation. *Microbe* 6: 479-485.
5. Sieber, J. R., M. J. McInerney, and R. P. Gunsalus. 2012. Genomic insights into syntrophy: the paradigm for anaerobic metabolic cooperation. *Ann. Rev. Microbiol.* V 66: (published online ahead of press, publication date October)
6. McInerney, M.J., T. M. Hoehler, R. P. Gunsalus, B. Schink. 2010. Introduction to microbial hydrocarbon production: bioenergetics. In: K. N. Timmis, V. de Lorenzo, T. McGenity, and J. R. van der Meer (eds.): *Handbook of Hydrocarbon and Lipid Microbiology. Hydrocarbons, Oils, Lipids: Diversity, Properites, and Formation*, Springer-Verlag GmbH, Heidelberg, Germany. Chapter 21, pp. 322-335. ISBN: 978-3-540-77588-1
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8. Hoehler, T.M., R. P Gunsalus and M. J. McInerney. 2010. Environmental constraints that limit methanogenesis. In: K. N. Timmis, V. de Lorenzo, T. McGenity, and J. R. van der Meer (eds.): *Handbook of Hydrocarbon and Lipid Microbiology. Hydrocarbons, Oils, Lipids: Diversity, Properites, and Formation*, Springer-Verlag GmbH, Heidelberg, Germany. Chapter 51, pp. 636-654. ISBN: 978-3-540-77588-1

Submitted:

9. Plugge, C.M., A.M. Henstra, P. Worm, A.H. Paulitsch, J.C.M. Scholten, A. Lykidis, A.L. Lapidus, E. Goltsman, E. Kim, E. McDonald, L. Rohlin, B.R. Crable, R.P. Gunsalus, A.J.M. Stams, and M.J. McInerney. 2012. Complete genome sequence of *Syntrophobacter fumaroxidans* strain (MPOB^T). SIGS

Most recent notes concerning the project:

Several new opportunities were identified to speed and complement the original stated aims. We have recently received data from proteomic analysis of *S. wolfei* grown axenically with crotonate and syntrophically with *Methanospirillum hungatei* with crotonate or butyrate. The proteomic studies are described briefly in Aim 3, and the resulting findings should provide significant new insight regarding “syntrophic” functions needed for hydrogen transfer. Second, we are attempting to set up metabolomics for *A. hydrogeniformans* OS1 strain to identify pathway intermediates for pathway verification. Third, we have initiated development of an *E. coli* gene expression system for *S. wolfei* genes with the goal of obtaining biochemical amounts of selected proteins for additional characterization. While these complementary experiments are not fully vetted, they show considerable promise to speed the

gene/protein functional studies of the model organism. Finally, without interrupting key proposed experiments, we have begin exploratory cell plating experiments with *S. wolfei* and for *M. hungatei*, where the latter is a syntrophic partner for the fatty acid degrader. The goal is to lay the foundation to begin opening up a genetic system for study of these model organisms. While of low priority, these projects have potential to speed progress towards understanding the novel central carbon and/or electron flow proteins needed for bio-hydrogen production.

Project URL

none

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