UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

NADH-DEPENDENT, FERREDOXIN-INDEPENDENT HYDROGEN PRODUCTION IN

TWO MODEL SYNTROPHIC BACTERIA

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

Degree of

DOCTOR OF PHILOSOPHY

By

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Norman, Oklahoma
2019

NADH-DEPENDENT, FERREDOXIN-INDEPENDENT HYDROGEN PRODUCTION IN
TWO MODEL SYNTROPHIC BACTERIA

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF MICROBIOLOGY AND PLANT BIOLOGY

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# TABLE OF CONTENTS

List of Tables

# List of Figures

**Abstract**

The relationship between a fatty acid-oxidizing, hydrogen-producing bacterium and a hydrogen consuming methnogenic partner has longed served as a model system for anaerobic syntrophic metabolism. This process is dependent on low hydrogen and formate concentrations maintained by the methanogenic partner to allow the fatty acid-oxidizing bacterium to continually re-oxidize cofactors such as NADH that are generated by fatty acid oxidation. Two multimeric [FeFe]-hydrogenases from two different syntrophic model organisms, *Syntrophomonas wolfei* and *Syntrophus aciditrophicus* were produced by heterologously expressing the genes encoding for two [FeFe]-hydrogenase and genes for [FeFe]-hydrogenase maturation in *Escherichia coli*. The *S. wolfei* recombinant Hyd1ABC was found to consist of three subunits Hyd1A, Hyd1B, and Hyd1C with molecular weights of 63.0 kDa, 43.0 kDa and 13.0 kDa. *S. wolfei* Hyd1ABC was observed as a heterotrimer with an $\alpha\beta\gamma$ configuration and a molecular mass of 115 kDa. The *S. aciditrophicus* purified recombinant HydAB consisted of two subunits HydA and HydB with molecular weights 68.5 kDa and 66.0 kDa and suggested to consist of a $\alpha_2\beta_2$ configuration. Both the *S. wolfei* Hyd1ABC and *S. aciditrophicus* HydAB were found to re-oxidize the cofactor NADH in a ferredoxin-independent manner. These NADH-dependent, ferredoxin-independent hydrogen production mechanisms provide *S. wolfei* and *S. aciditrophicus* with an energetic advantage under syntrophic conditions compared to the alternative which is NADH-dependent, ferredoxin-dependent electron confurcating hydrogen production. It is proposed that several differences in conserved residues within the HydB subunits are responsible for the observed ferredoxin-independent nature of these enzymes. These results demonstrate that NADH-dependent, ferredoxin-independent hydrogen production occurs in two phylogenetically distant organisms, *S. wolfei* a member of the phylum Firmicutes and *S.*

*aciditrophicus* a Deltaproteobacteria. This suggests a unifying theme for syntrophic fatty acid-oxidizing bacteria, in the form of NADH-dependent, ferredoxin-independent hydrogen production.

Hydrogen partial pressures determined during pure culture and co-culture growth suggest that both *S. wolfei* and *S. aciditrophicus* must maintain high NADH/NAD$^+$ ratios for NADH-dependent, ferredoxin-independent hydrogen production. NADH-dependent, ferredoxin-independent hydrogen production completes the pathway for how high redox potential electrons generated from 3-hydroxyacyl-CoA oxidation are used to produce hydrogen. It could be suggested that the observed dependence of syntrophic metabolizers on a hydrogen consuming partner is the result of a dependence on cofactor re-oxidation methods that utilize specifically high redox potential electrons. Other syntrophic metabolisms may be predicted to rely on mechanisms for re-oxidizing cofactors that also specifically utilize high redox potential electrons for hydrogen and formate production or direct interspecies electron transfer.

# CHAPTER 1: Hydrogen Production During Syntrophic Fatty Acid and Aromatic Acid Oxidation

**Importance of Anaerobic Syntrophy in Global Carbon Cycling**

Methane is of interest currently because it is a potent greenhouse gas and because of its potential as a renewable energy source (Stams and Plugge, 2009; Angelidaki et al., 2011). Methane-producing microorganisms, called methanogens, are obligately anaerobic Archaea that produce methane from a variety of substrates including hydrogen, formate, methyl-containing compounds and acetate. Methanogenesis plays a key role in the global carbon cycle due to the production of methane in anaerobic environments where it is estimated that approximately one gigaton of methane is produced each year (Thauer et al., 2008). Methanogenesis becomes the only process for anerobic decomposition of organic matter when all other terminal electron acceptors (such as nitrate, sulfate, oxygen) are depleted (Stams and Plugge, 2009). The process of methanogenesis in anaerobic environments involves multiple steps. The first step is hydrolysis of polymeric substrates such as polysaccharides, lipids, and proteins followed by primary fermentation to produce various organic compounds including alcohols, organic acids (acetate, propionate, lactate, butyrate), hydrogen, formate and $CO_2$ (McInerney et al., 2009). Some of these primary fermentation products (hydrogen, formate, acetate, and certain methyl-containing compounds) can be utilized directly by methanogens. Other primary fermentation products such as primary alcohols, propionate, longer chain fatty acids, and lactate cannot be utilized directly by methanogens. The conversion of these remaining primary fermentation products to substrates suitable to methanogenesis (hydrogen, formate or acetate) is performed by a group of metabolic specialists in a process termed syntrophy (McInerney et al., 2009). There are a number of types of microbial associations that have been referred to as syntrophy such as amino acid cross feeding (Nelson, 1951), the sharing of other metabolites required for biosynthesis (Pacheco et al., 2018), and other mutualistic interactions (Morris et al., 2013). However, for the remainder of this

document, I use the term syntrophy to refer to a specific interaction that occurs under anaerobic conditions between a bacterium oxidizing an organic compound (a primary fermentation product) that is dependent on having low hydrogen/formate levels maintained by a hydrogen/formate-consuming microorganism such as a methanogen or sulfate reducer (McInerney et al., 2009). Syntrophy involving the oxidation of these primary fermentation products and the transfer of the resulting high redox potential electrons to methanogens is the rate-limiting step in anaerobic decomposition of carbon (McInerney et al., 2009). Microorganisms that grow on these primary fermentation products deal with the difficult task of producing hydrogen and formate from high redox potential electrons while also conserving energy for growth (Schink, 1997; McInerney et al., 2009). Syntrophic metabolizers perform a critical role in global carbon cycling but face unique thermodynamic challenges related to the production of hydrogen or formate and energy conservation.

**The Unique Challenge of Syntrophy: Re-Oxidizing the High Redox Potential Electron Carriers NADH and Electron Transfer Flavoprotein**

Understanding the unique metabolic machinery and physiology of syntrophic metabolizers, particularly related to their ability to produce hydrogen or formate from high redox potential electrons, is crucial to fully understanding the key steps that control global anaerobic carbon decomposition. During syntrophy, the oxidation of the primary fermentation products results in electrons of high redox potentials, which are carried by the reduced cofactors NADH and electron-transferring flavoprotein (Etf). Using electrons of such high redox potentials to produce formate and hydrogen presents a thermodynamic challenge. The oxidation-reduction potential of hydrogen under standard conditions (partial pressure of 1 atm) is ($E_0' = -414$ mV).

Similarly, the oxidation-reduction potential of formate under standard conditions (1 M) is ($E_0' = $ -432 mV). However, the electron donors used to produce hydrogen or formate are estimated to be much higher in potential, NADH ($E_0' = $ -320 mV (Thauer et al., 1977)) and $Etf_{red}$ ($E' = $ -10 mV to $E' = $ -126 mV depending on butytryl-CoA/crotonyl-CoA redox potential). Estimates of the butytryl-CoA/crotonyl-CoA pair have varied from as low as $E_0' = $ -126 mV (Gustafson et al., 1986), $E_0' = $ -79 mV (Fink et al., 1986), to as high as $E_0' = $ -10 mV (Sato et al., 1999). The process of oxidizing a high redox potential donor and transferring electrons to a lower redox potential donor is energetically unfavorable. However, in syntrophic co-cultures the hydrogenotrophic methanogen partner can maintain hydrogen concentrations as low as 1 Pascal which corresponds to a higher redox potential for hydrogen ($E' = $ -260 mV) (Sieber et al., 2012). This is also true for the redox potential of formate, which is $E' = $ -290 mV at 10 µM formate (Sieber et al., 2012). Thus, as long as hydrogen or formate are maintained at low concentrations, which raises their predicted redox potentials, it is thermodynamically favorable to produce hydrogen from electrons with a high redox potential. The maintenance of the low levels of hydrogen and formate in natural environments is likely the function of hydrogenotrophic methanogens. Producing large amounts of biomass from syntrophic metabolizers can prove challenging and as a result there have been a limited number of biochemical investigations. In many cases, the enzymatic machinery used by syntrophic metabolizers to convert high redox potential electrons to hydrogen or formate has been inferred from studies on primary fermenters. As primary fermenters produce reduced cofactors pools with electrons at much lower redox potentials such as reduced ferredoxin and typically produce much higher hydrogen concentrations than syntrophic metabolizers do, it is possible that these inferences about syntrophic metabolism may ultimately prove to be incorrect.

4

*Syntrophomonas wolfei* is a Gram-positive rod-shaped bacterium and is phylogenetically a member of the phylum Firmicutes (McInerney et al., 1981; Sieber et al., 2010). *S. wolfei* serves as a model for the syntrophic metabolism of the short-chain fatty acids such as butyrate to the methanogenic substrates: hydrogen, formate and acetate. The sequencing of the *S. wolfei* genome identified key enzymes required for syntrophic metabolism (Sieber et al., 2010) but did not detect genes needed to grow by primary fermentation or by respiration, suggesting *S. wolfei* may be an obligate syntrophic metabolizer. Physiological studies of *S. wolfei* have supported this conclusion as *S. wolfei* specializes in growing under syntrophic conditions with the notable exception of its ability to grow in pure culture on unsaturated fatty acids, such as crotonate (McInerney et al., 1981; Beaty and McInerney, 1987). It is unclear as to how available unsaturated fatty acids, such as crotonate are in the environment (Wilson et al., 2017), but it seems unlikely that crotonate metabolism is *S. wolfei*'s ecological function. Additional proteomic studies further support many of the earlier genomic predictions (Sieber et al., 2015).

Recently, experiments have suggested that members of the genus *Syntrophomonas* grow faster syntrophically when electroconductive materials are added (such as activated charcoal or magnetite) as evidenced by increased methane production rates (Salvador et al., 2017; Fu et al., 2018; Zhao et al., 2018). The stimulation of methanogenesis in the presence of conductive materials has suggested that syntrophic metabolizers such as *S. wolfei* may possess the ability to directly transfer electrons to the methanogenic partner, in a process called direct interspecies electron transfer, as opposed to the production of hydrogen or formate. Little is known about the components required for direct electron transfer. In addition, key details of the enzymatic machinery responsible for the production of hydrogen and formate are lacking, in particular, the physiological electron carriers that are used for hydrogen and formate production. Understanding

the mechanisms by which *S. wolfei* transfers electrons to its methanogenic partner is the key to understanding the nature of syntrophic metabolism.

*S. aciditrophicus* serves as a model for the syntrophic metabolism of benzoate to hydrogen, formate and acetate. *S. aciditrophicus* is a Gram-negative, deltaproteobacterium isolated from sewage sludge (Jackson et al, 1999). *S. aciditrophicus* has been studied due to its ability to metabolize benzoate, cyclohexane carboxylate and crotonate in syntrophic co-culture (Elshahed et al., 2001). Detailed studies have also investigated metabolism of crotonate and benzoate in pure culture (Elshahed and McInerney, 2001; Mouttaki et al., 2007). Evidence was presented that the synthessis of cyclohexane carboxylate and benzoate from acetate occurs during pure culture metabolism (Mouttaki et al., 2007). The study of syntrophic benzoate degradation is crucial to understanding anaerobic decomposition as benzoate and the metabolite benzoyl-CoA are often key intermediates in anaerobic decomposition of aromatic compounds (Harwood et al., 1998; McInerney et al., 2008; McInerney et al., 2009). The *S. aciditrophicus* genome was the first sequenced genome from a syntrophic specialist and served as a blueprint for interpretation of genomes for many syntrophic specialists (McInerney et al., 2007; Sieber et al., 2010). In addition, *S. aciditrophicus* has an unusual mechanism for ATP synthesis involving the use of pyrophosphate and AMP to make ATP during acetyl-CoA metabolism (James et al., 2016; James et al., 2019, in preparation). Recently, it has been reported that *S. aciditrophicus* possesses electrically conductive pili and can grow by direct interspecies electron transfer with *Geobacter sulfurreducens* (Walker et al., 2018). *S. aciditrophicus* possesses many unique traits; however, key details are unclear about the redox carriers and enzymes used to produce hydrogen and formate, or to transfer electrons by direct interspecies electron transfer.

Re-oxidation of NADH and Etf$_{red}$ to produce hydrogen and formate occurs by enzymes known as either hydrogenasese or formate dehydrogenases. Evidence for the importance of hydrogen and formate in syntrophic interactions is based on the requirement for the presence of the partner organism such as a hydrogenotrophic methanogen, which are limited to consuming hydrogen and formate (McInerney et al., 1981; Beaty and McInerney, 1987; Jackson et al., 1999; McInerney et al., 2009). In addition, the detection of low levels of hydrogen in syntrophic co-cultures and the inhibition of syntrophic metabolism by the addition of hydrogen support this conclusion (Boone et al., 1989; Stams and Dong, 1995).  Genes predicted to encode for hydrogenases and formate dehydrogenase have also been identified in the genomes of syntrophic organisms (McInerney et al., 2007; Sieber et al., 2010; Plugge et al., 2012) as well as the partner hydrogen consuming organisms (Gunsalus et al., 2016).

Hydrogenases catalyze the reversible reduction of protons with electrons to produce molecular hydrogen.  There are three types of known hydrogenases distinguished by the composition of metals in the active site: [FeFe], [NiFe] and [Fe] (Volbeda et al., 1995; Peters et al., 1998; Peters et al., 2015; Søndergaard et al., 2016). [Fe]-hydrogenases lack [Fe-S] centers, only reduce the cofactor methenyltetrahydromethanopterin, and are only found in methanogenic Archaea (Shima et al., 2008). [NiFe]-hydrogenases are present in Bacteria, Archaea and Eukaryotes and contain a nickel atom and an iron atom in the active site, which coordinate with a CN- group (Peters et al., 2015). [FeFe]-hydrogenases have been found in bacterial and eukaryote genomes and contain two iron atoms in the active site, which coordinates a cysteine thiolate group (Peters et al., 2015). [FeFe]-hydrogenases are irreversibly inactivated by exposure to oxygen while [NiFe]-hydrogenases can often be reactivated using reductants (Vignais and Billoud, 2007). [FeFe]-hydrogenases and [NiFe]-hydrogenases have been identified in the

genomes of syntrophic organisms and are proposed to catalyze hydrogen production from NADH and reduced flavin cofactors (McInerney et al., 2007; Sieber et al., 2010; Plugge et al., 2012; Sieber et al., 2012; Sedano-Núñez et al., 2018). It has been suggested that [FeFe]-hydrogenases catalyze hydrogen production at faster rates than [NiFe]-hydrogenases (Vignais and Billoud, 2007).

Formate dehydrogenase have also been identified in the genomes of syntrophic organisms and likely utilize the same electron donors as those used by hydrogenases. These enzymes cataylze the reversible reduction of $CO_2$ and an electron pair to produce formate. These enzymes contain [Fe-S] clusters and either a molybdenum or tungsten containing metallopterin cofactor (Niks and Hille, 2019). They are separate from another class of formate dehydrogenases, the cofactor-less NADH-dependent formate dehydrogenases, which are typically found in eukaryotes and which have not been suggested to be present in syntrophic metabolizers (Niks and Hille, 2019). Formate dehydrogenases are also used to re-oxidize reduced cofactors generated during syntrophic metabolism and can be used by a methanogenic partner to oxidize formate to produce methane. The difference between formate and hydrogen as the primary means of transferring electrons has been debated. Diffusion modeling of syntrophic co-culture conditions has suggested that formate can account for 95% of the total number of electrons transferred compared to hydrogen (Boone et al., 1989; Stams and Dong, 1995). However, the addition of hydrogenase inhibitors had a far more pronounced effect than formate dehydrogenase inhibitors on inhibiting syntrophic butyrate oxidation in a co-culture of *S. wolfei* and *Methanosprillium hungatei,* suggesting a crucial role for hydrogen (Sieber et al., 2014). It has been also proposed that both hydrogen and formate act simultaneously as electron transfer carriers between syntrophic metabolizers and methanogenic partners (Schink et al., 2017). At

least one study has suggested that small differences in redox potential exist between the two metabolites in anaerobic environments where syntrophic metabolism occurs (Montag and Schink, 2018).

**Flavin-Based Electron-Bifurcating Enzymes and the Rnf Complex**

Recently, the roles of a number of electron-bifurcating enzymes have been elucidated in anaerobic microorganisms. The discovery of electron-bifurcating enzymes has changed our concepts on how cellular electron carriers are re-oxidized in anaerobic microorganisms.

Electron-bifurcation allows two electrons of the same potential to be split so that one electron becomes more reduced (and thus is thermodynamically more favorable in additional reduction reactions) while the other electron becomes more oxidized (and thus less thermodynamically favored as a reductant) (Buckel and Thauer, 2018b; Buckel and Thauer, 2018a; Schuchmann et al., 2018). In the reverse direction, electron confurcation, such reactions facilitate the two-electron reduction of a quinone or flavin cofactor by coupling the thermodynamically unfavorable oxidation of a high redox potential donor with the thermodynamically favorable oxidation of a low potential donor in a process that involves the formation of a semiquinone reactive intermediate (Buckel and Thauer, 2018a). Such electron-bifurcation reactions can link different electron carrier pools inside of an anaerobic organism and allow the reduction of the low potential carrier ferredoxin. It has been suggested that in many aspects reduced ferredoxin represents a form of energy currency as ferredoxin's low reduction potential can be used to conserve energy by generating a chemiosmotic gradient. This gradient can in turn can be used to generate ATP by the Rnf Complex, which is explained later (Buckel and Thauer, 2018b; Buckel and Thauer, 2018a; Schuchmann et al., 2018). Detailed summaries of

9

these electron-bifurcating enzymes can be found in a number of recent reviews on the topic (Baymann et al., 2018; Buckel and Thauer, 2018b; Buckel and Thauer, 2018a). The flavin-based, electron-bifurcating enzymes have been proposed to belong to one of four groups: 1. EtfAB containing, 2. NADH-dependent, ferredoxin-NADP$^+$ oxidoreductase (NfnAB)-homolog containing, 3. NADH:ubiquionone oxidoreductase (NuoF)-homolog containing and 4. heterodisulfide reductase (HdrABC)-homolog containing (Buckel and Thauer, 2018b). Examples of EtfAB-containing, electron-bifurcating enzymes include the electron-bifurcating butyryl-CoA dehydrogenase from *Clostridium kluyveri* (Li et al., 2008), the electron-bifurcating caffeyl-CoA reductase from *Acetobacium woodii* (Bertsch et al., 2013), the electron-bifurcating lactate dehydrogenase from *A. woodii* (Weghoff et al., 2015), and an electron-bifurcating FixABCX complex from *Azotobacter vinelandii* (Ledbetter et al., 2017). NfnAB-containing electron-bifurcating complexes that have been studied include the electron-bifurcating transhydrogenase from *C. kluyveri* (Wang et al., 2010) and the electron-bifurcating transhydrogenase from *Pyrococcus furiosus* (Lubner et al., 2017). Electron-bifurcating enzymes that contain a NuoF-homolog include the electron-bifurcating [FeFe]-hydrogenases from *Thermotoga maritima* (Schut and Adams, 2009), *A. woodii* (Schuchmann and Muller, 2012), *Moorella thermoacetica* (Wang et al., 2013c), *Ruminococcus albus* (Zheng et al., 2014), *Caldanaerobacter tencongensis* (Soboh et al., 2004; Kelly et al., 2015) and *Desulfovibrio fructosovorans* (Kpebe et al., 2018). Other electron-bifurcating enzymes containing a NuoF-homolog include an electron-bifurcating, formate-dependent dehydrogenase from *Clostridium acidiurici* (Wang et al., 2013b) and an electron-bifurcating [FeFe]-hydrogenase in a functional complex with a formate dehydrogenase from *Clostridium autoethanogenum* (Wang et al., 2013a). The first well characterized example of an electron-bifurcating HdrABC containing

10

complex was the MvhADG/HdrABC complex from *Methanothermobacter marburgensis* (Kaster et al., 2011) which bifurcates electrons from hydrogen to a higher redox potential to reduce a disulfide bond (CoM-S-S-CoB, the oxidized form of coenzyme M and coenzyme B) and to a lower redox potential to reduce ferredoxin. Another example of an HdrABC-containing, electron-bifurcating complex is the heterodisulfide reductase from *Methanosarcina acetivorans* (Yan et al., 2017) which bifurcates electrons from coenzyme 420 ($F_{420}$) to a higher redox potential to reduce the CoM-S-S-CoB bond and to a lower redox potential to reduce ferredoxin.

There are only three detailed mechanisms of electron flow in electron-bifurcating enzymes: the NADH-dependent, ferredoxin-NADP$^+$ oxidoreductase from *Pyrococcus furiosus* (Nfn1) (Lubner et al., 2017), the electron-bifurcating butyryl-CoA dehydrogenase from *Acidaminococcus fermentans* (Bcd) (Chowdhury et al., 2014), and the cytochrome *bc*-1 complex of the respiratory chain (Mitchell, 1975; Buckel and Thauer, 2018b). Only the Nfn1 and *A. fermentans* Bcd systems are flavin-based as the cytochrome bc-1 complex involves quinone-based electron bifurcation. Common characteristics of flavin-based, electron-bifurcating enzymes include: the low potential electron carrier ferredoxin, multiple flavin cofactors per enzyme complex, iron-sulfur centers, and separate electron flow paths for the high redox potential electron donor and the low potential electron donor to the site of electron bifurcation.

Ferredoxins are small acidic proteins that contain one to three [Fe-S] centers of varying conformation ([2Fe-2S], [4Fe-4S], [3Fe-4S]) and are viewed as only being able to accomodate a single electron (Sticht and Rosch, 1998; Buckel and Thauer, 2018b). Reported redox potentials for ferredoxins vary from $E' = -340$ mV in *A. fermentans* (Thamer et al., 2003) to $E' = -500$ mV in *Hydrogenobacter thermophilus* (Li and Elliott, 2016), although $E_0' = -410$ mV is most often used in models of electron-bifurcating enzymes. It has long been considered that ferredoxins

from different organisms are interchangeable when used with enzymatic assays (Buckel and

Thauer, 2018b). Ferredoxin from *Clostridium pasteranium* is often used when assaying

ferredoxin-dependent enzymes as the use of this organism allows the convenient purification of

large amounts of ferredoxin (Buckel and Thauer, 2018b). Ferredoxin has been identified as one

of the electron donors in all currently known flavin-based electron-bifurcating enzymes

(Baymann et al., 2018; Buckel and Thauer, 2018b; Buckel and Thauer, 2018a). Electron-

bifurcating enzymes have been proven crucial to understanding anaerobic metabolisms in

methanogens, homoacetogens, and primary fermenting organisms. However, no electron-

bifurcating enzymes have been purified and characterized from syntrophic metabolizers and their

potential roles are mostly inferred from genomic evidence.

The Rnf complex (named for *Rhodobacter* nitrogen fixation) is a six-subunit complex

first discovered in *Rhodobacter capsulatus* that was suggested to play a crucial role in nitrogen

fixation (Schmehl et al., 1993). Detailed study and experimentation with the Rnf complex in *A.

woodii* (Biegel and Müller, 2010) showed that it is a membrane-bound, $Na^+$-translocating

ferredoxin:$NAD^+$ oxidoreductase. The Rnf complex has been suggested to be important in many

anaerobic organisms for conserving energy by generating a chemiosmotic gradient coupled to the

oxidation of ferredoxin and reduction of $NAD^+$. It has been now suggested that reduced

ferredoxin should be viewed as an energy-rich compound as it can be used by a Rnf complex to

generate an ion gradient which in turn can be used to produce ATP using an ATP synthase

(Buckel and Thauer, 2018b; Muller et al., 2018). The Rnf complex can also function to make

reduced ferredoxin from NADH using the ion gradient in order to provide reduced ferredoxin for

biosynthesis and benzoyl-CoA reduction in *S. aciditrophicus* (McInerney et al., 2007).

The electron-bifurcating [FeFe]-hydrogenases are part of the NuoF-homolog containing flavin-based electron-bifurcating enzymes. At least six enzymes of this type have been purified and enzymatically characterized; however, the electron flow within these multimeric enzymes is still a mystery. Putative electron-bifurcating [FeFe]-hydrogenases have been identified in the genomes of several syntrophic metabolizers and two such enzymes are the focus of this dissertation.

It has been observed that there are similarities between the beta subunit of the electron-bifurcating hydrogenases and the NuoF subunit of the NADH:ubiquinone oxidoreductase that is involved in NADH oxidation (Buckel and Thauer, 2018a). In particular they share predicted NADH- and FMN-binding sites. However, the only FMN predicted to be bound in the beta subunit of the electron-bifurcating hydrogenases is considered unsuitable as the site of electron bifurcation. This is due to the close proximity of FMN to the NADH-binding site, suggesting that the proposed role of this FMN is limited to facilitating the two-electron oxidation from NADH (Buckel and Thauer, 2013; Baymann et al., 2018; Buckel and Thauer, 2018a). In current proposed mechanisms for electron-bifurcation, the initial electron transfer involves a thermodynamically unfavorable transfer to FMN. The transfer of electrons from NADH to this FMN is thermodynamically favorable and does not match the proposed mechanism in other electron-bifurcating enzymes (Buckel and Thauer, 2018a). Instead, another cofactor must serve as the site of electron bifurcation. At least two different explanations have been proposed for electron bifurcation in NADH-dependent, ferredoxin-dependent [FeFe]-hydrogenases.

The first explanation derived from the study of the *A. woodii* electron-bifurcating [FeFe]-hydrogenase suggests that a second bound but, as yet, unidentified flavin cofactor (see Figure 1) is present in the beta subunit and serves as the site of electron bifurcation (Buckel and Thauer,

2018a). A slight modification of this scheme (Schuchmann et al., 2018) has also been suggested

that includes two additional bound flavins. The necessity for two additional flavins is due to the

limitation of iron sulfur centers to accommodate only a single electron at a time.

**Figure 1. Competing Proposals for Electron Flow in Electron-Bifurcating [FeFe]-Hydrogenases.** The left panel represents electron flow similar to that proposed by (Buckel and

Thauer, 2018a) where electron-bifurcation occurs at a second flavin present in the beta subunit.

The right panel represents electron flow similar to that proposed by (Peters et al., 2018) where

electron bifurcation occurs at the H-cluster. Arrows indicate electron flow. Purple stars indicate

cofactors acting as the site of electron-bifurcation.
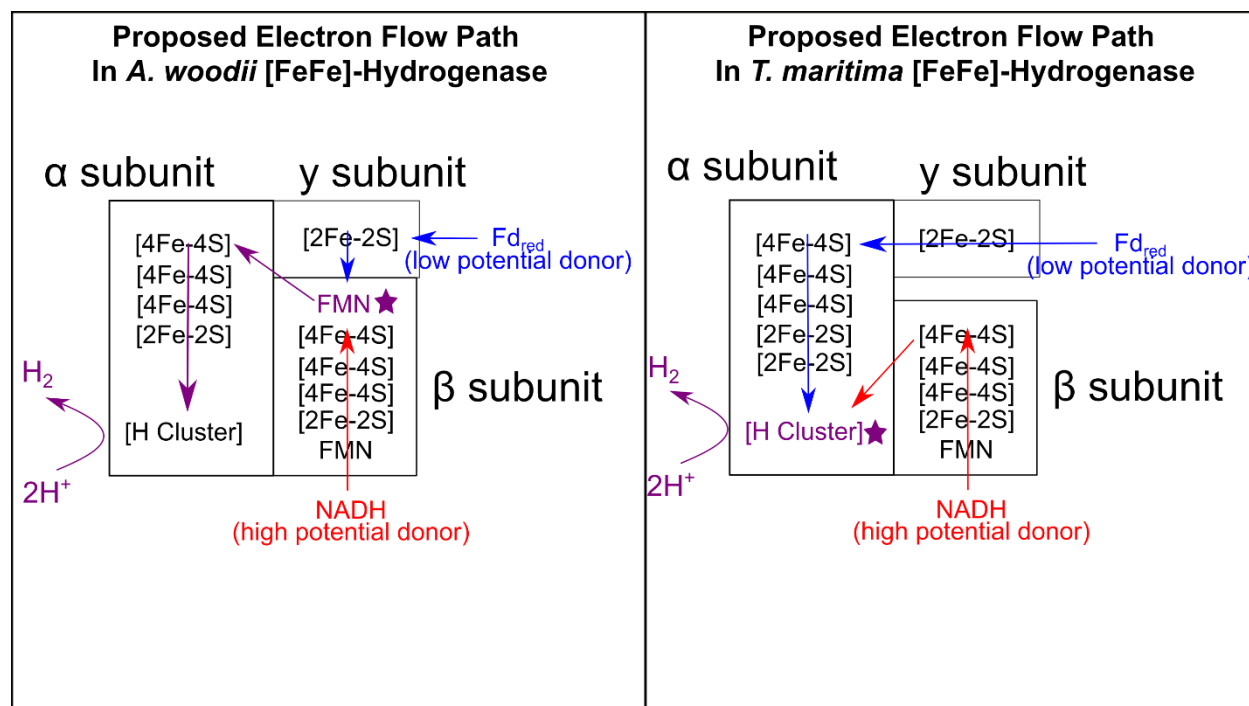


The second explanation suggests that the active site of the *T. maritima* [FeFe]-

hydrogenase, the [H]-cluster (Figure 1), serves as the site of electron bifurcation (Peters et al.,

2018). The explanation argues that the [H]-cluster, the catalytic site of proton reduction, is

14

capable of accommodating two electrons of different redox potential simultaneously. The metallopterin cofactor at the catalytic site of the electron-bifurcating formate dehydrogenases is also proposed to have the ability to accommodate two electrons of different redox potentials. Therefore, this electron flow scheme could also operate in electron-bifurcating formate dehydrogenases.

**Re-Oxidation of Reduced Cofactor Pools, NADH and Electron Transfer Flavoprotein in *S. wolfei* and *S. aciditrophicus***

During the oxidation of butyrate by *S. wolfei*, there are two oxidation-reduction steps that generate reduced cofactors. The first step, the oxidation of butyryl-CoA to crotonyl-CoA, is believed to produce reduced electron transfer flavoprotein ($ETF_{red}$) with a high redox potential ($E' = -125$ mV) (see Figure 2) (Gustafson et al., 1986; Sieber et al., 2010). The second step, the oxidation of 3-hydroxybutyryl-CoA to acetoacetyl-CoA ($E' = -250$ mV), has been shown to be coupled to the reduction of $NAD^+$ to NADH (Wofford et al., 1986). To continually oxidize butyrate, *S. wolfei* must re-oxidize NADH and $ETF_{red}$ by producing hydrogen or formate. For this to happen, the partner microorganism, such as a hydrogenotrophic methanogen, must continually remove hydrogen and formate so that cofactor re-oxidation remains thermodynamically favorable.

**Figure 2. Reduced Cofactor Generation during Syntrophic Butyrate Oxidation in *S. wolfei*.** Acyl-CoA oxidation is shown as coupled to Etf reduction and not by an electron-bifurcating mechanism.

Butyrate
↓ → ETF_red
Butyryl-CoA
↓
Crotonyl-CoA
↓
3-Hydroxybutyryl-CoA
↓ → NADH
Acetoacetyl-CoA
↓
Acetyl-CoA
↓
Acetate

Total Reduced Cofactors (per butyrate):
+1 NADH
+1 ETF_red
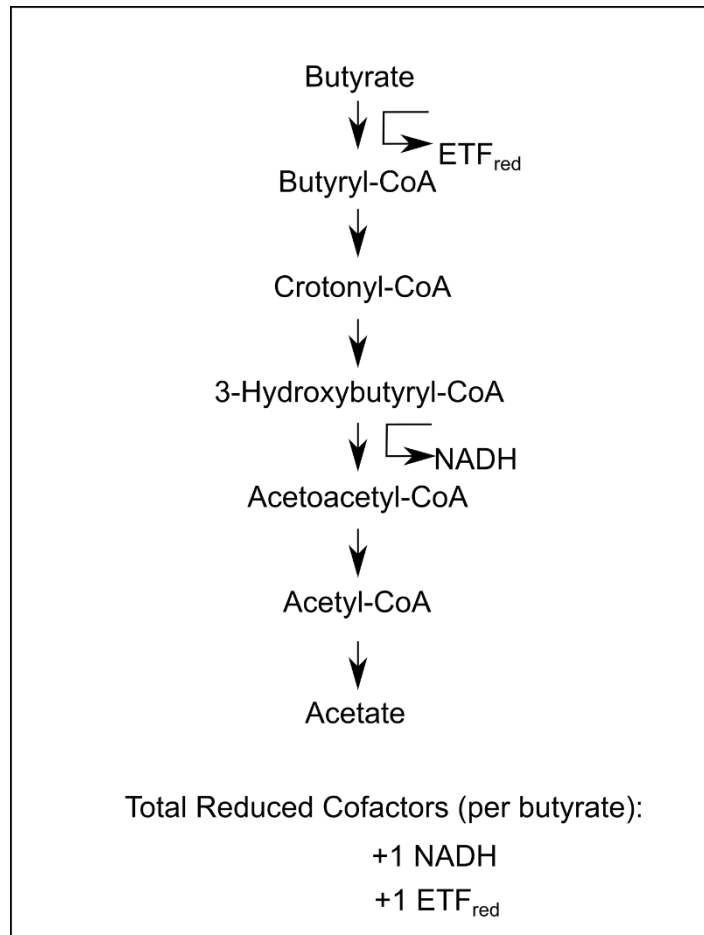
In *S. aciditrophicus*, enzymatic analyses suggest that NADH production (see Figure 3) is expected in the following 3-hydroxyacyl-CoA oxidation reactions: 3-hydroxybutyryl-CoA to acetoacetyl-CoA, 3-hydroxypimeyl-CoA oxidation to 3-oxopimelyl-CoA, and 6-hydroxycyclohex-1-ene-1carboxyl-CoA oxidation to 6-oxycyclohex-1-ene-1carboxyl-CoA (Elshahed and McInerney, 2001). The last oxidation step, the oxidation of glutaryl-CoA to glutaconyl-CoA would produce electrons of a similar potential to the butyryl-CoA to crotonyl-CoA oxidation (estimated $E' = -10$ mV to $E' = -126$ mV). This oxidation reaction has been suggested to occur by an electron-bifurcating acyl-CoA dehydrogenase (Djurdjevic, 2010) similar to an electron-bifurcating butyryl-CoA dehydrogenase. If the glutaryl-CoA dehydrogenase is electron-bifurcating, a pair of electrons from the low redox potential donor

ferredoxin (E′ = -410 mV) and a pair of high redox potential electrons from the oxidation of glutaryl-CoA to glutaconyl-CoA (E′ = - 126 mV) would be confurcated to an intermediate redox potential to produce two pairs of electrons at the redox potential of NADH (E′ = -320 mV). Alternatively, electrons from the oxidation of glutaryl-CoA to glutaconyl-CoA could be transferred to Etf$_{red}$ and then to a membrane-associated, iron-sulfur oxidoreductase system, which would reduce menaquinone (McInerney et al., 2007). Chemiosmotic energy would be used to lower the redox potential of the electrons in order to produce hydrogen or formate, similar to the mechanism proposed for *S. wolfei* (Crable, 2013). As with *S. wolfei*, *S. aciditrophicus* would require continual hydrogen or formate consumption by a partner organism to allow continual cofactor re-oxidation.

**Figure 3. Reduced Cofactors from Syntrophic Benzoate Oxidation in *S. aciditrophicus*.** Note that two NADH molecules are used to make two reduced ferredoxins needed for benzoyl-CoA reduction and have been subtracted from total NADH made during benzoyl-CoA metabolism to acetate and $CO_2$. The reduction of benzoyl-CoA is shown with the stoichiometry of an ATP-independent enzyme complex mediating two separate electron-bifurcation reactions as proposed by (Huwiler et al., 2019). Acyl-CoA oxidation is shown as being coupled to Etf reduction and not by an electron-bifurcating mechanism.

Benzoate

↓

Benzoyl-CoA

$2 \times Fd_{red}$ ⎤
$0.5\ NADH + 0.5\ Etf_{red}$ ←⎦ ↓

Cyclohexa-1,5-diene-1-carboxyl-CoA

↓

6-Hydroxycyclohex-1-ene-1-carboxyl-CoA

↓ → NADH

6-Oxocyclohex-1-ene-1-carboxyl-CoA

↓

3-Hydroxypimelyl-CoA

↓ → NADH

3-Oxopimelyl-CoA

↓

Glutaryl-CoA

↓ → $ETF_{red}$

Glutaconyl-CoA

↓

Crotonyl-CoA

↓

3-Hydroxybutyryl-CoA

↓ → NADH

Acetoacetyl-CoA

↓

Acetyl-CoA

↓

Acetate

Rnf Complex Conversion (per benzoate):

$2\ NADH \rightarrow 2\ Fd_{red}$

Total Reduced Cofactors (per benzoate):

$+1.5\ NADH$
$+1.5\ ETF_{red}$

Another important step during syntrophic benzoate metabolism in *S. aciditrophicus* is the reduction of benzoyl-CoA (see Figure 3). Evidence suggests that the mechanism is similar to the proposed reaction in *Geobacter metallireducens* involving the low redox potential electron

donor, reduced ferredoxin (Kung et al., 2009). However, a recent publication has shown that the

*G. metallireducens* benzoyl-CoA reductase is actually a one megadalton complex and benzoyl-

CoA reduction may involve two separate electron bifurcation steps (Huwiler et al., 2019). The

overall electron transfer scheme is the oxidation of two pairs of electrons from the low potential

donor ferredoxin ($E' = -410$ mV) with one pair being bifurcated to a lower redox potential ($E' = -$

580 mV) and used to reduce benzoyl-CoA. The second pair of electrons is bifurcated to a higher

redox potential ($E' = -200$ mV). It has been proposed that a second bifurcation reaction bifurcates

this higher redox potential electron pair to reduced NADH ($E' = -320$ mV) and reduced

menaquinone (MQ) ($E' = -70$ mV). However, there is much uncertainty over this second

bifurcation reaction. The membrane dependence of the reaction implicates a role for

menaquinone but menaquinone involvement has not been demonstrated. If *S. aciditrophicus*

utilizes the same enzyme complex for benzoyl-CoA reduction, as genomic analysis suggests

(Boll et al., 2014), this would alter the number of predicted reduced cofactors per mole of

benzoate oxidized from 2 NADH, 1 Etf$_{red}$ to 1.5 NADH and 1.5 Etf$_{red}$ as shown in Figure 3.

There are two different explanations for how NADH could be re-oxidized to produce

hydrogen (or formate) in *S. wolfei* and *S. aciditrophicus*. The first explanation argues that,

according to thermodynamic calculations, production of hydrogen using high redox potential

electrons from NADH ($E' = -320$ mV) can occur so long as the hydrogen partial pressure does

not exceed 60 pascals (see Figure 4) (Boone et al., 1989; Schink, 1997). As hydrogenotrophic

methanogens can maintain hydrogen partial pressures below 60 Pa, the process of producing

hydrogen from NADH would continue to function. The observed hydrogen partial pressures in

co-cultures of *S. wolfei* and a hydrogenotrophic methanogen (25 Pa) were agreement with this

proposal (Boone et al., 1989).

**Figure 4. Alternative Explanations for NADH Re-Oxidation in** *S. wolfei* **and** *S. aciditrophicus***.** A. NADH oxidation by non-electron bifurcating hydrogenase at low hydrogen partial pressures; B. NADH oxidation coupled to reduced ferredoxin oxidation by electron-bifurcating hydrogenases. Reduced ferredoxin could be made from electrons derived from NADH oxidation by the Rnf complex identified in the genome of *S. aciditrophicus* using the chemiosmotic gradient. In *S. wolfei*, FixABCX complex could bifurcate electrons from the high redox potential electron carrier NADH to the even higher potential electron carrier menaquinone to produce reduced ferredoxin. The high redox potential electron carrier menaquinone could be re-oxidized by a putative reverse quinone loop**.**

## A. NADH-Dependent Hydrogen Production

NADH
$(E' = -320\ mV)$    —Hydrogenase→    Hydrogen
Partial Pressure of 60 Pa
$(E'\ -320\ mV)$
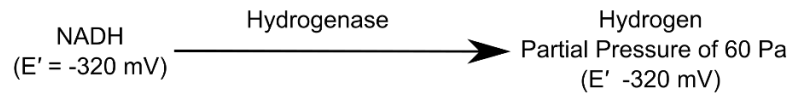
## B. Electron Confurcating Hydrogen Production

### Step 1. Reduce Ferredoxin with Electrons from NADH using Chemiosmotic Potential

Rnf Complex (*S. aciditrophicus*)

NADH

Na⁺ or H⁺

$Fd_{red}$
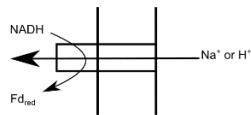
NADH
$(E'\ -320\ mV)$    Reverse Electron Transfer / or Electron-Bifurcation →    Ferredoxin
$(E'\ -410\ mV)$

FixABCX Complex (*S. wolfei*)

$Mq_{red}$

2 x NADH →

$Fd_{red}$
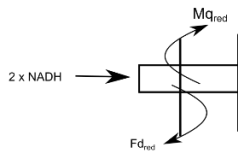
### Step 2. Electron Confurcating Hydrogen Production

NADH
$(E'\ -320\ mV)$

Confurcated Electrons
$(E'\ -365\ mV)$    —Hydrogenase→    Hydrogen
Partial Pressure of up to 1000 Pa
$(E'\ -365\ mV)$

Ferredoxin
$(E'\ -410\ mV)$

However, when the genomes of *S. wolfei* and *S. aciditrophicus* were sequenced, genes were identified that shared high homology to the multimeric [FeFe]-hydrogenase from *T. maritima*. The *T. maritima* [FeFe]-hydrogenase was initially presumed to utilize NADH for hydrogen production, but no production from NADH as a sole donor was observed (Verhagen et al., 1999). Later, it was discovered that the *T. maritima* hydrogenase is electron-bifurcating and required both reduced ferredoxin and NADH in 1:1 ratio to produce hydrogen (Schut and Adams, 2009). Characterization of additional multimeric [FeFe]-hydrogenases showed they were all capable of electron-bifurcation but could not utilize NADH alone. Therefore, the multimeric [FeFe]-hydrogenases in *S. wolfei* and other syntrophic metabolizers were widely assumed to operate only by electron-bifurcation and not capable of utilizing NADH as a sole donor (Stams and Plugge, 2009). However, for an electron-bifurcating [FeFe]-hydrogenase to function in *S. wolfei* there must be a source of reduced ferredoxin. However, the oxidation of fatty acids by *S. wolfei* was only predicted to produce NADH and reduced flavin cofactors. The genes for the Rnf complex capable of making reduced ferredoxin from electrons derived from NADH oxidation were identified in the *S. aciditrophicus* genome (McInerney et al., 2007). However, genes for the Rnf complex were not identified in the *S. wolfei* genome but instead another set of genes encoding proteins for the FixABCX, complex, which could perform a similar function (Sieber et al., 2010; Sieber et al., 2015). Recent studies of the FixABCX complex in *Azotobacter vinelandii* suggest it could provide a source of reduced ferredoxin by bifurcating electrons from NADH to the low potential electron carrier ferredoxin and to the high redox potential electron carrier menaquinone (Ledbetter et al., 2017). Thus, in both syntrophic model organisms, *S. wolfei* and *S. aciditrophicus*, complexes capable of supplying ferredoxin have been proposed. With a source of reduced ferredoxin, electron-bifurcating [FeFe]-hydrogenases could produce hydrogen from

22

NADH and reduced ferredoxin. From thermodynamic calculations, the redox potential of electrons confurcated from NADH and reduced ferredoxin would be close to E′ = -360 mV and capable of producing hydrogen partial pressures of at least 1000 pascals.

The physiological electron acceptors involved in the oxidation of acyl-CoA metabolites in *S. wolfei* and *S. aciditrophicus* have not been demonstrated experimentally. Enzymes that catalyze this reaction in primary fermenters such as *Clostridium kluyveri* have been found to use electron-bifurcation (Li et al., 2008). A glycine residue predicted to be required for binding of the electron-bifurcating flavin cofactor reaction is absent in the EtfB subunits present in the *S. aciditrophicus* and *S. wolfei* genomes (Garcia Costas et al., 2017) (Figure 5).

**Figure 5. Alignment** *S. wolfei* **and** *S. aciditrophicus* **of EtfB Subunits.** Red indicates residues proposed to be essential to NADH binding in electron-bifurcating EtfB subunits (Garcia Costas et al., 2017) while blue indicates a residue proposed to be required for flavin cofactor binding in electron-bifurcating EtfB subunits.  The *Paracoccus nitrificans* EtfB is not predicted to be electron-bifurcating, while the *Acidaminococcus fermentans*, *Acetobacterium woodii*, *Clostridium difficiile* and *Clostridium kluyveri* EtfBs are known to perform electron-bifurcation.

```
     Alignment Position 160                                                          Alignment Position 239
1 A. fermentans      LAVGADEAKLV--SDRAFGGADTLATSAAMANTIKHFGVPDLILCGRQAIDGDTAQVGPEIAEHLGLPQVTAALKVQVKD
2 A. woodii Copy 1   LAMGADEAYLL--SDRAFGGADTWATSATLAAGIKKVKKVDLVLAGRQAIDGDTAQVGSQIAQRLKMPVVTYVEDIKIED
3 A. woodii Copy 2   FYMGADEGCLL--SDRKFGGADVVATSYTLAQGTKRLGDFDLIICGKQTTDGDTAQVGPEMAEFLGIPHVTNVIKILAAD
4 C. difficile       LAMGADRGILL--TDRAFAGADTWATSSALAGALKNI-DFDIIIAGRQAIDGDTAQVGPQIAEHLNLPSITYAEEIKTEG
5 C. kluyveri        LAMGADEAILV--SDRAFAGADTQATSYALAGALKNL-EYDLIFAGRQAIDGDTAQVGPQIAEKLGIPQITYVEKVDVDG
6 S. aciditrophicus  LAMGADKGIHI--DDPALEGADAYATATALAAAIKGI-PYDVIFCGQRAIDDDSGQVGAILAELLGLPQLTVVTKEETDG
7 S. wolfei Copy 1   LAMGADRGILV--QQDT--AADEFARAVALAEAIKGE-NPDIILAGHVAADDGSSQVPTRVAEILGLPHVNVITAVEIAG
8 S. wolfei Copy 2   LAAGADEALLI--IDDELDKLGSAETAKILAAAIQRIEKVDLIIFGEGSGDNYSGQVGSRVAEILGLPQVGYASAIELQG
9 P. nitrificans     LAMGADRAILVVAADDVQQDIEPLAVAKILAAVARAE-GTELIIAGKQAIDNDMNATGQMLAAILGWAQATFASKVEIEG
```

Therefore, it is unlikely acyl-CoA oxidation during syntrophic metabolism in *S. wolfei* and *S. aciditrophicus* involves electron-bifurcation. Instead an alternative mechanism (see Figure 6) has been proposed in *S. aciditrophicus* (McInerney et al., 2007) and *S. wolfei* involving an iron-sulfur oxidoreductase in conjunction with a membrane-associated [FeFe]-hydrogenase or formate dehydrogenase to form a reverse quinone loop (Sieber et al., 2010; Crable, 2013; Schmidt et al., 2013; Sieber et al., 2015; Crable et al., 2016). The reverse quinone loop would utilize the potential energy from a proton gradient, generated by an ATP synthase to make hydrogen production from the high redox potential electrons generated during acyl-CoA oxidation more favorable (Figure 6).

**Figure 6. Proposed Mechanism of Etf Re-Oxidation in** *S. wolfei* **and** *S. aciditrophicus***.** The proposed redox values are similar to those described by (Schmidt et al., 2013). Redox potential

of Etf$_{red}$ is shown as the same of the butyryl-CoA/crontonyl-CoA redox value (Gustafson et al., 1986). MQ, Menaquinone.



1. Reverse Quinone Loop

Cytochrome-B Hydrogenase
(Oxidizes Menaquinone, Produces Hydrogen)

e$^-$ pair
(-326 mV)

Hydrogen
Partial Pressure of ~60 Pa
(E′ -326 mV)

2 H$^+$

MQ$_{ox}$

Quinone Translocation of 2 Protons
(Lowers redox of e- pair by -200 mV)

MQ$_{red}$ ← 2 H$^+$

Etf$_{red}$
(E′ -126 mV)

e$^-$ pair
(-126 mV)

Fe-S Oxidoreductase
(Oxidizes Etf$_{Red}$, reduces Menaquinone)

Cytoplasm

Exterior of cell
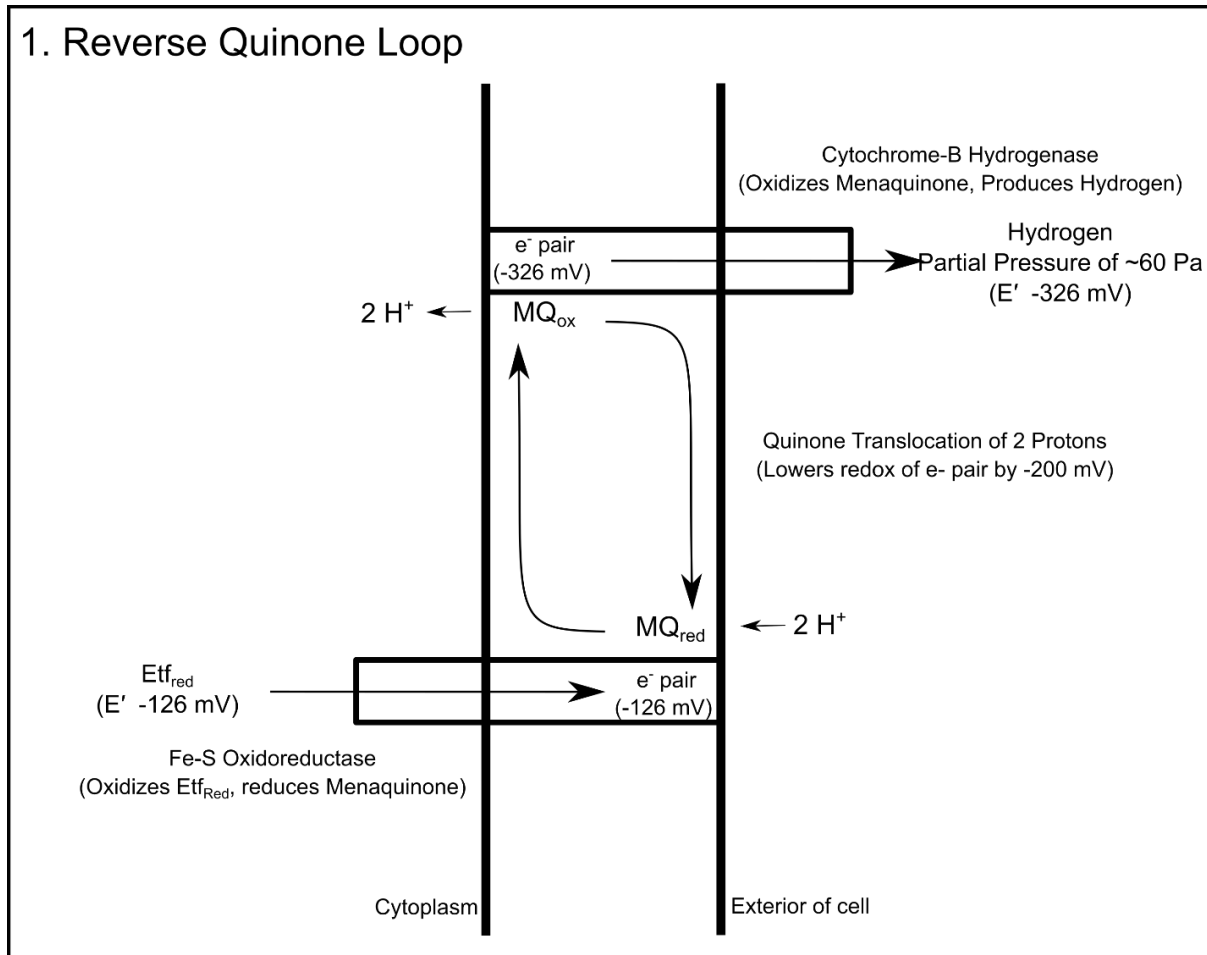
Previously, attempts have been made to investigate formate dehydrogenses and [FeFe]-hydrogenases from syntrophic microorganisms. One investigation of two formate dehydrogenases purified from the syntrophic propionate oxidizer *Syntrophobacter fumaroxidans* described multimeric complexes containing tungstate (de Bok et al., 2003). Both enzymes, FDH-1 and FDH-2, possessed high catalytic activity with the artificial electron acceptor benzyl viologen in both the formate-producing and formate-consuming reactions. However, a

physiological donor could not be identified for either enzyme. FDH-1 consists of three subunits with molecular weights of 89 kDa, 56 kDa, and 19 kDa and resembles the multimeric electron-bifurcating, formate dehydrogenase present in *Clostridium acidurici* (Wang et al., 2013b). Electron-bifurcating formate dehydrogenases require the involvement of both ferredoxin and NADH, which would not have been recognized at the time that the *S. fumaroxidans* formate dehydrogenases were characterized.

A second study described the purification of an enzyme activity with subunits predicted to encode for a multimeric [FeFe]-hydrogenase from *Syntrophomonas wolfei* (Müller et al., 2009). The three subunits for the [FeFe]-hydrogenase co-purified with subunits from a selenocysteine-containing formate dehydrogenase despite the use of various chromatographic steps. The purification was performed under oxic conditions that likely inactivated the catalytic sites of the [FeFe]-hydrogenase and selenocysteine-containing formate dehydrogenase. No hydrogen producing or formate producing activites with physiological electron donors were reported.

I attempted to purify formate dehydrogenases and [FeFe]-hydrogenases from the membrane and soluble fractions of *S. wolfei* without achieving homogeneous preparations. The enrichment of formate dehydrogenase activity from the membrane of *S. wolfei* using gel filtration and hydroxyapatite column chromatography indicated the partial purification of two subunits of predicted molecular weights 96 kDa and 30.8 kDa as identified by peptide mass spectrometry. Both subunits were predicted to be associated with a formate dehydrogenase. However, the predicted encoding genes from the *S. wolfei* genome (Swol_0798-Swol_0799) occur in an operon of at least four genes (Swol_0797-Swol_0800), suggesting additional subunits were lost during purification. In addition, difficulty was encountered in stabilizing formate dehydrogenase

activity during purification. The ability to identify a physiological electron donor and to place the role of enzyme within the metabolism of *S. wolfei* seemed unlikely and the approach was discontinued. Attempted purification of formate dehydrogenase and hydrogenase activity from the soluble portion of *S. wolfei* indicated that the two activities co-migrated despite the use of several common separation techniques including hydroxyapatite, anion-exchange, and gel-filtration chromatography. The inability to separate the two activities, along with large decrease in the activity during the multi-step purification suggested that another approach would be needed to obtain a pure protein.

The difficulties in purifying formate dehydrogenases and hydrogenases from syntrophic microorganisms were challenging and led to the use of a recombinant DNA approach to clone and express the proteins of interest in *Escherichia coli*. Production and characterization of [FeFe]-hydrogenases by a recombinant DNA approach had been demonstrated before and allowed the rapid purification of large amounts of active enzyme using affinity tag chromatography (Girbal et al., 2005; King et al., 2006). The research for this dissertation describes the properties of two multimeric [FeFe]-hydrogenases from *S. wolfei* and *S. aciditrophicus*. Recombinantly produced, affinity tagged multimeric [FeFe]-hydrogenases from *S. wolfei* and *S. aciditrophicus* were characterized to determine if they required reduced ferredoxin for NADH oxidation. My work shows that both enzymes reoxidize NADH coupled to hydrogen production without ferredoxin involvement so long as the hydrogen partial pressure was low. Thus, continual hydrogen production from NADH by these enzymes would explain the requirement syntrophic organisms have for low hydrogen partial pressures. This in turn explains the obligate requirement for the presence of hydrogen-consuming partner microorganisms during syntrophic fatty and aromatic acid metabolism.

**References**

Angelidaki, I., Karakashev, D., Batstone, D.J., Plugge, C.M., and Stams, A.J.M. (2011). "Chapter sixteen - Biomethanation and Its Potential," in *Methods in Enzymology,* eds. A.C. Rosenzweig & S.W. Ragsdale. Academic Press), 327-351.

Baymann, F., Schoepp-Cothenet, B., Duval, S., Guiral, M., Brugna, M., Baffert, C., et al. (2018). On the Natural History of Flavin-Based Electron Bifurcation. *Front Microbiol* 9**,** 1357.

Beaty, P.S., and McInerney, M.J. (1987). Growth of *Syntrophomonas wolfei* in pure culture on crotonate. *Arch Microbiol* 147(4)**,** 389-393. doi: 10.1007/BF00406138.

Bertsch, J., Parthasarathy, A., Buckel, W., and Muller, V. (2013). An electron-bifurcating caffeyl-CoA reductase. *J Biol Chem* 288(16)**,** 11304-11311. doi: 10.1074/jbc.M112.444919.

Biegel, E., and Müller, V. (2010). Bacterial Na+/-translocating ferredoxin:NAD+/ oxidoreductase. *Proc Natl Acad Sci Unit States Am* 107(42)**,** 18138-18142. doi: 10.1073/pnas.1010318107.

Boll, M., Löffler, C., Morris, B.E.L., and Kung, J.W. (2014). Anaerobic degradation of homocyclic aromatic compounds via arylcarboxyl-coenzyme A esters: organisms, strategies and key enzymes. *Environ Microbiol* 16(3)**,** 612-627. doi: 10.1111/1462-2920.12328.

Boone, D.R., Johnson, R.L., and Liu, Y. (1989). Diffusion of the Interspecies Electron Carriers $H_2$ and Formate in Methanogenic Ecosystems and Its Implications in the Measurement of $H_2$ or Formate Uptake. *Appl Environ Microb* 55(7)**,** 1735.

Buckel, W., and Thauer, R.K. (2013). Energy conservation via electron bifurcating ferredoxin reduction and proton/Na(+) translocating ferredoxin oxidation. *Biochim Biophys Acta* 1827(2)**,** 94-113. doi: 10.1016/j.bbabio.2012.07.002.

Buckel, W., and Thauer, R.K. (2018a). Flavin-Based Electron Bifurcation, A New Mechanism of Biological Energy Coupling. *Chem Rev* 118(7)**,** 3862-3886. doi: 10.1021/acs.chemrev.7b00707.

Buckel, W., and Thauer, R.K. (2018b). Flavin-Based Electron Bifurcation, Ferredoxin, Flavodoxin, and Anaerobic Respiration With Protons (Ech) or NAD+ (Rnf) as Electron Acceptors: A Historical Review. *Front Microbiol* 9**,** 401.

Chowdhury, N.P., Mowafy, A.M., Demmer, J.K., Upadhyay, V., Koelzer, S., Jayamani, E., et al. (2014). Studies on the Mechanism of Electron Bifurcation Catalyzed by Electron Transferring Flavoprotein (Etf) and Butyryl-CoA Dehydrogenase (Bcd) of *Acidaminococcus fermentans*. *J Biol Chem* 289(8)**,** 5145-5157. doi: 10.1074/jbc.M113.521013.

Crable, B. (2013). *Enzyme systems involved in interspecies hydrogen and formate transfer between syntrophic fatty and aromatic acid degraders and Methanospirillum hungatei.* Doctoral Dissertation, University of Oklahoma.

Crable, B.R., Sieber, J.R., Mao, X., Alvarez-Cohen, L., Gunsalus, R., Ogorzalek Loo, R.R., et al. (2016). Membrane Complexes of *Syntrophomonas wolfei* Involved in Syntrophic Butyrate Degradation and Hydrogen Formation. *Front Microbiol* 7**,** 1795. doi: 10.3389/fmicb.2016.01795.

de Bok, F.A.M., Hagedoorn, P.-L., Silva, P.J., Hagen, W.R., Schiltz, E., Fritsche, K., et al. (2003). Two W-containing formate dehydrogenases ($CO_2$-reductases) involved in

syntrophic propionate oxidation by *Syntrophobacter fumaroxidans*. *Eur J Biochem* 270(11)**,** 2476-2485. doi: 10.1046/j.1432-1033.2003.03619.x.

Djurdjevic, I. (2010). *Production of glutaconic acid in recombinant Escherichia coli.* Doctoral Thesis, Philipps-Universität Marburg.

Elshahed, M.S., Bhupathiraju, V.K., Wofford, N.Q., Nanny, M.A., and McInerney, M.J. (2001). Metabolism of Benzoate, Cyclohex-1-ene Carboxylate, and Cyclohexane Carboxylate by "*Syntrophus aciditrophicus*" Strain SB in Syntrophic Association with $H_2$-Using Microorganisms. *Appl Environ Microb* 67(4)**,** 1728-1738. doi: 10.1128/aem.67.4.1728-1738.2001.

Elshahed, M.S., and McInerney, M.J. (2001). Benzoate fermentation by the anaerobic bacterium *Syntrophus aciditrophicus* in the absence of hydrogen-using microorganisms. *Appl Environ Microbiol* 67(12)**,** 5520-5525. doi: 10.1128/AEM.67.12.5520-5525.2001.

Fink, C.W., Stankovich, M.T., and Soltysik, S. (1986). Oxidation-reduction potentials of butyryl-CoA dehydrogenase. *Biochemistry-US* 25(21)**,** 6637-6643.

Fu, L., Song, T., Zhang, W., Zhang, J., and Lu, Y. (2018). Stimulatory Effect of Magnetite Nanoparticles on a Highly Enriched Butyrate-Oxidizing Consortium. *Front Microbiol* 9(1480). doi: 10.3389/fmicb.2018.01480.

Garcia Costas, A.M., Poudel, S., Miller, A.F., Schut, G.J., Ledbetter, R.N., Fixen, K.R., et al. (2017). Defining Electron Bifurcation in the Electron-Transferring Flavoprotein Family. *J Bacteriol* 199(21). doi: 10.1128/jb.00440-17.

Girbal, L., von Abendroth, G., Winkler, M., Benton, P.M.C., Meynial-Salles, I., Croux, C., et al. (2005). Homologous and Heterologous Overexpression in *Clostridium acetobutylicum* and Characterization of Purified Clostridial and Algal Fe-Only Hydrogenases with High

Specific Activities. *Appl Environ Microb* 71(5)**,** 2777. doi: 10.1128/AEM.71.5.2777-2781.2005.

Gunsalus, R.P., Cook, L.E., Crable, B., Rohlin, L., McDonald, E., Mouttaki, H., et al. (2016). Complete genome sequence of *Methanospirillum hungatei* type strain JF1. *Stand Genomic Sci* 11**,** 2. doi: 10.1186/s40793-015-0124-8.

Gustafson, W.G., Feinberg, B.A., and McFarland, J.T. (1986). Energetics of beta-oxidation. Reduction potentials of general fatty acyl-CoA dehydrogenase, electron transfer flavoprotein, and fatty acyl-CoA substrates. *J Biol Chem* 261(17)**,** 7733-7741.

Harwood, C.S., Burchhardt, G., Herrmann, H., and Fuchs, G. (1998). Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. *FEMS Microbiol Rev* 22(5)**,** 439-458. doi: https://doi.org/10.1016/S0168-6445(98)00026-6.

Huwiler, S.G., Löffler, C., Anselmann, S.E.L., Stärk, H.-J., von Bergen, M., Flechsler, J., et al. (2019). One-megadalton metalloenzyme complex in *Geobacter metallireducens* involved in benzene ring reduction beyond the biological redox window. *Proc Natl Acad Sci Unit States Am***,** 201819636. doi: 10.1073/pnas.1819636116.

Jackson, B.E., Bhupathiraju, V.K., Tanner, R.S., Woese, C.R., and McInerney, M.J. (1999). *Syntrophus aciditrophicus* sp. nov., a new anaerobic bacterium that degrades fatty acids and benzoate in syntrophic association with hydrogen-using microorganisms. *Arch Microbiol* 171(2)**,** 107-114.

James, K.L., Kung, J.W., Crable, B.R., Mouttaki, H., Sieber, J.R., Nguyen, H.H., et al. (2019, in preparation). Syntrophus aciditrophicus uses the same enzymes in a reversible manner to syntrophically degrade fatty, aromatic and alicyclic acids and to synthesize aromatic and alicyclic acids from crotonate.

James, K.L., Rios-Hernandez, L.A., Wofford, N.Q., Mouttaki, H., Sieber, J.R., Sheik, C.S., et al. (2016). Pyrophosphate-Dependent ATP Formation from Acetyl Coenzyme A in *Syntrophus aciditrophicus*, a New Twist on ATP Formation. *MBio* 7(4). doi: 10.1128/mBio.01208-16.

Kaster, A.K., Moll, J., Parey, K., and Thauer, R.K. (2011). Coupling of ferredoxin and heterodisulfide reduction via electron bifurcation in hydrogenotrophic methanogenic archaea. *Proc Natl Acad Sci Unit States Am* 108(7)**,** 2981-2986. doi: 10.1073/pnas.1016761108.

Kelly, C.L., Pinske, C., Murphy, B.J., Parkin, A., Armstrong, F., Palmer, T., et al. (2015). Integration of an [FeFe]-hydrogenase into the anaerobic metabolism of *Escherichia coli*. *Biotechnol Rep (Amst)* 8**,** 94-104. doi: https://doi.org/10.1016/j.btre.2015.10.002.

King, P.W., Posewitz, M.C., Ghirardi, M.L., and Seibert, M. (2006). Functional Studies of [FeFe] Hydrogenase Maturation in an *Escherichia coli* Biosynthetic System. *J Bacteriol* 188(6)**,** 2163. doi: 10.1128/JB.188.6.2163-2172.2006.

Kpebe, A., Benvenuti, M., Guendon, C., Rebai, A., Fernandez, V., Le Laz, S., et al. (2018). A new mechanistic model for an O2-protected electron-bifurcating hydrogenase, Hnd from *Desulfovibrio fructosovorans*. *Biochim Biophys Acta Bioenerg* 1859(12)**,** 1302-1312. doi: https://doi.org/10.1016/j.bbabio.2018.09.364.

Kung, J.W., Loffler, C., Dorner, K., Heintz, D., Gallien, S., Van Dorsselaer, A., et al. (2009). Identification and characterization of the tungsten-containing class of benzoyl-coenzyme A reductases. *Proc Natl Acad Sci Unit States Am* 106(42)**,** 17687-17692. doi: 10.1073/pnas.0905073106.

Ledbetter, R.N., Garcia Costas, A.M., Lubner, C.E., Mulder, D.W., Tokmina-Lukaszewska, M.,
Artz, J.H., et al. (2017). The Electron Bifurcating FixABCX Protein Complex from
*Azotobacter vinelandii:* Generation of Low-Potential Reducing Equivalents for
Nitrogenase Catalysis. *Biochemistry* 56(32)**,** 4177-4190. doi:
10.1021/acs.biochem.7b00389.

Li, B., and Elliott, S.J. (2016). The Catalytic Bias of 2-Oxoacid:ferredoxin Oxidoreductase in
CO2: evolution and reduction through a ferredoxin-mediated electrocatalytic assay.
*Electrochim Acta* 199**,** 349-356. doi: https://doi.org/10.1016/j.electacta.2016.02.119.

Li, F., Hinderberger, J., Seedorf, H., Zhang, J., Buckel, W., and Thauer, R.K. (2008). Coupled
ferredoxin and crotonyl coenzyme A (CoA) reduction with NADH catalyzed by the
butyryl-CoA dehydrogenase/Etf complex from *Clostridium kluyveri*. *J Bacteriol* 190(3)**,**
843-850. doi: 10.1128/jb.01417-07.

Lubner, C.E., Jennings, D.P., Mulder, D.W., Schut, G.J., Zadvornyy, O.A., Hoben, J.P., et al.
(2017). Mechanistic insights into energy conservation by flavin-based electron
bifurcation. *Nat Chem Biol* 13(6)**,** 655-659. doi: 10.1038/nchembio.2348.

McInerney, M.J., Bryant, M.P., Hespell, R.B., and Costerton, J.W. (1981). *Syntrophomonas
wolfei* gen. nov. sp. nov., an Anaerobic, Syntrophic, Fatty Acid-Oxidizing Bacterium.
*Appl Environ Microbiol* 41(4)**,** 1029-1039.

McInerney, M.J., Rohlin, L., Mouttaki, H., Kim, U., Krupp, R.S., Rios-Hernandez, L., et al.
(2007). The genome of *Syntrophus aciditrophicus*: life at the thermodynamic limit of
microbial growth. *Proc Natl Acad Sci U S A* 104(18)**,** 7600-7605. doi:
10.1073/pnas.0610456104.

McInerney, M.J., Sieber, J.R., and Gunsalus, R.P. (2009). Syntrophy in anaerobic global carbon cycles. *Curr Opin Biotechnol* 20(6)**,** 623-632. doi: 10.1016/j.copbio.2009.10.001.

McInerney, M.J., Struchtemeyer, C.G., Sieber, J., Mouttaki, H., Stams, A.J., Schink, B., et al. (2008). Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. *Ann N Y Acad Sci* 1125**,** 58-72. doi: 10.1196/annals.1419.005.

Mitchell, P. (1975). The protonmotive Q cycle: a general formulation. *FEBS Lett* 59(2)**,** 137-139.

Montag, D., and Schink, B. (2018). Formate and Hydrogen as Electron Shuttles in Terminal Fermentations in an Oligotrophic Freshwater Lake Sediment. *Appl Environ Microb* 84(20)**,** e01572-01518. doi: 10.1128/AEM.01572-18.

Morris, B.E.L., Henneberger, R., Huber, H., and Moissl-Eichinger, C. (2013). Microbial syntrophy: interaction for the common good. *FEMS Microbiol Rev* 37(3)**,** 384-406. doi: 10.1111/1574-6976.12019.

Mouttaki, H., Nanny, M.A., and McInerney, M.J. (2007). Cyclohexane carboxylate and benzoate formation from crotonate in *Syntrophus aciditrophicus*. *Appl Environ Microbiol* 73(3)**,** 930-938. doi: 10.1128/AEM.02227-06.

Müller, N., Schleheck, D., and Schink, B. (2009). Involvement of NADH:Acceptor Oxidoreductase and Butyryl Coenzyme A Dehydrogenase in Reversed Electron Transport during Syntrophic Butyrate Oxidation by *Syntrophomonas wolfei*. *J Bacteriol* 191(19)**,** 6167. doi: 10.1128/JB.01605-08.

Muller, V., Chowdhury, N.P., and Basen, M. (2018). Electron Bifurcation: A Long-Hidden Energy-Coupling Mechanism. *Annu Rev Microbiol* 72**,** 331-353. doi: 10.1146/annurev-micro-090816-093440.

Nelson, T.C. (1951). Kinetics of genetic recombination in Escherichia coli. *Genetics* 36(2)**,** 162-175.

Niks, D., and Hille, R. (2019). Molybdenum- and tungsten-containing formate dehydrogenases and formylmethanofuran dehydrogenases: Structure, mechanism, and cofactor insertion. *Protein Sci* 28(1)**,** 111-122. doi: 10.1002/pro.3498.

Pacheco, A.R., Moel, M., and Segre, D. (2018). Costless metabolic secretions as drivers of interspecies interactions in microbial ecosystems. *bioRxiv***,** 300046. doi: 10.1101/300046.

Peters, J.W., Beratan, D.N., Schut, G.J., and Adams, M.W.W. (2018). On the nature of organic and inorganic centers that bifurcate electrons, coupling exergonic and endergonic oxidation–reduction reactions. *Chem Comm* 54(33)**,** 4091-4099. doi: 10.1039/C8CC01530A.

Peters, J.W., Lanzilotta, W.N., Lemon, B.J., and Seefeldt, L.C. (1998). X-ray crystal structure of the Fe-only hydrogenase (CpI) from *Clostridium pasteurianum* to 1.8 angstrom resolution. *Science* 282(5395)**,** 1853-1858.

Peters, J.W., Schut, G.J., Boyd, E.S., Mulder, D.W., Shepard, E.M., Broderick, J.B., et al. (2015). [FeFe]- and [NiFe]-hydrogenase diversity, mechanism, and maturation. *Biochim Biophys Acta Mol Cell Res* 1853(6)**,** 1350-1369. doi: https://doi.org/10.1016/j.bbamcr.2014.11.021.

Plugge, C.M., Henstra, A.M., Worm, P., Swarts, D.C., Paulitsch-Fuchs, A.H., Scholten, J.C.M., et al. (2012). Complete genome sequence of *Syntrophobacter fumaroxidans* strain (MPOB($^{T}$)). *Standards in genomic sciences* 7(1)**,** 91-106. doi: 10.4056/sigs.2996379.

Salvador, A.F., Martins, G., Melle-Franco, M., Serpa, R., Stams, A.J.M., Cavaleiro, A.J., et al. (2017). Carbon nanotubes accelerate methane production in pure cultures of methanogens

and in a syntrophic coculture. *Environ Microbiol* 19(7)**,** 2727-2739. doi: 10.1111/1462-2920.13774.

Sato, K., Nishina, Y., Setoyama, C., Miura, R., and Shiga, K. (1999). Unusually High Standard Redox Potential of Acrylyl-CoA/ Propionyl-CoA Couple among Enoyl-CoA/Acyl-CoA Couples: A Reason for the Distinct Metabolic Pathway of Propionyl-CoA from Longer Acyl-CoAs. *J Biochem* 126(4)**,** 668-675. doi: 10.1093/oxfordjournals.jbchem.a022501.

Schink, B. (1997). Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol Mol Biol Rev* 61(2)**,** 262-280.

Schink, B., Montag, D., Keller, A., and Müller, N. (2017). Hydrogen or formate: Alternative key players in methanogenic degradation. *Environ Microbiol Rep* 9(3)**,** 189-202. doi: 10.1111/1758-2229.12524.

Schmehl, M., Jahn, A., Meyer zu Vilsendorf, A., Hennecke, S., Masepohl, B., Schuppler, M., et al. (1993). Identification of a new class of nitrogen fixation genes in *Rhodobacter capsalatus*: a putative membrane complex involved in electron transport to nitrogenase. *Molecular and General Genetics MGG* 241(5)**,** 602-615. doi: 10.1007/BF00279903.

Schmidt, A., Muller, N., Schink, B., and Schleheck, D. (2013). A proteomic view at the biochemistry of syntrophic butyrate oxidation in *Syntrophomonas wolfei*. *PLoS One* 8(2)**,** e56905. doi: 10.1371/journal.pone.0056905.

Schuchmann, K., Chowdhury, N.P., and Müller, V. (2018). Complex Multimeric [FeFe] Hydrogenases: Biochemistry, Physiology and New Opportunities for the Hydrogen Economy. *Front Microbiol* 9(2911). doi: 10.3389/fmicb.2018.02911.

Schuchmann, K., and Muller, V. (2012). A bacterial electron-bifurcating hydrogenase. *J Biol Chem* 287(37)**,** 31165-31171. doi: 10.1074/jbc.M112.395038.

Schut, G.J., and Adams, M.W.W. (2009). The Iron-Hydrogenase of *Thermotoga maritima* Utilizes Ferredoxin and NADH Synergistically: a New Perspective on Anaerobic Hydrogen Production. *J Bacteriol* 191(13)**,** 4451.

Sedano-Núñez, V.T., Boeren, S., Stams, A.J.M., and Plugge, C.M. (2018). Comparative proteome analysis of propionate degradation by *Syntrophobacter fumaroxidans* in pure culture and in coculture with methanogens. *Env Microbiol* 20(5)**,** 1842-1856. doi: doi:10.1111/1462-2920.14119.

Shima, S., Pilak, O., Vogt, S., Schick, M., Stagni, M.S., Meyer-Klaucke, W., et al. (2008). The crystal structure of [Fe]-hydrogenase reveals the geometry of the active site. *Science* 321(5888)**,** 572-575. doi: 10.1126/science.1158978.

Sieber, J.R., Crable, B.R., Sheik, C.S., Hurst, G.B., Rohlin, L., Gunsalus, R.P., et al. (2015). Proteomic analysis reveals metabolic and regulatory systems involved in the syntrophic and axenic lifestyle of *Syntrophomonas wolfei*. *Front Microbiol* 6**,** 115. doi: 10.3389/fmicb.2015.00115.

Sieber, J.R., Le, H.M., and McInerney, M.J. (2014). The importance of hydrogen and formate transfer for syntrophic fatty, aromatic and alicyclic metabolism. *Environ Microbiol* 16(1)**,** 177-188. doi: 10.1111/1462-2920.12269.

Sieber, J.R., McInerney, M.J., and Gunsalus, R.P. (2012). Genomic Insights into Syntrophy: The Paradigm for Anaerobic Metabolic Cooperation. *Annu Rev Microbiol* 66(1)**,** 429-452. doi: 10.1146/annurev-micro-090110-102844.

Sieber, J.R., Sims, D.R., Han, C., Kim, E., Lykidis, A., Lapidus, A.L., et al. (2010). The genome of *Syntrophomonas wolfei*: new insights into syntrophic metabolism and biohydrogen

production. *Environ Microbiol* 12(8)**,** 2289-2301. doi: 10.1111/j.1462-2920.2010.02237.x.

Soboh, B., Linder, D., and Hedderich, R. (2004). A multisubunit membrane-bound [NiFe] hydrogenase and an NADH-dependent Fe-only hydrogenase in the fermenting bacterium *Thermoanaerobacter tengcongensis*. *Microbiology* 150(Pt 7)**,** 2451-2463. doi: 10.1099/mic.0.27159-0.

Søndergaard, D., Pedersen, C.N.S., and Greening, C. (2016). HydDB: A web tool for hydrogenase classification and analysis. *Sci Rep* 6**,** 34212. doi: 10.1038/srep34212 https://www.nature.com/articles/srep34212#supplementary-information.

Stams, A.J., and Plugge, C.M. (2009). Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nat Rev Microbiol* 7(8)**,** 568-577. doi: 10.1038/nrmicro2166.

Stams, A.J.M., and Dong, X. (1995). Role of formate and hydrogen in the degradation of propionate and butyrate by defined suspended cocultures of acetogenic and methanogenic bacteria. *Antonie Leeuwenhoek* 68(4)**,** 281-284. doi: 10.1007/BF00874137.

Sticht, H., and Rosch, P. (1998). The structure of iron-sulfur proteins. *Prog Biophys Mol Biol* 70(2)**,** 95-136.

Thamer, W., Cirpus, I., Hans, M., Pierik, A.J., Selmer, T., Bill, E., et al. (2003). A two [4Fe-4S]-cluster-containing ferredoxin as an alternative electron donor for 2-hydroxyglutaryl-CoA dehydratase from *Acidaminococcus fermentans*. *Arch Microbiol* 179(3)**,** 197-204. doi: 10.1007/s00203-003-0517-8.

Thauer, R.K., Jungermann, K., and Decker, K. (1977). Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol Rev* 41(1)**,** 100-180.

Thauer, R.K., Kaster, A.-K., Seedorf, H., Buckel, W., and Hedderich, R. (2008). Methanogenic archaea: ecologically relevant differences in energy conservation. *Nat Rev Microbiol* 6**,** 579. doi: 10.1038/nrmicro1931.

Verhagen, M.F.J.M., O'Rourke, T., and Adams, M.W.W. (1999). The hyperthermophilic bacterium, *Thermotoga maritima*, contains an unusually complex iron-hydrogenase: amino acid sequence analyses versus biochemical characterization. *Biochim Biophys Acta Bioenerg* 1412(3)**,** 212-229. doi: https://doi.org/10.1016/S0005-2728(99)00062-6.

Vignais, P.M., and Billoud, B. (2007). Occurrence, Classification, and Biological Function of Hydrogenases:  An Overview. *Chem Rev* 107(10)**,** 4206-4272. doi: 10.1021/cr050196r.

Volbeda, A., Charon, M.-H., Piras, C., Hatchikian, E.C., Frey, M., and Fontecilla-Camps, J.C. (1995). Crystal structure of the nickel–iron hydrogenase from *Desulfovibrio gigas*. *Nature* 373**,** 580. doi: 10.1038/373580a0.

Walker, D.J.F., Nevin, K.P., Holmes, D.E., Rotaru, A.-E., Ward, J.E., Woodard, T.L., et al. (2018). *Syntrophus* Conductive Pili Demonstrate that Common Hydrogen-Donating Syntrophs can have a Direct Electron Transfer Option. *bioRxiv***,** 479683. doi: 10.1101/479683.

Wang, S., Huang, H., Kahnt, J., Mueller, A.P., Köpke, M., and Thauer, R.K. (2013a). NADP-specific electron-bifurcating [FeFe]-hydrogenase in a functional complex with formate dehydrogenase in *Clostridium autoethanogenum* grown on CO. *J Bacteriol* 195(19)**,** 4373-4386. doi: 10.1128/JB.00678-13.

Wang, S., Huang, H., Kahnt, J., and Thauer, R.K. (2013b). *Clostridium acidurici* Electron-Bifurcating Formate Dehydrogenase. *Appl Environ Microb* 79(19)**,** 6176.

Wang, S., Huang, H., Kahnt, J., and Thauer, R.K. (2013c). A Reversible Electron-Bifurcating Ferredoxin- and NAD-Dependent [FeFe]-Hydrogenase (HydABC) in *Moorella thermoacetica*. *J Bacteriol* 195(6)**,** 1267.

Wang, S., Huang, H., Moll, J., and Thauer, R.K. (2010). NADP+ reduction with reduced ferredoxin and NADP+ reduction with NADH are coupled via an electron-bifurcating enzyme complex in *Clostridium kluyveri*. *J Bacteriol* 192(19)**,** 5115-5123. doi: 10.1128/jb.00612-10.

Weghoff, M.C., Bertsch, J., and Müller, V. (2015). A novel mode of lactate metabolism in strictly anaerobic bacteria. *Environ Microbiol* 17(3)**,** 670-677. doi: 10.1111/1462-2920.12493.

Wilson, R.M., Tfaily, M.M., Rich, V.I., Keller, J.K., Bridgham, S.D., Zalman, C.M., et al. (2017). Hydrogenation of organic matter as a terminal electron sink sustains high $CO_2$:$CH_4$ production ratios during anaerobic decomposition. *Org Geochem* 112**,** 22-32. doi: https://doi.org/10.1016/j.orggeochem.2017.06.011.

Wofford, N.Q., Beaty, P.S., and McInerney, M.J. (1986). Preparation of cell-free extracts and the enzymes involved in fatty acid metabolism in *Syntrophomonas wolfei*. *J Bacteriol* 167(1)**,** 179. doi: 10.1128/jb.167.1.179-185.1986.

Yan, Z., Wang, M., and Ferry, J.G. (2017). A Ferredoxin- and $F_{420}H_2$-Dependent, Electron-Bifurcating, Heterodisulfide Reductase with Homologs in the Domains Bacteria and Archaea. *mBio* 8(1)**,** e02285-02216. doi: 10.1128/mBio.02285-16.

Zhao, Z., Li, Y., Yu, Q., and Zhang, Y. (2018). Ferroferric oxide triggered possible direct interspecies electron transfer between *Syntrophomonas* and *Methanosaeta* to enhance

waste activated sludge anaerobic digestion. *Bioresour Technol* 250**,** 79-85. doi:

https://doi.org/10.1016/j.biortech.2017.11.003.

Zheng, Y., Kahnt, J., Kwon, I.H., Mackie, R.I., and Thauer, R.K. (2014). Hydrogen Formation

and Its Regulation in *Ruminococcus albus*: Involvement of an Electron-Bifurcating

[FeFe]-Hydrogenase, of a Non-Electron-Bifurcating [FeFe]-Hydrogenase, and of a

Putative Hydrogen-Sensing [FeFe]-Hydrogenase. *J Bacteriol* 196(22)**,** 3840.

# CHAPTER 2. *Syntrophomonas wolfei* Uses a NADH-Dependent, Ferredoxin-Independent, [FeFe]-Hydrogenase to Reoxidize NADH

The contents of this chapter were previously published as a manuscript in the journal Applied and Environmnetal Microbiology in 2017. This chapter represents a pre-print version of that manuscript with modifications to figure numbering, figure placement, and reference style to be consistent with the remainder of this dissertation.

**Abstract**

*Syntrophomonas wolfei* Uses a NADH-Dependent, Ferredoxin-Independent, [FeFe]-

Hydrogenase to Reoxidize NADH

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Running Head: NADH Reoxidation in *Syntrophomonas wolfei*.

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**Abstract**

*Syntrophomonas wolfei* syntrophically oxidizes short-chain fatty acids (four to eight carbon in

length) when grown in coculture with a hydrogen- and/or formate-using methanogen. The

oxidation of 3-hydroxybutyryl-CoA, formed during butyrate metabolism, results in the production of NADH. The enzyme systems involved in NADH reoxidation in *S. wolfei* are not well understood. The genome of *S. wolfei* contains a multimeric [FeFe]-hydrogenase that may be a mechanism for NADH reoxidation. The *S. wolfei* genes for the multimeric [FeFe]-hydrogenase (*hyd1ABC*; SWOL_RS05165, SWOL_RS05170, SWOL_RS05175) and [FeFe]-hydrogenase maturation proteins (SWOL_RS05180, SWOL_RS05190, SWOL_RS01625) were co-expressed in *Escherichia coli* and the recombinant Hyd1ABC was purified and characterized. The purified recombinant Hyd1ABC was a heterotrimer with an αβγ configuration and a molecular mass of 115 kDa. Hyd1ABC contained $29.2 \pm 1.49$ mole of Fe and 0.7 mole of FMN per mole enzyme. The purified, recombinant Hyd1ABC reduced $NAD^+$ and oxidized NADH without the presence of ferredoxin. The HydB subunit of the *S. wolfei* multimeric [FeFe]-hydrogenase lacks two iron-sulfur centers that are present in known confurcating NADH- and ferredoxin-dependent [FeFe]-hydrogenases. Hyd1ABC is a NADH-dependent hydrogenase that produces hydrogen from NADH without the need of reduced ferredoxin, which differs from confurcating [FeFe]-hydrogenases. Hyd1ABC provides a mechanism by which *S. wolfei* can reoxidize NADH produced during syntrophic butyrate oxidation when low hydrogen partial pressures are maintained by a hydrogen-consuming microorganism.

**Importance**

Our work provides mechanistic understanding of the obligate metabolic coupling that occurs between hydrogen-producing fatty and aromatic acid-degrading microorganisms and their hydrogen-consuming partners in the process called syntrophy (to feed together).  The multimeric [FeFe]-hydrogenase used NADH without the involvement of reduced ferredoxin. The multimeric

[FeFe]-hydrogenase would only produce hydrogen from NADH when hydrogen concentrations are low. Hydrogen production from NADH by *Syntrophomonas wolfei* would likely cease before any detectable amount of cell growth occurred. Thus, continual hydrogen production requires the presence of a hydrogen-consuming partner to keep hydrogen concentrations low and explains, in part, the obligate requirement that *S. wolfei* has for a hydrogen-consuming partner organism during growth on butyrate. We have successfully expressed genes encoding for a multimeric [FeFe]-hydrogenase in *E. coli* demonstrating that such an approach can be advantageous to characterize complex redox proteins from difficult to culture microorganisms.

**Introduction**

The model syntrophic bacterium, *S. wolfei,* oxidizes butyrate to acetate with the concomitant production of hydrogen and formate (McInerney et al., 1981). During syntrophic butyrate metabolism, the hydrogen- and/or formate-using methanogen maintains low levels of hydrogen and formate so that butyrate oxidation remains thermodynamically favorable (McInerney and Bryant, 1981; Schink, 1997). Butyrate oxidation by *S. wolfei* involves two different oxidation-reduction reactions, generating electrons at two different redox potentials, both of which are used to produce hydrogen or formate (Wofford et al., 1986; Sieber et al., 2010; Schmidt et al., 2013). The first set of electrons is generated during the oxidation of butyryl-CoA to crotonyl-CoA. Due to the high redox potential of this electron pair ($E^{0\prime}$ = -125 mV), it has been proposed that membrane complexes utilizing chemiosmotic energy are required for hydrogen or formate production (Sieber et al., 2010; Schmidt et al., 2013; Sieber et al., 2014; Sieber et al., 2015; Crable et al., 2016). The second pair of electrons is generated from the oxidation of 3-hydroxybutyryl-CoA to acetoacetyl-CoA ($E^{0\prime}$ = -250 mV) and is used to reduce $NAD^+$ to NADH

(E $^{0\prime}$ = -320 mV). NADH reoxidation must be coupled to hydrogen or formate production during syntrophic butyrate metabolism. Under the low hydrogen partial pressures maintained by hydrogenotrophic methanogens (<10 Pa), the redox potential (E') of hydrogen is about -260 mV, making hydrogen production directly from NADH thermodynamically feasible (Schink, 1997; Sieber et al., 2012).

The analysis of the *S. wolfei* genome identified a multimeric [FeFe]-hydrogenase encoded by the genes (*hyd1ABC*; SWOL_RS05165, SWOL_RS05170, SWOL_RS05175) that shared similar sequence identity to confurcating [FeFe]-hydrogenases (Schut and Adams, 2009; Sieber et al., 2010; Sieber et al., 2012), a NADPH-linked [FeFe]-hydrogenase (Malki et al., 1995), and NADH-dependent formate dehydrogenases (Hartmann and Leimkuhler, 2013). Most examples of multimeric [FeFe]-hydrogenases have been reported to function in either electron confurcation or the reverse (bifurcating) reaction. Confurcating [FeFe]-hydrogenases couple the unfavorable production of hydrogen from NADH with the favorable production of hydrogen from reduced ferredoxin (Schut and Adams, 2009), utilizing equimolar amounts of NADH and reduced ferredoxin to produce hydrogen, as was first demonstrated in the multimeric [FeFe]-hydrogenase from *Thermotoga maritima* (Schut and Adams, 2009). Additional multimeric [FeFe]-hydrogenases have been studied in *Acetobacterium woodii* (Schuchmann and Muller, 2012), *Moorella thermoacetica* (Wang et al., 2013b), and *Ruminococcus albus* (Zheng et al., 2014).

The beta-oxidation of butyrate by *S. wolfei* does not directly result in the formation of reduced ferredoxin. Thus, *S. wolfei*'s metabolism differs from the metabolism of organisms with reported confurcating/bifurcating [FeFe]-hydrogenases, which have ferredoxin-dependent

46

oxidoreductases, such as pyruvate:ferredoxin oxidoreductase that produce reduced ferredoxin during substrate degradation (Schut and Adams, 2009; Schuchmann and Muller, 2012; Wang et al., 2013b; Zheng et al., 2014). One potential source of reduced ferredoxin in *S. wolfei* is a putative Fix system, which could produce reduced ferredoxin from the oxidation of NADH (Sieber et al., 2010). However, the use of Fix to produce reduced ferredoxin would place a high energy demand on an organism that uses growth reactions that operate close to thermodynamic equilibrium (Jackson and McInerney, 2002). In the absence of an identifiable source of reduced ferredoxin and with syntrophic growth conditions that would be permissive for hydrogen production from NADH, it is likely that Hyd1ABC functions as a non-confurcating, NADH-dependent [FeFe]-hydrogenase.

We cloned and expressed the genes of a multimeric [FeFe]-hydrogenase (SWOL_RS05165, SWOL_RS05170, SWOL_RS05175) from *S. wolfei* and characterized the recombinant protein in order to understand the mechanisms by which *S. wolfei* reoxidizes NADH. To obtain an active enzyme, it was also necessary to co-express the genes required for [FeFe]-hydrogenase maturation (SWOL_RS05180, SWOL_RS05190, SWOL_RS01625). This strategy has been used successfully in the past to produce active [FeFe]-hydrogenases using *Escherichia coli* (Girbal et al., 2005; King et al., 2006) and allowed the production of an enzyme with the activity of a NADH-dependent [FeFe]-hydrogenase (Hyd1ABC: SWOL_RS05165, SWOL_RS05170, SWOL_RS05175 gene products).

**Results**

**Purification and molecular weight**. The recombinant gene products of SWOL_RS05165, SWOL_RS05170, SWOL_RS05175 were purified to apparent homogeneity using nickel-affinity and anion-exchange chromatography, resulting in an apparent yield of 12.7% (Table 1).

**Table 1. Purification of NADH-dependent [FeFe]-hydrogenase, Hyd1ABC.**

| Fraction | Protein (mg) | Specific activity[a] $H_2 \rightarrow MV_{ox}$ ($U \cdot mg^{-1}$) | Total activity[a] $H_2 \rightarrow MV_{ox}$ ($U \cdot mg^{-1}$) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Cell-free extract | 670 | 16.8 | 11200 | 1 | 100 |
| HisTrap HP | 4.0 | 655 | 2620 | 38.9 | 23.4 |
| UNO-Q1 | 0.43 | 3340 | 1430 | 199 | 12.7 |

[a]Activity was determined at 37 °C, pH 7.5; one unit of activity (U) equals 2 μmol of electrons transferred per min.

Approximately 0.55 mg of purified recombinant enzyme was obtained from 10.8 g (wet weight) of cells. SDS-PAGE analysis showed that the purified recombinant enzyme consisted of three subunits with molecular weights that were consistent with molecular weights predicted from the sequence of encoding genes; 63 kDa compared to the predicted 63.0 kDa (HydA1), 43 kDa compared to the predicted 43.9 kDa (HydB1), and 13 kDa compared to the predicted 17.6 kDa (HydC1) with the included molecular weight of the His-tag (MGSSHHHHHHSQDP, 1.6 kDa) (Figure 7).

48

**Figure 7. SDS-PAGE of NADH-Dependent [FeFe]-Hydrogenase, Hyd1ABC.** Lane 1, molecular weight markers, Lane 2, *E. coli* cell-free extract, Lane 3, HisTrap HP, Lane 4, UNO-Q1. The displayed molecular masses of Hyd1A-C peptides are predicted from the amino acid sequence.
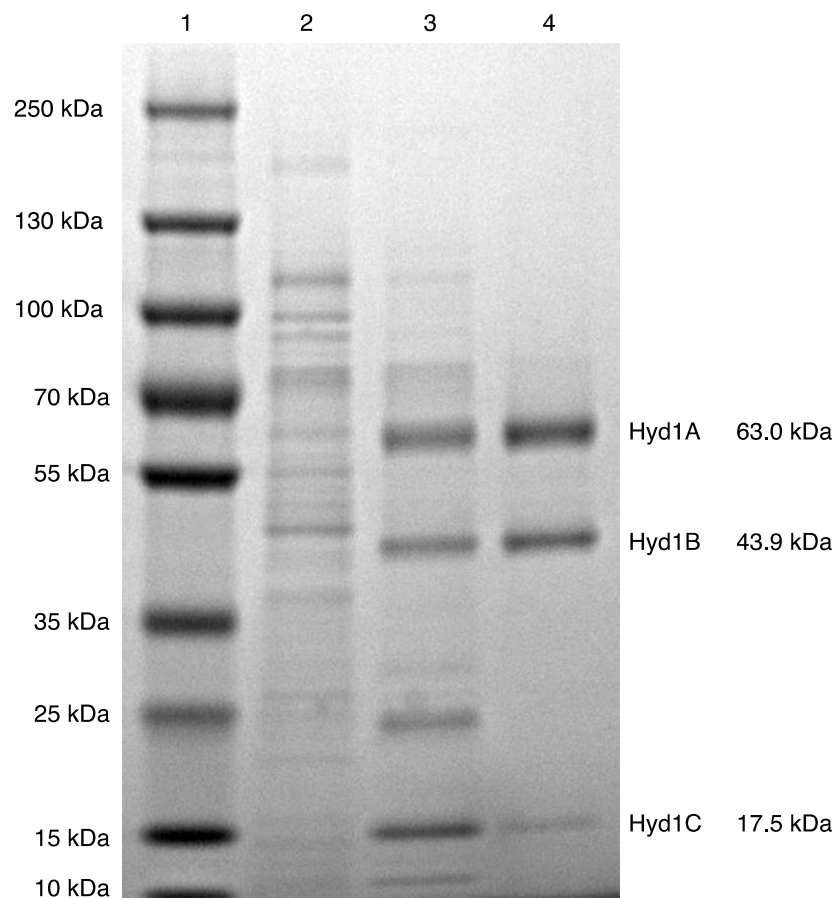


Native-PAGE gel electrophoresis analysis showed that the purified enzyme migrated as a single band (Figure 8) and size exclusion chromatography gave a molecular weight of 115 kDa.

**Figure 8. Native PAGE of the NADH-Dependent [FeFe]-hydrogenase**, **Hyd1ABC.** Lane 1: native molecular weight markers, Lane 2: *E. coli* cell-free extract, Lane 3: UNO-Q1 fraction of the purified, Hyd1ABC.
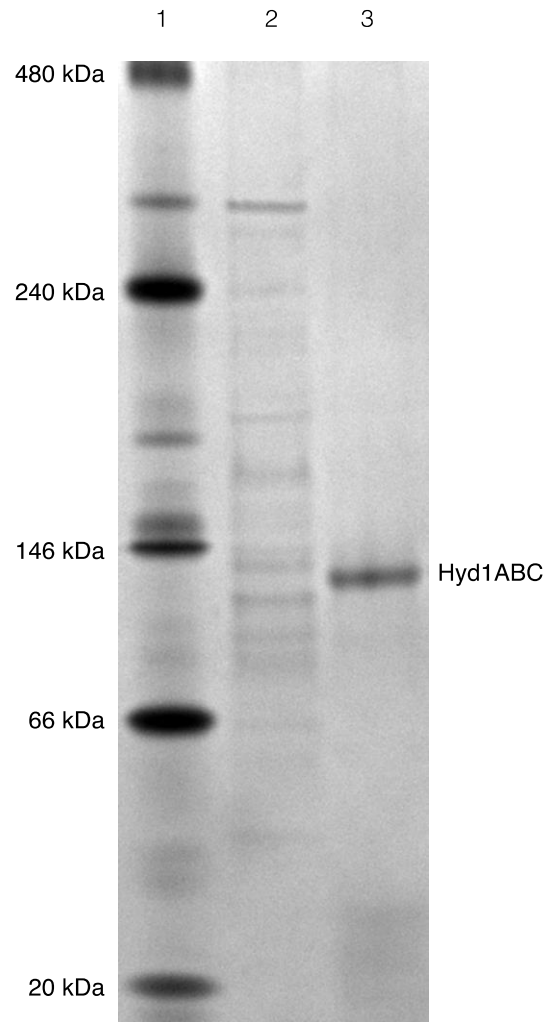


The single visible band from native gel electrophoresis when subjected to peptide analysis showed high coverage scores for the intended peptides (Table S1). Based on the subunit analysis and the native molecular weight, Hyd1ABC is a heterotrimer with an αβγ configuration and a molecular mass of 124.5 kDa as predicted by the sequence of the encoding genes. Iron

content was determined to be $29.2 \pm 1.49$ mole of Fe per mole of enzyme, which matches well with the predicted 30 mole Fe per mole of enzyme from conserved domain analysis, which indicated five [4Fe-4S]-clusters, two [2Fe-2S]-clusters and the six Fe in the [H-Cluster]. The flavin content was 0.7 mole of FMN per mole of enzyme, which agrees with the single flavin-binding site in the HydB subunit, predicted from conserved domain analysis.

**Activity**. The purified recombinant Hyd1ABC had high hydrogen-dependent, methyl viologen-reducing activity and reduced methyl viologen-oxidizing activity (Table 2), typical for many [FeFe]-hydrogenases.

**Table 2. Specific Activities of the Purified, NADH-Dependent [FeFe]-Hydrogenase, Hyd1ABC.**

| Reaction | Hydrogenase activity $(U \cdot mg^{-1})$ [a] |
|---|---|
| $H_2 \rightarrow MV_{ox}$ | 571 |
| $H_2 \rightarrow NAD^+$ | 94.5 |
| $H_2 \rightarrow NAD^+ + Fd_{ox}$ | 92.1[b] |
| $H_2 \rightarrow NADP^+$ | <0.01 |
| $H_2 \rightarrow NADP^+ + Fd_{ox}$ | <0.01 |
| $H_2 \rightarrow Fd_{ox}$ | <0.01 |
| $MV_{red} \rightarrow H_2$ | 24.3 |
| $NADH \rightarrow H_2$ | 6.6 |
| $NADH + Fd_{red} \rightarrow H_2$ | 6.0 |

| | |
|---|---|
| NADPH → H$_2$ | <0.01 |
| NADPH + Fd$_{red}$ → H$_2$ | <0.01 |
| Fd$_{red}$ → H$_2$ | <0.01 |

[a]Activity was determined at 37 °C, pH 7.5; one unit of activity (U) equals 2 μmol of electrons transferred per min.

[b] Activity is the rate of NAD$^+$ reduction at 340 nm in the presence of Fd$_{ox}$ and not the rate of Fd$_{ox}$ reduction (at 430 nm), which was not observed to occur.

The purified recombinant Hyd1ABC also had high NAD$^+$-reducing activity with hydrogen as the electron donor (specific activity of 94.5 U•mg$^{-1}$ at 37°C) and catalyzed hydrogen production from NADH with a specific activity of 6.6 U•mg$^{-1}$ at 37°C (Table 2). The enzyme did not oxidize NADPH nor reduce NADP$^+$ under any condition tested. Neither the rate of NAD$^+$ reduction nor the rate of NADH-dependent hydrogen production changed when oxidized or reduced clostridial ferredoxin, respectively, was added (Figure 9 and Figure 10).

**Figure 9. Reduction of NAD$^+$ by the Purified, NADH-Dependent [FeFe]-Hydrogenase, Hyd1ABC with Hydrogen as the Electron Donor.** Reactions were performed in 1.5 mL, sealed, anaerobic cuvettes containing 0.5 mL of 50 mM potassium phosphate buffer (pH 7.5 at 37°C) 5 μM FAD, 5 μM FMN, 2 mM DTE, 1 mm NAD$^+$ and 0.11 μg of Hyd1ABC. Clostridial ferredoxin was added at a concentration of 10 μM. The change in absorbance ($\Delta A_{340}$) before the addition of hydrogen was 0.129 A• min$^{-1}$ and after the addition of oxidized ferredoxin was 0.125 A•min$^{-1}$.
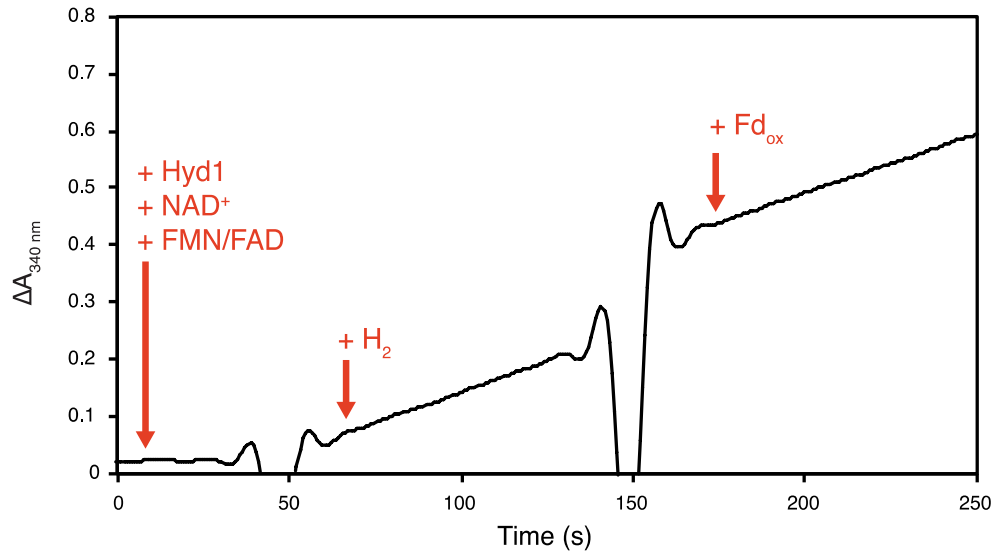
**Figure 10. The Effect of NADH and Reduced Ferredoxin on Hydrogen Production by Hyd1ABC.** Reactions were performed in 6.5 mL serum bottles containing 0.5 mL of 100 mM potassium phosphate buffer (pH 7.5 at 37°C), 5 µM FAD, 5 µM FMN, 2 mM DTE, 1 mM NADH and 1.3 µg of Hyd1ABC. A ferredoxin reduction system as indicated by +Fd consisted of 10 mM pyruvate, 1 mM CoA, 0.1 mM thiamine pyrophosphate, 5 µM clostridial ferredoxin and 0.1 U of clostridial pyruvate:ferredoxin oxidoreductase. NADH (1 mM) was added immediately after sampling time points indicated by +NADH.
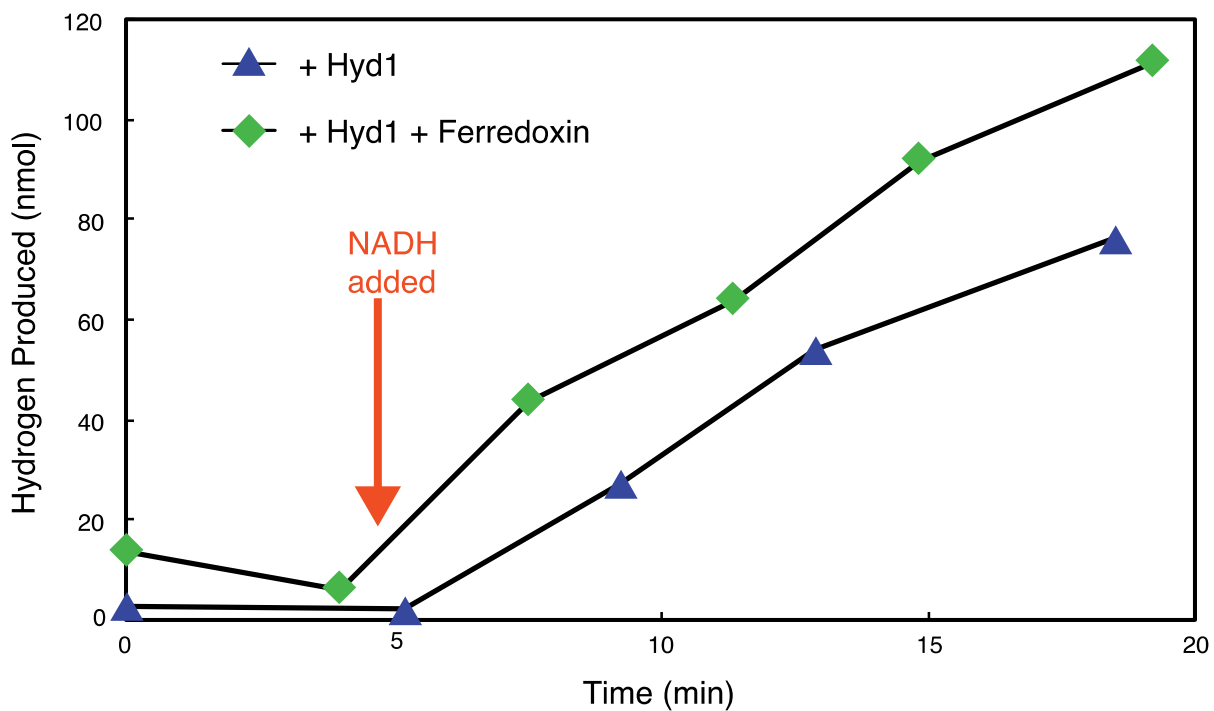


In addition, the purified recombinant Hyd1ABC did not reduce clostridial ferredoxin either alone or in the presence of $NAD^+$ with hydrogen as the electron donor. Hyd1ABC did not produce hydrogen from reduced clostridial ferredoxin in the absence of NADH, and the amount of hydrogen produced when both NADH and reduced ferredoxin were present was similar to that

when only NADH was present (Figure 10). The above experiments were repeated with the purified recombinant *S. wolfei* ferredoxin encoded by SWOL_RS10890 with results similar to those using the clostridial ferredoxin. That is, the recombinant *S. wolfei* ferredoxin did not appear to affect the rate of $NAD^+$ reduction or NADH oxidation. In addition, Hyd1ABC did not reduce the recombinant *S. wolfei* ferredoxin with hydrogen as the electron donor and did not produce hydrogen with reduced recombinant *S. wolfei* ferredoxin as the electron donor. Kinetics of $NAD^+$ reduction with hydrogen, where the $NAD^+$ concentration was varied, indicated a $K_m$ for $NAD^+$ of 520 µM, $V_{max}$ of 196 U•$mg^{-1}$, and a $k_{cat}$ of 406.7 $s^{-1}$, assuming a single catalytic site per a molecular mass of 124.5 kDa. (Figure S1). Hyd1ABC specific activities for methyl viologen and $NAD^+$ reduction and for NADH oxidation were similar to those reported for other multimeric [FeFe]-hydrogenases; however, the $K_m$ for reduction of $NAD^+$ by Hyd1ABC was higher than that of other multimeric [FeFe]-hydrogenases (Table S2).

In the above assays, the purified Hyd1ABC produced hydrogen from NADH under conditions where only a small amount of NADH would be oxidized to $NAD^+$ during the time course of the assay and thus the ratio of NADH/$NAD^+$ would remain high. To determine whether Hyd1ABC could produce hydrogen from NADH under more physiological conditions, we varied the ratio of NADH to $NAD^+$ and pH, but kept the total pyridine nucleotide concentration constant at 5 mM (Table 3).

**Table 3. Equilibrium Hydrogen Partial Pressures Produced by NADH-Dependent [FeFe]-Hydrogenase, Hyd1ABC at Different pH and NADH to NAD$^+$ ratios. [a]**

| Condition | Ratio of NADH/NAD$^+$ | Hydrogen Partial pressure (Pa)[b] | Hydrogen produced (nmol) |
|---|---|---|---|
| pH 6.5, 2.0 mM NAD$^+$, 3.0 mM NADH | 1.5 | 40.0 (6.9) | 95.4 |
| pH 6.5, 2.5 mM NAD$^+$, 2.5 mM NADH | 1.0 | 21.8 (8.7) | 51.8 |
| pH 7.0, 2.0 mM NAD$^+$, 3.0 mM NADH | 1.5 | 25.9 (0.8) | 61.9 |
| pH 7.0, 2.5 mM NAD$^+$, 2.5 mM NADH | 1.0 | 17.0 (11.3) | 40.4 |

[a]Assays were performed in 6.5 mL serum bottles with 0.5 mL of liquid volume containing 0.86 µg of protein and 6 mL of headspace.

[b] Values are the mean and the range (in parentheses) of measured hydrogen values in Pa.

In each case, more hydrogen was produced with a NADH to NAD$^+$ ratio of 1.5 compared to 1.0. More hydrogen was also produced at pH 6.5 compared to pH 7.0. Hydrogen partial pressures varied from 17.0 Pa with a range of 11.3 Pa to 40.0 Pa with a range of 6.9 Pa with higher partial pressures observed at lower pH values and higher NADH/NAD$^+$ ratios. Final hydrogen partial pressures detected in these experiments were below that predicted at thermodynamic equilibrium for the given NADH/NAD$^+$ ratios. With the given liquid and gas

volumes, a NADH/NAD$^+$ ratio of 1.5 and a pH of 7.0, the redox potential (E') of the

NADH/NAD$^+$ pair should be greater than -320 mV, even with the shift in NADH/NAD$^+$ ratio as

NADH is oxidized. The predicted equilibrium hydrogen partial pressure is in excess of 70 Pa for

these conditions; however, the measured hydrogen partial pressure was lower at 25.9 Pa, with a

range of 0.8, suggesting that thermodynamic equilibrium was not reached.

**Hydrogen levels in growing coculture.** Hydrogen partial pressures measured during growth of

*S. wolfei* in coculture with *M. hungatei* on either butyrate or crotonate were between 4.6 to 18.0

Pa (Figure S2), with the highest concentrations observed at day four and lower concentrations

observed during the later stages of growth. Hydrogen partial pressures measured for pure culture

*S. wolfei* grown on crotonate were between 27.4 to 40.0 Pa.

**Discussion**

The identification of a NADH-dependent [FeFe]-hydrogenase in *S. wolfei* provides an

explanation for how electrons derived from 3-hydroxybutyryl-CoA oxidation are used to make

hydrogen during syntrophic growth. Stoichiometrically, all electrons derived from the oxidation

of butyrate are converted to either hydrogen and formate as *S. wolfei* does not produce any other

reduced end products during syntrophic butyrate metabolism (McInerney et al., 1979; Amos and

McInerney, 1989). The electrons generated by the oxidation of 3-hydroxybutyryl-CoA to

acetoacetyl-CoA are used to reduce NAD$^+$ to NADH (Wofford et al., 1986). The presence of a

multimeric [FeFe]-hydrogenase and NADH-linked formate dehydrogenase genes in the genome

of *S. wolfei* (Sieber et al., 2010) suggested that NADH could be reoxidized using these enzyme

systems. Hyd1 represents the only hydrogenase encoded within the genome of *S. wolfei*

predicted to catalyze NADH oxidation. Of the two other hydrogenases present within the *S. wolfei* genome, Hyd2, encoded by SWOL_RS09950, is a membrane-bound [FeFe]-hydrogenase believed to interact with the menaquinone pool (Sieber et al., 2010; Sieber et al., 2015; Crable et al., 2016), while Hyd3, encoded by SWOL_RS12620, is a monomeric type [FeFe]-hydrogenase (Sieber et al., 2010), which has not been reported to interact with NADH (Poudel et al., 2016). *hyd1A* gene was upregulated under coculture conditions on butyrate compared to coculture conditions on crotonate (Sieber et al., 2014); and Hyd1ABC was confirmed to be present by proteomic techniques (Schmidt et al., 2013; Sieber et al., 2015) during syntrophic coculture growth on butyrate with *M. hungatei*. Thus, Hyd1ABC along with NADH-linked formate dehydrogenases are the likely routes of NADH reoxidation in *S. wolfei*.

The possibility that *S. wolfei* utilizes a NADH-dependent hydrogenase was suggested previously due to the closeness of the redox potentials of 3-hydroxybutyryl-CoA/acetoacetyl-CoA ($E^{0\prime}$ = -250 mV), NADH/NAD$^+$ ($E^{0\prime}$ = -320 mV), and $H_2/H^+$ at 1 Pa $H_2$ ($E^{\prime}$ = -261 mV) (Schink, 1997). The amino acid sequence comparisons suggested that Hyd1ABC might be a confurcating hydrogenase (Sieber et al., 2010; Sieber et al., 2012). Confurcating/bifurcating [FeFe]-hydrogenses produce hydrogen from NADH ($E^{0\prime}$ = -320 mV) and reduced ferredoxin ($E^{0\prime}$ = -398 mV or less) (Schut and Adams, 2009; Schuchmann and Muller, 2014). Coupling the unfavorable hydrogen production from NADH with the favorable hydrogen production from reduced ferredoxin would allow hydrogen production at partial pressures greater than 1000 Pa at equilibrium (equivalent to $E^{\prime}$ = -367 mV). The NADH/NAD$^+$ midpoint potential ($E^{0\prime}$ = -320 mV) would allow for a much lower hydrogen partial pressure of close to 70 Pa at equilibrium. Using fixed NADH/NAD$^+$ ratios of 1.0 and 1.5, Hyd1ABC produced partial pressures of

hydrogen from 17 to 40 Pa (Table 3). NADH/NAD$^+$ ratios reported for organisms grown under anaerobic conditions can vary with some values lower than those tested here, for example 0.27 for *Clostridium kluyveri*, 0.29 for *Clostridium welchii* and 0.4 for *E. coli* (Wimpenny and Firth, 1972), while other studies have reported higher ratios of 0.75 for *E. coli* (de Graef et al., 1999) and 1.16 for *Enterococcus faecalis* (Snoep et al., 1991). Thus, the production of hydrogen by Hyd1ABC from NADH/NAD$^+$ ratios of 1.0 or higher resemble physiological conditions for *S. wolfei* cells, although the actual NADH/NAD$^+$ ratios during syntrophic growth conditions have not yet been determined.

The partial pressure of hydrogen produced by Hyd1ABC was within the range of the hydrogen partial pressure (25 Pa) produced by cell suspensions of butyrate-grown *S. wolfei* cells when conditions allowed only hydrogen production from NADH (Wallrabenstein and Schink, 1994). Butyrate-oxidizing cocultures of *S. wolfei* and *Methanobacterium formicicum* maintained a partial pressure of hydrogen of 10.6 Pa (Boone et al., 1989), while hydrogen concentrations for cocultures of *S. wolfei* and *M. hungatei* ranged from 4.6 to 18.0 Pa (Figure S2). Measurements of partial pressures of hydrogen from methanogenic butyrate-degrading environments, such as lake sediment and sewage sludge, ranged from 3.7 to 26.9 Pa (Conrad et al., 1986). Hydrogen partial pressures from syntrophic, butyrate-oxidizing biogas reactors ranged from 1 to 4.5 Pa (Montag and Schink, 2016). Methanogenic syntrophic microcosms established from peat soil implicated members of the *Syntrophomonas* genus in butyrate oxidation had hydrogen partial pressures that ranged from 3 to 12.5 Pa (Schmidt et al., 2016). Thus, the hydrogen partial pressure that would allow for reoxidation of NADH during syntrophic butyrate metabolism is considerably less than that achieved by confurcating hydrogenases, but it is within the range of the hydrogen partial

59

pressures produced by Hyd1ABC from NADH alone (Table 3). Furthermore, such hydrogen partial pressures are observed in coculture, microcosms and environments where members of the *Syntrophomonas* genus would perform syntrophic butyrate oxidation. Thus, the hydrogen partial pressure achieved by Hyd1ABC from NADH would appear to be sufficient to allow syntrophic growth of *S. wolfei* on butyrate in coculture with a hydrogenotrophic methanogen.

Hyd1ABC was capable of both $NAD^+$ reduction with hydrogen and the oxidation of NADH to produce hydrogen in the absence of ferredoxin (Figure 9 and 10; Table 2), indicating the lack of a tight coupling that is reported for bifurcating/confurcating [FeFe]-hydrogenases (Schut and Adams, 2009; Schuchmann and Muller, 2012; Wang et al., 2013b). In addition, Hyd1ABC did not reduce ferredoxin even in the presence of $NAD^+$. Thus, if Hyd1ABC is capable of producing hydrogen using electrons from reduced ferredoxin under as yet unidentified conditions, the concomitant reduction of $NAD^+$ by hydrogen would result in hydrogen being equilibrated with the redox potential of the NADH/$NAD^+$ pool, preventing higher hydrogen partial pressures that would normally be achievable by confurcating hydrogenases. It is not surprising that Hyd1 does not interact with ferredoxin. Ferredoxin levels are very low in the proteome of *S. wolfei* (Sieber et al., 2015) and we did not detect ferredoxin using the traditional approach to purify ferredoxin (N. Losey and M. J. McInerney, unpublished data), e.g., elution from an ion exchange column after high salt treatment and monitoring of the $A_{390/280}$ ratio (Schönheit et al., 1978; Aono et al., 1989). Unlike other organisms known to possess confurcating hydrogenases (Schut and Adams, 2009; Schuchmann and Muller, 2012; Wang et al., 2013b; Zheng et al., 2014), *S. wolfei* does not have a clear mechanism or need to produce reduced ferredoxin in equivalent amounts to the amount of NADH made from butyrate (Sieber et al., 2010; Schmidt et al., 2013).

Microorganisms known to possess confurcating [FeFe]-hydrogenases such as *T. maritima* (Schut and Adams, 2009), *M. thermoacetica* (Wang et al., 2013b) and *R. albus* (Zheng et al., 2014) have fermentative metabolisms with ferredoxin-dependent enzymes such as pyruvate:ferredoxin oxidoreductase, which can produce reduced ferredoxin in amounts needed for the coupled hydrogen production from NADH. However, *S. wolfei* exclusively uses fatty acids as substrates for growth (McInerney et al., 1979; McInerney et al., 1981; Beaty and McInerney, 1987). Beta-oxidation of fatty acids by *S. wolfei* results in the formation of reduced electron transfer flavoprotein and NADH, but not directly in reduced ferredoxin (Wofford et al., 1986).

The presence of both HydB/HydC subunits has been suggested as being more diagnostic for identifying bifurcating [FeFe]-hydrogenases than the composition of the HydA subunit (Poudel et al., 2016). *S. wolfei* Hyd1ABC has two differences in iron-sulfur centers as predicted by NCBI's Conserved Domain Database (Marchler-Bauer et al., 2015) compared to known bifurcating/confurcating [FeFe]-hydrogenases that may relate to its inability to use ferredoxin as an electron donor (Figure 11).

**Figure 11. Comparison of Predicted Conserved Domains Present in Characterized Multimeric [FeFe]-Hydrogenases and Multimeric Formate Dehydrogenases.** The boxes highlight the heterogeneity in the number of conserved domains in HydB (β) subunits of characterized non-bifurcating NADH-dependent [FeFe]-hydrogensases and formate dehydrogenases compared to the similarity of conserved domains of the HydB (β) subunits of characterized bifurcating [FeFe]-hydrogenases and a bifurcating formate dehydrogenase. Heterogeneity in conserved domains is also present in two unstudied [FeFe]-hydrogenases from

syntrophic metabolizers *S. aciditrophicus* and *S. fumaroxidans* and a formate dehydrogenase

from *S. wolfei* previously found to co-purify with Hyd1ABC. The figure is a visual

representation of the conserved domains as predicted by NCBI's Conserved Domain Database

(40). Greek symbols at the top are the subunit designations. Accession numbers for the

sequences of the subunits are given in Table S3. Reported functions of enzymes were obtained

from following references:  *S. wolfei* hydrogenase, (this publication), *C. tengcongensis* (37), *D.*

*fructosivorans* (12), *R. capsulatus* (13), *T. maritima* (11), *A. woodii* (14), *M. thermoacetica* (15),

*R. albus* (16) and *C. acidurici* (36).

**α** **β** **γ** **δ**

**NADH-Dependent Hydrogenases**

*S. wolfei* — [2Fe-2S][4Fe-4S][4Fe-4S][4Fe-4S][H-Cluster] | [Flavin][4Fe-4S] | [2Fe-2S]

*C. tengcongensis* — [2Fe-2S][4Fe-4S][4Fe-4S][4Fe-4S][H-Cluster] | [2Fe-2S][Flavin][4Fe-4S][4Fe-4S] | [2Fe-2S]

**NADPH-Dependent Hydrogenase**

*D. fructosivorans* — [2Fe-2S][4Fe-4S][4Fe-4S][4Fe-4S][H-Cluster] | [Flavin][4Fe-4S][4Fe-4S] | [2Fe-2S] | [2Fe-2S]

**NADH-Dependent Formate Dehydrogenase**

*R. capsulatus* — [2Fe-2S][4Fe-4S][4Fe-4S][4Fe-4S][Molybdate] | [2Fe-2S][Flavin][4Fe-4S] | [2Fe-2S]

**Bifurcating NADH/Fd Hydrogenases**

*T. maritima* — [2Fe-2S][4Fe-4S][4Fe-4S][4Fe-4S][H-Cluster][2Fe-2S] | [2Fe-2S][Flavin][4Fe-4S][4Fe-4S] | [2Fe-2S]

*A. woodii* — [2Fe-2S][4Fe-4S][4Fe-4S][4Fe-4S][H-Cluster] | [2Fe-2S][Flavin][4Fe-4S][4Fe-4S] | [2Fe-2S] | [2Fe-2S]

*M. thermoacetica* — [2Fe-2S][4Fe-4S][4Fe-4S][4Fe-4S][H-Cluster] | [2Fe-2S][Flavin][4Fe-4S][4Fe-4S] | [2Fe-2S]

*R. albus* — [2Fe-2S][4Fe-4S][4Fe-4S][4Fe-4S][H-Cluster] | [2Fe-2S][Flavin][4Fe-4S][4Fe-4S] | [2Fe-2S]

**Bifurcating NADH/Fd Formate Dehydrogenase**

*C. acidurici* — [2Fe-2S][4Fe-4S][4Fe-4S][4Fe-4S][Molybdate] ; [2Fe-2S][4Fe-4S][4Fe-4S][4Fe-4S] | [2Fe-2S][Flavin][4Fe-4S][4Fe-4S] | [2Fe-2S]

**Uncharacterized Formate Dehydrogenase**

*S. wolfei* — [2Fe-2S][4Fe-4S][4Fe-4S][4Fe-4S][Molybdate] | [Flavin][4Fe-4S] | [2Fe-2S]

**Uncharacterized Hydrogenases**

*S. fumaroxidans* — [2Fe-2S][4Fe-4S][4Fe-4S][4Fe-4S][Molybdate] | [2Fe-2S][Flavin][4Fe-4S] | [2Fe-2S]

*S. acidotrophicus* — [2Fe-2S][4Fe-4S][4Fe-4S][4Fe-4S][Molybdate] | [2Fe-2S][Flavin][4Fe-4S][4Fe-4S]

The HydB1 subunit lacks a N-terminal [2Fe-2S] cluster and a C-terminal [4Fe-4S] cluster. The difference is reflected in overall peptide length of *S. wolfei* HydB1 of 407 amino

acid residues compared to the approximately 600 residues for HydB subunits of known bifurcating [FeFe]-hydrogenases. The reportedly non-confurcating, NADPH-dependent [FeFe]-hydrogenase from *Desulfovibrio fructosivorans* also lacks a N-terminal [2Fe-2S] cluster in its HydB subunit (Figure 11) (Malki et al., 1995). The number of iron-sulfur centers also seems to match with the ability to bifurcate in multimeric molybdopterin formate dehydrogenases. The multimeric, molybdopterin formate dehydrogenase from *Clostridium acidurici* has been shown to catalyze electron bifurcation and has the same iron-sulfur centers in its HydB and HydC equivalent subunits as known bifurcating [FeFe]-hydrogenases while a non-bifurcating, NADH-dependent formate dehydrogenase from *Rhodobacter capsulatus* lacks a C-terminal [4Fe-4S] cluster in its HydB subunit (Hartmann and Leimkuhler, 2013; Wang et al., 2013a). The NADH-dependent [FeFe]-hydrogenase from *Caldanaerobacter* (formerly *Thermoanaerobacter*) *tengcongensis* may be an example of an enzyme that has an equivalent number of iron-sulfur centers as bifurcating [FeFe]-hydrogenases but with the ability to reduce $NAD^+$ and oxidize NADH without ferredoxin (Soboh et al., 2004). However, the *C. tengcongensis* [FeFe]-hydrogenase was not tested under bifurcating/confurcating conditions by supplying ferredoxin in the presence of $NAD^+$ or NADH. While the number of examples of known bifurcating/confurcating and non-bifurcating [FeFe]-hydrogenases and formate dehydrogenases is small, it appears that the type and number of conserved iron-sulfur domains in the HydB/HydC subunits may be important for electron bifurcation activity. Electron bifurcation involves two independent paths of electron flow, either away from the bifurcation site for bifurcating enymes, or towards the bifurcation site for confurcating enzymes, (Chowdhury et al., 2014; Lubner et al., 2017). A change in the presence of an iron-sulfur cluster or its ability to facilitate the directionality of electron flow due to a change in redox potential or its distance from other redox

centers may affect the bifurcation reaction. It is also possible that the loss of the ability to bifurcate was independent of the change in the number of iron-sulfur clusters and such centers were eliminated overtime as they no longer served a critical function.

Multimeric [FeFe]-hydrogenases with the same number of iron-sulfur centers as *S. wolfei* Hyd1ABC are present in the genomes of other members of the *Syntrophomonadaceae* (see Table S3). This suggests that *Syntrophomonas zehnderi*, *Syntrophothermus lipocalidus,* and *Thermosyntropha lipolytica*, which perform syntrophic oxidation of fatty acids in coculture with hydrogenotrophic methanogens (Svetlitshnyi et al., 1996; Sekiguchi et al., 2000; Sousa et al., 2007), may also utilize non-confurcating, NADH-dependent hydrogen production. Future studies of additional multimeric [FeFe]-hydrogenases may allow the determination of the relationship, if any, between certain iron-sulfur centers in the HydB and HydC subunits and the ability to perform electron bifurcation.

The [FeFe]-hydrogenase in *Syntrophobacter fumaroxidans* lacks a C-terminal [4Fe-4S] domain in the HydB subunit, which differs from reported bifurcating [FeFe]-hydrogenases (Figure 11). However, the metabolism of propionate by the methylmalonyl-CoA pathway by *S. fumaroxidans* leads to pyruvate formation and the ability to produce reduce ferredoxin, which is required as the low potential donor for electron bifurcation (Worm et al., 2014). The [FeFe]-hydrogenase in *Syntrophus aciditrophicus* lacks the HydC subunit, which contains a [2Fe-2S] domain (Figure 11). *S. aciditrophicus* may also have the ability to generate reduced ferredoxin if the acyl-CoA dehydrogenases involved in cyclohex-1-ene-1-carboxylate and cyclohexane-1-carboxylate formation can catalyze electron bifurcation (Kung et al., 2013). The syntrophic metabolism of

ethanol and lactate are believed to generate reduced ferredoxin (Schink, 1997; Sieber et al., 2012; Meyer et al., 2013). The partial pressures observed during syntrophic ethanol oxidation by cocultures of *Pelobacter* species with *M. hungatei* exceeded 1000 Pa (Schmidt et al., 2014), which would not be thermodynamically favorable if NADH was the only electron donor for hydrogen production. Given the higher hydrogen partial pressures and presence of reduced ferredoxin-generating systems, it seems likely that some syntrophic oxidizers may utilize ferredoxin, possibly in a confurcating mechanism, for hydrogen production.

The *S. wolfei* Hyd1ABC was previously found to co-purify with a multimeric, formate dehydrogenase encoded by SWOL_RS03955, SWOL_RS03960, SWOL_RS03965, SWOL_RS03970 (Muller et al., 2009). The co-purification of Hyd1ABC with an apparent formate dehydrogenase suggested a possible formate dehydrogenase-hydrogenase complex, although an alternative would be that Hyd1 and the multimeric formate dehydrogenase are separate complexes that co-purify, perhaps due to the similar nature of the HydB/HydC subunits (Figure 11). Either as separate complexes or as a single larger complex, Hyd1 and the multimeric formate dehydrogenase could function to convert NADH to hydrogen and formate, respectively, which would mean that both metabolites are linked to the NADH/NAD$^+$ pool.

The identification of Hyd1ABC as a NADH-dependent hydrogenase suggests NADH-dependent hydrogen production occurs during syntrophic growth on butyrate by *S. wolfei* without the need of reduced ferredoxin. Ferredoxin-independent, hydrogen production from NADH would allow *S. wolfei* to avoid the energetically costly reaction to produce reduced ferredoxin from NADH. Based on the hydrogen partial pressures generated by Hyd1ABC (Table 3), continual NADH-

dependent hydrogen production would require the presence of a hydrogen-consuming organism

to maintain a low hydrogen partial pressure. NADH-dependent hydrogen production could

explain in part the obligate requirement *S. wolfei* has for a hydrogen-consuming organism such

as *M. hungatei* during growth on energetically poor substrates such as butyrate.

## Methods

**Plasmids and Strains.** Genes encoding for a putative multimeric [FeFe]-hydrogenase were

amplified by PCR from *S. wolfei* genomic DNA with Phusion High-Fidelity DNA Polymerase

(Thermo Fisher Scientific, Waltham, MA) using primers listed in Table 4.

**Table 4. List of Primers Used for Construction of pETDuet-1 SwHydABC, pCDFDuet-1**

**SwHydEFG and pCDFDuet-1 SwFd.**

| Primer[a] | Function |
|---|---|
| F: CGC<u>GGATCC</u>GATGATGGATTATAAGGAGATAATTGCCCAG | Amplify |
| R:  TT<u>GGCGCGCC</u>CTATAAGAATTTTTTATTCTTGGCATGG | SWOL_RS05165/SWOL |
| | _RS05170/SWOL_RS051 |
| | 75, addition of restriction |
| | cut sites for BamHI/AscI |
| F: TAAC<u>CATATG</u>CAGGATACTCCCAAAGCTA | Amplify |
| R: TAAC<u>CTCGAG</u>TTAAAGAATTGCTCGGACCCTG | SWOL_RS01625, |
| | addition of restriction cut |
| | sites for NdeI/XhoI |
| F: TTCA<u>CCATGG</u>AAGCGATATCAGTTAACC | Amplify |
| R: ACAT<u>GGATCC</u>TTAAACCTGCTTCAAAGG | SWOL_RS05180/ |
| | SWOL_RS05185/SWOL |

|  |  | _RS05190 addition of restriction cut sites for NcoI/BamHI |
| F: CTTA<u>GGATCC</u>GATGTCTTACATCATC | | Amplify SWOL_RS010890, addition of restriction cut sites for BamHI/HindIII |
| R: AGTGAT<u>AAGCTT</u>TTAGTCGTCCG | | |

[a]Underlined regions indicates restriction enzyme cut sites.

An amplicon with genes encoding for HydA, HydB, and HydC (SWOL_RS05165, SWOL_RS05170, SWOL_RS05175) with the addition of BamHI and AscI restriction sites was inserted into the first multiple clone site (MCS) of pETDuet-1 (Novagen, Merck KGaA, Darmstadt, Germany) (Figure S3) with the addition of a *N*-terminal (His)$_6$ residue on HydC to produce pETDuet-1 SwHydABC.

A second plasmid was created to express genes encoding for [FeFe]-hydrogenase maturation proteins. The Hyd maturases, HydE (SWOL_RS05180) and HydG (SWOL_RS05190), as well as a 348 base pair open reading frame with no identifiable conserved domains (SWOL_RS05185) were amplified from *S. wolfei* genomic template with the addition of NcoI and BamHI restriction sites and was inserted into the MCS of pCDFDuet-1 (Novagen, Merck KGaA, Darmstadt, Germany) (Figure S1). In addition, the gene encoding for the Hyd maturase, HydF (SWOL_RS01625), was amplified with restriction sites for NdeI and XhoI and inserted into the second MCS of pCDFDuet-1 to generate pCDFDuet-1 SwHydEFG.

In addition, a gene encoding for a putative ferredoxin, SWOL_RS010890, was amplified by PCR with the addition of BamHI and HindIII restriction sites and inserted into the first MCS of pCDFDuet-1 with the addition of a N-terminal (His)$_6$ residue to generate pCDFDuet-1 SwFd. SWOL_RS010890 is predicted to encode for a 6-kDa peptide containing a conserved domain for two [4Fe-4S] centers. The SWOL_RS010890 gene product shares 57% amino acid sequence identity to the ferredoxin from *Clostridium pasteurianum* (GenBank Nucleotide ID: M11214).

Due to changes in the locus tags for *S. wolfei* genes, a table listing the newly assigned locus tags for *S. wolfei* used in this publication, older *S. wolfei* locus tags and GenBank protein accession numbers is included as Table S4.

**Expression Conditions.** *E. coli* BL21(DE3) (Thermo Fisher Scientific, Waltham, MA) cells for expression of the genes for His-tagged hydrogenase and His-tagged ferredoxin were grown in LB medium with 50 mM potassium phosphate (pH 7.5) and 10 g$\cdot$l$^{-1}$ glucose with appropriate antibiotics at 37°C. Cultures were grown aerobically with shaking (200 rpm) to an OD$_{600}$ of 0.4 to 0.6 before induction by the addition of 0.5 mM IPTG, followed by the addition of 2 mM ferric ammonium citrate, 10 mM sodium fumarate, and 2 mM cysteine. After induction, the cultures were sealed with rubber stoppers and sparged with nitrogen gas for 6 hours until harvesting. Cells were harvested anaerobically by centrifugation at 6,000 • *g* for 20 minutes at 4°C, washed by resuspension in 50 mM potassium phosphate (pH 7.5) and then pelleted again by centrifuging at 6,000 • *g* for 10 minutes at 4°C. The final cell pellets were frozen in liquid N$_2$ until further use.

**Purification of His-Tagged NADH-Dependent Hydrogenase.** All manipulations, including

cell harvesting and protein purification, were performed inside a Coy Chamber with a 95%

nitrogen and 1-5% hydrogen atmosphere.  All purification buffers were prepared anoxically and

contained 2 mM dithioerythritol (DTE) and 5 µM of both FAD and FMN. Cell pellets (10.8 g)

were thawed, suspended in 20 ml of 50 mM Tris-HCl (pH 7.5) with 0.5 M NaCl, 30 mM

imidazole, and the cells broken by passage through a French pressure cell operating at an internal

pressure of 140 MPa. Cell debris was removed by centrifugation at 13,000 • $g$ for 20 minutes at

4ºC and passage through a 0.22 µm filter. Resulting cell lysate was applied to a HisTrap HP 5 ml

column (GE Healthcare Life Sciences, Pittsburgh, PA) equilibrated with the buffer used for cell

breakage, followed by a wash with 50 mM imidazole in the breakage buffer and His-tagged,

NADH-dependent hydrogenase was eluted with 250 mM imidazole in the breakage buffer.

Eluted fractions were then concentrated and desalted using an Amicon Ultra 0.5 ml Centrifugal

Filters (EMD Millipore) with a 100 kDa nominal molecular weight limit filter. The desalted

fractions were applied to a UNO-Q1 (Bio-Rad, Hercules, CA) column equilibrated with 50 mM

Tris-HCl (pH 7.5), and eluted with a gradient from 0 to 0.6 M NaCl over a 17-minutes period at

a flow rate of 2 ml/min. The purified protein was then aliquoted into several vials and flash

frozen in liquid nitrogen until used for further analyses.


His-tagged *S. wolfei* ferredoxin was prepared as described for the His-tagged NADH-dependent

hydrogenase, but the purification procedure ended after the HisTrap HP chromatography step.


**Enzymatic Assays.** All assays were performed at 37°C unless noted otherwise. Enzyme activity

measurements were determined in triplicate with varying concentrations of protein to ensure

activity was proportional to the amount of protein added. Hydrogenase-oxidizing activity was measured in rubber stopper-sealed, 1.4 ml quartz cuvettes (Nova Biotech, El Cajon, CA) with 600 µl of reaction mix and with hydrogen at a pressure of $1.2 \cdot 10^5$ Pa. The reaction mixture for hydrogen-oxidizing assays consisted of 50 mM Tris-HCl (pH 7.5) or potassium phosphate (pH 7.5), 2 mM DTE, 5 µm FAD, and 5 µm FMN. NAD(P)$^+$ reduction was tested with concentration of 1 mM NAD(P)$^+$ with and without 10 µM *C. pasteurianum* ferredoxin. Methyl viologen reduction assays were performed using a concentration of 10 mM methyl viologen. Reactions were initiated by either the addition of enzyme or the addition of $1.2 \cdot 10^5$ Pa of hydrogen to a 100% nitrogen headspace. The kinetic constants for NAD$^+$ reduction by hydrogen were determined with varying NAD$^+$ concentrations (0 to 2.0 mM). Spectrophotometric measurements for the reduction of NAD$^+$ and NADP$^+$ were performed at 340 nm ($\varepsilon = 6.2$ mM$^{-1}$ cm$^{-1}$). Clostridial ferredoxin reduction was measured using 430 nm ($\varepsilon_{\Delta ox\text{-}red} \approx 13.1$ mM$^{-1}$ cm$^{-1}$) and methyl viologen reduction at 600 nm ($\varepsilon = 10.0$ mM$^{-1}$ cm$^{-1}$).

Hydrogen-producing activities were measured in serum bottles (6.5 mL) sealed with butyl rubber stoppers with a 0.5 mL reaction mixture and shaken at 200 rpm. The reaction mix consisted of 100 mM potassium phosphate at pH 7.5, unless otherwise indicated, as well as 2 mM DTE, 5 µm FAD and 5 µm FMN. Reactions for hydrogen production were initiated by either the addition of enzyme or NADH. The headspace was sampled (0.4 mL) every 3.5 minutes and percent hydrogen in the gas phase was determined by a gas chromatograph equipped with a reductive gas analyzer. The concentration of NADH and NADPH was 1 mM. A reduced ferredoxin-generating system was used consisting of 10 mM pyruvate, 1 mM CoA, 0.1 mM thiamine pyrophosphate,

10 µM of clostridial or *S. wolfei* ferredoxin, and 0.1 U of clostridial pyruvate:ferredoxin oxidoreductase.

Assays were set up to determine final hydrogen concentration obtained at different NADH/NAD$^+$ ratios and pH values. The conditions tested included NADH/NAD$^+$ ratios of either 1.0 or 1.5 with a total pyridine nucleotide pool size of 5 mM with a buffer of 100 mM potassium phosphate at pH 6.5 or 7.0. The ratios of NADH to NAD$^+$ used in the above experiments were determined from the measured hydrogen partial pressures of growing *S. wolfei* cultures. Final hydrogen concentrations were determined in duplicate from sealed serum bottles after the reaction mixture was incubated for 24 hours at room temperature.

It has been previously reported that the presence of trace amounts of viologen dyes in cuvettes and stoppers can decouple bifurcating reactions (Kaster et al., 2011). Hydrogen production assays involving NADH were performed using serum bottles and rubber stoppers that were not previously exposed to viologen dyes. Hydrogen-oxidizing assays were performed with stoppers that were not previously exposed to viologen dyes and with extensively washed cuvettes. Methyl viologen reduction assays were done after all other assays were completed.

**Analytical Techniques.** Protein concentration was determined by using the Bradford protein assay with bovine serum albumin as the standard (Thermo Fisher Scientific, Waltham, MA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE analysis were performed using precast Novex 8-16% Tris-Glycine (Life Technologies Co., Carlsbad, CA.) according to the manufacturer's instructions. Gels were stained using Coomassie

Brilliant Blue G250 (Thermo Fisher Scientific, Waltham, MA). Bands from the native gel were excised for peptide identification. The protein in the excised band was digested with trypsin and subjected to high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) performed by the Laboratory for Molecular Biology and Cytometry Research at OUHSC (Oklahoma City, OK). Resulting peptide fragments were identified by MASCOT search and compared to the NCBInr database.

Molecular mass of recombinant Hyd1ABC was determined by size exclusion chromatography. Size exclusion chromatography was performed using a Superdex 200, 10/300 GL (GE Healthcare Life Sciences) calibrated with gel filtration standards (Bio-Rad Laboratories, Hercules, CA) using a buffer of 50 mM potassium phosphate (pH 7.5) with 0.5 M NaCl at a flow rate of 0.45 mL/min.

Iron content of the purified recombinant Hyd1ABC was determined using a ferrozine assay (Riemer et al., 2004). Identification and quantification of bound flavin was performed by high performance liquid chromatography (HPLC). The purified recombinant Hyd1ABC was washed 20-fold by ultrafiltration using flavin free 50 mM Tris-HCl (pH 7.5), boiled for 10 minutes, and denatured protein removed by centrifugation at 13,000 • $g$ for 10 minutes. The supernatant was then analyzed using a Kromasil 100-10-C18 Column (250 × 4.6 mm) using a buffer of 25% methanol in 50 mM ammonium formate similar to a previous HPLC technique (Seedorf et al., 2004) with a UV detector set to 275 nm. Retention times and peak areas were compared to FAD and FMN standards.

Hydrogen concentration was determined by a gas chromatograph (Seiler et al., 1980; Montag and Schink, 2016) equipped with a reductive gas detector Peak Performer RCP-910 (Peak Laboratories, Mountain View, CA.) Hydrogen concentrations were determined by comparing peak areas to standards containing hydrogen concentrations from 0.01% to 1%.

**Biochemicals and Enzymes.** NADH, $NAD^+$, FMN, FAD, pyruvate, thiamine pyrophosphate, methyl viologen and coenzyme A were purchased from Sigma-Aldrich (St-Louis, MO). Clostridial ferredoxin (Schönheit et al., 1978) and pyruvate ferredoxin oxidoreductase (Wahl and Orme-Johnson, 1987) were purified from *Clostridium pasteurianum* (DSM 525).

**Culture Growth Conditions.** Pure cultures of *S. wolfei* (DSM 2245B) and cocultures with *M. hungatei* strain JF-1 (ATCC 27890) were grown anaerobically with resazurin as redox indicator and cysteine sulfide (0.05%) as reducing agent. The culture medium consisted of 10 ml•l$^{-1}$ each of mineral, trace metal and vitamin solutions (Tanner, 2007) prepared anaerobically under a $N_2/CO_2$ (80:20) atmosphere with 3.5 g•l$^{-1}$ $NaHCO_3$ as buffering agent and either 20 mM butyrate or crotonate as substrate. Cultures were established in triplicate with 160 mL culture medium and 90 mL headspace volumes and incubated at 37°C with shaking (80 rpm). Growth was monitored at $OD_{600}$ with measurements for fatty acid, hydrogen and methane concentrations made at four-day intervals. Determination of butyrate, crotonate and methane concentrations were performed by HPLC and GC-FID as described previously (Sieber et al., 2014) while hydrogen concentrations were determined as described for hydrogen production assays.

**Acknowledgements**

74

**References**

Amos, D.A., and McInerney, M.J. (1989). Poly-β-hydroxyalkanoate in *Syntrophomonas wolfei*. *Arch Microbiol* 152(2)**,** 172-177. doi: 10.1007/BF00456097.

Aono, S., Bryant, F.O., and Adams, M.W. (1989). A novel and remarkably thermostable ferredoxin from the hyperthermophilic archaebacterium *Pyrococcus furiosus*. *J Bacteriol* 171(6)**,** 3433-3439.

Beaty, P.S., and McInerney, M.J. (1987). Growth of *Syntrophomonas wolfei* in pure culture on crotonate. *Arch Microbiol* 147(4)**,** 389-393. doi: 10.1007/BF00406138.

Boone, D.R., Johnson, R.L., and Liu, Y. (1989). Diffusion of the Interspecies Electron Carriers H(2) and Formate in Methanogenic Ecosystems and Its Implications in the Measurement of K(m) for H(2) or Formate Uptake. *Appl Environ Microbiol* 55(7)**,** 1735-1741.

Chowdhury, N.P., Mowafy, A.M., Demmer, J.K., Upadhyay, V., Koelzer, S., Jayamani, E., et al. (2014). Studies on the mechanism of electron bifurcation catalyzed by electron transferring flavoprotein (Etf) and butyryl-CoA dehydrogenase (Bcd) of *Acidaminococcus fermentans*. *J Biol Chem* 289(8)**,** 5145-5157. doi: 10.1074/jbc.M113.521013.

Conrad, R., Schink, B., and Phelps, T.J. (1986). Thermodynamics of $H_2$-consuming and $H_2$-producing metabolic reactions in diverse methanogenic environments under in situ conditions. *FEMS Microbiol Lett* 38(6)**,** 353-360. doi: http://dx.doi.org/10.1016/0378-1097(86)90013-3.

Crable, B.R., Sieber, J.R., Mao, X., Alvarez-Cohen, L., Gunsalus, R., Ogorzalek Loo, R.R., et al. (2016). Membrane Complexes of *Syntrophomonas wolfei* Involved in Syntrophic Butyrate Degradation and Hydrogen Formation. *Front Microbiol* 7**,** 1795. doi: 10.3389/fmicb.2016.01795.

de Graef, M.R., Alexeeva, S., Snoep, J.L., and Teixeira de Mattos, M.J. (1999). The Steady-State Internal Redox State (NADH/NAD) Reflects the External Redox State and Is Correlated with Catabolic Adaptation in Escherichia coli. *J Bacteriol* 181(8)**,** 2351-2357.

Girbal, L., von Abendroth, G., Winkler, M., Benton, P.M., Meynial-Salles, I., Croux, C., et al. (2005). Homologous and heterologous overexpression in *Clostridium acetobutylicum* and characterization of purified clostridial and algal Fe-only hydrogenases with high specific activities. *Appl Environ Microbiol* 71(5)**,** 2777-2781. doi: 10.1128/AEM.71.5.2777-2781.2005.

Hartmann, T., and Leimkuhler, S. (2013). The oxygen-tolerant and $NAD^+$-dependent formate dehydrogenase from *Rhodobacter capsulatus* is able to catalyze the reduction of CO2 to formate. *FEBS J.* 280(23)**,** 6083-6096. doi: 10.1111/febs.12528.

Jackson, B.E., and McInerney, M.J. (2002). Anaerobic microbial metabolism can proceed close to thermodynamic limits. *Nature* 415(6870)**,** 454-456.

Kaster, A.K., Moll, J., Parey, K., and Thauer, R.K. (2011). Coupling of ferredoxin and heterodisulfide reduction via electron bifurcation in hydrogenotrophic methanogenic archaea. *Proc Natl Acad Sci U S A* 108(7)**,** 2981-2986. doi: 10.1073/pnas.1016761108.

King, P.W., Posewitz, M.C., Ghirardi, M.L., and Seibert, M. (2006). Functional studies of [FeFe] hydrogenase maturation in an *Escherichia coli* biosynthetic system. *J Bacteriol* 188(6)**,** 2163-2172. doi: 10.1128/JB.188.6.2163-2172.2006.

Kung, J.W., Seifert, J., von Bergen, M., and Boll, M. (2013). Cyclohexanecarboxyl-Coenzyme A (CoA) and Cyclohex-1-ene-1-Carboxyl-CoA Dehydrogenases, Two Enzymes Involved in the Fermentation of Benzoate and Crotonate in *Syntrophus aciditrophicus*. *J. Bacteriol* 195(14)**,** 3193-3200.

Lubner, C.E., Jennings, D.P., Mulder, D.W., Schut, G.J., Zadvornyy, O.A., Hoben, J.P., et al. (2017). Mechanistic insights into energy conservation by flavin-based electron bifurcation. *Nat Chem Biol* 13(6)**,** 655-659. doi: 10.1038/nchembio.2348.

Malki, S., Saimmaime, I., De Luca, G., Rousset, M., Dermoun, Z., and Belaich, J.P. (1995). Characterization of an operon encoding an NADP-reducing hydrogenase in *Desulfovibrio fructosovorans*. *J Bacteriol* 177(10)**,** 2628-2636.

Marchler-Bauer, A., Derbyshire, M.K., Gonzales, N.R., Lu, S., Chitsaz, F., Geer, L.Y., et al.
(2015). CDD: NCBI's conserved domain database. *Nucleic Acids Res* 43(Database issue)**,**
D222-226. doi: 10.1093/nar/gku1221.

McInerney, M.J., and Bryant, M.P. (1981). "Basic Principles of Bioconversions in Anaerobic
Digestion and Methanogenesis," in *Biomass Conversion Processes for Energy and Fuels,*
eds. S.S. Sofer & O.R. Zaborsky. (Boston, MA: Springer US), 277-296.

McInerney, M.J., Bryant, M.P., Hespell, R.B., and Costerton, J.W. (1981). *Syntrophomonas
wolfei* gen. nov. sp. nov., an Anaerobic, Syntrophic, Fatty Acid-Oxidizing Bacterium.
*Appl. Environ. Microbiol.* 41(4)**,** 1029-1039.

McInerney, M.J., Bryant, M.P., and Pfennig, N. (1979). Anaerobic bacterium that degrades fatty
acids in syntrophic association with methanogens. *Arch Microbiol* 122(2)**,** 129-135. doi:
10.1007/BF00411351.

Meyer, B., Kuehl, J., Deutschbauer, A.M., Price, M.N., Arkin, A.P., and Stahl, D.A. (2013).
Variation among *Desulfovibrio* Species in Electron Transfer Systems Used for
Syntrophic Growth. *J Bacteriol* 195(5)**,** 990-1004.

Montag, D., and Schink, B. (2016). Biogas process parameters--energetics and kinetics of
secondary fermentations in methanogenic biomass degradation. *Appl Microbiol
Biotechnol* 100(2)**,** 1019-1026. doi: 10.1007/s00253-015-7069-0.

Muller, N., Schleheck, D., and Schink, B. (2009). Involvement of NADH:acceptor
oxidoreductase and butyryl coenzyme A dehydrogenase in reversed electron transport
during syntrophic butyrate oxidation by *Syntrophomonas wolfei*. *J Bacteriol* 191(19)**,**
6167-6177. doi: 10.1128/JB.01605-08.

Poudel, S., Tokmina-Lukaszewska, M., Colman, D.R., Refai, M., Schut, G.J., King, P.W., et al.
(2016). Unification of [FeFe]-hydrogenases into three structural and functional groups.
*Biochim. Biophys. Acta.* 1860(9)**,** 1910-1921. doi: 10.1016/j.bbagen.2016.05.034.

Riemer, J., Hoepken, H.H., Czerwinska, H., Robinson, S.R., and Dringen, R. (2004).
Colorimetric ferrozine-based assay for the quantitation of iron in cultured cells. *Anal Biochem* 331(2)**,** 370-375. doi: 10.1016/j.ab.2004.03.049.

Schink, B. (1997). Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol. Mol Biol Rev* 61(2)**,** 262-280.

Schmidt, A., Frensch, M., Schleheck, D., Schink, B., and Muller, N. (2014). Degradation of
acetaldehyde and its precursors by *Pelobacter carbinolicus* and *P. acetylenicus*. *PLoS One* 9(12)**,** e115902. doi: 10.1371/journal.pone.0115902.

Schmidt, A., Muller, N., Schink, B., and Schleheck, D. (2013). A proteomic view at the
biochemistry of syntrophic butyrate oxidation in *Syntrophomonas wolfei*. *PLoS One* 8(2)**,** e56905. doi: 10.1371/journal.pone.0056905.

Schmidt, O., Hink, L., Horn, M.A., and Drake, H.L. (2016). Peat: home to novel syntrophic
species that feed acetate- and hydrogen-scavenging methanogens. *ISME J* 10(8)**,** 1954-1966. doi: 10.1038/ismej.2015.256.

Schönheit, P., Wäscher, C., and Thauer, R.K. (1978). A rapid procedure for the purification of
ferredoxin from clostridia using polyethyleneimine. *FEBS Lett* 89(2)**,** 219-222. doi: 10.1016/0014-5793(78)80221-X.

Schuchmann, K., and Muller, V. (2012). A bacterial electron-bifurcating hydrogenase. *J Biol Chem* 287(37)**,** 31165-31171. doi: 10.1074/jbc.M112.395038.

Schuchmann, K., and Muller, V. (2014). Autotrophy at the thermodynamic limit of life: a model for energy conservation in acetogenic bacteria. *Nature Rev Micro* 12(12)**,** 809-821. doi: 10.1038/nrmicro3365

http://www.nature.com/nrmicro/journal/v12/n12/abs/nrmicro3365.html#supplementary-information.

Schut, G.J., and Adams, M.W. (2009). The iron-hydrogenase of *Thermotoga maritima* utilizes ferredoxin and NADH synergistically: a new perspective on anaerobic hydrogen production. *J Bacteriol* 191(13)**,** 4451-4457. doi: 10.1128/JB.01582-08.

Seedorf, H., Dreisbach, A., Hedderich, R., Shima, S., and Thauer, R.K. (2004). $F_{420}H_2$ oxidase (FprA) from *Methanobrevibacter arboriphilus*, a coenzyme $F_{420}$-dependent enzyme involved in $O_2$ detoxification. *Arch Microbiol* 182(2-3)**,** 126-137. doi: 10.1007/s00203-004-0675-3.

Seiler, W., Giehl, H., and Roggendorf, P. (1980). Detection of carbon monoxide and hydrogen by conversion of mercury oxide to mercury vapor.

Sekiguchi, Y., Kamagata, Y., Nakamura, K., Ohashi, A., and Harada, H. (2000). *Syntrophothermus lipocalidus* gen. nov., sp. nov., a novel thermophilic, syntrophic, fatty-acid-oxidizing anaerobe which utilizes isobutyrate. *Int J Syst Evol Microbiol* 50(2)**,** 771-779. doi: doi:10.1099/00207713-50-2-771.

Sieber, J.R., Crable, B.R., Sheik, C.S., Hurst, G.B., Rohlin, L., Gunsalus, R.P., et al. (2015). Proteomic analysis reveals metabolic and regulatory systems involved in the syntrophic and axenic lifestyle of *Syntrophomonas wolfei*. *Front Microbiol* 6**,** 115. doi: 10.3389/fmicb.2015.00115.

Sieber, J.R., Le, H.M., and McInerney, M.J. (2014). The importance of hydrogen and formate transfer for syntrophic fatty, aromatic and alicyclic metabolism. *Environ Microbiol* 16(1)**,** 177-188. doi: 10.1111/1462-2920.12269.

Sieber, J.R., McInerney, M.J., and Gunsalus, R.P. (2012). Genomic insights into syntrophy: the paradigm for anaerobic metabolic cooperation. *Annu Rev Microbiol* 66**,** 429-452. doi: 10.1146/annurev-micro-090110-102844.

Sieber, J.R., Sims, D.R., Han, C., Kim, E., Lykidis, A., Lapidus, A.L., et al. (2010). The genome of *Syntrophomonas wolfei*: new insights into syntrophic metabolism and biohydrogen production. *Environ Microbiol* 12(8)**,** 2289-2301. doi: 10.1111/j.1462-2920.2010.02237.x.

Snoep, J.L., Joost, M., de Mattos, T., and Neijssel, O.M. (1991). Effect of the energy source on the NADH/NAD ratio and on pyruvate catabolism in anaerobic chemostat cultures of *Enterococcus faecalis* NCTC 775. *FEMS Microbiol Lett* 81(1)**,** 63-66. doi: 10.1111/j.1574-6968.1991.tb04713.x.

Soboh, B., Linder, D., and Hedderich, R. (2004). A multisubunit membrane-bound [NiFe] hydrogenase and an NADH-dependent Fe-only hydrogenase in the fermenting bacterium *Thermoanaerobacter tengcongensis*. *Microbiology* 150(Pt 7)**,** 2451-2463. doi: 10.1099/mic.0.27159-0.

Sousa, D.Z., Smidt, H., Alves, M.M., and Stams, A.J.M. (2007). *Syntrophomonas zehnderi* sp. nov., an anaerobe that degrades long-chain fatty acids in co-culture with *Methanobacterium formicicum*. *Int J Syst Evol Microbiol* 57(3)**,** 609-615. doi: doi:10.1099/ijs.0.64734-0.

Svetlitshnyi, V., Rainey, F., and W, Juergen. (1996). *Thermosyntropha lipolytica* gen. nov., sp.

nov., a Lipolytic, Anaerobic, Alkalitolerant, Thermophilic Bacterium Utilizing Short- and Long-Chain Fatty Acids in Syntrophic Coculture with a Methanogenic Archaeum. *Int J of Syst and Evol Microbiol* 46(4)**,** 1131-1137. doi: doi:10.1099/00207713-46-4-1131.

Tanner, R.S. (2007). "Cultivation of Bacteria and Fungi," in *Manual of Environmental Microbiology, Third Edition*. American Society of Microbiology).

Wahl, R.C., and Orme-Johnson, W.H. (1987). Clostridial pyruvate oxidoreductase and the pyruvate-oxidizing enzyme specific to nitrogen fixation in *Klebsiella pneumoniae* are similar enzymes. *J Biol Chem* 262(22)**,** 10489-10496.

Wallrabenstein, C., and Schink, B. (1994). Evidence of reversed electron transport in syntrophic butyrate or benzoate oxidation by *Syntrophomonas wolfei* and *Syntrophus buswellii*. *Arch Microbiol* 162(1)**,** 136-142. doi: 10.1007/BF00264387.

Wang, S., Huang, H., Kahnt, J., and Thauer, R.K. (2013a). *Clostridium acidurici* electron-bifurcating formate dehydrogenase. *Appl Environ Microbiol* 79(19)**,** 6176-6179. doi: 10.1128/AEM.02015-13.

Wang, S., Huang, H., Kahnt, J., and Thauer, R.K. (2013b). A reversible electron-bifurcating ferredoxin- and NAD-dependent [FeFe]-hydrogenase (HydABC) in *Moorella thermoacetica*. *J. Bacteriol.* 195(6)**,** 1267-1275. doi: 10.1128/JB.02158-12.

Wimpenny, J.W.T., and Firth, A. (1972). Levels of Nicotinamide Adenine Dinucleotide and Reduced Nicotinamide Adenine Dinucleotide in Facultative Bacteria and the Effect of Oxygen. *J Bacteriol* 111(1)**,** 24-32.

Wofford, N.Q., Beaty, P.S., and McInerney, M.J. (1986). Preparation of cell-free extracts and the enzymes involved in fatty acid metabolism in *Syntrophomonas wolfei*. *J Bacteriol* 167(1)**,** 179-185.

Worm, P., Koehorst, J.J., Visser, M., Sedano-Núñez, V.T., Schaap, P.J., Plugge, C.M., et al.
(2014). A genomic view on syntrophic versus non-syntrophic lifestyle in anaerobic fatty
acid degrading communities. *BBA Bioenergetics* 1837(12)**,** 2004-2016. doi:
http://dx.doi.org/10.1016/j.bbabio.2014.06.005.

Zheng, Y., Kahnt, J., Kwon, I.H., Mackie, R.I., and Thauer, R.K. (2014). Hydrogen formation
and its regulation in *Ruminococcus albus*: involvement of an electron-bifurcating [FeFe]-
hydrogenase, of a non-electron-bifurcating [FeFe]-hydrogenase, and of a putative
hydrogen-sensing [FeFe]-hydrogenase. *J Bacteriol* 196(22)**,** 3840-3852. doi:
10.1128/JB.02070-14.

**Supplemental Figures and Tables**

**Table S1. Peptide Matches Obtained from the Excised Band from Native PAGE of the Purified, Expressed Hyd1ABC.**

| Old NCBI Locus Tag | New NCBI Gene Locus | Protein Accession Number | Annotation | Matched peptides | Protein sequence coverage |
|---|---|---|---|---|---|
| Swol_1 017 | SWOL_RS 05165 | WP_0116 40436 | Hyd1A, iron hydrogenase, small subunit | 56 | 70.6% |
| Swol_1 | SWOL_RS | WP_0116 | Hyd1B, NADH | 26 | 71.4% |

| | | | | | |
|---|---|---|---|---|---|
| 018 | 05170 | 40437 | dehydrogense subunit | | |
| Swol_1 019 | SWOL_RS 05175 | WP_0116 40438 | Hyd1C, iron-hydrogenase, gamma subunit | 5 | 53.4% |
| Swol_2 436 | SWOL_RS 12620 | WP_0116 41809 | Fe-hydrogenase, large subunit HymC | 5 | 5.7% |
| Swol_1 828 | SWOL_RS 09445 | WP_0116 41221 | NADH dehydrogenase (Quinone) | 2 | 4.2% |

**Table S2. Comparison of Properties of Multimeric [FeFe]-Hydrogenases**

| Source organism | Methyl viologen reducing activity (U•mg$^{-1}$) [a] | NAD$^+$ reducing activity (U•mg$^{-1}$) [a] | NADH oxidation activity (U•mg$^{-1}$) [a] | $K_M$ for NAD$^+$ (µM) | Molecular weight (kDa) | Assay temperature (°C) | Ref. No. |
|---|---|---|---|---|---|---|---|
| *S. wolfei* | 571 | 94.5 | 6.6 | 520 | 124.5 | 37 | This publication |
| *T. maritima* | NR | NR [b] | ~1.5 [c] | NR | NR | 80 | 11 |
| *A. woodii* | 711 | ~3.0 [c] | NR | 49 | 300 [d] | 30 | 14 |
| *M.* | 195 | 57.5 [c] | 4.6 [c] | 250 | 300 [d] | 45 | 15 |

*thermoacetic*

*a*

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *R. albus* | NA | 60 [c] | NR | NR | NR | 37 | 16 |
| *C.* | 1671 | 5 | 10 | 90 | NR | 70 | 37 |

*tengcongens*

*is*

[a] One unit of activity (U) equals 2 μmol of electrons transferred per min. See the referenced articles for assay conditions.

[b] NR, not reported.

[c] Ferredoxin present.

[d] Reported as a dimer of either αβγ heterotrimer or αβγδ heterotetramer.

**Table S3 Genbank Protein Accession Numbers of Subunits of Multimeric Formate Dehydrogenases and [FeFe]-Hydrogenases.**

| Source Organism | Subunit | GenBank Protein Accession Number |
|---|---|---|
| *Acetobacterium woodii* | HydA | AFA49450 |
| *Acetobacterium woodii* | HydB | AFA49451 |
| *Acetobacterium woodii* | HydC | AFA49452 |
| *Acetobacterium woodii* | HydD | AFA49454 |
| *Caldanaerobacter tencongensis* | HydA | AAM24150 |
| *Caldanaerobacter tencongensis* | HydB | AAM24149 |
| *Caldanaerobacter tencongensis* | HydC | AAM24148 |

| | | |
|---|---|---|
| *Clostridium acidurici* | FdhA | AFS79904 |
| *Clostridium acidurici* | FdhA | AFS79905 |
| *Clostridium acidurici* | FdhB | AFS79906 |
| *Clostridium acidurici* | FdhC | AFS79907 |
| *Desulfovibrio fructosivorans* | HydA | AAA87057 |
| *Desulfovibrio fructosivorans* | HydB | AAA87056 |
| *Desulfovibrio fructosivorans* | HydC | AAA87054 |
| *Desulfovibrio fructosivorans* | HydD | AAA87055 |
| *Moorella thermoacetica* | HydA | ABC20019 |
| *Moorella thermoacetica* | HydB | ABC20020 |
| *Moorella thermoacetica* | HydC | ABC20021 |
| *Rhodopsuedomonas capsulatus* | FdhA | ADE86759 |
| *Rhodopsuedomonas capsulatus* | FdhB | ADE86760 |
| *Rhodopsuedomonas capsulatus* | FdhC | ADE86761 |
| *Ruminococcus albus* | HydA | ADU23430 |
| *Ruminococcus albus* | HydB | ADU23431 |
| *Ruminococcus albus* | HydC | ADU23432 |
| *Syntrophobacter fumaroxidans* | HydA | ABK16541 |
| *Syntrophobacter fumaroxidans* | HydB | ABK16542 |
| *Syntrophobacter fumaroxidans* | HydC | ABK16543 |
| *Syntrophomonas wolfei* | FdhA | ABI68106/ABI68107 |
| *Syntrophomonas wolfei* | FdhB | ABI68105 |

| | | |
|---|---|---|
| *Syntrophomonas wolfei* | FdhC | ABI68104 |
| *Syntrophomonas wolfei* | HydA | ABI68331 |
| *Syntrophomonas wolfei* | HydB | ABI68332 |
| *Syntrophomonas wolfei* | HydC | ABI68333 |
| *Syntrophomonas zehnderi* | HydA | WP_046500146 |
| *Syntrophomonas zehnderi* | HydB | WP_046500148 |
| *Syntrophomonas zehnderi* | HydC | WP_046500150 |
| *Syntrophothermus lipocalidus* | HydA | WP_013174729 |
| *Syntrophothermus lipocalidus* | HydB | WP_013174328 |
| *Syntrophothermus lipocalidus* | HydC | WP_013174327 |
| *Syntrophus aciditrophicus* | HydA | ABC76974 |
| *Syntrophus aciditrophicus* | HydB | ABC76975 |
| *Thermosyntropha lipolytica* | HydA | WP_073091114 |
| *Thermosyntropha lipolytica* | HydB | WP_073091112 |
| *Thermosyntropha lipolytica* | HydC | WP_073091109 |
| *Thermotoga maritima* | HydA | AAD36496 |
| *Thermotoga maritima* | HydB | AAD36495 |
| *Thermotoga maritima* | HydC | AAD36494 |

**Table S4. Updated Locus Tag Designations of *S. wolfei* Genes.**

| Old NCBI Locus Tag | New NCBI Gene Locus | Protein Accession Number | Genome Location |
|---|---|---|---|
| Swol_1017 | SWOL_RS05165 | WP_011640436 | NC_008346 REGION: 1157124.1158848 |
| Swol_1018 | SWOL_RS05170 | WP_011640437 | NC_008346 REGION: 1158873.1160096 |
| Swol_1019 | SWOL_RS05175 | WP_011640438 | NC_008346 REGION: 1160156.1160602 |
| Swol_1020 | SWOL_RS05180 | WP_041427405 | NC_008346 REGION: 1160971.1162029 |
| Swol_1021 | SWOL_RS05185 | WP_041427406 | NC_008346 REGION: 1161998.1162378 |
| Swol_1022 | SWOL_RS05190 | WP_011640441 | NC_008346 REGION: 1162353.1163816 |
| Swol_0318 | SWOL_RS01625 | WP_011639769 | NC_008346 REGION: 384562.385770 |
| Swol_2105 | SWOL_RS10890 | WP_011641488 | NC_008346 REGION: 2424114.2424290 |
| Swol_1925 | SWOL_RS09950 | WP_011641315 | NC_008346 REGION: |

2197847.2199010

Swol_2436   SWOL_RS12620   WP_011641809   NC_008346 REGION:

2765177.2766868

**Figure S1. Kinetics of NAD$^+$ Reduction with Hydrogen by Hyd1ABC.**

Reactions were performed at 37°C in 1.6 ml cuvettes with 800 μl of 50 mM potassium phosphate

buffer (pH 7.5), with 5 μm FAD, 5 μm FMN and 86 μg of Hyd1ABC. The data were fit to the

reaction curve using non-linear regression with a $K_m$ of 0.52 mM NAD$^+$ and a $V_{max}$ of 196

μmole•min$^{-1}$•mg$^{-1}$of protein. A $k_{cat}$ of 406.7 s$^{-1}$ was determined using a single catalytic site per a

molecular mass of 124.5 kDa.
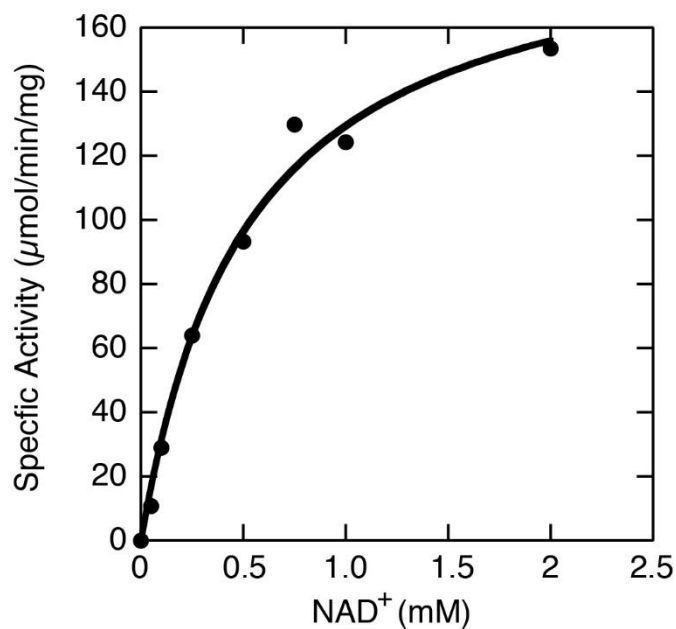
**Figure S2. Hydrogen Partial Pressures During Growth of *S. wolfei* in Pure culture and in Coculture with *M. hungatei*.** (A and B) *S. wolfei* grown in pure culture on crotonate; (C and D) *S. wolfei* grown in coculture with *M. hungatei* on crotonate; (E and F) *S. wolfei* grown in coculture with *M. hungatei* on butyrate. A, C, E, growth (diamonds) and hydrogen (Pa) (triangles); (B, D, F) substrate concentration (crotonate in B and D and butyrate in F) (circles) and methane concentration (squares).
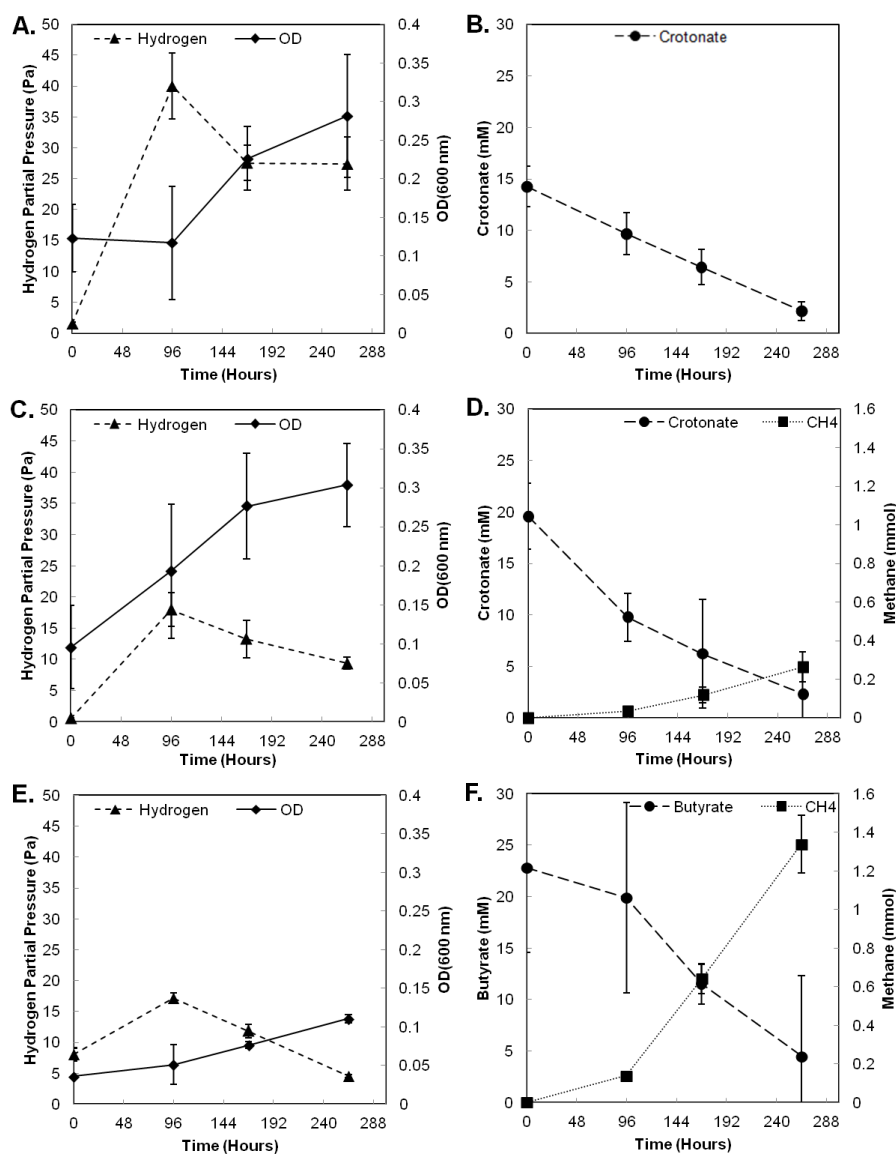
**Figure S3. Construction of Plasmids for Expression of Hyd1ABC and [FeFe]-Hydrogenase Maturation Proteins.**
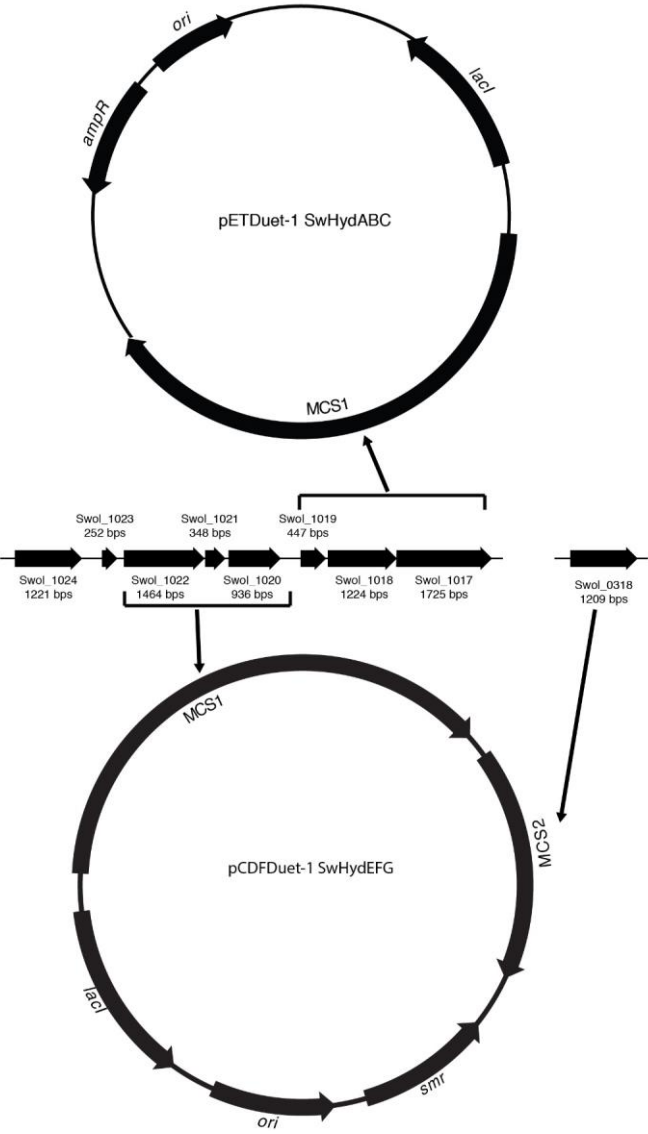
# CHAPTER 3. A NADH-Dependent, Ferredoxin-Independent [FeFe]-Hydrogenase from

## *Syntrophus aciditrophicus*

**Abstract**

     A NADH-dependent, ferredoxin-independent dimeric [FeFe]-hydrogenase (HydAB) from *Syntrophus aciditrophicus* was heterologously expressed in *Esherichia coli*, purified and characterized. The purified recombinant HydAB consisted of two subunits HydA and HydB with molecular weights 68.5 kDa and 66.0 kDa, respectively, as predicted from the encoding DNA sequences. The molecular weight of the HydAB complex as estimated by gel filtration chromatography (287 kDa) and native gel migration (323 kDa) suggested a $\alpha_2\beta_2$ configuration. The HydAB $\alpha_2\beta_2$ complex had $71.7 \pm 15.4$ mole of iron and 1.06 mole of FMN per mole of holoenzyme. HydAB catalyzed $NAD^+$ reduction coupled to hydrogen oxidation and produced hydrogen from NADH without the involvement of ferredoxin. Pure cultures of *S. aciditrophicus* and cocultures of *S. aciditrophicus* with *Methanospiriullum hungatei* generated hydrogen partial pressures of 5.9 to 36.6 Pa with crotonate, benzoate, and cylohex-1-ene-1-carboxylate as substrates which are the same magnitude as the hydrogen partial pressures (2.2 to 40.2 pascals) produced by the purified recombinant *S. aciditrophicus* HydAB at NADH to $NAD^+$ ratios of 1 to 5. Thus, the hydrogen partial pressures observed in metabolizing cultures and cocultures of *S. aciditrophicus* can be generated by HydAB if *S. aciditrophicus* maintains NADH to $NAD^+$ ratios greater than one. These results show that two phylogenetically distinct syntrophic fatty acid-oxidizing bacteria, *S. wolfei* a member of the phylum Firmicutes, and *S. aciditrophicus,* a Deltaproteobacteria, possess functionally similar hydrogenases that produce hydrogen from NADH during syntrophic fatty acid oxidation without the involvement of reduced ferredoxin. Most importantly, the work suggests that hydrogen production from NADH by ferredoxin-independent [FeFe]-hydrogenases may be a core feature of syntrophic fatty acid oxidization. This may be due to energetic advantages where the direct production of hydrogen from NADH

in a ferredoxin-independent mechanism allows the organisms to conserve more energy for growth. In contrast, electron confurcation of NADH and ferredoxin to produce hydrogen would require energy input to generate reduced ferredoxin from NADH. The reliance on a NADH-dependent, ferredoxin-independent [FeFe]-hydrogenase may explain the obligate requirement that syntrophic metabolizers have for a hydrogen-using partner microorganism when grown on fatty, aromatic and alicyclic acids.

**Introduction**

Benzoate and its activated form, benzoyl-CoA, are key intermediates in the degradation of organic matter in anaerobic environments (Harwood et al., 1998; McInerney et al., 2008; McInerney et al., 2009). In methanogenic environments, benzoate and other aromatic compounds are degraded to acetate, formate, $CO_2$, $H_2$ by syntrophic metabolizers. The degradation of benzoate and other aromatic and fatty acids to acetate, formate, $CO_2$, $H_2$ is thermodynamically unfavorable unless formate and $H_2$ are kept at low levels by formate- and $H_2$-using microorganisms such as methanogens. *Syntrophus aciditrophicus* is a metabolic specialist capable of oxidizing benzoate, alicyclic acids such as cyclohexane-1-carboxylate and fatty acids when paired with a hydrogen- or formate-consuming partner organism such as a methanogen or sulfate reducer (Jackson et al., 1999; Elshahed and McInerney, 2001). *S. aciditrophicus* serves as the model organism to study syntrophic aromatic and alicylic acid metabolism as its genome has been sequenced (McInerney et al., 2007) and methods have been developed to separate cells of *S. aciditrophcus* from its hydrogen- and formate-using partner (Sieber et al., 2015). Also, it can be grown in pure culture on crotonate (Jackson et al., 1999).

*S. aciditrophicus* syntrophically degrades benzoate and cyclohexane-1-carboxylate to acetate, $CO_2$, $H_2$ and formate via a 6-substituted cyclohex-1-ene-1-carboxyl-CoA intermediate

using a core set of enzymes (James et al., 2019, in preparation). *S. aciditrophicus* uses a unique

mechanism for energy generation from acetyl-CoA where an AMP-forming, acetyl-CoA

synthetase are used to make acetate, CoA and ATP from acetyl-CoA, AMP and pyrophosphate

by an acetyl-CoA synthetase (James et al., 2016). This enzyme requires unusually high

pyrophosphate and AMP levels within the cell to make ATP production from acetyl-CoA, AMP

and pyrophosphate thermodynamically favorable (James et al., 2016). As part of the metabolism

of benzoate or cyclohexane-1-carboxylate, reduced flavins (EtfAB) and NADH are generated

(McInerney et al., 2007; McInerney et al., 2009). *S. aciditrophicus* also contains genes for an Rnf

complex (McInerney et al., 2007) that can make reduced ferredoxin by oxidizing NADH using

energy from a membrane gradient (Schmehl et al., 1993; Biegel and Müller, 2010). The reduced

ferredoxin would be required by *S. acidotrophicus* to reduce benzoyl-CoA by an ATP-

independent mechanism (Fuchs et al., 2011; Kung et al., 2013). During syntrophic growth,

reduced EtfAB and NADH must be re-oxidized by the production of hydrogen or formate

(McInerney et al., 2007; McInerney et al., 2009). Hydrogen and formate production from

reduced flavin pools (EtfAB) requires energy input from a membrane gradient, a process termed

reverse electron transfer (McInerney et al., 2007; McInerney et al., 2009). A membrane-

associated iron-sulfur oxidoreductase in conjunction with a reverse quinone loop and membrane-

associated formate dehydrogenase may be responsible for EtfAB re-oxidation similar to the

mechanism proposed for *Syntrophomonas wolfei* (Schmidt et al., 2013; Sieber et al., 2015;

Crable et al., 2016). In *S. aciditrophicus,* the re-oxidation of NADH was previously proposed to

involve the use of the Rnf complex to oxidize NADH and produce reduced ferredoxin

(McInerney et al., 2007). The reduced ferredoxin would then be used to produce hydrogen or

formate (Sieber et al., 2012) by a mechanism referred to as electron bifurcation whereby for

every molecule of NADH oxidized, one molecule of reduced ferredoxin is also oxidized (Schut and Adams, 2009).The favorable production of hydrogen and formate using low potential electrons derived from reduced ferredoxin allows the unfavorable production of hydrogen and formate from the high redox potential electrons from NADH (Schut and Adams, 2009). If *S. aciditrophicus* uses electron-bifurcating hydrogenases and formate dehydrogenases, *S. aciditrophicus* would have to use the Rnf complex to make reduced ferredoxin from electrons derived from NADH oxidation, which consume potential energy from a membrane gradient, leaving less energy to be conserved for growth (Seedorf et al., 2008; Biegel and Müller, 2010).

The genome of *S. aciditrophicus* encodes two predicted hydrogenases, a cytoplasmic [NiFe]-hydrogenase and a dimeric cytoplasmic [FeFe]-hydrogenase (HydAB) (McInerney et al., 2007).  The latter is predicted to use NADH as the electron donor. The purpose of the work in this chapter was to determine if the dimeric cytoplasmic [FeFe]-hydrogenase (HydAB) produces hydrogen from NADH and whether this process requires reduced ferredoxin. To produce an active form of HydAB from *S. aciditrophicus*, a recombinant DNA approach was used to express HydAB in *Escherichia coli* and then characterize the purified recombinant HydAB.

**Results**

**Purification and characterization of *S. aciditrophicus* hydrogenase, HydAB**

The protein products of SYN_01369 and SYN_01370 (HydAB) were recombinantly produced in *E. coli* and purified using nickel-affinity chromatography and molecular weight cut off filters (100 kDa) (Table 5). All further enzymatic assays and properties were determined using this fraction. During purification, hydrogenase activity was followed by methyl viologen reduction with hydrogen as the electron donor. Recombinant HydAB was purified 36-fold with a 62% yield and a final specific activity of 13 U • mg$^{-1}$ (Table 5).

**Table 5. Purification of NADH-Dependent [FeFe]-Hydrogenase, HydAB.**

| Fraction | Protein (mg) | Specific Activity* $H_2 \rightarrow MV_{ox}$ (U/mg) | Total Activity* $H_2 \rightarrow MV_{ox}$ (U) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Cell free extract | 194 | 0.36 | 69.8 | 1 | 100 |
| Ni-Affinity | 3.3 | 13.0 | 42.9 | 36.1 | 62 |

*HydAB activity was followed by methyl viologen reduction with hydrogen as the electron donor at 37 °C and a pH of 7.5. One unit of activity (U) equals 2 μmoles of electrons transferred per min.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and non-denaturing polyacrylamide gel electrophoresis (Native PAGE) analysis indicated that a few contaminants were present in the final purified fractions. SDS-PAGE analysis (Figure 12) showed two bands which by the pattern of gel migration were estimated to be 68 kDa and 64 kDa in size. This compares favorably with the predicted molecular weights of HydA (68.5 kDa) and HydB (66.0 kDa) as estimated from their respective gene sequences. The HydA predicted molecular weight includes the additional N-terminal His tag (2.4 kDa) with the sequence MGSSHHHHHHSQDPNSSSARL. Analysis by high performance liquid chromatography coupled to tandem mass spectrometry (HPLC MS-MS analysis) of the peptides present in the purified HydAB fraction showed that many of the peptides that matched HydA and HydB although there were peptides that matched *E. coli* proteins (Table 6).

**Figure 12. SDS PAGE of Recombinant *S. aciditrophicus* HydAB Purified from *E. coli* by Nickel Affinity Chromatography.** Lane 1, molecular weight markers; Lane 2, *E. coli* cell-free extract; Lane 3, fraction after nickel-affinity chromatography. Molecular weights listed on gel image indicate the molecular weight as predicted from the encoding gene sequence.
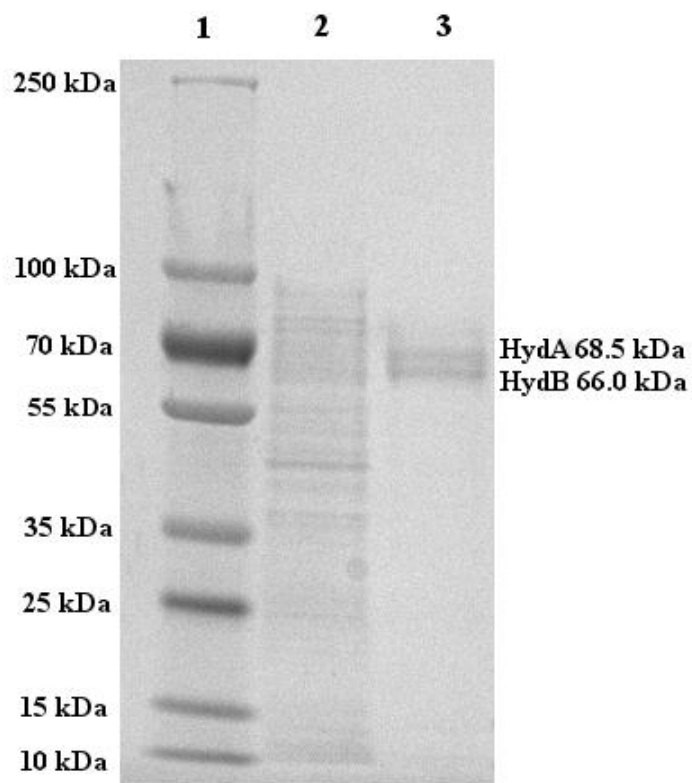
Further analysis of the purified recombinant HydAB was performed to determine its native molecular weight and cofactor content. Molecular weight determination by size exclusion chromatography indicated a protein of 286.96 kDa that would be consistent with a dimer of the αβ heterodimer of the HydAB subunits. Analysis by native PAGE (Figure 13) indicated a band migrating at a molecular weight of 322 kDa, which is consistent with a dimer of the αβ heterodimer (68.4 kDa • 2 + 65.9 kDa • 2). The iron content of the purified recombinant HydAB was $71.7 \pm 15.4$ moles of iron per mole per 287 kDa protein or $35.9 \pm 7.7$ moles of iron per mole of the single 143.5 αβ heterodimer. Based on domain analysis, HydAB is predicted to contain five [4Fe-4S]-clusters, two [2Fe-2S]-clusters and six Fe in the [H-Cluster] for an iron content of 30 moles of iron per mole of αβ heterodimer, which is lower than the experimentally determined iron content of $35.9 \pm 7.7$ moles of iron per mole of the 143.5 αβ heterodimer. The flavin content per dimer of the αβ heterodimer was 1.06 mole of flavin mononucleotide (FMN) per mole of 287 kDa or 0.53 FMN per 145.3 kDa αβ heterodimer.

**Figure 13. Native PAGE Analysis of Recombinant *S. aciditrophicus* HydAB Purified from *E. coli* by Nickel Affinity Chromatography.** Lane 1, molecular weight markers; Lane 2, *E. coli* cell-free extract; Lane 3, fraction after nickel-affinity chromatography.
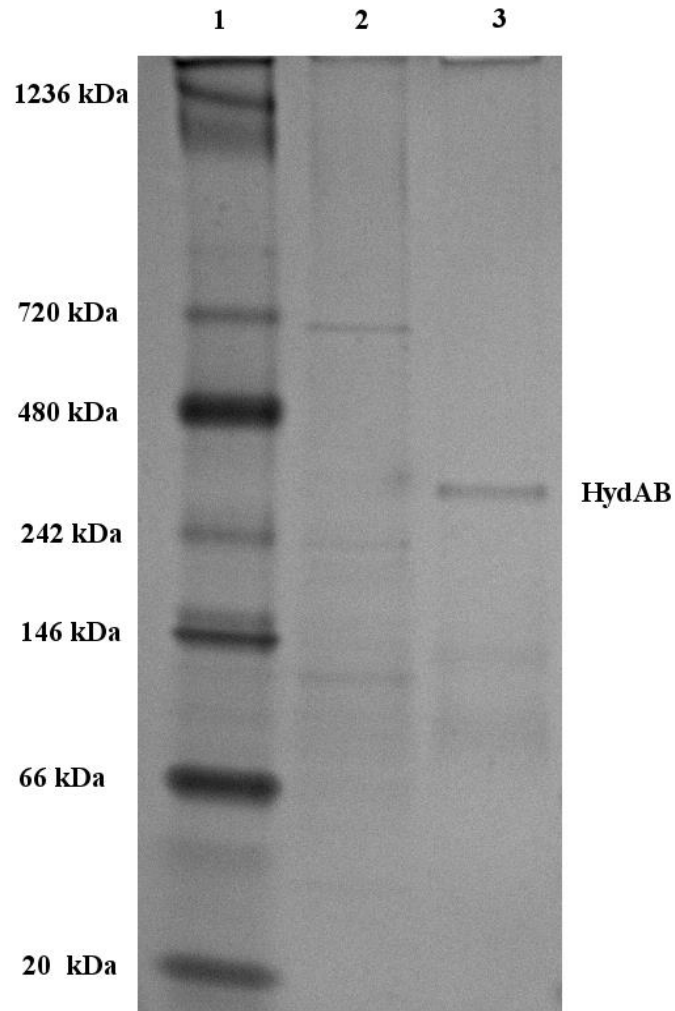
**Table 6. Peptides Detected by HPLC MS-MS Analysis of the Purified Recombinant HydAB Fraction.**

| SwissProt ID | Protein Description | Gene Name | Number of Matches to Gene Product | Protein Sequence Coverage (%) |
|---|---|---|---|---|
| Q2LSB7 | Iron only hydrogenase large subunit *Syntrophus aciditrophicus* (strain SB) | SYN_01370 | 805 | 81.16 |
| Q2LSB6 | NADH-quinone oxidoreductase chain F *Syntrophus aciditrophicus* (strain SB) | SYN_01369 | 840 | 65.07 |
| P0AG44 | 50S ribosomal protein L17 *Escherichia coli* (strain K12) | rplQ | 83 | 40.16 |
| P77398 | Bifunctional polymyxin resistance protein ArnA *Escherichia coli* (strain K12) | arnA | 195 | 36.67 |
| P0A9A9 | Ferric uptake regulation | fur | 56 | 41.22 |

| | | | | |
|---|---|---|---|---|
| | protein *Escherichia coli* (strain K12) | | | |
| P0ACJ8 | cAMP-activated global transcriptional regulator CRP *Escherichia coli* (strain K12) | crp | 107 | 66.67 |
| P17169 | Glutamine--fructose-6-phosphate aminotransferase [isomerizing] *Escherichia coli* (strain K12) | glmS | 191 | 48.77 |
| P0A9K9 | FKBP-type peptidyl-prolyl cis-trans isomerase SlyD *Escherichia coli* (strain K12) | slyD | 109 | 47.96 |
| P0A7S9 | 30S ribosomal protein S13 *Escherichia coli* (strain K12) | rpsM | 36 | 49.15 |
| P00393 | NADH dehydrogenase *Escherichia coli* (strain K12) | ndh | 89 | 35.94 |
| Q2LS97 | NADH-quinone | SYN_02139 | 90 | 12.7 |

| | | | | |
|---|---|---|---|---|
| | oxidoreductase chain F | | | |
| | *Syntrophus aciditrophicus* | | | |
| | (strain SB) | | | |
| P0CE47 | Elongation factor Tu 1 | tufA | 69 | 38.83 |
| | *Escherichia coli* (strain | | | |
| | K12) | | | |
| P0CE48 | Elongation factor Tu 2 | tufB | 67 | 36.04 |
| | *Escherichia coli* (strain | | | |
| | K12) | | | |
| P0A951 | Spermidine N(1)- | speG | 31 | 50.54 |
| | acetyltransferase | | | |
| | *Escherichia coli* (strain | | | |
| | K12) | | | |
| P0A7V0 | 30S ribosomal protein S2 | rpsB | 56 | 30.71 |
| | *Escherichia coli* (strain | | | |
| | K12) | | | |

**Partial purification of ferredoxin from *S. aciditrophicus* cell-free extracts**

To determine if purified recombinant HydAB is ferredoxin dependent, a protein fraction that contained *S. aciditrophicus* ferredoxin was obtained. After passage of *S. aciditrophicus* cell-free extracts through a DEAE Sepharose column, a dark brown band eluted at a concentration 0.62 M NaCl that contained ferredoxin. The ferredoxin-containing fraction had a 390/280

absorbance ratio of 0.082, which is much lower than the 390/280 absorbance ratio of 0.7 to 0.8

reported for other purified ferredoxins (Schonheit et al., 1978). Characterized ferredoxins have

an extinction coefficient of 30,000 $cm^{-1} M^{-1}$ at 390 nm (Schonheit et al., 1978) suggesting that

the concentration of the *S. aciditrophicus* ferredoxin fraction after ion exchange chromatography

was 3.7 µM with a total protein concentration of 0.25 mg• $ml^{-1}$. When the *S. aciditrophicus*

ferredoxin fraction was subjected to peptide identification by mass spectroscopy, a large number

of matches and high sequence coverage to the SYN_03059 gene product (Table 7) were

observed. SYN_03059 annotates as ferredoxin with two [4Fe-4S] clusters with a predicted 6.01

kDa molecular weight and a pI of 3.63.

**Table 7. HPLC MS-MS Identification Results of Partially Purified *S, aciditrophicus***

**Ferredoxin Fraction.**

| Swissprot ID | Protein Description | Gene Name | Number of Matches to Gene Product | Protein Sequence Coverage (%) (%) |
|---|---|---|---|---|
| Q2LUB2_SYNAS | Ferridoxin *Syntrophus aciditrophicus* (strain SB) | SYN_03059 | 24 | 98.31 |

| | | | | |
|---|---|---|---|---|
| Q2LR25_SYNAS | DNA-binding protein HU *Syntrophus aciditrophicus* (strain SB) | SYN_01724 | 35 | 27.47 |
| ACP_SYNAS | Acyl carrier protein *Syntrophus aciditrophicus* (strain SB) | acpP | 12 | 11.25 |
| Q2LU87_SYNAS | Hypothetical cytosolic protein *Syntrophus aciditrophicus* (strain SB) | SYN_02004 | 11 | 13.98 |
| Q2LSB7_SYNAS | Iron only hydrogenase large subunit *Syntrophus aciditrophicus* (strain SB) | SYN_01370 | 59 | 21.32 |
| Q2LWS6_SYNAS | Glycine cleavage system H protein *Syntrophus* | gcvH | 22 | 16.28 |

| | | | | |
|---|---|---|---|---|
| | *aciditrophicus* (strain SB | | | |
| Q2LSR6_SYNAS | Integration host factor beta-subunit | SYN_03200 | 9 | 9.68 |
| | *Syntrophus aciditrophicus* (strain SB) | | | |
| Q2LX26_SYNAS | Molybdenum transport regulatory protein_ lysR family | SYN_01042 | 9 | 20.66 |
| | *Syntrophus aciditrophicus* (strain SB) | | | |
| COAD_SYNAS | Phosphopantetheine adenylyltransferase | coaD | 15 | 13.33 |
| | *Syntrophus aciditrophicus* (strain SB) | | | |
| Q2LUY8_SYNAS | Acyl-CoA dehydrogenase_ | SYN_00480 | 10 | 10.7 |

| | | | | |
|---|---|---|---|---|
| | short-chain specific *Syntrophus aciditrophicus* (strain SB) | | | |
| Q2LXH8_SYNAS | ABC-type nitrate/sulfonate/bicarbonate transport systems_ periplasmic components *Syntrophus aciditrophicus* (strain SB) | SYN_00365 | 15 | 10.33 |
| Q2LUY7_SYNAS | Glutaconyl-CoA decarboxylase A subunit *Syntrophus aciditrophicus* (strain SB) | SYN_00481 | 15 | 7.97 |
| Q2LWR8_SYNAS | Acetyl-CoA synthetase *Syntrophus* | SYN_02635 | 20 | 9.91 |

*aciditrophicus*

(strain SB)

---

**Purification of *S. aciditrophicus* ferredoxin recombinantly produced in *E. coli***

The SYN_03059 gene product was recombinantly produced and purified from *E. coli* cell-free extracts. The protein was eluted with an imidazole concentration of 250 mM during nickel affinity chromatography and had a dark brown color characteristic of iron sulfur proteins. The 390/280 absorbance ratio of the nickel affinity-purified fraction was 0.63 with an estimated ferredoxin concentration of 34 µM based on the extinction coefficient at 390 nm for clostridial ferredoxin of 30,000 cm$^{-1}$ M$^{-1}$ and a molecular weight of 6,000 daltons (Hong and Rabinowtiz 1970). SDS-PAGE analysis of the fractions containing ferredoxin partially purified from *S. aciditrophicus* and ferredoxin recombinantly produced in *E. coli* indicated bands migrating closer to predicted molecular masses of 12 kDa (Figure 14). Negatively charged ferredoxins have been reported to exhibit anomalous migration during SDS-PAGE analysis, which has been proposed to be due to the interactions of the negatively charged peptide and SDS micelles (Darimont and Sterner, 1994; Huang et al., 2016; Kpebe et al., 2018).

**Figure 14. SDS-PAGE of Recombinantly Produced *S. aciditrophicus* Ferredoxin from *E. coli* and Ferredoxin Partially Purified from *S. aciditrophicus*.** Lane 1. molecular weight markers; Lane 2. nickel affinity purified recombinant SYN_03059 gene product; Lane 3. partially purified ferredoxin fraction from *S. aciditrophicus*.
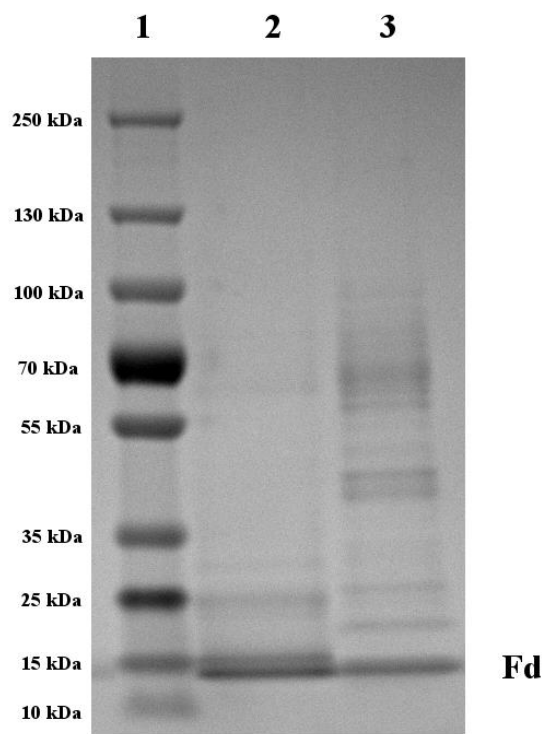


**Enzyme activities of the purified recombinant *S. aciditrophicus* HydAB**

The purified recombinant HydAB was capable of reducing methyl viologen (specific activity of 13.0 U•mg$^{-1}$ at 37°C) and NAD$^+$ (specific activity of 4.75 U•mg$^{-1}$ at 37°C) with hydrogen as the electron donor (Table 8). Characterized electron-bifurcating hydrogenases

require the addition of oxidized ferredoxin before reduction of $NAD^+$ occurs (Schuchmann and

Muller, 2012; Wang et al., 2013a). In contrast, the rate of $NAD^+$ reduction by purified

recombinant *S. aciditrophicus* HydAB was not affected by the presence of oxidized ferredoxin

(Figure 15). The change in absorbance ($\Delta A_{340}$) in the absence of ferredoxin was 0.275 A• $min^{-1}$

which was similar to the change in absorbance in the presence of clostridial ferredoxin 0.23 A•

$min^{-1}$. The specific $NAD^+$ reduction activity of HydAB was 4.75 U • $mg^{-1}$ of protein in the

absence of ferredoxin and was 3.78 • $mg^{-1}$ of protein in the presence of the clostridial ferredoxin.

The reduction of $NADP^+$ by purified recombinant HydAB was not observed either in presence or

the absence of clostridial ferredoxin. In addition, neither clostridial ferredoxin nor the

recombinantly produced *S. aciditrophicus* ferredoxin was reduced by HydAB in either the

presence or absence of $NAD^+$ (Table 8). The dependence of $NAD^+$ reduction on the presence of

*S. aciditrophicus* ferredoxin produced recombinantly in *E. coli* was tested only in a single assay

and not in triplicate. $NAD^+$ reduction before the addition of recombinantly produced *S.

aciditrophicus* ferredoxin was 1.61 U • $mg^{-1}$ at 37°C. After the addition of recombinantly

produced *S. aciditrophicus* ferredoxin the rate was 1.65 U • $mg^{-1}$ at 37°C.

**Figure 15. The Reduction of NAD$^+$ by HydAB is not Ferredoxin Dependent.** The reaction

was performed in 1.5-ml. stopper-sealed cuvettes with 50 mM potassium phosphate (pH 7.5), 5

µM FMN, 5 µM FAD, 2 mM dithioerythritol, and 1 mM NAD$^+$ and 8.1 µg of purified

recombinant HydAB as indicated by +H$_2$ase. The reaction was started with the addition of H$_2$.

Clostridial ferredoxin was added at a concentration of 10 µM as indicated by +Fd$_{ox}$. The rate of

reduction of NAD ($\Delta$A340) after the addition of hydrogen (0.275 $\Delta$A•min$^{-1}$) was similar to the

change after the addition of clostridial ferredoxin (0.23 $\Delta$A•min$^{-1}$).
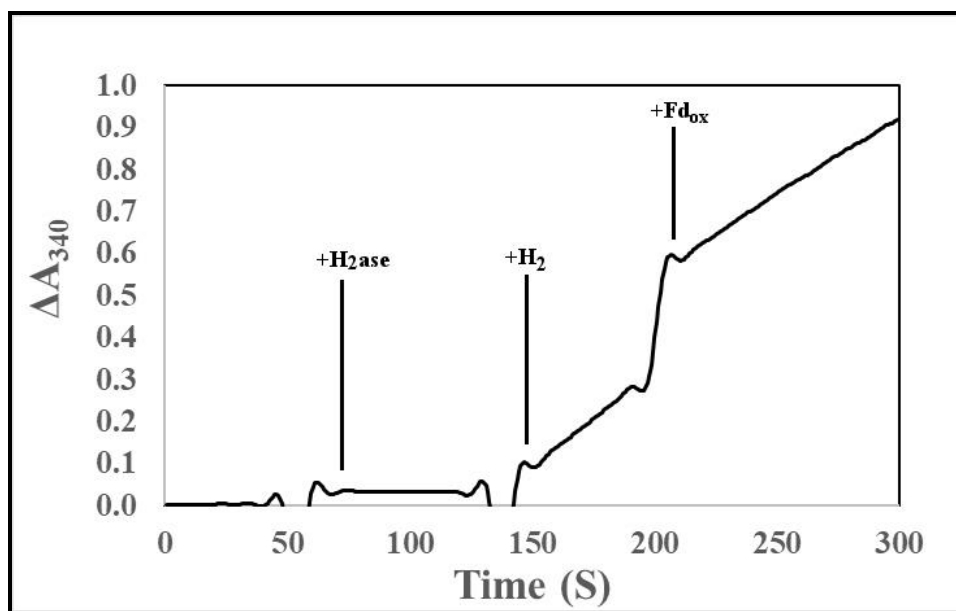


HydAB produced hydrogen from methyl viologen reduced by dithionite (specific activity

of 0.42 U•mg$^{-1}$ at 37°C) and from NADH (specific activity of 0.027 U•mg$^{-1}$) (Table 8). In

contrast to known electron-bifurcating hydrogenases (Schut and Adams, 2009; Schuchmann and

Muller, 2012; Wang et al., 2013c), the specific rate of hydrogen production from NADH was not

enhanced by the presence of reduced ferredoxin-generating system consisting of clostridial

pyruvate:ferredoxin oxidoreductase, pyruvate, and clostridial ferredoxin (Table 8). The specific

hydrogen production rate by HydAB was 0.014 U • mg$^{-I}$ with reduced clostridial ferredoxin

generating system compared to 0.027 U • mg$^{-I}$ without the reduced ferredoxin-generating system.

The maximum observed hydrogen production rate in the absence of the reduced ferredoxin-

generating system was 1.5 nanomol • min$^{-1}$ (Figure 16) compared to a maximum observed

hydrogen production rate of 0.6 nanomol • min$^{-1}$ in the presence of the reduced ferredoxin

generating system rate (Figure 17). The use of recombinantly produced *S. aciditrophicus*

ferredoxin (specific activity of 0.018 U • mg$^{-I}$) or ferredoxin partially purified from *S.

aciditrophicus* cell-free extracts as part of the reduced ferredoxin generating system did not

enhance hydrogen production activity compared to that in the absence of the reduced ferredoxin-

generating system (Table 8). Due to the limited yield of *S. aciditrophicus* ferredoxin that was

recovered during the partial purification from *S. aciditrophicus* cell-free extracts, only a single

hydrogen production assay was performed. In the presence of NADH and reduced ferredoxin

partially purified from the *S. aciditrophicus* the maximal hydrogen production was specific

activity of 0.013 U • mg$^{-I}$. The results indicated that regardless of the reduced ferredoxin source

(clostridial, recombinantly produced *S. aciditrophicus* ferredoxin or *S. aciditrophicus* ferredoxin

partially purified from *S. aciditrophicus* cell-free extracts), no enhancement of hydrogen

production rate was observed.


**Table 8. Specific Activities of Purified Recombinant HydAB.** [a] Assays were performed at 37

°C and a pH of 7.5; 1 unit of activity (U) equals 2 μmol of electrons transferred per min.

[b] The activity was measured as the rate of $NAD^+$ reduction ($\Delta$ 340 nm) in the presence of $Fd_{ox}$,

and not the rate of $Fd_{ox}$ reduction ($\Delta$ 430 nm), which was not observed to occur.

| Reaction | Hydrogenase Activity (U/mg)[a] |
|---|---|
| $H_2 \rightarrow MV_{ox}$ | 13.0 |
| $H_2 \rightarrow NAD^+$ | 4.75 |
| $H_2 \rightarrow NAD^+ +$ Clostridial $Fd_{ox}$ | 3.78 |
| $H_2 \rightarrow NADP^+$ | <0.01 |
| $H_2 \rightarrow NADP^+ +$ Clostridial $Fd_{ox}$ | <0.01 |
| $H_2 \rightarrow$ Clostridial $Fd_{ox}$ | <0.01 |
| $MV_{red} \rightarrow H_2$ | 0.42 |
| $NADH \rightarrow H_2$ | 0.027 |
| $NADH +$ Clostridial $Fd_{red} \rightarrow H_2$ | 0.014 |
| $NADH +$ Syntrophus $Fd_{red} \rightarrow H_2$ | 0.018 |
| Clostridial $Fd_{red} \rightarrow H_2$ | <0.001 |

**Figure 16. Production of Hydrogen from NADH by HydAB in the Presence of Reduced Ferredoxin Generating System.** Each assay was performed in a 6.5-ml serum bottle containing 1.0 ml of 50 mM Tris (pH 7.5) with 2 mM DTE, 5 µM FAD, 5 µM FMN, 25.4 µg HydAB, and a reduced ferredoxin-generating system consisting of 0.1 unit of pyruvate ferredoxin oxidoreductase, 20 µm clostridial ferredoxin, 10 mM pyruvate, 0.1 mM thiamine pyrophosphate, and 1 mM CoA. The reduced ferredoxin-generating system and HydAB were as added as

indicated by +Fd$_{red}$ and H$_2$ase, respectively. NADH was added to a final concentration of 1 mM as indicated by +NADH. The maximal rate of hydrogen production in the depicted assay was 24.6 nanomoles min$^{-1} \cdot$ mg$^{-1}$.
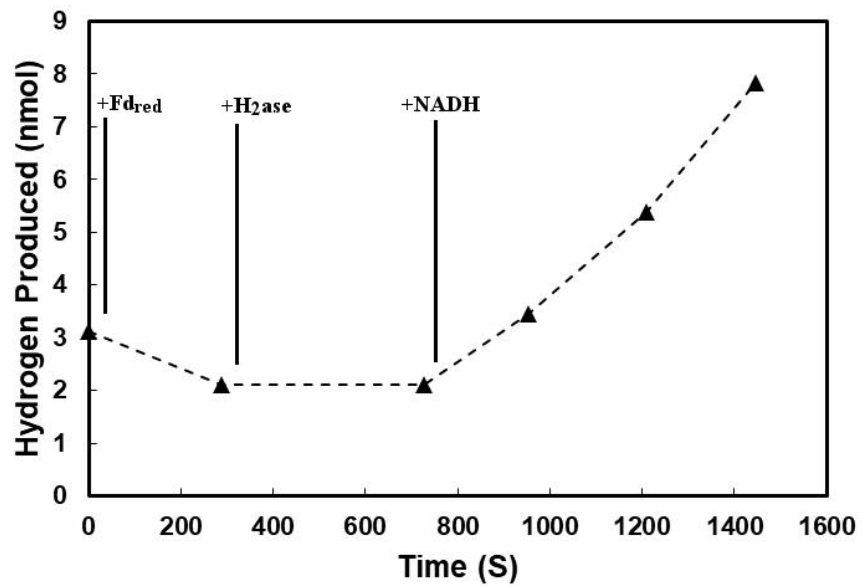
**Figure 17. Production of Hydrogen from NADH by HydAB Independent of Reduced Ferredoxin.** Enzyme assay was performed in a 6.5-ml serum bottle containing 1.0 ml of 50 mM Tris (pH 7.5) with 2 mM DTE, 5 µM FAD, 5 µM FMN, 50.7 µg HydAB (+H$_2$ase). NADH was added to a final concentration of 1 mM (+NADH). The maximal rate of hydrogen production in the enzyme assay reaction depicted was 30.1 nanomoles min$^{-1}$• mg$^{-1}$
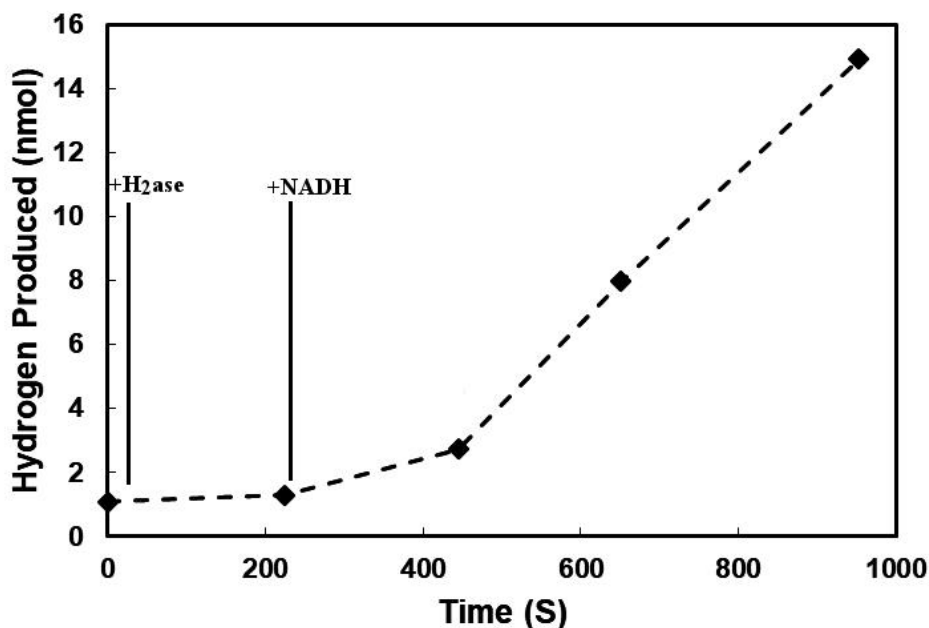


Equilibrium hydrogen partial pressures produced by HydAB varied with the NADH/NAD$^+$ ratio (Table 9), ranging from 40.17 ($\pm$ 3.2) pascals with a NADH/NAD$^+$ ratio of 5.0 to 2.2 ($\pm$ 0.4) pascals at a NADH/NAD$^+$ ratio of 0.2. These values are much lower than the equilibrium hydrogen partial pressures produced by electron bifurcating hydrogenases of 1,000 Pa at equilibrium (equivalent to E' = -365 mV). Higher hydrogen partial pressures were observed at higher NADH/NAD$^+$ ratios; however, hydrogen partial pressures never reached the thermodynamically predicted equilibrium pressures for the given NADH/NAD$^+$ ratio at pH 7.5

(see Table 9). A small amount of hydrogen was observed in the absence of NADH addition, which was likely due to carryover from manipulation of the enzyme inside the anaerobic chamber that contained hydrogen in the atmosphere.

Hydrogen partial pressures observed during the metabolism of crotonate, cyclohex-1-ene-1- carboxylate, and benzoate by pure cultures of *S. aciditrophicus* ranged from 8.3 to 36.6 pascals (Figure 18). Hydrogen partial pressures observed during crotonate, cyclohex-1-ene-1-carboxylate, and benzoate metabolism by cocultures of *S. aciditrophicus* with *M. hungatei* were between 6.6 and 19.1 pascals. Hydrogen partial pressures after 3 days of incubation continued to increase in pure cultures of *S. aciditrophicus* on benzoate and cyclohex-1-ene carboxylate while hydrogen partial pressures decreased after 3 days in cocultures on all three substrates. The hydrogen partial pressures produced by pure cultures and cocultures of *S. aciditrophicus* metabolizing crotonate, cyclohex-1-ene-1- carboxylate, and benzoate (6.6 to 36.6 pascals) could be generated by HydAB operating at $NADH/NAD^+$ ratios of 1.0 or higher (9.1 to 40.2 pascals).

**Table 9. Equilibrium Hydrogen Concentrations Produced by NADH-Dependent [FeFe]-Hydrogenase, HydAB with Varied $NADH/NAD^+$ ratios.**

| Condition[a] | Ratio of $NADH/NAD^+$ | Predicted Equilibrium Hydrogen Partial Pressure | Hydrogen Partial Pressure (Pa) | Hydrogen Produced (nanomol) |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| 1.0 mM NAD$^+$ + 5.0 mM NADH | 5.0 | 105.9 | 40.2 ± 3.2 | 101.8 ± 8.1 |
| 3.0 mM NAD$^+$ + 3.0 mM NADH | 1.0 | 21.3 | 9.1 ± 2.4 | 23.2 ± 6.0 |
| 5.0 mM NAD$^+$ + 1.0 mM NADH | 0.2 | 4.24 | 2.2 ± 0.4 | 5.4 ± 1.1 |
| 0.0 mM NAD$^+$ + 0.0 mM NADH | NA[c] | NA | 0.4 ± 0.2 | 1.0 ± 0.6 |

[a]Assays were performed at pH 7.5 in 6.5-mL serum bottles with 1.0 mL of liquid volume containing 69.6 µg of HydAB and 5.5 mL of headspace.

[b] Values are the mean ± the standard deviation of triplicate assays.

[c] NA, not applicable.

[d]The predicted equilibrium concentrations of hydrogen were calculated as described by (Gutekunst and Schulz, 2018). Equation and terms used were:

$$e^{((-0.094 \cdot n \cdot F)/(R \cdot T))} \cdot [NADH/NAD^+] \cdot 10^{(-pH + 7)} = [pH_2]$$

Faraday's constant = F = 96.485 J • V$^{-1}$ • mol$^{-1}$ of electron equivalents

R = 8.314 J • K • mol$^{-1}$ Gas Constant

n = 2, number of e$^-$ in transfer reaction

T = 298.15 Kelvin

 [pH$_2$] = Partial pressure of hydrogen

-0.094 = $\Delta E'$, difference in standard reduction potentials of $[H^+/H2]$ = -414 mV and

$[NADH/NAD^+]$ = -320 mV

**Figure 18. Hydrogen Partial Pressures During the Metabolism of Crotonate, Cyclohex-1-ene-1-carboxylate, and Benzoate by Pure Cultures of *S. aciditrophicus* and Co-cultures of *S. aciditrophicus* with *Methanospirillum hungatei*.** Panels A, B and C: hydrogen and substrate concentrations for metabolism of crotonate (A), cyclohex-1-ene carboxylate (B), and benzoate (C) by pure cultures of *S. aciditrophicus*. Panels D, E, and F: hydrogen and substrate concentrations during the metabolism of crotonate (D), cyclohex-1-ene carboxylate (E), and benzoate (F) by co-cultures of *S. aciditrophicus* with *M. hungatei*.
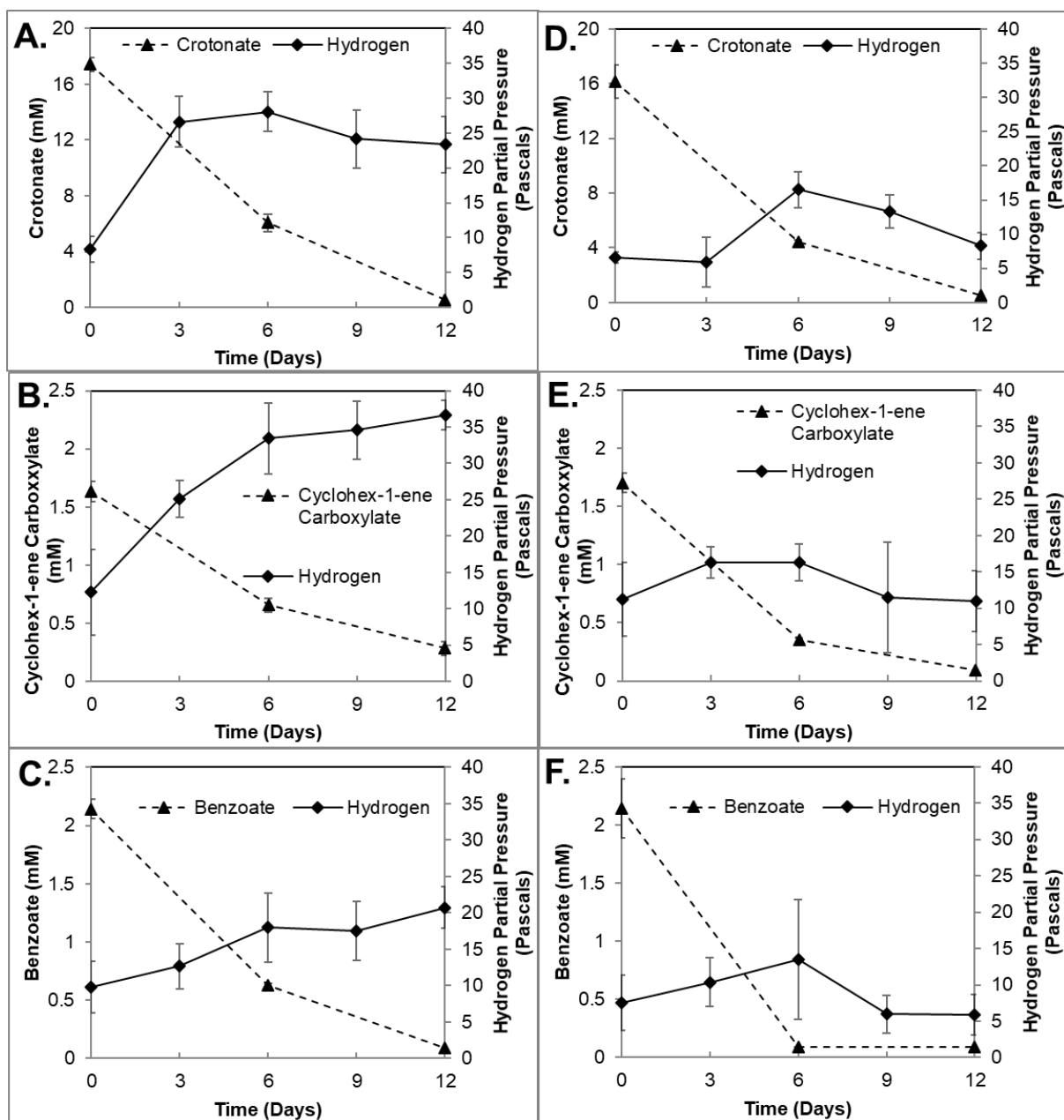
## Discussion

The results from the enzymatic assays show that HydAB from *S. aciditrophicus* is a NADH-dependent, ferredoxin-independent, dimeric [FeFe]-hydrogenase. HydAB functions in a similar manner as the NADH-dependent ferredoxin-independent [FeFe]-hydrogenase of *S. wolfei*, Hyd1ABC (Losey et al., 2017). Both enzymes function independently of ferredoxin to

produce hydrogen from NADH, as well as the reverse reaction, the reduction of $NAD^+$ coupled to hydrogen oxidation. The lack of ferredoxin dependence by these enzymes may be the evolutionary outcome unique to syntrophy (i. e., growth at very low hydrogen partial pressures). A NADH-dependent, ferredoxin-independent [FeFe]-hydrogenase provides both organisms with an efficient mechanism for hydrogen production without expending energy from a membrane gradient to generate reduced ferredoxin needed for electron bifurcation. These results are consistent with the known physiological properties of the syntrophic fatty acid oxidizer, *S. wolfei,* which lacks a system capable of generating sufficient amounts of reduced ferredoxin for NADH oxidation and does not have large amounts of ferredoxin (Losey et al., 2017). However, the result is somewhat surprising for *S. aciditrophicus* where ferredoxin (Table 7) and a reduced ferredoxin generating system in the form of the Rnf complex (McInerney et al., 2007) are both present. In addition to genomic evidence for the Rnf complex, experiments with inverted membrane vesicles of *S. aciditrophicus* have demonstrated Rnf activity (reduction of ferredoxin coupled to NADH oxidation dependent on a membrane gradient generated by ATP synthase, M. J. McInerney and N. Q. Wofford, unpublished results). Also, *S. aciditrophicus* has a clostridial like, negatively charged, low molecular weight ferredoxin that can be enriched by anion-exchange chromotography (Figure 14). The *S. aciditrophicus* ferredoxin is predicted to harbor two [4Fe4S]-clusters, as do other ferredoxins that have been used as low potential electron donors for the study of electron-bifurcating hydrogenases (Schut and Adams, 2009; Schuchmann and Muller, 2012; Wang et al., 2013c). It appears that, unlike *S. wolfei*, *S. aciditrophicus* has a mechanism for producing reduced ferredoxin but does not utilize it when producing hydrogen from NADH. This may suggest that ferredoxin-independent, hydrogen production from NADH is a more prevalent strategy than previously thought and may afford some advantage during

syntrophic metabolism. The likely function of the Rnf complex in *S. aciditrophicus* is to provide reduced ferredoxin for benzoyl-CoA reduction and certain biosynthetic reactions (McInerney et al., 2007; Fuchs et al., 2011; Kung et al., 2013).

Pure cultures of *S. aciditrophicus* and co-cultures of *S. aciditrophicus* with *M. hungatei* generated hydrogen partial pressures of 5.9 to 36.6 pascals (Figure 18), which is within the range of the hydrogen partial pressures (2.2 to 40.2 pascals) generated by the purified, recombinant *S. aciditrophicus* HydAB at NADH/NAD$^+$ ratios of 1 to 5 (Table 9). Thus, ferredoxin-independent, hydrogen production from NADH by HydAB can explain the hydrogen partial pressures observed during the metabolism of crotonate, benzoate, and cyclohex-1-ene by *S. aciditrophicus* but with the requirement of maintaining an intracellular NADH/NAD$^+$ ratio greater than one. Syntrophic metabolizers are known to require low hydrogen partial pressures for growth and metabolism (Sieber et al., 2012). The use of a ferredoxin-independent, NADH-dependent [FeFe]-hydrogenase would require continual hydrogen use by another microorganism to allow for continual hydrogen production from NADH and could explain, in part, the obligate requirement that *S. aciditrophicus* and *S. wolfei* have for a hydrogen-consuming organism during growth on substrates such as fatty and alicyclic acids. It should be noted that the pure culture metabolism of benzoate is dependent on the transfer of small amounts of crotonate with inoculum (Mouttaki et al., 2008), which likely occurred in the cultures used to for these hydrogen measurements.

Despite having similar functions, NADH-dependent ferredoxin-independent hydrogen production, the subunit composition of *S. aciditrophicus* HydAB differs from that of *S. wolfei* Hyd1ABC. The *S. wolfei* Hyd1ABC is a αβγ heterotrimeric complex with a molecular weight of ~120 kDa (Losey et al., 2017) while the *S. aciditrophicus* HydAB is a dimer of an αβ heterodimer with a molecular weight of ~ 280 kDa (Figure 13). A BLASTP comparison showed

a high homology (37% amino acid sequence identity) between the *S. wolfei* gamma subunit (Hyd1C) and the N-terminal portion (amino acid residues 15 to 152) of the *S. aciditrophicus* beta subunit (HydB). Another BLASTP comparison showed high homology (46% amino acid sequence identity) between the *S. wolfei* beta subunit (Hyd1B) to the C-terminal portion of the *S. aciditrophicus* beta subunit (HydB) (amino acid residues 177 to 585). These data indicate that the *S. aciditrophicus* beta subunit is a fusion of the smaller gamma subunit at the N-terminal portion of the peptide sequence to a beta subunit at the C-terminus (See Figure 19). A similar peptide fusion was reported in a multimeric NADH-dependent formate dehydrogenase (Laukel et al., 2003). A BLASTP comparison indicated that the fused beta subunit from this NADH-dependent formate dehydrogenase shared similarity (32% amino acid sequence identity) to the *S. aciditrophicus* [FeFe]-hydrogenase beta subunit. The differences in the beta subunits of the *S. aciditrophicus* [FeFe]-hydrogenase and *S. wolfei* [FeFe]-hydrogenase demonstrate that there are multiple arrangements of ferredoxin-independent, NADH-dependent [FeFe]-hydrogenases depending on the source organism (see Figure 19).

In addition to the *S. wolfei* and *S. aciditrophicus* [FeFe]-hydrogenases, two additional [FeFe]-hydrogenases were originally described to reduce $NAD(P)^+$, in a manner that did not involve electron-bifurcation or require ferredoxin. The first [FeFe]-hydrogenase from *Desulfovibrio fructosovorans* was originally described to reduce $NADP^+$ using hydrogen (Malki et al., 1995). However, a more recent study where the [FeFe]-hydrogenase was purified and characterized, indicated the enzyme is an electron-bifurcating enzyme, simultaneously reducing both ferredoxin and NADH when oxidizing hydrogen (Kpebe et al., 2018). The other [FeFe]-hydrogenase, from *Caldanaerobacter tencongensis,* was reported to be capable of producing hydrogen from NADH and the reduction of $NAD^+$ with hydrogen (Soboh et al., 2004). A

subsequent study where the *C. tencongensis* [FeFe]-hydrogenase was expressed heterologously in *E. coli* suggested that the enzyme requires reduced ferredoxin to produce hydrogen (Kelly et al., 2015), which indicates an electron-bifurcating mechanism. Therefore, we note that the only two prior examples of [FeFe]-hydrogenases reducing $NAD^+$ or $NADP^+$ in a ferredoxin-independent manner have been found later to be electron-bifurcating, ferredoxin-dependent enzymes. The *S. wolfei* and *S. aciditrophicus* [FeFe]-hydrogenases are the only known examples of NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases. We therefore focused on finding structural features that differentiate the *S. wolfei* and *S. acidotrophicus* NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases from the six known electron-bifurcating, NADH-dependent, ferredoxin-dependent [FeFe]-hydrogenases. As discussed below, we believe that differences in the composition of the beta subunit are key to determining whether the enzyme is or is not electron-bifurcating.

**Comparison of NADH-dependent, Ferredoxin-Independent Eznymes to Electron Bifurcating, NADH-Dependent, Ferredoxin Dependent [FeFe]-Enzymes**

We compared a group of NADH-dependent, ferredoxin-independent enzymes against a group of the known examples of electron-bifurcating NADH-dependent, ferredoxin-dependent formate dehydrogenases and [FeFe]-hydrogenases. In addition to the *S. wolfei* and *S. aciditrophicus* [FeFe]-hydrogenases, the NADH-dependent, ferredoxin-independent enzymes included NADH-dependent, ferredoxin-independent formate dehydrogenases and NADH:ubiquinone oxidoreductase subunits, Nqo1, Nqo2, and Nqo3. These enzymes share similar properties, the oxidation or reduction of NADH in a ferredoxin-independent manner. The

two groups, ferredoxin-dependent and ferredoxin-independent, were then compared to identify the features that may account for the difference in the requirement for ferredoxin.

**NADH-Dependent, Ferredoxin-Independent Formate Dehydrogenases**

For this analysis, other ferredoxin-independent enzymes with a composition similar to NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases were needed. One group of enzymes included five formate dehydrogenases that had been previously purified and characterized as NADH-dependent formate dehydrogenases. We suggest that it may be more appropriate that they be referred to as NADH-dependent and ferredoxin-independent formate dehydrogenases. This is to indicate that they are capable of formate oxidation coupled to the reduction of NADH without requiring ferredoxin. The distinction of ferredoxin-independence is important as there is at least one example of an electron-bifurcating formate dehydrogenase (Wang et al., 2013c) that utilizes both NADH and ferredoxin simultaneously. These NADH-dependent, ferredoxin-independent formate dehydrogenases were purified from *Cupriavidus oxalaticus*, (previously *Psuedomonas oxalaticus*) (Ruschig et al., 1976; Muller et al., 1978), *Methylosinus trichosporium* (Jollie and Lipscomb, 1991)*, Cupriavidus eutropha,* (previously *Alcaligenes eutropha)* (Friedebold and Bowien, 1993), *Methylobacterium extorquens* (Laukel et al., 2003), and *Rhodobacter capsulatus* (Hartmann and Leimkühler, 2013). With the exception of the *R. capsulatus* formate dehydrogenase, these enzymes were characterized before the first published examples of electron-bifurcating formate dehydrogenases (Wang et al., 2013b) and [FeFe]-hydrogenases (Schut and Adams, 2009), and as such the possibility of electron bifurcation was not recognized or tested. These five NADH-dependent, ferredoxin-independent formate dehydrogenases were reported to be oxygen tolerant and purified from organisms

126

capable of growth under aerobic conditions. In contrast, all electron-bifurcating formate

dehydrogenases and [FeFe]-hydrogenases have been purified from strict anaerobes (Soboh et al.,

2004; Schut and Adams, 2009; Schuchmann and Muller, 2012; Wang et al., 2013a; Wang et al.,

2013c; b; Zheng et al., 2014; Kpebe et al., 2018). The role of the NADH-dependent, ferredoxin-

independent formate dehydrogenases is proposed to be the generation of reduced NADH for

aerobic respiration in *C. oxalaticus* (Muller et al., 1978), *C. eutropha* (Friedebold and Bowien,

1993), *M. trichosporium* (Friedebold and Bowien, 1993) and *M. extorquens* (Laukel et al., 2003).

It was not stated what the proposed physiological role of the *R. capsulatus* formate

dehydrogenase was in the source organism (Hartmann and Leimkühler, 2013), although *R.

capsulatus* is capable of aerobic respiration (Zannoni, 1995). In contrast to the proposed role of

the NADH-dependent, ferredoxin-independent formate dehydrogenases as a source of reduced

NADH, we propose the opposite role for the NADH-dependent, ferredoxin-independent [FeFe]-

hydrogenases from *S. wolfei* and *S. aciditrophicus*, where instead the physiological role is

NADH oxidation.

The oxygen tolerant nature of the NADH-dependent, ferredoxin-independent formate

dehydrogenases is likely due to the use of of cysteine as a ligand to the metallopterin at the active

site as opposed to a selenocysteine (Hartmann and Leimkühler, 2013). The replacement of a

selenocysteine with a cysteine in the *E. coli* FdhF resulted in a less active enzyme (Axley et al.,

1991). However, formate dehydrogenases with a selenocysteine ligand are rapidly inactivated by

oxygen as opposed to cysteine-ligand versions (Ferry, 1990). Furthermore, it was observed that,

in the presence of oxygen, an electron bifurcating butyryl-CoA dehydrogenase generated

superoxide and hydrogen peroxide as byproducts (Chowdhury et al., 2015). This suggests that

operation of flavin-dependent electron-bifurcating enzymes in the presence of oxygen can

produce toxic, reactive oxygen compounds (Buckel and Thauer, 2018b). It could be argued that it is unlikely that oxygen-tolerant, NADH-dependent, ferredoxin-independent formate dehydrogenases have an unrecognized electron-bifurcation mechanism, as such as a mechanism would produce reactive oxygen compounds that would be toxic to aerobic organisms. However, recent evidence that the FixABCX complex from *Azotobacter vinelandii* performs electron bifurcation under aerobic conditions suggests electron-bifurcating enzymes are not restricted to anaerobes (Ledbetter et al., 2017). Despite incomplete experimental testing of the ability for electron-bifurcation, all current evidence suggests that the oxygen-tolerant, NADH-dependent formate dehydrogenases are ferredoxin-independent. Therefore, these five NADH-dependent, ferredoxin-independent formate dehydrogenases were included in the group of NADH-dependent, ferredoxin-independent enzymes along with the *S. wolfei* and *S. aciditrophicus* [FeFe]-hydrogenases.

**NADH:Quinone Oxidoreductase Subunits Nqo1, Nqo2, and Nqo3**

Sequence homology between the group of NADH-dependent, ferredoxin-independent formate dehydrogenase and the Nqo1, Nqo2, and Nqo3 (NADH:Quinone oxidoreductase) subunits that comprise the hydrophilic portion of Respiratory Complex I has been discussed extensively (Pilkington et al., 1991;Sazanov and Hinchliffe, 2006; Sieber et al., 2010). This portion of complex I (Nqo1-3) functions to transfer electrons from NADH to the remainder of Respiratory Complex I (Nqo4-15) (Sazanov and Hinchliffe, 2006). Sequence homology suggests that Nqo1, Nqo2, and Nqo3 are analogous to the beta, gamma, and alpha subunits of the NADH-dependent, ferredoxin-independent formate dehydrogenases respectively (Hille et al., 2014). Due to this homology, we included the Nqo1-Nqo3 subunits from *T. thermophilus* and the equivalent

128

*E. coli* NADH:Ubiquinone oxidoreductase subunits (NuoEFG) as the final members of the group of NADH-dependent, ferredoxin-independent enzymes.

**Electron-Bifurcating [FeFe]-Hydrogenases and Formate Dehydrogenases**

Electron-bifurcating, NADH- and ferredoxin-dependent enzymes include the [FeFe]-hydrogenases from *Thermotoga martima* (Schut and Adams, 2009), *Acetobacterium woodii* (Schuchmann and Muller, 2012), *Moorella thermoacetica* (Wang et al., 2013c), *Ruminococcus albus* (Zheng et al., 2014), *D. fructosovorans* (Kpebe et al., 2018), and *C. tengcongensis* (Soboh et al., 2004). In addition, there is an electron-bifurcating NADH- and ferredoxin-dependent formate dehydrogenase from *Clostridium acidurici* (Wang et al., 2013b) and a NADPH- and a ferredoxin-dependent [FeFe]-hydrogenase-formate dehydrogenase complex from *Clostridium autoethanogenum* (Wang et al., 2013a). The NADPH- and ferredoxin-dependent [FeFe]-hydrogenase-formate dehydrogenase complex consists of seven subunits; some of these subunits do not appear to have equivalents in any of the other electron-bifurcating enzymes.

**Figure 19. Differences in Cofactor Binding Sites between Electron-Bifurcating, NADH-Dependent and Ferredoxin-Dependent Enzymes and NADH-Dependent, Ferredoxin-Independent Enzymes.** Cofactor binding sites are shown as orientated from the N-terminal to C-terminal portion of the subunits. Dashed boxes indicate subunits that are fused together into a single polypeptide chain. Image depiction is not scaled to accurately reflect number of amino acids in each subunit. Nqo2/NuoE is homologous to the gamma subunits. Nqo1/NuoF is homologous to the beta subunit, and Nqo3/NuoG is homologous to the alpha subunit. Only a small region of 200 residues, predicted to bind a [2Fe-2S] cluster, and two [4Fe-4S], are

129

homologous between Nqo3, the alpha subunit of the formate dehydrogenases, and the alpha subunit of the [FeFe]-hydrogenases (Pilkington et al., 1991; Vignais et al., 2001; Sazanov and Hinchliffe, 2006) Delta subunits for the *A. woodii, C. tengcongensis, C. oxalaticus,* and *C. eutropha* are represented by an empty box, indicating that the subunits do not appear to have conserved domains predicted to bind cofactors.

## Electron Bifurcating [FeFe]-Hydrogenases/Formate Dehydrogenases

| Source Organism(s) | Reported Activity | α | β | γ | δ |
|---|---|---|---|---|---|
| T. maritima | Bifurcating NADH/Fd Hydrogenase | 2Fe-2S, 4Fe-4S, 4Fe-4S, 4Fe-4S, H-Cluster, 2Fe-2S | 2Fe-2S, Flavin, 4Fe-4S, 4Fe-4S, 4Fe-4S | 2Fe-2S | ☐ |
| A. woodii | Bifurcating NADH/Fd Hydrogenase | 2Fe-2S, 4Fe-4S, 4Fe-4S, 4Fe-4S, H-Cluster | 2Fe-2S, Flavin, 4Fe-4S, 4Fe-4S, 4Fe-4S | 2Fe-2S | |
| M. thermoacetica, R. albus | Bifurcating NADH/Fd Hydrogenase | 2Fe-2S, 4Fe-4S, 4Fe-4S, 4Fe-4S, H-Cluster | 2Fe-2S, Flavin, 4Fe-4S, 4Fe-4S, 4Fe-4S | 2Fe-2S | |
| C. tengcongenensis | Bifurcating NADH/Fd Hydrogenase* | 2Fe-2S, 4Fe-4S, 4Fe-4S, 4Fe-4S, H-Cluster | 2Fe-2S, Flavin, 4Fe-4S, 4Fe-4S, 4Fe-4S | 2Fe-2S | ☐ |
| C. acidurici | Bifurcating NADH/Fd Formate Dehydrogenase | 2Fe-2S, 4Fe-4S, 4Fe-4S, 4Fe-4S, 4Fe-4S, Mo / 2Fe-2S, 4Fe-4S, 4Fe-4S | 2Fe-2S, Flavin, 4Fe-4S, 4Fe-4S, 4Fe-4S | 2Fe-2S | |
| D. fructosovorans | Bifurcating NADH/Fd Hydrogenase* | 2Fe-2S, 4Fe-4S, 4Fe-4S, H-Cluster | Flavin, 4Fe-4S, 4Fe-4S, 4Fe-4S | 2Fe-2S | 2Fe-2S |

## NADH-Dependent, Ferredoxin-Independent Formate Dehydrogenases

| Source Organism(s) | Reported Activity | α | β | γ | δ |
|---|---|---|---|---|---|
| R. capsulatus | NADH-dependent Formate Dehydrogenase | 2Fe-2S, 4Fe-4S, 4Fe-4S, 4Fe-4S, 4Fe-4S, Mo | Flavin, 4Fe-4S | 2Fe-2S | ☐ |
| C. oxalaticus, C. eutropha, M. trichosporum | NADH-dependent Formate Dehydrogenase | 2Fe-2S, 4Fe-4S, 4Fe-4S, 4Fe-4S, 4Fe-4S, Mo | Flavin, 4Fe-4S | 2Fe-2S | |
| M. extorquens | NADH-dependent Formate Dehydrogenase | 2Fe-2S, 4Fe-4S, 4Fe-4S, 4Fe-4S, 4Fe-4S, W | Flavin, 4Fe-4S | 2Fe-2S | |

## NADH-Dependent, Ferrodoxin-Independent [FeFe]-Hydrogenases

| Source Organism(s) | Reported Activity | α | β | γ | δ |
|---|---|---|---|---|---|
| S. acidotrophicus | NADH-Dependent Hydrogenase | 2Fe-2S, 4Fe-4S, 4Fe-4S, H-Cluster | Flavin, 4Fe-4S | 2Fe-2S | |
| S. wolfei | NADH-Dependent Hydrogenase | 2Fe-2S, 4Fe-4S, 4Fe-4S, H-Cluster | Flavin, 4Fe-4S | 2Fe-2S | |

## NADH-Qunione Oxidoreductase (Nqo1-Nqo3)

| Source Organism(s) | Reported Activity | α | β | γ | δ |
|---|---|---|---|---|---|
| T. thermophilus | NADH-quinone Oxidorductase | 2Fe-2S, 4Fe-4S, 4Fe-4S | Flavin, 4Fe-4S | 2Fe-2S | |

**Comparison of the Alpha and Gamma Subunits of the Electron-Bifurcating Enzymes to**

**Those from the NADH-Dependent, Ferredoxin-Independent Enzymes**

As shown in Figure 19, the alpha subunits of the electron-bifurcating [FeFe]-hydrogenases and the NADH-dependent, ferredoxin-independent *S. wolfei* and *S. aciditrophicus* [FeFe]-hydrogenases share similar predicted cofactor binding sites: a single [2Fe-2S]-cluster, three [4Fe-4S]-clusters and the catalytic [H-cluster]. The *T. maritima* alpha subunit differs from others in containing an additional [2Fe-2S] cluster. The alpha subunits of the NADH-dependent, ferredoxin-independent formate dehydrogenase are similar to the alpha subunit of the electron-bifurcating formate dehydrogenase from *C. acidiurici* and are predicted to contain: a single [2Fe-2S] cluster, four [4Fe-4S] clusters and a metallopterin cofactor binding site. The alpha subunits are apparently similar between the electron-bifurcating NADH-dependent, ferredoxin-independent formate dehydrogenases and [FeFe]-hydrogenases and the NADH-dependent, ferredoxin-independent formate dehydrogenase and [FeFe]-hydrogenases.

 All of the compared enzymes regardless of ferredoxin dependence possess a similar gamma subunit that has a single [2Fe-2S] cluster (Figure 19). However, in the case of the *S. aciditrophicus* and the *M. extorquens* enzymes, it is proposed that the gamma subunit is fused to the N-terminal portion of the beta subunit. Based on the common presence of a gamma subunit between the ferredoxin-dependent and ferredoxin-independent enzymes, it is unlikely that the gamma subunit determines ferredoxin dependence.

In contrast to the alpha and gamma subunits, there are differences in the beta subunits between the electron-bifurcating NADH-dependent, ferredoxin-dependent [FeFe]-hydrogenases and formate dehydrogenases and the NADH-dependent, ferredoxin-independent formate dehydrogenases, [FeFe]-hydrogenase and the NADH dehydrogenase:ubiquinone oxidoreductase

132

(Figure 19). All beta subunits from the electron-bifurcating enzymes possess two [4Fe-4S] centers at the C-terminal portion of the protein that are not predicted to occur in any of the NADH-dependent, ferredoxin-independent enzymes. In addition, all of the beta subunits of the electron-bifurcating NADH-dependent, ferredoxin-dependent enzymes with the exception of the *D. fructosovorans* [FeFe]-hydrogenase, contain a N-terminal [2Fe-2S] center. Assuming a fused gamma peptide to the beta subunit for the *M. extorquens* formate dehydrogenase and the *S. aciditrophicus* [FeFe]-hydrogenase, then none of the beta subunits from the NADH-dependent, ferredoxin-independent enzymes possess a N-terminal [2Fe-2S] center. It appears that the N-terminal [2Fe-2S] center and two C-terminal [4Fe-4S] centers are present only in the beta subunits of the electron-bifurcating NADH-dependent, ferredoxin-dependent enzymes and not in the beta subunits of the NADH-dependent, ferredoxin-independent enzymes. Given the readily observable differences in the beta subunits that appeared to correlate with ferredoxin dependence we decided further investigate the beta subunits. This was done by building an alignment of the beta subunits to further investigate the primary amino acid sequences to identify if there were other differences that might differentiate the two groups.

**Amino Acid Sequence Differences in the Beta Subunits of Electron-Bifurcating and NADH-Dependent, Ferredoxin-Independent Enzymes**

There appears to be strongly conserved regions of amino acids near the predicted NADH binding site, FMN binding site (see Figure 20), and a proposed cofactor binding site (soluble-ligand-binding-beta-grasp or SLBB) that are shared amongst the beta subunits of the electron-bifurcating, NADH-dependent, ferredoxin-dependent enzymes but absent in the NADH-dependent, ferredoxin-independent enzymes. These observations were made by generating an

alignment of the beta subunits from seventeen different enzymes. Included in the alignment were beta subunits from the eight-known electron-bifurcating formate dehydrogenases and [FeFe]-hydrogenases, the five NADH-dependent, ferredoxin-independent formate dehydrogenase, two NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases, the Nqo1 subunit from *T. thermophilus,* and the equivalent NuoF subunit from *E. coli* strain K-12, substrain MG1655. The full alignment can be found in the supplemental figures section of this chapter.

A conserved sequence of DEGDPxxFM was observed in all the beta subunits of the electron-bifurcating, NADH-dependent, ferredoxin-independent enzymes (Figure 20 and 21) that corresponds with a region predicted to be involved in the binding of NADH in the *T. thermophilus* Nqo1 subunit (Walker, 1992; Sazanov and Hinchliffe, 2006). None of beta subunits of the NADH-dependent, ferredoxin-independent enzymes analyzed share a strong consensus sequence over the region. The beta subunits from electron-bifurcating, NADH-dependent, ferredoxin-dependent enzymes contain a methionine residue at the equvialent residue of $M^{289}$ in the *T. maritima* HydB sequence (Figure 20). The beta subunits of the NADH-dependent, ferredoxin-independent enzymes have lysine, serine or alanine at this position. The beta subunits from electron-bifurcating enzymes except the sequence from *D. fructosovorans* have alanine at the equivalent position to $A^{287}$ of the *T. maritima* HydB sequence while the NADH-dependent, ferredoxin-independent enzymes instead contiain a threonine or serine residue. The $M^{289}$ as found in the *T. maritima* HydB sequence and the other ferredoxin-dependent enzymes appears to be mutually exclusive to the presence of a serine or threonine residue (located two positions closer towards the N-terminus) which was found in all the ferredoxin-independent enzymes. In *T. thermophilus* Nqo1, the amino groups of $Ser^{96}$ and $Glu^{97}$ which are in close proximity to the locations of the mutually exclusive methionine or

134

serine/threonine are proposed to be involved in hydrogen bonding of NADH (Sazanov and

Hinchliffe, 2006; Sazanov, 2007). However, the proposed common threonine or serine in the

NADH-dependent, ferredoxin-independent enzymes, $Ser^{100}$ in the Nqo1 sequence, was not one of

the residues suggested to be involved with hydrogen bonding. In summary, in the compared

NADH-binding sites, those from the electron-bifurcating enzymes possess a methionine whereas

the methionine is absent, and a lysine, serine or alanine is present in the NADH-dependent,

ferredoxin-independent enzymes. In addition, in another position two residues closer to the N-

terminus, a threonine and serine are present in the NADH-dependent, ferredoxin-independent

beta subunits and absent in all electron-bifurcating enzyme beta subunits. It is unclear what roles

these residues might play, although the proximity to the NADH binding site would suggest some

involvement with the binding and oxidation of NADH.

**Figure 20. Conserved Regions in the NADH and FMN Binding Sites of the Beta Subunits of
Electron-Bifurcating [FeFe]-Hydrogenases and Formate Dehydrogenases.** Conserved

regions among the beta subunits of the eight electron-bifurcating enzymes are highlighted by

dashed box. The solid black box highlights residues that are suggested to be involved in NADH

and FMN binding (Walker, 1992; Sazanov and Hinchliffe, 2006). The yellow box highlights

amino acid residues where all sequences of the beta subunits of the electron-bifurcating enzymes

share a similar pattern and all the NADH-dependent, ferredoxin-independent beta subunits share

a different pattern. The electron-bifurcating beta subunits all share a methionine while all the

NADH-dependent, ferredoxin-independent beta subunits all lack the methionine but have a

lysine, serine or alanine. In the case of the FMN binding site, all electron-bifurcating beta

subunits possess a phenylamine while all the NADH-dependent, ferredoxin-independent beta

subunits possess a tyrosine. The alignment of full-length the beta subunits was performed in MEGA7 using the ClustalW algorithm. The alignment was inspected for regions where at least three consecutive amino acid residues were conserved among the sequences of the eight beta subunits of the electron-bifurcating enzymes. NADH-dependent, ferredoxin-independent beta subunit sequences were then inspected to determine if the conserved regions were unique to the electron-bifurcating NADH-dependent, ferredoxin-independent beta subunits.

Block 1 — positions 273–333 (NADH binding region)

```
         273   276 278 280 282 284 286 288 290 292 294 296 298 300 302 304 306 308 310 312 314 316 318 320 322 324 326 328 330   333
T. maritima HydB          G L K W E F T R K A Q G D - - I K F V V C N G D E G D P G A F M N R T L L E R D P H L V L E G M I I A G Y A V G A Q K G Y
C. tencongenesis HydB     G V K W E F A Y K Q K E T - - - P K Y V V V C N A D E G D P G A F M D R S I L E G D P H S V L E A M A I A G Y A I G A N H G Y
M. thermoacetica HydB     G L K W E F A Y R S P G P - - V K Y F V C N A D E G D P G A F M D R S I L E G D P H A V L E G M A I G A Y A I G A S Q G Y
A. woodii HydB            G L K W Q F A H D A V S E D G I K Y V A C N A D E G D P G A F M D R S V L E G D P H A V I E A M A I A G Y A V G A S K G Y
R. albus HydB             G R K W M F T K D A P G D - - V K Y V A C N A D E G D P G A F M D R S I L E G D P H A V I E A M T I A S Y A V G A H Q G Y
C. acidurici HylB         G L K W E F T A K A T G D - - Q K Y V L C N A D E G D P G A F M D R S I L E G D P H S V I E A M A L A G Y A I G S D Q G Y
C. autoethanogenum HytB   G R K W K T A A D I D T S - - P I Y V V C N G D E G D P G A F M D R S I M E G D P N S V I E G M T L C A Y A V G G T N G F
D. fructosovorans HydB    G I K W G I A L G N K A D - - Q K Y M V C N A D E G D P - E F M D R A V L E G D P H S V V E A M A I G G Y A I G A T R G T
S. wolfei HydB            G M K W S F V P K G E M Q - - K Y V I C N A D E G E P G T Y K D R L V L M E N D P F M L I E G M L L C G Y A I G A T L G Y
S. aciditrophicus HydB    G V K W S F V P K G E M Q - - - K Y V I C N A D E G E P G T Y K D R L V L M E E N P Q Q L I E G M L L C G Y A I G A T L G Y
C. necator FdhB           G I K W K T V L G A Q S A - - V K Y I V C N A D E G D S G T F S D R M V M E D D P F M L I E G M T I A A L A V G A E Q G Y
C. oxalaticus FdhB        G I K W K T V L G A Q S A - - V K Y I V C N A D E G D S G T F S D R M V M E D D P F M L I E G M T I A G L A V G A E Q G Y
M. extorquens FdhB        G R K W R S V R G E P G P - - R L M A V N G D E G E P G T F K D Q L Y L N T D P H R F L E G M L I G A H V V E A A D V Y
M. trichosporium FdhB     G I K W K T V A D A P A D - - R R Y V V T N A D E G D S G T F A D R M V M E G D P F V L I E G M T I C G Y A I G A S K G F
R. capsulatus FdhB        G L K W R T V A A A Q A D - - Q K Y I V C N V D E G D S G S F A D R M L I E G D P F C L V E G M A I A G H A V G A T R G Y
E. coli NuoF              G L K W S L M P K D E S M N - I R Y L L C N A D E M E P G T Y K D R L L M E Q L P H L L V E G M L I S A F A L K A Y R G Y
T. thermophilus Nqo1      G L K W S F M P K D D G K - - Q H Y L L C N A D E S E P G S F K D R Y I L E D V P H L L I E G M I L A G Y A I R A T V G Y
```

NADH binding region

Block 2 — positions 352–412 (FMN binding region)

```
         352   354 356 358 360 362 364 366 368 370 372 374 376 378 380 382 384 386 388 390 392 394 396 398 400 402 404 406 408 410   412
T. maritima HydB          E D A R K L G L L G E N I L G T G F S F D L E V K E G A G A F V C G E E T A L L A S I E G K R G M P R P K P P F P A Q S G
C. tencongenesis HydB     Q Q A R E Y G L L G K D I F G T G F D F D I E I R L G A G A F V C G E E T A L L N S I M G K R G E P R P R P P F P A V K G
M. thermoacetica HydB     S Q A R E Q G L L G K N L F N S G F D F D I D I R L G A G A F V C G E E T A L L A S I E G R R G E P R P R P P F P A V S G
A. woodii HydB            D Q A K E Y G L L G E N I F E T D F S F D L E I R L G A G A F V C G E E T A L M N S I E G K R G E P R P R P P F P A N K G
R. albus HydB             D E A K A K G L L G K D I F G T G F N F D M E I R L G A G A F V C G E E T A L L T S I E G N R G E P R P R P P F P A V K G
C. acidurici HylB         N K A K D E N L L G N N I L G T D F S F D I Q I V R G G A F V C G E E S T A L I A S I E G Q R G M P R N K P P F P A N K G
C. autoethanogenum HytB   D D A R E Y G L L G E N I F G S G F D F D I E L K Y G A G A F V C G E E T A L I R S M E G K R G E P V T K P P P S E G
D. fructosovorans HydB    N Q A K E K G L V K D - - - - - F D I E V R S G A G A Y V C G E E T A L L E S L I N S M E G K R G Y P R V R P P F P A A A G
S. wolfei HydB            D Q A R A K G F L G K K I F G S N F D F D I F V K E G G G A Y V C G E E T A L L E S L L N S L E G K R G V V R A K P P L P A L Q G
S. aciditrophicus HydB    G I A N A A G W L G D D I R G S G K R F H L E V R K G A G A Y V C G E E S S L I E S L E G K R G L P R H K P P F P F Q V G
C. necator FdhB           A I A H A A G W L G D D L R G S G K R F R L E V R K G A G A Y I C G E E T A L L E S L L E S L E G K R G I V R A K P P L P A H V G
C. oxalaticus FdhB        A K L P E G G - - - - - - T R I H L R R G A G A Y V C G E E S L L N S L L E S L E G K R G T V R A K P P L P A L K G
M. extorquens FdhB        E A A R K A G W L G P N V R G S G F A F D I E L R I G A G A Y I C G E E T A L I N S L E G R R A N P R S K P P F P A T S G
M. trichosporium FdhB     A M A K P F - - - - - L A E A G - F E M E V R V G A G R Y I C G E E T A L I N S L E G R R A N P R S K P P F P A T S G
R. capsulatus FdhB        A E A T E A G L L G K N I M G T G F D F E L F V H T G A G R Y L G K N L F G T D F S F D L H V H R G A G A Y I C G E E T A L M N S L E G L R A N P R L K P P F P A Q S G
E. coli NuoF
T. thermophilus Nqo1
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FMN binding region

The FMN binding site was a highly conserved region amongst all of the compared beta subunits. The electron-bifurcating NADH-dependent, ferredoxin-independent beta subunits all shared a conserved GxGAFVCGExTAL sequence. The phenylalanine residue present in the electron-bifurcating beta subunits (position 367 of *T. maritima* HydB; position 180 of *T. thermophilus* Nqo1) was replaced with tyrosine in the beta subunits of the NADH-dependent, ferredoxin-independent enzymes (Figure 20). This residue in *T. thermophilus* Nqo1, Tyr[180], was suggested to play an important role in NADH binding (Sazanov and Hinchliffe, 2006; Burroughs et al., 2007). Simultaneous mutation of the Tyr[180] residue and the Cys[182] residue was associated with a deficiency in mitochondrial complex I by presumably altering FMN or NADH binding (Benit et al., 2001; Sazanov, 2007). In summary, a tyrosine residue previously suggested to play a crucial role in NADH binding is present in the beta subunits of the NADH-dependent, ferredoxin-independent enzymes but is replaced by a phenylalanine residue in the beta subunits all of the electron-bifurcating enzymes.

Several other regions of amino acid residues near a SLBB domain (soluble-ligand-binding-Beta-grasp domain), (*T. thermophilus* Nqo1 positions 254-298, see Figure 21) were conserved in the electron-bifurcating beta subunits and absent in the NADH-dependent, ferredoxin-independent beta subunits. The, SLBB domain is a predicted ligand-binding domain present in Nqo1 (Burroughs et al., 2007). This domain has also been described as ubiquitin like (Sazanov and Hinchliffe, 2006), but it is not known to bind any cofactor in Nqo1. The conserved regions of amino acid residues near the SLBB domain shared among the electron-bifurcating beta subunits are GGPSG (*T. maritima* HydB positions 427-431), GSGG (*T. maritima* HydB positions 456-459) and CMV (*T. maritima* HydB positions 468-470) (Figure 21). There is an

additional group of conserved amino acid residues shared among the electron-bifurcating beta subunits, PNP (*T. maritima* HydB positions 538-540), that is located closer to the C-terminal [4Fe-4S] cluster than the SLBB domain. It is of interest that the electron-bifurcating subunits have conserved residues near the predicted SLBB domain whereas the NADH-dependent, ferredoxin-independent beta subunits do not. This could imply a property exclusively conserved in the electron-bifurcating beta subunits, such as the ability to bind a cofactor responsible for the electron-bifurcation reaction. An additional cofactor, such as FMN, has been proposed to be present in the beta subunit of the *A. woodii* electron-bifurcating [FeFe]-hydrogenase and serve as the site of electron-bifurcation (Buckel and Thauer, 2018). The number of conserved amino acid residues near the proposed SLBB domain in electron-bifurcating beta subunits suggests the domain binds the cofactor acting as the site of electron-bifurcation. As the NADH-dependent, ferredoxin-independent beta subunits do not share these conserved amino acid residues near the proposed SLBB domain, it would be predicted that they would not bind this additional cofactor, consistent with structural studies of Nqo1 that did not detect a cofactor at this location (Sazanov and Hinchliffe, 2006; Sazanov, 2007).

**Summary of Differences in the Beta Subunits of the Electron-Bifurcating Enzymes Compared to the NADH-Dependent, Ferredoxin-Independent Enzymes**

We propose that the ability of the NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases from *S. wolfei* and *S. aciditrophicus* to operate without electron-bifurcation is due to the fact that their beta subunits are more similar to other NADH-dependent, ferredoxin-independent than to the beta subunits of the electron-bifurcating [FeFe]-hydrogenases and formate dehydrogenases. We found several differences that separate the group of beta subunits

139

from the electron-bifurcating enzymes from the group of beta subunits from the NADH-dependent, ferredoxin-independent subunits. These differences (see Figure 21) include: (1) a N-terminal [2Fe-2S] cluster, (2) a methionine residue exclusive to a threonine or serine(located two residues closer to N-terminus), (3) a phenylalanine as opposed to a tyrosine, (4) conserved glycine rich regions near the SLBB domain, and (5) two C-terminal [4Fe-4S]-clusters. These differences are not a complete list of the differences between these two proposed groups of enzymes as we did not investigate regions that did not have at least three adjacent conserved residues and single site or two consecutive conserved residues could play important roles. In addition, we did not incorporate structural modeling in comparing these two groups that would likely indicate important differences between these two groups. However, we do believe that some of these differences could be responsible for the differing ability to perform electron-bifurcation between the two groups. Given the existing structural information for the Nqo1 structure, which likely encompasses the beta subunits of the other NADH-dependent, ferredoxin-independent enzymes, what is most urgently required is detailed structural information from the electron-bifurcating [FeFe]-hydrogenaes and formate dehydrogenases (Buckel and Thauer, 2018).

**Figure 21. Summary of Differences Between the Electron-Bifurcating [FeFe]-Hydrogenases and Formate Dehydrogenases and NADH-Dependent, Ferredoxin-Independent Enzymes**

| Category | Content |
|---|---|
| *T. martima* HydB Bifurcating Subunit AA Residues 1-626 | 1    100    200    300    400    500    600  626 |
| *T. thermophilus* Nqo1 Non-Bifurcating Subunit AA Residues 13-438 | TTNqo1 Residue 13 = TMHydB Residue 149   TTNqo1 Residue 438 = TMHydB Residue 569<br>13    100    200    300    400    438 |
| Location of Cofactor Binding Sites | [2Fe-2S] TM28-TM125<br><br>NADH Binding Residues TT64-TT100 TM196-232<br><br>FMN Binding Residues TT175-220 TM307-TM352<br><br>SLBB Domain TT254-TT298 TM386-TM432<br><br>[4Fe-4S] TT354-TT400 TM486-TM531<br><br>2x [4Fe-4S] TM569-TM676 |
| Location of Conserved Residues Unique to Bifurcating Subunits | TT94-TT102 TM226-TM234 Bifurcating Subunits: DEGDPxxFM Non-Bifurcating Subunits: DExxxGSxx or DExxxGTxx<br><br>TT176-TT188 TM308-TM320 Bifurcating Subunits: GxGAFVCGExTAL Non-Bifurcating Subunits: GxGxYxxGEExxC<br><br>TT293-TT296 TM427-TM431 Bifurcating Subunits: GGPSG Non-Bifurcating Subunits: GGxxx<br><br>TT324-TT327 TM456-TM459 Bifurcating Subunits: GSGG Non-Bifurcating Subunits: Gxxx<br><br>TT336-TT338 TM468-TM470 Bifurcating Subunits: CMV Non-Bifurcating Subunits: xxx<br><br>TT407-TT409 TM538-TM540 Bifurcating Subunits: PNP Non-Bifurcating Subunits: xxP |
| Summary of Differences Between Bifurcating Subunits and Non-Bifurcating Subunits | 1. N-terminal [2Fe-2S] Cluster<br>Bifurcating: Present (7/8)<br>Non-Bifurcating: Absent (9/9)<br><br>2. NADH Binding Site<br>Bifurcating: Methionine Residue (8/8)<br>Non-Bifurcating: Threonine or Serine Residue (9/9)<br><br>3. FMN Binding Site<br>Bifurcating: Pheylanine Residue (8/8)<br>Non-Bifurcating: Tyrosine Residue (9/9)<br><br>4. SLBB Domain<br>Bifurcating: GGPSG Residues (8/8)<br>Non-Bifurcating: GGxxx (9/9)<br><br>5. Two C-terminal [4Fe-4S] Clusters<br>Bifurcating: Present (8/8)<br>Non-Bifurcating: Absent (9/9) |

**Structural Similarities Between the Alpha Subunit of the NADH-Dependent, Ferredoxin-Independent Enzymes**

A region of roughly 200 amino acid residues at the N-terminal that is responsible for the binding of two [4Fe-4S] centers and a [2Fe-2S] center (Vignais et al., 2001; Sazanov and Hinchliffe, 2006; Hille et al., 2014) is shared between the NADH dehydrogenase subunit Nqo3, the alpha subunits of the NADH-dependent formate dehydrogenases, and monomeric, ferredoxin-dependent [FeFe]-hydrogenases. In addition, there is homology between the FMN, NADH, and [4Fe-4S] cluster binding sites within the beta subunit of the formate dehydrogenases and Nqo1. There is also homology between the [2Fe-2S] cluster containing gamma subunit of the formate dehydrogenases and Nqo3. In short Nqo1, Nqo2, and Nqo3 are directly comparable to the beta, gamma, and alpha subunits of the formate dehydrogenase (Hille et al., 2014). The *S. wolfei* and *S. acidotrophicus* [FeFe]-hydrogenases share this same similarity with the NADH-dependent, ferredoxin-independent formate dehydrogenases and Nqo1, Nqo2, and Nqo3 over a 200 amino acid region of the alpha subunit, over the entire beta subunit, and the entire gamma subunit. This similarity can be used to predict how electron flow may occur within the *S. wolfei* and *S. aciditrophicus* NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases.

Strong similarities exist between the NADH-dependent, ferredoxin-independent formate dehydrogenases and the NADH:ubiquinone oxidoreductase complex (Sazanov and Hinchliffe, 2006; Hille et al., 2014). These similarities allowed for homology modeling of the proposed structural model of the *Cupriavidus necator* (formerly *Ralstonia eutropha*) NADH-dependent, ferredoxin-independent formate dehydrogenase (Hille et al., 2014). The model was built using models of the *T. thermophilus* Nqo1, Nqo2 and Nqo3 subunits and *E. coli* FdhF and included a

proposed electron flow path from the NADH binding site in the beta subunit to the catalytic site in the alpha subunit. A V-shaped fork (see Figure 22) differentiates the proposed electron flow path in Nqo3 and the alpha subunit of the *C. necator* formate dehydrogenase (Hille et al., 2014). In Nqo3, electrons are proposed to first move from the N4 [4Fe-4S] cluster to the N5 [4Fe-4S] cluster and then to the N6A [4Fe-4S] cluster in the Nqo9 subunit cluster in a path away from the vestigial molybpoterin cofactor binding site near the N7 [4Fe-4S] cluster (Sazanov and Hinchliffe, 2006). However, in the *C. necator* formate dehydrogenase, the electron flow path differs after the N4 equivalent [4Fe-4S] cluster. Instead of moving to the N5 cluster as occurs in Nqo3, electrons are proposed to move to a [4Fe-4S] cluster present in the *C. necator* formate dehydrogenase alpha subunit that has been lost in Nqo3. This [4Fe-4S] cluster allows further electron transfer to the N7 [4Fe-4S] cluster and the active site with the molybdopterin cofactor. There is shared homology and a similar predicted cofactor binding sites between this formate dehydrogenase and the NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases. Therefore, it can be proposed that similar electron flow path occurs in both types of enzymes as diagrammed in Figure 22. Most of the electron flow path from the beta subunit to alpha subunit would be identical between these enzymes and would not a involve electron-bifurcation mechanism. The electron transfer from the NADH binding site in the beta subunit to the N4 equivalent [4Fe-4S] center in the alpha subunit would be the same, but different after this point. This is because the peptide sequences for the regions of the catalytic [H]-cluster and the closest [4Fe-4S] cluster in the *S. acidotrophicus* and *S. wolfei* [FeFe]-hydrogenases have little similarity to the molybpeterin cofactor binding site and the two closest [4Fe-4S] clusters in the *C. necator* formate dehydrogenase. Presumably, this additional [4Fe-4S] center allows electron flow from the N4 equivalent [4Fe-4S] center to the [H]-cluster. The N5 equivalent [4Fe-4S] center would

144

not be used for electron flow from NADH to the [H]-cluster in the NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases similar to the electron flow path of the *C. necator* formate dehydrogenase.

The structural similarities in the alpha, beta and gamma subunits of the *S. wolfei* and *S. aciditrophicus* NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases to the alpha, beta and gamma subunits of the NADH-dependent, ferredoxin-independent formate dehydrogenases and Nqo1, Nqo2, and Nqo3 argue that electron flow paths are similar. This electron flow path allows passage of electrons from the NADH binding site to the [H]-cluster catalytic site without electron input from another path as expected for electron bifurcation. Also, it argues against the [H]-cluster as the site of electron bifurcation as the alpha subunits of electron-bifurcating, ferredoxin-dependent [FeFe]-hydrogenases and NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases are similar in cofactor arrangement and conserved amino acid regions (Figure 19). Presumably, the proposed electron flow paths for the *S. wolfei* and *S. aciditrophicus* NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases does not occur in the electron-bifurcating [FeFe]-hydrogenases and formate dehydrogenases as these enzymes cannot reduce $NAD^+$ without also reducing ferredoxin.

**Figure 22. Proposed Electron Flow in NADH-Dependent, Ferredoxin-Independent [FeFe]-Hydrogenases.** Electron flow depicted for *T. thermophilus* Nqo1-Nqo and, *C. eutropha* formate dehydrogenase as illustrated by Hille et al. 2014. Blue arrows indicate electron flow.
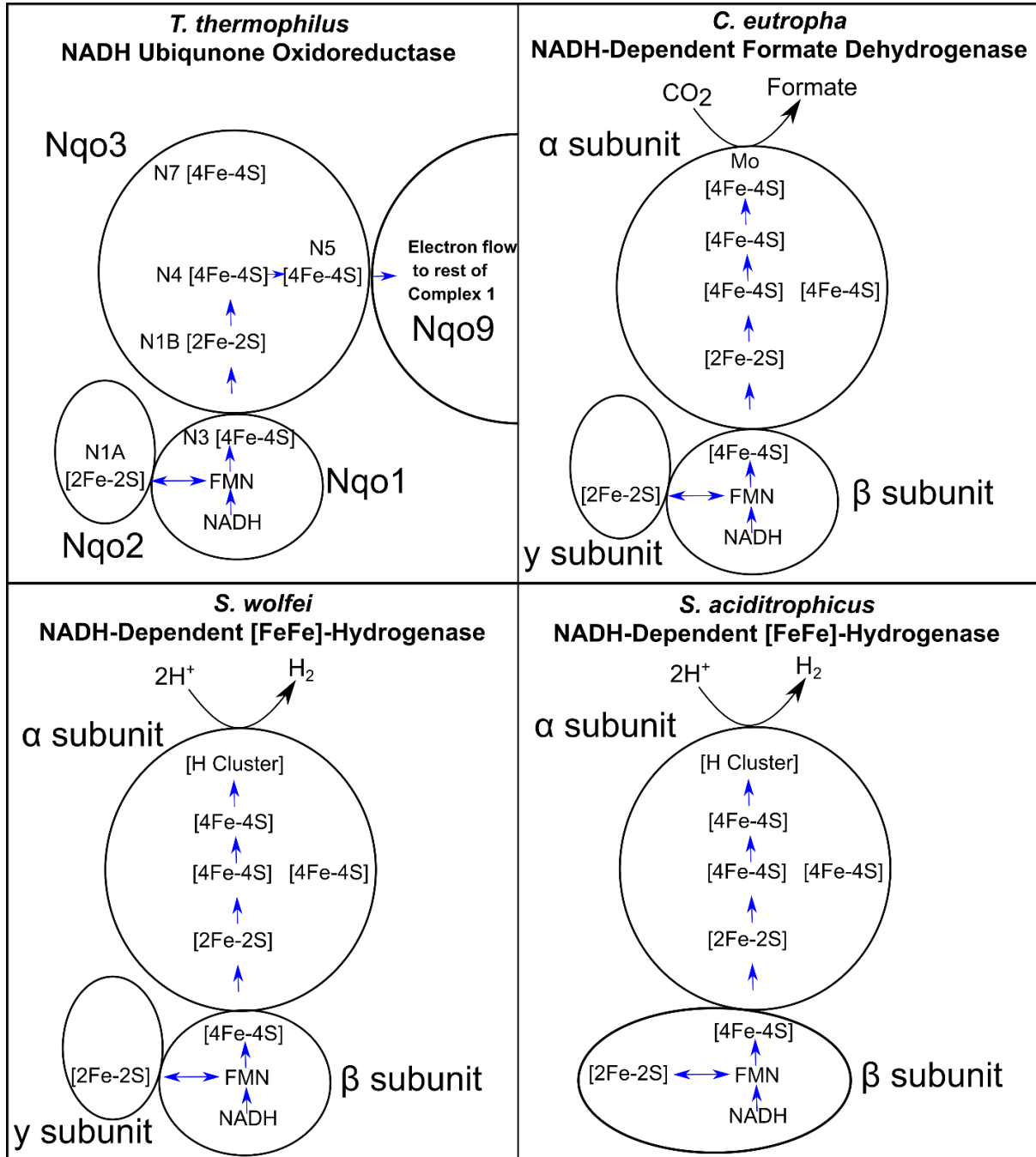
**Additional Example of a NADH-Dependent, Ferredoxin-Independent, [FeFe]-Hydrogenase**

Previously, a [FeFe]-hydrogenase from the hydrogenosome of the anaerobic cilliate *Nyctotherus ovalis* was proposed to be a NADH-dependent, ferredoxin-independent [FeFe]-hydrogenase (Boxma et al., 2007). This is unusual in that hydrogenosomes in many other anaerobic ciliates produce hydrogen from reduced ferredoxin and not from NADH (de Graaf et al., 2011). This is because anaerobic ciliates other than *N. ovalis* have the enzyme, pyruvate:ferredoxin oxidoreductase, which makes reduced ferredoxin, which is then used by a monomeric [FeFe] hydrogenase to make hydrogen (Peters et al. 1998). However, genes for pyruvate:ferredoxin oxidoreductase are absent in the *N. ovalis* genome (de Graaf et al., 2011). Instead, *N. ovalis* has the genes for pyruvate dehydrogenase, which produces NADH. The *N. ovalis* pyruvate-metabolizing enzyme has not been purified or enzymatically characterized, but is presumed to be NADH-dependent, ferredoxin-independent based on genomic analyses (Boxma et al., 2007). The *N. ovalis* [FeFe]-hydrogenase has the same conserved domains as both the *S. wolfei* and *S. aciditrophicus* NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases but consists of a fused peptide where the alpha, beta, and gamma subunits as found in the *S. wolfei* [FeFe]-hydrogenase are fused into a single peptide. This is in contrast to the *S. aciditrophicus* [FeFe]-hydrogenase where only the gamma and beta subunits are fused together. In addition, the *N. ovailis* hydrogenase (Genbank Protein Accession ID: AAU14235) possesses the features that we propose can be used to differentiate the NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases from the electron-bifurcating [FeFe]-hydrogenases (see Figure 21). Furthermore, as the production of hydrogen from NADH requires low hydrogen partial pressures to operate, the hydrogenosome would need to be in close proximity to a hydrogen-consuming organism to maintain low hydrogen partial pressures to allow continual NADH re-oxidation. A

147

hydrogenotrophic methanogen, presumably *Methanobrevibacter*, was found as an endosymbiont of *N. ovalis* and in close association to the hydrogenosome organelle (Gijzen et al., 1991). This fullfills the requirement that a hydrogen-consuming partner organism, in this case a methanogen, be present for hydrogen production from NADH. This [FeFe]-hydrogenase, despite being an apparent fusion of the equivalent *S. wolfei* [FeFe]-hydrogenase alpha, beta and gamma subunits into a single peptide, still shares key features that we propose distinguish NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases from the electron-bifurcating, ferredoxin-dependent [FeFe]-hydrogenases.

**Conclusion**

The results obtained from studying the recombinantly produced *S. aciditrophicus* HydAB indicate that it functions as a ferredoxin-independent, NADH-dependent [FeFe]-hydrogenase. In combination with the previous example of a ferredoxin-independent, NADH-dependent [FeFe]-hydrogenase, Hyd1ABC from *S. wolfei*, it appears that hydrogen production from NADH is a key feature of syntrophic fatty acid-oxidizing bacteria. Any organism producing hydrogen from NADH in a ferredoxin-independent manner could be viewed as a syntrophic metabolizer, as the process matches with the original definition of syntrophy in that a hydrogen or formate consuming microorganism is needed. Hydrogen production from NADH cannot be used to produce any useful amount of hydrogen under fermentative conditions in pure culture and only serves a function when used in combination with a hydrogen-consuming organism, such as a hydrogenotrophic methanogen or sulfate reducer. It remains to be fully demonstrated if the system for oxidizing reduced flavin cofactors (EtfAB) during syntrophic fatty acid metabolism also requires a low hydrogen partial pressure (<60 Pascals). While the concept of syntrophic

fatty, aromatic and alicyclic acid metabolism involves the use NADH-dependent, ferredoxin independent hydrogenses and formate dehydrogenase is based on only two examples, it does provide a biochemical explanation for syntrophy. The conclusion that a microorganism uses electron-bifurcating or non-electron bifurcating hydrogenasese or formate dehydrogenases should be made only after careful consideration of the organism's physiology and after appropriate enzymatic analysis. It would be prudent when attempting to classify multimeric [FeFe]-hydrogenases to make the comparisons described here to determine if the beta subunit more closely resembles that of a NADH-dependent, ferredoxin-indendent enzyme or electron bifurcating [FeFe]- hydrogenases.

NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases present in *S. wolfei* and *S. aciditrophicus* can be used to re-oxidize NADH without energy input needed to make reduced ferredoxin. This would allow more energy to be used for growth rather than NADH re-oxidation. Adaptations such as these that maximize energy conservation by utilizing high redox potential electrons to produce hydrogen or formate may be responsible for the defining requirement of syntrophic organisms, partners that maintain low hydrogen partial pressures.


## Methods

**Plasmid Design**

A DNA sequence encoding for the genes for the *S. aciditrophicus* dimeric [FeFe]-hydrogenase (SYN_01369 and SYN_01370) (HydAB) was ordered from Integrated DNA Technologies (Coralville, Iowa, USA) and delivered in a suitable plasmid for recombinant protein expression, including a T7 promoter region for protein expression. This plasmid was designated as pIDTKan-SbHyd. An additional amino acid sequence including six histidine

149

residues (MGSSHHHHHHSQDPNSSSARL) was added to the N-terminal region of SYN_01370 to allow for nickel affinity purification of the protein product. A homology search of the *Syntrophus acidotrophicus* genome using the *Clostridium pasteurianum* ferredoxin (GenBank Nucleotide ID: M11214) identified SYN_03059 as the most similar match. A DNA sequence encoding for SYN_03059 was also ordered from Integrated DNA Technologies for recombinant protein expression and the plasmid is referred to as pIDTAmp-SbFd.

**Table 10. List of Plasmids Used for the Expression of the *S. aciditrophicus* HydAB and Ferredoxin.**

| Plasmid | Function | Source |
|---------|----------|--------|
| pCDFDuet-1 SwHydEFG | Production of *S. wolfei* HydEFG | See (Losey et al., 2017) |
| pIDTKan-SbHyd | Production *S. aciditrophicus* HydAB | This work |
| pIDTAmp-SbFd | Production of *S. aciditrophicus* ferredoxin | This work |

[a] Underlined regions indicate restriction enzyme cut sites.

**Expression of HydAB in *E. coli***

     *E. coli* BL21(DE3) cells were co-transformed with pIDTKan-SBHydAB and pCDFDuet-1 SwHydEFG and used for expression (See Table 10). The latter plasmid contains maturation genes needed for the expression of a functional HydAB protein. The cells were grown at pH 7.5 in LB medium containing 50 mM potassium phosphate, 10 $g \cdot l^{-1}$ glucose, and the appropriate selective antibiotics. Expression conditions were similar to those used previously for expression

of the trimeric *S. wolfei* hydrogenase (see Chapter 2) although lower iron and cysteine concentrations were used. Briefly, cultures were incubated aerobically to an O.D. of 0.4-0.6 followed by induction by the addition of 0.5 mM IPTG. After induction, 10 mM sodium fumarate, 0.5 mM cysteine, and 0.5 mM ferric ammonium citrate were added. Cultures were then purged with nitrogen overnight to create anaerobic conditions and harvested by centrifugation (6,000 • *g* for 20 minutes at 4°C). Cell pellets were stored in liquid nitrogen until used. After induction, all additional manipulations of cell pellets and protein fractions were performed under anaerobic conditions inside a Coy anaerobic chamber operated with a 95% nitrogen and 1-5% hydrogen atmosphere.

**Purification of recombinant HydAB**

BL21(DE3) cells (3.05 g) induced for HydAB expression were resuspended in 30 mL of lysis buffer (50 mM potassium phosphate (pH 7.5), 0.5 M NaCl, 2 mM dithioerythritol 5 μM FAD, 5 μM FMN, 20 mM imidazole, 0.5 mg lysozyme, and 0.1% Triton X-100). The cells were passed through a French press cell operating at 140 MPa (Megapascals) and the resulting fluid was clarified by centrifugation at 13,000 • g for 10 minutes. The cell-free extract was then loaded onto a HisTrap Hp 5 ml column (GE Healthcare Life Sciences, Pittsburgh, PA) with an imidazole concentration of 20 mM, washed with 50 mM imidazole, and eluted with 250 mM imidazole. The fraction eluted with 250 mM was concentrated using an Amicon Ultra 0.5 ml centrifugal filter with a 100 kDa molecular weight cut-off (MWCO) filter and stored in liquid nitrogen until used for further analyses.

**Partial purification of ferredoxin from *S. acidotrophicus***

Approximately 5 grams of wet cell mass of *S. aciditrophicus* were disrupted by passage through a French pressure cell operating at 140 MPa. The lysis buffer consisted of 25 mM potassium phosphate (pH 6.5) with 2 mM DTE, 5 mg•l$^{-1}$ lysozyme, 0.125 mg•l$^{-1}$ DNase and 0.5% Triton X-100. The resulting extract was clarified by centrifugation at 12,000 • *g* for 10 minutes and loaded onto a DEAE-Sepharose anion exchange column and eluted with a gradient from 0 to 1.0 M NaCl. The last dark brown fraction to elute was collected and passed first through a 100 kDa MWCO filter to remove high molecular weight proteins and then concentrated and desalted using a 10 kDa MWCO filter.

**Production and purification of recombinantly produced *Syntrophus* ferredoxin**

E. coli BL21(DE3) cells (4.5 grams) transformed with pIDTAmp-SbFd were grown and induced using the same conditions as those used for production of *S. aciditrophicus* HydAB. The cells were lysed using a French pressure cell and the protein purified using nickel affinity chromatography as described for HydAB.

**Enzymatic assays**

Hydrogen-oxidizing assays were performed in 1.4 ml quartz cuvettes (Nova Biotech, El Cajon, CA) with gas tight rubber stoppers. The assays were performed in 1 ml volumes at 37°C using an assay buffer (50 mM Tris pH 7.5, 2 mM dithioerythritol, 5 µM FMN, and 5 µM FAD. NAD(P)$^+$ reduction was tested using a concentration of 1 mM NAD$^+$ or 1 mM NADP$^+$ with and without 10 µM clostridial ferredoxin in cuvettes that were never exposed to viologen dyes. Hydrogen-dependent clostridial ferredoxin reduction was tested using 30 µM clostridial ferredoxin. Hydrogen-dependent reduction of methyl viologen was monitored using 10 mM methyl viologen as electron acceptor. Hydrogen-oxidizing assays were initiated with the addition of hydrogen at a pressure of 1.2 X 10$^5$ Pa to a 100 % nitrogen headspace. NAD$^+$ and NADP$^+$

reduction was followed at 340 nm ($\varepsilon_{340}$ = 6.2 mM$^{-1}$ cm$^{-1}$); ferredoxin reduction was followed at

430 nm ($\varepsilon_{430}$ = 13.1 mM$^{-1}$ cm$^{-1}$); and methyl viologen reduction was followed at 600 nm ($\varepsilon_{600}$ =

10.0 mM$^{-1}$ cm$^{-1}$).

Hydrogen production assays were conducted using 6.5-ml serum bottles with a 1.0 ml

liquid assay volume with shaking (200 rpm) at 37°C. The reaction buffer contained 50 mM Tris

(pH 7.5), 2 mM DTE, 5 µM FMN, and 5 µM FAD. Hydrogen production was tested with either

1 mM NADH or a reduced ferredoxin generating system consisting of 0.1 U pyruvate ferredoxin

oxidoreductase, 10 mM pyruvate, 0.1 mM thiamine pyrophosphate, 1 mM CoA and either 0.5

µM ferredoxin partially purified from *S. aciditrophicus*, 5.0 µM of recombinantly ferredoxin

produced *S. aciditrophicus* ferredoxin, or 20 µM clostridial ferredoxin.

Hydrogen production assays to determine equilibrium hydrogen concentration at different

NADH/NAD$^+$ ratios were performed using 6.5 ml serum bottles with a 1.0 ml liquid assay

volume. Reaction buffer consisted of 10 mM Tris (pH 7.5), 2 mM dithioerythritol, 5 µM FMN,

and 5 µM FAD. The total concentration of NADH and NAD$^+$ was held constant at 6 mM with

variable NADH/NAD$^+$ ratios of 5.0, 1.0, and 0.2. Hydrogen production assay bottles were

incubated for 24 h at room temperature before measurement.

**Growth Conditions of Cultures for Hydrogen Partial Pressure Measurements**

Hydrogen concentrations were measured from triplicate cultures of either pure cultures of

*S. aciditrophicus* or cocultures of *S. aciditrophicus* with *M. hungatei* grown on the following

substrates: 20 mM crotonate, 2.5 mM sodium benzoate, or 2.5 mM cyclohex-1-ene-1-

carboxylate. The basal medium consisted of a mineral solution (10 ml • l$^{-1}$ ), a trace metal

solution (5 ml • l$^{-1}$ ), and a vitamin solution (10 ml • l$^{-1}$ ) (Tanner, 2007). The medium was

prepared anaerobically under a N$_2$/CO$_2$ (80:20) atmosphere with 3.5 g • liter$^{-1}$ sodium

153

bicarbonate as buffering agent. Each culture consisted of 20 ml medium in a 159-ml serum bottle. Cysteine sulfide (0.05%) was used a reductant and resazurin was used a redox indicator. The inoculum was a 10 to 15% (v/v) transfer of *S. aciditrophicus* grown in pure culture on 20 mM crotonate with the aforementioned basal medium (culture O.D.= 0.54). Cocultures were created by addition of 2.0 to 2.5% (volume to volume) of a *M. hungatei* culture grown on $H_2/CO_2$ (80:20), 0.05% sodium acetate (culture O.D.= 0.41) in the basal medium. Cultures were grown at 37°C but allowed to equilibrate to room temperature before hydrogen measurements were taken.

Crotonate, benzoate and cyclohex-1-ene-1-carboxylate concentrations were determined by high performance liquid chromatography (HPLC) analysis while methane measurements were made gas chromatography-flame ionization detector as previously described (Sieber et al., 2014). Hydrogen measurements were determined with a gas detector (Seiler et al., 1980) (Peak Performer RCP-910, Peak Laboratories, Mountain View, CA). Crotonate, benzoate and cyclohex-1-ene-1-carboxylate concentrations were determined every six days while hydrogen and methane concentrations were determined every 3 days.

**Analytical Techniques**

SDS-PAGE and native PAGE analysis were performed using precast 8-16% Tris-Glycine gels (Life Technologies Co., Carlsbad, CA) according to manufacturer's instruction and stained using Coomassie Brilliant Blue G-250. The Bradford protein assay (Life Technologies Co., Carlsbad, CA) was used to determine protein concentrations with bovine serum album as the standard. Protein fractions of purified HydAB and ferredoxin partially purified from *S. aciditrophicus* were sent to for peptide identification. Peptide identification was performed at the Laboratory for Molecular Biology and Cytometry Research at OUHSC (Oklahoma City, OK).

Peptides were digested by trypsin followed by high-performance, liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Peptides were identified by Mascot search of the NCBI nonredundant (nr) database.

The iron content, flavin content, and molecular mass of HydAB was determined as described previously for Hyd1AB from *S. wolfei* (Losey et al., 2017). Iron content determination was performed using the ferrozine assay (Riemer et al., 2004), flavin content was determined by HPLC analysis using a UV detector (Seedorf et al., 2004), and the molecular mass was determined by size-exclusion chromatography using a Superdex 200 10/300 GL (GE Healthcare Life Sciences) calibrated with gel filtration standards (Bio-Rad Laboratories, Hercules, CA).

Hydrogen concentrations were determined by comparison against hydrogen standards (0.01%-1.0%) using a reductive trace gas analyzer RCP-910 (Peak Laboratories, Mountain View, CA).

**Reagents and chemicals**

Biochemicals (NADH, NAD$^+$, FMN, FAD, pyruvate, thiamine pyrophosphate, methyl viologen, and coenzyme A) were purchased from Sigma-Aldrich (St. Louis, MO).

**References**

Benit, P., Chretien, D., Kadhom, N., de Lonlay-Debeney, P., Cormier-Daire, V., Cabral, A., et al. (2001). Large-scale deletion and point mutations of the nuclear NDUFV1 and NDUFS1

genes in mitochondrial complex I deficiency. *Am J Hum Genet* 68(6)**,** 1344-1352. doi: 10.1086/320603.

Biegel, E., and Müller, V. (2010). Bacterial Na+/-translocating ferredoxin:NAD+/ oxidoreductase. *Proc Natl Acad Sci Unit States Am* 107(42)**,** 18138-18142. doi: 10.1073/pnas.1010318107.

Boxma, B., Ricard, G., van Hoek, A.H., Severing, E., Moon-van der Staay, S.-Y., van der Staay, G.W., et al. (2007). The [FeFe] hydrogenase of *Nyctotherus ovalis* has a chimeric origin. *BMC Evol Biol* 7(1)**,** 230. doi: 10.1186/1471-2148-7-230.

Buckel, W., and Thauer, R.K. (2018). Flavin-Based Electron Bifurcation, A New Mechanism of Biological Energy Coupling. *Chem Rev* 118(7)**,** 3862-3886. doi: 10.1021/acs.chemrev.7b00707.

Burroughs, A.M., Balaji, S., Iyer, L.M., and Aravind, L. (2007). A novel superfamily containing the beta-grasp fold involved in binding diverse soluble ligands. *Biol Direct* 2**,** 4. doi: 10.1186/1745-6150-2-4.

Crable, B.R., Sieber, J.R., Mao, X., Alvarez-Cohen, L., Gunsalus, R., Ogorzalek Loo, R.R., et al. (2016). Membrane Complexes of *Syntrophomonas wolfei* Involved in Syntrophic Butyrate Degradation and Hydrogen Formation. *Front Microbiol* 7**,** 1795. doi: 10.3389/fmicb.2016.01795.

Darimont, B., and Sterner, R. (1994). Sequence, assembly and evolution of a primordial ferredoxin from *Thermotoga maritima*. *EMBO J* 13(8)**,** 1772-1781.

de Graaf, R.M., Ricard, G., van Alen, T.A., Duarte, I., Dutilh, B.E., Burgtorf, C., et al. (2011). The organellar genome and metabolic potential of the hydrogen-producing

mitochondrion of *Nyctotherus ovalis*. *Mol Biol Evol* 28(8)**,** 2379-2391. doi: 10.1093/molbev/msr059.

Elshahed, M.S., and McInerney, M.J. (2001). Benzoate fermentation by the anaerobic bacterium *Syntrophus aciditrophicus* in the absence of hydrogen-using microorganisms. *Appl Environ Microbiol* 67(12)**,** 5520-5525. doi: 10.1128/AEM.67.12.5520-5525.2001.

Friedebold, J., and Bowien, B. (1993). Physiological and biochemical characterization of the soluble formate dehydrogenase, a molybdoenzyme from *Alcaligenes eutrophus*. *J Bacteriol* 175(15)**,** 4719-4728.

Fuchs, G., Boll, M., and Heider, J. (2011). Microbial degradation of aromatic compounds — from one strategy to four. *Nat Rev Microbiol* 9**,** 803. doi: 10.1038/nrmicro2652.

Gijzen, H.J., Broers, C.A., Barughare, M., and Stumm, C.K. (1991). Methanogenic bacteria as endosymbionts of the ciliate *Nyctotherus ovalis* in the cockroach hindgut. *Appl Environ Microbiol* 57(6)**,** 1630-1634.

Gutekunst, K., and Schulz, R. (2018). "CHAPTER 4 The Physiology of the Bidirectional NiFe-hydrogenase in Cyanobacteria and the Role of Hydrogen Throughout the Evolution of Life," in *Microalgal Hydrogen Production: Achievements and Perspectives*. The Royal Society of Chemistry), 107-138.

Hartmann, T., and Leimkühler, S. (2013). The oxygen-tolerant and NAD+-dependent formate dehydrogenase from *Rhodobacter capsulatus* is able to catalyze the reduction of $CO_2$ to formate. *FEBS J* 280(23)**,** 6083-6096. doi: 10.1111/febs.12528.

Harwood, C.S., Burchhardt, G., Herrmann, H., and Fuchs, G. (1998). Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. *FEMS Microbiol Rev* 22(5)**,** 439-458. doi: https://doi.org/10.1016/S0168-6445(98)00026-6.

Hille, R., Hall, J., and Basu, P. (2014). The Mononuclear Molybdenum Enzymes. *Chem Rev* 114(7)**,** 3963-4038. doi: 10.1021/cr400443z.

Huang, H., Hu, L., Yu, W., Li, H., Tao, F., Xie, H., et al. (2016). Heterologous overproduction of 2[4Fe4S]- and [2Fe2S]-type clostridial ferredoxins and [2Fe2S]-type agrobacterial ferredoxin. *Protein Expr Purif* 121**,** 1-8. doi: 10.1016/j.pep.2015.12.019.

Jackson, B.E., Bhupathiraju, V.K., Tanner, R.S., Woese, C.R., and McInerney, M.J. (1999). *Syntrophus aciditrophicus* sp. nov., a new anaerobic bacterium that degrades fatty acids and benzoate in syntrophic association with hydrogen-using microorganisms. *Arch Microbiol* 171(2)**,** 107-114.

James, K.L., Kung, J.W., Crable, B.R., Mouttaki, H., Sieber, J.R., Nguyen, H.H., et al. (2019, in preparation). Syntrophus aciditrophicus uses the same enzymes in a reversible manner to syntrophically degrade fatty, aromatic and alicyclic acids and to synthesize aromatic and alicyclic acids from crotonate.

James, K.L., Rios-Hernandez, L.A., Wofford, N.Q., Mouttaki, H., Sieber, J.R., Sheik, C.S., et al. (2016). Pyrophosphate-Dependent ATP Formation from Acetyl Coenzyme A in *Syntrophus aciditrophicus*, a New Twist on ATP Formation. *MBio* 7(4). doi: 10.1128/mBio.01208-16.

Kelly, C.L., Pinske, C., Murphy, B.J., Parkin, A., Armstrong, F., Palmer, T., et al. (2015). Integration of an [FeFe]-hydrogenase into the anaerobic metabolism of *Escherichia coli*. *Biotechnol Rep (Amst)* 8**,** 94-104. doi: https://doi.org/10.1016/j.btre.2015.10.002.

Kpebe, A., Benvenuti, M., Guendon, C., Rebai, A., Fernandez, V., Le Laz, S., et al. (2018). A new mechanistic model for an O2-protected electron-bifurcating hydrogenase, Hnd from

*Desulfovibrio fructosovorans*. *Biochim Biophys Acta Bioenerg* 1859(12)**,** 1302-1312. doi: https://doi.org/10.1016/j.bbabio.2018.09.364.

Kung, J.W., Seifert, J., von Bergen, M., and Boll, M. (2013). Cyclohexanecarboxyl-Coenzyme A (CoA) and Cyclohex-1-ene-1-Carboxyl-CoA Dehydrogenases, Two Enzymes Involved in the Fermentation of Benzoate and Crotonate in *Syntrophus aciditrophicus*. *J Bacteriol* 195(14)**,** 3193. doi: 10.1128/JB.00322-13.

Laukel, M., Chistoserdova, L., Lidstrom, M.E., and Vorholt, J.A. (2003). The tungsten-containing formate dehydrogenase from *Methylobacterium extorquens* AM1: purification and properties. *Eur J Biochem* 270(2)**,** 325-333.

Ledbetter, R.N., Garcia Costas, A.M., Lubner, C.E., Mulder, D.W., Tokmina-Lukaszewska, M., Artz, J.H., et al. (2017). The Electron Bifurcating FixABCX Protein Complex from *Azotobacter vinelandii:* Generation of Low-Potential Reducing Equivalents for Nitrogenase Catalysis. *Biochemistry* 56(32)**,** 4177-4190. doi: 10.1021/acs.biochem.7b00389.

Losey, N.A., Mus, F., Peters, J.W., Le, H.M., and McInerney, M.J. (2017). *Syntrophomonas wolfei* Uses an NADH-Dependent, Ferredoxin-Independent [FeFe]-Hydrogenase To Reoxidize NADH. *Appl Environ Microb* 83(20)**,** e01335-01317. doi: 10.1128/AEM.01335-17.

Malki, S., Saimmaime, I., De Luca, G., Rousset, M., Dermoun, Z., and Belaich, J.P. (1995). Characterization of an operon encoding an NADP-reducing hydrogenase in *Desulfovibrio fructosovorans*. *J Bacteriol* 177(10)**,** 2628-2636.

McInerney, M.J., Rohlin, L., Mouttaki, H., Kim, U., Krupp, R.S., Rios-Hernandez, L., et al. (2007). The genome of *Syntrophus aciditrophicus*: life at the thermodynamic limit of

microbial growth. *Proc Natl Acad Sci U S A* 104(18)**,** 7600-7605. doi: 10.1073/pnas.0610456104.

McInerney, M.J., Sieber, J.R., and Gunsalus, R.P. (2009). Syntrophy in anaerobic global carbon cycles. *Curr Opin Biotechnol* 20(6)**,** 623-632. doi: 10.1016/j.copbio.2009.10.001.

McInerney, M.J., Struchtemeyer, C.G., Sieber, J., Mouttaki, H., Stams, A.J., Schink, B., et al. (2008). Physiology, ecology, phylogeny, and genomics of microorganisms capable of syntrophic metabolism. *Ann N Y Acad Sci* 1125**,** 58-72. doi: 10.1196/annals.1419.005.

Mouttaki, H., Nanny, M.A., and McInerney, M.J. (2008). Use of benzoate as an electron acceptor by *Syntrophus aciditrophicus* grown in pure culture with crotonate. *Environ Microbiol* 10(12)**,** 3265-3274. doi: 10.1111/j.1462-2920.2008.01716.x.

Muller, U., Willnow, P., Ruschig, U., and Hopner, T. (1978). Formate dehydrogenase from *Pseudomonas oxalaticus*. *Eur J Biochem* 83(2)**,** 485-498.

Riemer, J., Hoepken, H.H., Czerwinska, H., Robinson, S.R., and Dringen, R. (2004). Colorimetric ferrozine-based assay for the quantitation of iron in cultured cells. *Anal Biochem* 331(2)**,** 370-375. doi: 10.1016/j.ab.2004.03.049.

Sazanov, L.A. (2007). Respiratory complex I: mechanistic and structural insights provided by the crystal structure of the hydrophilic domain. *Biochemistry* 46(9)**,** 2275-2288. doi: 10.1021/bi602508x.

Sazanov, L.A., and Hinchliffe, P. (2006). Structure of the hydrophilic domain of respiratory complex I from *Thermus thermophilus*. *Science* 311(5766)**,** 1430-1436. doi: 10.1126/science.1123809.

Schmehl, M., Jahn, A., Meyer zu Vilsendorf, A., Hennecke, S., Masepohl, B., Schuppler, M., et al. (1993). Identification of a new class of nitrogen fixation genes in *Rhodobacter*

*capsalatus*: a putative membrane complex involved in electron transport to nitrogenase. *Molecular and General Genetics MGG* 241(5)**,** 602-615. doi: 10.1007/BF00279903.

Schmidt, A., Muller, N., Schink, B., and Schleheck, D. (2013). A proteomic view at the biochemistry of syntrophic butyrate oxidation in *Syntrophomonas wolfei*. *PLoS One* 8(2)**,** e56905. doi: 10.1371/journal.pone.0056905.

Schonheit, P., Wascher, C., and Thauer, R.K. (1978). A rapid procedure for the purification of ferredoxin from Clostridia using polyethyleneimine. *FEBS Lett* 89(2)**,** 219-222.

Schuchmann, K., and Muller, V. (2012). A bacterial electron-bifurcating hydrogenase. *J Biol Chem* 287(37)**,** 31165-31171. doi: 10.1074/jbc.M112.395038.

Schut, G.J., and Adams, M.W.W. (2009). The Iron-Hydrogenase of *Thermotoga maritima* Utilizes Ferredoxin and NADH Synergistically: a New Perspective on Anaerobic Hydrogen Production. *J Bacteriol* 191(13)**,** 4451.

Seedorf, H., Dreisbach, A., Hedderich, R., Shima, S., and Thauer, R.K. (2004). $F_{420}H_2$ oxidase (FprA) from *Methanobrevibacter arboriphilus*, a coenzyme $F_{420}$-dependent enzyme involved in $O_2$ detoxification. *Arch Microbiol* 182(2-3)**,** 126-137. doi: 10.1007/s00203-004-0675-3.

Seedorf, H., Fricke, W.F., Veith, B., Brüggemann, H., Liesegang, H., Strittmatter, A., et al. (2008). The genome of *Clostridium kluyveri*, a strict anaerobe with unique metabolic features. *Proc Natl Acad Sci Unit States Am* 105(6)**,** 2128.

Seiler, W., Giehl, H., and Roggendorf, P. (1980). Detection of carbon monoxide and hydrogen by conversion of mercury oxide to mercury vapor.

Sieber, J.R., Crable, B.R., Sheik, C.S., Hurst, G.B., Rohlin, L., Gunsalus, R.P., et al. (2015). Proteomic analysis reveals metabolic and regulatory systems involved in the syntrophic

and axenic lifestyle of *Syntrophomonas wolfei*. *Front Microbiol* 6**,** 115. doi: 10.3389/fmicb.2015.00115.

Sieber, J.R., Le, H.M., and McInerney, M.J. (2014). The importance of hydrogen and formate transfer for syntrophic fatty, aromatic and alicyclic metabolism. *Environ Microbiol* 16(1)**,** 177-188. doi: 10.1111/1462-2920.12269.

Sieber, J.R., McInerney, M.J., and Gunsalus, R.P. (2012). Genomic Insights into Syntrophy: The Paradigm for Anaerobic Metabolic Cooperation. *Annu Rev Microbiol* 66(1)**,** 429-452. doi: 10.1146/annurev-micro-090110-102844.

Soboh, B., Linder, D., and Hedderich, R. (2004). A multisubunit membrane-bound [NiFe] hydrogenase and an NADH-dependent Fe-only hydrogenase in the fermenting bacterium *Thermoanaerobacter tengcongensis*. *Microbiology* 150(Pt 7)**,** 2451-2463. doi: 10.1099/mic.0.27159-0.

Tanner, R.S. (2007). "Cultivation of Bacteria and Fungi," in *Manual of Environmental Microbiology, Third Edition*. American Society of Microbiology).

Vignais, P.M., Billoud, B., and Meyer, J. (2001). Classification and phylogeny of hydrogenases. *FEMS Microbiol Rev* 25(4)**,** 455-501.

Walker, J.E. (1992). The NADH:ubiquinone oxidoreductase (complex I) of respiratory chains. *Q Rev Biophys* 25(3)**,** 253-324.

Wang, S., Huang, H., Kahnt, J., Mueller, A.P., Köpke, M., and Thauer, R.K. (2013a). NADP-specific electron-bifurcating [FeFe]-hydrogenase in a functional complex with formate dehydrogenase in *Clostridium autoethanogenum* grown on CO. *J Bacteriol* 195(19)**,** 4373-4386. doi: 10.1128/JB.00678-13.

Wang, S., Huang, H., Kahnt, J., and Thauer, R.K. (2013b). *Clostridium acidurici* Electron-
Bifurcating Formate Dehydrogenase. *Appl Environ Microb* 79(19)**,** 6176.

Wang, S., Huang, H., Kahnt, J., and Thauer, R.K. (2013c). A Reversible Electron-Bifurcating
Ferredoxin- and NAD-Dependent [FeFe]-Hydrogenase (HydABC) in *Moorella
thermoacetica*. *J Bacteriol* 195(6)**,** 1267.

Zannoni, D. (1995). "Aerobic and Anaerobic Electron Transport Chains in Anoxygenic
Phototrophic Bacteria," in *Anoxygenic Photosynthetic Bacteria,* eds. R.E. Blankenship,
M.T. Madigan & C.E. Bauer.  (Dordrecht: Springer Netherlands), 949-971.

Zheng, Y., Kahnt, J., Kwon, I.H., Mackie, R.I., and Thauer, R.K. (2014). Hydrogen Formation
and Its Regulation in *Ruminococcus albus*: Involvement of an Electron-Bifurcating
[FeFe]-Hydrogenase, of a Non-Electron-Bifurcating [FeFe]-Hydrogenase, and of a
Putative Hydrogen-Sensing [FeFe]-Hydrogenase. *J Bacteriol* 196(22)**,** 3840.

**Supplemental Figures and Tables**


**Supplemental Figure S3.1 Full Alignment of Beta Subunits from NADH-Dependent, Ferredoxin-Independent [FeFe]-Hydrogenases and Formate Dehydrogenases, Electron-Bifurcating, NADH-Dependent, Ferredoxin-Dependent [FeFe]-Hydrogenases and Formate Dehydrogenases, and NADH:Quinone Oxidoreductases.** Residues highlighted in yellow correspond to residues that appeared to be differentiable conserved solely amongst the electron-bifurcating NADH-dependent, ferredoxin-dependent [FeFe]-Hydrogenases and Formate Dehydrogenases. The alignment of full-length the beta subunits was performed in MEGA7 using the ClustalW algorithm.

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T. maritima_HydB          --------------------------------------MFKNAKEFVQYAN 50
C. tencongenesis_HydB     -------------------------------------------------- 50
M. thermoacetica_HydB     ----------------------------------MIKSMEELQKFKESQL 50
A. woodii_HydB            -------------------------------------------------- 50
R. albus_HydB             ----------------------------------MTIEELNKIKAAKA 50
C. acidurici_HylB         ----------------------------------MITINDLKKIKEETL 50
C. autoethanogenum_HytB   -------------------------------------------------- 50
D. fructosovorans_HydB    -------------------------------------------------- 50
S. wolfei_HydB            -------------------------------------------------- 50
S. aciditrophicus_HydB    MITVQDIENVIAARGNAREHLMAILRDLENLSGRNVLDVSVLNTLAMKMD 50
C. necator_FdhB           -------------------------------------------------- 50
C. oxalaticus_FdhB        -------------------------------------------------- 50
M. extorquens_FdhB        --------------------------------------MSEASGTVRSFA 50
M. trichosporium_FdhB     -------------------------------------------------- 50
R. capsulatus_FdhB        -------------------------------------------------- 50
E. coli_NuoF              -------------------------------------------------- 50
T. thermophilus_Nqo1      -------------------------------------------------- 50


T. maritima_HydB          KLKTLRE-----------KKLNGVSIYVCVGTGCTAKGALKVYSAFEEEL 100
C. tencongenesis_HydB     -------------------MLYRSHVMVCGGTGCTSSGSDEVAERFIEEI 100
M. thermoacetica_HydB     EAMQSQ------------EAGAKAKIVVGMGTCGIAAGAREVMNAILDEV 100
A. woodii_HydB            ------------------MAYKRSQILICGGTGCTSSGSMVLVKELKKEL 100
R. albus_HydB             DIVKVRTIIAEEGEKLAKETGYRKQVLVCGGTGCQSSHSMDVLKALKEEL 100
C. acidurici_HylB         PKVSLRGDIEG-GEK--IESTYRKQILLCRGTGCTSSKSDKIEERFQELL 100
C. autoethanogenum_HytB   -------------------MSDKKTVNICCGTGCLAKGSMEVYEEMKAQI 100
D. fructosovorans_HydB    -------------------------------------------------- 100
S. wolfei_HydB            -------------------------------------------------- 100
S. aciditrophicus_HydB    LPQSAISGFTSFYTMFSTEPRAKFIIRVCKSGPCHVMGARTIFDVIENHL 100
C. necator_FdhB           -------------------MITITTIFVPRDSTALALGADDVARAIAREA 100
C. oxalaticus_FdhB        --------------MSAAVNSTVNTIFVPRDSTALALGADEVARAIEREA 100
M. extorquens_FdhB        HPGRGRNVARAVPKGRQVDPHAKVEIEELLGTRPRQRDLLIEHLHLIQDT 100
M. trichosporium_FdhB     ----------------------MTKVYVPRDMAALAVGAKRVLAAIEKEA 100
R. capsulatus_FdhB        ----------------------MKIWLPCDAAAKACGAEAVLAALRLEA 100
E. coli_NuoF              -------------------------------------------------- 100
T. thermophilus_Nqo1      -------------------------------------------------- 100


T. maritima_HydB          KKRNLLGQVTLEKIDDDKVTLNRTGCCGRCSSGPLVKIMPYR-------- 150
C. tencongenesis_HydB     KKAGLDKEILVV----------RTGCFGLCELGPVVVVYPEG-------- 150
M. thermoacetica_HydB     AKR-QLTGVTVS----------QTSCIGLCAQEPLVDVILPGQP------ 150
A. woodii_HydB            VKHDILDEVEVV----------TTGCFGLCELGPVVIVYPEG-------- 150
R. albus_HydB             AAKGIADEVLVV----------RTGCFGLCSLGPIVIVYPEG-------- 150
C. acidurici_HylB         KEKGIQDEVNVV----------RTGCFGLCEAGPIVVVYPGG-------- 150
C. autoethanogenum_HytB   AKLGANAEVNVK--------LKATGCDGLCEKGPVLKIYPDD-------- 150
D. fructosovorans_HydB    -------------------------------------------------- 150
S. wolfei_HydB            -------------------------------------------------- 150
S. aciditrophicus_HydB    GIRAGETTADGLFH------LEECECLGLCSAAPAMMVNYDMH------- 150
C. necator_FdhB           AARNEHVRIVRN-----------GSRGMFWLEPLVEVQTGAG------- 150
C. oxalaticus_FdhB        ARRGEAVRIVRN-----------GSRGMFWLEPLVEVQTEAG------- 150
M. extorquens_FdhB        YGQISADHLAALADEMSLAFAEVFETATFYAHFDVVKEGEADIPRLTIRV 150
M. trichosporium_FdhB     AARGEAIQIVRN-----------GSRGLLWLEPLVEVETPEG------- 150
R. capsulatus_FdhB        EKRGGALDIARN-----------GSRGMIWLEPLLEVETPAG------- 150
E. coli_NuoF              -------------------------------------------------- 150
T. thermophilus_Nqo1      ---------------------------MTGPILSG------------ 150
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165

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T. maritima_HydB          ---FFYSNVAPEDVPEIVDRTVLKGEPIERLFLTDPLTGE-KVPRIEDTT 200
C. tencongenesis_HydB     ---VFYSRVKPEYVPEIVEEHLLKGRPVRKYIYGESLEEK-AIKPLEETP 200
M. thermoacetica_HydB     --KVTYGKVDAAKAREIVGRHVVDGHIVTEWVVNRQGDAV---RPYTELP 200
A. woodii_HydB            ---TFYSRVEAADIPEMVEEHLVKGRPLDRLIYNEKGDGH-HPLSINELG 200
R. albus_HydB             ---AFYAQATPEGIKRIVDEHLVNGEICKDLLYQETVHEDGSIISLYETN 200
C. acidurici_HylB         ---TFYSHIKVEDVDQIVEEDLINNNVIKELLYKGSIDGD-KIKAISEVA 200
C. autoethanogenum_HytB   ---IAYFKVKVEDVEDVVKKTLMNGEIIEKLLYFETATKQ-RLRNHKESE 200
D. fructosovorans_HydB    ---------MAATTTE-------------------------------- 200
S. wolfei_HydB            ----------------------------------------------- 200
S. aciditrophicus_HydB    ------GNLSESNIKEILDSYSAREPAFKAEYGPG-------IEGRAVII 200
C. necator_FdhB           --RVAYGPVSAADVPGLFDAGLLQGGEHALSQG-----------VTEEIP 200
C. oxalaticus_FdhB        --RVAYGPVSAEDVPALFDAGLLQGGAHALAHG-----------LTEEIP 200
M. extorquens_FdhB        CDSITCAMFGADELLETLQRELASDAVRVVRAPCVGLCDHAPAVEVGHNF 200
M. trichosporium_FdhB     --RIGYGNVKPSDVPSLFDAGFLRGGAHPLSIG-----------KVEEHP 200
R. capsulatus_FdhB        --RIGFGPMTPADVPALFDA--LES--HPKALG-----------LVEEIP 200
E. coli_NuoF              -------------------------------------------MKNII 200
T. thermophilus_Nqo1      ----------------------------------------------- 200


T. maritima_HydB          LFKNQDFYIMEAIGESEC-------DSIEDYIARSGYESLVKALTS-MTP 250
C. tencongenesis_HydB     FFRKQRRIALRNCGVINP-------EDIREAIAFDGYKALAKVLTE-MTP 250
M. thermoacetica_HydB     FFKHQVRIALRNCGLIDP-------ESIGEYIAHDGYQALSKVLTT-MKP 250
A. woodii_HydB            FFKKQRRIALANCGVINP-------ENIDEYIGFDGYLALEKVLLT-MSP 250
R. albus_HydB             FYRKQKRIALRNCGVIDP-------EDIEEYIATDGYQALYKALTS-MTP 250
C. acidurici_HylB         FYAKQKRVSLKNCGLINP-------ECIDEYISHDGYFALHKALTE-MKP 250
C. autoethanogenum_HytB   FCKRQYKIALRNVGEIDP-------ISLEDYVERGGYKALKKAISS-MKP 250
D. fructosovorans_HydB    --KKQLRIATRNCGFIDP-------ESIDDYIALRGYEGLAKVLT--MTP 250
S. wolfei_HydB            -MAEEMRIVLRNYGKIDP-------LKIDDYLAQGGYKSLEKARA--MNT 250
S. aciditrophicus_HydB    NNSQQTRRLLENVGKVDP-------ASIESYLQNGGYEAARKAFAE-YSP 250
C. necator_FdhB           FLKQQERLTFARVGITDP-------LSLDDYRAHEGFAGLERALA--MQP 250
C. oxalaticus_FdhB        FLKKQERLTFARIGITDP-------LSLDDYRAHEGFAALERALS--MAP 250
M. extorquens_FdhB        LHRADLASVRAAVEAEDTHAHIPTYVDYDAYRAGGGYATLERLRSGELPV 250
M. trichosporium_FdhB     YWAKQTRVTFERVGVIDP-------VNLDDYVAHGGGQGLAKAFE--IGP 250
R. capsulatus_FdhB        FFKRQTRLTFARCGRIEP-------LSLAQFAAAEGWAGLRKALK--MTP 250
E. coli_NuoF              RTPETHPLTWRLRDDKQP-------VWLDEYRSKNGYEGARKALTG-LSP 250
T. thermophilus_Nqo1      LDPRFERTLYAHVGKEGS-------WTLDYYLRHGGYETAKRVLKE-KTP 250
                                             *          :

T. maritima_HydB          EEIIETVKASGLRGRGGGGFPTGLKWEFTRKAQGD--IKFVVCNGDEGDP 300
C. tencongenesis_HydB     EQVIEEVKKSGLRGRGGGGFPTGVKWEFAYKQKET--PKYVVCNADEGDP 300
M. thermoacetica_HydB     YEVIETIKKSGLRGRGGGGFPTGLKWEFAYRSPGD--VKYFVCNADEGDP 300
A. woodii_HydB            VDVINEVKASGLRGRGGGGFPTGLKWQFAHDAVSEDGIKYVACNADEGDP 300
R. albus_HydB             DEVVKEVLDSGIRGRGGAGFPTGRKWMFTKDAPGD--VKYVACNADEGDP 300
C. acidurici_HylB         EDVIETMKKSGLKGRGGGGFPTGLKWEFTAKATGD--QKYVLCNADEGDP 300
C. autoethanogenum_HytB   EDVLEEITKSGLRGRGGAGFPTGRKWKTAADIDTS--PIYVVCNGDEGDP 300
D. fructosovorans_HydB    AEVVDLVKRSGLRGRGGAGFPTGIKWGIALGNKAD--QKYMVCNADEGDP 300
S. wolfei_HydB            ADLIAEVKKSNLRGRGGAGFNCGMKWSFAAGAQAD--QKYVICNADEGEP 300
S. aciditrophicus_HydB    EQVIGIVKDSGLRGRGGAGFPVGVKWSFVPKGEMQ---KYVICNADEGEP 300
C. necator_FdhB           AEIVQEVTDSGLRGRGGAAFPTGIKWKTVLGAQSA--VKYIVCNADEGDS 300
C. oxalaticus_FdhB        AEIVQEVTDSGLRGRGGAAFPTGIKWKTVLGAQSA--VKYIVCNADEGDS 300
M. extorquens_FdhB        DDVLKVLDDGGLRGLGGAGFPTGRKWRSVRGEPGP---RLMAVNGDEGEP 300
M. trichosporium_FdhB     AKVIEEVTKSGLRGRGGAGFPAGIKWKTVADAPAD--RRYVVTNADEGDS 300
R. capsulatus_FdhB        AEVVEEVLASGLRGRGGAGFPTGIKWRTVAAAQAD--QKYIVCNVDEGDS 300
E. coli_NuoF              DEIVNQVKDAGLKGRGGAGFSTGLKWSLMPKDESMN-IRYLLCNADEMEP 300
T. thermophilus_Nqo1      DEVIEEVKRSGLRGRGGAGFPTGLKWSFMPKDDGK--QHYLICNADESEP 300
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166

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T. maritima_HydB          GAFMNRTLLERDPHLVLEGMIIAGYAVGAQKGYAYIRAEYPFAVKMFKKA  350
C. tencongenesis_HydB     GAFMDRSILEGDPHSVLEAMAIAGYAIGANHGYIYVRAEYPLAVKRLKIA  350
M. thermoacetica_HydB     GAFMDRSILEGDPHAVLEGMAIGAYAIGASQGYIYVRAEYPVAVQRLKLA  350
A. woodii_HydB            GAFMDRSVLEGDPHAVIEAMAIAGYAVGASKGYVYVRAEYPIAVNRLQIA  350
R. albus_HydB             GAFMDRSILEGDPHAVIEAMTIASYAVGAHQGYVYVRAEYPVAVNRLQIA  350
C. acidurici_HylB         GAFMDRSILEGDPHSVIEAMALAGYAIGSDQGYVYVRAEYPIAVKRLQIA  350
C. autoethanogenum_HytB   GAFMDRSIMEGDPNSVIEGMTLCAYAVGGTNGFAYIRDEYGLAVENMQKA  350
D. fructosovorans_HydB    -EFMDRAVLEGDPHSVVEAMAIGGYAIGATRGTVYIRAEYPLAIKRLKKA  350
S. wolfei_HydB            GTYKDRLIMENDPHTLIEGMAICAYAIGATQGYIYLRGEYPFLVSTLNTA  350
S. aciditrophicus_HydB    GTFKDRVLMEENPQQLIEGMLLCGYAIGATLGYIYVRGEYRRSIERLQSA  350
C. necator_FdhB           GTFSDRMVMEDDPFMLIEGMTIAALAVGAEQGYIYCRSEYPHAIAVLESA  350
C. oxalaticus_FdhB        GTFSDRMVMEDDPFMLIEGMTIAGLAVGAEQGYIYCRSEYPHAIAVLESA  350
M. extorquens_FdhB        GTFKDQLYLNTDPHRFLEGMLIGAHVVEAADVYIYLRDEYPISREILARE  350
M. trichosporium_FdhB     GTFADRMVMEGDPFVLIEGMTICGYAIGASKGFIYLRAEYPQCAEVLTEA  350
R. capsulatus_FdhB        GSFADRMLIEGDPFCLVEGMAIAGHAVGATRGYVVYIRSEYPDAIAVMRAA  350
E. coli_NuoF             GTYKDRLLMEQLPHLLVEGMLISAFALKAYRGYIFLRGEYIEAAVNLRRA  350
T. thermophilus_Nqo1      GSFKDRYILEDVPHLLIEGMILAGYAIRATVGYIYVRGEYRRAADRLEQA  350
                          : ::  ::   *  .:*.* :  .:: .    : * **     :

T. maritima_HydB          IEDARKLGLLGENILGTGFSFDLEVKEGAGAFVCGEETALLASIEGKRGM  400
C. tencongenesis_HydB     IQQAREYGLLGKDIFGTGFDFDIEIRLGAGAFVCGEETALLNSIMGKRGE  400
M. thermoacetica_HydB     ISQAREQGLLGKNLFNSGFDFDIDIRLGAGAFVCGEETALLASIEGRRGE  400
A. woodii_HydB            IDQAKEYGLLGENIFETDFSFDLEIRLGAGAFVCGEETALMNSIEGKRGE  400
R. albus_HydB             LDQAREYGLLGKNILGTGHDFDIEIRLGAGAFVCGEETALLTSIEGNRGE  400
C. acidurici_HylB         IDEAKAKGLLGKDIFGTGFNFDMEIRLGAGAFVCGEETALIASIEGQRGM  400
C. autoethanogenum_HytB   INKAKDENLLGNNILGTDFSFDIQIVRGGGAFVCGESTALMSSIEGMVGE  400
D. fructosovorans_HydB    IDDAREYGLLGENIFGSGFDFDIELKYGAGAFVCGEETALIRSMEGKRGE  400
S. wolfei_HydB            INQAKEKGLVKD--------FDIEVRSGAGAYVCGEETALIESIEGKRGE  400
S. aciditrophicus_HydB    IDQARAKGFLGKKIFGSNFDFDIFVKEGGGAYVCGEETSLINSMEGKRGY  400
C. necator_FdhB           IGIANAAGWLGDDLRGSGKRFHLEVRKGAGAYVCGEETALLESLEGRRGV  400
C. oxalaticus_FdhB        IAIAHAAGWLGDDLRGSGKRFRLEVRKGAGAYVCGEETALLESLEGKRGV  400
M. extorquens_FdhB        IAKLPEGG------------TRIHLRRGAGAYICGEESSLIESLEGKRGL  400
M. trichosporium_FdhB     LEAARKAGWLGPNVRGSGFAFDIELRIGAGAYVCGEETSLLESLEGKRGI  400
R. capsulatus_FdhB        IAMAKPF------LAEAG--FEMEVRVGAGAYVCGEETSLLNSLEGKRGT  400
E. coli_NuoF             IAEATEAGLLGKNIMGTGFDFELFVHTGAGRYICGEETALINSLEGRRAN  400
T. thermophilus_Nqo1      IKEARARGYLGKNLFGTDFSFDLHVHRGAGAYICGEETALMNSLEGLRAN  400
                          :                : :   *.* ::***.::*: *: *   .

T. maritima_HydB          PRPKPPFPAQSGLWGKPTLINNVETYANIPRILRDGVENYRKRGTE-NSP  450
C. tencongenesis_HydB     PRPRPPFPAVKGVWGKPTIINNVETFANIPPIILNGGEWFASIGTE-KSK  450
M. thermoacetica_HydB     PRPRPPFPAVSGLWGKPTVINNVETLANIPTIIRQGWEWFAGIGTE-KSK  450
A. woodii_HydB            PRPRPPFPANKGLFGKPTVLNNVETYANIPKIILNGAEWFASVGTE-KSK  450
R. albus_HydB             PRPRPPFPAVKGLFGKPTLLNNVETYANIAQIIRKGAAWYSAMGTE-KSK  450
C. acidurici_HylB         PRNKPPFPANKGLWDKPTLINNVETYANVPQIILKGADWFTSFGTK-DSP  450
C. autoethanogenum_HytB   PRAKYIHTTEKGLWGQPTVLNNVETWANVPIIIEKGGDWYHAIGTMEKSK  450
D. fructosovorans_HydB    PVTKPPFPAQSGYWEKPTIVNNVETFANIPAIIINGADWFSGIGTA-TSK  450
S. wolfei_HydB            PRFKPPYPPSEGLWSKPTIVNNVETFANIPVIIEKGADWYASIGNP-AYP  450
S. aciditrophicus_HydB    PRVRPPFPAAAGFMGMPSNVNNVETLSSIPMIVARGADWFKSVGTA-TCA  450
C. necator_FdhB           VRAKPPLPALQGLFGKPTVINNVISLATVPVILARGAQYYRDYGMG-RSR  450
C. oxalaticus_FdhB        VRAKPPLPALEGLFGKPTVINNVISLATVPVILARGAQYYRDYGMG-RSR  450
M. extorquens_FdhB        PRHKPPFPFQVGLFNRPTLINNIETLFWVRDLIERGAEWWKSHGRN-GRV  450
M. trichosporium_FdhB     VRAKPPLPAHVGFMSRPTVVNNVTLTATAPAILAKGGEYYASLGVG-RSR  450
R. capsulatus_FdhB        VRAKPPLPALKGLFGKPTVVNNLLSLAAVPWIIAHGAKAYESFGMD-RSR  450
E. coli_NuoF             PRSKPPFPATSGAWGKPTCVNNVETLCNVPAILANGVEWYQNISKS-KDA  450
T. thermophilus_Nqo1      PRLKPPFPAQSGLWGKPTTINNVETLASVVPIMERGADWFAQMGTE-QSK  450
                          :   . *    *: :**: :       ::  *   :  .

T. maritima_HydB          GTKMFSVAGPLKATGIIEVEFGTTLRDIIYNICGGFVEGEEFKAVQIGGP  500
C. tencongenesis_HydB     GTKVFALTGKVNNTGLIEVPMGTTLREIIYEIGGGIPGGKKFKAVQIGGP  500
M. thermoacetica_HydB     GTKVFALAGKINNNGLVEIPMGTSLRQVIYEIGGGIPGGKKFKAAQTGGP  500
A. woodii_HydB            GTKVFALGGKINNTGLLEIPMGTTLREIIYEIGGGIPNGKAFKAAQTGGP  500
R. albus_HydB             GTKVFALGGKITNVGLVEIPMGTTLREIIEEIGGGIPDGKAFKAAQTGGP  500
C. acidurici_HylB         GTKVFALGGKINNTGLVEIPMGTTLREVIYDVGGGIPDGKQFKAVQTGGP  500
C. autoethanogenum_HytB   GTKVFSLVGKVKNTGLVEVPMGTTLREIIYDIGGGVLNDRKFKAVQIGGP  500
D. fructosovorans_HydB    GTKVFALAGKIQNVGLIEVPMGISLREVIFDIGGGCPDGKAFKAVQTGGP  500
S. wolfei_HydB            GTKVFTLTGDINNRTFFEVPTNTTIREIVFQFGGGVPGDKKFKAVQIGGT  500
S. aciditrophicus_HydB    GTKLYCLSGKVNQTGLVELPMGATLRQMIDTFGGGMKSGSSFKFAQVGGA  500
C. necator_FdhB           GTLPFQLAGNIKQGGLVEKAFGVTLRELLVDYGGGTRSGRAIRAVQVGGP  500
C. oxalaticus_FdhB        GTLPFQLAGNIRQGGLVEKAFGVTLRELLVDYGGGTRSGRAIRAVQVGGP  500
M. extorquens_FdhB        GLRSYSVSGRVKEPGVKLAPAGLTIQELIDEYCGGISDGHSFAAYLPGGA  500
M. trichosporium_FdhB     GTMPLQIAGNVKFGGLYETPFG167GEIVNDIGGGSLSGRPVRAVQCGGP  500
```

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T. maritima_HydB          SGACLS--EDFIDMPLDYDTLKKADAMVGSGGIVVITKKTCMVEVARFFL 550
C. tencongenesis_HydB     SGGCIP--AELLDTPIDYDSLTSAGAMMGSGGLVVMDEDTCMVNVAKFFL 550
M. thermoacetica_HydB     SGGCIP--AEHLDAPIDYENLTALGTIMGSGGLIIMDEDTCMVDVAKFFM 550
A. woodii_HydB            SGGCLP--ESLLDTEIDYDNLIAAGSMMGSGGLIVMDEDNCMVDVARFFL 550
R. albus_HydB             SGGCIP--AQHIDTPIDYESLAALGSMMGSGGLIVMDEDNCMVDVSKFYL 550
C. acidurici_HylB         SGGCIT--ADYIDTEIDFATLNELGSMMGSGGMIVMDEDTCMVDIARFFL 550
C. autoethanogenum_HytB   SGGCLP--AEYLDLPVDYDTLVKADSMMGSGGMIVMDDRTCMVDVTRYYL 550
D. fructosovorans_HydB    SGGALA--NKDLDVAIDYESLAACKSIMGSGGMVVMDEDDCMVSVAKFFL 550
S. wolfei_HydB            SGAFIP--DALLDTPVAFDSMSAIGATLGSGATFVIDESRDIVDVATRIA 550
S. aciditrophicus_HydB    AGGILG--ADLMDLPLDIDSTIKAGVTLGSGVVLVCDQETCAVDFLLNVL 550
C. necator_FdhB           LGAYLP--ESRFDVPLDYEAYAAFGGVVGHGGIVVFDETVDMAKQARYAM 550
C. oxalaticus_FdhB        LGAYLP--ESRFDVPLDYEAYAAFGGVVGHGGIVVFDETVDMAKQARYAM 550
M. extorquens_FdhB        SGGILP--ASMNDIPLDFGTLEKYGCFIGSAAVVILSDQDDVRGAALNLM 550
M. trichosporium_FdhB     LGAYVP--PSLFDTPFDYEAFQKHDSLIGHGGLVVFDDTVDMAQMARFGM 550
R. capsulatus_FdhB        LGAYHP--VSDYHLPFCYEQFAGQGGLVGHAGLVVHDDTADMLKLARFAM 550
E. coli_NuoF              GTDFLT--EAHLDLPMEFESIGKAGSRLGTALAMAVDHEINMVSLVRNLE 550
T. thermophilus_Nqo1      STPPLPFTEEVLDTPMSYEHLQAKGSMLGTGGVILIPERVSMVDAMWNLT 550
                                        .   .       :*   .   .    .

T. maritima_HydB          DFTKRESCGKCVPCREGTM-QAYNILEKFTHGKATYEDLKTLEHLSKTIK 600
C. tencongenesis_HydB     EFTVDESCGKCAPCRIGTK-RMLELLDKITSGKGEEGDIEKLEELAKTIK 600
M. thermoacetica_HydB     DFVKDESCGKCTPCRIGTT-RMLEILNRITRGQGEEKDLDLLVELARQIK 600
A. woodii_HydB            DFTQDESCGKCPPCRIGTK-RMLEILERICDGKGVEGDIERLEELAVGIK 600
R. albus_HydB             NFTVDESCGKCTPCRVGTR-KLLQLLEKITDGKGEMEDLEKIQDLATHMK 600
C. acidurici_HylB         DFTVEESCGKCTPCREGTK-RMLELLEKITDGKGEMEDLDRLESLAETIK 600
C. autoethanogenum_HytB   SFLAEESCGKCVPCREGVK-RMLEILTDICNGDGKEGDIEELLEICSMTS 600
D. fructosovorans_HydB    DFTMDETCGKCTPCRIGSK-RLYEILDRITKGKGTRADLDRLKSLSEIIK 600
S. wolfei_HydB            GFFEHESCGKCAPCREGTA-RTAELMEKINEGRGSNKDVALLEKLGTVMS 600
S. aciditrophicus_HydB    NFFEHESCGQCIPCKLGTS-QLHYVAQKFAMREAEEKDIQLMIDTAKMMK 600
C. necator_FdhB           EFCAIESCGKCTPCRIGST-RGVEVMDRIIAGEQPVKHVALVRDLCDTML 600
C. oxalaticus_FdhB        EFCAIESCGKCTPCRIGST-RGVEVMDRIIAGEQPVKHVKLVRDLCDTML 600
M. extorquens_FdhB        KFFEDESCGQCTPCRSGTQ-------KARMLMENGVWDTDLLGELAQCMR 600
M. trichosporium_FdhB     EFCAIESCGKCTPCRIGST-RGVETIDRIVEGVDVEANIELLSDLCHTMK 600
R. capsulatus_FdhB        EFCAIESCGTCTPCRIGAV-RGVEVIDRIAAGD--ASAMPLLDDLCQTMK 600
E. coli_NuoF              EFFARESCGWCTPCRDGLP-WSVKILRALERGEGQPGDIETLEQLCRFLG 600
T. thermophilus_Nqo1      RFYAHESCGKCTPCREGVAGFMVNLFAKIGTGQGEEKDVENLEALLPLIE 600
                            *    *:** * **: *                             :

T. maritima_HydB          TA-SLCGLGKTAPNPILSTLKLFREEYIAHIEG-ECPSGMCTAFKKYVIN 650
C. tencongenesis_HydB     AT-ALCGLGQTAPNPVLSTLRYFRHEYEAHIKEKRCPAGVCTALLSFVID 650
M. thermoacetica_HydB     DT-ALCGLGQTAPNPVLSTITYFRDEYLAHIRDHRCPAHVCQELLSYVID 650
A. woodii_HydB            SS-ALCGLGQTAPNPVLSTIRFFRDEYEAHIRDKKCPAGVCKHLLDFKIN 650
R. albus_HydB             SS-SLCALGQSAPNPVLSTLQYFGDEYLAHIKEKKCPAGVCKNLLQYEII 650
C. acidurici_HylB         SS-SLCGLGQTAPNPVLSTLKYFRDEYEAHVKDKKCPAGACQSLLEFYIT 650
C. autoethanogenum_HytB   KA-SLCSLGKSAPNPVIASIRYFRDEFEEHIKNKRCRAGVCKKLTTFGID 650
D. fructosovorans_HydB    DT-ALCGLGQTMPNPILSTMDTFANEYEAHVDDKKCPAHVCTALLTYTID 650
S. wolfei_HydB            YS-CLCGLGQAAPAPVLTTIKHFKADYEAKFV------------------ 650
S. aciditrophicus_HydB    LA-SLCALGQSPILPIETMIRNFREEFVKHCDPDYECPQCDASLQAYYL- 650
C. necator_FdhB           NG-SLCAMGGMTPYPVLSALNEFPEDFGLASNPAKAA------------- 650
C. oxalaticus_FdhB        YG-SLCAMGGMTPYPVLSALNEFPEDFGLAPNPAHAAKAA---------- 650
M. extorquens_FdhB        DA-SICGLGQAASNPVSTVIKYFPDLFPEPRAVAAE-------------- 650
M. trichosporium_FdhB     FG-SLCALGGFAPYPVESALRHYPEDFRKATLPAAAE------------- 650
R. capsulatus_FdhB        LG-SLCALGGFTPYPVQSAIRHFPADFPCAREAAE-------------- 650
E. coli_NuoF              PGKTFCAHAPGAVEPLQSAIKYFREEFEAGIKQPFSNTHLINGIQPNLLK 650
T. thermophilus_Nqo1      GR-SFCPLADAAVWPVKGSLRHFKDQYLALAREKRPVPRPSLWR------ 650
                            :*  .      *:   :   : :  :
```

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T. maritima_HydB         PDICKGCGLCARSCPQNAITGERGKPYTIDQEKCVKCGLCASKCPFKAIE 700
C. tencongenesis_HydB    PEKCKACGICAKNCPVGAISGKPKTPYVIDQEKCIKCGTCIDKCPFGAIY 700
M. thermoacetica_HydB    AGKCTGCDACSRVCPVGAISGGKKEAHQIDPAACIKCGSCYEKCRFGAIT 700
A. woodii_HydB           ADTCKGCGICAKKCPADAISGEKKKPYNIDTSKCIKCGACIEACPFGSIS 700
R. albus_HydB            ADKCKGCTLCARNCPANAITGTVKNPHVIDTTKCIKCGVCMNNCKFGAII 700
C. acidurici_HylB        -DKCIGCTKCARQCPASCIDGKVKERHVINTSACVKCGACADVCPVNAVI 700
C. autoethanogenum_HytB  EDKCKGCDMCKKNCPADCITGEIKKPHTIDADKCLRCGNCMNICKFDAVK 700
D. fructosovorans_HydB   PAKCTGCGLCTRVCPVECISGTKKQPHTIDTTRCIKCGACYDKCKFDSII 700
S. wolfei_HydB           -------------------------------------------------- 700
S. aciditrophicus_HydB   -------------------------------------------------- 700
C. necator_FdhB          -------------------------------------------------- 700
C. oxalaticus_FdhB       -------------------------------------------------- 700
M. extorquens_FdhB       -------------------------------------------------- 700
M. trichosporium_FdhB    -------------------------------------------------- 700
R. capsulatus_FdhB       -------------------------------------------------- 700
E. coli_NuoF             ERW----------------------------------------------- 700
T. thermophilus_Nqo1     -------------------------------------------------- 700


T. maritima_HydB         LV- 703
C. tencongenesis_HydB    KK* 703
M. thermoacetica_HydB    RE- 703
A. woodii_HydB           KA- 703
R. albus_HydB            KK- 703
C. acidurici_HylB        KR- 703
C. autoethanogenum_HytB  VL- 703
D. fructosovorans_HydB   KQ- 703
S. wolfei_HydB           --- 703
S. aciditrophicus_HydB   --- 703
C. necator_FdhB          --- 703
C. oxalaticus_FdhB       --- 703
M. extorquens_FdhB       --- 703
M. trichosporium_FdhB    --- 703
R. capsulatus_FdhB       --- 703
E. coli_NuoF             --- 703
T. thermophilus_Nqo1     --- 703
```

**Supplemental Table S3.1 Genbank Accession Numbers for Protein Sequences in Chapter 3.**

| Source Organism | Subunit | GenBank Protein Accession Number |
|---|---|---|
| *Acetobacterium woodii* | HydA | AFA49450 |
| *Acetobacterium woodii* | HydB | AFA49451 |
| *Acetobacterium woodii* | HydC | AFA49452 |
| *Acetobacterium woodii* | HydD | AFA49454 |
| *Caldanaerobacter tencongensis* | HydA | AAM24150 |
| *Caldanaerobacter tencongensis* | HydB | AAM24149 |
| *Caldanaerobacter tencongensis* | HydC | AAM24148 |
| *Clostridium acidurici* | FdhA | AFS79904 |

| | | |
|---|---|---|
| *Clostridium acidurici* | FdhA | AFS79905 |
| *Clostridium acidurici* | FdhB | AFS79906 |
| *Clostridium acidurici* | FdhC | AFS79907 |
| *Clostridium autoethanogenum* | HytA | AGT29713 |
| *Clostridium autoethanogenum* | HytB | AGT29710 |
| *Clostridium autoethanogenum* | HytC | AGT29709 |
| *Clostridium autoethanogenum* | HytD | AGT29711 |
| *Clostridium autoethanogenum* | HytE1 | AGT29712 |
| *Clostridium autoethanogenum* | HytE2 | AGT29714 |
| *Clostridium autoethanogenum* | FdhA | AGT29705 |
| *Cupriavidus necator* | FdhA | WP_011614623 |
| *Cupriavidus necator* | FdhB | WP_011614622 |
| *Cupriavidus necator* | FdhC | WP_011614621 |
| *Cupriavidus necator* | FdhD | WP_010811246 |
| *Cupriavidus oxalaticus* | FdhA | WP_063236918 |
| *Cupriavidus oxalaticus* | FdhB | WP_063236917 |
| *Cupriavidus oxalaticus* | FdhC | WP_063236916 |
| *Cupriavidus oxalaticus* | FdhD | WP_063236919 |
| *Desulfovibrio fructosivorans* | HydA | AAA87057 |
| *Desulfovibrio fructosivorans* | HydB | AAA87056 |
| *Desulfovibrio fructosivorans* | HydC | AAA87054 |
| *Desulfovibrio fructosivorans* | HydD | AAA87055 |

| | | |
|---|---|---|
| *Escherichia coli str.* K-12 | NuoE | NP_416788 |
| *Escherichia coli str.* K-12 | NuoF | NP_416787 |
| *Escherichia coli str.* K-12 | NuoG | NP_416786 |
| *Methylorubrum extorquens* | FdhA | ACS42636 |
| *Methylorubrum extorquens* | FdhB | ACS42635 |
| *Methylosinus trichosporium* | FdhA | WP_003614381 |
| *Methylosinus trichosporium* | FdhB | WP_003614380 |
| *Methylosinus trichosporium* | FdhC | WP_003614379 |
| *Methylosinus trichosporium* | Fdhd | WP_003613007 |
| *Moorella thermoacetica* | HydA | ABC20019 |
| *Moorella thermoacetica* | HydB | ABC20020 |
| *Moorella thermoacetica* | HydC | ABC20021 |
| *Rhodopsuedomonas capsulatus* | FdhA | ADE86759 |
| *Rhodopsuedomonas capsulatus* | FdhB | ADE86760 |
| *Rhodopsuedomonas capsulatus* | FdhC | ADE86761 |
| *Ruminococcus albus* | HydA | ADU23430 |
| *Ruminococcus albus* | HydB | ADU23431 |
| *Ruminococcus albus* | HydC | ADU23432 |
| *Syntrophobacter fumaroxidans* | HydA | ABK16541 |
| *Syntrophobacter fumaroxidans* | HydB | ABK16542 |
| *Syntrophobacter fumaroxidans* | HydC | ABK16543 |
| *Syntrophomonas wolfei* | FdhA | ABI68106/ABI68107 |

(Swol_0783-Swol_0786)

| *Syntrophomonas wolfei* | FdhB | ABI68105 |
|---|---|---|

(Swol_0783-Swol_0786)

| *Syntrophomonas wolfei* | FdhC | ABI68104 |
|---|---|---|

(Swol_0783-Swol_0786)

| *Syntrophomonas wolfei* | FdhA | ABI68342/ ABI68343 |
|---|---|---|

(Swol_1024/Swol_1025

Swol_1028/Swol_1029)

| *Syntrophomonas wolfei* | FdhB | ABI68338 |
|---|---|---|

(Swol_1024/Swol_1025

Swol_1028/Swol_1029)

| *Syntrophomonas wolfei* | FdhC | ABI68339 |
|---|---|---|

(Swol_1024/Swol_1025

Swol_1028/Swol_1029)

| *Syntrophomonas wolfei* | FdhA | ABI69128/ ABI68343 |
|---|---|---|

(Swol_1828-Swol_1831)

| *Syntrophomonas wolfei* | FdhB | ABI69126 |
|---|---|---|

(Swol_1828-Swol_1831)

| *Syntrophomonas wolfei* | FdhC | ABI69127 |
|---|---|---|

(Swol_1828-Swol_1831)

| *Syntrophomonas wolfei* | HydA | ABI68331 |
|---|---|---|
| *Syntrophomonas wolfei* | HydB | ABI68332 |

| | | |
|---|---|---|
| *Syntrophomonas wolfei* | HydC | ABI68333 |
| *Syntrophomonas zehnderi* | HydA | WP_046500146 |
| *Syntrophomonas zehnderi* | HydB | WP_046500148 |
| *Syntrophomonas zehnderi* | HydC | WP_046500150 |
| *Syntrophothermus lipocalidus* | HydA | WP_013174729 |
| *Syntrophothermus lipocalidus* | HydB | WP_013174328 |
| *Syntrophothermus lipocalidus* | HydC | WP_013174327 |
| *Syntrophus aciditrophicus* (Syn_00629-Syn_00631) | FdhA | ABC76020/ABC76021 |
| *Syntrophus aciditrophicus* (Syn_00629-Syn_00631) | FdhB | ABC76022 |
| *Syntrophus aciditrophicus* (Syn_02137-Syn_02139) | FdhA | ABC76957/ABC76958 |
| *Syntrophus aciditrophicus* (Syn_02137-Syn_02139) | FdhB | ABC76959 |
| *Syntrophus aciditrophicus* | HydA | ABC76974 |
| *Syntrophus aciditrophicus* | HydB | ABC76975 |
| *Thermosyntropha lipolytica* | HydA | WP_073091114 |
| *Thermosyntropha lipolytica* | HydB | WP_073091112 |
| *Thermosyntropha lipolytica* | HydC | WP_073091109 |
| *Thermotoga maritima* | HydA | AAD36496 |
| *Thermotoga maritima* | HydB | AAD36495 |

| | | |
|---|---|---|
| *Thermotoga maritima* | HydC | AAD36494 |
| *Thermus thermophilus* | Nqo1 | AAA97943 |
| *Thermus thermophilus* | Nqo2 | AAA97942 |
| *Thermus thermophilus* | Nqo3 | AAA97944 |

# CHAPTER 4: NADH-Dependent, Ferredoxin-Independent Hydrogen Production as a

# Detailed Example of Syntrophic Metabolism

**Characteristics of Proposed NADH-Dependent, Ferredoxin-Independent Hydrogen and Formate Production by *S. wolfei* and *S. aciditrophicus***

In Chapter 3, I argued that the differences between the beta subunits of electron-bifurcating NADH-dependent, ferredoxin-dependent [FeFe]-hydrogenases and those of two NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases from *S. wolfei* and *S. aciditrophicus* could explain the observed ferredoxin dependence. The basis for this differentiation included a number of features found in ferredoxin-dependent [FeFe]-hydrogenases but not in ferredoxin-independent [FeFe]-hydrogenases such as: the presence of an additional N-terminal [2Fe-2S] center, two C-terminal [4Fe-4S] centers, specific residues near the predicted NADH and FMN binding sites, and a glycine-rich region that is a proposed to be a SLBB domain. Glycine-rich regions are characteristic of some flavin and NAPDH binding sites, as proposed for the *E. coli* NADPH:flavin oxidoreductase (Ingelman et al., 1999) and NADH:ubquinone oxidoreductase (Nqo1) (Sazanov, 2007; Berrisford and Sazanov, 2009), which suggests that the glycine-rich region may be a flavin binding site. The above listed features present only in the electron-bifurcating [FeFe]-hydrogenases could facilitate an alternative electron flow path in the beta subunit that mediates the electron-confurcating reaction from NADH and reduced ferredoxin.

During syntrophic metabolism in *S. wolfei* and *S. aciditrophicus*, the re-oxidation of NADH can also be coupled to the production of formate. The *S. wolfei* genome is predicted to encode three multimeric, cytoplasmic formate dehydrogenases (Sieber et al., 2010) while the *S. aciditrophicus* genome is predicted to contain two formate dehydrogenases (McInerney et al., 2007; Sieber et al., 2012). As subunit and domain composition and other features of the beta subunits of these formate dehydrogenases are similar to those of the NADH-dependent,

ferredoxin-independent [FeFe]-hydrogenases, I propose that the formate dehydrogenases in *S. wolfei* and *S. aciditrophicus* are also NADH-dependent, ferredoxin-independent (see Figure 23, see alignment in supplemental figures).

**Figure 23. Conserved Domains in Predicted Cytoplasmic *S. wolfei* and *S. aciditrophicus* Formate Dehydrogenases Suggest Function as NADH-Dependent, Ferredoxin-Independent.**

## Source Organism(s) | Locus Tags Of Encoding Genes | α | γ | β

*S. wolfei* NADH-Dependent, Ferrodoxin-Independent [FeFe]-Hydrogenases and Formate Dehydrogenases

*S. wolfei* — Swol_1017, Swol_1018, Swol_1019 — | 2Fe-2S | 4Fe-4S | 4Fe-4S | H-Cluster | | 2Fe-2S | | Flavin | 4Fe-4S |

*S. wolfei* — Swol_0783, Swol_0784, Swol_0785, Swol_0786 — | 2Fe-2S | 4Fe-4S | 4Fe-4S | 4Fe-4S | Mo or W | | 2Fe-2S | | Flavin | 4Fe-4S |

*S. wolfei* — Swol_1024, Swol_1025, Swol_1028, Swol_1029 — | 2Fe-2S | 4Fe-4S | 4Fe-4S | 4Fe-4S | Mo or W | | 2Fe-2S | | Flavin | 4Fe-4S |

*S. wolfei* — Swol_1828, Swol_1829, Swol_1830, Swol_1831 — | 2Fe-2S | 4Fe-4S | 4Fe-4S | 4Fe-4S | Mo or W | | 2Fe-2S | | Flavin | 4Fe-4S |

*S. acidotrophicus* NADH-Dependent, Ferredoxin-Independent [FeFe]-Hydrogenases and Formate Dehydrogenases

*S. acidotrophicus* — Syn_01369, Syn_01370 — | 2Fe-2S | 4Fe-4S | 4Fe-4S | H-Cluster | | 2Fe-2S | | Flavin | 4Fe-4S |

*S. acidotrophicus* — Syn_00629-Syn_00630, Syn_00631 — | 2Fe-2S | 4Fe-4S | 4Fe-4S | 4Fe-4S | Mo or W | | 2Fe-2S | | Flavin | 4Fe-4S |

*S. acidotrophicus* — Syn_02137-Syn_02138, Syn_02139 — | 2Fe-2S | 4Fe-4S | 4Fe-4S | 4Fe-4S | Mo or W | | 2Fe-2S | | Flavin | 4Fe-4S |

## Beta Subunit Properties

1. N-terminal [2Fe-2S]: Absent 7/7
2. NADH Binding Site (Serine/Threonine): 7/7
3. FMN (Tyrosine): 7/7
4. SLBB Domain (residues GGPSG): Absent 7/7
5. C-terminal 2 x [4Fe-4S]: Absent 7/7

178

These enzymes could allow both organisms to produce formate from NADH without requiring ferredoxin, similar to how both organisms produce hydrogen from NADH without ferredoxin. It is impossible to discern which observed differences between the NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases/formate dehydrogenaes and the electron bifurcating NADH-dependent, ferredoxin-dependent [FeFe]-hydrogenases/formate dehydrogenaes are ultimately responsible for the difference in ferredoxin-dependence. However, as all of the proposed differences for ferredoxin-independence were present in the *S. wolfei* and *S. aciditrophicus* formate dehydrogenases, it seems reasonable to conclude that they are likely NADH-dependent and ferredoxin-independent.

As detailed by the research presented in Chapters 2 and 3, *S. wolfei* and *S. aciditrophicus* both possess cytoplasmic [FeFe]-hydrogenases capable of producing hydrogen from NADH independently of ferredoxin. While less favorable for hydrogen production, NADH-dependent, ferredoxin-independent hydrogen production allows *S. wolfei* and *S. aciditrophicus* to re-oxidize NADH without having to convert NADH to reduced ferredoxin. This avoids the energy costly step of reverse electron transfer needed to make reduced ferredoxin from NADH, which could occur by either a Rnf Complex or FixABCX complex. By avoiding the reverse electron transfer from NADH to ferredoxin, more energy can be conserved for growth as exampled in the following discussion (Figures 24 and 25).

**Figure 24. Butyrate Oxidation by *S. wolfei* with NADH-Dependent, Ferredoxin-Independent Hydrogen Production.** Values shown with the assumption that hydrolysis of one ATP to ADP is equivalent to translocation of 3.35 protons. The actual ATP to proton ratio is dependent on the number of ion binding sites within the *c* ring of the ATP synthase (Müller and Hess, 2017) and has not been specifically determined for *S. wolfei*.
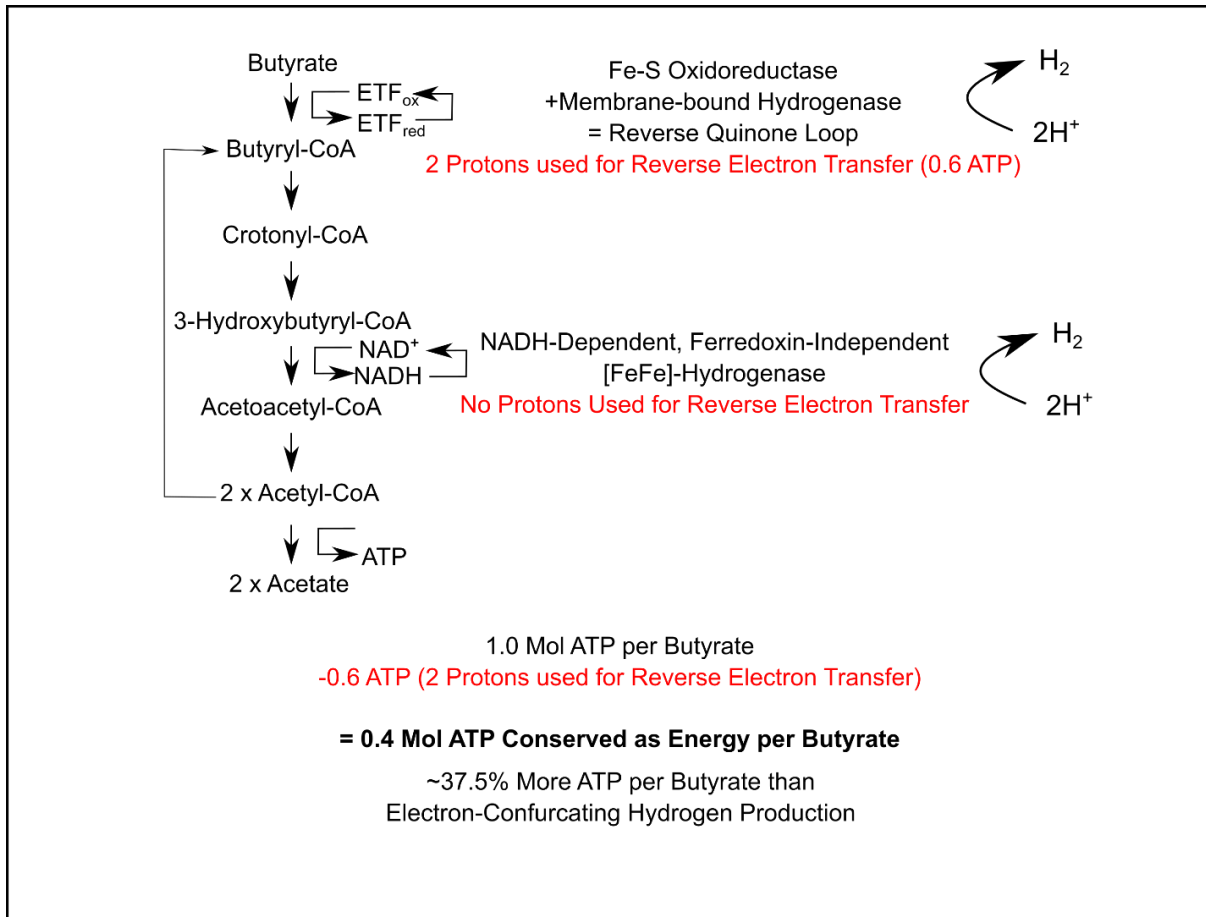
**Figure 25. Butyrate Oxidation by *S. wolfei* with NADH-Dependent, Ferredoxin-Dependent, Electron-Confurcating Hydrogen Production.** Values shown with the assumption that hydrolysis of one ATP to ADP is equivalent to translocation of 3.35 protons. Also assumes that one proton is used to convert one pair of electrons from NADH to reduced ferredoxin.



Butyrate

$ETF_{ox}$
$ETF_{red}$

Butyryl-CoA

Fe-S Oxidoreductase
+Membrane-bound Hydrogenase
= Reverse Quinone Loop

$H_2$

$2H^+$

2 Protons used for Reverse Electron Transfer (0.6 ATP)

Crotonyl-CoA

-CoA
Transfer

3-Hydroxybutyryl-CoA

$NAD^+$
NADH

Electron-Bifurcating
[FeFe]-Hydrogenase

$H_2$

$2H^+$

Acetoacetyl-CoA  0.5 Protons Used for Reverse Electron Transfer (0.15 ATP)
*Convert Half of NADH to $Fd_{red}$

2 x Acetyl-CoA

ATP

2 x Acetate

1.0 Mol ATP per Butyrate
-0.75 ATP (2.5 Protons used for Reverse Electron Transfer)

**= 0.25 Mol ATP Conserved as Energy per Butyrate**

~37.5% Less ATP per Butyrate than NADH-Dependent,
Ferredoxin-Independent Hydrogen Production

181

Syntrophic butyrate metabolism by *S. wolfei* when modeled using NADH dependent hydrogen production is predicted to produce 0.4 moles of ATP per mol of butyrate (Figure 24). In contrast, if half of the NADH is converted to ferredoxin as needed for electron bifurcation, the predicted ATP yield from butyrate is 0.25 moles of ATP per butyrate, which is 37.5% less than when a non-electron bifurcating hydrogenase is used (Figure 25). Thus, NADH-dependent, ferredoxin-independent hydrogen production allows more energy to be conserved per mole of butyrate.

It is unclear if NADH-dependent, ferredoxin-dependent, electron-confurcating hydrogen production would provide any advantage for a butyrate oxidizer like *S. wolfei*. This determination is based on the manner in which the $Etf_{red}$ cofactors are re-oxidized. The current model for $Etf_{red}$ reoxidation involves the use of a reverse quinone loop where the predicted redox potential of the electrons ($E' = -326$ mV) used to produce hydrogen or formate from $Etf_{red}$ would be similar to NADH ($E' = -320$ mV). Thus, the higher hydrogen partial pressures generated from confurcated electrons from NADH and ferredoxin ($E' = -365$ mV) by an electron-confurcating hydrogenase may inhibit $Etf_{red}$ re-oxidation. In order for $Etf_{red}$ re-oxidation to occur, hydrogen consumption by a hydrogen-consuming partner would have to lower the hydrogen partial pressure from ~1000 pascals ($E' = -365$ mV) to closer to 60 pascals ($E' = -326$ mV) that is currently predicted for the redox potential of the reverse quinone loop model. However, it should be noted that redox potential of electrons from the reverse quinone loop have not yet been determined. Regardless of what the redox potential of the quinone pool is, this example illustrates that the utilization of NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases and formate dehydrogenases as the primary means of NADH re-oxidation provides an energetic advantage during close association with hydrogenotrophic methanogens.

This dissertation argues that similarities in the beta subunit may explain why *S. wolfei* and *S. aciditrophicus* [FeFe]-hydrogenases and formate dehydrogenases utilize NADH to produce formate and hydrogen without requiring ferredoxin. However, it should be noted that several aspects of the alpha subunits from the *S. wolfei* and *S. aciditrophicus* [FeFe]-hydrogenases and formate dehydrogenases are shared with the electron-bifurcating formate dehydrogenases and [FeFe]-hydrogenases (Figure 26). More specifically, the nature of the catalytic sites for hydrogen and formate reduction are shared. The Nqo1 subunit has a vesitigial metallopterin binding site (Sazanov and Hinchliffe, 2006). The oxygen-tolerant, NADH-dependent, ferredoxin-independent formate dehydrogenases ligand the metallopterin cofactor with a cysteine (Muller et al., 1978; Jollie and Lipscomb, 1991; Laukel et al., 2003; Niks and Hille, 2019). However, the *S. wolfei* and *S. aciditrophicus* are predicted to use selenocysteine to ligand the metallopterin cofactor and such selenocysteine-cotaining formate dehydrogenases are known to be highly oxygen sensitive (Ferry, 1990). The *S. wolfei* and *S. aciditrophicus* NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases have the same [H]-cluster as the electron-bifurcating [FeFe]-hydrogenases (Figure 26). The H-cluster is also known to be highly oxygen sensitive and rapidly inactivated in the presence of oxygen (Peters et al., 2015). Thus, the alpha subunits of *S. wolfei* and *S. aciditrophicus* formate dehydrogenases and [FeFe]-hydrogenases share catalytic site properties with alpha subunit of the electron-bifurcating formate dehydrogenases and [FeFe]-hydrogenases that render them oxygen sensitive but differ in the type of beta subunit (see Figure 26).

**Figure 26. Features of *S. wolfei and S. aciditrophicus* [FeFe]-Hydrogenases and Formate Dehydrogenases Match Proposed Physiological Role, Hydrogen Production from NADH.**
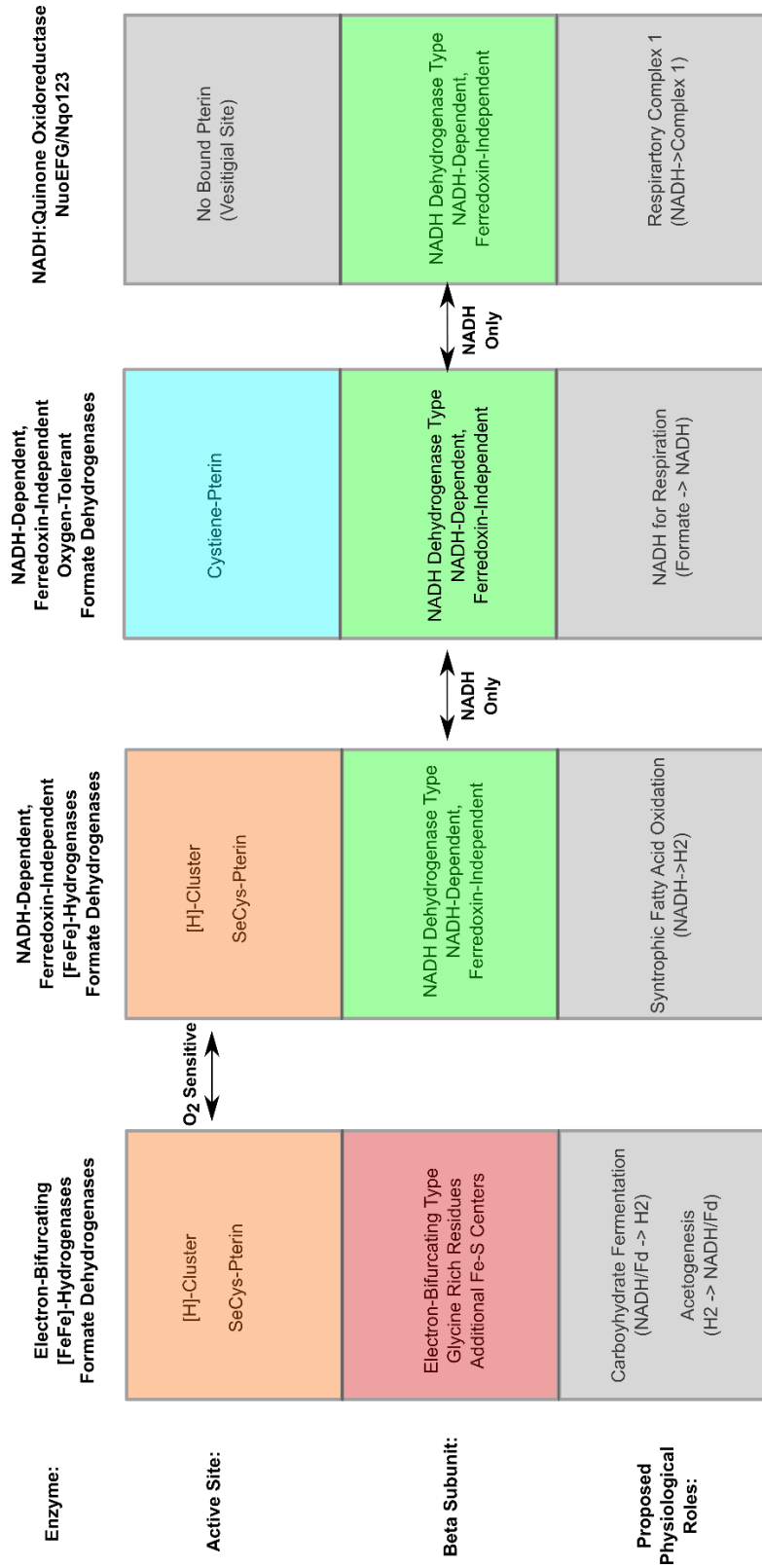
[FeFe]-hydrogenases and selenocysteine-ligand formate dehydrogenases allow faster hydrogen and formate production rates than cysteine-ligand formate dehydrogenases and NADH-dependent group 3 [NiFe]-hydrogenases. The trade-off of, inactivation by oxygen, may not be detrimental as the required partner, in this case a methanogen, is also obligately anaerobic. Group 3 [NiFe]-hydrogenases are notably more tolerant of oxygen and can catalyze the production of hydrogen coupled to oxidation of NADH (Peters et al., 2015). The group 3 [NiFe]-hydrogenaes have recently been suggested to be capable of electron-bifurcation (Gutekunst and Schulz, 2018; Preissler et al., 2018), and do not appear to be as specific for which reduced cofactors are used as physiological electron donors (Peters et al., 2015; Gutekunst and Schulz, 2018; Preissler et al., 2018). Group 3 [NiFe]-hydrogenaes also have different subunits compositions that the NADH-dependent [FeFe]-hydrogenases (Peters et al., 2015; Peters et al., 2018). It is interesting to note that the specific activity for hydrogen production from NADH by the *S. wolfei* Hyd1ABC [FeFe]-hydrogenase (6.6 U • mg$^{-1}$ at 37 °C) was higher than that of any NADH-dependent [NiFe]-hydrogenases (Preissler et al., 2018), with the highest specific activity for hydrogen production from being 2.81 U • mg$^{-1}$ at 60 °C with the HoxHYFUE from *Synechocystis* sp. PCC 6803 (Schmitz et al., 2002). The *S. wolfei* and *S. aciditrophicius* [FeFe]-hydrogenases and formate dehydrogenases possess combinations of oxygen sensitive active sites with superior catalysis rates for hydrogen and formate production that are combined with beta subunits that allow the oxidation of NADH without requiring reduced ferredoxin. This combination would be ideal for their proposed physiological role, the rapid re-oxidation of NADH to produce only low partial pressures of hydrogen and formate during syntrophic fatty acid oxidation.

186

A recent bioinformatic classification of over 714 [FeFe]-hydrogenase amino acid sequences did not identify NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases as a group separate from NADH-dependent, ferredoxin-dependent electron-bifurcating [FeFe]-hydrogenases, possibly due to the criteria used in the classification scheme (Poudel et al., 2016). Classifications schemes commonly used for [FeFe]-hydrogenaeses, classify the NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases with the Group 3A electron-bifurcating [FeFe]-hydrogenases due to the presence of subunits homologous to NuoE and NuoF (Peters et al., 2015; Poudel et al., 2016; Søndergaard et al., 2016). However, we suggest that more in-depth examination of the beta subunit, the NuoF homolog, may determine ferredoxin dependence.

The only NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases that we have identified are from the *Syntrophomonadaceae* (See Chapter 2), *S. aciditrophicus* (See Chapter 3), and *N. ovalis* (see Chapter 3). It is possible that NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases are relatively rare in comparison to NADH-dependent, ferredoxin-dependent electron-bifurcating [FeFe]-hydrogenases as they may only be useful under syntrophic conditions and less useful when performing primary fermentation which provides both NADH and reduced ferredoxin or under oxic conditions where their oxygen lability would favor [NiFe]-hydrogenases.

An unanswered question is if the NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases from *S. wolfei*, *S. aciditrophicus*, and *N. ovalis* share a common origin where they separate from the NADH-dependent, ferredoxin-dependent electron-bifurcating [FeFe]-hydrogenases or if the transition from electron bifurcating, ferredoxin-dependent to ferredoxin-independent occurred multiple times as a form of convergent evolution. The selective pressure could possibly be close association with hydrogenotrophic methanogens. The distribution of

NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases in different syntrophic organisms is currently unknown but their proposed energetic advantages over electron-bifurcating, NADH-dependent, ferredoxin-independent [FeFe]-hydrogenases suggests they may be present in other syntrophic metabolisms.

**Unanswered Questions: Acyl-CoA Oxidation Mechanisms in *S. wolfei* and *S. aciditrophicus*: and Intracellular NADH/NAD$^+$ Ratios**

While we have provided details on how NADH is re-oxidized in *S. wolfei* and *S. aciditrophicus*, the manner in which Etf$_{red}$ is re-oxidized, presumably a reverse quinone loop, remains undemonstrated by biochemical methods. Quinone loops have been proposed in additional syntrophic metabolisms such as syntrophic lactate oxidation by members of the genus *Desulfovibrio* (Li et al., 2011; Meyer et al., 2013) and syntrophic propionate oxidation by *Syntrophobacter fumaroxidans* (Müller et al., 2010; Plugge et al., 2012). The high redox potential of menaquinone, E′ = - 94 mV, when combined with a reverse quinone loop which involves the translocation of two protons (which lowers the redox potential by approximately - 200 mV) would indicate high redox potential electrons are still used for hydrogen or formate production.

As we can explain hydrogen production from NADH during syntrophic metabolism, the question turns to how to explain hydrogen production from Etf$_{red}$. *S. wolfei* appears to be capable of dissipating differences between the redox potentials of hydrogen and formate (Boone et al., 1989) and it presumambly produces both NADH-dependent, ferredoxin-independent [FeFe]-hydrogenase and NADH-dependent, ferredoxin-independent formate dehydrogenases simultaneously (Sieber et al., 2014; Sieber et al., 2015). Therefore, it seems unlikely that re-

oxidation of NADH and Etf$_{red}$ can be explained by one process using hydrogen production and the other process using formate production. Thus, either simultaneous hydrogen production from NADH and Etf$_{red}$ occurs or simultaneous production of formate from NADH and Etf$_{red}$ occurs. Either scenario would produce a situation where either hydrogen or formate production from NADH is equilibrated with hydrogen or formate production from the proposed reverse quinone loop. It is possible that for all intents and purposes, the redox potentials of the electrons from NADH and those involved in the reverse quinone loop are near equilibrium. This raises the question of how interactions between multiple cofactor re-oxidation processes simultaneously occur and are managed during syntrophic metabolism. This aspect of syntrophy cannot be addressed until a thorough understanding of the proposed reverse quinone loop is available.

Thermodynamic calculations for NADH-dependent, ferredoxin-independent hydrogen production by *S. wolfei* and *S. aciditrophicus* suggest both organisms need to maintain high NADH/NAD$^+$ ratios to generate the hydrogen partial pressures that were observed for both pure culture and co-culture growth. The other piece of the puzzle is the hydrogenotrophic methanogen partner, which in these experiments was *M. hungatei* which has a strongly contrasting situation in terms of intracellular cofactor pools. During the eight-electron reduction of $CO_2$ to methane, four pairs of electrons are consumed.

**Figure 27. Redox Reactions in Cytochrome-Free Hydrogenotrophic Methanogens such as** *Methanosprillium hungatei.* MF:methanofuran, THMPT:tetrahydromethanopterin, HS-CoB:coenzyme B, HS-CoM:coenzyme M, Fd:ferredoxin, $F_{420}$:Cofactor $F_{420}$.
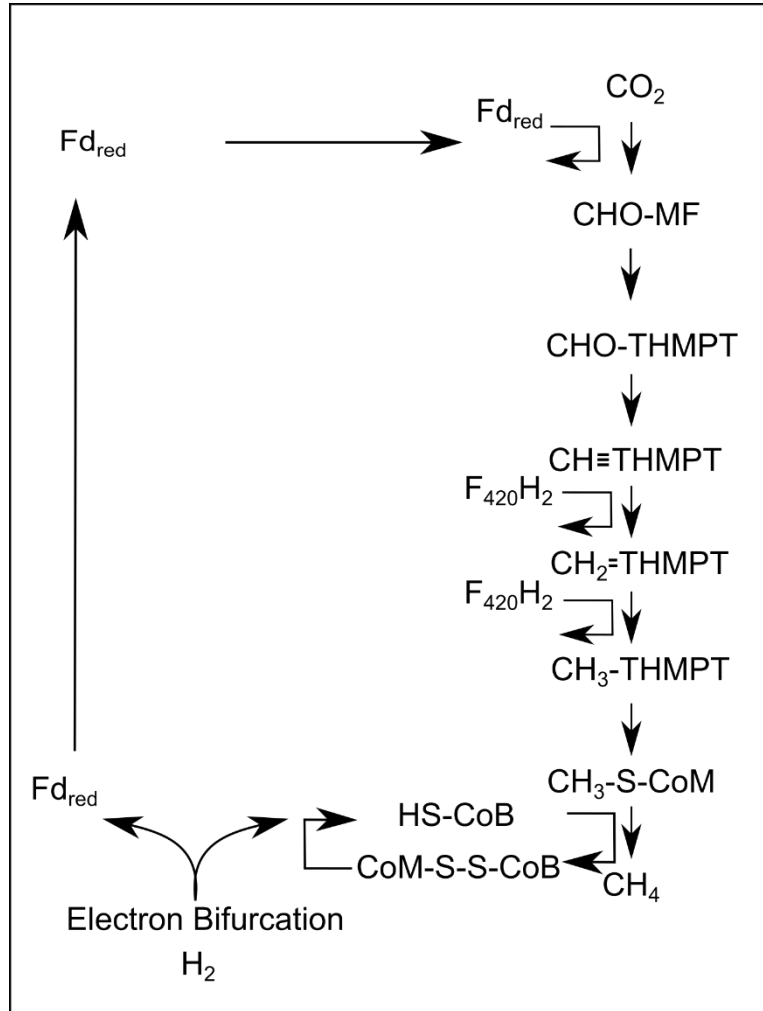


Two pairs of electrons come from reduced cofactor $F_{420}$, one pair from the low potential redox carrier ferredoxin, and one pair of electrons is from the oxidation of hydrogen coupled to the reduction of the disulfide bond between coenzyme B and coenzyme M (Figure 27). The low potential carrier, ferredoxin, is difficult to reduce by hydrogen at low hydrogen partial pressures and is regenerated by an electron-bifurcation mechanism involving CoM-S-S-CoB heterdisulfide reductase (Buckel and Thauer, 2013). The redox potential of NADH under standard conditions is

$E_0' = -320$ mV while the redox potential of the methanogenic cofactor $F_{420}$ under standard conditions is $E_0' = -360$ mV. For *S. wolfei* and *S. aciditrophicus* to produce the hydrogen at the partial pressures that, were observed, the NADH/NAD$^+$ ratio would have to be high, which would result in a redox potential of at least $E' = -320$ mV if not lower. In contrast, the cofactor $F_{420}$ reduced to oxidized (the $F_{420}H_2/F_{420}$) ratio in hydrogenotrophic methanogens would have to be low, below one, when hydrogen partial pressures are low. A fluorescence-based technique to measure the cofactor $F_{420}$ reduced to oxidized (the $F_{420}H_2/F_{420}$) ratio showed that it was below 1.0 in the hydrogenotrophic methanogen *Methanobacterium thermautotrophicus* when grown with hydrogen partial pressures below 200 pascals (de Poorter et al., 2005).

**Figure 28. Syntroph NADH/NAD$^+$ Ratio Compared to Methanogen $F_{420}H_2/F_{420}$ Ratio.**
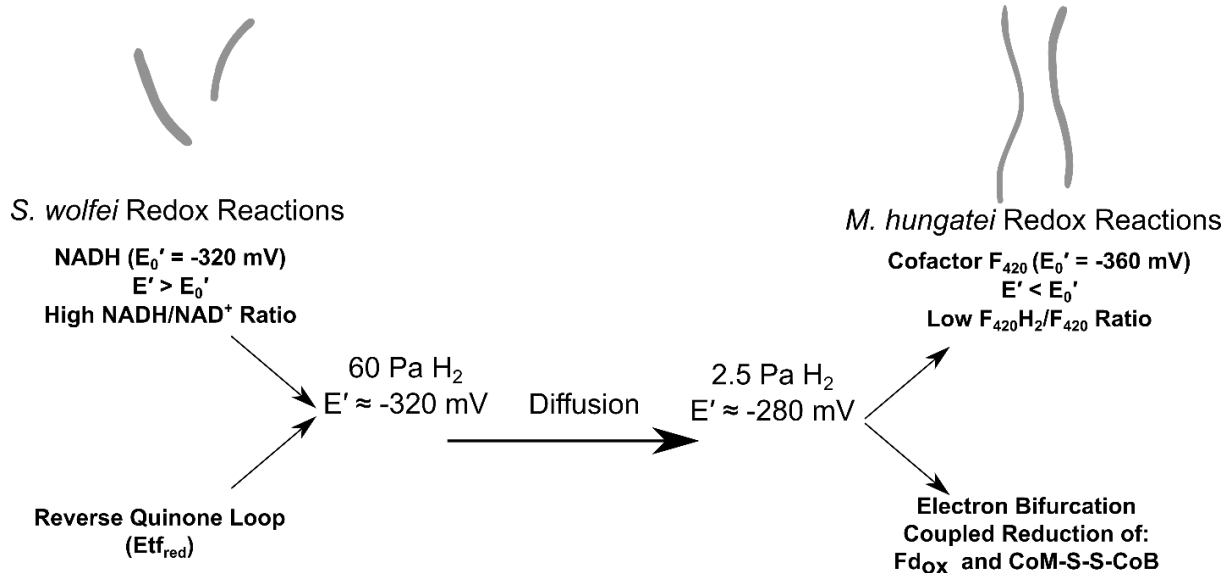


The reduced nature of the NADH/NAD$^+$ pool (where $E' > E_0'$) indicates that the syntrophic metabolizer is pushing the limits of this thermodynamically unfavorable process of producing hydrogen from NADH. This is stark contrast to cofactor $F_{420}$ pool, which is kept highly oxidized (where $E_0' > E'$). The apparent difference in the hydrogen redox potential (and

concentration) at the syntrophic metabolizer cell and the hydrogenotrophic methanogen cell may be important to the process of hydrogen diffusion, as the larger the difference between the concentration of hydrogen at the syntrophic metabolizer (the source) compared to the hydrogen concentration by the hydrogenotrophic methanogen (the sink), the faster the diffusion rate will be as predicted by Fick's law of diffusion (Schink and Thauer, 1988). This expected difference in hydrogen concentrations was observed when an ethanol-oxidizing, hydrogen-producing bacterium was separated by dialysis tubing from a hydrogen-consuming homoacetogen (Schink and Stieb, 1987). Separating the organisms resulted in a hydrogen concentration ten times higher in the compartment with the hydrogen-producing organism than when the organisms were grown in the same compartment. The observed hydrogen partial pressures measured in the *S. wolfei* and *M. hungatei* co-culture experiments are therefore likely intermediate between the hydrogen concentrations at each organism. The hydrogen concentrations at each organism should be reflected in the NADH/NAD$^+$ ratio of *S. wolfei* and the $F_{420}H_2/F_{420}$ ratio of *M. hungatei* and would offer an alternative mechanistic view on the syntrophic, hydrogen-based relationship.

**Summary: NADH-Dependent, Ferredoxin-Independent Hydrogen Production, Exemplifies the Intimate Nature of Syntrophic Metabolism in Co-Culture Between Syntrophic Metabolizers and Methanogenic Partners**

Syntropy was originally proposed to be dependent on low hydrogen and formate concentrations as explained by thermodynamics (McInerney et al., 1979; McInerney et al., 1981). This dependence in *S. wolfei* and *S. aciditrophicus* has been extensively detailed by physiological studies of co-cultures employing a wide range of culture-based (Wofford et al., 1986; Elshahed et al., 2001; Elshahed and McInerney, 2001), proteomic (Sieber et al., 2015;

James et al., 2019, in preparation), and transcriptional techniques (Sieber et al., 2014; James et al., 2019, in preparation). This dissertation suggests that from the perspective of the syntrophic metabolizer, syntrophy is the result of a dependence on cofactor re-oxidation mechanisms that utilize specifically high redox potential electrons to produce hydrogen or formate. NADH-dependent, ferredoxin-independent hydrogen production mechanisms in the syntrophic model organisms *S. wolfei* and *S. aciditrophicus* demonstrate how high redox potential electrons (E′ = -320 mV) from fatty acid oxidation (NADH) are used to produce hydrogen as had been predicted for *S. wolfei* nearly 30 years ago (Boone et al., 1989). The original observation that syntrophic metabolism is dependent on low hydrogen concentrations may be viewed alternatively as result of an obligate dependence on cofactor re-oxidation mechanisms that utilize high redox potential electrons. This argument is a rephrasing of the original thermodynamic argument, but with an additional emphasis on the physiological electron donors utilized by hydrogenases. The proposed cofactor re-oxidation mechanisms appear to agree with the original predicted thermodynamic constraints by allowing cofactor re-oxidation while maximizing the meager amount of energy that can be conserved for growth.

   Butyrate oxidation and benzoate oxidation by *S. wolfei* and *S. aciditrophicus* are among the most difficult syntrophic metabolisms as predicted by thermodynamic modelling and the organisms appear to require lower hydrogen and formate concentrations than many other proposed syntrophic interactions. For example, syntrophic ethanol oxidation by the genus *Pelobacter* (Schmidt et al., 2014) or the altered fermentation profile of *Ruminococcus albus* (Zheng et al., 2014) are dependent on a hydrogen-consuming organism but utilize electron-confurcating hydrogenases. However, NADH-dependent hydrogen production as occurs in *S. wolfei* and *S. aciditrophicus* is clearly much closer to the limits that a hydrogenotrophic

methanogen can accommodate than the hydrogen partial pressure that would be produced by

electron-confurcation from NADH and reduced ferredoxin (Figure 29).

**Figure 29. Narrow Hydrogen Partial Pressure Overlap Between Syntrophic Metabolizers and Methanogenic Partner.** Hydrogen partial pressures from *S. wolfei* and *S. aciditrophicus* cultures are from Chapters 2 and 3. The 2.5 pascal value was reported by (Cord-Ruwisch et al., 1988).

**Highest Hydrogen Partial Pressures by Syntrophs**

**Hydrogen Partial Pressures During Syntrophic Metaoblism**

**Lowest Hydrogen Partial Pressures by Methanogen**

40 Pa

36 Pa

17 Pa

13 Pa

5 Pa

4 Pa

2.5 Pa

Fd$_{red}$-Dependent (E' = -410 mV)

Confurcation NADH + Fd$_{red}$ (E'= -365 mV)

NADH-Dependent (E' = -320 mV)

*S. wolfei* Pure Culture On Crotonate

*S. aciditrophicus* Pure Culture On Crotonate

*S. wolfei* Co-Culture On Butyrate

*S. aciditrophicus* Co-Culture On Benzoate

*M. hungatei* H$_2$ + CO$_2$

10000 Pascal

1000 Pascal

100 Pascal

10 Pascal

1 Pascal

195

This exemplifies the degree to which these organisms as syntrophic specialists have adapted to an obligate dependence on hydrogenotrophic methanogenic partners. Other syntrophic metabolisms such as syntrophic acetate oxidation and propionate oxidation may involve similar high redox potential electron-donating, hydrogen-producing reactions such as NADH-dependent, ferredoxin-independent hydrogen production or the proposed reverse quinone loop. The work in this dissertation, demonstrates NADH-dependent, ferredoxin-independent hydrogen production in two obligately syntrophic model organisms and provides mechanistic understanding of the thermodynamic constraints placed on syntrophy. This suggests other high redox potential electron donor reactions should be expected for hydrogen production or other means of interspecies electron transfer in obligately syntrophic metabolizers.

**References**

Berrisford, J.M., and Sazanov, L.A. (2009). Structural Basis for the Mechanism of Respiratory Complex I. *J Biol Chem* 284(43)**,** 29773-29783. doi: 10.1074/jbc.M109.032144.

Boone, D.R., Johnson, R.L., and Liu, Y. (1989). Diffusion of the Interspecies Electron Carriers $H_2$ and Formate in Methanogenic Ecosystems and Its Implications in the Measurement of $H_2$ or Formate Uptake. *Appl Environ Microb* 55(7)**,** 1735.

Buckel, W., and Thauer, R.K. (2013). Energy conservation via electron bifurcating ferredoxin reduction and proton/Na(+) translocating ferredoxin oxidation. *Biochim Biophys Acta* 1827(2)**,** 94-113. doi: 10.1016/j.bbabio.2012.07.002.

Cord-Ruwisch, R., Seitz, H.-J., and Conrad, R. (1988). The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of

the terminal electron acceptor. *Arch Microbiol* 149(4)**,** 350-357. doi: 10.1007/bf00411655.

de Poorter, L.M., Geerts, W.J., and Keltjens, J.T. (2005). Hydrogen concentrations in methane-forming cells probed by the ratios of reduced and oxidized coenzyme $F_{420}$. *Microbiology* 151(Pt 5)**,** 1697-1705. doi: 10.1099/mic.0.27679-0.

Elshahed, M.S., Bhupathiraju, V.K., Wofford, N.Q., Nanny, M.A., and McInerney, M.J. (2001). Metabolism of Benzoate, Cyclohex-1-ene Carboxylate, and Cyclohexane Carboxylate by "*Syntrophus aciditrophicus*" Strain SB in Syntrophic Association with $H_2$-Using Microorganisms. *Appl Environ Microb* 67(4)**,** 1728-1738. doi: 10.1128/aem.67.4.1728-1738.2001.

Elshahed, M.S., and McInerney, M.J. (2001). Benzoate fermentation by the anaerobic bacterium *Syntrophus aciditrophicus* in the absence of hydrogen-using microorganisms. *Appl Environ Microbiol* 67(12)**,** 5520-5525. doi: 10.1128/AEM.67.12.5520-5525.2001.

Ferry, J.G. (1990). Formate dehydrogenase. *FEMS Microbiol Ecol* 7(3-4)**,** 377-382. doi: 10.1111/j.1574-6968.1990.tb04940.x.

Gutekunst, K., and Schulz, R. (2018). "CHAPTER 4 The Physiology of the Bidirectional NiFe-hydrogenase in Cyanobacteria and the Role of Hydrogen Throughout the Evolution of Life," in *Microalgal Hydrogen Production: Achievements and Perspectives*. The Royal Society of Chemistry), 107-138.

Ingelman, M., Ramaswamy, S., Nivière, V., Fontecave, M., and Eklund, H. (1999). Crystal Structure of NAD(P)H:Flavin Oxidoreductase from *Escherichia coli*. *Biochemistry* 38(22)**,** 7040-7049. doi: 10.1021/bi982849m.

James, K.L., Kung, J.W., Crable, B.R., Mouttaki, H., Sieber, J.R., Nguyen, H.H., et al. (2019, in preparation). *Syntrophus aciditrophicus* uses the same enzymes in a reversible manner to syntrophically degrade fatty, aromatic and alicyclic acids and to synthesize aromatic and alicyclic acids from crotonate.

Jollie, D.R., and Lipscomb, J.D. (1991). Formate dehydrogenase from *Methylosinus trichosporium* OB3b. Purification and spectroscopic characterization of the cofactors. *J Biol Chem* 266(32)**,** 21853-21863.

Laukel, M., Chistoserdova, L., Lidstrom, M.E., and Vorholt, J.A. (2003). The tungsten-containing formate dehydrogenase from *Methylobacterium extorquens* AM1: purification and properties. *Eur J Biochem* 270(2)**,** 325-333.

Li, X., McInerney, M.J., Stahl, D.A., and Krumholz, L.R. (2011). Metabolism of $H_2$ by *Desulfovibrio alaskensis* G20 during syntrophic growth on lactate. *Microbiology* 157(Pt 10)**,** 2912-2921. doi: 10.1099/mic.0.051284-0.

McInerney, M.J., Bryant, M.P., Hespell, R.B., and Costerton, J.W. (1981). *Syntrophomonas wolfei* gen. nov. sp. nov., an Anaerobic, Syntrophic, Fatty Acid-Oxidizing Bacterium. *Appl Environ Microbiol* 41(4)**,** 1029-1039.

McInerney, M.J., Bryant, M.P., and Pfennig, N. (1979). Anaerobic bacterium that degrades fatty acids in syntrophic association with methanogens. *Arch Microbiol* 122(2)**,** 129-135. doi: 10.1007/bf00411351.

McInerney, M.J., Rohlin, L., Mouttaki, H., Kim, U., Krupp, R.S., Rios-Hernandez, L., et al. (2007). The genome of *Syntrophus aciditrophicus*: life at the thermodynamic limit of microbial growth. *Proc Natl Acad Sci U S A* 104(18)**,** 7600-7605. doi: 10.1073/pnas.0610456104.

198

Meyer, B., Kuehl, J., Deutschbauer, A.M., Price, M.N., Arkin, A.P., and Stahl, D.A. (2013). Variation among *Desulfovibrio* Species in Electron Transfer Systems Used for Syntrophic Growth. *J Bacteriol* 195(5)**,** 990. doi: 10.1128/JB.01959-12.

Müller, N., Worm, P., Schink, B., Stams, A.J.M., and Plugge, C.M. (2010). Syntrophic butyrate and propionate oxidation processes: from genomes to reaction mechanisms. *Environ Microbiol Rep* 2(4)**,** 489-499. doi: doi:10.1111/j.1758-2229.2010.00147.x.

Muller, U., Willnow, P., Ruschig, U., and Hopner, T. (1978). Formate dehydrogenase from *Pseudomonas oxalaticus*. *Eur J Biochem* 83(2)**,** 485-498.

Müller, V., and Hess, V. (2017). The Minimum Biological Energy Quantum. *Front Microbiol* 8(2019). doi: 10.3389/fmicb.2017.02019.

Niks, D., and Hille, R. (2019). Molybdenum- and tungsten-containing formate dehydrogenases and formylmethanofuran dehydrogenases: Structure, mechanism, and cofactor insertion. *Protein Sci* 28(1)**,** 111-122. doi: 10.1002/pro.3498.

Peters, J.W., Beratan, D.N., Schut, G.J., and Adams, M.W.W. (2018). On the nature of organic and inorganic centers that bifurcate electrons, coupling exergonic and endergonic oxidation–reduction reactions. *Chem Comm* 54(33)**,** 4091-4099. doi: 10.1039/C8CC01530A.

Peters, J.W., Schut, G.J., Boyd, E.S., Mulder, D.W., Shepard, E.M., Broderick, J.B., et al. (2015). [FeFe]- and [NiFe]-hydrogenase diversity, mechanism, and maturation. *Biochim Biophys Acta Mol Cell Res* 1853(6)**,** 1350-1369. doi: https://doi.org/10.1016/j.bbamcr.2014.11.021.

Plugge, C.M., Henstra, A.M., Worm, P., Swarts, D.C., Paulitsch-Fuchs, A.H., Scholten, J.C.M., et al. (2012). Complete genome sequence of *Syntrophobacter fumaroxidans* strain (MPOB[T]). *Stand Genomic Sci* 7(1)**,** 91-106. doi: 10.4056/sigs.2996379.

Poudel, S., Tokmina-Lukaszewska, M., Colman, D.R., Refai, M., Schut, G.J., King, P.W., et al. (2016). Unification of [FeFe]-hydrogenases into three structural and functional groups. *Biochim Biophys Acta Gen Subj* 1860(9)**,** 1910-1921. doi: https://doi.org/10.1016/j.bbagen.2016.05.034.

Preissler, J., Wahlefeld, S., Lorent, C., Teutloff, C., Horch, M., Lauterbach, L., et al. (2018). Enzymatic and spectroscopic properties of a thermostable [NiFe]-hydrogenase performing $H_2$-driven NAD+-reduction in the presence of $O_2$. *Biochim Biophys Acta Bioenerg* 1859(1)**,** 8-18. doi: https://doi.org/10.1016/j.bbabio.2017.09.006.

Sazanov, L.A. (2007). Respiratory complex I: mechanistic and structural insights provided by the crystal structure of the hydrophilic domain. *Biochemistry* 46(9)**,** 2275-2288. doi: 10.1021/bi602508x.

Sazanov, L.A., and Hinchliffe, P. (2006). Structure of the hydrophilic domain of respiratory complex I from *Thermus thermophilus*. *Science* 311(5766)**,** 1430-1436. doi: 10.1126/science.1123809.

Schink, B., and Stieb, M. (1987). Cultivation of syntrophic anaerobic bacteria in membrane-separated culture devices. *FEMS Microbiol Ecol* 3(2)**,** 71-76. doi: 10.1111/j.1574-6968.1987.tb02341.x.

Schink, B., and Thauer, R. (1988). Energetics of syntrophic methane formation and the influence of aggregation. *Granular anaerobic sludge***,** 5-17.

Schmidt, A., Frensch, M., Schleheck, D., Schink, B., and Muller, N. (2014). Degradation of acetaldehyde and its precursors by Pelobacter carbinolicus and P-acetylenicus. *PLoS ONE.* 9. doi: 10.1371/journal.pone.0115902.

Schmitz, O., Boison, G., Salzmann, H., Bothe, H., Schütz, K., Wang, S.-h., et al. (2002). HoxE—a subunit specific for the pentameric bidirectional hydrogenase complex (HoxEFUYH) of cyanobacteria. *Biochim Biophys Acta Bioenerg* 1554(1)**,** 66-74. doi: https://doi.org/10.1016/S0005-2728(02)00214-1.

Sieber, J.R., Crable, B.R., Sheik, C.S., Hurst, G.B., Rohlin, L., Gunsalus, R.P., et al. (2015). Proteomic analysis reveals metabolic and regulatory systems involved in the syntrophic and axenic lifestyle of *Syntrophomonas wolfei*. *Front Microbiol* 6**,** 115. doi: 10.3389/fmicb.2015.00115.

Sieber, J.R., Le, H.M., and McInerney, M.J. (2014). The importance of hydrogen and formate transfer for syntrophic fatty, aromatic and alicyclic metabolism. *Environ Microbiol* 16(1)**,** 177-188. doi: 10.1111/1462-2920.12269.

Sieber, J.R., McInerney, M.J., and Gunsalus, R.P. (2012). Genomic Insights into Syntrophy: The Paradigm for Anaerobic Metabolic Cooperation. *Annu Rev Microbiol* 66(1)**,** 429-452. doi: 10.1146/annurev-micro-090110-102844.

Sieber, J.R., Sims, D.R., Han, C., Kim, E., Lykidis, A., Lapidus, A.L., et al. (2010). The genome of *Syntrophomonas wolfei*: new insights into syntrophic metabolism and biohydrogen production. *Environ Microbiol* 12(8)**,** 2289-2301. doi: 10.1111/j.1462-2920.2010.02237.x.

Søndergaard, D., Pedersen, C.N.S., and Greening, C. (2016). HydDB: A web tool for hydrogenase classification and analysis. *Sci Rep* 6**,** 34212. doi: 10.1038/srep34212

https://www.nature.com/articles/srep34212#supplementary-information.

Wofford, N.Q., Beaty, P.S., and McInerney, M.J. (1986). Preparation of cell-free extracts and the enzymes involved in fatty acid metabolism in *Syntrophomonas wolfei*. *J Bacteriol* 167(1), 179. doi: 10.1128/jb.167.1.179-185.1986.

Zheng, Y., Kahnt, J., Kwon, I.H., Mackie, R.I., and Thauer, R.K. (2014). Hydrogen Formation and Its Regulation in *Ruminococcus albus*: Involvement of an Electron-Bifurcating [FeFe]-Hydrogenase, of a Non-Electron-Bifurcating [FeFe]-Hydrogenase, and of a Putative Hydrogen-Sensing [FeFe]-Hydrogenase. *J Bacteriol* 196(22), 3840.

**Supplemental Figures**

**Supplemental Figure 4.1 Full Alignment of Beta Subunits from NADH-Dependent, Ferredoxin-Independent [FeFe]-Hydrogenases and Formate Dehydrogenases, Electron-Bifurcating, NADH-Dependent, Ferredoxin-Dependent [FeFe]-Hydrogenases and Formate Dehydrogenases, and NADH:Quinone Oxidoreductases Including Specifically Proposed NADH-Dependent, Ferredoxin-Independent Formate Dehydrogenases from *S. aciditrophicus* and *S. wolfei.*.** Residues highlighted in yellow correspond to residues that appeared to be differentiable conserved solely amongst the electron-bifurcating NADH-dependent, ferredoxin-dependent [FeFe]-Hydrogenases and Formate Dehydrogenases. The alignment of full-length the beta subunits was performed in MEGA7 using the ClustalW algorithm.

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T. maritima HydB            -------------------------------------------------- 50
C. tencongenesis HydB       -------------------------------------------------- 50
M. thermoacetica HydB       -------------------------------------------------- 50
A. woodii HydB              -------------------------------------------------- 50
R. albus HydB               -------------------------------------------------- 50
C. acidurici HylB           -------------------------------------------------- 50
C. autoethanogenum HytB     -------------------------------------------------- 50
D. fructosovorans HydB      -------------------------------------------------- 50
C. necator FdhB             -------------------------------------------------- 50
C. oxalaticus FdhB          -------------------------------------------------- 50
M. extorquens FdhB          -----------------------------------------------MSE 50
M. trichosporium FdhB       -------------------------------------------------- 50
R. capsulatus FdhB          -------------------------------------------------- 50
E. coli NuoF                -------------------------------------------------- 50
T. thermophilus Nqo1        -------------------------------------------------- 50
S. wolfei HydB              -------------------------------------------------- 50
Swol_0784                   -------------------------------------------------- 50
Swol_1024                   -------------------------------------------------- 50
Swol_1828                   -------------------------------------------------- 50
Syn_02139                   MTRWIRSARSPNIRSVLAKSKKFTCKEGIAMITTENIQEVINNRGKAREH 50
Syn_00631                   --------------------------MITEQLKNDIHRLLGRYPEK 50
S. aciditrophicus HydB      --------------------------MITVQDIENVIAARGNAREH 50


T. maritima HydB            -------------------MFKNAKEFVQYANKLKTLR----------- 100
C. tencongenesis HydB       -------------------------------------------------- 100
M. thermoacetica HydB       ----------------MIKSMEELQKFKESQLEAMQSQ----------- 100
A. woodii HydB              -------------------------------------------------- 100
R. albus HydB               ------------------MTIEELNKIKAAKADIVKVRTIIAEEGEKLA 100
C. acidurici HylB           ----------------MITINDLKKIKEETLPKVSLRGDIEG-GEK-- 100
C. autoethanogenum HytB     -------------------------------------------------- 100
D. fructosovorans HydB      -------------------------------------------------- 100
C. necator FdhB             -------------------------------------------------- 100
C. oxalaticus FdhB          ----------------------------------------------MSA 100
M. extorquens FdhB          ASGTVRSFAHPGRGRNVARAVPKGRQVDPHAKVEIEELLGTRPRQRDLLI 100
M. trichosporium FdhB       -------------------------------------------------- 100
R. capsulatus FdhB          -------------------------------------------------- 100
E. coli NuoF                -------------------------------------------------- 100
T. thermophilus Nqo1        -------------------------------------------------- 100
S. wolfei HydB              -------------------------------------------------- 100
Swol_0784                   -------------------------------------------------- 100
Swol_1024                   -------------------------------------------------- 100
Swol_1828                   -------------------------------------------------- 100
Syn_02139                   LMAILRDLENLSGNQQ--LSPETLNAVAEAMNLPQSTVAGFVGFYTMFST 100
Syn_00631                   QSALMPALMLAQKENANRLDQDDIRTVAELVDVPFGKAYGLATYYSMYNV 100
S. aciditrophicus HydB      LMAILRDLENLSGRNV--LDVSVLNTLAMKMDLPQSAISGFTSFYTMFST 100
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T. maritima HydB            EKKLNGVSIYVCVGTGCTAKGALKVYSAFEEELKKRNLLGQVTLEKIDDD 150
C. tencongenesis HydB       --MLYRSHVMVCGGTGCTSSGSDEVAERFIEEIKKAGLDKEILVVR---- 150
M. thermoacetica HydB       -EAGAKAKIVVGMGTCGIAAGAREVMNAILDEVAKR-QLTGVTVSQ---- 150
A. woodii HydB              -MAYKRSQILICGGTGCTSSGSMVLVKELKKELVKHDILDEVEVVT---- 150
R. albus HydB               KETGYRKQVLVCGGTGCQSSHSMDVLKALKEELAAKGIADEVLVVR---- 150
C. acidurici HylB           IESTYRKQILLCRGTGCTSSKSDKIEERFQELLKEKGIQDEVNVVR---- 150
C. autoethanogenum HytB     --MSDKKTVNICCGTGCLAKGSMEVYEEMKAQIAKLGANAEVNVKLKA-- 150
D. fructosovorans HydB      -------------------------------------------------- 150
C. necator FdhB             --MITITTIFVPRDSTALALGADDVARAIAREAAARNEHVRIVRNG---- 150
C. oxalaticus FdhB          AVNSTVNTIFVPRDSTALALGADEVARAIEREAARRGEAVRIVRNG---- 150
M. extorquens FdhB          EHLHLIQDTYGQISADHLAALADEMSLAFAEVFETATFYAHFDVVKEGE- 150
M. trichosporium FdhB       -----MTKVYVPRDMAALAVGAKRVLAAIEKEAAARGEAIQIVRNG---- 150
R. capsulatus FdhB          ------MKIWLPCDAAAKACGAEAVLAALRLEAEKRGGALDIARNG---- 150
E. coli NuoF                -------------------------------------------------- 150
T. thermophilus Nqo1        -------------------------------------------------- 150
S. wolfei HydB              -------------------------------------------------- 150
Swol_0784                   -------------------------------------------------- 150
Swol_1024                   -------------------------------------------------- 150
Swol_1828                   -------------------------------------------------- 150
Syn_02139                   R-PRAKFLIRVCKSGPCHVMGARTIFDYVEKHLGISPGQTTADGLFH--- 150
Syn_00631                   EKPVGRYHLQVDTNIPATLMGAGEILDHLEKTLNIRAGETTPDGLFT--- 150
S. aciditrophicus HydB      E-PRAKFIIRVCKSGPCHVMGARTIFDVIENHLGIRAGETTADGLFH--- 150


T. maritima HydB            KVTLNRTGCCGRCSSGPLVKIMPYR---FFYSNVAPEDVPEIVDRTVLKG 200
C. tencongenesis HydB       ------TGCFGLCELGPVVVVYPEG---VFYSRVKPEYVPEIVEEHLLKG 200
M. thermoacetica HydB       ------TSCIGLCAQEPLVDVILPGQPKVTYGKVDAAKAREIVGRHVVDG 200
A. woodii HydB              ------TGCFGLCELGPVVIVYPEG---TFYSRVEAADIPEMVEEHLVKG 200
R. albus HydB               ------TGCFGLCSLGPIVIVYPEG---AFYAQATPEGIKRIVDEHLVNG 200
C. acidurici HylB           ------TGCFGLCEAGPVVVVPGG---TFYSHIKVEDVDQIVEEDLINN 200
C. autoethanogenum HytB     ------TGCDGLCEKGPVLKIYPDD---IAYFKVKVEDVEDVVKKTLMNG 200
D. fructosovorans HydB      ---------------------------------MAATTTE--------- 200
C. necator FdhB             --------SRGMFWLEPLVEVQTGAG-RVAYGPVSAADVPGLFDAGLLQG 200
C. oxalaticus FdhB          --------SRGMFWLEPLVEVQTEAG-RVAYGPVSAEDVPALFDAGLLQG 200
M. extorquens FdhB          --ADIPRLTIRVCDSITCAMFGADELLETLQRELASDAVRVVRAPCVGLC 200
M. trichosporium FdhB       --------SRGLLWLEPLVEVETPEG-RIGYGNVKPSDVPSLFDAGFLRG 200
R. capsulatus FdhB          --------SRGMIWLEPLLEVETPAG-RIGFGPMTPADVPALFDA--LES 200
E. coli NuoF                -------------------------------------------------- 200
T. thermophilus Nqo1        ------------MTGPILSG---------------------------- 200
S. wolfei HydB              -------------------------------------------------- 200
Swol_0784                   -------------------------------------------------- 200
Swol_1024                   -------------------------------------------------- 200
Swol_1828                   -------------------------------------------------- 200
Syn_02139                   ---LEACECLGICSVAPAMMIN-----YDLHGNLTEERIATILDGYRSRE 200
Syn_00631                   ---LSEVECLASCGTCPVIQVN-----DVYYENMTREKVDSLLDSLR--- 200
S. aciditrophicus HydB      ---LEECECLGLCSAAPAMMVN-----YDMHGNLSESNIKEILDSYSARE 200
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204

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T. maritima HydB            EPIERLFLTDPLTGE-KVPRIEDTTLFKNQDFYIMEAIGESECDSIEDYI 250
C. tencongenesis HydB       RPVRKYIYGESLEEK-AIKPLEETPFFRKQRRIALRNCGVINPEDIREAI 250
M. thermoacetica HydB       HIVTEWVVNRQGDAV---RPYTELPFFKHQVRIALRNCGLIDPESIGEYI 250
A. woodii HydB              RPLDRLIYNEKGDGH-HPLSINELGFFKKQRRIALANCGVINPENIDEYI 250
R. albus HydB               EICKDLLYQETVHEDGSIISLYETNFYRKQKRIALRNCGVIDPEDIEEYI 250
C. acidurici HylB           NVIKELLYKGSIDGD-KIKAISEVAFYAKQKRVSLKNCGLINPECIDEYI 250
C. autoethanogenum HytB     EIIEKLLYFETATKQ-RLRNHKESEFCKRQYKIALRNVGEIDPISLEDYV 250
D. fructosovorans HydB      -------------------------KKQLRIATRNCGFIDPESIDDYI 250
C. necator FdhB             GEHALSQG-----------VTEEIPFLKQQERLTFARVGITDPLSLDDYR 250
C. oxalaticus FdhB          GAHALAHG-----------LTEEIPFLKKQERLTFARIGITDPLSLDDYR 250
M. extorquens FdhB          DHAPAVEVGHNFLHR------ADLASVRAAVEAEDTHAHIPTYVDYDAYR 250
M. trichosporium FdhB       GAHPLSIG-----------KVEEHPYWAKQTRVTFERVGVIDPVNLDDYV 250
R. capsulatus FdhB          --HPKALG-----------LVEEIPFFKRQTRLTFARCGRIEPLSLAQFA 250
E. coli NuoF                -------------------MKNIIRTPETHPLTWRLRDDKQPVWLDEYR 250
T. thermophilus Nqo1        ------------------------LDPRFERTLYAHVGKEGSWTLDYYL 250
S. wolfei HydB              ------------------------MAEEMRIVLRNYGKIDPLKIDDYL 250
Swol_0784                   ------------------------MAEEIRILLAHADQIDPAQAEDYI 250
Swol_1024                   ------------------------MAEEMRIVLRNYGKIDPLKIDDYI 250
Swol_1828                   ------------------------MAEEMRIVLRNYGKVDPLNIDSYT 250
Syn_02139                   PFFGEACGPEVEGRV-------CMLDEPGQTQRLTEKIGSIDPLSVDSYL 250
Syn_00631                   ----KGMMPESE----------TIYSFGSVCNVLLKNRGVKDATALAVAK 250
S. aciditrophicus HydB      PAFKAEYGPGIEGRA-------VIINNSQQTRRLLENVGKVDPASIESYL 250


T. maritima HydB            ARSGYESLVKALTS-MTPEEIIETVKASGLRGRGGGGFPTGLKWEFTRKA 300
C. tencongenesis HydB       AFDGYKALAKVLTE-MTPEQVIEEVKKSGLRGRGGGGFPTGVKWEFAYKQ 300
M. thermoacetica HydB       AHDGYQALSKVLTT-MKPYEVIETIKKSGLRGRGGGGFPTGLKWEFAYRS 300
A. woodii HydB              GFDGYLALEKVLLT-MSPVDVINEVKASGLRGRGGGGFPTGLKWQFAHDA 300
R. albus HydB               ATDGYQALYKALTS-MTPDEVVKEVLDSGIRGRGGAGFPTGRKWMFTKDA 300
C. acidurici HylB           SHDGYFALHKALTE-MKPEDVIETMKKSGLKGRGGGGFPTGLKWEFTAKA 300
C. autoethanogenum HytB     ERGGYKALKKAISS-MKPEDVLEEITKSGLRGRGGAGFPTGRKWKTAADI 300
D. fructosovorans HydB      ALRGYEGLAKVLT--MTPAEVVDLVKRSGLRGRGGAGFPTGIKWGIALGN 300
C. necator FdhB             AHEGFAGLERALA--MQPAEIVQEVTDSGLRGRGGAAFPTGIKWKTVLGA 300
C. oxalaticus FdhB          AHEGFAALERALS--MAPAEIVQEVTDSGLRGRGGAAFPTGIKWKTVLGA 300
M. extorquens FdhB          AGGGYATLERLRSGELPVDDVLKVLDDGGLRGLGGAGFPTGRKWRSVRGE 300
M. trichosporium FdhB       AHGGGQGLAKAFE--IGPAKVIEEVTKSGLRGRGGAGFPAGIKWKTVADA 300
R. capsulatus FdhB          AAEGWAGLRKALK--MTPAEVVEVLASGLRGRGGAGFPTGIKWRTVAAA 300
E. coli NuoF                SKNGYEGARKALTG-LSPDEIVNQVKDAGLKGRGGAGFSTGLKWSLMPKD 300
T. thermophilus Nqo1        RHGGYETAKRVLKE-KTPDEVIEEVKRSGLRGRGGAGFPTGLKWSFMPKD 300
S. wolfei HydB              AQGGYKSLEKARA--MNTADLIAEVKKSNLRGRGGAGFNCGMKWSFAAGA 300
Swol_0784                   KVGGFKGLEKARG--MTSKDLIEEVKKSGLRGRGGAGFNAGMKWSFVP-- 300
Swol_1024                   AAGGYKSLEKARS--MKQTEVIEEVKKSNLRGRGGAGFNAGMKWNFSYQV 300
Swol_1828                   QVGGYQALAKAKS--MSQTDLIEEVKKSGLRGRGGAGFNTGMKWSFSYGV 300
Syn_02139                   EKGGYAAIKKALAE-YQPDDVIAIVKDSGLRGRGGAGFPAGVKWSFLPKG 300
Syn_00631                   GNGAYQALAKAGTM-K-PEEILEEVKKANLRGRGGAGFPAGVKWGFIPKD 300
S. aciditrophicus HydB      QNGGYEAARKAFAE-YSPEQVIGIVKDSGLRGRGGAGFPVGVKWSFVPKG 300
                             .     :         .::  :  ..::* **..*   * **
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T. maritima HydB          QGD--IKFVVCNGDEGDPGAFMNRTLLERDPHLVLEGMIIAGYAVGAQKG 350
C. tencongenesis HydB     KET--PKYVVCNADEGDPGAFMDRSILEGDPHSVLEAMAIAGYAIGANHG 350
M. thermoacetica HydB     PGP--VKYFVCNADEGDPGAFMDRSILEGDPHAVLEGMAIGAYAIGASQG 350
A. woodii HydB            VSEDGIKYVACNADEGDPGAFMDRSVLEGDPHAVIEAMAIAGYAVGASKG 350
R. albus HydB             PGD--VKYVACNADEGDPGAFMDRSILEGDPHAVIEAMTIASYAVGAHQG 350
C. acidurici HylB         TGD--QKYVLCNADEGDPGAFMDRSILEGDPHSVIEAMALAGYAIGSDQG 350
C. autoethanogenum HytB   DTS--PIYVVCNGDEGDPGAFMDRSIMEGDPNSVIEGMTLCAYAVGGTNG 350
D. fructosovorans HydB    KAD--QKYMVCNADEGDP-EFMDRAVLEGDPHSVVEAMAIGGYAIGATRG 350
C. necator FdhB           QSA--VKYIVCNADEGDSGTFSDRMVMEDDPFMLIEGMTIAALAVGAEQG 350
C. oxalaticus FdhB        QSA--VKYIVCNADEGDSGTFSDRMVMEDDPFMLIEGMTIAGLAVGAEQG 350
M. extorquens FdhB        PGP---RLMAVNGDEGEPGTFKDQLYLNTDPHRFLEGMLIGAHVVEAADV 350
M. trichosporium FdhB     PAD--RRYVVTNADEGDSGTFADRMVMEGDPFVLIEGMTICGYAIGASKG 350
R. capsulatus FdhB        QAD--QKYIVCNVDEGDSGSFADRMLIEGDPFCLVEGMAIAGHAVGATRG 350
E. coli NuoF              ESMN-IRYLLCNADEMEPGTYKDRLLMEQLPHLLVEGMLISAFALKAYRG 350
T. thermophilus Nqo1      DGK--QHYLICNADESEPGSFKDRYILEDVPHLLIEGMILAGYAIRATVG 350
S. wolfei HydB            QAD--QKYVICNADEGEPGTYKDRLIMENDPHTLIEGMAICAYAIGATQG 350
Swol_0784                 PAE--VKYVVCNLDEGEPGTYKDRIICEKNAQALIEGMAICGVAINAKQG 350
Swol_1024                 QSE--EKYVVCNADEGEPGTYKDRIIMENDPQSVIEGMAICGYAIGSKKG 350
Swol_1828                 KAD--QKYVICNADEGEPGTYKDRIIMENDPQSVLEGMAICGYAIGANKG 350
Syn_02139                 -DMQ--KYVICNADEGEPGTYKDRILMEENPHGLLEGMMLCGYATGATVG 350
Syn_00631                 TDKP--VYLICNADEGEPGTYKDRQIMEYDPHLLIEGMAIAARAIGARQA 350
S. aciditrophicus HydB    -EMQ--KYVICNADEGEPGTFKDRVLMEENPQQLIEGMLLCGYAIGATLG 350
                              .  *  ** :.  : ::   :  .  .:*.*  :  .  .  .

T. maritima HydB          YAYIRAEYPFAVKMFKKAIEDARKLGLLGENILGTGFSFDLEVKEGAGAF 400
C. tencongenesis HydB     YIYVRAEYPLAVKRLKIAIQQAREYGLLGKDIFGTGFDFDIEIRLGAGAF 400
M. thermoacetica HydB     YIYVRAEYPVAVQRLKLAISQAREQGLLGKNLFNSGFDFDIDIRLGAGAF 400
A. woodii HydB            YVYVRAEYPIAVNRLQIAIDQAKEYGILGENIFETDFSFDLEIRLGAGAF 400
R. albus HydB             YVYVRAEYPVAVNRLQIALDQAREYGLLGKNILGTGHDFDIEIRLGAGAF 400
C. acidurici HylB         YVYVRAEYPIAVKRLQIAIDEAKAKGLLGKDIFGTGFNFDMEIRLGAGAF 400
C. autoethanogenum HytB   FAYIRDEYGLAVENMQKAINKAKDENLLGNNILGTDFSFDIQIVRGGGAF 400
D. fructosovorans HydB    TVYIRAEYPLAIKRLKKAIDDAREYGLLGENIFGSGFDFDIELKYGAGAF 400
C. necator FdhB           YIYCRSEYPHAIAVLESAIGIANAAGWLGDDIRGSGKRFHLEVRKGAGAY 400
C. oxalaticus FdhB        YIYCRSEYPHAIAVLESAIAIAHAAGWLGDDLRGSGKRFRLEVRKGAGAY 400
M. extorquens FdhB        YIYLRDEYPISREILAREIAKLPEGG------------TRIHLRRGAGAY 400
M. trichosporium FdhB     FIYLRAEYPQCAEVLTEALEAARKAGWLGPNVRGSGFAFDIELRIGAGAY 400
R. capsulatus FdhB        YVYIRSEYPDAIAVMRAAIAMAKPF------LAEAG--FEMEVRVGAGAY 400
E. coli NuoF              YIFLRGEYIEAAVNLRRAIAEATEAGLLGKNIMGTGFDFELFVHTGAGRY 400
T. thermophilus Nqo1      YIYVRGEYRRAADRLEQAIKEARARGYLGKNLFGTDFSFDLHVHRGAGAY 400
S. wolfei HydB            YIYLRGEYPFLVSTLNTAINQAKEKGLVK-------DFDIEVRSGAGAY 400
Swol_0784                 YIYCRGEYPFVVDLLRKAIQSAKDVGALG-------EFDIEVRMGAGAY 400
Swol_1024                 YIYCRGEYPYVVDILNQAIAQAKAKGVLG-------DFDVEVRMGGGAY 400
Swol_1828                 YIYCRGEYPYVVEILNKAIAQASEKGLLG-------DFNIEVRMGAGAY 400
Syn_02139                 YIYIRGEYRRSIERLQRAIDQAREKGILGDNIFGSSFRFDIFIKEGGGAY 400
Syn_00631                 FIYIRGEFAWIADILEKAIGEAKADGQLS-------ELDIIVHRGAGAY 400
S. aciditrophicus HydB    YIYVRGEYRRSIERLQSAIDQARAKGFLGKKIFGSNFDFDIFVKEGGGAY 400
                           : *  *:       :   :                      : :  *.*  :
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T. maritima HydB            VCGEETALLASIEGKRGMPRPKPPFPAQSGLWGKPTLINNVETYANIPRI 450
C. tencongenesis HydB       VCGEETALLNSIMGKRGEPRPRPPFPAVKGVWGKPTIINNVETFANIPPI 450
M. thermoacetica HydB       VCGEETALLASIEGRRGEPRPRPPFPAVSGLWGKPTVINNVETLANIPTI 450
A. woodii HydB              VCGEETALMNSIEGKRGEPRPRPPFPANKGLFGKPTVLNNVETYANIPKI 450
R. albus HydB               VCGEETALLTSIEGNRGEPRPRPPFPAVKGLFGKPTLLNNVETYANIAQI 450
C. acidurici HylB           VCGEETALIASIEGQRGMPRNKPPFPANKGLWDKPTLINNVETYANVPQI 450
C. autoethanogenum HytB     VCGESTALMSSIEGMVGEPRAKYIHTTEKGLWGQPTVLNNVETWANVPII 450
D. fructosovorans HydB      VCGEETALIRSMEGKRGEPVTKPPFPAQSGYWEKPTIVNNVETFANIPAI 450
C. necator FdhB             VCGEETALESLEGRRGVVRAKPPLPALQGLFGKPTVINNVISLATVPVI 450
C. oxalaticus FdhB          VCGEETALLESLEGKRGVVRAKPPLPALEGLFGKPTVINNVISLATVPVI 450
M. extorquens FdhB          ICGEESSLIESLEGKRGLPRHKPPFPFQVGLFNRPTLINNIETLFWVRDL 450
M. trichosporium FdhB       VCGEETSLLESLEGKRGIVRAKPPLPAHVGFMSRPTVVNNVLTLATAPAI 450
R. capsulatus FdhB          VCGEETSLLNSLEGKRGTVRAKPPLPALKGLFGKPTVVNNLLSLAAVPWI 450
E. coli NuoF                ICGEETALINSLEGRRANPRSKPPFPATSGAWGKPTCVNNVETLCNVPAI 450
T. thermophilus Nqo1        ICGEETALMNSLEGLRANPRLKPPFPAQSGLWGKPTTINNVETLASVVPI 450
S. wolfei HydB              VCGEETALIESIEGKRGEPRFKPPYPPSEGLWSKPTIVNNVETFANIPVI 450
Swol_0784                   VCGEETALIESIEGHRGEPRFKPPFPGVAGLWGVPTVVNNVETFACLPYI 450
Swol_1024                   VCGEESALIESIEGHRGEPRFKPPFPPVIGLWQKPTIVNNVETFANIPII 450
Swol_1828                   VCGEESALIESIEGHRGEPRFKPPFPPVIGLWGKPTIVNNVETFANIPAI 450
Syn_02139                   VCGEESSLMNSMEGKRGYPRFRPPFPAGAGFLAKPSNVNNVETYASVPMI 450
Syn_00631                   VCGEETALIESIEGKRGQPRIRPPFPAVEGLYGCPTIVNNVETLASVPFI 450
S. aciditrophicus HydB      VCGEETSLINSMEGKRGYPRVRPPFPAAAGFMGMPSNVNNVETLSSIPMI 450
                            :***.::*: *: * .     :   .  *     *: :**: :        :

T. maritima HydB            LRDGVENYRKRGTE-NSPGTKMFSVAGPLKATGIIEVEFGTTLRDIIYNI 500
C. tencongenesis HydB       ILNGGEWFASIGTE-KSKGTKVFALTGKVNNTGLIEVPMGTTLREIIYEI 500
M. thermoacetica HydB       IRQGWEWFAGIGTE-KSKGTKVFALAGKINNNGLVEIPMGTSLRQVIYEI 500
A. woodii HydB              ILNGAEWFASVGTE-KSKGTKVFALGGKINNTGLLEIPMGTTLREIIYEI 500
R. albus HydB              IRKGAAWYSAMGTE-KSKGTKVFALGGKITNVGLVEIPMGTTLREIIEEI 500
C. acidurici HylB           ILKGADWFTSFGTK-DSPGTKVFALGGKINNTGLVEIPMGTTLREVIYDV 500
C. autoethanogenum HytB     IEKGGDWYHAIGTMEKSKGTKVFSLVGKVKNTGLVEVPMGTTLREIIYDI 500
D. fructosovorans HydB      IINGADWFSGIGTA-TSKGTKVFALAGKIQNVGLIEVPMGISLREVIFDI 500
C. necator FdhB             LARGAQYYRDYGMG-RSRGTLPFQLAGNIKQGGLVEKAFGVTLRELLVDY 500
C. oxalaticus FdhB          LARGAQYYRDYGMG-RSRGTLPFQLAGNIRQGGLVEKAFGVTLRELLVDY 500
M. extorquens FdhB          IERGAEWWKSHGRN-GRVGLRSYSVSGRVKEPGVKLAPAGLTIQELIDEY 500
M. trichosporium FdhB       LAKGGEYYASLGVG-RSRGTMPLQIAGNVKFGGLYETPFGLTLGEIVNDI 500
R. capsulatus FdhB          IAHGAKAYESFGMD-RSRGTIPLQIGGNVKRGGLFETGFGITLGELVEDI 500
E. coli NuoF                LANGVEWYQNISKS-KDAGTKLMGFSGRVKNPGLWELPFGTTAREILEDY 500
T. thermophilus Nqo1        MERGADWFAQMGTE-QSKGMKLYQISGPVKRPGVYELPMGTTFRELIYEW 500
S. wolfei HydB              IEKGADWYASIGNP-AYPGTKVFTLTGDINNRTFFEVPTNTTIREIVFQF 500
Swol_0784                   LTNGADWFASIGAP-KYPGTKVLTLTGDVNNPTYFEVPTNYILSDVIYKL 500
Swol_1024                   IEKGGDWYKAIGAP-NYPGTKVLTLTGDVNNRTFFEVPTNTTIREVIFGL 500
Swol_1828                   VDKGADWYKGIGAA-GYPGTKVMTLTGDIVNRTVIEVPTNTTIRQVLDDF 500
Syn_02139                   IEKGAAWYKSVGLE-TCTGTKLYCLSGKLNRTGLVEMPMGTTLREIIETF 500
Syn_00631                   IEKGAEAFKQWGFE-NNYGFKLFGISGCVNKPGVYEYPLGVSFNELMEAA 500
S. aciditrophicus HydB      VARGADWFKSVGTA-TCAGTKLYCLSGKVNQTGLVELPMGATLRQMIDTF 500
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T. maritima HydB         CGGFVEGEEFKAVQIGGPSGACLS--EDFIDMPLDYDTLKKADAMVGSGG 550
C. tencongenesis HydB    GGGIPGGKKFKAVQIGGPSGGCIP--AELLDTPIDYDSLTSAGAMMGSGG 550
M. thermoacetica HydB    GGGIPGGKKFKAAQTGGPSGGCIP--AEHLDAPIDYENLTALGTIMGSGG 550
A. woodii HydB           GGGIPNGKAFKAAQTGGPSGGCLP--ESLLDTEIDYDNLIAAGSMMGSGG 550
R. albus HydB            GGGIPDGKAFKAAQTGGPSGGCIP--AQHIDTPIDYESLAALGSMMGSGG 550
C. acidurici HylB        GGGIPDGKQFKAVQTGGPSGGCIT--ADYIDTEIDFATLNELGSMMGSGG 550
C. autoethanogenum HytB  GGGVLNDRKFKAVQIGGPSGGCLP--AEYLDLPVDYDTLVKADSMMGSGG 550
D. fructosovorans HydB   GGGCPDGKAFKAVQTGGPSGGALA--NKDLDVAIDYESLAACKSIMGSGG 550
C. necator FdhB          GGGTRSGRAIRAVQVGGPLGAYLP--ESRFDVPLDYEAYAAFGGVVGHGG 550
C. oxalaticus FdhB       GGGTRSGRAIRAVQVGGPLGAYLP--ESRFDVPLDYEAYAAFGGVVGHGG 550
M. extorquens FdhB       CGGISDGHSFAAYLPGGASGGILP--ASMNDIPLDFGTLEKYGCFIGSAA 550
M. trichosporium FdhB    GGGSLSGRPVRAVQCGGPLGAYVP--PSLFDTPFDYEAFQKHDSLIGHGG 550
R. capsulatus FdhB       CGGTASGRPVKAVQVGGPLGAYHP--VSDYHLPFCYEQFAGQGGLVGHAG 550
E. coli NuoF             AGGMRDGLKFKAWQPGGAGTDFLT--EAHLDLPMEFESIGKAGSRLGTAL 550
T. thermophilus Nqo1     AGG--PLEPIQAIIPGGSSTPPLPFTEEVLDTPMSYEHLQAKGSMLGTGG 550
S. wolfei HydB           GGGVPGDKKFKAVQIGGTSGAFIP--DALLDTPVAFDSMSAIGATLGSGA 550
Swol_0784                GGGIKNGRKFKAVQVGGTSGAFIP--EQNLNTSIDFDSMSAIGAALGSGA 550
Swol_1024                GGGVAGGKKFKAVQIGGTSGGFIP--ESNLDTPIDFDSMNSIGAVLGSGA 550
Swol_1828                GGGITGGKKFKAVQIGGTSGGFIP--ESLLDTPIDFDSMSAIGATLGSGA 550
Syn_02139                GKGIKNGKTFKFAQVGGSAGGILG--PELMDLPLDIDSTIKAGVTLGSGV 550
Syn_00631                G-GVKG--KLKAAIVGGLSVAILKA-EELQDLTMDYDSCAKHGTGLGSGG 550
S. aciditrophicus HydB   GGGMKSGSSFKFAQVGGAAGGILG--ADLMDLPLDIDSTIKAGVTLGSGV 550
                              *        .     **             .   .          :* .


T. maritima HydB         IVVITKKTCMVEVARFFLDFTKRESCGKCVPCREGTM-QAYNILEKFTHG 600
C. tencongenesis HydB    LVVMDEDTCMVNVAKFFLEFTVDESCGKCAPCRIGTK-RMLELLDKITSG 600
M. thermoacetica HydB    LIIMDEDTCMVDVAKFFMDFVKDESCGKCTPCRIGTT-RMLEILNRITRG 600
A. woodii HydB           LIVMDEDNCMVDVARFFLDFTQDESCGKCPPCRIGTK-RMLEILERICDG 600
R. albus HydB            LIVMDEDNCMVDVSKFYLNFTVDESCGKCTPCRVGTR-KLLQLLEKITDG 600
C. acidurici HylB        MIVMDEDTCMVDIARFFLDFTVEESCGKCTPCREGTK-RMLELLEKITDG 600
C. autoethanogenum HytB  MIVMDDRTCMVDVTRYYLSFLAEESCGKCVPCREGVK-RMLEILTDICNG 600
D. fructosovorans HydB   MVVMDEDDCMVSVAKFFLDFTMDETCGKCTPCRIGSK-RLYEILDRITKG 600
C. necator FdhB          IVVFDETVDMAKQARYAMEFCAIESCGKCTPCRIGST-RGVEVMDRIIAG 600
C. oxalaticus FdhB       IVVFDETVDMAKQARYAMEFCAIESCGKCTPCRIGST-RGVEVMDRIIAG 600
M. extorquens FdhB       VVILSDQDDVRGAALNLMKFFEDESCGQCTPCRSGTQ-------KARMLM 600
M. trichosporium FdhB    LVVFDDTVDMAQMARFGMEFCAIESCGKCTPCRIGST-RGVETIDRIVEG 600
R. capsulatus FdhB       LVVHDDTADMLKLARFAMEFCAISCGTCTPCRIGAV-RGVEVIDRIAAG 600
E. coli NuoF             AMAVDHEINMVSLVRNLEEFFARESCGWCTPCRDGLP-WSVKILRALERG 600
T. thermophilus Nqo1     VILIPERVSMVDAMWNLTRFYAHESCGKCTPCREGVAGFMVNLFAKIGTG 600
S. wolfei HydB           TFVIDESRDIVDVATRIAGFFEHESCGKCAPCREGTA-RTAELMEKINEG 600
Swol_0784                VLVMDETRDIVDIVTRISKFFEHESCGKCNPCREGTF-RCREIMEKINSG 600
Swol_1024                VFVMDESRDIVNVVARIAKFFEHESCGKCSPCREGTK-RMHEMMERLNAG 600
Swol_1828                FFVMDETRDIVDVIDRISKFFAHESCGKCTPCREGTQ-RMHEMLHRVKNG 600
Syn_02139                VLVCDEDTCPVDFLLDVLNFFEHESCGQCVPCRVGTR-QLHHLARKFATG 600
Syn_00631                IMVISEDFSIPELALRTIKFYAHESCGKCVPCREGSY-TLVKILHKLLSG 600
S. aciditrophicus HydB   VLVCDQETCAVDFLLNVLNFFEHESCGQCIPCKLGTS-QLHYVAQKFAMR 600
                            .   .               *    *:** * **: *
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208

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T. maritima HydB            KATYEDLKTLEHLSKTIKTA-SLCGLGKTAPNPILSTLKLFREEYIAHIE  650
C. tencongenesis HydB       KGEEGDIEKLEELAKTIKAT-ALCGLGQTAPNPVLSTLRYFRHEYEAHIK  650
M. thermoacetica HydB       QGEEKDLDLLVELARQIKDT-ALCGLGQTAPNPVLSTITYFRDEYLAHIR  650
A. woodii HydB              KGVEGDIERLEELAVGIKSS-ALCGLGQTAPNPVLSTIRFFRDEYEAHIR  650
R. albus HydB               KGEMEDLEKIQDLATHMKSS-SLCALGQSAPNPVLSTLQYFGDEYLAHIK  650
C. acidurici HylB           KGEMEDLDRLESLAETIKSS-SLCGLGQTAPNPVLSTLKYFRDEYEAHVK  650
C. autoethanogenum HytB     DGKEGDIEELLEICSMTSKA-SLCSLGKSAPNPVIASIRYFRDEFEEHIK  650
D. fructosovorans HydB      KGTRADLDRLKSLSEIIKDT-ALCGLGQTMPNPILSTMDTFANEYEAHVD  650
C. necator FdhB             EQPVKHVALVRDLCDTMLNG-SLCAMGGMTPYPVLSALNEFPEDFGLASN  650
C. oxalaticus FdhB          EQPVKHVKLVRDLCDTMLYG-SLCAMGGMTPYPVLSALNEFPEDFGLAPN  650
M. extorquens FdhB          ENGVWDTDLLGELAQCMRDA-SICGLGQAASNPVSTVIKYFPDLFPEPRA  650
M. trichosporium FdhB       VDVEANIELLSDLCHTMKFG-SLCALGGFAPYPVESALRHYPEDFRKATL  650
R. capsulatus FdhB          D--ASAMPLLDDLCQTMKLG-SLCALGGFTPYPVQSAIRHFPADFPCARE  650
E. coli NuoF                EGQPGDIETLEQLCRFLGPGKTFCAHAPGAVEPLQSAIKYFREEFEAGIK  650
T. thermophilus Nqo1        QGEEKDVENLEALLPLIEGR-SFCPLADAAVWPVKGSLRHFKDQYLALAR  650
S. wolfei HydB              RGSNKDVALLEKLGTVMSYS-CLCGLGQAAPAPVLTTIKHFKADYEAKFV  650
Swol_0784                   RATEADIDNLLLLARVMKRA-ALCGLGQAAPVPITSTIEHFGHEYRRKLL  650
Swol_1024                   EGNAEDVELLGRLGKVMSVA-CLCGLGQAAPAPVLTTIKNFNADYQAKFN  650
Swol_1828                   GAVAGDLDYMERLGSVMSKA-CLCGLGQAAPAPVLTTLKHFRNDYTAKFN  650
Syn_02139                   TAVPGDLDLMVEKAKLMKN--SLCALGQSPILPITTMLKYFREDFLRHCD  650
Syn_00631                   QGEAADIEKILGICNTVRGL-TLCPTGEAFAVPIQAMVEKFRSEFDALIK  650
S. aciditrophicus HydB      EAEEKDIQLMIDTAKMMKLA-SLCALGQSPILPIETMIRNFREEFVKHCD  650
                                   :         :*  .    *:  :  :    :

T. maritima HydB            G-ECPSGMCTAFKKYVINPDICKGCGLCARSCPQNAITGERGKPYTIDQE  700
C. tencongenesis HydB       EKRCPAGVCTALLSFVIDPEKCKACGICAKNCPVGAISGKPKTPYVIDQE  700
M. thermoacetica HydB       DHRCPAHVCQELLSYVIDAGKCTGCGACSRVCPVGAISGGKKEAHQIDPA  700
A. woodii HydB              DKKCPAGVCKHLLDFKINADTCKGCGICAKKCPADAISGEKKKPYNIDTS  700
R. albus HydB               EKKCPAGVCKNLLQYEIIADKCKGCTLCARNCPANAITGTVKNPHVIDTT  700
C. acidurici HylB           DKKCPAGACQSLLEFYIT-DKCIGCTKCARQCPASCIDGKVKERHVINTS  700
C. autoethanogenum HytB     NKRCRAGVCKKLTTFGIDEDKCKGCDMCKKNCPADCITGEIKKPHTIDAD  700
D. fructosovorans HydB      DKKCPAHVCTALLTYTIDPAKCTGCGLCTRVCPVECISGTKKQPHTIDTT  700
C. necator FdhB             PAKAA---------------------------------------------  700
C. oxalaticus FdhB          PAHAAKAA-----------------------------------------  700
M. extorquens FdhB          VAAE---------------------------------------------  700
M. trichosporium FdhB       PAAAE--------------------------------------------  700
R. capsulatus FdhB          AAE-----------------------------------------------  700
E. coli NuoF                QPFSNTHLINGIQPNLLKERW-----------------------------  700
T. thermophilus Nqo1        EKRPVPRPSLWR-------------------------------------  700
S. wolfei HydB              -------------------------------------------------  700
Swol_0784                   QYA-----------------------------------------------  700
Swol_1024                   -------------------------------------------------  700
Swol_1828                   -------------------------------------------------  700
Syn_02139                   PSLCCPECANSLAHYYRGH-------------------------------  700
Syn_00631                   K-------------------------------------------------  700
S. aciditrophicus HydB      PDYECPQCDASLQAYYL--------------------------------  700
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T. maritima HydB          G-ECPSGMCTAFKKYVINPDICKGCGLCARSCPQNAITGERGKPYTIDQE 700
C. tencongenesis HydB     EKRCPAGVCTALLSFVIDPEKCKACGICAKNCPVGAISGKPKTPYVIDQE 700
M. thermoacetica HydB     DHRCPAHVCQELLSYVIDAGKCTGCGACSRVCPVGAISGGKKEAHQIDPA 700
A. woodii HydB            DKKCPAGVCKHLLDFKINADTCKGCGICAKKCPADAISGEKKKPYNIDTS 700
R. albus HydB             EKKCPAGVCKNLLQYEIIADKCKGCTLCARNCPANAITGTVKNPHVIDTT 700
C. acidurici HylB         DKKCPAGACQSLLEFYIT-DKCIGCTKCARQCPASCIDGKVKERHVINTS 700
C. autoethanogenum HytB   NKRCRAGVCKKLTTFGIDEDKCKGCDMCKKNCPADCITGEIKKPHTIDAD 700
D. fructosovorans HydB    DKKCPAHVCTALLTYTIDPAKCTGCGLCTRVCPVECISGTKKQPHTIDTT 700
C. necator FdhB           PAKAA--------------------------------------------- 700
C. oxalaticus FdhB        PAHAAKAA------------------------------------------ 700
M. extorquens FdhB        VAAE---------------------------------------------- 700
M. trichosporium FdhB     PAAAE--------------------------------------------- 700
R. capsulatus FdhB        AAE----------------------------------------------- 700
E. coli NuoF              QPFSNTHLINGIQPNLLKERW----------------------------- 700
T. thermophilus Nqo1      EKRPVPRPSLWR-------------------------------------- 700
S. wolfei HydB            -------------------------------------------------- 700
Swol_0784                 QYA----------------------------------------------- 700
Swol_1024                 -------------------------------------------------- 700
Swol_1828                 -------------------------------------------------- 700
Syn_02139                 PSLCCPECANSLAHYYRGH------------------------------- 700
Syn_00631                 K------------------------------------------------- 700
S. aciditrophicus HydB    PDYECPQCDASLQAYYL--------------------------------- 700


T. maritima HydB          KCVKCGLCASKCPFKAIELV- 721
C. tencongenesis HydB     KCIKCGTCIDKCPFGAIYKK* 721
M. thermoacetica HydB     ACIKCGSCYEKCRFGAITRE- 721
A. woodii HydB            KCIKCGACIEACPFGSISKA- 721
R. albus HydB             KCIKCGVCMNNCKFGAIIKK- 721
C. acidurici HylB         ACVKCGACADVCPVNAVIKR- 721
C. autoethanogenum HytB   KCLRCGNCMNICKFDAVKVL- 721
D. fructosovorans HydB    RCIKCGACYDKCKFDSIIKQ- 721
C. necator FdhB           -------------------- 721
C. oxalaticus FdhB        -------------------- 721
M. extorquens FdhB        -------------------- 721
M. trichosporium FdhB     -------------------- 721
R. capsulatus FdhB        -------------------- 721
E. coli NuoF              -------------------- 721
T. thermophilus Nqo1      -------------------- 721
S. wolfei HydB            -------------------- 721
Swol_0784                 -------------------- 721
Swol_1024                 -------------------- 721
Swol_1828                 -------------------- 721
Syn_02139                 -------------------- 721
Syn_00631                 -------------------- 721
S. aciditrophicus HydB    -------------------- 721
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