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Proteomic analysis reveals metabolic and regulatory systems involved in the syntrophic and axenic lifestyle of Syntrophomonas wolfei

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Microbial syntrophy is a vital metabolic interaction necessary for the complete oxidation of organic biomass to methane in all-anaerobic ecosystems. However, this process is thermodynamically constrained and represents an ecosystem-level metabolic bottleneck. To gain insight into the physiology of this process, a shotgun proteomics approach was used to quantify the protein landscape of the model syntrophic metabolizer, Syntrophomonas wolfei, grown axenically and syntrophically with Methanospirillum hungatei. Remarkably, the abundance of most proteins as represented by normalized spectral abundance factor (NSAF) value changed very little between the pure and coculture growth conditions. Among the most abundant proteins detected were GroEL and GroES chaperonins, a small heat shock protein, and proteins involved in electron transfer, beta-oxidation, and ATP synthesis. Several putative energy conservation enzyme systems that utilize NADH and ferredoxin were present. The abundance of an EtfAB2 and the membrane-bound iron-sulfur oxidoreductase (Swol_0698 gene product) delineated a potential conduit for electron transfer between acyl-CoA dehydrogenases and membrane redox carriers. Proteins detected only when S. wolfei was grown with M. hungatei included a zinc-dependent dehydrogenase with a GroES domain, whose gene is present in genomes in many organisms capable of syntrophy, and transcriptional regulators responsive to environmental stimuli or the physiological status of the cell. The proteomic analysis revealed an emphasis on macromolecular stability and energy metabolism by S. wolfei and presence of regulatory mechanisms responsive to external stimuli and cellular physiological status.

Keywords: syntrophy, Syntrophomonas wolfei, interspecies electron transfer, reverse electron transfer, hydrogen, methanogenesis

INTRODUCTION

The metabolic cooperation called syntrophy is a thermodynamically-based interaction between two or more microorganisms, which must rely on each other to maintain pool sizes of exchanged metabolites at sufficiently low concentrations so that the overall catabolic conversion is thermodynamically favorable (Schink and Stams, 2006; McInerney et al., 2008). Syntrophy is an essential intermediary step in the anaerobic degradation of natural polymers such as polysaccharides, proteins and lipids where syntrophic associations are needed to convert the products of fermentative microorganisms, fatty acids, alcohols and aromatic compounds, to methanogenic growth substrates acetate, H₂ and formate (McInerney et al., 2008; Stams and Plugge, 2009). Thus, syntrophic fatty acid metabolism accounts for much of the carbon flux and methane production in methanogenic environments (McInerney et al., 2008). Despite the ubiquity of syntrophy processes in anoxic environments, little is known about mechanisms by which syntrophic consortia regulate their metabolism.

S. wolfei is a metabolic specialist that syntrophically metabolizes a very limited number of fatty acids from four to eight carbons in length to acetate, H₂ and formate (McInerney et al., 1979, 1981; Beaty and McInerney, 1987). It can grow axenically on unsaturated fatty acids such as crotonate by oxidizing part of the molecule to acetate and reducing to remainder to the respective saturated fatty acid (Beaty and McInerney, 1987; Amos and McInerney, 1990). However, to oxidize saturated fatty acids, S. wolfei requires the presence of a suitable H₂- and/or formate-consuming partner (i.e., a methanogen) to maintain H₂ and formate at sufficiently low levels so that saturated fatty acid degradation is thermodynamically favorable (Schink, 1997). This allows S. wolfei to reoxidize its reduced electron carriers by forming H₂ and formate rather than by using the unsaturated fatty acid as an electron acceptor. Thus, the interaction between S. wolfei...
and *M. hungatei* during growth on crotonate is beneficial to each species but not obligatory as when *S. wolfei* grows syntrophically with *M. hungatei* on butyrate.

A critical physiological feature of *S. wolfei* during syntrophic growth on saturated fatty acids is the requirement for reverse electron transfer to produce H2 (E′ of −261 mV at 1 Pa H2) and formate (E′ of −258 mV at 1 μM formate) from electrons generated in the oxidation of acyl-CoA intermediates to their respective enoyl-CoA intermediates (E′ of −10 mV) (Sato et al., 1999). This redox reaction is energetically unfavorable (ΔE′ of −250 mV) and requires energy input to drive the reaction forward. The use of inhibitors showed that a chemiosmotic gradient is required for hydrogen production from butyrate (Wallrabenstein and Schink, 1994). A reverse quinone loop involving a membrane-bound, electron transfer flavoprotein (EtfAB):menaquinone oxidoreductase and either a membrane-bound hydrogenase or formate dehydrogenase has been hypothesized to use the proton motive force to produce H2 or formate, respectively, from electrons derived from the oxidation of butyryl-CoA (Schink, 1997; Sieber et al., 2012; Schmidt et al., 2013). The reverse quinone loop model for syntrophic reverse electron transfer is supported by the more than 100-fold higher expression of a membrane-bound hydrogenase, hyd2 (Sieber et al., 2014), and the presence of a membrane-bound formate dehydrogenase, Fdh2 (Schmidt et al., 2013) when *S. wolfei* is grown with *M. hungatei* on butyrate. In addition, a membrane-bound, iron-sulfur protein that may function as an EtfAB:menaquinone oxidoreductase and EtfAB2 were detected in the *S. wolfei* proteome (Schmidt et al., 2013). However, the genome of *S. wolfei* contains other possibilities for reverse electron transfer including the Fix system and a bifurcating, butyryl-CoA dehydrogenase (Bcd):EtfAB1 (Sieber et al., 2010).

Unlike organisms capable of syntrophy such as sulfate and iron reducers, *S. wolfei* cannot use alternative electron acceptors for growth (Sieber et al., 2010). The limited metabolic potential of *S. wolfei* makes it an ideal model organism for identifying the essential machinery of syntrophy, but makes it difficult to use genetic approaches to identify syntrophic processes. The genomes of *S. wolfei* (Sieber et al., 2010) and *M. hungatei* (NCBI Reference Sequence: NC_007796) have been recently sequenced and annotated, which has opened the investigation of syntrophy to high-throughput analyses. Genomic analyses of *S. wolfei* revealed metabolic specialization and nutritional self-sufficiency consistent with its limited metabolic potential (Sieber et al., 2010). Thus, *S. wolfei* appears to be genetically “hard-wired” for syntrophy. As a metabolic specialist that survives on reactions close to thermodynamic equilibrium, we hypothesize that *S. wolfei* is physiologically adapted to fatty acid metabolism and hydrogen and/or formate production and the shift from axenic to syntrophic growth involves a limited number of enzyme systems rather than the large global changes in gene expression that have been detected with sulfate reducers (Meyer et al., 2013a,b). In this study, we used whole cell proteomic analyses of *S. wolfei* grown alone and in coculture with *M. hungatei* grown axenically to identify the major metabolic systems used for axenic and syntrophic lifestyles.

**MATERIALS AND METHODS**

**ORGANISMS AND GROWTH CONDITIONS**

Pure cultures of *Syntrophomonas wolfei* (DSM 2245B) (McInerney et al., 1981) and cocultures of *S. wolfei* with *Methanospirillum hungatei* strain JF1 (ATCC 27890) were grown anaerobically as described previously (McInerney et al., 1979). *S. wolfei* cultures were grown in medium with 20 mM crotonate. *S. wolfei*- *M. hungatei* cocultures were grown in medium with 20 mM crotonate or 20 mM butyrate. Media were prepared using a modified Balch technique (Balch and Wolfe, 1976). All cultures were grown in 75 ml volumes in 160 ml serum bottles in triplicate. The headspace was N2/CO2 (80:20 v/v). All cultures were incubated at 37°C without shaking. Culture purity was checked daily by microscopic examination and inoculation of a thioglycolate medium. Cultures were transferred repeatedly until the growth rate and/or methane production rate among the replicates of a given growth condition were nearly equal. Cells were harvested at 50% substrate loss, which was in the late exponential phase of growth as determined by change in absorbance for pure cultures and methane production for cocultures.

Growth was monitored by measuring optical density at 600 nm. One milliliter of samples was taken daily to measure substrate depletion and product formation. Methane formation by cocultures was measured by daily headspace analysis. Methane was measured by gas chromatography with a flame ionization detection equipped with Porapak Q, 80/100 column (6 feet × 1/8 inch) (Supelco, Bellefonte, PA). The injector temperature was set at 100°C, the column at 100°C and the detector at 125°C. Helium was used as a carrier gas.

The concentrations of crotonate, butyrate, and acetate were determined by high performance liquid chromatography with a Prevail Organic acid column (250 by 4.6 mm; particle size 5 μm; Alltech Inc, Deerfield, Ill.) at a flow rate of 1 ml/min. The isocratic mobile phase consisted of 25 mM KH2PO4 (pH 2.5) to measure acetate concentrations. A mobile phase of 60% (v/v) KH2PO4 (25 mM, pH 2.5):40% (v/v) acetonitrile was used to quantify crotonate and butyrate. The UV absorbance detector was set at 210 nm to detect acetate and butyrate, and 254 nm for crotonate.

**SAMPLE PREPARATION**

Duplicate cultures of the three growth conditions, e.g., *S. wolfei* pure culture on crotonate, *S. wolfei*- *M. hungatei* on crotonate and *S. wolfei*- *M. hungatei* on butyrate, were harvested at 50% substrate loss by centrifugation (14,300 × G, 20 min, 4°C) and processed separately shotgun proteomics analysis. Cell pellet wet weights were 90 mg and 73 mg for *S. wolfei* pure cultures, 105 and 79 mg for *S. wolfei*- *M. hungatei* cocultures on crotonate, and 61 and 67 mg for *S. wolfei*- *M. hungatei* coculture on butyrate. Cell pellets were processed by generally following a protocol optimized for measurements of small bacterial samples (Thompson et al., 2008). Cell pellets were lysed and proteins denatured by incubating each cell pellet overnight at 37°C in 250–400 μL of 6 M guanidine and 10 mM dithiothreitol (DTT) (larger volumes used for larger cell pellets). Lysates were cooled to ambient temperature, and diluted with 50 mM Tris with 10 mM CaCl2.
to decrease the guanidine concentration to \( \sim 1 \text{ M} \). Ten micro-
grams of trypsin (sequencing grade, Promega, Madison WI) was
added to each lysate, followed by a 5-h incubation at 37°C. An
additional 10 μg trypsin was added, followed by a further
overnight incubation at 37°C. Remaining disulfide bonds were
reduced by adding additional DTT to a final concentration of
10 mM and incubation for 1 h at 37°C. Desalting was performed
using reverse-phase solid-phase extraction cartridges (Sep-Pak
Lite C18, Waters, Milford MA), with final elution using 0.1%
formic acid in acetonitrile. Solvent transfer to aqueous 0.1%
formic acid was performed by vacuum centrifugation, with final
volume adjusted to 150 μL. Particulates and remaining cellular
debris were removed by centrifugation through 0.45 μm pore fil-
ters (Ultrafree-MC, Millipore, Billerica MA). Samples were frozen
at \(-80°C\) until further use.

**LC-MS-MS Analysis**

Tryptic peptide mixtures were analyzed by two-dimensional liq-
uid chromatography/tandem mass spectrometry (2D LC-MS-
MS), using the MudPIT approach (Washburn et al., 2001; Wolters
et al., 2001) implemented as previously described in further detail
(Hervey et al., 2009). Two LC-MS-MS analyses were performed
on the tryptic digest from each cell pellet. Thus, for each growth
condition, two technical replicates were analyzed for each of the
two biological replicates. Aliquots (50 μL) were loaded via a pres-
sure cell (New Objective, Woburn MA) onto a “back” column
fabricated from 150 μm internal diameter (ID) fused silica tubing
(Polymicro Technologies, Phoenix AZ) packed with a \( \sim 4 \text{ cm} \)
long bed of reverse-phase chromatographic phase (Jupiter C18, 3 μm
datale size, Phenomenex, Torrance CA) upstream of a \( \sim 4 \text{ cm} \)
bed of strong cation exchange material (5 μm particle size SCX,
Phenomenex).

After sample loading, the back column was attached via a filter
union (Upchurch Scientific, Oak Harbor WA) to a “front” analyt-
cal column fabricated from a 100 μm ID PicoTip Emitter (New
Objective), packed with a \( \sim 14 \text{ cm} \) bed of reverse-phase material
(Jupiter C18, 3 μm particle size, Phenomenex). Two-dimensional
LC was performed via 12 step gradients of increasing salt (ammo-
nium acetate) concentration, with the eluted peptides from each
strong cation exchange step subsequently resolved via a sepa-
rate reverse-phase gradient (Accela HPLC, ThermoScientific, San
Jose CA). The LC eluent was interfaced via a nanospray source
(Proxeon, Odense, Denmark) with a linear-geometry quadrupole
ion trap mass spectrometer (LTQ-XL, ThermoScientific, San Jose
CA). Data acquisition was performed in data-dependent mode
under the control of XCalibur software. Up to five tandem mass
spectra were acquired from the most abundant parent ions in full-
scan mass spectra; dynamic exclusion was enabled with a repeat
count of 1 and duration of 60 s.

**Proteomics Data Analysis**

Peptide identifications were obtained from tandem mass spectra
using Sequest software (version 27) (Eng et al., 1994), and protein
identifications were compiled from peptide identifications using
DTASelect (version 1.9) (Tabb et al., 2002). A multiple-species
protein FASTA file was constructed from individual FASTA files
for S. wolfei subspecies wolfei Göttingen, M. hungatei JF-1, and
*Syntrophus aciditrophicus* strain SB downloaded from the DOE
Joint Genome Institute website. The sequence-reversed analog
of each protein sequence was appended to the FASTA file to allow
estimation of the false discovery rate of peptide identification
(Moore et al., 2002; Elias and Gygi, 2007). Sequences of 36 com-
mon contaminant proteins were also appended to the FASTA file.
The complete FASTA file contained 18006 entries. Peptide identi-
fications were retained for XCorr \( \geq 1.8 \) (charge state \( z = 1 \)), \( \geq 2.5 \)
(\( z = 2 \)), or \( \geq 3.5 \) (\( z = 3 \)), with DeltaCN \( \geq 0.08 \). Protein identifica-
tions required identification of two peptides, or a single peptide
in two different charge states. The false discovery rate for pep-
tides was generally \( \leq 1\% \). Estimates of protein abundance were
Calculated using normalized spectral abundance factors (NSAF)
(Zybailov et al., 2006).

Non-metric multidimensional scaling (NMDS) was per-
formed using Bray-Curtis dissimilarities of NSAFs from different
growth conditions. Bray-Curtis utilizes a shared presence absence
matrix to score similarity, which is why it has been well used in
ecology to assess the similarity of two communities. Rare or
missing proteins were included within the analysis as these may
have biological significance, with the caveat that if a protein was
detected in only in a single replicate of a treatment it was left
out. NMDS was calculated using the vegan package (Oksanen
et al., 2011) as implemented in R (R Development Core Team, 2011).

**Results and Discussion**

**Proteomic Overview of S. Wolfei**

The repertoire of proteins involved in syntrophic and axenic
growth of S. wolfei was characterized by growing S. wolfei
in pure culture on crotonate and in coculture with *M. hungatei*
on either crotonate or butyrate (Supplemental Figure 1). The
genome of *S. wolfei* contains 2574 protein encoding genes
(Sieber et al., 2010) and the proteomic analysis detected a
total of 790 proteins among the three growth conditions. Of
these, 106 are proteins without a known function (Supplemental
Data Set 1).

NMDS ordination using distance metrics! missing proteins were included within the analysis as these may have biological significance, with the caveat that if a protein was detected in only in a single replicate of a treatment it was left out. NMDS was calculated using the vegan package (Oksanen et al., 2011) as implemented in R (R Development Core Team, 2011).

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of which 113 appear to be constitutively present (i.e., less than 0.5-fold change among all conditions; Supplementary Table 1). Four open reading frames previously predicted to be pseudogenes (Swol_0818, Swol_1580, Swol_2335, and Swol_2574) were found to be protein encoding. Remarkably, the protein abundance, as represented by NSAF of most proteins changed very little between the pure and coculture growth conditions (Figure 2).

**HIGHLY ABUNDANT PROTEINS DURING ALL GROWTH CONDITIONS**

Highly abundant proteins were those involved in major pathways and processes within the cell. Nine proteins had an NSAF greater than 0.01 under all three growth conditions including two chaperonins (GroEL and GroES), one small heat shock protein (Swol_0588 gene product), two paralogs of the DNA-binding proteins HU, rubrerythrin (a protein employed during oxidative stress), two transcription factors, and the Swol_0133 gene product, annotated as a putative copper amine oxidase (Table 1).

The Swol_0133 gene product is a predicted cytoplasmic protein although a role in cell envelope function was recently proposed (Schmidt et al., 2013). Other abundant proteins had annotated functions involved in beta-oxidation, electron transfer and energy production (Table 1). GroEL and GroES were among the most abundant proteins in *Escherichia coli* (See datasets in Lu et al., 2007; Mancuso et al., 2012). The types of abundant proteins detected emphasize the importance of macromolecular stability and energy metabolism to *S. wolfei*.

**BETA-OXIDATION ENZYMES**

The abundance of beta-oxidation proteins reflect the metabolic specialization of *S. wolfei* as a bacterium that metabolizes short-chain, saturated and unsaturated fatty acids (Figure 3) (McInerney et al., 1979). Genomic analysis showed that *S. wolfei*’s genome contained multiple paralogs for each step in beta-oxidation (Sieber et al., 2010) and our whole-cell proteome analysis showed that *S. wolfei* expressed and translated multiple, paralogous, beta-oxidation enzymes. Seven acyl-CoA dehydrogenases were detected in the proteome (Figure 3; Supplemental Data Set 2). The Swol_2052 gene product was the most abundant acyl-CoA dehydrogenase and was detected in all growth conditions. Swol_2052 and Swol_1933 gene products were detected in the dominant Bcd activity purified from butyrate-grown *S. wolfei* cells (Müller et al., 2009) and subsequent proteomic analysis detected these two Bcds in crotonate-grown pure cultures and butyrate-grown cocultures of *S. wolfei* (Schmidt et al., 2013). Multiple CoA transferases, enoyl-CoA dehydratases, 3-hydroxyacyl-CoA dehydrogenases and acetyl-CoA acetyltransferases, whose abundance varied with growth condition, also were detected (Figure 3; Supplemental Data Set 2). Interestingly, gene products corresponding to a set of adjacent beta-oxidation genes and an acetate kinase (Swol_1483-1486) were not detected under any growth condition. A 3-hydroxybutyryl-CoA dehydrogenase (Swol_2030 gene product) was among the more abundant proteins detected, suggesting a specific role in oxidation/reduction when crotonate is metabolized in the absence of a suitable partner.

**INTERSPECIES ELECTRON TRANSFER PROTEINS**

Interspecies electron transfer is necessary for degradation of butyrate (Schink, 1997), and depending on the growth condition, *S. wolfei* can utilize either hydrogen (Sieber et al., 2014) or formate (Schmidt et al., 2013). The *S. wolfei* genome contains three hydrogenases and five formate dehydrogenases (Sieber et al., 2010). All three hydrogenases were detected in all growth conditions (Figure 3; Supplemental Data Set 2), suggesting the reoxidation of reduced electron carriers (NADH, reduced flavoproteins, and reduced ferredoxin) may involve different enzyme systems. The detected hydrogenases include the electron confurcating hydrogenase, Hyd1, which is predicted to use NADH and ferredoxin; a ferredoxin-dependent hydrogenase (Hyd3); and a membrane-bound hydrogenase (Hyd2), which may interact with the quinone pool. In contrast, the abundance of the four detected formate dehydrogenases was much lower than that of the hydrogenases (Figure 3; Supplemental Data Set 2). Hydrogenases were abundant in this study, but formate dehydrogenases were abundant.
butyrate-grown, protein was present in highly purified, Bcd preparations from abundant than Swol_0696-Swol_0698 gene products (could function in reverse electron transfer but these were less butyrate. 

et al., 2013). Swol_0696-Swol_0698 gene products likely form a complex (Swol_2121 and Swol_2122 gene products), FixX, a bound FeS oxidoreductase (Swol_0698), which is postulated to all growth conditions (Schmidt et al., 2013; Sieber et al., 2014), suggesting that relative importance of interspecies hydrogen vs. formate transfer depends on growth condition.

### PROTEINS NECESSARY FOR REVERSE ELECTRON TRANSFER

Swol_0697 and Swol_0696 gene products, which comprise the Etf complex EtfAB2, were among the most abundant proteins in the proteome in all growth conditions (Table 1; Figure 3), consistent with previous proteomic work (Schmidt et al., 2013). Adjacent to Swol_0696 and Swol_0697 is a gene for a membrane-bound FeS oxidoreductase (Swol_0698), which is postulated to be an EtfAB:menaquinone oxidoreductase (Sieber et al., 2012). The Swol_0698 gene product was highly abundant in under all growth conditions (Figure 3, Schmidt et al., 2013). This protein was present in highly purified, Bcd preparations from butyrate-grown, S. wolfei cells (Müller et al., 2009; Schmidt et al., 2013). Swol_0696-Swol_0698 gene products likely form a complex that functions to reduce menaquinone with electrons derived from acyl-CoA intermediates when S. wolfei grows on butyrate.

Proteomic analysis detected additional protein systems that could function in reverse electron transfer but these were less abundant than Swol_0696-Swol_0698 gene products (Figure 3). The Fix complex consists of FixAB, which is the EtfAB complex (Swol_2121 and Swol_2122 gene products), FixX, a ferredoxin (Swol_2123 gene product) and FixC, an Etf:quinone oxidoreductase (Swol_2124 gene product). The Fix proteins were ten-fold less abundant than the Swol_0696-Swol_0698 gene products under all growth conditions, suggesting that Fix may not function as a major catabolic system. However, it could function to supply reduced ferredoxin for biosynthetic processes, e.g., pyruvate synthesis from acetyl-CoA and CO₂ or hydrogen or formate production by confurcating hydrogenases and formate dehydrogenases (Figure 3). A heterodisulfide reductase (Hdr) was also detected in the proteome. In hydrogenotrophic methanogens, this enzyme couples the unfavorable reduction of ferredoxin with electrons from hydrogen or formate to the favorable reduction of CoM-S-S-CoB heterodisulfide with electrons derived from hydrogen or formate (Costa et al., 2010; Kaster et al., 2011). We also detected proteins (Swol_0400 and Swol_402 gene products) encoded by the genes adjacent to hdrABC. The Swol_0402 gene product annotates as a FAD and NAD⁺-binding oxidoreductase while the Swol_0400 gene product annotates as an iron-sulfur protein. Lastly, Swol_0266-Swol_0268 gene products could function as an electron bifurcating BcdEtfAB1 complex to produce reduced ferredoxin from crotonyl-CoA and NADH (Li et al., 2008).

Key proteins needed for ATP synthesis were also abundant in all growth conditions (Figure 3; Table 1). Given that S. wolfei lacks respiratory systems to create a proton motive force (Sieber et al., 2012), we suggest that the ATP synthase

### Table 1 | The most abundant peptides detected in each condition.

<table>
<thead>
<tr>
<th>Locus tag</th>
<th>Gene description</th>
<th>Crotonate</th>
<th>Crotonate with M. hungatei</th>
<th>Butyrate with M. hungatei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swol_0047</td>
<td>Transcriptional regulator, AbrB family</td>
<td>0.010 ± 0.002</td>
<td>0.011 ± 0.004</td>
<td>0.011 ± 0.001</td>
</tr>
<tr>
<td>Swol_0063</td>
<td>DNA-binding protein HU</td>
<td>0.008 ± 0.0004</td>
<td>0.011 ± 0.003</td>
<td>0.007 ± 0.002</td>
</tr>
<tr>
<td>Swol_0133</td>
<td>Copper amine oxidase</td>
<td>0.027 ± 0.002</td>
<td>0.042 ± 0.010</td>
<td>0.026 ± 0.003</td>
</tr>
<tr>
<td>Swol_0435</td>
<td>3-hydroxybutyril-CoA dehydrogenase</td>
<td>0.026 ± 0.002</td>
<td>0.002 ± 0.000</td>
<td>0.006 ± 0.001</td>
</tr>
<tr>
<td>Swol_0436</td>
<td>Coenzyme A transferase</td>
<td>0.011 ± 0.0004</td>
<td>0.008 ± 0.002</td>
<td>0.005 ± 0.001</td>
</tr>
<tr>
<td>Swol_0588</td>
<td>Small heat shock protein</td>
<td>0.011 ± 0.001</td>
<td>0.015 ± 0.004</td>
<td>0.011 ± 0.002</td>
</tr>
<tr>
<td>Swol_0648</td>
<td>DNA-binding protein HU</td>
<td>0.009 ± 0.0003</td>
<td>0.012 ± 0.002</td>
<td>0.008 ± 0.001</td>
</tr>
<tr>
<td>Swol_0670</td>
<td>Ruberythrin</td>
<td>0.008 ± 0.001</td>
<td>0.010 ± 0.003</td>
<td>0.006 ± 0.001</td>
</tr>
<tr>
<td>Swol_0696</td>
<td>Electron transfer flavoprotein β-subunit</td>
<td>0.022 ± 0.003</td>
<td>0.022 ± 0.005</td>
<td>0.010 ± 0.001</td>
</tr>
<tr>
<td>Swol_0697</td>
<td>Electron transfer flavoprotein α-subunit</td>
<td>0.016 ± 0.001</td>
<td>0.021 ± 0.004</td>
<td>0.009 ± 0.001</td>
</tr>
<tr>
<td>Swol_0767</td>
<td>Phosphate acetyltransferase</td>
<td>0.009 ± 0.0003</td>
<td>0.010 ± 0.001</td>
<td>0.008 ± 0.0003</td>
</tr>
<tr>
<td>Swol_0768</td>
<td>Acetate kinase</td>
<td>0.007 ± 0.001</td>
<td>0.013 ± 0.002</td>
<td>0.008 ± 0.001</td>
</tr>
<tr>
<td>Swol_1190</td>
<td>Molybdenum-pterin-binding protein</td>
<td>0.007 ± 0.001</td>
<td>0.011 ± 0.003</td>
<td>0.007 ± 0.001</td>
</tr>
<tr>
<td>Swol_1244</td>
<td>Polyhydroxyalkanoate synthesis regulator</td>
<td>0.001 ± 0.001</td>
<td>0.027 ± 0.003</td>
<td>0.023 ± 0.002</td>
</tr>
<tr>
<td>Swol_1727</td>
<td>Zn-dependent dehydrogenase</td>
<td>ND</td>
<td>0.019 ± 0.004</td>
<td>0.028 ± 0.005</td>
</tr>
<tr>
<td>Swol_1855</td>
<td>60 kDa chaperonin GROEL</td>
<td>0.042 ± 0.001</td>
<td>0.027 ± 0.004</td>
<td>0.014 ± 0.001</td>
</tr>
<tr>
<td>Swol_1856</td>
<td>10 kDa chaperonin GROES</td>
<td>0.049 ± 0.004</td>
<td>0.031 ± 0.007</td>
<td>0.022 ± 0.003</td>
</tr>
<tr>
<td>Swol_2030</td>
<td>3-hydroxybutyril-CoA dehydrogenase</td>
<td>0.021 ± 0.002</td>
<td>0.003 ± 0.001</td>
<td>0.005 ± 0.001</td>
</tr>
<tr>
<td>Swol_2051</td>
<td>Acetyl-CoA acetyltransferase</td>
<td>0.035 ± 0.001</td>
<td>0.010 ± 0.001</td>
<td>0.010 ± 0.0005</td>
</tr>
<tr>
<td>Swol_2148</td>
<td>Branched-chain amino acid aminotransferase</td>
<td>0.004 ± 0.001</td>
<td>0.006 ± 0.001</td>
<td>0.009 ± 0.002</td>
</tr>
<tr>
<td>Swol_2296</td>
<td>Hypothetical protein</td>
<td>0.005 ± 0.001</td>
<td>0.012 ± 0.001</td>
<td>0.010 ± 0.001</td>
</tr>
<tr>
<td>Swol_2382</td>
<td>Sodium-transporting two-sector ATPase</td>
<td>0.009 ± 0.0004</td>
<td>0.012 ± 0.002</td>
<td>0.008 ± 0.001</td>
</tr>
<tr>
<td>Swol_2386</td>
<td>FoF₁-type ATP synthase subunit B</td>
<td>0.012 ± 0.001</td>
<td>0.010 ± 0.0004</td>
<td>0.009 ± 0.002</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not detected.

when S. wolfei was grown under different growth conditions (Schmidt et al., 2013; Sieber et al., 2014), suggesting that relative importance of interspecies hydrogen vs. formate transfer depends on growth condition.
(Swol_2381-Swol_2388) most likely functions to hydrolyze ATP to create the proton motive force.

**IDENTIFICATION OF PROTEINS EXCLUSIVE TO INTERSPECIES INTERACTIONS**

We identified 15 *S. wolfei* proteins unique to interspecies interactions with *M. hungatei* (Supplemental Table 2; Supplemental Figure 2). The genes for these proteins are distributed throughout the chromosome and are not co-localized or within a genomic island. A putative zinc-dependent dehydrogenase (Swol_1727 gene product) was among the most abundant proteins detected (NSAF > 0.02) when *S. wolfei* was grown with *M. hungatei* on either crotonate or butyrate (Table 1; Figure 2). The high abundance of this gene product is surprising because *S. wolfei* is not known to degrade or produce alcohols (McInerney et al., 1979). Interestingly, Swol_1727 contains a GroES chaperonin domain and its deduced amino acid sequence is very similar (BLAST E-value of 5e-104) to that of SYN_01269 found in another syntrophic metabolizer, *Syntrophus aciditrophicus* (McInerney et al., 2007). Swol_1727 orthologs are also found in other sequenced syntrophic metabolizers, regardless of phylogenetic lineage, including *Syntrophobacter fumaroxidans*, *Pelotomaculum thermopropionicum*, *Syntrophothermus lipocalidus*, and *Syntrophobotulus glycolicus*. The abundance of the Swol_1727 gene product in the proteome of *S. wolfei* when grown with *M. hungatei* and the occurrence of closely related genes in genomes of organisms known to be capable of syntrophic metabolism suggests that it has an important function in syntrophy.

Analysis of the other 14 proteins exclusive to interspecies interactions did not reveal any feature suggestive of unique interspecies interactions (Supplemental Table 2). Two proteins have annotated functions in beta-oxidation (Swol_1935 and Swol_1936 gene products) and one has an annotated function in poly-(3-hydroxyalkanoate) synthesis [poly-(3-hydroxyalkanoic acid) synthase, Swol_1241 gene product]. Two proteins with unknown function (Swol_1036 and Swol_2364 gene products) were detected. Swol_1036 has a nucleotidylltransferase domain and Swol_2364 has a nucleoside triphosphate pyrophosphohydrolase domain, suggesting housekeeping functions. Other proteins detected have predicted functions in cell biosynthesis (Swol_0643, Swol_0965, Swol_0975, Swol_1727, Swol_1958, and Swol_1851 gene products), energy production (Swol_1030 gene product) and replication (Swol_0001 and Swol_0002 gene products).

Eighty-three *S. wolfei* proteins were unique to the syntrophic growth on butyrate where the activity of *M. hungatei* is obligatory (Supplemental Figure 2; Supplemental Table 3). The function of 33 of these proteins is unknown (Supplemental Table 3).
NADH/NAD product is a redox sensitive transcriptional regulator, which in other acid metabolism and transport based on COG functional classification of these regulatory proteins suggests the importance of sensing environmental and physiological signals during interspecies interactions.

Seven putative transcriptional regulatory proteins were detected only in butyrate-grown \textit{S. wolfei} cells. The function for many of these is unknown, but they likely serve important roles in modulating the physiological responses of \textit{S. wolfei} required for syntrophic growth. The \textit{Swol_1040} gene product is signal transduction histidine kinase that contains domains similar to those of an Fe-only hydrogenase and a ferredoxin. The \textit{Swol_1040} may be part of a two-component regulator involved in the regulation of hydrogen production. \textit{Swol_0456} gene product is one of three paralogous proteins in \textit{S. wolfei} that has PAS, sigma-54 and DNA-binding domains. PAS domains function as signal input modules in proteins that sense environmental stimuli by detecting changes in the electron transport system (Taylor and Zhulin, 1999). The \textit{Swol_1645} gene product is a redox sensitive transcriptional regulator, which in other organisms modulates transcription in response to shifts in the NADH/NAD$^+$ ratio (Brekasis and Paget, 2003; Gyan et al., 2006). Five receiver only domain proteins were also identified. The detection of these regulatory proteins suggests the importance of sensing environmental and physiological signals during interspecies interactions.

Other proteins detected only in butyrate-grown cells included proteins involved in amino acid metabolism and transport, lipid metabolism and transport, nucleotide metabolism and transport and cofactor transport and metabolism (Supplemental Table 3). The number of peptides assigned to proteins involved in amino acid metabolism and transport based on COG functional classification was higher in butyrate-grown, coculture \textit{S. wolfei} cells than crotonate-grown, pure culture \textit{S. wolfei} cells (Supplemental Figure 3). The importance of biosynthetic capability in slow growing syntrophic coculture was unexpected. Interestingly the up-regulation of genes involved in amino acid synthesis has been detected in cocultures of termite gut spirochetes (Rosenthal et al., 2013; Sieber et al., 2014), suggesting that relative importance of interspecies hydrogen vs. formate transfer depends on growth condition. A GroES domain-containing, zinc-dependent dehydrogenase (\textit{Swol_1727} gene product) and several transcriptional regulators, responsive to environmental stimuli or cellular physiological status, were detected when \textit{S. wolfei} was grown the \textit{M. hungatei}. Overall, the proteomic analysis revealed an emphasis energy metabolism and macromolecular stability by the metabolic specialist, \textit{S. wolfei}, and the involvement of regulatory proteins responsive to environmental and physiological signals during interspecies interactions.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: [http://www.frontiersin.org/journal/10.3389/fmicb.2015.00115/abstract](http://www.frontiersin.org/journal/10.3389/fmicb.2015.00115/abstract)

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Nine proteins unique to \textit{S. wolfei}-\textit{M. hungatei} coculture growth on crotonate were detected (Supplemental Table 4). Here, the presence of \textit{M. hungatei} is not obligatory for crotonate metabolism by \textit{S. wolfei}. The proteins found exclusively during coculture growth on crotonate include a transcriptional regulator with a HD-GYP domain, which may function as a phosphodiesterase to control cyclic nucleotide levels (Marinez et al., 2002), a putative NAD(P)H-flavin oxidoreductase function (\textit{Swol_1523} gene product), and three proteins with unknown functions (Supplemental Table 3).

This extensive proteomic analysis defines the physiological response of \textit{S. wolfei} to the syntrophic lifestyle. NMDS analysis showed that \textit{S. wolfei} adjusted its physiology in response to the methanogen. An uncharacterized, membrane-bound iron-sulfur oxidoreductase and EtfAB2 were abundant under all growth conditions and may provide the conduit for electron transfer between Bcd and the menaquinone pool. Reoxidation of menaquinol by a membrane-bound hydrogenase (Hyd2) provides a mechanism for the reverse electron transfer of electrons derived from butyryl-CoA oxidation to hydrogen using the proton motive force (Figure 3). Hydrogenases were abundant in this study, but formate dehydrogenases were abundant when \textit{S. wolfei} is grown under different growth conditions (Schmidt et al., 2013; Sieber et al., 2014), suggesting that relative importance of interspecies hydrogen vs. formate transfer depends on growth condition. A GroES domain-containing, zinc-dependent dehydrogenase (\textit{Swol_1727} gene product) and several transcriptional regulators, responsive to environmental stimuli or cellular physiological status, were detected when \textit{S. wolfei} was grown the \textit{M. hungatei}. Overall, the proteomic analysis revealed an emphasis energy metabolism and macromolecular stability by the metabolic specialist, \textit{S. wolfei}, and the involvement of regulatory proteins responsive to environmental and physiological signals during interspecies interactions.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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