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### **Title**

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**The Electron Transfer System of Syntrophically Grown *Desulfovibrio vulgaris***

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Running Title: SRM Syntrophic Metabolism

1 **ABSTRACT**

2 Interspecies hydrogen transfer between organisms producing and consuming hydrogen  
3 promotes the decomposition of organic matter in most anoxic environments. Although  
4 syntrophic couplings between hydrogen producers and consumers are a major feature of  
5 the carbon cycle, mechanisms for energy recovery at the extremely low free energies of  
6 reactions typical of these anaerobic communities have not been established. In this study,  
7 comparative transcriptional analysis of a model sulfate-reducing microbe, *Desulfovibrio*  
8 *vulgaris* Hildenborough, suggested the use of alternative electron transfer systems  
9 dependent upon growth modality. During syntrophic growth on lactate with a  
10 hydrogenotrophic methanogen, *D. vulgaris* up-regulated numerous genes involved in  
11 electron transfer and energy generation when compared with sulfate-limited  
12 monocultures. In particular, genes coding for the putative membrane-bound Coo  
13 hydrogenase, two periplasmic hydrogenases (Hyd and Hyn) and the well-characterized  
14 high-molecular weight cytochrome (Hmc) were among the most highly expressed and  
15 up-regulated. Additionally, a predicted operon coding for genes involved in lactate  
16 transport and oxidation exhibited up-regulation, further suggesting an alternative pathway  
17 for electrons derived from lactate oxidation during syntrophic growth. Mutations in a  
18 subset of genes coding for Coo, Hmc, Hyd and Hyn impaired or severely limited  
19 syntrophic growth but had little affect on growth via sulfate-respiration. These results  
20 demonstrate that syntrophic growth and sulfate-respiration use largely independent  
21 energy generation pathways and imply that understanding of microbial processes  
22 sustaining nutrient cycling must consider lifestyles not captured in pure culture.  
23

## 1 INTRODUCTION

2 Nutrient cycling on earth is determined primarily by cooperative interactions among  
3 microorganisms. The sharing of available energy within communities is particularly  
4 important in anaerobic systems, where limited energy is divided among highly  
5 specialized and metabolically interdependent populations (36, 37, 39). In the absence of  
6 exogenous electron acceptors such as sulfate and nitrate, the mineralization of organic  
7 matter in anoxic environments yields primarily carbon dioxide and methane - a process  
8 controlled by the synergistic activities of multiple anaerobic microbial populations. To  
9 better understand the metabolic basis and ecological significance of these syntrophic  
10 associations, we constructed an archetypical "community of two" by pairing  
11 *Desulfovibrio vulgaris* Hildenborough with a hydrogenotrophic methanogen,  
12 *Methanococcus maripaludis* strain S2.

13

14 *D. vulgaris* is a representative of sulfate-reducing microorganism, coupling the oxidation  
15 of characteristic substrates such as H<sub>2</sub>, lactate or ethanol with the reduction of sulfate to  
16 sulfide (for review see (32). In the absence of sulfate, *D. vulgaris* (and SRM in general)  
17 ferment organic acids and alcohols, producing hydrogen, acetate and carbon dioxide by  
18 forming syntrophic associations with hydrogen consuming populations (3, 23, 40). These  
19 alternative lifestyles might be sustained by distinct metabolic systems, possibly reflected  
20 in part by the large number of hydrogenases and electron transfer complexes described in  
21 past biochemical studies and more recently revealed in the *D. vulgaris* Hildenborough  
22 genome sequence (14, 32). Thus, even though sulfate-respiration and syntrophic growth  
23 both produce the same oxidized end products (acetate and carbon dioxide), mechanistic  
24 differences in electron transfer pathways likely exist. These differences were evaluated  
25 by comparing whole-genome transcriptional profiles of *D. vulgaris* Hildenborough grown

1 continuously on lactate under two culture conditions: syntrophic cocultures (lacking  
2 sulfate) and sulfate-limited monocultures. Complementary mutant studies showed that  
3 among genes highly up-regulated during syntrophic growth, at least two (coding for the  
4 Coo hydrogenase and the high-molecular weight cytochrome complex) were required for  
5 efficient syntrophic growth but not for sulfate respiration.

6

## 7 **MATERIALS AND METHODS**

### 8 *Strains*

9 Transcriptional analyses were performed using *Desulfovibrio vulgaris* Hildenborough  
10 and *Methanococcus maripaludis* S2. Additionally, four mutant strains (3 described in  
11 previous investigations and 1 described here) of *D. vulgaris* were used during phenotypic  
12 growth comparisons. Details of all six strains are provided in Table 1.

13

### 14 *Biomass production*

15 Three biological replicates of cocultures and sulfate-limited *D. vulgaris* monocultures  
16 were grown in a chemostat in coculture medium (CCM) containing 30 mM sodium DL-  
17 lactate (coculture and monoculture) and 10 mM Na<sub>2</sub>SO<sub>4</sub> (monoculture only). CCM also  
18 contained a basal salt solution consisting of the following components per liter: 2.17 g  
19 NaCl, 5.5 g MgCl<sub>2</sub>•6H<sub>2</sub>O, 0.14 g CaCl<sub>2</sub>•2H<sub>2</sub>O, 0.5 g NH<sub>4</sub>Cl and 0.335 g KCl. The  
20 medium was buffered using 1.1 mM of K<sub>2</sub>HPO<sub>4</sub> and 30 mM NaHCO<sub>3</sub> with 1 ml of  
21 nonchelated trace elements (46) and 1 ml of vitamin solution amended with 2.0 g/l  
22 choline chloride (2) added as growth supplements. L-cysteine•HCl (1 mM) and sulfide  
23 (1 mM Na<sub>2</sub>S•9H<sub>2</sub>O) were added as reducing agents. Resazurin (1 mg/L) was added as a  
24 redox indicator. Stock solutions of K<sub>2</sub>HPO<sub>4</sub> (1 M), NaHCO<sub>3</sub> (6.0 M), L-cysteine•HCl (1  
25 M), Na<sub>2</sub>S•9H<sub>2</sub>O (1 M) and the nonchelated trace elements and vitamin mixtures were

1 prepared under anoxic conditions. Medium was made by adding salts, sodium DL-  
2 lactate, resazurin and Na<sub>2</sub>SO<sub>4</sub> (if applicable) to water, then autoclaving. After  
3 sterilization, the chemostat or medium reservoir bottle was connected to N<sub>2</sub>:CO<sub>2</sub> (90:10)  
4 and allowed to cool to room temperature before adding the remaining components.  
5  
6 A 1 ml glycerol stock of previously grown coculture or monoculture was used to  
7 inoculate 100 ml of CCM (amended with sulfate for monocultures) in a 200 ml serum  
8 vial. Cultures were incubated in the dark at 37 °C with a shaking speed of 250 rpm.  
9 When the cultures reached an O.D.<sub>600</sub> of 0.27 ± 0.01, they were transferred to a 3 L  
10 FairMenTec chemostat (Wald, Switzerland) filled with 2 L of CCM (amended with  
11 sulfate for monocultures). Following inoculation, the chemostat was run in batch mode  
12 at 37°C with a stirring speed of 250 rpm. The pH was maintained at 7.0 – 7.2 via  
13 bicarbonate buffer and small automated additions of 0.1 M NaOH or HCl as needed. A  
14 blanket of N<sub>2</sub>:CO<sub>2</sub> (90:10) gas mixture was flushed through a sterile cotton plug before  
15 entering the headspace of the reactor, with the flow rate maintained at 0.20 ml/min using  
16 an Alicat Scientific mass controller (MC-20SCCM-D, Tucson, AZ). Headspace  
17 concentrations of CH<sub>4</sub>, CO<sub>2</sub>, H<sub>2</sub>, H<sub>2</sub>S, O<sub>2</sub> and N<sub>2</sub> were monitored at 30 min intervals  
18 using a Hiden Analytical QIC-20 mass spectrometer (Warrington, U.K.). Lactate,  
19 acetate, ethanol, glycerol and formate were measured enzymatically as previously  
20 described (40). Continuous culture operation was initiated after absorbance  
21 measurements reached approximately 0.27 (OD<sub>600</sub>). A dilution rate of 0.039 h<sup>-1</sup> was  
22 maintained and biomass harvested when the variance of O.D.<sub>600</sub> readings was less than  
23 10% over a period of three retention periods. Samples were taken regularly for direct cell  
24 counts and protein measurements. *Desulfovibrio*:*Methanococcus* cell ratios were  
25 determined through DAPI-stained cell counts. Total protein measurements were

1 determined using the Coomassie Plus Assay (Pierce, Rockford, IL). Cells were harvested  
2 using an ice-chilled sterile stainless steel tube connected to the chemostat medium  
3 exhaust line. Culture fluid was transferred to Falcon tubes (50 ml) that had been stored in  
4 an anoxic chamber and pre-chilled on ice. The tubes were centrifuged for 15 min at  
5 3,220 x g at 4°C. After centrifugation, the supernatant was poured off and tubes  
6 immediately frozen at -80 °C.

7

#### 8 *Transcriptional analysis*

9 Whole genome microarrays containing 3,482 of the 3,531 protein-coding sequences for  
10 *D. vulgaris* Hildenborough were synthesized as previously described (4) onto UltraGAPS  
11 glass slides (Corning Life Sciences, Corning, NY) using a BioRobotics Microgrid II  
12 microarrayer (Genomic Solutions, Ann Arbor, MI). Each slide contained duplicate spots  
13 for each protein-coding sequence and each biological replicate was hybridized to at least  
14 three slides. Thus each log<sub>2</sub> expression level described within this study were obtained  
15 from triplicate biological replicates, each with at least six technical replicates (duplicate  
16 on-chip technical replicates and at least three microarray slide replicates).

17

18 RNA isolation, quantification and transcription were performed as previously described  
19 (4). Briefly, total cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad,  
20 CA), purified using the RNeasy Mini kit (Qiagen, Valencia, CA) with on-column DNase  
21 digestion using an RNase-free DNase set (Qiagen, Valencia, CA). The cDNA probes  
22 were generated from 10 µg of purified total RNA using reverse transcriptase and then  
23 labeled (43). Random hexamers (Invitrogen, Carlsbad, CA) were used for priming and  
24 the fluorophore Cy5-dUT (Amersham Biosciences, Piscataway, NJ) was used for  
25 labeling. After labeling, RNA was removed by NaOH treatment and cDNA immediately

1 purified using a Qiagen PCR Mini kit. Genomic DNA was extracted from cell pellets  
2 using the Qbiogene FastDNA SPIN Kit for Soil (Mp Biomedicals, Solon, OH). Extracted  
3 gDNA was labeled with the fluorophore Cy3-dUTP (Amersham Biosciences,  
4 Piscataway, NJ). Labeling efficiencies were routinely monitored by measuring  
5 absorbances at 260 nm (for DNA concentrations), 550 nm (for Cy3) or 650 nm (for Cy5).  
6  
7 Cy3-dUTP-labeled genomic DNA for *D. vulgaris* was aliquoted for triplicate arrays and  
8 co-hybridized with Cy5 labeled cDNA (41, 47). Co-hybridization using dried probes  
9 mixed and resuspended in 35-40  $\mu$ l of hybridization solution containing 50% (v/v)  
10 formamide, 5x saline-sodium citrate (SSC; 1x SSC is 0.15 M NaCl, 0.015 M sodium  
11 citrate, pH 7.0), 0.1% (w/v) sodium dodecyl sulfate (SDS) and 0.1 mg/ml herring sperm  
12 DNA (Invitrogen, Carlsbad, CA). The hybridization solution was incubated at 95 to 98°C  
13 for 5 min, centrifuged briefly, incubated at 50°C and applied onto microarray slides.  
14 Hybridization was carried out in hybridization chambers (Corning Life Sciences,  
15 Corning, NY) at 45°C overnight (16-20 h). At each end of the microarray slide, 10  $\mu$ l of  
16 3x SSC solution was added to maintain proper humidity and probe hydration. Slides  
17 were washed twice in a solution containing 2x SSC and 0.1% (w/v) SDS at 42°C for 5  
18 min, twice in a solution containing 0.1x SSC and 0.1% (w/v) SDS at room temperature  
19 for 10 min and twice in 0.1x SSC at room temperature for 1 min. After drying under a  
20 stream of N<sub>2</sub>, the slides were scanned for Cy5 and Cy3 fluorophores using the ScanArray  
21 Express microarray analysis system (Perkin Elmer, Waltham, MA). Fluorescence  
22 intensities for each spot were determined using 16-bit TIFF scanned images and  
23 quantified with ImaGene software (v 6.0, Biodiscovery, Marina Del Rey, CA). Any spot  
24 with fewer than 75% of pixels or more than 3 standard deviations above the local  
25 background in both channels was rejected (10).



1  
2 For each array, signal intensities, spot quality and background intensities of each spot  
3 were quantified with ImaGene software (v 6.0, Biodiscovery, Marina Del Rey, CA).  
4 Computational analyses to determine the expression ratios, log ratios, Z scores, as well  
5 operon-based estimates of local accuracy were done as previously described (4, 25).  
6 Briefly, the  $\log_2$  expression was normalized globally by calculating the net signal for each  
7 spot. This was done by subtracting the background and adding a pseudosignal of 100,  
8 thereby enforcing a positive value. If the resulting net signal was less than 50, a value of  
9 550 was used. Following this, the expression levels for each spot were calculated from  
10 the ratio of the mRNA over gDNA (Cy3 channel over Cy5 channel). Expression levels  
11 for each replicate were normalized such that the total expression over the present spots  
12 was identical. Mean expression levels and standard deviations of each spot were  
13 estimated, requiring  $n > 1$ , where  $n$  is the number of scorable replicates. To estimate the  
14 differential gene expression between the control and treatment conditions, normalized log  
15 ratios were calculated. Each log ratio was calculated as  $\log_2(\text{coculture}) -$   
16  $\log_2(\text{monoculture})$ . This log ratio was normalized using locally weighted scatterplot  
17 smoothing (LOWESS) on the difference versus the sum of the log expression level (7).  
18 Since sector-based artifacts were detected, each log ratio was further normalized by  
19 subtracting the median of all spots within each sector. The final normalized log ratio  
20 ( $\log_2R$ ) was calculated from the average ratio of spots for each gene. The significance of  
21 the normalized log ratio was assessed using a Z score calculated as follows (where 0.25 is  
22 a pseudovariance term):

23

$$Z = \frac{\log_2(\text{coculture/monoculture})}{\sqrt{0.25 + \sum \text{variance}}}$$

24 Z scores were determined using operon-based estimates of local accuracy as a guide,  
25 where each point represents a group of 100 predicted significant changers with similar Z

1 scores. The estimated accuracy of each changer group was derived by inspecting other  
2 genes in the same operon as these changers. For random changers, the transcripts for  
3 50% of these genes should have been regulated in the same direction, while for perfect  
4 changers 100% of the genes should have been regulated in the same direction. Members  
5 of the operons without a consistent signal across replicates ( $Z < 0.25$ ) were excluded.  
6 The operon-based estimates of local accuracy calculated for this experiment suggested  
7 absolute values of Z scores greater than 1.0 signified statistically significant up- or down-  
8 regulation (Supplemental Figure 1).

9

### 10 *Mutant construction*

11 The generation of the *hmc*, *hyd*, and *hyn* deletion mutants have been previously described  
12 (6, 11, 31). The *coolL* transposon mutant was generated by conjugation between *D.*  
13 *vulgaris* and *E. coli* BW20767 (pRL27) (19). The conjugation protocol was a modified  
14 method of Fu and Voordouw (9). Briefly, cultures of *D. vulgaris* were grown to mid-  
15 exponential phase, and combined in a 3:1 or 6:1 ratio with the *E. coli* donor grown to  
16 early exponential phase in LC medium (1.0% [wt/vol] tryptone, 0.5% [wt/vol] yeast  
17 extract, and 0.5% [wt/vol] NaCl). Mating mixtures were concentrated by centrifugation.  
18 The concentrated cells were placed onto filter discs (0.22  $\mu\text{m}$  pore diameter, GSWP,  
19 Millipore Billerica, MA), the discs placed on the surface of solidified LS4 (LS4D with  
20 1% [wt/vol] yeast extract added), and incubated for sixteen hours at 34°C (25). The cells  
21 were then washed from the membrane with 2 ml LS4 medium. After six hours of  
22 incubation, antibiotic G418 (400  $\mu\text{g/ml}$ ) was added to select for the transposon mutants  
23 and nalidixic acid (200  $\mu\text{g/ml}$ ) was added to select against the *E. coli* donor. Cells were  
24 then spread onto LS4 agar (100 - 500  $\mu\text{l/plate}$ ) containing both antibiotics and incubated  
25 in an anaerobic growth chamber at 34°C for at least four days for colony growth.

1  
2 The chromosomal localization of the transposon insertions was identified by sequencing  
3 DNA after semi-random PCR amplification using a variation of a previously described  
4 protocol (5). One microliter of a 50- $\mu$ l boiled single-colony suspension in distilled H<sub>2</sub>O  
5 was used as the template DNA in a 20- $\mu$ l PCR mixture containing primer tpnRL17-1 (5'-  
6 AAC AAG CCA GGG ATG TAA CG-3') and either primer CEKG 2A (5'-GGC CAC  
7 GCG TCG ACT AGT AC(N)<sub>10</sub> AGA G-3'), CEKG 2B (5'-GGC CAC GCG TCG ACT  
8 AGT AC(N)<sub>10</sub> ACG CC-3') or CEKG 2C (5'-GGC CAC GCG TCG ACT AGT AC(N)<sub>10</sub>  
9 GAT AT-3'). One microliter of a 1:5 dilution of this reaction mixture was used as the  
10 template DNA for a second PCR performed with primers tpnRL17-2 (5'-AGC CCT TAG  
11 AGC CTC TCA AAG CAA-3') and CEKG 4 (5'-GGC CAC GCG TCG ACT AGT AC-  
12 3'). Thermocycler conditions were as previously described (5). Samples that produced  
13 distinct PCR products on an agarose gel after the second reaction were cleaned with a  
14 PCR purification kit (Qiagen, Valencia, CA) and sequenced by using primer tpnRL17-1.  
15 The chromosomal locations of the insertions were identified by BLAST analysis of the  
16 sequences adjacent to the transposon.

17

### 18 *Phenotypic growth analyses*

19 All phenotypic growth assays were carried out in 17 ml Hungate tubes equipped with  
20 rubber stoppers and screw-tops. Cultures were incubated at 37 °C in the dark with a 300  
21 rpm shaking speed. Each tube contained 8 ml of CCM amended with 30 mM of electron  
22 donor (lactate or pyruvate) and 30 mM of sulfate (if applicable). The headspace  
23 contained an overpressure of 180 mbar of N<sub>2</sub>:CO<sub>2</sub> (80:20). Dilution series out to 10<sup>-8</sup>  
24 were initiated from 1 ml glycerol stocks of each *Desulfovibrio* mutant and  
25 *Methanococcus*. *Desulfovibrio* cultures were grown in CCM amended with 30 mM

1 sulfate. *Methanococcus* cultures were grown in CCM lacking lactate and amended with 5  
2 mM acetate and 250 mbar overpressure of H<sub>2</sub>:CO<sub>2</sub> (80:20). Cocultures were established  
3 by combining 1 ml of exponentially growing *Desulfovibrio* and 0.5 ml of exponentially  
4 growing *Methanococcus* from the highest dilutions. Cocultures were transferred (1%  
5 v/v) three times to ensure dilution of any residual sulfate/acetate or H<sub>2</sub> before inoculating  
6 triplicate tubes for growth experiments. Tubes were monitored for growth using O.D.<sub>600</sub>  
7 readings blanked against uninoculated medium. Biomass concentrations were estimated  
8 using the previously published conversion values of 1.0 O.D.<sub>600</sub> = 0.385 g dry coculture  
9 biomass/L (40) and 1.0 O.D.<sub>600</sub> = 0.309 g dry monoculture biomass/L (45). Growth  
10 yields were estimated using the maximum O.D.<sub>600</sub> value, which corresponded with  
11 complete consumption of the 30 mM electron donor present. Errors represent the  
12 standard deviation of triplicate samples. Lactate-to-pyruvate, lactate<sub>coculture</sub>-to-  
13 lactate<sub>monoculture</sub> and pyruvate<sub>monoculture</sub>-to-pyruvate<sub>monoculture</sub> growth yield ratios were  
14 calculated using these estimated yields. The estimated yields and ratios were compared  
15 with previously published values of *D. vulgaris* grown in monoculture and in coculture  
16 with *Methanosarcina barkeri* (44).

17

## 18 RESULTS

19 Continuous cultures of syntrophically grown *D. vulgaris* and *M. maripaludis* on 30 mM  
20 lactate (without sulfate) were maintained using a dilution rate of 0.039 hr<sup>-1</sup> (Figure 1).  
21 Steady-state concentrations of lactate ranged from 3 – 5 mM during continuous  
22 operation, indicating nearly complete utilization of the 30 mM addition. Acetate (24-27  
23 mM) was the principal by-product produced, although small quantities of ethanol (0.02-  
24 0.1 mM) were detected throughout batch and continuous culture. Glycerol and formate,  
25 other potential by-product of lactate fermentation, were not detected at the 0.1 mM limit

1 of detection. Methane production began within 12 hours of inoculation and remained  
2 relatively stable during steady-state growth, although some fluctuations were routinely  
3 observed. These fluctuations occurred immediately after initiation of continuous culture  
4 operations and were likely a result of pressure differentials contained in the sampling  
5 inlet, not biological variation. The gas concentration values displayed in Figure 1 are  
6 lower than the predicted stoichiometry of lactate oxidation, reflecting loss as dissolved  
7 methane in the liquid effluent and in the headspace by N<sub>2</sub>/CO<sub>2</sub> gas flow during operation  
8 of the reactor. The methane values primarily served to assess steady-state operation. A  
9 *Desulfovibrio*-to-*Methanococcus* cell ratio of 4:1 was maintained during steady-state  
10 growth.

11

12 Whole-genome transcriptional analysis revealed highly divergent profiles between  
13 *Desulfovibrio* growing in coculture versus sulfate-limited monoculture (Supplemental  
14 Tables 1 and 2). Syntrophically grown *D. vulgaris* up-regulated 169 open-reading frames  
15 (ORFs) and down-regulated 254 ORFs compared with sulfate-limited monocultures  
16 grown at the same generation time. ORFs were considered to have statistically  
17 significant up- or down-regulation if the absolute value of the Z-score was greater than  
18 1.0, as determined through operon-based estimates of local accuracy (Supplemental  
19 Figure 1). Clustering into orthologous groups (COGs) showed that those associated with  
20 energy production and conservation were the most highly up-regulated, both by  
21 proportion and number (20% and 42 respectively, see Supplemental Figure 2). The  
22 largest number of down-regulated genes were in the signal transduction group (46 out of  
23 273) and inorganic ion transport and metabolism (23 out of 105). Every COG exhibited  
24 at least one statistically significant changer, based on an absolute Z-score of greater than  
25 one.

1  
2 Among the largest expression increases were in operons coding for three multisubunit  
3 transmembrane proteins associated with electron transfer reactions: the high-molecular  
4 weight cytochrome (Hmc, DVU0531-6), a cytoplasmic hydrogenase (Coo, DVU2286 –  
5 93,  $\log_2R = 1.1-1.7$ ), and a putative heterodisulfide reductase (Hdr, DVU2399-405,  $\log_2R$   
6 = 1.0-1.6). Significant increases in expression were also observed for genes coding for a  
7 transmembrane three-subunit molybdopterin-oxidoreductase (DVU0692-4;  $\log_2R = 1.0$ ),  
8 two periplasmic hydrogenases (*hydAB*, DVU1769-70 and *hynAB-1*, DVU1921-2;  $\log_2R =$   
9 1.0-2.0), an alcohol dehydrogenase (Adh, DVU2405,  $\log_2R = 3.0$ ), and an aldehyde-  
10 ferredoxin oxidoreductase (Aor, DVU1179;  $\log_2R = 1.9$ ). Only two genes known to be  
11 associated with electron transfer reactions were significantly down-regulated, a  
12 flavodoxin gene (DVU2680,  $\log_2R = -5.2$ ) and an adjacent hypothetical gene  
13 (DVU2681).

14  
15 Syntrophy was also associated with changes in the transcription of genes in a predicted  
16 operon (DVU3024-DVU3033) coding for lactate uptake and oxidation. The enzymes in  
17 this pathway are predicted to produce acetate, CO<sub>2</sub>, ATP, and reduced electron carriers  
18 (lactate permease, DVU3026; a putative lactate dehydrogenase related to the membrane-  
19 bound glycolate oxidase of *E. coli*, DVU3027-3028; a monomeric pyruvate:ferredoxin  
20 oxidoreductase, DVU3025; phosphate acetyltransferase, DVU3029; acetate kinase,  
21 DVU3030). The lactate permease and pyruvate oxidase in this operon-like arrangement  
22 were clearly up-regulated ( $\log_2R = 1.2$  and 2.0, respectively) during syntrophic growth, as  
23 was a second lactate permease (DVU2285,  $\log_2R = 1.4$ ) located up-stream and directly  
24 adjacent to the gene for the Coo hydrogenase. The genes coding for the lactate permease  
25 (DVU3026) and contiguous lactate dehydrogenase (DVU3027-3028) are conserved in

1 another lactate-oxidizing syntroph (*Pelomaculum thermopropionicum*) in an operon-like  
2 arrangement of the same gene order as in *D. vulgaris* Hildenborough (18).

3

4 Despite trace concentrations of sulfate in the growth medium, key genes of sulfate  
5 respiration (ATP sulfurylase, adenylyl-sulfate reductase, dissimilatory sulfite reductase,  
6 pyrophosphatase, and thiosulfate reductase) were also up-regulated during syntrophic  
7 growth, consistent with previous observations of constitutive expression (13, 49).

8 However, none of the sulfate permeases were up-regulated and one (DVU0053) was  
9 significantly down-regulated.

10

11 Although most differentially expressed genes (ca. 400) have no assigned function  
12 (lipoproteins, hypotheticals and conserved hypotheticals), some of the more highly up-  
13 regulated have homologs (possible orthologs) in the genomes of characterized bacterial  
14 syntrophs. For example, comparative analysis of one up-regulated hypothetical gene  
15 cluster (DVU2648-55) found no informative BLAST matches except for DVU2655  
16 encoding a putative D-alanyl-D-alanine carboxypeptidase (*dacA*). The *D. vulgaris* *DacA*  
17 shares highest similarity to proteins in *Syntrophobacter fumaroxidans* and *Syntrophus*  
18 *aciditrophicus* (1). Another cluster of up-regulated and highly expressed ORFs of  
19 unknown function (DVU0144-50) share high similarity to genes found in *S.*  
20 *fumaroxidans* (SFUM0625-9).

21

22 Complementary analyses of *D. vulgaris* mutants with deletions or disruptions in the *coo*,  
23 *hmc*, *hyd* and *hyn* operons (all up-regulated during growth in coculture) provided direct  
24 evidence for function in syntrophy. These mutants affected almost exclusively the  
25 capacity for syntrophic growth, either greatly inhibiting ( $\Delta cooL$  and  $\Delta hmc$ ) or slowing

1 and slightly inhibiting ( $\Delta hyd$  and  $\Delta hyn-1$ ) growth in coculture (Figure 2, Table 2).  
2 Notably, among these mutants, only the  $\Delta hmc$  strain showed slightly impaired respiratory  
3 growth (approximately 60% maximum cell density when compared with wildtype). The  
4  $\Delta cooL$  and  $\Delta hmc$  mutants were capable of only sparse growth in coculture on lactate,  
5 with a maximum cell density approximately 10% of the wildtype. However, when grown  
6 in coculture on pyruvate, only the  $\Delta hmc$  mutant was impaired (< 15% maximum cell  
7 density of wildtype). Cocultures established with the  $\Delta hyd$  and  $\Delta hyn-1$  mutants achieved  
8 cell densities comparable to the wildtype on both lactate and pyruvate but at reduced  
9 growth rates. Recovery of *Methanococcus* from all cocultures through addition of H<sub>2</sub> and  
10 acetate demonstrated an active hydrogenotrophic population, confirming attribution of  
11 the observed growth defects to mutations in *Desulfovibrio*.

12

### 13 **DISCUSSION**

14 A conceptual model for electron transfer during syntrophic growth that captures the  
15 transcription and mutant data is shown in Figure 3, providing a framework for the  
16 following discussion of the electron transfer reactions and energetics of syntrophic  
17 growth. Lactate is transported from the periplasm via a dedicated lactate permease  
18 (DVU3026) and oxidized to pyruvate by a putative lactate dehydrogenase (DVU3027-8),  
19 likely functions primarily during syntrophic growth. The extracted electrons reduce an  
20 unknown electron carrier and are shuttled to the Coo hydrogenase, subsequently forming  
21 H<sub>2</sub> while concomitantly translocating protons (or sodium) across the cytoplasmic  
22 membrane. Pyruvate is oxidized by the pyruvate-oxidoreductase, generating reduced  
23 ferredoxin. The membrane associated Hmc complex then couples the oxidation of  
24 reduced ferredoxin to the reduction of a periplasmic cytochrome and/or hydrogenases  
25 (Hyn-1 and Hyd), yielding hydrogen as a final product.



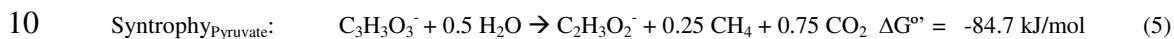
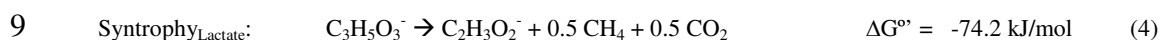
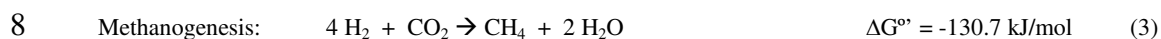
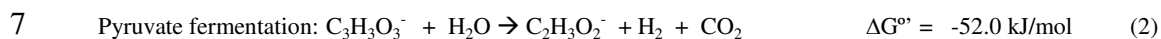
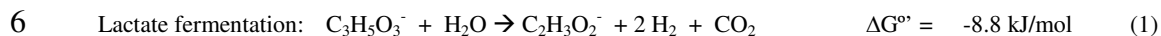
1

2 The standard free energy yields for syntrophic growth on either lactate or pyruvate are

3 well above the generally accepted minimum energy needed to support the two

4 populations (36), but lower for growth on lactate than pyruvate (Equations 4 and 5).

5



11

12 Using the concentrations observed during steady-state ([lactate] = 4 mM, [acetate] = 26

13 mM,  $[H_2] = 2.5 \cdot 10^{-5} \text{ atm}$ ,  $[CO_2] = 0.05 \text{ atm}$ ,  $[CH_4] = 0.0006 \text{ atm}$ ,  $T = 310 \text{ K}$ ) the free

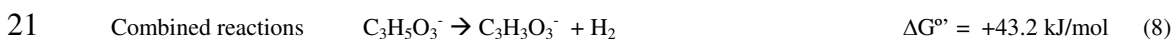
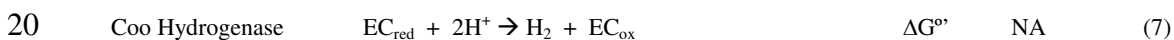
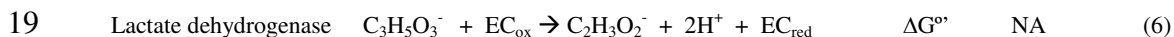
14 energy yields for lactate fermentation (-67.3 kJ/mol, Equation 1) and syntrophic growth

15 on lactate (-82.8 kJ/mol, Equation 4) become more favorable. The lower free energy

16 available for growth on lactate (Equation 4) is determined primarily by the energy cost of

17 the two-electron oxidation of lactate to pyruvate and hydrogen (Equation 8).

18



22

23 Reverse electron flow is thought necessary to sustain lactate oxidation to pyruvate during

24 respiratory growth (42). This cost is also reflected by the lower biomass yield on lactate

25 versus pyruvate for either growth modality (Table 2), as previously observed for *D.*26 *vulgaris* Hildenborough paired with a different methanogen (44). Since syntrophic

27 growth on lactate provides considerably less energy than is available through respiration,

1 we anticipated mechanistic differences in electron transfer reactions governing the initial  
2 two-electron oxidation of lactate.

3

4 These mechanistic differences were further suggested by the global up-regulation of  
5 genes associated with energy conservation and electron transfer during syntrophic  
6 growth. Up-regulation of genes in a predicted operon coding for lactate uptake and  
7 oxidation suggested that the immediate fate of electrons derived from lactate oxidation  
8 differs for syntrophic and respiratory growth. Notably, this lactate dehydrogenase is  
9 homologous to a membrane-bound glycolate oxidase in *E. coli* that is directly coupled to  
10 the electron transport chain (20, 26, 34). Additionally, the *coo* genes are up-regulated  
11 and encode for a protein homologous to those found in other *Bacteria* and *Archaea* that  
12 function as proton (or sodium) translocating hydrogenases, strongly suggesting a similar  
13 electrogenic role in *Desulfovibrio* (8, 16, 22, 35). Another highly up-regulated  
14 transmembrane protein (Hmc) likely shuttles electrons from the cytoplasm to/from  
15 soluble periplasmic carriers such as cytochrome  $c_3$  (28, 33), thus providing a possible link  
16 between cytoplasmic oxidation and periplasmic hydrogenases. There is no evidence that  
17 the Hmc has a function in direct proton translocation.

18

19 Mutants in a subset of these up-regulated genes served to confirm a direct involvement in  
20 syntrophy. The growth phenotype of the  $\Delta cooL$  mutant is of particular significance in the  
21 proposed mechanism of syntrophic growth. This mutant affected only the lactate-grown  
22 coculture, having no affect on respiratory growth with either lactate or pyruvate, nor any  
23 significant affect on the pyruvate-grown coculture. As represented by the model  
24 presented in Figure 3 and reactions 6 and 7, electrons derived from the oxidation of  
25 lactate may be shuttled via an undefined electron carrier (EC, likely the quinone pool) to

1 the Coo hydrogenase. The combined reaction (reaction 8) is only favorable at very low  
2 concentrations of H<sub>2</sub> and pyruvate. Continuous consumption of these compounds either  
3 internally (pyruvate) or externally via the methanogen (hydrogen) contributes to a more  
4 energetically favorable condition for continued lactate oxidation. Since internal  
5 concentrations of lactate and pyruvate likely remain relatively stable, fluctuations in the  
6 hydrogen concentration primarily governs the thermodynamic feasibility of lactate  
7 oxidation. Increases in hydrogen concentration inhibit lactate oxidation by preventing the  
8 Coo hydrogenase from re-oxidizing the electron carrier. For example, at the steady-state  
9 hydrogen concentrations observed (5-6 Pa) and assuming no contribution of reverse  
10 electron flow, lactate oxidation ceases at an intracellular lactate:pyruvate ratio of  
11 approximately 1000. However, without measurements of intracellular metabolite  
12 concentrations it is not possible to constrain the requirement for reverse electron flow in  
13 the initial oxidation of lactate, as shown by the absence of free. If reverse electron flow  
14 is required for lactate oxidation, the Coo hydrogenase might provide a mechanism – if  
15 this hydrogenase uses the PMF to evolve hydrogen (rather than functioning to export  
16 protons as depicted in Figure 3).

17

18 Since a mutant in the Coo hydrogenase impairs only growth on lactate in coculture, this  
19 hydrogenase appears to be part of a dedicated system for syntrophic growth on lactate. A  
20 similar function is indicated for the Hmc, required for the reoxidation of reduced  
21 ferredoxin generated by the activity of a pyruvate-ferredoxin oxidoreductase (Por,  
22 DVU3025) (30). This activity accounts for the defective growth of this mutant in  
23 coculture on both lactate and pyruvate, with the reduced impact upon respiratory growth  
24 previously attributed to compensation by alternative transmembrane electron carriers  
25 (e.g., the Tmc and Rnf complexes) apparently specific to sulfate-reduction (6, 29, 45).

1 Both the  $\Delta hyd$  and  $\Delta hyn-1$  mutants demonstrated small differences in maximum cell  
2 density during syntrophic growth on both lactate and pyruvate, possibly because  
3 alternative periplasmic hydrogenases (Hyn-2 and Hys) masked these lesions. Their  
4 impact upon the overall coculture, but not monoculture, growth rate suggests incomplete  
5 compensation by the two alternative hydrogenases under this growth modality. The  
6 differential coculture growth rates observed likely result from the varied affinities and  
7 activities of the Hyd and Hyn-1 hydrogenases that would affect only the hydrogen  
8 production rate (27).

9

10 Some evidence suggests a role for Hdr in ethanol production and consumption (13). In  
11 association with Adh and Aor, it may reduce acetyl-CoA and produce the small quantities  
12 of ethanol produced during syntrophic growth (Figure 3, orange box). As observed in  
13 other SRM, this pathway could transiently serve as an alternative electron transport  
14 mechanism to maintain redox balance during periods of elevated hydrogen concentration  
15 (21). However, both accumulation of toxic by-products such as ethanol or acetaldehyde,  
16 and the lack of energy recovered from acetyl-CoA reduction to ethanol, make this  
17 alternative pathway unfavorable for continued growth..

18

19 Although many genes encoding electron transfer functions are up-regulated, the single  
20 mostly highly down-regulated gene in coculture codes for a flavodoxin ( $\log_2 R = -5.2$ ).  
21 The down-regulation of this gene appears to be related to a general down-regulation of  
22 genes involved in iron and metal uptake when *D. vulgaris* is grown in coculture. This is  
23 almost certainly an indirect consequence of the absence of appreciable sulfide production  
24 by syntrophically grown *Desulfovibrio*. The formation of metal sulfides during growth  
25 by sulfate respiration greatly reduces metal availability, as reflected by the higher

1 expression levels in monoculture of genes for iron transport (*feoA*, *feoB*, *tonB*),  
2 molybdenum uptake (*modB*), and zinc uptake (*znuAB*; see Supplemental Table 2). A  
3 related flavodoxin in the cyanobacterium *Anabaena* replaces ferredoxin as electron  
4 carrier from photosystem I to ferredoxin-NADP<sup>+</sup> reductase under iron-deficient  
5 conditions (12). Thus, although the *Desulfovibrio* flavodoxin has been implicated as a  
6 constitutive component of the sulfate reduction pathway, its highly repressed expression  
7 in coculture suggests that it may function primarily under conditions of iron-limitation  
8 (17). Chemotaxis-related functions also comprise a general category of genes that tend to  
9 be down regulated in coculture (e.g., *cheA*, *cheY*, and multiple genes coding for methyl-  
10 accepting chemotaxis proteins [MCPs]; Supplemental Table 2), although one MCP  
11 (DVU0344) was up-regulated about 5-fold. Similar down-regulation of genes for MCPs  
12 was observed for *Rhizobium leguminosarum* following its differentiation into a plant-  
13 associated symbiotic bacteroid, suggesting that the transition from monoculture to  
14 coculture by the *Desulfovibrio* is also associated with comparable physiological change  
15 (48).

16

17 In contrast to the down-regulation of many genes for chemotaxis, several genes encoding  
18 for parts the flagellar system basal body and filament (*flgC*, *flgB*, *flgL*; Supplemental  
19 Table 2) are significantly up-regulated in coculture. Although we have no immediate  
20 explanation for these expression trends, recent studies of a similar syntrophic couple  
21 between a bacterium (*Pelotomaculum thermopropionicum*) and an archaeon  
22 (*Methanothermobacter thermautotrophicus*) have shown that the bacterial flagellum  
23 mediates physical association between the two species (38). In addition to promoting a  
24 close physical association thought to enhance syntrophy, the presence of the filament cap  
25 protein (FliD) alone induced expression of methanogen genes required for syntrophic

1 growth. Thus, available data are highly suggestive of specific metabolic and protein-  
2 mediated systems of communication between evolutionarily unrelated species of  
3 microorganisms.

4

5 The existence of independent electron transfer systems for syntrophic versus respiratory  
6 growth in *Desulfovibrio* also has relevance to the evolution of microorganisms  
7 functioning primarily as syntrophs. The evolutionary history of “obligate” syntrophs is  
8 closely intertwined with that of sulfate-reducers, with syntrophs appearing to have  
9 diverged on more than one occasion from sulfate-reducing ancestry. Independent energy  
10 conservation pathways functioning during sulfate-respiration and syntrophy may have  
11 permitted this evolution. The genomes of *Syntrophus aciditrophicus* (a Gram-negative  
12 deltaproteobacterium) and *Pelotomaculum thermopropionicum* (related to gram-positive  
13 *Desulfotomaculum* species) tentatively support this hypothesis as both contain  
14 homologues of enzymes functioning in the syntrophic growth of *Desulfovibrio* -  
15 including Coo and Hmc, electron transfer, and ferredoxin recycling (18, 24). More  
16 importantly, *P. thermopropionicum* and several related species contain vestiges of an  
17 ancestral sulfate-reducing pathway, suggesting relatively recent adaptation to low sulfate  
18 environments (15). While environmental fluctuations of sulfate likely contributed  
19 towards evolution of an independent “syntrophic” metabolism, stable anaerobic  
20 environments lacking terminal electron acceptors may promote genomic loss of  
21 functional abilities, such as observed in the facultative syntrophs *P. thermopropionicum*  
22 and *P. schinkii*. These stable environments may assist in developing more specialized  
23 ecological niches, increasingly segregating independent energy generation pathways  
24 within divergent microbial species.

25

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10 Microbes Online ([www.microbesonline.org](http://www.microbesonline.org)) and under GEO reference ###.

1 **Figure 1.** Representative biomass (squares), hydrogen (diamonds) and methane (crosses)  
2 profiles for a single biological coculture replicate. Continuous culture was started at hour  
3 72 (arrow).

4

5 **Figure 2.** (A) Growth curves of wildtype and mutant *D. vulgaris* cocultures on lactate.  
6 Error bars represent standard deviation of triplicate cultures. (B) Maximum cell densities  
7 (as determined by O.D.<sub>600</sub> measurements) of wildtype and mutant *D. vulgaris* cocultures  
8 and monocultures. Maximum cell densities values represent the largest O.D.<sub>600</sub> readings  
9 observed after exponential growth. Legend symbols refer to growth curves in (A);  
10 shading refers to cell densities in (B).

11

12 **Figure 3.** Proposed metabolic model for syntrophic growth for *D. vulgaris*  
13 Hildenborough. Color scheme refers to transcriptional changes of individual genes  
14 during coculture growth versus sulfate-limited monoculture. EC represents an unknown  
15 electron carrier interacting with Ldh. The lactate permease is represented by DVU3026.  
16 Abbreviations: Ldh – lactate dehydrogenase (likely DVU3027), Por –  
17 pyruvate:ferredoxin oxidoreductase (DVU3025), Pta – phosphate acetyltransferase  
18 (DVU3029), Ack – acetate kinase (DVU3030), Aor – aldehyde:ferredoxin  
19 oxidoreductase (DVU1179), Adh – alcohol dehydrogenase (DVU2405), Hdr – putative  
20 heterodisulfide reductase (DVU2399 – 2404), Fd – reduced or oxidized ferredoxin, Coo –  
21 cytoplasmic hydrogenase (DVU2286-93), Hmc – high molecular weight cytochrome  
22 complex (DVU0531-6), Hyn1 – [NiFe] hydrogenase isozyme 1 (DVU1921-2), Hyd –  
23 [Fe] hydrogenase (DVU1769-70). The red box highlights unique lactate oxidation  
24 enzymes functioning during syntrophic growth. The orange box depicts the proposed  
25 hypothetical pathway of ethanol production (via hydrogen consumption).



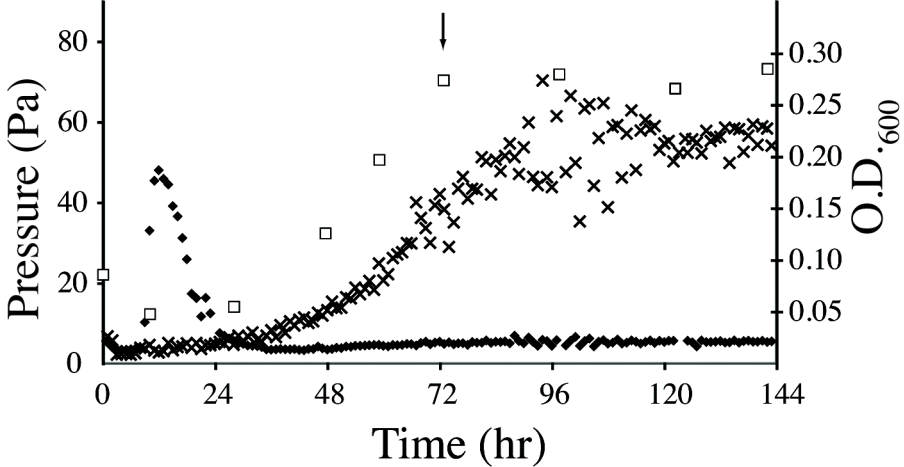
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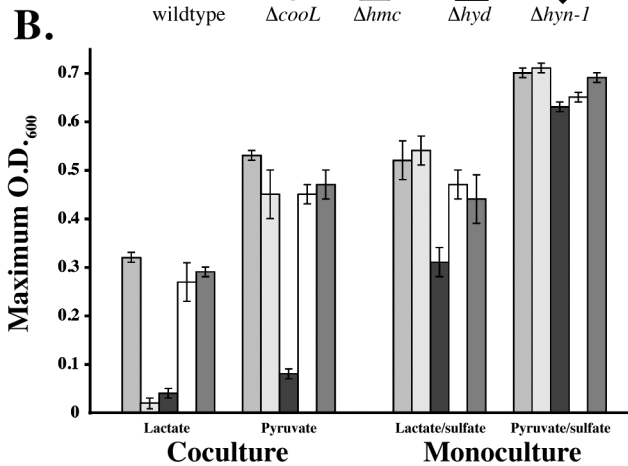
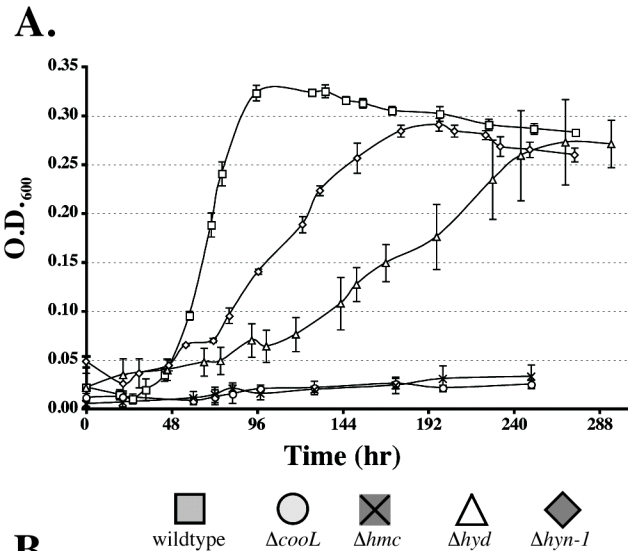
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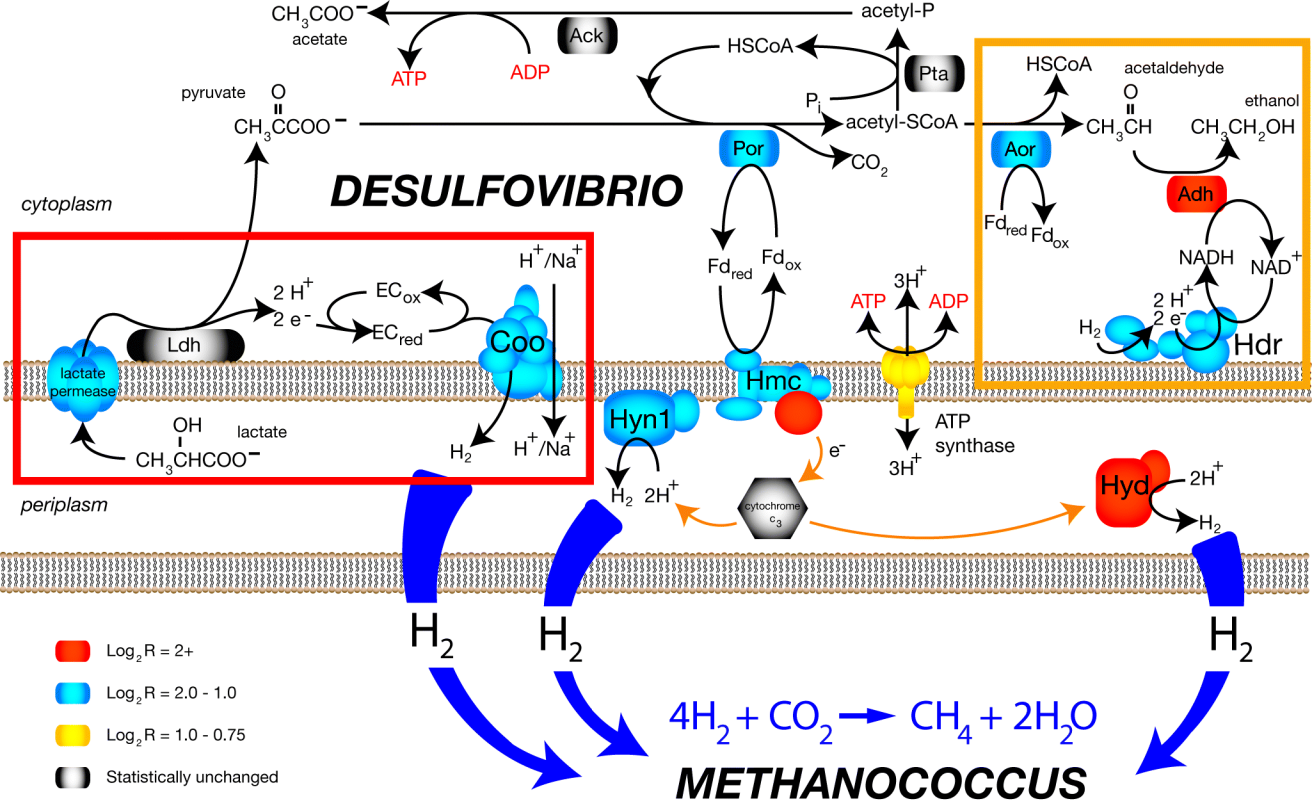
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**Table 1: Strains used during this investigation.**

<b>Strain</b>	<b>Mutant</b>	<b>Gene(s)</b>	<b>Description</b>	<b>Source</b>
<i>Desulfovibrio vulgaris</i> Hildenborough (ATCC 29579)	<i>n.a.</i>	<i>n.a.</i>	Wildtype strain	ATCC
JW3040	$\Delta$ <i>cooL</i>	DVU2288	Transposon-interruption of 3rd gene in CO-induced hydrogenase	This study
H801	$\Delta$ <i>hmc</i>	DVU0532-5	Deletion mutant of hmcBCDE	Dolla <i>et al.</i> , 2000
Hyd100	$\Delta$ <i>hyd</i>	DVU1679-70	Deletion mutant of hydAB	Pohorelic <i>et al.</i> , 2002
NiFe100	$\Delta$ <i>hyn</i>	DVU1921-2	Deletion mutant of hynAB-1	Goenka <i>et al.</i> , 2004
<i>Methanococcus maripaludis</i> S2	<i>n.a.</i>	<i>n.a.</i>	Wildtype strain	Whitman <i>et al.</i> , 1986



**Table 2: Estimated growth yields (g dry cell biomass/mol substrate) of wildtype cocultures and monocultures grown on lactate or pyruvate (30 mM). Monocultures were grown with 30 mM sulfate. Coculture from Traore *et al.*, contained *D. vulgaris* and *Methanosarcina barkeri*. All lactate:pyruvate, lactate<sub>coculture</sub>:lactate<sub>monoculture</sub> and pyruvate<sub>coculture</sub>:pyruvate<sub>monoculture</sub> ratios calculated based on growth yields listed here. Error represents the standard deviation of triplicate samples.**

Strain	COCULTURE			MONOCULTURE			Lactate <sub>CO</sub> :Lactate <sub>MONO</sub>	Pyruvate <sub>CO</sub> :Pyruvate <sub>MONO</sub>
	Lactate	Pyruvate	Lactate:Pyruvate	Lactate	Pyruvate	Lactate:Pyruvate		
wildtype	4.2 ± 0.1	6.8 ± 0.3	0.61 ± 0.05	5.0 ± 0.4	7.2 ± 0.1	0.69 ± 0.08	0.83 ± 0.08	0.94 ± 0.05
Traore <i>et al.</i> , 1983	5.3 ± 0.9	6.3 ± 0.9	0.84 ± 0.22	6.7 ± 1.2	10.1 ± 1.7	0.66 ± 0.25	0.79 ± 0.25	0.62 ± 0.22