Genetics and molecular biology of the electron flow for sulfate respiration in *Desulfovibrio*

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Progress in the genetic manipulation of the *Desulfovibrio* strains has provided an opportunity to explore electron flow pathways during sulfate respiration. Most bacteria in this genus couple the oxidation of organic acids or ethanol with the reduction of sulfate, sulfite, or thiosulfate. Both fermentation of pyruvate in the absence of an alternative terminal electron acceptor, disproportionation of fumarate and growth on H_2 with CO_2 during sulfate reduction are exhibited by some strains. The ability to produce or consume H_2 provides *Desulfovibrio* strains the capacity to participate as either partner in interspecies H_2 transfer. Interestingly the mechanisms of energy conversion, pathways of electron flow and the parameters determining the pathways used remain to be elucidated. Recent application of molecular genetic tools for the exploration of the metabolism of *Desulfovibrio vulgaris* Hildenborough has provided several new datasets that might provide insights and constraints to the electron flow pathways. These datasets include (1) gene expression changes measured in microarrays for cells cultured with different electron donors and acceptors, (2) relative mRNA abundances for cells growing exponentially in defined medium with lactate as carbon source and electron donor plus sulfate as terminal electron acceptor, and (3) a random transposon mutant library selected on medium containing lactate plus sulfate supplemented with yeast extract. Studies of directed mutations eliminating apparent key components, the quinone-interacting membrane-bound oxidoreductase (Qmo) complex, the Type 1 tetraheme cytochrome c_3 (Tp1-c_3), or the Type 1 cytochrome c_3:menaquinone oxidoreductase (Qrc) complex, suggest a greater flexibility in electron flow than previously considered. The new datasets revealed the absence of random transposons in the genes encoding an enzyme with homology to Coo membrane-bound hydrogenase. From this result, we infer that Coo hydrogenase plays an important role in *D. vulgaris* growth on lactate plus sulfate. These observations along with those reported previously have been combined in a model showing dual pathways of electrons from the oxidation of both lactate and pyruvate during sulfate respiration. Continuing genetic and biochemical analyses of key genes in *Desulfovibrio* strains will allow further clarification of a general model for sulfate respiration.

Keywords: *Desulfovibrio*, sulfate respiration, sulfite reduction, Coo hydrogenase, cytochrome c_3

INTRODUCTION

The ability to obtain energy from substrate oxidation coupled with sulfate reduction is a unique mechanism that is shared by a heterogeneous group of microbes that include proteobacteria, firmicutes and archaea; mesophiles, thermophiles, and psychrophiles. While this means of energy generation is restricted to anaerobes, these sulfate-reducing microbes (SRM) are found in a wide variety of environments including oxic waters and soils. Whereas oxygen at high concentrations is toxic to SRM, large variations in sensitivity are observed (Sass and Cypionka, 2007). Oxygen is reduced by many SRM perhaps as a protective mechanism because sustained growth supported through oxygenic respiration has not been demonstrated (Marschall et al., 1993; Hansen, 1994a).

Of this group of microbes, members of the δ-proteobacterial genus *Desulfovibrio* have been most intensively studied because of their rapid growth and ease of manipulation. *Desulfovibrio vulgaris* Hildenborough was the first of the sulfate-reducing bacteria to have its genome sequenced (Heidelberg et al., 2004). *D. vulgaris* was also the first to be genetically manipulated through conjugation (Powell et al., 1989; van den Berg et al., 1989) and the second to be modified by marker exchange mutagenesis (Fu and Voordouw, 1997), after *Desulfovibrio fructosovorans* (Rousset et al., 1991). Genome sequences of many more SRM are now available.[1]
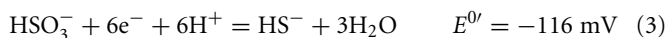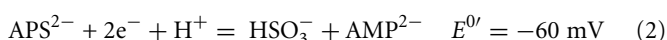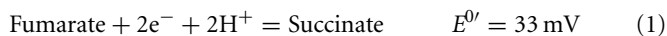
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[1] http://www.ncbi.nlm.nih.gov/sites/entrez

Improved genetic tools have been developed (Wall et al., 1993; Rousset et al., 1998; Bender et al., 2007; Keller et al., 2009; Zane et al., 2010), transposon mutant libraries have been generated (Groh et al., 2005; Deutschbauer, personal communication, and this lab) and systems biology tools have been applied (Mukhopadhyay et al., 2006; Redding et al., 2006; Tang et al., 2007; Pereira et al., 2008; He et al., 2010). Therefore this chapter will focus on the contributions of molecular genetics to our understanding of energy conversion in the *Desulfovibrio* strains. Confirmation that these insights might be applicable to other SRM genera remain to be established.

Our discussion will consider energy conversion by *Desulfovibrio* strains grown with lactate as carbon and electron source and sulfate as electron acceptor. Because electron flow from lactate oxidation with sulfate reduction provides robust growth of *Desulfovibrio*, data from systems biology approaches are now available from cells grown on these substrates. Strengthening relevance, the released fermentation products, lactate and H_2, are likely to be the sources of electrons found in natural environments where these bacteria flourish. Currently many transcriptomic data and some proteomic data have been generated[2] and transposon libraries have been selected with cells grown on these substrates (Deutschbauer, personal communication, and Wall lab).

As a terminal electron acceptor, sulfate has four properties that together give the SRM access to growth niches unavailable to other microbes. First, sulfate is a thermodynamically stable oxidized form of sulfur and must be activated prior to being reduced. Second, sulfate is reduced by soluble enzymes in the cytoplasm making active transport of the sulfate necessary because it is an ionized species at physiological pHs (H_2SO_4; pKa1, −3; pKa2, 2). Third, sulfide, HS^- (H_2S, pKa 6.9) the reduced product, is toxic at elevated concentrations (Caffrey and Voordouw, 2010) and must be transported out of the cell or defense systems invoked. Fourth, the fact that the reductive enzymes are soluble and not membrane-bound suggests that reduction of sulfate may not be uniquely or tightly associated with an electron transport system in the membrane.

The common sources of carbon and electrons used by SRM are fermentation products from facultative anaerobes. Most *Desulfovibrio* strains are incomplete oxidizers of organic acids and alcohols producing acetate in quantities nearly stoichiometric to the added substrate (Postgate, 1984a; Rabus et al., 2006). Therefore, about 95% of the carbon substrate oxidized by *Desulfovibrio* strains is used for energy generation and the residual used to produce cell material (Noguera et al., 1998; Rabus et al., 2006). The inability of these strains to oxidize acetate has been suggested to result from the high reduction potential of the fumarate/succinate couple ($E^{0'} = 33$ mV, Eq. 1, from Thauer et al., 1977) in the standard TCA cycle (Thauer, 1989). Therefore, reduction of the activated form of sulfate, adenosine 5′-phosphosulfate (APS, Eq. 2) or bisulfite (Eq. 3) cannot be coupled with succinate oxidation.

$$\text{Fumarate} + 2e^- + 2H^+ = \text{Succinate} \qquad E^{0'} = 33 \text{ mV} \qquad (1)$$

$$APS^{2-} + 2e^- + H^+ = HSO_3^- + AMP^{2-} \qquad E^{0'} = -60 \text{ mV} \qquad (2)$$

$$HSO_3^- + 6e^- + 6H^+ = HS^- + 3H_2O \qquad E^{0'} = -116 \text{ mV} \qquad (3)$$

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[2]www.microbesonline.org

For those strains of the genus *Desulfobacter* or *Desulfobacterium* that do completely oxidize organic acids to CO_2 with sulfate as electron acceptor, the standard TCA cycle is not used (Hansen, 1994a).

The *Desulfovibrio* pathway for lactate oxidation is through pyruvate to acetyl-CoA and from acetyl-CoA to acetate via acetylphosphate which is of sufficient energy to generate ATP by substrate-level phosphorylation (See Eqs 4–8). Early researchers believed that the SRM were restricted to substrate-level phosphorylation for ATP generation. However, the demonstration that *Desulfovibrio* strains could grow with H_2 oxidation, with carbon supplied by CO_2 and acetate, and sulfate as terminal electron acceptor (Butlin et al., 1949; Peck, 1960) firmly established the capacity for electron transport-linked phosphorylation. In addition, Peck (1966) convincingly argued that a mechanism, in addition to substrate-level phosphorylation, was essential during lactate plus sulfate growth. Peck pointed out that the two moles of ATP generated during the oxidation of two moles of lactate would be consumed during sulfate activation. Therefore, energy to support growth must be provided by an additional mechanism.

HYDROGEN CYCLING MODEL

Odom and Peck (1981a) proposed an unusual and controversial model for increasing the energy budget called the "hydrogen cycle." This model was proposed, in part, as an explanation for the observation of a H_2 transient seen in cultures of *Desulfovibrio* inoculated into medium with lactate or pyruvate as electron donors and sulfate as electron acceptor. They proposed that electrons and protons generated from organic acid oxidation could serve as substrates for a cytoplasmically located hydrogenase. The resulting H_2 could diffuse through the cytoplasmic membrane and be reoxidized by periplasmic hydrogenases. The electrons produced by the H_2 oxidation would be delivered to the *c*-type cytochrome pool for return to the cytoplasm through transmembrane protein complexes and used for sulfate reduction. The protons released would contribute to the chemiosmotic potential.

In contrast, Lupton et al. (1984) proposed that trace H_2 was produced and consumed in the initial growth phases of *Desulfovibrio* cultures on organic acids plus sulfate to control redox state of electron carriers. These researchers also provided evidence that they interpreted as eliminating the argument that the purpose of the transient H_2 production was to allow fermentation to make ATP for sulfate activation and reduction in growth initiation. They showed that with thiosulfate as terminal electron acceptor, a substrate that does not require activation prior to reduction, H_2 was still produced and consumed when cells were introduced into fresh medium.

Evidence both for and against the hydrogen cycling model has been published. Odom and Peck (1981a) demonstrated that spheroplasts produced from *Desulfovibrio gigas* were unable to oxidize lactate and reduce sulfate without the restoration of the periplasmic proteins, hydrogenase and the type I cytochrome c_3 (Tp1-c_3). From this result it was inferred that electrons were routed from lactate through the periplasm and back to sulfate. In addition, Peck et al. (1987) were able to trap and measure H_2 released by *D. vulgaris* from pyruvate oxidation coupled to sulfate reduction with membrane-inlet mass spectrometry. On the other hand, the existence of free H_2, predicted by the model to be a periplasmic

intermediate in the oxidation of lactate or pyruvate, would lead to the expectation that a H$_2$ atmosphere would strongly inhibit oxidation of these substrates. Lupton et al. (1984) showed that there was no inhibition of lactate or pyruvate oxidation by H$_2$ during sulfate respiration. This lack of inhibition of substrate oxidation by H$_2$ was confirmed by Pankhania et al. (1986) for cells with lactate. Also, a mutant of *Desulfovibrio desulfuricans* ATCC27774 was isolated that could grow on lactate plus sulfate but not H$_2$ plus sulfate (Odom and Wall, 1987). This result is incongruent with periplasmic H$_2$ as an intermediate in lactate oxidation during sulfate reduction. In addition, if the cycling process – H$_2$ production, membrane diffusion and H$_2$ oxidation – were essential for sulfate respiration with organic acids, SRM should all have hydrogenases. *Desulfovibrio sapovorans* is one example that lacks hydrogenase (Postgate, 1984b).

More recent reviews have diminished the putative role of hydrogen cycling in the metabolism of the SRM. Widdel and Hansen (1991) suggested that H$_2$ production is simply an accident of having constitutive hydrogenases active in the cell. Rabus et al. (2006) proposed that constitutive hydrogenases would produce hydrogen whenever there was an imbalance between reductant produced or consumed by the cells. An alternative suggestion of the latter researchers derived from the postulation that in natural environments the SRM participate routinely in interspecies H$_2$ transfer when sulfate is absent. As a result, SRM might not be able to redirect electrons completely when sulfate was available, thus driving the release of electrons as H$_2$ (Rabus et al., 2006). In contrast to these theories, Noguera et al. (1998) elegantly generated and tested a model for H$_2$ production and consumption by *D. vulgaris* growing on lactate plus sulfate. These researchers proposed that dual pathways were operating, simultaneously, for electron flow and that the free energy available determined the distribution of electron flow. Their model predicted 52% of the electron flow would move from lactate through H$_2$ and finally to sulfate; whereas, 48% would move to sulfate without H$_2$ formation. After subtraction of the reductant moving to biomass, their experiments showed that 47% of the remaining electrons were directly coupled to sulfate reduction and 53% formed H$_2$ before being used for sulfate reduction (Noguera et al., 1998).

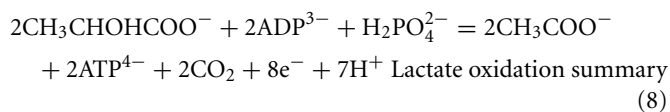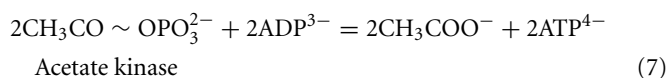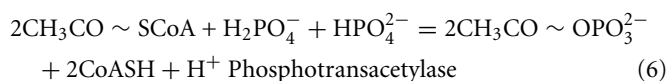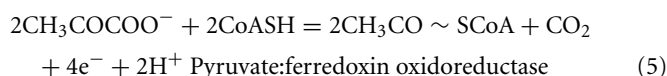## SULFATE REDUCTION AND LACTATE OXIDATION
REACTIONS
The predicted pathways of sulfate reduction and lactate oxidation are presented in the equations below (Eqs 4–16). Dissimilatory sulfate reduction occurs by a two step process requiring a minimum of four enzymes (**Table 1A**). First sulfate is activated to form APS at the expense of two ATP equivalents by sulfate adenylyltransferase. Two electrons are then used by APS reductase to reduce APS to HSO$_3^-$ (Eqs 10 and 12). The activation reaction is pulled by the hydrolysis of the released pyrophosphate (Eq. 11). Then bisulfite reductase reduces HSO$_3^-$ with six electrons to HS$^-$, in one step (Eq. 13). The equations show protonated species considering the published pKa's of the compounds[3] and assuming that the internal pH is ca. 7.5 (Cypionka, 1995). It should be noted that the pKa's of HSO$_3^{2-}$, H$_2$S, and H$_2$PO$_4^-$ are 7.2, 7.0, and 7.2, respectively,

and therefore the protonated and deprotonated forms were considered to be present in about equal quantities. The net result for sulfate reduction (Eq. 14) indicates a consumption of eight moles of electrons with eight and a half moles of protons.
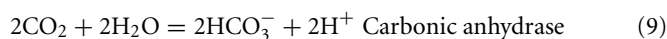
Lactate oxidation by the incomplete oxidizers in the *Desulfovibrio* genus, proceeds with pyruvate and acetyl-CoA as intermediates. Reductant is generated from lactate through lactate dehydrogenase and pyruvate:ferredoxin oxidoreductase (Eqs 4 and 5), while acetyl-CoA is the source for substrate-level phosphorylation producing ATP (Eqs 6 and 7). Seven protons were produced in the reactions as shown (Eq. 8). By interconversion of the adenylate nucleotides (Eq. 15) and summing Eqs 8, 9, 14, and 15, the combined oxidation/reduction reaction for growth with lactate plus sulfate is shown as Eq. 16. With the assumptions made here, 0.5 mole of protons are produced in excess of those consumed during reduction of one mole of sulfate. Clearly nitrogen metabolism, biomass production, transport functions and/or small variations in internal pH that affect the concentrations of protonated species will all affect the actual balance of electrons and protons (Hooper and DiSpirito, 1985).
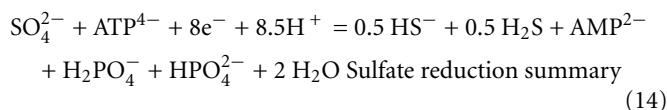
EQUATIONS
Lactate oxidation:

$$2CH_3CHOHCOO^- = 2CH_3COCOO^- + 4e^- + 4H^+$$

Lactate dehydrogenase $\quad$ (4)

$$2CH_3COCOO^- + 2CoASH = 2CH_3CO \sim SCoA + CO_2$$
$$+ 4e^- + 2H^+ \text{ Pyruvate:ferredoxin oxidoreductase} \quad (5)$$

$$2CH_3CO \sim SCoA + H_2PO_4^- + HPO_4^{2-} = 2CH_3CO \sim OPO_3^{2-}$$
$$+ 2CoASH + H^+ \text{ Phosphotransacetylase} \quad (6)$$

$$2CH_3CO \sim OPO_3^{2-} + 2ADP^{3-} = 2CH_3COO^- + 2ATP^{4-}$$

Acetate kinase $\quad$ (7)

$$2CH_3CHOHCOO^- + 2ADP^{3-} + H_2PO_4^{2-} = 2CH_3COO^-$$
$$+ 2ATP^{4-} + 2CO_2 + 8e^- + 7H^+ \text{ Lactate oxidation summary}$$
$$(8)$$

Dissolved CO$_2$:

$$2CO_2 + 2H_2O = 2HCO_3^- + 2H^+ \text{ Carbonic anhydrase} \quad (9)$$

Sulfate reduction:

$$SO_4^{2-} + ATP^{4-} + H^+ = APS^{2-} + HP_2O_7^{3-}$$

Sulfate adenylyltransferase $\quad$ (10)

$$HP_2O_7^{3-} + H_2O = H_2PO_4^- + HPO_4^{2-}$$

Inorganic pyrophosphatase $\quad$ (11)

$$APS^{2-} + 2e^- + 0.5H^+ = 0.5 HSO_3^- + 0.5 SO_3^{2-} + AMP^{2-}$$

APS reductase $\quad$ (12)

$$0.5 HSO_3^- + 0.5 SO_3^{2-} + 6e^- + 7H^+ = 0.5 HS^- + 0.5 H_2S$$
$$+ 3 H_2O \text{ Bisulfite reductase} \quad (13)$$

$$SO_4^{2-} + ATP^{4-} + 8e^- + 8.5H^+ = 0.5 HS^- + 0.5 H_2S + AMP^{2-}$$
$$+ H_2PO_4^- + HPO_4^{2-} + 2 H_2O \text{ Sulfate reduction summary}$$
$$(14)$$

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[3]http://chemweb.unp.ac.za/Chemistry/Physical_data/pKa_compilation.pdf

Table 1 | Expression of genes encoding enzymes putatively involved in Lactate/Sulfate growth of *Desulfovibrio vulgaris* Hildenborough.

Locus DVU No.	Gene	Annotation[a]	Microarray data (Log$_2$ R)[b]			Average Log$_2$ RNA/DNA[f]	Tn in gene[g]
			Stat/Expo[c]	LThio/LS[d]	PS/LS[e]		
A. SULFATE REDUCTION ENZYMES							
1295	sat	Sulfate adenylyltransferase (ATP-sulfurylase)	0.45	−1.07	−0.33	4.44	N
1636	ppaC	Pyrophosphatase	0.52	−1.17	−0.24	2.52	N
h⌐ 0846	apsB	Adenylsulfate reductase ß subunit	−0.56	−0.99	0.39	4.90	N
↓ 0847	aspA	Adenylsulfate reductase a subunit	−0.58	−1.81	0.04	4.72	N
⌐ 0402	dsrA	Dissimilatory sulfite reductase alpha subunit	−0.07	−0.33	0.69	4.32	N
0403	dsrB	Dissimilatory sulfite reductase beta subunit	−1.23	−0.18	0.88	4.19	N
↓ 0404	dsrD	Dissimilatory sulfite reductase D	−1.88	0.91	0.47	4.89	N
2776	dsrC	Dissimilatory sulfite reductase, gamma subunit	3.48	0.07	−0.87	3.39	N
B. CARBON METABOLISM ENZYMES							
0253	D-ldh	Lactate dehydrogenase, Glycolate oxidoreductase, FAD/iron-sulfur cluster-binding domain protein	−0.16	0.33	0.39	2.09	Y
0390	glcD	FAD/FMN-containing dehydrogenase, glycolate oxidase, subunit GlcD, putative	−0.97	0.28	0.26	0.11	Y
0600	ldh	L-lactate dehydrogenase	1.20	0.22	−0.14	−1.32	Y
↑ 0826	NA[i]	Glycolate oxidase, iron-sulfur subunit, putative	0.47	−0.51	−0.28	−0.05	Y
⌐ 0827	NA	Glycolate oxidase, subunit GlcD, putative, GO:0009339 glycolate oxidase complex	−0.19	−0.58	−0.02	−0.05	Y
1412	NA	D-isomer specific 2-hydroxyacid dehydrogenase family protein	0.15	−0.42	−0.36	0.42	Y
↑ 1782	NA	Iron-sulfur cluster-binding protein	0.29	−0.22	−0.21	−0.02	Y
⌐ 1783	NA	Cysteine-rich domain protein; GO:0009339 glycolate oxidase complex	1.19	0.02	−0.61	0.40	Y
2784	L-ldh	Dehydrogenase, FMN-dependent family	−0.84	−1.10	−0.45	0.07	Y
3071	NA	Oxidoreductase, FAD/iron-sulfur cluster-binding domain protein	−0.71	1.02	0.39	0.25	N
⌐ 3025	por	Pyruvate:ferredoxin oxidoreductase	−0.57	−0.26	0.37	2.83	N
3027	glcD	D-lactate dehydrogenase, glycolate oxidase subunit	1.10	0.25	−0.01	1.99	Y
3028	glpC	D-lactate dehydrogenase, iron-sulfur cluster-binding protein	1.39	0.47	−0.34	1.44	Y
3029	pta	Phosphotransacetylase	−0.33	−0.50	−0.29	1.80	N
↓ 3030	ackA	Acetate kinase	−0.97	−0.78	−0.30	1.79	N
2824	pfl-l	Pyruvate formate lyase	0.36	−0.40	−0.41	−1.44	Y
⌐ 1569	porA	Pyruvate-ferredoxin-oxidoreductase, alpha subunit	0.80	−0.56	−0.12	0.53	Y
↓ 1570	porB	Pyruvate-ferredoxin-oxidoreductase, beta subunit	−0.02	−0.25	−0.06	−0.81	N
C. PERMEASES AND TRANSPORTERS							
0053	sulP-1	Sulfate permease, putative	1.49	−0.23	−0.12	−0.21	Y
0279	sulP-1	Sulfate permease family protein	−2.28	−0.46	0.38	0.40	Y
1999	sul-1	Sulfate transporter family protein	−0.13	−0.06	−0.01	−0.75	Y
2110	b2975	L-lactate permease	0.79	0.65	−0.13	−0.85	Y
2285	lctP-1	L-lactate permease family protein	−2.34	−0.31	−0.20	0.13	Y
2451	lctP-2	L-lactate permease family protein	−1.05	−0.07	0.28	0.69	Y
2683	lldP-1	L-lactate permease family protein	−1.41	−0.27	0.25	0.47	Y

(Continued)

Table 1 | Continued

Locus DVU No.	Gene	Annotation[a]	Microarray data (Log$_2$ R)[b]			Average Log$_2$ RNA/DNA[f]	Tn in gene[g]
			Stat/Expo[c]	LThio/LS[d]	PS/LS[e]		
3026	*lldP-2*	L-lactate permease	0.23	0.11	0.13	0.74	Y
3284	*b2975*	L-lactate permease	1.28	1.07	0.51	−1.68	Y
0446	*dhlC*	Sodium/solute symporter family protein	1.51	−0.09	0.23	−1.98	Y
0088	*panF*	Sodium/pantothenate symporter	1.16	0.61	0.07	−0.09	Y
D. MEMBRANE-BOUND ELECTRON TRANSFER COMPLEXES							
0848	*qmoA*	Quinone-interacting membrane-bound oxidoreductase, Flavin protein	−0.49	−1.30	0.46	2.64	N
0849	*qmoB*	Quinone-interacting membrane-bound oxidoreductase, Flavin protein	−1.25	−1.30	0.004	1.62	N
0850	*qmoC*	Quinone-interacting membrane-bound oxidoreductase, Membrane FeS protein	−0.15	−1.26	0.42	0.83	N
1286	*dsrP*	Integral membrane protein	1.40	−1.07	0.66	2.06	N
1287	*dsrO*	Periplasmic (Tat), binds 2[4Fe-4S]	−1.57	−1.47	0.81	1.43	N
1288	*dsrJ*	Periplasmic (Sec) triheme cytochrome *c*	−1.31	−1.47	0.46	1.42	N
1289	*dsrK*	Cytoplasmic, binds 2 [4Fe-4S]	−1.98	−1.46	0.58	1.04	N
1290	*dsrM*	Inner membrane protein binds 2 heme *b*	−1.84	ND[j]	ND	1.37	N
0692	*qrcD*[k]	Molybdopterin oxidoreductase, transmembrane subunit	−0.46	−0.81	0.47	1.11	Y
0693	*qrcC*	Molybdopterin oxidoreductase, iron sulfur cluster binding subunit, containing cytochrome *c* heme	−1.65	−0.67	0.05	1.10	N
0694	*qrcB*	Molybdopterin oxidoreductase, molybdopterin binding subunit	−0.95	−0.35	0.10	1.06	Y
0695	*qrcA*	Cytochrome *c*, (six hemes)	−0.72	0.06	0.34	1.34	N
0263	*tmcA*	Transmembrane complex, tetraheme cytochrome *c$_3$*	−0.31	−0.17	0.28	1.69	N
0264	*tmcB*	Transmembrane complex, ferredoxin, 2 [4Fe-4S]	0.5	−0.44	0.39	2.21	Y
0265	NA	Membrane protein, putative	0.58	−0.50	0.26	1.41	Y
0531	*hmcF*	52.7 kd protein in *hmc* operon	0.59	0.29	−1.49	−1.90	Y
0532	*hmcE*	25.3 kd protein in *hmc* operon	0.19	1.53	0.35	−1.54	Y
0533	*hmcD*	5.8 kd protein in *hmc* operon	0.78	−0.55	−1.1	−1.97	N
0534	*hmcC*	43.2 kd protein in *hmc* operon	2.33	0.16	−0.33	−1.07	Y
0535	*hmcB*	40.1 kd protein in *hmc* operon	1.55	1.79	ND	−1.21	Y
0536	*hmcA*	High-molecular-weight cytochrome *c*	0.14	1.61	−0.3	−1.44	N
2791	*dhcA*	Decaheme cytochrome *c* associated with Rnf complex	−0.19	−0.14	0.96	1.03	Y
2792	*rnfC*	NADH:quinone oxidoreductase subunit RnfC	−0.37	−0.26	0.91	0.21	Y
2793	*rnfD*	Electron transport complex protein RnfD, putative	1.10	−0.57	1.00	−0.43	Y
2794	*rnfG*	NADH:quinone oxidoreductase subunit RnfG	0.14	−0.60	0.94	−0.05	Y
2795	*rnfE*	NADH:quinone oxidoreductase subunit RnfE	1.08	−0.71	0.38	−1.03	N
2796	*rnfA*	NADH:quinone oxidoreductase subunit RnfA	1.38	−0.52	1.09	−0.75	N
2797	*rnfB*	NADH:quinone oxidoreductase subunit RnfB	−0.13	−0.03	0.73	0.05	Y
3143	*ohcB*	Iron-sulfur cluster-binding protein	1.36	−0.11	0.59	−2.33	N
3144	*ohcA*	Cytochrome *c* family protein	1.12	−0.15	0.54	−1.74	Y
3145	*ohcC*	Hydrogenase, *b*-type cytochrome subunit, putative	0.78	0.48	0.64	−1.75	Y

(Continued)

Table 1 | Continued

Locus DVU No.	Gene	Annotation[a]	Microarray data (Log$_2$ R)[b]			Average Log$_2$ RNA/DNA[f]	Tn in gene[g]
			Stat/Expo[c]	LThio/LS[d]	PS/LS[e]		
E. PERIPLASMIC HYDROGENASES							
1769	hydA	Periplasmic [Fe] hydrogenase, large subunit	−0.47	1.27	−0.003	−0.06	Y
1770	hydB	Periplasmic [Fe] hydrogenase, small subunit	−0.42	1.86	0.74	−0.29	Y
1917	hysB	Periplasmic [NiFeSe] hydrogenase, small subunit	−0.93	0.25	0.31	1.88	Y
1918	hysA	Periplasmic [NiFeSe] hydrogenase, large subunit, selenocysteine-containing	−1.32	ND	ND	2.43	Y
1921	hynB-1	Periplasmic [NiFe] hydrogenase, small subunit, isozyme 1	0.82	−0.56	0.74	0.74	Y
1922	hynA-1	Periplasmic [NiFe] hydrogenase, large subunit, isozyme 1	0.57	−0.57	0.84	0.39	Y
2524	NA	[NiFe] hydrogenase	−0.23	−1.79	−0.34	−2.22	Y
2525	hynB-2	Periplasmic [NiFe] hydrogenase, small subunit, isozyme 2	−0.1	−1.46	−0.49	−0.96	Y
2526	hynA-2	Periplasmic [NiFe] hydrogenase, large subunit, isozyme 2	2.02	−1.36	−0.64	−0.44	Y
F. CYTOPLASMIC HYDROGENASES AND CARBON MONOXIDE DEHYRDOGENASES							
0429	echF	Ech hydrogenase, subunit EchF, putative	0.55	0.65	1.34	−1.30	Y
0430	echE	Ech hydrogenase, subunit EchE, putative	ND	ND	ND	ND	Y
0431	echD	Ech hydrogenase, subunit EchD, putative	−0.07	0.64	1.23	−1.14	N
0432	echC	Ech hydrogenase, subunit EchC, putative	1.35	0.25	0.77	−0.83	N
0433	echB	Ech hydrogenase, subunit EchB, putative	0.51	0.33	1.44	−1.33	Y
0434	echA	Ech hydrogenase, subunit EchA, putative	−0.08	−0.18	0.63	−0.34	Y
2286	cooM	Hydrogenase, CooM subunit, putative	−1.59	0.25	−0.10	1.30	N
2287	cooK	Hydrogenase, CooK subunit, selenocysteine-containing, putative	−0.47	−0.01	−0.78	1.52	N
2288	cooL	Hydrogenase, CooL subunit, putative	−0.70	−0.11	−0.73	1.69	N
2289	cooX	Hydrogenase, CooX subunit, putative	−0.91	0.09	−0.54	1.73	N
2290	cooU	Hydrogenase, CooU subunit, putative	−0.10	−0.21	−0.51	2.10	N
2291	cooH	Carbon monoxide-induced hydrogenase CooH, putative	−0.88	−0.34	−0.70	1.82	N
2292	hypA	Hydrogenase nickel insertion protein	1.09	0.50	−0.44	2.82	N
2293	cooF	Iron-sulfur protein	1.04	0.21	−0.74	2.70	N
2098	cooS	Carbon monoxide dehydrogenase	ND	ND	ND	0.54	Y
2099	cooC	Carbon monoxide dehydrogenase	0.19	1.52	1.52	−1.58	Y
G. PERIPLASMIC FORMATE DEHYDROGENASES							
0587	fdnG-1	Formate dehydrogenase, alpha subunit, selenocysteine-containing	1.87	ND	ND	0.29	Y
0588	hybA	Formate dehydrogenase, beta subunit	0.93	1.31	0.39	−0.27	N
2481	fdoH	Formate dehydrogenase, beta subunit	0.49	−0.17	−0.52	0.65	Y
2482	fdnG-2	Formate dehydrogenase, alpha subunit, selenocysteine-containing	0.34	ND	ND	0.00	Y
2483	cfdE	Formate dehydrogenase, cytochrome *c* family protein	0.09	−0.55	−0.27	0.54	Y
2484	cfdD	Formate dehydrogenase, cytochrome *c* family protein	−0.18	−0.59	−0.08	−0.62	N
2485	cfdC	Formate dehydrogenase, membrane protein, putative	0.53	−0.35	−0.33	0.15	Y

(Continued)

Table 1 | Continued

Locus DVU No.	Gene	Annotation[a]	Microarray data (Log$_2$ R)[b]			Average Log$_2$ RNA/DNA[f]	Tn in gene[g]
			Stat/Expo[c]	LThio/LS[d]	PS/LS[e]		
↑2809	NA	Cytochrome c_3	−0.83	−0.09	0.84	−1.51	Y
2810	fdhE	Formate dehydrogenase formation protein FdhE, putative	0.16	0.06	0.77	−1.70	Y
2811	COG-HybA	Formate dehydrogenase, beta subunit	−0.16	−0.82	0.66	−1.95	Y
2812	fdnG-3	Formate dehydrogenase, alpha subunit, selenocysteine-containing	−0.03	−0.33	0.70	−1.75	Y
H. PERIPLASMIC CYTOCHROMES							
3107	occ	Cytochrome c family protein (eight hemes)	2.63	−2.33	0.14	−0.10	Y
3171	cycA	Cytochrome c_3 (Tpl-c_3; four hemes)	0.29	−0.73	0.81	4.30	N
1817	cyf	c_{553} cytochrome (one heme)	0.90	−0.04	−0.12	2.54	Y
3041	NA	c_{553} cytochrome (one heme)	−0.13	0.89	−0.10	0.13	N
0702	NA	c_{554} cytochrome (four hemes)	−0.30	0.26	−0.17	0.99	N
0922	NA	c_{554} cytochrome (four hemes)	0.20	−0.05	0.14	−1.60	Y
0624	nrfH	Cytochrome c nitrate reductase, small subunit (four hemes)	−0.34	0.08	−0.01	−0.58	Y
↓0625	nrfA	Cytochrome c nitrite reductase, catalytic subunit (five hemes)	−0.49	−0.90	0.46	−2.37	Y
I. OTHER							
1777	cynT	Carbonic anhydrase	−1.08	0.15	−0.39	0.50	Y
2013	HCP	Hybrid cluster protein	ND	ND	ND	0.74	Y
2543	b0873	Hybrid cluster protein	0.67	0.07	0.01	0.66	Y
↑2402	hdrA	Heterodisulfide reductase, A subunit	−0.3	−0.36	0.23	1.61	Y
2403	hdrB	Heterodisulfide reductase, B subunit	−0.1	−0.17	0.54	2.14	Y
2404	hdrC	Heterodisulfide reductase, C subunit	0.8	−0.30	−0.11	2.48	N

[a] Gene annotation was obtained from http://www.microbesonline.org

[b] Log$_2$ R, where R is the ratio of transcripts in the experimental versus the control.

[c] Stat/Expo is the ratio of transcripts from cells grown in lactate/sulfate (60 mM/50 mM) comparing stationary cells to exponentially growing cells (samples T5/T1 as presented in *Clark et al., 2006*).

[d] LThio/LS is the ratio of transcripts from cultures growing exponentially (OD$_{600}$ ≈ 0.3) on lactate/thiosulfate (60 mM/30 mM) compared to those from lactate/sulfate (60 mM/30 mM) grown cells.

[e] PS/LS is the ratio of transcripts from cultures growing exponentially (OD$_{600}$ ≈ 0.3) on pyruvate/sulfate (60 mM/15 mM) compared to those from lactate/sulfate (60 mM/30 mM) grown cells.

[f] Average Log$_2$ RNA/DNA hybridization is a measure of the relative abundance of transcripts present for a given gene in an exponentially growing lactate/sulfate culture (OD$_{600}$ ≈ 0.3; *Wall et al., 2009*). Abundances were averaged from 173 data points with an average standard deviation of ± 1.54. The average gene expression was arbitrarily given a Log$_2$ R value of 0.

[g] Tn in gene, 'Y' indicates that at least one transposon insertion has been recovered in the gene and 'N', that a transposon insertion has not been recovered. The transposon used is the modified Tn5-RL27 (*Oh et al., 2010*).

[h] Arrows in the first column indicate the direction of transcription of the genes within a predicted operon.
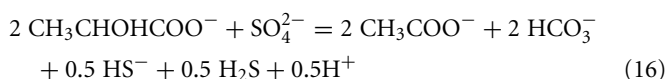
[i] NA = Not annotated with a gene name.

[j] ND = No data, insufficient useable data for given condition.

[k] Most current annotation of DVU0692–0695 was used (*Venceslau et al., 2010*).

Adenylate nucleotide interchange:

$$AMP^{2-} + ATP^{4-} = 2ADP^{3-} \quad \text{Adenylate kinase} \qquad (15)$$

Summary lactate plus sulfate metabolism:

$$2\,CH_3CHOHCOO^- + SO_4^{2-} = 2\,CH_3COO^- + 2\,HCO_3^-$$
$$+\ 0.5\,HS^- + 0.5\,H_2S + 0.5H^+ \qquad (16)$$

TRANSPORT FUNCTIONS

Transport functions of substrates and products have been addressed experimentally in variable detail. Cypionka (1995) has summarized his detailed work on sulfate transport in SRM. In freshwater SRM strains, sulfate was shown to be symported with protons, two if sulfate were in excess and three electrogenically if sulfate were limited. Since the genetic experiments to be described here with *D. vulgaris* were carried out with excess sulfate, transport

of sulfate likely occurs with the electroneutral symport of two electrons. Energy for the sulfate transport, i.e., the two protons used, is compensated by the diffusion of H_2S from the cell. However, in the slightly alkaline pH of the cell much of the sulfide would be ionized, HS^-. Little is known about the export of this charged ion. Cypionka (1995) suggests that it may be impermeable or symported with one proton. If true, energy spent transporting sulfate into the cell would be balanced by the electroneutral export of sulfide or diffusion of the protonated form.

Transport of the monocarboxylates, lactate and acetate, into and out of the cell, respectively, is likely to be by symport with one proton (Dong et al., 1993). Thus, the reciprocal transport of these compounds will not affect the proton balance of the cell. Although acetic acid in its protonated form can apparently diffuse across the cell membrane (Thauer and Morris, 1984), its continued production against a chemical gradient of acetate during oxidation of lactate or pyruvate would suggest an energy linked export process. Six genes have been annotated as lactate permeases in *D. vulgaris*, five for L-lactate and one for D-lactate (**Table 1**); whereas, no genes were annotated as acetate transporters. BLAST analysis[4] of the *D. vulgaris* genome showed that DVU0446 is 61% identical and DVU0088 is 23% identical to the acetate permease (*actP*, b4067) from *Escherichia coli* str. K-12 substr. MG1655. Further experimentation will be needed to establish whether these genes encode transporters for acetate and whether they might be essential.

THERMODYNAMICS

The model of Noguera et al. (1998) is predicated on the existence of two routes for electron flow during lactate plus sulfate growth and controlled by a thermodynamic switch that responds to free energy availability. This is consistent with the differences in reduction potential for the two oxidation steps generating electrons from lactate. For the first oxidation step, the $E^{0\prime}$ (mV) of the pyruvate/lactate couple is -190 mV; whereas, for pyruvate oxidation, the acetyl-CoA + CO_2/pyruvate couple is -498 mV. When sulfate is terminal electron acceptor, the $E^{0\prime}$ (mV) of SO_4^{2-}/HS^- is -200 (calculated for [HS^-] of 0.1 mM; Thauer et al., 2007). The standard reduction potential of the lactate dehydrogenases is above that of the H^+/H_2 couple ($E^{0\prime}$ of -414 mV), even if H_2 at more environmental concentrations is considered ($E^{0\prime}$ of -270 mV at 1 Pa:H_2), and would require energy-driven reverse electron flow to accomplish H_2 generation. In contrast, pyruvate oxidation with ferredoxin reduction has sufficient redox potential to generate H_2. Thus it is tempting to suggest that the two pathways for electron transport coincide with electrons from lactate and those from pyruvate.

It is clear that the lactate dehydrogenases are membrane-bound flavoproteins capable of delivering electrons directly to the menaquinone pool in the membrane (Reed and Hartzell, 1999; Thauer et al., 2007). In contrast, the pyruvate:ferredoxin oxidoreductase is a soluble enzyme in the cytoplasm that delivers electrons to the soluble electron carrier, ferredoxin (Kletzin and Adams, 1996; Garczarek et al., 2007). Indeed, these electron pools must mix

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[4]http://blast.ncbi.nlm.nih.gov/Blast.cgi

when the SRM grow on lactate in coculture with hydrogenotrophic methanogens, since lactate can be completely oxidized without the production of detectable pyruvate (Walker et al., 2009). During this syntrophic growth by *D. vulgaris*, the Coo membrane-bound hydrogenase has been shown to be needed, although it was not essential for monoculture on lactate plus sulfate (Walker et al., 2009). Interestingly, this protein complex belongs to a family of transmembrane complexes that function as proton (or sodium) – translocating hydrogenases and that use reduced ferredoxin as electron donors (Welte et al., 2010; **Figure 1**). The recently proposed mechanism of flavin-based electron bifurcation (Herrmann et al., 2008) could offer a possible solution to the necessity of lactate supported hydrogen production. This mechanism would suggest that, when concentrations of H_2 are maintained at very low levels, the exergonic reduction of protons to form H_2 from the reduced ferredoxin ($E^{0\prime} \leq 420$ mV) could be coupled with the endergonic formation of hydrogen with electrons from menaquinone ($E^{0\prime}$ -75 mV). The Coo membrane-bound hydrogenase complex is a flavoprotein that could allow bifurcation of electrons from the two sources, reduced ferredoxin and menaquinone. This model (**Figure 1**) would also provide an explanation for the decrease or cessation of electron flow in this pathway as hydrogen concentrations increased. Currently no experiments have been conducted to explore electron bifurcation during lactate oxidation and sulfate reduction in *Desulfovibrio*.

GENOMES

In 2004, the genome sequence of *D. vulgaris* (Heidelberg et al., 2004) became available. In the ensuing few years, several additional SRM genomes have become accessible. If hydrogen cycling were a major mechanism for augmenting the energy budget of the SRM, one might predict that conserved hydrogenases would be found among these bacteria for this process. By making the simple comparison of the genomes of two species that from physiological analyses were thought to be closely related, *D. vulgaris* and *Desulfovibrio alaskensis* G20 (Hauser et al., 2011), it can be seen that the candidate cytoplasmic hydrogenases from *D. vulgaris* (Ech hydrogenase, DVU0429–0434, and Coo hydrogenase, DVU2286–2293) are not conserved in the G20 strain. However, that does not preclude the existence of multiple electron pathways to sulfate in the SRM.
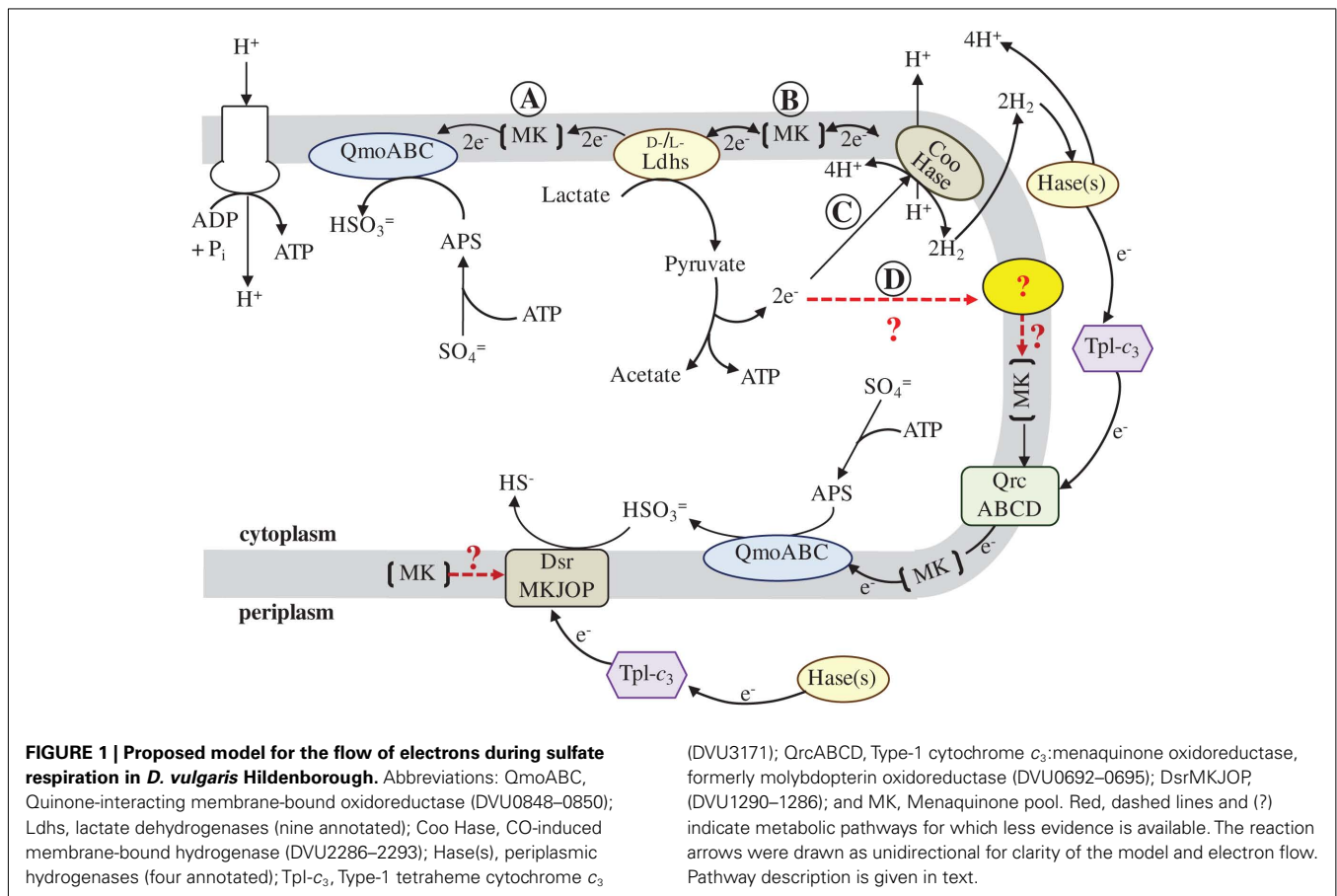
EXPRESSION AND MUTANT ANALYSIS OF GENES FOR CENTRAL REDOX PATHWAYS OF D.VULGARIS HILDENBOROUGH

Here we examine *D. vulgaris* gene expression data from microarrays of cells grown under different nutrient conditions, the relative abundance of transcripts from cells grown on lactate plus sulfate, and a random transposon mutant library to determine whether genes important for enzymes needed for energy generation in this growth mode become evident (**Table 1**).

MICROARRAY DATA

Microarray data provide information about the changes in expression of genes in response to environmental changes but do not allow absolute levels of gene expression to be determined. Changes in gene expression for *D. vulgaris* have been generated through

FIGURE 1 | Proposed model for the flow of electrons during sulfate respiration in *D. vulgaris* Hildenborough. Abbreviations: QmoABC, Quinone-interacting membrane-bound oxidoreductase (DVU0848–0850); Ldhs, lactate dehydrogenases (nine annotated); Coo Hase, CO-induced membrane-bound hydrogenase (DVU2286–2293); Hase(s), periplasmic hydrogenases (four annotated); Tpl-c_3, Type-1 tetraheme cytochrome c_3 (DVU3171); QrcABCD, Type-1 cytochrome c_3:menaquinone oxidoreductase, formerly molybdopterin oxidoreductase (DVU0692–0695); DsrMKJOP, (DVU1290–1286); and MK, Menaquinone pool. Red, dashed lines and (?) indicate metabolic pathways for which less evidence is available. The reaction arrows were drawn as unidirectional for clarity of the model and electron flow. Pathway description is given in text.

the Virtual Institute for Microbial Stress and Survival (VIMSS) program[5] supported by the US Department of Energy and are available at www.microbesonline.org (Dehal et al., 2010). These data are $\log_2$ ratios of experimental versus control transcript abundances from gene probe microarrays with 12 replicates for each ratio. All transcript preparations were made from cells that were in the mid-exponential growth phase (OD_{600} of ca. 0.3) with the exception of the experiment following gene expression changes throughout the growth curve (Clark et al., 2006).

Our simple interpretations of the differential expression of genes in microarrays (**Table 1**) are as follows. Comparison of transcripts from cultures in stationary phase versus exponential phase for cells growing with lactate (60 mM) plus sulfate (50 mM; Clark et al., 2006) represent a lactate-limiting condition and preparation for a non-growing state. We have assumed that an indicator for gene importance in the metabolism of lactate plus sulfate as cells enter stationary phase, at the substrate concentrations given, would be an increase in expression of carbon acquisition genes. A decrease in those genes needed for sulfate reduction and energy generation might also be expected since the cells are slowing growth and sulfate is plentiful. For cultures grown to mid-exponential phase with thiosulfate versus sulfate as electron acceptor, we would predict that transcript changes would

reflect a decrease for the sulfate-reducing enzymes but that sulfite reduction and carbon and electron metabolism would remain unchanged. For cultures growing exponentially with pyruvate as carbon and electron source compared to lactate, the genes differentially transcribed should include those required for lactate transport and oxidation. Clearly a direct connection between transcript changes and enzyme levels is often not evident (Torres-García et al., 2009). However, because energy conversion is such a large proportion of the metabolism of these bacteria, the possibility of correlated changes was tacitly assumed.

RELATIVE ABUNDANCE OF TRANSCRIPTS

All microarray experiments in the database of the VIMSS program have been normalized to DNA signals on the microarrays. As a result, a robust measure of the relative abundance of mRNA for each gene has been accumulated during microarray experiments, with 173 data points in the calculation for each (Wall et al., 2009). We determined an average expression level from the mRNA/DNA signals and arbitrarily set the average for all genes to 1 or $\log_2 = 0 \pm 1.54$. Thus a transcript value that is one SD above the average is a gene expressed approximately three fold more highly than average and is in the top 16% expression level of all genes. Because so much of the metabolic capacity of anaerobes is devoted to energy conversion, we assumed that the genes for the proteins and enzymes responsible would likely be among those above the average in expression.

RANDOM TRANSPOSON MUTANTS

To reveal genes needed for growth of *D. vulgaris* on lactate plus sulfate, the final information source comes from mutation analysis. The creation of a random transposon library in this bacterium with a modified Tn*5*-RL27 transposon (Larsen et al., 2002; Oh et al., 2010) has been initiated. To date, the Wall lab has 8,869 *D. vulgaris* mutants in which the insertion site of the transposon has been identified by sequencing that can be seen on the following website http://desulfovibriomaps.biochem.missouri.edu/mutants/. Most mutants were selected on lactate (60 mM) plus sulfate (30 mM) medium supplemented with yeast extract to avoid loss of auxotrophs. The library currently has mutations in 2208 unique genes or 62% of the ca. 3500 genes predicted in the genome. Our assumption for interpreting these data was that if a gene were essential for this growth mode, no mutations would be found in the gene. Of course, essential genes not specific to lactate/sulfate growth would also be identified among those not mutated. Conversely, genes actually needed, but whose loss could be compensated by expression of others, would likely be found to be mutated. As yet the library is not saturating for non-essential genes and the data must be interpreted with that caveat in mind.

Figure 2 provides two examples of the transposon mutant analysis. The enzymes for sulfate reduction have been biochemically confirmed (Peck and LeGall, 1994) and, as a result, the gene annotations are firm (**Table 1A**). There are apparently single copies of these genes in the genome and we would not expect mutations to be found in cells growing by sulfate respiration. In **Figure 2A**, a section of the genome with the genes for the bisulfite reductase is illustrated with the insertion sites of the transposon indicated. There are no mutations in *dsrABD* while the flanking genes were successfully mutated. In the second example (**Figure 2B**), the genes putatively encoding the cytoplasmic hydrogenase associated with carbon monoxide dehydrogenase, *cooMKLXUH*, also lack insertions of the transposon, a result not entirely expected. This is a region is about 8.2 kb. With 8869 transposon insertions, on average we should expect an insertion about every 425 bp (with the conservative assumption that the entire genome is an appropriate target). Interestingly the region in which transposon mutations have not been recovered is precisely congruent with the Coo hydrogenase operon. It is probable that this region is needed for growth on lactate/sulfate or to survive the manipulations of the mutagenesis process.

INFLUENCE OF GROWTH CONDITIONS ON THE EXPRESSION OF GENES

SULFATE UTILIZATION GENES

The molecular information about genes potentially involved in lactate/sulfate growth of *D. vulgaris* – differential expression in defined culture conditions, relative abundance of mRNA and essentiality during growth with lactate/sulfate – are considered for the different categories of functions (**Table 1**). First, the genes known to encode APS reductase (*apsBA*) and bisulfite reductase (*dsrABD*), tended to decrease in expression in stationary phase cells limited for electron donor where growth rate was decreasing (**Table 1A**). The expression levels of *apsBA* were also decreased when thiosulfate replaced sulfate. This result might be predicted

since the substrate for the APS reductase was absent. Also as expected, *dsrABD* mRNA did not decrease with thiosulfate, since the initial two electron reduction of thiosulfate generates sulfide and bisulfite (Thauer et al., 2007). Measurements of the dissimilatory bisulfite reductase genes in *Desulfobacterium autotrophicum* grown in lactate/sulfate compared to lactate/thiosulfate (lactate limiting in both media) showed that early exponential phase expression of the *dsrAB* genes with sulfate was slightly higher than that observed with bisulfite (Neretin et al., 2003). As these *D. autotrophicum* cells entered stationary phase, the *dsrAB* expression in bisulfite medium exceeded that with sulfate. Major changes were not seen in the production of *dsrAB* transcripts in *D. vulgaris* with alternative electron acceptors consistent with the report of constitutive expression of these genes (Brusseau, 1998, as reported in Neretin et al., 2003).

Neither *apsBA* nor *dsrABD* was responsive to the change in electron donor from lactate to pyruvate. The predictions of abundant mRNAs for these genes (**Table 1A**) and the absence of transposon mutations in the library (**Figure 2A**) were accurate and serve as the proof of principle for the use of these features as predictive measures. The transcript abundances for these genes were among the top 1% of all *D. vulgaris* genes.

For the highly expressed genes encoding sulfate adenylyltransferase and pyrophosphatase, *sat* and *ppaC*, the microarray data showed that transcription decreased with thiosulfate as terminal electron acceptor (**Table 1A**). Because these enzymes function primarily for sulfate utilization, the mRNA decrease for the encoding genes might reflect a control by sulfate concentrations. No transposon mutations in these genes were isolated from cells grown on lactate/sulfate medium consistent with an essential function.

Because sulfate carries two charges at physiological pHs, transport functions would be expected. However, all growth conditions from which molecular and genetic information were obtained had plentiful sulfate, where electroneutral uptake with two protons might be expected (Cypionka, 1995). Three candidate sulfate permease genes were annotated, but no single one was apparently essential since transposon mutants lacking each were recovered (**Table 1C**). The abundance level of the mRNA of each was near that of the average for *D. vulgaris* genes. Microarray changes also did not indicate a unique ortholog among the genes, although DVU0279 was strongly decreased in expression in stationary phase when carbon and electron flow was limited.

CANDIDATE GENES FOR LACTATE METABOLISM

For genes encoding possible enzymes for lactate transport and oxidation, the story is not clear. While some biochemical analysis for lactate dehydrogenase has been accomplished (Odom and Peck, 1981b; Ogata et al., 1981; Stams and Hansen, 1982), the genes encoding the enzyme(s) responsible for either oxidation or transport have not been identified. Of the six genes annotated as putative lactate permeases, only two showed increases in transcription as the cells were being limited for carbon, DVU2110 and DVU3284. Neither of these was essential alone since mutations were found in each in lactate/sulfate grown cells and the mRNA abundance of both was well below average (**Table 1C**). The two most highly transcribed orthologs were still only 1.6–1.7 times the average gene in expression.
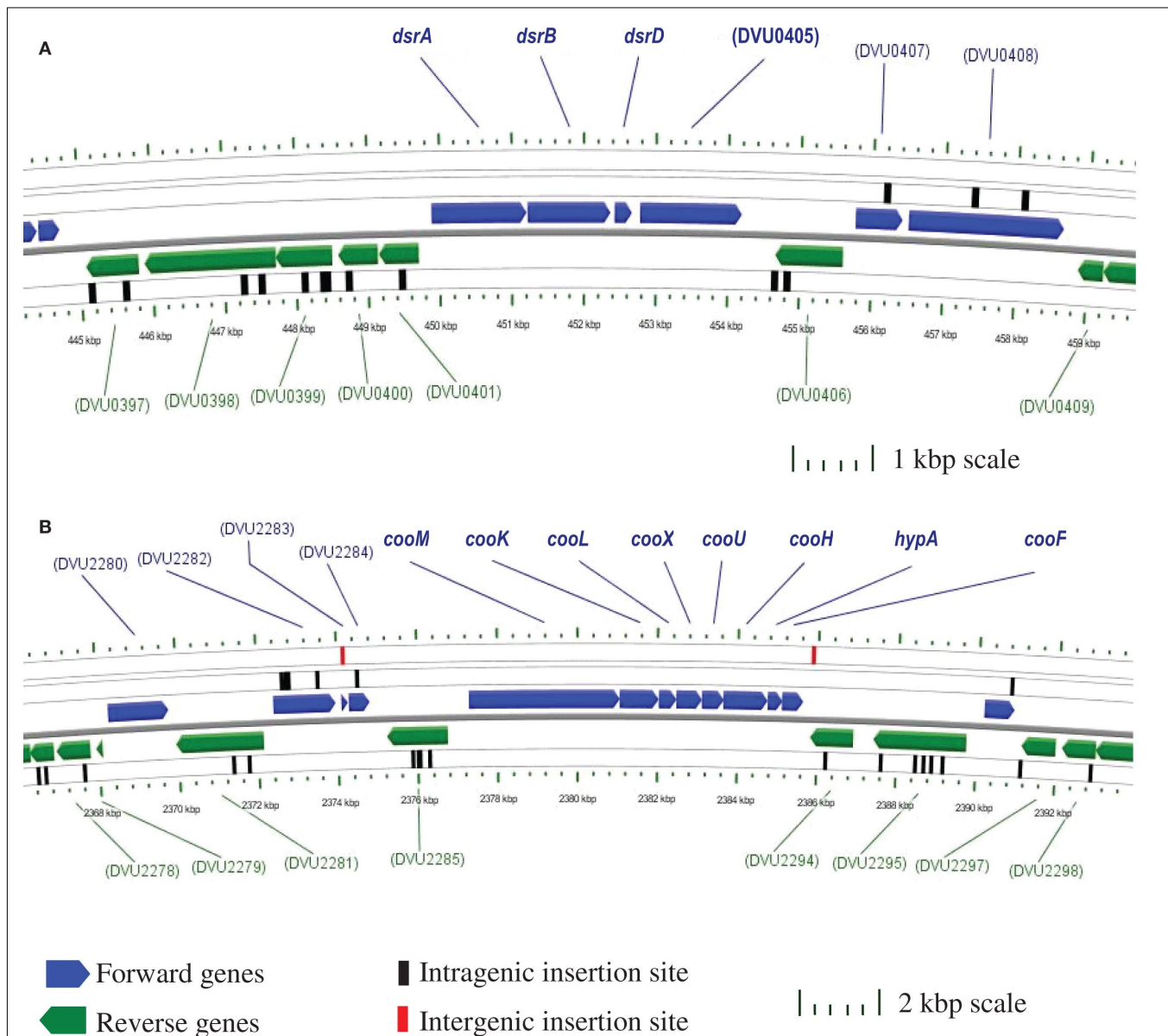
FIGURE 2 | Chromosomal maps generated with CGView (Stothard and Wishart, 2004) indicating the location of transposon insertions in the *Desulfovibrio vulgaris* Hildenborough genome. Open reading frames are designated by TIGR DVU numbers. **(A)** No intragenic transposons are within the 4.2 kb four gene dissimilatory bisulfite reductase (*dsr*) operon; however within the 5 kb regions flanking the operon there are 14 intragenic insertions. **(B)** No intragenic transposons are within the 8.2 kb eight gene putative CO-induced membrane-bound hydrogenase (*coo*) operon. Also, there are 21 intragenic transposon insertions within the 10 kb regions up- and down-stream of the operon, as well as two intergenic transposon insertions.

The number of candidate genes encoding the lactate dehydrogenase (LDH) activities that have been annotated is about nine (**Table 1B**), including those annotated as glycolate oxidases. Because many *Desulfovibrio* strains can readily use both L- and D-lactate (Stams and Hansen, 1982), at least two enzymes would seem to be necessary. Biochemical analysis of the LDHs showed that all are membrane-bound and NAD(P)$^+$-independent (Hansen, 1994b). However, the L- and D-LDH differ in their sensitivity to oxygen with the L-LDH from *Desulfovibrio baculatus* HL21 DSM 2555 (formerly, *Desulfovibrio desulfuricans* HL21) being extremely unstable (Stams and Hansen, 1982; Hansen, 1994a); whereas, the D-LDH from *D. vulgaris* Miyazaki is apparently much more stable when exposed to oxygen (Ogata et al., 1981).

Of the nine *ldh* orthologs and paralogs in *D. vulgaris*, none was increased in response to a change in electron donor from lactate to pyruvate (**Table 1B**). Genes for three enzymes were increased from 2.1 to 2.6 fold upon entering stationary phase presumably because of carbon and electron donor limitation, DVU0600, DVU1783, and the complex DVU3027–3028. Of the three, only the latter complex had more abundant transcripts than the average gene.

This complex is also part of a nine gene operon that includes *por,* encoding the primary pyruvate:ferredoxin oxidoreductase; *pta,* phosphotransacetylase; and *ackA,* the acetate kinase; and is preceded by a gene annotated as a lactate permease, DVU3026. Interestingly, *por, pta,* and *ackA* have not been mutated by the insertion of the transposon; whereas, seven transposon insertion sites have been identified within the coding sequences of the putative permease and lactate dehydrogenase, DVU3026, 3027, and 3028. We suggest that the transposon data mean that these genes are either not involved in lactate metabolism or that there are other enzymes able to compensate for the mutations in these genes.

TRANSMEMBRANE COMPLEX OPERONS

The genes encoding the transmembrane electron transport complexes (TMC) that are proposed to function with APS reductase and bisulfite reductase are *qmoABC* and *dsrMKJOP,* respectively (**Table 1D**; Matias et al., 2005; Pereira et al. (2007)). They are strictly conserved among sulfate reducers, underscoring their importance for sulfate reduction. The assignment of QmoABC and DsrMKJOP as electron carriers to their respective reductases also receives support from the location of their encoding genes (Pereira et al., 2007). *qmoABC* are immediately adjacent to and downstream of *apsBA.* Although the *dsrMKJOP* operon is not in the proximity of the structural genes for bisulfite reductase in *D. vulgaris,* it is contiguous with orthologs of the structural genes in the sulfur oxidizer, *Allochromatium vinosum* (Dahl et al., 2005). The genes for both of these TMCs were down regulated when *D. vulgaris* entered stationary growth phase caused by electron donor limitation (**Table 1D**), as suggested for genes encoding functions involved in energy generation. Curiously both are also decreased in expression when thiosulfate replaced sulfate as terminal electron acceptor, even though DsrMKJOP would be predicted to be essential for thiosulfate use (Thauer et al., 2007). A change in electron donor did not appear to impact the transcription of the genes for either of the TMCs importantly. The relative mRNA abundances for these genes were among the top 20% of all genes in the bacterium and no transposon mutants were identified, supporting the supposition that their function is essential for growth on lactate plus sulfate.

A deletion of the *qmoABC*-DVU0851 was recently constructed by marker exchange mutagenesis in *D. vulgaris* (Zane et al., 2010). This mutant confirmed the essential role of the encoded complex in sulfate reduction. The deletion mutant was unable to grow with sulfate as terminal electron acceptor with any electron donor including H_2 or formate, although growth with sulfite or thiosulfate was unimpaired. The hypothetical gene, DVU0851, was shown not to be necessary for sulfate reduction. Interestingly no other TMC of *D. vulgaris* was able to compensate for the loss of QmoABC.

Recently an additional four protein transmembrane complex, QrcDCBA or Type 1 cytochrome c_3:menaquinone oxidoreductase, has been proposed to be essential to carry electrons coming from H_2 or formate to the cytoplasmic membrane for delivery to APS reductase in *D. vulgaris* (Venceslau et al., 2010). The Qrc complex was suggested to be necessary because the QmoABC complex apparently has no periplasmic interface that would allow electrons from the soluble cytochrome *c* matrix to traverse the membrane to reach the sulfate-reducing enzymes. Curiously 10 of 12 fully sequenced *Desulfovibrio* strains have highly conserved *qrc* operons[2]; however, *D. desulfuricans* ATCC 27774 and *Desulfovibrio piger* do not. Because these two species still have a conserved Qmo complex, another complex may be needed to serve the Qrc function of delivery of periplasmic electrons to the membrane for sulfate reduction (Venceslau et al., 2010).

A transposon mutation in the *D. alaskensis* G20 gene Dde_2933 corresponding to *qrcB,* was unable to grow with H_2 or formate as electron donor (Li et al., 2009). In *D. vulgaris,* QrcDCBA was also decreased in expression during stationary phase when energy generation was decreased (**Table 1D**) and was found to have above average transcript abundance. Surprisingly, transposon insertions in the genes for this complex were isolated in cells growing on lactate plus sulfate, showing that either this complex was not required for electrons from all donors or that compensation for this complex was possible.

A deletion of DVU0692–0694, Δ*qrcBCD,* has been constructed in *D. vulgaris* (Wall lab, unpublished). Preliminary experiments showed that this mutant was capable of growing on lactate plus sulfate but the rate was slower and the yield was about 40% less than that of wild-type cells. As for the *D. alaskensis* G20 mutant, the *D. vulgaris* deletion could not grow with H_2 or formate with sulfate as electron acceptor, consistent with the predicted role for this complex by Venceslau et al. (2010). A need for the Qrc complex should be bypassed if electrons from the periplasmic cytochrome pool could be transferred to bisulfite reductase through a cytochrome *c* containing complex. Because DsrJ of the DsrMKJOP complex is a periplasmic cytochrome *c* and the complex is predicted to be the electron conduit to bisulfite reductase, this complex would be predicted to be able to accept periplasmically generated electrons from H_2 or formate carried by the abundant Tp1-c_3. Sulfite does support growth of the *qrc* mutant on formate. The inability of the Δ*qmoABC* mutant to grow on lactate, H_2 or formate during sulfate respiration would suggest that the Qrc complex acts prior to Qmo. The possible role of QrcABCD must be further investigated.

Of the remaining TMCs characterized or recognized in the *D. vulgaris* genome, the High molecular weight cytochrome (Hmc) complex has been proposed by many researchers to be a conduit for electrons from H_2 to sulfate (Peck, 1993; Dolla et al., 2000; Pereira et al., 2007). A mutant deleted for *hmcBCDE* was impaired in syntrophic growth with *Methanococcus maripaludis* (Walker et al., 2009). The low level of transcript abundance for this complex during growth on lactate/sulfate (**Table 1D**) would not appear compatible with a primary role for Hmc for energy conversion on these substrates. In addition, the presence of multiple transposon insertions within the Hmc coding sequences in the mutant library casts doubt on its importance. The inference that a functional Hmc is not essential for *D. vulgaris* in lactate plus sulfate medium is consistent with monoculture growth, albeit slower, of the deletion mutant (Dolla et al., 2000; Walker et al., 2009). These observations support a role for this TMC when H_2 must be maintained at a low level.

A role for the decaheme cytochrome *c* and the RnfCDGEAB complex in *Desulfovibrio* has not been established. This complex was first identified in *Rhodobacter capsulatus* as complex required

for nitrogen fixation (Schmehl et al., 1993). It is also in the family of electron bifurcating flavoproteins that are NADH ferredoxin oxidoreductases (Herrmann et al., 2008).Transcript changes and abundances were not particularly informative. However, several transposon mutations have been identified in the encoding genes indicating that the complex is not essential to *D. vulgaris* cells growing on lactate plus sulfate. A deletion of *rnfC* constructed by marker replacement confirmed a role for this complex in the ability of *D. vulgaris* to grow with molecular nitrogen as the sole nitrogen source (Wall lab, unpublished).

Finally, *tmcABCD* and *ohcABC* (Pereira et al., 2007) have been identified as encoding TMCs but a role for each is not yet known. The *tmc* genes are expressed at about the same level as the *dsrMKJOP* operon suggesting a role in the metabolism (**Table 1D**), but preliminary characterization of a mutant deleted for the genes had no apparent phenotype (Wall lab, unpublished). This was consistent with finding transposon mutations within the structural genes for the complex. In contrast, *ohcABC* transcripts were in the lower 15% abundance of all *D. vulgaris* genes, perhaps indicating a non-critical role in energy metabolism in cells growing with lactate plus sulfate.

HYDROGENASE OPERONS

The presence and activity of cytoplasmic hydrogenases were predicted to be necessary for energy augmentation by hydrogen cycling in sulfate reducers (Odom and Peck, 1981a). Although the distribution of cytoplasmic hydrogenases in *Desulfovibrio* strains is not conserved, there are candidate enzymes that could function in most cases. *D. vulgaris* has two putative energy-converting hydrogenases with subunits related to the proton-pumping NADH:ubiquinone oxidoreductase (complex I), Ech hydrogenase (Friedrich and Scheide, 2000) and the Coo hydrogenase, CooH, originally described in *Rhodospirillum rubrum* (Kerby et al., 1992). These enzyme complexes are reversible, oxidizing reduced ferredoxin with the production of H_2 while generating an electrochemical proton potential or using reversed electron transport to catalyze reduced ferredoxin from H_2 (Thauer et al., 2007). These complexes are encoded in DVU0429–0434 and DVU2286–2293, respectively (**Table 1F**). Transcription expression changes were not dramatic for these complexes, but the transcript abundance difference between the two was striking. The *cooMKLZUHhypAcooF* operon is among the top 15% of expressed genes; whereas, the *echABCDEF* operon is well below average in expression. As seen earlier in **Figure 1**, mutations in this *coo* operon were not recovered in cells grown with lactate/sulfate, suggesting that this cytoplasmic hydrogenase is needed for this growth mode. It should be noted that the putative structural genes for the CO dehydrogenase enzyme, *cooSC,* were found to carry transposon insertions, as did the *ech* operon.

Through examination of a mutation in *cooL,* the Coo hydrogenase has also been shown to be essential for syntrophic growth of *D. vulgaris* with *Methanococcus* on lactate but not pyruvate (Walker et al., 2009). However, there was no requirement for this hydrogenase for respiration of lactate with sulfate. This observation is curiously in conflict with the absence of transposon mutations in these genes when selection was carried out on

lactate/sulfate medium. Interestingly, Coo hydrogenase belongs to a family of transmembrane complexes that function as proton (or sodium) – translocating hydrogenases and that use reduced ferredoxin as electron donors (Welte et al., 2010; **Figure 1**). The recently proposed mechanism of flavin-based electron bifurcation (Herrmann et al., 2008) could offer a possible solution to the necessity of lactate supported hydrogen production. This mechanism would suggest that, when concentrations of H_2 are maintained at very low levels, the exergonic reduction of protons to form H_2 from the reduced ferredoxin ($E^{0\prime} \leq 420$ mV) could be coupled with the endergonic formation of hydrogen with electrons from menaquinone ($E^{0\prime}$ -75 mV). The Coo membrane-bound hydrogenase complex is a flavoprotein potentially allowing bifurcation of electrons from the two sources, reduced ferredoxin and menaquinone. While the energy available for pumping a proton (or sodium ion) might be dissipated through the bifurcation, the overall contribution to the proton gradient from the oxidation of the additional H_2 might compensate. This model (**Figure 1**) would also provide an explanation for the cessation of electron flow in this pathway as hydrogen concentrations increased. Currently no experiments have been conducted to explore electron bifurcation function during lactate oxidation and sulfate reduction in *Desulfovibrio*.

While the four periplasmic hydrogenases have been well characterized (Fontecilla-Camps et al., 1997; Vignais and Billoud, 2007), no single enzyme has been shown to be essential for uptake or production of H_2 (Casalot et al., 2002; Caffrey et al., 2007). While the transcript abundance of the [NiFeSe] hydrogenase appears to be the highest of the four, this may reflect the inclusion of selenium in the medium trace elements which strongly increases the production of the [NiFeSe] hydrogenase while repressing the [FeFe] and [NiFe]-1 hydrogenases (Valente et al., 2006). Multiple deletions and various combinations will be needed to determine the roles of these enzymes.

GENES FOR *c*-TYPE CYTOCHROMES

Of the annotated *c*-type cytochromes that are not apparently associated with large TMCs, the Type I tetraheme cytochrome c_3 (Tp1-c_3, DVU3171) and the monoheme c_{553} cytochrome (DVU1817) have highly abundant transcripts (**Table 1H**) suggesting a major role in electron flow during energy conversion. The high expression levels are congruent with the abundance of the cytochromes as well (Moura et al., 1991; Pollock and Voordouw, 1994; Aubert et al., 1998). Tp1-c_3 has been suggested to be the electron acceptor for each of the periplasmic hydrogenases and to serve as a capacitor for electrons in the periplasm of the SRM (Heidelberg et al., 2004). Interestingly, there is little expression modulation of the gene encoding, Tp1-c_3, *cycA*, upon entry into stationary phase or in response to altered electron acceptors or donors as has previously been reported (Payne et al., 2002). No transposon mutations have been recovered that would eliminate Tp1-c_3, although the small size of *cycA* may be a factor in mutant isolation. However, the successful construction of a marker exchange deletion of the encoding gene (Semkiw et al., 2010), showed that Tp1-c_3 is not essential for growth on lactate/sulfate. Similar mutants have been generated in *D. alaskensis* G20 (Rapp-Giles et al., 2000; Li et al., 2009). The *D. vulgaris* Δ*cycA* mutant was unable to grow

with H_2 or formate with sulfate or sulfite as electron acceptor (Semkiw et al., 2010). Growth was slowed with lactate plus sulfate or sulfite but the biomass produced was not different from wild-type. For cells growing with sulfate respiration, the observations mirror those for the Δqrc mutant of this bacterium or transposon interruptions in the homologs found in *D. alaskensis* G20 (Li et al., 2009). These results suggest a pathway for periplasmically generated electrons through Tp1-c_3 and the Qrc complex to the Qmo. Whether both the cytochrome and the Qrc complex are needed to transfer electrons for bisulfite reduction remains to be established.

The gene for the abundant cytochrome c_{553} (*cyf*, DVU1817) is located just upstream of the cytochrome *c* oxidase, a putative heme-copper oxygen reductase. It has been suggested that the cytochrome c_{553} might serve as an electron donor to that membrane-bound complex (Dolla et al., 2007). No major differences in expression were apparent in the microarray data. It might be predicted that constitutive expression could be advantageous if this cytochrome plays a role in oxygen protection. A deletion of *cyf* grew well on lactate plus sulfate (Wall lab, unpublished) confirming that this cytochrome is not a key electron carrier for energy conversion under these conditions. A possible role in oxygen tolerance will require further experimentation.

The function of other *c*-type cytochromes is still undetermined and will likely require multiple deletions and biochemical analysis to gain insight. The lack of transposon mutations in a number of the encoding genes may simply reflect the small size of the genes and the lack of saturation in the library of mutants.

SUMMARY OF KEY OBSERVATIONS FROM TRANSCRIPTS AND MUTANTS

In summary, the data in **Table 1** show that the transcriptional changes in the microarray data suggest that genes for both sulfate and sulfite reduction are not constitutively expressed but respond to the energy demands of the cell, decreasing in expression in stationary phase and when thiosulfate is provided as electron acceptor. The operons for the proposed transmembrane complexes for donating electrons to APS reductase and that for bisulfite reductase were similarly decreased under these conditions; whereas, operons for other putative transmembrane complexes were not uniformly changed in transcription in these conditions. The genes and operons for sulfate and sulfite reduction were among the most highly transcribed genes in the cells and, as would be predicted while growing with lactate plus sulfate, transposon insertions in these genes were not recovered. Transposons were recovered in almost all other operons and genes listed with the interesting exception of the Coo hydrogenase complex which showed a surprisingly low number of insertions. This complex has been shown not to be essential for growth on lactate plus sulfate but it appears to be needed for syntrophic growth with *Methanococcus maripaludis* with lactate as sole source of carbon and reductant (Walker et al., 2009) and for the efficient recovery of transposon mutants. Because this complex is a member of the energy-converting [NiFe] hydrogenases (Hedderich, 2004), it is predicted that this complex is integral to the energy budget at some stage of culturing for single colony formation on medium with lactate as electron donor and sulfate as electron acceptor.

MUTAGENESIS TOOL DEVELOPED FOR *DESULFOVIBRIO*

Important information about electron carriers has been gained from constructed deletions and from transposon insertions. However, the genes encoding a number of the enzymes believed to be needed for electron flow in *D. vulgaris* have not been specifically identified in part because multiple ORFs have been similarly annotated. Examples include the periplasmic hydrogenases, formate dehydrogenases, lactate permeases, lactate dehydrogenases, and others. When multiple genes encode isozymes, the loss of one enzyme by mutation of its encoding gene may be compensated by others. This compensation or suppression could explain why transposon mutations are observed in genes for what would be predicted to be key enzymes (**Table 1**). To elucidate the function of individual members of protein families, multiple genes will likely need to be eliminated simultaneously. Until recently, the number of sequential deletions in the same strain of *D. vulgaris* was limited by the number of reliable antibiotic resistant markers available to use for marker exchange deletion construction. A markerless deletion system has now been developed based on the counterselection of the gene for uracil phosphoribosyltransferase, *upp*, by resistance to the toxic uracil analog 5-fluorouracil (Keller et al., 2009). This method allows the generation of in-frame deletions without a residual antibiotic resistance gene. The application of this technique may contribute critical information about any unique roles of the four periplasmic hydrogenases. The flexible electron flow pathways and mechanisms of energy conversion in *D. vulgaris* will require the combined application of genetic, biochemical and systems biology approaches.

MODEL OF ELECTRON TRANSPORT FOR *D. VULGARIS* HILDENBOROUGH

To provide a framework for organizing the information obtained to date for electron flow from lactate to sulfate, a model is presented in **Figure 1**. This scheme draws heavily from the models presented by Noguera et al. (1998) and Walker et al. (2009) and is driven by the observations of two proteins putatively involved in the electron pathways, the Tp1-c_3 and the Coo hydrogenase. First, the phenotype of a deletion of the *cycA* gene encoding the Tp1-c_3 indicates that electrons from periplasmic H_2 (or formate) are exclusively dependent on this cytochrome for reaching sulfate. Thus the ability of the $\Delta cycA$ mutant to grow on organic acids with sulfate and produce sulfide means that some proportion of the electrons generated from substrate oxidation must not cycle as molecular H_2. Thus the hydrogen cycling hypothesis may contribute but is not essential to the energy budget of SRM grown with organic acids. Second, a mutant in the Coo hydrogenase complex *cooL*::Tn*5*-RL27 was unable to grow by syntrophy with a methanogen on lactate but could grow in monoculture with lactate plus sulfate (Walker et al., 2009). An interpretation of these observations is that the mutation in the Coo hydrogenase complex prevented H_2 generation from lactate but did not block electron flow to sulfate. With pyruvate as electron donor, syntrophy was established as well as growth of monocultures (Walker et al., 2009). Thus a path for H_2 production is available for electrons from pyruvate that is not available to electrons from lactate. In addition, the apparent lack of recovery of transposon mutations in the *cooMKLXUHhypAcooF* genes in the recently generated library, points to Coo hydrogenase

as potentially important, but not necessarily essential, player in electron flow from lactate to sulfate.

The model accommodates these seemingly contradictory observations by suggesting two pathways for the electrons from lactate (**Figure 1A and B**) and from pyruvate (**Figure 1C, and D**). In each case, one pathway has the potential for transfer of electrons into the menaquinone pool for delivery to the membrane associated complex, Qmo, that is dedicated for the first step in sulfate reduction (pathways A and D). Whereas the DsrMKJOP complex is thought to be the transmembrane complex for electron donation to bisulfite reductase, it may also receive electrons directly from the menaquinone pool. The second path in each case, B and C, provides a route for the electrons to generate H_2. The model is already too simple for electron flow from pyruvate since H_2 generation in the absence of a functional Coo hydrogenase is documented (Walker et al., 2009). The question marks in the model indicate the components that have a high uncertainty, including the membrane associated component(s) accepting electrons from pyruvate oxidation.

A future effort will be to determine the gatekeepers for directing electrons into one path or another. Will this be just the availability of acceptors or will the rate of flux through the pathways determine the choices? The complexity of this simple system continues to impress. It is already evident that there will not be a single wiring circuit for electron flow from these substrates in the SRM as it does not extend to the physiologically similar *D. alaskensis* G20 strain that lacks orthologs encoding the cytoplasmic hydrogenases of *D. vulgaris*.

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