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Energy metabolism in *Desulfovibrio vulgaris* Hildenborough: insights from transcriptome analysis

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Abstract Sulphate-reducing bacteria are important players in the global sulphur and carbon cycles, with considerable economical and ecological impact. However, the process of sulphate respiration is still incompletely understood. Several mechanisms of energy conservation have been proposed, but it is unclear how the different strategies contribute to the overall process. In order to obtain a deeper insight into the energy metabolism of sulphate-reducers whole-genome microarrays were used to compare the transcriptional response of *Desulfovibrio vulgaris* Hildenborough grown with hydrogen/sulphate, pyruvate/sulphate, pyruvate with limiting sulphate, and lactate/thiosulphate, relative to growth in lactate/sulphate. Growth with hydrogen/sulphate showed the largest number of differentially expressed genes and the largest changes in transcript levels. In this condition the most up-regulated energy metabolism genes were those coding for the periplasmic [NiFeSe] hydrogenase, followed by the Ech hydrogenase. The results also provide evidence for the involvement of formate cycling and the recently proposed ethanol pathway during growth in hydrogen. The pathway involving CO cycling is relevant during growth on lactate and pyruvate, but not during growth in hydrogen as the most down-regulated genes were those coding for the CO-induced hydrogenase. Growth on lactate/thiosulphate reveals a down-regulation of several energy metabolism genes similar to what was observed in the presence of nitrite. This study identifies the role of several proteins involved in the energy metabolism of *D. vulgaris* and highlights several novel genes related to this process, revealing a more complex bioenergetic metabolism than previously considered.

Keywords Bioenergetic metabolism · Hydrogen metabolism · Microarrays · Respiratory complexes · Sulphate-reducing bacteria

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Abbreviations

HS	Hydrogen/sulphate medium
LS	Lactate/sulphate medium
LT	Lactate/thiosulphate medium
PS	Pyruvate/sulphate medium
P	Pyruvate medium
SRB	Sulphate-reducing bacteria
$TpIc_3$	Type I cytochrome c_3

Introduction

Sulphate-reducing bacteria (SRB) are anaerobic prokaryotes widespread in natural habitats like marine or freshwater sediments, soil, and also the gastrointestinal tract of many animals, including man (Rabus et al. 2001). They are particularly abundant in habitats with high concentration of sulphate such as sea water, effectively linking the global sulphur and carbon cycles (Truper 1984; Hamilton 1998). SRB use sulphate or other sulphur oxo-anions as electron acceptors for the anaerobic oxidation of inorganic or organic substrates such as hydrogen, lactate, acetate, ethanol and propionate. As a consequence of this metabolism, large amounts of sulphide are produced and accumulated in their natural habitats. This aggressive metabolic end-product poses an important economic problem for the oil industry due to its involvement in biocorrosion of metal structures (Dinh et al. 2004) in addition to souring of oil and gas deposits (Hamilton 1998). However, SRB can also reduce a wide variety of other substrates, including heavy metals and radionuclides. Examples include U(VI), Tc(VII), As (V) and Cr (VI), making these bacteria interesting targets to use for bioremediation of contaminated anaerobic sediments and groundwater (Lloyd 2003; Wall and Krumholz 2006). In situ stimulation of microbial populations using specific electron donors or acceptors is an attractive strategy for environmental bioremediation processes. A better understanding of energy metabolism of SRB growing with different energy sources or electron acceptors is thus crucial for exploring their potential in bioremediation, as well as to control their activity wherever it leads to undesirable consequences.

Despite numerous studies, the bioenergetic mechanism of sulphate respiration is still far from being understood. Odom and Peck (1981) proposed a bioenergetic pathway to explain energy conservation in *Desulfovibrio*, the most studied genus of SRB, which involved cycling of hydrogen during growth in lactate/sulphate. The proposal considers that protons and electrons resulting from the oxidation of lactate to acetate are converted to hydrogen in the cytoplasm by a cytoplasmic hydrogenase. The hydrogen then diffuses across the membrane to the periplasm where it is converted to protons and electrons by a periplasmic hydrogenase. Transfer of the resulting electrons back to the cytoplasm for sulphate reduction results in a transmembrane proton gradient that can drive ATP synthesis by the ATP synthase complex. This mechanism has been disputed because hydrogen formation from lactate oxidation to pyruvate is energetically unfavourable, even in conditions where there is a large excess of lactate in the presence of small hydrogen concentrations (Pankhania et al. 1988; Traoré et al. 1981). Furthermore, this mechanism requires the presence of a cytoplasmic hydrogenase, which is already known to be absent in the genome of some SRB such as *Desulfovibrio desulfuricans* G20 (www.jgi.doe.gov) and *Desulfotalea psycrophila* (Rabus et al. 2004). Alternative chemiosmotic models have been proposed in which electrons generated from substrate oxidation are transported through membrane-bound electron carriers to sulphate reduction (vectorial electron transport), and in the process translocate protons to the periplasm (vectorial proton transport) (Lupton et al. 1984; Wood 1978). This electron transport chain most likely involves the participation of menaquinone (Maroc et al. 1970), plus two membrane-bound redox complexes that are conserved in sulphate reducers, the Qmo and Dsr complexes (Haveman et al. 2004; Pires et al. 2003, 2006). There is also evidence that cycling of other reduced intermediates like CO or formate may also be involved in the energy conservation mechanisms of *Desulfovibrio vulgaris* Hildenborough (referred hereafter as *D. vulgaris*) (Voordouw 2002; Heidelberg et al. 2004; Tang et al. 2007). Biochemical, genetic and genomic studies of *D. vulgaris* have allowed the identification and initial characterization of several of the participants in the electron transfer network involved in sulphate respiration (Pereira et al. 2007). However, the genome reveals a large number of genes involved in energy metabolism with an unknown function. Furthermore, different electron transport pathways seem to be involved in the oxidation of

pyruvate, lactate or hydrogen (Voordouw 2002; Rapp-Giles et al 2000), and different metabolic strategies may be used by different organisms (Pereira et al. 2007). In this work, the global gene expression profile of *D. vulgaris* grown in different electron donors and acceptors was investigated in order to obtain a broad view of the transcriptional response of the energy metabolism genes. It has been shown that there is a good correlation between mRNA levels and protein abundance of Central Intermediary Metabolism and Energy Metabolism genes (Nie et al. 2006), thus enabling a physiological interpretation of the transcriptional response. This study complements a previous one comparing transcriptional changes for growth in formate/sulphate relative to lactate/sulphate (Zhang et al. 2006a). The results provide several insights into the bioenergetic pathways that are operative under the different growth conditions.

Materials and methods

Growth conditions

D. vulgaris (DSM 644) was grown in modified medium C (Postgate 1984) at 37°C, under an atmosphere of N_2. The modified medium C contained 25 μM $FeSO_4.7H_2O$ and 1 μM $NiCl_2 \cdot 6H_2O$. It was previously shown that in medium C without supplements the amount of adventitious selenium and nickel is below the detection limit using atomic absorption spectrometry (<0.04 mg/l and <0.01 mg/l, respectively) (Valente et al. 2006). Medium C-LS contained 40 mM lactate, 40 mM sulphate, and was used as a control. Pyruvate (40 mM) and thiosulphate (40 mM) were used as an alternative electron donor and acceptor, respectively. Both pyruvate and thiosulphate were added from stock solutions in sterile conditions with a 0.2 μm filter. Growth with limiting sulphate was carried out with pyruvate (40 mM), using a 10% pyruvate/sulphate grown innoculum. Media (1000-ml) were dispensed in 2000-ml DURAN® SHOTT bottles, flushed with N_2 and sealed with butyl rubber stoppers. Cultures with hydrogen as electron donor were grown in a 3 l fermentor containing 2 l of growth medium with 40 mM acetate, gassed with a mixture of 80% (vol/vol) H_2, 20 % (vol/vol) CO_2 at 900 ml/min, stirred at 250 rpm and with a constant pH of 6.8. As a control, *D. vulgaris* was grown in the same conditions but in Medium C-LS, and gassed with N_2. In both procedures a 10% (vol/vol) inoculum of a freshly grown culture of *D. vulgaris* in the same conditions of the experiment was used, with exception to pyruvate culture where the inoculum was grown in pyruvate/sulphate medium. The optical density of the cultures at 600 nm (OD_{600}) was determined with a Shimadzu UV-1603 spectrophotometer. Cultures were harvested by centrifugation (10,000 × *g* for 15 min) in the mid-exponential phase with an OD_{600} of 0.47 for lactate/sulphate (LS), 0.42 for H_2+CO_2+acetate/sulphate (HS), 0.45 for lactate/thiosulphate (LT), 0.6 for pyruvate/sulphate (PS) and 0.47 for growth on pyruvate (P). The cells were stored at −70°C until used.

Global expression analysis

Microarray expression profiling was carried out using *D. vulgaris* whole genome microarrays which contain 3,482 of the 3,531 protein-coding sequences of the *D. vulgaris* genome (He et al. 2006). All microarray procedures including total RNA extraction, cDNA labelling, microarray hybridization and washing were performed using previously published protocols (He et al. 2006). RNA extraction, purification, and labelling were performed independently on each cell sample. Four samples of each total RNA preparation were labelled, two with Cy3-dUTP and another two with Cy5-dUTP, for microarray hybridization. To hybridize microarray glass slides, the Cy5-dUTP-labelled cDNA targets from one lactate/sulphate culture (control culture) were mixed with the Cy3-dUTP-labelled cDNA targets from one culture grown with a different electron donor or acceptor and vice versa (dye swap). As a result, each biological sample was hybridized to four microarray slides. The arrays were visualized by ScanArray Express confocal laser scanner (Perkin Elmer), and hybridization signal intensities were quantified using ImaGene software version 6.0 (Biodiscovery, Marina Del Rey, CA).

Two biological replicates obtained from independently grown *D. vulgaris* cultures were used in the gene expression analysis for all growth conditions. In addition, each microarray slide contained duplicate sets of gene fragments and the RNA isolated from each replicate sample was hybridized with four

microarray slides. This provided a total of 16 data points per experimental condition enabling the use of rigorous statistical tests to determine significant changes in gene expression. The resulting data files were subjected to Lowess intensity-based normalization and further analyzed using GeneSpring version 5.1 (Silicon Genetics, Redwood City, Calif.). Lowess normalization was performed on each microarray slide, and results of the two replicate cultures of each experimental condition were used for statistical analysis. To assess the statistical significance of individual data points, the Student *t*-test was used to calculate a p-value to test the null hypothesis that the expression level was unchanged. Gene expression changes observed in these arrays were extensively validated by RT-PCR in previous studies (He et al. 2006; Clark et al. 2006; Mukhopadhyay et al. 2007; Caffrey et al. 2007). The annotation of the ORFs was obtained from the comprehensive microbial resource at The Institute for Genomic Research (TIGR). The data were deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE8069.

Results and discussion

Gene expression profiling was performed using cells from *D. vulgaris* collected at mid-exponential phase (Fig. 1). The transcriptomic profiles of *D. vulgaris* cultures grown in these conditions were determined using DNA microarrays representing approximately 99% of the total protein-coding capacity of the *D. vulgaris* genome (Heidelberg et al. 2004; He et al. 2006). Relative expression ratios were derived by comparing mRNA abundance levels in cells grown in HS, PS, LT and P relative to mRNA levels in LS grown cells. The number of genes displaying more than two fold change in transcript abundance, either up or down and corresponding to a *P* value of 0.05 or smaller was 761 in HS, 272 in LT, 73 in PS, and 96 in P (Fig. 2). Of the 3,379 chromosomal genes in *D. vulgaris*, 2,315 genes have so far been classified into one of the 20 clusters of orthologous groups of functional categories (COG) (Tatusov et al. 1997, 2003). Growth on HS shows considerable changes in the transcription profile versus the LS reference across all COGs, whereas the most significant changes found during growth on LT, PS and P as compared to LS, involve primarily genes

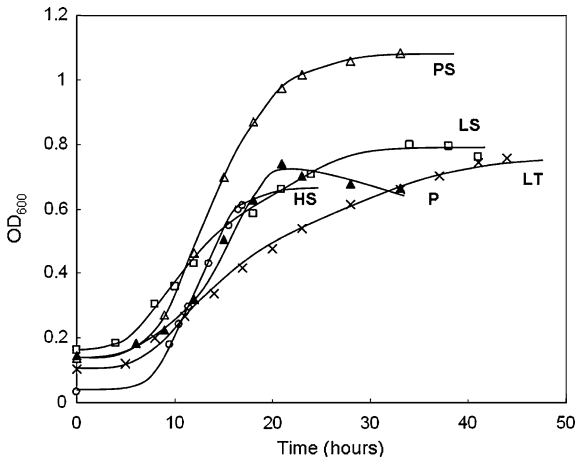

Fig. 1 Growth curves of *D. vulgaris*. □-LS; ◯-HS; Δ-PS; ▲-P; ×-LT. Points are averages of two independent growth experiments

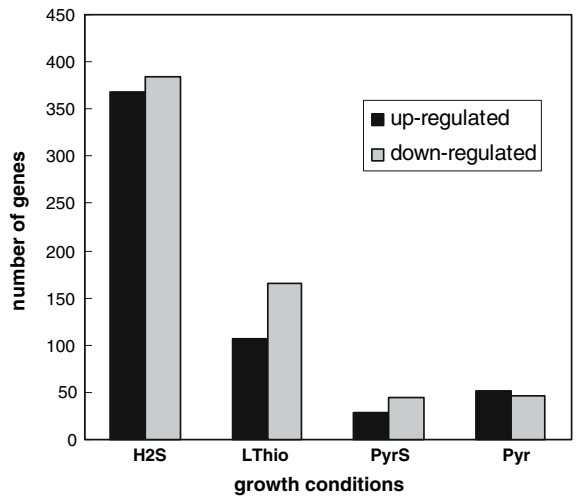

Fig. 2 - Distribution of up- and down-regulated genes in *D. vulgaris* Hildenborough as a function of different growth conditions. Only those genes with *P*-value ≤0.05 and a ratio value ≤0.5 and ≥2.0 are included in the plot

with functions in energy and central intermediary metabolism (Table 1). A detailed list of energy metabolism genes that display changes in their expression levels is presented in Table 2.

Changes in gene expression using hydrogen as electron donor

The use of hydrogen as electron donor lead to very significant changes in gene expression relative to

Table 1 Distribution of up- and down-regulated genes in *D. vulgaris* grown with different electron donors and acceptors for the COG functional categories presenting greater changes

COG functional category	HS		LT		PS		P	
	Total 761		Total 272		Total 73		Total 96	
	No. of genes		No. of genes		No. of genes		No. of genes	
	up	down	up	down	up	down	up	down
Cell envelope	22	29	7	14	1	3	9	3
Energy and central intermediary metabolism	45	35	14	27	7	4	20	3
Protein synthesis	2	42	1	8	0	0	1	0
Regulatory functions	22	17	8	7	3	3	3	2
Transport and binding proteins	34	31	4	25	0	6	1	8
Hypothetical proteins	129	120	36	39	11	18	12	21
Other or unknown function	115	118	37	45	7	10	5	8

The total number refers to the number of the all genes changed in each growth condition. Only those genes with *P*-value ≤ 0.05 and a ratio value ≤ 0.5 and ≥ 2.0 are included

growth with lactate, with approximately 30% of the genes involved in energy metabolism being affected (Table 2).

Periplasmic hydrogenases

As might be expected, some of the most affected genes include those that code for hydrogenases. The genome of *D. vulgaris* includes four periplasmic-facing hydrogenases, the [FeFe] hydrogenase (DVU1769-70; *hydAB*), two [NiFe] hydrogenase isoenzymes (DVU1921-22; *hynAB*-1 and DVU2525-26; *hynAB*-2) and a [NiFeSe] hydrogenase (DVU1917-18; *hysAB*). The genome also contains genes for two cytoplasmically oriented hydrogenases, the Ech hydrogenase (DVU0429-34; *echABCDEF*) and the Coo hydrogenase (DVU2286-93; *cooMKLXUHF*). It has not been clearly established if these hydrogenases may have specific roles, or if this redundancy allows functional compensation under different conditions (Goenka et al. 2005; Pohorelic et al. 2002). Recently, it was shown that expression of the three main hydrogenases detected in *D. vulgaris* ([FeFe], $[NiFe]_1$ and [NiFeSe]) is affected by the metals available in the growth medium, and that the [NiFeSe] hydrogenase is dominant when nickel and selenium are available (Valente et al. 2006). In the absence of selenium the [NiFeSe] hydrogenase is not detected and the major hydrogenases present are the $[NiFe]_1$ and the [FeFe] hydrogenases. In the present case the *hysAB* genes displayed a strong increase in transcript level with growth on HS, whereas the *hydA* gene transcript was slightly down-regulated and the *hynAB-1* genes showed no change. These data show that transcription of the [NiFeSe] hydrogenase gene is strongly up-regulated when hydrogen is supplied as electron donor, even when selenium is not available in the medium and thus the protein is not synthesised. This indicates that the [NiFeSe] is the most responsive hydrogenase to the presence of hydrogen. Western blot in similar growth conditions reveals a modest increase of both the $[NiFe]_1$ and [FeFe] hydrogenases when comparing HS to LS conditions (Valente et al. 2006). However, when nickel is present and selenium is not, the $[NiFe]_1$ hydrogenase is also strongly increased with growth in hydrogen relative to lactate. Very recently, it was also described that the transcription levels of the different hydrogenases of *D. vulgaris* are affected by the hydrogen concentration in the gas phase (Caffrey et al. 2007). The *hysAB* transcript level is higher with hydrogen at 5% (v/v) than with 50% and lowest with LS, whereas the opposite is observed for *hydAB*. These results, corroborated with studies of knock-out mutants, suggest that the high-activity, low-affinity [FeFe] hydrogenase is preferred when H_2 is plentiful, whereas the lower-activity, higher-affinity [NiFeSe] hydrogenase is preferred when there is a low concentration of H_2. In our studies the hydrogen concentration used was 80% and the *hysAB* genes were strongly up-regulated, but the other differences in growth conditions may preclude a direct comparison of the results. Overall, these studies reveal that the regulation of the periplasmic-facing

Table 2 Changes in transcription levels of energy metabolism genes from *D. vulgaris* Hildenborough grown with different electron donors and acceptors, relative to LS. Numbers in bold highlight changes larger than four fold up- or down

ORF	TIGR	Annotation	Symbol	HS		LT		PS		P	
				Ratio[a]	*P-value*[b]	Ratio[a]	*P-value*[b]	Ratio[a]	*P-value*[b]	Ratio[a]	*P-value*[b]
Periplasmic hydrogenases											
ORF01868	DVU1769	periplasmic [FeFe] hydrogenase, large subunit	HydA	0.46	2.3 E–03	2.92	4.3 E–06				
ORF01870	DVU1770	periplasmic [FeFe] hydrogenase, small subunit	HydB			**4.41**	2.9 E–07				
ORF02120	DVU1917	periplasmic [NiFeSe] hydrogenase, small subunit	HysB	3.42	3.1 E–03						
ORF02121	DVU1918	periplasmic [NiFeSe] hydrogenase, large subunit	HysA	**15.77**	2.0 E–02						
ORF03170	DVU2524	cytochrome c_3, putative	HynC3			0.27	5.9 E–04			2.65	8.5 E–04
ORF03171	DVU2525	periplasmic [NiFe] hydrogenase, small subunit, isozyme 2	HynB			0.36	2.6 E–04			2.14	2.3 E–03
ORF03173	DVU2526	periplasmic [NiFe] hydrogenase, large subunit, isozyme 2	HynA			0.43	3.2 E–03				
Cytoplasmic hydrogenases											
ORF05360	DVU0429	Ech hydrogenase, electron transfer protein	EchF	**11.60**	6.6 E–06			2.55	1.1 E–04		
ORF05364	DVU0431	Ech hydrogenase, unknown function	EchD	**5.20**	7.5 E–05			2.38	7.3 E–03	2.52	8.7 E–06
ORF05365	DVU0432	Ech hydrogenase, hydrogenase small subunit	EchC	3.34	6.4 E–04						
ORF05366	DVU0433	Ech hydrogenase, membrane protein	EchB	**10.23**	2.2 E–06			2.71	7.1 E–04	2.36	1.5 E–03
ORF05368	DVU0434	Ech hydrogenase, membrane protein	EchA	3.34	1.5 E–06						
ORF02767	DVU2286	hydrogenase, CooM subunit, putative	CooM	**0.22**	9.7 E–07						
ORF02769	DVU2287	hydrogenase, CooK subunit, selenocysteine-containing	CooK	**0.05**	8.4 E–010						
ORF02771	DVU2288	hydrogenase, CooL subunit, putative	CooL	**0.11**	2.7 E–08						
ORF02772	DVU2289	hydrogenase, CooX subunit, putative	CooX	**0.09**	1.0 E–011						
ORF02773	DVU2290	hydrogenase, CooU subunit, putative	CooU	**0.13**	2.7 E–08						

Table 2 continued

ORF	TIGR	Annotation	Symbol	HS		LT		PS		P	
				Ratio[a]	*P-value*[b]	Ratio[a]	*P-value*[b]	Ratio[a]	*P-value*[b]	Ratio[a]	*P-value*[b]
ORF02775	DVU2291	carbon monoxide-induced hydrogenase, putative	CooH	**0.14**	3.9 E–08						
ORF02777	DVU2292	hydrogenase nickel insertion protein	CooA	**0.19**	1.9 E–05						
ORF02778	DVU2293	iron-sulfur protein	CooF	0.27	1.4 E–06						
Periplasmic cytochromes											
ORF04139	DVU3107	octaheme cytochrome *c*	Occ			**0.16**	6.8 E–06				
ORF04250	DVU3171	type I cytochrome c_3	TpIc3							2.46	1.4 E–03
Membrane-bound electron transfer complexes											
ORF05546	DVU0531	hmc complex, electron transfer protein	HmcF	**0.22**	2.6 E–03					2.97	1.9 E–02
ORF05549	DVU0533	hmc complex, membrane protein	HmcD	**0.14**	1.5 E–05			0.48	4.1 E–03	2.10	4.3 E–02
ORF05551	DVU0535	hmc complex, electron transfer protein	HmcB	**0.20**	1.3 E–02	3.17	8.8 E–03				
ORF05554	DVU0536	hmc complex, high-molecular weight cytochrome *c*	HmcA	0.27	1.2 E–02					2.33	3.9 E–04
ORF03607	DVU2793	Rnf complex, membrane protein	RnfD					2.00	3.4 E–05		
ORF03609	DVU2795	Rnf complex, membrane protein	RnfE	2.25	6.5 E–06						
ORF03611	DVU2796	Rnf complex, membrane protein	RnfA	2.62	3.2 E–07			2.15	3.2 E–05		
ORF03613	DVU2797	Rnf complex, electron transfer protein	RnfB	3.19	5.6 E–07						
ORF05080	DVU0263	type II cytochrome c_3	TmcA	2.17	3.2 E–05						
ORF01067	DVU1286	Dsr complex, transmembrane protein	DsrP	0.48	2.2 E–04	0.40	4.2 E–05				
ORF01068	DVU1287	Dsr complex, iron-sulfur binding protein	DsrO	0.31	3.5 E–06	0.33	1.7 E–08				
ORF01069	DVU1288	Dsr complex, cytochrome *c* family protein	DsrJ	0.48	3.9 E–04	0.35	1.1 E–07			2.09	6.1 E–03
ORF01070	DVU1289	Dsr complex, iron-sulfur binding subunit	DsrK	0.37	2.9 E–06	0.36	3.2 E–06			2.16	1.8 E–04
ORF01072	DVU1290	Dsr complex, membrane cytochrome *b* protein	DsrM	0.29	6.1 E–07	0.38	7.3 E–05				

Table 2 continued

ORF	TIGR	Annotation	Symbol	HS		LT		PS		P	
				Ratio[a]	*P-value*[b]	Ratio[a]	*P-value*[b]	Ratio[a]	*P-value*[b]	Ratio[a]	*P-value*[b]
ORF00341	DVU0848	Qmo complex, flavin protein	QmoA	0.37	1.0 E–06	0.44	1.4 E–02			2.27	2.3 E–02
ORF00343	DVU0849	Qmo complex, flavin protein	QmoB			0.46	8.7 E–03			2.36	1.6 E–02
ORF00345	DVU0850	Qmo complex, membrane iron-sulfur protein	QmoC			0.47	1.2 E–02				
ORF04199	DVU3143	iron-sulfur cluster binding protein	OhcB							2.63	1.6 E–04
ORF04200	DVU3144	octaheme cytochrome *c*	OhcA							2.13	2.5 E–04
ORF00078	DVU0692	molybdopterin oxidoreductase, transmembrane subunit								2.05	5.0 E–02
ORF00079	DVU0693	molybdopterin oxidoreductase, iron-sulfur cluster-binding subunit								2.45	8.9 E–03
Formate dehydrogenases											
ORF05640	DVU0587	formate dehydrogenase, α subunit	FdhA	**4.05**	5.7 E–03	3.89	3.2 E–07	0.43	1.3 E–04	0.44	1.4 E–04
ORF05642	DVU0588	formate dehydrogenase, β subunit	FdhB	3.00	1.5 E–03	3.07	1.0 E–06				
ORF03099	DVU2481	formate dehydrogenase, β subunit	CfdB	2.73	4.0 E–06						
ORF03102	DVU2482	formate dehydrogenase, α subunit	CfdA	2.11	6.2 E–03	0.49	4.6 E–03				
ORF03638	DVU2809	cytochrome c_3	FdhC3	3.95	7.1 E–04						
ORF03639	DVU2810	formate dehydrogenase, formation protein, putative	FdhE	3.10	3.6 E–03						
ORF03640	DVU2811	formate dehydrogenase, β subunit	FdhB	**5.24**	8.9 E–05						
ORF03641	DVU2812	formate dehydrogenase, α subunit	FdhA	**5.07**	6.9 E–05						
Enzymes involved in the reduction of sulfur oxo-anions											
ORF00336	DVU0846	adenylyl sulfate reductase , β subunit	ApsB			0.49	4.9 E–04			2.47	5.7 E–03
ORF00338	DVU0847	adenylyl sulfate reductase , α subunit	ApsA			0.29	6.0 E–04			2.25	2.8 E–02

Table 2 continued

ORF	TIGR	Annotation	Symbol	HS		LT		PS		P	
				Ratio[a]	*P-value*[b]	Ratio[a]	*P-value*[b]	Ratio[a]	*P-value*[b]	Ratio[a]	*P-value*[b]
ORF04933	DVU0172	thiosulfate reductase, iron-sulfur binding protein	phsB	2.14	1.2 E–04	0.42	1.1 E–05				
Electron transfer proteins											
ORF00292	DVU0819	iron-sulfur flavoprotein	Isf	3.12	1.3 E–04	**4.15**	1.5 E–05	3.66	1.7 E–07	2.58	3.5 E–05
ORF02976	DVU2404	heterodissulfide oxidoreductase, subunit C	hdrC	2.01	1.2 E–03						
ORF03423	DVU2680	flavodoxin	Fla					0.32	1.3 E–06	0.37	9.8 E–05
Oxidative stress responsive proteins											
ORF04274	DVU3185	rubredoxin-oxygen oxidoreductase	roO	0.43	2.0 E–04						
ORF04112	DVU3093	rubredoxin-like protein	rdl	2.45	1.8 E–04						
ORF01341	DVU1457	thioredoxin reductase	trxB	**4.81**	1.5 E–07						
ORF01989	DVU1839	Thioredoxin	trxA	2.55	3.6 E–05						
ATP synthase											
ORF00217	DVU0774	ATP synthase, F1 ε subunit	AtpC			0.50	4.9 E–03				
ORF00219	DVU0775	ATP synthase, F1 β subunit	AtpD			0.43	1.9 E–03				
ORF00220	DVU0776	ATP synthase, F1 γ subunit	AtpG	0.37	1.4 E–04						
ORF00223	DVU0777	ATP synthase, F1 α subunit	AtpA			0.29	7.9 E–04				
ORF00224	DVU0778	ATP synthase, F1 δ subunit	AtpH			0.46	5.2 E–03				
ORF00226	DVU0779	ATP synthase, F0, B subunit, putative	AtpF2			0.46	7.1 E–04				
ORF00462	DVU0918	ATP synthase, F0, A subunit	AtpB	0.32	2.5 E–07						
Carbon metabolizing enzymes											
ORF05664	DVU0600	L-lactate dehydrogenase	ldh	2.85	1.2 E–05						
ORF03593	DVU2784	Lactate dehydrogenase, FMN-dependent family	lldD	**4.29**	1.7 E–10	0.36	3.5 E–02				
ORF02745	DVU2271	Pyruvate:formate-lyase	pflA	**4.43**	7.6 E–05						
ORF02977	DVU2405	alcohol dehydrogenase	adh	2.21	1.3 E–02						

[a] mRNA abundance levels in cells grown in HS, PS, LT and P relative to mRNA levels in cells grown in LS

[b] probability that the mRNA abundance remained unchanged

hydrogenases of *D. vulgaris* is quite intricate and fine-tuned to changes in the environmental conditions. The presence of several hydrogenases with different properties and cofactors undoubtedly provides this organism with a great flexibility in terms of hydrogen metabolism, which is a crucial metabolite in anaerobic environments and also plays an essential role in its energetic metabolism.

Cytoplasmic facing hydrogenases

The two membrane-bound cytoplasmic-facing hydrogenases showed opposite response when hydrogen was used as electron donor, with the *echABCDEF* prominent among the gene clusters up-regulated and the *cooMKLXUHF* prominent among those down-regulated. A similar situation was reported for cells grown in formate/sulphate (Zhang et al. 2006a). In *Methanosarcina barkeri* the Ech hydrogenase complex has been shown to be multifunctional, with one of its roles being to drive ferredoxin reduction with H_2, which is used for CO_2 fixation (Meuer et al. 2002; Hedderich and Forzi 2005). This is a crucial activity for *D. vulgaris* cells grown in hydrogen, since acetate and CO_2 are the only carbon sources available. Thus, the increased expression of the Ech hydrogenase in these conditions points to its role in reducing ferredoxin for carbon fixation. Unfortunately, no replicates were obtained for the ferredoxin I gene and therefore its transcriptional response cannot be evaluated. The down-regulation of genes coding for the Coo hydrogenase suggests that the CO pathway is operative during growth with lactate, but does not function when H_2 is used as electron donor.

Formate dehydrogenases

All three formate dehydrogenases encoded in the *D. vulgaris* genome (DVU0587-88; DVU2481-85; DVU2809-12) and the pyruvate:formate lyase activating enzyme (DVU2271; *pflA*) showed increased expression during growth on hydrogen, as reported also for growth on formate (Zhang et al. 2006a). This suggests that formate cycling is occurring during growth with HS, providing an alternative pathway for energy generation: CO_2 and acetate are converted to formate, which is transported across the membrane and is oxidised in the periplasm by the formate dehydrogenases, thus contributing to the proton motive force as electrons are transferred back to the cytoplasm to reduce sulphate (Heidelberg et al. 2004; Tang et al. 2007; Badziong et al. 1979).

Transmembrane complexes

Contrary to most organisms, the terminal reductases of SRB are located in the cytoplasm, and therefore, are not directly involved in charge translocation across the membrane. The electrons generated in the periplasm from hydrogen oxidation have to be transported across the membrane to be used in the reduction of sulphate. *Desulfovibrio* spp. contain several membrane-bound redox complexes that can accept electrons from an abundant pool of periplasmic cytochromes *c* that act as electron acceptors for the hydrogenases and formate dehydrogenases (Pereira et al. 2007). The first such complex to be identified was the transmembrane Hmc complex of *D. vulgaris*, which was proposed to accept electrons from periplasmic hydrogenases via the type I cytochrome c_3 (TpIc_3), and to transfer them to the cytoplasmic reduction of sulphate (Rossi et al. 1993). In support of this proposal increased expression of the Hmc complex was observed when using hydrogen as electron donor (Keon et al. 1997), and a Δhmc mutant where the *hmc* operon was knocked-out grew slower than the wild type in these conditions (Dolla et al. 2000). Contrary to these results in the present study the *hmc* operon was one of those more down-regulated with growth on H_2. It is possible that the conditions used here for HS growth (constant pH and with H_2/CO_2 bubbled through the medium, removing H_2S), which were very different from the referred studies, led to down-regulation of the *hmc* genes. Our observation is corroborated by recent results from the same group obtained with *D. vulgaris* grown with 5% and 50% hydrogen showing also a reduced transcript level of the *hmc* operon relative to lactate growth conditions (Caffrey et al. 2007). An alternative transmembrane electron transfer pathway may involve the Tmc complex, whose protein subunits are homologous to those of Hmc (Pereira et al. 2006), given that the gene encoding the cytochrome *c* subunit of this complex (*tmcA*, DVU0263) was up-regulated in HS relative to LS. The TmcA cytochrome is actually a much better electron acceptor for the periplasmic hydrogenases via TpIc_3 than HmcA (Pereira et al. 1998; Valente et al. 2001).

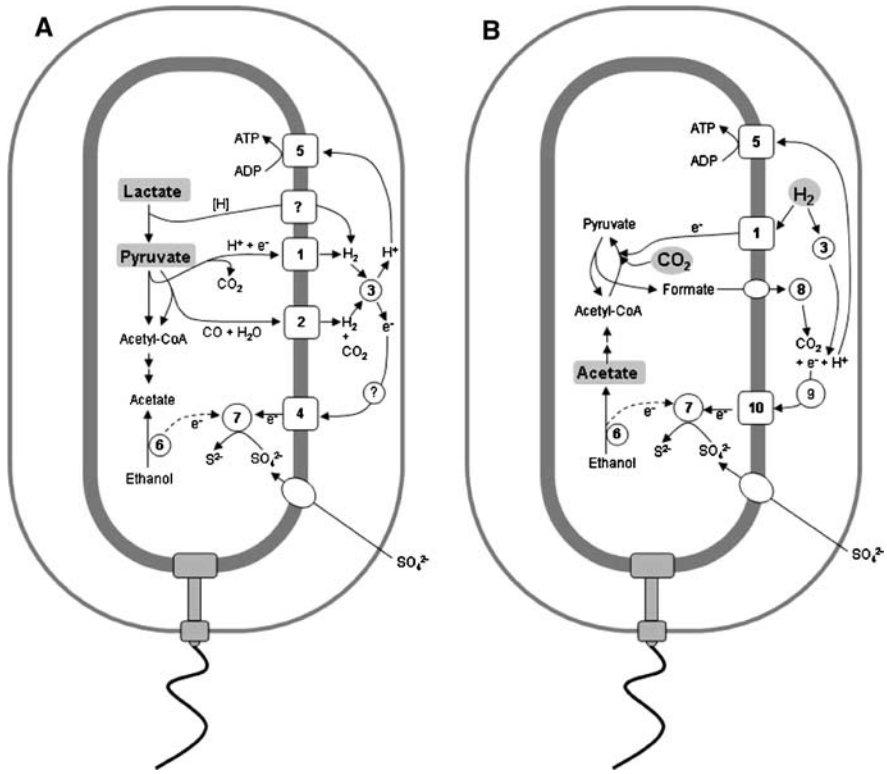

Fig. 3 Comparative scheme of the bioenergetic pathways operative in *D. vulgaris* grown in different conditions**.** Energy and/or carbon sources are in a grey background. [H] represents hydrogen equivalents. The pathway that accepts electrons from lactate oxidation or the pathway leading to the production of ethanol have not been elucidated. For the sake of simplicity the role of the membrane menaquinone pool is not considered. (**a**) Growth with lactate or pyruvate and sulphate. Numbers correspond to the following enzymes or proteins: 1—Ech hydrogenase, 2—CO-dehydrogenase and associated hydrogenase, 3—Periplasmic hydrogenases, 4—Electron transfer complexes including Dsr and Qmo, 5—ATP synthase, 6—Alcohol dehydrogenase and other proteins of the pathway, 7—Sulphate reducing enzymes. (**b**) Growth with hydrogen as electron donor and acetate/CO_2 as carbon sources. Numbers correspond to the following enzymes or proteins: 1—Ech hydrogenase, 3—Periplasmic hydrogenases, 5—ATP synthase, 6—Alcohol dehydrogenase and other proteins of the pathway, 7—Sulphate reducing enzymes, 8—Formate dehydrogenases, 9—Pool of periplasmic cytochromes *c*, 10—Transmembrane electron transfer complexes including Tmc

The two membrane complexes QmoABC and DsrMKJOP, conserved in all SRB sequenced to date, are thought to be involved in the sulphate reduction pathway as electron donors to the enzymes APS reductase and sulphite reductase, respectively (Haveman et al. 2004; Pires et al. 2003, 2006). Genes from both these complexes were down-regulated in H_2-grown cells, indicating that electron transport through Qmo and Dsr complexes is less important during growth on hydrogen than on lactate. This may be related to the up-regulation of the genes of the Tmc complex that may provide an alternative route for the flow of electrons to the cytoplasmic terminal reductases. The three complexes, Tmc, Hmc and Dsr have a homologous cytoplasmic subunit, which displays spectroscopic features indicative of the presence of a special $[4Fe4S]^{3+}$ center (Pires et al 2006; Pereira et al. 2006), and this suggests this subunit may play similar roles in the cytoplasm. The transmembrane electron flow through the Tmc complex may allow a reduced electron flow through the Qmo and Dsr-associated pathways. This proposal is in agreement with the fact that the genes encoding the APS reductase (DVU0846/7) and sulphite reductase (DVU0402/4) did not display significant differences in transcript levels.

Another membrane redox complex of unknown function, RnfCDGEAB (DVU2792-97), showed up-regulation for several genes. This complex may interact with a decaheme cytochrome *c* encoded in an

adjacent gene (DVU2791; DhcA). The *rnf* genes may be associated with different functions in different organisms. They were first identified in *Rhodobacter capsulatus* as being involved in nitrogen fixation (Schmehl et al. 1993). The three integral membrane subunits RnfADE, and the cytoplasmic RnfG subunit, show similarity to subunits of the Nqr complex of *Vibrio* spp., a Na^+—translocating NADH:quinone oxidoreductase (Steuber 2001). In *E. coli* the Rnf complex is named Rsx and is involved in keeping the redox-sensitive transcriptional factor SoxR in its inactive reduced state during aerobic growth (Koo et al. 2003). There is so far no information as to the possible role of the Rnf complex in *Desulfovibrio* spp.

Ethanol pathway

Some studies have suggested the involvement of an additional bioenergetic pathway in *D. vulgaris* involving ethanol, which can be oxidised by an alcohol dehydrogenase and the reducing equivalents transferred to sulphate reduction, through a still uncertain mechanism involving *hdrABC* and other gene products (Haveman et al. 2003). A relationship between ethanol and H_2 metabolism was first revealed by the fact that a *D. vulgaris* mutant lacking the [FeFe] hydrogenase had very low levels of the DVU2405-encoded alcohol dehydrogenase, which is one of the most highly expressed proteins in several growth conditions (Haveman et al. 2003; Zhang et al. 2006b). This pathway is more active during exponential than stationary growth phases (Zhang et al. 2006a). In HS-grown cells there is an up-regulation of this alcohol dehydrogenase gene (DVU2405; *adh*), as previously reported (Haveman et al. 2003), and the gene for a subunit of a putative heterodisulphide reductase (DVU2404; *hdrC*), suggesting that this pathway is more important than in LS growth conditions. This pathway provides an alternative route for electron transfer to sulphate reduction, and may also be associated with the down-regulation of genes encoding the Qmo and Dsr complexes, which contain subunits homologous to Hdr proteins.

Changes in gene expression using pyruvate as energy source

The number of energy metabolism genes with modified expression in PS relative to LS was quite small and all changes were below four-fold either up or down (Table 2). This agrees with the fact that oxidation of lactate proceeds via pyruvate. Three of the *ech* genes were up-regulated. In these growth conditions carbon fixation is not required and it is more likely that the Ech hydrogenase is acting to generate H_2 from ferredoxin, which is reduced by the pyruvate:ferredoxin oxidoreductase. This process is associated with energy conservation as proposed in the hydrogen-cycling hypothesis, and suggests a greater relevance for this pathway with pyruvate than with lactate. The observed up-regulation of the *ech* operon in HS and PS conditions suggests that in *D. vulgaris*, as in *M. barkeri* (Meuer et al. 2002), the Ech hydrogenase is able to function bi-directionally depending on the growth conditions. Among the few energy metabolism genes down-regulated were the *hmc* genes (DVU0533; *hmcD*), the *fhdA* gene (DVU0587) coding for the catalytic subunit of the periplasmic FdhAB formate dehydrogenase, and the gene coding for the redox protein flavodoxin (DVU2680; *fla*).

In cells grown with pyruvate and a restricted amount of sulphate more energy metabolism genes were up-regulated than in PS-grown cells, but all expression ratios were below three-fold (Table 2). *D. vulgaris* does not grow on pyruvate alone unless a small amount of sulphate is present at the beginning of growth, probably due to the inhibitory effect of accumulation of reduced compounds during the fermentation burst (Voordouw 2002; Magee et al. 1978). In this study a small amount of sulphate was present in the medium because the inoculum was made using cells grown in PS conditions. Interestingly, and contrary to our expectation, in these sulphate-limited conditions the genes for several proteins thought to be involved in sulphate respiration showed increased expression. These included APS reductase, some subunits of the Qmo and Dsr complexes, the $TpIc_3$ (DVU3171), the $[NiFe]_2$ hydrogenase (DVU2524-25), and several genes of the Hmc complex. The genes for two other membrane redox complexes of unknown function were also up-regulated (DVU0692/3 and DVU3143/4). The increased expression of the Hmc complex agrees with published experiments, which showed that in similar growth conditions a Δhmc mutant accumulates large amounts of hydrogen, in contrast to the wild type (Voordouw 2002). These results suggest that during growth in pyruvate with limiting sulphate, cycling of hydrogen is also occurring. Only two energy metabolism genes were

down-regulated, *fla* and *fhdA* genes as observed also in PS. The reduced level of transcription of the gene coding for flavodoxin in both pyruvate growth conditions suggests a role for this protein in the lactate oxidation pathway upstream of pyruvate.

Changes in gene expression using thiosulphate as electron acceptor

Although previous studies have addressed the effect of different electron donors in the transcriptional and translational response of *D. vulgaris* (Zhang et al. 2006a; Haveman et al. 2003) this is the first time that the effect of a different electron acceptor is explored. In contrast to sulphate, thiosulphate does not require prior activation to be used as electron acceptor and therefore a higher cell yield would be expected. However, the yield of cells grown in LT is lower than in LS as previously reported (Magee et al. 1978). The down-regulation of genes coding for ATP synthase (DVU0774-79), APS reductase (DVU0846-47; *apsAB*), the Qmo and Dsr complexes, and the $[NiFe]_2$ hydrogenase (Table 2) suggests a low energy state of the cells, which agrees with the slower growth rate (Fig. 1). Reduction of thiosulphate yields sulphite, which at high concentrations is toxic (Badziong and Thauer 1978). It is possible that accumulation of this compound led to down-regulation of genes involved in the sulphate reduction pathway as observed for nitrite, which inhibits the sulphite reductase and thus leads to an accumulation of sulphite (Haveman et al. 2004; He et al. 2006). In agreement with this proposal several of the genes mentioned above were also down-regulated in *D. vulgaris* cells grown in the presence of nitrite (Haveman et al. 2004; He et al. 2006). Some genes of the membrane-bound (DVU2482; *fdnG*) and the three-subunit (DVU2811; *fdhB*) formate dehydrogenases were also down-regulated, whereas the genes coding for the periplasmic two-subunit formate dehydrogenase (DVU0587/8; *fdhAB*) were up-regulated indicating different regulation for these proteins in LT, in contrast with what was observed in HS conditions. The up-regulation of the genes coding for the [FeFe] hydrogenase, which displays the highest rates of hydrogen uptake when compared with the other periplasmic hydrogenases (Valente et al. 2005), may serve to improve energy recovery through H_2 cycling. The gene coding for a periplasmic octaheme cytochrome *c* (DVU3107) of unknown function is the most down-regulated energy metabolism gene in these conditions. The physiological role of this cytochrome is not known but the considerable down regulation in these conditions and lack of transcriptional response of this gene in the other conditions tested suggests a role in sulphate reduction. This is supported by the significant down-regulation of this gene in the transition from exponential to stationary phase in *D. vulgaris* cells grown with lactate/sulphate and with formate/sulphate (Zhang et al. 2006a). Unexpectedly, the gene for one of the subunits of the putative thiosulphate reductase (DVU0172; *phsB*) was down-regulated, as well as a putative lactate dehydrogenase (DVU2784; *lld*D), which suggests that these genes may code for proteins with different functions from those indicated in the genome annotation.

Finally, the gene for an iron-sulphur flavoprotein (DVU0819; *isf*) is noteworthy because it was up-regulated in all conditions tested. This protein is homologous to the Isf protein of *Methanosarcina thermophilus*, where it plays a role in electron transport during fermentation of acetate to methane by accepting electrons from ferredoxin (Zhao et al. 2001). The role of Isf in *Desulfovibrio* metabolism has not been investigated.

Overall view of the bioenergetic metabolism of *D. vulgaris* from the transcriptional studies

In this work, the global gene expression response of *D. vulgaris* to growth on different electron donors and acceptors provided several important insights into the bioenergetic pathways of this organism, and its response to different growth conditions. A simplified scheme of these pathways deduced from the transcriptional results in this work, and taking into account previously published results (Voordouw 2002; Zhang et al. 2006a) is presented in Fig. 3. The change from an organic carbon and energy source (lactate) to H_2 as energy source and CO_2/acetate as carbon source results in a strong shift in the transcriptional pattern of *D. vulgaris*. Since hydrogen is a ubiquitous metabolite in anaerobic environments, and it is likely to be an important energy source for *Desulfovibrio* spp. in their natural habitats, these results are of great physiological significance. The evidence indicates a shift in metabolic

trafficking involving different bioenergetic pathways when changing from lactate to H_2. The formate cycling pathway is more relevant for growth with H_2 than lactate, whereas the contrary is observed for the CO cycling pathway. The results provide also evidence for the existence of a pathway involving ethanol that is up-regulated during growth in H_2, and provides a soluble route for electron transfer to sulphate reduction. This pathway involves several novel proteins of unknown function that should be further investigated. Regarding the complexes involved in the transmembrane electron transfer the evidence indicates that the Tmc is preferred during growth with H_2, whereas the Qmo and Dsr are more relevant during growth with carbon sources.

An interesting observation from the present results is that when several, seemingly redundant, isoenzymes are present, as in the case of hydrogenases or formate dehydrogenases, there are different transcriptional responses to the changes in growth conditions, indicating that these isoenzymes have specialized roles and are not completely interchangeable. All the studies published so far indicate that there is a fine-tuning of the activity of the periplasmic hydrogenases, which should permit a rapid adaptation to changing environmental conditions, including different intracellular and extracellular concentrations of H_2 or metals available. The present study also gives strong support for a bifunctional role of the cytoplasmic Ech hydrogenase that may reduce ferredoxin for carbon fixation during growth in H_2, or oxidize ferredoxin forming H_2 for hydrogen cycling during growth in PS.

Finally, the results reported in this work identified several novel gene products that are involved in energy metabolism and that merit further study to clarify their function. Examples are the Rnf complex (and other membrane redox complexes), the proteins involved in the ethanol pathway, the octaheme cytochrome *c*, the Isf protein, flavodoxin and several others. Our study highlights the high complexity and plasticity of the *D. vulgaris* energetic metabolism and show that further studies are warranted before sulphate respiration can be fully understood.

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