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Transcriptional response of *Desulfovibrio vulgaris* Hildenborough to oxidative stress mimicking environmental conditions

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Abstract Sulfate-reducing bacteria (SRB) are anaerobes readily found in oxic–anoxic interfaces. Multiple defense pathways against oxidative conditions were identified in these organisms and proposed to be differentially expressed under different concentrations of oxygen, contributing to their ability to survive oxic conditions. In this study, *Desulfovibrio vulgaris* Hildenborough cells were exposed to the highest concentration of oxygen that SRB are likely to encounter in natural habitats, and the global transcriptomic response was determined. Three hundred and seven genes were responsive, with cellular roles in energy metabolism, protein fate, cell envelope and regulatory functions, including multiple genes encoding heat shock proteins, peptidases and proteins with heat shock promoters. Of the oxygen reducing mechanisms of *D. vulgaris* only the periplasmic hydrogen-dependent mechanism was up-regulated, involving the [NiFeSe] hydrogenase, formate dehydrogenase(s) and the Hmc membrane complex. The oxidative defense response concentrated on damage repair by metal-free enzymes. These data, together with the down-regulation of the ferric uptake regulator operon, which restricts the availability of iron, and the lack of response of the peroxide-sensing regulator operon, suggest that a major effect of this oxygen stress is the inactivation and/or degradation of multiple metalloproteins present in *D. vulgaris* as a consequence of oxidative damage to their metal clusters.

Keywords Oxidative stress · *Desulfovibrio* · Genomics · Metalloproteins · Fur · PerR · Thioredoxin · Hmc

Abbreviations

D	*Desulfovibrio*
PMF	Proton motive force
ROS	Reactive oxygen species
SRB	Sulfate-reducing bacteria

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Introduction

Sulfate-reducing bacteria (SRB) are anaerobic microorganisms capable of coupling the oxidation of a variety of electron donors to the reduction of sulfate (Rabus et al. 2000). Recently, SRB have become very actively investigated for their use in bioremediation, namely in decontamination of sites polluted by toxic metals and radionuclides, or aromatic and chlorinated compounds (Wall and Krumholtz 2006). Although SRB are classified as strict anaerobes, the activity of these bacteria is well documented in oxic zones of their habitats [reviewed in (Dolla et al. 2006)]. For example, measurements of bacterial sulfate reduction in marine and fresh water environments have revealed that it occurs consistently within well-oxygenated photosynthetic zones of microbial mats, where SRB have to deal with oxygen concentrations up to 1 mM (Sigalevich et al. 2000). Also, it was recently shown that *Desulfovibrio desulfuricans* ATCC27774 can grow in the presence of oxygen at nearly atmospheric partial pressure (Lobo et al. 2007).

Studies of how SRB cope with exposure to molecular oxygen provide important insights into their ecological significance in natural habitats as well as into their practical application in bioremediation. Three different oxygen-reducing systems have been characterized in *Desulfovibrio* spp., a group of widely distributed SRB that have been intensively studied as model organisms for these bacteria: (1) a cytoplasmic electron transport chain terminating with the flavo-diiron protein rubredoxin-oxygen oxidoreductase (Roo) (Chen et al. 1993); (2) a membrane-bound system involving a terminal cytochrome *bd* oxidase (Cyd) (Lemos et al. 2001) and (3) a periplasmic hydrogen-dependent system involving hydrogenases and *c*-type cytochromes (Baumgarten et al. 2001; Fournier et al. 2004). The genome sequences of *D. vulgaris* Hildenborough (Heidelberg et al. 2004) and *D. desulfuricans* G20 (http://www.jgi.doe.gov) confirmed the presence of these pathways in both organisms, and also of a heme-copper-type terminal cytochrome *c* oxidase (Cox). Also, various enzymes for scavenging reactive oxygen species (ROS) such as hydrogen peroxide and superoxide have been reported in SRB. Two disproportionating enzymes, the superoxide dismutase (Sod) and catalase (Kat), are broadly distributed in SRB (Hatchikian and Henry 1977; dos Santos et al. 2000; Fournier et al. 2003). The superoxide reductases (Sor) and rubrerythrins catalyzing the reductive elimination of superoxide and hydrogen peroxide, respectively, are also present in these organisms (Pierik et al. 1993; Voordouw and Voordouw 1998; Coulter et al. 1999; Lombard et al. 2000). Two structurally different types of Sor are found in SRB, the rubredoxin oxidoreductase (Rbo) (also named desulfoferredoxin) (Moura et al. 1990; Voordouw and Voordouw 1998; Lombard et al. 2000) and neelaredoxin (Abreu et al. 2002). Three copies of rubrerythrin peroxidases are present in the *D. vulgaris* and *D. desulfuricans* G20 genomes. More recently, several thiol-specific peroxidases (thiol-peroxidase, BCP-like protein and putative glutaredoxin) were found to be up-regulated under oxygen exposure in *D. vulgaris* (Fournier et al. 2006). In addition, the hybrid-cluster protein (Hcp), also present in several organisms, has been shown to be involved in oxidative stress defense and to have peroxidase activity (Almeida et al. 2006).

In addition to detoxification of oxygen or its reaction products SRB also show behavioral responses to oxygen such as flocculation (Sigalevich et al. 2000), migration to anoxic zones (Krekeler et al. 1997, 1998) and a complex interplay of positive and negative aerotaxis (Eschemann et al. 1999). Furthermore, the *D. vulgaris* genome encodes a family of genes for methyl-accepting chemotaxis proteins (MCPs) (Deckers and Voordouw 1996), including those for DcrA (DVU3182) (Fu et al. 1994) and DcrH (DVU3155) (Xiong et al. 2000), proposed to be involved in sensing the oxygen concentration or redox potential of the environment (Dolla et al. 1992; Fu et al. 1994).

Therefore, the response of *Desulfovibrio* spp. to oxidative stress is expected to be broad. Studies of transcriptomic response of *D. vulgaris* to low levels of oxygen have revealed minimal perturbation, e.g., exposure to 0.1% oxygen caused the up-regulation of 12 genes (Mukhopadhyay et al. 2007). Exposure to air for 1 h elicited a mild response with significant changes in the abundance of mRNA from 130 genes and no significant effect on cell viability (Zhang et al. 2006). In contrast, exposure to air for 4 h resulted in an extreme response with the up-regulation of 393 genes and down-regulation of 454 genes and a dramatic reduction in cell viability of approximately 90% (Mukhopadhyay et al. 2007). Cells exposed to 1 h of pure oxygen showed 57 proteins with significant differences in abundance and cell viability reduced approximately five times (Fournier et al. 2006). In the present work we investigated the transcriptional response of *D. vulgaris* to these latter conditions, since they mimic the upper range of oxygen concentration that this organism is likely to encounter in its natural habitats.

Materials and methods

Strains, media, culture and oxidative stress condition

Desulfovibrio vulgaris (DSM 644) cultures (4 × 1,000 ml) were initiated with a 10% (vol/vol) inoculum of a freshly grown culture in modified medium C (Postgate 1984) at 37°C. The modified medium C contained 40 mM lactate and 40 mM sulfate as electron donor and acceptor, respectively, and was supplemented with 25 μM $FeSO_4 \cdot 7H_2O$ and

1 μM $NiCl_2 \cdot 6H_2O$. Cultures were grown in 2,000-ml DURAN® SHOTT bottles closed with black rubber stoppers and the headspace flushed with N_2. Cell densities were measured with a Shimadzu UV-1603 spectrophotometer as the optical density at 600 nm (OD_{600}). When the cultures reached mid-exponential phase ($OD_{600} \sim 0.48$), oxidative stress was applied by flushing the culture broth with pure oxygen at room temperature for 1 h (Fournier et al. 2006), whereas control cultures were kept anoxic at room temperature for the same time. Under these conditions, the final dissolved oxygen concentration was approximately 1 mM, estimated using the Bunsen adsorption coefficient (Weiss 1970). Cell mass samples were harvested by centrifugation (10,000 × *g* for 15 min) and stored at −70°C until use.

Aliquots of oxygen-flushed and anaerobic cultures were collected and transferred to the anaerobic chamber, serially diluted on modified medium C, and plated on medium E (Postgate 1984) supplemented with 1 μM $NiCl_2 \cdot 6H_2O$. Plates were incubated at 37°C in an anaerobic jar and colonies were counted after 5 days as the number of surviving colony-forming units/ml (CFU/ml) and compared with the surviving CFU/ml obtained from cells that were not exposed to oxygen.

Microarray analysis

A previously described whole-genome oligonucleotide DNA microarray, covering more than 98.6% of the annotated protein-coding sequences of the *D. vulgaris* genome, was used for global transcriptional analysis of oxidative stress response (He et al. 2006). The accuracy of these microarrays in global transcriptional profiling have been extensively validated in previous studies on various stress response pathways in *D. vulgaris* (Chhabra et al. 2006; Clark et al. 2006; He et al. 2006; Mukhopadhyay et al. 2006). All microarray procedures including total RNA extraction, cDNA labeling, microarray hybridization and washing were performed using previously published protocols (He et al. 2006). Four samples of each total RNA preparation were labeled, two with Cy3-dUTP and another two with Cy5-dUTP, for microarray hybridization. To hybridize microarray glass slides, the Cy5-dUTP-labeled cDNA targets from one untreated control culture were mixed with the Cy3-dUTP-labeled cDNA targets from one oxygen-treated culture and vice versa (dye swap). As a result, each biological sample was hybridized to four microarray slides. Two biological replicates obtained from independently grown *D. vulgaris* cultures were used in the gene expression analysis. 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 resulted in a total of 16 data points per experimental condition enabling the use of rigorous statistical tests to determine significant changes in gene expression. To determine signal fluorescence intensities of each spot, 16-bit TIFF scanned images were analyzed by application of the software ImaGene, version 6.0 (Biodiscovery, Marina Del Rey, CA, USA) to quantify spot signal, spot quality and background fluorescence intensities. Empty spots, poor spots and negative spots were flagged according to the instruction of the software and removed in subsequent analysis. The resulting data files were subjected to Lowess intensity-based normalization and further analyzed using GeneSpring version 5.1 (Silicon Genetics, Redwood City, CA, USA). Lowess normalization was performed on each microarray slide 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. The annotation of the ORFs was obtained from the comprehensive microbial resource at The Institute for Genomic Research (TIGR).

Results and discussion

Pair-wise comparison of gene expression profiles between the controls and the oxygen-treated samples using a cutoff criterion of 2.0-fold change and 95% confidence showed significant changes in 307 genes, with 192 genes up-regulated and 115 genes down-regulated (Supplementary Table 1). The number of genes affected is smaller than reported in a recent study where *D. vulgaris* cells were exposed to air during 4 h [393 genes up-regulated and 454 down-regulated (Mukhopadhyay et al. 2007)]. The genes with altered expression levels were grouped into functional role categories according to the annotation of the *D. vulgaris* genome sequence by TIGR (Peterson et al. 2001; Heidelberg et al. 2004) (Fig. 1). A large proportion of the highly up-regulated genes are grouped into cellular roles involved in protein fate, cell envelope, transport/binding and regulatory functions, whereas the functional group showing more pronounced down-regulation of genes was that of energy metabolism. Cell viability upon plating showed that counts are reduced to 30% (0.9×10^7 CFU/ml *versus* 3×10^7 CFU/ml) in agreement with a prior study using a similar treatment that reported a reduction to 23% in the number of CFU/ml (Fournier et al. 2004).

Genes associated with oxidative stress response pathways

In line with previous studies (Fournier et al. 2003, 2006; Zhang et al. 2006; Mukhopadhyay et al. 2007; Scholten et al. 2007), few of the genes known to be directly involved in oxidative stress response displayed significant up-regulation (Table 1). Of the three oxygen-reducing systems described for *D. vulgaris* only the hydrogen-dependent

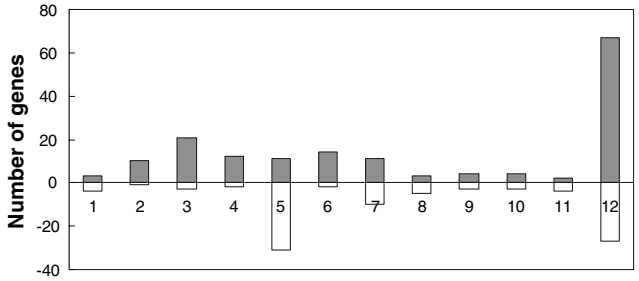

Fig. 1 Functional profiling of the transcriptional response of *D. vulgaris* to 1 h of oxygen exposure. The functional role category annotation is that provided by TIGR (http://www.tigr.org). *Each column* represents the number of genes in a selected functional category showing significant changes in mRNA abundance. *Positive and negative values* indicate up- and down-regulation, respectively. Columns: *1* protein synthesis, *2* cellular processes, *3* protein fate, *4* regulatory functions, *5* energy metabolism, *6* cell envelope, *7* transport and binding proteins, *8* central intermediary metabolism, *9* DNA metabolism, *10* amino acid biosynthesis, *11* biosynthesis of cofactors, prosthetic groups and carriers, *12* hypothetical proteins

system is significantly up-regulated, as deduced from the genes coding for the catalytic subunit of the NiFeSe hydrogenase (*hysA*) and several subunits of the Hmc transmembrane complex (see Table 2). The genes coding for the metal containing peroxidases rubrerythrin (DVU3094; *rbr*) and nigerythrin (DVU0019; *ngr*), catalase (DVUA0091; *katA*), superoxide reductase (DVU3183; *sor*) and rubredoxin:oxygen oxidoreductase (DVU3185; *roo*) exhibited no significant change, whereas the *rbr*2 (DVU2318), *b0873* (DVU2543) and the quinol oxidase *cydAB* (DVU3270-71) genes were down-regulated. Under mild oxidative conditions the *sor*, *sodB* and *katA* genes also showed no change (Zhang et al. 2006), and a down-regulation of genes *sor*, *rbr* and *rbr2* in identical oxidative conditions to those of the present work was observed by RT-PCR (Fournier et al. 2006).

In contrast, thioredoxin (DVU1839; *trxA*) and thioredoxin oxidoreductase, (DVU1457; *trxB*) genes were up-regulated, as also observed in cells exposed to air during 1–4 h (Zhang et al. 2006; Mukhopadhyay et al. 2007). For aerobic and facultative anaerobic bacteria it has been well established that thioredoxin plays an important role in the oxidative stress response by reducing a number of very important proteins including peroxiredoxins, directly reducing H_2O_2, scavenging hydroxyl radicals and quenching singlet oxygen, besides maintaining the intracellular thiol-disulfide balance (Zeller and Klug 2006). Thioredoxin also serves as electron donor to methionine sulfoxide reductases (Zeller and Klug 2006) that were also up-regulated (DVU1984, MsrA and DVU0576, MsrB). Side chains of cysteines and methionines usually suffer oxidation during oxidative stress leading to protein damage, lower cellular antioxidant capacity, altered protein function and interference with signal transduction. Methionine sulfoxide reductases are able to repair the damaged proteins by reduction of the altered amino acids (Moskovitz 2005).These results indicate that in strongly oxidative conditions *D. vulgaris* increases expression of metal-free oxidative defense proteins, rather than of the metal-containing proteins, like Sor and rubrerythrins. Under the experimental conditions used, ROS may also arise from the reaction of the sulfide present in the medium with the added oxygen, and from the reaction of the oxygen with reduced metalloproteins. A switch to metal-free oxidative defense proteins reduces the possibility for release of metal cations from protein degradation, which could contribute significantly to production of further ROS, as previously suggested (Dolla et al. 2006; Fournier et al. 2006).

Besides the functions in oxidative stress defense, thioredoxin can also influence the expression of ferric uptake regulator (Fur) genes by repressing them in oxidative stress conditions (Zeller and Klug 2006). In *D. vulgaris* the Fur

Table 1 Effect of oxygen exposure on genes involved in oxygen damage repair, aggregate formation and aerotaxis mechanisms in *D. vulgaris*

Gene identifier	Annotation	Expression ratio (treatment/control)	*P*-value
Damage repair			
DVU0576	Methionine sulfoxide reductase (*msrB*)	5.23	1.9E-05
DVU0664	Cysteine desulfurase (*nifS*)	3.12	3.6E-04
DVU0665	Nitrogen fixation protein NifU homolog (*nifU*)	3.84	3.5E-04
DVU1457	Thioredoxin reductase (*trxB*)	3.29	1.6E-03
DVU1839	Thioredoxin (*trxA*)	2.48	2.1E-05
DVU1984	Methionine sulfoxide reductase (*msrA*)	6.14	1.2E-07
Aggregation formation and aerotaxis mechanisms			
DVU2073	Chemotaxis protein CheY (*cheY-2*)	5.36	1.9E-07
DVU2076	Chemotaxis protein methyltransferase (*cheR-2*)	2.49	7.0E-04
DVU2309	Methyl-accepting chemotaxis protein, putative	2.31	1.5E-05
DVU2893	Flagellar basal-body rod protein, putative	9.34	8.3E-04

Table 2 Effect of oxygen exposure on the transcriptional responses of *D. vulgaris* genes involved in energy metabolism

Gene identifier and response	Annotation	Expression ratio (treatment/control)	*P*-value
Up-regulation			
DVU1918	Periplasmic [NiFeSe] hydrogenase, large subunit, (*hysA*)	2.27	1.0E-04
DVU2481	Formate dehydrogenase, β subunit, putative	2.15	3.4E-03
DVU2809	Cytochrome c_3 subunit of formate dehydrogenase	2.23	1.4E-05
DVU0531	hmc Operon protein 6, (*hmcF*)	2.94	9.3E-07
DVU0532	hmc Operon protein 5, (*hmcE*)	3.40	2.6E-07
DVU0533	hmc Operon protein 4, (*hmcD*)	2.19	2.0E-04
DVU0536	High-molecular-weight cytochrome *c*, (*hmcA*)	3.38	4.9E-04
Down-regulation			
DVU0692	Molybdopterin oxidoreductase, transmembrane subunit, putative	0.45	1.4E-04
DVU0693	Molybdopterin oxidoreductase, iron–sulfur cluster-binding subunit, putative	0.34	1.4E-03
DVU0694	Molybdopterin oxidoreductase, molybdopterin-binding subunit, putative	0.48	1.2E-03
DVU0775	ATP synthase, F1 β subunit, (*atpD*)	0.27	2.5E-04
DVU0776	ATP synthase, F1 γ subunit, (*atpG*)	0.26	7.6E-06
DVU0777	ATP synthase, F1 α subunit, (*atpA*)	0.19	8.6E-06
DVU0778	ATP synthase, F1 delta subunit, (*atpH*)	0.35	2.0E-05
DVU0918	ATP synthase F0, A subunit, (*atpB*)	0.46	8.9E-05
DVU0779	ATP synthase F0, B subunit, putative, (*atpF2*)	0.26	1.3E-04
DVU0780	ATP synthase F0, B subunit, putative, (*atpF1*)	0.39	5.4E-03
DVU0848	Quinone-interacting membrane-bound oxidoreductase, putative, (*qmoA*)	0.32	8.5E-05
DVU0849	Quinone-interacting membrane-bound oxidoreductase, iron–sulfur-binding subunit, (*qmoB*)	0.28	1.9E-04
DVU0850	Quinone-interacting membrane-bound oxidoreductase, transmembrane subunit, (*qmoC*)	0.48	7.4E-06
DVU1287	Reductase, iron–sulfur binding subunit, (*dsrO*)	0.42	1.7E-03
DVU1288	Cytochrome *c* family protein, (*dsrJ*)	0.42	1.2E-03
DVU1289	Reductase, iron–sulfur binding subunit, (*dsrK*)	0.37	1.1E-03
DVU1290	Nitrate reductase, γ subunit, (*dsrM*)	0.48	6.2E-03
DVU1921	Periplasmic [NiFe] hydrogenase, small subunit, isozyme 1, (*hynB*-1)	0.46	4.9E-06
DVU2514	Pyruvate kinase, (*pyk*)	0.46	7.7E-03
DVU2525	Periplasmic [NiFe] hydrogenase, small subunit, isozyme 2, (*hynB*-2)	0.49	7.9E-04
DVU2526	Periplasmic [NiFe] hydrogenase, large subunit, isozyme 2, (*hynA*-2)	0.50	2.9E-03
DVU2795	Electron transport complex protein RnfE, putative	0.50	2.0E-04
DVU3171	Cytochrome c_3, (tpIc_3)	0.40	8.1E-04
DVU0846	Adenylylsulfate reductase, β subunit, (*apsB*)	0.28	3.5E-05
DVU0847	Adenylylsulfate reductase, α subunit, (*apsA*)	0.24	3.3E-04
DVU1295	Sulfate adenylyltransferase, (sat)	0.33	1.2E-02
DVU1636	Inorganic pyrophosphatase, manganese-dependent, (*ppaC*)	0.47	5.2E-03

regulon responds to a variety of stresses (Bender et al. 2007) and some of the genes that were predicted to be controlled by Fur at the transcriptional level (Rodionov et al. 2004) were down-regulated (Table 3) suggesting a role for this regulon in preventing ROS damage in *D. vulgaris*. Since the Fur regulons of many bacteria are involved in iron homeostasis (Escolar et al. 1999; Hantke 2001), repression of the Fur regulon in oxidative stress implies a link between this stress and iron depletion, probably to reduce the extent of radical generation through the Fenton reaction. The genes predicted to be regulated by the peroxide-sensing regulator PerR (Rodionov et al. 2004), that belongs to the same family as Fur, were also examined in the present study and showed no change. Exposure to air for up to 4 h also showed less that twofold reduction in the average change in transcript abundance for the genes of the PerR regulon (Mukhopadhyay et al. 2007). The Fur and PerR regulons are both believed to be regulated by ferrous

Table 3 Effect of oxygen exposure on the transcriptional responses of *D. vulgaris* genes in the predicted Fur regulon (Rodionov et al. 2004)

Gene identifier	Annotation	Expression ratio (treatment/control)	*P*-value
DVU0273	Conserved hypothetical protein	**0.36**	3.6E-07
DVU0304	Hypothetical protein	**0.46**	3.2E-03
DVU0763	GGDEF domain protein	**0.42**	1.0E-04
DVU2378	Transcriptional regulator, AraC family	**0.45**	1.7E-05
DVU2574	Ferrous ion transport protein, putative	1.24	3.2E-01
DVU2680	Flavodoxin	**0.30**	4.4E-05
DVU3123	HD domain protein	0.58	7.0E-04
DVU3330	Conserved hypothetical protein	0.92	6.5E-01

Significant changes are indicated in bold

iron through a common mechanism (Fuangthong et al. 2002). Both regulons were up-regulated in nitrite stress as well in heat shock response (Chhabra et al. 2006; He et al. 2006), but our results suggest that there is another mechanism that controls the expression of these two regulons. This would account for the lack of response of the PerR regulon and down-regulation of the Fur regulon.

The iron–sulfur clusters of several proteins are major targets for ROS, inducing inactivation of crucial enzymes and release of free iron, which in turn perpetuates oxidative stress through the production of hydroxyl radicals. The observed up-regulation of the *nifU* and *nifS* genes, which are involved in the biosynthesis and repair of iron–sulfur clusters (Johnson et al. 2005) serves to counter this effect.

In addition to the molecular strategies described above, a behavioral response was observed with induction of some flagellar and chemotaxis proteins (Table 1). A putative flagellar basal-body rod protein was up-regulated 9.34-fold following the oxidative stress. Two chemotaxis proteins (DVU2073 and DVU2076) were also up-regulated, but no change was observed for the genes of the chemoreceptors DrcA and DcrH. In contrast, another putative MCP (DVU2309) that shows 25% identity and 43% similarity with DrcA and 22% identity and 36% similarity with DcrH sequences, was up-regulated. This protein is likely to be involved in the signal transduction mechanisms that follow exposure to the oxidative conditions used.

Genes involved in energy metabolism

In this work a very significant number of genes with important roles in energy metabolism had their expression affected, as observed in Table 2. A similar result was observed in the transcriptional response of *D. vulgaris* to 4 h exposure to air (Mukhopadhyay et al. 2007). Several genes involved in the sulfate reduction pathway were down-regulated, in particular two transmembrane complexes believed to be essential for sulfate reduction, *qmoABC* (DVU0848-50) (Pires et al. 2003) and *dsrMKJOP* (DVU1287-90) (Haveman et al. 2004; Pires et al. 2006). Another transmembrane complex encoded by the operon DVU0692-94 was down-regulated as well as the inorganic pyrophosphatase (DVU1636; *ppaC*), sulfate adenylyltransferase (DVU1295; *sat*) and APS reductase (DVU0846-7; *apsAB*). The ATP synthase genes (DVU0775-80) also showed a very strong down-regulation. These observations point to a decrease in sulfate reduction and energy production and agree with the biochemical studies that demonstrated inhibition of growth and sulfate reduction by oxygen in *Desulfovibrio* spp. (Cypionka 2000). Interestingly, the sulfate reduction pathway was also down-regulated in response to heat shock (Chhabra et al. 2006) and nitrite stress conditions (Haveman et al. 2004; He et al. 2006; Mukhopadhyay et al. 2006). In heat shock, the explanation might be due to cell death caused by high temperature, whereas in nitrite stress there is a competition for electrons to reduce nitrite. In oxidative stress two mechanisms can contribute to the down-regulation of bioenergetic pathways: (1) reduced expression of proteins containing metal clusters sensitive to degradation by oxygen or/and ROS to limit the extent of damage imparted by the released metals, and (2) competition for electrons to reduce ROS or oxygen, that are thus diverted from the respiratory chain, as observed in nitrite stress.

In contrast to the general trend, the genes encoding the Hmc complex (DVU0531-36; *hmcABCDEF*), which is a membrane electron transfer complex proposed to link periplasmic hydrogen oxidation to cytoplasmic sulfate reduction (Rossi et al. 1993), were up-regulated. This agrees with earlier biochemical studies that showed an increase of the cytochrome *c* content (Fournier et al. 2004) since this complex includes the 16-heme cytochrome HmcA. The increase in the cytochrome content cannot be attributed to the type I cytochrome c_3 (DVU3171), the main cytochrome present in the periplasm (Louro 2007), because its gene was down-regulated. It has been shown that a ΔHmc mutant displayed slower growth of single cells into colonies than the wild-type, indicating that the Hmc complex may be involved in establishment of a low redox potential necessary for growth (Dolla et al. 2000). The results described here support this

proposal and are different from those reported for the exposure of *D. vulgaris* cells to air during 4 h where heat maps of transcriptomic response show a slight decrease for the genes of this operon (Mukhopadhyay et al. 2007).

It was previously reported that oxidative conditions similar to those employed in this study lead to an increase in the content of periplasmic [Fe] hydrogenase that was proposed to be involved in oxygen reduction (Fournier et al. 2004, 2006). In the present work the genes for the periplasmic [Fe] hydrogenase (*hydAB*) showed no change, whereas the gene of the catalytic subunit of the [NiFeSe] hydrogenase (DVU1918; *hysA*) was up-regulated. In addition, the two periplasmic [NiFe] hydrogenases genes (*hynAB*-1 and *hynAB*-2) were down-regulated in oxygen stress. The expression of periplasmic hydrogenases in *D. vulgaris* is influenced by the availability of the metal ions needed for their catalytic sites (Valente et al. 2006). The growth medium contained nickel, which leads to a significant reduction in the amount of [Fe] hydrogenase and an increase in the transcripts of both the [NiFe] and [NiFeSe] hydrogenases (Valente et al. 2006). Since the [NiFeSe] hydrogenase is much less sensitive to oxygen than the other periplasmic hydrogenases (Valente et al. 2005), it makes sense that it should be up-regulated in these conditions.

Genes associated with protein damage and repair

Several of genes with the largest increases in expression in this work were also up-regulated in the heat shock stress response (Chhabra et al. 2006) (Table 4). It is known that both prokaryotic and eukaryotic organisms respond to environmental stresses by inducing the synthesis of heat shock proteins (HSPs) (Lindquist and Craig 1988). These HSPs include molecular chaperones that assist folding of nascent and misfolded proteins, and ATP-dependent proteases that degrade damaged proteins (Yura and Nakahigashi 1999). The up-regulated operon DVU1874-76 (*dnaJ-dafA-clpB*) encodes three heat shock-responsive proteins that play an important role for cell survival by preventing aggregation and assisting refolding of proteins (Bukau and Horwich 1998). Two genes (DVU2441-42) encoding Hsp20 proteins, which act as chaperones to protect other proteins against denaturation and aggregation, were up-regulated. Another HSP that showed its gene up-regulated in this study was HslVU (DVU1467). Other genes also implicated in heat shock response that show a significant up-regulation include several peptidases (DVU2494, DVU1430, DVU3395 and DVU0759), a membrane-associated zinc metalloprotease (DVU0864-5), ATP-dependent proteases [*clp*S (DVU1339), *clp*A (DVU1601) and *clp*X (DVU1602)], multidrug resistance proteins (DVU2816-7; DVU2325) and transcriptional regulators, including the *marR* operon (DVU0525-26), which in *Escherichia coli* has been proposed to activate the multiple antibiotic resistance and oxidative stress regulons (Ariza et al. 1994). The transcriptional regulator rrf2 (DVU0529) had its gene up-regulated in this study, as was also observed in heat shock (Chhabra et al. 2006) and salt stress conditions (Mukhopadhyay et al. 2006). The *rrf2* gene is found downstream of the operon encoding the Hmc transmembrane redox complex, and has been proposed to be involved in Hmc transcriptional regulation (Rossi et al. 1993). The *hmc* operon was up-regulated in agreement with this proposal (see Table 2).

The *D. vulgaris* genome has heat shock promoter elements corresponding to the DNA binding sites for alternate sigma factors σ^{32} and σ^{54} acting in a similar fashion to what has been described for *E. coli*, as well as CIRCE elements as observed in *Bacillus subtilis* (Chhabra et al. 2006). Our results showed up-regulation of several genes with heat shock promoter elements, as well as with the CIRCE elements (Table 5). In δ-proteobacteria, σ^{32} seems to play a central role in the regulation of the heat shock response (Rodionov et al. 2004), and our results showed up-regulation of a chaperonin (DVU1977) and a down-regulation of a sigma 70 family protein (DVU1584). The GroE (DVU1977) chaperonin system and the heat-inducible transcription repressor HrcA that contain CIRCE elements, also showed up-regulation. In *B. subtilis* and other bacteria the GroE chaperonin modulates the activity of the HrcA repressor by sensing non-native proteins (Mogk et al. 1997; Wiegert and Schumann 2003). In addition, several genes under the control of σ^{54} were up-regulated. The products of σ^{54}-dependent regulation span a wide variety of functions, including response to heat and phage shock (Buck et al. 2000). The phage shock protein A (pspA) that is induced in *E. coli* cells under extreme stress conditions has been proposed to respond to extracytoplasmic stress by playing a role in maintaining cytoplasmic membrane integrity and/or the proton-motive force, as well as a role in protein export to the periplasm (Darwin 2005). In *D. vulgaris* the observed up-regulation of the σ^{54}-promoted pspA indicates that this is also involved in the response to severe oxidative stress. The energy status of the cell is probably low due to the stress severity and due to deviation of electrons for reducing ROS with subsequent dissipation of the proton motive force one of the proposed inducing signals for the PspA response (Kleerebezem et al. 1996). Enhanced export of proteins to the periplasm is also probably required to replace those inactivated by ROS.

Conclusions

Oxidative stress arising from the highest oxygen concentration likely to be found by *D. vulgaris* in its natural habitat, resulted in a milder response compared to that observed in

Table 4 Effect of oxygen exposure on the transcriptional responses of *D. vulgaris* in heat shock stress-related genes

Gene identifier	Annotation	Expression ratio (treatment/control)	*P*-value
DVU0864	Glycoprotease family protein, putative	2.77	8.1E-04
DVU0865	Membrane-associated zinc metalloprotease, putative	2.56	1.2E-04
DVU1430	Peptidase, M16 family	2.55	3.8E-04
DVU2494	Peptidase, M48 family	4.13	1.9E-03
DVU3395	Peptidase, M23/M37 family	2.68	1.5E-04
DVU1601	ATP-dependent Clp protease adaptor protein, (*clp*S)	3.72	7.1E-05
DVU1602	ATP-dependent Clp protease, (*clpA*)	2.33	1.8E-03
DVU1336	ATP-dependent Clp protease, (*clpX*)	2.36	7.6E-07
DVU1874	ATP-dependent Clp protease, (*clpB*)	2.05	3.9E-04
DVU1875	dafA Protein	6.11	8.7E-06
DVU1876	dnaJ Protein, putative	2.85	2.9E-05
DVU0813	Heat-inducible transcription repressor (*hrcA*)	2.14	7.0E-03
DVU1467	Heat shock protein HslVU, (*hslU*)	2.42	2.6E-06
DVU2441	Heat shock protein, Hsp20 family, (*hspC*)	13.15	3.2E-08
DVU2442	Heat shock protein, Hsp20 family	7.72	3.5E-03
DVU0525	Transcriptional regulator, MarR family	8.22	8.3E-12
DVU0526	Drug resistance transporter, putative	3.59	8.1E-09
DVU0529	Transcriptional regulator, rrf2 protein, putative	2.94	4.1E-03
DVU2325	Mercuric transport protein periplasmic component	6.68	3.0E-10
DVU2816	Multidrug resistance protein	4.18	2.0E-03
DVU2817	Multidrug resistance protein, (*acrA*)	5.50	5.3E-04

The list shows genes that responded in previous heat-shock studies of *D. vulgaris* (Chhabra et al. 2006) and that were also affected in this study

Table 5 Genes predicted to have σ^{32}, σ^{54} promoters or CIRCE sites in the *D. vulgaris* genome (Chhabra et al. 2006) that were affected in the present study

Promoter or site and gene identifier	Annotation	Expression ratio (treatment/control)	*P*-value
σ^{32}-Dependent promoters			
DVU1584	σ^{70} Family protein	0.50	3.9E-04
DVU1977	Chaperonin, 10 kDa	2.15	4.0E-04
CIRCE sites			
DVU1977	Chaperonin, 10 kDa, (*groES*)	2.15	4.0E-04
DVU0813	Heat-inducible transcription repressor (*hrcA*)	2.14	7.0E-03
σ^{54}-Dependent promoters			
DVU0036	Hypothetical protein	2.42	4.5E-03
DVU0360	Acetolactate synthase, large subunit, biosynthetic type, (*ilvB*-1)	3.69	2.9E-03
DVU0759	Peptidase, M29 family	3.79	5.1E-04
DVU2489	Hypothetical protein	2.17	9.3E-03
DVU2548	Acyl carrier protein phosphodiesterase, (*acpD*)	3.54	9.1E-07
DVU2988	Phage shock protein A, (*pspA*)	2.34	4.2E-04
DVU3283	Hypothetical protein	3.29	4.3E-05

exposure to air during 4 h (Mukhopadhyay et al. 2007), which is reflected in a much smaller number of genes affected. Of the oxygen reducing mechanisms of *D. vulgaris* only the hydrogen-dependent mechanism is up-regulated and the cellular response appears to concentrate on damage repair by metal-free enzymes and behavioral responses, represented schematically in Fig. 2. When cells cannot escape the oxidizing environment as in the case of our assays, this level of oxidative stress causes inevitably protein degradation, as in the case of heat shock, but the results indicate that this process may be more significant for metal-containing proteins, which are particularly abundant in *D. vulgaris*. Given the scenario of metalloproteins as the key targets for oxidative stress, a generalized down-regulation

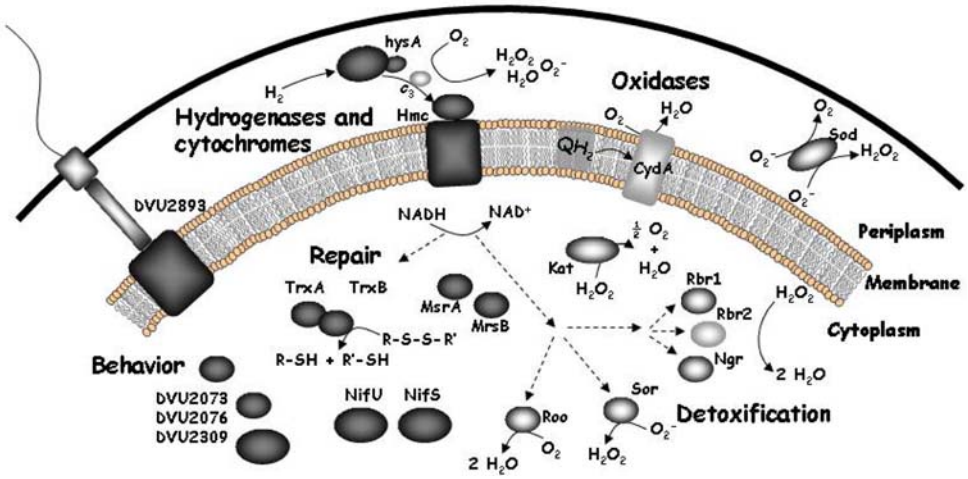

Fig. 2 Schematic representation of the transcriptional response of *D. vulgaris* genes discussed in the text. *Dark gray* indicates genes that were up-regulated, *medium gray* indicates genes that show no significant changes, and *light gray* indicates genes that were found down-regulated. Protein names are those found in the genome annotation: *cydA* quinol oxidase, *hysA* periplasmic NiFeSe hydrogenase large subunit, *Hmc* hmc complex, *Kat* catalase, *MsrA and B* methionine sulfoxide reductases, *NifU* nitrogen fixation protein, *NifS* cysteine desulfurase, *Ngr* nigerythrin, *Rbr1* rubrerythrin 1, *Rbr2* rubrerythrin 2, *Roo* rubredoxin-oxygen oxidoreductase, *Sod* superoxide dismutase, *Sor* superoxide reductase, *TrxA* thioredoxin, *TrxB* thioredoxin reductase

of the enzymes involved in energy metabolism and of the metal-based ROS detoxifying enzymes is an effective strategy to limit the availability of sensitive redox cofactors that would enhance the damage. Nonetheless, protein degradation and repair is an obvious consequence of oxidative damage and provides a rational for the similarities between oxygen and heat shock transcriptional responses.

Down-regulation of the Fur operon limits iron availability and therefore restricts the opportunity for further damage. The different transcriptional response of the Fur and PerR regulons suggests a more complex mode of regulation than previously described. Further studies of the way these regulons are modulated is warranted given the broad effect that they have in *D. vulgaris* metabolism and the insights that those results might have for other organisms.

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